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Biotechnological approaches to increase drought tolerance and nutritional quality in tomato

Ph.D. Dissertation
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1. Introduction

1.1 The tomato

Tomato (*Solanum lycopersicum*) belongs to the *Solanaceae* family, also known as the nightshade family, which contains about 2800 species and includes many economically important crops, such as potato, pepper and eggplant, as well as ornamental plants such as Petunia *spp.* and Nicotiana *spp.*, and medicinal plants, such as Datura *spp.*, Capsicum *spp.* and Nicotiana *spp.* (Foolad, 2007).

Due to their significance in our diet, *Solanaceae* plants represent one of the three most economically important groups of plants, besides grasses and legumes, to humankind (Kumar and Khurana, 2014).

All tomato species are native to a small area of South America, between Ecuador and Chile, even though they have evolved and are adapted to some of the most diverse and extreme habitats.

Tomato is used both as a fresh market and processed product, such as paste, juice, sauce, powder or whole. With worldwide production reaching almost 164 million tons in 2013, tomato is the seventh most important crop species after maize, rice, wheat, potatoes, soybeans and cassava. Asia dominates the tomato market with China ranking first, followed in decreasing order by India, USA, Turkey, Egypt, Iran, Italy, Brazil and Spain (Fig. 1.1, FAOSTAT 2013, http://faostat.fao.org).

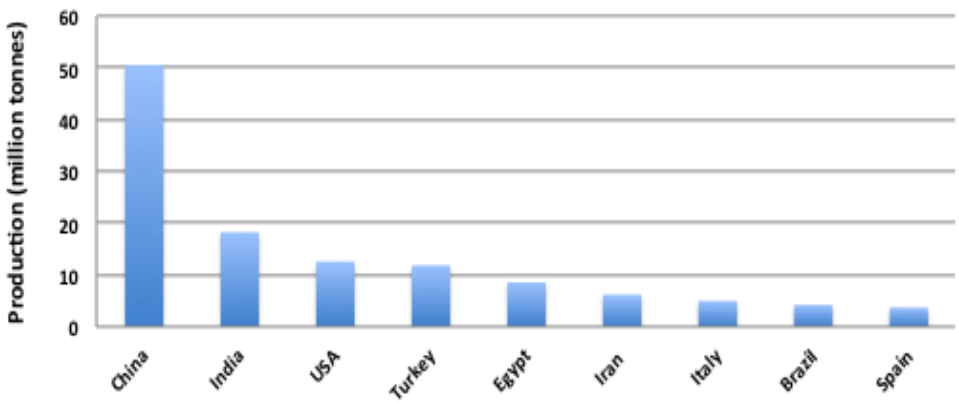

Figure 1.1 – Tomato production (million tonnes). Our elaboration on FAOSTAT data.

In terms of genetic and genomic resources, tomato is a model plant since it exhibits a reduced genomic size (950 Mb), a short generation time, and routine transformation technologies. Moreover, it shares with the other *Solanaceous* plants the same haploid chromosome number and a high level of conserved genomic organization (Barone *et al*., 2009).

Various biological aspects of tomato have been investigated with molecular biological approaches, and a sequencing of tomato genome was completed and a highly accurate and well-annotated reference sequence with other findings was published in May 2012 (Yano *et al.*, 2007; TGC, 2012).

Traditional tomato genetic resources include nine wild and related species.

They are all diploids (2n=2X=24), are similar in chromosome number and structure.

1.2 Tomato fruit quality

Fruit quality attributes can be divided in three different categories, namely chemical-phisical properties (firmness, pH, total soluble solids), organoleptic properties (taste and aroma) and nutritious content (Tab. 1.1). The research carried out within this thesis focus the attention on total soluble solids and firmness and the level of bioactive compounds such as AsA, phenolics and carotenoids.

Table 1.1 – Tomato (*S. lycopersicum*). Nutritional value referred to 100 g.

Water (g)	94.52
Energy (kcal)	18.00
Protein (g)	0.88
Total lipid (g)	0.20
Carbohydrate, by difference (g)	3.89
Fiber, total dietary (g)	1.2
Sugars, total(g)	2.63
Minerals	
Calcium (mg)	10.00
Iron (mg)	0.27
Magnesium (mg)	11.00
Phosphorus (mg)	24.00
Potassium (mg)	237.00
Sodium (mg)	5.00
Zinc (mg)	0.17
Vitamins	
Vitamin C, total ascorbyc acid (mg)	13.7
Thiamin (mg)	0.04
Riboflavin (mg)	0.02
Niacin (mg)	0.59
Vitamin B-6 (mg)	0.08
Folate, DFE (µg)	15.00
Vitamin A (IU)	833.00
Vitamin E (mg)	0.54
Vitamin K (µg)	7.09

Source: USDA National Nutrient database.

1.2.1 Total Soluble Solids

Economic success of a cultivar in the processed foodstuff market is dictated in part by a combination of total fruit yield and fruit soluble solids content. Fruit with high soluble solids contain less water and therefore require less processing to generate derivatives of the appropriate consistency for consumer tastes. In addition, since sugar is a major constituent of soluble solids content (SSC), such fruit are also likely to be sweeter and therefore require the addition of less sugar during processing.

These processing savings can have a significant bearing on the profitability of processed tomato products and, thus, from a commercial standpoint, there is considerable interest in manipulating the soluble solids content of tomato varieties

(Baxter *et al.*, 2005).

The cultivated variety of tomato, *Solanum lycopersicum*, has large, red fruit that predominantly accumulate hexose sugars. In contrast, the wild relative, *Solanum pennellii*, has small, green fruit that predominantly accumulate sucrose and have higher soluble solids content.

In tomato fruit, the dominant soluble metabolites are sugars and organic acids (Grierson and Kader, 1986; Roessner-Tunali *et al.*, 2003). Their overall content can be expressed as SSC that is measured by a refractive index called Brix degree.

1.2.2 Firmness

Texture is an important aspect of consumer perception of fresh tomatoes (Causse *et al.*, 2003; Serrano-Megias and Lopez-Nicolas, 2006). Texture traits include flesh firmness, mealiness, meltiness, crispness, and juiciness (Harker *et al.*, 1997; Redgwell and Fischer, 2002; Szczesniak, 2002). Previous research has focused on creating tomatoes with greater firmness in order to have greater disease resistance and longer shelf life (Hongsoongnern and Chambers, 2008). The amount of gel and seeds within locules are unique aspects of tomato perceptions (Chaib *et al.*, 2007).

Firmness to touch was considered an important attribute by the majority of participants. Oltman and co-authors conducted a series of focus group in order to know how the tomato consumer choices and they saw that most participants would only buy tomatoes that were firm (Oltman *et al.*, 2014).

Tomatoes that are not firm are susceptible to chilling injury (Jackman *et al.*, 1990). Firmness may be used to evaluate a tomato for the extent of its chilling injury, which consumers may be able to visualize.

A number of works have been published on tomato fruit firmness measurements, and different instruments for measuring firmness has been illustrated (Batu, 1998). Instrumental measurements are generally used to evaluate firmness related to the mechanical properties of fruit tissues. The most widely used measurement of fruit

texture is the Magness–Taylor firmness test, which assesses the maximum force needed to compress the sample in a specific way (Abbott, 2004). This system was mainly developed to evaluate commercial quality with respect to the tactile assessment of fruit firmness by consumers at the time of purchase. Many other types of destructive or non-destructive mechanical measurements exist, including compression, puncture, tension, and vibration. Rheological measurements inform about the mechanical properties of the fruit or of a localized part of the fruit (peel, pericarp, etc.).

1.2.3 Ascorbic acid

In addition to its roles in cardiovascular function, immune cell development, and iron utilization, vitamin C (L-ascorbic acid or AsA) serves as a water-soluble antioxidant in humans (Sies *et al.*, 1995; Levine *et al.*, 1986; Levine *et al.*, 1995).

Despite the fact that most mammals can synthesize L-ascorbic acid (AsA), humans are an exception as a result of a mutation to L-gulono-1,4-lactone oxidase, the last enzyme in the animal AsA biosynthetic pathway (Chatterjee, 1973). Because AsA is water-soluble, it is not stored and is readily excreted from the body. Therefore, AsA must be obtained regularly from dietary sources. The National Academy of Sciences has recommended 90 mg/day of the vitamin for adult males and 75 mg/day for adult females. Although vitamin C can be obtained from the consumption of fresh meat, it is destroyed by heating and is more typically obtained from plant sources (Gallie, 2013). According to the Centers for Disease Control and Prevention, good sources of vitamin C include broccoli, tomatoes, leafy greens, apricots and pineapple (http://www.cdc.gov/nccdphp/dnpa/5ADay/index.htm).

Tomatoes contain moderate amounts of vitamin C, but especially in the summer, because of the large consumption of fresh fruit provides consumers with significant AsA intake (Stevens *et al.*, 2006).

The Average ascorbic acid levels found in market-purchased products ranged

beetwen 0.08 and 0.10 g/kg on a wet weight base (Franke *et al.*, 2004; Nagarajan and Hotchkiss, 1999; Rickman *et al.*, 2007).

AsA is also an essential compound for plants, having a primary role as an antioxidant preventing oxidative stress as well as playing a role in plant development and hormone signalling (Pastori *et al.*, 2003), the activation of the cell cycle (Potters *et al.*, 2002) and possibly cell wall loosening during cell expansion or fruit ripening (Fry, 1998).

A recent plethora of evidences suggests that it may play a role in protection of plant against several environmental stresses such as metal action, salinity, weedicides, O_3, UV-B and pathogenesis (Shalata *et al.*, 2001). The endogenous level of AsA is determined by both *de novo* AsA biosynthesis and recycling of the oxidized forms of AsA, monodehydroascorbate (MDA) and dehydroascorbate (DHA) via MDA reductase and DHA reductase, respectively (Conklin and Barth, 2004).

Plants and animals employ distinct pathways for the synthesis of L-ascorbic acid (Fig. 1.2).

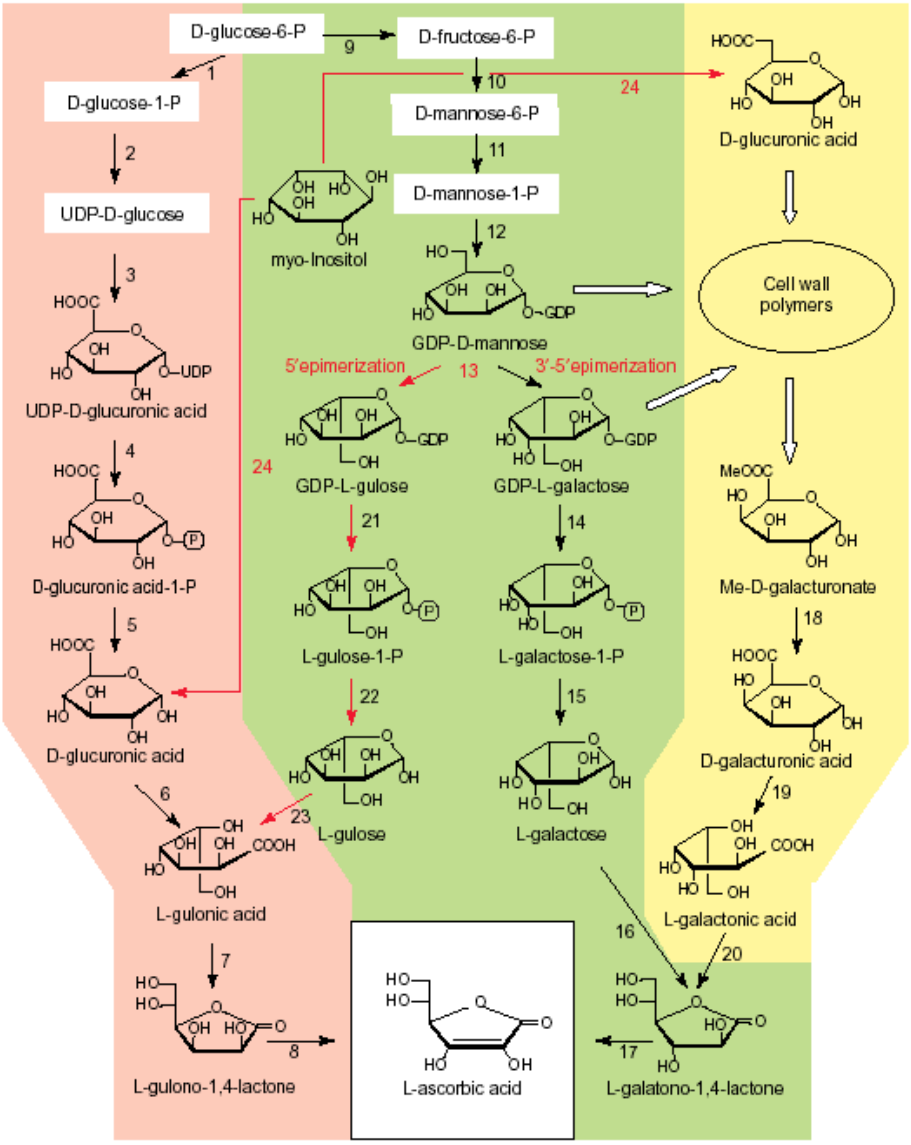

Figure 1.2- Proposed biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8) and plants (reactions 9–24). Red arrows indicate enzymatic activities of the new pathways leading to L-ascorbic acid biosynthesis [15,19]. Enzymes catalyzing the numbered reactions are: 1, phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDP-glucose dehydrogenase; 4, glucuronate-1-phosphate uridylyltransferase; 5, glucurono kinase; 6, glucuronate reductase; 7, aldono-lactonase; 8, gulono-1,4-lactone dehydrogenase; 9, glucose-6-phosphate isomerase; 10, mannose-6-phosphate isomerase; 11, phosphomannomutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); 13, GDP-mannose-3',5'-epimerase; 14, phosphodiesterase; 15, sugar phosphatase; 16, L-galactose dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methylesterase; 19, D-galacturonate reductase; 20, aldono-lactonase; 21, phosphodiesterase; 22, sugar phosphatase; 23, L-gulose dehydrogenase; 24, myo-Inositol oxygenase. Adapted from Valpuesta and Botella (2004).

The AsA pathway in mammals begins with D-glucose and proceeds through D-glucose-1-P, UDP-glucose, UDP-D-glucuronic acid, UDP-D-glucuronic acid-1-P, D-glucuronic acid, L-gulonic acid, and finally gulono-1,4-lactone (Fig. 1.2). Gulono-1,4-lactone oxidase then converts gulono-1,4-lactone into 2-keto-gulono-γ-lactone which spontaneously converts to AsA (Burns, 1967).

In contrast to this single AsA pathway in mammals, there are at least four

biosynthetic pathways suggested to date in plants but the contribution of each one to the synthesis of AsA varies between different species, organs and developmental stages (Cruz-Rus *et al.*, 2011). The first discovered was the Smirnoff-Wheeler pathway in which AsA synthesis originates with L-galactose (Wheeler *et al.*, 1998) (Fig. 1.2).

L-Galactose is produced from mannose-1-phosphate through the intermediates guanosine diphosphate (GDP)-mannose and GDP-L-galactose (Wolucka *et al.*, 2001). L-Galactose then undergoes oxidation to L-galactono-1,4-lactone catalyzed by the NAD-dependent L-galactose dehydrogenase followed by oxidation to L-ascorbic acid by the mitochondrial-localized L-galactono-1,4-lactone dehydrogenase (Siendones *et al.*, 1999; Bartoli *et al.*, 2000). The first plant alternative pathways includes D-galacturonic acid as key intermediate which generated from the breakdown of pectin during fruit ripening, serves as the starting point for AsA synthesis and is reduced to L-galactonic acid as catalyzed by the NADPH-dependent D-galacturonic acid reductase (GalUR). A rule of pectin catabolism was also hypnotised by Di Matteo and co-authors (2010). L-Galactonic acid spontaneously converts to L-galactono-1,4 lactone which L-galactono-1,4-lactone dehydrogenase converts to AsA (Valpuesta and Botella, 2004).

In the second alternative pathway, GDP-mannose 3′,5′-epimerase, which catalyzes conversion of GDP-D-mannose to GDP-L-galactose in the L-galactose pathway, also operates in the 5′-epimerization of GDP-D-mannose to produce GDP-L-gulose. Conversion of GDP-L-gulose to L-gulonic acid allows AsA to be synthesized essentially as in the animal pathway although evidence for this is still lacking. The presence of L-gulonic acid and L-gulono-1,4-lactone dehydrogenase activity supports the existence of this pathway in plants (Wagner *et al.*, 2003).

The third alternative pathway involves D-glucuronic acid, an intermediate of the animal pathway which in plants can be generated by *myo*-inositol oxygenase. Support for this pathway in plants comes from the observation that overexpressing an *Arabidopsis* gene having homology to a porcine *myo*-inositol oxygenase increased

AsA content (Lorence *et al.*, 2004).

Although multiple AsA biosynthetic pathways may exist in plants, the observation that mutants affected in the Smirnoff–Wheeler pathway result in substantial reductions in AsA content does indicate that the alternative pathways are unable to compensate for the loss in AsA biosynthetic capacity in Smirnoff–Wheeler pathway mutants. Thus, these alternative pathways may make only minor contributions to AsA biosynthesis and strategies focusing on these other pathways may be limited to increasing AsA in specific organs or at specific developmental stages.

The regulation of ascorbate levels in cells is therefore tightly controlled by the level of synthesis and recycling as well as degradation (Pallanca and Smirnoff, 2000; Green and Fry, 2005) and the transport of this molecule within the cell (Horemans *et al*., 2000).

The transcription of genes encoding biosynthetic enzymes such as D-galacturonate reductase and myo-inositol oxygenase and the AsA recycling enzymes MDHAR are positively correlated with the increase in AsA during plant ripening.

The recent cloning of several genes that regulate AsA synthesis and recycling has facilitated the generation of transgenic plants with enhanced AsA levels and in some cases as much as six-fold increases in AsA relative

to wild-type plants have been achieved.

All genes involved in the Smirnoff–Wheeler pathway have been characterised in Arabidopsis (Wheeler *et al*., 1998; Conklin *et al.*, 1999; Wolucka and Van Montagu, 2003; Laing *et al*., 2004, 2007; Dowdle *et al*., 2007; Linster *et al.*, 2007). Also, mutations in the D-Man/L-Gal pathway result in a significantly decreased AsA content, whereas the overexpression of genes in this pathway increases the AsA levels (Conklin *et al*., 2000; Gatzek *et al*., 2002; Wolucka *et al.*, 2003; Dowdle *et al*., 2007; Linster *et al*., 2008). For example, the GDP–D-Man pyrophosphorylase (VTC1) vtc1-1 mutant displays a decrease in the synthesis of AsA to only one-third of the amount in wild-type plants (Conklin *et al*., 2000). Two homologous genes, VTC2 and VTC5, encode GDP–L-Gal phosphorylase in Arabidopsis (Dowdle *et al*.,

2007; Linster *et al*., 2007, 2008) and the double mutant (vtc2/vtc5), which has almost no GDP–L-Gal phosphorylase activity, displays significantly decreased AsA levels and impaired growth (Dowdle *et al*., 2007). The overexpression of the tomato GDP–Man-3,5-epimerase gene SIGME increases AsA content and enhances tolerance to salt and cold (Zhang *et al*., 2011). Two master regulators of this pathway, Arabidopsis AMR1 (Zhang *et al*., 2009) and AtERF98 (Zhang *et al*., 2012) have been recently reported as well.

In addition to *de novo* synthesis, AsA recycling also affects the AsA level (Smirnoff *et al*., 2001; Chen *et al*., 2003; Stevens *et al*., 2008). As the most abundant water-soluble antioxidant, one of the important roles of AsA is to scavenge reactive oxygen species (ROS) (Smirnoff and Wheeler, 2000) that are normally generated during photosynthesis and aerobic metabolism in plants (Conklin and Barth, 2004; Foyer and Noctor, 2009). During ROS scavenging, AsA is oxidised into monodehydroascorbate (MDHA) by AsA peroxidase (APX), and MDHA can be recycled to AsA by monodehydro-ascorbate reductase (MDAR) or disproportionate to dehydro-ascorbate (DHA) and AsA. The DHA can be recycled to AsA by dehydroascorbate reductase (DHAR) or hydrolysed to 2,3-diketogulonic acid (Smirnoff and Wheeler, 2000). The over-expression of wheat DHAR significantly increased the levels of AsA in tobacco and maize, which indicates that AsA recycling also plays an important role in the regulation of AsA levels in plants (Chen *et al*., 2003). Thus, MDAR and DHAR play important roles in recapturing AsA and regulating the redox state of plant cells (Chen *et al*., 2003; Eltayeb *et al*., 2007; Stevens *et al*., 2008).

Beside genes controlling AsA biosynthesis within the Smirnoff-Wheeler pathway, the overexpression of a myo-inositol oxygenase (MIOX) gene in *Arabidopsis thaliana* leads to plants with 2–3-fold increase in foliar AsA content (Lorence *et al*., 2004).

1.2.4 Phenolic compounds

A number of clinical trials and cohort studies have suggested a role of dietary polyphenols in the prevention of several major chronic diseases such as cardiovascular diseases, cancers, diabetes, neurodegenerative diseases, and osteoporosis (Arts *et al*., 2005; Scalbert *et al*., 2005). Plants are the most important dietary source of phenolic compounds for human.

As a general rule recently proposed by Quideau *et al.* (2011), the term 'plant phenolics' should be strictly used to refer to secondary natural metabolites arising biogenetically from the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids (Fig. 1.3), or the 'polyketide' acetate/malonate pathway, which can produce simple phenols, or both of them. These pathways produce a bewildering array of monomeric and polymeric structures (the term 'polyphenols' defining those with more than one phenolic ring) that fulfill a very broad range of physiological roles in plants.

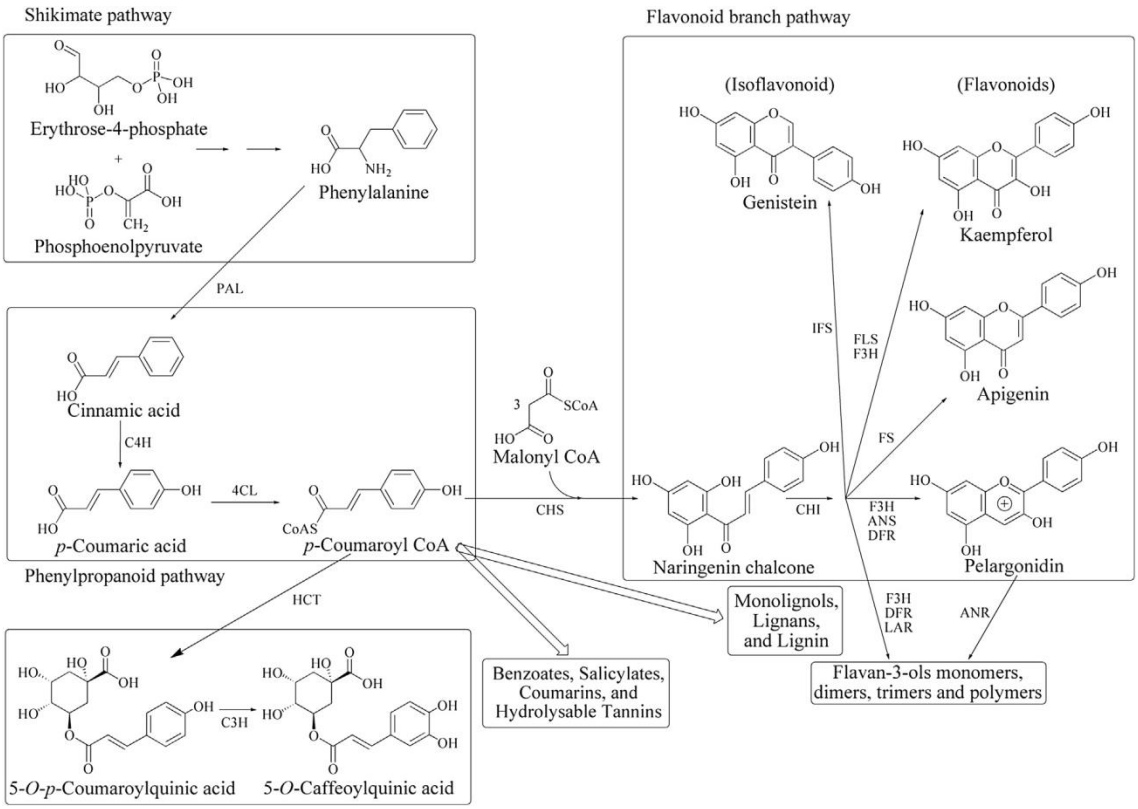

Figure 1.3 - Schematic of the major branch pathways of (poly)phenol biosynthesis. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; HCT, hydroxycinnamoyl transferase; C3H, p-coumarate-3-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; ANS, anthocyanidin synthase; DFR, dihydroflavonol reductase; FS, flavone synthase; FLS, flavonol synthase; F3H, flavanone 3-hydroxylase; IFS, isoflavone synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase (Cheynier *et al*., 2013).

It has been estimated that around 8000 different phenolic compounds are synthesized by plants, via a variety of polymerization, hydroxylation, methylation, glycosylation, acylation, prenylation, and condensation reactions (Pourcel *et al.*, 2006) and more than 500 different phenolic compounds are known in foods, from low-molecular-weight phenolic acids to highly polymerized proanthocyanidins. Several classes of phenolics have been categorized on the basis of their basic skeletons: C6 (simple phenols, benzoquinones), C6-C1 (phenolic acids and aldehydes), C6-C2 (acetophenones, phenylacetic acids), C6-C3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C6-C4 (naphthoquinones), C6-C1-C6 (xanthones), C6-C2-C6 (stilbenes, anthraquinones), C6-C3-C6 (flavonoids, isoflavonoids, neoflavonoids), $(C6\text{-}C3\text{-}C6)_{2,3}$ (bi-, triflavonoids, proanthocyanidin dimers, trimers), $(C6\text{-}C3)_2$ (lignans, neolignans), $(C6\text{-}C3)_n$ (lignins), $(C6)_n$ (catechol melanins, phlorotannins), $(C6\text{-}C3\text{-}C6)_n$ (condensed tannins).

Dietary polyphenols belong to four main classes of flavonoids, phenolic acids, stilbenes, and lignans, that are largely present in a glycosidic form (glycosides of flavonoids, lignans, and stilbenes) or as esters (phenolic acids esterified to polyols such as quinic acid).

These compounds are involved in a broad range of physiological and ecological processes, ranging from recruitment of pollinators and seed dispersers, interactions with pathogens, nitrogen-fixing bacteria and parasitic roots in the rhizosphere, control of male fertility, UV-tolerance, antioxidant based defence, auxin transport, and defence against microbes and grazers (Grotewold, 2006).

However the levels of phenolics differ among cultivars, maturity stages, technological processes and storage.

The investigations to date indicate that *chi* gene activity appears to be key to flavonol accumulation in tomato peel, whilst *chs* and *fls* activities are required for the production of flavonols in flesh tissue. Therefore, it was reasoned that, to achieve increased flavonol accumulation throughout the tomato fruit, ectopic expression of three genes encoding the biosynthetic enzymes CHS, CHI and FLS would be

sufficient. Indeed a cross harbouring these three genes accumulates increased levels of quercetin‑glycosides in peel and kaempferol‑glycosides in flesh. It is also noteworthy that a similar phenotype can be achieved by crossing tomatoes containing Lc and C1 transgenes with tomatoes containing the CHI transgene. Accumulation of phenolic compounds in plant tissues is a distinctive characteristic of plant stress. This accumulation is due to an increased enzyme activity of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and other enzymes, activity of phosphoenolpyruvate (PEP)-carboxylase also increases, suggesting a shift from sucrose production to processes in support of defense and repair.

The control of the production of polyphenols involves a matrix of potentially overlapping regulatory signals. These include developmental signals, such as during lignification of new growth or the production of anthocyanins during fruit and flower development, and environmental signals for protection against abiotic and biotic stresses. For some polyphenols, such as the flavonoids, there is now an excellent understanding of the nature of those signals and how the signal transduction pathway connects through to the activation of the biosynthetic genes. The regulation of flavonoid production occurs principally through changes in the transcription rate of the biosynthetic genes. This is achieved through the action of transcriptionfactors (TFs).

Central to the direct regulation of anthocyanin and proanthocyanidin (PA) biosynthetic genes are core ‘MBW’ regulation complexes, comprising of specific members of the R2R3MYB and basic helix-loop-helix (bHLH) TF families in conjunction with a WD-repeat (WDR; tryptophan-aspartic acid (W-D) dipeptide repeat) protein. Variant MBW complexes can form from different MYB and bHLH components, and these can have different target genes and vary in their activation or repression actions.

Outside of the anthocyanins, PAs and flavonols, there are only a few polyphenol biosynthetic pathways for which TFs have been characterized. Most notably, the regulation of lignin biosynthesis has been extensively studied in *A. thaliana*, and to a

lesser extent the grasses (Gray *et al*., 2012; Zhao *et al*., 2011; Cheynier *et al*., 2013). Also, it has been demonstrated that ethylene and other hormones can modulate the intensity and the direction of regulative effects on metabolism and the final level of phenolics in plant tissues. Several authors reported that the production of phenolic compounds can be affected by exogenous treatments with ethylene and/or by biotic and abiotic stresses, and that this effect could be mediated by ethylene responsive elements (EREs) and MYB factors. Indeed, by using various *Arabidopsi*s mutants, it has been recently demonstrated that auxin and ethylene regulate flavonol biosynthesis through MYB12-mediated signalling and that ethylene modulates flavonoid accumulation in the roots.

The tomato fruit is an important source of phenolics. In general, the level of total soluble phenols (TSP) has been reported twice higher in the exocarp than in the mesocarp during all stages of ripening and it normally increases significantly in both tissues during ripening, more pronouncedly in exocarp (Minoggio *et al*., 2003; Kacjan *et al*., 2011). The TSP content has been reported to reach highest levels (10.39 mg 100 g FW^{-1}; Carrillo-Lòpez and Yahia, 2013) in cherry-type. In addition to the flavonoid naringenin chalcone that accumulates up to 1% dry weight of the tomato fruit cuticle, various other flavonoids accumulate in tomato fruit such as rutin (quercetin-3-rutinoside), kaempferol-3-O-rutinoside and a quercetin-trisaccharide. Quercetin accumulates at level order of 10 μg gFW^{-1} (Hertog *et al*., 1993; Crozier *et al.*, 1997) and its actual concentration vary according to the genotype, the organ, the developmental stage and the environment.

1.2.5 Carotenoids and colours

Carotenoids are the second most abundant naturally occurring pigments on earth, with more than 750 members. They range from colorless to yellow, orange, and red, with variations reflected in many fruits, and vegetables, which contribute to their economic value as well. The colours of fruits and vegetables depend on conjugated

double bonds and the various functional groups contained in the carotenoid molecule (Rodriguez-Amaya and Kimura, 2004).

Naturally, most of the carotenoids occur as trans-isomer in plants. However, cis-isomers may increase due to the isomerization of the trans -isomer of carotenoids during food processing (Schieber *et al.*, 2005). Heating at 60 °C and 80 °C favored the isomerization of lycopene (Lee and Chen, 2002). However lycopene is responsible for red colour while other carotenoids lead to yellow and orange colours. The rate beetween different carotenoids is an important index in the colour definition (Khoo *et al.*, 2011).

Oltman and co-workers (2014) showed that one of the most determined attribute for tomato purchase was colour: in three focus group most people liked dark-red coloured tomatoes, with only a few people (<20%) mentioning they would not mind a lighter red, or yellow, or orange colour.

Provitamin A carotenoids play essential roles in animals as precursors for the synthesis of retinoid, retinol (vitamin A), retinal (main visual pigment), and retinoic acid (which controls morphogenesis) (Fraser and Bramley, 2004; Krinsky and Johnson, 2005). In humans, carotenoids also serve as antioxidants and reduce age-related macular degeneration of the eye, the leading cause of blindness in the elderly worldwide (Johnson and Krinsky, 2009; Fiedor and Burda, 2014). Lycopene, the most abundant carotenoid in ripe tomato, has been involved in treating chronic diseases and lowering risk of cancer and cardiovascular disease (Sandmann *et al.*, 2006; Ford and Erdman, 2012; Liu *et al.*, 2015).

Chemically, carotenoids are mainly C_{40} lipophilic isoprenoids and can be divided in two subgroups: xanthophylls (they present oxigen) and carotenes.

Carotenoids are synthesized in all photosynthetic organisms (bacteria, algae, and plants), as well as in some non-photosynthetic bacteria and fungi. Carotenoids and their oxidative and enzymatic cleavage products called apocarotenoids are crucial for various biological processes in plants, such as assembly of photosystems and light harvesting antenna complexes for photosynthesis and photoprotection, and regulation

of growth and development (Cazzonelli and Pogson 2010; Ruiz-Sola and Rodrıguez-Concepciona, 2012; Havaux, 2014; Nisar *et al.*, 2015).

Apocarotenoids are also proposed to serve as signaling molecules and have been implicated in the interactions of plants with their environment (Walter and Strack, 2011; Cazzonelli, 2011).

Carotenoids are formed and sequestered at very high levels in plastids, mainly chloroplasts and chromoplasts (Egea *et al.*, 2010). In green chloroplast-containing tissues, carotenoid biosynthesis mostly takes place in the envelope and the thylakoid membrane, and xanthophylls are predominantly produced to perform photosynthetic functions (Deruere *et al.*, 1994; Vishnevetsky *et al.*, 1999; Joyard *et al.*, 2009).

However, specific carotenoid in the form of lycopene crystalloid is accumulated at a high level in membrane-shaped structures in chromoplast-containing tissues during tomato fruit ripening accompanied by breakdown of the thylakoid membrane, the appearance of carotenoid-containing crystalloids, the synthesis of new membranes of sites of formation of carotenoid crystals, as well as an increase in the number and size of plastoglobules (Harris and Spurr, 1969; Simkin *et al.*, 2007). ZDS, LCY-B, and two β-carotene β-hydroxylase proteins were detected in the proteome of pepper (*Capsicum annuum*) plastoglobules, while ZDS was identified in the proteome of tomato chromoplasts (Barsan *et al.*, 2010).

Carotenoid biosynthesis starts with the condensation of two geranyl-geranyl pyrophosphate (GGPP) molecules by phytoene synthase (PSY) to form phytoene as a 15-cis isomer. There is only one PSY gene in *Arabidopsis*, while three tissue-specific isoforms exist in tomato with PSY1, which contributes to carotenoid production in tomato fruit (Fig. 1.4) (Bramley, 2002; Giorio *et al.*, 2008).

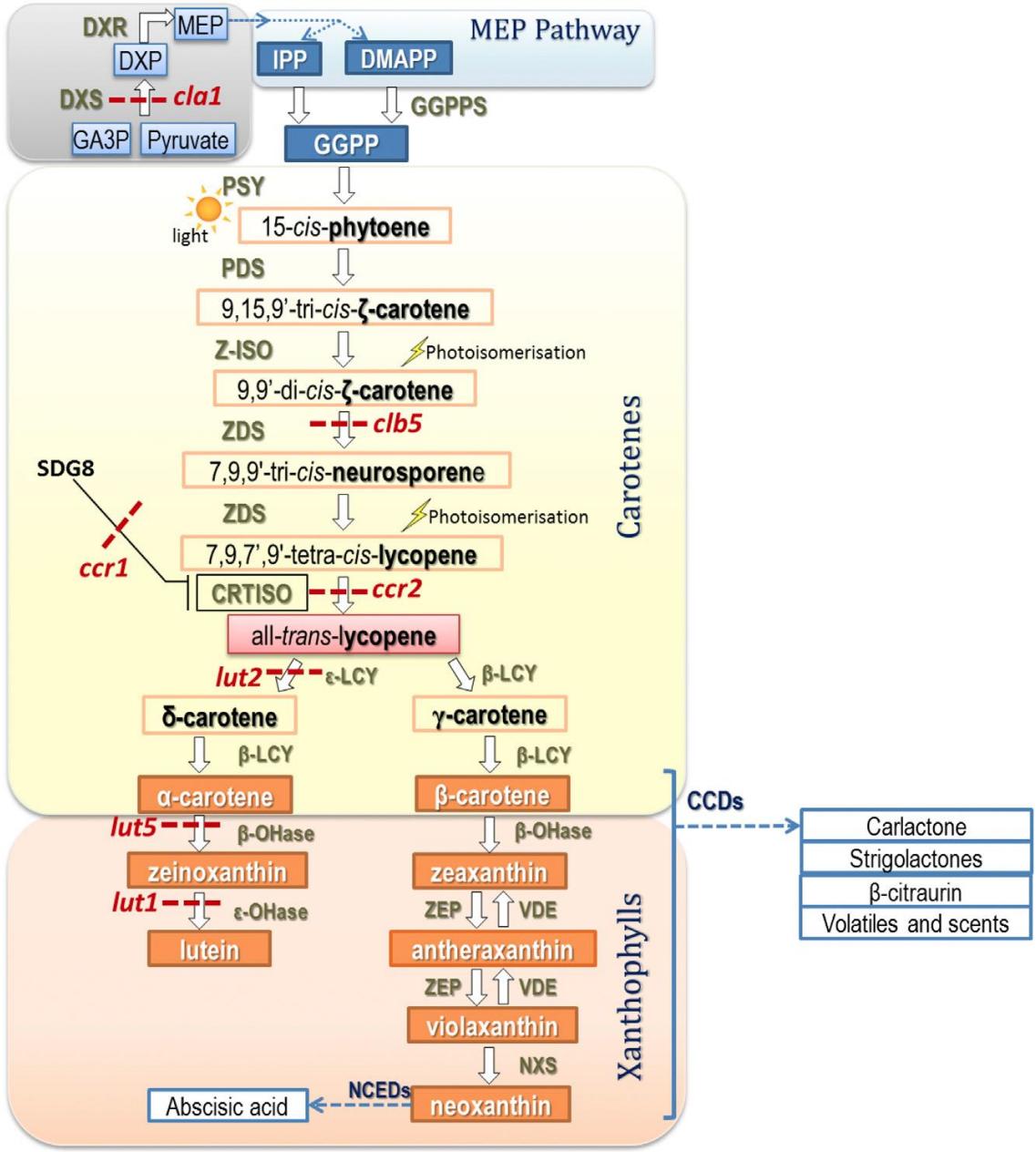

Figure 1.4 - Schematic Carotenoid BiosyntheticPathway.
The pathway shows the primary steps found in nearly all plant species. The C40 carotenoid phytoene is derived by condensation of two molecules of the C20 geranyl-geranyl pyrophosphate (GGPP), produced from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Phytoene is converted into lycopene via a series of desaturation and isomerization. Lycopene is cyclized by b-LCY and ε-LCY or b-LCY to produce a-carotene or b-carotene. These carotenes are further hydroxylated to produce xanthophylls (e.g. lutein and zeaxanthin). The cleavage of b-carotene branch by CCDs and NCEDs produces various volatiles (e.g. b-citraurin, etc.) and phytohormones (strigolactones and abscisic acid). Arabidopsis mutants defective in carotenogenesis (cla1; altered chloroplast 1, clb5; chloroplast biogenesis 5, ccr1, and ccr2; carotenoid and chloroplast regulation-1 and 2, lut1, lut2, and lut5; lutein deficient-1, 2, and 5) are shown in red. b-LCY, b-cyclase; b-OHase, b-carotene hydroxylase; CCD, carotenoid cleavage dioxygenase; CRTISO, carotenoid isomerase; ; ε-LCY, ε-cyclase; ε-OHase, ε-carotene hydroxylase; GA3P, glyceraldehyde-3-phosphate; GGPPs, GGPP synthase; NCED, 9-cis-epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; SDG8, SET2 histone methyltransferase; VDE, violaxanthin de-epoxidase; ZDS, z-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, zcarotene isomerase (Nisar *et al*., 2015).

Four double bonds are introduced into phytoene via two phylogenetically related

enzymes: phytoene desaturase (PDS) and z-carotene desaturase (ZDS). These enzymes catalyze two symmetric dehydrogenation reactions converting 15-cis phytoene to tetra-cis-lycopene. Both of these enzymes are encoded by single-copy genes in tomato, grape and *Arabidopsis*. The all-trans isomer of lycopene in higher plants demonstrates a requirement of specific isomerase enzymes. A carotenoid isomerase (CRTISO) that is capable of isomerizing cis bonds at 7, 9 and 70, 90 positions has been demonstrated to convert tetra-cislycopene to all-trans-lycopene.

The cyclization of lycopene is a crucial step in carotenoid metabolism and generates carotenoid diversity distinguished by different cyclic end groups: either the addition of beta (b-ring) and/or epsilon (ε-type ring). These rings are generated by lycopene b-cyclase (b-LCY) and lycopene ε-cyclase (ε-LCY), respectively (Cunningham *et al.*, 1993; Cunningham *et al.*, 1996; Pecker *et al.*, 1996; Ronen *et al.*, 1999).

One route leads to b-carotene, zeaxanthin, violaxanthin, and neoxanthin, providing precursors for the synthesis of ABA and strigolactones. The alternative pathway leads to a-carotene and lutein.

Carotenoid accumulation is an outcome of its synthesis and degradation. A metabolic equilibrium between biosynthesis and catabolism of carotenoids is essential for maintaining their content and composition in photosynthetic tissues (Beisel *et al.*, 2010).

Tomato contains a complex mixture of bioactive components, serving as a dietary source of nutrients, such as a mixture of carotenoids, including lycopene, b-carotene, and lutein.

Abushita and co-authors (2000) analysed the lycopene content in twelve tomato cultivars and observed that it ranged between 16-36 μg/100 gr of fresh weight.

Besides their functions as pigments and nutrients, carotenoids are also the precursors of many important volatile flavor compounds in plants, conferring the sensory attribute that can be detected by consumers (Vogel *et al.*, 2010).

Some apocarotenoids have commercial value in the food and cosmetic industries as aromas, flavors, and pigments (Giuliano *et al.*, 2003).

1.3 Drought stress

Drought is defined as "a marked deficiency of rain compared to that usually occurring at the place and season under consideration" (Walker, 1999.

A distinction between drought and aridity occur: droughts usually last less than five years (and mostly less than two), whereas persistent dry conditions lasting more than a decade reflect a shift to aridity. (Alwynne and Beaudoin, 2002).

Among the abiotic constraints, drought is the first responsible that limits the global productivity of major crops and, thus, it is among a major topics of plant research and breeding.

A major challenge for current agricultural biotechnology is to satisfy an ever increasing demand in food production facing a constantly increasing world population that will reach more than 9 billion in 2050 (Godfray *et al*., 2010; Tester and Langridge, 2010). This growing demand for food is paralleled by dramatic losses of arable land due, among others to increasing severity of soil destruction by abiotic environmental conditions and human activities. In particular drought and salinity are the two major environmental constraints that adversely affect plant growth and development and have a crucial impact on agricultural productivity and yields. Drought is a consequence of water shortage, it is critical for crop production in large agronomic areas worldwide and it is usually coped with extensive irrigations. Although earth is rich in water, most water resources are highly salinized whereas high quality fresh water that is suitable for irrigation is often extremely limited. Accordingly, not only drought but also soil salinity becomes increasingly an agricultural problem due to extensive spreading of agricultural practices as irrigation (Flowers, 2004). These shortages in water suitable for irrigation are predicted to get even worst as a consequence of global warming and population growth.

Plants develop a range of strategies to avoid or tolerate drought stress.

In the case of stress avoidance, plants maintain high water status either by efficient water absorption from roots or by reducing evapo-transpiration from aerial parts.

Drought tolerant plants maintain turgor and continue metabolism even at low water potential, e.g. by protoplasmic tolerance or synthesis of osmoprotectants, osmolytes or compatible solutes (Turner *et al.*, 2001).

The initial period of drought stress or soil water-deficit induces stomatal closure to reduce water loss by a process mediated by abscisic acid (ABA) (Leung *et al.*, 1998; Koornneef *et al.*, 1982; Bartels *et al.*, 1990).

Furthermore, the closed stomata decrease CO2 supply to the mesophyll cells so that plant photosynthetic activity is also reduced (Barker *et al.*, 2004; Mishra *et al.*, 2012).

Drought tolerance is a polygenic trait that involves a cascade of responses ranging from physiological changes to transcriptional regulation.

The adaptive mechanisms in response to drought in plants involve reduction in water loss, reduction in radiation absorption, reduction in evaporation surfaces, and lowering tissue water potential. Other adaptive mechanisms include maintaining cell turgor pressure and reduction water loss by the accumulation of compatible solute molecules such as betaine, proline, sorbitol, and so on (Seki *et al.*, 2007).

Drought stress is not static; it can occur at any time during the crop growth, it can vary in severity and it frequently occurs in conjunction with other environment stresses, such as heat and salinity (Wheat, 2010).

Different tissues or organs of the plant will respond differently to drought stress and the level of stress will fluctuate diurnally, high stress during peak photosynthetic periods and low stress overnight (Wilkins *et al.*, 2005). The nature of a plant response will also vary greatly depending on whether the plant is entering stress for the first time or after several exposures and whether they are recovering from stress after a rainfall or irrigation event (Barbazuk *et al.*, 2007).

Shinozaki and colleagues (2003) investigated the molecular response to drought by microarray analyses in *Arabidopsis* and they classified responsive genes into two groups. The first group includes genes coding for proteins that most probably function in abiotic stress tolerance. These include molecules such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, mRNA-

binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases.

The second group is comprised of regulatory proteins, i.e. protein factors involved in further regulation of signal transduction and stress-responsive gene expression.

These include various transcription factors (bZIP, WRKY, MYB, and AP2/EREBP), protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signalling molecules such as calmodulin-binding protein. Many transcription factor genes were stress inducible, suggesting that various transcriptional regulatory mechanisms may function in regulating drought, cold, or high salinity stress signal transduction pathways. These transcription factors could govern expression of stress-inducible genes either cooperatively or independently.

In a recent work on tomato, when comparing differentially expressed genes under drought stress, prominent genes could be identified in the drought-tolerant line as compared to the susceptible line. The upregulated genes in the drought-tolerant line were related to energy, plant hormones, and cation transporters (Sadder *et al*., 2014).

Orrellana and co-authors (2010) found that an AREB-type transcription factors, SIAREB1, was correlated with the degree of drought and salt tolerance in transgenic tomato. Its overexpression resulted in the activation of stress related genes leading to an improved tolerance (Orellana *et al.,* 2010).

In a study on abiotic tolerance in tomato, the gene SpUSP revealed to play a critical role, especially in drought tolerance. It was related to an increase in ABA content, suggesting that SpUSP has a function in regulating stomatal opening and thus improves drought resistance by ABA (Loukehaich *et al.*, 2012).

In another study where tomatoes underwent to different water stress treatment, an increase in ascorbate peroxidase (APX) activity was specially noted in fruits at mature green and breaker stages. SlAPXcyto and SlAPXt transcripts increased under most water stress treatments in fruits at all development stages.

This increase in SlAPXcyto and SlAPXt transcripts was not correlated with changes in APX activity, suggested a post-transcription regulation (Murshed *et al*., 2013).

That being said, a reduction in fruit yield in soluble solids is expected as a consequence of drought in tomato and this reduction would occur at a different extend in susceptible and tolerant plants.

1.4 Genetic resources and QTLs

Fruit quality traits are very complex traits controlled by Quantitative Trait Loci (QTL) including many genetic elements all potentially contributing to the expression of the trait. QTLs share high "genotype x environment" interaction affecting expression of the traits through various physiological and metabolic components. Indeed, fruit quality traits are influenced by varietal differences, the nutritional regime of plants, stage of ripening at harvest and post-harvest storage conditions. The term QTL was coined by Gelderman (1975) to denote 'a region of the genome that is associated with an effect on a quantitative trait'.

The principles and various methodologies of QTL analysis have been reviewed by various authors (Gupta, 2002; Mackay, 2004; Flint *et al.*, 2005) However, it must be stressed that all these methodologies rely on meiotic recombination among markers and detection of the recombinants in the segregating mapping populations. For such analyses, large population with accurate genotyping and phenotyping are required (Keurentjes *et al.*, 2011).

Traditionally, quantitative trait mapping studies in plants and animals involve whole genome segregating populations, but epistatic interactions in F2 or recombinant inbred lines (RILs) make it difficult to fully define and characterize individual loci. Epistatic interactions and morphological traits represent only 5 % of the whole genome.

Moreover multiple segregating quantitative traits loci (QTL) at the whole-genome level tend to mask the effects of one another by introducing high variances in statistical analyses. In sharp contrast, Introgression lines (ILs) ILs are identical for the

entire genome except for a single introgressed region, and therefore all the phenotypic variation in these lines is associated with the introduced segment.

Specifically, ILs are a set of nearly isogenic lines developed through a succession of backcrosses, where each line carries a single marker-defined chromosome segment from a divergent genome (Fig. 1.5; Zamir, 2001).

The principles of the IL approach were first demonstrated in tomato. *Solanum lycopersicum* (the domesticated tomato) is one of the 17 core species in the tomato clade. The small green-fruited desert species *Solanum pennellii* is a distant relative of *S. lycopersicum*, having evolved unique adaptations in terms of morphology, mating system, chemistry (especially secondary compounds) and responses to biotic/abiotic stress. Despite these drastic ecological differences, *S. pennellii* is sexually compatible and produces fertile hybrids with *S. lycopersicum*, making it the founding donor parent of the first IL population used for interspecific QTL identification, cloning, and plant breeding. Thus, to enhance the progress of tomato breeding, it has been developed a population of 76 segmental ILs that are composed of marker-defined genomic regions of the wild species *S. pennellii* (accession code LA716), substituting for the homologous intervals of the cultivated variety M82 (*S. lycopersicum*; the taxonomic classification of tomato in the genus *Solanum* is available in (Knapp *et al.*, 2004). The ILs that represent the entire genome partition the genetic map into 107 bins, which are defined by singular or overlapping segments (Liu *et al.*, 2003).

Consequently, these populations are very effective in identifying and stabilizing QTLs, because any phenotypic difference between an IL and the recurrent parent is attributed solely to one or more donor parent genes within the introgressed chromosomal segment (Lippman *et al.*, 2007). The use of such targeted population structures increased the identification power for QTL by several times in both plants and animals (Eshed and Zamir, 1995; Singer *et al.*, 2004).

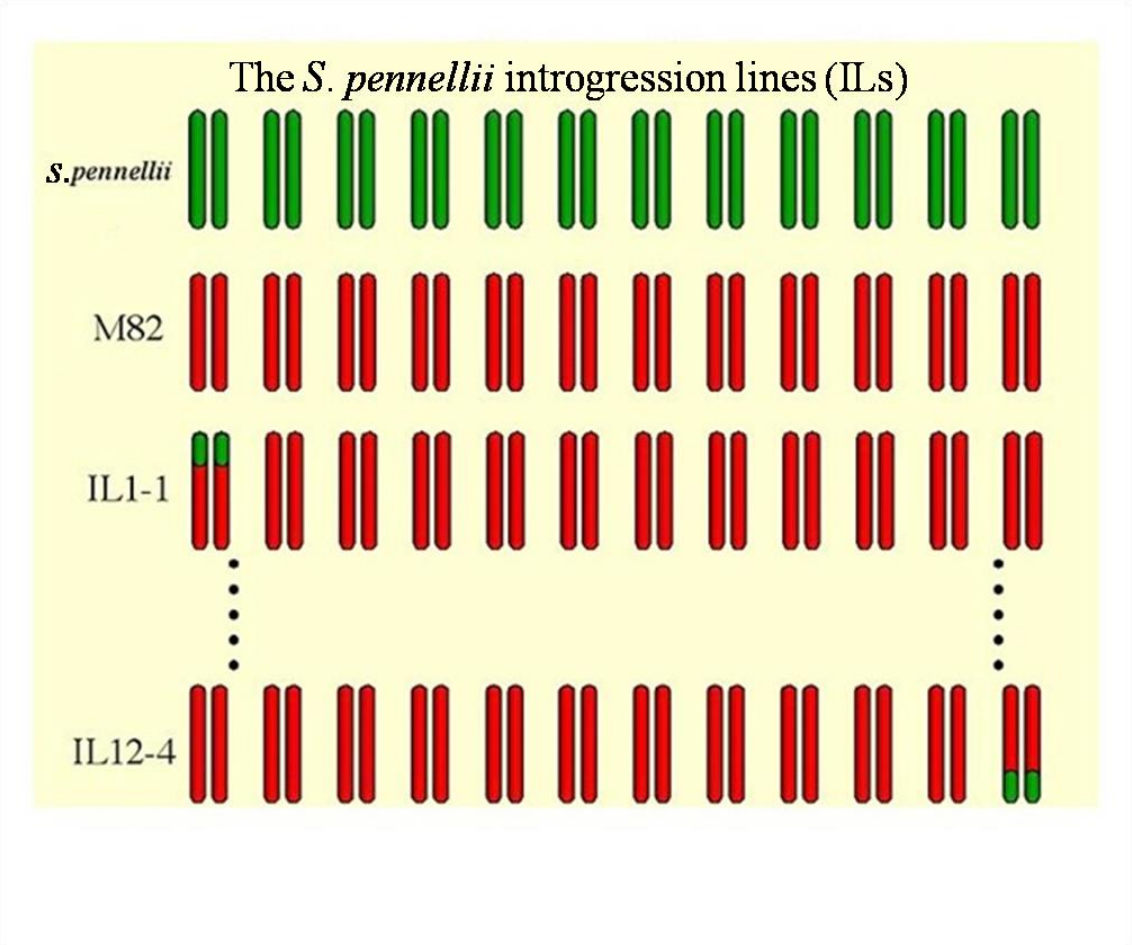

Figure 1.5 - Development of *S. pennellii* introgression lines. The ILs have been produced through successive introgression backcrossing and marker-assisted selection to generate a set of recurrent parent lines with a single homozygous introgressed segment. Sublines were obtained as the product of backcrossing selected introgressed lines with M82, so generating smaller introgressed sections. The *S. pennelli* ILs thus subdivide the tomato genome into 107"bins" (Eshed and Zamir, 1995).

1.5 Aims and scope

This thesis mainly aims at the development of new models of sustainable agriculture based on crop management procedures with reduced levels of water restitution. To achieve this general goal the research of this thesis has focused on the development of new tomato genotypes that can reconcile a reduced yield loss and an increase of the organoleptic and nutritional quality of food and derivative outcomes.

Specific objectives of this thesis are:

a) to survey the fruit quality variability within the most cultivated tomato hybrids;
b) to identify wild QTLs combining drought tolerance and higher fruit quality;
c) to assess the effects of reduced water restitution regimes in susceptible and tolerant tomato plants on both the yield, the quality of fresh fruit, the stability of the quality over post-harvest storage and the quality of the tomato sauce derivative;
d) to dissect QTLs by transcriptomic approach for the identification of candidate genes;
e) to develop markers for the QTL pyramiding in advanced tomato breeding lines.

2. Materials and methods

2.1 Comparisons among commercial processing tomatoes

In order to understand how commercial processing tomatoes perform in terms of nutritional quality, a set of cultivated hybrids were analysed.
These tomatoes, chosen for their features of productivity, disease resistance, shape and colour were kindly provided by different seed companies (Tab. 2.1).

Table 2.1 - Hybrid tomatoes cultivated in Eboli over the 2013 growing season.

Hybrid	**Seed company**	**Shape**
Gordes	Monsanto	Round
6803	Heinz	Round
5508	Heinz	Round
Impact	ISI	Round
Gong	ISI	Round
Salsero	Esasem	Round
Pietrarossa	Clause	Round
Vulcan	Nunhems	Round
Perfectpeel (PS1296)	Monsanto (Peto seed)	Round
Docet	Monsanto	Elongated
Discovery	ISI	Elongated
Smart F1	ISI	Elongated
Taylor F1	Nunhems	Elongated
Dres	Clause	Elongated

2.2 Tomato response to drought stimuli

2.2.1 Tomato breeding lines

Introgression lines (IL*s*) selected among the progenies from the initial *S. pennellii* x *S. lycopersicum* hybridization, include a set of single marker-defined introgressed genomic regions from the wild green-fruited species *S. pennellii*, native from the dry Andean plateau (Acc LA0716), in the genomic background of the cultivated *S.*

lycopersicum inbred variety M82 (Acc LA3475).

