UNIVERSITÁ DEGLI STUDI DI CATANIA

Dipartimento di Scienze Biologiche, Geologiche e Ambientali

PHD IN

SCIENZE GEOLOGICHE, BIOLOGICHE E AMBIENTALI

Coordinatore: Prof.ssa A. Di Stefano

CARMEN ARLOTTA

Study of bioactive compounds and genes involved in their biosynthesis in *Punica granatum* L.

Ph.D. thesis

Tutor: Prof. S. A. Raccuia

XXXI cycle

AA.AA. 2015÷2018

Riassunto

Il frutto della pianta di melograno (*Punica granatum* L.) è un importante alimento nutraceutico ricco di composti fenolici. Tutte le parti del frutto presentano rilevanti proprietà benefiche per la salute attribuibili all'alto contenuto di questi composti, il quale può variare in base ai genotipi, alle condizioni ambientali di crescita ed alla tecnica agronomica adottata. Diverse accessioni siciliane e internazionali di melograno sono state studiate ed è stata effettuata la caratterizzazione di una collezione rappresentativa di germoplasma di melograno siciliano. Caratteristiche morfologiche sono state misurate sul frutto intero, sulla buccia, sugli arilli e sul succo, inoltre su quest'ultimo sono stati valutati il pH, i solidi solubili totali (°Brix), il colore, il contenuto fenolico totale (TPC), l'attività antiossidante (AA), gli zuccheri e i sali minerali. Quattro nuovi marcatori SSR polimorfici coinvolti nella via delle antocianine sono stati identificati e studiati in aggiunta a sette SSR nucleari presenti in letteratura, al fine di analizzare la diversità genetica tra le cultivars. Inoltre, è stata studiata anche la fisiologia della germinazione dei semi valutandone la risposta a trattamenti con acido gibberellico e con KNO_3. Il profilo polifenolico, comprendente antocianine, ellagitannini e gallotannini è stato rilevato mediante LC-PDA Orbitrap FTMS in diversi succhi di melograno, in foglie, fiori e durante le fasi di sviluppo del frutto di due accessioni differenti per il colore degli arilli (Wonderful e Valenciana). Inoltre, l'effetto della sovraespressione di un importante complesso regolatorio della biosintesi dei flavonoidi, MYB-bHLH-WD40, è stato studiato mediante agroinfiltrazione su diversi gruppi metabolici di *Nicotiana benthamiana*. Il livello di espressione di questo complesso e di alcuni geni strutturali coinvolti nella via delle antocianine è stato esaminato in foglie, fiori e durante le fasi di sviluppo del frutto del melograno di entrambe le accessioni colorate.

Lo studio ha evidenziato un'elevata diversità tra i genotipi siciliani, i quali si sono differenziati rispetto ai genotipi internazionali in resa in arilli e in succo, mostrando valori più alti rispetto al Wonderful One, ed inoltre significative differenze sono emerse anche in TPC e AA. Glucosio e fruttosio si sono confermati i principali zuccheri presenti nel succo con rapporto glucosio/fruttosio compreso tra 0,9 e 1,3. Tra i sali minerali analizzati il potassio è risultato l'elemento più abbondante. Una correlazione positiva è stata riscontrata tra i dati morfologici, biochimici e i microsatelliti, mentre un'alta percentuale di germinazione dei semi è stata osservata in alcuni dei genotipi siciliani. Differenze significative nei composti fenolici sono state trovate tra i diversi succhi esaminati, in

particolare la cultivar Wonderful ha mostrato il maggiore quantitativo di antocianine, mentre punicalagine α e β sono risultate prevalenti nel succo di Wonderful One. Inoltre, Valenciana ha mostrato alcune caratteristiche bioagronomiche e di composizione del succo simili a quelle di alcuni dei genotipi siciliani studiati, ma differenti da Wonderful. Interessanti risultati sono emersi anche dall'analisi metabolomica dei composti fenolici in foglie, in fiori e in diverse fasi di sviluppo del frutto delle cultivars Valenciana e Wonderful. In particolare, in ambedue le cv, durante l'accrescimento degli arilli, è stato osservato un aumento del contenuto di antocianine e una diminuzione di ellagitannini e gallotannini, mentre a maturazione commerciale gli arilli di Wonderful risultavano più ricchi in derivati della cianidina e delfinidina, differenziandosi da quelli di Valenciana che erano più ricchi in pelargonidin-3-glucoside.

Tra i geni studiati in questo lavoro mediante agroinfiltrazione nelle foglie di *N. benthamiana*, *PgMYB2* in congiunzione con *PgbHLH* ha determinato una produzione significativa di diidroflavonoli tipici della via biosentetica dei flavonoidi. Dalle successive prove di espressione genica durante le fasi di sviluppo del frutto di melograno è emerso che l'espressione di *PgMYB2* diminuisce con la maturazione degli arilli, prevalentemente espresso nel fiore ed in parte nel frutto immaturo. Questi risultati insieme a quelli della sovraespressione in Nicotiana suggeriscono che i geni *PgMYB2* e *PgbHLH* sono coinvolti nelle prime fasi della via di biosintesi delle antocianine nella produzione di flavonoidi, ed in particolare di diidroflavonoli. Di contro, i livelli di espressione di *PgMYB1*, *PgMYB1.2, PgCHS*, *PgF3'5'H, PgDFR* e *PgUFGT* che aumentano con lo sviluppo del frutto della cv Wonderful, seguendo lo stesso andamento delle antocianine, suggeriscono che questi geni sono coinvolti nella biosintesi delle antocianine.

Abstract

The pomegranate (*Punica granatum* L.) plant fruit is an important nutraceutical food rich in phenolic compounds. All parts of its fruit exhibit important health-beneficial properties attributed to the high content of phenolic compounds, which can differ according to genotypes, environmental growth conditions and the agronomic technique adopted. Several Sicilian and international pomegranate accessions were studied, and a core of Sicilian pomegranate germplasm was characterized. Morphological features were measured in whole fruit, in peel, in arils and juice, moreveor on this latter pH, total soluble solids (°Brix), total phenolic content (TPC), antioxidant activity (AA), sugars and mineral salts were analysed. Four new polymorphic SSR markers involved in the anthocyanin pathway, were developed and used in addition to literature-derived seven nuclear SSRs to analyse genetic diversity among cultivars. Furthermore, seed germination physiology was also investigated, evaluating the response to Gibberellic acid and KNO_3 treatments. The polyphenolic profiles, including anthocyanins, ellagitannins and gallotannins were detected by LC-PDA Orbitrap FTMS in some pomegranate juices and in leaves, flower and during fruit development of two different pomegranate cultivars differing for their arils colour (Wonderful and Valenciana). Furthermore, the effect of overexpression of an important flavonoid regulatory genes complex, MYB-bHLH-WD40, on *Nicotiana benthamiana* was investigated by agroinfiltration. Expression levels of this complex and of structural genes involved in anthocyanins pathways was examined in leaf, flower and during pomegranate fruit developmental stages of both coloured accessions.

The study showed a high variability between Sicilian genotypes, which differed from the international genotypes in aril and in juice yields, showing higher values than Wonderful One, and in addition significant differences in TPC and AA were observed between them. Glucose and fructose were confirmed as the main sugars in juice with a glucose / fructose ratio between 0.9 and 1.3. Among the mineral salts analyzed potassium was found the most abundant element. A positive correlation was found between morphological, biochemical and microsatellite data and a high percentage of seed germination was observed in some of Sicilian genotypes. Significant differences in phenolic compounds were found among different juices examined and the greatest quantity of anthocyanins was found in Wonderful cultivar, while punicalagin α and β were prevalent in Wonderful One. In addition, Valenciana showed some bio-agronomic and juice composition characteristics similar to

those of some Sicilian genotypes studied, but different from Wonderful. Interesting results emerged from the metabolomic analysis of phenolic compounds in leaves, flowers and at different stages of fruit development of Valenciana and Wonderful cultivars. In particular, we observed an increase of anthocyanins and a decrease of ellagitannins and gallotannins during fruit ripening stages both cv, while at maturation the Wonderful arils were richer in derivatives of cyanidin and delphinidin differentiating from those of Valenciana that were rich in pelargonidine-3-glucoside.

Among *PgMYB* genes agroinfiltrated in *N. benthamiana* leaves, *PgMYB2* with *PgbHLH* resulted in a significant production of dihydroflavonols typical compounds of the flavonoids pathway. Gene expression assays during the fruit development stages showed that the expression of *PgMYB2* decreased with aril ripening, while it is mainly expressed in flower and partly in unripe fruit. These results together with those of overexpression in Nicotiana suggest that the *PgMYB2* and *PgbHLH* genes are involved in the early stage of anthocyanin biosynthesis pathway in the production of flavonoids, and in particular dihydroflavonols. On the other hand, the expression levels of *PgMYB1, PgMYB1.2*, *PgCHS*, *PgF3'5'H*, *PgDFR* and *PgUFGT* increased with the fruit development of Wonderful cv, following the same trend of anthocyanins, suggesting that these genes are involved with the biosynthesis of anthocyanins.

Acknowledgments

I would like to begin by expressing my thanks to my tutor Dr. S. A. Raccuia for the valuable lessons he gave me during these three years of PhD, enriching my knowledge.
I thank Dr. Giuseppe Puglia for what he explained and taught me with great willingness and patience, supporting me in my doctorate path.
I thank Dr. Claudia Genovese and Dr. Valeria Toscano for their availability and for their support and help during this period.
I thank Dr. Jules Beekwilder and Dr. Ric de Vos and Team Metabolic System for welcoming at the University of Wageningen and helping me to enrich my research work.
I then want to thank my colleagues, but especially friends, who in different times and in various ways, have given me their help and their friendship.
Finally, I wish to thank with love my family and my boyfriend for having always been near me in the good and bad moments of life, teaching me how to overcome difficulties, believing in me and supporting me day after day.

Index

Preface

This work has been carried out in collaboration with:

CNR AGROFOOD Department - Institute for Mediterranean Agriculture and Forest Systems of Catania, which funded all this work.

UNIVERSITY & RESEARCH, University of Wageningen. A part of this work has been carried out thanks to the supervision of Dr. Jules Beekwilder and Dr. Ric de Vos, Team Metabolic System (BU), University of Wageningen, Netherlands.

1 Introduction

1.1 Nutraceuticals foods

Plants have been one of the main food sources since the first appearance of man on earth. In foods of plant origin such as fruits and vegetables, bioactive compounds are naturally present; these compounds can have nutritional value and can exhibit a biological activity usually referred to as chemo-preventive property: antioxidant, anti-carcinogenic and anti-inflammatory. For this reason, rather than nutritional components, these molecules are considered compounds with a pharmacological activity and they are often referred to as "nutraceuticals". The term nutraceutical was coined from "nutrition" and "pharmaceutical" by Dr. Stephen DeFelice MD, founder and chairman of the foundation for innovation in medicine (FIM) Cranford, New Jersey, in 1989 (DeFelice, 1989). Nutraceuticals are also called **functional foods** (Fig. 1), which are defined as "food products to be taken as part of the usual diet in order to have beneficial effects that go beyond basic nutritional function" (Chauhan et al., 2013).

Fig. 1. Functional foods.

These bioactive compounds are grouped into different classes: polyphenols the most important among these compounds (isoflavones, lignans, flavonoids, carotenoids, tannins etc.) vitamins (vitamin C, vitamin E, vitamin B9), fatty acids (as punicic acid), Omega3 and Omega6, minerals, prebiotics and a lot of others. Among the nutraceutical foods, the pomegranate (*Punica granatum* L.) plant fruit, exhibits important health properties attributed to the high content of bioactive molecules in fruit.

1.2 Pomegranate (*Punica granatum* L.)

1.2.1 Origin and diffusion

P. granatum belongs to the Punicaceae (or Lythraceae) family and it is an ancient and appreciated fruit plant (Fig. 2). The name ‘‘pomegranate’’ follows the Latin name of the fruit *Malum granatum*, which means “grainy apple”. Punicaceae contains only two species, *Punica granatum* L. and *Punica protopunica* Balf. which is endemic to the Socotra Island (Yemen) (Mars, 2000; Levin, 2006).

Fig. 2. Pomegranate tree.

Pomegranate is native to Afghanistan, Iran, China and the Indian subcontinent. From the west of Persia (modern day Iran), pomegranate cultivation spread through the Mediterranean region to the Turkish European borders and American southwest, California and Mexico (Celik et al., 2009; Lansky and Newman, 2007). It is considered an excellent fruit for arid environments and can grow in different geographical regions included Mediterranean basin (North Africa, Egypt, Israel, Syria, Lebanon, Turkey, Greece, Cyprus, Italy, France, Spain, Portugal), Asia and California (Holland et al., 2009) (Fig. 3).

Mediterranean-like climate provide the optimal growth conditions for pomegranate. This climatic condition includes high exposure to sunlight, mild winters with minimal temperatures not lower than -12 °C and dry hot summers without rain during the last stages of the fruit development (Levin, 2006). Under such conditions, the fruit will develop to its best size and optimal colour and sugar accumulation without the danger of splitting (Holland et al., 2009).

Fig. 3. Pomegranate production in the world (http://www.freshplaza.it/article/80301/Salvare-il-reddito-alla-produzione-diversificando-il-programma-sul-melograno-di-Terremerse).

Contrary to what happens for some other minor species, the official statistical sources of FAO do not report data on the area used (ha) for the production of pomegranate. An annual world production of about 1.5 million tons has been estimated, over 90% of which is supplied by the three main producing countries: Iran, India and China (Holland et al., 2008).

In Italy, ISTAT (National Institute of Statistics) reported an area of just 7 hectares for 2008 and a production of about 690 tons per year mainly in Calabria and Sicily regions; there is a growing import of fruit from Israel, Spain and Turkey. In the last few years in Italy there has been a rapid expansion of the areas used that reached about 819 ha at the end of 2017 (www.istat.it). Pomegranate has always been limited and considered as a minor crop, although in the last ten years there has been a renewed cultivation, commercial and scientific interest thanks to its health properties (Bellini et al., 2010).

The main cultivars now widespread in the world are Wonderful and Akko, the first obtained in America and the second in Israel. Many other varieties have been described in recent years in the various producing countries.

Today the main producers and exporters of the world are India, Iran, China, Turkey, United States, Spain, South Africa, Peru, Chile and Argentina. The global pomegranate production is around 2 million tons. The European Union (EU) is the leading importer of pomegranates. In 2013, another 67 thousand tons were added to the European market. in addition to local production (World Pomegranate Market Supply & Forecast, 2015).

Italian production covers only 30% of the market with an income for farmers between 15.000 and 25.000 euros per hectare. However, the south is the most suitable area for production: Sicily, Sardinia, Puglia, Calabria, Campania and Lazio are the regions most affected by this sudden expansion (www.ilsole24ore.com/art/impresa-e-territori/2015-11-10). In Sicily interesting resources of pomegranate germplasm are present in semi-abandoned orchards or as sparse plants. A recent evaluation of different Italian and Spanish varieties highlighted the promising traits of a Sicilian accession, called 'Primosole' which is currently being diffused (La Malfa et al., 2009, Todaro et al., 2016).

1.2.2 Botanical traits of pomegranate

1.2.2.1 Leaves and flowers

Pomegranate is a deciduous tree. Young leaves have a lanceolate-obovate shape, tend to have a reddish colour that turns green when the leaf matures. In varieties with young pink-purple bark, this colour appears also on the lower part of the central vein, and in the leaf margins.

Mature leaves usually have a special glossy appearance and they are green, entire, smooth, and hairless with short petioles (Holland et al., 2009).

Flowering occurs about 1 month after bud break. In the northern hemisphere flowering occurs in April-May but may continue until end of summer. Flowers can appear solitary, in pairs, or in clusters of 3-5 (Fig. 4) (Holland et al., 2009).

Fig. 4. Pomegranate flowers. It is possible to observe solitary or clusters flowers.

The flower, in the early balloon stage, seems a small pear with a greenish colour that becomes orange-red in flower matures red. The petals are orange-red or pink and rarely white (Feng et al., 1998; Wang 2003; Levin 2006). Pomegranate flowers develop as hermaphrodite flowers ("vase shape") or as male flowers ("bell shape"). Both types have several hundred stamens. The stigma of the hermaphrodite is at the anthers height or emerging above them. This position allows for self-pollination as well as pollination by insects mainly bees. Wind pollination is reported to occur but infrequently (Morton 1987). Pomegranate flowers can self-pollinate and produce normal fruit (Nalawadi et al. 1973; Karale et al., 1993; Mars 2000; Levin 2006; Holland et al., 2009) but the degree of fruit set by self-pollination varies among different pomegranate cultivars (Levin 1978; Kumar et al., 2004).

1.2.2.2 Fruits

The fruit is a fleshy berry connected to the tree through the petiole, it is crowned by the prominent calyx opened or closed depending on the variety and on the stage of ripening.
During the development the colour of the sepals' skin changes continuously from green to orange-red. In the ripe fruit the external colour ranges from yellow, green, or pink overlain with pink to deep red or indigo to fully red, pink or deep purple cover, depending on the variety and stage of ripening (Holland et al., 2009). The pomegranate fruit peel has two parts: an outer, hard pericarp, and an inner, spongy mesocarp (Fig. 5). Membrane walls (septum) of the mesocarp are organized as chambers, which comprises the fruit inner wall where arils attach, containing the seed (Stover & Mercure 2007). The interior network of membranes comprising almost 26–30% of total fruit weight.
The bitegmic ovules of pomegranate possess two integuments that develop into the distinct inner and outer seed coats of pomegranate seeds (Qin et al., 2017). The juicy edible outer coat of arils develops entirely from outer epidermal cells of the seed, which elongate in a radial direction (Fahan, 1976) and it is highly vacuolized and rich in organic acids, sugars, minerals and anthocyanins (Fawole and Opara, 2013a). The compact cells of the inner seed coat show little to no vacuolization and they are enriched in lignin and fiber (Qin et al., 2017).

Fig. 5. Different parts of pomegranate fruit (http://fsi.colostate.edu/pomegranates/).

The arils vary in size and the seeds vary in hardness among different varieties (soft, semi-soft, semi-hard and hard). The number of seeds in a pomegranate can vary from 200 to about 1.400. There is no correlation between the outer skin colour of the rind and the colour of the arils. These colours could be very different or similar, depending on the variety. The outer skin colour does not indicate the extent of ripening degree of the fruit or its readiness for consumption because it can attain its final colour long before the arils are fully ripened (Holland et al., 2009).

1.3 Bioactive compounds

Plants carry out an intense synthesis of compounds called "secondary metabolites", also known as "phytochemical" or “bioactive compounds”. There are approximately 100,000 compounds known to be derived from plants and the number is constantly growing. The plants, in fact, interact continuously with the surrounding environment because being immobile they can’t escape the biotic stresses (due to the presence and / or attack of animals and / or pathogenic and non-pathogenic microorganisms) and abiotic (due to environmental factors: temperature, salinity, UV radiation, humidity, presence of pollutants). So, plants have evolved chemical defence systems that allow them to deal with the different ones dangers. Many metabolites act as specific or non-specific deterrents to herbivores, micro-organisms and viruses. Some plants can produce compounds with an antibiotic, antifungal, antiviral function or to build barriers against the entry of pathogens and parasites. On the other hand, many plant species, require insects for pollination and produce "attractors" compounds. In addition to the colour of the flowers, the plants synthesize volatile aromatic compounds that attract insects which thus guarantee entomophilous pollination. Furthermore, plants can produce specific secondary metabolites that attract the natural

enemies of their predators. Thanks to a cross-talk based synthesis of compounds, the plants can establish symbiosis with soil microorganisms and finally defend themselves from abiotic stresses such as excessive leaf temperatures and UV radiation. The Mediterranean climate characterized by rainy winters and hot/dry summers, elicits in many plants as pomegranate, considerable levels of these metabolites as protective agents mainly from abiotic stresses. Secondary metabolites can be divided into three large classes of compounds: isoprenoids, alkaloids and polyphenols.

1.3.1 Isoprenoids

Isoprenoids are a vast and heterogeneous class of compounds such as terpenes and steroids having a base structure with repeats of five carbon atoms (isoprene), the play important roles in plants. Terpenes are the most abundant and most widely used natural products in plants.

1.3.2 Alkaloids

Another class of secondary metabolites is represented by alkaloids. They comprise a heterogeneous group of compounds having a nitrogenous group deriving from amino acids such as lysine, histidine and tryptophan. They are generally distributed in the form of salts in all the organs of the plant, but mostly in seeds, leaves, bark and root.

1.3.3 Polyphenols

Polyphenols are one of the most important and the most copious group of phytochemicals belonging to a large and heterogeneous family of natural organic polymeric substances; among them there are lipophilic molecules, carboxylic acids, water soluble glucosides and large insoluble polymers Polyphenols confer resistance against biotic and abiotic stress. They also have antibacterial, antioxidant, anti-inflammatory and anti-tumour properties. Physiological and environmental factors induce the polyphenols production (Rolland et al., 2006; Catala et al., 2011). In these regulations distinct hormone signalling pathways have been involved e.g. induction by abscisic acid (ABA), jasmonate (JA), or cytokinins, and repression by gibberellic acid (GA), ethylene, or brassinosteroids (BRs) (Peng et al., 2011).

Polyphenols are characterized by the presence of multiple phenolic groups associated in complex structures low molecular weight (phenolic acids and flavonoids) or high molecular weight (tannins) with structure common benzene-derived chemistry with one or more hydroxyl groups associated to the ring. Currently over 8,000 phenolic structures are known, of which more than 4,000 belong to the flavonoid class, but also phenolic acid and tannins are abundant. Some of phenolic compounds are represented in the following Fig. 6.

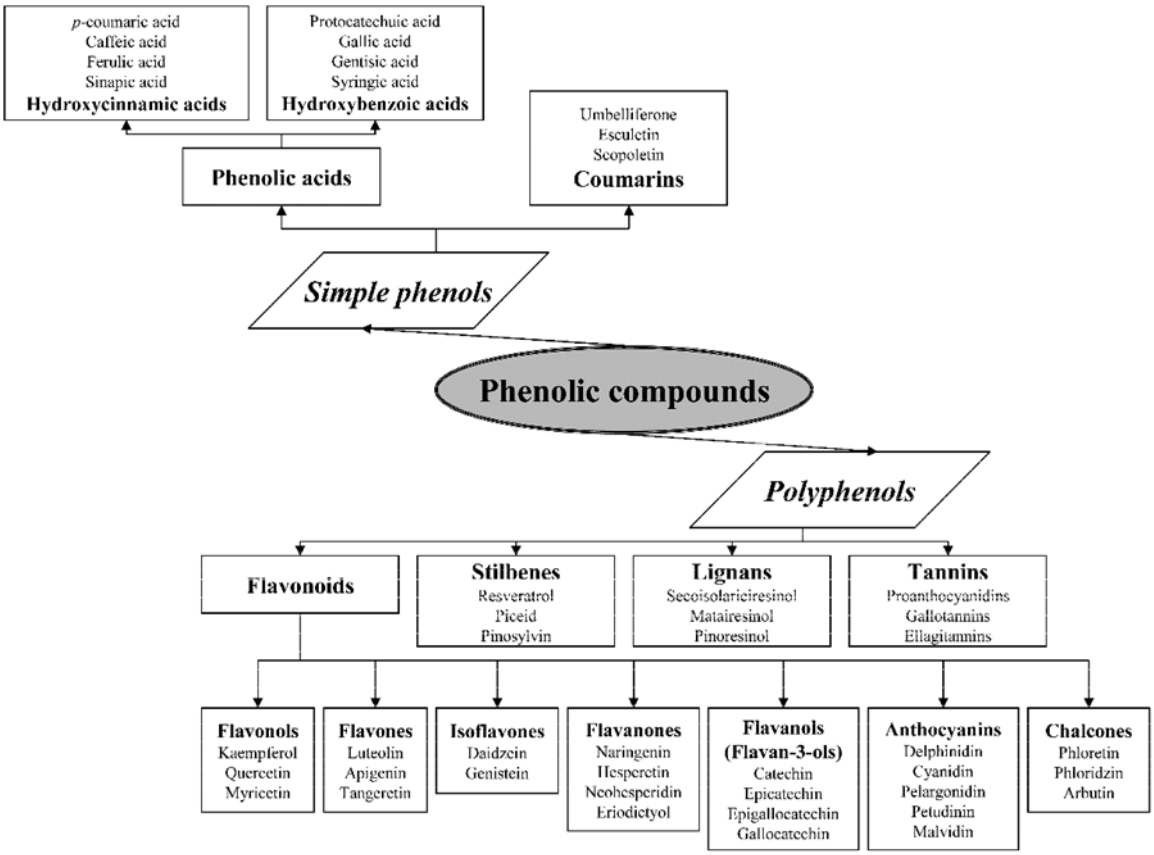


Fig. 6. Classification of phenolic compounds (Soto et al., 2015).

1.3.3.1 Phenolic acid

Phenolic acids can be divided into two subclasses: hydroxybenzoic acids derivative from benzoic acid and hydroxycinnamic acids derivative from cinnamic acid.

Gallic acid is one of the polyphenols representatives of hydroxybenzoic acid, while the most common of hydroxycinnamic acid is caffeic acid.

1.3.3.2 Tannins

Tannins are phenolic compounds with a high mass and high affinity for proteins with which they form insoluble compounds. There are three major groups: hydrolysable tannins, condensed tannins (procyanidins and proanthocyanidins) and tannoids.

The hydrolysable tannins (decomposable in water, with which they react to form other substances) are esters of gallic acid, at the centre of a hydrolysable tannin molecule, there is a carbohydrate (usually D-glucose but also cyclitols like quinic or shikimic acids). The hydroxyl groups of the carbohydrate are partially or totally esterified with phenolic groups such as gallic acid in gallotannins or ellagic acid in ellagitannins. In pomegranate the high antioxidant activity is attributed also to the content of hydrolysable tannins as punicalagins (in form α and β), punicalins, ellagic acid, gallotannins and other ellagitannin-based compounds, these compounds can contribute to reduce rates of cardiovascular disease, diabetes, and prostate cancer (Johanningsmeir and Harris, 2011).

Condensed tannins are polymers of flavan-3-ols such as catechin and epicatechin. The condensed tannins are not very soluble in water and are responsible for the red colour of many roots. Tannoids are derivatives of chlorogenic acid (caffeic acid ester with quinic acid). Tannins have astringent, anti-inflammatory, anti-bacterial and anti-oxidant properties.

1.3.3.3 Flavonoids

The flavonoids consist of two aromatic rings bound to a benzopyranic ring to generally form an oxygenated heterocycle (C6-C3-C6). Generally, flavonoids carry conjugated double bonds which, by absorbing light, make the coloured flavonoids appear. The term flavonoid derives in fact from the latin word flavus (yellow) and refers to the role they play as pigments in flowers, fruits and leaves. Flavonoids are the most widespread polyphenolic compounds in the plant kingdom, normally in the form of glycosides, and are used by plants mainly as visual and olfactory attractants necessary for pollination and seed dispersal and, thanks to their antioxidant power, to protect themselves from damage caused by UV rays. The flavonoids have high antioxidant activity, this probably depends on the hydroxyl groups present on the basic structure.

Through methylation reactions, glycosylation, isoprenylation, different types of flavonoids are formed: flavones, flavanones, flavonols, isoflavones and anthocyanins.

The flavonols are the main class of flavonoids, among them the most representative are quercetin and kaempferol. Flavonols are found in many fruits and vegetables such as

tomatoes, onions, tea leaves, blueberries, capers, red grapes and therefore also in wine. They have an antioxidant activity and anti-cancer effect, for example quercetin inhibits the oxidation of lipids, and anti-inflammatory activity by inhibition of enzymes such as phospholipase A2, while kaempferol has been found to induce apoptosis in breast cancer cells through extracellular signal-regulated kinase activation and up-regulation of p53 (Calderòn-Montano et al., 2011).

The flavanols can be found in monomeric form, taking the name of catechins, or in polymer form, such as proanthocyanidins. Catechins are mainly found in apples, plums, beans, lentils, strawberries, cherries, and have multiple biological activities including vasodilatory, anti-tumor, anti-inflammatory, antibacterial and immune system stimulation effects. The proanthocyanidins are found in the seeds and in the grape skin and also have an antioxidant effect.

Antocyanins are the most important pigments in nature and they colour flowers and fruits. Food plants rich in anthocyanins include the pomegranate, blueberry, raspberry, black rice, and black soybean, among many others that are red, blue, purple, or black. Some of the colours of autumn leaves are derived from anthocyanins

1.4 Benefits of pomegranate

The pomegranate fruit has been used for thousands of years as medicine thanks to the natural source of phytochemicals present in the fruit and in the juice. The first who noticed the "medicinal" properties of the pomegranate was Hippocrates, prescribeding it as an anti-inflammatory, anti-diarrhea, antibacterial and anthelmintic. These healing properties, centuries later, have been confirmed by medical research.

Some clinical research studies suggest that pomegranate plays a role in reducing the risk of different tumour forms (prostate, breast, lung, skin, colon cancer, multiple myeloma) (Malik and Mukhtar, 2006; Khan et al., 2007; Seeram et al., 2007, Tibullo et al., 2016), coronary disease (Sumner et al., 2005), Alzheimer's disease (Singh et al., 2008) and erectile dysfunction in male patients (Forest et al., 2007). The juice consumption changes the blood parameters such as LDL, HDL, and cholesterol (Kaplan et al., 2001; Aviram et al., 2000, 2004) and increases the prostate specific antigen (PSA) (Pantuck et al., 2006) (Fig. 7).

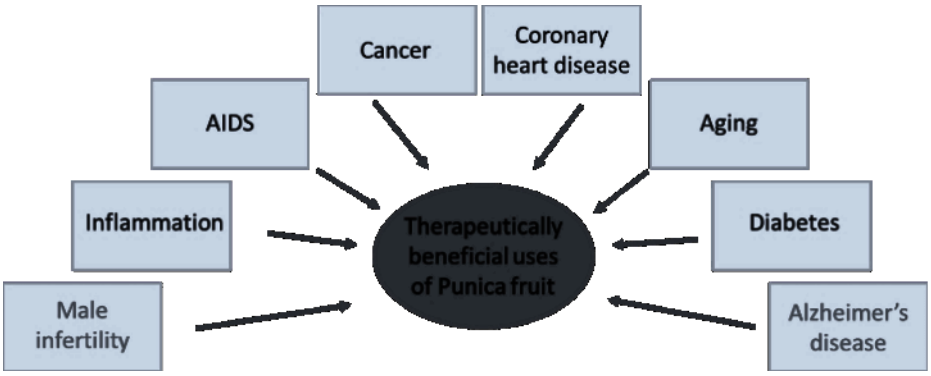


Fig. 7. Therapeutically beneficial uses of pomegranate fruit.

Extracts of all parts of the pomegranate fruit including the fruit juice, peel, arils, flowers, and bark exhibit therapeutic properties (Lansky and Newman, 2007) and antimicrobial activities. The most activities can be related to ellagic acid and hydrolysable tannins, such as punicalagin (Howell and D'Souza, 2013), (Tab. 1).

Tab. 1. Principal constituent of pomegranate tree and fruit (Sreekumar et al., 2014).

Pomegranate peel	Pomegranate juice	Pomegranate root and bark	Pomegranate flower	Pomegranate leaves	Pomegranate seed
(i) Gallic acid (ii) Ellagic acid (iii) Punicalin (iv) Punicalagin (v) Caffeic acid (vi) Ellagitannins (vii) Pelletierine alkaloids (viii) Luteolin (ix) Kaempferol (x) Quercetin	(i) Simple sugars (ii) Aliphatic organic acids (iii) Gallic acid (iv) Ellagic acid (v) Quinic acid (vi) Flavonols (vii) Amino acids (viii) Minerals (ix) EGCG (x) Ascorbic acid	(i) Ellagitannins (ii) Piperidine alkaloids (iii) Pyrrolidine alkaloid (iv) Pelletierine alkaloids	(i) Gallic acids (ii) Ursolic acid (iii) Triterpenoids (iv) Fatty acids	(i) Carbohydrates (ii) Reducing sugars (iii) Sterols (iv) Saponins (v) Flavanoids (vi) Tannins (vii) Piperidine alkaloids (viii) Flavone (ix) Glycoside (x) Ellagitannins	(i) 3,3′-Di-*O*-methylellagic acid (ii) 3,3′,4′-Tri-*O*-methylellagic acid (iii) Punicic acid (iv) Oleic acid (v) Palmitic acid (vi) Stearic acid (vii) Linoleic acid (viii) Sterols (ix) Tocopherols (x) Sex steroids

1.4.1 Healthy properties of pomegranate juice

Pomegranate arils are mainly consumed as fresh fruit and juice, also in salads and desserts. Pomegranate juice can be obtained from the squeezing of the arils alone (natural juice) or of the whole fruit (arils and peel), the latter has a greater quantity of phenols because the peel has many bioactive compounds, like punicalagin (Jurenka, 2008). Punicalagin from the peel represents the compound that gives the major contribution to the total antioxidant capacity of pomegranate juice (Gil et al., 2000; Tzulker et al., 2007).

The taste of the juice is variable, in general, the flavour is intermediate with a sweet base, a sour bottom and a light bitter and astringent tone given by the tannic component of the aril. The juice concentration, the content of sugars, the colour, the polyphenol content and the quality of the product depend on the variety and degree of ripeness of fruits, climatic and environmental condition can influence these aspects. The most suitable varieties are those which have fruits with a more acidic flavour, rich in phenols with important health properties (Cristofori et al., 2011). The red colour of juice can be attributed to anthocyanins, such as delphinidin, cyanidin, and pelargonidin glycosides. Generally, an increase in juice pigmentation occurs during fruit ripening (Hernàndez et al., 1999), (Fig. 8).

Fig. 8. Pomegranate juice. The colour is conferred by the presence of anthocyanins.

The juice represents on average the 30% of the total fruit weight and is a good source of minerals (potassium, phosphorus, calcium, iron and magnesium), glucose 0.36 mg / ml and fructose 3.3 mg / ml (Tezcan et al., 2009) and fibers. Moreover, it contains organic acids (citric acid, malic acid), water, vitamin C, vitamin E, coenzyme Q and polyphenols such as ellagitannins (punicalagin) and anthocyanins. From a purely nutritional point of view, the daily dose of pomegranate juice for human consumption is about 250 g, which provides about 134 Kcal (Tab. 2).

Tab. 2. Nutritional value per 100 g of pomegranate Juice (source USDA).

Nutrients	Unit	Value per 100 g
Water	g	85.95
Energy	Kcal	54.00
Protein	g	0.15
Total lipid (fat)	g	0.29
Carbohydrate, by difference	g	13.13
Fiber, total dietary	g	0.10
Sugar, total	g	12.65
Minerals		
Calcium, Ca	mg	11.00
Iron, Fe	mg	0.10
Magnesium, Mg	mg	7.00
Phoshorus, P	mg	11.00
Potassium, K	mg	214.0
Sodium, Na	mg	9.00
Zinc, Zn	mg	0.09
Vitamins		
Vitamin C, total ascorbic acid	mg	0.10
Thiamin	mg	0.02
Riboflavin	mg	0.02
Niacin	mg	0.23
Vitamin B6	mg	0.04
Folate	mg	24.00
Vitamin E (alpha-tocopherol)	mg	0.38
Lipids		
Fatty acids, total saturated	g	0.08
Fatty acids, total monounsaturated	g	0.06
Fatty acids, total polyunsaturated	g	0.05
Cholesterol	mg	0.00

1.4.1.1 Antioxidant activity

Beneficial effects and the antioxidant activity of the juice and fruit in general are attributed to the presence of polyphenols. Their main role is to act against free radicals, molecules that are highly harmful to the cells themselves. The most known free radicals are those that contain oxygen, called "reactive oxygen species" (ROS, from the English Reactive Oxigen Species), responsible for oxidative damage. Among the most common ROS there are the superoxide anion (O2 • -), hydrogen peroxide (H_2O_2) and the hydroxyl radical (• OH).

Polyphenols are characterized by an ideal chemical structure for the radical scavenger activity and it has been shown that these types of molecules perform a higher antioxidant

activity in vitro compared to vitamins C and E1. The structural characteristic responsible for their antioxidant action, and therefore radical-scavenger, is precisely the presence of the phenolic hydroxyl group. These molecules are able to donate the hydrogen atom present in the phenolic OH to the free radicals, thus interrupting the propagation of the chain reactions typical of oxidative processes.

Preclinical studies have found that these bioactive compounds have important protective activities against many pathological conditions, defending the body from cellular aging, cancer, some diseases of the cardiovascular system, diabetes, multiple sclerosis, Parkinson's disease and Alzheimer.

The antioxidant capacity of foods against free radicals present in our organism is evaluated by a scale called ORAC table (Oxygen Radical Absorbance Capacity) (Fig. 14). Refering to this data pomegranate is one of the richest fruits of antioxidants with an ORAC index equal to 10,470 or 3 times higher than that of blueberries and 12 times that of oranges (Okonogi et al., 2007). It provides 20% more antioxidants than other beverages like cherry juice, orange juice, red wine, iced tea (Gil et al., 2000; Lansky et al., 2007; Seeram et al., 2008).

1.4.1.2 Anticarcinogenic properties

Different studies suggest that pomegranate juice and extract of fruit can play an important role in cancer prevention in prostate, breast, colon, lungs, skin and myeloma multiple, probably inhibiting cancer cell proliferation or through apoptosis. A recent study conducted with prostate cancer cells has shown that juice and extracts of fruit can counteract angiogenesis and metastatic process (Wang and Martins-Green, 2014). This can be demonstrated with the turnover of arachidonic acid, which is a good indicator of cancer cells invasiveness and metastasis. Inhibition of tumour creeping is due to the suppression of regulatory molecules of the G1 phase (cicline).

Following a clinical trial conducted by the Department of Biology and Neuroscience of the University of California and performed on patients with prostate cancer, it has been observed that some components extracted from pomegranate juice, such as luteolin, ellagic acid and punicic acid (Wang et al., 2012) are effective in inhibiting the growth of prostate carcinoma showing a significant decrease in PSA (specific prostatic antigen). This because they interfere with numerous biological processes involved in tumour cell metastasis such as cell growth suppression, cell migration inhibition and chemotaxis of proteins important in the formation of metastases in prostate cancer.

On the other hand, in *vitro* studies conducted on breast cancer cell lines (MCF-7) have verified the chemo-procursive action of ellagic acid extracted from pomegranate juice also in this tissue (Kim et al., 2002). In addition, a recent study on the multiple myeloma cells showed the anti-proliferative potential of pomegranate juice and its ability to induce G0/G1 cell cycle block and its anti-angiogenic effects. Sequential combination of bortezomib/PGJ improved the cytotoxic effect of the proteosome inhibitor and an inhibitory effect on the tube formation, microvessel outgrowth aorting ring and decreased cell migration and invasion as showed by wound-healing and transwell assays, respectively. Analysis of angiogenic genes expression in endothelial cells confirmed the anti-angiogenic properties of pomegranate. Therefore, pomegranate juice administration could represent a good tool to identify novel therapeutic strategies for multiple myeloma treatment (Tibullo et al., 2016).

1.4.1.3 Antihypertensive and anti-coronary heart disease

In *vivo* and in *vitro* studies on human have examined the effects of a series of pomegranate constituents on the prevention of arteriosclerosis, on the reduction of LDL oxidation (Fuhrman et al., 2010). Evidence suggests that polyphenolic antioxidants in juice can reduce oxidative stress and atherogenesis and can help to prevent various cardiovascular risk factors including hypertension, cholesterolemia, hyperglycemia and inflammation. The studies that conducted on the consumption of pomegranate juice and the possible effects on blood pressure have shown that the intake of juice in hypertensive subjects is able to significantly reduce the levels of systemic blood pressure (systolic and diastolic), a decrease in systolic blood pressure of about 3 mmHg may lead to a reduction in the risk of myocardial infarction (5.5%) and stroke (7%) (Sleight et al., 2001). The possible mechanisms that favour the reduction of blood pressure associated with pomegranate juice consumption can be exerted by the flavonoids contained in the pomegranate. The down-regulation of endothelial NOS induced by low-density oxidized lipoproteins in human coronary endothelial cells has been shown to be neutralized by pomegranate juice (De Nigris et al., 2006). From this point of view, there is growing evidence that isolated phenols and flavonoid-rich foods can have beneficial effects on oxidative stress, endothelial function and the renin-angiotensin-aldosterone system (Grassi et al., 2009, 2010). Considering these results pomegranate juice can be considered as an important component of the diet for patients with high risk of hypertension and cardiovascular disease.

1.4.2 Pomegranate seed oil

Pomegranate Seed Oil (PSO), obtained by cold pressing of the seeds separated from the pulp is drawing attention as precious source of healthy substances, because of its richness in fatty acids. PSO represents 12-20% of the total weight of the pomegranate seed and the main constituents are fatty acids. The total lipid content varies between 7.9 and 16%, PSO is composed of large amounts of conjugated linoleic acids, such as punicic acid, polyunsaturated fatty acid, in a range of 74-85% of the total fatty acid content (Verardo et al., 2014) and promotes its biological potential and healthy effects. Other content in fatty acids is represented by oleic, linoleic and palmitic acids. Furthermore, tocopherol was found in the seeds, in particular ɣ-tocopherol (87-95%). Thanks to the particular composition in fatty acids, phenolic compounds and phytosterols and for the known antioxidant, anti-inflammatory properties, there has been a growing production of cosmetic products based on pomegranate seed oil, because its bioactive components such as punicic acid act in skin care protecting it from UV rays, able to slow down the degenerative processes of aging skin (Jadoon et al., 2015), protecting the skin from the harmful effects of ROS, stimulating the production of collagen and elastin, increasing the skin tone.

1.4.3 Pomegranate peel

The peel is the third co-product obtained from the pomegranate, making up approximately 60% of the total weight of the fruit (Lansky and Newman 2007). Comparing it with the bioactive contents of the different parts of the fruit, it presents the highest quantity in phenolic compounds and the greatest antioxidant activity. In particular, high molecular weight phenolic compounds, proanthocyanidins, ellagitannins and polycosaccharides complexes are present; also low molecular weight phenolic compounds such as flavonoids among which anthocyanins that give the tipical colour of peel, and appreciable quantities of minerals are detected (Dikmen et al., 2011). Punicalagins and punicalin present in the pomegranate peel are ellagitannins. Punicalagin has a high antioxidant and slow-release activity in the blood where it is hydrolyzed and then conjugated in the liver and finally eliminated with urine (Lansky and Newman 2007). Instead, punicalin can be hydrolyzed to ellagic acid by the intestinal flora. The presence of antioxidant activity in the skin and its bioactive compounds play a fundamental role for human health and they have also many

possible applications in the industrial field (*e.g.* as food preservatives, stabilizers, supplements).

1.4.3.1 Antibacterial and anti-inflammatory action

Many studies confirmed that peel extracts compared with other parts of the fruit exhibit a greater antibacterial activity since they contain hydrolysable tannins useful for natural treatments of bacterial and viral infections. Recent studies have highlighted the inhibitory effect of bacterial growth in *Straphylococcus aureus* and *Escherichia coli* (Pagliarulo et al., 2016). In 2012 Fawole and his collaborators testing the antibacterial activity of peel extract with methanol have drawn the conclusion that it could hinder the growth of Gram-positive and Gram-negative bacteria.

As regards to anti-inflammatory action of pomegranate, the punicalagin and punicalin present in the skin significantly reduce the production of nitric oxide and prostaglandins by inhibiting the expression of inflammatory proteins (Lee et al., 2008).

1.4.4. Leaves and flowers

Leaves and flowers have also important health property for their content of polyphenols as tannins and ellagic acid in both tissues and punicalagin and punicalin in flowers (Lan et al., 2009; Kaur et al., 2006; Aviram et al., 2008). Leaves are also rich in fatty acids (Ercisli et al., 2007). A report suggests that extracts from pomegranate leaves can inhibit the development of obesity and hyperlipidaemia in high-fat diet induced obese mice, this extract may be a novel appetite suppressant (Lei et al., 2007).

One study reported that pomegranate flowers and fruit extracts exhibited high activity on lowering circulation lipid and modifying heart disease risk factors in diabetic animals and humans with hyperlipidaemia (Kaplan et al., 2001; Huang et al., 2005). Furthermore, studies in vivo and in vitro reported the antiatherogenic properties and mechanisms of action of all pomegranate fruit parts: peels, arils, seeds, and flowers, in comparison to whole fruit juice. This study showed an attenuation of atherosclerosis development thanks to some of the pomegranate extracts, in particular flower extracts could be related to beneficial effects on serum lipids levels and on macrophage atherogenic properties (Aviram et al., 2008).

1.5 Principal compounds in pomegranate and their biosynthesis

1.5.1 General biosynthesis of polyphenols

Polyphenol biosynthesis starts from two primary metabolites deriving from the pentose phosphate pathway and from glycolysis, respectively the erythro-3-phosphate and the phosphoenolpyruvate. By means of the shikimic acid route these compounds form the chorismic acid which is the precursor of the synthesis of the aromatic amino acids phenylalanine, tryptophan and tyrosine. Phenylalanine is the precursor of all phenolic compounds synthesized by plants (Fig. 9).

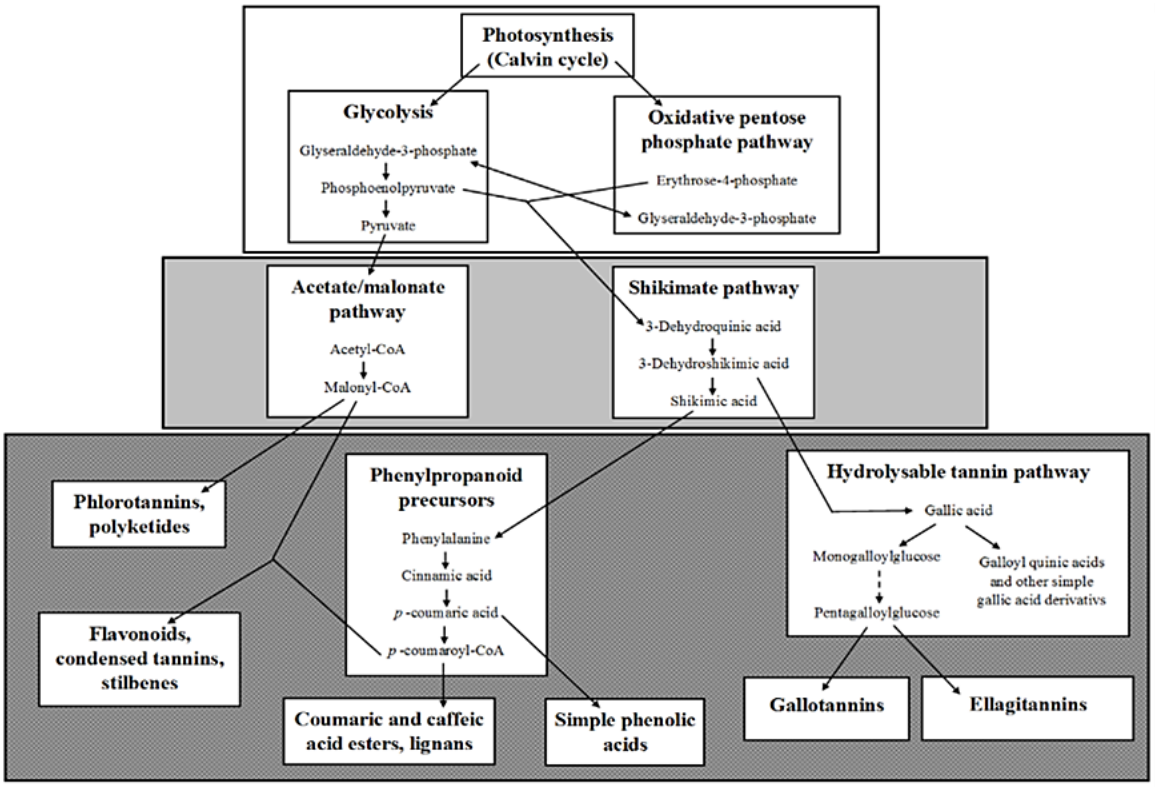


Fig. 9. General phenolic biosynthesis pathway (http://naturalchemistry.utu.fi/).

1.5.2 Hydrolysable tannins

Gallic acid is a common precursor for gallotannins and ellagitannins synthesis (hydrolysable tannins), although its metabolism in plants is still an open question (Fig. 10). Derivatives of these compounds have been identified in different pomegranate tissues, but their metabolism has not yet been fully characterized (Ono et al., 2011, Qin et al., 2017; Yuan et al., 2017). Gallotannins include galloyl glucose (glucogallin), digalloyl glucoses, trigalloyl glucose, tetragalloyl glucose, pentagalloyl glucose and other compounds.

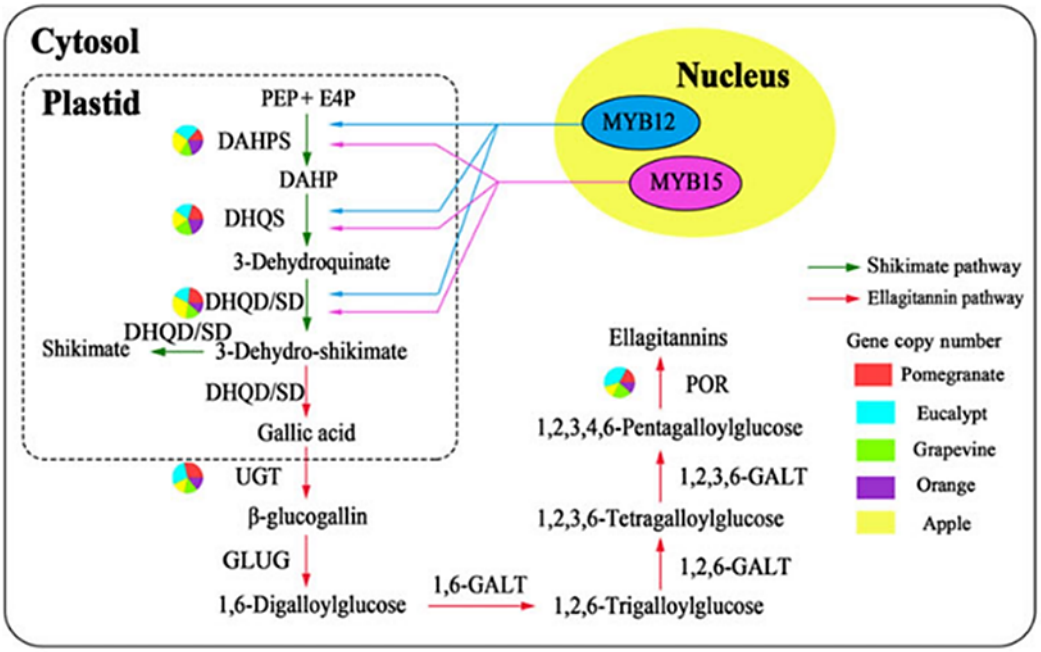


Fig. 10. Ellagitannins biosynthesis pathway. Green and red arrows represent the shikimate and ellagitannin pathways, respectively (Yuan et al., 2017).

Punicalagins are the predominant ellagitannins found in pomegranate fruit and juice (Cerda et al., 2003). Punicalagins exist in two reversible anomer α and β, they are a gallagic moiety and an ellagic moiety bound to glucose. Punicalagins are water-soluble and hydrolyse into smaller phenolic compounds, such as ellagic acid that in turn is gradually metabolized by the intestinal microbiota to produce different types of urolithins (Cerda et al., 2003) (Fig. 11). In pomegranate ellagic acid and punicalagins have important antioxidant activity and they have been implicated as potent anticancer and anti-atherosclerotic agents (Adams et al., 2010; Usta et al., 2013). Levels of punicalagins, ellagic acid and gallic acid decrease during pomegranate fruit development (Lingling Han et al., 2015).

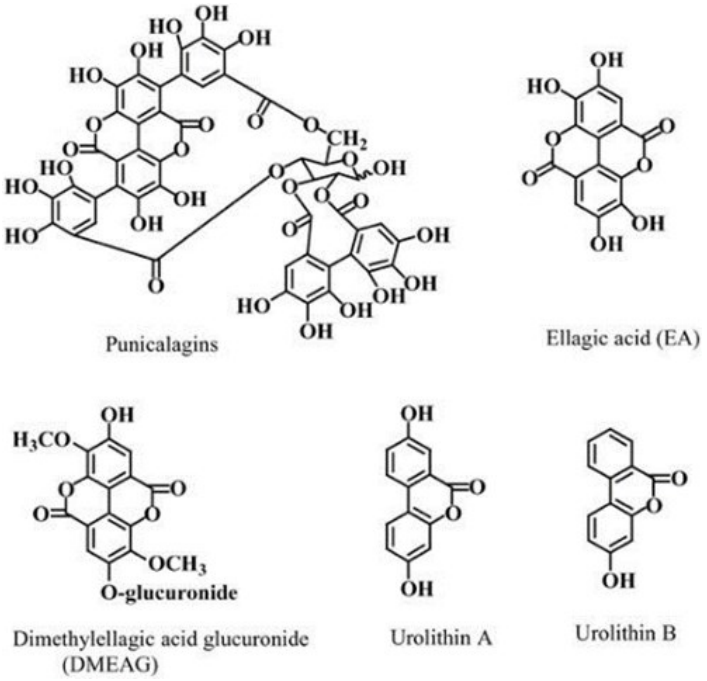


Fig. 11. Chemical structures of punicalagin isomers and its metabolites, EA, DMEAG, urolithin A and urolithin B (Seeram et al., 2006).

1.5.3 Anthocyanins

An important group belonging to family of flavonoids is represented by the anthocyanins, which is a class of water-soluble pigments responsible for the red, purple, orange and blue colour of many flowers, vegetables and fruits. Free anthocyanins are neutral or violet in colour, while acid salts of anthocyanins are red and alkali salts are blue. The intensity of the typical red colour of pomegranate fruit depends on the concentration of the anthocyanins (Gil et al., 1995). These secondary metabolites are important in plants and in human health, their beneficial effects have been documented in many in *vivo* and in *vitro* studies as reported previously in this thesis.

Anthocyanins are present in nature mainly in the sugar-free anthocyanidin aglycones and the anthocyanin glycosides. Anthocyanins are anthocyanidins with sugar group(s), are mostly 3-glucosides of the anthocyanidins. These molecules are the aglycon forms of anthocyanins based on the flavylium ion or 2-phenylchromenylium, and comprise hydroxyl and methoxyl groups in different positions (Tab. 3). More than 635 different anthocyanins have been identified. Among them, the six most mentioned anthocyanidins in plants are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (de Pascual-Teresa and Sanchez-

Ballesta, 2008) and in pomegranate, pelargonidin, cyanidin and delphinidin are present (Fig. 12).

