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STUDY OF ROOTSTOCK-SCION INTERACTIONS IN GRAPEVINE

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To my husband, Vincenzo

… now we can go forward into the Future

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Abstract

The grapevine is one of the fruit-crops most threatened by climate change. Despite the phenotypic plasticity that enables the adaptation to different limiting environments, the vines that experience severe abiotic stresses can suffer from serious metabolic damage, with negative consequences on grape production and quality.

Grafting in viticulture is a widely used technique that allows overcoming the problem of phylloxera. But, beyond conferring to *Vitis vinifera* the tolerance against the pest *Daktulosphaira vitifoliae*, the rootstocks can influence the scion's phenotype by increasing its vigor and enhancing the resistance to drought. For this reason, the exploitation of rootstocks is counted among the most affordable strategies that can be used to mitigate the detrimental effects of global warming.

This Ph.D. research project investigated in detail rootstock-scion interactions in grapevine, using an integrated molecular and biochemical approach. In particular, the attention was focused on the rootstock influence on grape berry secondary metabolism, the accumulation trends of phenolic compounds, and the molecular networks involved in the process, both in conditions of optimal irrigation and water stress.

The whole research activity was performed using an experimental system that included potted Pinot noir vines grafted on two rootstocks with opposite characteristics (1103 Paulsen - P, highly vigorous and highly tolerant to drought, and Mgt 101-14 - M, less vigorous and susceptible to drought), as well as not grafted vines as a control (NGC).

The research work was built on the results of the Italian – Israeli bilateral project named "RINGO" (Rootstock-scion INteraction in Grape: an Omics perspective), carried out on the same experimental system. Starting from a Next-Generation Sequencing approach that evaluated the transcriptomic profile both of mRNA and small RNAs, the Ph.D. research project implemented and elaborated the data obtaining valuable results, that were enriched by a phenotyping activity on grape quality during two vegetative seasons (2012-2013).

To deepen the study of rootstock-scion interactions on the basis of the results collected at first, further experiments were set up. During the vegetative season 2017, the vines were maintained under the same conditions of the previous vintages and accurate additional

measurements, not performed before, on vine phenology, physiology, and productivity were carried out, alongside molecular and chemical analyses on the grapes produced.

During the vegetative season 2018, the same experimental protocol was repeated, but adding a controlled *pre*-veraison water stress period to test the rootstock influence on plant physiology and grape quality, in the event of water shortage.

In both the 2017 and 2018 seasons, an in-depth phenotyping work was carried out on the phenolic composition of grapes and the expression levels of some genes and miRNAs belonging to the phenylpropanoid pathway or involved in stress response were assessed. In particular, ten genes and five miRNA were selected to analyze by qRT-PCR because already detected as differentially expressed in berry skins between the three root systems (M, P, NGC) in the previous deep-sequencing.

The results collected during this multi-year study about rootstock-scion interactions highlighted that grafting *per se* had a strong influence on berry skin transcriptome, mostly at maturity. In general, the main differences were detected in grafted (M and P) compared to not grafted vines, both at the molecular and biochemical levels. Some genetic determinants (both genes and miRNAs) involved in the phenylpropanoid pathway and stress response were identified as influenced by the rootstock.

The rootstocks used on Pinot Noir vines, in the absence of stress factors, did not cause alterations in the scion in terms of development, photosynthetic efficiency, and primary metabolism. On the contrary, the main effects on grape quality were charged to the secondary metabolism, which was more significantly modulated during grape ripening in the plants grafted on 1103 Paulsen than in those grafted on Mgt 101-14. Finally, the obtained data suggested a rootstock-dependent response in case of water stress, which caused clear metabolic responses in the grapes, strongly impacting on gene expression and phenolic compounds accumulation.

Given the complexity of the topic studied, further investigation is needed to discover new details about the molecular network that regulates the interaction between rootstock and scion, particularly concerning grape quality.

1. Introduction

1.1 Economic importance, origins and features of *Vitis vinifera*

The grapevine (*Vitis vinifera* L.) is one of the oldest and most important fruit crops, whose cultivation covered about 7.5 million hectares worldwide in 2016, considering both wine grapes, table grapes, and *raisins* (O.I.V., 2017). Among the producing countries, only 5 represent the 50% of the global vineyard and, after Spain, China, and France, Italy has a central role, reaching 9% of the total surface (about 690,000 hectares). The annual production of grapes in Italy amounted to about 7.9 million tons in 2016, of which over 85% was represented by wine grapes. Furthermore, in the same year, Italy turned out to be the first wine-producing country (with 50.9 million hectoliters, and a turnover of approximately EUR 11 million), confirming the significant value of this product for the National economy (O.I.V., 2017).

Vine cultivation has a very ancient history, that is closely connected with the evolution and movements of human society. According to some studies, the cultivation of grapevines for winemaking dates back at least 4,000 years before Christ. It is assumed that it was firstly domesticated in the area located around Mount Ararat, in the Caucasus region (Eurasia), and then spread towards the Mediterranean basin Countries (Greece, France, Spain, and the Italian Magna Grecia). In Italy, in particular, the grapevine was domesticated by the Etruscans around 1000 b.C. and has always been one of the dominant crops for the subsequent civilizations (Buono and Vallariello, 2002; Scienza, 2007).

Vitis vinifera is a perennial, woody, climbing plant, belonging to the family of *Vitaceae* and the genus *Vitis*. Over the centuries, *Vitis vinifera* has undergone processes of strong selection that led to obtaining plants with hermaphroditic flowers (self-fertile), good fruitfulness,

propagation capacity, root tolerance to limestone, but most of all good grape quality for winemaking. Nowadays, the almost unique grapevine species extensively used in the wine industry is *Vitis vinifera sub. sativa*, a domesticated form of the dioecious climbing *Vitis vinifera sub. sylvestris* (Keller, 2010).

The reference taxonomic unit for the vine is the "cultivar" (or cultivated variety). The total number of grapevine cultivars is estimated between 6,000 and 10,000 (Galet, 2000), and a large part of them are held in germplasm collections around the world. Despite this wide genetic variability, only fewer than 400 varieties are commercially important, and, among these, the most widespread international cultivars are very limited, including Cabernet Sauvignon, Merlot, Tempranillo, Chardonnay, Syrah, Sauvignon blanc, Pinot noir (O.I.V., 2017). The genetic complexity within a grapevine cultivar is also increased by the presence of clones and biotypes, which may have very different phenotypic outcomes (Pelsy, 2010).

The genome of *Vitis vinifera* L. is fully mapped since 2007, thanks to two parallel international projects that applied different strategies to get to the same goal (Jaillon *et al*., 2007; Velasco *et al*., 2007). The grapevine genome is highly heterozygous and has a diploid chromosome number (2n = 38); its size is around 475 - 500 Mb (approximately four times the size of *Arabidopsis thaliana*), and contains a set of 30,434 protein-coding genes (Jaillon *et al*., 2007; Velasco *et al*., 2007). At that time, the grapevine genome was the first accessible for a fruit crop and has soon become a model plant for non-climacteric fruit research. Moreover, the grapevine is still assumed as a reference woody species to study the molecular regulation of stress response, thanks to its ability to tolerate multiple stresses and quite extreme environments, implementing molecular and physiological adaptation strategies (Pagliarani *et al*., 2017).

1.2 Grapevine development and berry ripening

The annual growth cycle of the grapevine is composed of different phenological phases, distributed throughout the year (bud break, anthesis, fruit set, veraison, harvest, and leaf abscission); the period that includes all the developmental stages is conventionally comprised between April 1st and October 31st in the Northern Hemisphere (Winkler *et al*., 1974), and it is followed by winter dormancy.

The grapevines produce both fruit and foliage from the same buds, which are particularly numerous and complex. After bud break, the gradual and continuous development of shoots and leaves takes place, forming a suitable canopy for capturing sunlight and acting as a source for sending carbohydrates to the bunches. At the same time, after bloom, fertilized flowers set fruit, and as the berries develop, they become the sink for photosynthetic products (Failla, 2007; Keller, 2010).