The genotypes used in this research were the tomato cv. M82 (Acc LA3475) and the IL9adm whose seeds were provided by the Tomato Genetics Resource Centre (TGRC) (http://tgrc.ucdavis.edu/), University of California (Davis, USA). The latter is a subline selected at the University of Naples, deriving from the IL9-2-5, harboring in the genomic context of the cv. M82, a homozygous DNA segment around 8 cM-long from *S. pennellii* (starting from 28 and ending to 36 cM), located on chromosome 9.

It was decided to use the IL9adm for this investigation because the parental IL9-2-5 was well-known for carrying a QTL controlling a high content in soluble solids (Fridman *et al*., 2004). Moreover, it was shown to be tolerant to drought upon we carried out experiments dedicated. The M82 represented the control.

2.2.2 Trials

Seeds exploited in all trials that took place during the summer 2013, were sown directly in the alveolar plateau containing soil and compost and the seedlings were grown under controlled condition in a suitable growth chamber (22°C with 16 h/8 h daylight/dark).

Plants of commercial hybrids were transplanted in the field in Eboli (Salerno), where they were grown in parcels, 20 plants in each one, according to an experimental design fit a randomized complete block with three replications. The plants were reared on the basis of local common agricultural practices.

Moreover, two different experiments were carried out in two next years–to analyze the response in terms of fruit yield and quality by both M82 and IL9adm under controlled conditions of water supplied over the growth cycle. The first year-trial (2013) was carried out in open field in Acerra (Naples), while the second year-trial (2014) was arranged-in tunnel at the University of Naples Federico II in Portici.

For the experiment which took place in Portici, three plants for each of both genotypes were transplanted in one out of twelve plastic lisymeters 60 centimeter sized in diameter (Fig. 2.1). Two different level of irrigation regimes were imposed on the basis of the-volume of water lost, referred to-the field capacity: restitution of a volume capable of setting the full field capacity (100%) and half of the same volume (50%).

The field capacity (FC) was estimated according to Rawl and Brakensiek (1985), as follow:

FC = 0.3486 – 0.0018 x % sand + 0.0039 x % clay + 0.0228 x % organic matter/0.67 – 0.0738 x bulk density

The water loss was estimated by measuring the reduction of water content in the soil relatively to the field capacity using the formula below:

{FC-[(FW-DW)/FW]} x (*Vol* + *d*) + *p*

Where FW represents the Fresh Weight of the soil collected 24 hours before the water supply, DW is the Dry Weight after treating the soil for 24 hours at 105 °C in the oven, *Vol* is the Volume of soil in the tank, *d* represents the soil density and *p* is the estimation of water loss within 24 hours.

Water restitution treatments were applied starting since the time when the majority of the plants showed fruit set on the first inflorescences (Fig. 2.1).

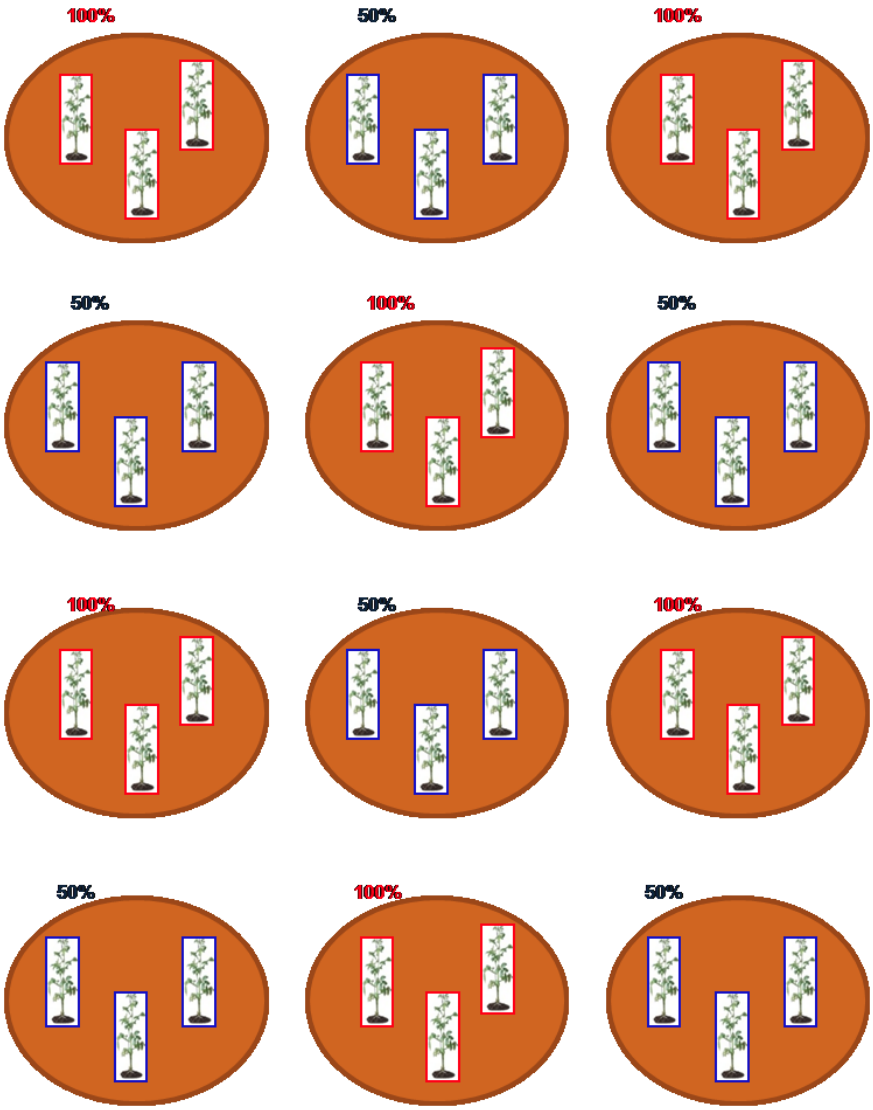

Figure 2.1 – Experimental design inherent the trial aimed to study the tomato response to controlled drought, carried out in the lisymeters in Portici during the growing season 2014.

In the experiment set up under irrigation controlled condition in open field, plots containing twenty plants each, were arranged according to a randomized complete block design with three replicates during the summer 2013 (Fig. 2.2).

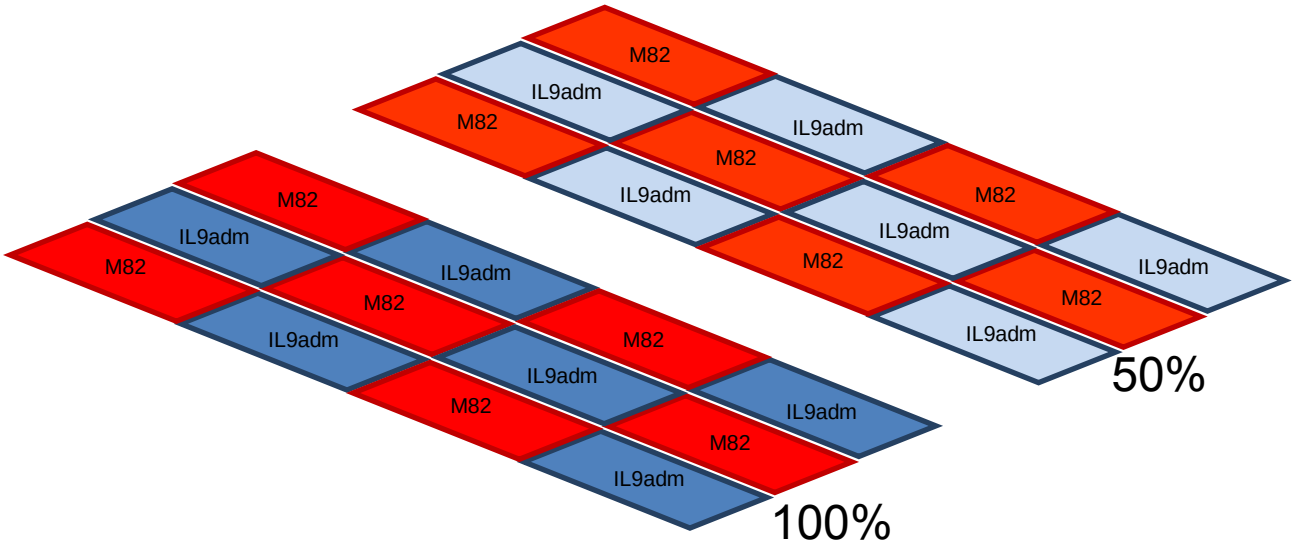

Figure 2.2 – Experimental design adopted to study the tomato response to controlled drought, carried out in open field conditions in Acerra during the growing season 2013.

Plants from both M82 and IL9adm genotypes were supplied by two different water restitution levels, the entire (100%) and half (50%) volume of evapotranspired (ET) water.

The right volume of irrigation water was calculated upon estimating the daily value of crop evapotranspiration (ET_c). It was assessed upon the determination of the potential evapotranspiration (ET_0) using the FAO standard method based (paper 56) on the Penman-Monteith (Allen *et al.*, 1998) formula, which asks for measuring a number of different meteorological parameters, such as wind speed at 2 meters above the ground surface, the temperature, solar radiation and others, all evaluated as daily mean values, monitored by a "watch dog" weather Station (Weather Vantage Pro2^{TM}, Davis).

It was possible to calculate the ET_c by using the equation $ET_c = k\ ET_0$, where k is a suitable crop coefficient whose value for the tomato crop during the stage of fruit development and set is estimated to be around 1.

The differential irrigation treatment was set out when the fruit set occurred among the majority of the plants grown (Fig. 2.3).

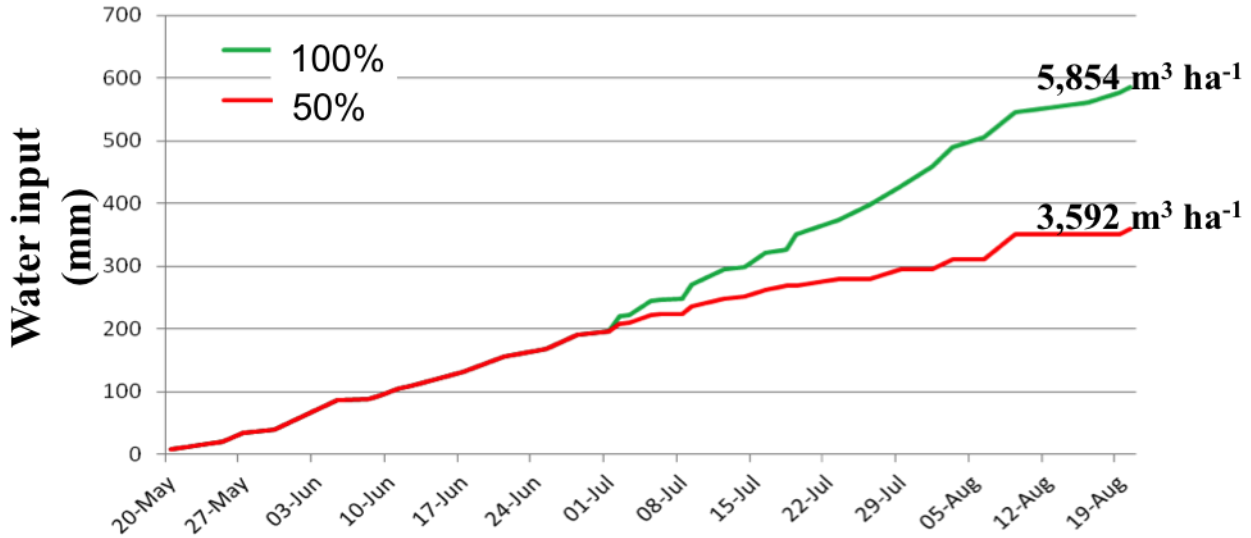

Figure 2.3 – The differential water restitution started since fruit set stage in Acerra experimental plots (2013). The volume of irrigation water was calculated upon estimating the ET_0 by a "Watch-dog" weather station.

For all the three experiments, fruits were harvested at the red ripe stage. Intact fruit were used for measuring both the firmness and the content in soluble solids.

Then, five to ten of whole fruits were longitudinally cut, seeds and locular jelly removed, and flesh frozen by immersion in liquid nitrogen.

Frozen flesh bits were ground through a laboratory WARUING® Commercial blender. Resulted powder was frozen in liquid nitrogen and stored at -80 °C until starting the molecular and biochemical analyses.

Total RNA from both M82 and IL9adm genotypes grown under the two water supply regimes during the two next growing seasons were purified, and a functional genomic analysis was carried out exploiting the microarray method to detect the set of genes responsive to drought in two different genetic background.

For that purpose three different biological replicates were taken into account for each of both genotypes undergoing the two water treatments and fruits were processed according to method above described before extracting total RNA.

2.3 Morphological and physiological analyses

In order to compare relevant aerial and hypogeal traits of plants from both M82 and IL9adm with the main purpose to get a fine morpho-physiological characterization for them, different assays were conducted.

In particular, on plants grown under water deficit and normal irrigation conditions, a desiccation test were performed to analyze the water loss rate, while the actual relative content of water (RWC) was measured in the leaves.

A root screening based on biometrical evaluations, was carried out on 45-day plants at flowering stage, grown in a hydroponic system.

2.3.1 Desiccation test

Desiccation trials were carried out on a representative number of plants. Water Loss Rate (WLR) in a short and a long period and Relative Water Content (RWC) were evaluated. Plants having similar leaf size were selected within each genotype and used for dehydration treatment; well-watered seedlings of the same genotypes were collected and used as control. Leaves were collected periodically and Fresh weight (FW) was immediately recorded (time 0). Then they were placed on dry filter paper for 24 h at 4°C and the weight (W 24) was measured again. Total dry weight (DW) was recorded after drying for 24 h at 80° C. The water loss rate for the long period was measured as described by Suprunova *et al.* (2004) using the formula:

$WLR\ (g\ h^{-1}g^{-1}DW) = (FW\text{-}W_{24})/(DW\ X\ 24)$.

While for the short period, WLR was calculated for each time point using the equation:

$WLR\ (g\ h^{-1}g^{-1}\ DW) = [(F_{Tx} - F_{Tx+2})\ X\ 60]\ /\ [DW\ X\ (T_{x+2}\text{-}T_x)]$.

The time [T_x (min); x=0] at which the blades of fully expanded first leaves were first weighed was considered as 0 min (T_0=0 min). The leaf blades were weighed five times. The time of each measurement (min) was recorded as time T_x where x = 2, 4, 6 and 8 (every 2 h), 2 indicating the time of the first measurement and 8 indicating the

time of the last measurement. Total dry weight (DW) was recorded after drying for 24 h at 80° C.

2.3.2 Relative water content survey

The RWC was measured according to Suprunova *et al*. (2004) using the formula:
RWC (%) = [(W-DW) / (TW-DW)] x 100
Where, W represents the sample fresh weight, TW is the sample turgid weight after 24 h socked under water and DW is the sample dry weight after 24 h at 80°C in the oven.

2.3.3 Reliefs on root traits

Three plants for each of both M82 and IL9adm genotypes, were grown in a tank filled with a mineral nutrient water solution (Fig. 2.4). Plants floated on water surface due to perlite stones which allow us to extract integral roots. Among the diverse traits, the length of taproot was measured and first order roots were counted. The root biomass was also evaluated.

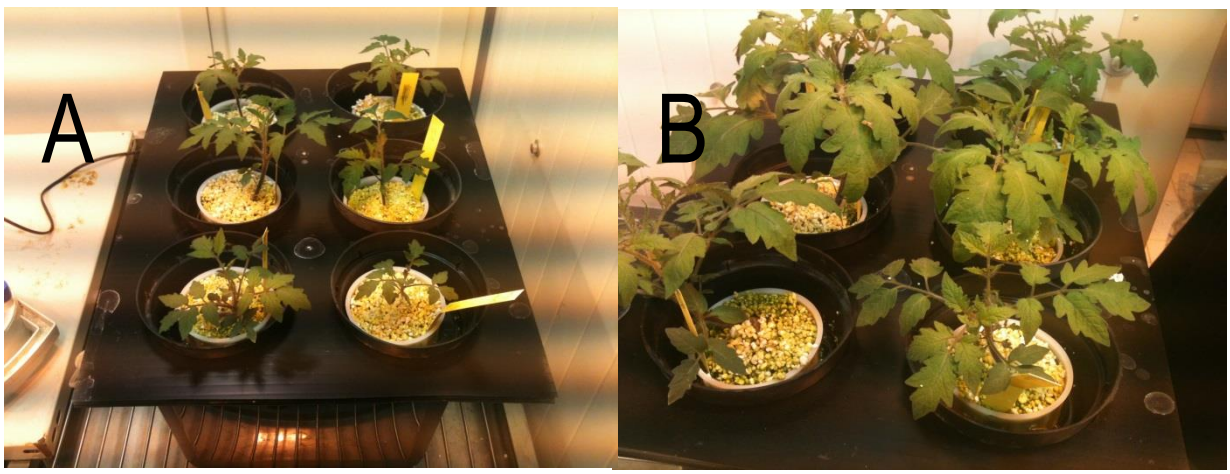

Figure 2.3 – Hydroponic system set up to measure different root traits in both M82 and IL9adm plantlets 15 (A) (A) and 30 days old (B).

2.4 Physical and chemical analyses on fruits and leaves

2.4.1 Content in soluble solids

The mesocarp from ripe fruit was squeezed and the content in soluble solids of the resulting juice measured on a refractometer ATAGO Model ATC-1.
The contents in soluble solids were expressed on a °Brix scale. For each genotype the average of 10-20 measures was considered.

2.4.2 Fruit firmness

The firmness value of tomatoes was determined by using a T.R. TURIONI penetrometer. A probe was applied at as many as possible points on the fruit equator, the displacement of the compressing probe on the fruit was recorded in terms of pressure (N mm^{-2}), and the average of 10-20 measures were used for each genotype.

2.4.3 Dry matter

Most of the results of our analyses were obtained on a dry weight basis. To this purpose 10 grams (three replicates) of fruit powder were weighed and get dried in the oven at 85 °C for 24 hours. The dry matter weight was estimated using the formula:
% DM= (C-T)/B-T x 100, where, "T" is the tare, "B" represents the tare + the fresh weight and "C" the tare + the dry weight.
The tomato humidity was evaluated as difference: % H_2O = 100 – DM

2.4.4 Specific gravity

The specific gravity was measured by putting tomato fruits in a graduated cylinder filled with a known level of water. Then we registered the water level variation which was equal to the tomato volume. The tomato weight was previously measured.

2.4.5 Colorimetric assay

Sauces were produced from mature fruits in collaboration with the Experimental Station for the Preserved Food (SSICA) located in Angri (Salerno) through exploiting a pivotal processing system.
Color was checked from the sauces using a Minolta apparatus giving tristimulus coordinates *L**, *a**, and *b** at SSICA laboratories.

2.4.6 Assay on hydrogen peroxide content

The level of hydrogen peroxide in the fruits, the related processed products and leaves was measured according to Baptista *et al*. (2001).
A volume of 0.75 ml of 0.1% w/v TCA was added to 0.25 g of ground sample and homogenized by using mortar and pestle.
The mix was transferred to a 1.5-ml eppendorf tube and incubated 10 min on ice.
Samples were next centrifuged (15,000 x *g* for 15 min at 4°C) and an aliquot of 0.5 ml from the supernatant was added to 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1.0 ml of 1 M KI.
After a gentle mix, the absorbance was read for 1 min at 405 nm wavelenght using 250 µl of the reaction mixture by a proper spectrophotometer (Victor).
In order to quantify the H_2O_2 content a calibration curve ($R^2 > 0.99$) was taking into account using solutions with H_2O_2 aliquots whose concentrations were known.

Results were expressed as μmol H_2O_2 g^{-1} fresh weight (FW). The blank probe consisted of 0.1% w/v TCA in the absence of tissue extract.

2.4.7 Assay on ascorbic acid content

Ascorbic acid (AsA) levels were measured using the procedure described by Kampfenkel *et al*. (1995) and modified according to Di Matteo (2010). Frozen tissue (250 mg) was placed in a 1.5-ml tube with a bead and 200 μL of cold 6% trichloroacetic acid (TCA) (Sigma), and was homogenized for two times at 50 Hz in a TissueLyzer (Qiagen) for 1 min. Samples were then incubated on ice for 10 min and centrifuged for 25 min at 25,000 × *g* at 4°C. A proper volume of 6% TCA was added to the supernatant up to a total volume of 500 μl, and samples centrifuged as above for 10 min.

Two different assays were performed to evaluate the content of both total (tot AsA) and reduced (AsA) ascorbic acid. The absorbance was read at 525 nm using water as reference with a UV spectrophotometer (Beckman DU-640).

For determining tot AsA, three different replicates of a volume of 20 μl of each sample were placed in three corresponding wells in a 96-well plate. To reduce oxidized form a volume of 20 μl of 5 mM DTT (diluted in 0.4 M Phosphate buffer, pH 7.4) was added. Samples were incubated for 20 min at 37 °C. A volume of 10 μl of N-ethyl maleimide (0.5% v/v NEM) was added to the reaction that subsequently was mixed and kept for 1 min at room temperature. Then 80 μl of color reaction were added to each sample and an incubation 40 min-long took place at 37 °C.

The coloring solution was obtained by adding 2.75 part of A solution and 1 part of B solution. The A solution was made of 31% orthophosforic acid (H_3PO_4), 46% v/v TCA, 0.6% v/v ferric chloride, while the B solution contained 4% 2,2-dipyridyl diluted in 70% ethanol.

To ascertain the content of AsA, a volume of 20 μl of sample was examined by three different replicates. A volume of 20 μl of phosphate buffer (pH 7.4) and 10 μl of water were added to each sample. Subsequently, 80 μl of color reaction (composed as

previously described) were added to samples and incubated for 40 min at 37 °C. Both total and reduced AsA concentrations were expressed in terms of mg per 100 g of FW, upon calculating standard curves designed over a dynamic range from 0 and 100 nmol AsA. The equations used for calculating both total and reduced AsA content were, Y (Abs) = 0.0118*x* - 0.0222 and Y (Abs) = 0.0115x - 0.023, respectively (R^2 > 0.99).

2.4.8 Assay on content in total phenolics

The amount of total phenolic compounds was determined according to Folin-Ciocalteu's procedure (Singleton and Rossi, 1965). A volume of 1 ml of 60% methanol was added to 250 mg of ground tissue in a 1.5 ml-tube containing a bead. Samples were homogenized twice in a Tissue Lyzer (Qiagen) at 50 Hz for 1 min. Extracts were next placed on ice for 3 min in the dark and vortexed. The extract was transferred in a 15 ml tube and a final volume of 5 ml was set by adding 60% methanol. Samples were centrifuged at 3,000 x *g* for 5 min. An aliquot of 62.5 µl of the supernatant was mixed to 62.5 µl of Folin-Ciocalteu's reagent (Sigma), and 250 µl of deionized water and the resulting mix incubated for 6 min.

After addition of 62.5 µl of 7.5% sodium carbonate and 500 µl of deionized water, samples were incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm. The total phenolics concentration was expressed in terms of µg of gallic acid equivalents per mg of FW, based on a gallic acid standard curve designed over a dynamic range from 0 to 125 nmol, by exploiting the equation Y (Abs) = 0.0234*x*– 0.0776 (R^2 > 0.99).

2.4.9 Assay on flavonoid content

Flavonoids were extracted and analyzed as previously described by Zhishen and co-authors (1999) with slight modifications. In particular, 5 ml of 80% methanol were added to 0.5 g of fresh, powdered tissue in dark conditions.

The mix was homogenized by using mortar and pestle, transferred to a 15 ml-tube and undergone to sonification for 20 min. Samples were then vortexed and stored 5 min on ice in the dark.

A volume of 2 ml of the extract were taken and centrifuged for 5 min at 14,000 rpm in a 4 °C pre-cooled centrifuge.

An aliquot of 100 µl was collected and analysed in triplicate. For that goal, a volume of 400 µl of deionized water and subsequently 30 µl of 5% $NaNO_2$ were added. Afterwards, the mixture was incubated for 5 min.

Thereafter, a volume of 30 µl of 10% $AlCl_3$ was added and the mixture incubated for 6 min. Finally, a volume of 200 µl of 1 M NaOH and 240 µl of deionized water were added and the mix absorbance was measured in the Victor spectrophotometer at 490 nm wavelength. Total flavonoid content was expressed as mg of quercetin equivalents (QE) per 100 g FW, upon creating a suitable set of standard curves (R^2 > 0.99) linking the absorbance and the concentrations of quercetin.

2.4.10 Analysis of carotenoids by High Performance Liquid Chromatography (HPLC)

About 0.1 g of freeze-dried fruit tissue were weighed and homogenized in liquid nitrogen. A tiny spatula spoon of $CaCO_3$ and the same amount of PVPP were mixed to the sample. It was extracted with 1 ml of pure acetone and transferred in a 1.5 ml-tube. It was centrifuged 5 min, 13,000 rpm at 4°C. This operation was repeated once more with the same volume of acetone and the supernatant was collected and stored at –20 °C until required. Samples were centrifuged and filtered before the injection in the HPLC. Carotenoid pigments were analyzed by RP-HPLC using ternary gradient elution and a Symmetry C_{18} column (4.6 x 150 mm I.D., 3.5µm) from Waters. The

chromatography system was equipped with a Shimadzu LC-10AT VP solvent delivery system and SPD-M 10° VP photodiode array detector (DAD). The mobile phase consisted of acetonitrile:methanol:ethyl acetate containing 0.05% triethylamine flowing at 0.8 ml/min. A gradient was applied from 88:8:4 to 48:26:26 in 25 min, and back to the initial condition (30 min). Volume injection was 20 µl. Detection was at the wavelengths of maximum absorption (max plot).

2.4.11 HPLC with UV detection coupled with electrospray ionization tandem mass spectrometry (MS/MS) assays

An amount of freeze-dried fruit tissue ranging from 0.1 to 0.15 g was ground with liquid nitrogen. Then, samples were homogenized with 1 ml of 100% methanol and transferred into a 1.5 ml-tube. After the centrifugation (5 min, 13,000 rpm at 4°C) the supernatant was poured in an amber eppendorf and the volume recorded. Thereafter, the supernatant was stored at -20 °C until required (at most for one week).
Samples were previously filtered and then loaded into the HPLC/UV- ESI MS MS analysis system (Shimadzu Prominence LC-20AD with a detector UV/VIS coupled with a mass spectrometer Applied Biosystems/MDS Sciex 3200 Qtrap equipped with a source of ionization for the Electrospray Turbo VTM). Table 2.2 shows the HPLC gradient imposed in the experiment, while Tab. 2.3 illustrates the source condition.

Table 2.2 - HPLC gradient for phenolic compounds analysis.

Time (min.)	Conc. A(%)	Conc. B(%)
0.10	95	5
10.0	95	5
40.0	5	95
50.0	5	95
55.0	95	5
60.0	95	5

Table 2.3 - Conditions exploited for the source.

Cur gas	20 psi
CAD gas	Medium
Gas 1	60 psi
Gas 2	30 psi
Ionization voltage	4500 V (positive polarization) -4500 V (negative polarization)
Temperature	380 °C

2.4.12 Assay on the antioxidant activity: ORAC

With the aim to evaluate the total antioxidant activity extant in tomato tissues, an assay based on the Oxygen Radical Absorbance Capacity (ORAC) was performed. Upon weighing and homogenizing 50 mg of freeze-dried tomato fruit, a volume of 500 µl of 80% methanol was added and the mixture incubated for 1 h at room temperature.

The preparations of reactives were made abiding by the following protocols:

Phosphate buffer pH 7.4

It was composed by two different A and B phosphate salts:

A. 13.064 g/100 ml (0.75 M) K_2HPO_4
B. 8.999 g/100 ml (0.75 M) NaH_2PO_4

Fluorescein (87 µM) (Fluorescein sodium salt - Sigma)

A quantity of 1.64 mg of fluorescein was dissolved into a final volume of 50 ml using phosphate buffer pH 7.4. Then, it was poured in an eppendorf tube wrapped in aluminium paper at 4 °C.

Trolox standard

A stock solution concentrated 1,000 mg/l was prepared in 95 % ethanol. A series of dilution were prepared.

A volume amounting to 100 µl of the samples was added into the wells in triplicate (the same as the blank).

Fluorescein was added in the injector 1 and the reactive AAPH 35 mM was added in the injector 2.

All the reactives needed to stay at 37 °C for the analysis. The experiment was designed using the software Gen5.
The Orac activity was referred in terms of trolox equivalent.

2.5 Shelf life

An experiment focused on assessing the quality extent upon harvesting and during the conservation time, better known as "shelf life", was carried out exploiting the mature fruits harvested at the red ripe stage from the open field trial hold in Acerra experimental plot during the growing season 2013, in order to figure out how a reduced restitution of irrigation water would affect the fruit quality components.
A number of fruits from both M82 and IL9adm were stored in a glass jar covered with a transpirant film at 20 °C.
Every week, fruits were carefully weighed with the aim to evaluate the loss in fresh weight and the set of fruits displaying a low firmness were discarded. Once that the weight was recorded, fruit were cleaned with a water bleach solution to avoid rots.

2.6 Tomato breeding

2.6.1 Plant material

With the goals to set up new tomato lines getting QTL*s* (Quantitative Trait Loci) from the wild *S. pennellii* capable of ameliorating both drought tolerance and fruit nutritional quality traits, four different advanced tomato lines, P011_156, P011_58, P011_85 and P011_77 (Fig. 2.4) were used as parents in a breeding program involving the IL9adm as source of the wild QTL*s*.
The above mentioned advanced breeding lines were selected at the University of Naples "Federico II" because of in agronomic trials carried out over different

growing seasons exhibited good agronomic performances. Furthermore, the mentioned tomato line displayed to be superior in comparison to several commercial tomato varieties in other important traits, such as amount of lycopene, firmness, yield and resistance to pathogens.

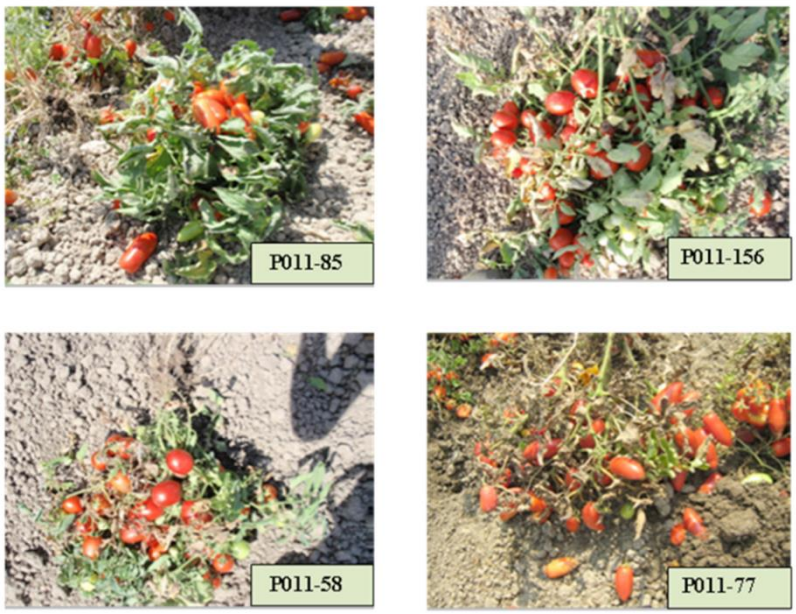

Figure 2.4 – Advanced breeding lines exploited as recurrent parents in a back-cross breeding scheme for transferring dissected superior QTL*s* from the wild *S. pennellii* in the tomato genetic background.

2.6.2 MAS based-breeding activities

The mentioned advanced tomato breeding lines (TBL*s*) were used as recurrent parents (acceptors) in a back-cross breeding (BC) scheme where IL9adm represented the initial parental donor of the wild QTL*s* (Fig. 2.5).

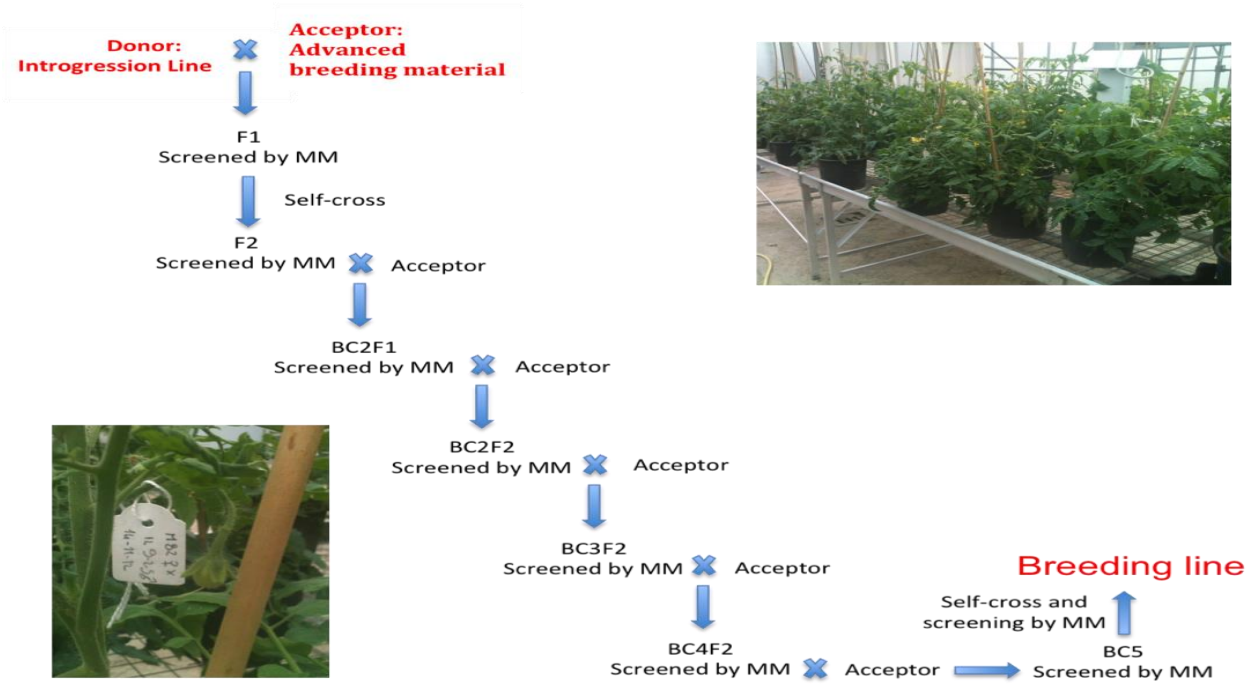

Figure 2.5 – A breeding program based on a back-cross scheme was set off implying the IL9adm as donor parent for wild dissected superior QTL*s* and four different advanced tomato breeding lines (P011_58, P011_77, P011_85 and P011_156) as acceptors for those QTL*s*.

According to a BC scheme, upon the initial cross IL9adm x TBL, the resulting F_1 plant were permitted to come to selfing producing F_2 plants. Starting from this F_2 generation, the plants of interest containing remarkable QTL*s* are used to pollinate cyclically the recurrent parent over the next BC generations with the double aims to establish in the genetic background of the acceptor line the desired QTL*s* and to increasingly reduce the segment of initial wild introgression. To finely map the alien QTL*s* within the introgression 9adm and drive the selection of desired genotypes taking those QTL*s* through exploiting a MAS (Marker Assisted Selection) approach, CAPS (Cleaved Amplified Polymorphic Sequences) markers have been developed spanning the wild introgression at intervals of around 1 cM, being able to give reliable DNA amplicons differently sized evidencing polymorphism between wild and cultivated genotypes. In order to detect primer pairs capable of displaying CAPS polymorphism, PCR amplicons from both the advanced tomato breeding lines and IL9adm were sequenced, and restriction enzymes discriminating the two different parental lines were selected, upon identifying either SNP (single nucleotide Polymorphism)- or chromosomic-based polymorphisms occurring in the restriction

sites. We designed six different primer pairs (Tab. 2.4 and Tab. 2.5) mapping throughout the 9adm genomic region. These markers were used to identify hybrid F_1 genotypes (harbouring alleles from both parents) as well as genotypes belonging to the different next BC generations getting the wild QTL*s*. Instead, plants without any wild introgression were not selected.

Table 2.4 – List of CAPS markers used in the MAS-breeding capable of distinguishing wild and cultivated QTL*s*.

Marker	Amplicon length (bp)	
	M82	IL9adm
TG223	1,200	1,200
TC217592	1,163	1,156
TC238140	1,026	1,036
DB708941	1,344	1,293
TC235285	1,172	1,184
TC227407	1,000	1,000

Seedlings were transplanted in 20 cm diameter pots and grow up in a cold greenhouse.

Leaves were collected from one month old plantlets and DNA extracted by using the "DNA plant mini kit" (Qiagen) according to manufacturer's instructions. The DNA was exploited as template for CAPS analyses and detect the plants harbouring the alien QTL*s*.

At each BC cycle, flowers of plants from selected recurrent breeding lines were emasculated at the upcoming flowering stage by removing stamen. Then, the isolated stigma were artificially pollinated by wiping grain pollen dust collected from donor genotypes on their surface.

2.7 Molecular analysis

2.7.1 RNA purification

Total RNA was isolated from homogenized powdered tomato flesh fruit stored at -80 °C according to Griffiths and co-workers' procedure (1999). An amount of 4 g of frozen powder were added to 12 ml of Extraction Buffer (Solution A) and an equal volume of phenol/chloroform (Solution B) in an Oakridge tube. Tubes were vigorously shaken and then centrifuged at 10,000 rpm (15 min at RT). The supernatant was recovered, added to 15 ml of Solution B in a clean Oakridge tube, vigorously shaken and the mix centrifuged at 10,000 rpm (15 min at RT). Hence, a volume of 11 ml of the aqueous phase was transferred to a clean Oakridge tube and the nucleic acid was precipitated for 1 h at -20°C with the addition of 27.5 ml of ice-cold pure ethanol and 1.1 ml of 3 M sodium acetate (pH 6.0). RNA was next pelletted by centrifuging at 10,000 rpm (15 min, 4 °C) and the pellet washed in 70% ethanol. Upon dissolving the pellets in 2 ml of DEPC-treated water by vortexing the tubes, 2 ml of 2X cetyltrimethylammonium bromide (CTAB) Extraction Buffer (Solution C) and 4 ml of CTAB Precipitation Buffer (Solution D) were added and the mixture centrifuged at 12,000 rpm (30 min at RT). The resulting pellet was re-suspended in 400 ml of 1.4 M NaCl and precipitated again at -20 °C (for 1 h to over-night) by the addition of 1 ml of ice-cold pure ethanol. The samples were then centrifuged at max speed (10 min, 4 °C), the pellets washed in 500 ml of 70% ethanol, re-dissolved in 400 ml of DEPC-treated water and incubated at 50 °C for 5 min. The RNA was next extracted twice by adding a volume of 400 ml of phenol/chloroform (Solution B). Thereafter, the nucleic acid was precipitated in 0.1 volume of 3 M sodium acetate (pH 6.0) and 3 volumes of ice-cold 100% ethanol at -20 °C (for 1 h to over-night). The samples were washed with 500 ml of 70% ethanol. The pellet was re-dissolved in 180 µl of DEPC-treated water and the remaining DNA was eliminated by treating the specimen with 1 µl of RQ1 DNase (Promega), working in 20 µl of RQ1 DNase Reaction Buffer (Solution E). The mixture was incubated at 37 °C for 30 min.

The purity of the RNA was determined using a Bio Rad SmartSpec 3000

spectrophotometer and integrity was confirmed using the AGILENT 2100 Nano Bioanalyzer with RNA 6000 nano chip.

RNA Extraction Buffer (Solution A)

6% (w/v) 4-aminosalicylic acid

1% (w/v) 1,5-naphthalenedisulphonic acid

50mM Tris-HCl pH 8.3

5% (w/v) Phenol Solution

Phenol Solution

100 g Phenol crystals

14 ml m-cresol

0.1 g 8-hydroxy-quinoline

30 ml DEPC-treated water

Phenol/Chloroform Solution (Solution B)

500 g Phenol crystals

0.5 g 8-hydroxy-quinoline

500 ml Chloroform

20 ml Iso-amyl alcohol

200 ml 100mM Tris-HCl pH 8.0

2X CTAB Extraction Buffer (Solution C)

1.4 M NaCl

2% (w/v) CTAB

0.1 M Tris-HCl pH 8.0

20 mM EDTA pH 8.0

CTAB Precipitation Buffer (Solution D)

1% CTAB (w/v)

50 mM Tris-HCl pH 8.0

10 mM EDTA pH 8.0

10X RQ1 DNase Reaction Buffer (Solution E)

400 mM Tris-HCl pH 8.0

100 mM $MgSO_4$

10 mM $CaCl_2$

2.7.2 ***DNA purification***

DNA extraction was performed starting by leaf tissue samples collected from seedlings 20-30 day old and frozen in liquid nitrogen, using the commercial kit DNeasy ® Plant Mini Kit (QIAGEN) according to manufacturer's instruction. Briefly, 80-100 mg of powdered tissue were homogenized with 400 µl of AP1 lysis buffer and 4 µl of RNase A in a 1.5 ml-microfuge tube. The resulting mixture was shaken and incubated for 25 min at 65 °C.

At the end of incubation, a volume of 130 µl of extraction buffer P3 was mixed to the initial mixture by pipetting up and down and upon incubating the final mix on ice for 15 min, a centrifugation 7 min-long took place at max speed and RT.

The supernatant was recovered and loaded onto a QIA shredder filtering column and centrifuged again for 2 min at max speed and RT.

Afterwards, the flow-through were moved into a new 1.5 ml-microfuge tube and the DNA precipitated by mixing the aqueous phase with 1.5 vol. of Washing buffer I.

The solution was next loaded onto a silica column able to hold the DNA. A centrifugation 1 min-long taking place at 8,000 x *g* and RT allowed to clean the DNA from remaining contaminants. The silica cartridge was next washed twice with 500 µl of Washing buffer II.

Eventually, the DNA was eluted in a final volume of 100 µL of elution buffer by centrifuging the silica cartridge at 8,000 x *g* and RT and evaluated in terms concentration and purity by using a spectrophotometer (Implen).

2.7.3 Sequencing DNA polymorphic regions for MAS based-breeding

DNA amplification was carried out by PCR using specific primer pairs designed on several gene sequences spanned throughout DNA region corresponding to the 9adm introgression on tomato chromosome 9 and deposited in GenBank and DFCI. PCR products were purified by the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. Amplified DNA fragments were sequenced using the BigDye® Terminator Cycle Sequencing Kit v.3.1 based on the Sanger method. Each

sequencing reaction took place in a final volume of 10 μl using 20 ng of amplified and purified DNA, 1.5 μl of BigDye buffer (containing gligerine and TRIS base), 1 μl of 3.3 pmol/μl unique primer, 1 μl of Ready Reaction 2.5X BigDye and a proper volume of mQ sterilized water.

Upon an initial DNA denaturation at 95 °C 5 min-long, 35 amplification cycles came as follow: 95 °C for 30 sec, 50 °C for 10 sec and 60 °C for 4 min with final temperature decrease to 0 °C. The reactions were carried out into a Verity thermocycler (Life Technologies)

Thereafter, samples were purified using Big Dye® XTerminator TM Purification Kit Applied Biosystems. The products of the sequencing reaction were loaded on a capillary sequencer ABI PRISM 3130 Genetic Analyzer with a length of 36 cm, using POP-7 Polymer and the sequences were elaborated by the related Sequencing Analysis v2.0 instrumental software.

The sequence analysis aimed to detect useful polymorphism was performed by the use of SeqScape v2.1 software (Life Technologies). Finally the detection of polymorphic restriction sites was made by exploiting the Webcutter tool from the Internet (http://rna.lundberg.gu.se/cutter2/).

2.7.4 CAPS analysis

An amount of 50 ng of DNA template was amplified by PCR in a final volume of 100 μl consisting in 20 μl of 5X Reaction buffer, 16 μl of 1.25 mM dNTPs, 5 μl of both forward and reverse primers and 0.5 of 5U μl^{-1} Taq DNA polymerase (GO Taq, Promega).

Upon denaturing DNA at 95 °C for 3 min, 30 amplification cycles were carried out according to the temperature profile over the time: 95 °C for 1 min, T_a (Tab. 2.5) for 30 sec and 72 °C for 30 sec with a final temperature decrease to 4 °C. The reactions were carried out into a Verity thermocycler (Life Technologies).

Table 2.5 - Details of CAPS marker primer pairs used in the MAS based-breeding program.

Marker	Chromosome SL2.40	Forward	Reverse	Ta (°C)
TG223	2779001-2781500	GGGTCGTAAACTCGCACATT	CTTGCAATCCTGCTTCTTCC	52
TC217592	2837901-2840400	GAGTCGGAGCAAGACCACTC	CGCGGGAATAGAAGTATGGA	50
TC238140	2931165-2930896	AAGCAGCAGCACTACAAGCA	ATTGTGAGGACCGACACACA	55
DB708941	3068101-3070600	TCCCTAGTGGCAAATCTTGG	CGCGGCTATGAGAAATGAAT	55
TC235285	3271995-3270698	GTAGCATGCCTCAAGCACAA	GATGCTGGCCTTGGTACATT	55
TC227407	3350429-3351298	AGCTCTCCGCGTAAATTCAA	ATCGTTTCACGAACGAATCC	55
TC217240	3477087 - 3479900	TGATCCTAGATGGGCTGACC	TCCAGGATTCGTCTCTGCTT	57
TC225638	3516931 - 3517854	TGATTTTAGAGCCCCTGTGC	TCATGATCTCCATTGCCAAA	55
TC188748	3680901 - 3681807	TTACATACACACGGCAACACA	TCTGGGAGAAGGGAGAGAAA	57
TC230608	3714701 - 3717900	GGAACTGCTCAGCCAACTTC	TCGTGCCTGTAAAACTGTGC	57

The products from PCR were next digested by exploiting one of the different restriction enzymes selected whose recognized sites was detected to be polymorphic. The final reaction volume was 20 μl containing the specific restriction enzyme (1 μl), the related 10X buffer (2 μl) and 15 μl of PCR product. The reaction lasted 2-3 h at the enzyme optimal temperature.

The products of digestions were run in a 1.0% agarose gel electrophoretic dyed with the SYBR Safe (Life Technologies) fluorescent color and visualized by the GelDoc (BioRad) acquisition image.

2.7.5 Microarray analysis

Chip TomatoArray1.0 was synthesized on CombiMatrix platform at University of Verona. This chip had 90k silicon electrodes that support 20,200 DNA probes synthesized *in situ* with 4 replicates, each randomly scattered in the array to evaluate variability of the experiment. Each probe was composed of oligonucleotides, made up of 30-35 nucleotides, designed to be specific for the different Tentative Consensus (TC) tomato sequences from TIGR (The Institute for Genomic Research) *S. lycopersicum* Gene Index Release 11.0 (2006). As negative controls, 9 probes provided by CombiMatrix were exploited.

Three replicates of hybridization were done for both M82 and IL9adm in the two experimental conditions of complete and half restitution of water lost, adding up a total of twelve hybridizations. In particular, each biological replication exploited total RNA isolated from fruits that came from a single plant. After hybridization and washing, the microarray platform was dipped in imaging solution, covered with glassy LifterSlip™, and then scanned using a ScanArray 4000XL (Perkin Elmer) and the accompanying acquisition software (ScanArray Express Microarray Analysis System v4.0). The resulting TIFF images were processed to extract raw data using the CombiMatrix Microarray Imager Software v5.8.0. Signal probe medians and standard deviations were imported into the SPSS software (IBM), and normalization was achieved by correcting each probe median based on the ratio between the median of the array and the average median of arrays. Following normalization and quality control, data were log_2 transformed. Finally, probe signals with a variability coefficient higher then 0.5 as well as spikes and factory probes were filtered out. Also, probes with signal intensities in the upper most and lower most 10% of values were eliminated.

2.7.5.1 Statistical and bioinformatic analyses

The signal differentially expressed between the two experimental condition of full and partial water restitution in each genotype, were identified by using a Student's t-test built in TMEV (TIGR Multiple Experiment Viewer, Saeed *et al*., 2003) software (version 4.4.0). In particular to compare transcriptional profile showed by each genotype in both water regimes, a significance threshold of $P< 0.05$ was chosen coupled with a 1,000 re-sampling based-bootstrap (Tusher et al., 2001).

Tentative Consensus sequences that showed a different expression profile were utilized as input in Blast2GO bioinformatic tool (Conesa *et al*., 2005) in order to get an automatic high-throughput annotation, gene ontology mapping and categorization of TC*s*.

To analyze the co-regulation among signals differentially expressed a hierarchical clustering was generated by using the CoExpression software, using a Pearson correlation with a threshold of 95%.

2.7.5.2 Experimental validation by RT-qPCR

TC expression profiles of genes considered to be key in controlling the major steps for AsA accumulation in tomato fruit (*GDP-mannose pyrophosphorolase*, *Vtc2*, *Modehydroascorbate reductase* and *Dehydroascorbate reductase*) in response to drought stress were validated by real-time quantitative RT-PCR (Tab 2.6) in a 7900HT Fast Real-Time PCR System (Applied Biosystems).

Amplification was performed in 12.5-μL reaction volume starting from the cDNA synthesized from total RNA using the SuperScript III H-Reverse Transcriptase (Life Technologies) in a Power SYBR® Green PCR Master Mix (Applied Biosystems). Relative quantification was achieved by the ΔΔCt method (Livak *et al*. 2001). Primer pairs were designated using Primer Express software (v 2.0).

Primer pairs were validated using a standard curve over a suitable dilution range (R^2>0.98 with a slope close to -3.3). For each TC*s* three biological replicates for

treatments were considered and for each experiment three technical replicates were performed.

As calibrator was used M82 at 100% water treatment and as internal control endogenous gene Elongation Factor 1 (*EF1*).

PCR primers were designed using the Primer3 Plus software available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/.

Sequences for RT-qPCR primers were retrieved from the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com) if available, or alternatively designed *ad hoc* by using Primer express software version 3.0 (Applied Biosystems). Sequences were processed by ABI PRISM SeqScape software.

3 Results

3.1 Field screening of tomato hybrids for fruit quality

Hybrid tomatoes expressed an average level of total soluble solids of 4.43 ± 0.19 (mean ± standard error) °Brix, ranging between 4.05 ± 0.05 for Gordes and 4.70 ± 0.11 for Perfect Peel (Fig. 3.1). The post hoc Duncan's test revealed five homogeneous subgroups. In particular, the subgroup with the highest fruit SSC mean includes Perfect Peel, Heinz 6803, Discovery, Docet, Gong, Pietrarossa, Smart, Salsero and Dres whereas the subgroup with the lowest SSC mean level includes 5508, Gong, Gordes, Impact, Taylor and Vulcan.

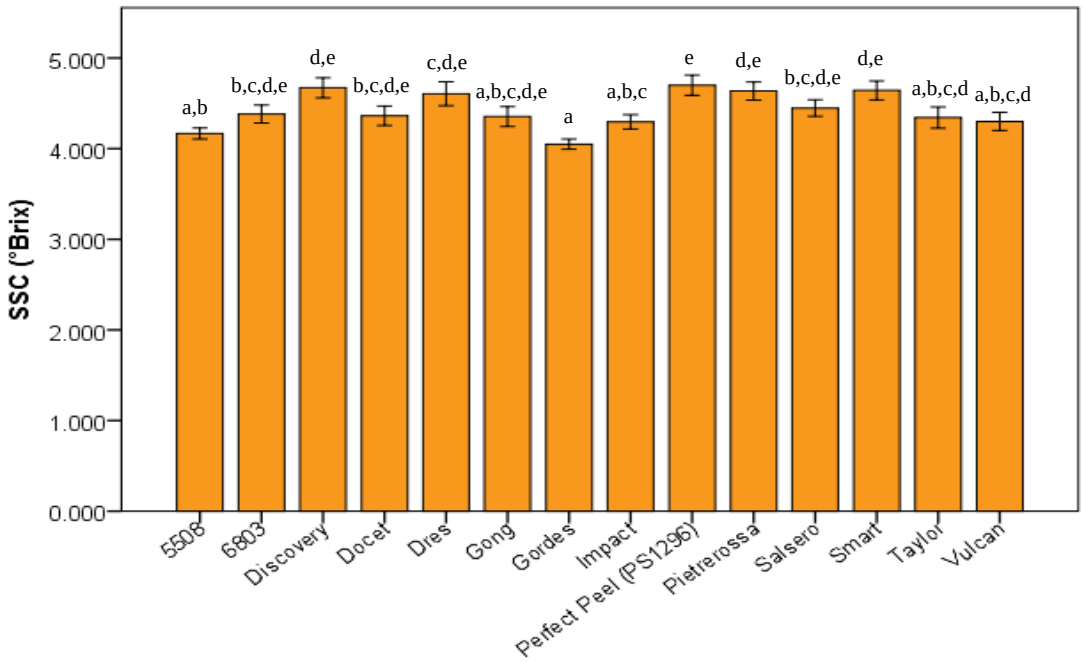

Figure 3.1 - Fruit Soluble Solids Content in 14 tomato hybrids. Same letters stand for differences statistically not significant at Duncan's post hoc test (P<0.05).

Fruit firmness in the tomato hybrids is reported in the Figure 3.2. The average fruit firmness was 31.12 ± 3.47 N · mm^{-2}, with Heinz 6803 performing the highest firmness (36.35 ± 0.91 N · mm^{-2}). The Duncan's post hoc test allowed hybrids to be

divided in in seven subgroups with Smart and Docet being assigned to the subgroup with the lowest mean and Heinz 5508, Heinz 6803, Dres, Impact, Perfect Peel and Salsero being assigned to the most firm subgroup.

As for the dry matter content, Dres performed on average 6.00 gDW · 100 gFW^{-1}, significantly higher than the dry matter content expressed by half of hybrid set members (Fig. 3.3).

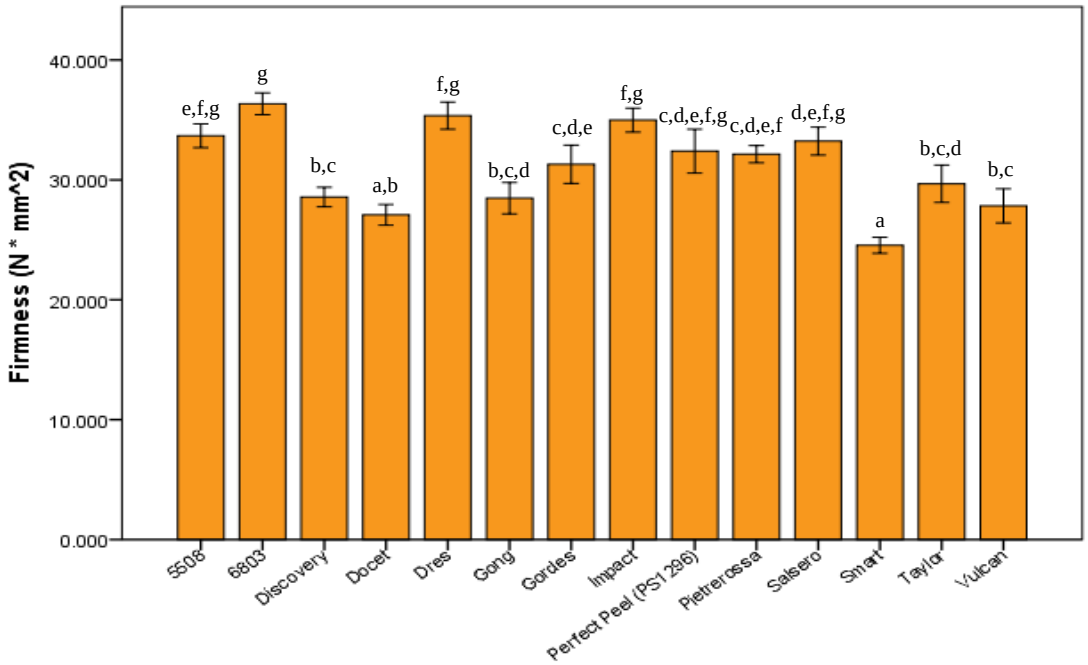

Figure 3.2 - Fruit firmness in 14 tomato hybrids. Same letters stand for differences statistically not significant at Duncan's post hoc test (P<0.05).