Tab. 3. Anthocyanidins and their substitutions

Anthocyanidins	R3'	R4'	R5'	R3	R5	R6	R7
Cyanidin	-OH	-OH	-H	-OH	-OH	-H	-OH
Delphinidin	-OH	-OH	-OH	-OH	-OH	-H	-OH
Pelargonidin	-H	-OH	-H	-OH	-OH	-H	-OH
Malvidin	$-OCH_3$	-OH	$-OCH_3$	-OH	-OH	-H	-OH
Peonidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	-OH
Petunidin	-OH	-OH	$-OCH_3$	-OH	-OH	-H	-OH

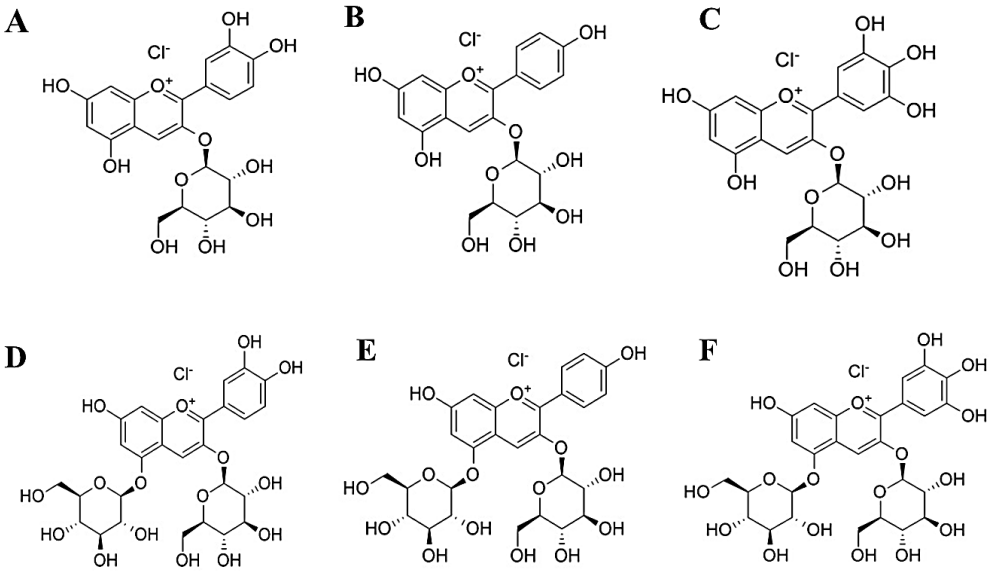


Fig. 12. Structure of anthocyanins: A) Cyanidin 3-glucoside; B) Pelargonidin 3-glucoside; C) Delphinidin 3-glucoside; D) Cyanidin 3,5-diglucoside; E) Pelargonidin 3,5-diglucoside; F) Delphinidin 3,5-diglucoside.

Previous studies reported 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin as the anthocyanins involved in the colouring of pomegranate aril colouring during maturation (Gil et al., 1995; Hernandez 1999). The fruit skin contains only the cyanidin and pelargonidin derivatives. During early maturation stages the 3,5-diglucosides are the main pigments and the delphinidin-based derivatives are the predominant compounds. In late maturation stages the proportion of monoglucosides increases up to reach

values like or higher than those of the diglucosides, and the cyanidin-based derivatives are the predominant substances (Gil et al., 1995).

In *Arabidopsis thaliana* flavonols are found in all tissues, whereas anthocyanin and proanthocyanidins specifically accumulate in vegetative tissues or the seed coat, respectively (Xu et al., 2014).

1.5.3.1 Structural genes

The flavonoid biosynthetic pathway (Fig. 13) is well known and it is conserved among different species in the plant kingdom (Winkel-Shirley 2001). Structural and regulatory genes involved in anthocyanin biosynthesis were isolated and functionally characterized from many plant species including maize, petunia, morning glory, snapdragon, Arabidopsis and more (Koes et al., 2005; Chopra et al., 2006).

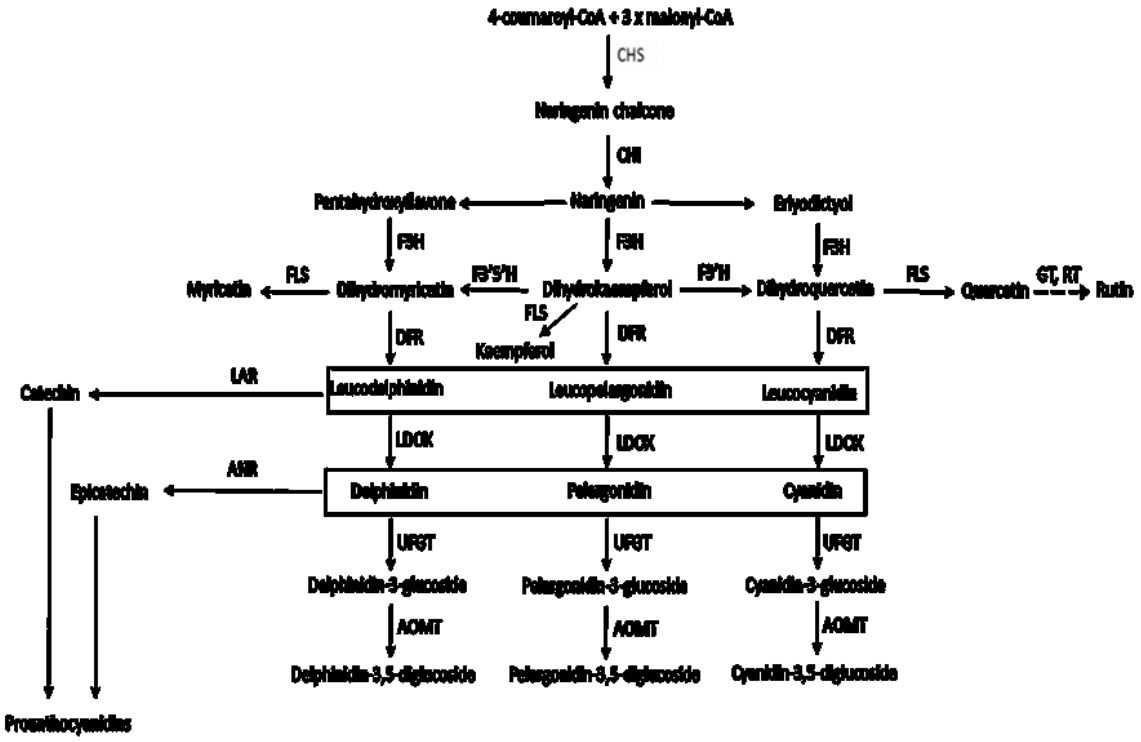


Fig. 13. Anthocyanins biosynthetic pathway.

Many genes involved in the pomegranate anthocyanins pathway have been isolated (Ben-Simhon et al., 2015; Zhao et al., 2015), but their expression has not been characterized in arils. Several plants were recognized as model genetic system to investigate gene function including *Arabidopsis thaliana* and *Nicotiana benthamiana.* Nicotiana is an interesting plant

to monitor cross-talk between different metabolite groups upon overexpression of TFs regulating secondary metabolism (Outchkourov et al., 2015) including phenolic compounds (Gaquerel et al., 2014) and anthocyanins (Deluc et al., 2006).

The main structural genes involved in anthocyanin biosynthesis include: phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), and *UDP* glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) (Schijlen et al., 2004; Saito et al., 2013).

The first committed enzyme in the pathway is Chalcone synthase (CHS) that catalyses the synthesis of an intermediate chalcone-like compound (naringin chalcone) from one molecule of 4-coumaroyl CoA and three molecules of malonyl CoA. The chalcone is rapidly and stereo-specifically isomerized to the colourless naringenin by chalcone isomerase (CHI). Naringenin is hydroxylated at the 3-position by flavanone 3-hydroxylase (F3H) to dihydrokaempferol, a hydroflavonol. F3H also catalyses the hydroxylation of eryodictyol and pentahydroxyl flavanones to dihydroquercetin and dihydromyricetin, respectively.

Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'- hydroxylase (F3'5'H), which are P450 enzymes, catalyse the hydroxylation of dihydrokaempferol (DHK) to form dihydroquercetin and dihydromyricetin, respectively. F3'H and F3'5'H determine the hydroxylation pattern of the B-ring of flavonoids and anthocyanins, and are necessary for cyanidin and delphinidin production, respectively. They are the key enzymes that determine the structures of anthocyanins and thus their colour (Tanaka, 2006). F3'H and F3'5'H catalyse the hydroxylation of flavanones, flavonols and flavones. There are many important floricultural crops, such as chrysanthemums (*Chrysanthemum morifolium*), roses (*Rosa hybrida*), and carnations, which do not produce delphinidin since they do not have a functioning F3'5'H gene and thus their flowers lack of violet/blue colours.

Dihydroflavonols are reduced to corresponding colourless leucoanthocyanidins by the enzyme dihydroflavonol 4- reductase (DFR). In some plant species, such as petunia (*Petunia hybrida*) and cymbidium (*Cymbidium hybrida*), DFR has strict substrate specificity and cannot utilize dihydrokaempferol. This is the reason why these species lack pelargonidin-based anthocyanins and thus their flowers lack of an orange/brick red colour.

Leucoanthocyanidins are precursors of the next enzyme, a dioxygenase referred to as anthocyanidin synthase (ANS, also called leucoanthocyanidin dioxygenase, LDOX) catalyses the synthesis of corresponding coloured anthocyanidins.

The resulting unstable anthocyanidins are further coupled to sugar molecules by UDP-glucoside: flavonoid glucosyltransferase (UFGT) (Kovinich et al., 2010) and anthocyanin O-methyl transferase (AOMT) that catalyse the O-glycosylation of anthocyanidins or anthocyanins to the final relatively-stable anthocyanins.

Variations in colour intensity can be attributed to differences in expression of structural or regulatory genes (Zhao et al., 2015). White grapes have arisen through mutations in two MYB transcription factors that specifically control UFGT expression (Kobayashi et al., 2004; Walker et al., 2007). In white Malay apple fruits (*Syzygium malaccense*), the absence of detectable levels of UFGT transcripts is responsible for the lack of anthocyanins (Kotepong et al., 2011). Yellow pears were observed to have substantially lower transcript levels for LDOX, UFGT, and PcMYB10 (a regulatory gene) compared to red pears (Pierantoni et al., 2010). In pomegranate the cause for the "white" phenotype is a mutation in the PgLDOX gene (Ben-Simhon et al., 2015).

1.5.3.2 Regulatory genes (MBW complex)

Anthocyanin biosynthetic genes are activated by the MBW complex consisting of R2R3 Myb regulatory protein domain, the bHLH helix-turn-helix transcription factors and the WD40 protein domain (Jaakola et al., 2013; Montefiori et al., 2015) (Fig. 14).

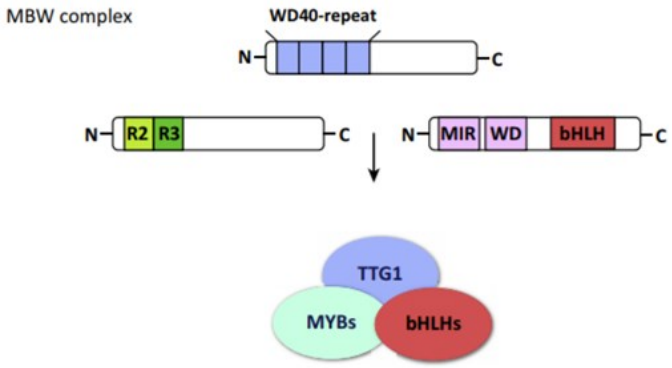


Fig. 14. MBW (MYB–bHLH–WD40) complex (Xu et al., 2014).

The combinations and interactions between these regulatory elements determine the set of genes to be expressed in positive or in negative (Hichri et al., 2011; Jaakola et al., 2013) and the production of the flavonoid pigments. In Arabidopsis, genes encoding enzymes in the early steps of the anthocyanin biosynthetic pathway that lead to the production of flavonols,

are activated by three R2R3-MYB regulatory genes (*AtMYB11*, *AtMYB12* and *AtMYB111*), whereas the activation of the late biosynthetic genes, leading to the production of anthocyanins, requires an MBW complex (Petroni et al., 2011).

In the late step of anthocyanins biosynthesis, the temporal and spatial expression of the structural genes depend on regulatory genes. In *Arabidopsis thaliana AtMYB75/ PAP1*, *AtMYB90/PAP2*, *AtMYB113* and *AtMYB114* genes control anthocyanin biosynthesis in vegetative tissues (Pelletier et al., 1999; Gonzalez et al., 2008; Xu et al., 2014).

In pomegranate three regulatory genes have been described: *PgAn2* (MYB), *PgAn1* (bHLH) and *PgWD40*. Activity of the pomegranate regulatory gene *PgWD40* was demonstrated in an Arabidopsis *ttg1-9* mutant and was shown to be dependent on MYB function (Ben-Simhon et al., 2011).

Other authors (Outchkourov et al., 2015) have used *Nicotiana benthamiana*, as plant model known for its ability to produce anthocyanins (Deluc et al., 2006), to investigate the effects of expression of transcription factors *ROS1* (MYB type) and *DEL* (bHLH type), from *Antirrhinum majus* on *Nicotiana benthamiana* metabolites, observing that the expression of both *ROS1* and *DEL* induced a single anthocyanin, delphinidin-3-rutinoside.

High correlations have been found between the expression levels of the three pomegranate regulatory genes and those of two key biosynthetic genes of the anthocyanin pathway: *PgDFR* and *PgLDOX*. The expression of *PgAn2* (MYB) and *PgWD40* genes is also highly correlated with cyanidin accumulation in the pomegranate fruit skin (Ben-Simhon et al., 2011).

Yuan *et al*. (2017) identified six R2R3-MYB genes, nine bHLH genes, and thirteen WD40 genes that were highly expressed in the peel and aril suggesting their roles in regulating anthocyanin production in pomegranate fruit. During the flowering and ripening of the fruit these genes show different patterns of expression in different species. In pomegranate the relationship between anthocyanin content and gene expression patterns in flowers, peel and arils at different developmental stages were previously analysed, even with a semi-quantitave approach and through a transcriptome analysis (Ben-Simhon et al., 2015; Qin et al., 2017). The importance of MYB transcription regulators in anthocyanin biosynthesis in this specie has been suggested. The R2R3-MYB gene family of TFs in plants is characterized by the MYB domain (Khaksar et al., 2015). This domain consists of two imperfect repeats referred to as R2 and R3, each forming a helix-turn-helix structure of about 53 amino acids (Du et al., 2009). Different studies in Arabidopsis suggest that MYB binding sites recognized by MBW complexes can be classed in two major groups: the MYB core elements (5' to 3',

CNGTTR$_3$, also called MBSI) and AC-rich elements ([A/C]CC[A/T]A[A/C]), while bHLH are known to bind to the E/G-box (CANNTG/CACGTG) (Ishida et al., 2007; Kang et al., 2009; Song et al., 2011; Xu et al., 2014). Analysis in promoter regions of structural genes *CHS*, *F3H* and *UGFT* in Arabidopsis, *CHS* in grapevine and corn showed that a CCAAT-box (CAACGG motif) is present in these promoter regions. In a study based on homology modelling (Khaksar et al., 2015) pomegranate MYB protein presents the DNA consensus motif (AACNG) and it can bind the motif in promoter regions of structural genes. Therefore, it is expected that *MYB* transcription factors from pomegranate regulate the anthocyanin pathway over fruit developmental stages and it is affected by environmental conditions (Khaksar et al., 2015).

Unfortunately their role in the control of biosynthesis of anthocyanins and on regulatory elements involved in the flavonoid metabolism is still far to be completely understood especially in pomegranate. The knowledge of the genes involved in the production of anthocyanins as for other bioactive compounds is crucial for the development of new cultivars with a higher healthy compounds content and to design new drugs that reduce or eliminate serious side effects.

2 Aim of work

In recent years the growing interest of pomegranate as nutraceutical compounds source mainly active in the prevention of cancer, cardiovascular and hypertensive diseases, has given great importance to the study of antioxidant metabolism in this species and to the discovery of new genotypes with ameliorate properties. Among antioxidant molecules present in pomegranate, the anthocyanins are very important compounds due to their health-beneficial properties and attractive colourful pigments, playing also a role in protecting plant tissues against UV irradiation.

Although the understanding on pomegranate nutraceutical compounds production has considerable increased in the last years, the characterization of genetic regulation and metabolomics of phenolic compounds has not been totally determined in this species. Therefore, other studies need to be performed on these crucial issues to fully exploit healthy pomegranate properties.

To examine the potentiality of overlooked local pomegranate varieties and investigate molecular mechanisms of phenolic metabolism we:

1) Characterized a core Sicilian pomegranate germplasm through an integrated morphological, biochemical and molecular approach and evaluated the seed germination physiology;
2) Examined the metabolomic profile in pomegranate juice at commercial fruit maturation;
3) Evaluated the pomegranate metabolomic profile in leaf, flower and fruit during development stages;
4) Investigate the role of pomegranate complex regulatory genes of anthocyanins pathway in *Nicotiana benthamiana* and evaluated the expression level of genes involved in this pathway in different tissues of pomegranate.

3 Materials and methods

During this study, a characterization of a Sicilian pomegranate germplasm core collection, was carried through the analysis of seed germination behaviour, fruit bio-agronomical traits, physico-chemical properties and genetic diversity among sampled genotypes. Since the prominent interest about pomegranate juice as source of nutraceutical compounds, a metabolomic and functional genetic investigation was undertaken to investigate, respectively, polyphenolic composition and the anthocyanins genetic pathway expression profile during fruit developmental stages. Furthermore, agro-infiltration of the *MYB-bHLH-WD40* genes in *Nicotiana benthamiana* was used to investigate the effects of overexpression of pomegranate anthocyanins regulatory complex on polyphenolic metabolism.

3.1 Plant material

For the different trials of this study the sampling was carried on: fruits and leaves from eleven pomegranate Sicilian genotypes including nine new clones from "Genotype 1" (Gen.1) to "Genotype 9" (Gen.9), two Sicilian commercial cultivars "Primosole" and "Dente di Cavallo"; and three international commercial varieties "Valenciana" (Spanish origin), "Wonderful" (American origin) and "Wonderful One" (Israeli clone of Wonderful) collected in Sicily in October 2015, 2016 and 2017 (Fig. 15). The international genotypes evaluated are native of Mediterranean environment, therefore they are adapted to the Sicilian pedoclimatic conditions. All cultivars were harvested from a germplasm collection of CNR-ISAFOM, section of U.O.S. Catania (Italy), grown under the same environmental conditions and with the same applied agronomic practices.

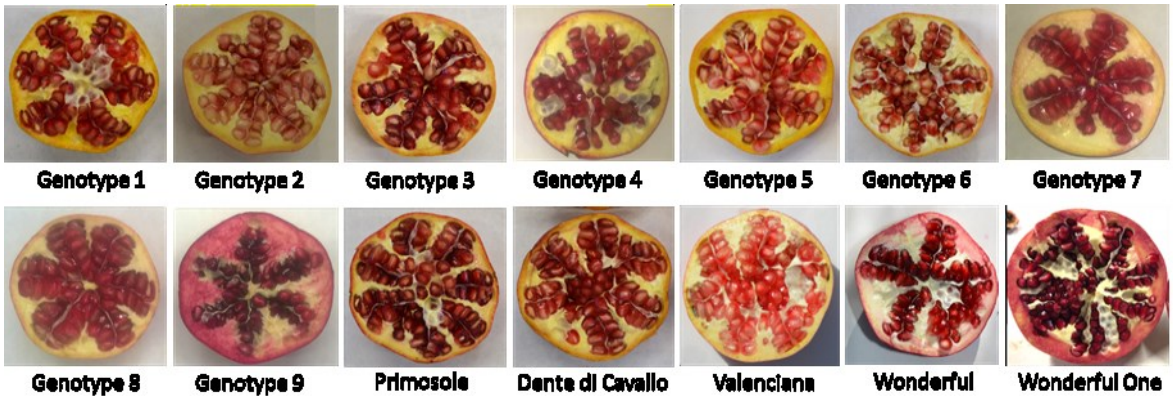


Fig. 15. Fourteen genotypes: 11 Sicilian genotypes (Gen.1 to Gen.9, Primosole and Dente di Cavallo) and 3 international genotypes (Valenciana, Wonderful and Wonderful One).

Plant of two weeks of *Nicotiana benthamiana* were used to carry out agro-infiltration experiments. Plants were grown from seeds on soil in the "Wageningen University greenhouse" with minimum of 16 hours light/day. Temperature was approximately 28 °C during the day and 25 °C in the night.

3.2 Characterization of Sicilian germplasm

In order to carry out a characterization of Sicilian germplasm, a first screening based on bio-agronimic, phisyco-chemical and biochemical (total phenolic content and antioxidant activity) characters was carried out on fourteen genotypes: eleven Sicilian genotypes and three international cultivars as reference.
Based on this initial screening and on the stability of the material during the years, nine genotypes, including seven Sicilian cultivars (Gen.1, Gen.2, Gen.3, Gen.5, Gen.6, Primosole and Dente di Cavallo) and two international cultivars (Valenciana and Wonderful) were selected to carry out the subsequent analyses (sugar content, ionic content, genetic diversity and germination ecophysiology). For each accession, five fruits at commercially ripe were randomly collected around the canopy of one tree per cultivar.

3.2.1 Chemicals

Folin-Ciocalteau, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fructose, glucose, sodium hydroxide solution 50% and standard solutions for IC 1000 mg/l of sodium, potassium, magnesium, calcium, fluoride, chloride, sulfate, phosphate and potassium nitrate (KNO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanesulphonic acid, sodium carbonate 0.5M and sodium bicarbonate 0.5M were obtained from Thermo Scientific (Waltham, Massachusetts, USA). Gallic acid was provided by Extrasynthese (Genay, France). Water Type I reagent grade was produced using a Milli-Q water purification system (Millipore, MA, USA). Gibberellic Acid (GA) was provided by Duchefa Biochemie (Netherlands).

3.2.2 Bio-agronomic and physico-chemical characteristics

Five mature fruits per genotype were analysed. Arils were manually separated from the peel and morphometric measurements were carried out on whole fruits, peel, arils and seeds. Nineteen quantitative fruit morphological traits were examined, including fruit weight (g), fruit diameter (mm), fruit length (without calix) (mm), fruits circumference (mm), number of septums, number of arils per fruit, aril weight (g), weight of 100 arils per fruit (g) and weight of one aril (g), peel weight (g), juice weight (g). Diameter and length were measured using a calibre with an accuracy of 0.01 mm and the weight was detected on a balance with an accuracy of 0.001 g. Peel yield (%) was calculated as the ratio between peel weight and fruit weight, multiplied by 100. In the same way aril yield, juice yield and seed yield were calculated and expressed in %. Furthermore, dry peel yield (%) was calculated as the ratio between dry peel weight and fresh peel weight, multiplied by 100. The same condition was used to calculate dry aril yield. Peel and arils were dried for 48 h in a stove at 105 °C. The biochemical features of juice were identified measuring total soluble solids (TSS), pH, juice colour L, juice colour a, juice colour b. The pomegranate juice (PJ) was obtained by manual squeezing of arils through sterile gauze.

Total soluble solids (TSS) was determined using a digital refractometer DBR 45 (Giorgio Bormac srl, Carpi, Italy) and results were reported as degrees Brix (°Brix) at 20 °C. The juice pH was measured using a digital pH meter XS Instruments mod. PC510 at 20 °C.

Juice colour was measured with a Chroma Meter CR-400 (Konica Minolta, Osaka, Japan), using the CIELAB colour system (Moss and Otten, 1989). Three colour measurements were made and L*, a*, b*, C* and h° units of colour space was detected. L* is the lightness (0 = black; 100 = white); positive values of a* indicate amounts of red while negative values indicate amounts of green; positive values of b* indicate amounts of yellow while negative values indicate amounts of blue. C* is the chroma, a measure of the intensity/purity of colour (0 = achromatic); h° is the hue angle on the colour wheel (0° = red; 90° = yellow; 180° = green; 270° = blue).

3.2.3 Total Phenolic Content

For the determination of the total phenolic content (TPC) the Folin-Ciocalteau reagent was used, a yellow oxidizing mixture based on phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). The phenolic compounds in the basic environment are oxidized by the molybdenum and tungsten metals present in the complexes and assume

the blue colour due to the reduction of metals, measured by a spectrophotometer at a wavelength of 760 nm.

The Total Phenol Content (TPC) was determined through the method described by Dewanto *et al.* (2002), with modifications as follows: 125 µl of PJ appropriately diluted with ultrapure water was mixed with 625 µl of Folin–Ciocalteu reagent 5-fold-diluted with ultrapure water. The mixture was allowed to stand at room temperature for 6 min and then 1.25 ml of Na_2CO_3 7% were added. The final volume was adjusted to 3 ml with ultrapure water. After incubation in dark for 60 min at room temperature, the absorbance was read at 760 nm against water as a blank. Results were expressed as gallic acid equivalents per litre of juice (mg GAE l^{-1}) through the calibration curve of gallic acid (25-200 mg/l) and the samples were analysed in triplicates.

3.2.4 Antioxidant activity

Free radicals are responsible for oxidative damage and are known to cause various degenerative disorders such as mutagenesis, carcinogenesis, cardiovascular disorders and aging, antioxidants that occur naturally in many foods act against them and therefore it is important to know their content and their effectiveness for storage or protection against oxidative damage.

The determination of the antioxidant activity was carried out through the DPPH method described by Brand-William *et al.* (1995) with some modifications.

This method involves the use of a solution of DPPH (2,2-diphenyl-1-picrylhydrazyl), a slightly stable nitrogenous organic radical in respect of solar radiation, since the unpaired electron located on the nitrogen atom is stabilized by resonance on the aromatic ring, moreover the presence of sterically cumbersome groups such as the phenols and the nitro groups, give further stability to the same radical (Fig. 16).

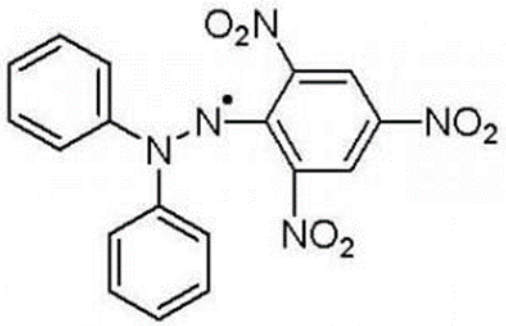


Fig. 16. 2,2-difenil-1-picrilidrazile.

The DPPH • is reacted with an antioxidant compound able to yield a hydrogen atom to the radical compound, in this way there is a discoloration of the solution due to the disappearance of the radical that will be measured through the spectrophotometer at the wavelength of the maximum of absorbance (λ_{max} = 517 nm). The measurement takes place at room temperature to eliminate the risk of thermal degradation of the molecules tested.

To measure antioxidant activity, a 0.1 mM DPPH solution in methanol was prepared, conserved in the dark until used. The juice was appropriately diluted with methanol, vortexed and centrifuged at 5000 rpm for 4 min. Afterwards, 100 µl of supernatant was mixed with 2 ml of 0.1 mM DPPH in methanol. After incubating at room temperature for 30 min in the dark, the absorbance of the mixture was measured at 517 nm. The reaction mixture without DPPH was used for the blank. Trolox was used as a reference (calibration range 10–200 mmol/l). All determinations were performed using a spectrophotometer (Eppendorf, Italy) and all samples were analysed in triplicates. The results are expressed as mmol Trolox per liter.

3.2.5 Sugars

The determination of the main sugars (fructose and glucose) in PJ was established in the current study. The pomegranate juices were diluted in the ratio 1:1000 with deionized water, filtered with a syringe filter 0.45 µm and subjected to analysis.

Carbohydrates analysis were performed using a High-Performance Anion-Exchange chromatography with Pulsed Amperometric Detection (HPAE-PAD), Dionex ICS3000 (Thermo Scientific) (Fig. 17), consisting in a quaternary gradient inert pump, a pulsed amperometric detector, a metal-free injection valve with a 20 µl sample loop, AS40 automated sampler, a Dionex CarboPac PA10 analytical column (250x4 mm i.d.) and CarboPac PA10 guard column (50x4 mm i.d.). The detection cell contained a gold working electrode (1.0 mm diameter) and an Ag/AgCl reference electrode. All experiments were carried out at 30 °C under isocratic elution using NaOH 100 mM with a flow-rate of 0.8 ml/min. Run time was 30 min, 20 min for analysis and 10 min for conditioning. All analyses were performed in triplicate for each agronomic sample, quantified by calibration curve (range 0.5-100 mg/l; $r > 0.999$) and the results are reported in mg/l. Relative standard deviations (RSD%) of peak retention times were < 0.7 %.

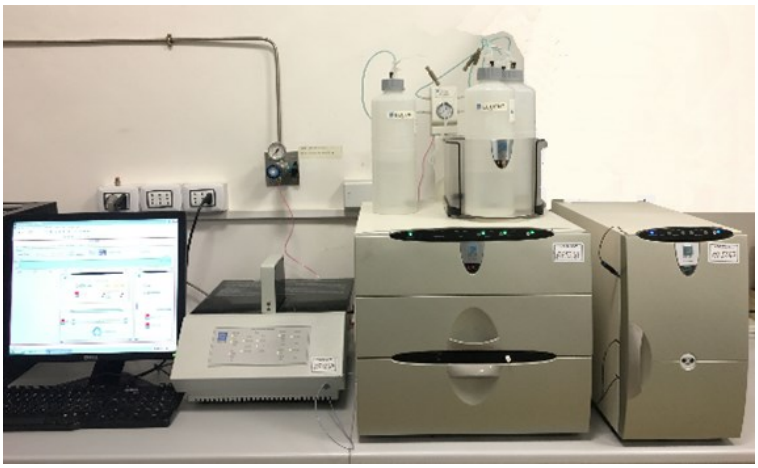


Fig. 17. Dionex ICS3000 (Thermo Scientific) Ion Chromatography System with Pulsed Amperometric Detection (line 1) and Conductometric Detection (line 2).

3.2.6 Mineral salts

For the determination of the mineral content of pomegranate juices, the ion chromatography method was used with a conductivity detector in the presence of a suppressor. The most important inorganic anionic and cationic constituents were analyzed: F^-, Cl^-, PO_4^{3-} and SO_4^{2-} for anions, Na^+, K^+, Mg^{2+} and Ca^{2+} for cations.

All chromatographic analysis were performed using a Thermo Scientific Dionex ICS3000 ion chromatograph (Sunnyvale, CA, USA) (Fig. 16), composed of an isocratic pump, a cationic or anionic suppressor, a conductance detector equipped with a temperature compensated conductivity cell, an injection valve with a 25 µl loop and a column thermostat compartment.

The ion separation was carried out with two ion-exchange columns: anions were separated on a Dionex IonPac AS22 column (250x4 mm i.d.) protected by an IonPac AG22 guard column (50x4 mm i.d.); cations were determined using an IonPac CS12A column (250x4 mm i.d.) equipped with IonPac CG12A guard column (50x4 mm i.d.).

An aqueous solution containing 20 mM methanesulphonic acid was used for elution of cations. The anionic mobile phase containing 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate was prepared from 500 mM stock standard solutions. Eluents were prepared daily. Flow rate of 1.0 ml/min and 1.2 ml/min was used for the separation of cations and anions respectively, maintaining the column temperature at 30°C during analysis. The chromatographic run had a duration of 20 min and the peaks were identified on the basis of the comparison of retention times of external standards. Quantification was performed by

creating calibration curves (range 0.5-100 mg/l). The calibration curves of the peak areas showed a good correlation with r > 0.999. Relative standard deviations (RSD%) of peak retention times ranged from 0.4% to 2.1%. Results were reported as mean ± standard deviation of three replicates and expressed as mg/l of pomegranate juice.

3.2.7 Genetic diversity

As regards to the analysis of the genetic diversity of pomegranate Sicilian germplasm, leaf samples were collected in October 2015 from 7 pomegranate Sicilian genotypes (Gen.1, Gen.2, Gen.3, Gen.5, Gen.6, Primosole and Dente di Cavallo) and from 2 international cultivars (Valenciana and Wonderful) used as out-group. For each genotype the leaves from five biological replicas were used.

3.2.7.1 DNA extraction

Genomic DNA was extracted from 100 mg of leaf pomegranate using CTAB method described by Allen *et al.* (2006). Samples quantity and quality were assessed by checking them on a 1% agarose gel and by measuring with an Eppendorf BioSpectrometer® (Eppendorf AG, Hamburg, Germany) their absorbance at 260 nm and 280 nm to assess DNA purity.

3.2.7.2 PCR and primer design

Sixty-four *loci* (Tab. 4) characterized in previous studies (Ebrahimi et al., 2010; Pirseyedi et al., 2010; Currò et al., 2010; Hasnaoui et al., 2012; Zai-Hai et al., 2012; Parvaresh et al., 2012; Rania et al., 2012) were considered for the analysis of genetic relationships among cultivars. Out of 64 *loci*, 7 markers were selected based on heterozygosity, number of alleles, allele size and amplification reproducibility, preferring tetra- and tri-nucleotides compared to di-nucleotides. Furthermore 4 new gene-derived primer pairs were designed with Websat web software (Martins et al., 2009) using putative *P. granatum* R2R3 MYB gene sequence (HM056531.1) involved in the biosynthesis of anthocyanins (Tab. 5).

DNA was diluted to 30 ng/µl for PCR amplification. PCR assays were performed in a reaction mixture of 25 µl including: 90 ng of genomic DNA, 0.25 µM of each primer, 200 µM dNTPs, 1 U of Q5 High-Fidelity DNA polymerase (Biolabs, New England) and 4 µl of 5x Q5 Reaction Buffer. DNA amplifications were performed in a thermocycler with the

following cycling program: an initial denaturation step of 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, at optimal annealing temperature for 30 s (Tab. 5), 72 °C for 30 s and a final extension at 72 °C for 2 min.

Tab. 4. Primer sets of microsatellites from literature. In bold are presented the selected primer pairs used in this study.

Locus	Primer sequence Forward (5'-3')	Primer sequence Reverse (5'-3')	Repeat type	No. Of alleles	Allelic size	He	Ho	PIC	Reference
pg4	CTGATGTAATGGCTGAGCAAA	GCACTTGAACAAAGAGAATGC	$(TC)_{12}$ TT$(TC)_{20}$	5	220-245	0.74	1	0.696	Ebrahimi et al. 2010
pg10(a)	TGCTAGACAGAACTG GGAGAAC	AGAGAGTGGGGTTTCCATTG	$(AG)_9$ GG$(AG)_{14}$	4	224-245	0.627	1	0.554	Ebrahimi et al. 2010
pg14	GCACATTTCTTCCACCTTCC	GGTTACAATGCACAGAGTCCAC	$(AG)_{32}$	4	227-245	0.653	1	0.600	Ebrahimi et al. 2010
pg21	CAAGACAGAAGCACCATCCA	TCTCCCAAATCAGACCAACC	$(AG)_7$	4	210-225	0.66	1	0.595	Ebrahimi et al. 2010
pg22	CCCCGCACTTAGAATCTATTA	TCCAGTTCCAATCGACAGAC	$(ACAT)_3$ $(AT)_3$ $(AG)_{22}$ $(AT)_3$	3	230-240	0.52	1	0.410	Ebrahimi et al. 2010
Pg17	CATCAGACTACGATGGCACT	GCATAATAGCCTTCAATTTACA	$(TCA)_{14}$	4		0.63	1	0.560	Parvaresh et al. 2010
Pom047	GCCTATCTCGTGATCACATC	AATGGGAGCGGACTAACTAT	$(CT)_{24}$	3	205, 210, 213	0.51	0.97		Rania et al. 2012
Pom021	GACTGGAAGAAGCAGAGACT	GAAAAGGAAGTAGCAGAGCA	$(AC)_{28}$	6	190, 195, 198, 200, 205, 2010	0.61	0.89		Rania et al. 2012
ABRII-MP42	GAGCAGAGCAATTCAATCTC	AACAA TTTCCCA TGTTTGAC	$(GA)_9$	4	194/200-220	0.61	0.87	0.540	Pirseyedi et al. 2010
ABRII-MP30	CCCAGTTTGTAGCAAGGTA	AAGCTGACA TTCTTTGAAGC	$(TGAGC)_3$	3	175/160-190	0.60	0.85	0.520	Pirseyedi et al. 2010
Pom010	CCTCATTGCTGATGAATCTT	ACTCGAGAAGCTCTGTGAAG	$(AG)_{19}$	3	234, 236, 248	0.61	0.85		Rania et al. 2012
ABRII-MP04	CAGGTGATTGACTACTTGG	CAGATCTACAATAACATCAC	$(GT)_7$	2	201/195-215	0.48	0.82	0.360	Pirseyedi et al. 2010
ABRII-MP34	GGAAGAAGCAGAGCAATAGA	GTCCTGAGTAACCTGAGCTG	$(GAA)_3$	3	210/180-220	0.48	0.75	0.360	Pirseyedi et al. 2010
POM_AGC5	TTCGATATTGTTTATTGTGTCG	CAACGAACTAGACGACACAC	$(TA)_6$ $(TG)_6$	3	121–125		0.71	0.710	Currò et al. 2010
ABRII-MP33	TCTGTTTATTGCTGAAAGGG	TCTTCTTCTTCTCCACCGTA	$(AG)_{12}$	2	105/80-120	0.43	0.65	0.340	Pirseyedi et al. 2010
POM_AGC11	CGTCATCCCTTATGTTCTTC	CTGGGGAAGTCGACGAAG	$(CT)_{10}$ TTCT$(CA)_6$	3	196–200		0.53	0.530	Currò et al. 2010
ABRII-MP12	TTGAGTCCCGATCATATCTC	TCAA TCTGTCAGGAACAACA	$(CA)_{11}$	3	270/240-270	0,42	0.6	0.340	Pirseyedi et al. 2010
Pom024	GGAGATTTGAATTGGGAAGT	GTGGACTAACTCAAGCAAGG	$(AG)_{27}$	3	230–242	0.50	0.59		Hasnaoui et al. 2010
EPS16	AGGCTTCATGACCCATCATC	AGGAAGAGTTCGGGTTCGAT	$(TGG)_8$	2	191		0.55	0.459	Zai-Hai et al. 2012
EPS13	GCTAGCGAATGAAATGTCTT	GAGTATAGCAGTAGGGGAGATG	$(TA)_9$	2	178		0.55	0.301	Zai-Hai et al. 2012
EPS08	TTCCCGAGAAAGTTGCATATCT	TAGTCCGTGAGGATTTTGTCCT	$(AG)_{18}$	5	204		0.62	0.656	Zai-Hai et al. 2012
Pom024	GGAGATTTGAATTGGGAAGT	GTGGACTAACTCAAGCAAGG	$(AG)_{27}$	3	225, 230, 240	0.46	0.53		Rania et al. 2012

Tab .4. (*continued*).

Locus	Primer sequence Forward (5'-3')	Primer sequence Reverse (5'-3')	Repeat type	No. Of alleles	Allelic size	He	Ho	PIC	Reference
ABRII-MP46	AGTTGATCTGATGGACAAGG	CAGTACGGTGCTCAATACAA	$(GTT)_4$	2	270/250-300	0.38	0.51	0.310	Pirseyedi et al. 2010
EPS19	TGGGGATTATCGTTGTCTTCA	TCCAAGCTGAACTCGTTCCT	$(CT)_9$	3	236		0.5	0.594	Zai-Hai et al. 2012
Pg18	TCTAAGGGCAGAATGGCACT	TGGCACTAGATCCGTAAATCTC	$(TCA)_{14}$	6		0.63	0.49	0.559	Parvaresh et al. 2010
Pom021	GACTGGAAGAAGCAGAGACT	GAAAAGGAAGTAGCAGAGCA	$(AC)_{28}$	3	201–205	0.54	0.48		Hasnaoui et al. 2010
Pg23	ACCACTCCCACCATTATTGC	GGAGGGAAGAGACGAGCATT	$(TC)_{16}$	2		0.5	0.48	0.374	Parvaresh et al. 2010
EPS09	TAATCCCATTCCAAACAAGTCC	ATATTGACGGAGGCTTCACTGT	$(AG)_{15}$	4	199		0.48	0.509	Zai-Hai et al. 2012
EPS17	TGTGGGTGTGGGAACATAATAA	TCAACAGGACAAAGGATGAAGA	$(TCC)_5$	3	275		0.48	0.559	Zai-Hai et al. 2012
Pg8	CACCATAGACTTAAACGAGCACAA	GAAGCTCCATTGCCTCGTCC	$(AG)_{20}$	3		0.52	0.40	0.452	Parvaresh et al. 2010
ABRII-MP07	GATTAACAGCAAAGCCTAGAGG	AGTAGCTGCAACAAGATAAGG	$(AT)_9 (GT)_7$	2	181/180-190	0.32	0.39	0.390	Pirseyedi et al. 2010
EPS01	TCTATTCCACATAGAAAGAGGGG	ATGATGTCTATGCAATTGGCTG	$(GGA)_7$	3	121		0.38	0.461	Zai-Hai et al. 2012
ABRII-MP26	TTTCTCGAAGAATTGGGTAA	CTGAGTAAGCTGAGGCTGAT	$(AG)_{25}$	5	166/145-160	0.62	0.37	0.600	Pirseyedi et al. 2010
EPS06	AAATCGCATCCCTCCGTCT	CTGTTCGCCAGGGTAAAGA	$(TC)_{13}$	4	147		0.36	0.432	Zai-Hai et al. 2012
EPS10	TAGCACAGGGGAAATCTGAAAT	GGAAGAGTTTGGTTCAGGATTG	$(GAT)_6$	3	130		0.36	0.461	Zai-Hai et al. 2012
EPS12	TCTCTCTCCCTCATTTCCTCTG	GTCTCTACCATCTCAGCAGCCT	$(CT)_9$	3	199		0.36	0.431	Zai-Hai et al. 2012
POM_AAC14	CGAGAACCGTTAGTCATGC	AGTGACGGCAGGACAAGAAC	$(CA)_7$	2	156–158		0.32	0.320	Currò et al. 2010
EPS24	CAAACGCCTTCATGAAACTACA	GACAAAAATTTCCAGCTCCATC	$(GT)_7$	2	104		0.31	0.387	Zai-Hai et al. 2012
ABRII-MP39	AGTCTCTGAAGTTTGTCGGA	CCTGAGTAAAGCATCTCACTG	$(GA)_8 (TTTTCT)_2$	2	252/250-305	0.29	0.30	0.260	Pirseyedi et al. 2010
pg18(b)a	TCTAAGGGCAGAATGGCA CT	TGGCACTAGATCCGTAAATCTC	$(TCA)_{14}$	3	335-347	0.32	0.30	0.269	Ebrahimi et al. 2010
EPS04	AAAGGGGAAAAAGACGAAGAAG	CCCTGTCCTTAAGTCTGAGTGG	$(CT)_{10}$	2	257		0.29	0.308	Zai-Hai et al. 2012
Pom055	GAGACAATTGGGATCAGAAA	AGTCGACGAACTGTGAAATC	$(GCC)_5$ GCACTCA$(TC)_8$	2	245, 250	0.33	0.28		Rania et al. 2012
Pom006	TACTAGGTGGAACCGAACTT	CCTTGACAACCTCATCTCAT	$(GA)_4$ ACT $(GA)_{26}$	5	153, 155, 159, 165, 171	0.49	0.26		Rania et al. 2012
Pom010	CCTCATTGCTGATGAATCTT	ACTCGAGAAGCTCTGTGAAG	$(AG)_{19}$	2	231–233	0.28	0.26		Hasnaoui et al. 2010

Tab. 4. (*continued).*

Locus	Primer sequence Forward (5'-3')	Primer sequence Reverse (5'-3')	Repeat type	No. Of alleles	Allelic size	He	Ho	PIC	Reference
Pom013	CACACCCTTCATCAAAAGAT	GGACTAACAACCAGCCATAG	$(CT)_{19}$	4	156, 158, 162, 164	0.56	0.25		Rania et al. 2012
Pom045	ATGAATGAGGAAGACGAAAA	GTGCTCCATCCATACAAAAT	$(CA)_{10}$	2	150. 158	0.35	0.25		Rania et al. 2012
pg18(a)	TCTAAGGGCAGAATGGCACT	TGGCACTAGATCCGTAAATCT C	$(TCA)_{14}$	3	186-200	0.37	0.25	0.326	Ebrahimi et al. 2010
EPS05	TTGTTGGGTATTCCTCTTCTC	ACATCATACACCTTGCCCTC	$(CTT)_{11}$	2	197		0.24	0.210	Zai-Hai et al. 2012
Pom006	TACTAGGTGGAACCGAACTT	CCTTGACAACCTCATCTCAT	$(GA)_4$ ACT (GA)26	4	145–171	0.21	0.22		Hasnaoui et al. 2010
POM_AAC1	GGGTCTTCCTAATTCTCTGG	TACAACTTCGGACTCACTTGC	$(CT)_9$ $(TA)_8$	3	174–178		0.21	0.290	Currò et al. 2010
Pom046	CTTCCTCCTACCGAACTATG	CCCACTTTGACACTTCTACC	$(GA)_{21}$	2	215, 240	0.22	0.21		Rania et al. 2012
pg23	ACCACTCCCACCATTATTGC	GGAGGGAAGAGACGAGCATT	$(TC)_{16}$	3	227-235	0.34	0.20	0.314	Ebrahimi et al. 2010
ABRII-MP28	A TCCTCTGTCTTTGTGTTCG	TGAGTAATTCCGGTCAGAAG	$(GAGG)_3$ $(GA)_{19}$	3	349/350-390	0.65	0.15	0.580	Pirseyedi et al. 2010
EPS03	CGCTGGTCACACTACTTACTCG	TTGTAGTGGAAGACACAGCAGC	$(AT)_{12}$	2	263		0.14	0.172	Zai-Hai et al. 2012
EPS11	AACTTCTGGTGTCTCTTCCACC	GTGTGGAGTTGAAGATCGATGA	$(AT)_9$	2	147		0.12	0.091	Zai-Hai et al. 2012
Pom013	CACACCCTTCATCAAAAGAT	GGACTAACAACCAGCCATAG	$(CT)_{19}$	3	165–169	0.11	0.11		Hasnaoui et al. 2010
Pom014	CGCATTTGGTTGTAGAAGAC	AGGAGCGTCTGTTTTAATCTT	$(GA)_{27}$	3	197–207	0.11	0.11		Hasnaoui et al. 2010
Pom014	CGCATTTGGTTGTAGAAGAC	AGGAGCGTCTGTTTTAATCTT	$(GA)_{27}$	4	192, 198, 200, 205	0.33	0.11		Rania et al. 2012
POM_AAC13	TCTCCCGACAACAAATCAC	CCCGACACAACACATACTTCAG	$(AC)_9$ $(AT)_6$ $(AC)_6$..$(TA)_{11}$	2	275-277		0.09	0.090	Currò et al. 2010
POM_AAC3	TGATGAAACCATGTAACTCG	CTCCGATAACGTCTCCAAGC	$(TC)_6$	2	204–206		0.06	0.060	Currò et al. 2010
Pom004	TCCTTTTTACCCAATTTTCA	TGCACATCTTTTGCTGTAAG	$(CT)_{10}$	3	112, 116, 120	0.2	0.06		Rania et al. 2012
Pom039	TAGTTGAATAGGCCACATCC	CTATACAGTCCGAGGACCAC	$(CA)_{12}$	2	140,146	0.04	0.05		Rania et al. 2012
Pom046	CTTCCTCCTACCGAACTATG	CCCACTTTGACACTTCTACC	$(GA)_{21}$	2	241–245	0.037	0.04		Hasnaoui et al. 2010
Pom055	GAGACAATTGGGATCAGAAA	AGTCGACGAACTGTGAAATC	$(GCC)_5$ GCACTCA$(TC)_8$	2	245–257	0.037	0.04		Hasnaoui et al. 2010

Tab.5. Primer sets used to investigate genetic diversity among pomegranate cultivars and the annealing temperature (Ta) chosen for each primer.

Locus	Repeat motif	Primer sequence (5'-3')	Ta °C	Reference
pg4	$(TC)_{12}$ TT$(TC)_{20}$	F: CTGATGTAATGGCTGAGCAAA R: GCACTTGAACAAAGAGAATGC	63	Ebrahimi et al. 2010
pg10(a)	$(AG)_9$ GG$(AG)_{14}$	F: TGCTAGACAGAACTGGGAGAAC R: AGAGAGTGGGGTTTCCATTG	63	Ebrahimi et al. 2010
pg14	$(AG)_{32}$	F: GCACATTTCTTCCACCTTCC R: GGTTACAATGCACAGAGTCCAC	62	Ebrahimi et al. 2010
pg21	$(AG)_7$	F: CAAGACAGAAGCACCATCCA R: TCTCCCAAATCAGACCAACC	62	Ebrahimi et al. 2010
pg22	$(ACAT)_3$ $(AT)_3$ $(AG)_{22}$ $(AT)_3$	F: CCCCGCACTTAGAATCTATTA R: TCCAGTTCCAATCGACAGAC	56	Ebrahimi et al. 2010
Pg17	$(TCA)_{14}$	F: CATCAGACTACGATGGCACT R: GCATAATAGCCTTCAATTTACA	57	Parvaresh et al. 2010
Pom047	$(CT)_{24}$	F: GCCTATCTCGTGATCACATC R: AATGGGAGCGGACTAACTAT	57	Rania et al. 2012
MYBmp01	$(CT)_9$	F: GATGAAGATGACAAAACACCCC R:TGGGAGCTAGACAGAGTGACAA	60	Present study
MYBmp02	$(GA)_{12}$	F: TCCTCAAGCAGACCCAGAAA R: TGCTGTTCTTGTTACGCCTT	62	Present study
MYBmp03	$(AGC)_4$	F: AGGCGTAACAAGAACAGCAA R: AGCAACAGTCTTCCACCTCC	62	Present study
MYBmp04	$(GAG)_4$	F: CTCGCTTGTCTTGCTAAAGGAT R: CGAGGAACTTATTGACCCACTC	57	Present study

3.2.7.3 Capillary Electrophoresis

The amplification products were analysed by Capillary Electrophoresis using QIAxcel High Resolution Gel Cartridge (Qiagen, Hilden, Germany). This is a nucleic acid separation apparatus based on their length by capillary electrophoresis. The separation takes place at the level of a capillary of prefabricated gel cartridge. Each sample is automatically loaded into a single capillary where it crosses an electric field; the nucleic acids negatively charged with the positive pole, like a normal agarose gel electrophoresis, the low-weight molecules migrate faster than those with high molecular weight. When the molecules pass through the capillary, they pass through a detector that reveals a fluorescent signal. A photomultiplier converts the signal emitted into an electrical datum that is transferred to a computer that passes through the software QIAxcel Screengel produces the electropherogram or gel image (Fig. 18).

Fig. 18. Capillary Electrophoresis: QIAxcel system.

For the genetic diversity analysis GeneALEx version 6.5 (Peakall & Smouse, 2012) was used to obtain a pairwise population matrix calculated through Nei's genetic distance, while expected heterozygosity (H_e), observed heterozygosity (H_o), number effective alleles (N_e), Shannon index (I), the number of alleles and UPGMA dendogram were calculated with Popgene program, version 1.32 (Yeh et al., 1997). The number of alleles, major allele frequency, number of genotypes and polymorphism information content (PIC) value for each primer was obtained using Powermarker software, version 3.25 (Liu & Muse, 2005).

3.2.8 Seed germination

For germination assays, seeds obtained from five Sicilian varieties (Gen.1, Gen.2, Gen.6, Primosole and Dente di Cavallo) and two international commercial varieties (Valenciana and Wonderful) were considered. Seeds were separated from the flesh, dried in a humidifier at 35°C and stored in a climatic chamber at 15 °C with 16% of humidity until analysis.

Four hundred and fifty seeds per genotype were surface-sterilized with 1% (w/v) sodium hypochlorite for 15 minutes, followed by three thorough rinses with sterile water and used for germination tests sowing them on 3 Petri dishes, fifty per plate, with two layers of filter paper. They were subjected to different treatments: 150 seeds were sowed with sterile water and used as control (CTRL); 150 seeds with 20 mM of Potassium Nitrate (KNO_3) and other 150 seeds with 0.1 mM of Gibberellic Acid (GA). The seeds were incubated in growth chamber under a 12/12 h light/dark cycle photoperiod at 20 °C and germination was periodically monitored. The seeds were considered germinated when there was a 1 mm radicle protrusion through the seed coat (Fig. 19).

Fig. 19. Seed germination of pomegranate.

The germination rate (GR) was calculated as the reciprocal value of the time until 50% germination (t50) using the Boltzmann equation.

3.3 Metabolomic profile in pomegranate juice

In order to evaluate the metabolic differences between Sicilian and international genotypes, five genotypes were considered for these analyses. Two Sicilian genotypes (Gen.1 and Gen.2), considered commercial interesting, and three international cultivars (Valenciana, Wonderful and Wonderful One). For each accession, six fruits of uniform size were randomly collected around the canopy of one tree per cultivar.

3.3.1 Chemicals

All solvents and reagents were of analytical or HPLC grade.
Standards used for the analyses were: delphinidin-3-O-glucoside, pelargonidin-3-O-glucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, punicalagin purchased from Sigma-Aldrich (St. Louis, MO, USA); cyanidin-3-O-glucoside, delphinidin-3,5-diglucoside, ellagic acid, rutin and quercetin dehydrate provided by Extrasynthese (Genay, France). Acetonitrile, HPLC supra-gradient grade and methanol absolute, HPLC supra-gradient grade were purchased from Biosolve Chimie (Dieuze, France), while formic acid (FA) for analysis, 98–100% from Merck-KGaA (Darmstadt, Germany). Ultrapure water was produced using a water purification system (Elga Maxima, Bucks, UK).

3.3.2 Metabolites extraction and LC-PDA-MS analysis

For metabolite analysis 3 replicas of 300 µl of pomegranate juice were used. The samples were extracted for every single replica with 900 µl of 95% of MeOH and 5% of FA. Extracts were sonicated for 15 min, centrifuged at max speed for 15 min. The supernatant was put into HPLC vials to analyse.
Samples were analysed on Acquity Ultra Performance Liquid Chromatography system with a photodiode array (UPLC-PDA; Waters) coupled to an LTQ Ion Trap-Orbitrap Fourier Transformed Mass Spectrometer (FTMS; Thermo) (Fig. 20). Samples were separated on a Luna C18-reversed phase column (150×2 mm, 3µm; Phenomenex). As solvents were used ultrapure water with 0.1% formic acid (eluent A) and acetonitrile acidified with 0.1% formic acid (eluent B), using a linear gradient from 5 to 35% eluent B at a flow rate of 0.19 ml/min for 45 min, following by washing and stabilization. A mass resolution of 60,000 was

employed for data acquisition. Samples were analyzed using a mass range of m/z 150-2000, in negative ionization mode. Identification of detected compounds was based on retention time, accurate masses of both the parent and fragment ions, in combination with any PDA absorbance spectra (recorded at 240–600 nm). Data analysis was carried out in an untargeted manner as described by De Vos *et al.* (2007). Visualization of the HPLC-PDA-FTMS data was performed using Xcalibur 2.1 software (Thermo). The MetAlign software package was used for baseline correction, noise estimation and mass peak alignment (Lommen, 2009). Threshold for peak detection was set at 10,000 ions per scan. MSClust software (Tikunov et al., 2012) was used to clustering mass peaks originating from the same metabolite, including adducts, fragments and isotopes so to reconstruct metabolites.

Quantification of selected compounds (delphinidin-3-O-glucoside, pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside delphinidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, cyanidin-3,5-diglucoside, punicalagin, ellagic acid, gallic acid, rutin and quercetin dehydrate) was performed using standards dilution in acidified aqueous methanol at a final concentration of 75% methanol (v/v) and 0.1% formic acid (v/v). The calibration curve of standards mix was created in seven points from 22.7 to 83.3 ppm.