The grape berry (pericarp) anatomy is characterized by three major types of tissue: skin (or exocarp), flesh (mesocarp and endocarp), and seed (coat, endosperm, and embryo). In particular, berry skins constitute 5-20% of fresh berry weight and consist of two anatomically different regions, the outermost epidermis, and the innermost hypodermis (Jackson, 2000). Epidermis (a single cell layer), is coated with a waxy cuticle (pruin), a first protective hydrophobic barrier against environmental stress and water loss, while hypodermis (that consists of a variable number of cell layers, commonly 10, that decrease to 4-5 during maturation), is where phenolic compounds accumulate in relatively high concentrations during ripening (Failla, 2007; Keller, 2010).

The berry is supplied through the pedicel by xylem, with a dominant role in the early stages of development, it remains active up to veraison, and phloem, limitedly active at the beginning of berry growth, it becomes the main vasculature after veraison (Coombe and Mc Carthy, 2000). As a matter of fact, in the berry tissues different types of substances accumulate: primary metabolites (as water, sugar, amino acids, minerals, and micronutrients) that are essential for plant survival, and secondary metabolites, organic compounds that act as defense molecules against pathogens, antioxidants facing UV photo-oxidative damages, and visual or olfactory signals for seed dispersers. The main chemical compounds contained in the berry and their localization within the different tissues is illustrated in Fig.1.

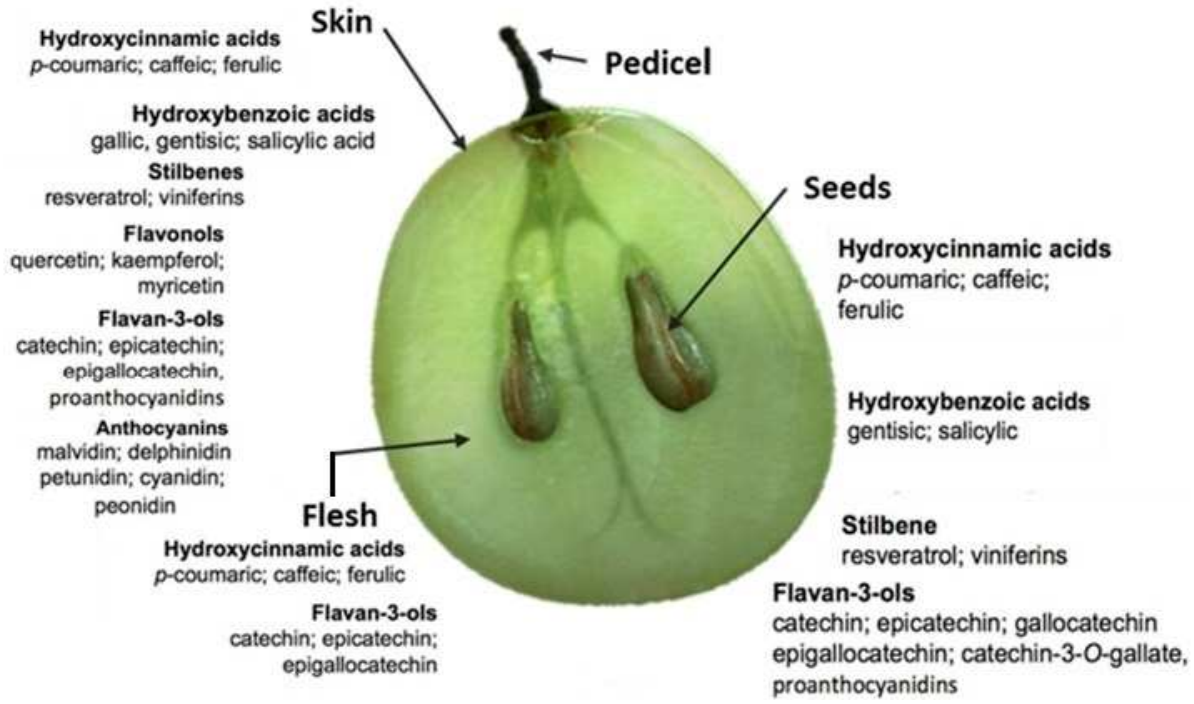

Fig.1 - Schematic structure of a ripe grape berry and phenolic compounds distribution between several organs and tissues (indicated by arrows). Adapted from Cosme (*et al.*, 2018).

It is well known that grape berry development has a typical double sigmoid pattern, composed of two phases that are separated by a lag period (Harris *et al*., 1968; Coombe and Mc Carthy, 2000; Ollat *et al*., 2002). Berry development begins immediately after anthesis and ends at harvest (Fig.2).

Phase I (or herbaceous phase) is characterized by an intense cell division activity. At this juncture, the total number of cells within the berry is established, and it has a dominant influence on the final berry size, and several solutes start to accumulate in the different tissues (tartaric and malic acids, hydroxycinnamic acids, tannins, and monomeric catechins, as well as aroma compounds like methoxypyrazine). Throughout this phase, sugars remain low and chlorophyll is the main pigment. Subsequently, the lag phase (ending with veraison) provides for berry softening with a decline in turgor, and skin color changes from green to purple in the dark-skinned varieties, due to the degradation of chlorophyll and the accumulation of anthocyanins in the exocarp; in white grape cultivars, the berries acquire a translucent appearance and the skin color is owed to carotenoids and pale yellow flavonols. In the lag phase, there is no increase in berry size.

Phase II (or ripening phase) consists of a change in the mechanical properties of the berry and an almost doubling of the berry volume only due to cell distension, which initially occurs in a very rapid way and it progressively stops towards fruit maturity. The concentration of some chemical compounds accumulated in Phase I decreases by dilution effect, depending on environmental factors (malic and tartaric acids, hydroxycinnamates, pyrazines) or to condensation reactions between molecules (tannins). On the contrary, the amount of other metabolites increases exponentially, for example sugars (glucose and fructose), amino acids (mainly proline and arginine) and secondary metabolites such as anthocyanins (in red

cultivars), stilbenes, flavonols, volatile flavors (in white varieties) and aroma precursors (Failla, 2007; Keller, 2010).

The seed maturation is simultaneous and coordinated with berry growth phases. In the first stage the seeds rapidly increase in size, then, 10-15 days before veraison, they reach the final volume and, at the beginning of the lag phase, the embryo comes to maturity and the endosperm continues to accumulate reserves until the seeds turn dormant. Moreover, the seeds change color after desiccation of their tegument and become viable only at the end of the vegetative season, when the grapes are theoretically attractive for seed-dispersing mammals and birds or, more realistically, ready for winemaking (Failla, 2007; Keller, 2010).