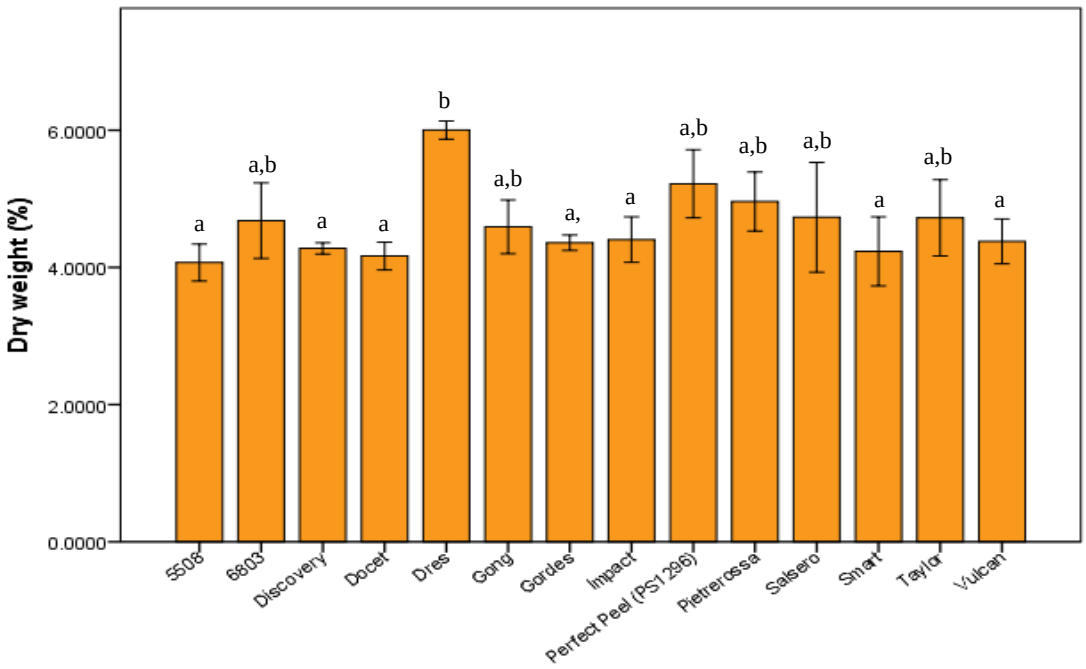

Figure 3.3 - Dry weight in 14 tomato hybrids. Same letters stand for differences statistically not significant at Duncan's post hoc test (P<0.05).

Details on fruit levels of analysed antioxidants within the hybrid set are reported in the Table 3.1. In terms of total ascorbic acid, Vulcan and Pietrarossa showed the highest concentrations with 35.4 ± 1.05 and 35.31 ± 2.69 mg · 100 g^{-1} on a fresh weight basis, respectively. The average fruit content in phenolics ranged from 8.50 mg of gallic acid equivalent · 100g FW^{-1} of Taylor to 38.33 mg of gallic acid equivalent · 100g FW^{-1} in Heinz 6803 (Tab. 3.1).

A low variability was observed among hybrids in terms of flavonoids and carotenoids content. In fact, the flavonoid concentration ranged beetwen 6.83 ± 0.62 mg of Pietrarossa and 5.72 ± 0.07 mg of Quercetin Equivalent (QE) 100 g FW^{-1} for Dres.

As for the carotenoid content in the hybrids, Discovery performed with the highest average level (83.04 ± 2.96 µg ·100 g FW^{-1}). For both traits, the fruit levels in flavonoids and those in carotenoids, significant differences among hybrids were not observed at Duncan's post hoc test (Tab 3.1).

Table 3.1 - Levels of antioxidant compounds in tomato fruit harvested from hybrids grown in the field trial at Eboli over the 2013 growing season.

	Tot AsA (mg · 100 g FW^{-1})		Phenolics (mg GAE · 100 g FW^{-1})		Flavonoids (mg QE · 100g FW^{-1})		Carotenoids (µg · g FW^{-1})	
Genotype	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Heinz5508	27.70^{a,b}	1.39	27.67^{b,c}	4.05	6.44^{a}	0.55	58.66^{a}	6.52
Heinz6803	29.13^{a,b,c}	1.14	38.33^{c}	1.86	6.52^{a}	0.18	79.69^{a}	0.17
Discovery	25.24^{a,b}	2.06	27.33^{b,c}	2.67	5.79^{a}	0.17	83.04^{a}	2.96
Docet	27.45^{a,b}	2.55	28.50^{b,c}	2.50	5.99^{a}	0.65	63.57^{a}	1.50
Dres	27.60^{a,b}	1.73	27.50^{b,c}	0.50	5.72^{a}	0.07	60.07^{a}	10.91
Gong	28.81^{a,b,c}	2.25	22.00^{b}	4.73	6.16^{a}	0.15	63.54^{a}	12.64
Gordes	27.90^{a,b}	0.17	23.50^{b}	7.50	6.48^{a}	0.24	64.06^{a}	7.21
Impact	31.55^{b,c}	2.13	29.33^{b,c}	2.60	6.07^{a}	0.05	66.10^{a}	6.93
PerfectPeel	31.09^{b,c}	2.58	33.00^{b,c}	6.11	6.56^{a}	0.22	67.17^{a}	3.54
Pietrarossa	35.31^{c}	2.69	30.00^{b,c}	3.51	6.83^{a}	0.62	64.70^{a}	1.98
Salsero	24.93^{a,b}	2.19	28.66^{b,c}	4.91	6.24^{a}	0.37	73.63^{a}	4.71
Smart	29.12^{a,b,c}	1.20	20.33^{b}	1.86	6.71^{a}	0.23	60.07^{a}	13.98
Taylor	22.57^{a}	0.44	8.50^{a}	2.50	5.94^{a}	0.52	60.50^{a}	1.89
Vulcan	35.40^{c}	1.04	26.00^{c}	3.21	6.04^{a}	0.37	58.87^{a}	7.47

In order to describe performances of the tomato hybrids in terms of all the quality-related variables, the Principal Component Analysis was used to extract four components explaining about 88% of the overall variability. Components 1 and 2 and components 3 and 4 are plotted each one against the other in Figure 3.4. The component 1 mainly accounted for the variability in the level of total AsA and flavonoids. The component 2 mainly described variability in firmness, dry weight and the content of phenolic compounds. The component 3 was associated to the fruit level of phenolic compounds and carotenoids, while the component 4 accounted for the SSC. The fruit shape well discriminate genotypes in the bidimensional space component 1/2.

Within the round shaped tomatoes, Perfect Peel performed higher scores particularly for components 1, 2 and 4. On the other hand, Docet had more equilibrate scores on all four components within the group of elongated hybrids.

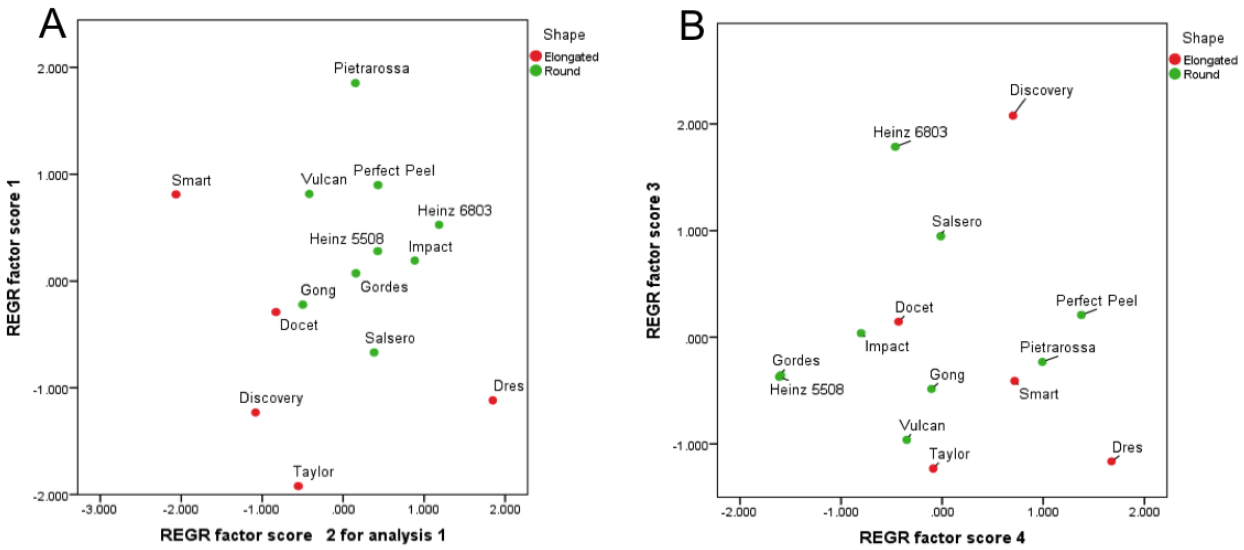

Figure 3.4 – Graphical representation of fruit quality-related performances of 14 field-grown hybrids based on the Principal Component Analysis. The component 1 was associated to the level of total AsA and flavonoids, the component 2 to firmness, dry weight and the content of phenolic compounds, the component 3 to the fruit level of phenolic compounds and carotenoids, while the component 4 to SSC.

3.2 Plant response to drought in open field

In the framework of a research activity carried out in the Laboratory of Functional Genomics and Breeding at the University of Naples Federico II since the 2012 the introgression 9-2-5 from the wild *Solanum pennellii* has been dissected and stabilized in a number of sub-ILs in order to fine-map overlapping QTLs. Within this set of sub-ILs, the IL9adm harbouring an homozygous wild introgression of ca 9 cM from the marker TG223 to the marker TC227407 (details on molecular marker used in this thesis will be given in the section 3.3), was selected based on preliminary results indicating an higher drought tolerance. Therefore, in order to prove a differential physiological response to drought in the IL9adm controlled by the wild introgression, the IL9adm was compared in its performances to the parental line M82 in a filed trial. This trial was carried out in the 2003 at Acerra (Naples, Southern Italy) and involved two different water treatments, the one providing plants with all the evapotranspired water (namely 100%) and the other one with the 50% of that volume (namely 50%).

The figure 3.5 illustrates changes in the relative water content (RWC) in leaves from M82 and IL9adm at 100% and 50% water restitution treatments. Although no

significant effects of the genotypes were observed (Tab 3.2), the ANOVA revealed a statistical significant reduction (from 89 % to 79 % and from 88 % to 84 % in M82 and IL9adm respectively) in leaf RWC due to 50% reduction of the water restitution (Tab. 3.2). The different response of M82 and IL9adm to a reduced water restitution in terms of RWC is confirmed by a significant interaction "Genotype x Water restitution" (Tab. 3.2).

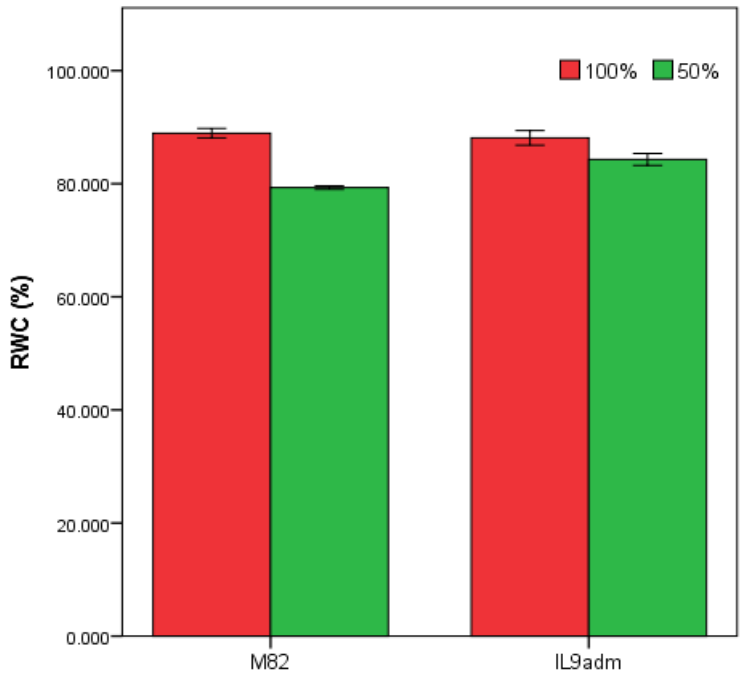

Figure 3.5 – Leaf Relative water content in M82 and IL9adm grown under two different water level restitutions. The RWC was evaluated two weeks before fruit harvesting.

Table 3.2 - ANOVA statistics for leaf RWC in M82 and IL9adm grown under two different water level restitutions.

Source of variability	F	P
Genotype	3.79	0.09
Water restitution	39.91	$3.3 \cdot 10^{-4}$
Genotype x water restitution	7.36	0.03

In order to confirm the higher drought tolerance of the IL9adm and investigate on leaf physiological mechanisms supporting its differential drought response, plants from the field trial in Acerra were used to estimate the loss of water over the time

from young 10 cm-long shout detached from the mother plant (Fig. 3.6). The water loss rate (WLR) was evaluated after 2 h, from 2 to 4 h, from 4 to 6 h and from 6 to 24 h and expressed as g water · h^{-1} · g^{-1} DW. IL9adm showed a lower WLR in both water treatments over all time points with the exception of the last two time points for the 50% water treatment. As for the 100% treatment, after 2 h a WLR of 0.33 ± 0.001 g · h^{-1} · g^{-1} DW was recorded for IL9adm which subsequently decreased up to 0.08 ± 0.004 from 6 to 24 h. On the other hand, the IL9adm grown with a 50% water treatment performed a WLR ranging from 0.27 ± 0.03 to 0.07 ± 0.001. The ANOVA test confirmed that the WLR was significantly affected both by the genotype, the water treatment and decreased along with time points (Tab. 3.3).

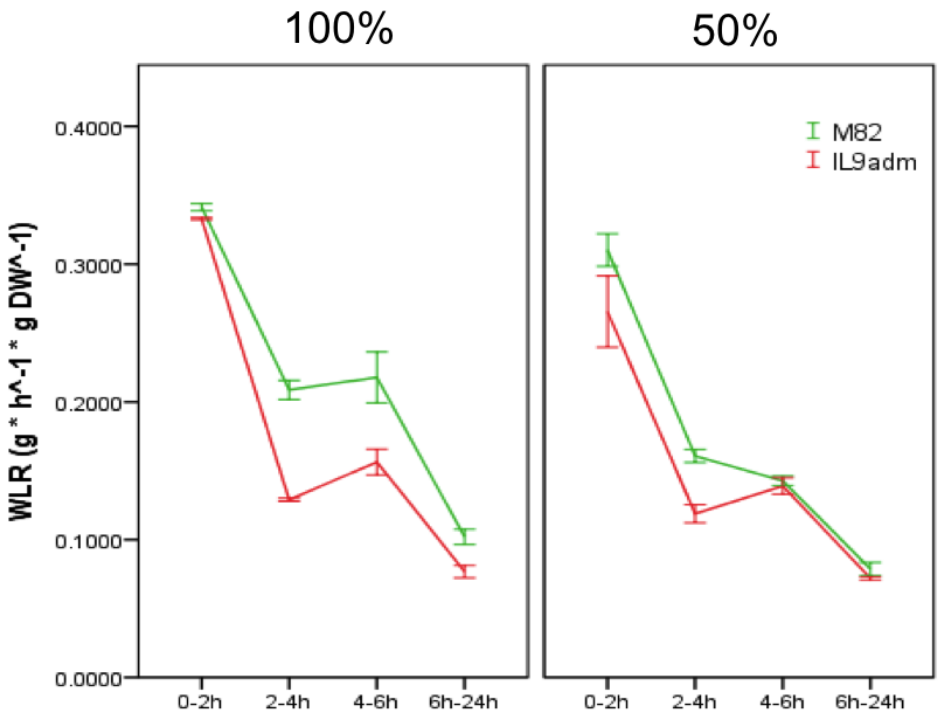

Figure 3.6 – Shoot water loss rate (WLR) in M82 and IL9adm plants grown under two different water restitution levels.

Table 3.3 - ANOVA statistics for water loss rate (WLR) in M82 and IL9adm plants grown under two different water restitution levels.

Source of variability	df	F	*P*
Genotype	1	42.46	$6.5 \cdot 10^{-7}$
Water restitution	1	44.17	$4.7 \cdot 10^{-7}$
Time point	3	337.49	$6.2 \cdot 10^{-21}$
Genotype x water restitution	1	3.45	0.07
Genotype x time point	3	3.30	0.04
Water restitution x time point	3	2.50	0.08

The differential amplitude of response in leaves of M82 and IL9adm to a reduced water restitution regime was also confirmed by analysing the leaf hydrogen peroxide level (Fig. 3.7). In particular, the reduction of the water restitution increased the leaf level of H_2O_2 and the increase was far higher in M82 than IL9adm.The ANOVA test allowed to identify the genotypes, the water treatment and the interaction genotype x water treatment as significant source of variation (Tab 3.4).

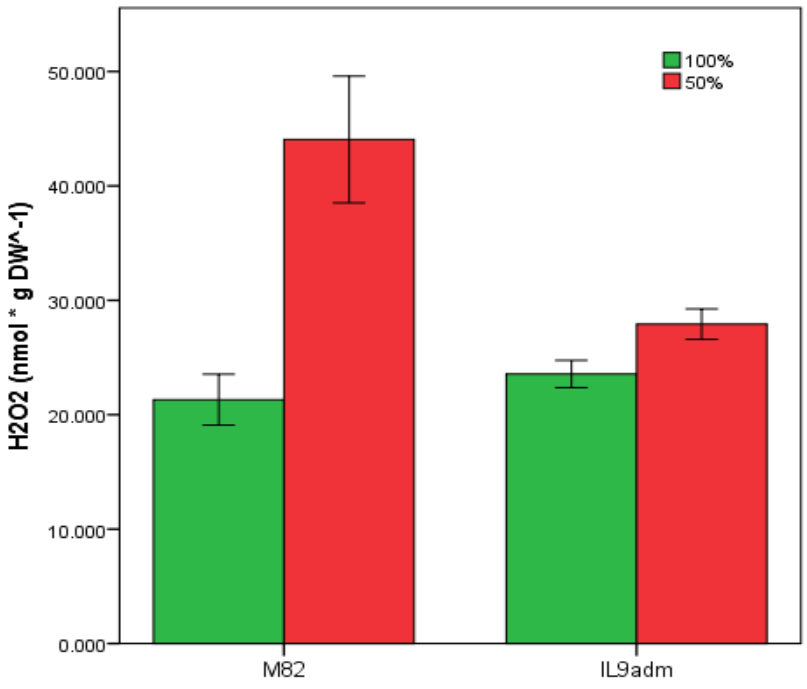

Figure 3.7 – Leaf hydrogen peroxide level in M82 and IL9adm grown under two different water restitution levels.

Table 3.4 – ANOVA statistics to evaluate effects of genotype and water restituion levels on leaf hydrogen peroxide.

Source of variability	F	*P*
Genotype	5.40	0.07
Water restitution	20.59	0.01
Genotype x water restitution	9.46	0.03

3.3 - Effects of drought on yield and fruit quality in open field

Yield performances for both M82 and IL9adm plants undergone to 100% and 50% water treatments are reported in Table 3.5. In particular, performances are expressed as marketable yield, total yield, scrap fruit, unripe fruit, % of unripe out of scrap fruit, soluble solids in marketable yield and soluble solids in total yield.

IL9adm performed a marketable yield of 1.90 kg · plant^{-1} under 100% water restitution treatment, very close to that performed by M82, 2.11 kg · plant^{-1}. By contrast, when the 50% of water restitution treatment was applied the marketable yield decreased up to 1.19 ± 0.15 kg · plant^{-1} and 1.32 ± 0.004 kg · plant^{-1} in M82 and IL9adm, respectively. According to the ANOVA (Tab. 3.6), the genotype was a significant source of variability for total yield, scrap fruit, unripe tomatoes and total yield of soluble solids. The latter was significantly affected by the water treatment.

The marketable yield was not significantly affected by the genotype, but it reduced significantly when the lower water treatment was applied.

Only the unripe/scrap ratio showed a significant interaction "genotype x water treatment".

Table 3.5 - Mean and standard error (SE) for yield parameters recorded on M82 and IL9adm grown under two different water restitution levels (100% and 50%) in Acerra over the 2013 growing season.

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Marketable yield ($kg \cdot plant^{-1}$)	2.11	0.17	1.19	0.15	1.90	0.19	1.32	0.004
Total yield ($kg \cdot plant^{-1}$)	2.55	0.12	1.53	0.15	2.90	0.14	1.81	0.004
Total scrap ($kg \cdot plant^{-1}$)	0.44	0.07	0.33	0.01	1.00	0.25	0.49	0.002
Unripe ($kg \cdot plant^{-1}$)	0.28	0.06	0.09	0.02	0.86	0.25	0.36	0.007
Unripe out of Scrap (%)	62.46	5.32	28.16	6.33	84.68	4.52	73.23	1.46
Marketable yield in SS ($g \cdot plant^{-1}$)	96.67	5.01	66.45	4.17	107.04	6.29	88.83	5.88
Total yield in SS ($g \cdot plant^{-1}$)	117.97	4.12	82.26	4.59	163.89	3.77	119.49	5.29

Table 3.6 – ANOVA statistics to evaluate effects of genotype and water restitution levels on yield features.

Source of variability	Dependent variable	F	*P*
Genotype	Marketable yield	0.09	0.77
	Total yield	6.82	0.03
	Scrap	7.77	0.02
	Unripe	10.98	0.01
	Unripe/Scrap	49.78	$1.0 \cdot 10^{-4}$
	Marketable yield of soluble solids	1.02	0.34
	Total yield of soluble solids	22.39	$1.0 \cdot 10^{-3}$
Water restitution	Marketable yield	25.79	$1.0 \cdot 10^{-3}$
	Total yield	76.09	$2.3 \cdot 10^{-5}$
	Scrap	5.707	0.04
	Unripe	7.133	0.03
	Unripe/Scrap	23.014	$1.0 \cdot 10^{-3}$
	Marketable yield of soluble solids	10.461	0.01
	Total yield of soluble solids	41.598	$1.9 \cdot 10^{-4}$
Genotype x water restitution	Marketable yield	1.274	0.29
	Total yield	0.071	0.78
	Scrap	2.355	0.16
	Unripe	1.511	0.25
	Unripe/Scrap	5.742	0.04
	Marketable yield of soluble solids	0.458	0.52
	Total yield of soluble solids	0.337	0.58

The effect of drought in both M82 and IL9adm on yield performances was comparatively expressed as Blum indexes that is the percentage loss of the specific yield parameter (Fig. 3.8). The Student's t test coupled with 2000 bootstrapping re-sampling confirmed significant differences (P<0.05) between M82 and IL9adm for all the considered yield parameters except for the "unripe fruit". In particular, the reduced water restitution decreased the marketable yield of 49.45 ± 0.23 % in M82 and of 30.68 ± 0.23 % in IL9adm with a significant higher reduction of scrap in the drought tolerant genotype.

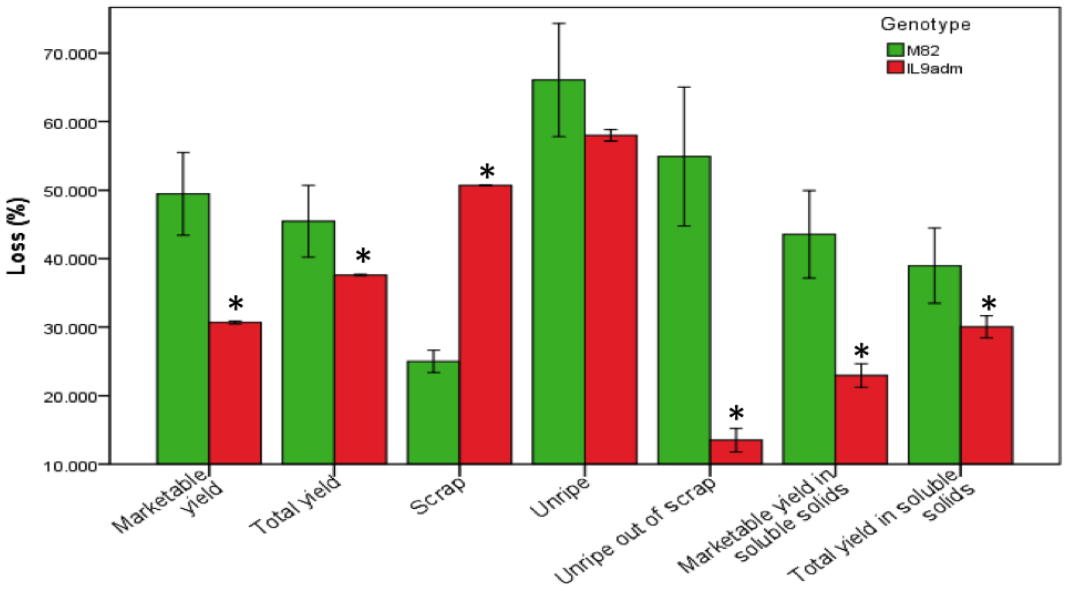

Figure 3.8 - Blum indexes for yield parameters. * statistically significant difference between M82 and IL9adm at Student's t test coupled with 2000 bootstrapping re-sampling.

Main chemico-phisycal properties of tomato fruits and their statistics are summarized in table 3.7 and table 3.8.

The specific gravity was not significantly affected by both the genotype and the water restitution level although it was higher in IL9adm in the 50% treatment.

On the other hand the SSC was significantly higher in the tolerant genotype and, as we could expect, it was affected by the water restitution level (P<0.05; Tab. 3.8).

Finally, the firmness was significantly increased in the 50% water restitution treatment (Tab. 3.8).

Table 3.7 – Fruit chemico-phisycal properties in M82 and IL9adm growen at two different water restitution levels in the field at Acerra over the 2013 growing season.

Variabiles	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Specific gravity ($g \cdot ml^{-1}$)	0.98	$6.0 \cdot 10^{-3}$	0.98	$1.0 \cdot 10^{-3}$	0.97	0.01	0.99	$3.0 \cdot 10^{-3}$
Soluble solids content (°Brix)	4.82	0.14	5.44	0.05	5.50	0.20	6.15	0.15
Firmness ($N \cdot mm^{-2}$)	31.09	0.90	34.35	0.73	28.28	1.43	33.92	0.89

Table 3.8 – ANOVA statistics for specific gravity, soluble solid content (SSC) and firmness.

Source of variability	Dependent variable	F	*P*
Genotype	Specific gravity	0.26	0.62
	SSC	22.37	$1.2 \cdot 10^{-3}$
	Firmness	2.51	0.15
Water restitution	Specific gravity	4.44	0.07
	SSC	18.93	$2.0 \cdot 10^{-3}$
	Firmness	18.87	$2.0 \cdot 10^{-3}$
Genotype x water restitution	Specific gravity	0.45	0.52
	SSC	0.01	0.91
	Firmness	1.36	0.28

As for the content in antioxidants, the level of AsA in fruit at red ripe stage is reported in the table 3.9. According to the ANOVA test, the only source of significant variation was the genotype an igher level f AsA in red ripe fruit from the tolerant IL9adm (Tab. 3.10).

Table 3.9 - Mean and standard error AsA content ($mg \cdot 100g\ DW^{-1}$) at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
516.55	12.01	547.34	18.57	588.90	19.62	556.88	12.45

Table 3.10 – ANOVA statistics to evaluate effects of genotype and water restitution levels on the level of AsA at red ripe stage (RR).

Source of variability	F	*P*
Genotype	5.59	0.05
Water restitution	$1.1 \cdot 10^{-3}$	0.97
Genotype x water restitution	3.29	0.11

As for phenolics, the level recorded in fresh red ripe fruit and relevant statistics are summarized in the Table 3.11 and Table 3.12. Due to the high variability was observed, both genotype and water restitution level were not significant source of variation according to ANOVA test.

Table 3.11 – Mean and standard error for the level of total phenolics (mg GAE · 100g DW^{-1}) at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
620.15	108.35	617.60	96.81	532.18	74.40	498.67	6.90

Table 3.12 – Effects of genotype and water restitution levels on total phenolic compounds at red ripe stage (RR) atANOVA test.

Source of variability	F	P
Genotype	1.60	0.24
Water restitution	0.05	0.83
Genotype x water restitution	0.04	0.85

Analysis of the total level of flavonoids in red ripe fruit revealed an average content of 138.08 ± 9.56 and 129.33 ± 7.37 mg of quercetin equivalents (QE) per 100 g DW^{-1} in M82 and IL9adm, respectively, when a complete restitution of lost water (100%) was operated (Tab. 3.13). Neverthless, the total flavonoids concentration was not significantly affected by the genotype, the water treatment and their interaction (Tab. 3.14).

Table 3.13 - Mean and standard error of of total flavonoids (mg QE · 100 g DW^{-1}) at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
138.08	9.56	129.30	10.56	129.33	7.37	114.73	12.08

Table 3.14 – ANOVA statistics to evaluate effects of genotype and water restitution levels on fruit total flavonoid concentration at red ripe stage (RR).

Source of variability	F	*P*
Genotype	1.35	0.28
Water restitution	1.35	0.28
Genotype x water restitution	0.08	0.78

In order to gain a separation of individual molecules of the phenolics pool, an HPLC/UV - ESI MS MS approach and most abundant fractions are reported in table 3.15 while their ANOVA statistics are summerized in table 3.16.

The most rapresentative flavonoid was the rutin, ranging between 133.06 ± 16.71 of IL9adm 100% WR and 209.04 ± 24.75 μg g DW^{-1}. On the other hand, phenolic acids were mostly represented by chlorogenic acid and, at a far lower extend, by caffeic acid (Tab. 3.15). With the exception of chlorogenic acid, rutin, quercetin and caffeic acid significantly increased their levels in response to a lower water restitution rate (Tab. 3.16).

Table 3.15 - Tomato phenolic compounds detected by HPLC/UV- ESI MS MS analysis in M82 and IL9adm red ripe fruit under two water restitution level.

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rutin (μg g DW^{-1})	163.41	14.33	208.24	29.03	133.05	16.71	209.04	24.75
Quercetin (μg g DW^{-1})	0.77	0.16	1.42	0.14	1.01	0.07	1.79	0.12
Caffeic acid (μg g DW^{-1})	0.24	0.03	0.31	0.05	0.28	0.03	0.40	0.03
Chlorogenic acid (μg g DW^{-1})	15.76	2.45	12.14	0.84	15.72	2.32	17.92	1.33

Table 3.16 - ANOVA statistics to evaluate effects of genotype and water restitution levels onflavonoids and phenolic acids at red ripe stage (RR).

Dependent variable	Source of variability	F	*P*
Rutin	Genotype	0.45	0.52
	Water restitution	7.52	0.03
	Genotype x water restitution	0.50	0.50
Quercetin	Genotype	5.41	0.05
	Water restitution	30.77	$5.4 \cdot 10^{-4}$
	Genotype x water restitution	0.25	0.63
Caffeic acid	Genotype	3.82	0.09
	Water restitution	8.12	0.02
	Genotype x water restitution	0.53	0.49
Chlorogenic acid	Genotype	2.38	0.16
	Water restitution	0.14	0.71
	Genotype x water restitution	2.44	0.16

In terms of fruit total carotenoid content, M82 and IL9adm displayed differences statistically not significant when the completed restitution of the lost water was operated (100%) (Tab. 3.17). Neverthless, the level of total carotenoids was significantly decreased in both tomato lines when the 50% water restitution was applied (Tab. 3.17 and Tab. 3.18).

Table 3.17 – Mean and standard error of total carotenois ($\mu g \cdot g\ DW^{-1}$) at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
1420.78	248.10	1170.59	227.29	2193.61	297.45	1325.19	128.81

Table 3.18 – Effects of genotype and water restitution levels on the fruit total carotenoids concentration at red ripe stage (RR) evaluated by ANOVA test.

Source of variability	F	*P*
Genotype	3.94	0.08
Water restitution	5.73	0.04
Genotype x water restitution	1.75	0.22

Again, carotenoid fractions were separated using an HPLC approach. In particular, major peaks detected in red ripe fruit are reported in table 3.19. Their sum was also evaluated as well as the lycopene/carotene ratio because its association to the fruit colour.

β-carotene was the most abundant carotenoid with the highest concentration in M82 fruit undergone to 100% water treatment (261.56 ± 17.69 μg · g DW^{-1}; Tab. 3.19). The ANOVA confirmed a significant effect of the genotype on the level of α-carotene and β-carotene, on the sum of peaks and the lycopene/carotene ratio (Tab. 3.20).

Fruit lycopene concentration at RR stage ranged between 6.63 ± 0.45 μg · g DW^{-1} in M82 undergone to the 100% water treatment and 8.95 ± 0.72 in the same genotype when a 50% water restitution was operated. A similar trend was observed in the IL9adm and the ANOVA test confirmed the significant effect of a reduced water restitution on the increase of lycopene (Tab. 3.20).

Also, the β-carotene concentration, the sum of peaks and the lycopene/carotene ratio were affected by a significant interaction "genotype x water treatment). The worst lycopene/carotene ratio was observed in M82 under the 100% water restitution treatment. This ratio was significantly enhanced by the reduced water restitution treatment in M82 while it remained essencially unmodified in IL9adm red ripe fruit (Tabb. 3.19 and 3.20).

Table 3.19 – Average and standard error of main carotenoids detected at red ripe stage (RR) by HPLC analysis.

	M82				IL9adm			
	100%		50%		100%		50%	
Compound	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Lutein (μg · g DW^{-1})	16.45	1.19	22.73	1.09	20.22	1.13	22.31	1.5
Lycopene (μg · g DW^{-1})	6.63	0.45	8.95	0.72	7.22	0.71	7.59	0.07
α-carotene (μg · g DW^{-1})	16.11	1.29	21.41	1.75	23.78	2.1	26.19	1.8
β-carotene (μg · g DW^{-1})	261.56	17.69	214.8	11.89	140.83	3.55	183.16	9.07
Sum of peaks	300.74	16.33	267.88	15.38	192.05	7.27	239.25	9.97
Lycopene/carotene	0.02	$2.9 \cdot 10^{-4}$	0.04	$1.5 \cdot 10^{-4}$	0.04	$3.0 \cdot 10^{-3}$	0.04	0.002

Table 3.20 - Effects of genotype and water restitution levels on carotenoid fractions at red ripe stage (RR) evaluated by ANOVA test.

Source of variability	Dependent variable	F	*P*
Genotype	Lutein	1.83	0.21
	Lycopene	0.47	0.51
	α-carotene	12.53	$7.6 \cdot 10^{-3}$
	β-carotene	42.27	$1.9 \cdot 10^{-4}$
	Sum of peaks	28.77	$6.7 \cdot 10^{-4}$
	Lycopene/carotene	27.35	$7.9 \cdot 10^{-4}$
Water restitution	Lutein	11.45	$9.6 \cdot 10^{-3}$
	Lycopene	5.88	0.04
	α-carotene	4.8	0.06
	β-carotene	0.04	0.85
	Sum of peaks	0.31	0.6
	Lycopene/carotene	3.47	0.09
Genotype x water restitution	Lutein	2.85	0.13
	Lycopene	3.12	0.12
	α-carotene	0.67	0.44
	β-carotene	14.45	$5.2 \cdot 10^{-3}$
	Sum of peaks	9.78	0.01
	Lycopene/carotene	36.11	$3.2 \cdot 10^{-4}$

Results related to the fruit total antioxidant activity evaluated by the ORAC procedure are reported in the table 21 and table 22. In particular, the ANOVA test confirmed the significant effect of the genotype. In fact, the antioxidant activity was higher in both M82 than in IL9adm at 100% and 50% water restitution treatments ($P<0.05$; Tab. 3.21 and Tab. 3.22).

The ORAC value was not significantly affected by the different levels of water restitutions but a significant interaction between genotype and water restitution was recorded (Tab. 3.22). In fact, M82 and IL9adm had an opposite response to the reduced water restitution regime, the former decreasing the fruit total antioxidant activity and the latter increasing the antioxidant activity in the red ripe mesocarp.

Table 3.21 – Mean and standard error of fruit total antioxidant activity (μmol TE g $DW^{-}1$) in M82 and IL9adm under two water restitution levels at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
1048.48	28.91	907.26	54.11	381.10	33.163	616.41	86.84

Table 3.22 – ANOVA statistics to evaluate effects of genotype and water restitution levels on fruit total antioxidant activity at red ripe stage (RR).

Source of variability	F	P
Genotype	74.02	$2.5 \cdot 10^{-5}$
Water restitution	0.71	0.42
Genotype x water restitution	11.43	0.01

Table 3.23 reported the fruit level of H_2O_2 at red ripe stage. Its average concentration significantly increased, irrespective of the genotype, at a reduced water restitution level (Tab. 3.24). Neighter the genotype nor the interaction between genotype and water treatment significantly affected the level of hydrogen peroxide in the fruit.

Table 3.23 – Mean and standard error of hydrogen peroxide amount (nmol · g DW^{-1}) in M82 and IL9adm under two water restitution levels at red ripe stage (RR).

M82				IL9adm			
100%		50%		100%		50%	
Mean	SE	Mean	SE	Mean	SE	Mean	SE
0.35	0.16	1.28	0.22	0.55	0.15	1.14	0.13

Table 3.24 – ANOVA statistics to evaluate effects of genotype and water restitution levels on hydrogen peroxide amount at red ripe stage (RR).

Source of variability	F	*P*
Genotype	0.02	0.88
Water restitution	18.24	$3.7 \cdot 10^{-3}$
Genotype x water restitution	0.90	0.37

3.4 Effects of open field drought on the quality of tomato puree

Tomatoes harvested from the open field trial in Acerra across the 2013 growing season were delivered to the SSICA, in Angri, where they underwent processing to puree by mean of a scaled processing facility. Table 3.25 summarize some yield parameters and quality indexes of resulting sauces.

The genotype significantly affected the industrial yield that was higher for IL9adm than M82 and as we could aspect, the 50 % water restitution treatment significantly reduced the yield in both the genotypes. The discarded peel was significantly affected by the genotype and the water restitution and its highest value was 2.60% recorded for IL9adm in the 50% water treatment.

The component "L" of the colour was affected by the genotype wherease the component "a" was significantly affected by the genotype and the water restitution. Components "a", "b" and "L" had a significant interaction between genotype and water treatment (Tab. 3.26).

They all concurred to discriminate genotypes and water treatments (Fig. 3.9).The juice Ph was not affectd by any of considered source of variation (Tab. 3.26).

Table 3.25 – Tomato sauce quality and yield processing parameters from fruit of M82 and IL9adm grown at two different water restitution levels.

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Pre-consumer waste (%)	1.30	0.01	3.60	0.01	1.20	0.05	2.90	0.02
Discarded peel (%)	2.00	0.03	1.50	0.01	1.60	0.02	2.60	0.03
Industrial yield (%)	96.70	0.51	93.90	0.42	97.20	0.63	94.50	0.84
Colour Hunter Lab 2°C a/L	2.23	0.03	2.24	0.04	2.170	0.03	2.19	0.05
Colour Hunter Lab 2°C L	25.12	0.12	24.92	0.08	25.74	0.21	26.13	0.25
Colour Hunter Lab 2°C a	31.18	0.15	30.69	0.09	30.34	0.34	31.26	0.33
Colour Hunter Lab 2°C b	13.96	0.20	13.73	0.03	13.98	0.22	14.26	0.07
Juice pH	4.11	0.03	4.18	0.04	4.13	0.03	4.230	0.03

Table 3.26 - ANOVA statistics of tomato sauce quality and yield processing parameters from fruit of M82 and IL9adm grown at two different water restitution levels .

Source of variability	Dependent variable	F	P
Genotype	Pre-consumer waste (%)	48.00	$1.2 \cdot 10^{-4}$
	Discarded peel (%)	36.75	$3.0 \cdot 10^{-4}$
	Industrial yield (%)	90.75	$1.2 \cdot 10^{-5}$
	Colour Hunter Lab 2°C a/L	0.91	0.37
	Colour Hunter Lab 2°C L	251.17	$2.5 \cdot 10^{-7}$
	Colour Hunter Lab 2°C a	5.47	0.05
	Colour Hunter Lab 2°C b	22.70	$1.1 \cdot 10^{-3}$
	Juice pH	0.37	0.56
Water restitution	Pre-consumer waste (%)	1200.00	$5.2 \cdot 10^{-10}$
	Discarded peel (%)	18.75	$3.0 \cdot 10^{-3}$
	Industrial yield (%)	2268.75	$4.1 \cdot 10^{-11}$
	Colour Hunter Lab 2°C a/L	0.07	0.80
	Colour Hunter Lab 2°C L	2.71	0.14
	Colour Hunter Lab 2°C a	13.87	$6.0 \cdot 10^{-3}$
	Colour Hunter Lab 2°C b	0.19	0.70
	Juice pH	2.17	0.18
Genotype x water restitution	Pre-consumer waste (%)	27.00	$8.2 \cdot 10^{-4}$
	Discarded peel (%)	168.75	$1.1 \cdot 10^{-6}$
	Industrial yield (%)	0.75	0.41
	Colour Hunter Lab 2°C a/L	0.01	0.93
	Colour Hunter Lab 2°C L	26.19	$1.1 \cdot 10^{-3}$
	Colour Hunter Lab 2°C a	149.19	$1.8 \cdot 10^{-6}$
	Colour Hunter Lab 2°C b	19.59	$2.3 \cdot 10^{-3}$
	Juice pH	0.07	0.80

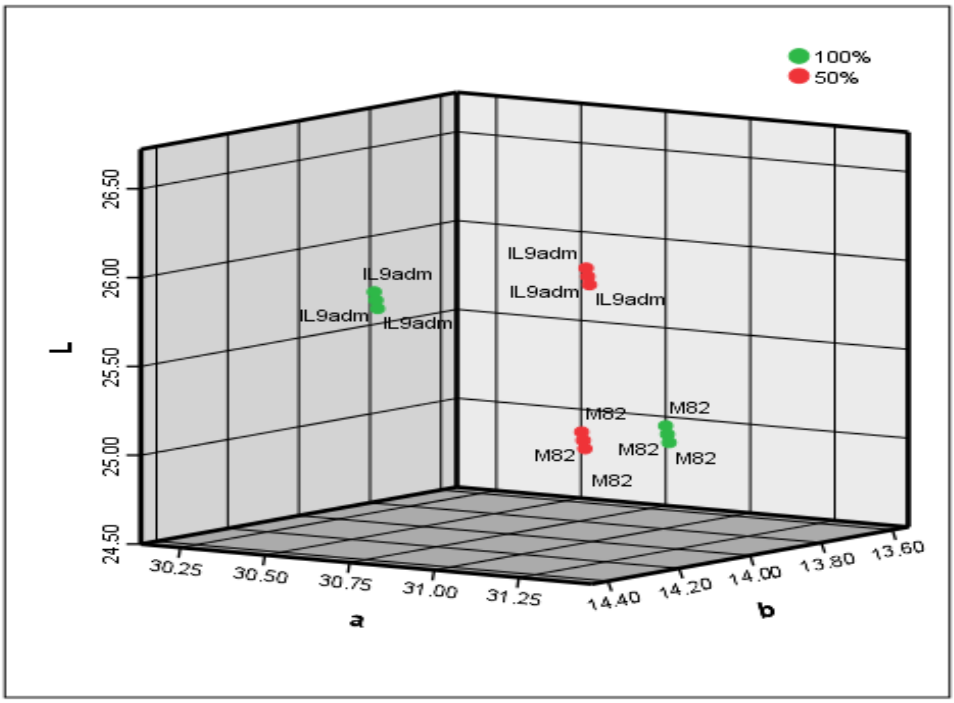

Figure 3.9 – Three dimension graph showing colour features of tomato sauces got from the processing of M82 and IL9adm grown under two different water level restitutions.

As for the level antioxidants, the genotype did not affect the concentration of AsA wherease the water treatment did (Tab. 3.27 and Tab. 3.28). Specifically, a dramatic decrease of AsA was observed upon processing and the extend of the decay was significantly affected by the water treatment. In particular, the reduced water restitution (50%) was associated to a lower decay of AsA (Tab. 3.27).

Table 3.27 - Mean and standard error AsA content (mg · 100g DW^{-1}) in tomato sauce (PP) and its variation upon processing compared to fresh red ripe stage (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	241.85	4.65	369.08	26.56	249.91	5.44	330.49	12.03
Δ PP (%)	-53.18	0.90	-32.57	4.85	-57.56	0.92	-40.65	2.16

Table 3.28 – ANOVA statistics to evaluate effects of genotype and water restitution levels on AsA concentration in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability	Dependent variable	F	P
Genotype	PP	0.71	0.43
	Δ PP (%)	3.56	0.11
Water restitution	PP (%)	32.99	$1.0 \cdot 10^{-3}$
	Δ PP (%)	32.23	$1.0 \cdot 10^{-3}$
Genotype x water restitution	PP	1.66	0.24
	Δ PP (%)	0.31	0.59

As for the level of total phenolics, a significant effect of the genotype and the water treatment was detected by the ANOVA test (Tab. 3.29 and Tab. 3.30). Processing modified the level of total phenolics in the puree compared to the red ripe fruit and the variation was significantly affected by the genotype, the water treatment and the interaction between them. In fact, in M82 the processing reduced the total level of phenolics and the decay was larger in the 50% water treatment than in 100%. On the other hand, the puree from IL9adm had a slite increase in the total level of phenolics when the 100% water restitution treatment occurred wherease it remained unchanged when the 50% water treatment was imposed (Tab. 3.29 and Tab. 3.30).

Table 3.29 – Mean and standard error of phenolic compounds (mg GAE · 100g DW^{-1}) in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	553.84	6.83	446.74	6.27	589.50	8.36	493.95	5.03
Δ PP (%)	-10.69	1.10	-27.67	1.02	10.77	1.57	-0.95	1.01

Table 3.30 – ANOVA statistics to evaluate effects of genotype and water restitution levels on total phenolic compounds and in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability		F	*P*
Genotype	PP	37.89	$2.7 \cdot 10^{-4}$
	Δ PP (%)	405.04	$3.9 \cdot 10^{-8}$
Water restitution	PP	226.59	$3.7 \cdot 10^{-7}$
	Δ PP	143.61	$2.2 \cdot 10^{-6}$
Genotype x water restitution	PP	0.74	0.42
	Δ PP (%)	4.82	0.06

The level of total flavonoids in tomato puree is summarized in Table 3.31 and Table 3.32. The ANOVA test underlined that the level of total flavonoids was significantly affected by the genotype, the water treatment and the interaction between these sources of variation. In particular, the level of total flavonoids was higher in IL9adm fruit at 100% water treatment but it remained essentially unchanged in M82 when the reduced water restitution treatment was imposed wherease it dramatically lowered in IL9adm. Also, processing caused the level of total flavonoids to decay and again the extend of this decay was significantly affected by the genotype, the water treatment and the intereaction between the genotype and the water restitution with the lowest decay occurring in IL9adm treated with 100% water restitution and the highest decay occurring in IL9adm challenged with 50% water restitution.

Table 3.31 - Mean and standard error of of total flavonoids (mg QE · 100 g DW^{-1}) in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	84.10	0.68	82.41	0.63	100.12	0.83	62.60	0.51
Δ PP (%)	-39.09	0.49	-36.27	0.49	-22.58	0.65	-45.44	0.44

Table 3.32 – ANOVA statistics to evaluate effects of genotype and water restitution levels on total flavonoid levels in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability		F	*P*
Genotype	PP	7.93	0.02
	Δ PP (%)	49.32	$1.1 \cdot 10^{-4}$
Water restitution	PP	849.18	$2.0 \cdot 10^{-9}$
	Δ PP (%)	368.19	$5.6 \cdot 10^{-8}$
Genotype x water restitution	PP	708.61	$4.2 \cdot 10^{-9}$
	Δ PP (%)	604.69	$7.9 \cdot 10^{-9}$

An HPLC/UV - ESI MS MS analysis was carried out in order to quantify individual phenolic fractions in the tomato puree and evaluate variations in their content occurring as a consequence of the processing. Again, the most representative flavonoids were the rutin and quercetin and the most abundant phenolic acids were chlorogenic acid and caffeic acid (Tab. 3.33). For all of them, the absolute level was significantly affected by the genotype (with the exception of caffeic acid), the water treatment and the interaction between the genotype and the water treatment (Tab. 3.34). In general, the level of all four fractions increased massively upon processing in the range 31.05% to 65.57% for rutin, 8.83% to 100.45% for quercetin, 362.45% to 710.55% for caffeic acid nd 112.01% to 150.41% for chlorogenic acid and these increases were significantly affected by the genotype, the water treatment and the interaction between them.

Table 3.33 - Phenolic compounds in M82 and IL9adm under two water conditions in tomato sauce (PP) and its variation upon processing (Δ PP (%)) detected by an HPLC/UV- ESI MS MS approach.

		M82				IL9adm			
		100%		50%		100%		50%	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rutin (μg g DW^{-1})	PP	251.53	0.06	344.77	0.06	174.36	0.06	322.56	0.06
	Δ PP (%)	53.92	0.04	65.57	0.03	31.05	0.04	54.31	0.03
Quercetin (μg g DW^{-1})	PP	1.40	0.06	2.85	0.06	1.74	0.06	1.94	0.06
	Δ PP (%)	81.56	7.47	100.45	4.06	73.05	5.73	8.83	3.23
Caffeic acid (μg g DW^{-1})	PP	1.94	0.06	2.07	0.06	2.30	0.06	1.86	0.06
	Δ PP (%)	699.85	23.85	562.19	18.43	710.55	20.38	362.45	14.32
Chlorogenic acid (μg g DW^{-1})	PP	38.42	0.06	30.40	0.06	38.07	0.06	38.00	0.06
	Δ PP (%)	143.83	0.37	150.41	0.48	142.13	0.37	112.01	0.32

Table 3.34 - ANOVA statistics to evaluate effects of genotype and water restitution levels on flavonoids and phenolic acids fractions in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Dependent variable		Source of variability	F	*P*
Rutin		Genotype	740634.20	$3.7 \cdot 10^{-21}$
	PP	Water restitution	4372310.17	$3.1 \cdot 10^{-24}$
		Genotype x water restitution	226601.55	$4.2 \cdot 10^{-19}$
		Genotype	249872.15	$2.9 \cdot 10^{-19}$
	Δ PP	Water restitution	261218.09	$2.4 \cdot 10^{-19}$
		Genotype x water restitution	28927.59	$1.6 \cdot 10^{-15}$
Quercetin		Genotype	24.13	$1.2 \cdot 10^{-3}$
	PP	Water restitution	203.33	$5.7 \cdot 10^{-7}$
		Genotype x water restitution	116.53	$4.8 \cdot 10^{-6}$
		Genotype	86.79	$1.4 \cdot 10^{-5}$
	Δ PP	Water restitution	17.78	$2.9 \cdot 10^{-3}$
		Genotype x water restitution	59.79	$5.6 \cdot 10^{-5}$
Caffeic acid		Genotype	1.69	0.23
	PP	Water restitution	6.46	0.03
		Genotype x water restitution	24.33	$1.1 \cdot 10^{-5}$
		Genotype	23.36	$1.3 \cdot 10^{-3}$
	Δ PP	Water restitution	154.29	$1.6 \cdot 10^{-6}$
		Genotype x water restitution	28.96	$6.6 \cdot 10^{-4}$
Chlorogenic acid		Genotype	3925.92	$4.7 \cdot 10^{-12}$
	PP	Water restitution	4906.52	$1.9 \cdot 10^{-12}$
		Genotype x water restitution	4739.63	$2.2 \cdot 10^{-12}$
		Genotype	2683.60	$2.1 \cdot 10^{-11}$
	Δ PP	Water restitution	925.31	$1.5 \cdot 10^{-9}$
		Genotype x water restitution	2248.24	$4.3 \cdot 10^{-11}$

As for the content of total carotenoids (Tab. 3.35), its variation was significantly affected by the genotype, the water restitution treatment and the interaction between them. Same sources of significant variation affected variability in the effect of processing. In general, the processing increased the level of total carotenoids with the exception of IL9adm treated with the 100% water restitution whose puree showed a reduction in the level of total carotenoids (Δ PP equal to -35.57%) when compared to the level observed in the red ripe fruit.

Table 3.35 – Mean and standard error of total carotenois (μg · g DW^{-1}) in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	1660.64	0.06	1650.39	0.06	1413.45	0.06	1975.95	0.06
Δ PP (%)	16.88	$3.3 \cdot 10^{-3}$	40.99	0.01	-35.57	$3.3 \cdot 10^{-3}$	49.11	0.003

Table 3.36 – ANOVA statistics to evaluate effects of genotype and water restitution levels on the total carotenoids content in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability	Dependent variable	F	*P*
Genotype	PP	460608.43	$2.4 \cdot 10^{-20}$
	Δ PP (%)	29481666.67	$1.4 \cdot 10^{-27}$
Water restitution	PP	22874204.42	$4.0 \cdot 10^{-27}$
	Δ PP	177496326.00	$1.1 \cdot 10^{-30}$
Genotype x water restitution	PP	24603726.82	$3.0 \cdot 10^{-27}$
	Δ PP (%)	55024816.67	$1.2 \cdot 10^{-28}$

An HPLC strategy was applied in order to separate individual carotenoid peaks. In particular, major peaks detected tomato puree are reported in table 3.37. Their sum was also evaluated as well as the lycopene/carotene ratio.

β-carotene was the most abundant carotenoid with the highest expression in M82 when the lost water was fully reintegrated (100%). Its level was significantly affected by the genotype, the water treatment and the interaction between them. Same applied for the level of lutein, lycopene, α-carotene, the sum of all detected carotenoid peaks wherease the lycopene/carotene ratio only changed according to the genotype effect (Tab. 3.38). Also, the processing affected changes (Δ PP) in the level of lutein, lycopene, α-carotene, β-carotene, the sum of all detected carotenoid peaks and the lycopene/carotene ratio and all these effects were significantly mediated by the genotype, the water treatment and their interaction.