Fig. 20. Acquity Ultra Performance Liquid Chromatography system with a photodiode array (UPLC-PDA; Waters) coupled to LTQ Ion Trap-Orbitrap FTMS.

3.4 Metabolomic profile during different pomegranate fruit development stages

Based on the data obtained in the pomegranate juice we decided to investigate the metabolic profile during fruit development stages of only two cultivars: Valenciana and Wonderful. Leaves, flowers and fruit during maturation were analysed.

3.4.1 Samples

In 2017, from June to October, leaves, flowers and fruits at different development stages were harvested from three tree per plant of Valenciana and Wonderful. The two cultivars were grown under the same environmental conditions and with the same applied agronomic practices in a randomized block field.

Four growth stages were identified: flower (stage 1-ST1), unripe fruit (stage 2 - ST2), turning fruit (stage 3 - ST3), ripe fruit (stage 4 - ST4). During the last sampling (October) mature leaves were also collected (Fig. 21). Three flowers/fruits from three replicate trees of each pomegranate accession were collected at each stage. During each sampling the peel was removed from the fruit and arils, flowers and leaves were immediately immersed in liquid nitrogen and transported to the laboratory (Fig. 16 - A). Before the analysis leaves and flowers were ground in a fine power with liquid nitrogen. Also arils were ground with liquid nitrogen but following two different ways depending on the stage of arils: 1) an amount of whole frozen arils of ST2, ST3 and ST4 were ground entirely (flesh plus seed, F+S); 2) the remaining part of arils of ST3 and ST4 were firstly separated in flesh and seeds by frozen pestle and mortar and afterwards only flesh (F) was ground. At ST2 it was not possible to separate flesh from seeds because it was not quite development. The shredded samples were stored at -80 °C for the future analysis.

Fig. 21. Different development stages of Valenciana and Wonderful pomegranates: leaf, flower (ST1), unripe fruit (ST2), turning fruit (ST3) and ripe fruit (ST4). Tree biological replicas were used.

3.4.2 LC-PDA-Orbitrap FTMS

For the analysis 300 mg of leaf, ST1, ST2, ST3 F+S and ST4 F+S of Valenciana and Wonderful were used. Three biological replicas of each tissue were extracted with 900 µl of 95% of MeOH and 5% of FA. The procedure was the same as that described in Paragraph 3.3.1. Data analysis from HPLC-PDA-Orbitrap FTMS was performed in an untargeted manner, essentially as described by De Vos *et al*. (2007). Chromalynx software (Waters) was used to compare the chromatographic profile in the different samples. Visualization of the data was performed using Xcalibur 2.1 software (Thermo). QualBrowser was used for identified compounds in the different tissues.

3.5 Functional genetics

The study aimed at defining properties and function of single genes and gene variations has been recently referred as “functional genetics” which is a functional branch of genetics (Marchetti et al., 2012). In this study the analysis of the genes’ function involved in the biosynthetic pathway of anthocyanins was investigated through two different strategies:

1) over-expression of the pomegranate regulatory complex (*PgMYB-PgbHLH-PgWD40*) in *Nicotiana benthamiana* to analyse its effect on polyphenolic metabolism; 2) expression profile analysis of genes involved in the biosynthesis of anthocyanins in leaves, flowers, unripe (ST2) and ripe (ST4 F+S) arils in the two *P. granatum* cultivars with different metabolomics profile.

3.5.1 Identification of genes associated with anthocyanins biosynthesis

In order to investigate the genetic regulation of the anthocyanins pathway a set of genes involved in the biosynthesis of these compounds were identified and their levels of expression were estimated during fruit ripening in Valenciana and Wonderful cultivars.

3.5.1.1 RNA Isolation

In order to isolate the selected genes' transcripts total RNA was extracted as described by Zarei *et al*. (2012) from leaves, flowers and two fruit developmental stages (ST2 and ST4 F+S) of Valenciana and Wonderful (as illustrated in Fig. 21, Paragraph 3.4.1).

The samples were ground in a mortar with a pestle to obtain a fine powder. About 100 mg of powdered leaves or flowers and around 300 mg of powdered arils were transferred to a 2 ml tube and 1 ml of pre-heated (65 °C) extraction buffer I (2% CTAB, 25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2.0 M NaCl, 2% PVP). This solution was autoclaved (121 °C for 20 min) and prior to use 50 μg/ml proteinase K (Qiagen, Hilden, Germany) was added to the prepared aliquot, while 20 μl of β-mercaptoethanol were added directly to each tube. Afterwards samples were mixed by inverting and vortexing and then incubated at 65 °C for 15 min. During incubation, the solution was shaken and vortex-mixed every few minutes. Tubes were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was transferred to a new tube, an equal volume of chloroform:isoamyl alcohol 24:1 was added, and the suspension was vortex-mixed and centrifuged at 13,000 × g for 10 min (this step was repeated at least twice). The upper aqueous phase was transferred to a fresh tube and 1/4 volume of 10 M LiCl was added. Tubes was inverted gently to mix the solution and incubated at 4 °C overnight. Samples were centrifuged at 20,000 × g for 40 min at 4 °C. The supernatant was decanted, and the pellet was washed with 500 μl of 70% ice-cold ethanol, centrifuged briefly and ethanol was decanted (this step was repeated twice). Three hundred μL of pre-warmed at 65 °C extraction buffer II (1.0 M NaCl, 10 mM Tris-HCl (pH 8.0), 1

mM EDTA (pH 8.0), 0.5% SDS) was added to the pellet and left for 15 min to dissolve it. An equal volume of chloroform:isoamyl alcohol 24:1 was added and, after vortexing, the mixture was centrifuged at 13,000 × g for 10 min at 4°C (this step was repeated twice). A 0.1 volume of 3 M NaOAc (pH 5.2) and 0.6 volume of isopropanol was added. This mixture was gently mixed and incubated at -20 °C for at least 2 h. Tubes were centrifuged at 20,000 × g for 30 min at 4 °C, the pellet was washed with ice-cold 70% ethanol and dried for 15 min. The RNA pellet was resuspended in 30 µl DEPC-treated water.

The extracts were analysed with QIAxcel instrument by the mean of capillary electrophoresis, that provides a RNA Integrity Score (RIS) as function of the ratio of 18S and 28S rRNA peaks.

3.5.1.2 Synthesis of cDNA

Two different methods were used to synthesize the cDNA.

1) 1.0 µg of total RNA extracted from Wonderful cultivar leaves was used to synthesize first–strand cDNA using SuperscriptII Reverse transcriptase (Invitrogen, San Giuliano Milanese, MI, Italy) in 20 µl total volume as follows: 1 µg of RNA, 1 µl of dT21 (12 µM) and steril water until 5 µl of total volume were mixed and incubated at 70 °C for 2 min and in ice for 2 min. Thereafter, 2 µl of 5x first strand buffer, 1 µl of DTT (20 mM), 1 µl of dNTP's (10 mM) and 1 µl of SuperscriptII Reverse Transcriptase were added and the total was mixed and incubated at 42°C for 90 min., at 85 °C for 5 min, at 4 °C for 5 min, and at 85°C for 5 min. At the end sterile MQ water was added up to 20 µl of total volume. This method was used for the amplification of selected gene transcripts to be used for cloning, sequencing and primer designing for qPCR assays;

2) While samples to be used for quantitative Real time PCR (see Fig. 21) were reverse transcribed by using QuantiTect Reverse Transcription Kit (QIAGEN, Germany). This method allows carrying out the cDNA synthesis and the gDNA removal at the same time and it is specifically designed to be used for Syber Green amplification that take place on a Rotor Gene-6000 (Qiagen, Germany). For this protocol 0.8 µg of total RNA was used with gDNA Wipeout Buffer to perform genomic DNA elimination reaction and incubated at 42 °C for 2 min. The Reverse-transcription reaction was carried out using reverse transcription master mix (Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix) and template RNA obtained from the previous reaction, incubated at 42 °C for 15 min, to synthesize cDNA. Quantiscript Reverse Transcriptase was inactivated at 95 °C for 3 min.

3.5.1.3 Designing of cloning primers and amplification

Five transcription factors (TFs) that take part of the regulatory complex of anthocyanins (*PgMYB1*, *PgMYB2*, *PgMYB4*, *PgbHLH*, *PgWD40*) and three genes (*PgDFR*, *PgLDOX*, *PgUFGT*) involved in anthocyanin biosynthesis pathway were considered in this study.

Oligomeric primers for *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgWD40*, *PgbHLH, PgDFR*, *PgLDOX* and *PgUFGT* were designed basing on the DNA sequences of *P. granatum* L. available in the literature and in the National Centre for Biotechnology Information (NCBI) Nucleotide database (Tab. 6). We designed *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgWD40*, *PgbHLH* cloning primers in order to cover the CDS of relative genes to be used for agro-infiltration experiments.

The Oligo Calc and Primer3 webservers resources (Rozen et al., 1999) were used for primers designing.

Tab. 6. Cloning primer designing to isolate partial or complete CDS.

Gene name	Name in this study	Primer name	Primer sequence (5'-3')	Ta °C	Expected Product size (bp)	Accession number	Reference
PgMYB	*PgMYB1*	PgMYB1_F	F: ATGGAGGAAGCTGCTTCTTTTCGAAGAGTAAGG	56	1361	KP726347.1	In this study
		PgMYB1_R	R: TTATATTATCCCCATCTCTTCTTGGTCGGCACATAG				
	PgMYB2	PgMYB2_F	F: ATGACGGCACCAACAAGGCG	56	1135	HM056531.1	In this study
		PgMYB2_R	R: TTAAAATCGCTTATCAGCGGTCAACATGTCCTC				
	PgMYB4	PgMYB4_F	F: ATGGGAAGGTCTCCTTGCTGTGAGAAAG	56	792	KM881712.1	In this study
		PgMYB4_R	R: TCATTTCATCTCCAAACTTCTGTAATCCAATACGCC				
PgbHLH	*PgbHLH*	PgbHLH_F	F: ATGGCTGTGCCGCCCAGTAG	56	2070	KF874658.1	In this study
		PgbHLH_R	R: CTAAGAGTCAGTGTGGGGTATAAGCTGTTGG				
PgWD40	*PgWD40*	PgWD40_F	F: ATGGACAACTCGACTCAGGAGTCCCAC	58	1005	HQ199314.1	In this study
		PgWD40_R	R: TCAAACTTTCAATAACTGCATTTTGTTTGAGAATGAGACAGC				
PgLDOX	*PgLDOX*	PgLDOX_F	F: TTCGAGGAGGAGAAGAGGGA	60	728	KF841619.1	In this study
		PgLDOX_R	R: AGGATCTCGATTGTGTCCCC				
PgDFR	*PgDFR*	PgDFR_F	F: CAAAGACGCACCTTAGCCTG	60	824	KF841618.1	In this study
		PgDFR_R	R: ATGGGAAAGAGGGAGAAGCC				
PgUFGT	*PgUFGT*	PgUFGT_F	F: CGGTGTTAGCTTTCCCCTTC	<50	1184	KF841620.1	In this study
		PgUFGT_R	R: CACATCCCCGATCATCCTCC				

The PCR was performed with 50X Advantage 2 Polymerase (Advantage 2 PCR Kit Clontech, Takara Bio USA). The PCR conditions were as follows: start denaturation at 95 °C for 1 min, 30 cycles with denaturation at 94 °C for 15 sec, annealing 30 sec at different genes temperature (See Table 2), extension at 68 °C for 2 min and final extension at 68 °C for 1 min. The PCR products were check by the mean of electrophoresis on agarose gel at 1.5%, and Gel Red is used as intercalates. The PCR products were purified by QIAquick PCR Purification Kit or by QIAquick Gel Extraction Kit (Qiagen, Germany).

3.5.1.4 pJET Cloning

The PCR products of *PgDFR*, *PgLDOX* and *PgUFGT* genes amplification that showed a single band in agarose gel, were cloned into the pJET vector (CloneJET PCR Cloning Kit, Thermo Scientific). This vector contains a lethal gene *eco47IR* that enables positive selection of recombinant plasmid, which is disrupted by ligation of a DNA insert into the cloning site (Fig. 22).

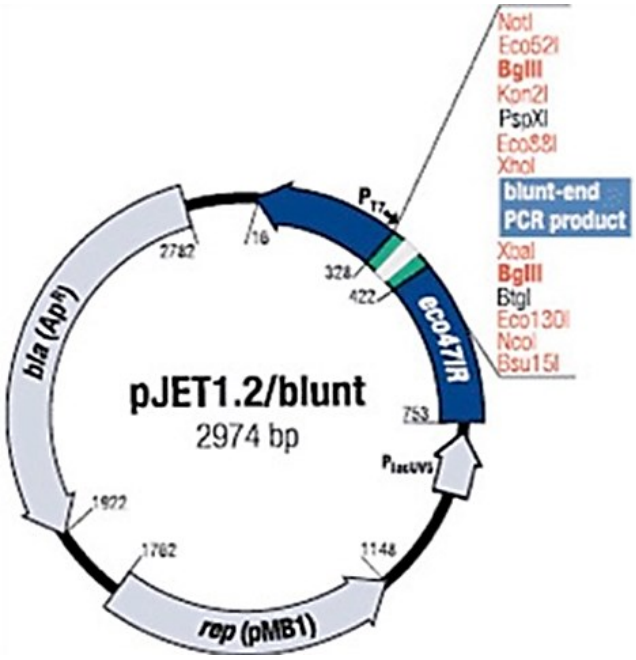


Fig. 22. Map of pJET1.2/blunt vector, with the restriction sites evidenced. Eco47IR is the lethal gene, *bla* is the gene that caused the Ampicillin resistance.

Only the cells with recombinant plasmids were able to propagate. The growth medium was LB - ampicillin (50 mg/l) plates, pH 7.0. After 12h-long incubation at 37 °C the colony picking and colony PCR techniques were used for isolated colonies which were transferred

in LB - ampicillin (50 mg/l) plate. The colony PCR amplification was carried out with MyTaq DNA Polymerase™ (Bioline, London, UK) at T_a of 60 °C. The vector primers used were: pJET1.2 forward sequencing primer 5'- CGACTCACTATAGGGAGAGCGGC- 3' and pJET1.2 reverse sequencing primer 5'- AAGAACATCGATTTTCCATGGCAG- 3' that are located respectively at the positions 310 and 428 of the vector sequence. The PCR products were verified on agarose gel at 1.5%. Positive clones were incubated at 37 °C for 16 h in LB Lennox liquid broth with ampicillin. Plasmid DNA was extracted by the means of QIAprep Miniprep Kits and amplified using vector primers and MyTaq DNA Polymerase™ (Bioline, London, UK). Quantitative and qualitative analyses of extracted plasmid DNA were performed by using spectrophotometer. The DNA sequences showed in Appendix were obtained with the mean of Sanger sequencing method using vector primers as sequencing primers, the contigs sequences were obtained with CodonCode Aligner Vers. 8.0.1 program and then used for BLAST searches (Supplement 1).

3.5.1.5 TOPO TA Cloning

In order to isolate genes of regulatory complex and to prepare the relative constructs for agro-infiltration in *N. benthamiana* the purified PCR products of each *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgWD40*, *PgbHLH* CDS amplification were cloned into the TOPO TA cloning vector (pCR™8/GW/TOPO® TA Cloning® Kit, Invitrogen, Thermo Fisher Scientific). This vector contains the *att* sites for rapid recombination into a variety of Gateway (TM) destination vectors, GW primer sites, within the *att* regions, located less than 55 base pairs from the PCR product insertion site for convenient sequencing, EcoR I sites flanking the PCR product insertion site for easy excision of inserts. This vector contains Spectinomycin resistance for easy antibiotic selection (Fig. 23).

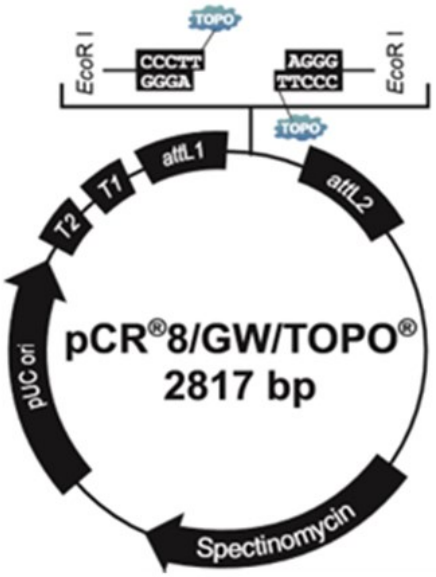


Fig. 23. Map of pCR™8/GW/TOPO® TA cloning vector, with the *attL1* and *attL2* sites highlighted.

3.5.1.6 Chemical Transformation in *Escherichia coli*

The transformation in One Shot or DH5α competent cell has been carried out using the protocol described in the TOPO TA Cloning Kit (Invitrogen, San Giuliano Milanese, MI, Italy), as follows:

1. Add 2 µl of the TOPO® Cloning reaction from Perform the TOPO® Cloning reaction, into a vial of One Shot® Chemically Competent E. coli and mix gently. Do not mix by pipetting up and down.
2. Incubate on ice for 5–30 min.
3. Heat-shock the cells at 42°C for 30 sec without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37 °C for 1 h.
7. Spread 50 and 150 µl from each transformation on two prewarmed selective LB (Luria-Bertani: 1% tryptone, 0.5% yeast extract, 1% NaCl, 15 g/l agar, pH 7.0) plate with spectinomycin 100 mg/ml.
8. Incubate plates at 37 °C.

3.5.1.7 Colony PCR and miniprep

To verify if the transformation was successful, colony screening was performed using the Colony PCR technique.

After 16-18 h at 37 °C the colonies were picked, put in 1 ml sterile water vortexed for 10 sec and a part stored in a LB – Spectinomycin plates for the future analyses. 10 µl of water with colony, were used as template for the screening of the recombinant clones during colony PCR. The amplification was carried out with Super Taq (Thermo Fisher Scientific) at Ta of 56 °C. The vector primers used were: pCR8_GW forward sequencing primer 5'-GTTGCAACAAATTGATGAGCAATGC- 3' and reverse sequencing primer of each examined. The PCR products were verified on agarose gel at 1.2%. Positive clones were incubated for 16 h at 37 °C in LB liquid broth with Spectinomycin. Plasmid DNA was extracted by the means of QIAprep Miniprep Kits (QIAGEN, Germany), that uses silica membrane technology (Fig. 24), digested with EcoRI HF and verified on agarose gel 1.2%. Quantitative and qualitative analyses of extracted plasmid DNA were performed by using NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

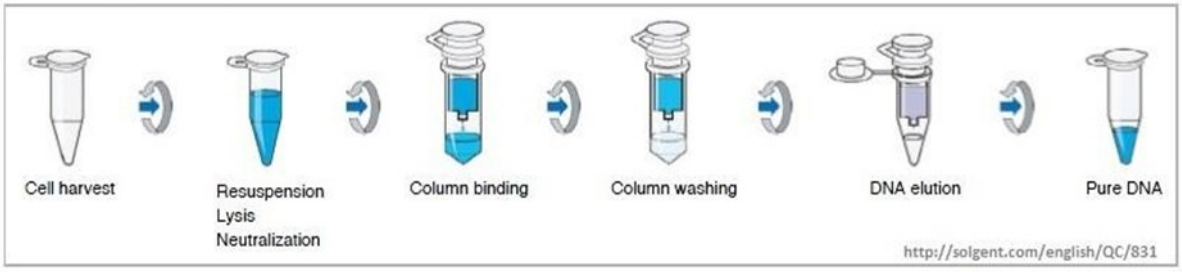


Fig. 24. Scheme of miniprep protocol, with the resuspension, column binding, column washing, DNA elution phases showed.

The sequences were obtained using Sanger sequencing method using with gene specific primers (Tab. 7), the contigs sequences were obtained with CodonCode Aligner Vers. 8.0.1 program and then used for BLAST searches (Supplement 2).

Tab. 7. Primers designed in order to sequencing. Some of these are the same of primers cloning.

Gene name	Name in this study	Primer name	Primer sequence (5'-3')	Accession number
PgMYB	***PgMYB1_seq***	PgMYB1_F1	F: CCAAGTACTAATAATCAGGGCTTCGTAGATTGTAAG	KP726347.1
		PgMYB1_R1	R: CAGCCACCTCAAACGACAGC	
	PgMYB2_seq	PgMYB2_F	F: ATGACGGCACCAACAAGGCG	HM056531.1
		PgMYB2_R1	R: CCCAAGGGGATTCGAACCCGG	
	PgMYB4_seq	PgMYB4_F	F: ATGGGAAGGTCTCCTTGCTGTGAGAAAG	KM881712.1
		PgMYB4_R1	R: GATGTTCGTCACGGGTTCCCCTGTC	
PgbHLH	***PgbHLH_seq***	PgbHLH_F1	F: CTGACAGTGAACCAAGCAGGCAAC	KF874658.1
		PgbHLH_R1	R: CCACGACGCCGTCTAGCAGAGG	
PgWD40	***PgWD40_seq***	PgWD40_F	F: ATGGACAACTCGACTCAGGAGTCCCAC	HQ199314.1
		PgWD40_R1	R: CTTCCCCCCACGCAATGTCGTACAC	

3.5.1.8 Gateway cloning

The gateway cloning ((Invitrogen, Thermo scientific) was necessary to carry out an efficient gene transfer between pCR8 vector containing the specific gene (entry clone) and the binary Gateway™ overexpression vector pGD625 (destination vector) in order to express selected TFs in agrobacterium cells (Fig. 25).

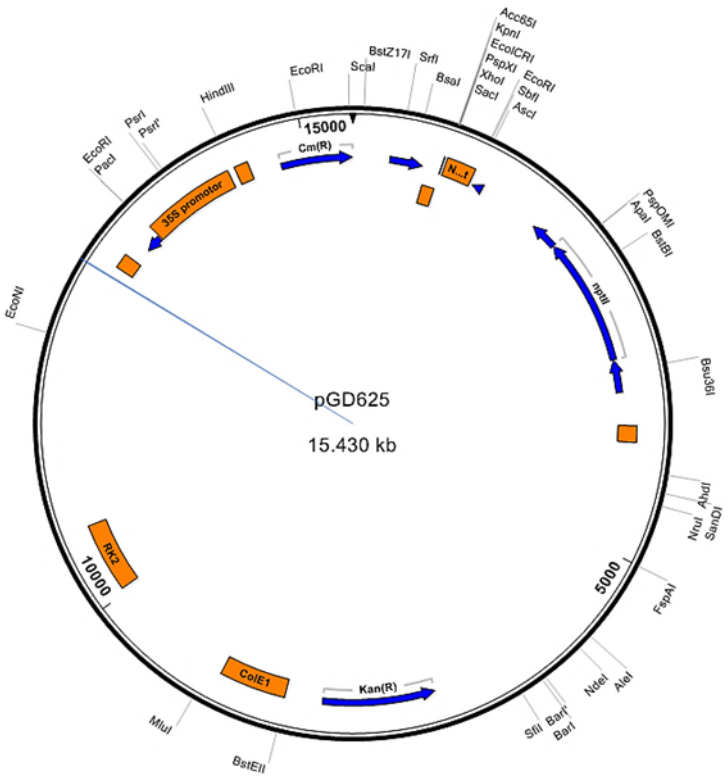


Fig. 25. Map of pGD625 vector contained Gateway cassette, *attR1* and *attR2* recombination sites, ccdB gene and the 35S promoter.

The L+R reaction takes place between the *attL* sites of the generated entry clone and the *attR* sites of the destination vector. This reaction is catalysed by the LR Clonase enzyme mix. As a result, an expression clone with the DNA of interest flanked by *attB* sites is generated. As in the BP reaction, a DNA fragment containing the *ccdB* gene is excised from the destination vector (Fig. 26).

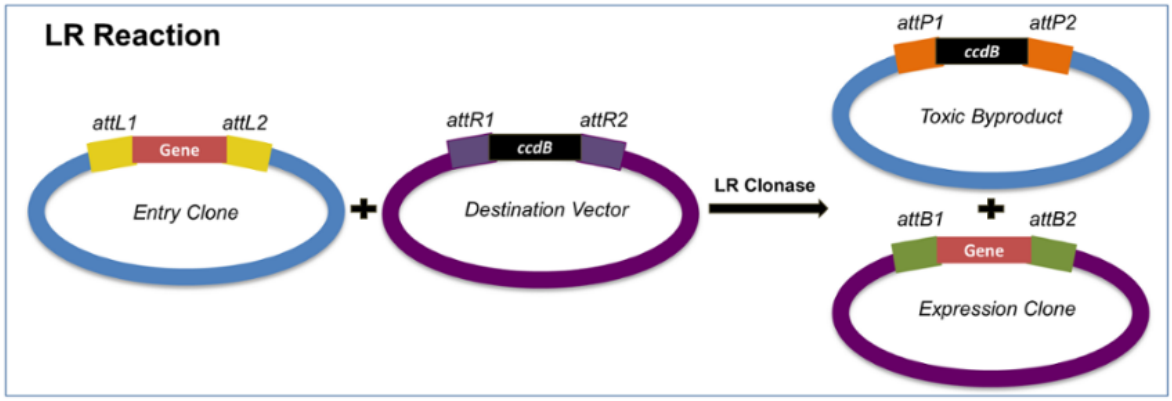


Fig. 26. The Gateway system adopts phage integration into the BP and LR reactions. (https://blog.addgene.org/plasmids-101-gateway-cloning).

In the reaction 1-2 µl (50-150 ng) pGD625-binary vector were added to 1-2 µl (150 ng/ µl) miniprep pCR8 vector + gene, 1 µl LR clonise II and 1 µl TE buffer fill 5 µl. The mix was left 2 hours at room temperature then 0.3 µl Proteinase K was added and incubated at 37 °C for 10 min. For transformation in *E. coli* 2.5 µl of the reaction was used as explained in the Paragraph 3.5.1.6.

3.5.1.9 Electroporation transformation in Agrobacterium tumefaciens

After transformation in *E. coli* correct recombinant Plasmid DNA was extracted by the means of QIAprep Miniprep Kits (QIAGEN, Germany) and used to do transformation in *Agrobacterium tumefaciens* AGL0 cells (Lazo et al., 1991). AGLO harboring pBINPLUS (pBIN) plasmid (van Engelen et al., 1995) was used as a negative control.

Electrocompetent Agrobacterium cells were prepared as follow:

1. A 25 ml preculture of Agrobacterium was grown in liquid LB with appropriate antibiotic at 28 °C, 200 rpm, 48 h.

2. The preculture was shaken vigorously to dissociate the aggregates and take OD600 (>1). An inoculum of 200 ml LB culture was added with the required volume to give a final OD600 of 0.02. Grow at 28 °C, 200 rpm.
3. When the OD600 reach 0.6 (circa 8 hrs of culture), put the culture on ice for 15 min.
4. Centrifuge the culture in 500 ml tubes 4000g, at 4 °C for 10 min.
5. Discard surnatant and resuspend the pellet in 500 ml of ice cold SDW.
6. Centrifuge 4000g, at 4 °C, for 10 min.
7. Repeat the SDW wash twice (step 5 and 6).
8. Discard the supernatant and resuspend the pellet in 40 ml of ice-cold glycerol 10%.
9. Centrifuge at 4000g for 10 min in 50 ml tube.
10. Resuspend the pellet in an equal volume of ice-cold glycerol 10% (300 to 500 µl).
11. Freeze 40 µl aliquots in liquid nitrogen on dry ice. Store at - 80 °C.

Electroporation of *Agrobacterium tumefaciens* was carried out as follow:

1. Pre-chill 2mm gap electroporation cuvettes.
2. Let the frozen electrocompetent cells to thaw on ice for 10 min.
3. Add 100 nano of plasmid prep (in up to 4 µl) to 40 µl of electrocompetent cells. Keep on ice 2 to 5 min.
4. Electroporate at 2500-3000 volts (15000 nvolts/cm), 25 µF, shunt 201 Ohms 5msec.
5. Remove rapidly the cells from the cuvette with a P200 and add them to 960 µl of liquid LB in a 2 ml Eppendorf, incubate flat at 28 °C, shaking 200 rpm, 2 h. (Immerse immediately the cuvette in a beaker of distilled water).
6. Plate 200 µl on LB supplemented with appropriate antibiotics (Rifamycin 25 mg/ml and Kanamycin 100 mg/ml).
7. Grow at 28 °C until colonies are visible (2 days).

To verify the presence of the gene strains in the vector a screening of agrobacterium colony was carried out by Colony PCR methods using detection primers (Tab. 8). Positive clones were used for agroinfiltration in *N. benthamiana* leaves.

Tab. 8. Detection primers designed at the centre of each sequence contig (S1) to identify positive agrobacterium clones.

Gene name	Name in this study	Primer name	Primer sequence (5'-3')	Product size (bp)
PgMYB	***PgMYB1_det***	PgMYB1_F3	F: AAGGAAAGGTGCATGGACTG	726
		PgMYB1_R3	R: AGCTTGTGAAGACGGACGAT	
	PgMYB2_det	PgMYB2_F3	F: GATCTCAGCATTGCACGAAA	217
		PgMYB2_R3	R: GGTGCTGGAACTTCTCTGGA	
	PgMYB4_det	PgMYB4_F3	F: GTCCTTGATTGCTGGTCGAT	240
		PgMYB4_R3	R: TTGAACCCTTCGGCATTATC	
PgbHLH	***PgbHLH_det***	PgbHLH_F3	F: GAGGCAGACCAGAGGTCAAG	154
		PgbHLH_R3	R: CTTGGGACTCCACAGCTTTC	
PgWD40	***PgWD40_det***	PgWD40_F3	F: CTCGGTTCTCAACAACAGCA	241
		PgWD40_R3	R: AATTCTCACCGACCCATCAG	

3.5.2 AGRO-INFILTRATION: transient expression in leaves of *Nicotiana benthamiana*

Agroinfiltration was used to induce transient expression of genes of regulatory anthocyanins complex from *P. granautm* in plants of *N. benthamiana*, in order to produce a transient overexpression of isolated pomegranate transcription factors.

Agrobacterium positive strains and empty pBIN agrobacterium vector were grown 220 rpm at 28 °C for 24 h in 10 ml of LB media with Rifamycin 25 mg/ml and Kanamycin 100 mg/ml. Then samples were centrifuged 3000 rpm at 20 °C for 10 min and the pellet was resuspended in 10 ml of infiltration media by rolling (in roller bank) for 2 h at room temperature.

Infiltration Media was prepared with 10 mM MES buffer (dissolve in MQ water), 10 mM $MgCl_2$ and 100 μM of acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone, Sigma). When the pellet was resuspended, the solution was dissolved with media until to get OD600=0.5A. Before infiltration equal volumes were mixed of the Agrobacterium strains.

The bacterium suspension was injected slowly into the leaves (2 leaves per plant) of two week-old *N. benthamiana* by pressing a 1 ml syringe without metal needle towards the underside of the leaf (Fig. 27 A). The plants were grown under greenhouse conditions (special cabinet) until the harvest (Fig. 27 B).

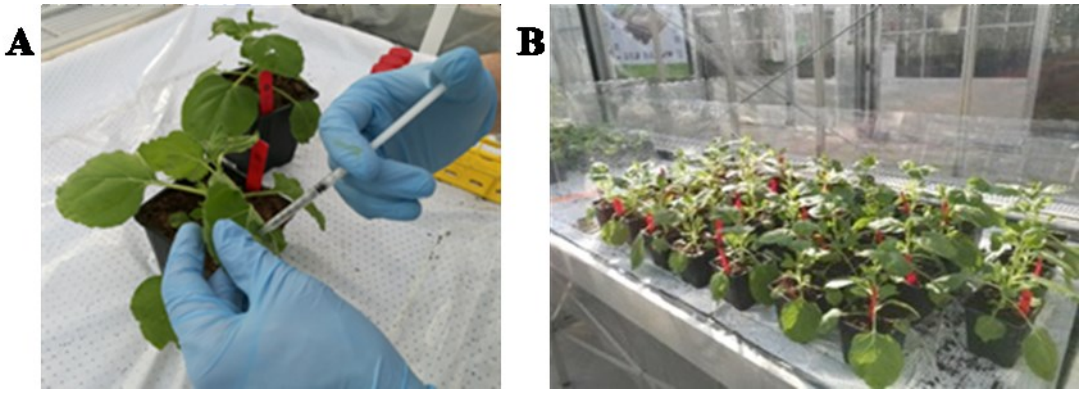


Fig. 27. (A) Agroinfiltration of Nicotiana leaf with agrobacterium strains. (B) Plants of *Nicotiana benthamiana* in the greenhouse.

Two different experiments were carried out. In the first experiment the different constructs combination (Tab. 9) was tested. The infiltration was carried out in two plants per constructs, two leaves per plant. All plants infiltrated with *PgMYB4*'s constructs were harvested three days after agroinfiltration, because leaves were suffering. The other leaves were harvested five days after agroinfiltration.

Tab. 9. Different construct combinations.

	PgMYB1	***PgMYB2***	***PgMYB4***
pBIN+pBIN+pBIN	PgMYB1+pBIN+pBIN	PgMYB2+pBIN+pBIN	PgMYB4+pBIN+pBIN
PgbHLH+pBIN+pBIN	PgMYB1+PgbHLH+pBIN	PgMYB2+PgbHLH+pBIN	PgMYB4+PgbHLH+pBIN
PgWD40+pBIN+pBIN	PgMYB1+PgWD40+pBIN	PgMYB2+PgWD40+pBIN	PgMYB4+PgWD40+pBIN
PgbHLH+PgWD40+pBIN	PgMYB1+PgbHLH+PgWD40	PgMYB2+PgbHLH+PgWD40	PgMYB4+PgbHLH+PgWD40

In the second experiment only the combination PgMYB2+PgbHLH was considerated with the combination of ROS1+DEL as positive and pBIN as negative control. AGL0-ROS1 and AGL0-DEL were obtained from Outchkourov *et al.* (2014).

Six *N. benthamiana* plants per each construct were used and two leaves per plants were agroinfiltrated. First three plants per constructs were harvested four days after agroinfiltration, the last three plants per constructs were harvested six days after agroinfiltration and ground in liquid nitrogen.

3.5.2.1 LC-PDA-Orbitrap FTMS

For the analysis 300 mg of *N. benthamiana* leaves were used. The samples were in double for the first experiment and in triplicate for second. Samples were extracted with 900 µl of 95% of MeOH and 5% of FA. The procedure and data process were the same as that described in the Paragraph 3.3.1. MetAlign and MSClust software were used to obtain reconstruct metabolites derivates from cluster of mass peaks originating from the same metabolite, including adducts fragments and isotopes. Zero values for compound intensity, i.e., absent in sample, were replaced by random values between 0 and 1.

3.5.3 Gene expression analysis

To assess the expression levels of the genes associated with anthocyanins biosynthesis during fruit development stages, leaves, flowers and two fruit developmental stages (ST2 and ST4 F+S) of Valenciana and Wonderful (Fig. 21, Paragraph 3.4.1) were used to perform quantitative PCR assays.

3.5.3.1 Primer designing for qPCR

The qPCR primers were designed with Primer3 and Primer-Blast using the contigs sequences obtained from pJET cloning for *PgDFR*, *PgLDOX* and *PgUFGT* genes, while the for *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgWD40* and *PgbHLH* genes the relative qPCR primers were designed basing on the complete CDS obtained from TA cloning. As regards to *PgCHS*, *PgF3'H* and *PgF3'5'H* genes the relative qPCR primers were designed basing on sequences available at NCBI Nucleotide database. The primer sequences of *PgRPSII* (Ribosomial Protein S) and *PgActin* housekeeping genes were taken from relevant literature (Ben-Simhon et al., 2015; Zhao et al., 2015) (Tab. 10). For all qPCR primers the selected designing parameters were an amplicones length of 90-150 bp and 59-64 °C as melting temperature range.

Tab. 10. qPCR primers designed based on sequence contigs and sequence presents in NCBI database.

Gene name	Transcript name for qPCR used in this study	Primer name	Primer sequence (5'-3')	GC %	Ta °C	Product size (bp)	Accession number	Reference
PgCHS	*PgCHS_01*	PgCHS_F01	F: CGCTAGGCAGGACATTGTGG	60	58	101	KF841615.2	In this study
		PgCHS_R01	R: GGGTGATCTTCGACTTGGGC	60				
PgF3'5'H	*PgF3'5'H_01*	PgF3'5'H_F01	F: GGCACTCCTTTTGGTATGTCC	52	58	101	KU058892.1	In this study
		PgF3'5'H_R01	R: TGTCGAGGACCTGATTACGC	55				
PgF3'H	*PgF3'H_01*	PgF3'H_F01	F: CACCCATCAACCCCACTCTC	60	58	104	KC430328.1	In this study
		PgF3'H_R01	R: GCCCACACGTTGACCAAAAG	55				
PgDFR	*PgDFR_01*	PgDFR_F01	F: CACAGAGAAGGTGTCGTTCTC	52	58	118	PgDFR_contig	In this study
		PgDFR_R01	R: GAGAAGCCCCTTCTCCCTG	63				
PgLDOX	*PgLDOX_01*	PgLDOX_F01	F: CCGGGCCGAGCTCAAGAAGG	70	58	103	PgLDOX_contig	In this study
		PgLDOX_R01	R: CTCCCCGGCCTTCTTGACCC	70				
PgUGTF	*PgUGTF_01*	PgUFGT_F01	F: GGCCGATCATTCCCACGAGCA	62	58	130	PgUFGT_contig	In this study
		PgUFGT_R01	R: GCAGCAGGTTCAAAGGCCCG	65				
PgMYB	*PgMYB1*	PgMYB1_F01	F: TGGATTGGTACGTATTTCACTTCC	42	57	109	PgMYB1_contig	In this study
		PgMYB1_R01	R: TCTTCCGGACCATGTGAGTG	55				
	PgMYB1.2	PgMYB1_F01.2	F: GCTTCTAGGGAACAGATGGACG	55	57	91	KF841621.1	In this study
		PgMYB1_R01	R: TCTTCCGGACCATGTGAGTG	55				
	PgMYB2_01	PgMYB2_F01	F: ATGCGGGAAGAGTTGCAGG	58	57	107	PgMYB2_contig	In this study
		PgMYB2_R01	R: GCCTATGGAGCCTGAGAATCA	52				
	PgMYB4_01	PgMYB4_F01	F: GGATCAACTACCTGCGTCCC	60	58	116	PgMYB4_contig	In this study
		PgMYB4_R01	R: TCGACCAGCAATCAAGGACC	55				
PgbHLH	*PgbHLH_01*	PgbHLH_F01	F: CTTCATCGACCACCAACCCC	60	58	101	PgbHLH_contig	In this study
		PgbHLH_R01	R: GCGAGTTGAAACGAGGGTGG	60				
PgWD40	*PgWD40_01*	PgWD40_F01	F: CATCGAGGAGTACACCAACCG	57	58	94	PgWD40_contig	In this study
		PgWD40_R01	R: GGGGTGATCGAAGGAGAGGG	65				
PgACT	*PgACT*	PgACT_F0	F: GATTCTGGTGATGGTGTGAG	50	58	168	GU376750	Zhao et al., 2015
		PgACT_R0	R: GACAATTTCCCGTTCAGCAG	50				
PgRPSII	*PgRPSII*	PgRPSII_F0	F: TCAATTTGTGAGGGTCGTTCT	43	58	111	Ophir et al., 2014	Ben-Simhon et al., 2015
		PgRPSII_R0	R: GATTCAAGAGTAGTAACCGATTCCA	40				

3.5.3.2 Quantitative PCR assays

To assess the expression level of the genes associated with anthocyanins biosynthesis pathway 80 ng of cDNA from leaf, flower (ST1), unripe aril (ST2), and ripe aril (ST4 F+S) from Valenciana and Wonderful were used as template with QuantiNova™ SYBR® Green PCR Kit (QIAGEN, Germany). Three biological replicates were used per each stage. The RT–qPCRs were run on a Rotor Gene-6000 (QIAGEN, Germany) with the following condition: first step at 95 °C for 2 min and afterwards 40 cycles alternating between 95 °C for 5 sec and 60 °C for 10 sec at. Each 20 µl reaction mixture consisted of: 10 µl of 2x QuantiNova SYBR Green PCR Master Mix, 1.4 µl each of forward and reverse primer (10 µM), and 1 µl of cDNA (80 ng). The melting curves were assessed for the different primers couples. The Take Off Point (TOP) and the amplification value of the primers were obtained automatically by the Rotor-Gene system selecting the "comparative quantitation analysis of cycling" method. These values were used to calculate the relative expression ratio by the Pfaffl method (Pfafft, 2001). The expression levels were compared with the *PgRPSII* housekeeping gene and the unripe aril of Valenciana was considered as calibrator tissue and set to value of 1.

3.6 Statistical analysis

All data were submitted to the Barlett's test for the homogeneity of variance and the data that were not homogeneous were logarithmically transformed. All the data homogeneous were analysed using analysis of variance (ANOVA) by CoSTAT program.

For morphological and physico-chemical characterization of all genotypes analysed a correlation table was created by CoSTAT program.

For nine cultivars (Gen.1, Gen.2, Gen.3, Gen.5, Gen.6, Primosole, Dente di Cavallo, Valenciana and Wonderful) used in the genetic diversity analysis and also in the morphological characterization we used a Mantel permutation test with PastProject program 3.04 version (Hammer et al., 2001) to estimate molecular genetic distances between pairs of populations and analyse its correlation with the Euclidean distances obtained from morphological traits using Euclidean similarity index as computation method. Significantly different traits (fruit weight, aril weight, juice yield, pH values, °Brix, Chroma, phenolic content and antioxidant activity) were used to calculate a Principal Component Analysis of the individuals with Morphotools, a set of R (R Core Team, 2015) functions (Koutecký, 2015).

As regards to germination tests, the effects of treatments on final germination was analysed by

fitting factorial generalized linear models (GLMs, logit link function, binomial distribution) to the germination data using dedicated R set of functions as described in Puglia *et al.* (2015).

For metabolomics features (in juice, in development pomegranate fruit stages and in *N. benthamiana*) the principal component analysis (PCA) was obtained by SIMCA tool version 14 (MKS Umetrics AB, 1992-2015). The relative intensity levels of compounds resulting from MSClust software were used for Student's t-test.

4 Results and discussion

4.1 Characterization of Sicilian germplasm

In this study, an integrated morphological, biochemical and molecular approach was used to obtain a complete characterization of a core of Sicilian germplasm. From these data were elaborated basic information to compare genotypes for breeding or to evaluate growth under different climatic conditions (Dafny-Yalin et al., 2010; Al-Maamari et al., 2016).

4.1.1 Bio-agronomic and physico-chemical characteristics

Fruit size is an important trait attracting consumers for the fresh market; here significant morphometric differences among studied genotypes were found. Indeed, the average fruit weight found in this study was 380.62 g with a high variability among genotypes ranging from 232.55 g in Gen.7 to a maximum of 825.82 g observed in Wonderful (Tab. 10). Variation of fruit weight could depend on the cultivar and ecological condiction (Shulman et al., 1984). These data showed that as regards the two genotypes used as witness Valenciana and Wonderful, the average fruit weight was greater than reported in the literature (La Malfa et al., 2006; Wetzstein et al., 2011, Chater et al., 2018), while the data of Primosole and Dente di Cavallo were lower than reported by La Malfa *et al.* (2006).

Also, fruit circumference showed significant differences among genotypes which average was 293.80 mm. However, Wonderful and Gen.6 showed the highest values (368 mm and 362 mm respectively), while the lowest value was observed in Gen.7 (248 mm). Accordingly, Wonderful and Gen.6 displayed also the highest fruit length and diameter values, while the lowest values were found in Gen.7, as shown in Tab. 10. The average fruit size of Wonderful grown in Sicily was greater than the Wonderful cultivated in California as previously reported (Wetzstein et al., 2011; Chater et al., 2018). This could probably due to the different climate, temperature and humidity conditions in the two environments. No significant differences among considered genotypes were observed for fruit shape (FL/FD). The smallest value of septum number was in Wonderful One (5.23), while the greatest in Gen.6 (8.20). According to the fruit weight, Wonderful showed also the highest weight of peel and arils (430.34 and 395.48 g respectively) (Tab. 11). The latter result was in agreement with the value reported for Wonderful cultivar grown in Spain (Alcazar-Mármol et al., 2017), but substantially differs with what obtained for

the same cultivar grown in America (Chater et al., 2018) probably, once again, due to dissimilar environmental growth conditions. The value reported for aril weight of our Valenciana (217.45 g) was lower than reported by Alcazar-Mármol *et al*. (2017).

However, percentage of the dry aril yield was highest in Genotype 9 (26.14%), while dry peel yield was found to be highest in Dente di Cavallo (36.05%), indicating that the percentage of humidity in these fruits, for the parameters analaysed, is lower than the other cultivars examined. Dente di Cavallo showed the highest aril yield (64.52%), while the lowest was observed in Wonderful One (41.36%). On the contrary, the latter genotype showed the highest peel yield (58.29%) while Dente di Cavallo was the lowest (35.49%), showing that aril and peel yields are inversely correlated. The newly characterized Sicilian genotypes had intermediate values both for yield in arils and for yield in peel among commercial genotypes (both Sicilian and international). Furthermore, Gen. 8 showed the highest weight of 100 arils, instead Genotype 9 presented the lowest values (23.83 g and 0.24 g) (Tab. 11). The data obtained for Wonderful (38.60 g) was higher than reported by Chater *et al.* (2018). Gen.2 showed the highest seed yield (20.90%) while Gen.8 the lowest (12.64%) with Valenciana (13.44%).

Tab. 10. Morphologic characteristics of fruit and peel.

Cultivar	Fruit weight (g)	Fruit circumference (mm)	Fruit lenght (mm)	Fruit diameter (mm)	Fruit shape (FL/FD)	Septum number	Peel weight (g)	Peel yield (%)	Dry peel yield (%)
Genotype 1	285.72±28.61de	271.40±8.65ef	71.40±2.71def	82.60±2.41ef	0.87±0.02a	6.60±0.55bc	106.78±10.39e	37.52±3.53de	28.67±3.49cde
Genotype 2	385.92±29.97c	303.20±10.24c	79.64±1.68c	98.08±8.55c	0.82±0.07a	6.60±0.55bc	162.15±17.11cd	41.10±2.22bcde	27.53±1.76def
Genotype 3	304.07±34.41de	276±10.84def	74.4±2.31cde	83.20±2.78ef	0.90±0.03a	5.80±0.45bc	111.92±11.63e	37.06±4.74de	29.49±1.87cde
Genotype 4	372.59±37.03cd	295.67±7.10cde	78.04±3.48cd	90.70±1.69cde	0.87±0.03a	6.67±0.58bc	205.35±12.06c	55.62±7.92a	24.50±0.11fg
Genotype 5	255.87±23.26e	263.00±7.88f	70.24±2.57def	80.64±3.74ef	0.88±0.04a	7.00±1.00b	121.954±22.29e	47.76±8.64abcd	33.02±1.69ab
Genotype 6	632.42±105.24b	362.00±21.68b	90.74±3.00b	106.44±6.09b	0.86±0.04a	8.20±0.45a	295.72±40.29b	47.27±6.11abcd	19.75±1.21h
Genotype 7	232.56±23.45e	248.00±7.55f	65.37±2.86f	77.47±2.51f	0.85±0.02a	5.34±0.58bc	104.97±4.62e	45.35±3.42abcde	24.21±2.38fg
Genotype 8	448.86±25.64c	312.18±6.02c	81.88±1.80c	97.10±2.79cd	0.85±0.04a	6.25±0.96bc	165.23±25.18cd	36.80±5.28de	24.28±1.28fg
Genotype 9	261.85±58.22e	262.67±16.57f	65.37±8.05f	81.20±4.49ef	0.81±0.08a	5.67±0.58bc	131.49±14.93de	51.21±7.19abc	30.6±0.97bcd
Primosole	297.85±54.84de	276.00±16.74def	67.60±2.41ef	82.40±3.58ef	0.83±0.02a	6.20±0.45bc	117.74±16.10e	40.07±6.06cde	33.96±2.38ab
Dente di Cavallo	359.42±46.66cd	295.00±11.73cde	79.56±5.49c	90.62±4.65de	0.88±0.07a	7.00±0.00b	127.79±23.83de	35.49±4.41e	36.05±0.97a
Valenciana	378.51±28.24c	297.60±3.37cd	80.98±6.05c	90.00±2.47de	0.91±0.08a	7.00±0.00b	161.06±24.53cd	42.49±4.97bcde	31.28±2.91bc
Wonderful	825.83±142.80a	386.00±19.62a	105.05±8.61a	114.65±5.18a	0.92±0.06a	6.75±1.50bc	430.34±70.61a	52.24±3.02ab	20.97±1.54gh
Wonderful One	287.31±29.39de	264.45±5.75f	75.13±1.96cde	83.23±0.57ef	0.91±0.03a	5.23±0.77c	166.95±10.47cd	58.29±3.16a	25.66±1.41ef
Mean value	380.63	293.80	77.53	89.88	0.87	6.45	172.10	44.88	27.86

Values represent average of five fruits per genotype, ± standard deviation. Different capital letters within a column refer to a difference significant at $P \leq 0.05$.

Tab. 11. Morphologic characteristics of arils and seeds.

Cultivar	Number of arils	Aril weight (g)	Weight of 100 arils (g)	Aril yield (%)	Dry aril yield (%)	Seed weight (g)	Seed yield %
Genotype 1	464.20±79.8bc	178.94±25.61de	39.98±1.96cd	62.48±3.54ab	16.35±0.83g	54.59±10.8bc	19.04±2.58abc
Genotype 2	568.60±50.23bc	223.78±17.42cde	43.00±4.32c	58.02±2.23abcd	16.73±0.76fg	80.42±3.89b	20.90±1.21a
Genotype 3	479.40±107.12bc	192.15±31.89de	41.84±3.46c	62.95±4.74ab	17.17±0.87fg	53.15±3.78bc	17.59±1.58abc
Genotype 4	229.03±5.97d	167.25±47.29de	61.14±0.08a	44.38±7.93e	19.27±0.09cd	52.48±4.65bc	14.09±0.15bc
Genotype 5	372.20±87.48c	133.92±26.77de	38.76±4.3cd	52.24±8.64bcde	17.97±0.26def	42.68±8.07c	16.59±1.86abc
Genotype 6	674.20±146.24b	336.70±85.89b	50.87±1.86b	52.73±6.12bcde	16.93±0.56fg	123.63±22.48a	19.54±1.34ab
Genotype 7	339.63±69.24cd	127.58±20.71de	37.81±2.18cd	54.65±3.42abcde	20.02±0.29c	34.34±5.71c	14.72±1.11bc
Genotype 8	449.51±73.82bc	283.63±27.15bc	63.71±5.51a	63.2±5.28ab	18.51±0.56de	56.68±6.04bc	12.64±1.3c
Genotype 9	566.11±269.35bc	130.37±47.38de	23.83±2.52e	48.8±7.20cde	26.14±0.82a	50.3±22.83c	18.65±4.54abc
Primosole	510.80±158.46bc	180.11±45.77de	38.49±5.93cd	59.94±6.06abc	18.99±1.27cd	46.29±9c	15.66±2.85bc
Dente di Cavallo	550.60±78.25bc	231.63±31.62cd	43.85±2.74c	64.52±4.41a	18.03±0.45def	57.32±5.81bc	16.02±1.17abc
Valenciana	543.20±104.58bc	217.45±22.1cde	43.01±6.53c	57.52±4.97abcd	17.29±0.74efg	50.6±14.03c	13.44±4.16c
Wonderful	1028.72±219.96a	395.48±80.39a	38.6±3.42cd	47.77±3.02de	23.08±0.51b	118.51±34.84a	14.19±2.16bc
Wonderful One	382.92±87.83cd	120.36±20.88e	33.4±4.17d	41.36±2.38e	23.32±0.35b	49.74±15.34c	17.35±4.46abc
Mean value	511.37	208.53	42.74	55.04	19.27	62.19	16.46

Values represent average of five fruits per genotype, ± standard deviation. Different capital letters within a column refer to a difference significant at $P \leq 0.05$.

Gen.8 and Dente di Cavallo showed the highest juice yield with 50.57% and 48.50% respectively (Tab. 12), instead Wonderful One showed the lower value with 24.00%. The average yield of fruit juice of Wonderful in this study (33.58%) was lower than 37% of the total fruit mass of Wonderful cultivar grown in Australia (Weerakkody et al., 2010). In general Sicilian genotypes have showed values of yield in arisl and in juice higher or similar compared to the international genotypes (Valenciana, Wonderful and Wonderful One). This classifies Sicilian accessionsas good products for the market and juice production.

Determination of total soluble solids (TSS or °Brix) is important to establish the organoleptic quality of the juice and it is the major parameter determining the cultivars that can be used for wine preparation (Sezer et al., 2007). The range of °Brix values found was from 15.57 in Gen.4 to 18.30 in Gen.9, all variety tested had a °Brix value higher than the minimum threshold generally required for commercial use (>12%) (Barone et al., 2001; Zaouay et al., 2014). The mean value found in our genotypes was similar with Sicilian (Dente di Cavallo) and international (Valenciana and Wonderful, Wonderful One) cultivars (Ferrara et al. 2014; Beaulieu et al., 2015; Alcaraz-Mármol et al., 2017; Adiletta et al., 2018), while the value obtained for Wonderful was higher than reported by Chater *et al.* (2018). This may be due to environment conditions. In reverse the highest pH value was measured for Gen.4 and the lowest for Gen.9. These pH values were in agreement with Ferrara *et al.* (2014) and Beaulieu *et al.* (2015) for Wonderful One and Wonderful respectively and with Todaro *et al.* (2016) for Primosole, Dente di Cavallo and Valenciana, growth in the experimental farm of the Catania University (Italy, Sicily), but they were lower than reported by Alcaraz-Mármol *et al.*(2017) and Adiletta *et al.* (2018) for Wonderful and Dente di Cavallo. . pH relates to the free hydrogen ions in solution indicating the alkaline/acidity balance, a low pH leads to more stable colour, anthocyanin is stable at pH low with red colour (Wahyuningsih et al., 2017).

It was observed also differences among juice colour in the genotypes (Tab. 12), Gen.3 showed the highest values of lightness of colour (L*) (22.40), whereas Gen.9, Wonderful and Wonderful One showed the lowest (13.47, 13.59 and 14.63 respectively). The highest values of colour a* (tending to red colour) were observed in Wonderful One (11.04), Gen.7 (11.05), Gen.5 (10.96) and Gen.3 (10.49). Gen.6 (4.49) and Gen. 3 (4.36) showed the greatest value of colour b*, means that the juice colour tending to yellow. As regard value C* was observed that Wonderful One was the genotype with the most intensity/purity of the colour with a value of 12.39. Also, for value of h° significant difference were found between cultivars, very high value

was found in Gen.3 (25.06). According to the CIELAB colour parameters cultivars with values of colour a* positive and colour b* negative can have pigmentation from red to blue (Gen.1, Gen.2, Gen.4, Gen.8, Primosole, Dente di Cavallo), while cultivars with values of colour a* and colour b* positives can have pigmentation from red to yellow (Gen.5, Gen.6, Gen.7, Gen.9, Wonderful and Wonderful One). Valenciana showed the lowest value of colour a* and colour b*, C*and h°, which means that its juice tends towards a clear pink colour.