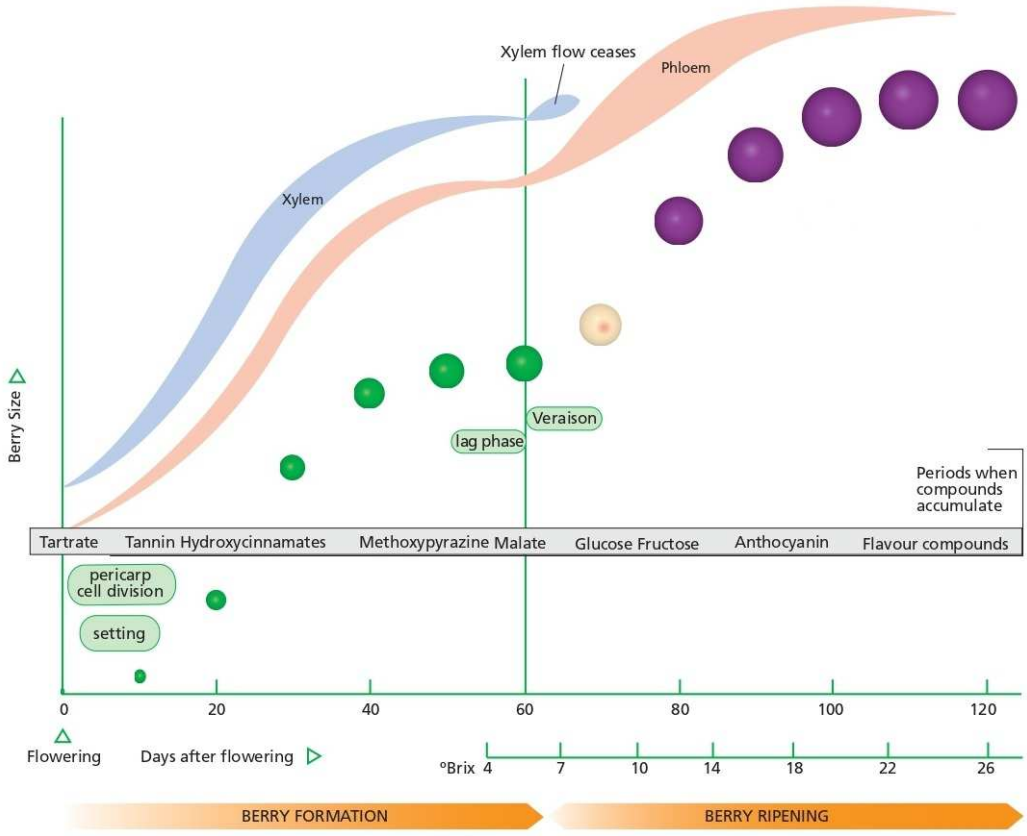

Fig.2 - Graphic representation of the double sigmoid pattern of berry development (10-day intervals after bloom). In the picture are shown the changes in berry size and color during the ripening, the inflow rates of xylem and pholem sap, the accumulation periods of the different chemical compounds and the sugar levels (°Brix). Adapted from Coombe (2001).

The physiological maturity is reached when berries have completed maturation, their vascular connection with the plant ceases, and the elaborated sap is no more loaded to the sinks. Subsequently, a process of grape over-ripeness can occur, with a gradual loss of water and a consequent concentration of the chemical compounds contained in berries (Failla, 2007).

The final grape quality is directly correlated to maturity at the end of the season, and the different compounds that have accumulated during ripening determine the main organoleptic characteristics of the wine. However, it is difficult to define "quality" and "optimal maturity" univocally, since both these parameters are closely related to the wine style desired and enological target (Poni *et al*., 2018).

The technological requirements for winemaking are related to an adequate accumulation of sugars and organic acids (and the relevant pH value) in the grapes, while the characteristics of phenolic maturity are linked to the accumulation of phenolic compounds, mainly in berry skins and seeds (Guillaumie et al., 2011).

At the molecular level, the onset of berry ripening is driven by a transcriptomic shift, which evolves until full maturation. The metabolomic changes are determined by the expression profiles of thousands of structural and regulatory genes involved in complex biosynthetic pathways (an example in Fig.6), that are considerably modulated according to each developmental stage (Deluc *et al*., 2007; Fasoli *et al*., 2012). Recently, some key transcriptional changes were determined alongside the berry ripening program and can be considered as putative biomarkers for the different critical stages of maturation (Fasoli *et al*., 2018).

Dynamics of gene expression during berry ripening are extremely variable among the grapevine varieties (Massonnet *et al*., 2017), and even within the same cultivar, it is possible to highlight strong differences due to the vintage effect and the interaction genotype-environment (Dal Santo *et al*., 2018). However, the expression of some variety-dependent genes can determine specific phenotypic traits (e.g. in berry color, anthocyanin profiles or the synthesis of specific aroma compounds), inherited from parent genotypes through the numerous domestication crosses (Massonnet *et al*., 2017; Fasoli *et al*., 2018).

1.3 Grape phenolic compounds

Phenolic compounds are secondary metabolites that influence organoleptic attributes of grapes and wines (color, flavor, texture, astringency and antioxidant properties) which are distributed in the various berry tissues and accumulate at different times during ripening (Fig.1 and Tab.1).

Grape phenolics have multiple biological activities, they protect leaves or fruits against UV photo-oxidative damage, are free radicals scavengers, play a role in defense against biotic and abiotic stresses, and also act as visual signals for pollinator recruiters or seed dispersers. Moreover, these metabolites are termed "nutraceutical compounds" thanks to their wide range of demonstrated beneficial effects on human health (Georgiev *et al*., 2014).

Grape phenolics are derived from the aromatic amino acid L-phenylalanine, a product of the shikimate pathway, and their biosynthesis has been described in detail and reviewed by different authors (Adams, 2006; Kennedy *et al*., 2006; Conde *et al*., 2007; Teixeira *et al*.,

2013), while the complex transport mechanisms in *Vitis vinifera* remain still unclear (Gomez *et al*., 2011).

Phenolic compounds have a common chemical structure composed of a phenyl ring backbone with a hydroxyl group or other substitutes and are divided between non-flavonoids (with a simple C6 backbone: hydroxycinnamic acids, hydroxybenzoic acids, and stilbenes) and flavonoids (anthocyanins, flavonols, and flavan-3-ols). The different chemical structures are shown in Fig.4.

Non-flavonoid compounds:

- Hydroxycinnamic acids (the third most abundant class of soluble phenolics in grapes, after proanthocyanidins and anthocyanins) are derivatives of cinnamic acid and the predominant are p-coumaric, caffeic, and ferulic acids. They are accumulated mainly in flesh, but also skin and seed tissues, and are mostly synthesized before veraison; then the accumulation stops and the content per berry remains almost constant. Hydroxycinnamic acids are usually present as *trans* isomers, and, normally are esterified with tartaric acid (*trans*-*p*-coumaroyl-tartaric acid or coutaric acid, *trans*-caffeoyl-tartaric acid or caftaric acid, and *trans*-feruloyl-tartaric acid or fertaric acid).
- Hydroxybenzoic acids and their derivatives are present in low concentrations compared to hydroxycinnamic acids. The hydroxybenzoic acids are mainly found in their free form in grape berries and the most common are gallic acid (the major), gentisic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, and salicylic acid. This last one is involved in plant signalling, in particular in the response to stress. The accumulation pattern is variable between the different

molecules during berry ripening. The hydroxybenzoic acids are present in skins and in seeds, where gallic acid can esterify flavan-3-ols.

- Stilbenes are compounds naturally occurring in a limited number of plant families among which stand out *Vitaceae* (Jeandet *et al*., 2010). In grapevine, stilbenes are primarily found in ripen berry skins, with some accumulation differences according to the grapevine cultivar (Gatto *et al*., 2008). The constitutive concentration is generally low, and the synthesis increases starting from veraison towards maturity, but it can burst in case of pathogen attack or at the onset of abiotic stresses. Hence, stilbenes intervene as phytoalexins triggering some mechanisms of grape resistance (Jeandet *et al*., 2010). The most studied stilbene in grapes and wines is resveratrol, a molecule that is gaining attention for its medical and pharmacologic properties since it has shown preventive powers on different kinds of human diseases (Snopek *et al*., 2018; Amor *et al*., 2018). Resveratrol can be present in its free form as a monomeric unit, *trans*-resveratrol (3,5,4'-trihydroxystilbene), which is the precursor of more complex molecules. Resveratrol can be glycosylated, generating *trans*- and *cis*-piceids (3-O-β-D-glucoside) or dimethylated, originating *trans*-pterostilbene (3,5-dimethoxy-4'-hydroxystilbene). Polymerization by oxidative coupling through peroxidase activity leads to the formation of viniferins (α- β- γ- δ- ε), the main group of resveratrol cyclic oligomers.

Tab.1 - Phenolic compounds produced and accumulated in grape berries.
Very abundant compound (+++) to absent (-). Adapted from Teixeira (*et al.*, 2013).