Table 3.37 – Mean and standard error of carotenoid fractions in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

		M82				IL9adm			
		100%		50%		100%		50%	
Compound		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Lutein ($\mu g \cdot g\ DW^{-1}$)	PP	7.52	0.06	6.45	0.06	6.44	0.06	4.35	0.06
	Δ PP	-54.31	0.35	-71.64	0.25	-68.15	0.29	-80.52	0.26
Lycopene ($\mu g \cdot g\ DW^{-1}$)	PP	7.23	0.06	6.57	0.06	6.96	0.06	5.19	0.06
	Δ PP	9.16	0.87	-26.58	0.65	-3.60	0.80	-31.65	0.76
α-carotene ($\mu g \cdot g\ DW^{-1}$)	PP	26.76	0.06	16.78	0.06	25.48	0.06	14.53	0.06
	Δ PP	66.14	0.36	-21.62	0.27	7.15	0.24	-44.53	0.22
β-carotene ($\mu g \cdot g\ DW^{-1}$)	PP	192.42	0.06	184.28	0.06	161.01	0.06	122.42	0.06
	Δ PP	-26.43	0.02	-14.21	0.03	14.32	0.04	-33.16	0.03
Sum of peaks	PP	233.93	0.23	214.07	0.23	199.89	0.23	146.48	0.23
	Δ PP	-22.21	0.08	-20.09	0.09	4.08	0.12	-38.77	0.10
Lycopene/carotene	PP	0.03	$2.5 \cdot 10^{-4}$	0.03	0	0.04	$2.9 \cdot 10^{-4}$	0.04	$3.4 \cdot 10^{-4}$
	Δ PP	38.42	1.03	-13.52	0.71	-14.60	0.66	4.07	1.07

Table 3.38 - ANOVA statistics to evaluate effects of genotype and water restitution levels on carotenoid fractions in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

		PP		Δ PP	
Source of variability	Dependent variable	F	*P*	F	*P*
Genotype	Lutein	758.32	$3.3 \cdot 10^{-9}$	1537.32	$2.0 \cdot 10^{-10}$
	Lycopene	204.36	$5.6 \cdot 10^{-7}$	132.80	$2.9 \cdot 10^{-6}$
	α-carotene	932.81	$1.4 \cdot 10^{-9}$	21720.48	$5.0 \cdot 10^{-15}$
	β-carotene	652510.39	$6.2 \cdot 10^{-21}$	122413.43	$5.0 \cdot 10^{-18}$
	Sum of peaks	48417.17	$2.0 \cdot 10^{-16}$	1559.50	$1.9 \cdot 10^{-10}$
	Lycopene/carotene	248.73	$2.6 \cdot 10^{-07}$	399.00	$4.1 \cdot 10^{-8}$
Water restitution	Lutein	750.22	$3.4 \cdot 10^{-9}$	2623.58	$2.3 \cdot 10^{-11}$
	Lycopene	447.81	$2.6 \cdot 10^{-8}$	1700.45	$1.3 \cdot 10^{-10}$
	alfa-caroten	32874.71	$9.6 \cdot 10^{-16}$	62975.59	$7.1 \cdot 10^{-17}$
	β-carotene	163749.86	$1.6 \cdot 10^{-18}$	320121.74	$1.1 \cdot 10^{-19}$
	Sum of peaks	25163.92	$2.8 \cdot 10^{-15}$	44701.21	$2.8 \cdot 10^{-16}$
	Lycopene/carotene	0.1	0.76	351.81	$6.7 \cdot 10^{-8}$
Genotype x water restitution	Lutein	78.32	$2.1 \cdot 10^{-5}$	73.04	$2.7 \cdot 10^{-5}$
	Lycopene	92.47	$1.1 \cdot 10^{-5}$	24.74	$1.1 \cdot 10^{-3}$
	α-carotene	71.05	$3.0 \cdot 10^{-5}$	4215.27	$3.5 \cdot 10^{-12}$
	β-carotene	69476.18	$4.8 \cdot 10^{-17}$	917797.06	$1.6 \cdot 10^{-21}$
	Sum of peaks	5273.61	$1.4 \cdot 10^{-12}$	54527.93	$1.3 \cdot 10^{-16}$
	Lycopene/carotene	2.07	0.19	1584.93	$1.7 \cdot 10^{-10}$

According to the ANOVA test, the absolute level of total antioxidant activity in the tomato puree was significantly affected by the genotype, the water treatment and their interaction. The puree for IL9adm showed an higher antioxidant activity and this activity increased when the reduced water restitution treatment was applied in both the genotypes. In general, the processing led to a dramatic decrease of the toal antioxidant activity in all the factor combinations, but the reduction was of significant less extent in IL9adm puree (Tab. 3.39 and Tab. 3.40).

Table 3.39 – Mean and standard error of fruit total antioxidant activity (μmol TE g DW^{-1}) in M82 and IL9adm under two water restitution levels in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	280.67	0.58	329.82	0.57	169.23	0.577	273.72	0.57
Δ PP (%)	-73.23	0.05	-63.64	0.06	-55.59	0.151	-55.60	0.09

Table 3.40 – ANOVA statistics to evaluate effects of genotype and water restitution levels on fruit total antioxidant activity in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability	Dependent variable	F	*P*
Genotype	PP	21052.85	$5.6 \cdot 10^{-15}$
	Δ PP (%)	17005.29	$1.3 \cdot 10^{-14}$
Water restitution	PP	17704.87	$1.1 \cdot 10^{-14}$
	Δ PP (%)	2367.21	$3.5 \cdot 10^{-11}$
Genotype x water restitution	PP	2296.82	$3.9 \cdot 10^{-11}$
	Δ PP (%)	2367.21	$3.5 \cdot 10^{-11}$

The level of H_2O_2 in tomato puree from M82 and IL9adm grown at 100% and 50% water restitution treatments and relative ANOVA statistics are reported in Table 3.41 and Table 3.42, respectively. Overall, the absolute level of H_2O_2 did not change significantly according to genotype and water treatment. Neverthless, it increased massively upon the processing and these increases were significantly affected by the genotype, the water restitution level and their interaction. In particular, the highest increase in hydrogen peroxide was observed in M82 under 100% water restitution and the lowest increase in puree from M82 under the 50% water restitution treatment.

Table 3.41 – Mean and standard error of hydrogen peroxide concentration (nmol · g DW^{-1}) in M82 and IL9adm under two water restitution levels in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PP	2.17	0.11	2.39	0.11	1.95	0.33	2.48	0.20
Δ PP (%)	512.96	30.22	87.45	8.76	255.14	60.49	118.13	17.28

Table 3.42 – ANOVA statistics to evaluate effects of genotype and water restitution levels on hydrogen peroxide concentration in tomato sauce (PP) and its variation upon processing (Δ PP (%)).

Source of variability		F	*P*
Genotype	PP	0.11	0.74
	Δ PP	10.43	0.01
Water restitution	PP	3.23	0.11
	Δ PP (%)	63.96	$4.4 \cdot 10^{-5}$
Genotype x water restitution	PP	0.55	0.48
	Δ PP (%)	16.82	$3.4 \cdot 10^{-3}$

3.5 Effects of drought in open field trial on post-harvested fruit quality

The susceptible variety M82 and the IL9adm performed differential decay of fruit firmness over 53 days of post harvest (Fig. 3.10) and the decay was affected by the water restitution treatment. As a general remark, the decay significantly increased with the length of post harvest time (Tab. 3.48) and was significantly higher at the 50% water restitution treatment. Nevertheless, the % of firm fruit had a significant interaction "genotype x time" meaning that the two tomato lines performed differently depending on the length of the post harvest period. Also, the % of firm fruit had a significant interaction "genotype x water restitution", explaining the contrasting behaviour of the two tomato lines when the two water restitution treatment were individually considered.

Moreover we measured the loss of fruit weight during the 53 days of post harvest (Fig. 3.11 and Tab. 3.44). Although the fruit weight showed a steady decrease over the post harvest, significant effects of the two genotypes and of the two water treatments were not revealed by an ANOVA approach (Tab. 3.44).

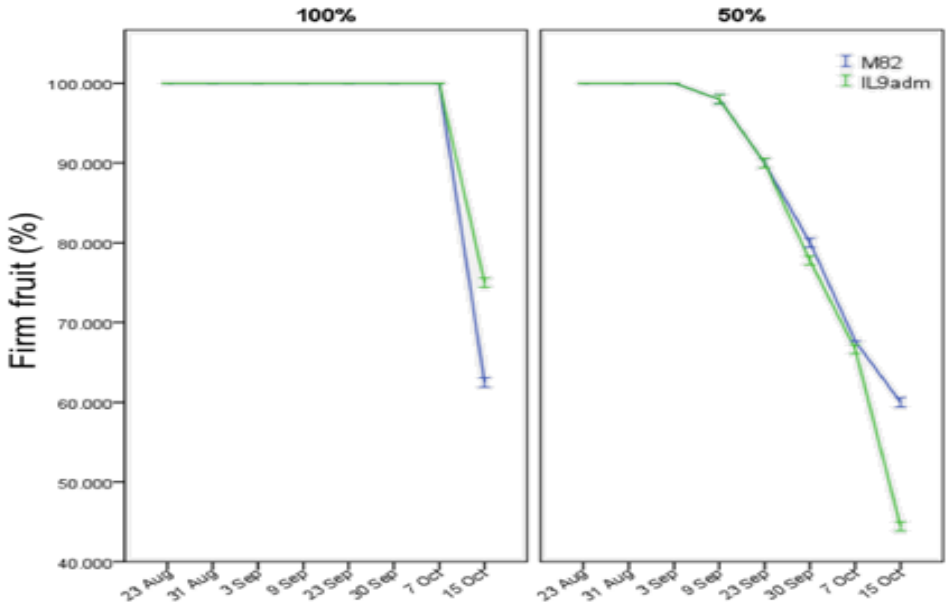

Figure 3.10 – Decay of firm fruit (%) in M82 and IL9adm grown under two different water restitution levels over a post harvest of 53 days.

Table 3.43 – ANOVA statistics to evaluate effects of genotype, water restituion levels and time on fruit firmness in M82 and IL9adm across 53 days of post harvest (53 dph).

Source of variability	df	F	*P*
Genotype	1	10.75	$1.6 \cdot 10^{-3}$
Water restitution	1	7420.00	$6.7 \cdot 10^{-68}$
Time	7	6540.44	$8.5 \cdot 10^{-89}$
Genotype x time	7	3.22	$5.4 \cdot 10^{-3}$
Genotype x water restitution	1	266.81	$1.1 \cdot 10^{-17}$
Water restitution x time	7	1309.92	$1.5 \cdot 10^{-15}$

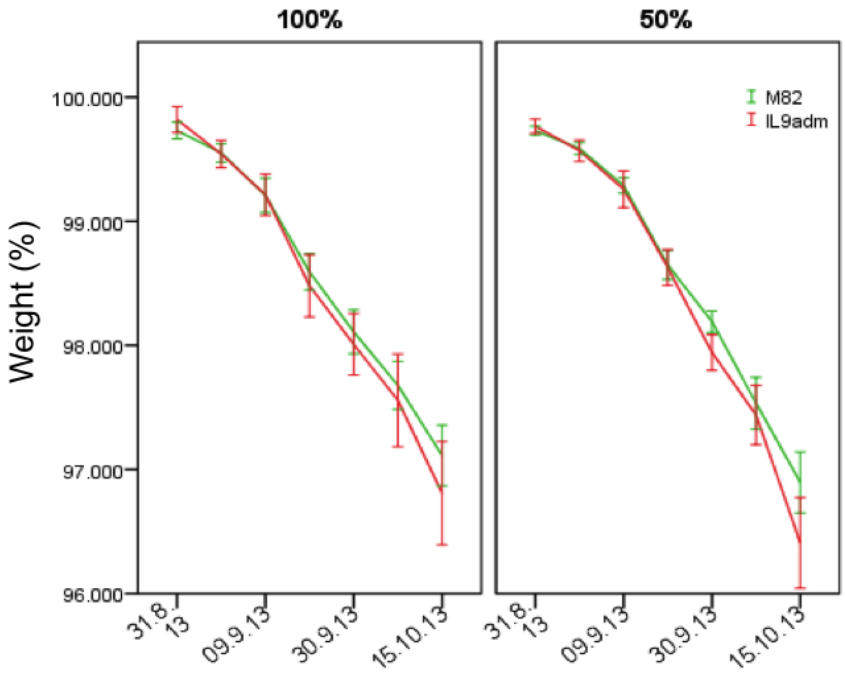

Figure 3.11 - Fruit weight variation (%) of M82 and IL9adm grown under two different water restitution levels, across 53 days post harvest.

Table 3.44 – ANOVA statistics to evaluate effects of genotype, water restituion levels and time on fruit weight of M82 and IL9adm, across 53 days of post harvest (53 dph).

Source of variability	df	F	P
Genotype	1	2.52	0.11
Water restitution	1	0.35	0.55
Time	6	158.29	$1.2 \cdot 10^{-15}$
Genotype x water restitution	1	0.012	0.73
Genotype x time	6	0.68	0.66
Water restitution x time	6	0.59	0.74

As for the fruit antioxidant content, 53 days after harvesting tomato fruit had a level of AsA in the mesocarp significantly affected by the genotype, the water restitution level and the interaction between the two main factors. Same was for the relative variation compared to the AsA concentration at harvesting in the flesh of red ripe fruit (Tab. 3.45 and Tab. 3.46). In particular, fruit from the variety M82 treated with 50% water restitution showed a 68% increase in AsA concentration compared to the

red ripe stage reaching 923.82 mg of AsA in 100g of DW. Conversely, the level of AsA in M82 fruit from the 100% water restitution treatment remained essentially unchanged across the post harvest storage. On the other hand, fruits from the IL9adm increased their AsA level of 20.77% if mother plant underwent to the 100% water restitution treatment wherease steadly maintained the same level of AsA as that showed at red ripe stage after 53 days from harvesting.

Table 3.45 - Mean and standard error AsA content (mg · 100g DW^{-1}) at 53 days post harvest (53 dph) its variation across storage (Δ 53 dph (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	487.39	1.25	923.82	1.38	711.21	1.54	565.55	0.97
Δ 53 dph (%)	-5.65	0.24	68.78	0.25	20.77	0.26	1.56	0.18

Table 3.46 – ANOVA statistics to evaluate effects of genotype and water restitution levels on the AsA concentration at 53 days post harvesting (53 dph) and its variation across storage (Δ 53 dph (%)).

Source of variability	Dependent variable	F	*P*
Genotype	53 dph	2471.08	$3.4 \cdot 10^{-14}$
	Δ 53 dph (%)	7555.66	$3.4 \cdot 10^{-13}$
Water restitution	53 dph	11835.78	$1.46 \cdot 10^{-12}$
	Δ 53 dph (%)	13835.76	$3.05 \cdot 10^{-14}$
Genotype x water restitution	53 dph	47255.10	$1.15 \cdot 10^{-14}$
	Δ 53 dph (%)	39791.92	$4.4 \cdot 10^{-16}$

The level of phenolic compounds after post harvest storage is significantly affected by the genotype, the water restitution level and the interaction "genotype x water restitution" (Tab. 3.47 and Tab. 3.48). Upon storage, total phenolics increased and the extend of the increase varied from 7.83% to 54.42%, significantly depending on the genotype, the water restitution treatment and the interaction between the two factors.

Table 3.47 – Mean and standard error of total phenolics (mg GAE · 100g DW^{-1}) at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	757.92	0.03	811.73	13.84	821.82	15.91	537.70	9.99
Δ 53 dph (%)	22.22	0.07	31.43	2.24	54.42	2.99	7.83	2.00

Table 3.48 – ANOVA statistics to evaluate effects of genotype and water restitution levels on total phenolics at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

Source of variability		F	P
Genotype	53 dph	81.09	$1.8 \cdot 10^{-5}$
	Δ 53 dph (%)	4.12	0.08
Water restitution	53 dph	97.42	$9.4 \cdot 10^{-6}$
	Δ 53 dph (%)	77.74	$2.2 \cdot 10^{-5}$
Genotype x water restitution	53 dph	209.73	$5.1 \cdot 10^{-7}$
	Δ 53 dph (%)	173.33	$1.1 \cdot 10^{-6}$

The level of total flavonoids in the tomato flesh after 53 days of storage varied significantly according to the water restitution treatments (Tab. 3.49 and Tab. 3.50). Nevertheless, the response to the 50% reduction of the water restitution was different in the two genotypes (the interaction "genotype x water treatment" was significant; Tab. 3.49 and Tab. 3.50). In fact, the 50% treatment increased the fruit level of total flavonoids after post harvest storage in M82 and had the opposite effect in IL9adm. Compared to the level of total flavonoids in the flesh of red ripe fruit at harvesting, the level of total flavonoids upon storage changed significantly according to the genotype, the water treatment and the interaction between them.

Table 3.49 - Mean and standard error of total flavonoids (mg QE · 100 g DW^{-1}) at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	129.30	1.25	148.97	1.38	165.26	1.54	115.61	0.98
Δ 53 dph (%)	-6.36	0.90	15.21	1.06	27.78	1.19	0.76	0.85

Table 3.50 – ANOVA statistics to evaluate effects of genotype and water restitution levels on the total flavonoid level at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

Source of variability		F	P
Genotype	53 dph	0.99	0.35
	Δ 53 dph (%)	94.83	$1.0 \cdot 10^{-5}$
Water restitution	53 dph	132.58	$2.9 \cdot 10^{-6}$
	Δ 53 dph (%)	7.26	0.03
Genotype x water restitution	53 dph	708.89	$4.2 \cdot 10^{-9}$
	Δ 53 dph (%)	577.67	$9.5 \cdot 10^{-9}$

An HPLC/UV - ESI MS MS approach allowed to quantify within the pericarp of fruit stored for 53 days, among most representative fractions, the flavonoids rutin and quercetin and two phenolic acids, caffeic acid and chlorogenic acid (Tab. 3.51). Analysis was carried out in order to know the phenolic compound composition. Specifically, the level of rutin, upon storage, was significantly affected by the genotype, the water restitution and the interaction between factors. It increased when the 50% water restitution treatment was applied and the increase was higher in IL9adm than M82. Also, the 53 day-storage decreased the level of rutin in all genotype x water restitution combinations except for IL9adm treated with 50% water restitution where the rutin level increased of 48.28% compared to the red-ripe stage. Moreover, the level of quercetin was not genotype-dependent and was significantly affected only by the water treatment. With the exception of tomato fruit from M82 plants undergone to 100% water restitution, the pericarp concentration of quercetin decreased upon post harvest storage from 31.35 to 43.81% (Tab. 3.51 and Tab. 3.52). As for phenolic acids, the level of chlorogenic acid at 53 days after harvesting was significantly affected by the genotype, the water treatment and the interaction between fixed factors (Tab. 3.52). In particular, it increased at 50% water treatment and underwent to a reduction from 60.43 to 82.26% upon storage when compared to the red ripe fruit.

Table 3.51 - Phenolic compounds in M82 and IL9adm under two water conditions at 53 days post harvesting (53 dph) and its variation across storage (Δ 53 dph (%)) detected by HPLC/UV-ESI MS MS.

		M82				IL9adm			
		100%		50%		100%		50%	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rutin (μg g DW^{-1})	53dph	155.00	0.06	176.85	0.06	113.61	0.06	309.96	0.06
	Δ 53 dph (%)	-5.14	0.04	-15.07	0.03	-14.61	0.04	48.28	0.03
Quercetin (μg g DW^{-1})	53dph	0.81	0.06	0.98	0.06	0.66	0.06	1.00	0.06
	Δ 53 dph (%)	5.36	7.47	-31.35	4.06	-34.55	5.73	-43.81	3.23
Caffeic acid (μg g DW^{-1})	53dph	0.04	0.01	0.06	0.01	0.05	0.01	0.05	0.01
	Δ 53 dph (%)	-84.52	2.39	-81.92	1.84	-82.38	2.04	-87.89	1.43
Chlorogenic acid (μg g DW^{-1})	53dph	2.79	0.06	3.75	0.06	4.92	0.06	7.09	0.06
	Δ 53 dph (%)	-82.26	0.37	-69.11	0.48	-68.68	0.37	-60.43	0.32

Table 3.52 - ANOVA statistics to evaluate effects of genotype and water restitution levels on phenolic fractions at 53 days post harvesting (53 dph) and its variation across storage (Δ 53 dph (%)).

Dependent variable		Source of variability	F	*P*
Rutin	53dph	Genotype	630925.40	$7.1 \cdot 10^{-21}$
		Water restitution	3570582.46	$6.9 \cdot 10^{-24}$
		Genotype x water restitution	2283794.94	$4.1 \cdot 10^{-23}$
	Δ 53 dph	Genotype	622737.40	$7.4 \cdot 10^{-21}$
		Water restitution	601393.00	$8.5 \cdot 10^{-21}$
		Genotype x water restitution	1136937.84	$6.7 \cdot 10^{-22}$
Quercetin	53dph	Genotype	1.23	0.30
		Water restitution	19.17	$2.4 \cdot 10^{-3}$
		Genotype x water restitution	2.50	0.15
	Δ 53 dph	Genotype	23.74	$1.2 \cdot 10^{-3}$
		Water restitution	18.29	$2.7 \cdot 10^{-3}$
		Genotype x water restitution	6.52	0.03
Caffeic acid	53 dph	Genotype	0.16	0.70
		Water restitution	2.46	0.16
		Genotype x water restitution	3.07	0.12
	Δ 53 dph	Genotype	0.96	0.36
		Water restitution	0.55	0.48
		Genotype x water restitution	4.30	0.07
Chlorogenic acid	53dph	Genotype	2243.23	$4.4 \cdot 10^{-11}$
		Water restitution	731.81	$3.7 \cdot 10^{-9}$
		Genotype x water restitution	110.20	$5.9 \cdot 10^{-6}$
	Δ 53 dph	Genotype	827.09	$2.3 \cdot 10^{-9}$
		Water restitution	764.92	$3.1 \cdot 10^{-9}$
		Genotype x water restitution	40.13	$2.2 \cdot 10^{-4}$

As for the total level of carotenoids after post harvest storge, a significant effect of the genotype, the water restitution and the interaction "genotype x watee restitution was detected (Tab. 3.53 and Tab. 3.54). Compared to the pericarp at red ripe stage, stored fruit after 53 days denoted an increase in the total level of carotenoid with the exception of IL9adm challenged with 50% water restitution treatment. Indeed, variation in the level of total carotenoids across storage was significantly affected by genotype, water treatment and interaction "genotype x water treatment".

Table 3.53 – Mean and standard error of of carotenois (μg · g DW^{-1}) at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	2659.82	0.06	2670.32	0.06	2844.96	0.06	1179.43	0.06
Δ 53 dph (%)	87.21	0.01	128.12	0.01	29.69	0.003	-11.00	0.01

Table 3.54 – ANOVA statistics to evaluate effects of genotype and water restitution levels on the total carotenoids amountat red ripe stage (RR), at 53 days post harvest (53 dph) and in tomato sauce (PP), its variation across storage (Δ 53 dph (%)) and its variation upon processing (Δ PP (%)).

Source of variability	Dependent variable	F	*P*
Genotype	53 dph	127872162.99	$4.1 \cdot 10^{-30}$
	Δ 53 dph (%)	347993808.10	$7.6 \cdot 10^{-32}$
Water restitution	53 dph	205433594.44	$6.2 \cdot 10^{-31}$
	Δ 53 dph (%)	422.50	$3.2 \cdot 10^{-8}$
Genotype x water restitution	53dph	210679174.56	$5.6 \cdot 10^{-31}$
	Δ 53 dph (%)	59931936.10	$8.6 \cdot 10^{-29}$

Table 3.55 reports the level of HPLC carotenoid fractions at 53 days after storage and their variation upon storage. Their sum was also evaluated and the lycopene/carotene ratio calculated. All carotenoid fractions as well as their sum and the lycopene/carotene ratio, were significantly affected by the genotype, the water treatment and the interaction between the two factors (Tab. 3.56). The only exception was the lycopene that resulted significantly affected by the genotype and the

interaction "genotype x water treatment". After 53 dph, the β-carotene content decreased cospicuosly in M82 with 100% water restitution and and IL9adm with 50% of water reintegration, while an increment of about 49% was observed in IL9adm 100%.

As for lycopene, it increased after 53 dph, with the highest increment observed for IL9adm at 100% of water restitution (43.67%).

Table 3.55 – Average and standard error of main carotenoids at at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

		M82				IL9adm			
		100%		50%		100%		50%	
Compound		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Lutein ($\mu g \cdot g\ DW^{-1}$)	53 dph	16.28	0.06	26.36	0.06	21.7	0.06	15.15	0.06
	Δ 53 dph	-1.05	0.35	15.97	0.25	7.36	0.29	-32.09	0.26
Lycopene ($\mu g \cdot g\ DW^{-1}$)	53 dph	6.69	0.06	9.24	0.06	10.38	0.06	7.64	0.06
	Δ 53 dph	0.92	0.87	3.29	0.65	43.67	0.80	0.68	0.76
α-carotene ($\mu g \cdot g\ DW^{-1}$)	53 dph	22.71	0.06	30.73	0.06	39.06	0.06	18.42	0.06
	Δ 53 dph	41.02	0.36	43.54	0.27	64.26	0.24	-29.68	0.22
β-carotene ($\mu g \cdot g\ DW^{-1}$)	53 dph	164.76	0.06	203.84	0.06	209.83	0.06	132.63	0.06
	Δ 53 dph	-37.01	0.02	-5.10	0.03	48.99	0.04	-27.59	0.03
Sum of peaks	53 dph	210.44	0.23	270.16	0.23	280.98	0.23	173.84	0.23
	Δ 53 dph	-30.03	0.08	0.85	0.09	46.30	0.12	-27.34	0.10
Lycopene/carotene	53 dph	0.04	0.001	0.04	0.001	0.04	0.001	0.05	0.001
	Δ 53 dph	49.62	1.20	4.29	0.60	-4.63	0.49	39.00	0.94

Table 3.56 - ANOVA statistics to evaluate effects of genotype and water restitution levels on HPLC carotenoid fractionss at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

		53 dph		Δ 53 dph	
Source of variability	Dependent variable	F	*P*	F	*P*
Genotype	Lutein	2504.66	$2.8 \cdot 10^{-11}$	4676.21	$2.3 \cdot 10^{-12}$
	Lycopene	328.14	$8.9 \cdot 10^{-8}$	673.38	$5.2 \cdot 10^{-9}$
	α-carotene	1224.45	$4.9 \cdot 10^{-10}$	8090.92	$2.6 \cdot 10^{-13}$
	β-carotene	51224.78	$1.6 \cdot 10^{-16}$	1038782.35	$9.6 \cdot 10^{-22}$
	Sum of peaks	3115.56	$1.2 \cdot 10^{-11}$	62447.15	$7.4 \cdot 10^{-17}$
	Lycopene/carotene	1000.05	$1.1 \cdot 10^{-9}$	130.48	$3.1 \cdot 10^{-6}$
Water restitution	Lutein	933.43	$1.4 \cdot 10^{-9}$	1496.83	$2.2 \cdot 10^{-10}$
	Lycopene	2.62	0.14	689.50	$4.8 \cdot 10^{-9}$
	α-carotene	11971.24	$5.4 \cdot 10^{-14}$	27070.43	$2.1 \cdot 10^{-15}$
	β-carotene	108996.33	$7.9 \cdot 10^{-18}$	513892.34	$1.6 \cdot 10^{-20}$
	Sum of peaks	10538.27	$9.1 \cdot 10^{-14}$	49283.32	$1.9 \cdot 10^{-16}$
	Lycopene/carotene	535.53	$1.3 \cdot 10^{-8}$	0.98	$3.5 \cdot 10^{-1}$
Genotype x water restitution	Lutein	20736.15	$6.0 \cdot 10^{-15}$	9484.65	$1.4 \cdot 10^{-13}$
	Lycopene	2098.04	$5.7 \cdot 10^{-11}$	859.23	$2.0 \cdot 10^{-9}$
	α-carotene	61607.06	$7.8 \cdot 10^{-17}$	30137.48	$1.4 \cdot 10^{-15}$
	β-carotene	1014148.4	$1.1 \cdot 10^{-21}$	3030245.32	$1.3 \cdot 10^{-23}$
	Sum of peaks	130512.74	$3.9 \cdot 10^{-18}$	294415.79	$1.5 \cdot 10^{-19}$
	Lycopene/carotene	90.28	$1.2 \cdot 10^{-5}$	2704.50	$2.1 \cdot 10^{-11}$

In order to comprehensively evaluate the overall level of antioxidants, the total antioxidant activity was estimated by the ORAC procedure (Tab. 3.57 and Tab 3.58). The post harvest storage led to a significant decrease of the antioxidant activity and this reduction operated at a far less extent in IL9adm fruit when plants were reintegrated with all the evapotranspired water 100% treatment). In general, the total antioxidant activity varied significantly with the genotype, the water treatment and the interaction between the two independent variables although the exent of the mean difference was relatively low.

Table 3.57 – Mean and standard error of fruit total antioxidant activity (µmol TE g $DW^{-}1$) in M82 and IL9adm under two water restitution levels at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	319.38	0.58	365.89	0.58	364.64	0.577	366.50	0.57
Δ 53 dph (%)	-69.54	0.05	-51.63	0.05	-1.57	0.055	-23.83	0.05

Table 3.58 – ANOVA statistics to evaluate effects of genotype and water restitution levels on fruit total antioxidant activity at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

Source of variability	Dependent variable	F	*P*
Genotype	53 dph	1578.20	$1.7 \cdot 10^{-10}$
	Δ 53 dph (%)	756156.98	$3.4 \cdot 10^{-21}$
Water restitution	53dph	1754.75	$1.1 \cdot 10^{-10}$
	Δ 53 dph (%)	1568.00	$1.8 \cdot 10^{-10}$
Genotype x water restitution	53dph	1495.08	$1.2 \cdot 10^{-10}$
	Δ 53 dph (%)	133040.31	$3.5 \cdot 10^{-18}$

Upon storage, the level of pericarp hydrogen peroxide showed a massive increase with increments accounting for genotype, water restitution and interaction significant effects (Tab. 3.59 and Tab. 3.60). Also, pericarp samples from plant treated with 50% water restitution showed an increase only in M82 whose absolute levels were higher than IL9adm.

Table 3.59 – Mean and standard error of hydrogen peroxide concentration (nmol · g DW^{-1}) in M82 and IL9adm under two water restitution levels at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
53 dph	2.71	0.01	2.79	0.01	2.42	0.01	2.32	0.01
Δ 53 dph (%)	664.17	1.63	118.52	0.45	341.46	1.05	103.96	0.51

Table 3.60 – ANOVA statistics to evaluate effects of genotype and water restitution levels on fruit hydrogen peroxide level at 53 days post harvest (53 dph) and its variation across storage (Δ 53 dph (%)).

Source of variability	Dependent variable	F	*P*
Genotype	53 dph	4335.80	$3.1 \cdot 10^{-12}$
	Δ 53 dph (%)	26952.72	$2.1 \cdot 10^{-15}$
Water restitution	53 dph	5.71	0.04
	Δ 53 dph (%)	145325.71	$2.5 \cdot 10^{-18}$
Genotype x water restitution	53 dph	257.71	$2.3 \cdot 10^{-7}$
	Δ 53 dph (%)	22500.44	$4.4 \cdot 10^{-15}$

3.6 – Root phenotyping in an hydroponic trial

In order to research morphological and physiological mechanism leading to an higher drought tolerance in IL9adm a comparative study of the root system was carried out in hydroponic culturing. The figure 3.12 displays the number, the weight and the linear development of adventicious roots, the weight and the length of the taproot and the aboveground fresh mass.

The ANOVA test (Tab. 3.61) showed a significant higher taproot weight in IL9adm plants than M82.

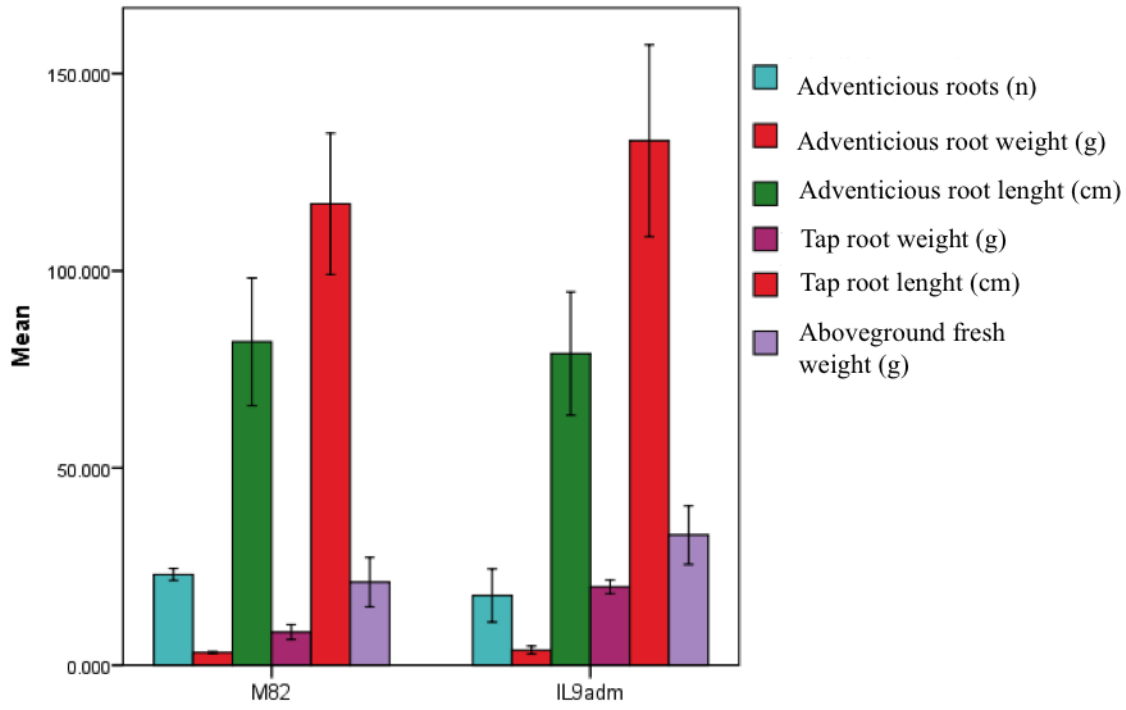

Figure 3.12 – Root features of M82 and IL9adm grown in an hydroponic system.

Table 3.61 – ANOVA statistics to evaluate effects of the genotype on M82 and IL9adm root morphology in an hydroponic system.

Source of variability	Dependent variable	F	*P*
Genotype	Adventicious roots (No.)	0.60	0.48
	Adventicious root weight (g)	0.37	0.57
	Adventicious roots length (cm)	0.02	0.90
	Tap root weight (g)	19.92	0.01
	Tap root length (cm)	0.28	0.62
	Aboveground fresh weight (g)	1.51	0.29

3.7 Effects of drought in lysimeter trials

In order to evaluate the drought effects on M82 and IL9adm fruits in more finely controlled conditions, plants were grown in lysimeters where water treatments (100% and 50% water restitutions) were applied under a plastic tunnel and fruits were colleted at harvesting. Yield performaces were express in terms of marketable yield, total tield, scrap fruit, marketable fruit in terms of soluble solids and total yield in terms of soluble solids (Tab. 3.62). According to the ANOVA test, the genotype was

significant source of variation only for scrap fruit wherease the water treatments significantly affected the marketable yield, the total yield and the total yielded soluble solids (Tab. 3.63). Therefore, when the yield performances were considered in terms of percent of reduction at 50% water treatment compared to 100% (Blum index; Fig. 3.13), scrap fruit, total yielded soluble solids and marketable yielded soluble solids significantly differentiated the response in M82 and IL9adm.

Table 3.62 – Mean value and standard error (SE) of yield features performances in M82 and IL9adm grown under two different water restitution levels (100% and 50%) in lysimeters.

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Marketable yield (kg · plant^{-1})	0.62	0.01	2.68	0.10	0.63	0.11	0.34	0.10
Total yield (kg · plant^{-1})	0.72	0.04	3.36	0.07	0.82	0.12	0.57	0.01
Scrap (kg · plant^{-1})	0.012	0.002	0.02	0.007	0.09	0.04	0.15	0.008
Marketable yield in SS (g · plant^{-1})	42.09	2.44	28.51	5.99	47.51	8.52	31.22	6.90
Total yield in SS (g · plant^{-1})	48.22	0.41	31.73	3.93	62.16	11.54	52.00	5.06

Table 3.63 - ANOVA statistics to evaluate effects of genotype and water restituion levels on yield features registered on tomato plants allevated in lysimeters.

Source of variability	Dependent variable	F	*P*
Genotype	Marketable yield	0.24	0.64
	Total yield	2.56	0.15
	Total scrap	19.79	$3.0 \cdot 10^{-3}$
	Marketable yield in soluble solids	1.32	0.29
	Total yield in soluble solids	6.93	0.03
Water restitution	Marketable yield	11.55	0.01
	Total yield	9.67	0.02
	Total scrap	1.75	0.23
	Marketable yield in soluble solids	6.30	0.04
	Total yield in soluble solids	4.51	0.07
Genotype x water restitution	Marketable yield	0.13	0.73
	Total yield	0.48	0.51
	Total scrap	1.10	0.33
	Marketable yield in soluble solids	0.07	0.79
	Total yield in soluble solids	0.62	0.46

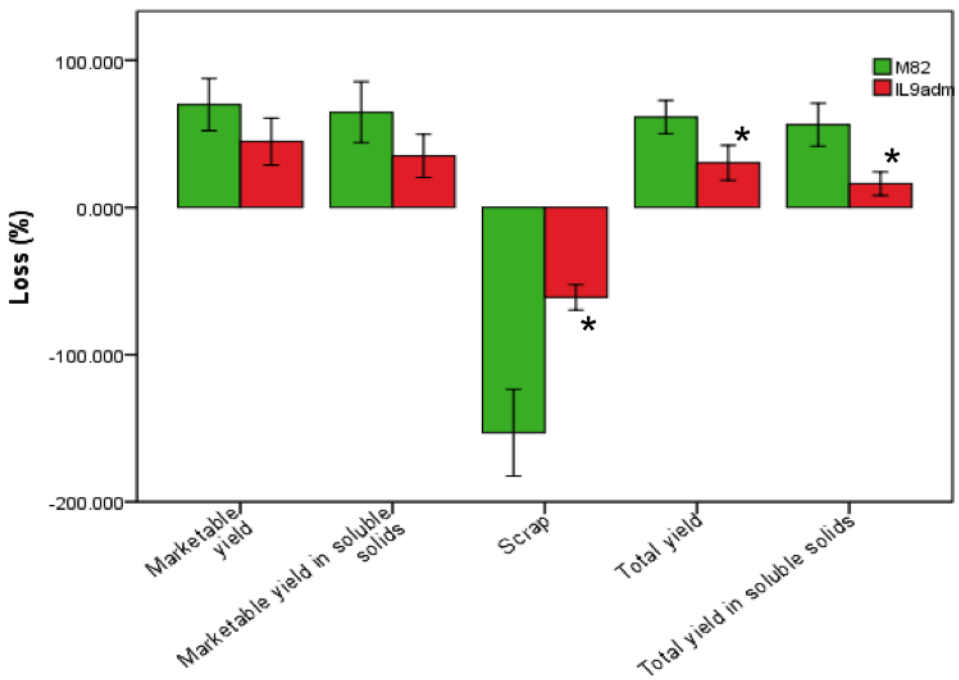

Figure 3.13 - Blum indexes of yield performances. * statistically significant difference between M82 and IL9adm at Student's t test coupled with 2000 bootstrapping re-sampling.

The fruit soluble solid content (SSC) increased from 6.65 ± 0.29 °Brix to 7.64 ± 0.21 °Brix in M82 when a 50% reduction of the water reintegration was applied. Conversely, higher levels of fruit SSC were achieved in the more tolerant IL9adm ranging from 7.56 ± 0.13 °Brix to 9.19 ± 0.67 °Brix at 100% and 50% water treatment, respectively (Tab. 3.64) The ANOVA test highlighted a significant effect of the genotype and the water restitution on SSC (Tab. 3.65).

On average, the firmness was 25.65 ± 2.26 and 31.16 ± 2.20 N · mm^{-2} for M82 and IL9adm, respectively. A 50% reduction in water reintegration caused contrasting effects on firmness in M82 and IL9adm and all observed effects resulted not significant at the ANOVA test (Tab. 3.65).

Table 3.64 – Mean and standard error of soluble solid content (SSC) and firmness of M82 and IL9adm fruits grown under two different water restituion levels.

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
SSC (°Brix)	6.65	0.29	7.64	0.21	7.56	0.13	9.19	0.67
Firmness (N · mm^{-2})	25.65	2.26	30.35	2.08	31.16	2.20	26.82	2.36

Table 3.65 - ANOVA statistics to evaluate effects of genotype and water restituion levels on soluble solid content (SSC) and firmness.

Source of variability	Dependent variable	F	*P*
Genotype	SSC	17.00	$4.4 \cdot 10^{-3}$
	Firmness	0.27	0.62
Water restitution	SSC	19.28	$3.1 \cdot 10^{-3}$
	Firmness	0.01	0.93
Genotype x water restitution	SSC	1.16	0.32
	Firmness	5.62	0.05

Table 3.66 summarizes the fruit level of total AsA, total carotenoids and total phenolics detected in M82 and IL9adm grown under 100% and 50% water restitution treatments. The 50% reduction in water reintegration caused a significant increase of total AsA only in IL9adm.

Table 3.66 – Mean and standard error of total ascorbic acid (Tot AsA), total carotenoids and total phenolic compounds in M82 and IL9adm grown under two different water restitution levels (100% and 50%). * statistically significant difference of 50% water restitution vs 100% at Student's t tests coupled with 2000 bootstrapping re-sampling ($P< 0.05$); # statistically significant difference of IL9adm vs M82 at Student's t tests coupled with 2000 bootstrapping re-sampling ($P< 0.05$).

	M82				IL9adm			
	100%		50%		100%		50%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tot AsA ($mg \cdot 100\ g\ DW^{-1}$)	355.59	43.87	414.45	38.80	288.10	25.81	513.56*	129.71
Tot carotenoids ($\mu g\ g\ DW^{-1}$)	5.78	0.89	9.83*	0.12	7.35	2.15	10.49	2.57
Total phenolics ($mgGAE \cdot 100\ g\ DW^{-1}$)	81.50	8.43	76.90	2.61	73.87	4.11	107.87*#	3.21

The reduced water restitution treatment also increased the fruit level of total carotenoids in M82 and the total level of phenolics in IL9adm. The fruit level of total penolics in IL9adm under 50% water treatment reached 107.87 ± 3.21 mg GAE $\cdot$ 100 g DW^{-1} that was significantly higher than the level observed in fruit from M82 at the same conditions

3.8 – Identification of candidate genes mediating fruit quality and response to drought

In order to investigate the molecular network controlling fruit quality-related processes in response to water deficit, a transcriptomic analysis was performed on a 90k Combimatrix TomatArray 1.0 comparing red-ripe fruit from IL9adm and M82 at two different water treatments grown in the lysimeter trial hereabove described.
The statistical elaboration of transcriptomic data through a two-way ANOVA enabled the identification of 456 transcripts differentially expressed between genotypes, 541 transcripts differentially expressed between water treatments ad 546 transcipts showing significant interaction genotype x water treatment (Fig. 3.14).

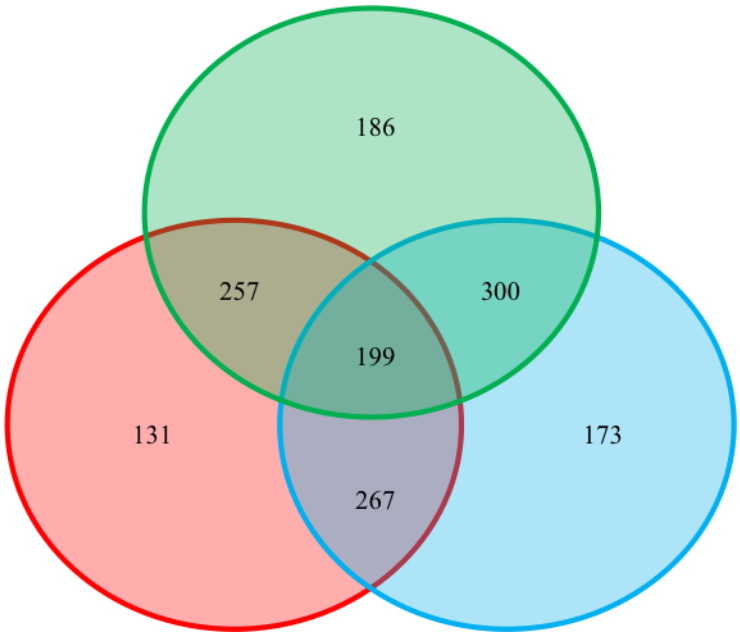

Figure 3.14 - Venter's diagram illustrating transcripts with significant effect of the genotype (red ellipse), transcript with significant effect of the water restitution level (blu ellipse) and transcripts with significant interaction «Genotype x Water restitution» (green ellipse) at ANOVA test ($P< 0.01$).

The set of 546 transcripts with significant interaction represented about 2.69 % of the total transcripts located on the chip and their distribution across "Gene Onthology" (GO) terms are displayed in the figure 3.15.

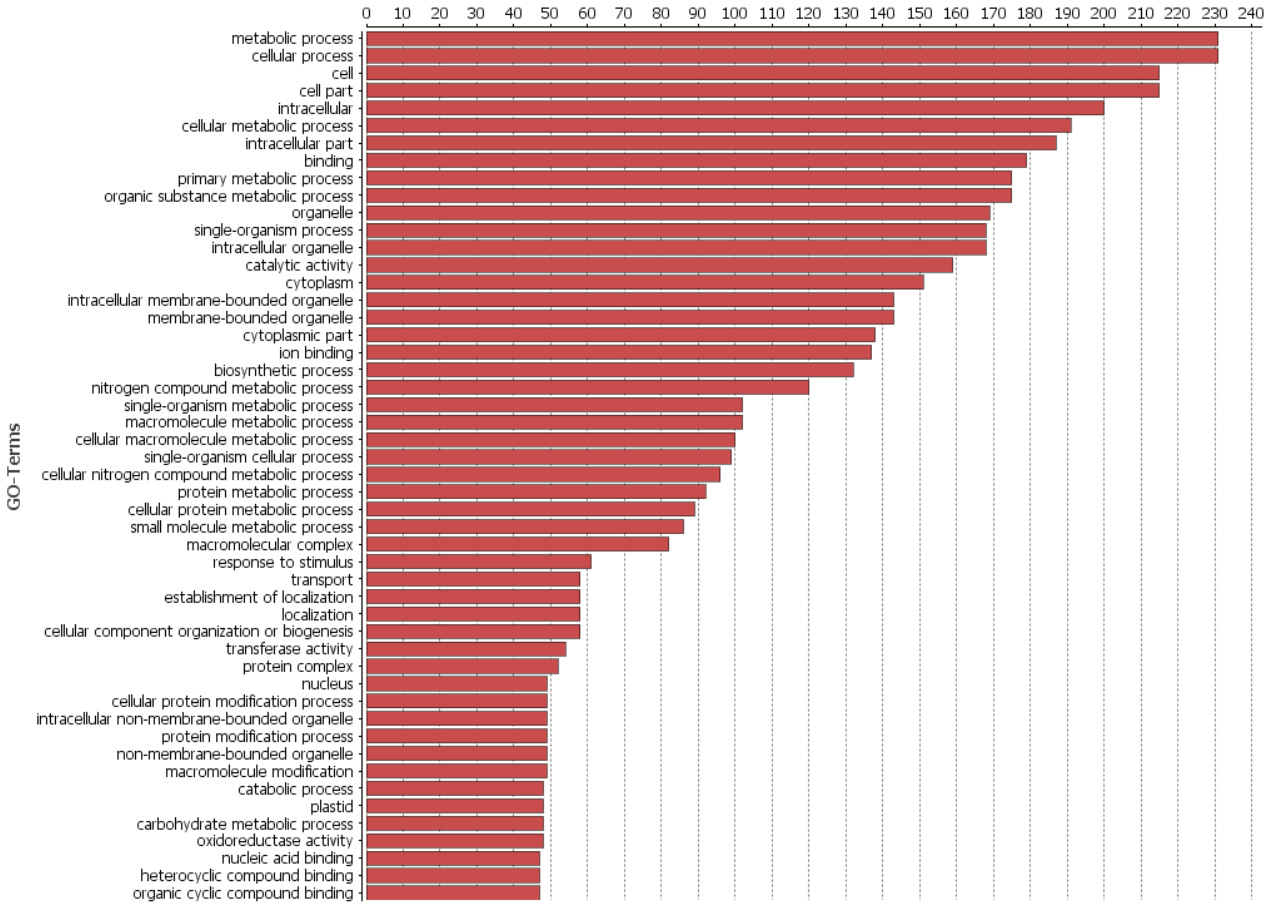

Figure 3.15 - Distribution of 546 transcripts with significant interaction «Genotype x Water treatment» over GO terms computed by using Blast2GO software suite.

The enrichment analysis of the 546 differentially expressed genes was performed by using the software AgriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) against the Solyc gene models (www.solgenomics.org) represented on the microarray slide and returned no enriched GO categories. Figure 3.16 illustrates the comparative distribution (expressed as percentage) of GO annotations in the set of 546 transcripts with significant interaction «Genotype x Water treatment» and in the overall list of GO annotations represented on the microarray slide.

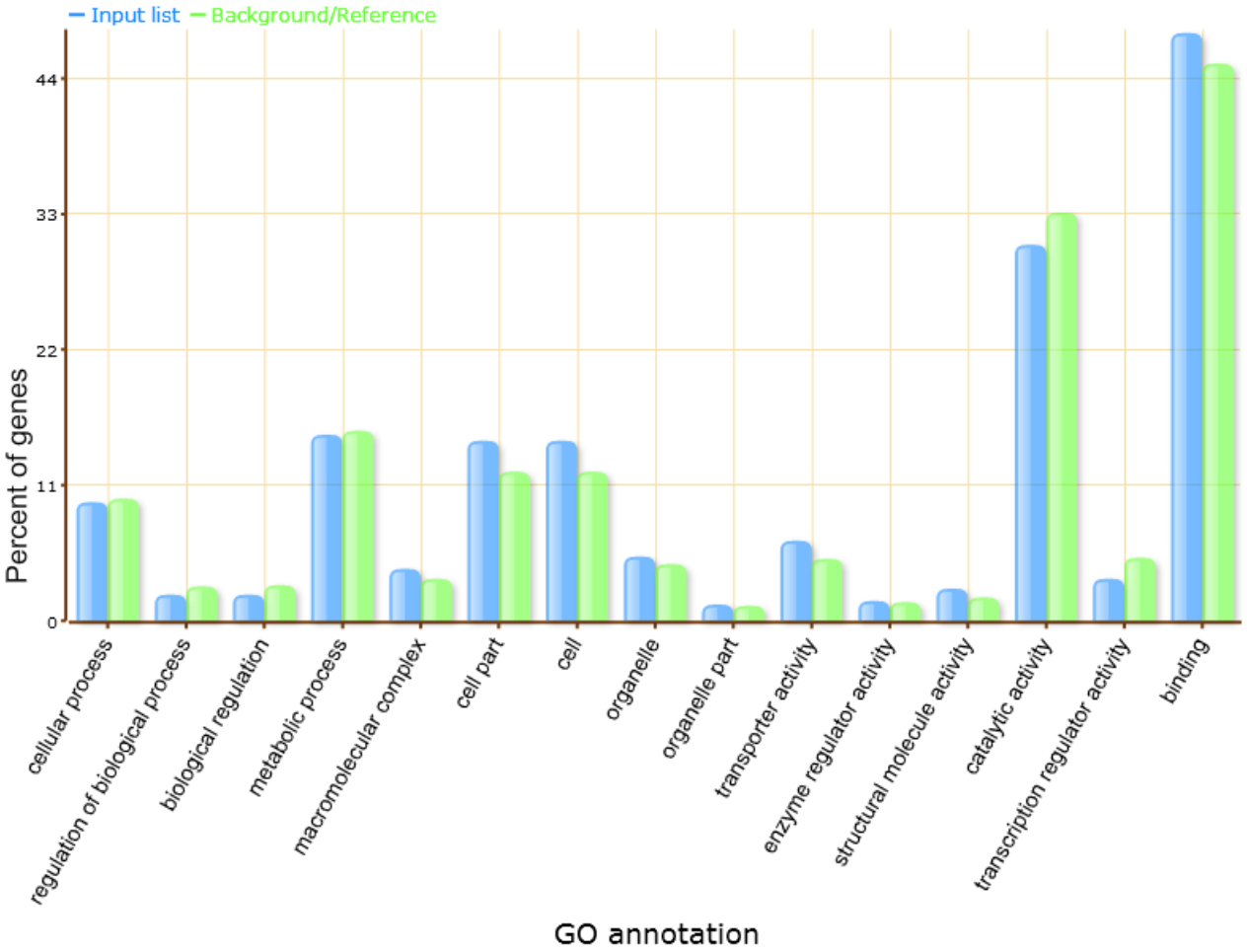

Figure 3.16 - Comparative distribution of gene models over GO annotations in the set of 546 transcripts with significant interaction «Genotype x Water treatment» (input list) and in the overall list of gene models represented on the microarray slide (Background/Reference) as computed by AgriGO singular enrichment analysis (SEA - http://bioinfo.cau.edu.cn/agriGO/analysis.php).

The set of 546 transcripts with significant interaction were mapped on metabolic pathways by using the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml) and candidate tanscripts controlling fruit quality traits and their modulation in response to drought were selected (Fig. 3.17, 3.18, 3.19, 3.20, 3.21, 3.22 and Tab. 3.67).

In particular, an ADP-glucose pyrophoshorylase was mapped to the starch biosynthesis pathway while an alpha-amylase and an hexokinase gene were mapped to the starch degradative pathway (Fig. 3.17).

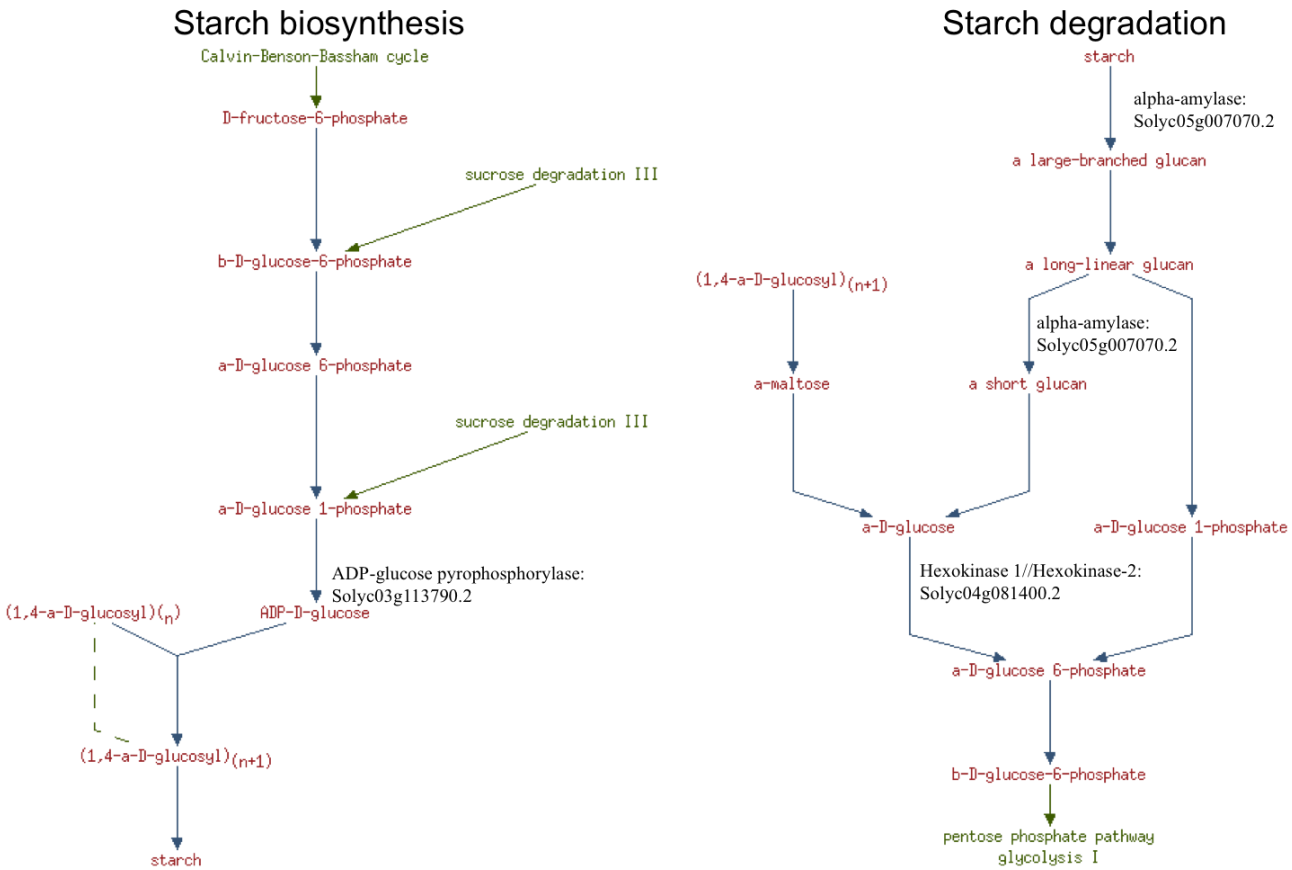

Figure 3.17 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the starch biosynthetic and degradative pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

As for pectin and cellulose metabolism, potentially involved in mediating mainly the fruit firmness, a glycosyltransferase gene was mapped on the pectin biosynthesis pathway, a polygalacturonase gene to the pectin catabolism and two cellulose synthase genes and an endoglucanase gene were mapped to the cellulose metabolism (Fig. 3.18).