Tab. 13 shows the correlation between morphologic and physico-chemical characteristic among cultivars analysed. Fruit weight, circumference, length, diameter, peel weight, aril number, aril weight, seed weight, and juice weight were highly correlated with each other ($r \geq 0.6$), but the harder correlation was found between fruit weight with circumference, length, diameter,peel weight, aril weight, seed weight and juice weight with $r \geq 0.8$ indicating that larger fruit are heavier and have greater total aril weight. Overall among the analyzed genotypes Wonderful was the largest in fruit size with the highest weight in arils, seeds and peel, while Gen.7 was the smallest in fruit size, seed and peel weight. The rest of the genotypes showed intermediate values.

Tab. 12. Physico-chemical characteristics of juice.

Cultivar	Juice weight (g)	Juice yield (%)	pH	Total soluble solids (°Brix)	Lightness (L*)	Color a*	Color b*	Chroma (C*)	Hue (h°)
Genotype 1	124.34±16.87def	43.43±2.47abc	3.63±0.08cd	16.06±0.19c	17.70±0.93b	6.84±0.67bcd	-1.62±0.43c	7.05±0.58cd	-13.51±4.39de
Genotype 2	143.35±15.72def	37.12±2.57bcd	3.76±0.10c	15.76±0.63c	17.77±0.8b	5.86±0.6de	-1.70±0.19c	6.11±0.58cd	-16.27±2.19de
Genotype 3	139.00±30.61def	45.35±6.1ab	3.77±0.08c	16.06±0.58c	22.40±4.05a	10.49±3.42ab	4.49±1.47a	11.21±3.02ab	25.06±8.27a
Genotype 4	114.77±42.68def	30.28±8.06de	4.15±0.10a	15.57±0.67c	21.25±0.85ab	8.38±1.83abcd	-1.30±0.23bc	8.48±1.84abc	-8.85±0.87cde
Genotype 5	91.23±20.76f	35.64±7.47cd	3.54±0.06d	17.00±0.37bc	18.69±1.33b	10.93±1.47a	2.52±3.52a	11.51±2.51a	10.82±13.15ab
Genotype 6	213.06±64.13bc	33.18±5.34de	3.15±0.05f	16.08±0.51c	21.06±5.51ab	10.68±3.37ab	4.36±1.94a	11.40±4.07ab	19.36±7.84ab
Genotype 7	93.24±15.12ef	39.93±2.44abcd	3.33±0.05e	16.67±0.82bc	17.46±0.94b	11.05±0.95a	0.75±0.17abc	11.08±0.93ab	3.95±1.21bc
Genotype 8	226.95±21.45b	50.56±4.05a	3.95±0.06b	15.65±0.3c	20.01±1.38ab	7.52±0.47abcd	-1.21±0.37bc	7.62±0.46abc	-9.11±2.81cde
Genotype 9	80.07±25.12b	30.14±2.93de	2.88±0.02g	18.30±0.6a	13.47±0.76c	8.26±1.47abcd	1.49±0.4ab	8.35±1.53abc	8.33±2.02ab
Primosole	133.81±40def	44.27±5.39abc	3.72±0.07c	17.52±0.85ab	14.86±0.27b	8.75±1.1abc	-1.33±0.09bc	8.81±1.09abc	-8.6±1.2cd
Dente di Cavallo	174.31±26.58bcd	48.50±3.78a	3.64±0.03cd	17.54±0.21ab	18.07±1.78b	6.66±1.03cd	-0.83±1.01bc	6.78±0.95cd	-7.7±9.23cde
Valenciana	166.85±20.15cde	44.08±4.11abc	3.67±0.10cd	17.58±0.6ab	19.59±1.5ab	4.88±1.34e	-1.59±0.64c	5.19±1.19d	-19.3±9.41e
Wonderful	276.98±51.18a	33.58±2.57de	3.14±0.20f	17.75±0.86ab	13.59±0.74c	7.08±1.67abcd	1.30±0.37ab	7.20±1.71bcd	10.32±1.15ab
Wonderful One	70.61±16.95f	24.00±5.14e	3.08±0.06f	17.07±0.39abc	14.63±0.41c	11.04±1.36ab	2.06±0.66a	12.39±0.85a	12.42±1.14ab
Mean value	146,33	38,58	3,53	16,76	17,90	8,46	0,53	8,80	0,49

Values represent average of five fruits per genotype, ± standard deviation. Different capital letters within a column refer to a difference significant at $P \leq 0.05$.

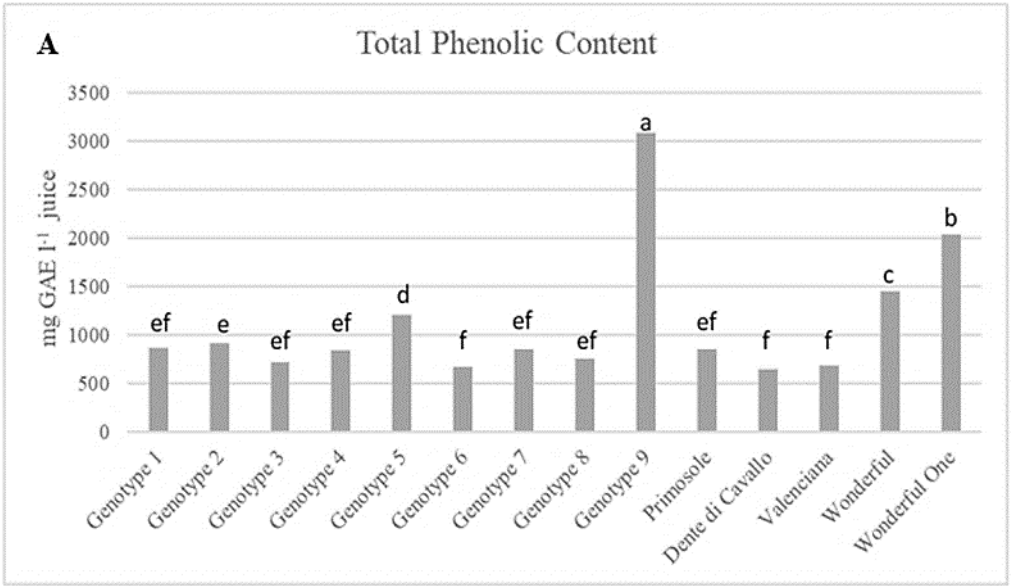
Tab. 13. Correlation matrix (Pearson correlation coefficient, r) of morphologic parameters of 14 different pomegranate cultivars.

Parameters	**FW**	**FC**	**FL**	**FD**	**Sn.**	**PW**	**An.**	**AW**	**AW100**	**SW**	**JW**
FW	**1**										
FC	0.980***	**1**									
FL	0.933***	0.919***	**1**								
FD	0.927***	0.952***	0.884***	**1**							
Sn.	0.441***	0.508***	0.418***	0.463***	**1**						
PW	0.940***	0.903***	0.875***	0.861***	0.405**	**1**					
An.	0.792***	0.755***	0.718***	0.689***	0.262*	0.665***	**1**				
AW	0.935***	0.935***	0.874***	0.877***	0.422***	0.758***	0.823***	**1**			
AW100	0.269*	0.345**	0.315*	0.369**	0.290*	0.198ns	-0.246ns	0.308*	**1**		
SW	0.886***	0.886***	0.810***	0.850***	0.488***	0.803***	0.781***	0.860***	0.134ns	**1**	
JW	0.870***	0.870***	0.821***	0.801***	0.351**	0.666***	0.765***	0.971***	0.361**	0.713***	**1**

FW, fruit weight; FC, fruit circumference; FL, fruit length; FD, fruit diameter; Sn., Septum number; PW, peel weight; An, Arils number; AW, aril weight; AW100, weight of 100 arils; SW, seed weight; JW, juice weight. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

4.1.2 Total Phenolic Content and Antioxidant activity

Total phenolic content (TPC) and antioxidant activity (AA) of 14 different pomegranate juices (PJs) are shown in Fig. 28. The average value of TPC was 1108.44 mg/l showing to be significantly influenced by the genotype ($p < 0.05$). The PJs exhibited high amount of TPC (Fig. 28A) with a range between 645.59 and 3083.13 mg/l. In particular, Genotype 9 showed the highest phenolic content following by Wonderful One and Wonderful among international genotypes and by Genotype 5 among Sicilian genotypes. The genotypes with lower values were Dente di Cavallo, Genotype 6 and Valenciana. The value obtained for Wonderful One (2037.57 mg/l) was similar to reported by Ferrara *et al.* (2014) for the same genotype (2337 mg/l) grown in Lecce (Italy), while values of Valenciana and Wonderful were lower than reported by Mena *et al*. (2011) grown in Spain. In comparison with previous literature data, Sicilian genotypes often showed TPs lower than some Croatian cultivars (Radunic et al., 2015) and higher values than some Iranian pomegranate (Tehranifar A. et al., 2010). The antioxidant activity followed the same trend of the TPC with mean value of 12.66 mmol/l, varying significantly ($p < 0.05$) between 6.24 in Dente di Cavallo and 35.56 in Genotype 9 (Fig. 28B). Wonderful One and Wonderful showed the highest AA among international cultivars while Genotype 5 among Sicilian cultivars. DPPH value of Wonderful in this study was similar or higher than some Wonderful cultivars reported by Mena *et al*. (2011).



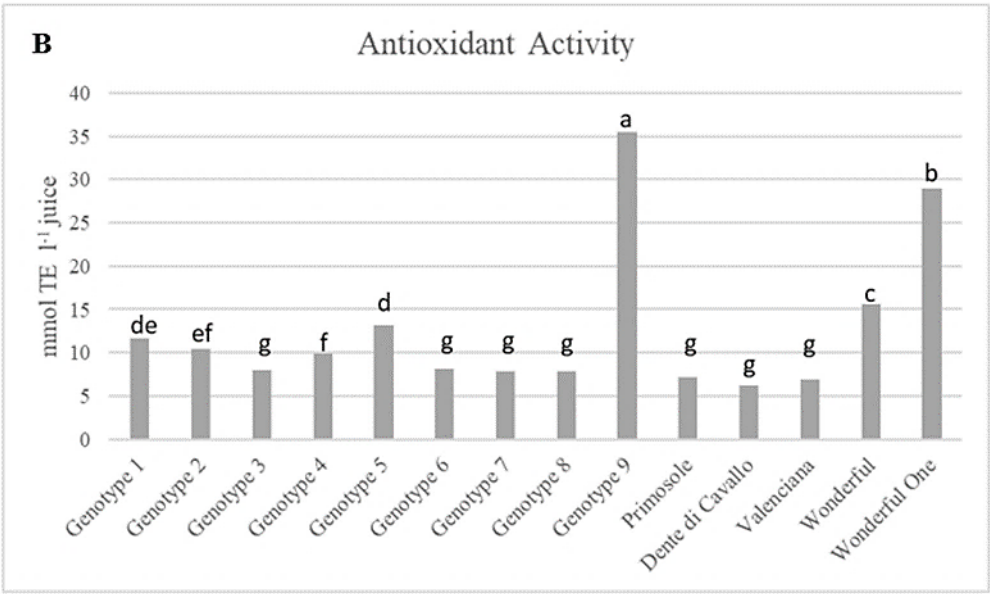


Fig. 28. TPC (A) and AA (B) in pomegranate juices. Value bars indicated with different letters are significantly different ($P < 0.05$).

The AA and TPC levels were positively and significantly correlated (r = 0.974) (Tab. 14). It is evident that as the total phenolic concentration increases, the antioxidant activity against DPPH radically increases. This means that the phenolic compounds detected in the samples are those that exhibit antioxidant properties. Positive correlation was found among colour a* and colour b* (0.659) and among these with chroma (0.976 and 0.726 respectively) and hue (0.640 and 0.949 respectively). A negative correlation between colour L* and TPC and AA was observed, indicating that genotypes with darker colour showed values highest of TPC and AA (Gen.9, Wonderful and Wonderful One); the same situation for pH, where negative correlation was observed.

Tab. 14. Correlation matrix (Pearson correlation coefficient, r) of physico-chemical and biochemical (TPC and AA) parameters of 14 different pomegranate cultivars.

Parameters	°Brix	pH	L*	a*	b*	C*	h°	TPC	AA
°Brix	**1**								
pH	-0.421***	**1**							
L*	-0.501***	0.542***	**1**						
a*	-0.024ns	-0.232ns	0.153ns	**1**					
b*	-0.048ns	-0.429***	0.156ns	0.659***	**1**				
C*	-0.055ns	-0.253ns	0.184ns	0.976***	0.726***	**1**			
h°	-0.018ns	-0.462***	0.045ns	0.640***	0.949***	0.687***	**1**		
TPC	0.518***	-0.688***	-0.751***	0.156ns	0.250ns	0.170ns	0.321*	**1**	
AA	0.433**	-0.679***	-0.725***	0.182ns	0.275ns	0.218ns	0.330ns	0.974***	**1**

°Brix, total soluble solids; L*, lightness; a*, color a*; b*, color b*; C*, chroma; h°, hue; TPC, total phenolic content; AA, antioxidant activity. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

4.1.3 Sugars and mineral salts

Based on the morphological and physico-chemical screening, nine genotypes were considered commercially interesting and stable over the years, between these seven Sicilian genotypes (Gen.1, Gen.2, Gen.3, Gen.5, Primosole, dente di Cavallo) and two internationals genotypes (Valenciana and Wonderful). On these cultivars the subsequent analyzes were carried out.

4.1.3.1 Sugars

Carbohydrates were quantified in juice of seven Sicilian and two international pomegranates. The main sugars detected in juices were glucose and fructose (Tab. 15). The glucose concentration was mainly greater than fructose, with the ratio glucose/fructose taking values from 0.9 to 1.3. Similar profiles were previously described in other cultivars, one of this was Primosole (Ozgen et al., 2008; Cam et al., 2009; D'Aquino et al., 2010). In contrast, it is reported that the Spanish genotypes have almost always higher levels of fructose than glucose (Melgarejo et al., 2000) as reported for Wonderful and Valenciana grown in Spain (Mena et al., 2011; Alcaraz-Mármol et al., 2017).

In this work the highest amount of glucose was detected in Genotype 2 and in Wonderful with 64.16 g/l and 62.54 g/l respectively, the lowest in Genotype 6 with 36.21 g/l. Similar trend was for fructose with Genotype 2 showed also the highest amount of fructose (68.90 g/l) while Genotype 6 the lowest (27.84 g/l). Juices that contain equal amounts of glucose and fructose are the most recommended. High levels of fructose also lead to higher LDL level and greater insulin resistance, while a high level of glucose did not show this effect (Stanhope et al., 2009).

Sugar profile contributes to potential health benefits (Aarabi et al., 2008) and determines the sensory attributes of pomegranate, juice red pomegranate cultivars usually have a sourer taste than pink-white genotypes. Fig. 29 shows the chromatogram of one of the PJs (Dente di Cavallo).

Tab. 15. Sugar contents (g/l) of pomegranate juices.

Cultivar	Glucose	Fructose	Total sugars
		(g/l)	
Genotype 1	45.33±0.79de	33.54±0.39de	78.87
Genotype 2	64.16±0.09a	68.90±0.24a	133.06
Genotype 3	43.18±1.20ef	32.03±0.80e	75.20
Genotype 5	46.21±2.29d	34.44±1.54d	80.65
Genotype 6	36.83±0.96g	27.84±0.63g	64.67
Primosole	48.69±0.28c	35.51±0.15d	84.20
Dente di Cavallo	52.81±1.16b	38.36±1.36c	91.17
Valenciana	40.96±2.62f	29.89±1.72f	70.85
Wonderful	62.54±0.23a	62.50±0.04b	125.04

Data are expressed as mean ± SD (n=3). Significant differences ($P < 0.05$) are indicated by different letters.

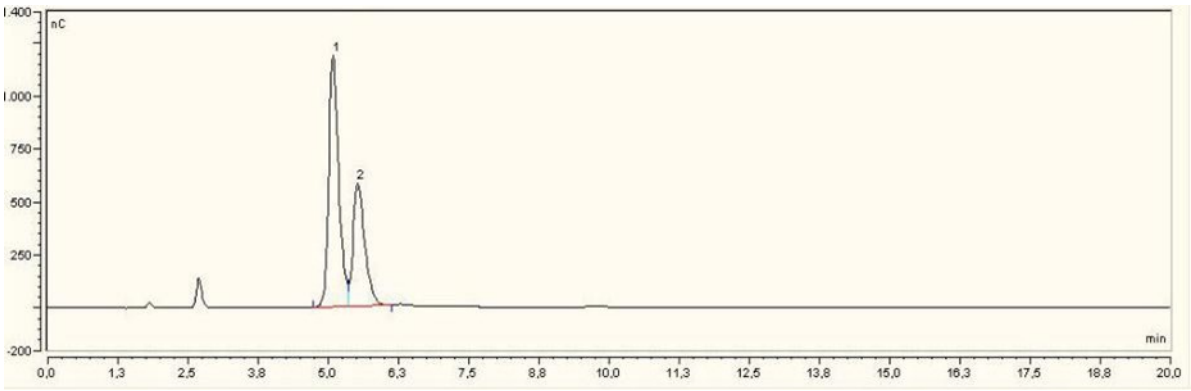


Fig. 29. HPAE-PAD chromatogram of pomegranate juice (Dente di Cavallo) showing the glucose (1) and fructose (2) peaks.

4.1.3.2 Mineral salts

Minerals are substances required by the body in small quantities to perform many vital functions. These include for example the formation of bones and teeth or take part in the composition of body fluids and tissues, as well as the function of enzymatic systems and nervous function. The mineral salts in pomegranate juice, shown in Tab. 16, varied significantly among the genotypes. The highest content of macro-elements present in all samples of juices was potassium, followed by chlorides and phosphates. Al-Maiman and Ahmad et. al. (2002) reported that the potassium and sodium content were the highest among the mineral elements in pomegranate juice.

Concerning anions (Fig. 30), the highest values were found in the genotype Dente di Cavallo (1367.6 mg/l) followed by Wonderful (1234.20 mg/l). Lower values were generally found in the Sicilian genotypes, especially in Genotype 1 (740.47 mg/l). The relative order of concentration of anions was $Cl^- > PO_4^{3-} > SO_4^{2-} > F^-$. The content of chlorides, sulphates and fluorides was greater in Dente di Cavallo, whereas the content of phosphates in Wonderful.

Regarding cations (Fig. 31) the greatest results were generally found in Wonderful (2350.17 mg/l) and Dente di Cavallo (2241.74 mg/l), the lowest value was in Genotype 5 (1362.84 mg/l). The order of concentration of cations was $K^+ > Mg^{2+} > Na^+ > Ca^{2+}$. Potassium, sodium and calcium were predominant in Wonderful, whereas magnesium in Genotype 3. Overall, among the genotypes analysed Dente di Cavallo and Wonderful showed the highest concentration of minerals, 3609.34 and 3584.37 mg/l respectively. The pomegranate juice appears to be a good source of nutrients and variation in mineral composition could originate from the pomegranate genotypes as well as agro-climatic conditions, handling practices and manufacturing conditions (Melgarejo et al., 2000; Vardin and Fenercioğlu, 2003).

Tab. 16. Mineral salts (mg/l) of pomegranate juices.

Cultivar	Fluorides	Chlorides	Phosphates	Sulphates	Sodium	Potassium	Magnesium	Calcium
Genotype 1	42.97±1.52e	365.17±7.88f	262.80±5.95h	69.53±0.40i	9.21±0.92e	1710.42±19.95d	54.32±1.26c	8.66±1.69c
Genotype 2	41.80±0.72e	296.07±2.02h	333.40±3.95d	83.70±1.51g	13.33±0.49c	1819.33±13.83c	57.57±0.54b	7.67±1.06c
Genotype 3	48.27±1.33c	317.63±2.87g	310.87±7.49f	113.20±1.45d	13.58±0.24c	1679.56±68.23d	72.08±3.02a	6.98±0.40c
Genotype 5	45.60±1.52d	380.83±3.86e	294.47±4.91g	90.93±2.01f	12.81±0.31cd	1287.99±70.45e	53.98±2.81c	8.05±0.69c
Genotype 6	41.67±0.29e	285.70±1.04h	317.17±2.72e	139.97±1.40b	11.70±1.05d	2056.63±19.41b	69.58±0.61a	30.38±1.20b
Primosole	39.03±1.22f	489.63±7.94c	387.67±0.61c	126.60±0.44c	14.08±0.14c	1668.04±0.56d	49.00±1.02d	7.29±0.53c
Dente di Cavallo	59.90±0.61a	637.10±12.00a	463.87±8.28b	206.73±3.23a	18.53±1.69a	2142.66±16.43b	70.60±0.73a	9.95±1.18c
Valenciana	42.37±0.42e	422.00±1.67d	387.80±0.36c	93.97±1.21e	16.09±0.88b	1749.37±28.68d	48.75±0.93d	8.15±1.60c
Wonderful	51.33±0.75b	557.97±6.33b	545.30±8.31a	79.60±1.47h	18.84±0.86a	2236.56±51.49a	57.84±1.85b	36.93±1.20a

Data are expressed as mean ± SD (n=3). Significant differences ($P < 0.05$) are indicated by different letters.

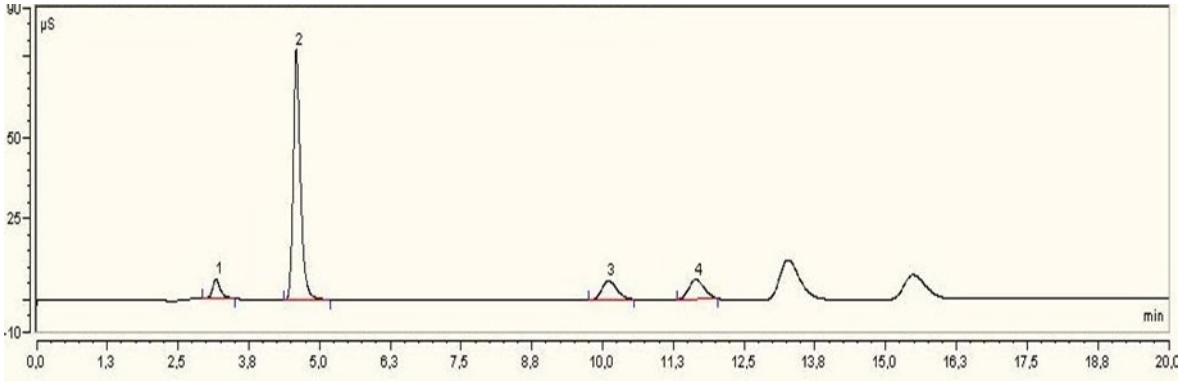


Fig. 30. IC chromatogram of pomegranate juice (Dente di Cavallo) showing the fluorides (1), chlorides (2), phosphates (3) and sulfates (4) peaks.

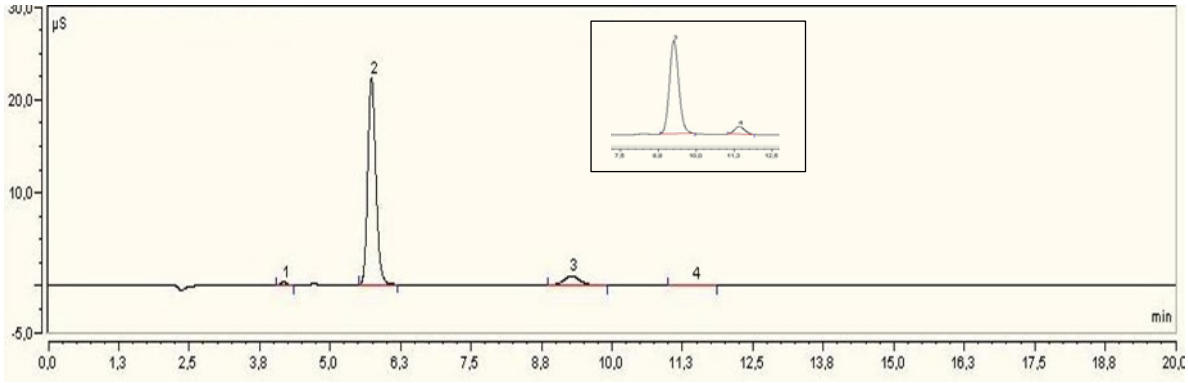


Fig. 31. IC chromatogram of pomegranate juice (Dente di Cavallo) showing the sodium (1), potassium (2), magnesium (3) and calcium (4) peaks.

4.1.4 Sicilian germplasm genetic diversity evaluation

On the seven Sicilian genotypes and the two international genotypes analyzed for sugars and mineral salts, genetic diversity was also evaluated.

The purity of extracted DNA, expressed as ratio of A260/A280, was between 1.75 and 1.86 and the concentration ranged from 164.7 to 670 ng/µl (Fig. 32). Basing on these values, all the samples were considered suitable for downstream analyses.

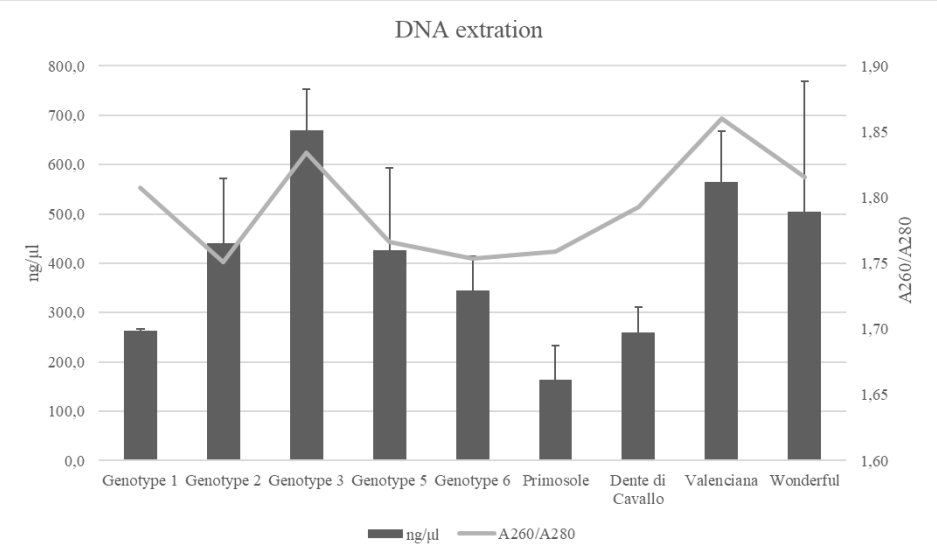


Fig. 32. DNA extraction concentration (ng/ul), on the left axis, and purity (A260/A280 absorbance), on the right axis. Bars follow the left axis, while the trend line refers to right axis. The error bars indicate the Standard Deviation of five biological replicates.

Out of 75 primer pairs screened on pomegranate genotypes, 11 (7 literature-derived and 4 newly designed) were selected basing on heterozygosity, number of alleles, allele size and amplification reproducibility, preferring tetra- and tri-nucleotides compared to di-nucleotides. The analysed markers produced a total of 96 alleles, with maximum number of alleles per locus ranging from 4 to 14, and a length of the amplified bands comprised between 130 bp to 367 bp (Fig. 33). The polymorphic information content (PIC) was used to measure the genetic diversity. High, medium or low loci polymorphism is in accordance with PIC > 0.5, 0.5 > PIC > 0.25 and PIC < 0.25, respectively (Vaiman et al., 1994; Parvaresh et al., 2012). A high PIC for all the markers used in this study was observed, on average 0.753, ranging from 0.469 of *pg4* to 0.891 of *pg14* (Tab. 17) higher than the previous analysis with the same microsatellites (Ebrahimi et al., 2010; Parvaresh et al., 2012).Among the new microsatellite markers developed in this study, *MYBmp04* and *MYBmp01* displayed a very high variability among the studied genotypes with PIC values of 0.875 and 0.775 respectively (Tab. 15). Although with a less degree, *MYBmp02* and *MYBmp03* markers showed relevant variability and all of them could be recommended for further genetic analyses aimed at detecting molecular diversity in pomegranate germplasm collection. Shannon index (*I*), number of effective alleles (N_e) and heterozigosity (H_e) values were consistent with the PIC trend. The Nei's genetic diversity and Shannon's information index in Sicilian pomegranates in the present study were higher in comparison to Indian pomegranates, based on ISSR markers that reported by Narzary *et al.*

(2010). It can be showed that the SSR marker is a trustworthy technique for assessing genetic diversity in pomegranate genotypes. The average number of alleles was 8.727 which is significantly higher respect to what reported for Iranian pomegranate genotypes analysed with chloroplast SSRs (Norouzi et al., 2012) and even respect to previous analyses with nuclear microsatellites as well (Ebrahimi et al., 2010; Soriano et al., 2011). This set of 4 new SSR Myb along with the other 7 SSRs might be useful for population genetic analyses, such as genotyping and linkage mapping.

Tab. 17. Microsatellite allele data obtained using 11 polymorphic microsatellite loci in 9 pomegranate genotypes.

Locus	Major Allele Frequency	Genotype No	Allele No	Ho[a]	He[b]	Nei[c]	I[d]	PIC[e]
pg4	0.667	4	4	0.000	0.521	0.510	0.961	0.469
pg10(a)	0.458	7	7	0.542	0.704	0.689	1.392	0.642
pg14	0.167	14	14	0.000	0.918	0.899	2.463	0.891
pg21	0.25	14	11	0.458	0.887	0.869	2.205	0.862
pg22	0.25	10	10	0.000	0.876	0.858	2.109	0.843
Pg17	0.313	13	9	0.417	0.836	0.819	1.902	0.797
Pom047	0.208	10	10	0.000	0.894	0.875	2.178	0.862
MYBmp01	0.292	8	8	0.000	0.819	0.802	1.794	0.775
MYBmp02	0.458	7	7	0.000	0.723	0.708	1.516	0.671
MYBmp03	0.542	5	5	0.000	0.653	0.639	1.258	0.597
MYBmp04	0.208	11	11	0.000	0.904	0.885	2.279	0.875
Average	0.347	9.364	8.727	0.129	0.794	0.778	1.823	0.753

[a] Observed heterozygosity; [b] Expected heterozygosity; [c] Effective number of alleles;

[d] Shannon's Information index; [e] Polymorphism information content.

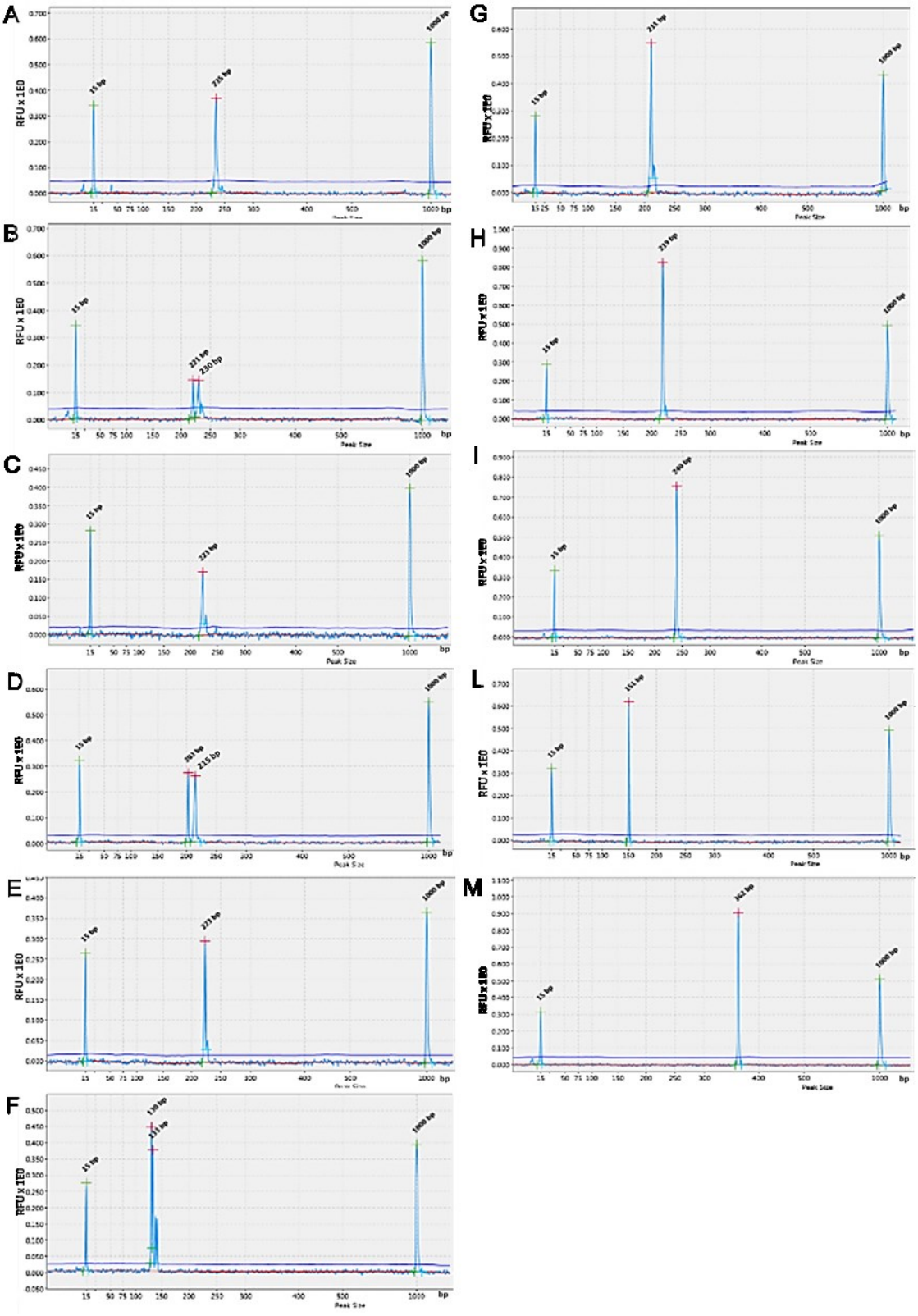


Fig. 33. Capillary electrophoresis run of the PCR amplification products of Wonderful variety with literature derived ones pg4 (A), pg10 (B), pg14 (C), pg21 (D), pg22 (E), Pg17 (F), Pom047 (G) and new developed markers MYBmp01 (H), MYBmp02 (I), MYBmp03 (L), MYBmp04 (M) and. MYB derived markers were always homozygous, while pg17, pg21 and pg10 were heterozygous. Peaks at 15 and 1000 bp correspond to the alignment marker used in the analysis.

Cluster analysis (Fig. 34) obtained from all 11 microsatellites, showed that there is a genetic differentiation among the international genotypes, the nine accessions were clustered into 3 main groups. The first one was composed of Dente di Cavallo, Genotype 1, Genotype 2 and Valenciana while the second one included Genotype 3, Genotype 5, Primosole and Wonderful. On the other hand, Genotype 6 was distinctly separated, forming an outgroup, from all the other tested varieties.

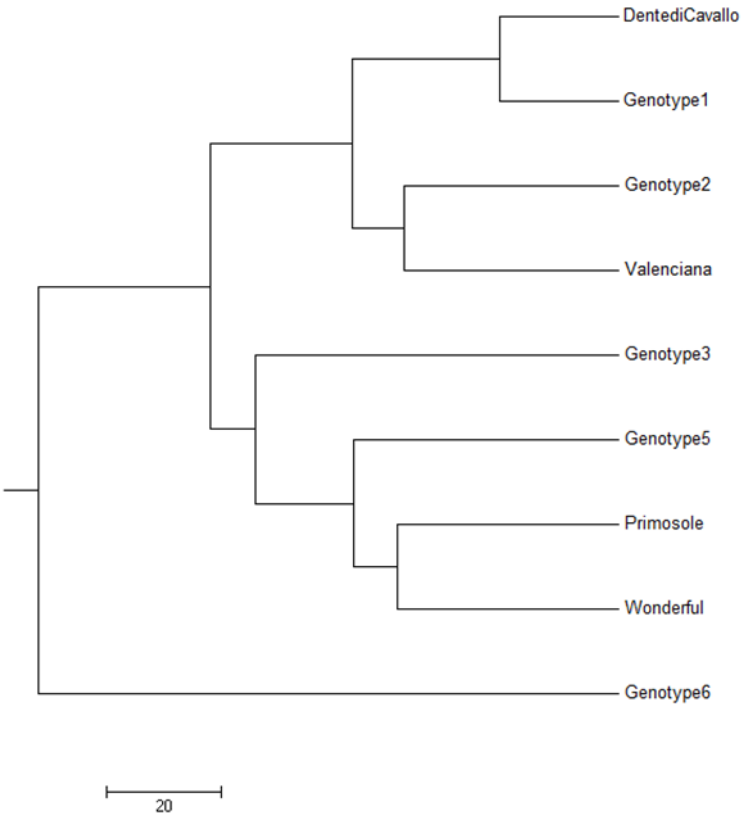


Fig. 34. UPGMA dendrogram of 9 pomegranate genotypes based on all nuclear microsatellite (SSR) markers described in Table 15.

Analysing separately the 7 nuclear microsatellites and the 4 *Myb* microsatellites it is observed a different behaviour among the genotypes. The UPGMA on the 7 SSR showed a first cluster that include international genotypes with three Sicilian genotypes, a second cluster including Genotype 3 and Genotype 5 and a last cluster with Genotype 2 and Genotype 6 (Fig. 35A). While the UPGMA obtained only on the subset comprising 4 *Myb* SSR (Fig. 35B) exhibited three different clusters the first with Dente di Cavallo associated with Genotype 1, the second with Sicilian Genotypes comprising also Valenciana and a last separate cluster with Wonderful

and Primosole. The different clusterization of the two international commercial varieties observed in this dendogram could arose from the highly different phenolic compounds composition between Wonderful and Valenciana as reported along this study.

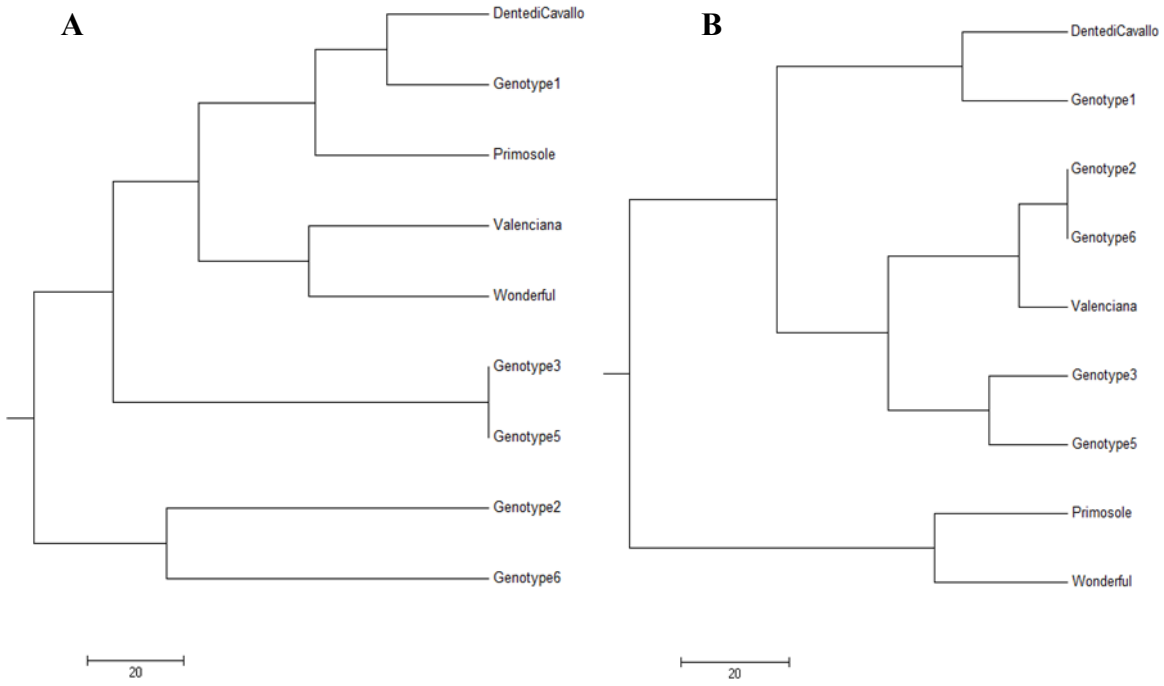


Fig. 35. UPGMA dendrogram of 9 pomegranate genotypes based on A) 7 SSR and B) 4 *Myb* microsatellite (SSR) markers described in Table 16.

Considering the UPGMA analyses, it can be observed that Dente di Cavallo and Genotype 1 always clustered together also Genotype 5 and Genotype 3, Wonderful and Primosole in general are closely related, while Valenciana showed association with Sicilian genotypes in particular with Genotype 1, Genotype 2 and Dente di Cavallo. The affinity between Valenciana and the most of Sicilian genotypes could be due to a common genetic origin since they have been domesticated within the Mediterranean basin; similarly the clustering of Primosole together with Wonderful could be explained by a common genetic matrix between the two as shown by Parvaresh *et al.* (2012) referring to “Palermo” genotype.

4.1.5 Principal component analysis

The UPGMA analysis is in accordance with the PCA (Fig. 36) elaboration obtained using morphological and biochemical analyses of nine genotypes. Indeed, the two data sets seems to be positively correlated (r= 0.43) with a significant extent (p= 0.019) that supports the presence of correlation between morphological and microsatellite data. The isolation of the Genotype 6 from the compact cloud composed by Dente di Cavallo, Genotype 1, Genotype 2 and Valenciana shown in the PCA elaboration based on fruit characters is coherent with the UPGMA dendrogram considering all primers and the literature-derived primers elaborations. In the PCA this behaviour could be due to the fruit weight and aril weight which may have influenced its distribution; while in the UPGMA dendrograms this can be due to a slightly different genetic origin. Moreover, the isolation of Wonderful from the other cultivars observed in the PCA is in agreement with the MYB related genes UPGMA. This behaviour could be affected by the use of microsatellites derived from *MYB* related genes, which are linked to anthocyanins production in pomegranate fruits. On the other hand, the compact cloud including Sicilian genotypes and Valenciana shows how these accessions are very similar each other either from a genetic and a physico-chemical point of view. As regards to Genotype 5, while the dendrograms show the common genetic matrix with the other Sicilian accessions, in the PCA it exhibits an intermediate stance that seems to be influenced by its peculiar juice – chemical characteristics (TPC and AA) as reported in this study.

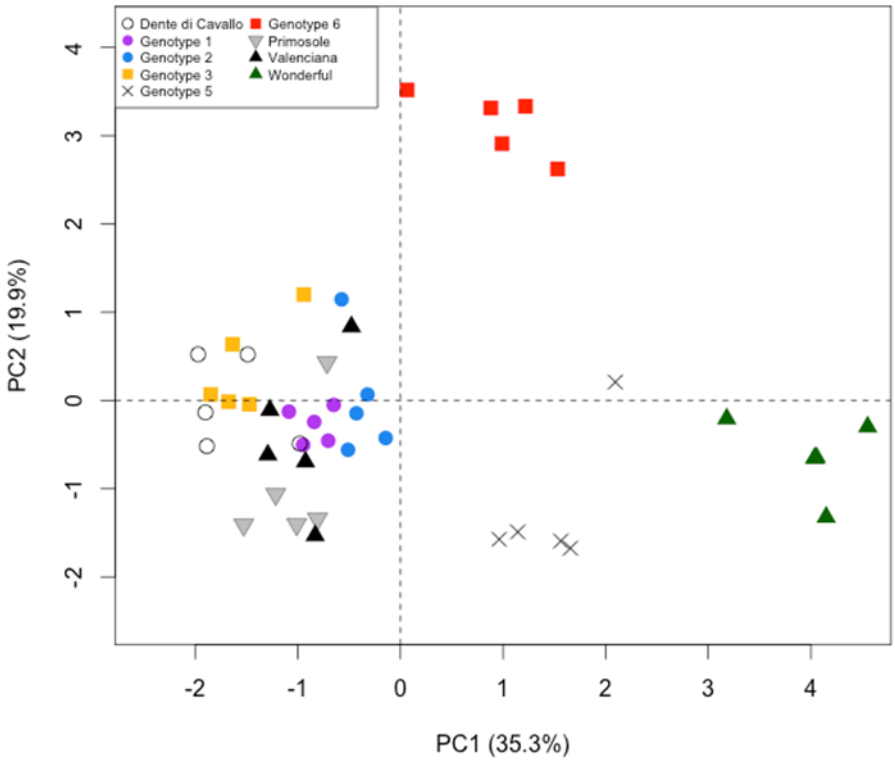


Fig. 36. Principal component analysis of 9 individuals per each population where the first two axes explain the 55.2% of the total variance.

4.1.6 Seed germination

Seed dormancy is controlled by exogenous (e.g., light, temperature, and moisture) and endogenous factors, these are primarily hormones, but also include small molecules such as reactive oxygen species and nitric oxide (NO). Dormancy is often broken naturally by the after-ripening process, but it can also be ended artificially through stratification (subjecting seeds to moisture and cold) (Bewley and Black 1994).

The seeds of different varieties of pomegranate analysed did not show any dormancy, but a marked variability was observed (Fig. 37). In particular, Dente di Cavallo exhibited the lowest germination (42.9%), while Primosole and Valenciana the highest (93.9% and 94% respectively). Addition of Gibberellic acid did not alter the germination behaviour increasing germinability only for Genotype 6 (89.8%) in keeping to what previously found in Chinese pomegranate seed germination (Shalimu et al., 2015). Nitrate addition did not alter significantly the germination performances of the sampled populations.

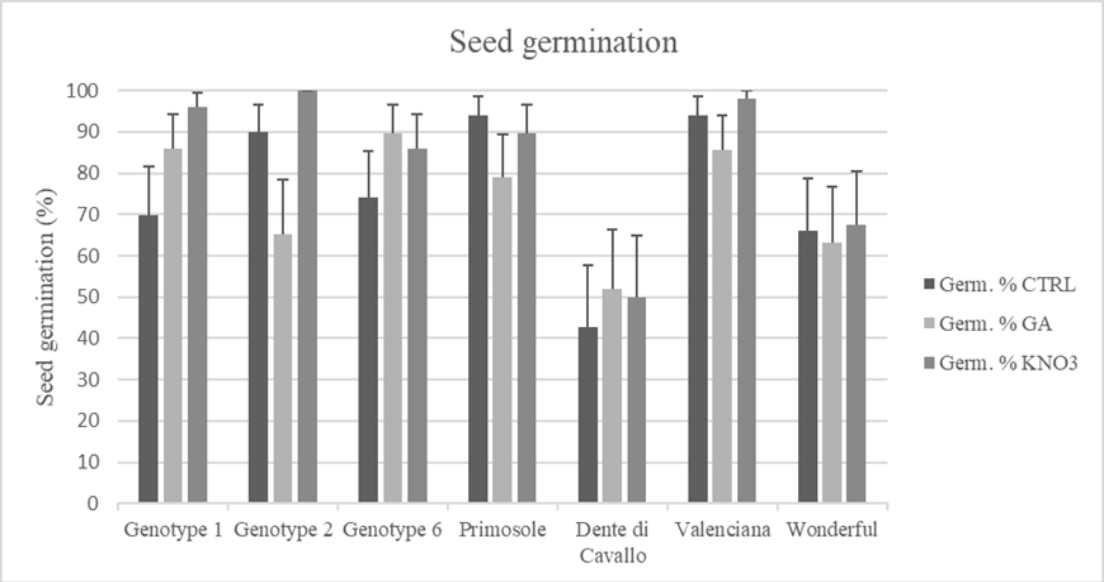


Fig. 37 Mean final germination (%) of seed from 7 cultivars treated with GA, KNO_3 and the control with H_2O, CTRL incubated at 20 °C. The lower and upper bars show the binomial confidence.

Tab. 18 Generalized linear model of 7 cultivars.

Model		**Coefficient**	**SE**	**Z**	**P value**
AIC= 166.92	**Treat: GA 100 µM**	-0.36	0.19	-1.92	0.05 .
	Treat: KNO3 20 mM	0.00	0.19	-0.03	0.98
	Genotype 1	1.06	0.28	3.82	0.00 ***
	Genotype 2	1.17	0.29	4.08	0.00 ***
	Genotype 6	0.65	0.24	2.75	0.01 **
	Primosole	1.39	0.3	4.57	0.00 ***
	Dente di Cavallo	-0.65	0.24	-2.75	0.01 **
	Valenciana	1.96	0.36	5.51	0.00 ***
	Wonderful	0.06	0.24	0.24	0.81

AIC (Akaike Information Criterion). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

The results of Generalized linear model (Tab. 18) show that germination is more influenced by the genotype rather than by treatment. The Gen. 1, Gen.2, Primosole and Valenciana exhibited the most significant differences respect to the others. Among the studied varieties, remarkable germination performance differences were observed between the Sicilian and international cultivars. The greatest germination variation among genotypes was observed in the germination rate measurement (GR) where the studied varieties showed substantial differences. In

particular, among the international varieties, Valenciana showed the highest GR with germination time of 50% (t50) of the seeds (Fig. 38) in 6 days for CTRL and 6.6 days for treatments, exceeding 85% of final germination in all the tests. As regards to the Sicilian varieties, the Genotype 1 stands out with germination speed with time of 50% in 10.50 with KNO_3, 11 and 11.83 for the CTRL and GA respectively, followed by the Genotype 2 that despite the slower germination rate of the Genotype 1, reaches the 100% of the final germination with KNO_3. Seeds of "Dente di Cavallo" presented a low germination rate with germination time of 50% in 59 days with GA and 54 with KNO_3, followed by "Wonderful" with about 33 days for all treatments. The last two cultivars exhibited a lower seed vitality compared to the other accessions. While Primosole and Genotype 6 showed intermediate germination levels between the tested cultivars.

These data are the first results on Sicilian genotypes and they indicate that germination response vary highly across cultivars. Among commercial varieties, the major differences were found between Dente di Cavallo and Valenciana. This feature could be attributed to the seed coat hardness which is known to be high in Dente di Cavallo and very low in Valenciana which is considered as a soft-seed variety. The woody character of the inner seed coat significantly hamper the embryo expansion and consequent radicle protrusion producing a slower germination.

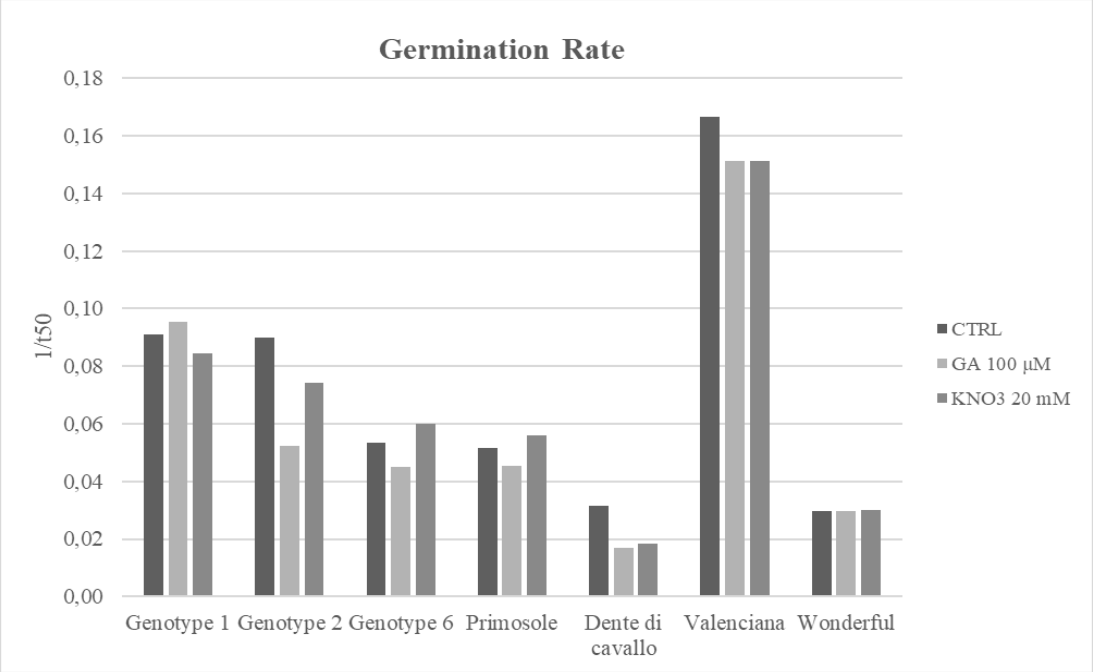


Fig. 38. Germination rate (GR) of 7 cultivars treated with GA, KNO_3 and the control with H_2O, CTRL.

4.2 Metabolomic profile in pomegranate juice

The liquid chromatography method associated with the Orbitrap allowed the identification and quantification of phenolic compounds in pomegranate juice of five different cultivars, two Sicilian genotypes Gen.1, Gen.2, selected based on commercial interest, and tree commercial cultivars Valenciana (VL), Wonderful (WD) and Wonderful One (WD1).

4.2.1 Identification of phenolic compounds

The identification of phenolic compounds was done by LC-PDA Orbitrap FTMS in negative ion mode. A database was constructed based on literature research (Fisher et al., 2011a; Mena et al., 2012; Calani et al., 2013; Gómez-Caravaca et al., 2013; Wyrepkowski et al., 2014; Brighenti et al., 2017) to include metabolites reported to be present in juice. Ten standards (see Paragraph 3.3.1) were injected and compared for retention time, accurate mass, and PDA spectra with LC peaks of six anthocyanins, ellagic acid, gallic acid, punicalagins and rutin, while other compounds (ellagitannins, gallotannins and some flavonoids) were tentatively identified by combining the information obtained with PDA and MS detectors in this study and with literature data. A single LC-MS in the negative ionization mode was used to analyse anthocyanins and non-anthocyanins phenolic compounds. Traditionally anthocyanins analysis in MS is carried out in the positive ion mode only as reported in most of literature (Mena et al., 2012, Gomez-Caravana et al., 2013) and few cases in negative (Falani et al., 2016). The mass spectrometric behaviours of anthocyanins in negative ion mode were studied in detail (Sun et al., 2006), this method can provide rapid and reliable identification and differentiation of anthocyanins and non-anthocyanin polyphenols generated from a single LC with or without the use of an UV/Vis detector (Sun et al., 2006). According to what obtained from Sun *et al.* (2012) we found different characteristic ions $[M–2H]^-$ and $[M–2H+H_2O]^-$ doublet ions and adduct with formic acid $[M–2H+HCOOH]^-$. Typically ion chromatography in negative mode of some anthocyanins was reported in Fig. 39 and $[M-2H+H_2O]^-$ was found the most predominant ion in negative mode.