Compound	Level of synthesis			Location	Berry phenological scale			
	Skin	Flesh	Seed		Blooming	Green stage	*Veraison*	Ripening
Nonflavonoids								
Hydroxycinnamic acids	++	+++	++	Hypodermal cells and placental cells of the pulp; primarily in the vacuoles of mesocarp cells.	+++	+++	+	+
Hydroxybenzoic acids	+	–	++					
Stilbenes	+++	+	++	Berry skin and seeds.	–	+	++	+++
Flavonoids								
Flavonols	++	–	–	Dermal cell vacuoles of the skin tissue and cell wall of skin and seeds.	++	+	+++	++
Flavan-3-ols	++	+	+++	Specific vacuoles of hypodermal skin cells and seed coat soft parenquima.	+	++	+++	++
Anthocyanins	+++	–	–	Cell layers below the epidermis; storage confined to the vacuoles and cytoplasmic vesicles named anthocyanoplasts.	–	–	+	+++

Flavonoid compounds:

Flavonoids represent the most significant phenolic fraction in grapes (except for hydroxycinnamic acids) and are synthesized via the general phenylpropanoid pathway, so-called due to the C6–C3 scaffold resulting from the first step of their biosynthesis. In particular, flavonoid molecules have a very specific three-ring structure (Fig.3): two hydroxylated benzene rings (A and B) are joined by a three-carbon chain which is part of the heterocyclic ring (C). Depending on the oxidation state of the C-ring, the compounds belong to the structural classes described below.

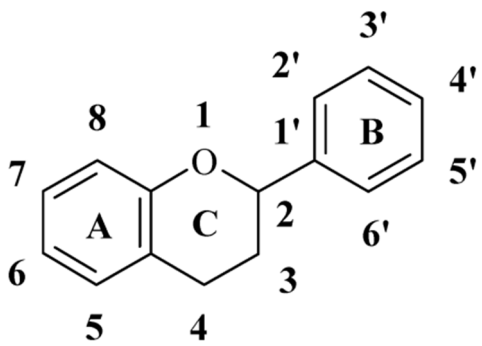

Fig.3 - Flavonoid ring structure and numbering. From Farkas (*et al.*, 2004).

- Flavonols, a class of flavonoids located in the skin outermost layer, act as a natural sunscreen for the cluster and are responsible for bitterness and astringency in wines (Waterhouse, 2002). Flavonols have a 3-hydroxyflavone backbone and are differentiated according to the number and type of substituents on the B ring. The main grape flavonols are quercetin, myricetin, kaempferol, and the methylated form isorhamnetin, laricitrin and syringetin. The effective flavonol profile is cultivar-dependent (e.g. in Pinot noir is characterized by a high content of quercetin, followed by lower amounts of myricetin and minimum quantities of kaempferol and isorhamnetin - Mattivi *et al*., 2006). Flavonols are always found in a glycoside-form (glucosides, galactosides, rhamnosides or glucuronides), with a sugar molecule attached to position 3 of the flavonoid skeleton. Flavonols synthesis is carried out mainly during the early stages of ripening and it stops at veraison (Teixeira *et al*., 2013).
- Flavan-3-ols are the most abundant class of phenolic compounds in grapes; they contribute to the mouthfeel and palatability of wines and confer bitterness and astringency. Flavan-3-ols are present at the highest concentrations in seeds, then in skins and flesh only in traces.

  Flavan-3-ols in grapes are five: (+)catechin and its isomer (−)epicatechin, (+)gallocatechin, (−)epigallocatechin and catechin-3-*O*-gallate and their molecules have a hydroxyl group at position 3 of C ring. Flavan-3-ols can be monomeric (tannins) or polymeric structures of various sizes, with up to 40 subunits linked by 4-6 and 4-8 interflavan bonds (proanthocyanidins or condensed tannins).

- Anthocyanins are the molecules responsible for the color of grapes (from red to blue pigmentation) and are stored in the vacuoles of hypodermal cells of berry skins. Their chemical structure (Fig.5) was firstly described in 1959 (Ribéreau-Gayon, 1959). The basic molecule has a flavylium core, with the typical C6-C3-C6 skeleton, that contains one heterocyclic benzopyran ring (C), one fused aromatic ring (A) and the phenyl constituent (B). In the cation form, anthocyanidins have two double bonds in the C ring and carry a positive charge. The common aglycones anthocyanins in grapes are five (cyanidin, peonidin, petunidin, delphinidin, malvidin), and are different from each other due to the presence of hydroxyl or methoxyl substitutions in position 3' and 5' on the B ring (He *et al*., 2010).

  Anthocyanins are anthocyanidins with a covalent bond with one (3-monoglucoside, in *Vitis vinifera*) or two (3,5-diglucosides in non-*vinifera Vitis* species) sugar molecules. Moreover, anthocyanins can be found in an acylated form, if esterified with acetic, coumaric, or caffeic acids at 6’ position of the glucose bonded to the 3’ of the C ring (Teixeira *et al*., 2013).

  Each grapevine cultivar has a conserved and distinctive anthocyanin profile (e.g. Pinot noir is characterized by the prevalence of malvidin-3-glucoside and peonidin-3-glucoside and the total absence of acylated anthocyanins), which can considerably vary based on environmental conditions (Guidoni *et al*., 2008). In general, malvidin-3-glucoside, along with its acylated forms, is the major anthocyanin present in grapes (Kennedy *et al*., 2006).

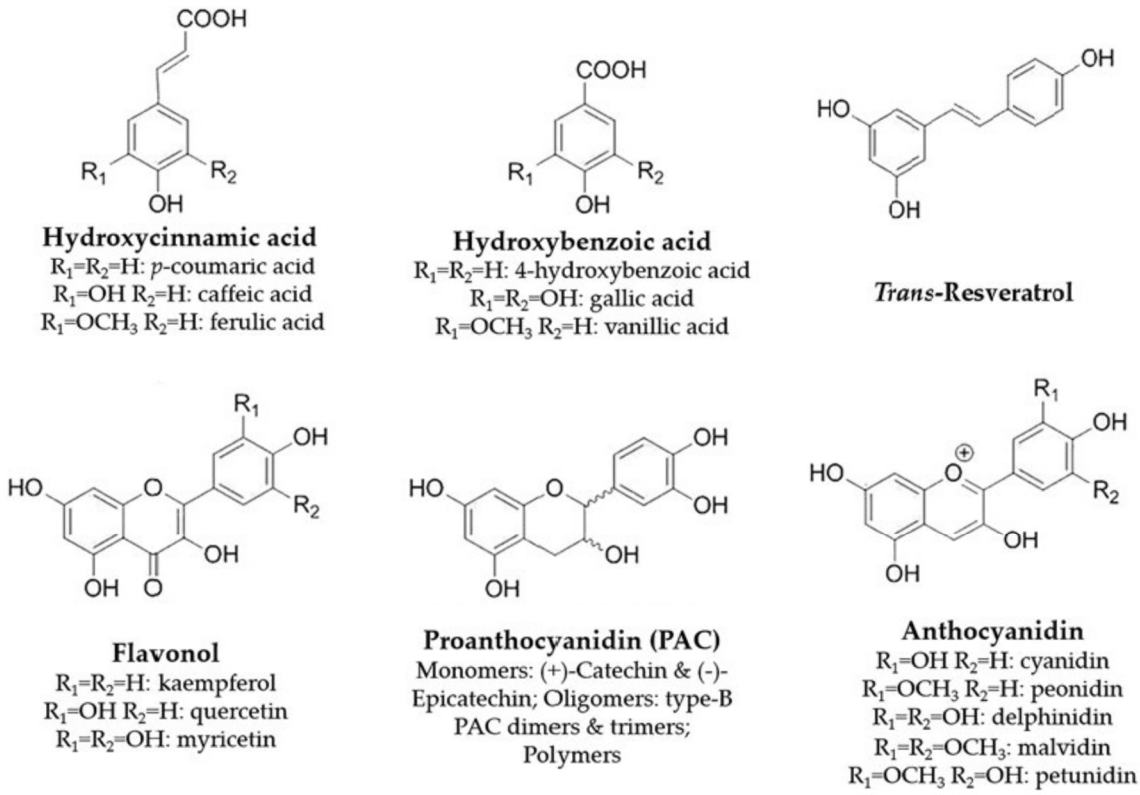

Fig.4 - Example of the chemical structure of the non-flavonoid and flavonoid compounds described. Adapted from Zhao (*et al.*, 2019).