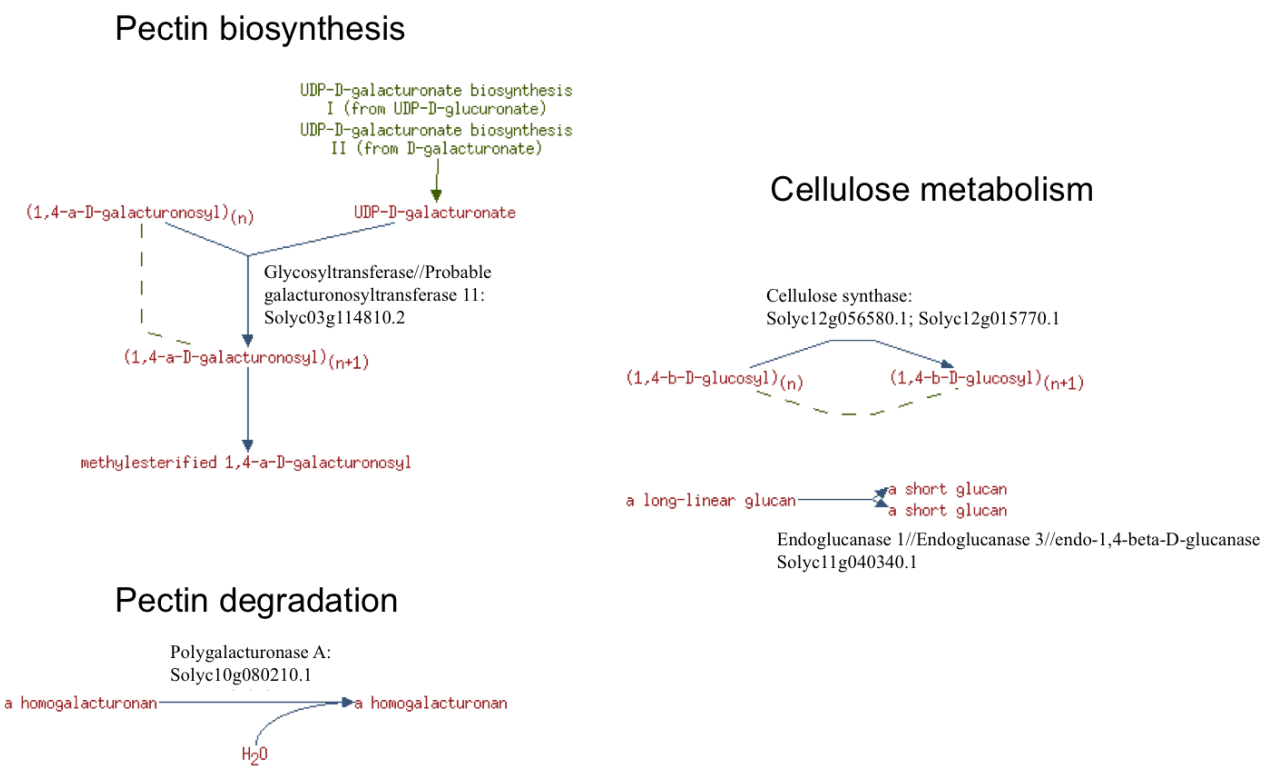

Figure 3.18 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the pectin and cellulose metabolic pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

A cytosine-specific methyltransferase gene and two 1-aminocyclopropane-1-carboxylate oxidase genes were mapped to the ethylene biosynthetic pathway that may act as master regulator mechanism in mediating the fruit response to drought (Fig 3.19).

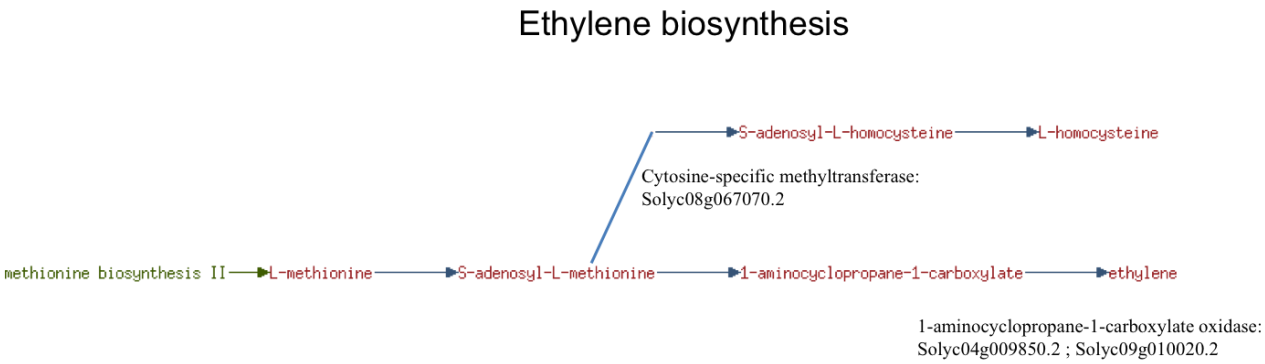

Figure 3.19 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the ethylene pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

Although no genes within the set of 546 transcripts with significant interaction were mapped to the carotenoids pathway, a 3-ketoacyl-CoA thiolase, a 3-hydroxy-3-methylglutaryl coenzyme A synthase and a diphosphomevalonate decarboxylase genes were mapped to the mevalonate pathway that is involved in synthesizing precursors committed to the carotenoids pathway (Fig 3.20).

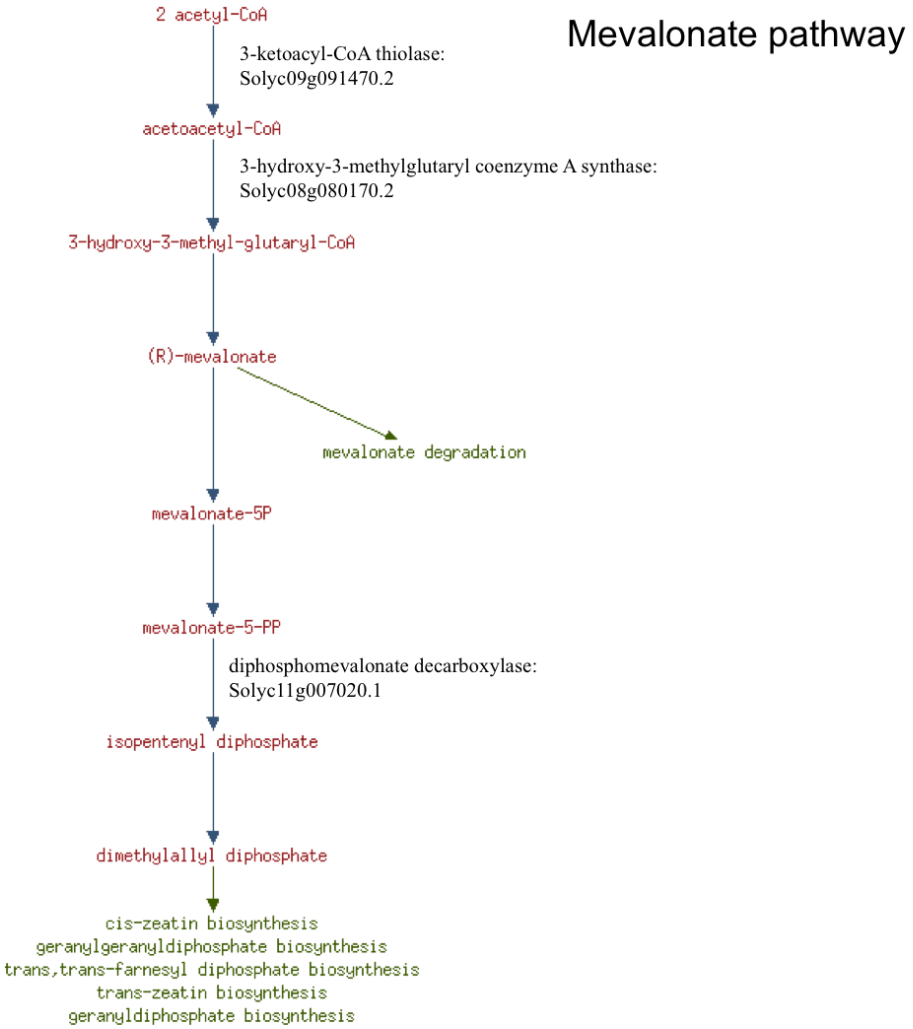

Figure 3.20 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the mevalonate pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

Seven differentially expressed transcripts were involved in the AsA pathways (Fig. 3.21). In particular, a GDP-D-mannose pyrophosphorylase, a GDP-L-galactose phosphorylase and an hexokinase genes were mapped to the Smirnoff Wheeler pathway, an UDP-D-glucose dehydrogenase and a galactokinase genes were mapped to the myo-inositol pathway, a polygalacturonase gene was mapped to the pectin degradation pathway and a gene annotated as L-ascorbate oxidase was mapped to the asA recycling pathway. All these gene are candidate in mediating the control of ascorbate accumulation in the response to drought.

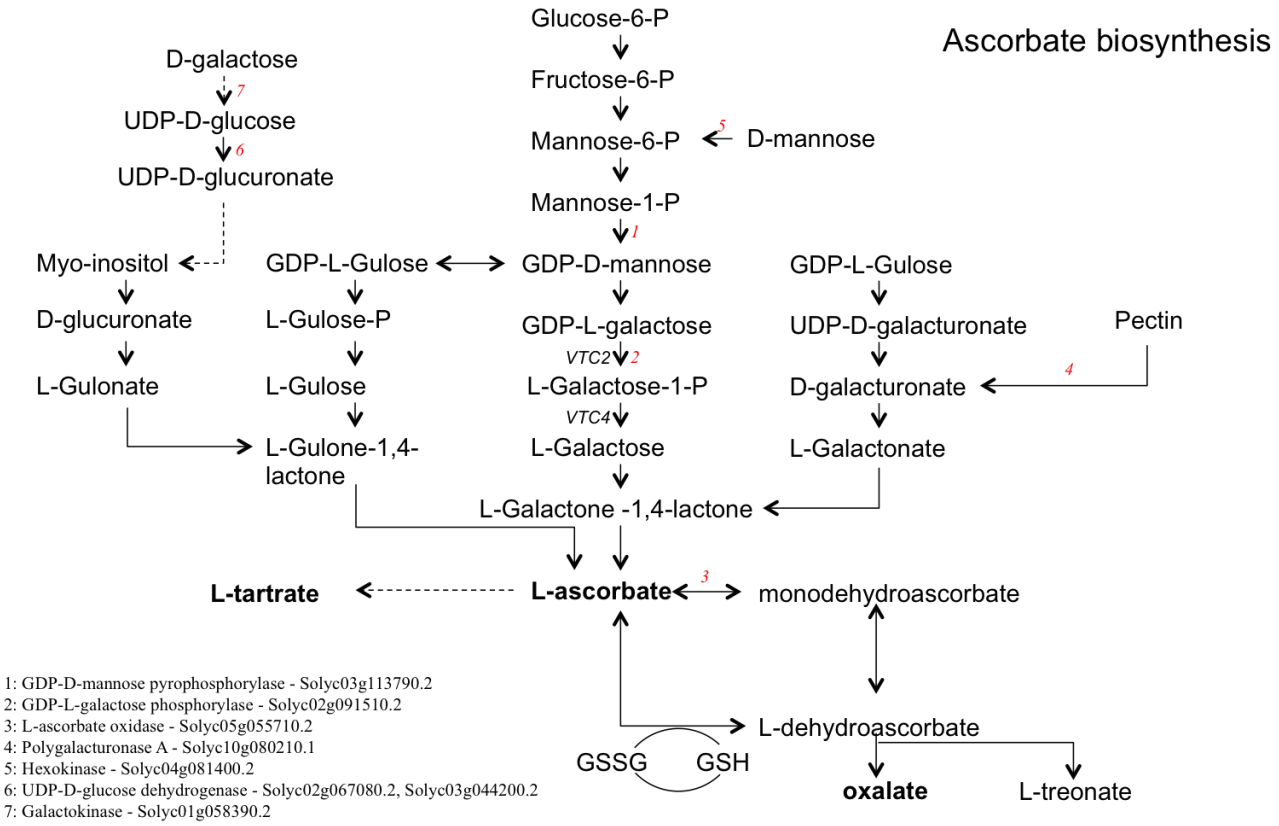

Figure 3.21 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the ascorbate alternative pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

As for mechanisms controlling the accumulation in the fruit of phenolics, among genes with significant interaction 'genotype x water treatment' two cytochrome P450, a dihydroflavonol-4-reductase, and an acyl-transferase genes were mapped to the phenylpropanoid metabolism (Fig. 3.22). In particular, the cytochrome P450s are

involved in the synthesis of the luteolin from the apigenin, in the synthesis of trans dihydroquercetin and in the formation of the flavanone eriodictyol from the naringenin (Fig. 3.22).

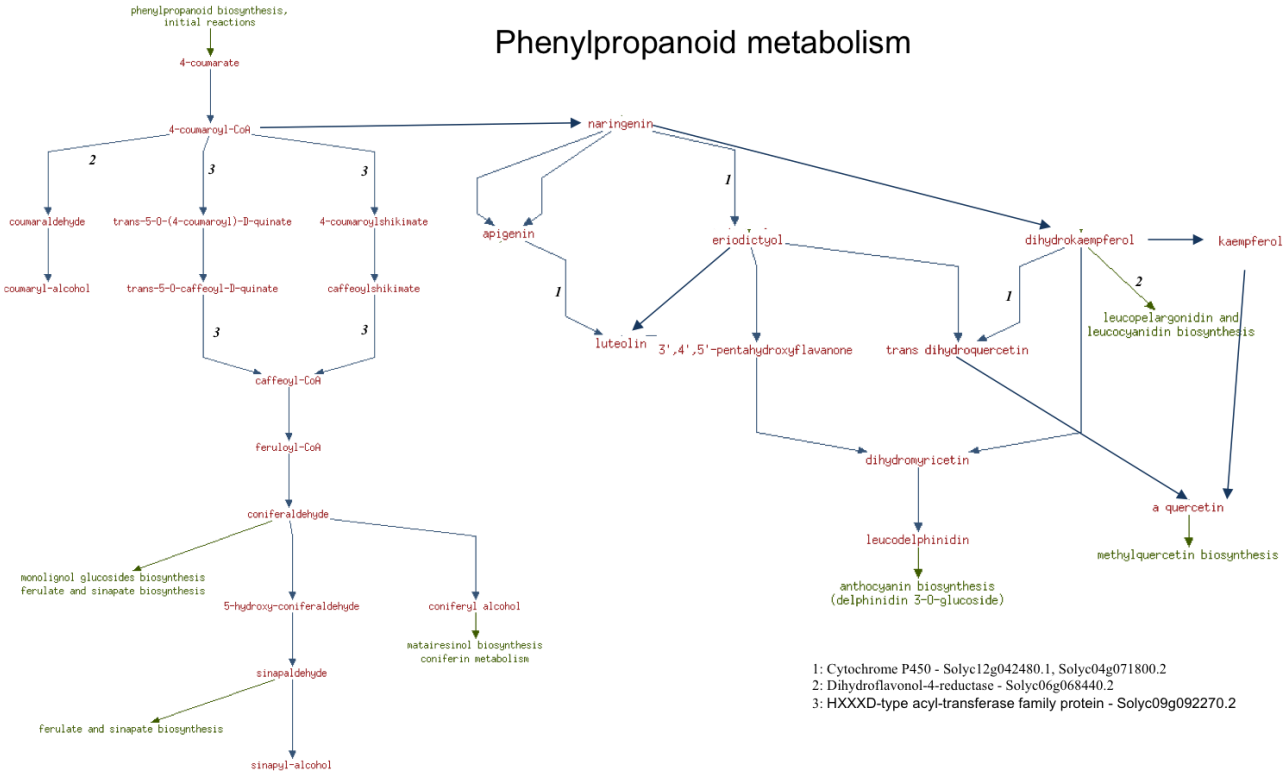

Figure 3. 22 - Schematic representation of transcripts with significant interaction «Genotype x Water treatment» mapped on the phenylpropanoid metabolic pathways by the Omics Data Analysis available at the Solcyc server (http://solcyc.solgenomics.net/overviewsWeb/celOv.shtml).

All candidate genes presumably involved in controlling fruit quality in response to drought were selected from the 546 transcripts with significant interaction are reported in (Tab. 3.67). The expression level of candidate genes listed in Table 3.67 were validated by RTq-PCR and the fold change of the transcript abundance is reportred in the same table.

Table 3.67 - Annotation details of selected candidate gene in the microarray experiment showing significant interaction "Genotype x Water treatment" at the ANOVA test ($P< 0.01$). Change in the transcript levels are reported according to RTq-PCR validation.

Gene model	Functional descritpion	Solcyc pathway	Chromosome assignment	Fold change (FC) expression compared to M82 100% (mean ± standard error)		
				M82 50%	IL9adm 100%	IL9adm 50%
Solyc01g058390.2.1	Galactokinase	galactose degradation II; UDP-galactose biosynthesis (salvage pathway from galactose using UDP-glucose)	SL2.40ch01	1.35 ± 0.27	0.53 ± 0.05	0.31 ± 0.05
Solyc02g091510.2.1	GDP-L-galactose phosphorylase 2	ascorbate biosynthesis I (L-galactose pathway)	SL2.40ch02	-1.61 ± 0.17	-0.17 ± 0.27	-0.31 ± 0.07
Solyc03g044200.2.1	Alcohol dehydrogenase	formaldehyde oxidation II (glutathione-dependent); galactose degradation II; glycine betaine biosynthesis II (Gram-positive bacteria); isoleucine degradation II; leucine degradation III; oxidative ethanol degradation I; phenylalanine degradation III; phenylethanol biosynthesis; UDP-D-xylose biosynthesis	SL2.40ch03	1.07 ± 0.09	-0.03 ± 0.05	-0.35 ± 0.14
Solyc03g113790.2.1	Mannose-1-phosphate guanyltransferase	ascorbate biosynthesis I (L-galactose pathway); colanic acid building blocks biosynthesis; GDP-mannose biosynthesis I; GDP-mannose biosynthesis II; GDP-mannose metabolism; glycogen biosynthesis I (from ADP-D-Glucose); starch biosynthesis	SL2.40ch03	-1.35 ± 0.23	-0.45 ± 0.19	-0.15 ± 0.24
Solyc03g114810.2.1	Glycosyltransferase	homogalacturonan biosynthesis	SL2.40ch03	1.55 ± 0.24	-0.05 ± 0.1	-0.68 ± 0.00
Solyc04g009850.2.1	1-aminocyclopropane-1-carboxylate oxidase-like protein	ethylene biosynthesis from methionine	SL2.40ch04	-1.31 ± 0.15	-0.31 ± 0.2	0.12 ± 0.46
Solyc04g071800.2.1	Cytochrome P450	flavonol biosynthesis; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis; luteolin biosynthesis; syringetin biosynthesis	SL2.40ch04	-1.62 ± 0.14	0.04 ± 0.19	0.15 ± 0.28
Solyc04g081400.2.1	Hexokinase 1	GDP-glucose biosynthesis; GDP-mannose metabolism; glycolysis III; mannitol degradation II; starch degradation; sucrose degradation I; sucrose degradation III	SL2.40ch04	1.38 ± 0.22	0.27 ± 0.18	0.10 ± 0.01
Solyc05g007070.2.1	Alpha amylase 2	starch degradation	SL2.40ch05	1.45 ± 0.07	-0.06 ± 0.12	-0.39 ± 0.17
Solyc05g055710.2.1	Ascorbate oxidase	ascorbate glutathione cycle	SL2.40ch05	2.09 ± 0.05	-0.22 ± 0.27	0.00 ± 0.17
Solyc06g068440.2.1	Cinnamoyl-CoA reductase	capsiconiate biosynthesis; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis; phenylpropanoid biosynthesis	SL2.40ch06	-1.79 ± 0.07	-0.88 ± 0.31	-0.29 ± 0.41
Solyc08g067070.2.1	Cytosine-specific methyltransferase	methionine degradation I (to homocysteine)	SL2.40ch08	1.42 ± 0.1	-0.14 ± 0.11	-0.25 ± 0.29
Solyc09g010020.2.1	1-aminocyclopropane-1-carboxylate oxidase	ethylene biosynthesis from methionine	SL2.40ch09	1.02 ± 0.13	0.31 ± 0.1	-0.03 ± 0.22
Solyc09g092270.2.1	Hydroxycinnamoyl-CoA transferase	chlorogenic acid biosynthesis I; chlorogenic acid biosynthesis II; phenylpropanoid biosynthesis; simple coumarins biosynthesis	SL2.40ch09	2.88 ± 0.22	-0.02 ± 0.08	-0.32 ± 0.35
Solyc10g080210.1.1	Polygalacturonase A	homogalacturonan degradation	SL2.40ch10	1.55 ± 0.06	0.82 ± 0.25	0.61 ± 0.07
Solyc11g007020.1.1	Diphosphomevalonate decarboxylase-like protein	mevalonate pathway	SL2.40ch11	-2.24 ± 0.23	-0.55 ± 0.18	-0.30 ± 0.4
Solyc11g040340.1.1	Endoglucanase 1	cellulose biosynthesis	SL2.40ch11	-2.01 ± 0.19	-0.16 ± 0.13	-0.21 ± 0.07
Solyc12g015770.1.1	Cellulose synthase	cellulose biosynthesis	SL2.40ch12	-1.45 ± 0.07	-0.33 ± 0.21	-0.03 ± 0.27
Solyc12g042480.1.1	Cytochrome P450	flavonol biosynthesis; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis; luteolin biosynthesis	SL2.40ch12	1.71 ± 0.18	-0.37 ± 0.09	-0.12 ± 0.05
Solyc12g056580.1.1	Cellulose synthase	cellulose biosynthesis	SL2.40ch12	0.85 ± 0.1	-0.03 ± 0.03	-0.03 ± 0.1

As for the set of 544 transcripts with significant effect of the "water restitution" treatment, the enrichment analysis did not result in any over-represented GO category. The comparative distribution (expressed as percentage) of gene models over GO annotations in the set of 544 transcripts with significant effect of the water treatment and of the overall list of gene models represented on the microarray slide is displayed in the Figure 3.23.

Also, differentially expressed transcripts were mapped to metabolic pathways and candidate genes for fruit quality traits are listed in the Table 3.68. Among 17 selected genes, 4 were mapped to the ethylene metabolism, 3 were mapped to the cellulose biosynthesis, 1 to the mevalonate pathway, 2 to the phenylpropanoid metabolism and 7 to the AsA metabolism.

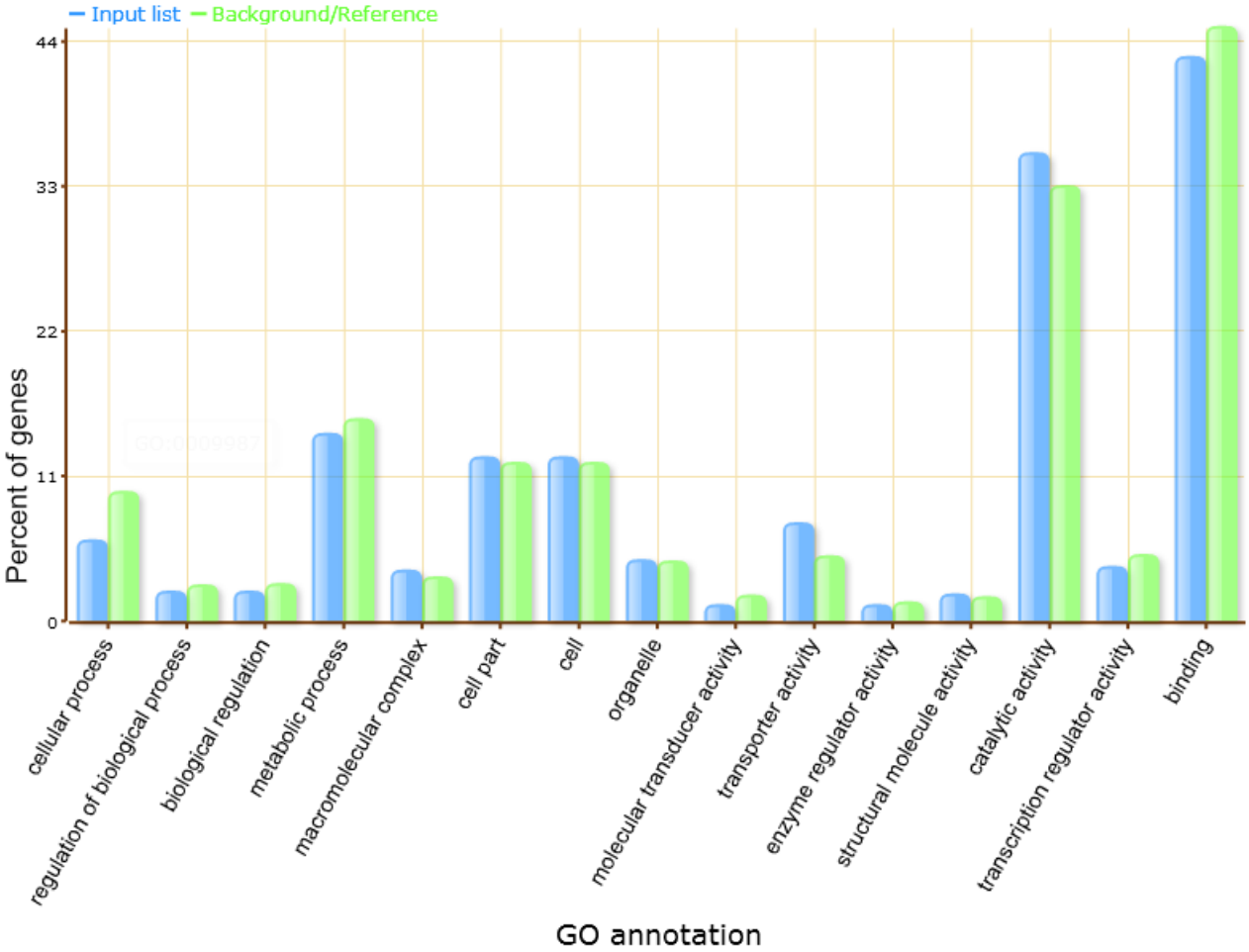

Figure 3.23 - Comparative distribution of gene models over GO annotations in the set of 544 transcripts with significant «Water treatment» (input list) effect and in the overall list of gene models represented on the microarray slide (Background/Reference) as computed by AgriGO singular enrichment analysis (SEA - http://bioinfo.cau.edu.cn/agriGO/analysis.php).

Table 3.68 - Annotation details of selected candidate gene in the microarray experiment showing a significant effect of the "water treatment" at the ANOVA test ($P< 0.01$). Change in the transcript levels are reported according to RTq-PCR validation.

Gene model	Functional descritpion	Solcyc pathway	Chromosome assignment	Fold change (FC) expression compared to M82 100% (mean ± standard error)		
				M82 50%	IL9adm 100%	IL9adm 50%
Solyc08g067070.2	Cytosine-specific methyltransferase	methionine degradation I (to homocysteine)	SL2.40ch08	1.42 ± 0.1	-0.14 ± 0.11	-0.25 ± 0.15
Solyc12g100330.1	Cytosine-specific methyltransferase	methionine degradation I (to homocysteine)	SL2.40ch12	0.89 ± 0.11	-0.2 ± 0.08	0.11 ± 0.15
Solyc02g071380.2	1-aminocyclopropane-1-carboxylate oxidase 3	leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis	SL2.40ch02	0.92 ± 0.2	-0.31 ± 0.19	0.47 ± 0.2
Solyc07g049550.2	1-aminocyclopropane-1-carboxylate oxidase	ethylene biosynthesis from methionine; flavonol biosynthesis; syringetin biosynthesis	SL2.40ch07	1.38 ± 0.14	-0.65 ± 0.22	-0.52 ± 0.02
Solyc11g040340.1	Endoglucanase 1	cellulose biosynthesis	SL2.40ch11	-2.01 ± 0.19	-0.16 ± 0.13	-0.21 ± 0.04
Solyc12g056580.1	Cellulose synthase	cellulose biosynthesis	SL2.40ch12	0.85 ± 0.1	-0.03 ± 0.03	-0.03 ± 0.05
Solyc08g061100.2	Cellulose synthase	cellulose biosynthesis	SL2.40ch08	1.47 ± 0.23	-0.21 ± 0.2	0.09 ± 0.08
Solyc08g080170.2	3-hydroxy-3-methylglutaryl coenzyme A synthase	mevalonate pathway	SL2.40ch08	1.09 ± 0.09	0.02 ± 0.02	0.27 ± 0.03
Solyc09g092270.2	HXXXD-type acyl-transferase	chlorogenic acid biosynthesis I; chlorogenic acid biosynthesis II; phenylpropanoid biosynthesis; simple coumarins biosynthesis	SL2.40ch09	2.88 ± 0.22	-0.02 ± 0.08	-0.32 ± 0.18
Solyc12g042480.1	Cytochrome P450	flavonol biosynthesis; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis; luteolin biosynthesis; syringetin biosynthesis	SL2.40ch12	1.71 ± 0.18	-0.37 ± 0.09	-0.12 ± 0.03
Solyc02g067080.2	UDP-D-glucose dehydrogenase	colanic acid building blocks biosynthesis; galactose degradation II; UDP-D-xylose biosynthesis	SL2.40ch02	-2.49 ± 0.23	-0.01 ± 0.14	-0.66 ± 0.04
Solyc02g091510.2	GDP-L-galactose phosphorylase	ascorbate biosynthesis I (L-galactose pathway)	SL2.40ch02	-1.61 ± 0.17	-0.17 ± 0.27	-0.3 ± 0.04
Solyc03g096050.2	GDP-L-galactose phosphorylase	ethylene biosynthesis from methionine; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis	SL2.40ch03	-3.71 ± 0.01	0.17 ± 0.29	-0.4 ± 0.46
Solyc05g055710.2	L-ascorbate oxidase homolog	ascorbate glutathione cycle	SL2.40ch05	2.09 ± 0.05	-0.22 ± 0.27	0.00 ± 0.08
Solyc07g052230.2	L-ascorbate oxidase	ascorbate glutathione cycle	SL2.40ch07	1.14 ± 0.22	0.09 ± 0.22	0.39 ± 0.12
Solyc11g008860.1	L-ascorbate oxidase	ascorbate glutathione cycle	SL2.40ch11	-1.6 ± 0.08	-0.34 ± 0.08	-0.92 ± 0.05
Solyc12g094620.1	Catalase	oxidative ethanol degradation III; removal of superoxide radicals	SL2.40ch12	-1.47 ± 0.11	-0.59 ± 0.07	-0.68 ± 0.07

Concerning the set of 456 transcripts with significant effect of the “genotype”, again, the enrichment analysis did not result in any over-represented GO category. The comparative distribution (expressed as percentage) of gene models over GO annotations in the set of genes significantly affected in their expression by the genotype and of the overall list of gene models represented on the microarray slide is displayed in the Figure 3.24. Mapping transcripts differentially expressed in IL9adm pericarp vs M82 allowed the selection of candidate genes potentially involved in controlling fruit quality (Tab. 3.69). In particular, 2 genes were mapped to the cellulose biosynthesis, 2 were mapped to the starch metabolism, 2 were mapped to the pectin metabolism, 4 to the ethylene metabolism, 2 to the AsA metabolism, 2 to the mevalonate pathway and 3 to the phenylpropanoid pathway. Change in the expression of candidate transcripts were validated by RT-qPCR (Tab. 3.69).

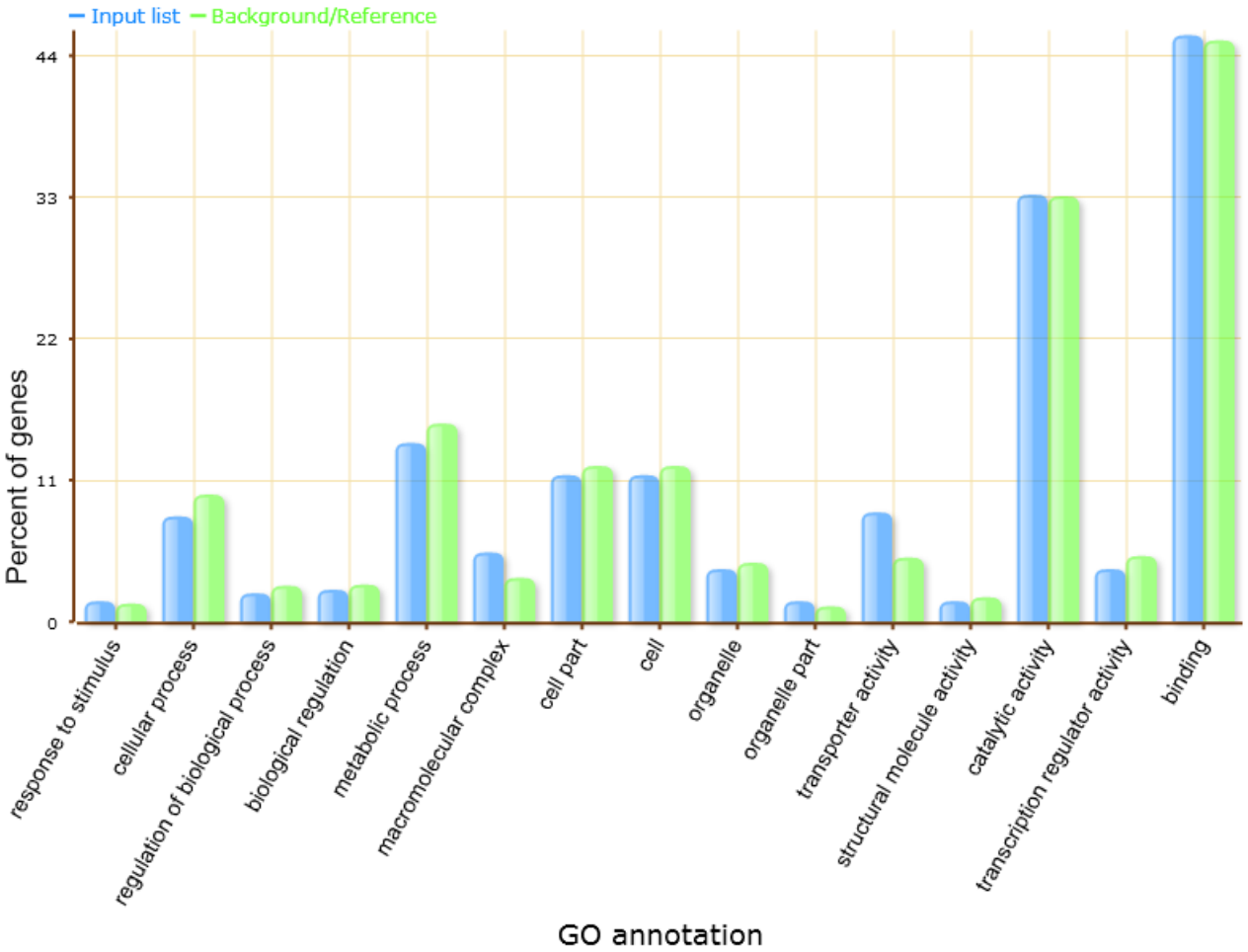

Figure 3.24 - Comparative distribution of gene models over GO annotations in the set of 456 transcripts with significant «Genotype» (input list) effect and in the overall list of gene models represented on the microarray slide (Background/Reference) as computed by AgriGO singular enrichment analysis (SEA - http://bioinfo.cau.edu.cn/agriGO/analysis.php)

Table 3.69 - Annotation details of selected candidate genes in the microarray experiment showing a significant "Genotype" effect at the ANOVA test ($P< 0.01$). Change in the transcript levels are reported according to RTq-PCR validation.

Gene model	Functional descritpion	Solcyc pathway	Chromosome assignment	Fold change (FC) expression compared to M82 100% (mean ± standard error)		
				M82 50%	IL9adm 100%	IL9adm 50%
Solyc01g079180.2	pectinesterase	homogalacturonan degradation	SL2.40ch01	1.31 ± 0.17	1 ± 0.01	-0.15 ± 0.09
Solyc02g067080.2	UDP-D-glucose dehydrogenase	colanic acid building blocks biosynthesis; galactose degradation II; UDP-D-xylose biosynthesis	SL2.40ch02	-2.46 ± 0.23	1.00 ± 0.01	-0.63 ± 0.08
Solyc02g071040.2	starch synthase IV precursor	starch biosynthesis	SL2.40ch02	1.24 ± 0.37	0.93 ± 0.01	-0.17 ± 0.03
Solyc03g096050.2	1-aminocyclopropane-1-carboxylate oxidase 1	ethylene biosynthesis from methionine; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis	SL2.40ch03	-3.66 ± 0.01	1.02 ± 0.02	-0.35 ± 0.91
Solyc03g114810.2	Glycosyltransferase	homogalacturonan biosynthesis	SL2.40ch03	1.58 ± 0.24	1.00 ± 0.01	-0.65 ± 0.00
Solyc04g009860.2	1-aminocyclopropane-1-carboxylate oxidase	ethylene biosynthesis from methionine	SL2.40ch04	1.01 ± 0.11	0.96 ± 0.00	-0.18 ± 0.17
Solyc04g071800.2	CYP92B3	flavonol biosynthesis; leucodelphinidin biosynthesis; leucopelargonidin and leucocyanidin biosynthesis; luteolin biosynthesis; syringetin biosynthesis	SL2.40ch04	-1.62 ± 0.14	1.00 ± 0.01	0.15 ± 0.28
Solyc05g007070.2	alpha-amylase	starch degradation	SL2.40ch05	1.48 ± 0.07	1.00 ± 0.01	-0.36 ± 0.17
Solyc05g055710.2	L-ascorbate oxidase	ascorbate glutathione cycle	SL2.40ch05	2.13 ± 0.05	0.98 ± 0.03	0.04 ± 0.17
Solyc07g049550.2	1-aminocyclopropane-1-carboxylate oxidase	ethylene biosynthesis from methionine; flavonol biosynthesis; syringetin biosynthesis	SL2.40ch07	1.42 ± 0.14	0.94 ± 0.02	-0.48 ± 0.05
Solyc08g061100.2	Cellulose synthase	cellulose biosynthesis	SL2.40ch08	1.48 ± 0.23	0.98 ± 0.02	0.09 ± 0.16
Solyc08g067070.2	Cytosine-specific methyltransferase	methionine degradation I (to homocysteine)	SL2.40ch08	1.44 ± 0.1	0.99 ± 0.01	-0.22 ± 0.29
Solyc08g080170.2	3-hydroxy-3-methylglutaryl coenzyme A synthase	mevalonate pathway	SL2.40ch08	1.09 ± 0.09	1.00 ± 0.00	0.27 ± 0.06
Solyc09g092270.2	HXXXD-type acyl-transferase family protein	chlorogenic acid biosynthesis I; chlorogenic acid biosynthesis II; phenylpropanoid biosynthesis; simple coumarins biosynthesis	SL2.40ch09	2.96 ± 0.22	1.01 ± 0.01	-0.23 ± 0.35
Solyc10g086180.1	Phenylalanine ammonia-lyase	flavonol biosynthesis; suberin biosynthesis	SL2.40ch10	1.2 ± 0.09	0.91 ± 0.05	-0.69 ± 0.33
Solyc11g010850.1	1-deoxy-D-xylulose-5-phosphate synthase	methylerythritol phosphate pathway; pyridoxal 5'-phosphate biosynthesis	SL2.40ch11	2.33 ± 0.09	0.98 ± 0.02	-0.16 ± 0.08
Solyc11g040340.1	Endoglucanase 1	cellulose biosynthesis	SL2.40ch11	-2.00 ± 0.19	0.99 ± 0.01	-0.21 ± 0.07

In order to further investigate transcription mechanisms controlling fruit quality traits, a correlation analysis was performed and correlations higher than 0.97 were represented as network (Fig. 3.25). In particular, the network highlighted corrrelations involving candidate transcripts for fruit quality, transcripts for ethylene regulation, transcripts encoding for transporters and transcrips encoding for transcription factors. Among all, the network illustrates the relationship among the Solyc02g08830.2 (early-responsive to dehydration), the Solyc09g075020.2 (ABC transporter) and the Solyc12g056580.1 (cellulose synthse) possibly involving the link between the fruit response to drought, phenolic accumulation and firmness.

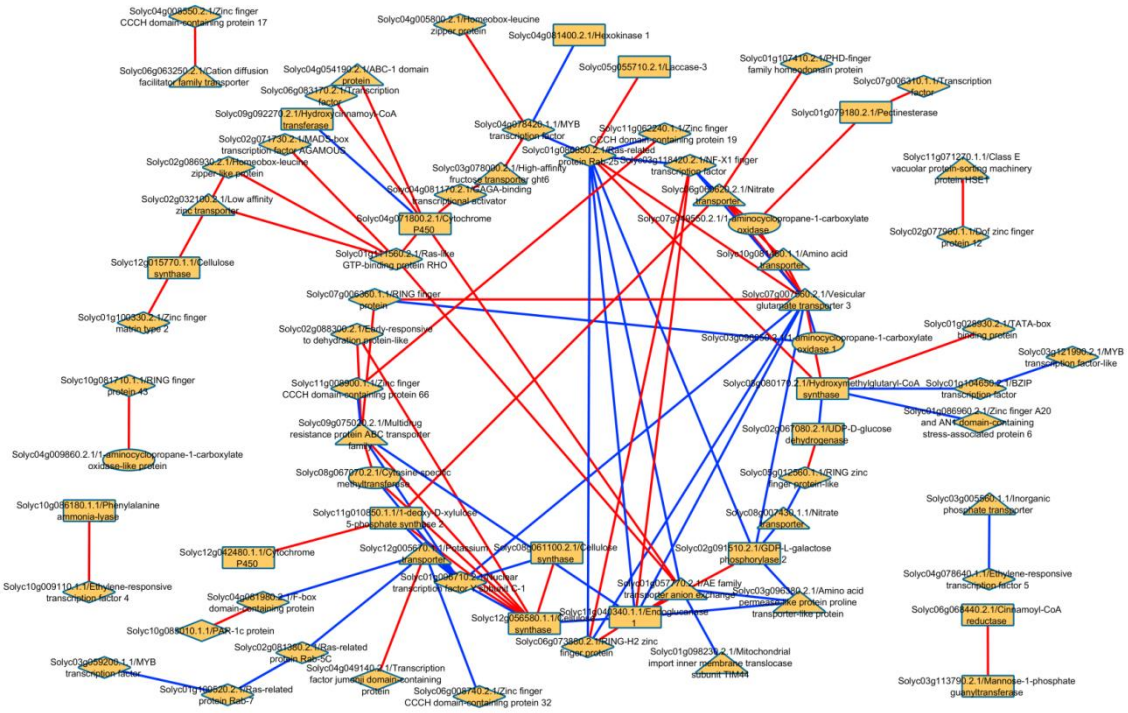

Figure 3.25 - Network illustrating correlation relationship among differentially expressed selected transcripts. Lines linking nodes indicate significant Pearson's correlation between corresponding transcripts (cut off at 0.97; red lines stand for positive correlations, blu lines stand for inverse correlations). In particular, corrrelations among candidate transcripts for fruit quality (rectangles), transcripts for ethylene regulation (ovals), transcripts encoding for transporters (triangles) and transcrips encoding for transcription factors (diamonds) are visualized. The network was build by Cytoscape software suite (v. 3.2.0).

3.9 Marked assisted selection in tomato for transfer the QTL9adm to breeding lines

The introgression line IL9adm from *S. pennellii* is well-known to be not only more tolerant to drought, but also for displaying finer qualitative traits related to fruit production, in comparison to the parental M82 tomato line.

Data from the trials we carried out in open field, showed that the IL9adm exhibited higher content in both soluble solids, and total AsA content than M82, when the plants were supplied with both the complete and partial volume of consumed water during the fruit set and ripining stage.

Those phenotypic evidences proved as wild QTL*s* from IL9adm would enhance the ability to produce larger amount of soluble solids and total AsA in tomato lines if they were transferred in those lines.

For that reason, we decided to use a set of several advanced tomato breeding lines, P011_58, P011_77, P011_85 and P011_156, selected for their higher agronomic performances as well as high both quantitative and qualitative fruit productions in comparison to M82, as parental male materials (♂) to cross to the IL9adm, used as female (♀), in order to get on a MAS-based backcross breeding program heading to set the wild and dissected QTL*s* from IL9adm in the recipient genomic background of P011 tomato lines.

3.9.1 Identification of molecular markers targeting wild QTLs located on 9adm genomic region

Upon selecting the set of DNA sequences within the genomic regions associated with 9adm introgression, each of them was amplified by PCR and the corresponding DNA amplicons were sequenced from M82 and the set of tomato P011 tomato lines as well as from IL9adm and the wild parental species *S. pennellii* (*Sp*).

By aligning the set of wild and cultivated DNA amplified fragments, different molecular polymorphisms, such as SNP*s*, DNA deletions and insertions, were

identified to be placed on restriction sites. Enzymes capable of cutting diversely whether targeting either a wild or the corresponding cultivated restriction site, were next detected. The set of resulting CAPS molecular markers (TG223, TC238140, DB708941, TC235285, TC227407) able to evidence polymorphism between wild and cultivated QTL*s* are listed and detailed in Tab. 3.70.

Table 3.70 – List of CAPS markers detected among DNA sequences included in the chromosomic region 9adm. The set of CAPS markers were used in the MAS-based breeding due to their ability to distinguish wild and cultivated alleles and QTL*s*.

Marker	Amplicon length (bp)		Restriction enzyme	Incubation Temperature (°C)	Cultivated allele fragments	Wild allele fragments
	M82	IL9adm				
TG223	1,200	1,200	Tru1 I	65	700	750
TC238140	1,026	1,036	Hind III	37	420+600	420+511+105
DB708941	1,344	1,293	Bspe I	37	1344	865+428
TC235285	1,172	1,184	Msc1	37	1,093+73	697+414+73
TC227407	1,000	1,000	Pst I	37	700+300	1,000

The theoretical patterns predicted *in silico* for each of the set of CAPS markers were confirmed *in vitro* upon PCR and digestion of the selected amplicons with the proper restriction enzyme and the resulting electrophoretic profiles are displayed in Fig. 3.26. In fact, upon running the products of digestion of each of amplicons through an agarose electrophoretic gel, it was assessed how:

i) TG223 amplicon generated two different DNA fragments, the bigger (the corresponding band evidencing a 750 bp-fragment in the gel pic) from M82 and the P011 tomato lines, slighty heavier than the DNA fragment (whose band was associated to a near 700 bp-fragment in the gel pic) from both *Sp* and IL9adm (Fig. 3.26A),

ii) TC238140 originated two different DNA fragments (whose molecular weights were about 400 and 600 bp according to the bands in the gel pic) from M82 and the P011 tomato lines, and three fragments (the associated bands weighed roughly 420, 500 and 100 bp in the gel pics) from both *Sp* and IL9adm (Fig. 3.26B),

iii) DB708941 was not cut by BspeI when coming from M82 and the P011*s*, as expected, while two diverse DNA fragments (around 430 and 870 bp in the gel pic) were formed upon digestion in both *Sp* the IL9adm (Fig. 3.26C),

iv) TC235285 was cut in two different DNA fragments (one weighing 1,100 bp and one smaller than 100 bp, according to the corresponding gel bands) when coming from M82 and the P011*s* and three in the case of both *Sp* and IL9adm (about 700, 400 and 100 bp) (Fig. 3.26D),

v) TC227407 presented two different DNA fragments (nearly 300 and 700 bp as displayed in the gel pic) in M82 and the P011*s*, while no cleavage occurred in both *Sp* and IL9adm (Fig. 3.26E).

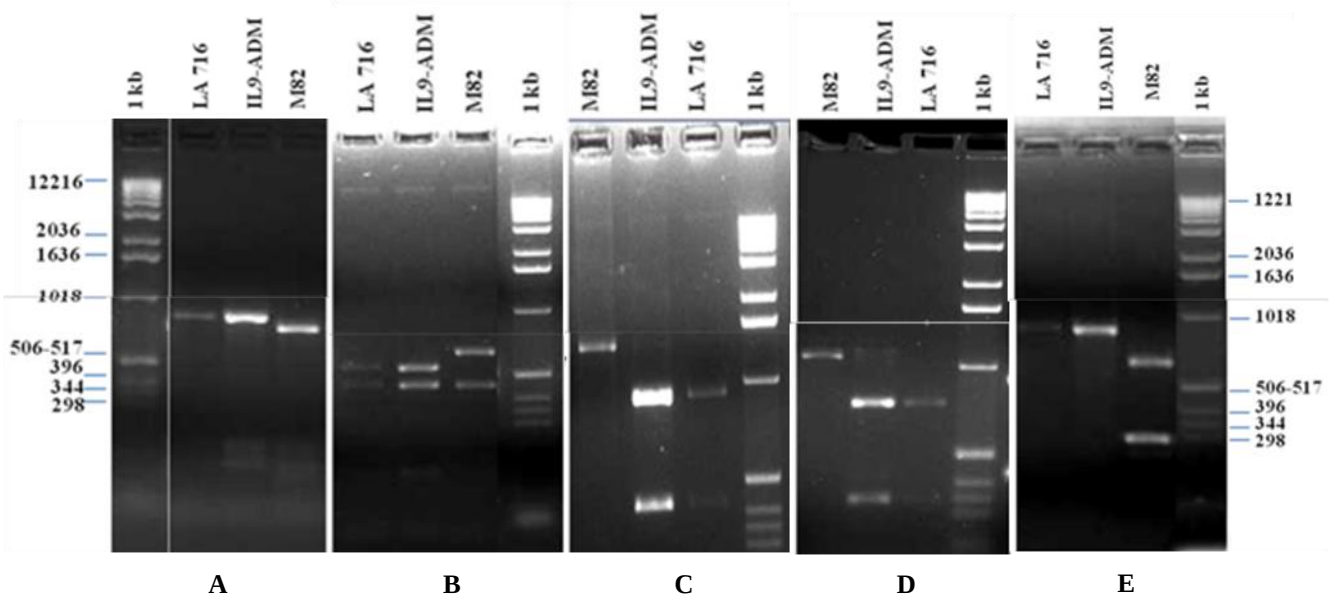

Figure 3.26 – Gel electrophoreic patterns exhibited by the CAPS molecular markers built in this study confirmed experimentally their theoretical abilty at being able to distinguish wild (*S. pennelli* LA716 and IL9adm) and cultivated (M82) alleles in the MAS-based breeding program.
A: TG223, B: TC238140, C: DB708941, D: TC235285 and E: TC227407. 1kb: 1kb DNA ladder.

Due to their demonstrated capacity to discern both wild and cultivated alleles, the detected CAPS molecular markers were exploited to assist the selection of superior genotypes getting wild and dissected QTL*s* over the next backcross generations.

3.9.2 MAS-based selection of hybrid plants F_1 progenies originated from the cross between P011 tomato lines and IL9adm

The hybrid identities of a selected set of F_1 plants originated from the cross involving each of the four different P011*s* and IL9adm were assessed through exploiting all of the five CAPS markers above described. Among the entire populations of plant discended by crossing IL9adm with P011_58, P011_77, P011_85 and P011_156, different plants were examined on molecular basis, to establish whether they were actual F_1 hybrids or not. A total of four plants were considered in the cases of the first and third crossess, five for the second and fourth crossess. The total of the investigated plants resulted to belong to F_1 progenies because they exhibited heterozygous genotypes at each of the loci analyzed by CAPS markers, except a plant from the cross involving the P011_156 parental line that, in effect, displayed to be homozygous at each of CAPS loci, hence possessing only the cultivated allele (Fig. 3.27).

		CSPS markers								CSPS markers				
Cross	Plant	TG223	TC238140	DB708941	TC235285	TC227407	Cross	Plant	TG223	TC238140	DB708941	TC235285	TC227407
P011-58 x IL9-ADM	F1a	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F1b	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F1c	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F1c	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F1d	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F1d	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F1n	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F1e	+/-	+/-	+/-	+/-	+/-
							P011-77 x IL9-ADM	F1l	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F1b	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F1c	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F1d	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F1d	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F1e	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F1e	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F1i	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F1h	-/-	-/-	-/-	-/-	-/-
							P011-156 x IL9-ADM	F1m	+/-	+/-	+/-	+/-	+/-

Figure 3.27 – MAS-based selection in the F_1 progenies derived by crossing the IL9adm (♀) with four different tomato advanced breeding lines (♂): P011_58, P011_77, P011_85 and P011_156. The two + and - symbols indicate the wild and cultivated alleles, respectively. The orange shadow marks the set of plants which were demonstrated to be F_1 hybrids on the basis of their heterozygous genotype at each of the CAPS loci.

3.9.3 MAS-based selection of plants in the F_2 progenies originated from the cross between P011 tomato lines and IL9adm

Once F_1 plants were detected by using the set of CAPS markers, they were permitted to self in order to get four different F_2 progenies each deriving from one of the P011 recurrent parental line. The entire population of F_2 plants were analyzed by mean of the five CAPS markers, with the purpose to detect the group of plants harboring the integral 9adm introgression in homozygous condition, the group of plants lacking the 9adm introgression or any part of it, and the group of plants displaying possible recombinations occurring on the 9adm introgression. In Fig. 3.28 are indicated the genotypes assessed at the each of the five different CAPS loci for each F_2 plant.

Cross	Plant	CAPS markers TG223	TC238140	DB708941	TC235285	TC227407	Cross	Plant	CAPS markers TG223	TC238140	DB708941	TC235285	TC227407
P011-58 x IL9-ADM	F2a	+/+	+/+	+/+	+/+	+/+	P011-77 x IL9-ADM	F2a	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2b	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2b	-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM	F2c	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2c	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2d	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2d	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2e	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2e	+/+	+/+	+/+	+/+	+/+
P011-58 x IL9-ADM	F2f	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2f	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2g	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2g	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2h	+/+	+/+	+/+	+/+	+/+	P011-77 x IL9-ADM	F2h	-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM	F2i	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2i	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2l	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2l	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2m	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	F2m	+/+	+/+	+/+	+/+	+/+
P011-58 x IL9-ADM	F2n	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	F2n	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2o	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	F2o	+/+	+/+	+/+	+/+	+/+
P011-58 x IL9-ADM	F2p	+/+	+/+	+/+	+/+	+/+	P011-77 x IL9-ADM	F2p	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	F2q	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	F2q	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	F2a	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	F2a	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	F2b	+/+	+/+	+/+	+/+	+/+	P011-156 x IL9-ADM	F2b	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2c	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2c	+/+	+/+	+/+	+/+	+/+
P011-85 x IL9-ADM	F2d	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2d	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	F2e	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	F2e	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2f	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2f	+/+	+/+	+/+	+/+	+/+
P011-85 x IL9-ADM	F2g	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2g	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	F2h	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2h	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2i	+/+	+/+	+/+	+/+	+/+	P011-156 x IL9-ADM	F2i	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2l	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2l	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2m	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	F2m	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2n	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2n	+/+	+/+	+/+	+/+	+/+
P011-85 x IL9-ADM	F2o	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2o	+/+	+/+	+/+	+/+	+/+
P011-85 x IL9-ADM	F2p	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2p	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	F2q	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	F2q	+/-	+/-	+/-	+/-	+/-

Figure 3.28 – MAS-based selection in the F_2 progenies setting up by crossing the IL9adm (♀) with four different tomato advanced breeding lines (♂): P011_58, P011_77, P011_85 and P011_156. The two + and - symbols indicate the wild and cultivated alleles, respectively. The red shadow evidences the set of plants detected harboring entirely the introgression 9adm in homozygous condition and selected for being crossed to the recurrent tomato lines. The orange shadow marks the plants heterozygous at each CAPS locus and potentially useful for setting in homozygous condition each CAPS locus upon selfing, and finally the plants not evidenced in any colors were not selected as lacking any wild QTLs.