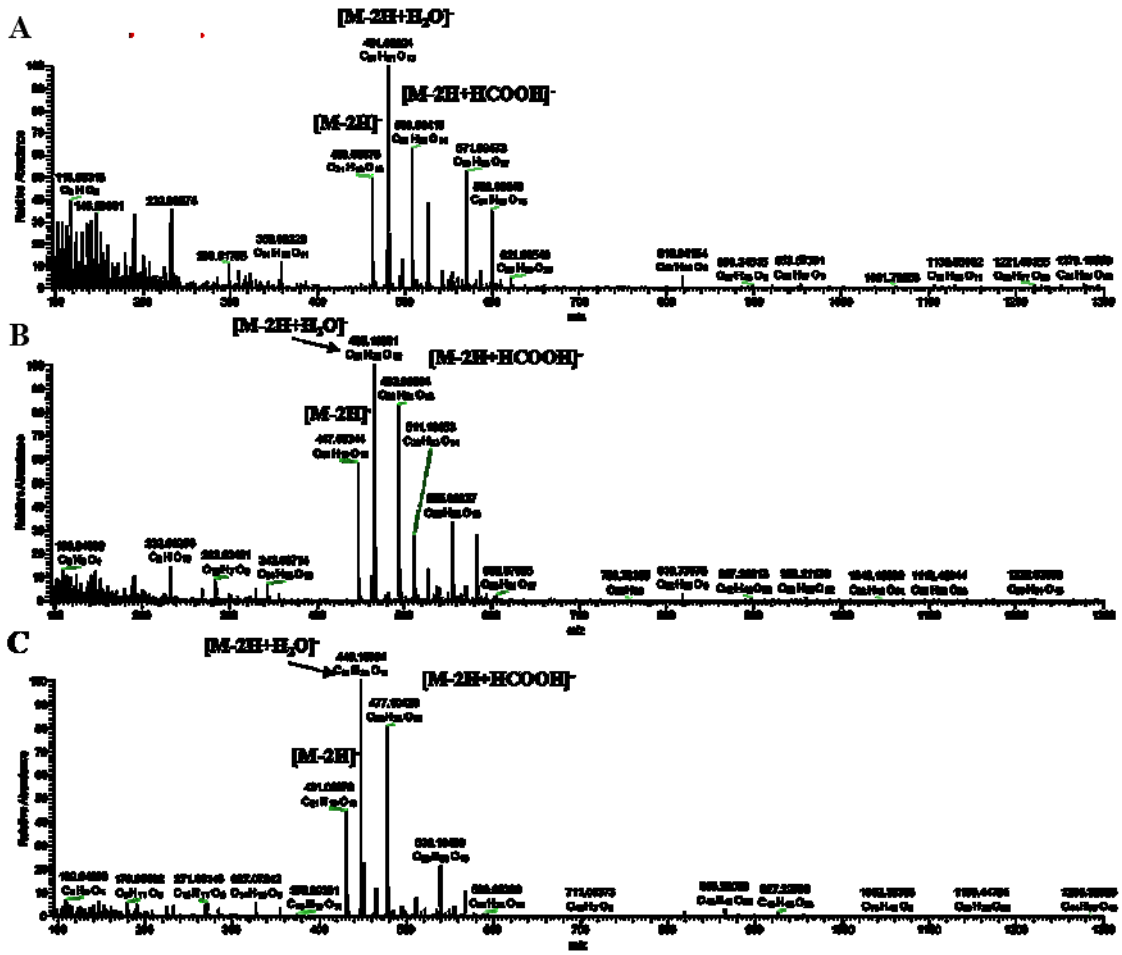


Fig. 39. Typical MS spectra of delphinidin 3-glucoside (A), cyanidin 3-glucoside (B), pelargonidin 3-glucoside (C) in negative ion mode.

Anthocyanins and hydrolyzable tannins are the most abundant phenolic compounds in pomegranate juices and include ellagitannin as punicalagins, gallotannins and gallogyl esters (Gil et al., 2000; Fisher et al., 2011). Anthocyanins are pigments responsible for the red, purple, orange and blue colour of many flowers, vegetables and fruits; the intensity of the typical red colour of pomegranate fruit depends on the concentration of the anthocyanins (Gil et al., 1995). Anthocyanins are important in human health, for their beneficial effects (Wang and Martins-Green, 2014, Tibullo et al., 2016).

In this study six anthocyanins, nine ellagitannins, five gallotannins and three flavonoids were detected in five different juice although at different levels, except for gallic acid and rutin no peak was found (Tab. 18 and 19). Data obtained for pelargonidin-3-glucoside in WD1, pelargonidin-3,5-diglucoside in Gen.1 and Gen.2 and for trigalloylglucose in Gen.1, Gen.2 and VL were below the detectable limit (< LOD) set to 10,000 ions per scan.

Tab. 18. Identification of anthocyaninsin juices.

Compound	Assignment	rt (min)	HPLC-PDA λmax (nm)	$[M-2H+H_2O]^-$ *m/z*	Gen. 1	Gen. 2	VL	WD	WDI
1	Delphinidin-3-glucoside [a]	8.27	276, 521	481.0984	x	x	x	x	x
2	Cyanidin-3-glucoside [a]	9.99	280, 323, 516	465.1038	x	x	x	x	x
3	Pelargonidin-3-glucoside [a]	11.78	277, 329, 427, 500	449.1086	x	x	x	x	
4	Delphinidin-3,5-diglucoside [a]	5.02	276, 517	643.1509	x	x	x	x	x
5	Cyanidin-3,5-diglucoside [a]	6.54	278, 515	627.1563	x	x	x	x	x
6	Pelargonidin-3,5-diglucoside [a]	8.31	276, 498	611.1613			x	x	x

[a] Compounds identified by comparing retentions time, HPLC-PDA and MS data with reference standards

Tab. 19. Identification of phenolic compounds in juices.

Compound	Assignment	rt (min)	HPLC-PDA λmax (nm)	$[M-H]^-$ m/z	Gen. 1	Gen. 2	VL	WD	WDI
	Ellagitannins								
7	Punicalagin α [a]	8.29	258, 382	1083.0601	x	x	x	x	x
8	Punicalagin β [a]	10.64	258, 379	1083.0604	x	x	x	x	x
9	Ellagic acid [a]	21.96	253, 368	300.9991	x	x	x	x	x
10	Ellagic acid pentoside	21.03	253, 362	433.0419	x	x	x	x	x
11	Ellagic acid deoxyhexose	21.45	253, 362	447.0575	x	x	x	x	x
12	Ellagic acid hexoside	16.41	253, 362	463.0526	x	x	x	x	x
14	Pedunculagin monomer II	6.42	n.d.	783.0698	x	x	x	x	x
15	Pedunculagin dimer III [b]	13.26	n.d.	1567.1464	x	x	x	x	x
17	Galloyl HHDP hexose	14.83	n.d.	633.0743	x	x	x	x	x
	Gallotannins								
19	Gallic acid [a]	4.48	271	169.0144					
20	Gallotannins (Galloyl glucose)	3.50	n.d.	331.0675	x	x	x	x	x
22	Digalloyl hexose I isomer II	10.87	n.d.	483.0791	x	x	x	x	x
23	Digalloyl hexose I isomer III	11.43	n.d.	483.0790	x	x		x	x
24	Digalloyl hexose I isomer IV	13.06	n.d.	483.0790	x	x	x	x	x
25	Trigalloylglucose	12.04	n.d.	635.0900				x	x
	Flavonoids								
31	Naringin	26.7	283, 334	579.1728	x	x	x	x	x
32	Rutin [a]	22.21	255, 355	609.1466					
33	Catechin	11.62	n.d.	289.0722	x	x	x	x	x
34	Epicatechin	15.2	n.d.	289.0722	x	x	x	x	x

Compounds identified by comparing with reference compounds in literature. [a] Compounds identified by comparing retentions time, HPLC-PDA and MS data with reference standards. [b] Compound appears as doubly-charged molecular ions. x, detected; n.d. λ not detected.

In Fig. 40 MS intensity signal of phenolic compounds are illustrated. The content of phenolic compounds was different among cultivars as well as reported from Gomez-Caravaza *et al.* (2016). High levels of ellagitannins (7-12, 15, 17) in particular ellagic acid hexoside (12) and galloyl glucose (20) were found in Wonderful One as well as narigenin (31). The astringent taste of this cultivar may be associated with the high phenolic compounds (Vardin et al., 2009). In contrast high levels of anthocyanins (1, 2, 4, 5) were shown in Wonderful comparable with those described in literature (Brighenti et al., 2017). Sicilian genotypes seem have the same profile, similar to Valenciana. Among flavonoids epicatechin (34) were major than catechin (33) and in particular in Gen.1, Gen. 2 and Wonderful.

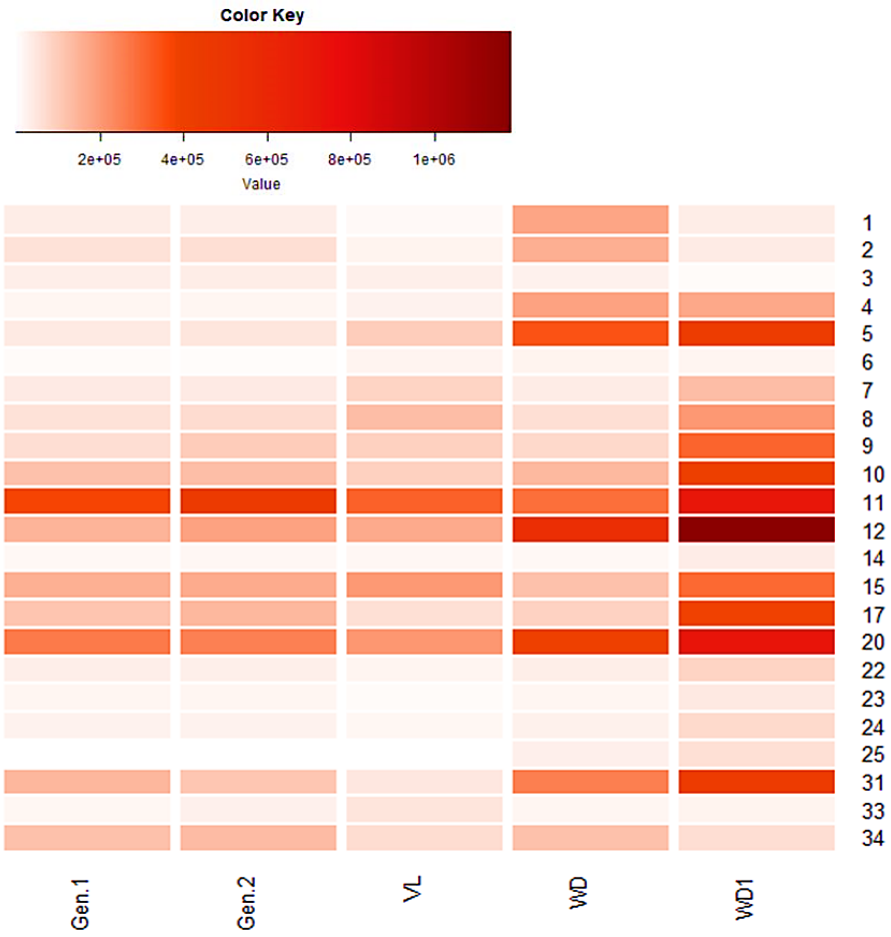


Fig. 40. MS intensity signal heat map of phenolic compounds found in Genotype1 (Gen.1); Genotype 2 (Gen.2); Valenciana (VL); Wonderful (WD); Wonderful One (WD1). Number of compounds correspond to number reported in Tabs. 18 and 19.

4.2.2 Quantification of phenolic compounds

Four phenolic compounds (gallic acid, ellagic acid, punicalagin α and punicalagin β) and six anthocyanins (delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside and pelargonidin-3,5-diglucoside) identified with negative mode were quantified (Tab. 20) in juice of five pomegranate accessions.

The characteristic MS spectra in negative ion mode of anthocyanins is more reliable than the traditional methodology of using a UV/Vis detector, especially when an anthocyanin is coeluting with another compound or the anthocyanins are present in low levels that a UV/Vis detector is not sufficient (Sun et al., 2012). Quantification was carried out using the absolute mass signal intensities expressed as peak height obtained by MetAlign software. The typical chromatogram of anthocyanins recorded at 510 nm and coeluting of delphinidin 3-glucoside and pelargonidin-3,5-diglucoside are shown in Fig. 41.

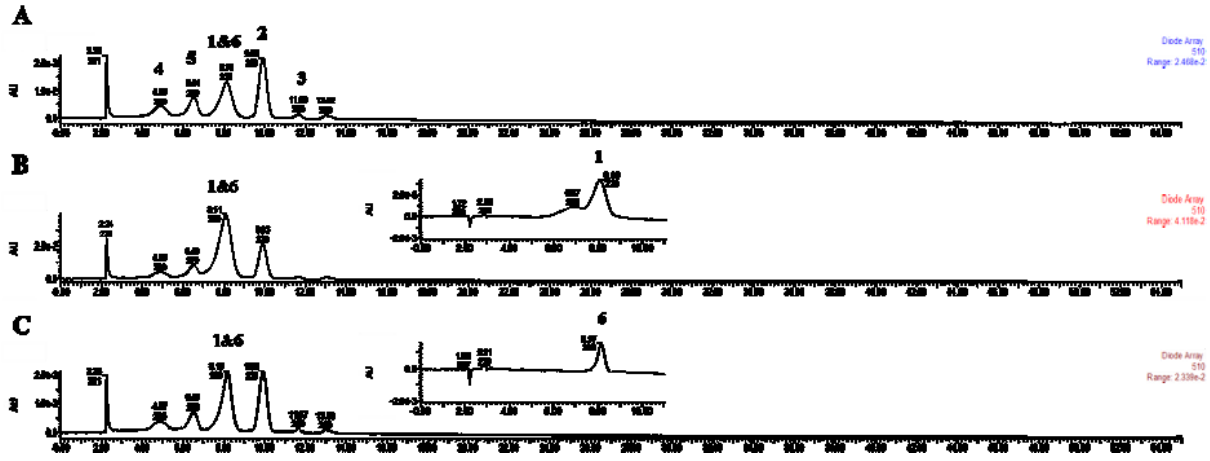


Fig. 41. A) HPLC-PDA chromatogram recorded at 510 nm showing the anthocyanins peaks of Wonderful juice and overlap of 1 and 6 peaks. Delphinidin-3-glucoside (1). Cyanidin-3-glucoside (2). Pelargonidin-3-glucoside (3). Delphinidin-3.5-diglucoside (4). Cyanidin-3.5-diglucoside (5). Pelargonidin-3.5-diglucoside (6). B) Wonderful juice plus delphinidin 3-glucoside standard (1). C) Wonderful juice plus pelargonidin 3.5-diglucoside standard (6).

The results from the present study showed large differences in anthocyanin composition and content among genotypes (Tab. 20, Fig. 42). Punicalagin (isomers α and β) is the most abundant

compounds among the pomegranate polyphenols (Lu et al., 2008), which have important antioxidant and atherosclerotic biological properties (Vegara et al., 2013), among polyphenol examined punicalagins had the highest concentration according to what reported in the literature (Nuncio-Jáuregui et al., 2015; Brighenti et al., 2017). In contrast to reported by Gomez-Caravaza *et al.* (2013) where Valenciana had a major quantitative of punicalagins than Wonderful One, in this study we found punicalagins mostly in Wonderful One with average value of 244.03 mg/l followed by Valenciana 140.74 mg/l. Sicilian genotypes and Wonderful had similar values. Ellagic acid showed the highest level in Wonderful One (3.10 mg/l).

To order anthocyanins profiles, significant differences in concentrations were found in accessions, and, as demonstrated by Fisher *et al.* (2011a) and Gomez-Caravaca *et al.* (2016), some anthocyanins compounds could not be detected in some cultivars. Wonderful showed the highest total anthocyanin content 248.11 mg/l against the lower content was found in Valenciana 26.87 mg/l. Pelargonidin-3,5-diglucoside was found the least abundant compounds among the analysed cultivars (5.05 mg/l) as observed in other Sicilian and Spanish cultivars (Todaro et al., 2016). In contrast cyanidin-3,5-diglucoside was found to be the predominant anthocyanin pigment (137.71 mg/l) in agreement with results obtained in peruvian juices and Chelfi pomegranates grown in Tunisia (Gil et al., 1995a; Fisher et al., 2011), while delphinidin-3-glucoside was found predominant in Portuguese pomegranates (Miguel et al., 2004). Anthocyanin composition and content can depend on plant origin and maturation, e.g. anthocyanin composition increases during ripening of the fruits (Gil et al., 1995). Different glycosylation of anthocyanins was found by Borochov-Neori *et al.* (2011) dependent on the harvest data and environmental conditions. It is possible to distinguish the pigmentation of pomegranate juices by their content in anthocyanins: high values of anthocyanins should be correlated with high value of colour a* (Dafny-Yalin et al., 2010). High concentration of cyanidin-3,5-diglucoside was found in Wonderful and Wonderful One which showed (see Paragraph. 4.1.1) high value of colour a*, positive value of colour b* and low values of L* confirm gloomy red colour of these cultivars.

Wonderful and Wonderful One, Genotype 1 and Genotype 2 reported Cyanidin-3-glucoside as the first compound and delphinidin-3-glucoside as second, Valenciana were characterized by the presence of cyanidin 3,5 glucoside, cyanidin-3-glucoside as first and second compound respectively. Delphinidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-glucoside and cyanidin-3,5-diglucoside were more concentrated in Wonderful than other cultivars. Wonderful One contained two major anthocyanin cyanidin-3,5-diglucoside and delphinidin-3,5-

diglucoside. Pelargonidin-3-glucoside and pelargonidin-3,5-glucoside were below the detectable limit in Wonderful One and Sicilian genotype respectively.

Tab. 20. Quantitative results of six anthocyanins and tree non-anthocyanins compounds in five genotypes.

Compounds	Assignment	Gen.1	Gen.2	VL	WD	WDI	total
1	Delphinidin-3-glucoside	9.71±1.94	8.74±0.83	3.04±1.22	74.00±13.84	7.88±2.82	103.36
2	Cyanidin-3-glucoside	13.28±2.38	15.40±2.41	4.81±2.23	54.02±5.97	8.60±0.81	96.07
3	Pelargonidin-3-glucoside	2.57±0.28	2.76±1.12	2.51±1.37	2.01±0.36	<LOD	10.38
4	Delphinidin-3,5-diglucoside	3.23±0.69	3.19±0.52	4.22±0.72	52.78±7.16	27.59±19.91	91.01
5	Cyanidin-3,5-diglucoside	3.83±0.70	4.61±0.47	10.80±1.01	63.64±5.37	54.83±30.69	137.71
6	Pelargonidin-3,5-diglucoside	<LOD	<LOD	1.49±0.40	1.66±0.30	1.06±0.40	5.05
	total anthocyanins	33.06	35.07	26.87	248.11	100.48	
7	Punicalagin α	33.28±8.35	33.84±11.11	76.070±10.14	31.27±3.91	127.70±13.83	302.16
8	Punicalagin β	25.49±7.94	31.15±5.22	64.67±14.11	28.55±3.45	116.33±19.50	266.20
9	Ellagic acid	0.50±0.06	0.79±0.29	0.73±0.11	0.58±0.07	3.10±1.64	5.69
	total phenols	59.28	65.78	141.47	60.39	247.12	

Gen.1: Genotype 1. Gen.2: Genotype 2. VL: Valenciana. WD: Wonderful. WDI: Wonderful One. Data are expressed as mean ± SD (n=3).

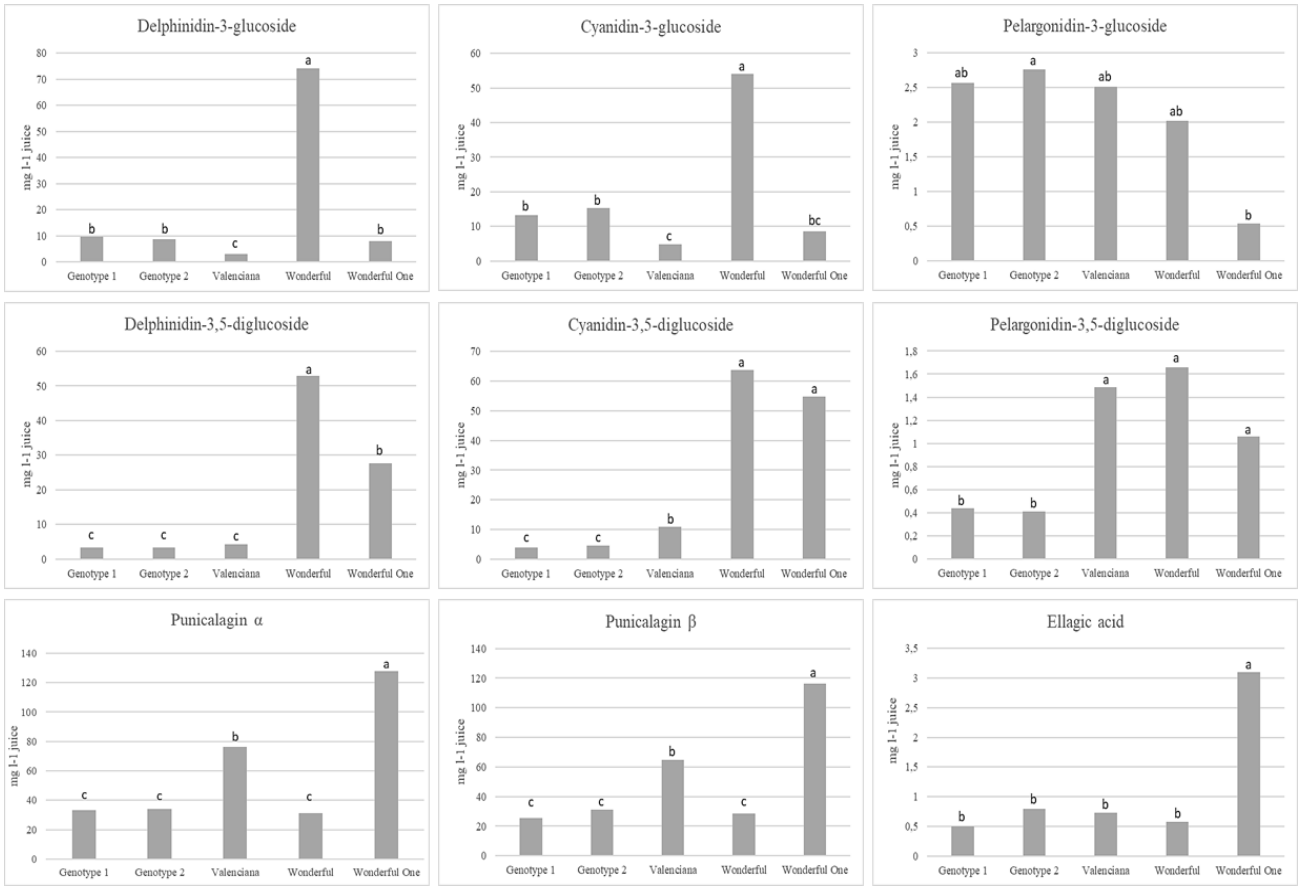


Fig. 42. Anthocyanins and non-anthocyanins profile of juice of 5 pomegranate cultivars. Values are the average of triplicate. Different letters indicate significant differences at P ≤0.05.

4.2.3 Principal component analysis (PCA) in pomegranate juice

In order to enable a simple visual interpretation of all untargeted metabolites found in different juices by Metalign-Galaxy workflow method, a principal component analysis (PCA) was performed. T1, T2 and T3 explained the 97.2% of the total variance among pomegranate cultivars, as reported in the screen plot (Fig. 43). T1 and T2 explained the major percentage in the variability of samples (88.1%). Two cluster were identified: the first cluster includes Valenciana, Genotype 1 and Genotype 2 and a second cluster comprises Wonderful and Wonderful One. The PCA showed Wonderful and Wondeful One closely together, while it was observed that these cultivars displayed large differences in the profiles of some of the major phenolic compounds e.g. punicalagins and cyanidin-3-glucoside. Apparently, the general metabolic profile of these cultivars did not follow these metabolites.

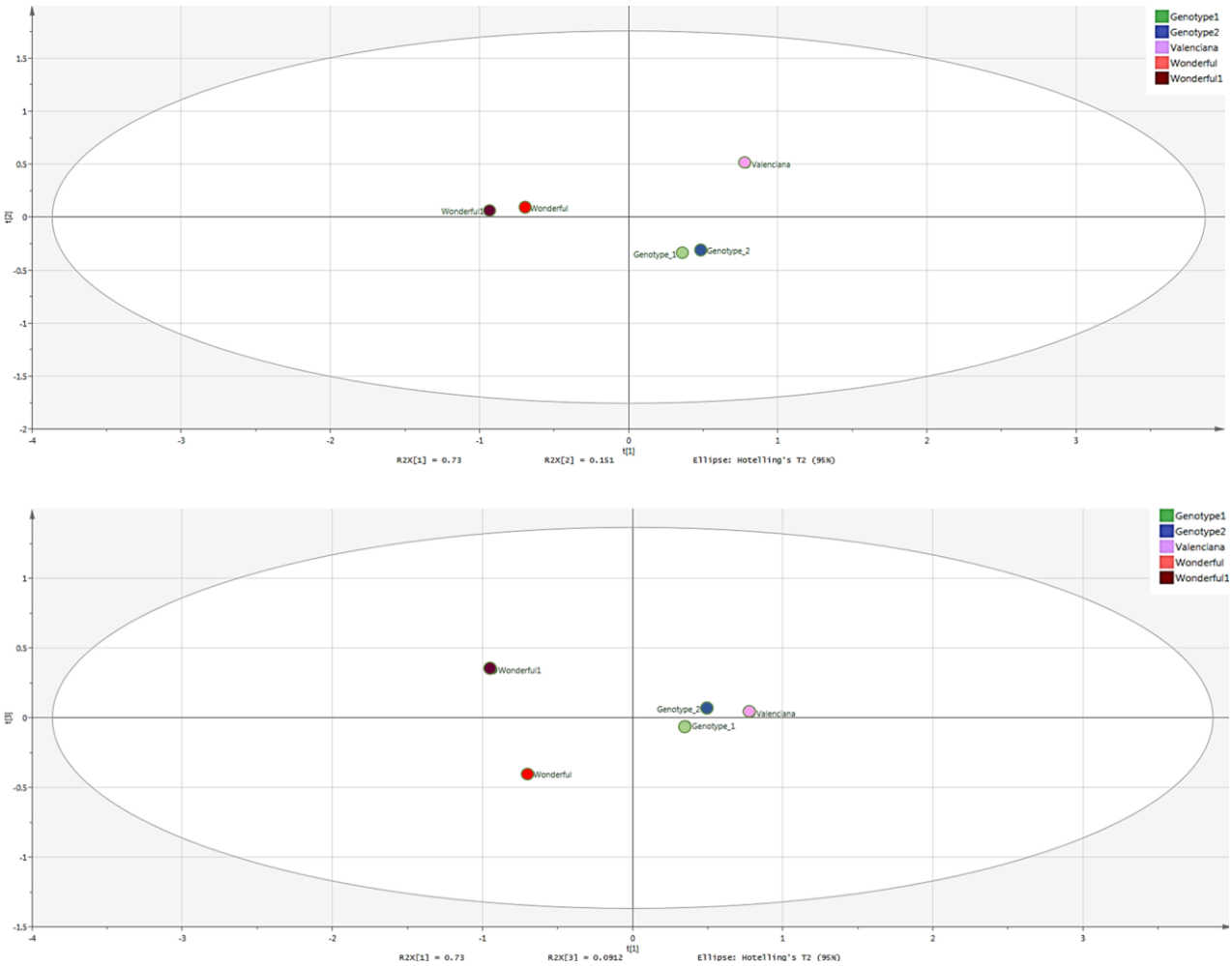


Fig. 43. Principal component analysis (PCA) of 5 genotypes: Genotype 1 (Blue); Genotype 2 (Green); Valenciana (Pink); Wonderful (Red); Wonderful One (Purple) of 262 metabolites

4.3 Metabolomic profile in different pomegranate fruit development stages

Based on the data obtained on the juices and using the same database, but more extensive, phenolic compounds were identified in two cultivars, one pink, Valenciana (VL), and the other red, Wonderful (WD), during some development stages: leaf, flower (stage-ST1), unripe arils (stage-ST2), turning arils (stage-ST3 F+S and stage-ST3+F) and ripe arils (stage-ST4 F+S and stage-ST4+F). The development stages and respectively extractions are shown in Fig. 44.

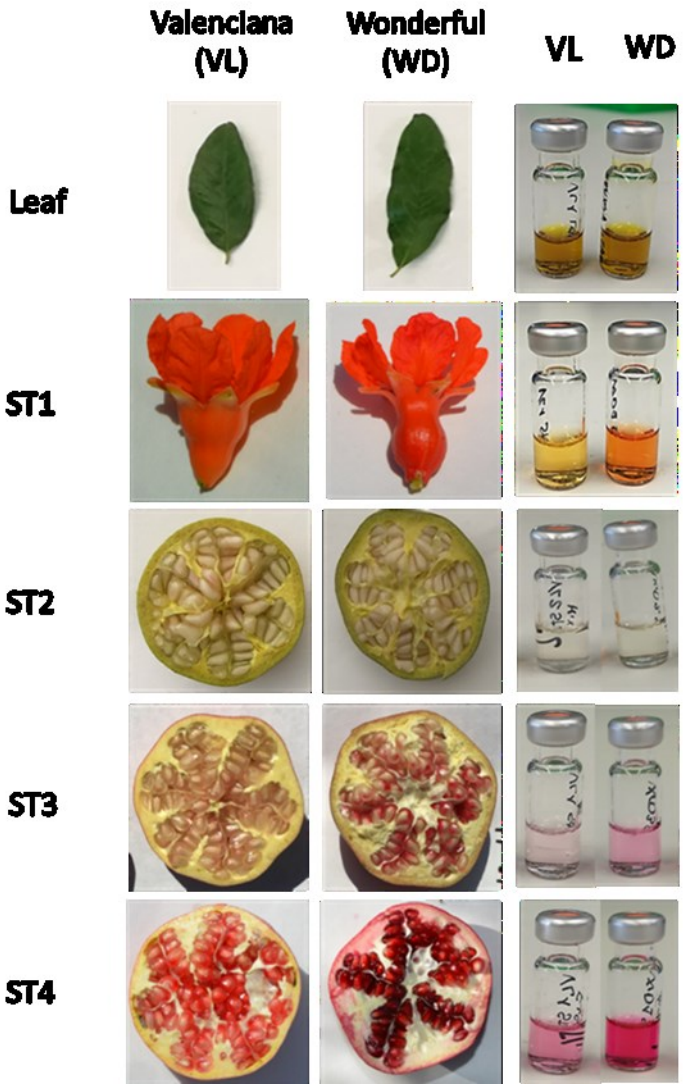


Fig. 44. Different development stages: leaf, flower (ST1), unripe arils (ST2), turning arils (ST3) and ripe arils (ST4) and respectively extractions for LC-MS analysis (95% of MeOH and 5% FA).

Colour in pomegranate peel and flesh tissues play an important role in its marketing (Pala et al., 2011). Fruit pigmentation increase during ripening (Gil., 1995) and it could be used as index of maturation. The majority of the studies carried out on the pomegranate examine anthocyanin contents in peel (Ben-Simhon et al., 2011, Zhao et al., 2015; Khaksar et al., 2015; Rouholamin et al 2015), that is known to be more abundant than juice (Fisher et al., 2011), but few studies (Ben-Simhon et al., 2015) evaluate the content of phenolic compounds during aril ripening stages and only in flesh.

In this study six anthocyanins, twelve ellagitannins, ten gallotannins, one galloyl ester and four flavonoids were identified during aril development distinguishing the whole aril (F+S) from the alone flesh (F). Retention time, HPLC-PDA and MS data are shown in Tab. 21. Area of peak obtained by QualBrowser was different between compounds and cultivars and showed in Fig. 45. According to the data obtained by Ben-Simihon *et al.* (2011) and Zhao *et al.* (2015) anthocyanins increase during fruit development stages, behaving differently depending on the genotype, except for perlargonidin-3,5-diglucoside which was found mostly in flower. Anthocyanin profile was examined also in leaves, but data obtained was low compared to the ripe stage. In leaves of Wonderful and Valenciana cyanidin-3,5-diglucoside and delphinidin-3,5-glucoside were found, while cyanidin-3-glucoside only in WD and very low signal of perlargonidin-3,5-diglucoside in VL were obtained. Also Ben-Simhon *et al.* (2015) evaluated anthocyanins in leaves, but they found only cyanidin-3,5-diglucoside, probably because they worked with young leaves.

Delphinidin-3-glucoside (purple colour) was present mainly in red-coloured fruit genotype (Wonderful) except for leaf and unripe aril stage, while in the rose-coloured fruit genotype (Valenciana) it was found only in ripe stages. Cyanidin-3-glucoside (red colour) followed the same trend as delphinidin-3-glucoside, but it was also found in turning stage of Valenciana. Opposite situation was found for pelargonidin-3-glucoside that was more abundant in pink genotype except for flower that was present only in red genotype. Pigments diglycosidic delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside increased during fruit maturation in both genotypes, but mostly in Wonderful; delphinidin diglucoside could not be detected in the red ST1 and ST2. On the contrary pelargonidin-3,5-diglucoside was more abundant in flower, mainly in the red fruit genotype in agrees with what reported by Ben-Simhon *et al.* (2011). No differences were found among stages Flesh plus seed (F+S) and only flesh (F), confirming that anthocyanins are present in the flesh tissue.

The obtained results of the anthocyanins during development of the aril show the presence of pelargonidin 3,5 glucoside in arils. Interestingly, major level of cyanidin-3,5-diglucoside were

found in Wonderful, compared to obtained by Ben-Simhon *et al.* (2015). Also, delphinidin 3,5-diglucoside was found in flower, but not cyanidin-3-glucoside.

Among the two cultivars examined, Wonderful showed major pigmentations during fruit stages with high value of cyanidin-3,5-diglucoside, while anthocyanin content in Valenciana was much lower, except for monodiglucoside of pelargonidin (orange pigment), while diglucoside of pelargonidin was similar among cultivars according to reported.

Tab. 21. Anthocyanins identified during different fruit development stage in Valenciana (VL) and Wonderful (WD).

Compound	Assignment	rt (min)	HPLC-PDA λmax (nm)	$[M-2H+H_2O]^-$ m/z	Leaf		ST1		ST2		ST3 F+S		ST3 F		ST4 F+S		ST4 F	
					VL	WD	VL	WD	VL	WD	VL	WD	VL	WD	VL	WD	VL	WD
1	Delph-3-glc [a]	8.1	276, 521	481.0994				x				x		x	x	x	x	x
2	Cyanidin-3-glc [a]	10.06	280, 323, 516	465.1039		x					x	x	x	x	x	x	x	x
3	Pelar-3-glc [a]	11.67	277, 329, 427, 500	449.1093				x				x		x	x	x	x	x
4	Delph-3,5-diglc [a]	4.9	276, 517	643.1504	x	x			x		x	x	x	x	x	x	x	x
5	Cyanidin-3,5-diglc	6.55	278, 515	627.1578	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6	Pelar-3,5-diglc [a]	8.2	276, 498	611.1624			x	x			x	x	x	x	x	x	x	x

[a] Compounds identified by comparing retentions time, HPLC-PDA and MS data with reference standards. Abbreviation: delphinidin (delph); pelargonidin (pelar); glucoside (glc). x, detected.

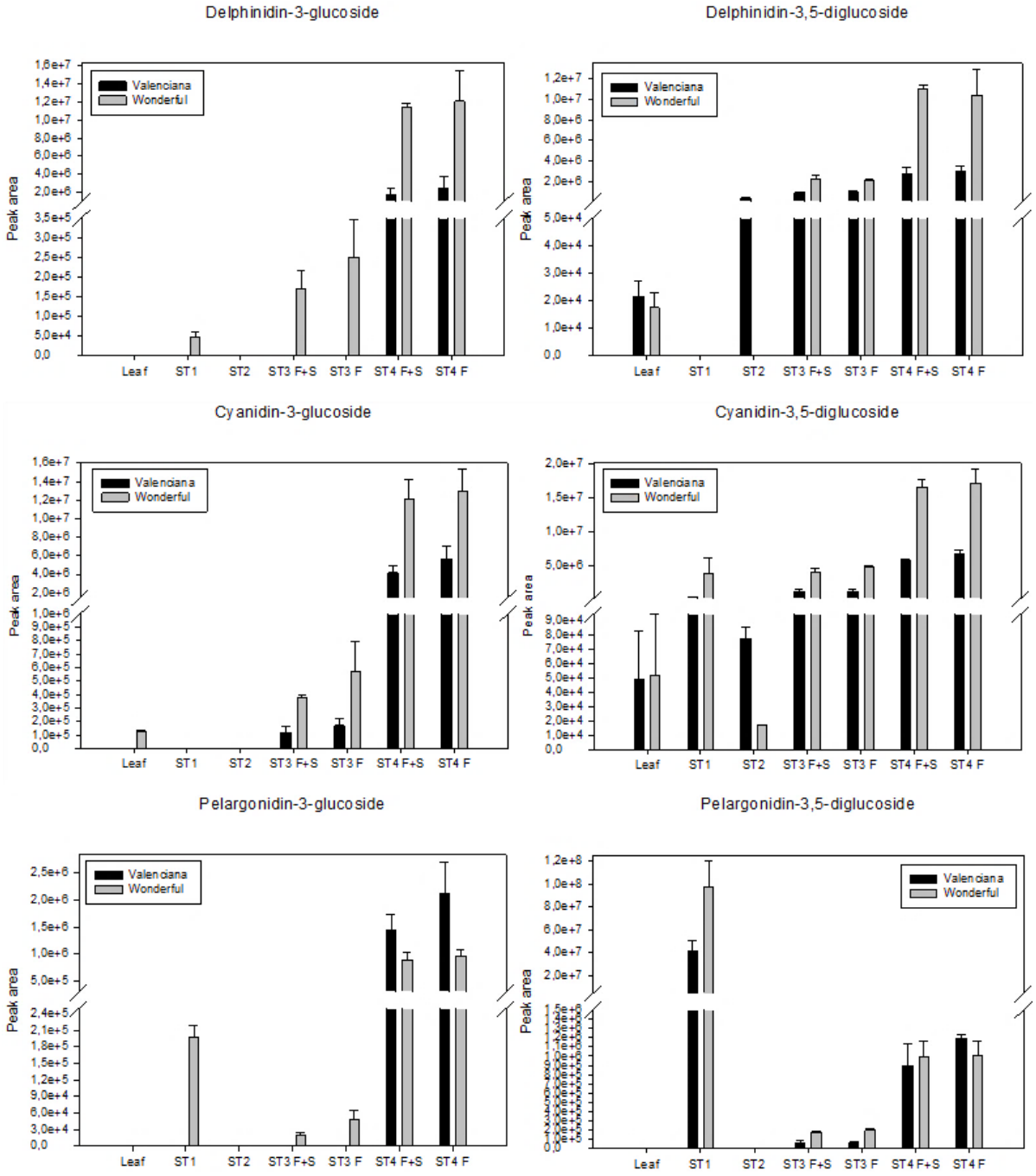


Fig. 45. Histograms of anthocyanins found in Valenciana (VL) and Wonderful (WD). Leaf (L) flower (stage-ST1), unripe arils (stage-ST2), turning arils (stage-ST3 F+S and stage-ST3+F) and ripe arils (stage-ST4 F+S and stage-ST4+F). Number of compounds correspond to number reported in tab. 19.

To analyze hydrolyzable tannins, ellagitannins and gallotannins and other phenolic compounds were tentatively identified during fruit development stages of Valenciana and Wondeful. Different compounds including trigalloglucose, tetragalloglucose, pentagalloglucose

(Wyrepkowski et al., 2014) involved in biosynthesis of ellagitannins were tentatively identified in pomegranate (Tab. 22, Fig. 46).

In contrast to what is reported for anthocyanins, the ellagitannins and gallotannins decrease with the fruit development stages, resulting in a higher presence in leaves and flowers. This result is in agreement with Han *et al.* (2015) which reported that same phenolic compounds include punicalagins, ellagic acid and gallic acid decrease during fruit development stages. We confirmed that some secondary metabolites such as ellagic acid were found in only low concentrations in pomegranate juice, while gallic acid was not found at all juices. High amounts of punicalagin α and punicalagin β were found in the flower of both cultivars, mainly in Valenciana. Differences were observed among stages F+S and F. The peak area of ellagic acid and ellagic acid hexoside changed depending on the presence or not of the seed, showing to be major with seed. Naringin was found mainly in unripe and turning fruits of Wonderful. For rutin high mass signals were observed in leaf of Valenciana, and a stronger decrease during fruit ripening compared to Wonderful was observed. Concerning catechin and epicatechin, it was observed that catechin was major in fruit, especially in seed, compared to leaf and flower, but decreased during fruit development. The opposite situation was observed for epicathechin, which increased during development stages with a decrease from stage ST2 to stage ST3 to increase in stage ST4 (Fig. 47). Wonderful showed a major intensity of both compounds.

Gallotannins (19-29) precursors of ellagitannins showed to be more abundant in the first stage of fruit development. Punicalagins the most important ellagitannins in pomegranate were high in the first stages. This indicate that in the early fruit development stage there was more synthesis of these compounds and this later gradually slowed or stopped and increase production of anthocyanins in agreement with reported by Han *et al.* (2015).

Tab. 22. Identification of phenolic compounds during fruit development stages in Valenciana (VL)and Wonderful (WD).

Compound	Assignment	rt (min)	HPLC-PDA λmax (nm)	[M-H]⁻ m/z	Leaf		ST1		ST2		ST3 F+S		ST3 F		ST4 F+S		ST4 F	
					VL	WD	VL	WD	VL	WD	VL	WD	VL	WD	VL	WD	VL	WD
	Ellagitannins																	
7	Punicalagin α [a]	8.29	258, 382	1083.0601	x	x	x	x	x	x	x	x	x	x	x	x	x	x
8	Punicalagin β [a]	10.64	258, 379	1083.0604	x	x	x	x	x	x	x	x	x	x	x	x	x	x
9	Ellagic acid [a]	21.96	253, 368	300.9991	x	x	x	x	x	x	x	x	x	x	x	x	x	x
10	Ellagic acid pentoside	21.03	253, 362	433.0419	x	x	x	x	x	x	x	x	x	x	x	x	x	x
11	Ellagic acid deoxyhexose	21.45	253, 362	447.0575	x	x	x	x	x	x	x	x	x	x	x	x	x	x
12	Ellagic acid hexoside	16.41	253, 362	463.0526	x	x	x	x	x	x	x	x	x	x	x	x	x	x
13	Pedunculagin monomeric I	5.55	n.d.	783.0699	x	x	x	x	x	x	x	x	x	x	x	x	x	x
14	Pedunculagin monomer II	6.42	n.d.	783.0698	x	x	x	x	x	x	x	x	x	x	x	x	x	x
15	Pedunculagin dimer III [b]	13.26	n.d.	1567.1464	x	x	x	x	x	x	x	x	x	x	x	x	x	x
16	Pedunculagin dimer IV [b]	15.17	n.d.	1567.1464	x	x	x	x	x	x	x	x	x	x	x	x	x	x
17	Galloyl HHDP hexose	14.83	n.d.	633.0743	x	x	x	x	x	x	x	x	x	x	x	x	x	x
18	HHDP glucose	3.02	n.d.	481.0629	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Gallotannins																	
19	Gallic acid [a]	4.48	271	169.0144	x	x	x	x			x							
20	Gallotannins (Galloyl glucose)	3.5	n.d.	331.0675	x	x	x	x	x	x	x	x	x	x	x	x	x	x
21	Digalloyl hexose I isomer I	9.79	n.d.	483.0786	x	x	x	x	x	x	x	x	x	x	x	x	x	x
22	Digalloyl hexose I isomer II	10.87	n.d.	483.0791	x	x	x	x	x	x	x	x	x	x	x	x	x	x
23	Digalloyl hexose I isomer III	11.43	n.d.	483.0790	x	x	x	x	x	x	x	x	x	x	x	x	x	x
24	Digalloyl hexose I isomer IV	16.06	n.d.	483.0789														
25	Trigalloylglucose	12.04	n.d.	635.0900	x	x	x	x	x	x	x	x	x	x	x	x	x	x
26	Tetragalloylglucose isomer I	20.41	n.d.	787.1015	x	x	x	x	x	x	x	x		x	x	x	x	x
27	Tetragalloylglucose isome II	21.15	n.d.	787.1018	x	x	x	x	x	x	x	x	x	x	x	x	x	x
28	Tetragalloylglucose isomer III	21.87	n.d.	787.1016	x	x	x	x	x	x	x	x	x	x	x	x	x	x
29	Pentagalloylglucose	24.49	n.d.	939.1130	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Gallagyl esters																	
30	Punicalin	4.62	n.d.	781.0543	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Flavonoids																	
31	Naringin	26.7	283, 334	579.1728	x	x	x	x	x	x	x	x	x	x	x	x	x	x
32	Rutin [a]	22.21	255, 355	609.1466	x	x	x	x	x	x	x	x	x	x	x	x	x	x
33	Catechin	11.62	n.d.	289.0722	x	x	x	x	x	x	x	x	x	x	x	x	x	x
34	Epicatechin	15.2	n.d.	289.0722	x	x		x	x	x	x	x	x	x	x	x	x	x

Compounds identified by comparing with reference compounds in literature. [a] Compounds identified by comparing retentions time, HPLC-PDA and MS data with reference standards. [b] Compound appears as doubly-charged molecular ions. x, detected; n.d. λ not detected.

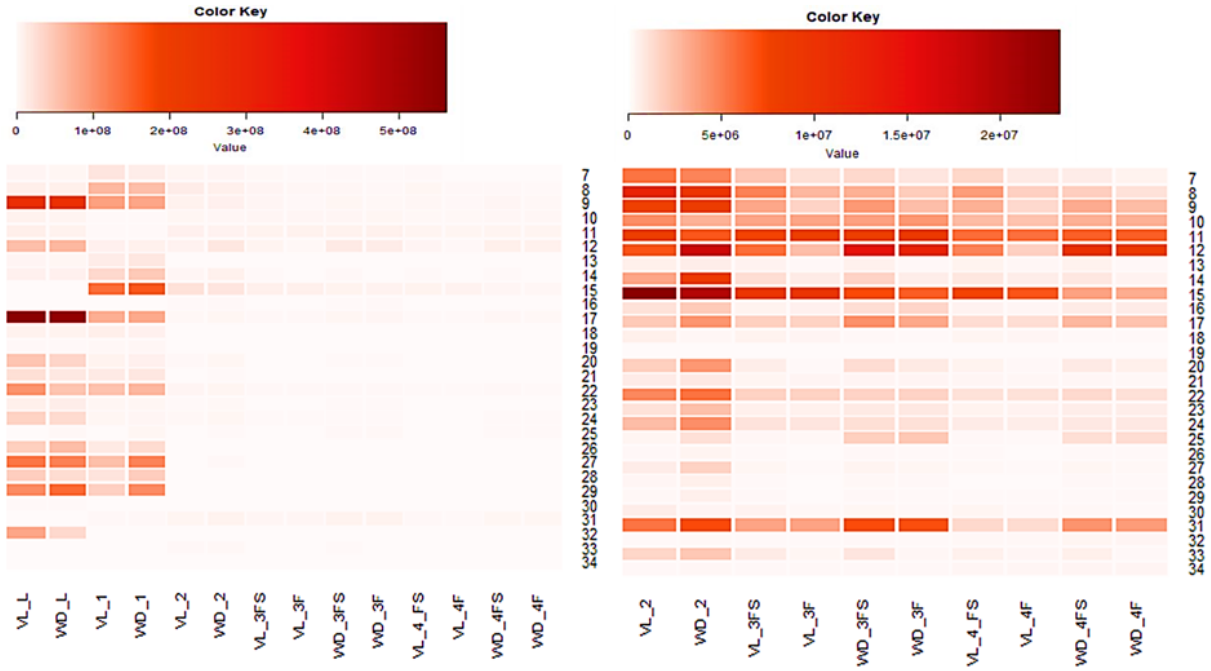


Fig. 46. Peak area heat map of phenolic compounds found in Valenciana (VL) and Wonderful (WD). Leaf (L) flower (stage-ST1), unripe arils (stage-ST2), turning arils (stage-ST3 F+S and stage-ST3+F) and ripe arils (stage-ST4 F+S and stage-ST4+F). Number of compounds correspond to number reported in Tab. 20.

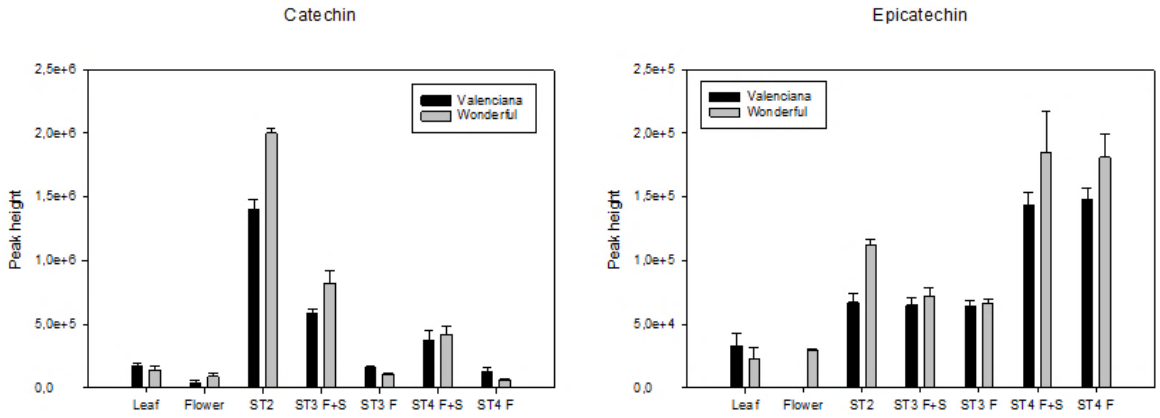


Fig. 47. Diagrams showing the peak height of catechin and epicatechin identified during different fruit development stages.

4.3.1 Principal component analysis (PCA) in pomegranate tissues

In order of enabling a view on the metabolic changes that occurs during fruit maturation, two principal component analysis of the data set examined were performed and showed in Fig. 48A-B. The samples derived from various stages of developments were clearly separated based on metabolites. Leaves, flowers, unripe arils, turning arils and ripe arils of the two genotypes followed the same behaviour (Fig. 48A), but in separately cluster, showing to be metabolically different from each other (Fig. 48B).

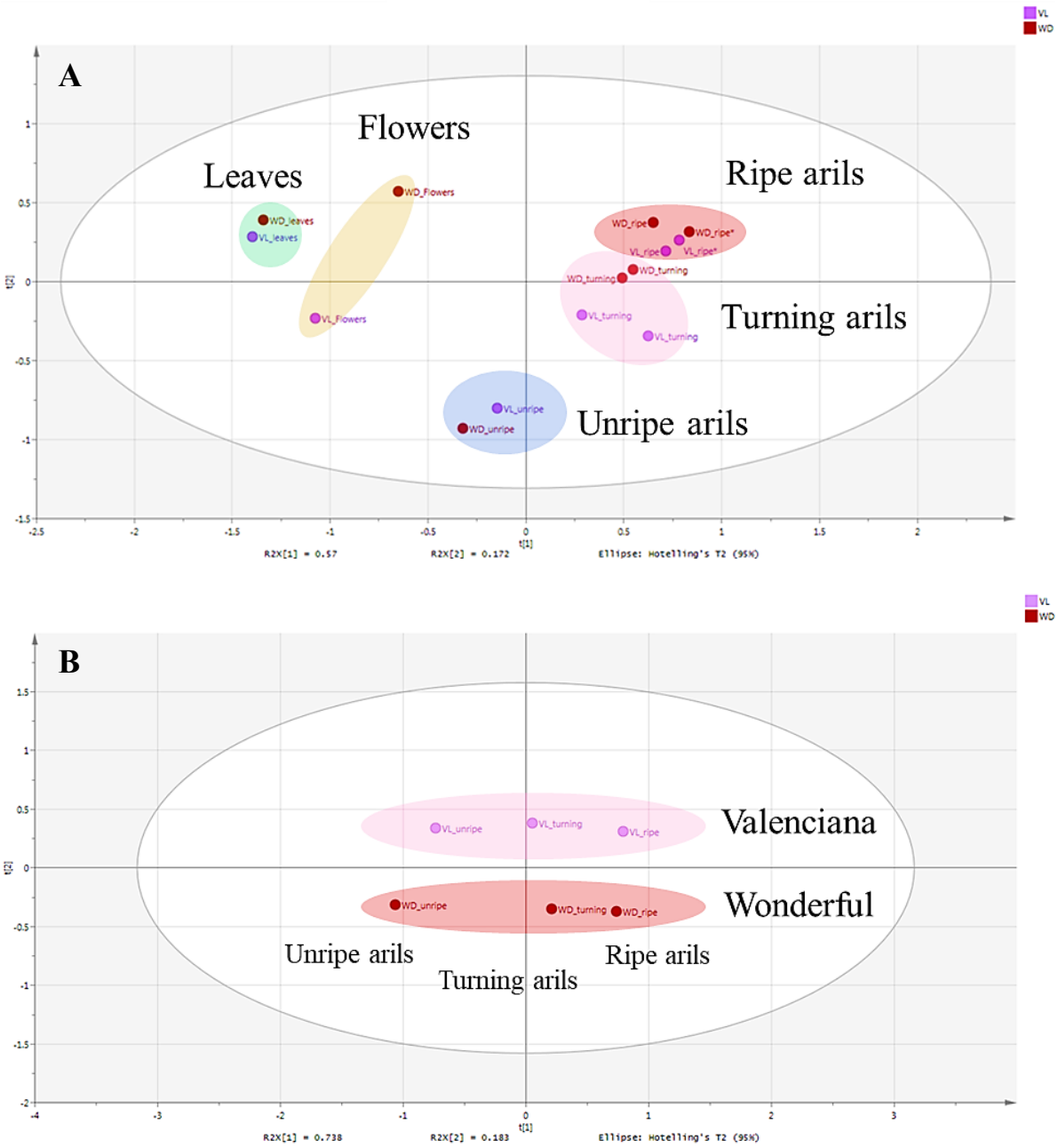


Fig. 48. Principal component analysis (PCA) of 2 genotypes Valenciana (VL) and Wonderful (WD) during different development tissues (PCA-A). during ripening arils (PCA-B). The stars indicate stage ST4 F+S.

4.4 Functional genetics

Based on the anthocyanin profile obtained during the fruit development stages of Valenciana and Wonderful, we investigated the effects of overexpression of anthocyanins regulatory complex on polyphenolic metabolites in *N. benthamiana*. Moreover, the gene expression levels of the anthocyanins regulatory complex and other genes involved in the anthocyanin biosynthesis in leaf, flower, unripe (ST2) and ripe (ST4 F+S) of the Valenciana and Wonderful cultivars were evaluated.

4.4.1 RNA Isolation

Total RNA was extracted from leaves, flowers and different fruit developmental stages of Valenciana and Wonderful (as illustrated in Fig. 44). The extracts were analysed by capillary electrophoresis (Fig. 49), while an example of electropherograms with 18S and 28S rRNA peaks is shown in Fig. 50. The measures of RIS values (Tab. 23) shown a value always greater than 5 for all the samples taken into consideration.

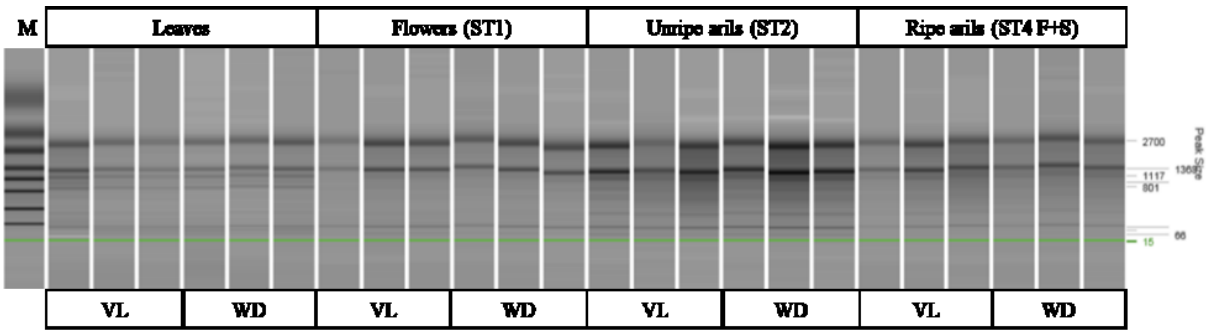


Fig. 49. Gel view of RNA runs of Leaves. flowers and different fruit arils development stage of Valenciana (VL) and Wonderful (WD) cultivars. Peaks at 15 correspond to the alignment marker used in the analysis. ST1, flower; ST2, unripe aril (flesh plus seed); ST4 F+S, ripe aril flesh plus seed.