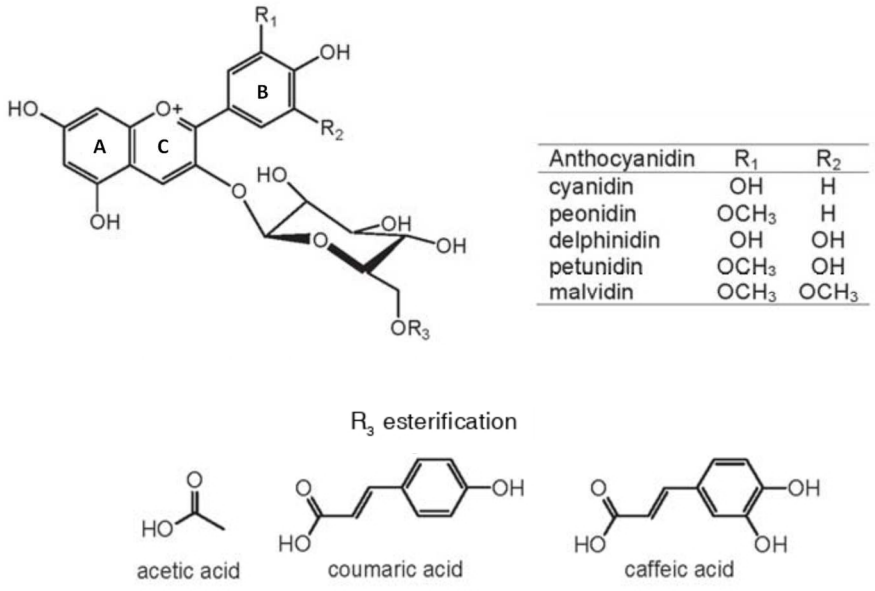

Fig.5 - Chemical structure of grape anthocyanins. Adapted from Kennedy (*et al.*, 2006).

1.4 Biosynthesis pathway of phenolic compounds in grapes

Flavonoids are synthesized via the general phenylpropanoid pathway, from a common precursor, the amino acid L-phenylalanine (Fig.6). This intricate pathway involves numerous enzymes or multienzyme complexes and it branches into several key points for the production of different classes of chemical compounds (Boss *et al*., 1996; He *et al*., 2010).

The first reaction involves the deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL), producing cinnamic acid, which is then converted by hydroxylation in p-coumaric acid by the enzyme cinnamate-4-hydroxylase (C4H); subsequently, p-coumaric acid is condensed with an acetyl-CoA molecule to 4-coumaroyl-CoA by the enzyme 4-coumaroyl:CoA-ligase (4CL). Alternatively, 3-hydroxylation of p-coumaric acid carried out by the enzyme coumarate-3-hydroxylase (C3H), originates caffeic acid, which can be further converted into ferulic acid through 3-methylation by a caffeic acid *O*-methyltransferase (COMT) (Conde *et al*., 2007).

The intermediate product 4-coumaroyl-CoA is the common substrate for both stilbene synthase (STS) and chalcone synthase (CHS), the enzymes that operate the entry point into stilbene or flavonoid pathways. At this stage, two chemically different intermediates are produced following three condensation reactions with malonyl-CoA: resveratrol by the enzyme STS or tetrahydroxychalcone (also known as naringenin chalcone) by the enzyme CHS (Teixeira *et al*., 2013).

The flavonoid pathway continues with the conversion of naringenin chalcone in naringenin (naringenin flavanone) by the enzyme chalcone isomerase (CHI), through a stereo-specific ring closure. Naringenin flavanone can be further hydroxylated by flavonoid 3'-hydroxylase

(F3’H) or flavonoid 3’5’-hydroxylase (F3’5’H) to produce eriodictyol or pentahydroxyflavanone, respectively. Otherwise, naringenin is hydroxylated at position 3 by flavanone 3-hydroxylase (F3H) to form dihydrokaempferol, precursor of the flavonols.

The competition between F3’H and F3’5’H activity establishes a primary bifurcation that leads to the production of cyanidin-like (3’,4’-hydroxylated) or delphinidin-like anthocyanins (3’,4’,5'-hydroxylated), precursors of red or blue skin pigments (Bogs *et al*., 2006; Castellarin *et al*., 2006).

Dihydrokaempferol is an intermediate molecule that can be processed by three different enzymes: flavonol synthase (FLS, a 2-oxoglutarate dependent dioxygenase), to produce directly a flavonol aglycone (kaempferol); flavonoid-3’-hydroxylase (F3’H) or flavonoid-3’5’-hydroxylase (F3’5’H) to synthesize, through the hydroxylation of the B ring of in 3’ or 3’5’ positions, dihydroquercetin or dihydromyricetin, respectively. These last two molecules are then substrates of FLS for the synthesis of the flavonols quercetin and myricetin or substrates for dihydroflavonol 4-reductase (DFR) that reduces dihydroflavonols to the corresponding leucoanthocyanidins (leucocyanidin or leucodelphinidin), deviating towards the biosynthesis branch of anthocyanins and proanthocyanidins (Downey *et al*., 2003). In fact, leucoanthocyanidins can be reduced to flavan-3-ols ((+)-catechin) by the action of leucoanthocyanidin reductase (LAR) or can be catalyzed by leucoanthocyanidin dioxygenase (LDOX) to produce cyanidin or delphinidin.

Subsequently, cyanidin and delphinidin are further processed by two alternative enzymes: anthocyanidin reductase (ANR) for the synthesis of (-)-epicatechin and (-)-epicatechin-3-O-gallate, or UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT, an enzyme expressed only in dark-skinned varieties) that adds a glucose residue at the 3 position of the C ring of

anthocyanidins, producing more stable anthocyanins (cyanidin-3-glucoside or delphinidin-3-glucoside).

Finally, cyanidin-3-glucoside or delphinidin-3-glucoside can be further processed by the enzyme O-methyltransferase (OMT) to deliver peonidin-3-glucoside (a cyanidin-like, disubstituted anthocyanin), petunidin-3-glucoside and malvidin-3-glucoside (two delphinidin-like trisubstituted anthocyanins) (Fournier-Level *et al*., 2011).

The possible acylation of the anthocyanins occurs in certain grape varieties with the addition of an aliphatic acetyl group or an aromatic *p*-coumaroyl group to the 6' position of the 3-*O*–glucoside (Fig.5) and is carried out by anthocyanin and flavonoid acyltransferases, which belong to two different categories (aliphatic and aromatic acyltransferases) on the basis of the acyl-donor specificity (Boss *et al*., 1996; He *et al*., 2010; Kuhn *et al*., 2013).

The synoptic illustration of the reactions that take place along the phenylpropanoid pathway in grapevine, with the acronyms of the related enzymes involved and the chemical compound synthesized, is shown in Fig.6.