As it is shown in Fig. 3.28, a total of 12 different plants were detected to exhibit a homozygous genotype for the wild allele at each of the five CAPS loci, three from

the P011_58, two from the P011_85, three from the P011_77 and four from the P011_156. Those plants were selected and next used as female parents in the first backcross to the corresponding tomato line. However, the majority of the plants were heterozygous for the entire 9adm introgression. The seeds from a group of these plants were selected to save the chance to set the introgression 9adm in the homozygous status upon a new self cycle.

3.9.4 MAS-based selection of plants in the BC_1F_2 progenies originated from the cross between *P011 tomato lenes and IL9adm*

From crossing the selected F_2 plants to the corresponding P011 tomato lines, a total of 35 plants belonging to BC_1F_2 progenies were analyzed by CAPS (Fig. 3.29).

		CAPS markers								CAPS markers				
Cross	Plant	TG223	TC238140	DB708941	TC235285	TC227407	Cross	Plant	TG223	TC238140	DB708941	TC235285	TC227407
P011-58 x IL9-ADM	BC1a	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	BC1a	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	BC1b	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1b	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	BC1c	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1c	-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM	BC1d	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	BC1d	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	BC1e	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1e	-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM	BC1f	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1f	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	BC1g	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1g	-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM	BC1h	-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM	BC1h	+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM	BC1i	+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM	BC1i	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1a	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	BC1a	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1b	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	BC1b	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	BC1c	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	BC1c	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1d	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	BC1d	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1e	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	BC1e	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	BC1f	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	BC1f	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1g	-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM	BC1g	-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM	BC1h	+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM	BC1h	+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM	BC1i	+/-	+/-	+/-	+/-	+/-							

Figure 3.29 – MAS-based selection in the BC_1F_2 progenies originated by the initial cross between IL9adm (♀) with four different tomato advanced breeding lines (♂): P011_58, P011_77, P011_85 and P011_156. The two + and - symbols indicate the wild and cultivated alleles, respectively. The orange shadow evidences plants harboring the integral introgression 9adm in heterozygous condition as expected to be for real BC_1F_2 plants and for that reason selected for being crossed to the recurrent tomato lines.

Six plants originated from each of the tomato lines, P011_58, P011_77, P011_85 and P011_156, were selected in the corresponding BC_1F_2 populations due to their heterozygous condition at each of the CAPS locus located on the 9adm genomic region, revealing their possess of wild QTL*s*, and further crossed to the proper P011 line in order to get the BC_2F_2 progenies.

3.9.5 MAS-based selection of plants in the BC_2F_2 progenies originated from the cross between P011 tomato lines and IL9adm

Upon crossing plants picked up in the BC_1F_2 population back to the four different recipient parental tomato lines, a total of 52 plants belonging to BC_2F_2 progenies, thirteen from each of the P011 lines, were analyzed by mean of CAPS markers (Fig. 3.30).

CAPS markers							CAPS markers						
Cross	Plant	Marcatori CAPS					Cross	Plant	Marcatori CAPS				
Genotipo		TG223	TC238140	DB708941	TC235285	TC227407	Genotipo		TG223	TC238140	DB708941	TC235285	TC227407
P011-58 x IL9-ADM BC2a		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2a		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2b		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2b		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2c		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2c		-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM BC2d		-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM BC2d		-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM BC2e		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2e		-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM BC2f		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2f		-/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM BC2g		-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM BC2g		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2h		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2h		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2i		-/-	-/-	-/-	+/-	+/-	P011-77 x IL9-ADM BC2i		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2l		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2l		+/-	-/-	-/-	-/-	-/-
P011-58 x IL9-ADM BC2m		-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM BC2m		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2n		-/-	-/-	-/-	-/-	-/-	P011-77 x IL9-ADM BC2n		+/-	+/-	+/-	+/-	+/-
P011-58 x IL9-ADM BC2o		+/-	+/-	+/-	+/-	+/-	P011-77 x IL9-ADM BC2o		-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2a		-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM BC2a		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2b		+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM BC2b		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2c		+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM BC2c		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2d							P011-156 x IL9-ADM BC2d		-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2e		-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM BC2e		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2f		+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM BC2f		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2g		-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM BC2g		-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2h		+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM BC2h						
P011-85 x IL9-ADM BC2i		-/-	-/-	-/-	+/-	+/-	P011-156 x IL9-ADM BC2i		-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2l		+/-	+/-	+/-	-/-	-/-	P011-156 x IL9-ADM BC2l		+/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2m		-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM BC2m		+/-	+/-	+/-	+/-	+/-
P011-85 x IL9-ADM BC2n		-/-	-/-	-/-	-/-	-/-	P011-156 x IL9-ADM BC2n		-/-	-/-	-/-	-/-	-/-
P011-85 x IL9-ADM BC2o		+/-	+/-	+/-	+/-	+/-	P011-156 x IL9-ADM BC2o		+/-	+/-	+/-	+/-	+/-

Figure 3.30 – MAS-based selection in the BC_2F_2 progenies from the initial cross between IL9adm (♀) with four different tomato advanced breeding lines (♂): P011_58, P011_77, P011_85 and P011_156. The two + and - symbols indicate the wild and cultivated alleles, respectively. The orange shadow evidences plants harboring the integral introgression 9adm in homozygous condition and for that reason selected for being next crossed to the recurrent tomato lines. Several plants displayed recombination between wild and cultivated alleles along the 9adm genomic region, as shown by the orange shadow covering partially the different CAPS loci.

Nine plants from the P011_58, seven from P011_77, eight from P011_85 and P011_156 possessed the 9adm introgression in the heterozygous condition, but very interestingly in one plant from P011_58, in two different plants from P011_77, and in one plant from P011_85 and P011_156 each, the introgression was maintained partially (Fig. 3.30). Those findings carry in remarkable genetic meanings because indicate as diverse recombination events took place during the meiosis I in the BC_1F_2 plants between wild and cultivated alleles located on the 9adm genomic region. The precious consequence is represented by the dissection of wild QTL*s* into single genetic factors in the BC_2F_2 plants. That is the first step to their setting in the genetic background of tomato advanced breeding lines through further backcross cycles.

Fruits at red ripe stage were harvested from different heterozygous and homozygous plants belonging to BC_2F_2 progenies from each of the four P011 tomato parental lines, pooled within hetozygous and homozygous categories, and the content in both soluble solids (Fig. 3.31A) and total AsA (Fig. 3.31B) were measured and compared.

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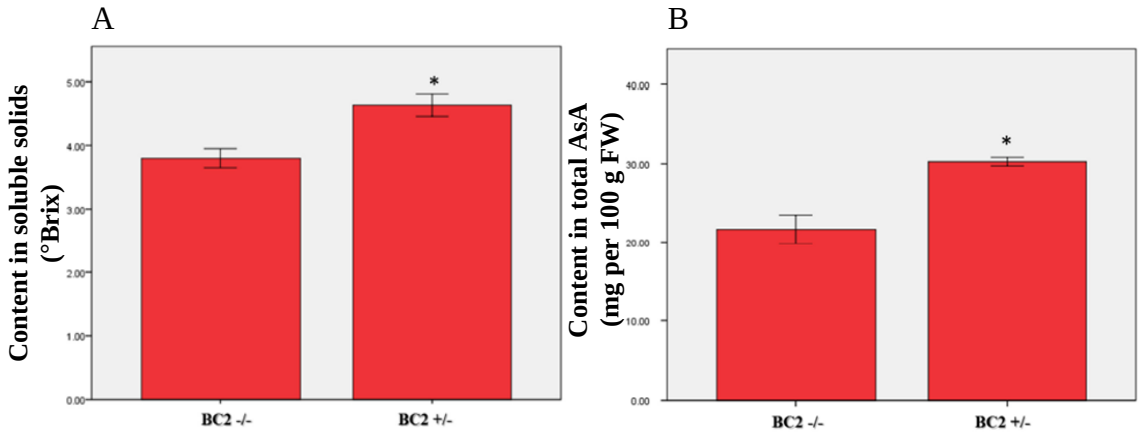

Figure 3.31 – Content in soluble solids (A) and total AsA (B) was ascertained in the BC_2F_2 progenies originated by the initial cross between IL9adm (♀) with four different tomato advanced breeding lines (♂): P011_58, P011_77, P011_85 and P011_156. Mature fruit were harvested from both heterozygous (BC2 +/-) and homozygous (BC2 -/-) plants deriving from each of the four P011 tomato lines, pooled within each of both categories, and then processed for the analytical evaluations.

The levels in both soluble solids and total AsA were assessed to be higher in fruit from heterozygous than homozygous plants, suggesting that wild QTL*s* located on the 9adm introgression contribute to enhance both traits not only when working in concert with the other related wild genes in the genetic context of the parental

IL9adm introgression line, but also when dissectected and taken away from the original 9adm introgression. Those findings seem to be suggesting as robust improvement in the fruit content of both soluble solids and total AsA and perhaps in other important traits associated with both quantitative and qualitative production in tomato, are strongly supposed to be dragged upon the backcross breeding program will be coming to harbor in next future.

4 Discussion

An initial objective of the project was to identify the variability in the tomato processing fruit quality. In a recent past the food company choices pointed at an higher content of soluble solids and at a good firmness. These two features are still very important because on one hand an increase in soluble solids of just 1% represents ~20% increase in yield of certain processed products (Foolad *et al*., 2007) and on the other hand softening considerably influences post-harvest performance like transportation and storage (Brummel and Harpster, 2001).

Among others, components of the tomato nutritional quality are the biomolecules (carotenoids, vitamins, and phenols) with antioxidant properties present in fruits, leading to the beneficial role of tomato intake in human diseases. Several studies showed a role of tomato fruits in the healing and prevention of some illnesses. Their consumption is associated with a reduced risk of cancer, inflammation, and chronic noncommunicable diseases including cardiovascular diseases such as hypertension, coronary heart disease, diabetes, and obesity (Zu *et al*., 2014; Wilkinson and Chodak, 2003; Cohen, 2002).

Consumers are more sensible than in the past about the role of fruits and vegetables in the human health and some authors refer to tomato as a functional food (Canene-Adams *et al*., 2005)

Therefore, currently the nutritional quality of the fruit constitutes one of the major objectives of tomato breeding.

Within this context we set out a first open field trial with the aim of assessing the phytochemical content of commercial tomato hybrids.

Regarding the soluble solid content, hybrids ranged between 4.05 and 4.70 °Brix. These results match those observed in earlier studies: Kavitha and co-authors reported a range between 3.7 and 5.2 °Brix in commercial varieties and hybrids (2013).

In reviewing the literature, only few data were found allowing the comparison between hybrids and commercial and introgression lines. As a consequence we thought to compare antioxidant values of our hybrids to either introgression lines or commercial hybrids.

The choise to show the composition of tomatoes on a fresh matter basis (FW) was taken because in this trial it is essential to consider the nutritional value. The dry matter content of microconstituents is more pertinent when comparisons of the effect of the mode of colture on the composition plant are made (as we made for the next trials we are going to show).

We found great variability in terms of total phenolic compounds. The range was 8.50-38 mg GAE · 100 g FW^{-1}. Other authors registered values significantly lower, 1.89-3.08 mg 100 g FW^{-1} in eleven cultivars or 2.24-3.75 mg 100 g FW in twelve tomato advanced breeding lines and six open pollinated cultivars (Kacjan Marsic *et al*., 2011; Frusciante *et al*., 2007). Carillo-Lopez and Yahia (2013) recorded an average value of about 50 mg GAE · 100 g FW^{-1} with the highest amount in the exocarp.

Di Matteo and co-workers (2013) found 44.39 mg GAE · 100 g FW^{-1} in the homozygote tomato Red Setter, while Rigano *et al.* (2014) recorded an amount of about 9 and 14 mg GAE · 100 g FW^{-1} in two tomato lines.

The flavonoid content observed in our study (5.72 - 6.71 mg QE · 100 g FW^{-1}) was very similar to the amount found by Davilla-Avina *et al.* (2012) in a cultivar grown in greenhouse, but 2 folds higher than Frusciante *et al.* (2007).

Di Matteo *et al.* (2013) registered a flavonoid amount slightly higher than ours in a homozygote line, while Rigano *et al.* (2014) observed an amount about 2 times lower in two tomato introgression lines.

Among the water-soluble antioxidants than we analyzed, Ascorbic acid (AsA) showed results in line with common commercial varieties (about 20 mg · 100 g FW^{-1}) (Gould, 1992). However the AsA amount in our Hybrid trial was higher than some cultivars presented by Caris-Veyrat and co-workers (2004). Sacco *et al.* (2012) recorded an high value of AsA in two ILs (35 - 40 mg · 100 g FW^{-1}) when they were

grown in greenhouse, while when they were allevated in open field, values were similar to the ours.

Shifting our attention on lipid-soluble bioactive compounds, we analyzed the level of carotenoids in the commercial hybrids. The average value we found, was equal to 65.98 ± 7.66 μg · g FW^{-1}. As mentioned in literature review, the relative contribution of lipid-soluble antioxidants to the total antioxidant activity in tomato fruits is much lower than the contribution from water-soluble antioxidants (Frary *et al*., 2010). Rigano *et al.* (2014) registered a range between 70.15 and 130.56 μg · g FW^{-1} in an introgression line, while great genotypic variation was observed for total carotenoids among tomato cultivars, hybrids, cherry tomatoes and wild species in the paper by Kavitha and co-workers (2013).

The results of Duncan test analysis conducted on our tomatoes didn't show any significant variability among our hybrids in terms of total carotenoids.

We used a pricipal component analysis (PCA) to discriminte plants based on the overall fruit qualities. However, the PCA didn't show a sharp difference among Hybrids. Only Perfect Peel performed higher with respect to the components one and two who explayned AsA, flavonoids, dry weight and phenolic compounds.

One interesting finding from the PCA was the relation observed between the shape and the fruit quality given the discrimination effectively operated by fruit shape on compents one and two.

This result is in keeping with a previous study by Kacjan Marsic *et al.* (2011) where a significant effect of the shape was observed among fruit quality traits.

The general result of this study indicates a narrow variability in terms of nutritional quality in the tomato commercial hybrids.

According to this evidence, wild germoplasm (i.e. introgression lines) could be a precious resource to enhance the nutritional quality variability. These lines could be used as a valuable resource to improve several important traits, such as fruit yield, nutritional value and tolerance to enviromental stresses of tomato cultivars when wild QTLs are transferred and fixed in the tomato genetic background. Marker assisted

breeding may help in identifying backcross populations with higher content in antioxidants and more tolerant to abiotic stresses (Kavitha *et al*., 2013). Among these, drought has a major impact on plant growth and development, limiting crop production throughout the world. Soils too dry for crop production cover 28% of the earth's land (Bray, 2004). Moreover challenge for current agricultural biotechnology is to satisfy an ever increasing demand in food production facing a constantly increasing world population that will reach more than 9 billion in 2050 (Godfray *et al*., 2010; Tester and Langridge, 2010).

According to these evidences, we selected the introgression line 9adm which was shown to have some interesting features, in terms of fruit quality and drought tolerance, in previous studies carried out in our laboratory.

Consiquently the second question of our research aimed to determine the effect of water shortage on tomato fruit quality and yield in the domesticated genotype M82 and in the above mentioned IL9adm. The latter harbors a segment of about seven centimorgans derived from the wild tomato related species *Solanum pennellii*. Two trials were designed either in open field or in more environmental controlled conditions, in tunnel. In both the experiments M82 and IL9adm were grown applying two different water regimes: full water restitution (100% WR) and half water restitution (50% WR).

Once tomatoes reached the red ripe (RR) stage, their antioxidant content was analized on fresh tomato, upon trasformation in tomato sauce (PP) and after 53 days post harvest (53 dph).

To check the water depletion effects on plants from the open field trial, leaves were analyzed. First of all, we measured the hydrogen peroxide (H_2O_2) amount, cosidered one of the most important oxidative indicator of stress. H_2O_2 was found to be significantly higher in M82 under the 50% WR condition. Under stress condition, plants develop abscisic acid (ABA) and once it is imported into guard cells this triggers the production of reactive oxygen species (ROS) such as H_2O_2 (Jarzyniak and Jasinski, 2014). The H_2O_2 content was also measured in fruits at RR stage. The

results confirmed a significant effect of the water restitution. From the microarray analysis we observed that the gene Solyc12g094620.1 (a catalase) was more down-regulated under stress condition. A less amount of catalase could emphasize the H_2O_2 action. Then the relative water content (RWC) analysis was carried out in order to clarify if the less water supply in the soil corresponded to a decrease in the leaf water content. It showed a significant effect of the water restitution. Stressed leaves got more water and data are in agreement with Sacco *et al*. (2013). Beside the RWC, we carried out an analysis of water loss rate (WLR). IL9adm lost significantly less water than M82 across several time points. Therefore, we concluded that IL9adm was much effective in controlling leaf water loss and this might contribute to its higher tolerance. These differential behaves can be compared to results published by Liu and co-workers (2015). They studied the drought stress in transgenic tomato lines carrying drought tolerant genes and within the control. They discovered that transgenic plant had lower WLR and higher RWC (Liu *et al*., 2015). It has been reported that solutes like carbohydrates, play an important roles in enhancing plant tolerance to a wide range of environmental stresses (Chen *et al*., 2007). The solute accumulation mantein cellular osmotic adjustment and membrane integrity and may also enhance the water retantion capacity of plant under stress condition. The higher RWC and lower WLR we found in IL9adm could be explained by the significant higher content of soluble solids (SSC) in IL9adm, constituted in majority by sucrose and other carbohydrates. Altough our observations on the SSC were limited to fruit we suggest that a differential attitude to control osmotic adjustment might contribute to the higher tolerance in IL9adm. The tolerant plant had higher SSC than M82 in both open-field or tunnel trials. According to Helyes *et al*. (2012) and Barbagallo *et al*. (2013) the SSC increased when the 50% WR was applied. An increased synthesis of sugar and polyol compounds is triggered by various abiotic stresses. However, accumulated sugars may differ among plant species and nature of abiotic stressors (Zhuo *et al*., 2013; Folgado *et al*., 2014; Moyankova *et al*., 2014).

As reported in the chapter two, the genotype IL9adm is a subline of the parental line

IL9-2-5, a tomato line of the cultivated species, *Solanum lycopersium*, carrying a 9 cM introgression from the wild species, *Solanum pennellii*. IL9-2-5 produces fruit with high soluble solids content (Brix), an important determinant of fruit quality for processing. Two quantitative trait loci relating to fruit soluble solids content have been identified within the introgressed segment. One of these QTLs, the, the Brix-9-2-5, has been shown to encode a fruit apoplastic invertase (Lin5) with altered kinetic properties (Fridman *et al*., 2002; Baxter *et al*., 2005). In our study, we proved that the gene Lin5, involved in an higher fruit SSC, is outside of the introgressed wild segment in IL9adm. In the same time, IL9adm showed always an higher amount of SSC than M82 and consequently it could suggest that other genes might be involved in the higher fruit SSC accumulation.

The current study found that the restitution of only the half the evapotranspirated water significantly affects the total yield per plant and the marketable yield per plant. The 50% WR led to an important reduction of yield in our trials, but IL9adm showed a significant less loss with respect to M82.

In previous study on tomatoes, the drought stress condition negatively affected the total yield which was less than half than that produced under full irrigation. The loss in marketable yield was statistically significant too (Barbagallo *et al*., 2013). The same evidences were confirmed by Helyes *et al.* (2012) evaluating the effect of different water level irrigations on processing tomatoes. The number of marketable fruits per plant was reduced as soil water tension during fruit development and maturation growth stages increased, mainly since many small green fruits aborted or did not enlarge under drier conditions (Marouelli and Silva, 2007).

Taken together, the above mentioned results of our study suggest that the genotype IL9adm is more tolerant than the genotype M82 in condition of half water supply.

These results are in line with the fact that IL9adm harbors a shoot-specific QTL (PW 9-2-5) derived from its parental subline, the plant IL9-2-5. This QTL accounts for an altered growth habit resulting in increases in plant weight, yield and Brix units. Investigating the drought effect on M82 and IL9-2-5, Rigano *et al.* (2014) observed a

better response to water deprivation in IL9-2-5. The latter genotype maintained an higher percentage of fruit weight and an aboveground biomass (Rigano *et al*. 2014). Althogh we didn't find any significant difference in terms of aboveground mass, an higher tap root was noticed in IL9adm in the hydroponic trial aiming to compare some biometric measures in our two genotypes. The difference was statistically significant and point out the contribution of root morphology to the higher drought tolerance in IL9adm. Further work is required to establish this.

Studying novel loci regulating interspecific variation in root morphology and cellular development in tomato, Ron and co-authors (2013) discovered that a number of *S. pennellii* loci provided a long root phenotype compared with the corresponding loci in M82.

If we focus our attention on antioxidant content in tomato berries, we can observe that while the ascorbic acid (AsA) content was not significantly affected by the water restitution in the open field trial, it was shown to be significantly higher in IL9adm 50% WR in the lysimeter trial and this increase was associatd to changes in the expression of a number of AsA biosynthetic genes. It is generally assumed that plant tolerance to environmental stresses is positively correlated with AsA content (Knörzer *et al*. 1996; Tambussi *et al*. 2000). Neverthless reviewing the literature, discordant results emerged.

Barbagallo *et al*. (2013) found an AsA content significantly greater in tomatoes grown under very limited soil water conditions, while Helyes *et al*. (2012) measured significantly higher content of AsA with optimum water supply conditions. Dumas *et al*. (2013) reported that vitamin C production is promoted by water limitation in processing tomato, although the extent of this effect may be cultivar-dependent. Another study revealed that ascorbate content decreased in tomato fruits that were shaded during ripening (Gautier *et al*., 2008).

The transcriptomic analysis of fruit pericarp allowed the identification of a number of candidate genes for controlling differently the fruit level of AsA in the two genotypes in response to drought. Among transcripts with significant interaction "genotype x

water treatment", a GDP-L-galactose phosphorylase gene and a mannose-1-phosphate guanyltransferase gene were significantly downregulated in M82 under 50% of water restitution thus negatively correlating the AsA accumulation. These results support the hypothesys that the Smirnoff-Wheel pathway might not contribute to the increase in AsA fruit level in drought challenged plants. Similarly, the increase in the transcript level of an ascorbate oxidase gene may lead to an higher rate of AsA usage that may not explain the increase in AsA in M82 treated with 50% water restitution. On the other hand, the fact that the transcription level of two UDP-D-glucose dehydrogenase and of an galactokinase gene correlated with the fruit AsA level point out the involvement of the L-gulonate pathway in controlling the AsA level in response to drought. Also, a polygalacturonase gene showed a transcription level correlating to the level of AsA supporting the hypothesis that the alternative D-galacturonate pathway may give its contribution to the AsA pool in response to drought.

A differential effect of storage on AsA was observed. While AsA in M82 100% slightly decreased, the storage led to a great enhancement in the 50% WR (+ 68%). In IL9adm after 53 days post harvest (53 dph), the increase was observed in the 100 % WR condition (+ 20%), instead.

Vinha *et al*. (2013) investigated the effect of a 15 day storage at different temperatures on antioxidants in several cultivars. They discovered an increase in AsA, but its value was cultivar dependent.

Conversely the vitamin C showed a decay of about 45% after 12 days of storage (Moneruzzaman *et al*., 2009). A possible explation of the AsA variation during the storage is that ripening phenomen are still in action during the post harvest and it may lead to a change in the antioxidant composition (Valverde *et al*., 2011).

As aspected, the processing led to a significant decrease of AsA in both the genotypes and treatments. Vitamin C losses during food processing are likely to be due to a combination of (endogenous) enzymatic activity (e.g., ascorbic acid oxidase) and heat treatment (Lopez-Sanchez *et al*., 2015).

Abuscita *et al.* observed that Just 45% of the initial content of ascorbic acid was retained in the final tomato paste using an hot break processing, the same of our study (Abushita *et al*., 2000).

Phenolic compounds ranged between 498 and 620 mg GAE · 100g DW^{-1} in tomatoes from the open field trial.

A slight decrease was caused by the 50% WR, but the ANOVA didn't show any significant effect of the genotype or of water restitution. Under tunnel no variation occurred in M82, while there was a significant increase in IL9adm. A moderate reduction in therms of total phenolics compounds was also displayd by Helyes *et al*. (2012) while an increase was led by drought conditions in the experiment by Barbagallo *et al*. (2013). In addition, the analysis of gene expression allowed to correlate the expression of a phenylalanine ammonia-lyase gene, a key function in controlling phenolics biosynthesis, with an aminocyclopropane-1-carboxylate oxidase gene, thus highlighting the role of ethylene and fruit ripening in controlling the accumulation of phenolics in response to stress.

In general phenolics rised during the storage according to Davila-Avina *et al*. (2012), but in contrast with Vinha *et al*. (2013).

The processing led to a decay of phenolics, but it didn't happen in IL9adm which showed almost no variations in the WR 50% and an increase (10%) in 100% WR.

Conflicting results for total phenolics, flavonoids, and the total hydrophilic antioxidant activities of processed tomato samples (Capanoglu *et al*., 2008) were found in the current literature.

Some studies indicate that a considerable loss of hydrophilic antioxidants is caused by the processing approach. In experiments conducted by Chang *et al*. (2006), two tomato varieties showed that the total phenolic content increased by 13% and 50% in two different heating treatments respectively, when compared to the corresponding levels in fresh tomatoes (Chang *et al*., 2006).

Among the phenolic acids, the chlorogenic acid was the major compound, while the most abundant flavonoid and phenolic compound in general, was the rutin according

to Martinex-Huelamo *et al*. (2015). Taken individually, all the phenolic compounds increased upon the transformation, in our experience. Rutin was more than twice higher in the tomato sauce than in the raw tomato while chlorogenic acid and caffeic acid showed a slight decrease (Martinex-Huelamo *et al*., 2015).

We registered a general increase in quercetin (on average + 66%). Processing into the end product can increase the content of free quercetin by up to 30%, a change that may be brought about by enzymatic hydrolysis of quercetin conjugates (Stewart *et al*., 2000). Opposite evidences were found by Crozier *et al*. (1997) who studied the effect of cooking on the quercetin content of onions and tomatoes. With both vegetables, boiling reduced the quercetin content by 80%, microwave cooking by 65%, and frying by 30% (Crozier *et al*., 1997).

Despite the storage led to an increase in total phenolic compounds, a decrease for each of the phenolic compounds was observed except for the rutin in IL9adm 50% WR and M82 WR 100%. The increase in Folin-Ciocalteau reactive products can be explained by the formation of Maillard reaction products (MRPs) as a consequence of nonenzymatic browning. It is known that intermediate as well as final MRPs melanoidins exert antioxidant activity (Lee *et al*., 1992). Rutin was shown to be stable in an experiment of dried tomato storage at 4 °C (Giovannelli and Paradiso, 2002).

The total carotenoid content rised under the 50 % WR and this result is consistent with earlier studies (Barbagallo *et al*., 2013). The total level of carotenoid in red ripe fruit was unaffected by the genotype. As for the lysimeter trial, a great enhancement of carotenoids was observed in M82 in the 50% WR condition with a significant effect of the water treatment. In agreement with this result, the microarray analysis displayd an over-expression of the gene Solyc08g080170.2 that is annotated as 3-hydroxy-3-methylglutaryl coenzyme A synthase and wasmapped tothe mevalonate pathway which synthesizes isoprenoids that are precursors of carotenoids.

Except for IL9adm 50% the storage led to an increase in total carotenoids.

Abushita *et al*. (2000) reported a decay of total carotenoids upon hot break processing

from 1430 to 1318 μg · g DW^{-1}, but with no significant change in the individual carotenoid composition.

Lycopene is by far the most studied antioxidant compund in tomato for its ability of prevention in prostate cancer (Mucci *et al*., 2014). Despite the pre-dominance of dietary all-trans-lycopene, cis-isomers account for 58-73% of total lycopene in human serum. Very recently Cooperstone and co-workers (2015) observed that all-trans-lycopene accounted for 90% of total lycopene in their tomato juice. Nevertless they found that cys-lycopene was by far the more bioavailable in a clinical study in human (Cooperstone *et al*., 2015).

In our study we found a maximum lycopene content of 8.65 μg · g DW^{-1} in M82 50% WR consistent with a range of 1.86-14.62 of mg per 100 g of FW previously reported (Frusciante *et al*., 2007). The drought stress led to a significant enhancement of this compound in the open field trial and this corroborates the increase of total carotenoids observed in the tunnel trial.

A decrease in its content was observed in tomato sauces, except for M82 100% WR. Page *et al*., (2012) discovered that upon an hot break, tomato sauce from M82 lost about 20% of lycopene, while IL9adm lost about 35% of its lycopene content.

Takeoka and co-workers (2001) reported no consistent changes in lycopene levels as the fresh tomatoes were processed into hot break juice.

In the current study, in general after 53 dph lycopene increased (of 43 % in IL9adm). The present findings mirror those of the previous study by Davila Avina *et al*. (2012).

To measure the total antioxidant activity we used the ORAC assay that revealed a very slight reduction in M82 when stressed, but contrary to this, it was twice higher in IL9adm 50% WR. The latter result was in agreement with Barbagallo *et al*. (2013) who observed that the antioxidant activity was positively affected by water shortage.

The antioxidant activity in tomato has been mainly associated to ascorbic acid (28 - 38%) and phenolics (60 - 70%).

Both the storage and the processing led to a decrease in the antioxidant activity.

A breeding program was primed in 2012 aiming to transfer useful dissected wild QTL*s* from *S. pennellii* (*Sp*) into four different tomato advanced breeding lines, P011_58, P011_77, P011_85 and P011_156 (P011*s* afterwards) selected at the University of Naples "Federico II" because of their superior agronomic performances.

Among the 76 different introgression lines (IL*s*) produced by the initial *S. pennellii* x *S. lycopersicum* cv. M82 hybridization and harboring as many as isolated and dispersed DNA introgression bits from *Sp*, collectively covering the entire tomato genome (Eshed *et al*. 1992), the IL9-2-5, possessing a 9 cM-long wild DNA insertion (Fridman *et al*. 2002), is renowned for possessing several QTL*s* able to confer elevated traits in relation to fruit quality, such as the content in soluble solids (Eshed and Zamir 1995, Baxter *et al*. 2005) and total AsA (Stevens *et al*. 2007, Stevens *et al*. 2008).

In fact, two different major genes controlling the level of soluble solids were mapped on the 9-2-5 introgression, a *Lin5* gene encoding for a fruit apoplastic *invertase* (Fridman *et al*. 2000) with enhanced kinetic activity favoring at last, the accumulation of both simple sugars and organic acids in the mature fruits (Baxter *et al*. 2005), and a *PW9-2-5* gene determining a high level of photoassimilates via an increased shoot mass (Fridman *et al*. 2002) and a QTL ruling the AsA content, involving three different DNA regions located on 9-2-5 region, of which one about 0.1 cM-long containing a gene encoding for the the *Monodehydroascorbate reductase* (MDHAR), an enzyme working in the recycling path of AsA (Stevens *et al*. 2008).

Different sub-lines were selected from the parental IL9-2-5 carrying in a reduced piece of the original 9-2-5 introgression, to explore the chance to dissect the group of *Sp* QTL*s* located on it and separately transfer them into the tomato genome (Stevens *et al*. 2008).

In this study, we used the IL9adm, a IL9-2-5 sub-line selected at the Department of Agriculture of the University of Naples "Federico II", for inserting wild QTL*s* in the genetic background of each of the four tomato P011*s*.

The IL9adm differs from the parental IL9-2-5 due to lacking a chromosomal proximal fragment of the introgression, where the *Lin5* gene was mapped (Friedman *et al*. 2004, Stevens *et al*. 2008). In fact, PCR-based molecular analysis carried out by means of CAPS markers which we built on the basis of the tomato whole genome sequence (TWGS) SL2.40 blueprint, capable of integrally marking the 9-2-5 introgression (Tab. 3.36), evidenced as several CAPS loci (TC217240, TC225638, TC188748 and TC230608) were homozygous for the wild allele in the IL9-2-5, but homozygous for the alternative cultivated allele in the IL9adm.

In particular, the *Lin5* gene has been mapped slightly internal in the 9-2-5 introgression than the TC217240 locus, that allowed us to delimitate the wild fragment absent in the IL9adm to around 2-3 cM, starting from the centromeric border.

In spite of being missing of *Lin5* gene, the IL9adm showed higher content in soluble solids than the parental tomato M82 line, suggesting as the *PW9-2-5* gene could also take relevantly part in driving the accumulation of both simple sugars and organic acids in the mesocarp of mature fruits, even though previous study has appointed *Lin5* as the more remarkable gene in promoting those processes (Baxter *et al*. 2005).

Furthermore, a set of different genes involved in the carbohydrate metabolism, such as those affecting sugar transport and breakdown, starch metabolism and glycolysis, and therefore potentially able to affect the level of soluble solids in mature fruits, have been mapped to 9-2-5 introgression (Causse *et al*. 2004).

The unchanged ability exhibited by IL9adm to produce soluble solids could hence hint the involvement of one or more of those genes in the promotion of biosynthesis and accumulation of soluble solids.

For the reasons above deployed, we found exploiting the IL9adm, source of useful *Sp* genes, a powerful breeding approach, in order to couple the promotion of the

accumulation of soluble solids (*PW9-2-5*) and total AsA (MDHAR) in fruit mesocarp in the P011*s* tomato lines, but in the same time reducing the extent of wild and unwanted chromatin inserted into those lines.

We therefore have been aiming to: *1*) transferring the integral 9adm introgression from the M82 genetic background conferring not appreciable agronomic performances into the P011 genetic background, proved to be superior for many different agronomic traits, *2*) dissecting the *Sp* 9adm chromatin to be inseterted as single and isolated QTL*s* into the P011 genetic background.

We set off a backcross-based breeding program, by setting on the initial hybridization between IL9adm and each of the four P011*s*. The F_1 plants were permitted to go self-pollinated, then the F_2 plants were crossed back to each of the corresponding recipient P011 parental line and plants belonging to BC_1F_2 progenies were again crossed back to the same tomato parental line to get plants in the BC_2F_2, current step of the breeding program.

By using the set of CAPS markers mapping on 9adm genomic region, built and tuned in this study, evidencing polymorphism between the wild and cultivated alleles, we have been able to properly drive the selection of desired genotypes over the next generations of the breeding program.

To date, we selected a total of 32 different plants belonging to the BC_2F_2 progenies resulted to be heterozygous for the wild and cultivated alleles at CAPS loci.

We selected not only plants possessing the entire wild introgression in the P011 genetic background, that indicates a positive pursuit of goal *1*, but also plants from each of the four backcross paths in which only a bit of the initial 9adm introgression was held, indicating as several recombination events happened in the mother cells at meiosis I in the BC_1F_2 plants between wild and cultivated 9adm chromatin, gaining the noteworthy goal *2* to promote the dissection of wild QTL*s* into single genetic factors. That represents the first step to get stable isogenic lines from P011*s*, possessing the desired *Sp* alleles of *PW9-2-5* and *MDHAR* and other interesting genes isolated.

The analyses about the content in soluble solids and total AsA carried out on heterozygous and homozygous plants in BC_2F_2, revealed higher content of both in the fruit mesocarp from the former genotypes in comparison to the latter genotypes, prompting that the improved expression of fruit content of both soluble solids and total AsA and perhaps other important quality traits, would be drown upon coming to an end in the backcross breeding program.

5 Conclusion

This study was carried out to identify genomic regions involved in the control of fruit quality traits in tomato, headed to select new genotypes fitting the increasingly high public demand for a sustainable agriculture asking for limited levels of energy inputs, such as mainly the water supply.

To gain this objective, we compared several commercial hybrid tomatoes for evaluating their nutritional quality-related traits with the purpose to study the narrow genetic variability underlying tomato fruit quality and identify, among others, cultivated hybrids meeting the high-yielding expectation and superior fruit quality.

We studied the contribution to drought tolerance and fruit quality by wild QTLs from the tomato relative *S. pennellii* when two different water regimes were imposed, in both open field and tunnel. This allowed us to examinate the plant physiological response under a low water supply and how it affected the fruit quality behaviour in a susceptible and in a more tolerant genomic background.

Through a transcriptome analysis, we identified candidate genes which were involved in mediating the plant response to drought and the effect on the level of soluble solids and firmness and on the biosynthesis of carotenoids, phenols and AsA, thus in the fruit quality.

We developed a breeding program to transfer wild dissected QTLs controlling quality traits, such as the content in soluble solids and AsA into advanced breeding tomato lines selected for displaying high agronomic performances.

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Appendix:
Research papers

Nutraceuticals for Protection and Healing of Gastrointestinal Mucosa

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Abstract: Natural medicinal products have been used for millennia for the treatment of several ailments. Although many have been superseded by conventional pharmaceutical approaches, there is currently a resurgence in the interest in natural products by the general public and the use of complementary and alternative medicine is increasing rapidly in developed countries. Also, pharmaceutical industries are more and more interested in examining their potential as sources of novel medicinal compounds which may act as growth factor or show immunomodulatory or anti-microbial activity. The subgroup of natural bioactive compounds that bridge the gap between food products and drugs are termed nutraceuticals or functional foods. In contrast with most standard medicinal compounds, nutraceuticals are generally used to prevent rather than to treat disease. Many of the claims for such products are supported by very limited scientific evidence. However, there has recently been a great interest at evaluating the mechanism by which natural products exert their beneficial effects in the gastrointestinal tract. In particular, a major area of interest is for the use of biologically active chemical components of plants, i.e. phytochemicals, in a number of gastrointestinal disorders. While the major focus of phytochemical research has been on cancer prevention, several products of plant origin are being used and/or under study for a variety of other gastrointestinal problems. In this review we discuss the scientific evidence supporting the potential use of nutraceuticals as agents capable to prevent or accelerate healing of gastrointestinal mucosal damage, with a focus on polyphenol extracts obtained from apple.

Keywords: Apple polyphenols, gastric protection, intestinal protection, antioxidants, nutraceuticals, phytochemicals.

INTRODUCTION

The term "Nutraceutical" was firstly coined by DeFelice, chairman of the Foundation for Innovation in Medicine (FIM) [1, 2], and was later defined by Health Canada as, " A *product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food… is demonstrated to have a physiological benefit or provide protection against chronic disease*." [3]. Nutraceuticals include a variety of dietary supplements (mineral, vitamin and antioxidant supplements) but also probiotics, herbs, essential oils, fortified foods and drinks.

For their ancient use among population as natural remedy against several diseases, phytochemicals, i.e. plant components with activities towards animal biochemistry and metabolism, are being widely studied for their ability to provide health benefits and to justify their use as nutraceuticals [4]. Phytochemicals are reported to exert several biological activities: i) participating as substrates and as cofactors or inhibitors of enzymes in biochemical reactions; ii) influencing absorption and stability of nutrients as well as scavenging and eliminating toxic compounds along the gastrointestinal tract (GIT); iii) being selective growth factors and fermentation substrates for beneficial oral and gastrointestinal bacteria as well as inhibitors of deleterious intestinal bacteria [4]. In the body phytochemicals may act at both systemic level (in organs outside the GIT) or inside the gastrointestinal tract, also if they are bound to insoluble dietary fibre [5]. In the case of benefits at systemic level (if any) phytochemicals effects are mediated by their bioavailability and/or by the activity of metabolites and biotransformation products. For long time these effects and the underlying mechanisms have been supposed on the basis of *in vitro* studies on cell or tissue culture performed using high concentrations of the compounds that *in vivo*, by oral exposure, could never occur. Unfortunately, in many cases, the effects found *in vitro* were also transferred to the food containing the bioactive compound, without considering the low amounts achievable by dietary intake and the bioavailability issue. A clear example is the case of resveratrol, which was demonstrated to have several biological effects *in vitro* at concentrations of 1-100 μM [6], that is unlikely to be obtained *in vivo* by consuming moderate amount of red wine (containing resveratrol at 0-10 ppm) due to the rapid metabolism and urinary excretion of the molecule [7].

On the other hand poor attention was paid to the potential role of phytochemicals inside the GI tract where bioavailability is not an issue for their bioactivity. In the mouth, stomach and upper intestine, it is possible the definition of efficacy dosages of individual phytochemicals for specific effect. In Table **1** the results obtained by human and animal long term intervention studies published during the last 10 years and dealing with the effects of polyphenols inside the GIT were summarized. The Table highlights the dosage, the source, the target and the main outcomes of the studies.

Curcumin was the polyphenol majorly studied in humans in the last years. Due to its ascertained ability to inhibit NF-κB activation [9] curcumin has been proposed as potential therapeutic agent against several non communicable chronic diseases having an inflammatory origin such as neurodegenerative diseases (Alzheimer's and Parkinson's disease, multiple sclerosis, epilepsy), CVD, diabetes, obesity, allergies and certain types of cancer [10]. Anyway results from intervention studies are still not conclusive. As regards treatment of colon cancer no effect was found in the study performed by [11] administering to patients 4 g/d curcumin in capsules per 4 months. The authors reported some systemic effects of curcumin but no direct effect on mucosa [11]. Similarly no effect was found by treatment with soy isoflavones in the study by [12] and in eradication of *Helicobacter pylori* in patients with chronic gastritis treated with curcumin [13]. On the contrary amelioration of intestinal mucosa inflammation was shown with low dose of curcumin (1.1 g/d) in patients with inflammatory bowel diseases [14] and, similarly, a big study on 87 patients with resected colon cancer or polyps showed that a mixture of flavonoids, although not effective in reducing colon cancer, reduced neoplasia [15].

These features highlight the actual general assumption (derived from evidences in animals) that in the case of GIT cancers and diseases, phytochemicals may better function as pathology preventive but curative agent. In fact, in the animal studies using accelerated model of pathologies, as $Apc^{Min/+}$ mice, which spontaneously develop multiple intestinal polyps within a few weeks of birth, the inclusion in the diet or in drinking water of curcumin or EGCG just after weaning was demonstrated to retard or reduce tumorogenesis at polyphenol dosage that was absolutely affordable by humans with diet [16-18]. Moreover animal studies

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Table 1. Effects of Food Bioactive Polyphenols Inside the Gastrointestinal Tract (GIT)

Subjects (n)	Phenolic compound and source	Dose and experiment conditions	Target	Results	Major findings	Reference
20 Colorectal cancer patients	Pure resveratrol in capsules	2.2 – 4.4 mmol/d x 8 days	Colon cancer	Tumor cell proliferation is 5% reduced	Resveratrol (0.5-1.0 g/d) produces levels in the human GIT sufficient to elicit anticarcinogenic effects.	[8]
87 Patients with resected colon cancer or polyps	Mixture of flavonoids in tablets (apigenin and EGCG)	40 mg/d x 2-5 years	Colorectal cancer	No significant effect in cancer and polyp recurrence rates Significantly reduced recurrence rate for neoplasia in treated subjects	Sustained long-term treatment with a flavonoid mixture could reduce the recurrence rate of neoplasia in patients with resected colon cancer.	[15]
15 colorectal cancer patients	Curcuminoids in capsules	Curcuminoids from 0.5 to 4 g/d x 4 months	Colorectal cancer	No partial responses to treatment. Reduction (by ~60%) of inducible PGE2 production in blood 1 h after higher dose	Systemic pharmacological properties with 4g curcuminoids / die	[11]
36 Chronic gastritis H. pylori^{+} patients	Curcumin tablets *vs* Antibiotics	Curcumin 120 mg/d x 4 weeks	H. pylori infection	Bacteria eradication: 1/17 pts with curcumin 15/19 pts with antibiotics	It is unlikely that curcumin alone has antibactericidal effect on H. pylori.	[13]
5 with proctitis 5 with Crohn's	Curcumin in capsules	1.1-1.7 g/d x 1 month 1.1 g/d x 3 months	Inflammatory bowel disease	Amelioration of proctitis Reduction of Crohn's Disease Activity Index	Curcumin at dosage of 1.1 g/d can improve inflammatory bowel disease	[14]
150 Patients with adenomatos polyps	Soy-drink powder	Isoflavones 83 mg/d *vs* 3 mg/d (control) x 12 months	Colorectal epithelial cell proliferation	In colon: ↑ cell proliferation and number of crypt labeled nuclei In cecum/colon: ↑ proliferation count. No effect on proliferation distribution and crypt height	Soy isoflavones has no effect on some markers of colorectal cancer	[12]
35 Apc$^{Min/+}$ mice	Curcumin	Curcumin 150-300-750 mg/kg pd Vs Control diet x 8 weeks	Intestinal tumorogenesis	Curcumin 300 mg/kg pd prevented/retarded adenoma formation	Curcumin is efficacy at a dose that in humans would be of 1.6 g per 70 kg person	[16]
141 Apc$^{Min/+}$ mice	Pure EGCG Caffeine	EGCG 0.02-0.32% *Vs* Caffeine 0.044% *Vs* control in drinking water x 6-7 weeks	Intestinal tumorogenesis	EGCG decreases small intestinal tumorogenesis in a dose-dependent manner In small intestinal tumors increases levels of E-cadherin and decreases several trascriptor factors of oncogenic genes Caffeine has no effect	EGCG inhibits intestinal tumorigenesis in Apc$^{Min/+}$ mice	[17]
27 Apc$^{Min/+}$ mice	EGCG or ECG	EGCG or ECG 0.01% Vs vehicle in drinking water x 2 months	Intestinal tumorogenesis	Only EGCG reduces the size and number of tumors and the total number of polyps and tumor load compared with controls. suppresses bFGF in tissue	EGCG may suppress intestinal tumorigenesis *in vivo* by reducing bFGF expression and angiogenesis	[18]
78 piglets	Apple pomace diet (AP) Red-wine pomace diet (RWP) Vs Control diet (CD)	Total polyphenols ingestion (mg/d): AP ➔ 26.0 – 110 RWP ➔ 57.2 – 242 CD ➔ 16.9 – 71.5 x 4 weeks	Morphology of GI tract	Villus length of jejunum and ileum in: CD decrease after weaning AP and RWP never decrease Villus breadth of jejeum in: CD increase by 73% AP and RWP increase by 10% Peyer's patches area: CD increase AP and RWP no effect	RWP ➔ inhibitory effect on the jejunum villi growth AP and RWP ➔ stimulating effect on crypt size in piglet colon can reduce the GALT activation via the Peyer's patches in the ileum.	[19]
32 rats	Apple polyphenol extracts (APE) in drinking water	10 mL APE 10^{-4} M/d (phenols 0.9 mg/kg) x 10 days before indomethacin treatment	Injury of gastric mucosa	APE pretreatment decreased the extent of macroscopic and microscopic injury vs control.	APE prevents indomethacin injury on gastric mucosa in rats.	[20]
98 Apc$^{Min/+}$ mice Wild type mice	Apple polyphenol extracts (APE) in drinking water	APE 8 μM catechin eq/kg b.w. *Vs* water x 12 weeks + Western Diet (WD) + Balanced Diet (BD)	Familial adenomatous polyposis (FAP)	APE: prevents cachexia in Apc$^{Min/+}$ WD reduces polyp number and eliminates high-grade dysplasia in Apc$^{Min/+}$ mice influences polyp growth has antioxidant and anti-lipoperoxidation properties protects against DNA hypomethylation	APE is a candidate chemopreventive agent for clolorectal cancer, particularly for high risk populations (such as FAP) eating a WD	[22]

demonstrated, accordingly with epidemiological evidences, that mixture of phytochemicals, as found in natural products, may function even better than single pure compounds, due to synergisms among individual molecules and/or nutrients which can lead to modification of GIT morphology, as found by [19] in piglets administered with apple or red wine pomace. This fact together with biochemical and epigenetic activities of compounds in the GI environment and mucosa can explain the ability of phytochemicals to counteract dietary chemical toxics or the detrimental long term effect of nutritionally imbalanced diet, as demonstrated in studies relative to stomach [20, 21] and colorectal [22] mucosa protection by apple polyphenols (APE), respectively.

In this framework and starting from the ancient proverb that "an apple a day takes the doctor away", in this review scientific evidence supporting the potential use of nutraceuticals as agents capable to prevent or accelerate healing of gastrointestinal mucosal damage will be discussed majorly focusing on apple polyphenols.

NUTRACEUTICALS IN THE PROTECTION AND HEALING OF GASTRIC MUCOSA DAMAGE

In the stomach there is a massive production of reactive oxygen species (ROS), their concentration being 1000-fold higher than that in other tissues or plasma [23]. Generation of ROS contributes to exogenous injury to the gastric mucosa, including damage brought about by ethanol or nonsteroidal anti-inflammatory drugs (NSAIDs) [24,25]. Moreover, ROS play a major role in the multistep process leading to the development of gastric cancer [26].

Naturally occurring anti-oxidants exert protective biochemical effects in a number of biological experimental systems and are known to scavenge oxygen and nitrogen free radicals breaking lipid chain peroxidation reaction. In particular, phenolic compounds, which are widely distributed in vegetable foods, are considered to play an important role in the prevention of oxidative damage in living systems [27]. Beside their action as radical scavengers, phenolic compounds also have several indirect effects, in fact they are able to inhibit lipoxygenase [28], to reduce platelet aggregation [29] and to reduce the bioavailability of food carcinogens [30]. Certain flavonoids or compounds with flavonoid-like properties have anti-ulcer activity and prevent gastric mucosal lesions brought about by a number of ulcerogens [31-33]. This effect has been related to their ability to inhibit lipid peroxidation, to scavenge ROS, and/or to modulate leukocyte function [34,35]. Singh and colleagues [36] demonstrated that melatonin and β-carotene were protective against indomethacin-induced gastric injury in the rat and that this effect was mediated by scavenging of oxygen-derived free radicals. Similarly, Alarcon de la Lastra *et al.* [31] showed that extra-virgin olive oil-enriched diets prevented indomethacin-induced gastric damage in rats. Finally, the synthetic flavonoid mecianadol has been shown to prevent ethanol- and aspirin-induced gastric injury in the rat [37].

A long list of phytochemicals has been demonstrated to exert gastroprotective effects in *in vivo* experimental model.

Aloe vera, a medicine since before Roman times, contains several potentially bioactive compounds, including salycilates, lupeol, campestrol, β-sitosterol, γ-linolenic acid, aloctinA, and anthraquinones. Acemannan, a component of *Aloe vera*, has been reported to prevent stress-induced gastric ulceration in the rats [38]. *In vivo* and *in vitro* studies have provided evidence that the wound healing properties of *Aloe vera* depend on its ability to stimulate collagen synthesis and fibroblast activity, thus enhancing the remodeling phase of the ulcer healing process [39,40].

Curcumin, the yellow pigment of turmeric (*Curcuma longa*), a widely used spice in Indian and Thai cuisine, has been shown to improve endoscopic healing of peptic ulcers as well as symptoms of patients with non-ulcer dyspepsia [41]. A new zinc(II)-curcumin complex has been demonstrated to prevent cold-restraint stress-induced gastric ulcers in mice and this gastroprotective effect was associated to increased mRNA expression of HSP70 and attenuation of increased iNOS mRNA expression in the mucosa [42] (ok). Also, curcumin prevents indomethacin-induced gastric ulcerations in the rat and this effect is mediated by preventing peroxidase inactivation and by enhancing ROS scavenging [43].

The Bael (*Aegle marmelos*) tree grows in tropical and subtropical countries. Luvangetin, a pyranocoumarin isolated from the seed of Aegle marmelos, has been shown to protect rodents gastric mucosa in multiple model of gastric culceration through a nonidentified prostaglandin-independent pathway [44].

Garlic derivatives are commonly used by the public for a variety of condition including hypercholesterolemia. Also, ingestion of garlic results in the formation of diallyl disulphide from its organosulphur constituents, thus causing an increase in the tissue activities of phase II detoxification enzymes quinine reductase and glutathione transferase [45]. Garlic oil has been shown capable of preventing ethanol-induced gastric injury in rats and this protective effect has been attributed to its anti-oxidant activity [46]. Infact, garlic oil decreased lipid peroxidation and ameliorated the decrease in anti-oxidant enzyme levels brought about by ethanol [46].

Several other plant-derived extracts are of potential interest in the protection of gastric mucosa against axogenous injury. Often these extracts derive from sources such as South America folklore products. One example is provided by Dragon's blood (i.e. Sangre de grado). This viscous red tree sap is used by indigenous cultures of the Amazon river basin because of its healing properties. Sangre de grado derives from several *Croton* species and is used orally for gastritis and gastric ulcer [47]. In particular, sangre de grado was able to accelerate the healing of acetic acid-induced gastric ulcers in rats, reducing myeloperoxidase activity, ulcer size and bacterial content of the ulcer. Also, sangre de grado attenuated the ulcer-induced overexpression of proinflammatory genes such as TNF-α, iNOS, IL-1β, IL-6, and COX2 [47].

Natural honey and *Nigella sativa* have been in use as a natural remedy for over thousand years in various part of the world. In particular, honey has been used to advantage as a topical preparation for wound healing due to its capacity to stimulate tissue growth, enhance re-epithelization and minimize scar formation [48]. These effects are ascribed to honey's acidity, hydrogen peroxide content, osmotic effect, antioxidant and immuno-stimulatory effect [48]. A study by Bukhari and coworkers has recently shown that both natural honey and *Nigella sativa* seeds were equally effective in healing gastric ulcers induced by acetylsalicylic acid in rats at the macroscopic and microscopic level [49]. Phytomedicines have shown great promise in the treatment of several intractable infectious diseases, including opportunistic AIDS infections. In particular, honey can inhibit the growth of *H. pylori*, the main etiologic agent of gastritis, peptic ulcer and gastric adenocarcinoma [50], thus raising the possibility of using honey orally for the treatment of *H. pylori* infection.

In the past few years, we have been interested in evaluating the potential gastroprotective effect of a polyphenolic extract obtained from a variety of apple (i.e. *Annurca* Apple) tipycal of Southern Italy. We have shown that this apple polyphenol extract (APE), rich in catechin and epicatechin, is able to prevent ROS- or indomethacin-induced injury to gastric epithelial cells *in vitro* [19]. Also, APE is able to prevent indomethacin- or aspirin-induced injury to the rat stomach *in vivo* both macroscopically and at the histological level [19,20]. This effect is associated with a significant increase in the intracellular anti-oxidant activity and to a decrease in drug-induced lipid peroxidation as assessed by determination of malondialdheyde, thus suggesting that the APE protective effect is mainly contributed by its anti-oxidant activity. Moreover, APE

counteracted the increased expression both at the mRNA and protein level, of COX-2 and HB-EGF, observed following administration of a damaging dose of aspirin [20]. More interestingly, the gastroprotective effect exerted by APE was not associated to a decrease in gastric acid secretion [20] (Fig. **1**). This has potential clinical relevance, because prolonged acid suppression, which is the currently adopted method to prevent gastric damage induced by NSAIDs or aspirin, has been reported to be associated with increased susceptibility to infections, spontaneous hip fracture and formation of gastric polyps mainly in the fundus of the stomach [51-53].

Cordyceps sinensis (Cordyceps caterpillar mushroom) is a traditional Chinese medicine and health food used to support many organ systems. It is commercially produced in a liquid medium or on a solid (grain/potato) phase. Marchbank *et al.* have recently demonstrated that Cordyceps sinensis extract is able to prevent indomethacin –induced injury to the rat stomach, the results being similar to those obtained with the potent gastroprotective agent EGF [54].

Fermentation is a commonly used process in the standard food industry as well as in the bioactive food field. Fermentation of food products has many effects including partial degradation of protein constituents which, as well as aiding absorption from the gut, may also influence its biological activity. Recently, Fitzgerald *et al.* have demonstrated that a commercial fish protein hydrolysate preparation significantly prevents indomethacin-induced gastric injury in the rat and accelerates epithelial cell migration and proliferation (i.e. the main events leading to gastrointestinal mucosal repair) [55].

In conclusion, the use of food bioactive compounds to reduce drug-induced gastric injury is a reliable approach well acceptable by the general public and that can be particularly effective for stomach disorders, as food bioactive compounds are present in the stomach at the same concentration as in the original foods without uncertainties related to the bioavailability and biotransformation. In particular, the use of NSAID, including low-dose aspirin, continues to be associated with an unacceptable risk for GI ulceration and bleeding. The most effective strategy to reduce NSAID-associated gastric toxicity consists of the use of anti-secretory agents, such as H2-receptor antagonists or proton pump inhibitors (PPI). In particular, the use of NSAID combined with PPI has been adopted widely in clinical practice and it is estimated that PPI use could significantly reduce the rate of endoscopic NSAID-related ulcers. However, prolonged use of PPI is costly and might carry some risk (51-53). That dietary polyphenols, such as those extracted from apple, are able to prevent NSAID injury to the stomach without altering gastric homeostasis, indicates that there might be low cost, effective and safe means of addressing the issue of the prevention of NSAID gastropathy.