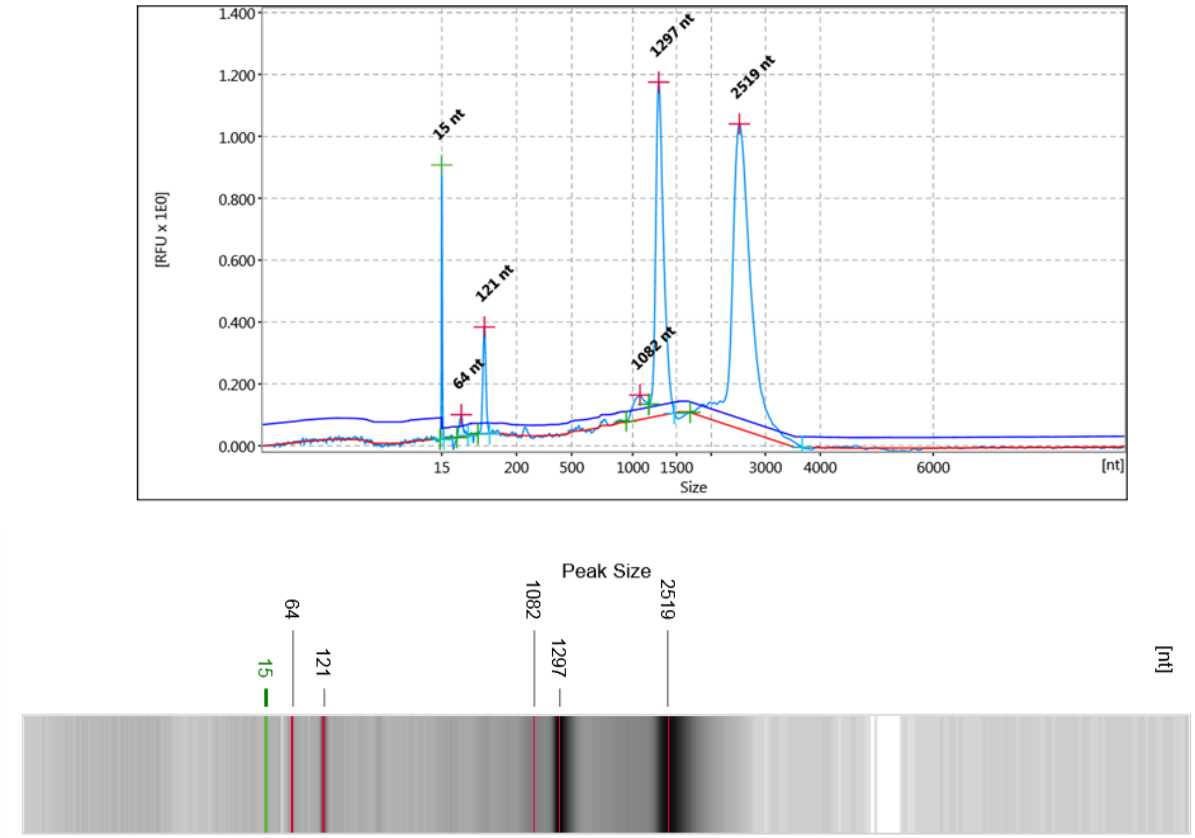


Fig. 50. Example of electropherogram of replica of one pomegranate sample VL-ST1. Size 1297 nt corresponds to 18S RNA while size 2519 nt corresponds to 28S RNA. Peak at 15 correspond to the alignment marker used in the analysis.

Tab. 23. RNA Integrity Score (RIS) of RNA extraction of Valenciana and Wonderful in different development stage.

Cultivar	Tissue	RIS
Valenciana	**Leaf**	5.3
		5.3
		5.6
	Average	5.4
	ST1	6.3
		6.1
		6.2
	Average	6.2
	ST2	5.1
		7.1
		5.2
	Average	5.8
	ST4 F+S	6.6
		5.6
		6.6
	Average	6.3
Wonderful	**Leaf**	5.3
		5.9
		6.2
	Average	5.8
	ST1	6.2
		6.0
		5.9
	Average	6.0
	ST2	7.5
		5.2
		8.0
	Average	6.9
	ST4 F+S	6.6
		6.9
		7.1
	Average	6.9

. Values of three biological replicas.

4.4.2 Identification and isolation of genes associated with anthocyanins biosynthesis

Oligonucleotide primers designed were used for the isolation and characterization by PCR of pomegranate genes associated with biosynthesis of anthocyanins (see Tab. 6, Paragraph 3.5.).

After pJET cloning, TA cloning (Fig. 51) and sequencing, we identified *PgMYB1_contig, PgMYB2_contig*, *PgMYB4_contig*, *PgbHLH_contig*, *PgWD40_contig*, *PgDFR_contig*, *PgLDOX_contig* and *PgUFGT_contig*. All contigs are reported in Supplements 1 and 2. For *PgMYB2_contig*, two introns were found and a *PgMYB2_splice_contig* was obtained by the in silico removal of predicted introns.

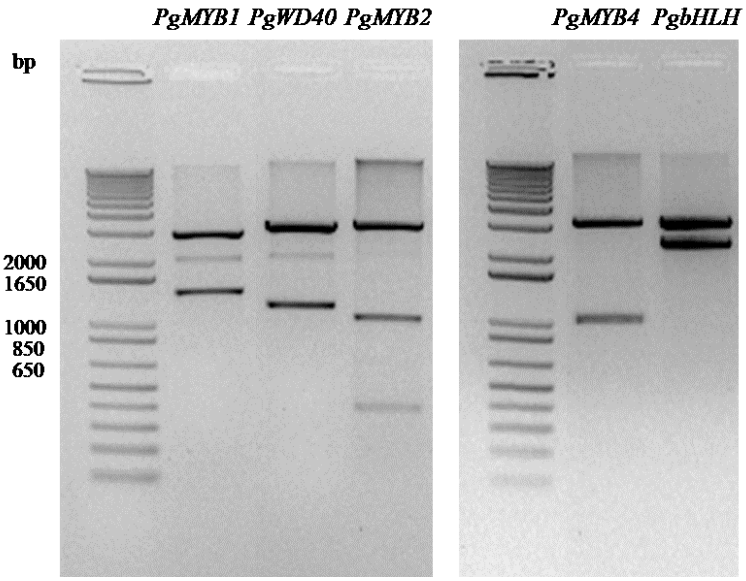


Fig. 51. Digestion with EcoRI HF of anthocyanins transcription factors *PgMYB1*. *PgMYB2*. *PgMYB4*. *PgWD40* and *PgbHLH*. The fragment with the highest size (2817 bp) is the pCR™8/GW/TOPO® TA cloning vector. Marker used is 1 kb plus DNA Ladder (Invitrogen).

The obtained contigs were compared with nucleotide sequences of pomegranate by BLASTn platforms and the relative results are shown in Tabs. 24 and 25. The BLASTn search of the obtained sequences against *P. granatum* database, resulted in a percentage of identities that was always higher than 97%. Alignments of *PgMYB2_contig* and *PgMYB2_splice_contig* with *P. granatum* sequences matching are reported in appendix (Supplements 3, 4 and 5). These results and the score obtained by BLAST confirmed the isolation of complete CDS for anthocyanin TFs and the partial CDS for *PgDFR*, *PgLDOX* and *PgUFGT* genes.

The query cover is a percent of the query sequence that overlaps the subject sequences. The Expect value (E) is a parameter that describes the number of hits that one 'expects' to see by chance when searching a database of a given size. It decreases exponentially as the Score (S) of the match increases.

Tab. 24 BLASTn of the TFs sequence against *Punica granatum* database.

	Results of sequence alignment (blastn)				
Gene	**Total score**	**Query cover**	**Expect**	**Identities**	**Accession number**
PgMYB1_contig	2471	100%	0.0	99%	KP726347.1
	1360	54%	0.0	99%	MF063017.1
	1360	54%	0.0	99%	KF841621.1
	501	21%	3E-62	98%	JF747151.1
PgMYB2_contig	1964	100%	0.0	98%	HM056531.1
	1696	81%	0.0	99%	KF631414.1
	774	41%	6E-168	98%	GU371444.1
	420	19%	2E-118	100%	FN677589.1
PgMYB2_splice_contig	763	71%	0.0	97%	GU371444.1
	862	72%	1E-176	99%	KF631414.1
	1094	100%	3E-168	98%	HM056531.1
	331	27%	5E-92	100%	FN677589.1
PgMYB4_contig	1468	88%	0.0	100%	KM881712.1
PgbHLH_contig	*3795*	100%	0.0	99%	KF874658.1
	604	15%	8E-174	100%	JF747152.1
	604	15%	2E-116	100%	KF631416.1
PgWD40_contig	*1857*	100%	0.0	100%	HQ199314.1
	953	51%	0.0	100%	KT821069.1
	953	51%	0.0	100%	KT821068.1
	953	51%	0.0	100%	KT821067.1
	953	51%	0.0	100%	KT821065.1
	953	51%	0.0	100%	KT821064.1
	953	51%	0.0	100%	KT821063.1
	953	51%	0.0	100%	KT821062.1
	953	51%	0.0	100%	KT821061.1
	953	51%	0.0	100%	KT821060.1
	953	51%	0.0	100%	KT821059.1
	953	51%	0.0	100%	KT821058.1

Tab. 25. BLASTn of the partial sequences of *PgDFR*, *PgLDOX* and *PgUFGT* against *Punica granatum* database.

	Results of sequence alignment (blastn)				
Gene	**Total score**	**Query cover**	**Expect**	**Identities**	**Accession number**
PgDFR	1504	100%	0.0	100%	KC430327.1
	1498	100%	0.0	99%	KF841618.1
	577	39%	7E-166	99%	JN316028.1
	418	28%	4E-118	99%	JF747150.1
	1508	100%	7E-101	100%	KP726344.1
PgLDOX	1339	100%	0.0	99%	KF841619.1
	747	57%	0.0	99%	GU376749.1
	1342	100%	0.0	99%	KP726345.1
	562	42%	4E-161	99%	KT779433.1
	405	30%	7E-114	99%	JF747149.1
PgUFGT	1336	98%	0.0	99%	KF841620.1
	1092	82%	0.0	99%	GU371443.1
	1342	98%	0.0	99%	KP726346.1
	927	82%	0.0	93%	KF631415.1

Furthermore, using BLASTx tool the obtained contigs were either searched against NCBI Protein database including all species and limiting to *Arabidopsis thaliana* species (Supplements 6, 7, 8 and 9). As regards to the first sequence hit results, the identities of all contigs examined against all database resulted > 99% with *P. granatum*. The detected putative conserved domains resulted consistent with what we expected.

The *PgMYB1* gene cloned in this study showed similarity amino acidic of 99-97% with P. *granatum* protein OWM84991.1, AHZ97876.1 ALT22077.1 PKI68397.1 AVW85507.1. The query cover of 54%, is due to the presence of an intron in the sequence contig. Homology was found also with other fruit species such as *Morella rubra* and *Vitis vinifera*.

Blastx against *A. thaliana* protein database reported similarity of 80% and 75% with AtMYB66/WER (NP_196979.1) and AtMYBGL1 (AFD94023.1) respectively, which are associated with determination of epidermal cell type (Oppenheimer et al., 1991; Lee et al., 1999). In pomegranate the epidermal cells of the fleshy edible part of the fruit protruded from the outer integument of the seed could be regarded as orthologous to trichomes (Fahn, 1982). Identity of 80% was also found between *PgMYB1* and AtMYB114 (NP_001321375.1) (not

shown in table) which is known to be involved in control anthocyanin biosynthesis with AtMYB90/PAP2 and AtMYB113 in vegetative tissues (Gomez et al., 2008).

From the blastx, the *PgMYB2_splice* sequence showed a high degree of 99% similarity with protein OWM86420.1 (Qin et al., 2017) and 72% with the proteins AHB18402.1 (KF631414.1) and ADG65150.1 (HM056531.1). In blastx against *A. thaliana* 57% protein sequence identity was found with AtMYB23 (NP_198849.1) and AtMYB66 (NP_196979.1), while 58% with AtMYB5 (NP_187963.1). From the literature it is known that AtMYB23 regulates trichome extension and branching in combination with AtMYB5 (Kikir et al., 2005; Li et al., 2009). AtMYB5 also regulates outer seed coat differentiation and it was proposed to be partially redundant with AtMYB123/TT2 in regulating tannin biosynthesis (Gonzalez et al., 2009; Li et al., 2009).

Concerning to *PgMYB4*, 99% identify of protein sequence was found with *P. granatum* protein KM881712.1 and identity of 98-88% with Arabidopsis MYB4 (OAO97731.1) and MYB3 protein (NP_564176.2) respectively, which encode transcriptional repressors in Arabidopsis (Jin et al., 2000; Preston 2004; Dubos et al., 2008).

The homology of 99% of *PgbHLH* with *P. granatum* basic helix-loop-helix regulatory protein (AIS24255.1) and OWM77890.1 (Qin et al., 2017) and their identity with bHLH of other species (81-74%), also with TT8 (CAC14865.1) of *A. thaliana* confirms its identity. Also the identity of *PgWD40* contig was confirmed with homology amino acid sequence of 100-80% with *P. granatum* protein ADV40946.1 and *A. thaliana* protein NP_197840.1 respectively.

Protein sequence identity of 100% with *P. granatum* proteins PKI49859.1, AET74075.2 and 99% identity with AHZ97873.1, ALT22074.1 and OWM75048.1 was found in *PgDFR_contig*, while 99% identity was found for *PgUFGT_contig* with proteins of *P. granatum* ALT22076.1, AHZ97875.1.

The DNA plasmid containing *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgbHLH*, *PgWD40* was used to carry out the Gateway cloning by a binary vector and transformation in Agrobacterium (Fig. 52). The Agrobacterium positive strains and empty pBIN agrobacterium vector were injected into the leaves (2 leaves per plant) of two week-old *N. benthamiana*.

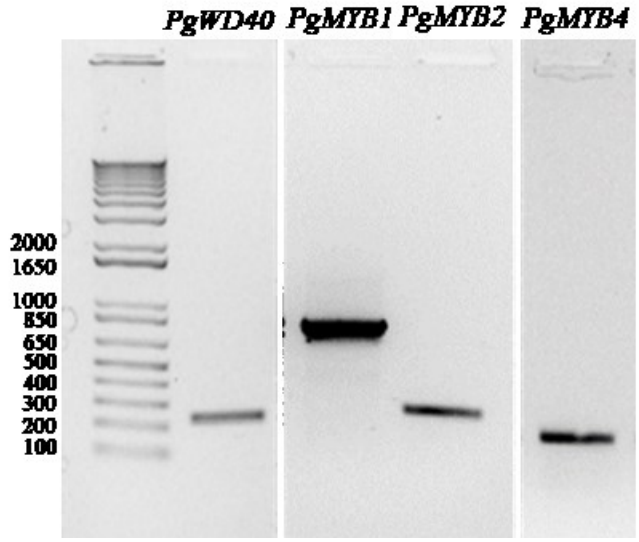


Fig. 52. Some positive agrobacterium clones of *PgWD40*. *PgMYB1* and *PgMYB2* sequences screened by detection primers (Tab. 7). Clones were used in agro-infiltration.

4.4.3 Leaves of *N. benthamiana* agroinfiltrated with pomegranate genes

The effects of the expression of three TFs from pomegranate on *N. benthamiana* metabolites were estimated using untargeted LC-MS analysis (De Vos et al., 2007). In particular complete CDS of *PgMYB1*, *PgMYB2*, *PgMYB4*, *PgbHLH* and *PgWD40* obtained by cloning were agroinfiltrated in *N. benthamiana* leaves.
Two experiments were carried out to investigate the metabolomic products of the flavonoid biosynthesis in the presence of overexpression of the MYBs constructs:

In the first, different combinations of the three PgMYBs TFs with PgWD40 and PgbHLH were investigated and pBIN vector was used as negative control (Paragraph 3.5.2, Tab. 9). Leaves were harvested five days after agroinfiltration except for PgMYB4's constructs that were harvested three days after agroinfiltration, because leaves were wilting, as showed in Fig. 51 Combination of PgMYB2+bHLH and PgMYB2+bHLH+WD40 showed a change of colour in the agroinfiltrated areas as showed in Fig. 53. The rest of constructs including PgMYB1's and combination without PgMYBs FTs did not show a significant change in colour and in metabolites. This behaviour was confirmed in the PCA (Fig. 54) of untargeted metabolites obtained by LC-MS in negative mode, where three different clusters were observed. In fact, a first cluster in the middle, close to pBIN's metabolites, included PgMYB1's, PgbHLH's and PgWD40's combinations and PgMYB2 and PgMYB2+PgW40 constructs, proving to have the same metabolic profile of plants treated with an empty vector. While, the other two clusters positioned in opposite vertices containing different metabolic profiles were referable to the construct containing PgMYB4 and the other one was related to the PgMYB2+PgbHLH and PgMYB2+PgbHLH+PgWD40 constructs combination. The presence of metaboloties referable to PgMYB4 construct appeared to be produced by the wilting process which can lead to many pleiotropic changes in the metabolome.

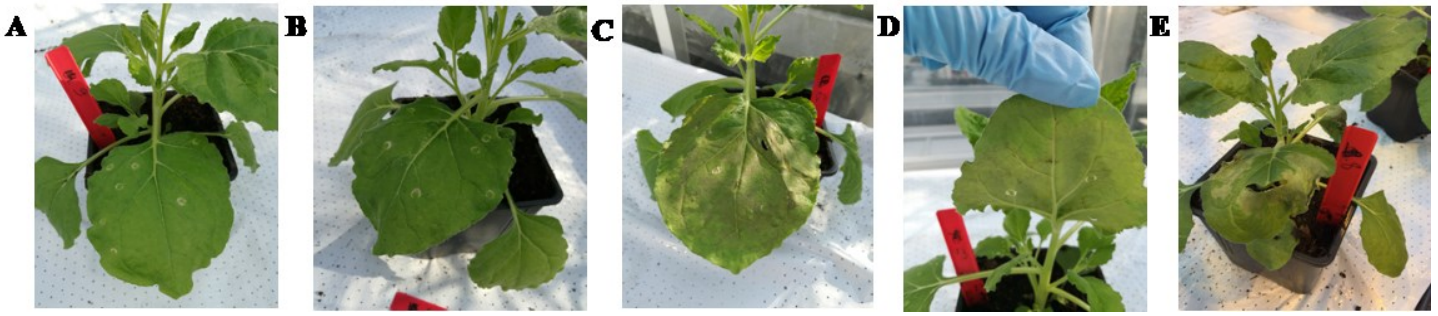


Fig. 53. Nicotiana leaves agroinfiltrates with pBIN empty vector (A), *PgMYB1* (B), *PgMYB2+PgbHLH+PgWD40* construct (C), *PgMYB2+PgbHLH* construct (D) or with *PgMYB4* (E) construct.

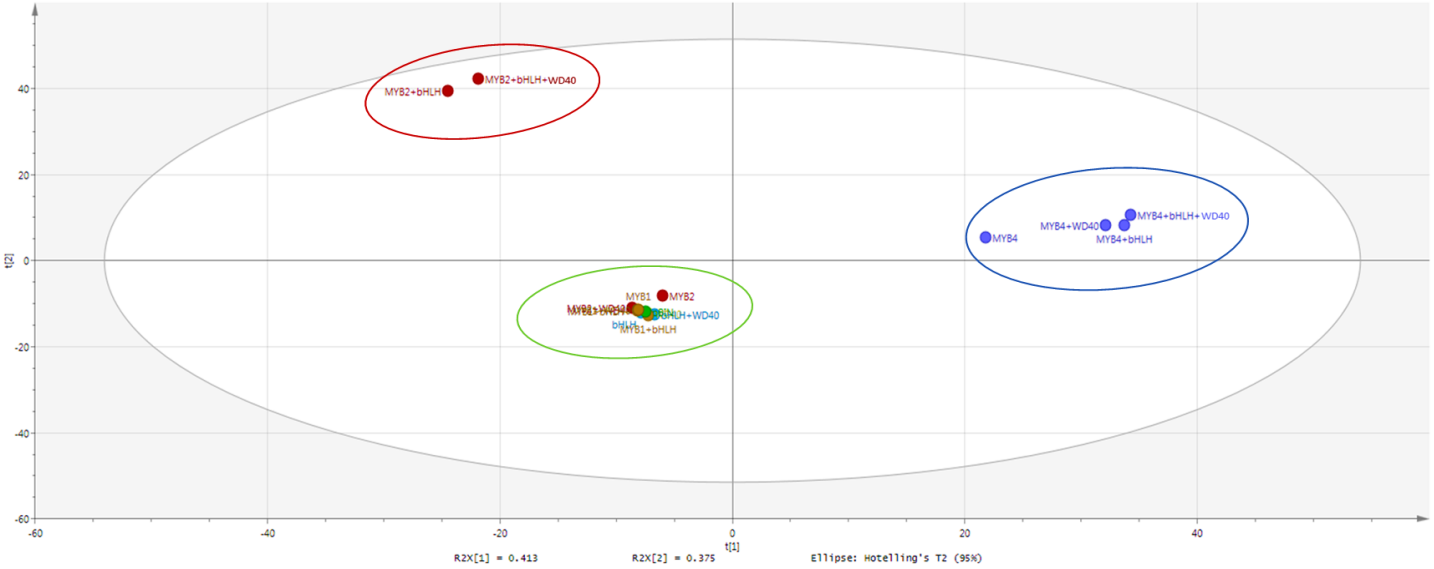


Fig. 54. Principal component analysis based on metabolites obtained from first experiment of agroinfiltration. PgMYB2+PgbHLH and PgMYB2+PgbHLH+PgWD40 constructs (Red circle); PMYB4 constructs (blue circle) and the rest of constructs with empty vector (green circle).

On the other hand, the cluster including PgMYB2+bHLH and PgMYB2+PgbHLH+PgWD40 constructs resulted in a marked difference in the metabolic content respect to the other constructs. The difference among the produced metabolites will be discussed within the second experiment results description. This difference was also found in the phenotypes appearance of these two constructs respect to the others (Fig. 53) and confirmed by PCA, in particular in the combination between PgMYB2 and PgbHLH. The peak intensity of some metabolites was found greater in PgMYB2+PgbHLH than in PgMYB2+PgbHLH+PgWD40 as shown in Fig. 55.

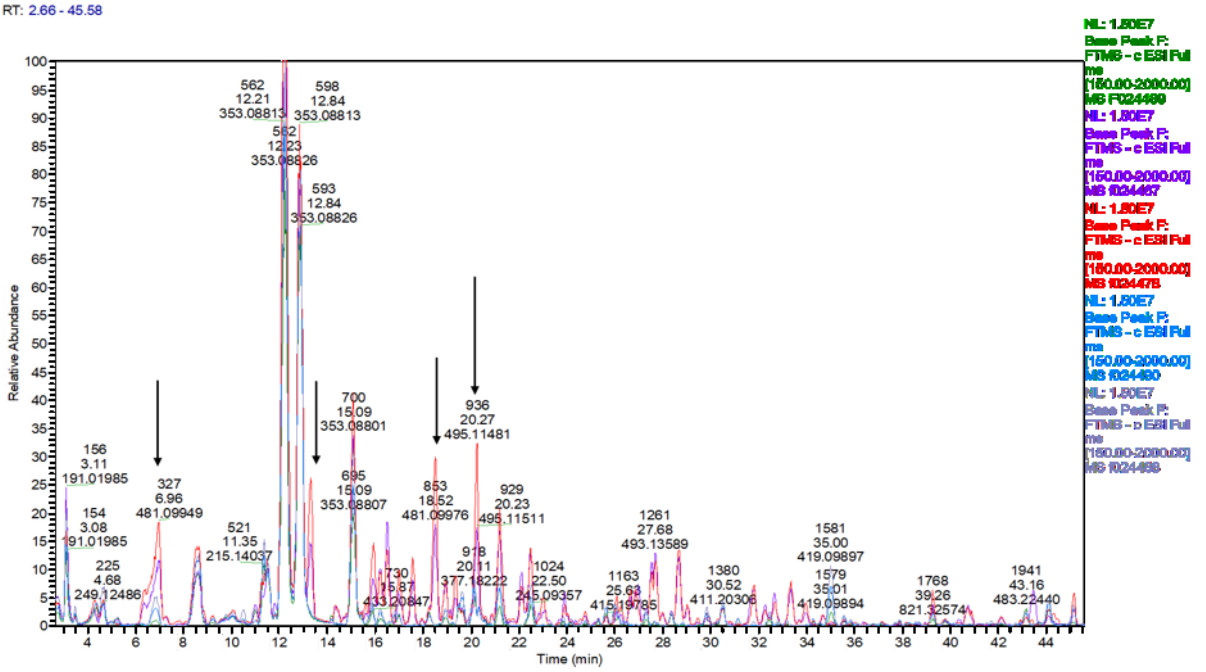


Fig.55. Mass spectrum of pBIN empty vector (green colour), PgMYB2+PgbHLH+PgWD40 (purple colour), PgMYB2+ PgbHLH (red colour), PgMYB2+PgWD40 (blue colour) and PgMYB2 (grey colour). The arrows show some metabolites present in PgMYB2+PgbHLH and PgMYB2+PgbHLH+PgWD40 constructs compared to pBIN empty vector and the other constructs.

A second experiment was carried out using only PgMYB2+bHLH construct in comparison with pBIN empty vector and with the regulation complex of ROS1+DEL TFs obtained from *Antirrhinum majus*. Aprevious study demonstrated that overexpression of *A. majus* ROS1 (a MYB type TF) and DEL (bHLH type) in *N. benthamiana* induced the formation of a single anthocyanin, Delphinidin-3-rutinose (Outchkourov et al., 2014).

Two harvest times were performed; three plants per constructs were harvested four days after agroinfiltration and three plants after six days. It was observed a change in colour of Nicotiana leaves in both construct combinations, brown colour in PgMYB2+PgbHLH and purple colour

in ROS1+DEL (Fig. 56), where the leaf colouring intensity increased more with the harvest time. By a microscopy analysis of epidermal cells of PgMYB2+PgbHLH and wild type plants brown areas were found in agroinfiltrated plants indicating possible metabolite vacuolar inclusions (Fig. 56).

Observing the PCA of expressed metabolites a clear deviation of the treated plants from the wild type was detected Fig. 57. The metabolites amount increases with time focusing on the coloured part (represented in PCA with a star).

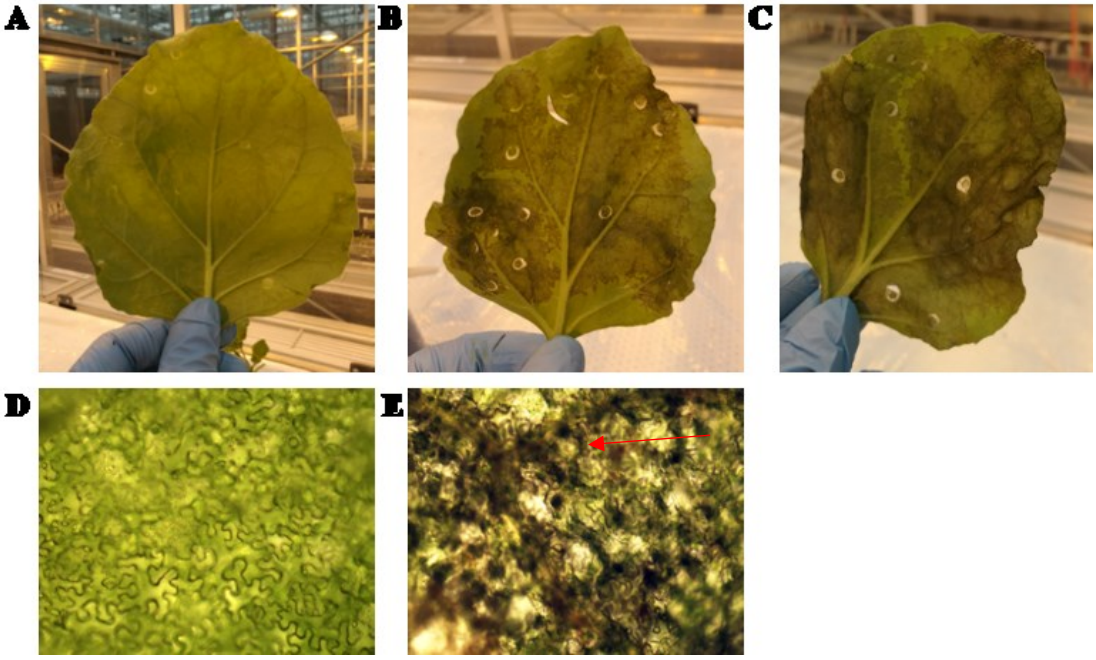


Fig. 56. Nicotiana leaves agroinfiltrated with pBIN empty vector (A), PgMYB2+bHLH (B), PgROS1+DEL (C). Microscopy epidermal cells of pBIN (D) and PgMYB2+PgbHLH (E) plants, arrow indicate possible metabolite vacuolar inclusions.

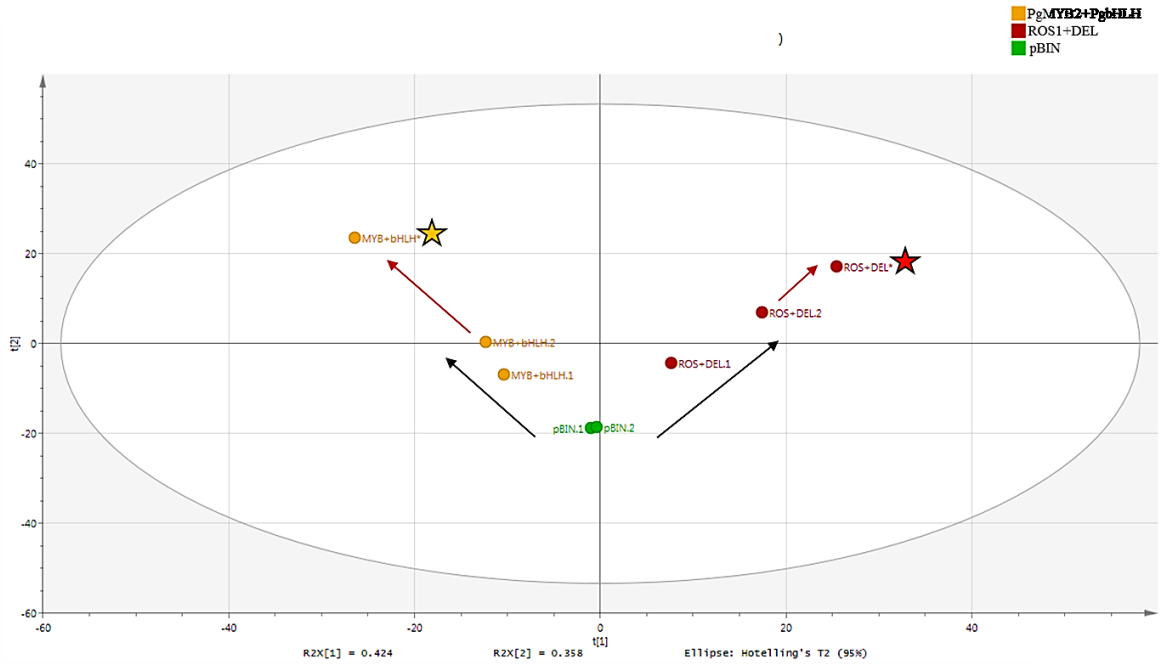


Fig. 57. Principal component analysis based on metabolites obtained from the second experiment of agroinfiltration with PgMYB2+PgbHLH (yellow colour); ROS1+DEL (red colour) and pBIN empty vector (green colour). The black arrows infidicate the harvest time from first to second, the red arrows and stars indicate coloured area of leaves agroinfiltrated of the second harvest.

At 510 nm, the typical anthocyanins detection wavelength, these were not observed in PgMYB2+PgbHLH plants compared to ROS1+DEL plants, where delphinidin-3-rutinoside was found (Fig. 58A) confirming results obtained by Outchkourov *et al.* (2014), while analysing the spectrum at 280 nm of PgMYB2+PgbHLH lines we found compounds which are derived from the anthocyanin pathway (Fig. 58B).

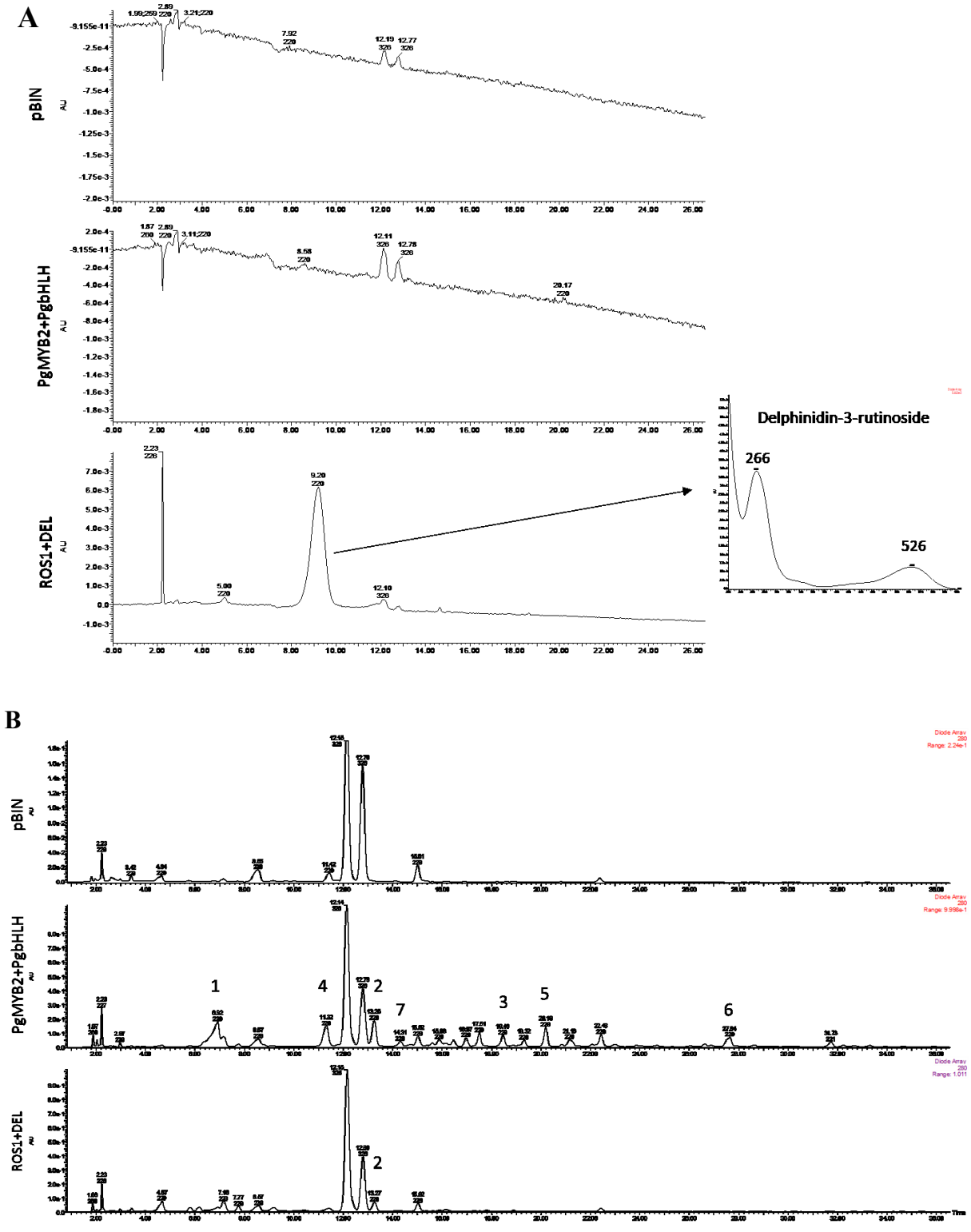


Fig. 58. (A) HPLC-PDA detection at 510 nm of *N. benthamiana* leaves extracts infiltrated with pBIN (control), PgMYB2&PgbHLH and ROS1&DEL. Inserted panel shows the UV-Vis absorbance spectrum of the delphinidin-3-rutinoside (D3R) peak. (B) HPLC-PDA detection at 280 nm of samples described in (A). Indicated peak numbers refer to Table 26.

Among these compounds a class of flavonoids, the dihydroflavonols, was detected which included dihydromyricetin, dihydrokampferol and dihydroquercetin conjugated with hexose (Tab. 26). The brown colour of Nicotiana leaves with PgMYB2+PgbHLH can be an index of absence of anthocyanins, represented by the pourple colour in transient leaves with ROS1+DEL. Probably the brown colour was due to oxidation processes indicated by the presence of oxalated compounds found in these samples or to possible formation of proanthocyanidins derived from the leucoanthocyanidins produced by the dihydroflavonols (Fig. 54). Further analysis to assess the presence of these compounds in Nicotiana leaves must be conducted.

Tab. 26. Dihydroflavonols, significantly compounds (students t-test, $p < 0.05$) found in the PgMYB2&PgbHLH infiltrated leaves as compared to pBIN empty vector and ROS1&DEL.

Compound	Assignment	rt (min)	HPLC-PDA λmax (nm)	[M-H]-	pBIN	ROS1& DEL	PgMYB2& PgbHLH
1	Dihydromyricetin hex. isomer I	6.96	290	4.810.992		x	x
2	Dihydromyricetin hex. isomer II	13.29	290	4.810.992		x	x
3	Dihydromyricetin hex. isomer III	18.52	290	4.810.992		x	x
4	Dihydromyricetin-methyl ether hex. isomer I	11.37	n.d.	4.951.149		x	x
5	Dihydromyricetin-methyl ether hex. isomer II	20.25	n.d.	4.951.148		x	x
6	Dihydroquercetin di-methyl ether hex.	27.68	n.d.	4.931.359			x
7	Dihydrokampferol hex.	14.39	n.d.	4.491.094		x	x

Hex., hexoside.

The data reported in Fig. 59 show the relative changes in content induced by MYB2&bHLH infiltration compared to empty vector pBIN and ROS1&DEL infiltration. During harvesting, the intensity of the peaks increases, concentrating especially in the most coloured areas of the leaf. All the analysed compounds were up-regulated in PgMYB2&PgbHLH agroinfiltrated plants compared to the wild type lines (Fig. 59). In particular compounds dihydromyricetin hexoside isomer II and dihydromyricetin-methyl ether hexoside isomer II were highly up-regulated compared to the empty vector pBIN. Also, in ROS1&DEL infiltrated, these compounds were found but with less intensity than pomegranate TFs, furthermore two compounds were very low or not expressed, dihydromyricetin-methyl ether hexoside isomer I and dihydroquercetin di-methyl ether hexoside respectively. All compounds showed a strong expression in presence of PgMYB2&bHLH compared to ROS1&DEL snapdragon genes. While *PgMYB2* showed a metabolomic profile similar to empty vector, the presence of *bHLH*

associated with *PgMYB2* is essential to induce accumulation of different dihydroflavonols in *N. benthamiana*, suggesting their cooperation in this stage of anthocyanin biosynthesis.

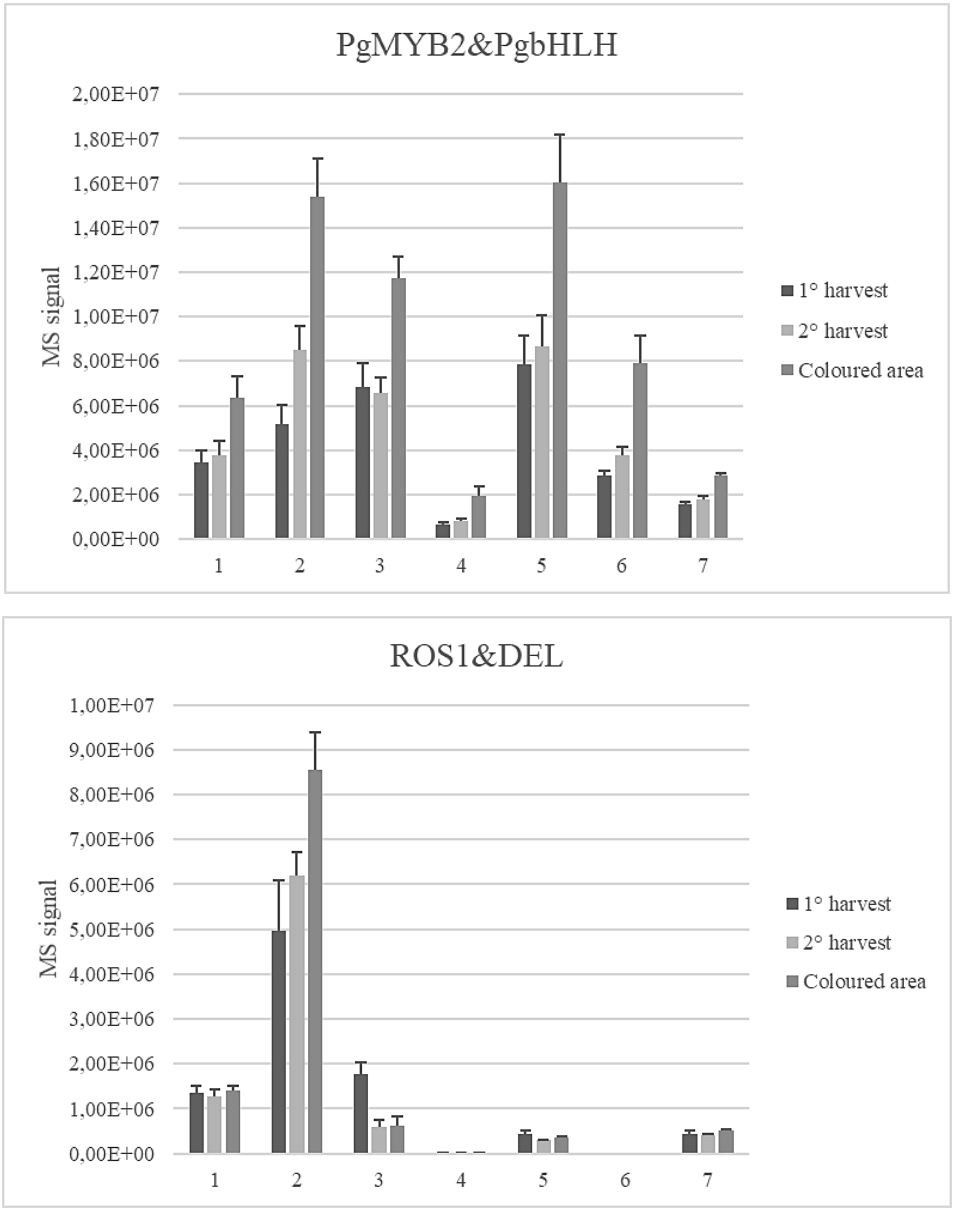


Fig. 59. Diagram showing the ratios of the MS signals of significantly changed ($P < 0.05$) compounds in the PgMYB2&PgbHLH infiltrated leaves as compared to the empty vector pBIN and ROS1&DEL. The bars indicate standard deviation of three biological replicas. Dihydromyricetin hexoside isomer I (1); dihydromyricetin hexoside isomer II (2); dihydromyricetin hexoside isomer III (3); dihydromyricetin-methyl ether hexoside isomer I (4); dihydromyricetin-methyl ether hexoside isomer II (5); dihydromyricetin di-methyl ether hexoside (6); dihydrokampferol hexoside (7).

4.4.4 Gene expression analysis

In conjunction with metabolomic data obtained from agroinfiltration in *N. benthamiana* and the polyphenolic profile obtained in different tissues of Valenciana and Wonderful during development stages, we investigate the levels of expression of some genes involved in anthocyanins pathway in these two genotypes.

Six transcription factor genes (*PgMYB1*, *PgMYB1.2*, *PgMYB2*, *PgWD40, PgbHLH*) and six anthocyanin biosynthetic structural genes (*PgDFR*, *PgLDOX*, *PgUFGT*, *PgCHS*, *PgF3'H*, *PgF3'5'H*) were expressed in leaf, flower (ST1) and in two different fruit arils ripening stages (uripe-ST2 and ripe-ST4 F+S) of the rose-coloured fruit pomegranate Valenciana cultivar and red-coloured fruit pomegranate Wonderful. From the qPCR data *PgMYB1*, *PgMYB1.2*, *PgMYB2*, *PgbHLH*, *PgCHS*, *PgF3'5'H*, *PgDFR*, and *PgUFGT* primers were suitable for the transcriptional analyses, while *PgMYB4*, *PgWD40*, *PgF3'H, PgLDOX* and *PgACT* were discarded because its dissociation curve showed more than one peak.

All the data were normalized against *PgRPSII* (Ribosomial Protein S) used as reference gene. The relative expression ratio represents how many times the gene is expressed compared to unripe ST2 from Valenciana.

4.4.4.1 *PgMYB1* expression profile

The role of *MYB* in the timing of colour was described in several studies including apple (Espley et al. 2007; Lin-Wang et al. 2010), grape (Boss et al., 1996; Cutanda-Perez et al., 2009), Chinese bayberry (Niu et al., 2010) and pear (Feng et al., 2010).

In this study the expression levels of the ***PgMYB1*** were mostly influenced by the genotypes and the tissues. The interaction between genotype and tissue was statistically significant (Tab. 27). In Wonderful, transcriptional levels were highest in ripened stage (ST4 F+S) than the other tissues, with 9.2 times more up-regulated than the same stage of rose-coloured pomegranate accession (Valenciana), which, in turn, was similar to the control (ST2) (Fig. 60), this was in agreement with the increase of anthocyanins in Wonderful ripe fruit. *PgMYB1* expression in Valenciana leaf and flower was slightly higher than in Wonderful, it could be linked to the production of other flavonoids and not only to the anthocyanins that were more present in Wonderful flower (see Paragraph 4.3). Therefore, from these data *PgMYB1* gene appears to be more expressed in the ripe stage following the same trend reported by Ben-Simhon *et al.* (2011, 2015), but here, even with a limited extent, the expression was found also in leaf tissue. This

finding partly reflects the transcriptomic data obtained by Qin *et al.* (2017) reporting a high number of transcripts in flower and seed outer coat.

Based on these data and the protein homology of *PgMYB1_contig* with proteins AtMYB66 and AtMYB114 (Paragraph 4.4.2), *PgMYB1* could play a role in the formation of the aril flesh and in the biosynthesis of anthocyanins.

Tab. 27. Analysis of variance of gene expression ratio of *PgMYB1.2* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Souce of Variation	***PgMYB1***			
	df	Absolute value		% of total
Genotype (G)	1	0.49	*	42.83
Tissue (T)	3	0.39	*	34.02
G*T	3	0.26	*	23.15

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

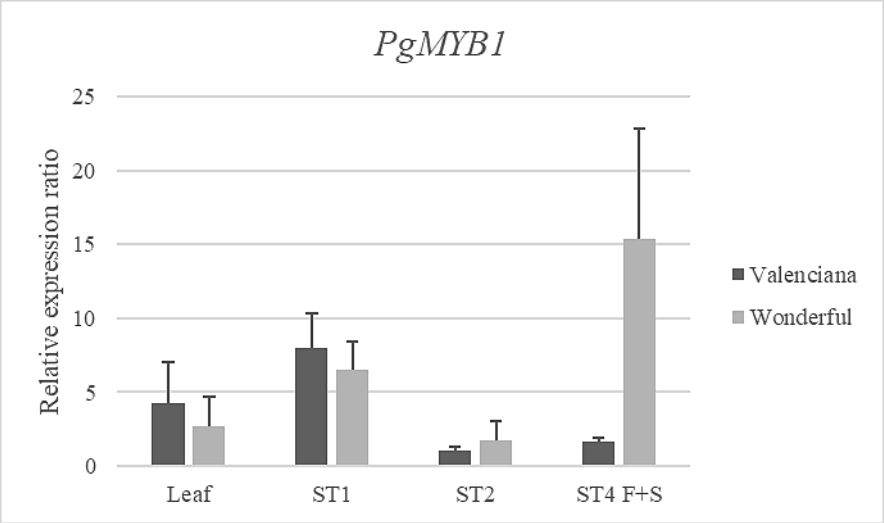


Fig. 60. The level of gene expression of *PgMYB1* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.2 *PgMYB1.2* expression profile

The transcriptional gene levels of ***PgMYB1.2*** showed to be highly significant in the two genotypes, in their tissues and in the interaction between them (Tab. 28). The transcript

abundance increased strongly with arils colour ripening in red-coloured pomegranate cultivar resulting overexpressed compared to the ripe (ST4 F+S) of pink-coloured variety that was below the control (VL-ST2) as well as in all other stages (Fig. 61) and it was absence in leaf. The expression of the transcript was similar in flower of both cvs, but it was greater in unripe fruit of Valenciana and higher in ripe fruit of Wonderful.

The expression pattern of *PgMYB1.2* observed in this study differs respect to what reported by Ben Simhon *et al.* (2011, 2015) and Zhao *et al*. (2015) in peel fruit, where peaks were found in flower or turning stage and in ripe stage. High *PgMYB1.2* expression levels in ripe fruits in particular in Wonderful could indicate their main contribution in the late stages of the anthocyanin biosynthesis pathway. Given this difference in behaviour respect to the other *PgMYB* genes analysed and the still limited knowledge about the pomegranate MYB isoforms a further isolation of *PgMYB1.2* sequences would be desirable to understand whether this sequence represents a new *PgMYB* isoform or not.

Tab. 28. Analysis of variance of gene expression ratio of *PgMYB1.2* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Souce Variation of	***PgMYB1.2***			
	df	Absolute value		% of total
Genotype (G)	1	0.52	***	14.00
Tissue (T)	3	1.68	***	45.13
G*T	3	1.53	***	40.87

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

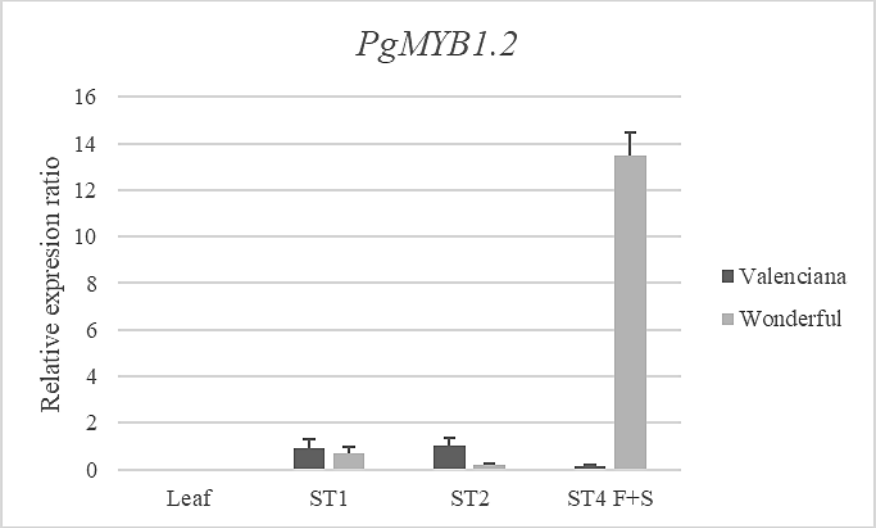


Fig. 61. The level of gene expression of *PgMYB1.2* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.3 *PgMYB2* expression profile

In contrast to gene *PgMYB1.2*, the statistical analysis ***PgMYB2*** showed that gene transcription levels were highly significant in tissues contributing to the 87.48% of the total variance. No significance was found for the genotypes and the interaction between the genotype and the tissue (Tab. 29). The value of relative expression, in the average of the genotypes, was higher in flower about 10 times more than the control in both cultivars. Subsequently, it significantly reduced in fruit in growth, reaching a value of 3.37 in ST2 of Wonderful and a value similar to the control in ripe fruit of both accessions (Fig. 62).

The expression pattern of *PgMYB2* was higher in the flower and decreased with the maturation of the aril in accordance to what reported by Qin *et al.* (2017) for the Pgr021507.1, which protein sequence OWM86420.1 showed a 100% identity with the contig obtained in this study. However, the expression levels of *PgMYB2* in flower and unripe aril together with the protein homology with *AtMYB5* (see Paragraph 4.4.2) and high production of dihydroflavonols (DHK, DHQ and DHM) but not of anthocyanins, in *PgMYB2*+*PgbHLH* agroinfiltrated *N. benthamiana* plants (see Paragraph 4.4.3) suggest that *PgMYB2* and *PgbHLH* genes could play a role in the early steps of biosynthesis of anthocyanins to induce synthesis of flavonoids. Dihydroflavonols are used as substrates by FLS to synthesize flavonols and by DFR to synthesize

leucoanthocyanidins, which can be used as substrates by LAR (leucoanthocyanidin reductase) to synthesize catechin or anthocyanidins by LDOX.

PgMYB2 could induce synthesis of catechin in seed, that as showed in this study, decreases with fruit development (see Paragraph 4.3) or of flavonoids in flower, as flavonols. In apple callus and ectopic expression was observed that *MYB12* could interact with *bHLH3* and *bHLH33* and played an essential role in proanthocyanidin synthesis, while overexpression of *MYB22* promoted flavonol accumulation in red apple callus and decreased anthocyanin accumulation (Wang et al., 2017). In *A. thaliana AtTT2* and *AtMYB5* genes were proposed to regulate PA biosynthesis in seeds (Baudry et al., 2006; Gonzalez et al., 2008, 2009; Appelhagen et al., 2011a, Xu et al. 2013), while *AtMYB11*, *AtMYB12* and *AtMYB111* control flavonol biosynthesis in all tissues of *A. thaliana* (Stracke et al., 2007).

Therefore, an important regulation role of MYB TFs could be inferred in the synthesis of flavonoids.