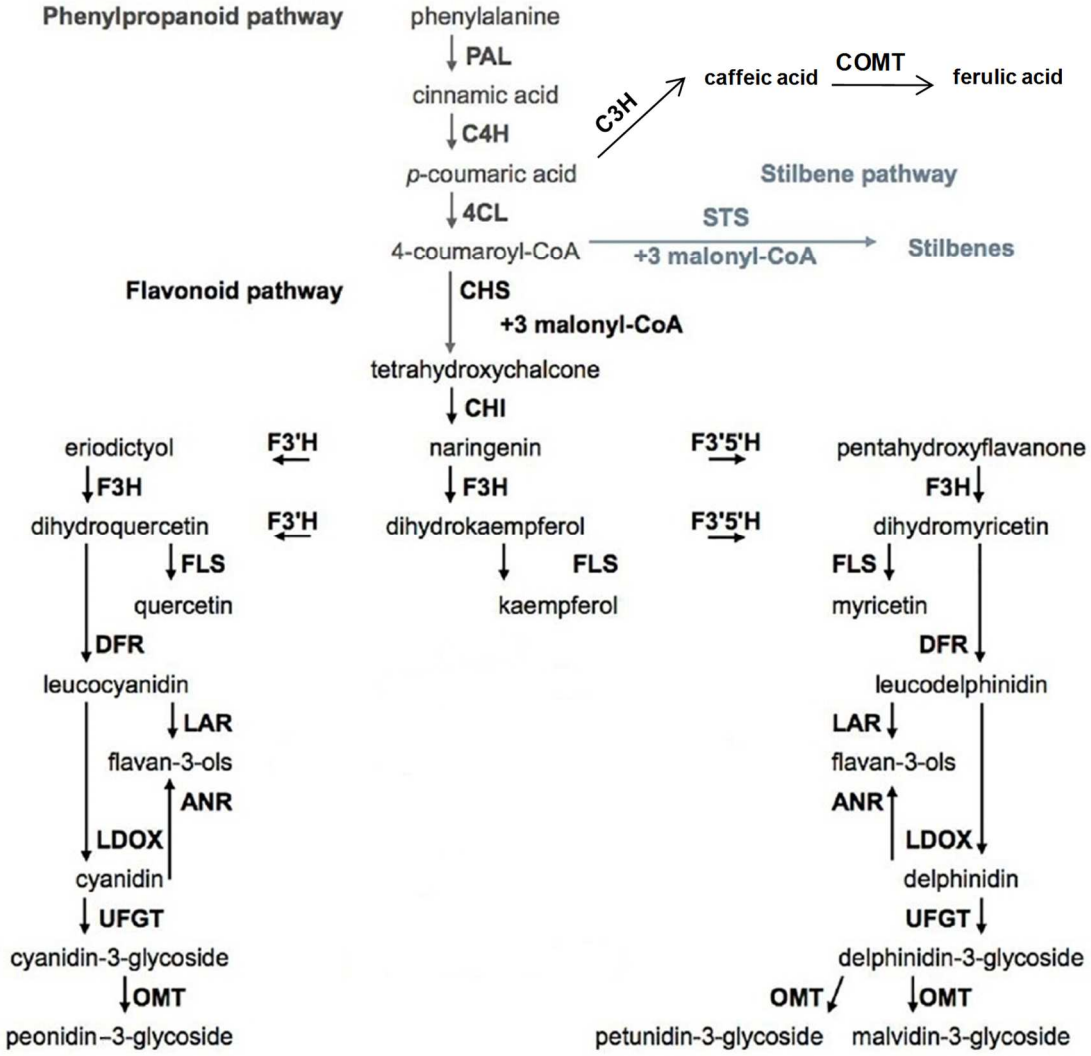

Fig.6 - Biosynthetic phenylpropanoid pathway in grapes.

Enzyme acronyms: Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), coumarate-3-hydroxylase (C3H), caffeic acid O-methyltransferase (COMT), 4-coumaroyl:CoA-ligase (4CL), stilbene synthase (STS), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), flavanone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), leucoanthocyanidin dioxygenase (LDOX), dihydroflavonol 4-reductase (DFR), UDP-glucose flavonoid glucosyltransferase (UFGT), O-methyltransferase (OMT). Adapted from Teixeira (*et al*., 2013).

1.5 Regulation of flavonoid biosynthesis

In plants, the transcriptional or post-transcriptional regulation of the structural genes involved in the phenylpropanoid biosynthetic pathway is finely controlled, at multiple levels. The most common regulatory mechanisms are the MBW complex, composed of MYB transcription factors, basic helix-loop-helix (bHLH) and WD40 proteins (Hichri *et al*., 2011) or RNA interference, where miRNAs are key players (Sharma *et al.*, 2016; Qiao *et al.*, 2017).

Transcription factors:

Transcription factors (TFs) are proteins that bind a specific sequence of DNA adjacent to the gene that they regulate, conditioning its transcription. The TFs act by promoting (as activators), or blocking (as repressors) gene expression.

The MYB superfamily is the most abundant group of TFs described in plants and includes several members, both positive or negative regulators. MYB TFs are involved with a central role in the main physiological processes, for example, organ development, response to biotic stresses, resistance to pathogens (Matus *et al*., 2008).

MYB proteins are characterized by the so-called N-terminal MYB domain, consisting of 1 to 3 imperfect repeats of almost 52 amino acids (R1, R2, R3): each repeat has a helix-turn-helix structure that is involved in DNA binding and connects to the regulatory elements in the promoter, while the C-terminal region establishes protein-protein interactions with the components of the transcriptional machinery (Matus *et al*., 2008).

In grapevine, R2R3-MYBs are by far the most important TFs that control flavonoid and stilbene accumulations during ripening, at the different spatiotemporal level (Deluc *et al*., 2006; Czemmel *et al.*, 2012; Cavallini *et al*., 2015).

One of the best-known examples in the literature is the regulation of the UDP-glucose:flavonoid 3-o-glucosyltransferase (UFGT) gene (see Fig.6) by the MYBA transcription factors, which favor anthocyanin accumulation in dark-skinned grapes. According to several authors, the appearance of grapevine sports with white berry skins was due to different kind of alterations (e.g. mutations or transposon insertions) in the sequence of MYBA genes (Kobayashi *et al*., 2004; Walker *et al*., 2007; Furiya *et al*., 2009; Shimazaki *et al*., 2011).

Another interesting example of a pathway regulated by MYB TFs is resveratrol biosynthesis, whose feedback regulation has been recently illustrated in grapevine leaves (Jeandet *et al.*, 2019). In the presence of UV-C rays during the ripening period, MYB14, together with another transcription factor (WRKY3), up-regulates the activity of a STILBENE SYNTHASE gene (STS29), resulting in resveratrol accumulation, useful for the dissipation of oxidative stress caused by solar radiation. On the contrary, when resveratrol level increases too much, MYB14 is down-regulated by another TF (WRKY8) and the transcription of STS29 is blocked, causing a stop in the synthesis of this metabolite (Jeandet *et al*., 2019).

Likewise, another TF gene family specific to plants that is attracting the attention of many research groups, less characterized than MYB, is NAC (NAM-ATAF1,2-CUC2). A total of 74 different NAC genes have already been described in *Vitis vinifera* (Wang *et al*., 2013), and have different roles in plant development, berry ripening and the response to biotic and abiotic stresses. The precise functions of each NAC gene are not yet completely clear, but it is known that they are often tissue-specific and can be activators or repressors of the

expression of their target genes, depending on the environmental conditions (Le Hénanff *et al*., 2013).

miRNAs:

miRNAs are non-coding endogenous small RNAs (19-24 nucleotides long), with broad phylogenetic conservation in eukaryotes. In plants, they are coded by specific MIR genes and their biogenesis from miRNA precursor to mature miRNAs requires multiple steps (Fig.7), that take place in the cell nucleus and the cytoplasm, with the involvement of numerous enzymes (Rogers and Chen, 2013; Yu *et al*., 2017).

Mature miRNAs perform Post-Transcriptional Gene Silencing (PTGS), acting as sequence-specific guides for either cleavage or translational block of target transcripts (Chuck *et al*., 2009; Solofoharivelo *et al*., 2014).

In recent years, some studies have revealed the central role of miRNAs in grapevine metabolism. The availability of the grapevine genome sequence (Velasco *et al.*, 2007; Jaillon *et al.*, 2007), in fact, has allowed the identification, or prediction *in silico*, of hundreds of miRNAs, which are released on the official miRNA Database (www.mirbase.org). miRNA targets in *Vitis vinifera* belong mainly to different transcription factors families or are functional genes involved in development, tissue differentiation, secondary metabolism, resistance and adaptation to biotic and abiotic stress (Mica *et al*., 2010; Wang *et al.*, 2011; Belli Kulhan *et al*., 2015; Pantaleo *et al*., 2016).

miRNAs expression is strongly influenced by environmental conditions and developmental stages; miRNAs may act as cell-to-cell or systemic signal molecules, also performing long-range movements and efficient information exchanges between tissues. The transition of

miRNAs through the junction point of grafted grapevines has been already established (Pant *et al.*, 2008; Cookson *et al.*, 2014; Maré *et al.*, 2016). In fact, grafting can alter miRNAs abundance in the scion, as their movement through the vascular system is coupled with stress signals, causing changes in the final phenotype (Pagliarani *et al.*, 2017; Yang *et al.*, 2015).