ROLE OF NUTRACEUTICALS IN THE LOWER GASTROINTESTINAL TRACT

Foods contain complex mixtures of components [56]; to understand their impact on human health, their nature, origin, amount in the diet, bioavailability and microbial metabolization in the colon need to be investigated. In this respect, gaining understanding of the metabolization pathways of polyphenols and dietary fibre by the microbiota and the kind of bioactive metabolites that are formed during this process is of great interest. Moreover, the effects of such metabolites on the composition of the microbiota itself might be investigated to prepare new strategies that could be an important tool for future therapeutic approaches.

Several studies in the past decade have supported evidence for an alternative approach to the treatment of inflammatory bowel disease (IBD) *via* manipulation of the resident enteric microflora. This may be achieved with pre- and pro-biotics, and with exogenous butyrate. Increasing appreciation of the pivotal role of the enteric microflora in the maintenance of a healthy gut, and in the pathogenesis of IBD, [57,58] has supported the interest in this area. IBD, mainly ulcerative colitis (UC) and Crohn's disease (CD), is a chronic relapsing disorder associated with uncontrolled inflammation within the gastrointestinal tract [59], which has been shown to predispose to the development of colorectal cancer later in life [60]. Recently, it has been estimated that IBD affects

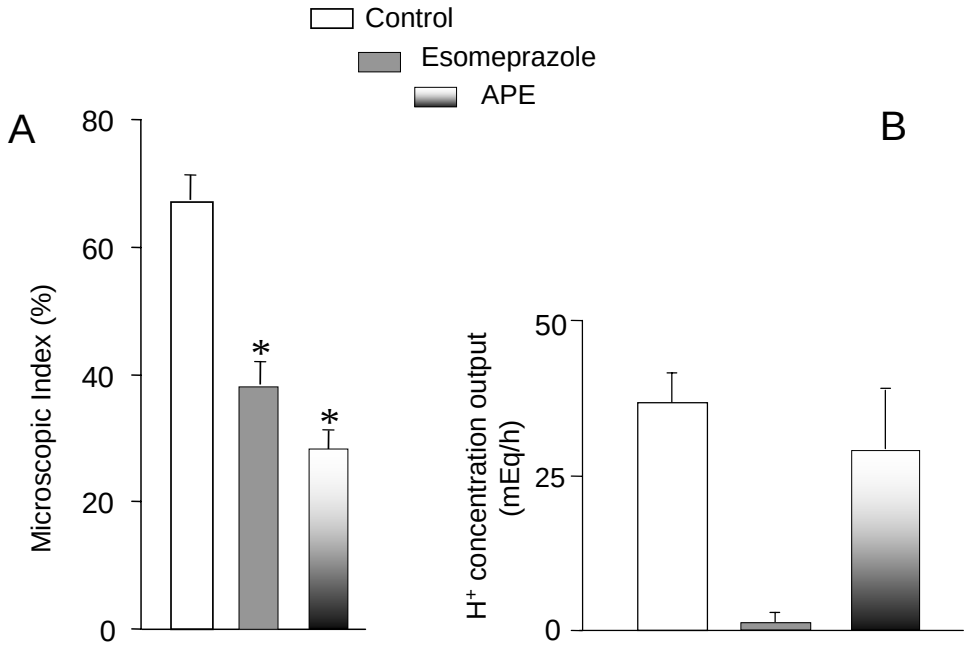

Fig. (1). Effects of APE or esomeprazole, a proton pump inhibitor, on gastric mucosal damage induced by aspirin (panel **A**) and on gastric acid secretion (panel **B**) in rats. APE or esomeprazole were equally effective in preventing aspirin-induced gastric damage (panel **A**). This was at the expenses of profound acid inhibition with esomeprazole, whereas the protective dose of APE did not alter gastric acid secretion (panel **B**).

approximately one million people in USA alone [61]. An underlying factor in the development of these conditions appears to be a dysregulated immune response to the host microbiota in genetically susceptible individuals [62]. Whilst CD and UC both fall under the collective term IBD, these conditions can be quite distinct, with different pathogenesis, underlying inflammatory profiles, symptoms and treatment strategies. CD is predominantly a Th1-driven immune response, characterised initially by increased interleukin (IL)-12 expression, followed by interferon (IFN)-γ and tumour necrosis factor (TNF)-α [63]. CD can occur in any region of the gastrointestinal tract and is characterised by transmural, granulomatous inflammation. In contrast, UC is believed to be a Th2 immune response, leading to increased production of proinflammatory cytokines. UC is restricted to the colon and generally begins in the rectum and spreads proximally, dependent upon disease severity [63]. To further complicate IBD diagnosis, approximately 10% of IBD patients present with symptoms that cannot be categorized as typical of UC or CD. These conditions are referred to as indeterminate colitis, and may develop into UC or CD as the disease progresses [59]. Whilst the aetiology of IBD is not well understood, environmental, genetic and immunological factors appear to play a role in the development of both diseases [64].

As the intestinal microbiota has been linked to the pathogenesis of IBD, probiotic treatment is a consequent choice for therapeutic intervention. An alternative is *via* the administration of prebiotics. Prebiotics are indigested dietary fibres producing: short-chain fatty acids, including butyrate, propionate and acetate in the colon as a consequence of fermentation by luminal bacteria. Short-chain fatty acids are readily absorbed by the intestinal mucosa and are an important source of substrate for metabolism of colonocytes; they are trophic to the intestinal mucosa, stimulate water and sodium absorption in the colon, and induce enzymes that promote mucosal restitution [65,66]. A direct anti-inflammatory role for butyrate, the most extensively studied of the short-chain fatty acids, has been suggested [67]; butyrate enemas have been shown to be of benefit in the management of ulcerative colitis [68] and in animal models of colon inflammation. Germinated barley foodstuff (GBF), a dietary component high in glutamine-rich protein and hemi-cellulose-rich dietary fibre, has demonstrated prebiotic characteristics in the DSS model of rat colitis, as it decreased the incidence of bloody diarrhoea and mucosal injury [69]. In animal and human studies, ingestion of resistant fibre has resulted in an increase in the population of Bifidobacillus and Lactobacillus in the colon and an increase in faecal butyrate concentration; GBF has been shown to attenuate inflammation in dextran sodium sulphate, trinitrobenzene sulfonic acid (TNBS) and HLA transgenic animal models of colitis [70-73]. Improvement in clinical and endoscopic indices has been reported in a pilot study of 10 patients with active ulcerative colitis with 4 weeks of treatment with GBF that was well tolerated and did not show significant adverse events [74]. Furthermore, the fibre also contributes to increase stool consistency due to its high avidity for water and possibly its adsorption of luminal bile salts [72,75]. An open-label, parallel-group, randomized study of another fibre, Platago ovata seeds, also reported equal efficacy to mesalazine in maintaining remission in patients with ulcerative colitis [76]. GBF was also shown to display a greater capacity to reduce the symptoms of DSS colitis than a probiotic mixture of lactobacilli and C. butyricum [77]. More recently, it was reported that a mixture of long-chain inulin and oligosaccharide was able to reduce macroscopic scores and inflammatory histological scores in the colon [78]. Oligosaccharides are other prebiotics which have shown a capacity to reduce DSS colitis in rats [79]. There is definitive evidence from animal models that prebiotic supplementation of the diet may provide a therapeutic option in the treatment of IBD. The rationale behind prebiotic use is to elevate the endogenous numbers of beneficial bacterial strains including lactobacillus and bifidobacterium [80]. This increase leads to the beneficial effects seen by probiotic administration, including an increase in short chain fatty acids production, particularly butyrate, which can provide fuel for colonocytes, production of anti-bacterial substances, and decreased luminal pH [62]. Our previous study [81] demonstrated that serum and tissue transglutaminase (TG) activity correlate with the severity of inflammation in the TNBS-model of colitis; moreover butyrate stimulates TG activity in several cultured cell lines [82]. Based on these evidences, we treated TNBS rats with butyrate, mesalamine, or butyrate in combination with mesalamine enemas, obtaining an improvement of histology more marked in the presence of butyrate that also increases TG activity in the colon. Further evidences have shown that different isoforms of TG play a key role in tissue healing in UC [83] (Fig. **2**).

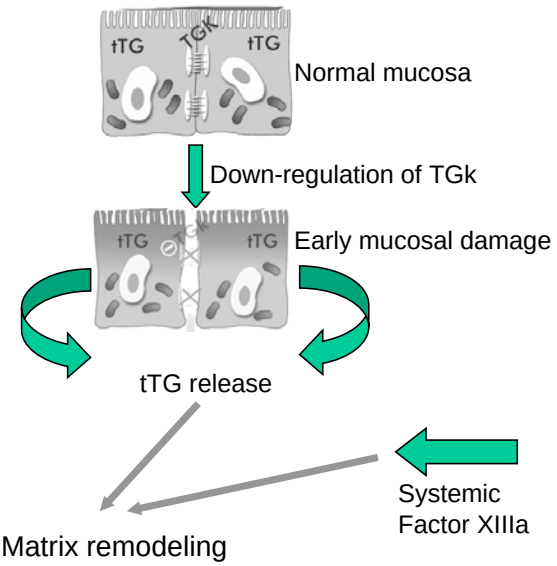

Fig. (2). Role of different transglutaminases in the colon: in the normal colon, Keratinocyte transglutaminase (TGk) contribute to intracellular tight junction formation while tissue transglutaminase (tTG) is preferentially localised within the cell cytosol. In the presence of pathogens, TGk is downregulated allowing cell separation and antigens to penetrate and perpetrate the mucosal damage. At that stage, tTG is upregulated and massively released in the inflamed area together with the circulating transglutaminase factor XIIIa; they promote cross linking of proteins in the extracellular matrix (ECM) initiating the healing process.

The research involving the use of prebiotics to treat IBD is not currently as extensive as that regarding probiotic therapy. Probiotics are provided in processed foods or in dietary supplements as live bacteria. Yogurt is the most common probiotic-carrying food; however, cheese, fermented and unfermented milks, juices, smoothies, cereal, nutrition bars, and infant/toddler formula all are vehicles for probiotic delivery. The main probiotic supplements on the market utilize lactobacilli, streptococci and bifidobacteria, which are normal constituents of the human gastrointestinal microflora. However, studies are also investigating potential probiotic roles of other microbes such as yeast (*Saccharomyces boulardii*), which are not normally found in the gastrointestinal tract [84,85]. Probiotic microorganisms do not act exclusively in the large intestine by affecting the intestinal flora but also affect other organs, either by modulating immunological parameters, intestinal permeability and bacterial translocation, or by providing bioactive metabolites [86]. As the microbial environment has been shown to play a role in the development of IBD, targeting of the microbiota presents an option for therapeutic intervention [87]. One potential method to manipulate the intestinal microbiota in an attempt to reduce the inflammatory response is *via* the administration of probiotics. Probiotics have been used in the treatment of a number

of inflammatory conditions including arthritis [88], atopic eczema [89], pouchitis [90], radiationinduced [91], and NSAID-induced enteropathy [92], chemotherapy-induced mucositis [93], ulcerative colitis [94], Crohn's disease [95], antibiotic-induced diarrhoea [96] and experimental colitis [97]. The mode of action of probiotics is complex and not completely understood; there have been a large number of known probiotic species, most of which show differing mechanisms of action. However there are a number of common mechanisms present in a wide variety of probiotic strains. One such mechanism is adherence to the intestinal mucosal surface which prevents colonisation of pathogenic bacteria through a form of competition between the two species [98]. Evidence for this mode of action has been shown in numerous *in vitro* model systems for example, pre-incubation with Lactobacillus rhamnosus GG (LGG) has been shown to prevent the adherence of B. vulgatus to mouse epithelial IEC-6 cells [99]. A further common mode of action is *via* stimulation of the intestinal immune system. Probiotics are believed to be involved in the modulation of cytokine levels by inhibiting production of pro inflammatory cytokines (including TNF-α and IL-1β) and promoting production of anti-inflammatory cytokines (including IL-10) [99]. Probiotics are also believed to function *via* the modulation of cell proliferation and apoptosis [100]. The increase in epithelial cell proliferation is believed to be due to the ability of probiotic strains to produce short chain fatty acids *via* the fermentation of polysaccharides. The antiapoptotic effects of probiotics have been shown in human and mouse colon cells by the activation of the anti-apoptotic Akt pathway and by the inhibition of the proapoptotic p38/MAPK pathway [101]. Therefore, probiotics have the potential to be beneficial in the treatment of IBD due to their capacity to prevent the colonisation of pathogenic bacteria [98], reduce inflammatory cytokine expression [99], enhance epithelial cell proliferation [100], inhibit apoptosis [101] and provide metabolic energy for colonocytes [98].

A number of clinical trials have focused on the use of probiotics in IBD, however, there is a deficiency of large, randomised, double-blind, placebo-controlled clinical trials investigating the efficacy of candidate probiotic species or combinations thereof. One promising study involving the use of probiotics in IBD treatment was in the setting of pouchitis, an inflammatory condition which often arises following surgical resection as a treatment for UC. The study involved patients deemed to be in clinical remission from pouchitis following surgical resection for UC. Patients received daily treatment with probiotics or placebo and were periodically assessed for signs of relapse. Only 15% of treated patients showed signs of relapse compared to 100% of the placebo group [90]. This study indicated that probiotics have the potential to prevent inflammatory conditions in humans. Based on the results from studies in both animal models and clinical trials, there is evidence that a number of probiotic species assist in the reduction of inflammation and intestinal damage whilst others have no effect, depending on the disease setting. For probiotics to become a legitimate therapeutic option for the treatment of IBD there needs to be more focus on the determination of which probiotic strains have the greatest efficacy in a specific disease setting and whether these candidate probiotics are more effective alone, or in conjunction with other pro- or prebiotics. Moreover more consideration of possible adverse side effects and knowledge of the effect of the probiotics on immune regulation in the intestinal mucosa is claimed. An important point is the time that the species remains in the gastrointestinal tract, as this will determine the frequency and dose requirements [102]. It may also be the case that our genetic profile may predispose our responsiveness to probiotic treatment, as is the case with chemotherapy [103]. Identification of these "probiotic responsiveness genes" may lead to screening to determine whether a patient will be responsive to probiotic therapy, and to which probiotics they would respond more efficiently.

There is currently a growing interest in the use of natural bioactive products by the general public, with many healthy subjects and patients taking them for the prevention and treatment of multiple conditions, including gastrointestinal disorders. The gastroprotective effect of a polyphenolic extract (APE) obtained from a variety of apple prompted us to test the possibility that APE may be effective in the treatment of experimental colitis. In TNBS rats APE reduced macroscopic and microscopic disase activity and reduced the expression of pro-inflammatory cytochine [104]. As the long lasting inflammation has been shown to predispose to the development of colorectal cancer, the chemopreventive effect of APE in intestinal polyps formation in ApcMin/+ Mice [22] warrants future investigations for APE in IBD. Furthermore, in a recent article, Shapiro *et al.* [105], suggest that the addition of polyphenols to artificial nutritional formulas would improve the outcome of patients with IBD and acute pancreatitis in need of enteral or parenteral nutrition.

Zinc carnosine (ZnC), is a health food product claimed to possess health-promoting and gastrointestinal supportive activity. A recent study [106] demonstrated that ZnC stimulated cell migration and proliferation, and that oral ZnC decreased gastric and small intestinal injury in animals. No significant increase in gut permeability was seen in volunteers treated with indomethacin when ZnC was co-administered.

Pharmacological options to reduce problems related to gastrointestinal symptoms including cramps, diarrhoea, nausea, and bleeding are limited, particularly in competitive athletics. One product that is attracting great interest is bovine colostrum. Colostrum is the first milk produced after birth and is particularly rich in immunoglobulins, antimicrobial peptides and other bioactive molecules including growth factors [107]. Some studies suggest it may be of value in eliminating infection and stimulating growth of the neonatal gastrointestinal tract [108,109]. Its value in the prevention and treatment of adult gastrointestinal injury has now been suggested [110]. Using a combination of a clinical trial and *in vitro* experiments, this study has shown that bovine colostrum reduces the exercise-induced increase in gut permeability, possibly through mechanisms including reducing temperature-induced apoptosis and induction of heat shock protein.

CONCLUSIONS

Many healthy subjects and patients are taking potentially bioactive products for the prevention and treatment of multiple conditions, including gastrointestinal disorders. This forms the basis of a world-wide, multi-million dollar major commercial industry. While the scientific validity of the use of a number of these products is lacking, in the past few years much effort has been made to provide solid knowledge of the mechanisms underlying the beneficial effects of nutraceuticals. Scientifically rigorous research is warranted in order to identify novel compounds to be used alone or in combination with standard drugs in gastrointestinal disorders. Also, future research should be aimed at further increasing the efficacy of a promising nutraceutical, trying to use it as a chemical template for combinatorial synthesis. Finally, researchers in this area should focus on the understanding of the mocecular action of each nutraceutical and test the possible synergistic effects with other nutraceuticals and or derivatives, food components, or conventional drug. However, one must keep in mind that just because isolated compounds start from a natural food, they are not necessarily safe and natural and, therefore strict quality control and regulatory issues are mandatory. Future clinical studies are necessary to determine whether these compounds will be as interesting as preventive or therapeutic agents as they are in pre-clinical studies.

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Human bioavailability of flavanols and phenolic acids from cocoa-nut creams enriched with free or microencapsulated cocoa polyphenols

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Abstract

Human bioavailability of cocoa flavanols and phenolic acids from a cocoa-nut cream (CC) and from CC enriched with a 1·5 % (w/w) cocoa polyphenol extract in free form (FPC) or encapsulated with a gastric-resistant high-amylose maize starch (EPC), was studied. In a randomised cross-over protocol, with 1-week wash-out in between, twelve healthy volunteers had three portions/d of each cream, providing approximately 190 μmol/d of total flavanols and 12 μmol/d of total phenolic acids with CC and 385 and 28 μmol/d with both FPC and EPC, respectively. Blood, urine and faecal samples were analysed by HPLC/MS/MS. Serum (epi)catechin was absent at baseline and after CC consumption, while 22·1 (SEM 2·62) and 1·59 (SEM 0·22) nmol ($P < 0·05$) were found after FPC and EPC, respectively. The EPC increased faecal excretion of total flavanols compared to FPC (151·0 (SEM 54·6) *v.* 28·0 (SEM 14·0) nmol; $P < 0·05$). Within 6 h after consumption, serum phenolic acid content was 50-fold higher than (epi)catechin; no difference between CC and FPC was observed, but a significant reduction after EPC (1954 (SEM 236·3) and 1459 (SEM 137·6) *v.* 726·8 (SEM 73·4) nmol, $P < 0·05$) was recorded. Short-term phenolic acid urinary excretions were significantly higher after FPC than CC and EPC, the values being 11·4 (SEM 5·1) *v.* 3·1 (SEM 1·7) and 0·9 (SEM 0·5) μmol, respectively. Faecal phenolic acids were approximately 60-fold reduced after FPC (8·1 (SEM 0·13) nmol) and EPC (14·7 (SEM 2·7) nmol) consumption compared to CC (641·4 (SEM 99·1) nmol) consumption. The data demonstrated that: (i) (epi)catechin was absorbed from CC; (ii) cocoa polyphenols' consumption increased circulating phenolic acids; and (iii) encapsulated ingredient increased flavanol delivering into the gut. Further studies should evaluate whether encapsulated cocoa polyphenols may be a functional prebiotic ingredient.

Key words: Bioavailability: Cocoa polyphenols: Encapsulation: Functional food

Epidemiological studies associate cocoa and chocolate consumption to a reduced risk of CVD, and attribute this effect to their polyphenol moiety[1,2]. Cocoa polyphenols include a sub-class of flavonoids, namely flavan-3-ols, occurring as monomers, mainly epicatechin and catechin, oligomers (procyanidins B_1, B_2 and C_1) and polymers (up to ten units), known as procyanidins[3–5]. Monomers account for 5–10 % of total cocoa flavanols, while oligomers and polymers constitute ≥90 %[6]. They are the major cocoa polyphenols, being estimated to be more than 3 g/100 g cocoa powder, while cocoa phenolic acids and flavonols are 100 times less abundant, estimated at 62 mg and 30 mg/100 g, respectively[7]. Small amounts of gallocatechin and epigallocatechin have also been quantified in cocoa[5]. The abundance of flavanols compared to the other polyphenols has justified the major scientific interest shown up till now in the bioavailability of these compounds. All in all, bioavailability studies demonstrated that, whereas monomers are readily absorbed in the stomach and small intestine, the absorption of dimeric procyanidins in human subjects is very limited[8,9]. Indeed, polymeric procyanidins mainly reach the colon, where they are largely metabolised by the local micro-organisms to produce several phenolic acids[10–12]. Once formed, they are absorbed, further metabolised in the liver and excreted in urine[8,13,14]. Thus, the general consensus is that cocoa flavanol bioavailability is dependent on the ingested dose, the glucuronidated, sulphated and methylated metabolites being the most abundant compounds within 6 h after consumption, while phenolic acids (in the free forms or further metabolised by the liver) predominate later on. These *in vivo* studies were mainly

Abbreviations: CC, control cream; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; HACS, high-amylose maize starch.

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performed using chocolate or cocoa, consumed alone and/or with milk, and often highlighted some differences in bioaccessibility and biotransformation of cocoa polyphenols, depending on the composition of the dietary matrices such as the presence of proteins that generally retard polyphenol absorption as well as the fat content that may modulate relative absorption of individual compounds and/or metabolites[8,15–21].

In some cases, the effects of cocoa or chocolate consumption were reported, a few hours after intake, on some markers of cardiovascular function (such as NO synthesis, flow-mediated vasodilation and peripheral arterial tonometry responses) and associated with the increased amount of catechin phase I/II metabolites in the blood[22]. However, the biological effects of flavanol-3-ol-conjugated metabolites have been mostly studied *in vitro* using concentrations that not always have physiological relevance; thus, the *in vivo* bioactivity of these compounds is still a highly debated matter[23]. On the other hand, the potential role of other classes of cocoa polyphenols, such as phenolic acids, in exerting short-term bioactive effects has never been explored, despite a growing interest in phenolic acids formed from the catabolism of flavanols by colon microflora emerging in the scientific literature[23].

The knowledge of the relationship between the physiological response to foods and their composition is fundamental to formulate new ingredients and foods having a nutritional advantage, compared to the existing ones[24]. In general, due to the extensive metabolism and rapid excretion of cocoa flavanols, increasing their intake without increasing overall energy intakes may be nutritionally advantageous. Enrichment of cocoa-containing foods (e.g. chocolate bars, creams or drinks) with flavanol-rich extracts obtained from different cocoa bean fractions may be a reliable appropriate solution[25]. Unfortunately, catechin and procyanidin enrichment over a certain level may impart an undesired bitter and astringent taste to the final product. Encapsulation of cocoa polyphenol extract may overcome this limitation. In fact, during the last 10 years, this technology has rapidly enlarged its application in the food industry, thus allowing food addition with several bioactive compounds, guaranteeing their protection during food processing, tailoring their release over time and/or at particular sites and masking unwanted tastes and flavours of core materials[26–28].

In this framework, the objectives of the present study were to evaluate human bioavailability of cocoa flavanols and phenolic acids upon consumption of nut creams containing 20 % of cocoa (CC) and to evaluate whether, and to what extent, cocoa-nut cream enrichment with a cocoa polyphenol extract in free (FPC) or in encapsulated (EPC) form influenced this issue. For this purpose, nut creams were developed and produced. A total of twelve healthy volunteers participated in the study, consumed the creams in a randomised manner and blood, urine and faeces were collected at specific time points over 24 h. Biological samples were analysed by HPLC/MS/MS to monitor free native flavanol-3-ols (monomers and dimers) and several phenolic acids (both present in cocoa-nut creams as well as known to originate from cocoa flavanols and flavonols).

Materials and methods

Chemicals and reagents

All chemicals and reagents were of analytical grade. Methanol, water, acetonitrile and hexane were from Merck; only for cream chemical characterisation, water was obtained from a MilliQ water purification system (Millipore Corporation). Ethyl acetate, glacial acetic acid and HCl were from Clean Consult International; formic acid (98 % purity), butylated hydroxytoluene and salts used for PBS preparation were obtained from Sigma. All analytical standards, 5-caffeoylquinic acid (95 %, chlorogenic acid), *trans*-4-hydroxy-3-methoxycinnamic acid (99 %, ferulic acid), 3,4-dihydroxybenzoic acid (≥97 %, protocatechuic acid), 4-hydroxybenzoic acid (99 %, *p*-hydroxybenzoic acid), 3,4-dihydroxycinnamic acid (≥98 %, caffeic acid), 4-hydroxyphenylacetic acid (98 %), 3-(4-hydroxyphenyl)propionic acid (98 %), 4-hydroxy-3-methoxybenzoic acid (97 %, vanillic acid), naringenin (98 %) and quercetin (≥98 %) were purchased from Sigma. (+)-Catechin (≥99 %), (−)-epicatechin (≥97 %), procyanidin B_1 and B_2 (≥90 %), apigenin (≥99 %), luteolin (≥97 %) and kaempferol (≥99 %) were obtained from Fluka.

Preparation of free and encapsulated cocoa polyphenol extract

Polyphenol extract from cocoa nibs was produced by La Morella Nut, according to the procedure described by Ortega *et al.*[25]. It was partly microencapsulated by KARMAT using a technological process based on the formation of nanocomplexes with a high-amylose maize starch (HACS) as coating agent of cocoa polyphenols and on their aggregation in microcomplexes. In particular, the following steps were performed: (1) solubilisation of the coating material in an alkaline solution (pH 12) kept at high temperature (80°C) and stirred continuously; (2) chilling this material up to 30°C; (3) addition, under stirring, of the cocoa extract up to 10 % of HACS (w/w); (4) pressurisation into an homogenator together with an acid solution until a pH of approximately 5 is reached and, finally, spray-drying using 200°C as inlet temperature, approximately 100°C outlet temperature and a flow rate of 10 l/h to obtain fine particles.

The final ingredient contained cocoa polyphenol extract–HACS in a 1:9 (w/w) ratio.

Preparation of cocoa-nut creams

Once the polyphenol-rich ingredients were obtained, a nut cream containing 20 % (w/w) cocoa (control cream, CC) and ten prototypes of polyphenol-enriched cocoa-nut creams containing free or encapsulated polyphenols ranging from 0·5 to 2·5 % (w/w) were prepared by a pilot-scale apparatus located at La Morella Nuts. The CC was prepared by the sequential addition of individual ingredients through continued mixing, in order to obtain a well-mixed and refined cream (particle sizes about 30 μm). To this basic cream, the free or encapsulated polyphenol extract was slowly added and mixed gently to homogenously disperse the ingredients.

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Once the homogeneous products were obtained, creams were immediately packaged in 33-g portions and labelled with alpha-numeric codes.

Sensory analysis of creams

To establish what was the maximum enrichment of the creams achievable with the polyphenol-rich ingredients, a sensory analysis of the ten cream prototypes was performed. A total of thirty untrained subjects, selected among students and staff of the Department of Food Science at the University of Naples based on medical status, absence of allergies and habitual consumption of nut/chocolate creams and spreads, were enrolled to participate in the study. The selected subjects were healthy, of both sexes (fifteen male and fifteen female), between 25 and 35 years of age and were of normal weight (BMI 22 (SEM 2) kg/m^2). A total of ten prototype formulations, prepared as described earlier, were compared to CC: they contained 0·5, 1, 1·5, 2 and 2·5 % of the cocoa polyphenol extract, in free or encapsulated form. At different days for each test, and always at least 2 h after having breakfast, three types of creams were tested in blinded and randomised manner. Approximately 10 g of each cream, placed in transparent small cups, were provided to subjects together with a glass of room temperature water and a slice of white bread for palate cleansing between sample testing. The panelists were asked to taste and to assign a score to their hedonic of the following sensory attributes: sweetness, bitterness, creaminess, fatness, granularity and overall palatability. Ratings were based on a nine-point hedonic scale (0 = extremely dislike, 9 = extremely like).

Composition of the cocoa-nut creams

The composition of CC, FPC and EPC is summarised in Table 1, reporting for each parameter the mean of three measures obtained by triplicate analysis and SEM. Water, lipids, proteins and carbohydrates were determined by official methods of analysis (AOAC 9321.04, AOAC 963.15, AOAC 939.02, AOAC 980.13, respectively), while flavanols (monomers, oligomers and polymers) as well as phenolic acids and flavonols were measured according to the method described by Ortega *et al.*[25] with slight modifications. Briefly, approximately 3 g of sample were weighted and fat was removed by using 3 ml of hexane. Polyphenols were extracted from the defatted pellet using a total volume of 9 ml (3 × 3 ml) acetone–water (1:1) solution. After addition of extraction solvent to the pellet, the whole mixture was sonicated for 10 min at 4°C to improve the polyphenols' extraction efficiency and obtain a recovery of 98 % of polyphenols from the encapsulated ingredient (as found in preliminary experiments whose data are not shown). All acetone/water phases were collected and acetone removal by rotary evaporator was followed by freeze-drying of resulting aqueous solutions. Finally, approximately 10 mg of dried extracts were suspended in 2 ml of a methanol–water (1:1) solution and 20 µl of this suspension were injected in to a HPLC system (Shimadzu LC-10A Series) equipped with two pumps (LC-10AD), a controller (SCL-10A) and a diode-array detector (SPD-M10A). Chromatography separation was carried out with a Prodigy 5 µm ODS-3 100 Å column, size 250 × 4·60 mm C18 column, purchased from Phenomenex, with a mobile phase flow rate of 0·8 ml/min, consisting of acidified water with 0·2 % of formic acid (phase A) and an acetonitrile–methanol solution in the ratio 60:40 (v/v, phase B). The elution gradient was set as follows: time 0 = 20 % B; 0–6 min 30 % B; 6–16 min 40 % B; 16–24 min 50 % B; 24–32 min 98 % B; 32–35 min 98 % B; 35–40 min 20 % B; and 40–45 min 20 % B. Catechin, epicatechin, procyanidin B_2, *p*-hydroxybenzoic acid, vanillic acid, apigenin, naringenin, quercetin arabinoside, luteolin glucoside, quercetin glucoside and kampferol-rutinoside were detected at 280 nm; protocatechuic acid at 254 nm; and chlorogenic acid,

Table 1. Composition of experimental creams (per 100 g)
(Mean values with their standard errors (*n* 3))

	CC		FPC		EPC	
	Mean	SEM	Mean	SEM	Mean	SEM
Water (g)	1·5	0·1	1·5	0·1	1·3	0·2
Protein (g)	7·9	0·3	7·8	0·6	6·7	0·5
Carbohydrates (g)	49·7	2·1	50·0	1·8	57·3*	2·8
Dietary fibre	40·0	3·0	40·1	2·6	47·2*	2·0
Lipids (g)	40·9	2·9	40·8	3·1	34·8	1·9
Total flavanols (µmol)	190·0	2·2	385·1†	5·3	385·3†	4·9
Monomers‡	176·0	2·1	336·8†	5·0	337·0†	4·4
Dimers§	14·0	0·1	48·3†	0·3	48·3†	0·5
Total phenolic acids (µmol)‖	12·0	0·9	28·1†	1·0	27·9†	0·2
Total flavonols (µmol)¶	9·2	0·0	12·9†	0·1	13·0†	0·4

CC, control cream; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract.
* Mean values were significantly different from that of FPC ($P<0·05$; Bonferroni test).
† Mean values were significantly different from that of CC ($P<0·05$; Bonferroni test).
‡ Catechin and epicatechin.
§ Procyanidin B_1 and B_2.
‖ *p*-Hydroxybenzoic acid, vanillic acid, protocatechuic acid, chlorogenic acid and caffeic acid.
¶ Apigenin, naringenin, quercetin arabinoside, luteolin glucoside, quercetin glucoside, kampferol-rutinoside and rutin.

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caffeic acid and rutin at 330 nm. The peak area was integrated by means of Class-VP software (version 7.3) obtained from Shimadzu. Each compound was quantified using specific calibration curves obtained with the reference standard compounds, as reported previously; for glycosides, the calibration curves of the respective aglycones were used.

Bioavailability study

Study design. The present study was a single-blind study, and had a randomised, cross-over design with three arms.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving subjects were approved by the Ethics Committee of 'Federico II' University of Naples (Ethic Approval Number 37/10). Written informed consent was obtained from all subjects before entering into the study.

A total of twelve healthy volunteers (four males/eight females, mean age 24 (SEM 3) years, BMI 23·1 (SEM 1·5) kg/m^2) were selected among the students of the Agricultural Faculty, University of Naples. Subjects reporting symptoms of gastrointestinal disorders (including frequent diarrhoea episodes or constipation), having metabolic diseases (diabetes, metabolic syndrome, etc.), taking non-steroidal anti-inflammatory drugs or having undergone controlled dietary regimens over the last 6 months were considered ineligible for the present study. The selected volunteers signed a written informed consent before starting the study. The experimental design is schematised in Fig. 2. For the 3 d before and over each experiment day, subjects were asked to follow a polyphenol-free diet. It consisted, in exclusion of the habitual diet, of all polyphenol-rich foods and beverages such as fruits, vegetables, chocolate, tea, coffee, wine, beer, supplements, herbal extracts and wholegrain-based foods. Consumption of non-steroidal anti-inflammatory drugs and antibiotics was also avoided during 1 week and 1 month before treatments, respectively. On the experiment day, after fasting for 12 h, the subjects reached the laboratory at 08.00 hours and were randomised to receive one of the three experimental creams, which was consumed within 15 min together with three slices of toasted bread and a glass of room temperature water. They left the research centre 6 h later and were allowed to have lunch (within 14.45 hours) and dinner (within 22.00 hours), including, in both occasions, another cream portion. Each subject was invited to consume lunch and dinner during the three experimental sessions, always constituting the same foods, so that no influence of dietary pattern on bioavailability of compounds in the three treatments might occur. In particular, they were invited to choose among the following foods: rice or pasta with butter and cheese or with tuna, meat or fish or bread with ham and/or cheese. After 24 h, subjects returned to consume their own habitual diet for a 1-week wash-out period. After this week, the experimental design was repeated. In particular, subjects again followed a 3-d polyphenol-free diet and were randomised for another treatment.

Thus, all subjects participated in the three experimental sessions (one for each type of cream) with a 1-week interval between each other, and on each test day, they consumed three portions (99 g) of the test cream they were randomised for.

Sample collection. At fasting conditions and at 30 min, 1, 2, 4 and 6 h after breakfast consumption, blood samples were drawn. Then, 24 h urine was collected over 0–2, 2–4, 4–6, 6–8, 8–10, 10–24 h time intervals, after ingestion of the first cream portion, and the volume was measured. The 10 ml samples of urine collected before breakfast and at each time interval were stored for analysis. The day after the experiment, participants returned to the laboratory after fasting for 12 h and their blood samples were taken (24 h from the first cream consumption), while the faecal sample was collected on the experiment day. No subject was constipated or had diarrhoea episodes over the study period; thus, faecal samples were always collected from each volunteer.

Biological sample treatment, storage and analysis. Blood samples were collected in a Vacutainer tube for gel separation, and immediately centrifuged at 4000 rpm for 10 min at 4°C. Urine samples were immediately treated with 0·005 % of butylated hydroxytoluene. Faeces were diluted in the ratio 1:10 (w/v) in PBS (10 mM) containing 0·005 % of butylated hydroxytoluene, vortexed and centrifuged at 4000 rpm for 15 min at 4°C. Serum, urine and faecal supernatants were stored at −40°C until the analysis. Procyanidins, metabolites and phenolic acids were extracted by 1·5 ml of ethyl acetate from 500 μl of serum and 1·5 ml of urine and faecal suspensions (twice and for three times, respectively). The collected supernatants were dried under nitrogen flow and the dry extracts were dissolved in 50 μl methanol–water (70:30); 30 μl were injected into HPLC/MS/MS apparatus. Each sample was extracted in duplicates. Quantitative determination of total extracted analytics was performed using a HPLC system consisting of two micropumps by Perkin Elmer Series 200, coupled with an API 3000 Triple Quadrupole mass spectrometer (Applied Biosystem Sciex). Elution was achieved with a Phenomenex Luna 3 μ C18(2) 100 A (50 × 2·00 mm) column and by using the following mobile phases: A = water–acetonitrile–formic acid 94·9:5:0·1 (by vol.) and B = acetonitrile–formic acid 99·9:0·1 (v/v); the flow rate was 200 μl/min. The linear gradient for chromatographic separation was: 0–1 min, 4–40 % B; 1–3 min, 40–100 % B; 3–5 min, 100 % B; and 6–10 min, 4 % B. Selected compounds in the native form were detected and quantified through electrospray ionisation MS/MS analysis. After ionisation in negative mode, transitions of parent and product ions specific for each compound were tracked in multiple reaction monitoring mode. For each compound, all MS parameters (declustering potential, focusing potential, collision energy, collision cell exit potential), set as previously described[12,29] and then optimised through direct infusion experiments, are reported in Table 2, together with limits of detection and quantification. When analytical standards were not available, compounds were identified comparing molecular weight and fragmentation patterns with those reported in the literature[12,29]. Epigallocatechin was quantified using the calibration curve built with epicatechin; hippuric, dihydroferulic and hydroxybenzoic acids were quantified using the calibration curve built

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Table 2. MS parameters, negative ions and transition product ions analysed by HPLC/MS/MS

Compound	M−H	Product ions	DP	FP	CE	CXP 1	CXP 2	LOD (ng/ml)	LOQ (ng/ml)
Procyanidin B_2	577	289; 425; 407	−45	−300	−35; −25; −31	−7		2·5	5·0
(Epi)catechin	289	245	−40	−300	−21	−7		5·0	10·0
EGC*†	305	179	−40	−375	−30	−7		–	–
Protocatechuic acid	153	109	−45	−400	−21	−10		0·5	1·0
Vanillic acid	167	152; 108; 123	−45	−250	−22; −26	−9	−11	2·5	5·0
Ferulic acid	192·8	133·9; 177·9	−35	−250	−22; −17	−10		0·5	1·0
Chlorogenic acid	353	191	−35	−250	−21	−8		0·5	1·0
Caffeic acid	179	135	−49	−350	−35	−8		0·5	1·0
Hippuric acid*‡	178	134; 77	−45	−350	−20	−7		–	–
Homovanillic acid	180·8	136·7; 122	−50	−350	−10; −18	−7		25·0	100·0
Hydroxybenzoic acid*‡	137	93	−50	−350	−25	−7		–	–
Coumaric acid	163	119·1	−40	−350	−23	−5		2·5	5·0
Di-HCA	181·1	109; 137	−50	−300	−25; −14	−9		25	50
Di-HFA*‡	195	136	−40	−350	−20	−6		–	–
DHPA	167	123·1	−30	−250	−11	−7		0·5	1·0
HPA	151	107; 78·9	−35	−250	−16; −25	−7		3·0	5·0
HPP	164·9	121; 105·9; 76·7	−25	−250	−10; −20; −10	−7		25·0	100·0
DHPV*§	207	163; 122	−35	−350	−25	−7		–	–

CXP, collision cell exit potential; DP, declustering potential; FP, focusing potential; CE, collision energy; LOD, limit of detection; LOQ, limit of quantification; EGC, epigallocatechin; Di-HCA, dihydrocaffeic acid; Di-HFA, dihydroferulic acid; DHPA, 3,4-dihydroxyphenylacetic acid; HPA, 4-hydroxyphenylacetic acid; HPP, 3-(4-hydroxyphenyl)propionic acid; DHPV, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.
* Identified based on molecular weight and specific fragmentation patterns, as reported by Fogliano *et al.*[12] and Urpi-Sarda *et al.*[29].
† Reference calibration curve was epicatechin.
‡ Reference calibration curve was ferulic acid.
§ Reference calibration curve was HPA.

with ferulic acid; and dihydroxyphenyl-γ-valerolactone was quantified using the calibration curve built with hydroxyphenylacetic acid.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS for Windows (version 15; SPSS Inc.). The results from HPLC/MS/MS analysis were analysed and expressed as the absolute changes from the baseline to reduce possible effects of inter-subject fasting variability. The AUC for each compound from baseline to 6 h after first cream portion consumption in the case of serum samples and to 0–6 and 6–24 h for urine samples were estimated using the linear trapezoidal rule. As data were normally distributed and presented homogeneous variance (Levene test), they were analysed by one-way ANOVA for repeated measures; the subjective time curves for all measured compounds were compared and tested for the effect of treatment and of time as factors. For all tests, following a significant main effect in the ANOVA, individual means were compared using the Bonferroni test ($P < 0·05$). Results were considered significant at $P < 0·05$. All values were reported as means with their standard errors.

Results

Sensory analysis of creams

The products enriched with 2 and 2·5 % (w/w) of polyphenol extracts obtained an overall acceptability score of 4·6 (SEM 1·3) and 4·6 (SEM 1·1) (2·0 % polyphenols) and 4·12 (SEM 1·0) and 4·2 (SEM 1·2) (2·5 % polyphenols) for free cocoa polyphenol and encapsulated extract, respectively. These low scores, compared to the one attributed to the CC (7·5 (SEM 1·3)), were conditioned by the bitterness perception for the creams enriched with 2·0 and 2·5 % of the free extract (3·5 (SEM 1·2) and 2·9 (SEM 1·3), respectively) and the granularity for those containing the same amounts of encapsulated extract (3·7 (SEM 1·2) and 3·0 (SEM 1·3), respectively). The 1·5 %-enriched creams obtained a mean score for total acceptability of approximately 6·5 (6·4 (SEM 1·3) and 6·6 (SEM 1·2), when contained free cocoa polyphenol or encapsulated cocoa polyphenol extract, respectively), while hedonic for bitterness was even scored slightly higher for EPC (6·0 (SEM 0·3)) than for FPC (4·8 (SEM 0·2)) and, on the contrary, granularity being scored 5·0 (SEM 0·2) and 6·3 (SEM 0·3), respectively (Fig. 1).

Thus, 1·5 %-enriched creams were selected as the final products to be tested in the bioavailability study and will be

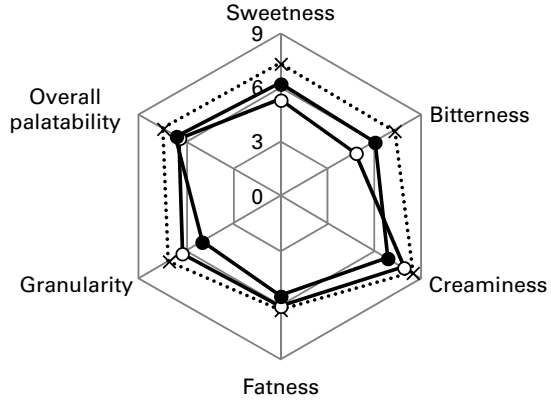

Fig. 1. Hedonic profile of control cream (-×-), and experimental creams enriched with 1·5 % of free (FP, -○-) and encapsulated (EP, -●-) cocoa polyphenol extract. Values are mean scores (n 30) anchored by 0 (extremely disliking) to 9 (extremely liking).

here-after indicated as FPC (the one containing 1·5 % free cocoa polyphenol extract) and EPC (the one containing 1·5 % encapsulated cocoa polyphenol extract).

Bioavailability study

Serum. Fig. 3 shows the mean serum concentration–time curves of total flavanols and phenolic acids found in subjects following consumption of experimental cocoa-nut creams.

None of monitored flavanols (i.e. (epi)catechin, epigallocatechin and procyanidin B_1) was found in serum at baseline. Only (epi)catechin was found in serum of subjects after consumption of FPC and EPC, while it was absent after CC consumption. In particular, (epi)catechin reached a concentration peak (C_{max} = 4·1 (SEM 2·3) nmol/l) 1 h after FPC consumption and it slowly returned to baseline value at 6 h post-consumption. After EPC consumption, the increase of serum (epi)catechin from baseline was significantly lower (C_{max} = 0·9 (SEM 0·6) nmol/l) than after FPC and was found only within the first hour after consumption. Accordingly, AUC_{0-6} of (epi)catechin after FPC was 13·9-fold higher than that found after EPC (7·4 (SEM 4·4) *v.* 0·5 (SEM 0·3) nmol/l × h, respectively; $P<0·05$).

Serum concentration of total phenolic acids at baseline was always about 135·2 (SEM 19·1) nmol/l, without the differences among treatments; after consumption of the creams, it was always higher than baseline. At 30 min after FPC consumption, serum phenolic acid concentration was higher than that found after EPC and CC (198·3 (SEM 101·0) *v.* 37·2 (SEM 17·2) and 24·8 (SEM 5·2) nmol/l, respectively), while the latter cream guaranteed a much higher concentration of phenolic acids (approximately 150 nmol/l) at both 6 and 24 h after consumption compared to the cocoa polyphenol-enriched creams (approximately 25 nmol/l).

The phenolic acids retrieved in serum samples and the relative amounts are reported in Table 3.

No significant difference was recorded in serum total phenolic acid concentrations following CC and FPC consumption, while a significantly lower amount was found after EPC consumption (726·8 (SEM 73·44) *v.* 1954·26 (SEM 236·33) and 1459·37 (SEM 137·63) nmol, respectively).

Urine. Fig. 4 shows the mean urinary excretion–time curves of total catechins and phenolic acids found in subjects following consumption of experimental cocoa-nut creams.

(Epi)catechins and procyanidins were never found in the urine of subjects at baseline, while they were retrieved after consumption of all the creams. In particular, within 4 h from CC consumption, their total concentration reached a plateau value that was maintained for up to 8 h, returned to baseline value at 10 h and was again higher than baseline at 24 h. After FPC consumption, only one concentration peak within the 2 h from consumption was found, while after EPC, catechin and procyanidin excretion was always negligible. However, only EPC always elicited a lower excretion of total flavanols than CC and FPC, while urinary excretion of total flavanols after FPC was significantly reduced compared to that after CC, only after the consumption of the second cream portion (AUC_{6-24} being 9·1 (SEM 6·3) and 62·0 (SEM 42·0) nmol/l × h, respectively).

According to serum data, the concentration of phenolic acids that were retrieved from baseline urine samples (approximately 25·3 (SEM 2·0) μmol/l, with no differences between treatments) and after cream consumption was approximately 1000-fold higher than that of total flavanols. Moreover, EPC elicited the lowest excretion, both over the first 6 h and over the 24 h after consumption, while a significantly higher excretion of phenolic acids following FPC than CC consumption was recorded only within 6 h after consumption (see Table 4). Apart from those found in serum samples, urinary phenolic acids also comprised of ferulic acid, dihydroferulic acid, protocatechuic acid, coumaric acid, caffeic acid and dihydrocaffeic acid.

Faeces. Flavanols were never found in faeces collected at baseline, and their amount after consumption of EPC (150·97 (SEM 54·65) nmol) was higher than after consumption of FPC (27·98 (SEM 13·97) nmol) and CC (4·27 (SEM 4·52) nmol) (Table 5).

Table 3. Amount (nmol) of serum-free native flavanols and phenolic acids over the time intervals 0–6 h following consumption of the three cocoa creams

(Mean values with their standard errors (*n* 12))

	CC		FPC		EPC	
	Mean	SEM	Mean	SEM	Mean	SEM
Flavanols (nmol)						
(Epi)catechin	ND		22·07*	2·62	1·59*†	0·22
Phenolic acids (nmol)						
Vanillic acid	32·57	2·29	7·53*	0·86	15·31*†	1·51
Chlorogenic acid	5·48	0·67	13·93*	1·59	4·19*	0·53
Hippuric acid	1386	196·1	994·7*	90·89	318·7*†	42·32
Hydroxybenzoic acid	480·2	32·64	318·8*	27·64	332·9*†	24·13
DHPV	16·87	0·82	115·2*	15·36	29·18*†	2·47
HPA	16·78	1·82	3·04*	0·43	5·63*	0·67
HPP	15·82	2·01	6·12*	0·87	20·92*	1·81
Total	1954	236·3	1459	137·6	726·8*†	73·44

CC, control cream; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract; ND, not determined; DHPV, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; HPA, 4-hydroxyphenylacetic acid; HPP, 3-(4-hydroxyphenyl) propionic acid.

* Mean values were significantly different from that of CC ($P<0·05$; Bonferroni test).

† Mean values were significantly different from that of FPC ($P<0·05$; Bonferroni test).

Table 4. Amount (nmol) of parental flavanols and phenolic acids excreted in urine collected over the time intervals 0–6 and 6–24 h following consumption of the three nut-cocoa creams

(Mean values with their standard errors (*n* 12))

	CC				FPC				EPC			
	0–6 h		6–24 h		0–6 h		6–24 h		0–6 h		6–24 h	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Flavanols (nmol)												
(Epi)catechin	5·26	3·72	11·75	7·16	4·82	3·41	2·16*	1·53				
EGC	0·02	0·01	0·50	0·35					0·19	0·14	2·00†	1·42
Procyanidin	0·39	0·24	1·17	0·82	0·85	0·47	1·83	1·29			2·73	1·80
Total	5·68	3·97	13·42	8·33	5·67	3·88	4·00	2·82	0·19	0·14	4·73	3·22
Phenolic acids (nmol)												
Protocatechuic acid	42·0	15·2	420·4	139·3	22·9	9·0	548·2	180·0	9·6*	4·2	693·7	252·2
Ferulic acid	15·2	5·3	1340·4	533·6	67·9*	38·2	91·6	51·2	19·1	9·2	1024·1	315·9
Vanillic acid	6·3	2·9	338·4	110·4	34·4*	11·2	452·2	192·5	16·3	10·2	195·9	84·1
Caffeic acid	1·4	0·6	99·0	29·7	2·5	0·9	74·3	18·7	0·5	0·3	21·3	7·7
Coumaric acid	1·2	0·6	23·9	13·0	3·1	1·4	34·4	12·4	1·4	0·4	38·5	12·4
Chlorogenic acid	0·1	0·0	1·9	0·5	0·2	0·0	1·4	0·6	0·4	0·2	2·9	0·9
Hippuric acid	2279	1411	98 571	45 317	8562*	3854	44 321	20 004	341·0*†	241·1	4128	2919
Homovanillic acid	515·8	160·7	48 861	17 674	2023*	802·9	48 489	16 071	223·4	156·4	32 110	7995
DHPA	122·1	72·7	6205	2881	199·5	73·6	10 911	3986	117·2	48·6	3467	1139
Di-HCA	40·6	14·5	2106	896·2	94·6	31·3	2417	749·1	34·4†	11·5	1915	566·3
Hydroxybenzoic acid	22·2	12·3	470·7	156·2	31·1	19·8	269·0	189·2	42·4	19·5	1202	536·6
Di-HFA	10·1	4·5	449·7	318·0	302·1*	177·0	313·7	163·3	60·7*†	34·3	104·9	51·2
HPP	3·4	2·1	30·2	10·4	1·1	0·6	3·3*	1·5	0·1	0·0	2·2*	1·6
HPA	2·6	1·2	87·6	42·5	64·2*	37·7	78·0	40·0	24·3*	10·5	11·4	7·2
DHPV	0·3	0·1	9·2	3·0	0·4	0·2	3·1	1·4	0·9	0·3	3·2	1·5
Total	3062	1703	159 015	68 124	11 409*	5058	108 007	41 661	891·7*†	546·7	44 921	13 890

CC, control cream; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract; EGC, epigallocatechin; DHPA, 3,4-dihydroxyphenylacetic acid; Di-HCA, dihydrocaffeic acid; Di-HFA, dihydroferulic acid; HPP, 3-(4-hydroxyphenyl)propionic acid; HPA, 4-hydroxyphenylacetic acid; DHPV, dihydroxyphenyl-γ-valerolactone.

* Mean values were significantly different from that of CC ($P<0·05$; Bonferroni test).

† Mean values were significantly different from that of FPC ($P<0·05$; Bonferroni test).

Table 5. Amount (nmol) of parental flavanols and phenolic acids excreted in faeces collected the day after consumption of the three nut-cocoa creams

(Mean values with their standard errors (*n* 12))

	CC		FPC		EPC	
	Mean	SEM	Mean	SEM	Mean	SEM
Flavanols (nmol)						
(Epi)catechin	ND		10·25	5·13	31·54	12·44
EGC	4·27	4·52	ND		0·52	0·30
Procyanidin	ND		17·73*	8·84	118·9*†	41·91
Total	4·27	4·52	27·98	13·97	151·0*†	54·65
Phenolic acids (nmol)						
Ferulic acid	42·58	6·33	ND		ND	
PCA	25·13	6·31	4·69*	0·00	9·65*	0·11
Vanillic acid	8·46	1·91	ND		ND	
Coumaric acid	8·01	1·25	ND		4·56	2·34
Caffeic acid	1·67	0·00	2·67*	0·00	ND	
Chlorogenic acid	0·86	0·43	0·05*	0·01	0·04	0·00
Homovanillic acid	322·2	0·00	ND		ND	
Di-HCA	141·3	38·61	ND		ND	
Di-HFA	37·75	27·94	ND		ND	
HPP	53·44	16·28	ND		ND	
DHPV	ND		0·70	0·12	0·41	0·23
Total	641·4	99·07	8·11*	0·13	14·66*	2·68

CC, control cream; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract; ND, not determined; EGC, epigallocatechin; PCA, protocatechuic acid; Di-HCA, dihydrocaffeic acid; Di-HFA, dihydroferulic acid; HPP, 3-(4-hydroxyphenyl)propionic acid; DHPV, dihydroxyphenyl-γ-valerolactone.

* Mean values were significantly different from that of CC ($P<0·05$; Bonferroni test).

† Mean values were significantly different from that of FPC ($P<0·05$; Bonferroni test).

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On the contrary, the amount of phenolic acids retrieved in faeces at 24 h following CC consumption (641·43 (SEM 99·07) nmol) was the only one to be higher than that found at baseline (1767·64 (SEM 594·27) nmol, without differences among treatments) and after FPC (8·11 (SEM 0·13) nmol) and EPC (14·66 (SEM 2·68) nmol) consumption.

The bioavailability data have been summarised in Table 6 to gain the complete picture of the distribution of the ingested bioactive compounds.

Discussion

Enrichment of foods with catechin and epicatechin can be difficult due to their bitter taste and astringency in the mouth. Encapsulating flavanols with a coating material inaccessible by salivary enzymes, such as HACS, can overcome this problem. The cocoa-nut cream enriched with microencapsulated cocoa flavanols (EPC) used in the present study showed a slightly higher hedonic for bitterness than the cream added with the ingredient in the free form (FCP; see Fig. 1).

Testing the bioavailability of the encapsulated bioactive compounds from enriched foods is fundamental to foresee their *in vivo* bioactivity. To our knowledge, the present study is the first study evaluating cocoa flavanol and phenolic acid bioavailability from cocoa-nut creams in human subjects. The study was designed to follow the fate of parental and free cocoa polyphenols after the consumption of one portion of three cocoa-nut creams (33 g) differing in amount (CC *v.* FPC) and form (FPC *v.* EPC) of contained cocoa polyphenols and of a total of 99 g cream (three portions) distributed throughout the day (one portion at each daily meal, i.e. at breakfast, lunch and dinner; see Fig. 2). Thus, the comparison between CC and FPC allowed us to evaluate the effect of dose on bioavailability of free parental flavanols and of phenolic acids contained in the creams, while comparison between FPC and EPC allowed us to investigate the effect of microencapsulation of the bioactive compounds.