Tab. 29. Analysis of variance of gene expression ratio of *PgMYB2* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgMYB2***			
	df	Absolute value		% of total
Genotype (G)	1	0.05	ns	4.42
Tissue (T)	3	1.04	***	87.48
G*T	3	0.10	ns	8.09

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

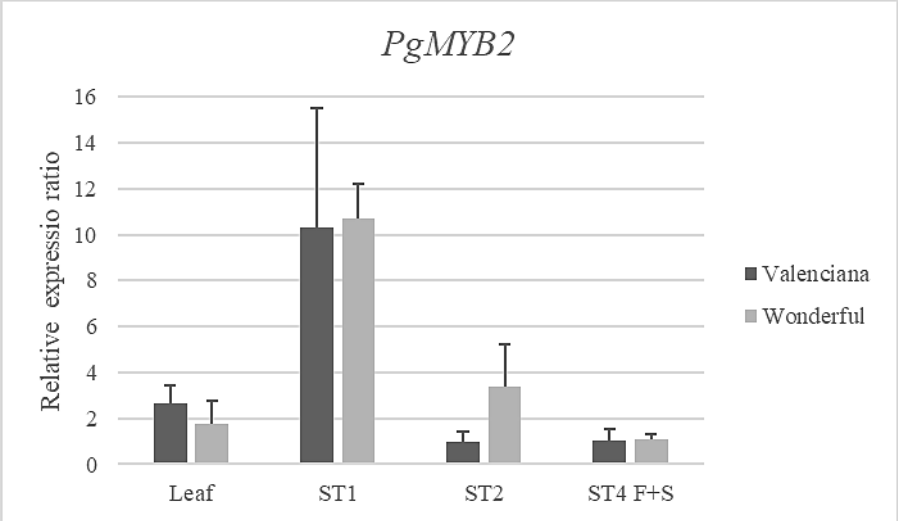


Fig. 62. The level of gene expression of *PgMYB2* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.4 *PgbHLH* expression profile

Statistical analysis of ***PgbHLH*** expression revealed consistent differences among the genotypes, the tissues and their interaction (Tab. 30). The trascriptional levels of *PgbHLH* were nearly constant in all the considered stages of red-coloured fruit cultivar except for leaf and ripe stage of Valenciana that a down-regulation was observed. ST1 stage of both genotypes showed a similar transcript abundance (Fig. 63).

Unlike what reported for Wonderful fruti peel by Ben Simhon *et al.* (2011), in present study the expression level of *PgbHLH* remained constant during all the fruit development stages in Wonderful, while it had a decreasing trend in Valenciana. However, these findings agree with Qin *et al.* (2017) where the most abundant transcripts were detected in flower and in different development stages of outer seed coat, even if the highest expression was found in outer seed coat 50 days after pollination (corresponding to ST2 stage used here). This can indicate its role in anthocyanins biosynthesis and in conjunction with *PgMYB2* in the regulation of dihydroflavonols production in *N. benthamiana* agroinfiltrated leaves (see Paragraph 4.4.3).

Tab. 30. Analysis of variance of gene expression ratio of *PgbHLH* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgbHLH***			
	df	Absolute value		% of total
Genotype (G)	1	1.07	***	53.35
Tissue (T)	3	0.67	***	33.72
G*T	3	0.26	***	12.93

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

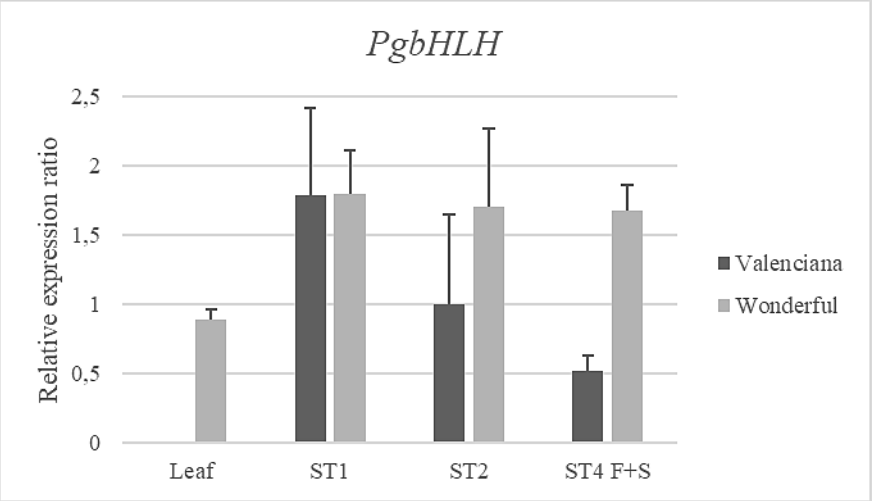


Fig. 63. The level of gene expression of *PgbHLH* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.5 *PgCHS* expression profile

The transcriptional levels of ***PgCHS*** showed to be influenced by the 3 sources of variation (genotype, tissue and interaction of both) (Tab. 31), similarly to what observed in *PgMYB1*. The expression pattern of PgCHS was detected similar in flower of both genotypes, as well as in leaf. Level of expression was very high in ripened arils of Wonderful, while no expression was observed for ripe stage of Valenciana (Fig. 64). Previously the expression level of *PgCHS*, was shown to be uncorrelated with the fruit peel colour and the timing of colour appearance in Wonderful and P.G.135-36 accessions (Ben-Simhon et al., 2011). Zhao *et al.* (2015) documented the presence of two peaks of *PgCHS* transcript abundance (at early development

and at fruit ripening) arguing that is expression was uncorrelated with the increase of anthocyanin biosynthesis.

Here we found higher expression levels in flower of both accessions and in ripened arils of Wonderful. In the latter case, the huge increase of *PgCHS* expression could be related to the increase of both anthocyanins and epicatechins, which were found in the ripe stage (Paragraph 4.3) with a slightly more amount in Wonderful. On the other hand, low expression levels reported for ST4 F+S stage in Valenciana may suggest that more CHS isoforms characterization needs to be carried out on this pomegranate variety. However, the role of this gene may vary according to fruit developmental stage and to genotypes in agreement with the statistical data shown in Tab. 31.

Tab. 31. Analysis of variance of gene expression ratio of *PgCHS* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgCHS***			
	df	Absolute value		% of total
Genotype (G)	1	2.36	***	48.61
Tissue (T)	3	0.57	***	11.63
G*T	3	1.93	***	39.76

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

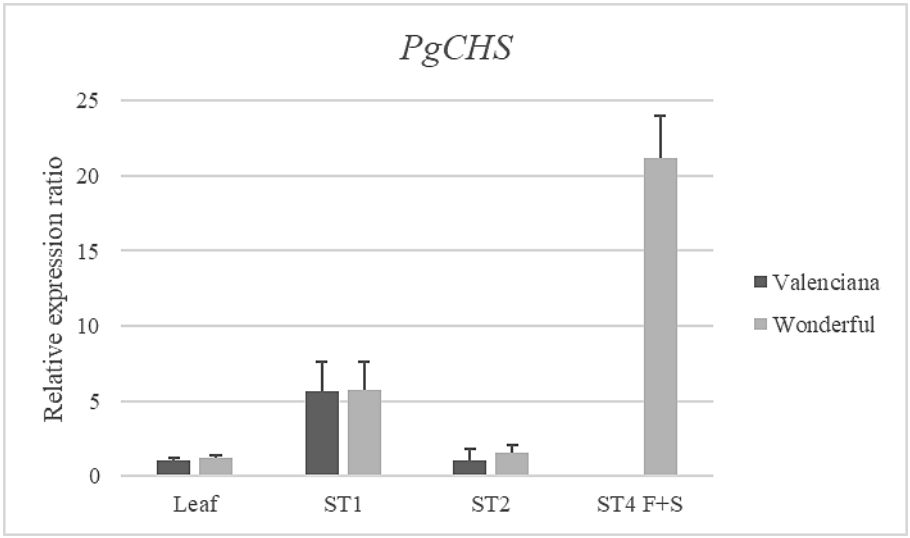


Fig. 64. The level of gene expression of *PgCHS* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.6 *PgF3'5'H* expression profile

In ***PgF3'5'H*** expression levels the interaction between the two cultivars and the tissues was extremely significant ($P \leq 0.001$), but no significant ($P > 0.05$) difference among genotypes and the tissues were observed (Tab. 32). Indeed, as shown in Fig. 65, the transcription level of *PgF3'5'H* was highest in Wonderful ripened arils stage than in Valenciana with 2.48 times up-regulated compared to the control. In contrast at flower stage *PgF3'5'H* expression was higher in Valenciana than in Wonderful similarly to what observed for the transcription levels of *PgMYB1*.

F3'5'H gene determines the hydroxylation pattern of the B-ring of flavonoids and anthocyanins and it is responsible for the production delphindin-based anthocyanins (Seitz et al., 2015). F3'5'H is a key enzyme in anthocyanins structure determination and thus in their colour (Tanaka, 2006). Given the absence of derivatives of delphinidin in Valenciana flower (see Paragraph 4.3) it is possible that in this stage of development the formation of other flavonoids, such as myricitin, is induced. Here a correlation was found between the accumulation of mono and diglucoside of delphinidin during aril ripening, especially in the red-coloured fruit genotype.

Tab. 32. Analysis of variance of gene expression ratio of PgF3'5'H gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgF3'5'H***			
	df	Absolute value		% of total
Genotype (G)	1	0.03	ns	13.46
Tissue (T)	3	0.02	ns	8.33
G*T	3	0.19	***	78.21

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

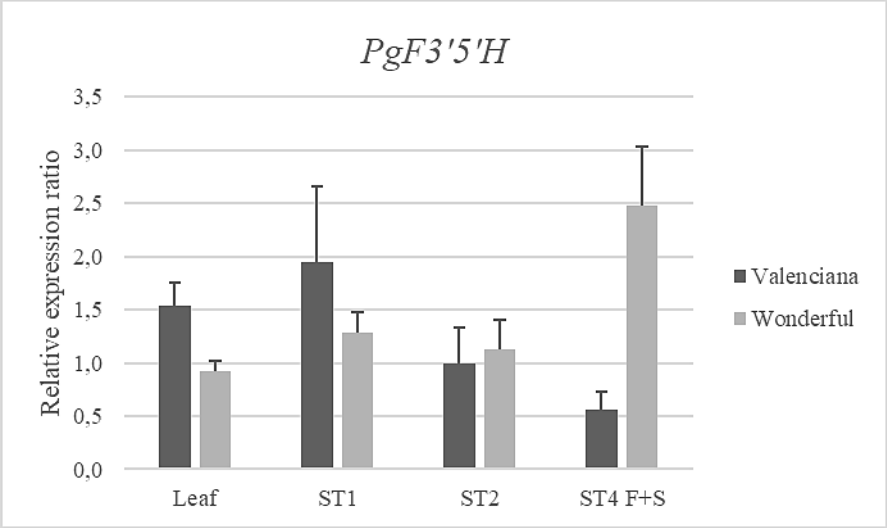


Fig. 65. The level of gene expression of *PgF3'5'H* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.7 *PgDFR* expression profile

Statistical analysis of ***PgDFR*** expression patterns revealed a strong influence by the 3 sources of variation (genotype, tissue and interaction of both) (Tab. 33). The transcriptional level showed to be abundant in flower (19.34 times up-regulated) and in arils ripe stage (20.05 times up-regulated) in red pomegranate compared to the control. A lesser extent it was also present in flower of rose-coloured accession, but not in ripe aril stage. In both cultivars the expression was down-expressed in leaf compared to the control. (Fig. 66)

The expression level of *PgDFR* on flower and aril development stages of red cultivar (Wondeful) follows the same trend reported by Ben-Simhon *et al.* (2011) for Wonderful peel fruit during ripening. Previously *DFR* showed the highest level of expression in dark peel pomegrante genotype compared to red, green and white cultivars (Rouholamin et al., 2015). In red cultivar an increase in expression during maturation is observed and it may indicate that *PgDFR* could be involved in the biosynthesis of anthocyanins in peel and in arils. The low expression of *DFR* in flower of Valenciana compared to Wonderful could support the hypothesis that in flower of Valenciana other flavonol compounds are produced.

Tab. 33. Analysis of variance of fold change of *PgDFR* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgDFR***			
	df	Absolute value		% of total
Genotype (G)	1	1.76	***	33.78
Tissue (T)	3	2.54	***	48.90
G*T	3	0.90	***	17.33

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

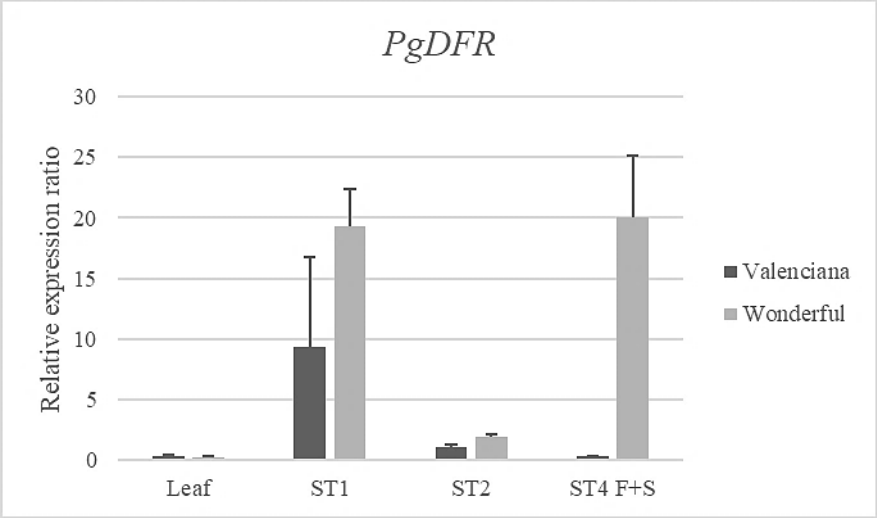


Fig. 66. The level of gene expression of *PgDFR* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

4.4.4.8 *PgUFGT* expression profile

Analysis of the variance of *PgUFGT* transcription levels showed significant influence by the tissue with 66.34% of the total of variation and less significance by the genotype with 28.34%, while no significant difference was found in the interaction between genotype and tissue (Tab. 34). In Fig. 67 it is possible to observe a high expression in ST4 of Wonderful which was 2.18 times up-regulated compared to Valenciana at the same stage. At the ST1 stage a similar gene expression level was observed between Valenciana (2.08 times respect to control) and Wonderful (2.04 times respect to control).

The *UFGT* is important in flavonoids biosynthesis beacouse stabilizes anthocyanidin by attaching sugar moieties to the anthocyanin aglycone (Prior and Wu, 2006). In peach flower the anthocyanin content and the expression levels of *DFR* and *UFGT* genes increase during development (Wen et al., 2014). Here the expression levels of *PgDFR* and *PgUFGT* follow a similar expression trend of Pgr021399 (OWM75048.1 protein) and Pgr022819 (OWM79407.1 protein) as reported by Qin *et al.* (2017) on flower and out seed coat of red pomegranate fruit, with 2 high expression peaks, one in flower and the other in the ripening stage. Valenciana, pink genotype shows only one peak in flower but then decreases with fruit development.

In pomegranate Qin *et al* (2017) evaluated the expression levels of different transcripts related to DFR and to BZ1 enzymes, while Yuan *et al.* (2017) identified 2 genes belonging to the family of DFR and 5 genes of the UFGT family. Among them they detected an expression profile variability depending on the gene during the developmental fruit stages. Therefore, we hypothesize that here different isoforms of *DFR* and *UFGT* were detected in the two genotypes. *UFGT* may have different substrate specificities in pigmented and non-pigmented tissues, as it belongs to an enzyme family that exhibits broad substrate specificity for flavonoids and anthocyanidins with 3-hydroxyl groups (Cultrone et al. 2010). Zhao *et al.* (2015) reported that although anthocyanin glycosylation is inhibited in white pomegranate, the detection of PgUFGT transcripts indicates that a functional glucosyl transferase is active. In red grape UFGT (VvGT1) is responsible for anthocyanin formation and in vitro is active on a range of flavonoids (Offen et al., 2006). In Arabidopsis *At5g17050* encodes an anthocyanidin 3-O-glucosyltransferase which specifically glucosylates the 3-position of the flavonoid C-ring. Anthocyanidins such as cyanidin and pelargonidin as well as flavonols such as kaempferol and quercetin are accepted substrates (Arabidopsis Information Resource, TAIR).

UFGT may be involved in the glucosylation of other metabolic pathways, which may explain why its gene expression pattern was different between Wonderful and Valenciana.

Tab. 34. Analysis of variance of fold change of *PgUFGT* gene expression respect to the CTRL and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	***PgUFGT***			
	df	Absolute value		% of total
Genotype (G)	1	0.15	*	28.34
Tissue (T)	3	0.35	***	66.34
G*T	3	0.03	ns	5.32

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; df (degrees of freedom).

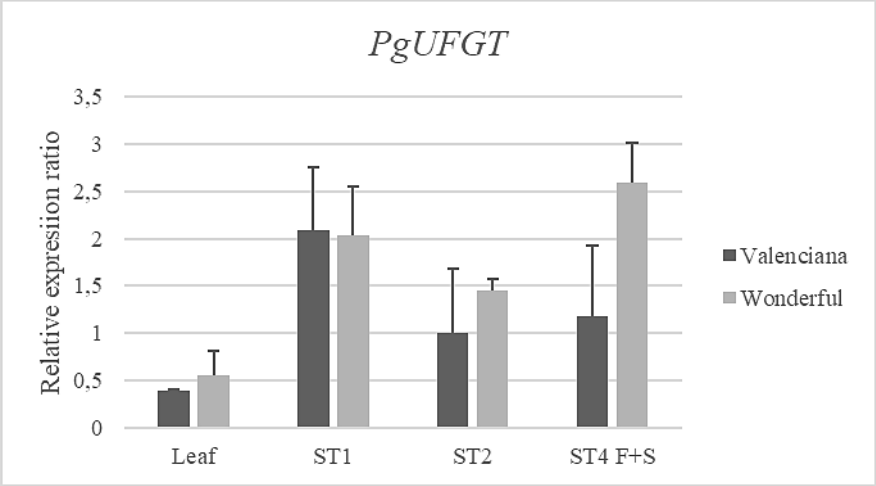


Fig. 67. The level of gene expression of *PgUFGT* in leaf, flower (ST1), unripe aril (ST2) and ripe aril (ST4 F+S) in Valenciana and Wonderful genotypes. The relative expression ratio is expressed as the fold increases relative to unripe of Valenciana that is considered 1. The error bars represent the error mean of three biological replicates.

5 Conclusions

In the present study were examined: the biodiversity of Sicilian pomegranate germplasm; the metabolomic profile in the juice and during fruit development and finally some of the molecular mechanisms of the anthocyanin biosynthesis, which are interesting compounds that mainly contribute to the peculiar nutraceutical properties of the juice.

Characterization of Sicilian local accessions were carried out through an integrated morphological, biochemical and molecular approach and seed germination behaviour was analysed. Significant differences were observed for many of the examined parameters and a great variability was detected across the investigated accessions with Sicilian ones showing important differences on relevant morphological traits respect to international varieties. In particular, all the Sicilian accessions exhibited smaller fruit size respect to Wonderful, while aril and juice yields were higher than those measured for Wonderful One with the Gen.8 and Dente di Cavallo showing the highest levels.

Total phenolic content and antioxidant activity were also significantly different among cultivars, the highest level was found in Sicilian Gen.9, which feature lower the pH value and confers a sourer taste. This accession, together with Wonderful One and Wonderful, presented the lowest L * value in accordance with their peculiar deep red colour.

As regards to biochemical analyses, for most of the assayed accessions glucose was more abundant than fructose with a ratio glucose/fructose taking values from 0.9 to 1.3. For the first time an evaluation of mineral content in Sicilian genotypes was performed showing a rich amount of Potassium (the highest element) with greater concentration in Dente di Cavallo and in Wonderful. The peculiar morphological and biochemical features of Sicilian pomegranates make these accessions promising for commercial exploitation as fresh or processed fruit.

New molecular markers linked with the anthocyanin biosynthesis are presented here and they can be used as new molecular tools for targeted phenolic composition germplasm screening. Indeed, the UPGMA analysis obtained using MYB-related-genes is in accordance with morphological and biochemical features of Wonderful, while Valenciana is genetically and physico-chemically closer to Sicilian genotypes. In general, the molecular and bio-morphological data sets are positively correlated. In fact, the isolation of Wonderful from the other cultivars observed in the PCA is in agreement with the MYB related genes UPGMA.

The seed germination assay carried out for the first time in Sicilian genotypes showed a high germination variability among the cultivars analysed. We found a high percentage of germination among Sicilian genotypes, except for Dente di Cavallo, while Valenciana was the

accession with the highest germination speed and rate. These results are very useful for further breeding efforts of Sicilian genotypes.

Juice metabolomic profile showed significant differences in phenolic composition among cultivars with greater presence of ellagitannins, such as punicalagins, in Wonderful One, while higher anthocyanins in Wonderful. Quantification of punicalagins, showed higher values for Gen.1, Gen.2, Valenciana and Wonderful One respect to anthocyanins content, while the opposite composition was observed in Wonderful. The analysis of PCA based on metabolic content showed two distinct clusters, a first with Valenciana close to the Sicilian genotypes (Gen. 1 and Gen. 2) and a second group that includes Wonderful and Wonderful One. This result is in agreement with the UPGMA and PCA morphological and biochemical data reported previously where Wonderful was separated from Valenciana, which, in turn, is close to Sicilian genotypes. In the metabolic PCA Wonderful and Wonderful One showed a close relation, however the phenolic composition substantially differed *e.g.* punicalagins and cyanidin-3-glucoside, as for TPC and AA levels between the two cultivars. Therefore, we suggest Wonderful as a good candidate for the study of anthocyanins biosynthesis, while Wonderful One for the ellagitannins biosynthesis.

The metabolomic profile during fruit development stages in Wonderful and Valenciana showed an increase of anthocyanins mainly in ripe aril. At this stage the hydrolysable tannins (ellagitannins and gallotannins) were poorly present while resulting highly concentrated in leaves, flower and unripe aril. High values of ellagic acid, gallic acid and punicalagins were observed in flower of both cultivars, mainly in Valenciana. Concerning catechin and epicatechin, we found that catechin decreased during fruit ripening, and it seems more concentrated in seed, while opposite situation was observed for epicatechin, which increased during fruit development stages. These condensed tannins were always with a greater intensity in Wonderful cv. While substantial differences between the two genotypes were found for the anthocyanin profile with Wonderful richer than Valenciana. Higher values of cyanidin (red pigment) and delphinidin (purple pigment) were found in Wonderful ripe aril, while the monodiglucoside of pelargonidin (orange pigment) was more concentrated in Valenciana. Whereas the diglucoside of pelargonidin was found much more in flower than ripe aril stages and in particular in Valenciana. These results confirm different colour in arils of the two cultivars and the orange colour of the flowers.

The investigation of pomegranate anthocyanins pathway complex regulatory genes (PgMBW), showed that among the different constructs combinations agroinfiltrated in *Nicotiana benthamiana,* the *PgMYB2&PgbHLH* led to a colour change in leaves. From their metabolomic

analysis a major role of the complex in the formation of dihydroflavonols (DHK, DHQ and DHM) was deduced, while the absence of anthocyanins compounds suggests no relation with their synthesis. The gene expression assays in pomegranate showed a high transcription level of *PgMYB2* at the flowering stage of Valenciana and Wonderful, while a decrease was observed during fruit ripening. Likewise, *PgbHLH* transcription levels were higher in flower and decreased in ripening stages for Valenciana, while remained stable for Wonderful. These higher expression levels observed for *PgMYB2* and *PgbHLH* in flower and partly in unripe fruit, together with the production in *N. benthamiana* leaves of dihydoflavonols, suggests that this regulatory complex could play a role in the early steps of the anthocyanins biosynthesis to induce the production of flavonoids especially dihydroflavonols. These compounds could be converted to flavonols in flower or to catechin in unripe fruit. However, as anthocyanins concentrations, at later stages of fruit ripening in Wonderful the expression levels of *PgMYB1, PgMYB1.1, PgCHS, PgF3'5'H,* and *PgUFGT* increased, while their expression decreased in Valenciana variety, which is characterized by a lower amount of anthocyanins. Moreover, similar *PgDFR* expression levels were observed in flower and ripe fruit for Wonderful in agreement with the anthocyanins profile observed in this accession. These data suggest the important role of *PgMYB1, PgMYB1.1, PgCHS, PgF3'5'H, PgUFGT* and *PgDFR* in the biosynthesis of anthocyanins especially for the reddish-fruit pomegranate genotype.

The results obtained in this work allow to expand the knowledge concerning Sicilian genotypes surveyed features; to examine the metabolites in the juice and during fruit development stages and to investigate the biosynthesis mechanisms of the anthocyanins in the pomegranate. These data may represent a starting point for further studies and insights on the metabolomics, *i.e.* composition of flavonols, and genomics, such as the characterization of new isoforms, linked to biosynthesis of compounds with high nutraceutical value of this important species.

6 Appendix

Supplement 1. Sequence contigs obtained from pJET cloning.

PgDFR_contig

CCTTAGCCTGTGGAGGGCCGATCTCAACGAAGCGGGAAGCTTCGATGAGCCAATCCAAGGGTGCACTGGCGTGTTCCACGTCGCGACCCCCATG
GACTTTGAATCCAAGGACCCTGAGAATGAAGTGATCAAGCCAACGATCGAAGGGATGCTCAGTATAATGAAGTCGTGTGTGAAGGCGAAGGTGA
GGAGACTGGTCTTCACGTCTTCCGCCGGGACTGTCAATGTTCAACCCGTCCAGAGACCTGTCCACGACGAGACCTCATGGAGTGACCTCGACTT
CGTCTGGGCGACCAAGATGACCGGTTGGATGTACTTTGTGTCCAAGACAATGGCAGAGCGAGCTGCCTGGAAATTCGCCGAAGAGAACAACCTT
GATTTCATCAGTATCATACCGACCCTCGTGGTCGGCCCGTTCCTCATGCCTTCTTTCCCTCCGAGCCTCATCACCGCGCTTTCCCCGATCACGA
GGAACGAAGCTCACTACGGGATCATAAGGCAGGGCCAGTTCGTTCACCTGGACGACCTATGCATGTCCCACATATTCCTGTACGAGCACCCAGA
GGCCAAGGGCCGGTACATCTGCTCATCGCACGATGAGAACATCACCGGCATCGCAAAGCTCCTAAGGGAGAAGTACCCCGAGTATGACATCCCA
ACAAAGTTCGAAGGGGTCGATGAGAACACAGAGAAGGTGTCGTTCTCTTCGAAGAAGCTAAGGGACTTGGGGTTCGAGTTCAAGCACAACTTGG
AGGACATGTTCGTCGGGGCTGTGGAGTGTTGCAGGGAGAAGGGGCTTCTCCCTCTTTCCCAT

PgLDOX_contig

TTCGAGGAGGAGAAGAGGGATGAGGGCCCTCAGGTCCCGACCATTGACCTAAGGGACATTGAGTCAGAGGACGAGGTTGTCCGGGAGAAGTGCC
GGGCCGAGCTCAAGAAGGCCGCCGCTGACTGGGGCGTCATGCACCTCGTGAACCACGGGATCCCTGACAACCTCATTGAGCGGGTCAAGAAGGC
CGGGGAGGAGTTCTTCAACCTCCCCATTGAGGAGAAGGAGAAGTACGCGAACGACCAGGCCTCGGGGAGGATCCAAGGGTATGGGAGCAAGCTC
GCCAACAATGCCAGCGGGCAGCTCGAGTGGGAGGACTACTTCTTCCACCTCGTGTTCCCGGAGGACAAACGCAACATATCGATCTGGCCTAAGA
TCCCGAGTGATTACAAGGCCGCGACCGCAGAGTATGCAAGGCTGCTACGGGCATTGGCGACGAGAGTGCTCTCGGCCCTCTCGCTTGGGCTGGG
CTTGGAGGAAGGCAGGCTGGAGAAGGAAGTTGGCGGGCTTGAGGAGATGCTGCTGCAGATGAAGATCAACTTCTACCCGAAGTGCCCGCAGCCG
GAGCTGGCCCTCGGCGTGGAGGCACACACCGACATCAGCGCCCTAACCTTCATCCTCCACAACATGGTCCCGGGCCTGCAGCTCTTCTACAAGG
GTAAGTGGGTGACCGCCAAGTGCGTTCCGAACTCGATTATCATGCATATTGGGGACACAATCGAGATCCT

PgUFGT_contig

GTCATTTCCACGGGAAGCCCCAGGAGGACATCGGCCTCTTCATGAGGGCTGCCCCCGAGGGCTTCCGTAAGGCTATGGAGGCTGCAGCCGTCGA
CTCCGGGAAGAGGATAACCTGTGTGGTGGCCGACGCCTTTTTCTGGTTCGCTGAGGAGATAGCTGCAGAGCTGCGGGTGCCCTGGGTTGCCTTC
TGGACCAGTGGACCCTCCTCCCTCGCCGCCCATCTCTACACTGACCTCCTCAGGAACAAACTCCATATCACCGACGGAATAGCGGGGCTCGGAG
ACGAGAGGCTTAATGACTTCATCCCTGGTATGTCAAATAAGATGCGACTCAAGGACCTCCCCGAAGGAGTCCTCTTCGGCAATTTGGAATCCCC
TTTCTCCCACATGCTGCTCCGCATGGGCCGATCATTCCCACGAGCAACCGCCGTTGCTCTCAACTCTTTCGAGGAGCTGGACCTTTCCCTTACC
CAACATCTCGAGTCCCACCACATCCCCACCTTGCTCAGCATCGGGCCTTTGAACCTGCTGCACCTGCAGCTCCACGGGACTCATAATTTAGTAG
CAGGAGCACCCAGAGGCTCCGATGAGTACGGGTGCCTCCCGTGGCTCGACAATCAGAAGCCGGCGCCTGCTTCCGTCGCCTACATCAGCTTTGG
GTCAGTGACAGTCCCTCCTAGGAGCGAGCTCGTGGCAATAGCAGAAGCCCTGGAGGCCAGCGGGGTGCCCTTC

Supplement 2. Sequence contigs obtained from TOPO TA cloning.

PgMYB1_contig

ATGGAGGAAGCTGCTTCTTTTCGAAGAGTAAGGAAAGGTGCATGGACTGAGGAAGAAGACGACCTTCTTCGGAAATGTGTAGATAAGTACGGAC
AAGGTGAATGGCGCCGAGTTCCTCTCAGAGCAGGTACACATATATACAGTTCTAACCCTCTCGTTATATATATTGAGAAAAATGAAAATACATC
GGCATGTATAAAATCTCCAACGTGAACATGCGCCAAGTTCGGGACAGTTTGAGTTAGCACACTCGTACTCTTTAATTTGCAACTGTAATGGATG
ATCTGCAGCCTAATTTGACACTTTGGCAGCTGAAACTTAACCCAAACTAAAGAAAAGATCATGACCCTGGTGGGATATATTATATCATATAGTC
ACTTTACATAATATATGTGTAATATATTCTGATGATTAGCCTTGCTTCAGTGGACTTGTTCGTTTTGCTTTTTCTATGGGTCTGCAATTTTTTT
CTGAACTAAATGAAGTCGGTAGAGAATTACTTAGCTTGTCGCATACGCATGCTTCCGTTTAACCTGCAGTGCTTCTGGTATTAATGAATTGCTT
CCAAGTACTAATAATCAGGGCTTCGTAGATTGTAAGTTTTCACTAACATGATCAaGaAACTGTGCGTATGAAGGGTTGAATAGGTGCAGGAAGA
GCTGTCGTTTGAGGTGGCTGAACTATTTAAGTCCAAACATAAAGAGAGGAGAGTTTACTGAAGATGAGATTGACATGATCGTCCGTCTTCACAA
GCTTCTAGGGAACAGGTAAGTTAAACATTCATGCGTGTGCTTTTATTGATCATAGTTAGGGTTTTAGTTTATGTCACTACCATAAAATGTGCTT
CTCTTTTTGATTTGTGGATTGGTACGTATTTCACTTCCAGATGGACGTTGATTGCCGGTATACTTTCAGGAAGAACTGCGAATGATGTCAAGAA
CTACTGGAACACTCACATGGTCCGGAAGAAGACAGTTCCTAATGAGGAAATCCATCAAAGGAAAACCAAAACGAAAACCAACATTATAATTAAA
CCTCGGCCTCGGACACTCTCAAAGGACTTCGGTAAAGCAAAACCTGCAGGAAATACTGCTGCAGCAGCAAGTAATAATACGATCCCACAAGACC
ATCTTGACGGGAATCCACCGTCAATGACACCGGAAGATGACACCGTGTGGTGGGAGAGCATGATCCTTGAGGAGGGCGTGGCAACCCCCAGATG
TAGAGGTGGGGCTGCCGGAGAGCCACCACCCACGAGCCTATGGAGTCTAGAGGGGGAGAAGGCTAGGGACAACGAGATGTCCTTTGATGTCGAC
ATATGGAATGCTCTATATGCCGACCAAGAAGAGATGGGGATAATATAA

PgMYB2_contig

ATGACGGCACCAACAAGGCGTAACAAGAACAGCAACAATAATGGTAATGAGCAGGGAAGCAGCAGCAGCAAGAAGGTGATGAACAGAGGGGCAT
GGACTGCTGAGGAAGACCGGAAGCTCTCTCAGTACATCGAAATCCACGGCCCCCGGAGGTGGAAGACTGTTGCTTCTAAATCAGGTACAATTGC
ATATGTATCTCATCTTCATGTACGTACAACACATTGTTGCTTTTACTTCATTCTGTACATGTACAATTAAATCTACCTATACATGATAATAATA
AGAAGGACTTTTGGTTTTTTTGGGTTGCAGGGTTGAACCGATGCGGGAAGAGTTGCAGGCTGAGGTGGCTGAACTATCTGAGGCCCAATATCAA
GAGAGGGAACATCTCGGACGAGGAAGAGGATTTGATTCTCAGGCTCCATAGGCTGCTGGGCAACAGGTAATAAATTACATTTCGACGAAAAGTT
TTTCCATCCCGAGAGCACGATGTATGTGTTCTTTTTTCGAGAATGCAGCCCAGCATCCTAAAAGGTTTACCGGACAAGTCAGGACGCAACCCCA
ACGGGTTCGACTTAGTAGTTAATGTGCACTCAAGTTTACACCGAGACCCGGGTTCGAATCCCCTTGGGACTAATTATCCGCTCCGTGCACCGCC
GGGCTTCACGAAGCTTCGGAAAGACAAGTGACAACACGTATTCCTAAGATCTCAGCATTGCACGAAACTCGCTTGTCTTGCTAAAGGATTTATC
TCGATAACGAAGTATTAACATTTTTGTTCGTGTTGTTTAATTATTACCAGGTGGTCCTTGATCGCTGGGAGGCTTCCAGGCAGGACTGATAACG
AGATTAAGAACTACTGGAATTCTCATTTGAGCAAAAAAATAAATCAAAAGGAGAAATCCAGAGAAGTTCCAGCACCTCAGGAGGCCACAACACC
GCCGCTTAAAACTCCCGAAAACTCTCAGGCCGAGGAGGAGGAGGGCGGCAATAAATTGGGGACCGGAAACTTGGATCCTAGCTTTGACGTGAAC
GAGTTCTTCGACTTCACTACCGAGGGATCTTACGGTCTCGAGTGGGTCAATAAGTTCCTCGAACTCGACGAGGACATGTTGACCGCTGATAGCG
AATTTTAA

PgMYB2_splice_contig

ATGACGGCACCAACAAGGCGTAACAAGAACAGCAACAATAATGGTAATGAGCAGGGAAGCAGCAGCAGCAAGAAGGTGATGAACAGAGGGGCAT
GGACTGCTGAGGAAGACCGGAAGCTCTCTCAGTACATCGAAATCCACGGCCCCCGGAGGTGGAAGACTGTTGCTTCTAAATCAGGGTTGAACCG
ATGCGGGAAGAGTTGCAGGCTGAGGTGGCTGAACTATCTGAGGCCCAATATCAAGAGAGGGAACATCTCGGACGAGGAAGAGGATTTGATTCTC
AGGCTCCATAGGCTGCTGGGCAACAGGTGGTCCTTGATCGCTGGGAGGCTTCCAGGCAGGACTGATAACGAGATTAAGAACTACTGGAATTCTC
ATTTGAGCAAAAAAATAAATCAAAAGGAGAAATCCAGAGAAGTTCCAGCACCTCAGGAGGCCACAACACCGCCGCTTAAAACTCCCGAAAACTC
TCAGGCCGAGGAGGAGGAGGGCGGCAATAAATTGGGGACCGGAAACTTGGATCCTAGCTTTGACGTGAACGAGTTCTTCGACTTCACTACCGAG
GGATCTTACGGTCTCGAGTGGGTCAATAAGTTCCTCGAACTCGACGAGGACATGTTGACCGCTGATAGCGAATTTTAA

PgMYB4_contig

ATGGGAAGGTCTCCTTGCTGTGAGAAAGCTCACATGAACAAAGGCGCCTGGACGAAGGAGGAAGACGACCGGCTCATCGCCTACATAAGGGCCC
ATGGGGAAGGCTGCTGGCGGTCCCTCCCGAAGGCCGCCGGCCTCCTGCGGTGCGGCAAGAGTTGCCGCCTCCGATGGATCAACTACCTGCGTCC
CGATCTCAAGCGCGGGAACTTCACCGAAGAGGAAGACGAGCTCATCATCAAGCTTCACAGCCTCCTCGGCAACAAGTTAGTATTTATTTATTAT
AAGCAATTTGGCCTTCATATTATCACAAATTATATAATATCTGTATTTTGTACATTTTTTGATGGTTTTTTTTTTGGGGGTAAAATAGGTGGTC
CTTGATTGCTGGTCGATTACCGGGCAGGACCGATAATGAAATAAAGAACTACTGGAACACGCACATAAGGAGGAAACTCTTGAACCGAGGCATC
GACCCTtCCACCCACCGGCCAATCAACGAGCTACCGGCGTCGACAGGGGAACCCGTGACGAACATCTCATTTTCCTCCGCCGCAGCAATGGTGT
CTAGAGTACAGCATCAAGAGAAGAAGGCCGATAATGCCGAAGGGTTCAAGATCAAGTTAGAGGAGAAGAACCCGGCTCATCAGGAGCAGTGCCC

GGACTTGAACCTCGAGCTCAGAATAAGCCCTCCCCATCAAACCCAATACCCTCAGGCCGATCAGCCGATGAAATCCGGCGGGAGAAACCACCTT
TGCTTCGTGTGCAGCCTAGGGTTGCAAAACAGCAAGGAGTGCAGTTGCAACATTAGCGGGGGCAGAGGCAGCGGCGCTTCTAGCAGTTATGACT
TTCTAGGGTTGAAAACCGGCGTATTGGATTACAGAAGTTTGGAGATGAAATGA

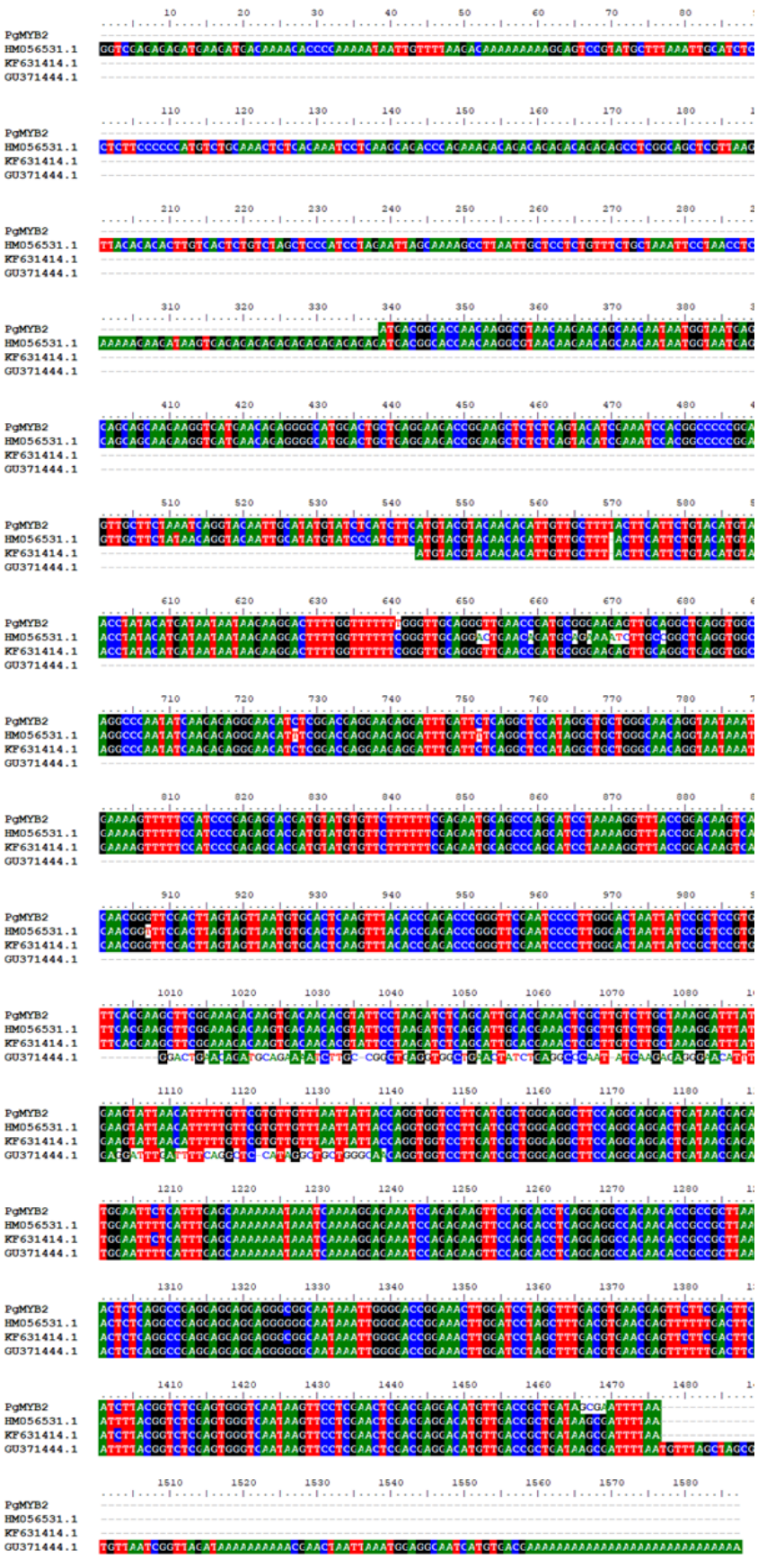
PgbHLH_contig

ATGGCTGTGCCGCCCAGTAGCCGCCTTCAAAGCATGCTGCAGGCAGCGGTCCaATCAGTTAAATGGACCTATAGTCTCTTCTGGCATATCTGTC
CTCAGCAAGGGATCTTAATATGGGGAGATGGGTACTACAACGGGCCAATCAAAACACGGAAGACAGTGCAGCCCATGGAAGTTAGTGCCGAGGA
GGCCTCGCTGCAGAGAAGCCAGCACCTCCGGGAGCTCTACGAGTCCCTCTCGGCCGGCGAGACAAACCAGCCTGCGAGGCGGCCTTGCGCCTCG
CTCTCGCCCGAGGACCTGACCGAGACAGAATGGTTCTACTTAATGTGCGTCTCTTTCTCATTTCCTCCCGGGGTAGGGTTACCGGGGAAAGCAT
ACGCGAGGAGGCAGCATGTATGGCTCACCGGAGCAAACGAAGTTGATAGCAAGACATTTTCAAGGGCTATCTTAGCCAAGAGCGCTCGTATCCA
GACAGTTGTGTGCATTCCTCTGCTAGACGGCGTCGTGGAGCTGGGGACAACAGAAAGGATACAAGAGGACATTGGATTTACAACCCATGTAAAG
AGCTTCTTCATCGACCACCAACCCCCTCTTCCACCAAAGCCGGCCCTCTCGGAGCACTCCACCTCCAATCCCGCAACGTCCTCCGACCACCCTC
GTTTCAACTCGCCGCCAGTCCCCTCTATTGTGTACGCTGCAGCTGCTGTGGATCCCCCAGTGGCTGCTCACCAAGCAGCAGATGATGAGGAGGA
GGACGAGGAAGAGGAAGACGAGGCTGAGTCTGACTCCGAGGCTGAGACTGGCCAAGCCGCCAGCCGACAGGCAGTAGGTCAGAACCCGCAAGCC
GGCGTGGTTGGGGCAGCCGAACAGAGCGAGCTCATGCAGCTCGAGATGTCAGAGGACATTCGGCTCGGCTCACCCGATGATGCGTCGAACAATC
TGGATTCAGATTTTCACATGCTGACAGTGAACCAAGCAGGCAACATTAACCAGGCAGACCACCAGCGACGAATCGATTCATTCAGGGCCGAGTC
GAGCCGGAGGTGGCAGATGATGCAAGATCCCCTGAGAAGCAGCCTTCAAGGTCCACCATCAGGCCCTCCGCAGTTGGATGAACTGTCACAAGAG
GATACGCACTACTCTCAGACCATCTCGACCATCCTCCAGAGCCAGCCAAACAGGTGGGCAGAGATATCATCGACTAGCTATGTCTCGTACTCGG
CTCAGTCGGCATTCTCTAAGTGGTCGACCCGGGCCGACCACCTACTCCATGTCCCAGCAGAGGGCACGTCCCAGTGGCTCCTCAAGTACATTCT
GTTCAGCGTGCCATTCCTGCATAACAAGTACAGGGATGAGCACTCACCCAAATCCCGGGATGGCGAATCCTCAACCCGGTTCCGCAAGGGCACT
CAGCAGGATGAGCCGAGCGCTAACCATGTGCTGGCAGAGAGACGGCGCCGGGAGAAGCTGAACGAGAGATTCATTATATTGAGGTCCCTGGTCC
CGTTTGTCACCAAAATGGACAAGGCCTCAATATTGGGCGACACGATCGAGTACGTTAAGCAGTTGCGCAAGAAGATTCAGGACCTTGAAGCAAA
GAATAGACAGATGGAGGCAGACCAGAGGTCAAGGCAGATCGGAGAACCCCAGAGGTCCGGCAGCGTTGACAGGGCTGCCCGCATGGCCCCGTTG
GCGACGGAAAAGAGGAAAATGAGAATCGTAGAGGGTAATGGCGGGGCAAAGCCGAAAGCTGTGGAGTCCCAAGCACCGCCACTGCCGCTGCCGC
CGCCAGCACCGGCACCGATGGAAACCAGCGCGCAGGTCTCGATCATAGAGAGCGATGGGTTAATCGAACTACAATGCCCACACAAAGAGGGTTT
GCTGCTCGATGTCATGCAGATGCTCCGAGAACTCAGGATCGAAATAACTGCAGTCCAATCGTCTCTCACTAACGGGTTCTTCGTGGCAGAATTG
AGAGCCAAGGTGAAGGAGAATTTAAATGGGAAGAAAGCAAGCATCGTTGAGGTGAAGAGGGCAATCCAACAGCTTATACCCCACACTGACTCTT
AG

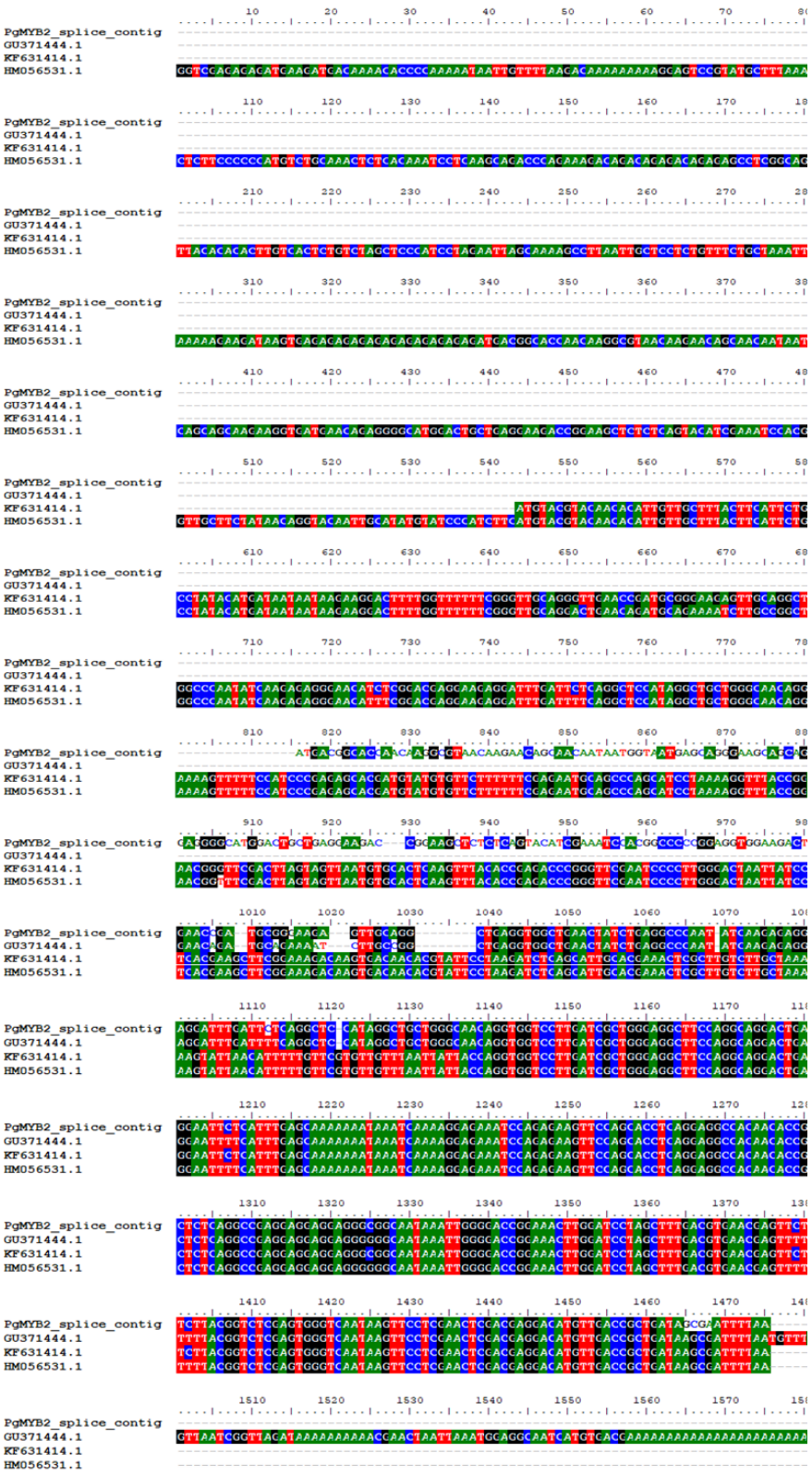
PgWD40_contig

ATGGACAACTCGACTCAGGAGTCCCACCACCTCCGATCTGAcCACTCCGCCACCTATGAGTCCCCCTACCCGCTCTATGCCATGGCCGTTTCCC
CCTCCCGTCACCACGGTCACAACCACCGCATCGCCGTCGGCAGCTTCATCGAGGAGTACACCAACCGGGTCGACGTCGTCTCCTTCGACCCCGA
CACCCTCACCCTCAAGCCCATCCCCTCCCTCTCCTTCGATCACCCCTACCCTCCCACGAAGCTCATGTTCCACCCCAAGTCCGACCTCCTCGCC
TCCTCCGGCGACTTTCTCCGCCTCTGGGAGGTCCGCGATTCCTCCGTGGAGCCCGTCTCGGTTCTCAACAACAGCAAAACCAGCGAGTTCTGCG
CCCCTTTGACCTCCTTCGATTGGAACGAGATCGAGCCCAAGAGGATCGGCACTTGTAGCATTGACACCACCTGCACGGTTTGGGACATTGAGAA
GAGTTGCGTCGAGACCCAATtGATAGCTCATGATAAGGAAGTGTACGACATTGCGTGGGGGGAAGCTCGGGTTTTCGCATCGGTCTCTGCTGAT
GGGTCGGTGAGAATTTTCGATTTGCGGGACAAGGAACACTCTACGATTATATACGAGAGCCCACAGCCCGATACCCCTTTGTTGAGATTGGCCT
GGAACAAGCAAGATTTGAGATACATGGCCACCATTTTGATGGATAGTAATAAAGTTGTGATCTTGGACATACGATCCCCTGCAATGCCGGTGGC
AGAGCTCGAGAGGCACCGGGCAAGTGTCAATGCGATAGCATGGGCTCCCCAGAGCAGTAGGCATATCTGCTCTGCTGGGGATGATACGCAGGCC
CTGATTTGGGAGCTTCCCACTGTTGCCGGACCCAATGGGATAGACCCAATGTCGATGTACTCTGCTGGTTCGGAGATTAACCAACTTCAATGGT
CTGCTGCTCAGCCTGACTGGATTGCTGTCTCATTCTCAAACAAAATGCAGTTATTGAAAGTTTGA

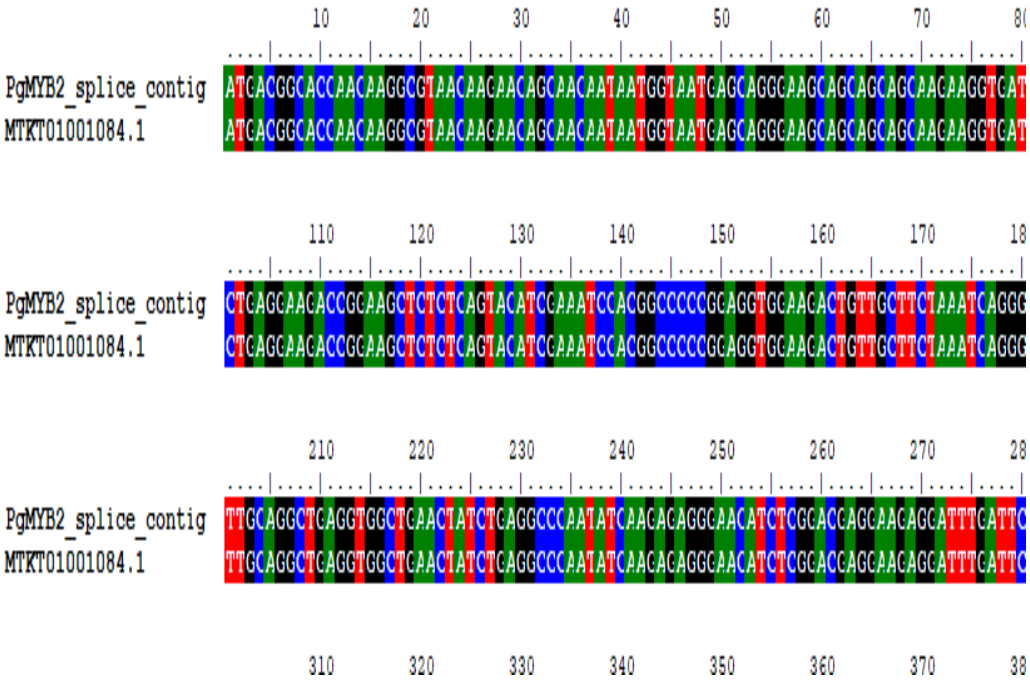
Supplement S3. Alignments of *PgMYB2_conting* against BLASTn of *Punica granatum* sequences (HM056531.1, KF631414.1, GU371444.1).



Supplement S4. Alignments of *PgMYB2_splice_conting* against BLASTn of *Punica granatum* sequences (HM056531.1, KF631414.1, GU371444.1).



Supplement S5. Alignments of *PgMYB2_splice_conting* against *Punica granatum* sequences MTKT01001084.1 (OWM86420.1 protein).



Supplement S6. BLASTx of *PgMYB1_contig* against all NCBI Protein database and limited to *A. thaliana* species. Results show first 8 protein sequences that were found against the relative contigs.