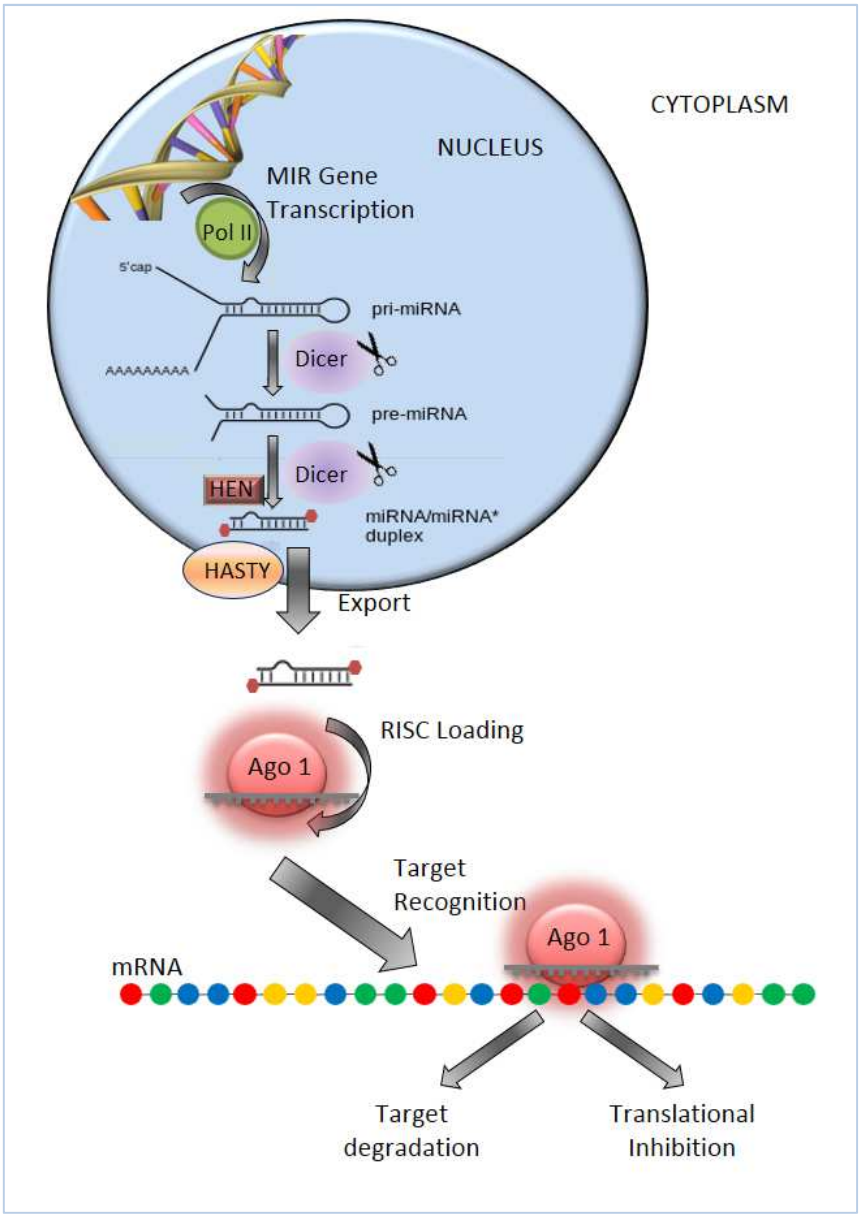

Fig.7 - The biogenesis and regulation mechanisms of plant miRNAs. Adapted from Zhang (*et al.*, 2006).

1.6 The problem of phylloxera and the role of rootstocks

"Imagine taking two organisms, cutting both of them in half and fusing them together to make a superior individual. Sounds like science fiction? With plants is reality"

Kümpers and Bishopp, 2015

Most of the grapevines cultivated in the world are grafted to overcome the problem of phylloxera, a harmful viticultural pest that spread in Europe at the end of the 19th century.

The causative agent of phylloxera is *Daktulosphaira vitifoliae,* an invasive soil-dwelling aphid that feeds solely on *Vitis* species. This monophagous parasite is native to eastern and south-eastern United States, and has coevolved in the same habitat with several American *Vitis* species, which suffer only weak lesions at the leaf level, but are almost resistant, while European grapevines are highly susceptible and show extensive damages in the event of an attack (Granett *et al*., 2001, Skinkis *et al*., 2009).

Grape phylloxera was discovered in California and first described in 1855 (Russel, 1974), but it became well-known only after being introduced in France in 1863. This vine epidemic colonized the Old Continent in few years, and rapidly devastated a large portion of the own-rooted vineyards (Pouget, 1990), threatening the existence of the European viticulture itself (Powell *et al*., 2013).

As a result of the explosion of phylloxera, in all the countries involved an intensive research activity started to find a solution as quickly as possible and to prevent all the vineyards from being wiped out. It took many years to understand the lifecycle of *Daktulosphaira vitifoliae,* but it became soon clear how the major damages occurred. The scientists discovered that the dangerous plant-sucking insect feeds on *Vitis vinifera* roots, eliciting the formation of the

characteristic hook-shaped galls on root tips or “nodosities” and swellings on mature roots or “tuberosities” (Eitle *et al*., 2017). Such pathogenic structures compromise the absorbent function (inhibiting water and mineral uptake, and source-sink translocation), the root growth and triggering secondary fungal infections on the wounds (Granett *et al*., 1998), that lead to a rapid decline of the vine. The very first attempts to fight *Daktulosphaira vitifoliae* using chemical insecticides (e.g. toxic fumigants or carbon bisulphide injected in the soil) or eccentric remedies (e.g. tobacco leaves, oil, sulfur, seawater, incense, and manures) had very modest effects, not solving the problem (Ordish, 1987). Once understood that some American *Vitis* species were not susceptible to phylloxera, the experts began to produce hybrids crossed with *Vitis vinifera* (called “hybrid direct producers”) with disappointing results in terms of grape quality. Finally, they sensed the possibility to bypass phylloxera with grafting (Fig.8), a propagation technique that involves the union of the root system from a plant (rootstock) with the shoot of another plant (scion).

This ingenious solution permitted to obtain composite grapevines with a resistant root system deriving from cuttings of American *Vitis* species, maintaining in the scion the peculiar characteristics of *Vitis vinifera* grapes. Grafting was a successful strategy: grafted vines had massive advantages over their parents and the issues caused by phylloxera ceased almost completely.

The rootstock selection was carried out in the early decades of the 20th century and, indeed, most of the different genotypes (that are still used) were developed before 1930. Since the rootstock was a phenomenally durable form of biological control against phylloxera, entomological researches were suspended although the understanding of the general biology of *Daktulosphaira vitifoliae* and its interaction with the host-plant remained limited.

The rootstock breeders realized soon that, in addition to phylloxera tolerance, grafting could provide to the scion other positive traits inherited from the parent genotypes (Jackson, 2000; Zavaglia *et al*., 2016), such as greater resistance to some biotic or abiotic stresses, vigor alteration, phenology delay.