Data indicated that flavanol absorption at 6 h after the consumption of cocoa-nut creams was dose-dependent, as previously reported after cocoa and chocolate consumption[8,15–20]. In fact, as summarised in Table 6, after consumption of one CC portion, flavanols were not detected in the bloodstream, while consumption of FPC, containing an almost doubled amount of free monomers, determined (epi)catechin presence in the serum. The time-course values of (epi)catechin concentration in serum and urine following FPC consumption (Figs. 3(a) and 4(a)) demonstrated a rapid absorption ($t_{max} = 1$ h) and excretion (within 2 h) upon consumption. The rapid absorption and serum clearance of parental (epi)catechin after cocoa-nut cream (t_{max} at 1 h and absence of compounds at 6 h) was consistent with literature data on the bioavailability of epicatechin from cocoa and chocolate, thus confirming the occurrence of gastric absorption and a rapid plasma clearance (within 6 h from consumption) of compounds[30–32]. This feature was corroborated by data obtained after consumption of EPC, which elicited a 10-fold lower amount of serum (epi)catechin than FPC. In fact, as about 50 % (195·3 μmol of a total 385·3 μmol/100 g of cream)

Table 6. Summary of bioavailability of cocoa polyphenols from the three cocoa-nut creams‡

	CC			FPC			EPC		
	Total flavanols	Total phenolic acids	Total polyphenols	Total flavanols	Total phenolic acids	Total polyphenols	Total flavanols	Total phenolic acids	Total polyphenols
Blood									
0–6 h (μmol)	0·000	1·977	1·977	0·022*	1·459	1·481	0·002	0·744*†	0·745*†
% Dose one portion	0·000	49·4	2·808	0·020	15·6	1·043	0·001	7·9	0·525
Urine									
0–6 h (μmol)	0·006	3·062	3·068	0·006	11·41*	11·41*	0·000	0·892*†	0·892*†
% Dose one portion	0·010	76·6	4·358	0·005	121·8	8·033	0·000	9·5	0·628
6–24 h (μmol)	0·013	159·0	159·03	0·004	108·0	108·0	0·005	44·92	44·93
0–24 h (μmol)	0·019	162·1	162·1	0·010	119·4	119·4	0·005	45·81*†	45·82*†
% Dose three portions	0·011	1351	76·75	0·003	425·0	28·01	0·0015	163·0	10·75
Faeces									
0–24 h (μmol)	0·004	0·641	0·646	0·028	0·008*	0·036*	0·151†	0·015*	0·134*
% Dose three portions	0·002	5·3	0·306	0·008	0·03	0·008	0·045	0·05	0·031

CC, control cream; FPC, cream enriched with 1·5 % (w/w) of the free cocoa polyphenol extract; EPC, cream enriched with 1·5 % (w/w) of the encapsulated cocoa polyphenol extract.
* Values were significantly different from that of CC ($P<0.05$; Bonferroni test).
† Values were significantly different from that of FPC ($P<0.05$; Bonferroni test).
‡ Subjects ingested on three different occasions three portions (33 g each)/d of CC (providing a total of approximately 190 μmol/d flavanols and acids 12 μmol/d total phenolic), FPC (providing a total of 385 μmol/d flavanols and 28 μmol/d total phenolic acids) or EPC (providing a total of 385 μmol/d flavanols and 28 μmol/d total phenolic acids).

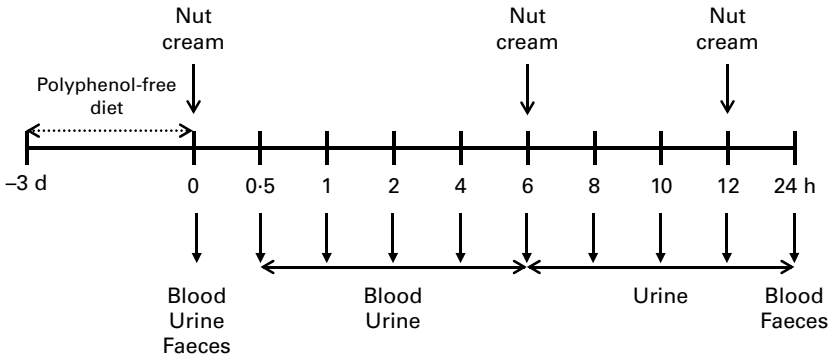

Fig. 2. Study design. Each subject followed the time schedule for each type of cocoa-nut cream by a cross-over randomised design. After a 1-week wash-out period during which subjects returned to their habitual diet, they switched to the 3-d polyphenol-free diet and were randomised for another treatment. A total of three portions (33 g each) of the cocoa-nut cream were consumed upon each treatment.

of cocoa flavanols in EPC were encapsulated by a coating material resistant to gastric digestion (HACS), their gastric absorption was reduced compared to FPC[33]. Moreover, we hypothesised that the higher amount of dietary fibre in EPC (+7·2 %, due to the HACS coating) might slow gastric emptying rate[34], thus blunting free epicatechin absorption compared to CC. A slower arrival of total flavanols (the half deriving from cocoa as in CC plus the half encapsulated) in the intestine after consumption of EPC might have also caused an increased formation of conjugated metabolites at the level of intestinal mucosa or liver, as a consequence of a lower amount of substrate per unitary time than after ingestion of CC and FPC. This hypothesis justified the missed detection in urine of the monitored cocoa native and free flavanols and the much lower procyanidin concentrations found after EPC consumption compared to CC and FPC consumption

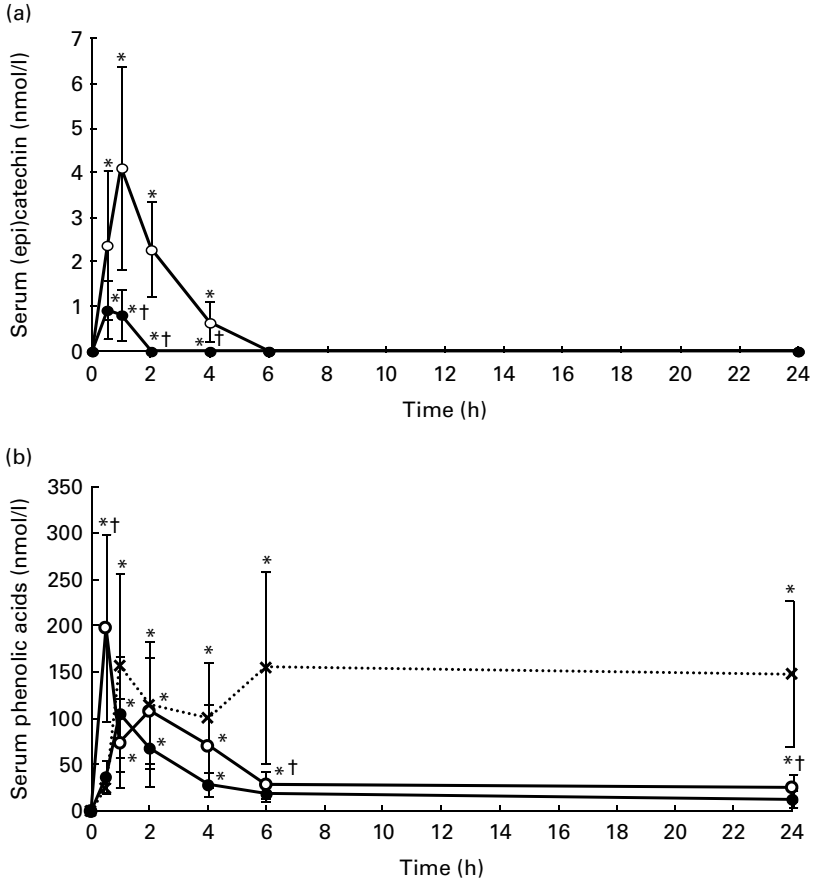

Fig. 3. Serum concentration–time curves of (a) (epi)catechin and (b) total phenolic acids over 24 h following consumption of the three types of cream (CC, control cream; FPC, free cocoa polyphenol cream; EPC, encapsulated cocoa polyphenol cream). (a) -○-, FPC; -●-, EPC. (b) -○-, FPC; -●-, EPC; ···×···, CC. Values are means, with their standard errors represented by vertical bars (n 12). * Mean values were significantly different from that of time 0 ($P<0·05$; Bonferroni test). † Mean values were significantly different from that of CC ($P<0·05$; Bonferroni test).

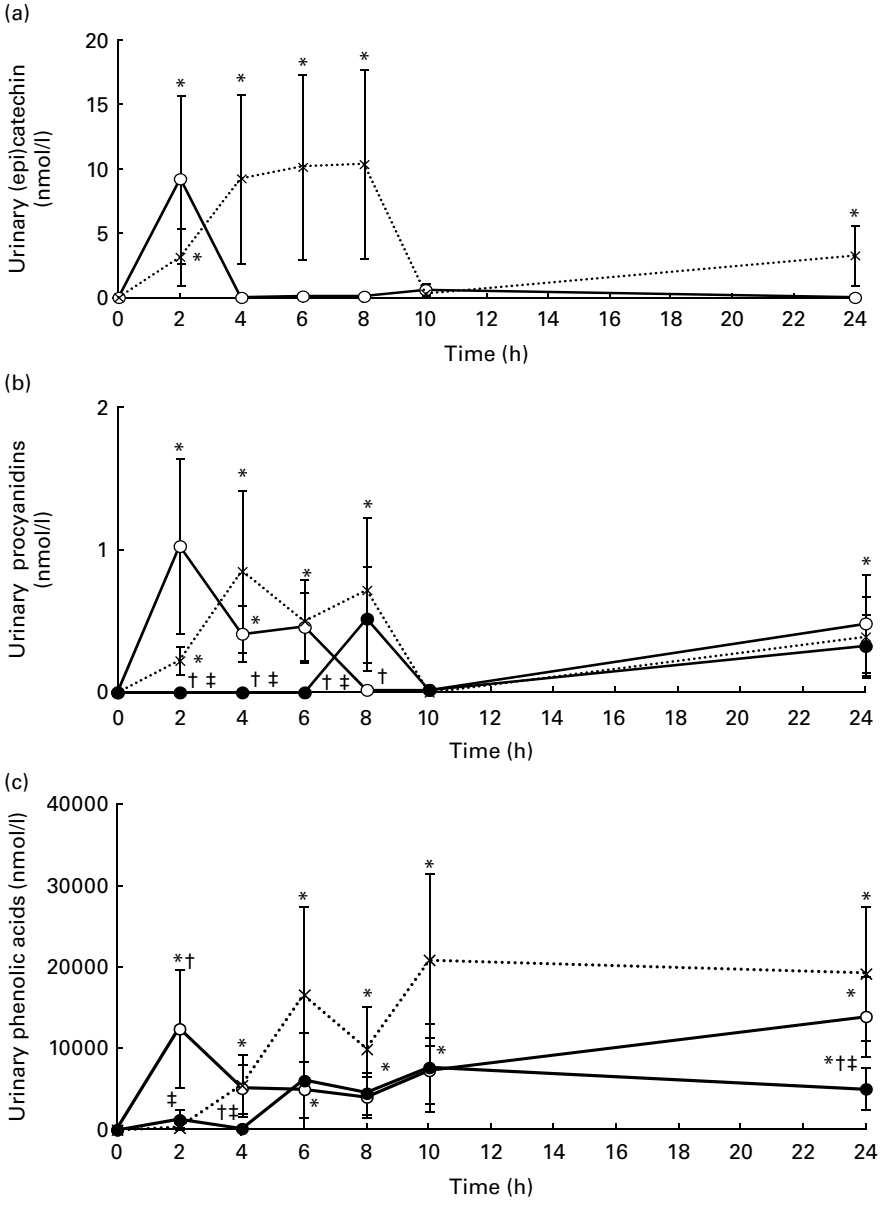

Fig. 4. Urinary excretions of (a) (epi)catechin, (b) procyanidins and (c) total phenolic acids over 24 h following consumption of the three types of cream (CC, control cream; FPC, free cocoa polyphenol cream; EPC, encapsulated cocoa polyphenol cream). Values are means, with their standard errors represented by vertical bars (*n* 12). (a) -○-, FPC; ···×···, CC. (b) -○-, FPC; -●-, EPC; ···×···, CC. (c) -○-, FPC; -●-, EPC; ···×···, CC. * Mean values were significantly different from that of time 0 ($P<0{\cdot}05$; Bonferroni test). † Mean values were significantly different from that of CC ($P<0{\cdot}05$; Bonferroni test).

(see Fig. 4(a) and (b)). Encapsulation of polyphenols might influence gastric emptying and in turn modify metabolism of the part of flavanols present in EPC that were not encapsulated.

Looking at the time-course values of phenolic acids in serum and in urine, a double concentration peak was found: the first at 30 min–1 h and the second at 4–6 h after consumption. They could account for the absorption of cocoanut cream parental phenolic acids from the stomach and for the absorption of compounds delivered in the intestine by the ring scission of procyanidins or flavones and/or by hydrolysis from cocoa fibres, as previously demonstrated or suggested *in vitro* [35,12]. In fact, Ortega *et al.*[35] demonstrated an increased amount of phenolic acids (mainly hydroxybenzoic, syringic and chlorogenic acids) in the bioaccessible fraction deriving from both gastric and duodenal digestion of cocoa liquor and cocoa powder, compared to the amount present in the food matrices. Accordingly, in a previous work, we showed that pancreatin digestion of water-insoluble cocoa dietary fibre led to a soluble fraction (correspondent to the bioaccessible moiety), exerting the same antioxidant capacity of pepsin fraction despite a reduced concentration of catechins, and thus suggesting that other antioxidant compounds might form in the medium at intestinal-simulated conditions[12].

Anyway, the total flavanols found in the parental form in blood and urine within 6 h from consumption, compared to

the dose ingested, were 0·010 % after CC, 0·025 % after FPC and 0·002 % after EPC. Percentages below 0·1 % of the ingested dose for native free flavanols were expected, as it is known that flavanols are mainly metabolised by the liver and intestine. In fact, authors reporting plasma concentration, of flavanols from 257 nmol/l up to 6–12 μmol/l, 1–2 h after consumption of 40–100 g of commercial cocoa or high-flavanol cocoa or chocolate, always analysed samples after treatment with glucuronidase, thus summing both free and metabolised compounds[9,18,19,30,32,36]. As we did not perform glucuronidase treatment on samples, we measured the compounds present in biological samples in the free form. Only in the study by Schroeter *et al.*[22], both parent compounds and their metabolite concentrations in plasma at 2 h after high-flavanol cocoa consumption were shown to be approximately 300 and 1400 nM, respectively. Thus, in that case, after ingestion of 604 μmol of flavanol monomers in cocoa, a concentration ratio between epicatechins and glucuronides of 1:4 could be calculated. Indeed, in the present study, after consumption of cocoa-nut creams containing 190–385 μmol of total flavanols and 12–28 μmol of phenolic acids per 100 g of cream, serum maximum concentration of (epi)catechin ranged between 0 and 4 nM, while concentration of total phenolic acids was between 100 and 200 nM within 2 h after consumption (see Fig. 3(b)). Thus, within 2 h after consumption of cocoa-nut creams, the concentration ratio between (epi)catechin and phenolic acids in serum was 1:50, much higher than that achievable for glucuronidated products.

Phenolic acids in serum, over a time-window shortly following cocoa or chocolate consumption, have never been evaluated in previous studies. Rios *et al.*[13] quantified phenolic acids in urine collected over 0–48 h after cocoa consumption. They reported that among the eleven aromatic acids retrieved (3,4-dihydroxyphenylpropionic acid, *m*-hydroxyphenylpropionic, ferulic acid, 3,4-dihydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, phenylacetic acid, vanillic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid and hippuric acid) only vanillic acid showed a peak excretion shortly after chocolate consumption (0–3 h), probably deriving from oxidation of vanillin present in the chocolate, while the other phenolic acids increased starting from 6 h after consumption. However, Urpi-Sarda *et al.*[37] successively showed that, although phenolic acids were most abundant in urine collected after 6 h from consumption, concentrations higher than baseline values were retrieved in the 0–6 h time interval for 3,4-dihydroxyphenylacetic acid, five hydroxybenzoic acids and two hydroxycinnamic acids; moreover, they found that when the cocoa beverage was prepared with milk, compared to water, vanillic acid was more abundant and majorly excreted over the first 6 h after consumption[37].

The different matrices tested (chocolate by Rios *et al.*[13]; Urpi-Sarda *et al.*[37] cocoa beverage prepared with water or milk and cocoa-nut creams in the present study) might account for the different results obtained by the studies. However, looking at 0–6 h urinary excretion of phenolic acids following CC and FPC consumption, the present data indicated a dose-dependence from ingested flavanol monomers and phenolic acids excreted. This feature was reinforced by the data recorded after EPC consumption: when cocoa polyphenols were partly encapsulated, phenolic acids were markedly reduced over 0–6 h time intervals, while an increased rate of excretion after 6 h was recorded. These results suggested that flavanols, other than cocoa dietary fibres, might contribute to the phenolic acid formation in the short term and confirmed the major role of gut microflora on phenolic acid formation in the long term[8,10–14]. In fact, the higher amount of flavanols in the faeces collected after consumption of EPC, than in those after FPC, together with an equivalent amount of phenolic acids indicated that the encapsulation allowed the delivery of bioactive compounds in the lower gut and the successive metabolism by local microflora (see Figs. 3(b) and 4(b), 24 h time point).

Despite the fact that FPC caused the highest phenolic acid excretion over the first 6 h after consumption, the amount of phenolic acids excreted in 24-h urine following its consumption did not differ by that recorded after CC, both being consistent with that observed by Rios *et al.*[13] after consumption of 80 g chocolate. It may be possible that a different concentration ratio of monomers (not absorbed and/or degraded in the upper gastro-intestinal tract) and oligomers plus polymers (naturally reaching the gut) in the gut after consumption of the three creams might differently influence microflora metabolism and absorption of metabolites over the 24 h.

All in all, the present data demonstrated that parental cocoa flavanols are absorbed by cocoa-nut creams in a dose-dependent manner and phenolic acids are the major metabolites in the short term, being at a concentration ratio of 50:1 *v.* (epi)-catechin. Encapsulation of cocoa polyphenols with HACS caused a reduced 24-h bioavailability of these compounds. On the other hand, encapsulation effectively masked bitter taste and allowed delivering of flavanol monomers into the gut. From the nutritional point of view, encapsulated cocoa polyphenols may be considered as a functional prebiotic ingredient, as evidenced in a recent human study[38]. Further studies should be performed to test this hypothesis and to evaluate the *in vivo* efficacy of this ingredient towards specific pathologies and functions.

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Eco-physiological response to water stress of drought-tolerant and drought-sensitive tomato genotypes

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ORIGINAL ARTICLE

Eco-physiological response to water stress of drought-tolerant and drought-sensitive tomato genotypes

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Abstract
Water stress is an increasing environmental constraint affecting tomato growth and yield in Mediterranean areas. *Solanum pennellii* is a wild tomato species that exhibits a higher water use efficiency compared with cultivated *S. lycopersicum*. In particular, a cultivated line carrying a small *S. pennellii* region on chromosome 9 (IL 9-2-5) was identified as more tolerant to water deficit. In this work, the tolerant (IL 9-2-5) and the susceptible (M82) genotypes were subjected to three different water regimes: irrigation with 100% (V1), 50% (V2) and 25% (V3) field capacity. To evaluate the physiological response of IL 9-2-5 and M82 to water deficit, leaf functional traits, plant biomass production and maximal PSII photochemical efficiency were measured together with photosynthetic pigments and phenolic compounds. The higher tolerance to water deficiency of IL 9-2-5 was associated with the development of a better antioxidant system, especially in treatment V3. In addition, IL 9-2-5 had higher values of sclerophylly and leaf dry matter content thus confirming that the tolerance of IL 9-2-5 can be attributed to traits related to leaf morphology and physiology. In future, identification of polymorphisms in key-genes controlling these traits can guide breeding efforts aimed at improving susceptible genotypes.

Keywords: *Functional leaf traits, phenolics, photochemical efficiency, tomato introgression lines, water stress*

Introduction

Drought leading to water stress is an environmental constraint in hot and dry climate (e.g. Mediterranean environments) affecting crop growth and yield, and reducing agricultural productivity. In a near future, losses of crop yields due to this abiotic stress may be amplified due to the threats of climate changes emerging from global warming as well as due to the growing scarcity of fresh water available for irrigation caused by urbanization and depletion of aquifers. The ultimate goal is to develop crop plants with improved water use efficiency in order to minimize drought-induced losses of yield and permit the use of cultivable land with limited water supplies (Vitale et al. 2011; Mishra et al. 2012).

Plants develop a range of mechanisms for dealing with low water availability that include: (1) stress escape by completing their life cycle before severe water deficit occurs; (2) stress avoidance by enhancing their capacity to absorb water and conserve it thanks to a large root system, a reduced leaf area and limited transpiration; (3) stress tolerance by improving osmotic adjustment ability and increasing cell wall elasticity; (4) stress resistance by altering metabolic pathways so that the plant can survive under severe stress conditions (e.g. increased antioxidant metabolism) (Xu et al. 2010; Clayes & Inzè 2013; Lawlor 2013). Exposure to water deficit often increases the production of reactive oxygen species and, as a consequence, promotes the concentration of antioxidant compounds as well as the activity of some antioxidant enzymes (ascorbate peroxidase, catalase and superoxide dismutase) (Garg & Manchanda 2009; Sanchez-Rodriguez et al. 2011; Barbagallo et al. 2012). In particular, phenolic compounds can act to detoxify free radicals (Sanchez-Rodriguez et al. 2011). In addition, carotenoids can reduce and eliminate the reactive oxygen damage, serve as precursors of ABA synthesis and

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also participate in photosynthesis as the chlorophylls (Gong et al. 2010).

Water deficit has imposed selective pressure in the evolution of plant morphology and physiology. Selection in water-limited environments can result in populations and/or species with traits that improve their relative fitness in response to drought. Such traits can improve tissue tolerance of desiccation allowing leaves to function longer under drought conditions or improve avoidance of water loss allowing leaves to maintain high water potential (Easlon & Richards 2009).

Tomato (*Solanum lycopersicum*), one of the most important vegetable crops worldwide, is also one of the crops most demanding in water. As with many crop plants, cultivated tomato carries only a very small fraction of the genetic variation that is available in related wild species and landraces (Tanksley & McCouch 1997). It has therefore become a goal of modern breeding to screen wild genetic resources for advantageous traits that could be introduced into modern varieties to enrich the genetic basis of cultivated plants with novel alleles that improve agricultural yield under optimal as well as less optimal field conditions. Many wild relatives of cultivated tomato *S. lycopersicum* exhibit different degrees of tolerances to abiotic and biotic stresses. *Solanum pennellii*, one of the crossable wild relatives of cultivated tomato that originated in the deserts of Peru, displays drought tolerance compared with cultivated *S. lycopersicum*. In particular, it was demonstrated that the desert-adapted *S. pennellii* has higher water use efficiency both in water-stressed and non-stressed conditions and reduced leaf stomatal conductance in response to drought (Easlon & Richards 2009). Eshed and Zamir (1995) generated a collection of introgression lines (ILs) in which defined genomic segments of the wild species *S. pennellii* replaced homologous region in the background of the cultivated variety *S. lycopersicum* M82. Overall, the population of ILs provides complete coverage of the wild-species genome and allows the reservoir of wild genes to be investigated. In particular, a set of such *S. pennellii* ILs has been extensively phenotyped for dissecting traits such as plant yield and fruit quality (Lippman et al. 2007; Alseekh et al. 2013). Previously, a shoot-specific QTL (PW 9-2-5) was identified in the line IL 9-2-5 carrying a 9 cM introgression from the wild species *S. pennellii*, which accounts for an altered growth habit resulting in increases in plant weight, yield and Brix units. Afterward, the tomato IL (IL 9-2-5) was identified as more tolerant to water deficit in terms of yield losses (Vasco et al. 2011). In this work, the tolerant genotype IL 9-2-5 and the drought-sensitive genotype M82 were subjected to three levels of irrigation and their eco-physiological responses were evaluated by measuring leaf functional traits together with photosynthetic pigments and phenolic compounds in order to gain a deeper understanding of the mechanisms that regulate the response to water stress in the selected IL.

Material and methods

Plant material and growth conditions

Plants were grown during the 2012 season at the Department of Agricultural Sciences, Portici, Naples, Italy. Seeds of IL 9-2-5 and M82 were kindly provided by the Tomato Genetics Resource Center (TGRC), University of California, Davis, USA (http://tgrc.ucdavis.edu/). The seeds were germinated in Petri dishes on water-soaked filter paper and subsequently transferred in peat-filled planting tray and incubated in a growth chamber at 22°C with 16 h/8 h light/dark. The plants were transplanted, at the four-leaf stage, in big pots of 100 cm diameter under a plastic cover. At mid-day, the environmental conditions ranged from 29–34°C temperature, 38–64% relative humidity and 1560–1700 μmol photons $m^{-2} s^{-1}$ photosynthetic photon flux densities (PPFDs). Three plants for each pot were used. The pots were filled with soil (characteristics in Table I) and received 300 g of Nitrophoska Blu Gold (N:P:K 12:12:17). The soil water holding capacity (WHC) was estimated according to Rawls and Brakensiek (1989). Soil organic matter (SOM) was evaluated according to Allen (1989) via loss on ignition at 550°C for 2 h of oven-dry samples (75°C). Soil density was calculated as the dry weight of soil divided by its volume. Pots were arranged according to a randomised complete block design with three replicates. Treatments were the genotype (M82 and IL 9-2-5) and the water restitution level. In particular, three water restitution levels were applied consisting of the restitution to plants of 100% (V1), 50% (V2) and 25% (V3) of the lost water, respectively. Lost water was estimated by measuring the reduction in the soil moisture relatively to the field capacity. Water restitution treatments were applied when most of the plants showed fruit set on the first inflorescences. Leaf samples were collected 1 (30 days after water stress, DAWS) and 2 months (60 DAWS) from the application of the first

Table I. Soil texture (sand, loam and clay percentage), SOM, bulk density (BD) and WHC of soil used to fill the experimental pots.

Sand (%)	Loam (%)	Clay (%)	SOM (%)	BD ($kg\,dm^{-3}$)	WHC (% vol/vol)	WHC (% p/p)
78.3	15.8	5.9	0.69	0.90	17.68	0.21

differentiating watering. Leaves were harvested, frozen immediately in liquid N2 and kept at −80°C until analyzed.

Photosynthetic pigments

Total chlorophyll and carotenoids were extracted in ethanol with calcium carbonate (0.3 mg ml^{-1}) and centrifuged at 13,000*g* for 5 min. Thereafter the absorbance of the supernatant was measured at 665, 649 and 470 nm. The chlorophyll *a*, chlorophyll *b* and carotenoids were estimated according to Lichtenthaler (1987).

Total phenolics content

Total phenolics content was assayed using a modified procedure of the Folin–Ciocalteu's test (Singleton & Rossi 1965). In brief, 250 mg of frozen ground tissue were homogenized in a mortar with pestle and extracted using 1 ml of 60% methanol. Samples were left on ice for 3 min in the dark. Crude extracts were transferred in a 15 ml tube and volume was increased to 5 ml adding 60% methanol. The samples were centrifuged at 3000*g* for 5 min; then, 62.5 μl of the supernatant, 62.5 μl of Folin–Ciocalteu's reagent (Sigma, St. Louis, MO, USA) and 250 μl of deionised water were mixed and incubated for 6 min; 625 μl of 7.5% sodium carbonate and 500 μl of deionised water were added to the samples and incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm. The concentration of total phenolics was expressed in terms of μg of gallic acid equivalents per 1 mg of fresh weight (FW).

Leaf traits determination

The evaluated leaf functional traits were specific leaf area (SLA), leaf dry matter content (LDMC) and leaf relative water content (RWC). LA for SLA determination was measured using the program Image J 1.45 (Image Analysis Software), whereas SLA, LDMC and RWC according to Cornelissen et al. (2003). More specifically, SLA, which represents the light-intercepting area per dry mass of leaf, was measured as the ratio of leaf area to leaf dry mass and expressed as cm^2 g^{-1} DW. For dry mass determination, leaves were dried at 70°C for 48 h. LDMC was measured as the oven-dry mass of a leaf divided by its water-saturated fresh mass and expressed as g DW g^{-1} FW. The saturated FW was determined by submerging the petiole of leaf blades in distilled water for 48 h in the dark. LDMC is related to the average density of the leaf tissues (Cornelissen et al. 2003). The RWC was expressed as percentage of (leaf fresh mass − leaf dry mass)/(leaf saturated fresh mass − leaf dry mass).

Fluorescence emission measurements

Chlorophyll *a* fluorescence measurements were carried out by means of a pulse amplitude modulate fluorometer (Mini-PAM, Walz, Germany) equipped with a leaf-clip holder (Leaf-Clip Holder 2030-B, Walz), able to record the incident PPFD on the leaf and abaxial leaf temperature. Measurements were performed in early morning (7:00–7:30) on 10 attached leaves *per* replicate of each genotype *per* each water regime, under natural conditions of temperature (20–24°C). More specifically, on 40 min dark-adapted leaves, the background fluorescence signal (F_o) was induced by light of about 0.5 μmol photons m^{-2} s^{-1} at the frequency of 0.6 kHz. Maximal fluorescence (F_m) was obtained by imposing to the leaf 1 s saturating flash of about 10,000 μmol photon m^{-2} s^{-1}. F_o and F_m were used to calculate the maximum photochemical efficiency of PSII as: $[F_v/F_m = (F_m - F_o)/F_m]$ (Maxwell & Johnson 2000).

Total biomass determination

Plants were harvested 95 days post-transplantation. In particular, root system was cut away at collar level and aboveground biomass was weighted, stems were split into main and lateral stems and stems and leaves were counted, as well as the length of main stems was measured. Total plant yield was also assayed for all the genotypes as weight of total collected fruits.

Statistical analysis

Statistical analyses were performed using a two-way ANOVA with treatments (V1, V2 and V3) and genotypes (M82 and IL 9-2-5) as grouping variables both at 30 and 60 DAWS. The Student–Newman–Keuls test was applied for all pairwise multiple comparison procedures. The package Sigma-Stat 3.5 was used (Jandel Scientific, San Rafael, CA, USA). Principal component analysis (PCA) was performed by using the Statistical package for Social Sciences (SPSS) Package 6, version 15.0.

Results and discussion

Antioxidant and photosynthetic pigment analyses

In this study, phenolic compounds and photosynthetic pigments were measured in order to gain a deeper understanding of the mechanisms that regulate the response to water stress in the tolerant genotype IL 9-2-5 and in the drought-sensitive genotype M82 under different watering conditions. At 30 DAWS, a significant increase in the amount of phenolic compounds was observed at V3 for the genotype M82 (Figure 1, Table II). At 60 DAWS, an increase was observed in both genotypes compared

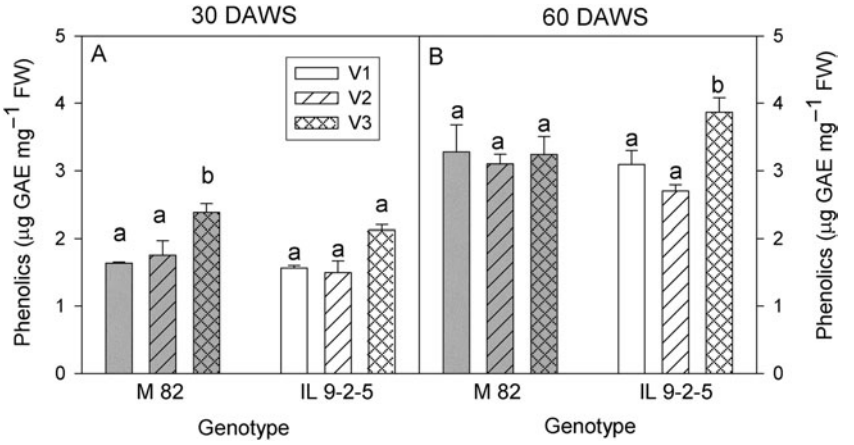

Figure 1. Total phenolics content in the leaves of two genotypes M82 and IL 9-2-5 in response to different water regimes V1, V2 and V3 following 30 (A) and 60 (B) DAWS. Mean (± SE) values are shown. Within each genotype, values marked with different letters indicate significant difference among treatments (Student–Newman–Keuls test, $p < 0.05$).

with the first sampling (Figure 1). In the second sampling, only in IL 9-2-5 a significant increase of total phenolics was measured in treatment V3 compared with treatments V2 and V1 (Figure 1, Table III). The general increase observed moving from 30 to 60 DAWS may be due to the establishment of multiple stresses on the plant. In fact, although in the treatment V1 the water is not a limiting factor, the condition of high temperature and irradiance are exacerbated with the progress of the summer season. Hence, the increase of total phenolics could be a defensive response of the plant to protect the photosystems from the excessive radiation and to mitigate the limited water availability (Ennajeh et al. 2009). The different behavior of M82 and IL 9-2-5 at 60 DAWS is in agreement with the studies from Sanchez-Rodriguez et al. (2011) who demonstrated that polyphenols play a significant role in water-stress tolerance in tomato and that moderate water stress can induce shikimate pathway in tolerant tomato cultivars.

As for the photosynthetic pigments, at 30 DAWS no significant differences in pigment concentrations were found between watering treatments for both genotypes (Figure 2, Table II). The genotype IL 9-2-5 did have a significant higher chlorophyll *a* and *b* concentration regardless of treatments (Table II). At 60 DAWS, for IL 9-2-5 genotype there was a significant increase in the concentration of chlorophyll *a*, chlorophyll *b* and carotenoids in plants subjected to V3 treatment compared with plants of V1 and V2 treatments whereas in M82 an opposite trend was found, thus evidencing a significant interaction between treatments and genotype (Table III). Reduction in leaf pigments induced by drought is considered to be an oxidative stress indicator, which might be attributed to pigment photo-oxidation, chlorophyll degradation and/or chlorophyll synthesis deficiency (Sanchez-Rodriguez et al. 2012). In particular, reduction of chlorophyll concentrations is identified as a drought response mechanism in order to minimize the light absorption by chloroplasts. Accordingly, the decrease in chlorophyll content and carotenoids in M82 and the increase in IL 9-2-5 at 60 DAWS demonstrate that both genotypes perceived the water stress but responded to it in opposite ways, thus confirming the higher tolerance of IL 9-2-5 to water deficiency.

Functional leaf traits

The different water treatments and the extent of the stress affected significantly the leaf functional traits, namely SLA, LDMC and RWC (Figure 3). In response to prolonged water stress conditions, the SLA decrease at 60 DAWS compared with 30 DAWS was evident for both tomato genotypes, and more specifically it was of 33% for M82 and 35% for IL 9-2-5. At 30 DAWS, no statistical difference has been observed in SLA of M82 and IL 9-2-5 among different water regimes as well as no interaction between genotype and treatments was evidenced (Table II). After 60 DAWS, IL 9-2-5 showed a significant reduction of SLA in treatments V2 and V3 compared with V1 (Figure 3B) whereas in M82 an increase of SLA was observed in V3 thus a significant interaction between genotype and treatments was observed (Table III). This result may be ascribed to the development of new generation leaves in both genotypes, which in response to limiting water supply have reduced the leaf expansion to avoid water loss by transpiration and increased the leaf hardness and rigidity (sclerophylly) to control leaf dehydration. Also in this framework, even if in the treatment V1 the water is not a limiting factor, the increasing temperature and irradiance with the progress of the

Table II. Summary of two-way ANOVA statistics for comparison of effects of three different water treatments on the two tomato genotypes: M82 and IL 9-2-5 at 30 DAWS incoming.

	Treatment				Genotype				T × G				Residual	
	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P	DF	MS
Phenolics	2	1.19	9.04	2×10^{-3}	1	0.03	0.20	0.66	2	0.16	1.20	0.33	16	0.13
Chl *a*	2	63.29	1.36	0.29	1	372.91	7.98	0.01	2	22.52	0.48	0.63	16	46.76
Chl *b*	2	20.52	1.65	0.22	1	278.13	22.31	<0.001	2	2.31	0.19	0.83	16	12.47
Car	2	4.80	1.04	0.38	1	7.45	1.62	0.22	2	0.23	0.05	0.95	16	4.61
SLA	2	476.80	0.38	0.69	1	756.15	0.60	0.44	2	1038.08	0.83	0.44	41	1253.03
LDMC	2	1.27×10^{-4}	0.22	0.81	1	8.61×10^{-3}	14.62	<0.001	2	1.33×10^{-4}	0.23	0.80	41	5.89×10^{-4}
RWC	2	156.38	0.86	0.43	1	750.12	4.14	0.05	2	62.93	0.35	0.71	41	181.39
F_v/F_m	2	5.42×10^{-3}	4.32	0.02	1	4.68×10^{-5}	0.037	0.85	2	1.28×10^{-3}	1.02	0.37	41	1.25×10^{-3}

Notes: Treatments: V1, V2, V3; genotypes: M82, IL 9-2-5. T × G: interactions between treatments (T) and genotype (G). SLA, specific leaf area; LDMC, leaf dry matter content; RWC, relative water content; F_v/F_m, maximal PSII photochemical efficiency.

Table III. Summary of two-way ANOVA statistics for comparison of effects of three different water treatments on the two tomato genotypes: M82 and IL 9-2-5 at 60 DAWS incoming.

	Treatment				Genotype				T × G				Residual	
	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P	DF	MS
Phenolics	2	1.24	7.11	0.01	1	0.26	1.48	0.25	2	0.35	1.99	0.18	12	0.18
Chl *a*	2	151.94	2.48	0.13	1	496.92	8.11	0.02	2	1557.28	25.41	<0.001	12	61.28
Chl *b*	2	9.55	0.47	0.64	1	213.72	10.44	0.01	2	250.10	12.21	1×10^{-3}	12	20.48
Car	2	17.33	1.73	0.22	1	7.12	0.71	0.41	2	132.07	13.19	<0.001	12	10.02
SLA	2	189.35	0.30	0.75	1	189.35	0.30	0.75	2	6709.45	10.44	<0.001	42	642.76
LDMC	2	1.89×10^{-3}	1.62	0.21	1	0.02	20.81	<0.001	2	4.55×10^{-4}	0.39	0.68	42	116×10^{-3}
RWC	2	151.20	2.54	0.09	1	222.89	3.74	0.06	2	193.32	3.24	0.05	42	59.61
F_v/F_m	2	1.84×10^{-3}	2.67	0.08	1	3.67×10^{-4}	0.53	0.47	2	8.08×10^{-5}	0.12	0.89	42	6.90×10^{-4}

Notes: Treatments: V1, V2, V3; genotypes: M82, IL 9-2-5. T × G: interactions between treatments (T) and genotype (G). SLA, specific leaf area; LDMC, leaf dry matter content; RWC, relative water content; F_v/F_m, maximal PSII photochemical efficiency.

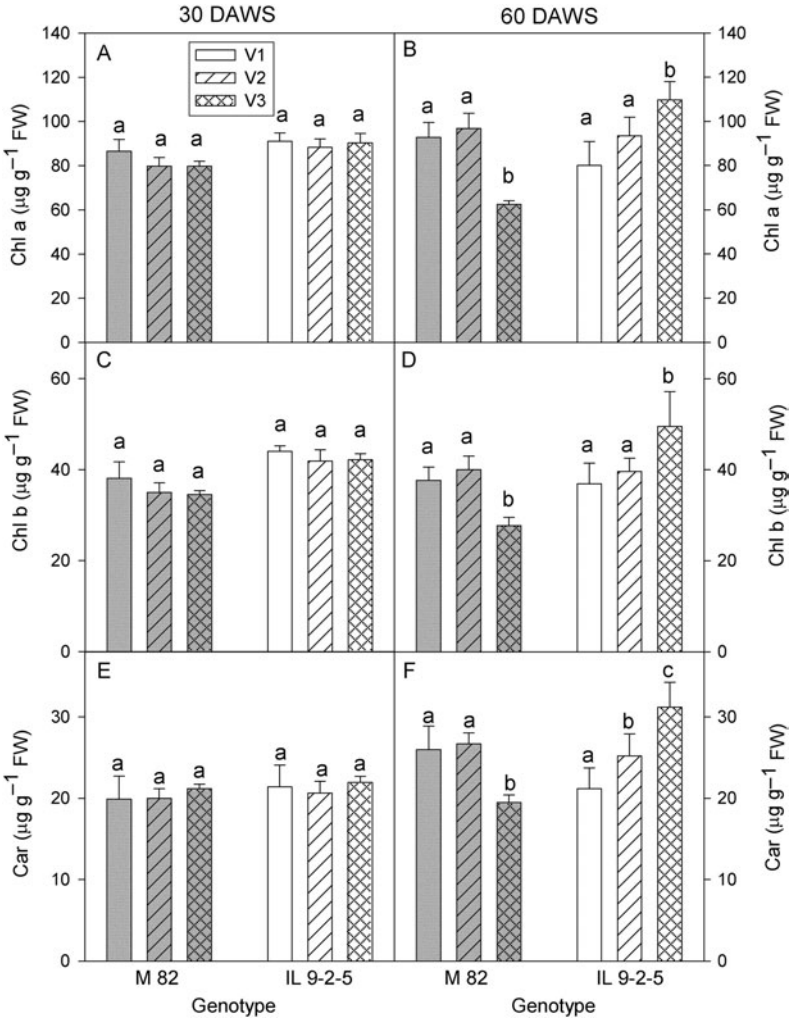

Figure 2. Chlorophyll *a* (A, B), chlorophyll *b* (C, D) and carotenoid content (E, F) in the leaves of two genotypes M82 and IL 9-2-5 in response to different water regimes V1, V2 and V3 following 30 and 60 DAWS. Mean (± SE) values are shown. Within each genotype, values marked with different letters indicate significant difference among treatments (Student–Newman–Keuls test, $p < 0.05$).

summer season may have triggered the formation of new leaves with small SLA. It is well known that the reduction of leaf area as well as the developing of mechanic tissues represents a useful way to cope with environmental harsh conditions, such as high irradiance, temperature and water stress, that may limit plant productivity (Hunt & Cornelissen 1997; Gulías et al. 2003; Poorter & Bongers 2006).

As expected, the LDMC showed an opposite trend compared with SLA (Figure 3(C),(D)). The comparison between the two genotypes demonstrated that IL 9-2-5 showed a significant higher LDMC compared with M82 in all tested water conditions at both 30 and 60 DAWS (Tables II and III). In particular, at 30 DAWS no significant difference was detected in both genotypes in response to different water regimes. At 60 DAWS, LDMC increased significantly (around 30%) in both tomato genotypes, although IL 9-2-5 maintained a higher value compared with M82. Since this parameter can be considered an index of tissue density (Poorter & Bongers 2006), its augment in response to prolonged water stress conditions could indicate the development of a strategy to cope with water stress that implicates the reduction of growth rate and the increase of leaf longevity in order to retain nutrients, as observed by other authors (Poorter & De Jong 1999; Ryser & Urbas 2000). Moreover, due to the higher LDMC observed in IL 9-2-5 under V3 treatment, it could be hypothesized that this genotype under limiting water supply allocates most new photosynthates towards the production of sclerenchymatic tissues in order to avoid leaf dehydration, rather than toward the growth.

The water status of leaves in response to different water treatments and their duration is indicated by RWC (Figure 3(E),(F)). At 30 DAWS, no statistical differences were evident in both tomato genotypes among different water regimes. Conversely, at 60

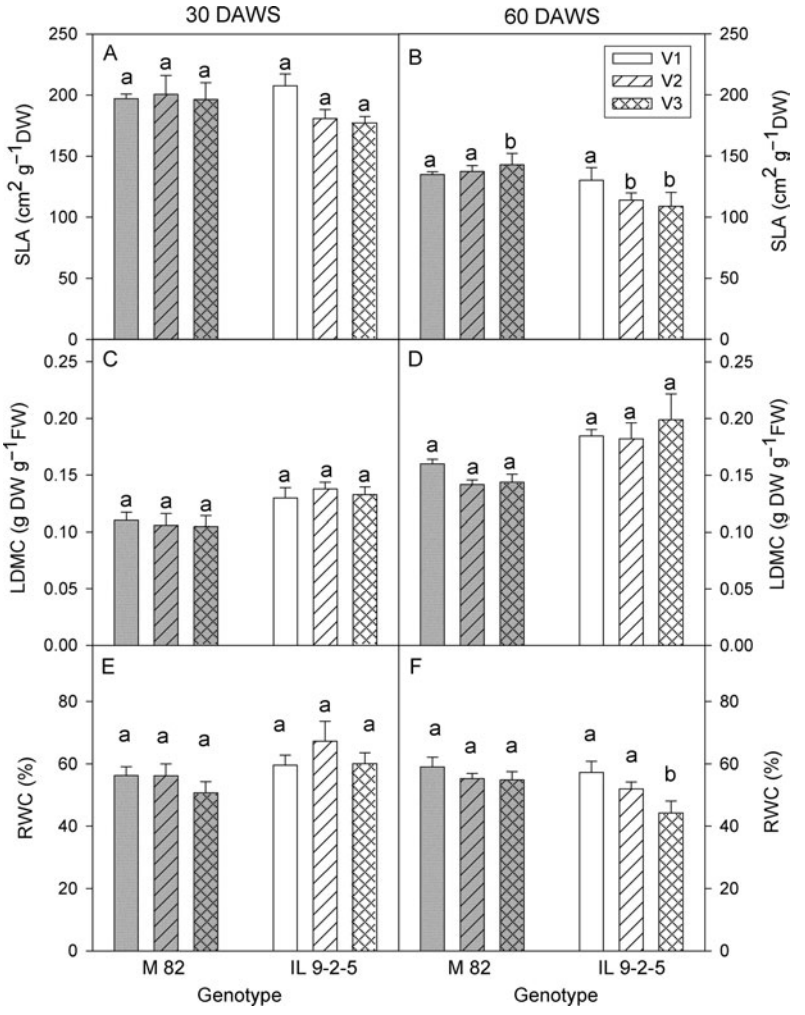

Figure 3. SLA (A, B), LDMC (C, D) and leaf relative water content RWC (E, F) in the leaves of two genotypes M82 and IL 9-2-5 in response to different water regimes V1, V2 and V3 following 30 and 60 DAWS. Mean (± SE) values are shown. Within each genotype, values marked with different letters indicate significant difference among treatments (Student–Newman–Keuls test, $p < 0.05$).

DAWS the genotype IL 9-2-5 showed a reduction of 23% of RWC in the treatment V3 compared with V2 and V1 whereas in M82 RWC values remained comparable to those observed at 30 DAWS. This result evidenced that under prolonged water stress conditions, leaves of IL 9-2-5 are more vulnerable to dehydration compared with the M82 genotype, despite its higher LDMC. The interaction between genotype and treatments was significant only at 60 DAWS (Table III).

Maximal PSII photochemical efficiency

In both tomato genotypes at 60 DAWS, no difference in maximal PSII photochemical efficiency (F_v/F_m) was detected independently of different water treatments (Figure 4). The values of F_v/F_m ratio were comparable for all water regimes and next to 0.8, which is considered the threshold value for plants in healthy status. This indicates that the photochemical apparatus of tomato does not lose its functionality in light conversion to reaction centers even under prolonged periods of limited water supply (Maxwell & Johnson 2000). This result can be considered important since it is well known that water scarcity represents one of the most severe constraints to tomato cultivation (Sanchez-Rodriguez et al. 2011).

Plant biomass production

Regardless of the higher biomass accumulation in IL 9-2-5 previously demonstrated (Fridman et al. 2000), here we confirm that IL 9-2-5 expresses a different response to water deprivation compared with M82 since it retains a higher proportion of its aboveground biomass under water stress (Table IV). In fact, the percentage of aboveground biomass in IL 9-2-5 was higher than M82 at both V2 and V3 and both the water restitution levels and the genotypes significantly affected the reduction in aboveground biomass (Table V). In addition, IL 9-2-5 maintained

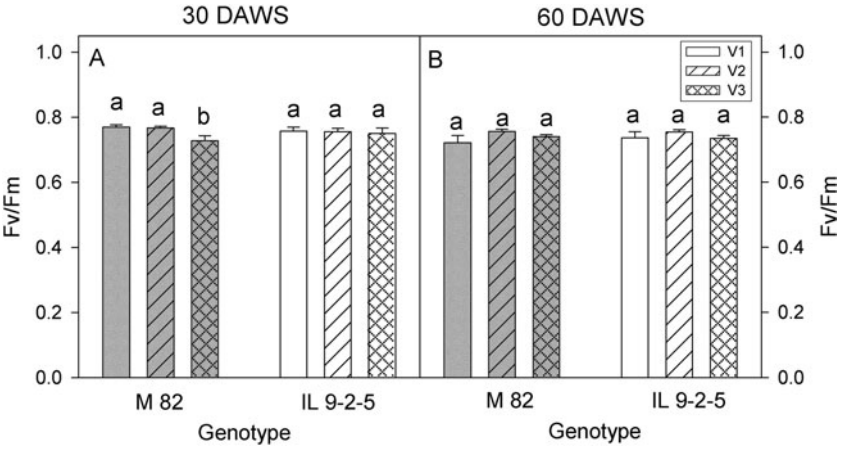

Figure 4. Maximal PSII photochemical efficiency (F_v/F_m) in the leaves of two genotypes M82 and IL 9-2-5 in response to different water regimes V1, V2 and V3 following 30 (A) and 60 (B) DAWS. Mean (± SE) values are shown. Within each genotype, values marked with different letters indicate significant difference among treatments (Student–Newman–Keuls test, $p < 0.05$).

Table IV. Effect of water stress on plant biomass production in M82 and IL 9-2-5.

		Genotypes	
Trait	Treatment	M82	IL 9-2-5
Aboveground biomass	V2	88.65 ± 0.34	93.83 ± 2.50
	V3	49.00 ± 1.47	57.0 ± 11.98
Main stems	V2	95.38 ± 1.78	72.73 ± 18.18
	V3	88.08 ± 6.45	59.09 ± 8.70
Main stem length	V2	107.89 ± 2.41	112.93 ± 14.69
	V3	96.67 ± 4.71	115.13 ± 5.53
Leaves per main stem	V2	115.06 ± 8.99	109.46 ± 12.16
	V3	84.41 ± 4.86	90.65 ± 5.49

Notes: Traits are expressed as a percentage of their values under fully irrigated treatments (V1). Mean (± SE) values are reported.

significantly lower proportions of main stems at reduced levels of water restitution even though this response did not significantly change between V2 and V3 (Tables IV and V). Finally, the response to water deprivation in terms of length of stems and of number of leaves per stem did not account for differences between genotypes. Changes in the length of main stems were statistically the same at V2 and V3 while the level of water reintegration significantly affected the variations in the number of leaves per stem (Table V). Overall, considering the aboveground biomass data reported, IL 9-2-5 expressed lower amplitude than M82 in the plant response to water stress. In addition, preliminary data demonstrates that the genotype IL 9-2-5 maintained a higher percentage of fruit biomass than M82 at reduced levels of water reintegration. In V2 conditions, the percentage of fruit weight compared with that in fully irrigated conditions (V1) was 69.66 ± 4.17 (mean ± SE) in IL 9-2-5 and 40.28 ± 4.18 in M82. The same pattern was observed in V3 conditions where this percentage was 43.86 ± 1.71 in IL 9-2-5 and 22.81 ± 2.15 in M82.

Principal component analysis

Recorded variables on M82 and IL 9-2-5 plants were integrated by a PCA (Figure 5) approach in order to discriminate the effects of genotypes and water restitution treatments (i.e. 100%, 50% and 25% restitution of water loss) on the performances of all traits analyzed. The main three visualized components account for 84% of the overall variability. Component 1 mainly explains variability in the SLA

Table V. Summary of two-way ANOVA statistics reporting effects of two reduced water restitution levels (V2 and V3) on the genotypes M82 and IL 9-2-5.

	Treatment				Genotype				T × G				Residual	
	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P	DF	MS
Aboveground biomass	1	3507.72	512.28	4.87×10^{-7}	1	104.44	15.25	7.93×10^{-3}	1	4.81	0.70	0.43	16	46.76
Main stems	1	138.59	0.53	0.49	1	1772.94	6.81	0.04	1	49.08	0.19	0.68	16	12.47
Main stem length	1	209.75	1.54	0.26	1	496.71	3.64	0.10	1	209.75	1.54	0.26	16	4.61
Leaves per main stem	1	1596.77	8.64	2.60×10^{-2}	1	22.73	0.12	0.74	1	180.69	0.98	0.36	16	0.13

Treatments: V2, V3. Genotypes: M82, IL 9-2-5. T × G: interactions between treatments (T) and genotype (G).

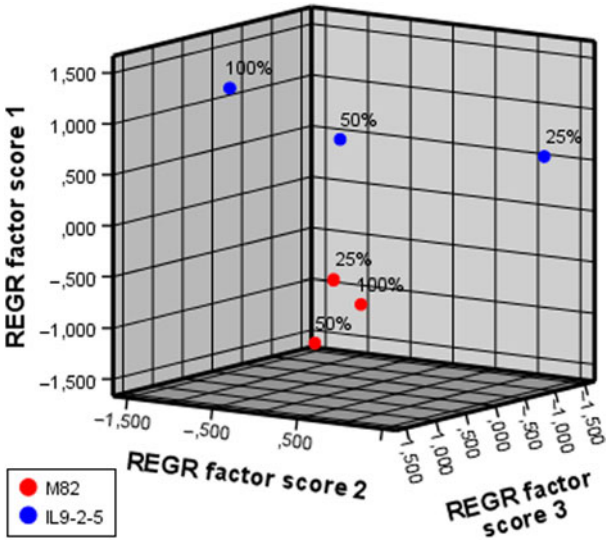

Figure 5. Discrimination of the overall response of M82 and IL 9-2-5 plants to reduced water supply (i.e. 100%, 50% and 25% restitution of water loss) by PCA. Component 1 (REGR factor score 1): SLA (30 and 60 DAWS), LDMC (30 and 60 DAWS), RWC (30 and 60 DAWS), chlorophyll *a* and *b* leaf contents (30 and 60 DAWS), leaf level of carotenoids (60 DAWS) and length of main stems. Component 2 (REGR factor score 2): F_v/F_m (60 DAWS), leaf level of total phenols (30 and 60 DAWS), green FW and number of main stems. Component 3 (REGR factor score 3): F_v/F_m (30 DAWS), level of carotenoids in leaves (30 DAWS) and number of leaves *per* main stem.

(30 and 60 DAWS), LDMC (30 and 60 DAWS), RWC (30 and 60 DAWS), chlorophyll *a* and *b* leaf contents (30 and 60 DAWS), leaf level of carotenoids (60 DAWS) and length of main stems. Component 2 mainly explains variability in the F_v/F_m (60 DAWS), leaf level of total phenolics (30 and 60 DAWS), green FW and number of main stems. Finally, component 3 mainly explains variability in the F_v/F_m (30 DAWS), the level of carotenoids in leaves (30 DAWS) and the number of leaves *per* main stem. The PCA output shows an evident separation between the tolerant genotype IL 9-2-5 and the susceptible genotype M82 that is mainly attributable to the component 1. In addition, in case of the tolerant genotype IL 9-2-5 the response to the three different water regimes can be mainly explained by the component 2. This allows us to point out at the latter traits as effective components of the complex response of IL 9-2-5 to limited water supply.

Conclusion

In this paper, we provided evidences that the IL 9-2-5 performs better than the genotype M82 to reduced water regimes. In particular, we demonstrated that both genotypes perceived the water stress since they react to it by modifying the photosynthetic pigment content in leaves; however, they react in a contrasting way evidencing a higher tolerance of IL 9-2-5 to water deprivation. We showed that the main factors responsible for this better response generally include a more efficient antioxidant system joined with the modification of functional leaf traits associated with an increase of leaf mechanical resistance (namely sclerophylly and LDMC). In addition, we showed that IL 9-2-5 subjected to the water regime V2 (irrigation with 50% field capacity) maintained a higher percentage of fruit weight and aboveground biomass compared with M82 suggesting that IL 9-2-5 could be cultivated in semi-arid environments with reduced losses of yield. Future studies will be required to evaluate if the cultivation of the genotype IL 9-2-5 under the regime V2 can, not only permit the use of marginal lands or cultivation with a more sustainable use of water, but also promote the quality and the nutritional properties of the tomato fruits. Finally, due to the information deriving from the complete sequencing of the tomato genome (Sato et al. 2012) the identification of candidate genes controlling the physiological and morphological traits analyzed is underway by exploring those mapping in the introgression region 9-2-5. Identification of polymorphisms in key-genes controlling these traits can guide in future the breeding efforts aimed at improving susceptible genotypes.

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Note

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