Gene	Results of sequence alignment (blastx)											
	Species name	**Accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	***Arabidopsis thaliana* accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	**Detected putative conserved domains**
PgMYB1_contig	*Punica granatum*	OWM84991.1	457	54%	3.00E-90	99%	NP_196979.1	159	25%	9.00E-22	80%	Myb-like DNA-binding domain
	Punica granatum	AHZ97876.1 ALT22077.1 PKI68397.1 AVW85507.1	453	54%	2.00E-89	97%	AFD94023.1	155	23%	7.00E-21	75%	
	Ecaliptus grandis	XP_018731301.1	218	54%	1.00E-28	75%	NP_001331609.1	120	17%	9.00E-21	80%	
	Morella ruba	ADG21958.1	212	46%	4.00E-28	82%	AAL01236.1	154	23%	2.00E-20	75%	
	Ecaliptus grandis	XP_010064838.2	216	52%	7.00E-28	86%	AFD94041.1	154	23%	2.00E-20	75%	
	Ecaliptus grandis	XP_010062250.1	214	50%	1.00E-27	89%	AAL01223.1	154	23%	2.00E-20	75%	
	Vitis vinifera	XP_002274992.2	210	50%	3.00E-27	82%	AFD94046.1	154	23%	2.00E-20	75%	
	Vitis vinifera	ACL97979.1	209	50%	3.00E-27	82%	AAC97388.1	154	23%	2.00E-20	75%	
PgMYB4_contig	*Punica granatum*	AJD79907.1	531	89%	4.00E-141	99%	OAO97731.1	327	89%	9.00E-83	93%	Myb-like DNA-binding domain
	Theobroma cacao	XP_007041863.1	393	89%	2.00E-99	63%	NP_195574.1	327	89%	1.00E-82	92%	
	Herrania umbratica	XP_021286892.1	389	89%	2.00E-98	61%	AAC83582.1	326	89%	1.00E-82	92%	
	Olea europaea var. sylvestris	XP_022864720.1	389	89%	2.00E-98	63%	NP_564176.2	267	78%	8.00E-65	88%	
	Camellia sinensis	ARB51599.1	387	89%	6.00E-98	63%	NP_192684.1	264	57%	6.00E-64	86%	
	Sesamum indicum	XP_011092988.1	386	89%	2.00E-97	61%	AAF18515.1	243	74%	2.00E-57	86%	
	Hevea brasiliensis	XP_021645677.1	384	89%	6.00E-97	60%	OAP08362.1	310	91%	1.00E-53	83%	
	Handroanthus impetiginosus	PIN19164.1	382	89%	3.00E-96	63%	NP_179263.1	313	91%	1.00E-53	83%	

Supplement 7. BLASTx of *MYB2_contig* and *MYB2_splice_contig* against all NCBI Protein database and limited to *A. thaliana* species. Results show first 8 protein sequences that were found against the relative contigs.

Gene	Results of sequence alignment (Blastx)											
	Species name	**Accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	***Arabidopsis thaliana* accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	**Detected putative conserved domains**
PgMYB2_contig	*Punica granatum*	OWM86420.1	385	54%	6.00E-70	96%	NP_198849.1	176	64%	5.00E-55	57%	Myb-like DNA-binding domain
	Punica granatum	PKI39776.1	376	48%	6.00E-70	96%	NP_196979.1	166	60%	2.00E-51	57%	
	Punica granatum	AHB18402.1	334	43%	8.00E-70	96%	NP_187963.1	166	65%	5.00E-51	58%	
	Punica granatum	ADB92614.1	291	38%	6.00E-68	94%	ABK28720.1	162	49%	4.00E-49	70%	
	Punica granatum	ADG65150.1	325	43%	6.00E-68	94%	NP_198405.1	162	49%	5.00E-49	70%	
	Punica granatum	PKI41545.1	297	46%	2.00E-40	71%	AAL01236.1	160	57%	5.00E-49	57%	
	Punica granatum	OWM88361.1	258	41%	3.00E-40	71%	AAL01243.1	158	62%	9.00E-49	52%	
	Eucaliptus grandis	KCW77811.1	217	37%	7.00E-37	70%	AAC97388.1	159	62%	1.00E-48	52%	
PgMYB2_splice_contig	*Punica granatum*	OWM86420.1	394	99%	6.00E-138	99%	NP_198849.1	176	64%	5.00E-55	57%	Myb-like DNA-binding domain
	Punica granatum	PKI39776.1	382	87%	1.00E-133	99%	NP_196979.1	166	60%	2.00E-51	57%	
	Punica granatum	AHB18402.1	315	72%	4.00E-107	98%	NP_187963.1	166	65%	5.00E-51	58%	
	Punica granatum	ADG65150.1	306	72%	1.00E-103	95%	ABK28720.1	162	49%	4.00E-49	70%	
	Punica granatum	ADB92614.1	303	71%	3.00E-103	96%	NP_198405.1	162	49%	5.00E-49	70%	
	Punica granatum	PKI41545.1	303	84%	4.00E-102	81%	AAL01236.1	160	57%	5.00E-49	57%	
	Quercus suber	XP_023887706.1	287	84%	6.00E-96	79%	AAL01243.1	158	62%	9.00E-49	52%	
	Eucaliptus grandis	XP_010053502.1	286	84%	6.00E-96	79%	AAC97388.1	159	62%	1.00E-48	52%	

Supplement 8. BLASTx of the *bHLH_contig* and *WD40_ splice_contig* against all NCBI Protein database and limited to *A. thaliana* species. Results show first 8 protein sequences that were found against the relative contigs.

Gene	Results of sequence alignment (blastx)											
	Species name	**Accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	***Arabidopsis thaliana* accession number**	**Total score**	**Query cover**	**Expect**	**Identities**	**Detected putative conserved domains**
PgbHLH_contig	*Punica granatum*	PKI60599.1	1239	99%	0.0	99%	NP_192720.2	513	84%	4.00E-103	74%	bHLH-MYC and R2R3-MYB transcription factor N-terminal and Helix-loop-helix domain
	Punica granatum	AIS24255.1	1238	99%	0.0	99%	CAC14865.1	513	84%	4.00E-103	74%	
	Punica granatum	OWM77890.1	1224	99%	0.0	99%	ABK28624.1	513	84%	5.00E-103	74%	
	Punica granatum	OWM69186.1	1012	99%	0.0	81%	OAO98324.1	502	84%	1.00E-97	72%	
	Eucaliptus grandis	XP_010070223.1	965	99%	0.0	78%	T04030	450	61%	7.00E-86	75%	
	Cephalotus follicularis	GAV82393.1	954	99%	0.0	74%	AAB72192.1	320	73%	4.00E-56	48%	
	Betula luminifera	AKG50134.1	952	99%	0.0	72%	NP_176552.1	320	73%	4.00E-56	48%	
	Theobroma cacao	EOX94796.1	940	99%	0.0	74%	NP_001332705.1	317	76%	5.00E-55	45%	
PgWD40_contig	*Punica granatum*	ADV40946.1	692	99%	0.0	100%	NP_197840.1	572	99%	0.0	80%	WD40 repeat
	Prunus avium	XP_021805418.1	608	99%	0.0	86%	CAC10524.1	570	99%	0.0	80%	
	Prunus persica	XP_007218262.1	608	99%	0.0	86%	BAD89974.1	550	96%	0.0	81%	
	Trema orientalis	PON83683.1	607	99%	0.0	86%	NP_172751.1	430	94%	3.00E-151	63%	
	Parasponia andersonii	PON71957.1	607	99%	0.0	86%	AAC18912.1	428	94%	2.00E-150	63%	
	Prunus mume	XP_008234637.1	607	99%	0.0	86%	AAM65213.1	425	94%	2.00E-149	63%	
	Prunus avium	XP_021805417.1	607	99%	0.0	86%	NP_189298.1	421	94%	7.00E-148	62%	
	Prunus persica	XP_020413133.1	607	99%	0.0	86%	AAO42231.1	421	94%	9.00E-148	62%	

Supplement 9. BLASTx of the *PgDFR*, *PgLDOX* and *PgUFGT* against all NCBI Protein database and limited to *A. thaliana* species. Results show first 8 protein sequences that were found against the relative contigs.

Gene	Results of sequence alignment (blastx)											
	Species name	Accession number	Total score	Query cover	Expect	Identities	*Arabidopsis thaliana* accession number	Total score	Query cover	Expect	Identities	Detected putative conserved domains
PgDFR_contig	*Punica granatum*	PKI49859.1	570	99%	0.0	100%	BAD95233.1	428	99%	4.00E-151	73%	Dihydroflavanol-4-reductase
	Punica granatum	AET74075.2	568	99%	0.0	100%	NP_199094.1	427	99%	6.00E-151	73%	
	Punica granatum	AHZ97873.1	568	99%	0.0	99%	CAP08822.1	427	99%	6.00E-151	73%	
	Punica granatum	ALT22074.1	565	99%	0.0	99%	CAP08818.1	426	99%	3.00E-150	72%	
	Punica granatum	OWM75048.1	520	99%	0.0	90%	CAP08805.1	426	99%	4.00E-150	72%	
	Eucaliptus grandis	XP_010060970.1	458	99%	2.00E-160	78%	CAP08819.1	426	99%	4.00E-150	72%	
	Herrania umbratica	XP_021283610.1	453	98%	2.00E-158	78%	CAP08813.1	425	99%	6.00E-150	72%	
	Corchorus capsularis	OMO87552.1	452	98%	1.00E-157	78%	AAA32783.1	425	99%	8.00E-150	72%	
PgLDOX_contig	*Punica granatum*	PKI64472.1	409	99%	3.00E-141	100%	BAC75819.1	348	99%	8.00E-121	80%	Leucoanthocyanidin dioxygenase
	Punica granatum	ALT22075.1	407	99%	1.00E-140	99%	BAC75818.1	347	99%	1.00E-120	80%	
	Punica granatum	AHZ97874.1 OWM71959.1	407	99%	1.00E-140	99%	2BRT_A	347	99%	3.00E-120	80%	
	Nekemias grossedentata	AGO02175.1	366	99%	3.00E-124	86%	NP_194019.1	347	99%	3.00E-120	80%	
	Vitis vinifera	NP_001268147.1	365	99%	4.00E-124	86%	OAP00169.1	347	99%	6.00E-120	80%	
	Jatropha curcas	XP_012087030.1	365	99%	5.00E-124	87%	CAD91994.1	345	99%	4.00E-119	80%	
	Vitis amurensis	ACN38270.1	365	99%	5.00E-124	86%	AEI99590.1	344	99%	8.00E-119	80%	
	Corchorus olitorius	OMO81016.1	365	99%	6.00E-124	84%	1GP4_A	336	99%	1.00E-115	79%	
PgUFGT_contig	*Punica granatum*	ALT22076.1	459	99%	7.00E-159	99%	NP_197207.1	197	99%	2.00E-60	50%	UPD-glycosiltransferase
	Punica granatum	AHZ97875.1	459	99%	7.00E-159	99%	NP_197205.1	175	98%	1.00E-51	47%	
	Punica granatum	OWM79407.1	458	99%	3.00E-158	99%	NP_564357.1	172	98%	8.00E-51	47%	
	Punica granatum	OWM63414.1	394	99%	5.00E-133	87%	AAM65321.1	172	98%	8.00E-51	47%	
	Punica granatum	ADB92613.1	380	77%	7.00E-130	99%	OAP13716.1	169	97%	2.00E-49	47%	
	Punica granatum	AHB18403.1	340	77%	2.00E-114	90%	NP_197206.2	166	97%	1.00E-48	46%	
	Syzygium malaccense	ADB43601.1	243	98%	2.00E-76	58%	CAC01717.1	166	97%	3.00E-48	46%	
	Eucalyptus grandis	XP_010063209.1	246	98%	4.00E-76	58%	OAO92492.1	166	97%	7.00E-48	47%	

7 Reference

Aarabi, A., Barzegar, M., & Azizi, M. H. (2008). Effect of cultivar and cold storage of pomegranate (Punica granatum L.) juices on organic acid composition. *ASEAN. Food. J, 15*, 45-55.

Adams, L. S., Zhang, Y., Seeram, N. P., Heber, D., & Chen, S. (2010). Pomegranate ellagitannin–derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. *Cancer Prevention Research, 3*(1), 108-113.

Adiletta, G., Petriccione, M., Liguori, L., Pizzolongo, F., Romano, R., & Di Matteo, M. (2018). Study of pomological traits and physico-chemical quality of pomegranate (Punica granatum L.) genotypes grown in Italy. *European Food Research and Technology, 244*(8), 1427–1438.

Alcaraz-Mármol, F., Nuncio-Jáuregui, N., García-Sánchez, F., Martínez-Nicolás, J. J., & Hernández, F. (2017). Characterization of twenty pomegranate (Punica granatum L.) cultivars grown in Spain: Aptitudes for fresh consumption and processing. *Scientia Horticulturae, 219*, 152–160.

Allen, G. C., Flores-Vergara, M. A., Krasynanski, S., Kumar, S., & Thompson, W. F. (2006). A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols, 1*(5), 2320–2325.

Al-Maamari, I. T., Khan, M. M., Ali, A., Al-Sadi, A. M., Waly, M. I., & Al-Saady, N. A. (2016). Diversity in phytochemical composition of Omani fenugreek (Trigonella foenum-graecum L.) accessions. *Pakistan Journal of Agricultural Sciences*, *53*(4).

Al-Maiman, S. A., & Ahmad, D. (2002). Changes in physical and chemical properties during pomegranate (Punica granatum L.) fruit maturation. *Food Chemistry, 76*(4), 437-441.

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Hayek, T., et al. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in the atherosclerotic apolipoprotein E-deficient mice. *American Journal of Clinical Nutrition, 71*, 1062–1076.

Aviram, M., Rosenblat, M., Gaitini, D., Nitecki, S., Hoffman, A., Dornfeld, L., et al. (2004). Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clinical Nutrition, 23*, 423–433.

Aviram, M., Volkova, N., Coleman, R., Dreher, M., Reddy, M. K., Ferreira, D., & Rosenblat, M. (2008). Pomegranate Phenolics from the Peels, Arils, and Flowers Are Antiatherogenic: Studiesin Vivoin

Atherosclerotic Apolipoprotein E-Deficient (E0) Mice andin Vitroin Cultured Macrophages and Lipoproteins. *Journal of Agricultural and Food Chemistry, 56*(3), 1148–1157.

Barone, E., Caruso, T., Marra, F. P., & Sottile, F. (2000). Preliminary observations on some Sicilian pomegranate (Punica granatum L.) varieties. *Options Méditerranéennes. Série A, Séminaires Méditerranéens*, (42), 137-141.

Baudry, A., Caboche, M., & Lepiniec, L. (2006). TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in Arabidopsis thaliana. *The Plant Journal*, *46*(5), 768-779.

Beaulieu, J. C., Lloyd, S. W., Preece, J. E., Moersfelder, J. W., Stein-Chisholm, R. E., & Obando-Ulloa, J. M. (2015). Physicochemical properties and aroma volatile profiles in a diverse collection of California-grown pomegranate (Punica granatum L.) germplasm. *Food chemistry, 181*, 354-364.

Bellini, E., Giordani, E., & La Malfa, S. (2010). I fruttiferi minori in Italia, una risorsa tradizionale per l'innovazione frutticola: il kaki e il melograno come casi di studio. *Italus Hortus, 17*(1), 75-90.

Ben-Simhon, Z., Judeinstein, S., Nadler-Hassar, T., Trainin, T., Bar-Ya'akov, I., Borochov-Neori, H., & Holland, D. (2011). A pomegranate (Punica granatum L.) WD40-repeat gene is a functional homologue of Arabidopsis TTG1 and is involved in the regulation of anthocyanin biosynthesis during pomegranate fruit development. *Planta, 234*(5), 865-881.

Ben-Simhon, Z., Judeinstein, S., Trainin, T., Harel-Beja, R., Bar-Ya'akov, I., Borochov-Neori, H., & Holland, D. (2015). A" White" anthocyanin-less pomegranate (Punica granatum L.) caused by an insertion in the coding region of the Leucoanthocyanidin Dioxygenase (LDOX; ANS) gene. *PLoS One, 10*(11), e0142777.

Bewley, J. D., Bradford, K., & Hilhorst, H. (2012). *Seeds: physiology of development, germination and dormancy*. Springer Science & Business Media.

Borochov-Neori, H., Judeinstein, S., Harari, M., Bar-Ya'akov, I., Patil, B. S., Lurie, S., & Holland, D. (2011). Climate effects on anthocyanin accumulation and composition in the pomegranate (Punica granatum L.) fruit arils. *Journal of agricultural and food chemistry, 59*(10), 5325-5334.

Boss, P. K., Davies, C., & Robinson, S. P. (1996). Analysis of the expression of anthocyanin pathway genes in developing Vitis vinifera L. cv Shiraz grape berries and the implications for pathway regulation. *Plant physiology*, *111*(4), 1059-1066.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - *Food Science and Technology, 28*(1), 25–30.

Brighenti, V., Groothuis, S. F., Prencipe, F. P., Amir, R., Benvenuti, S., & Pellati, F. (2017). Metabolite fingerprinting of Punica granatum L.(pomegranate) polyphenols by means of high-performance liquid chromatography with diode array and electrospray ionization-mass spectrometry detection. *Journal of Chromatography A, 1480*, 20-31.

Calani, L., Beghè, D., Mena, P., Del Rio, D., Bruni, R., Fabbri, A., & Galaverna, G. (2013). Ultra-HPLC–MS n (poly) phenolic profiling and chemometric analysis of juices from ancient Punica granatum L. cultivars: A nontargeted approach. *Journal of agricultural and food chemistry, 61*(23), 5600-5609.

Cam, M., Hisil, Y., & Durmaz, G. (2009). Characterisation of Pomegranate Juices from Ten Cultivars Grown in Turkey. *International Journal of Food Properties, 12*(2), 388–395.

Catala, R. et al. (2011) Integration of low temperature and light signaling during cold acclimation response in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A. 108*, 16475–16480.

Celik, I., Temur, A., & Isik, I. (2009). Hepatoprotective role and antioxidant capacity of pomegranate (Punica granatum) flowers infusion against trichloroacetic acid-exposed in rats. *Food and Chemical Toxicology, 47*(1), 145-149.

Cerdá, B., Cerón, J. J., Tomás-Barberán, F. A., & Espín, J. C. (2003). Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *Journal of agricultural and food chemistry, 51*(11), 3493-3501.

Chater, J. M., Merhaut, D. J., Jia, Z., Mauk, P. A., & Preece, J. E. (2018). Fruit quality traits of ten California-grown pomegranate cultivars harvested over three months. *Scientia Horticulturae, 237*, 11–19.

Chauhan, B., Kumar, G., Kalam, N., & Ansari, S. H. (2013). Current concepts and prospects of herbal nutraceutical: a review. *Journal of advanced pharmaceutical technology & research, 4*(1), 4.

Chopra, S., Hoshino, A., Boddu, J., & Iida, S. (2006). Flavonoid Pigments as Tools in Molecular Genetics. *The Science of Flavonoids*, 147–173.

CoSSI, F. (2017). Melograno, potenzialità e limiti di un antico frutto italiano. *Rivista di frutticoltura e di ortofloricoltura, 81*(12), 52-63.

Cristofori, V., Caruso, D., Latini, G., Dell'Agli, M., Cammilli, C., Rugini, E., ... & Muleo, R. (2011). Fruit quality of Italian pomegranate (Punica granatum L.) autochthonous varieties. *European Food Research and Technology, 232*(3), 397-403.

Cultrone, A., Cotroneo, P. S., & Recupero, G. R. (2010). Cloning and molecular characterization of R2R3-MYB and bHLH-MYC transcription factors from Citrus sinensis. *Tree genetics & genomes*, *6*(1), 101-112.

Currò, S., Caruso, M., Distefano, G., Gentile, A., & La Malfa, S. (2010). New microsatellite loci for pomegranate, Punica granatum (Lythraceae). *American journal of botany*, 9*7*(7), e58-e60.

Cutanda-Perez, M. C., Ageorges, A., Gomez, C., Vialet, S., Terrier, N., Romieu, C., & Torregrosa, L. (2009). Ectopic expression of VlmybA1 in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. *Plant molecular biology*, *69*(6), 633-648.

D'Aquino, S., Palma, A., Schirra, M., Continella, A., Tribulato, E., & La Malfa, S. (2010). Influence of film wrapping and fludioxonil application on quality of pomegranate fruit. *Postharvest Biology and Technology, 55*(2), 121–128.

Dafny-Yalin, M., Glazer, I., Bar-Ilan, I., Kerem, Z., Holland, D., & Amir, R. (2010). Color, Sugars and Organic Acids Composition in Aril Juices and Peel Homogenates Prepared from Different Pomegranate Accessions. *Journal of Agricultural and Food Chemistry, 58*(7), 4342–4352.

De Pascual-Teresa, S., & Sanchez-Ballesta, M. T. (2008). Anthocyanins: from plant to health. *Phytochemistry reviews, 7*(2), 281-299.

De Vos, R., Moco, C. S., Lommen, A., Keurentjes, J. J., Bino, R. J., and Hall, R. D. (2007). Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat. Protoc. 2*, 778–791.

DeFelice, S. L. (1989). The NutraCeutical Revolution: Fueling a powerful, new international market. *Harvard University Advanced Management Program in Biomedical Research and Development*.

Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., et al. (2006). Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiol, 140*, 499–511.

Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of agricultural and food chemistry, 50*(10), 3010-3014.

Dikmen, M., Ozturk, N., & Ozturk, Y. (2011). The Antioxidant Potency ofPunica granatumL. Fruit Peel Reduces Cell Proliferation and Induces *Apoptosis on Breast Cancer. Journal of Medicinal Food, 14*(12), 1638–1646.

Du, H., Zhang, L., Liu, L., Tang, X. F., Yang, W. J., Wu, Y. M., & Tang, Y. X. (2009). Biochemical and molecular characterization of plant MYB transcription factor family. *Biochemistry (Moscow), 74*(1), 1-11.

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I. & Lepiniec, L. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana. *The Plant Journal, 55*(6), 940-953.

Ebrahimi, S., SEYED, T. B., & SHARIF, N. B. (2010). Microsatellite isolation and characterization in pomegranate (Punica granatum L.). 156-163.

Espley, R. V., Hellens, R. P., Putterill, J., Stevenson, D. E., Kutty-Amma, S., & Allan, A. C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal, 49*(3), 414-427.

Fahan A (1976) The leaf. The flower. The seed. In: Plant anatomy. *Hakkibutz Hameuhad Publishing House Ltd, Jerusalem*, 171–212, 321–394, 419–430

Fahn, A. (1982). The seed. *Plant anatomy, 3rd edn. Pergamon Press Ltd, Oxford, UK*, 485-486.

Fanali, C., Belluomo, M. G., Cirilli, M., Cristofori, V., Zecchini, M., Cacciola, F., … Dugo, L. (2016). Antioxidant activity evaluation and HPLC-photodiode array/MS polyphenols analysis of pomegranate juice from selected italian cultivars: *A comparative study. ELECTROPHORESIS, 37*(13), 1947–1955.

Fawole, O. A., & Opara, U. L. (2013). Changes in physical properties, chemical and elemental composition and antioxidant capacity of pomegranate (cv. Ruby) fruit at five maturity stages. *Scientia Horticulturae, 150*, 37-46.

Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC complementary and alternative medicine, 12*(1), 200.

Feng, S., Wang, Y., Yang, S., Xu, Y., & Chen, X. (2010). Anthocyanin biosynthesis in pears is regulated by a R2R3-MYB transcription factor PyMYB10. *Planta*, *232*(1), 245-255

Feng, Y. Z., Chen, D. J., Song, M. T., Zhao, Y. L., & Li, Z. H. (1998). Assessment and utilization of pomegranate varieties resources. *Journal of Fruit Science, 15*(4), 370-373.

Ferrara, G., Cavoski, I., Pacifico, A., Tedone, L., & Mondelli, D. (2011). Morpho-pomological and chemical characterization of pomegranate (Punica granatum L.) genotypes in Apulia region, Southeastern Italy. *Scientia Horticulturae, 130*(3), 599–606.

Ferrara, G., Giancaspro, A., Mazzeo, A., Giove, S. L., Matarrese, A. M. S., Pacucci, C., … Gadaleta, A. (2014). Characterization of pomegranate (Punica granatum L.) genotypes collected in Puglia region, Southeastern Italy. *Scientia Horticulturae, 178*, 70–78.

Fischer, U. A., Carle, R., & Kammerer, D. R. (2011a). Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. *Food Chemistry, 127*(2), 807–821.

Fischer, U. A., Dettmann, J. S., Carle, R., & Kammerer, D. R. (2011). Impact of processing and storage on the phenolic profiles and contents of pomegranate (Punica granatum L.) juices. European Food *Research and Technology, 233*(5), 797.

Forest, C. P., Padma-Nathan, H., & Liker, H. R. (2007). Efficacy and safety of pomegranate juice on improvement of erectile dysfunction in male patients with mild to moderate erectile dysfunction: a randomized, placebo-controlled, double-blind, crossover study. International Journal of Impotence *Research, 19*(6), 564–567.

Fuhrman, B., Volkova, N., & Aviram, M. (2010). Pomegranate juice polyphenols increase recombinant paraoxonase-1 binding to high-density lipoprotein: Studies in vitro and in diabetic patients. *Nutrition, 26*(4), 359–366.

Gaquerel, E., Gulati, J., and Baldwin, I. T. (2014). Revealing insect herbivory-induced phenolamide metabolism: from single genes to metabolic network plasticity analysis. *Plant J. 79*, 679–692.

Gil, M. I., Cherif, J., Ayed, N., Artés, F., & Tomás-Barberán, F. A. (1995a). Influence of cultivar, maturity stage and geographical location on the juice pigmentation of Tunisian pomegranates. *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 201*(4), 361-364.

Gil, M. I., García-Viguera, C., Artés, F., & Tomás-Barberán, F. A. (1995). Changes in pomegranate juice pigmentation during ripening. *Journal of the Science of Food and Agriculture, 68*(1), 77–81.

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant Activity of Pomegranate Juice and Its Relationship with Phenolic Composition and Processing. *Journal of Agricultural and Food Chemistry, 48*(10).

Gómez-Caravaca, A. M., Verardo, V., Toselli, M., Segura-Carretero, A., Fernández-Gutiérrez, A., & Caboni, M. F. (2013). Determination of the major phenolic compounds in pomegranate juices by HPLC–DAD–ESI-MS. *Journal of agricultural and food chemistry, 61*(22), 5328-5337.

Gonzalez, A. et al. (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *Plant J 53*, 814–827.

Gonzalez, A. et al. (2009) TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation. *Dev. Biol. 325*, 412–421.

Gonzalez, A., Zhao, M., Leavitt, J. M., & Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *The Plant Journal*, *53*(5), 814-827.

Gonzalez, A., Zhao, M., Leavitt, J. M., & Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *The Plant Journal*, *53*(5), 814-827.

Grassi, D., Desideri, G., & Ferri, C. (2010). Flavonoids: *Antioxidants Against Atherosclerosis. Nutrients,* 2(8), 889–902.

Grassi, D., Desideri, G., Croce, G., Tiberti, S., Aggio, A., & Ferri, C. (2009). Flavonoids, Vascular Function and Cardiovascular Protection. *Current Pharmaceutical Design, 15*(10), 1072–1084

Gulimire, H.L. Dong, and Julaiti. 2003. The industry status and future development of pomegranate in Xinjiang. *Northw. Hort*. 6:7.

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). Paleontological statistics software: package for education and data analysis. *Palaeontologia Electronica*, (4).

Han, L., Yuan, Z., Feng, L., & Yin, Y. (2015). Changes in the composition and contents of pomegranate polyphenols during fruit development. *Acta Hortic*, *1089*, 53-61.

Hasnaoui, N., Buonamici, A., Sebastiani, F., Mars, M., Zhang, D., & Vendramin, G. G. (2012). Molecular genetic diversity of Punica granatum L. (pomegranate) as revealed by microsatellite DNA markers (SSR). *Gene, 493*(1).

Hernandez, F., Melgarejo, P., Tomas-Barberan, F. A., & Artes, F. (1999). Evolution of juice anthocyanins during ripening of new selected pomegranate (Punica granatum) clones. *European Food Research and Technology, 210*(1), 39-42.

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J Exp Bot 62*: 2465–2483.

Holland D, Bar-Ya'akov I (2008). The pomegranate: new interest in an ancient fruit. *Chron Hortic 48*:12–15

Holland, D., Hatib, K., & Bar-Ya'akov, I. (2009). Pomegranate: Botany, Horticulture, *Breeding. Horticultural Reviews*, 127–191.

Howell, A. B., & D'Souza, D. H. (2013). The pomegranate: effects on bacteria and viruses that influence human health. *Evidence-Based Complementary and Alternative Medicine*, 2013.

Huang, T. H.-W., Peng, G., Kota, B. P., Li, G. Q., Yamahara, J., Roufogalis, B. D., & Li, Y. (2005). Pomegranate flower improves cardiac lipid metabolism in a diabetic rat model: role of lowering circulating lipids. *British Journal of Pharmacology, 145*(6), 767–774.

Ishida, T. et al. (2007) Arabidopsis TRANSPARENT TESTA GLABRA2 is directly regulated by R2R3 MYB transcription factors and is involved in regulation of GLABRA2 transcription in epidermal differentiation. *Plant Cell 19*, 2531–2543.

Jaakola, L. (2013). New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends in plant science, 18*(9), 477-483.

Jadoon, S., Karim, S., Asad, M. H. H. B., Akram, M. R., Kalsoom Khan, A., Malik, A., … Murtaza, G. (2015). Anti-Aging Potential of Phytoextract Loaded-Pharmaceutical Creams for Human Skin Cell Longetivity. *Oxidative Medicine and Cellular Longevity, 2015*, 1–17

Jin, H. et al. (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. *The EMBO Journal 19*, 6150–6161.

Johanningsmeier, S. D., & Harris, G. K. (2011). Pomegranate as a functional food and nutraceutical source. *Annual review of food science and technology, 2*, 181-201.

Jurenka, J. (2008). Therapeutic applications of pomegranate (Punica granatum L.): a review. *Alternative medicine review, 13*(2), 128.

Kang, Y.H. et al. (2009) The MYB23 gene provides a positive feedback loop for cell fate specification in the Arabidopsis root epidermis. *Plant Cell 21*, 1080–1094.

Kaplan, M., Hayek, T., Raz, A., Coleman, R., Dornfeld, L., Vaya, J., et al. (2001). Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *The Journal of Nutrition, 131*, 2082–2089.

Karale, A. R., Supe, V. S., Kaulgud, S. N., & Kale, P. N. (1993). Pollination and fruit set studies in pomegranate. *Journal of Maharashtra Agricultural Universities (India).*

Kaur, G., Jabbar, Z., Athar, M., & Alam, M. S. (2006). Punica granatum (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. *Food and Chemical Toxicology, 44*(7), 984–993.

Khaksar, G., Tabatabaei, B. E. S., Arzani, A., Ghobadi, C., & Ebrahimie, E. (2015). Functional Analysis of a Pomegranate (Punica granatum L.) MYB Transcription Factor Involved in the Regulation of Anthocyanin Biosynthesis. *Iranian journal of biotechnology, 13*(1), 17.

Khan, N., Afaq, F., Kweon, M. H., Kim, K., & Mukhtar, H. (2007). Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice. *Cancer Research, 67*, 3475–3482.

Kim, H. S., Kim, B. G., Sung, S., Kim, M., Mok, H., Chong, Y., & Ahn, J. H. (2013). Engineering flavonoid glycosyltransferases for enhanced catalytic efficiency and extended sugar-donor selectivity. *Planta*, *238*(4), 683-693.

Kim, N. D., Mehta, R., Yu, W., Neeman, I., Livney, T., Amichay, A., Lansky, E. (2002). Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. *Breast Cancer Research and Treatment, 71*(3), 203–217.

Kirik, V. et al. (2005) Functional diversification of MYB23 and GL1 genes in trichome morphogenesis and initiation. *Development 132*, 1477–1485.

Kobayashi S, Goto-Yamamoto N, Hirochika H (2004) Retrotransposon-induced mutations in grape skin color. *Science 304*: 982. PMID: 15143274

Koes, R., Verweij, W., & Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends in Plant Science, 10(5), 236–242.

Kotepong P, Ketsa S, van Doorn WG (2011) A white mutant of Malay apple fruit (Syzygium malaccense) lacks transcript expression and activity for the last enzyme of anthocyanin synthesis, and the normal expression of a MYB transcription factor. *Funct Plant Biol 38*: 75–86.

Koutecký, P. (2014). MorphoTools: a set of R functions for morphometric analysis. Plant Systematics and Evolution, 301(4).

Kovinich, N., Saleem, A., Arnason, J. T., & Miki, B. (2010). Functional characterization of a UDP-glucose:flavonoid 3-O-glucosyltransferase from the seed coat of black soybean (Glycine max (L.) Merr.). *Phytochemistry, 71*(11-12), 1253–1263.

La Malfa, S., Gentile, A., Domina, F., & Tribulato, E. (2006, October). Primosole: A new selection from Sicilian pomegranate germplasm. In I International Symposium on Pomegranate and Minor Mediterranean *Fruits 818* (pp. 125-132).

Lan, J., Lei, F., Hua, L., Wang, Y., Xing, D., & Du, L. (2009). Transport behavior of ellagic acid of pomegranate leaf tannins and its correlation with total cholesterol alteration in HepG2 cells. *Biomedical Chromatography, 23*(5), 531-536.

Lansky, E. P., & Newman, R. A. (2007). Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *Journal of ethnopharmacology, 109*(2), 177-206.

Lazo, G. R., Stein, P. A., and Ludwig, R. A. (1991). A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Biotechnology 9*, 963–967.

Lee, M. M., & Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. *Cell, 99*(5), 473-483.

Lee, S.-I., Kim, B.-S., Kim, K.-S., Lee, S., Shin, K.-S., & Lim, J.-S. (2008). Immune-suppressive activity of punicalagin via inhibition of NFAT activation. *Biochemical and Biophysical Research Communications, 37*1(4), 799–803.

Lei, F., Zhang, X. N., Wang, W., Xing, D. M., Xie, W. D., Su, H., & Du, L. J. (2007). Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice. *International Journal of Obesity, 31*(6), 1023.

Levin, G. M. (2006). Pomegranate roads: a Soviet botanist's exile from Eden. Pomegranate Roads.

Levin, G.M., and E.A. Sokolova. 1979. Materials for the study of Punica protopunica (Punicaceae). *Botanicheskii Zhurnal 64*:998–1005.

Li, S.F. et al. (2009) The Arabidopsis MYB5 transcription factor regulates mucilage synthesis, seed coat development, and trichome morphogenesis. *Plant Cell 21*, 72–89.

Lin-Wang, K., Bolitho, K., Grafton, K., Kortstee, A., Karunairetnam, S., McGhie, T. K., ... & Allan, A. C. (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC plant biology*, *10*(1), 50.

Liu, K., & Muse, S. V. (2005). PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics, 21*(9).

Lommen, A. (2009). MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Anal. Chem. 81, 3079–3086.

Lu, J., Ding, K., & Yuan, Q. (2008). Determination of punicalagin isomers in pomegranate husk. *Chromatographia, 68*(3-4), 303-306.

M Calderon-Montano, J., Burgos-Morón, E., Pérez-Guerrero, C., & López-Lázaro, M. (2011). A review on the dietary flavonoid kaempferol. *Mini reviews in medicinal chemistry, 11*(4), 298-344.

Malik, A., & Mukhtar, H. (2006). Prostate cancer prevention through pomegranate fruit. *Cell Cycle, 5*, 371–373.

Marchetti, Pinotti M Lunghi B Casari C Bernardi F. Functional Genetics. T*hrombosis Research, 129*:3 2012.

Mars, M. (2000). Pomegranate plant material: Genetic resources and breeding, a review. Options Méditerranéennes, Ser. A, 42, 55-62. Martínez, J. J., Melgarejo, P., Hernández, F., Salazar, D. M., & Martínez, R. (2006). Seed characterisation of five new pomegranate (Punica granatum L.) varieties. *Scientia Horticulturae, 110*(3), 241–246.

Martinez-Nicolas, J. J., Melgarejo, P., Legua, P., Garcia-Sanchez, F., & Hernández, F. (2016). Genetic diversity of pomegranate germplasm collection from Spain determined by fruit, seed, leaf and flower characteristics. *PeerJ, 4*, e2214.

Martins, W. S., Lucas, D. C. S., Neves, K. F. S., & Bertioli, D. J. (2009). WebSat - A Web Software for MicroSatellite marker development. *Bioinformation, 3*(6).

Melgarejo, P., Salazar, D. M., & Artés, F. (2000). Organic acids and sugars composition of harvested pomegranate fruits. *European Food Research and Technology, 211*(3), 185–190.

Mena, P., Calani, L., Dall'Asta, C., Galaverna, G., García-Viguera, C., Bruni, R., ... & Del Rio, D. (2012). Rapid and comprehensive evaluation of (poly) phenolic compounds in pomegranate (Punica granatum L.) juice by UHPLC-MSn. *Molecules, 17*(12), 14821-14840.

Mena, P., García-Viguera, C., Navarro-Rico, J., Moreno, D. A., Bartual, J., Saura, D., & Martí, N. (2011). Phytochemical characterisation for industrial use of pomegranate (Punica granatum L.) cultivars grown in Spain. *Journal of the Science of Food and Agriculture, 91*(10), 1893-1906.

Montefiori, M., Brendolise, C., Dare, A. P., Lin-Wang, K., Davies, K. M., Hellens, R. P., & Allan, A. C. (2015). In the Solanaceae, a hierarchy of bHLHs confer distinct target specificity to the anthocyanin regulatory complex. *Journal of experimental botany*, *66*(5), 1427-1436.Morton, J. F. (1987). Fruits of warm climates. JF Morton.

Moss, J. R., & Otten, L. (1989). A relationship between colour development and moisture content during roasting of peanuts. *Canadian Institute of food science and technology journal, 22*(1), 34-39.

Nalawadi, U. G., Farooqui, A. A., Dasappa, R., Gubraiah, S., & Nalini, A. S. (1973). Studies on the floral biology of pomegranate (Punica granatum L.). *Mysore J. Agric. Sci*.

Niu, S. S., Xu, C. J., Zhang, W. S., Zhang, B., Li, X., Lin-Wang, K., ... & Chen, K. S. (2010). Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (Myrica rubra) fruit by a R2R3 MYB transcription factor. *Planta*, *231*(4), 887-899.

Nuncio-Jáuregui, N., Cano-Lamadrid, M., Hernández, F., Carbonell-Barrachina, Á., & Calín-Sánchez, Á. (2015). Comparison of Fresh and Commercial Pomegranate Juices from Mollar de Elche Cultivar Grown under Conventional or Organic Farming Practices. *Beverages, 1*(2), 34–44.

Offen, W., Martinez-Fleites, C., Yang, M., Kiat-Lim, E., Davis, B. G., Tarling, C. A., ... & Davies, G. J. (2006). Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. *The EMBO journal*, *25*(6), 1396-1405.

Okonogi, S., Duangrat, C., Anuchpreeda, S., Tachakittirungrod, S., & Chowwanapoonpohn, S. (2007). Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food Chemistry, 103*(3), 839–846.

Ono, N. N., Britton, M. T., Fass, J. N., Nicolet, C. M., Lin, D., & Tian, L. (2011). Exploring the Transcriptome Landscape of Pomegranate Fruit Peel for Natural Product Biosynthetic Gene and SSR Marker Discovery F. *Journal of integrative plant biology, 53*(10), 800-813.

Oppenheimer, D. G., Herman, P. L., Sivakumaran, S., Esch, J., & Marks, M. D. (1991). A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. *Cell, 67*(3), 483-493.

Outchkourov, N. S., Carollo, C. A., Gomez-Roldan, V., de Vos, R. C., Bosch, D., Hall, R. D., & Beekwilder, J. (2014). Control of anthocyanin and non-flavonoid compounds by anthocyanin-regulating MYB and bHLH transcription factors in Nicotiana benthamiana leaves. *Frontiers in plant science, 5*, 519.

Ozgen, M., Durgaç, C., Serçe, S., & Kaya, C. (2008). Chemical and antioxidant properties of pomegranate cultivars grown in the Mediterranean region of Turkey. *Food Chemistry, 111*(3), 703–706.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P., & Volpe, M. G. (2016). Inhibitory effect of pomegranate (Punica granatum L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic Staphylococcus aureus and Escherichia coli. *Food Chemistry*, 190, 824–831.

Pala, Ç. U., & Toklucu, A. K. (2011). Effect of UV-C light on anthocyanin content and other quality parameters of pomegranate juice. *Journal of Food Composition and Analysis, 24*(6), 790-795.

Pantuck, A. J., Leppert, J. T., Zomorodian, N., Aronson, W., Hong, J., Barnard, R. J., et al. (2006). Phase II study of pomegranate juice for men with rising prostatespecific antigen following surgery or radiation for prostate cancer. *Clinical Cancer Research, 12*, 4018–4026.

Parvaresh, M., Talebi, M., & Sayed-Tabatabaei, B. E. (2012). Molecular diversity and genetic relationship of pomegranate (Punica granatum L.) genotypes using microsatellite markers. *Scientia horticulturae*, *138*, 244-252.

Peakall, R. O. D., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular ecology notes*, *6*(1), 288-295.

Peng, Z. et al. (2011) Brassinosteroid enhances jasmonate-induced anthocyanin accumulation in Arabidopsis seedlings. *J. Integr. Plant Biol. 53*, 632–640.

Petroni, K., & Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant science*, *181*(3), 219-229.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, *29*(9), e45-e45.

Pierantoni L, Dondini L, De Franceschi P, Musacchi S, Winkel BSJ, Sansavini S (2010) Mapping of an anthocyanin-regulating MYB transcription factor and its expression in red and green pear, Pyrus communis. *Plant Physio Bioch 48*:1020–1026

Pirseyedi, S. M., Valizadehghan, S., Mardi, M., Ghaffari, M. R., Mahmoodi, P., Zahravi, M., … Nekoui, S. M. K. (2010). Isolation and Characterization of Novel Microsatellite Markers in Pomegranate (Punica granatum L.). *International Journal of Molecular Sciences, 11*(5).

Preston, J. et al. (2004) AtMYB32 is required for normal pollen development in Arabidopsis thaliana. *Plant J. 40*, 979–995.

Puglia, G., Grimaldi, S., Carta, A., Pavone, P., & Toorop, P. (2015). Pericarp structure of Glebionis coronaria (L.) Cass. ex Spach (Asteraceae) cypselae controls water uptake during germination. *Seed Science Research, 25*(03), 255–266.

Qin, G., Xu, C., Ming, R., Tang, H., Guyot, R., Kramer, E. M., ... & Gao, Z. (2017). The pomegranate (Punica granatum L.) genome and the genomics of punicalagin biosynthesis. *The Plant Journal, 91*(6), 1108-1128.

Radunić, M., Jukić Špika, M., Goreta Ban, S., Gadže, J., Díaz-Pérez, J. C., & MacLean, D. (2015). Physical and chemical properties of pomegranate fruit accessions from Croatia. *Food Chemistry, 177*, 53–60.

Rania, J., Salwa, Z., Najib, H., Amal, B. D., Messaoud, M., & Amel, S. H. (2012). Microsatellite polymorphism in Tunisian pomegranates (Punica granatum L.): Cultivar genotyping and identification. *Biochemical Systematics and Ecology, 44*, 27–35.

Rolland, F. et al. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu. *Rev. Plant Biol. 57*, 675–709.

Rouholamin, S., Zahedi, B., Nazarian-Firouzabadi, F., & Saei, A. (2015). Expression analysis of anthocyanin biosynthesis key regulatory genes involved in pomegranate (Punica granatum L.). *Scientia Horticulturae*, *186*, 84-88.

Rouholamin, S., Zahedi, B., Nazarian-Firouzabadi, F., & Saei, A. (2015). Expression analysis of anthocyanin biosynthesis key regulatory genes involved in pomegranate (Punica granatum L.). *Scientia Horticulturae, 186*, 84-88.

Rozen, S., & Skaletsky, H. (1999). Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics methods and protocols*, 365-386.

Santos, S.A.O.; Vilela, C.; Freire, C.S.R.; PascoalNeto, C.; Silvestre, A.J.D. Ultra-high performance liquid chromatography coupled to mass spectrometry applied to the identificatíon of valuable phenoliccompounds from Eucalyptus wood. *J. Chromatogr. B 2013*, 938, 65–74.

Sarkhosh A, Zamani Z, Fatahi R, Ranjbar H (2009) Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions by fruit characteristics and RAPD markers. *Sci Hortic 12*:313–319

Seeram, N. P., Aronson, W. J., Zhang, Y., Henning, S. M., Moro, A., Lee, R.-P., et al. (2007). Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localise to the mouse prostate gland. *Journal of Agriculture and Food Chemistry, 55*, 7732–7737.

Seitz, C., Ameres, S., Schlangen, K., Forkmann, G., & Halbwirth, H. (2015). Multiple evolution of flavonoid 3′, 5′-hydroxylase. *Planta, 242*(3), 561-573.

Sezer, E. D., Akçay, Y. D., İlanbey, B., Yıldırım, H. K., & Sözmen, E. Y. (2007). Pomegranate wine has greater protection capacity than red wine on low-density lipoprotein oxidation. *Journal of medicinal food, 10*(2), 371-374.

Shalimu, D., Li, K., Baskin, C. C., Baskin, J. M., & Liu, Y. (2015). Seed germination biology of four pomegranate (Punica granatum) cultivars from Xinjiang, *China. HortScience, 50*(6), 826-829.

Shulman, Y., Fainberstein, L., & Lavee, S. (1984). Pomegranate fruit development and maturation. *Journal of Horticultural Science, 59*(2), 265-274.

Singh, M., Arseneault, M., Sanderson, T., Murthy, V., & Ramassamy, C. (2008). Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. *Journal of agricultural and food chemistry, 56*(13), 4855-4873.

Sleight, P., Yusuf, S., Pogue, J., Tsuyuki, R., Diaz, R., & Probstfield, J. (2001). Blood-pressure reduction and cardiovascular risk in HOPE study. *The Lancet, 358*(9299), 2130–2131.

Song, S.K. et al. (2011) Cell fate in the Arabidopsis root epidermis is determined by competition between WEREWOLF and CAPRICE. *Plant Physiol. 157*, 1196–1208.

Stanhope, K. L., Schwarz, J. M., Keim, N. L., Griffen, S. C., Bremer, A. A., Graham, J. L., … Havel, P. J. (2009). Consuming fructose-sweetened, not glucose-sweetened, beverages increase visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *Journal of Clinical Investigation, 119*(5).

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., & Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. *The Plant Journal*, *50*(4), 660-677.

Stover, E. D., & Mercure, E. W. (2007). The pomegranate: a new look at the fruit of paradise. *HortScience, 42*(5), 1088-1092.

Sumner, M. D., Elliott-Eller, M., Weidner, G., Daubenmier, J. J., Chew, M. H., Marlin, R., et al. (2005). Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease. *American Journal of Cardiology, 96*, 810–814.

Sun, J., Lin, L. Z., & Chen, P. (2012). Study of the mass spectrometric behaviors of anthocyanins in negative ionization mode and its applications for characterization of anthocyanins and non-anthocyanin polyphenols. *Rapid Communications in Mass Spectrometry*, *26*(9), 1123-1133.

Tanaka, Y. (2006). Flower colour and cytochromes P450. *Phytochemistry Reviews, 5*(2-3), 283-291.

Tehranifar, A., Zarei, M., Nemati, Z., Esfandiyari, B., & Vazifeshenas, M. R. (2010). Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (Punica granatum L.) cultivars. Scientia Horticulturae, 126(2), 180–185.

Tezcan, F., Gültekin-Özgüven, M., Diken, T., Özçelik, B., & Erim, F. B. (2009). Antioxidant activity and total phenolic, organic acid and sugar content in commercial pomegranate juices. *Food Chemistry, 115*(3), 873–877.

Tibullo, D., Caporarello, N., Giallongo, C., Anfuso, C., Genovese, C., Arlotta, C., … Raccuia, S. (2016). Antiproliferative and Antiangiogenic Effects of Punica granatum Juice (PGJ) in Multiple Myeloma (MM). *Nutrients*, *8*(10), 611.

Tikunov, Y. M., Laptenok, S., Hall, R. D., Bovy, A., and de Vos, R. C. (2012). MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics* 8, 714–718.

Todaro, A., Cavallaro, R., La Malfa, S., Continella, A., Gentile, A., Fischer, U., & Spagna, G. (2016). Anthocyanin profile and antioxidant activity of freshly squeezed pomegranate (Punica granatum L.) juices of sicilian and spanish provenances. *Italian Journal of Food Science, 28*(3), 464-479.

Tohge, T., Nishiyama, Y., Hirai, M. Y., Yano, M., Nakajima, J. I., Awazuhara, M., ... & Noji, M. (2005). Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. *The Plant Journal*, *42*(2), 218-235.

Tzulker, R., Glazer, I., Bar-Ilan, I., Holland, D., Aviram, M., & Amir, R. (2007). Antioxidant Activity, Polyphenol Content, and Related Compounds in Different Fruit Juices and Homogenates Prepared from 29 Different Pomegranate Accessions. *Journal of Agricultural and Food Chemistry, 55*(23), 9559–9570.

Usta, C., Ozdemir, S., Schiariti, M., & Puddu, P. E. (2013). The pharmacological use of ellagic acid-rich pomegranate fruit. *International journal of food sciences and nutrition, 64*(7), 907-913

Vaiman, D., Mercier, D., Moazami-Goudarzi, K., Eggen, A., Ciampolini, R., Lépingle, A., ... & Lévéziel, H. (1994). A set of 99 cattle microsatellites: characterization, synteny mapping, and polymorphism. *Mammalian Genome, 5*(5), 288-297.

Van Engelen, F. A., Molthoff, J. W., Conner, A. J., Nap, J. P., Pereira, A., and Stiekema, W. J. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res. 4*, 288–290.

Vardin H, Fenerciog˘lu H (2009) Study on the development of pomegranate juice processing technology; the pressing of pomegranate fruit. *Acta Hort 818*:373–381

Vardin, H., & Fenercioğlu, H. (2003). Study on the development of pomegranate juice processing technology: clarification of pomegranate juice. *Food/nahrung, 47*(5), 300-303.

Vegara, S., Mena, P., Martí, N., Saura, D., & Valero, M. (2013). Approaches to understanding the contribution of anthocyanins to the antioxidant capacity of pasteurized pomegranate juices. *Food Chemistry, 141*(3), 1630–1636.

Verardo, V., Garcia-Salas, P., Baldi, E., Segura-Carretero, A., Fernandez-Gutierrez, A., & Caboni, M. F. (2014). Pomegranate seeds as a source of nutraceutical oil naturally rich in bioactive lipids. *Food research international, 65*, 445-452.

Wahyuningsih, S., Wulandari, L., Wartono, M. W., Munawaroh, H., & Ramelan, A. H. (2017, April). The Effect of pH and Color Stability of Anthocyanin on Food Colorant. *In IOP Conference Series: Materials Science and Engineering* (Vol. 193, No. 1, p. 012047). IOP Publishing.

Walker AR, Lee E, Bogs J, McDavid DA, Thomas MR, Robinson SP (2007) White grapes arose through the mutation of two similar and adjacent regulatory genes. *Plant J 49*: 772–785. PMID: 17316172.

Wang, H. X. (2003). The characteristics of Mudanhua pomegranate variety and its cultural techniques. *South China Fruits, 32*(1), 49-50.

Wang, L., Ho, J., Glackin, C., & Martins-Green, M. (2012). Specific Pomegranate Juice Components as Potential Inhibitors of Prostate Cancer Metastasis. *Translational Oncology, 5*(5), 344–IN5.

Wang, N., Xu, H., Jiang, S., Zhang, Z., Lu, N., Qiu, H., ... & Chen, X. (2017). MYB12 and MYB22 play essential roles in proanthocyanidin and flavonol synthesis in red-fleshed apple (Malus sieversii f. niedzwetzkyana). *The Plant Journal*, *90*(2), 276-292.

Wang, Y. S., Xu, Y. J., Gao, L. P., Yu, O., Wang, X. Z., He, X. J., ... & Xia, T. (2014). Functional analysis of flavonoid 3′, 5′-hydroxylase from tea plant (Camellia sinensis): critical role in the accumulation of catechins. *BMC plant biology, 14*(1), 347.

Weerakkody, P., Jobling, J., Infante, M. M. V., & Rogers, G. (2010). The effect of maturity, sunburn and the application of sunscreens on the internal and external qualities of pomegranate fruit grown in Australia. *Scientia Horticulturae, 124*(1), 57–61.

Wen, X. C., Han, J., Leng, X. P., Ma, R. J., Jiang, W. B., & Fang, J. G. (2014). Cloning and expression of UDP-glucose: flavonoid 3-O-glucosyltransferase gene in peach flowers. *Genetics and Molecular Research*, *13*(4), 10067-10075.

Wetzstein, H. Y., Zhang, Z., Ravid, N., & Wetzstein, M. E. (2011). Characterization of attributes related to fruit size in pomegranate. *HortScience, 46*(6), 908-912.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant physiology*, *126*(2), 485-493.

Wyrepkowski, C., Gomes da Costa, D., Sinhorin, A., Vilegas, W., De Grandis, R., Resende, F., … dos Santos, L. (2014). Characterization and Quantification of the Compounds of the Ethanolic Extract from Caesalpinia ferrea Stem Bark and Evaluation of Their Mutagenic Activity. *Molecules, 19*(10), 16039–16057.

Xu, W. et al. (2014) Complexity and robustness of the flavonoid transcriptional regulatory network revealed by comprehensive analyses of MYB–bHLH–WDR complexes and their targets in Arabidopsis seed. *New Phytol. 202*, 132–144.

Xu, W., Dubos, C., & Lepiniec, L. (2015). Transcriptional control of flavonoid biosynthesis by MYB–bHLH–WDR complexes. Trends in Plant Science, 20(3).

Xu, W., Grain, D., Le Gourrierec, J., Harscoët, E., Berger, A., Jauvion, V., ... & Salsac, F. (2013). Regulation of flavonoid biosynthesis involves an unexpected complex transcriptional regulation of TT 8 expression, in A rabidopsis. *New Phytologist*, *198*(1), 59-70.

Yuan, Z., Fang, Y., Zhang, T., Fei, Z., Han, F., Liu, C., ... & Zhang, M. (2018). The pomegranate (Punica granatum L.) genome provides insights into fruit quality and ovule developmental biology. *Plant biotechnology journal.*

Zaouay, F., Salem, H. H., Labidi, R., & Mars, M. (2014). Development and quality assessment of new drinks combining sweet and sour pomegranate juices. *Emirates Journal of Food and Agriculture*, 1-8.

Zarei, A., Zamani, Z., Mousavi, A., Fatahi, R., Alavijeh, M. K., Dehsara, B., & Salami, S. A. (2012). An effective protocol for isolation of high-quality RNA from pomegranate seeds. *Asian Aust J Plant Sci Biotechnol, 6*, 32-7.

Website:

https://www.arabidopsis.org/ (Arabidopsis Information Resource, TAIR)

http://naturalchemistry.utu.fi/

http://www.freshplaza.it/article/80301/Salvare-il-reddito-alla-produzione-diversificando-il-programma-sul melograno-di-Terremerse/

http://www.ilsole24ore.com/art/impresa-e-territori/2015-11-10/il-melograno-conquista-consumatori-italiani/

http://www.istat.it/

http://www.prospectiva2020.com/sites/default/files/report/files/re/ -pomegranate- feb 2015.pdf. World Pomegranate Market Supply, Demand and Forecast (2015)

https://blog.addgene.org/plasmids-101-gateway-cloning/

https://ndb.nal.usda/