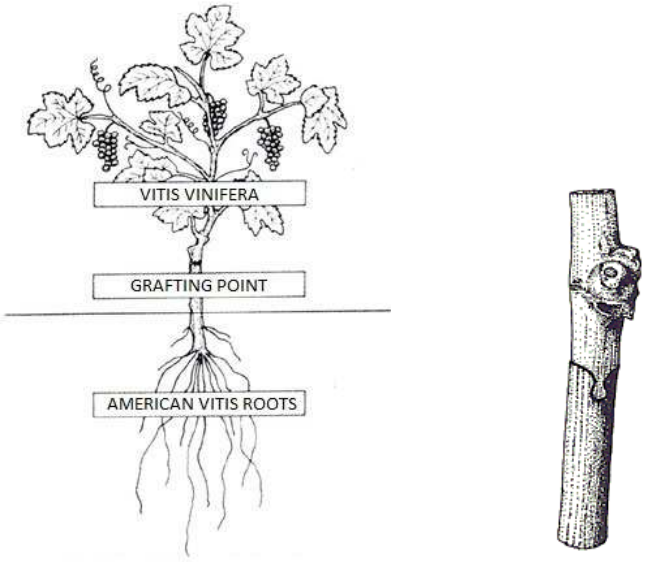

Fig.8 - Example of a grafted grapevine and an omega bench graft.

The American non-*vinifera* species most commonly used as parents in the rootstock breeding programs are: V. *berlandieri*, a genotype tolerant to limestone and drought; V. *riparia*, a mesophylic genotype, adapted to soils with water stagnation; V. *rupestris*, a xerophilic genotype, from arid climates and poor soils (Lovisolo *et al*., 2010).

In Italy, there are currently 45 varieties of rootstocks officially registered in the National Catalogue of Grapevine Cultivars (http://catalogoviti.politicheagricole.it/catalogo.php), as *Vitis* species or interspecific hybrids. Only 6 new rootstocks were obtained and added in the last decade, confirming that the breeding activity is a slow and a currently uncommon

process among the International research institutions. Nonetheless, some promising results were obtained at the University of Milan, where an interesting selection of rootstocks characterized by high tolerance to drought was carried out, obtaining the M4 genotype (Meggio *et al*., 2014; Merli *et al*., 2016).

Despite the great variability of environments and soils in the vineyards, the varieties of commercial rootstocks multiplied in the nurseries and actually employed are very limited. For example, in Italy, the most widespread are only: 1103 Paulsen, 110 Richter, 140 Ruggeri (*V. berlandieri* x *V. rupestris*) and Kober 5 BB, S.O.4, 420A (*V. berlandieri* x *V. riparia).* Less common are the rootstocks obtained from *V. vinifera* x *V. berlandieri* (Gravesac, Fercal) and *V. riparia* x *V. rupestris* (Mgt 101-14 and 3309 Couderc). Some examples of grapevine rootstocks genotypes and their parentages are shown in Fig.9.

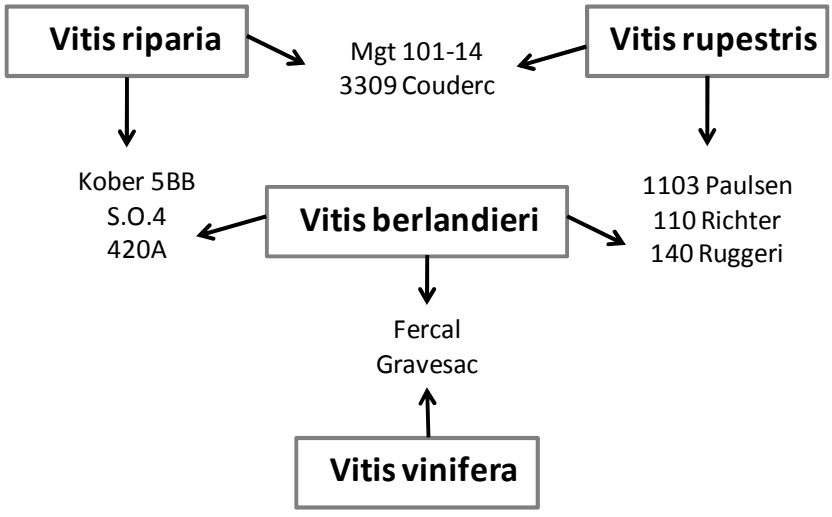

Fig.9 - Grapevine rootstock genotypes and their parentages. Adapted from Corso and Bonghi (2014).

1.7 Climate change impact on viticulture

"Wine is proving to be a canary in the coalmine for climate change"

Goode, Nature 2012

The issue of climate change, already forecasted by experts in the past decades, is nowadays a concrete and undeniable concern worldwide.

Climate change is due to the emission of greenhouse gases (mainly CO_2) deriving from human activities. It is expected to cause a steady global increase of air temperature (with warming up to 1.5° C between 2030 and 2052) and a strong modification in the precipitation pattern, but the latter is more difficult to predict (IPCC, 2018).

One of the supply chains within the agri-food sector that is likely to suffer the worst damages is certainly the wine industry since the grapevine is a fruit-crop very sensitive to deviations in climate (Winkler *et al*., 1974; Jones *et al*., 2005; Goode, 2012).

In fact, the grapevine needs precise thermal characteristics for the vegetative development (with a different optimum depending on the cultivar): a minimum basal temperature of 10° C for growth, and an average temperature between 12° C and 22° C for maturation, which can increase to 20° C - 30° C in summer. Besides, the major thermal limits are frost in spring, and heat peaks above 40° C in the hot season, which can be overcome only in case of optimal water availability (Jones, 2006; Cardell *et al*., 2019).

The main areas suited to viticulture are located in arid or semi-arid climates, characterized by drought and water deficit during the growing season. This kind of environmental conditions, if worsened, would no longer allow the grapevine cultivation (Jones *et al*., 2005). Almost all the wine districts are diffused exclusively at specific latitudes, namely between

the 30th and 50th parallels in the Northern Hemisphere and between the 30th and 45th parallels in the Southern Hemisphere (Fig.10). However, there is recent evidence showing that new cool-climate viticulture areas (such as the UK, Denmark, and southern Sweden) go beyond these latitudinal bands (Nesbitt *et al.*, 2018). Likewise, quality wines cannot be produced in tropical and subtropical regions or at high cold latitudes (Hannah *et al*., 2013; van Leeuwen and Darriet, 2016). It follows that such narrow climate zones are more subjected to possible weather alterations.

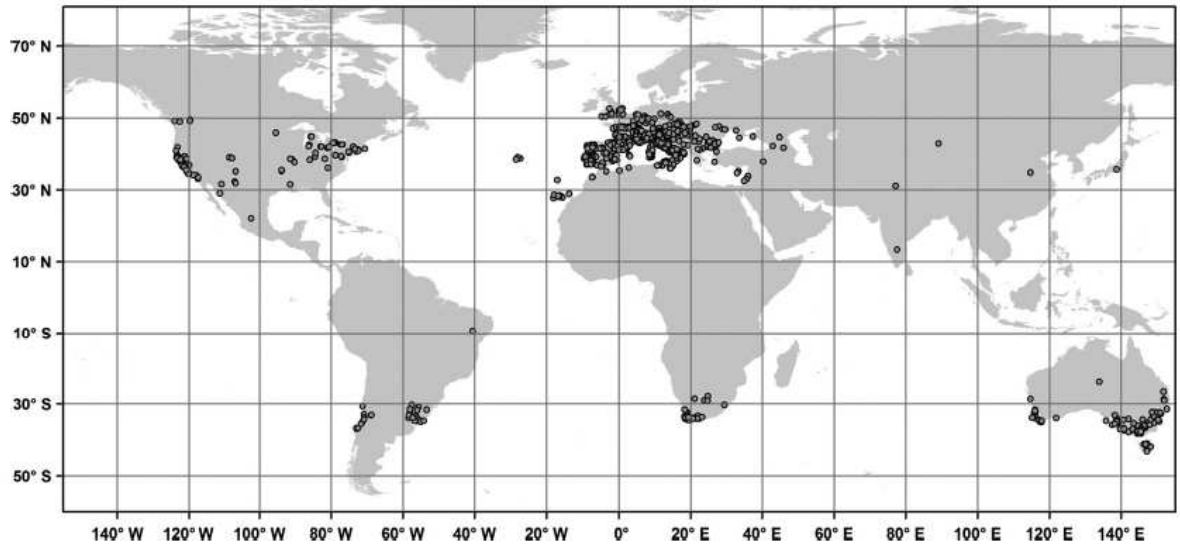

Fig.10. World distribution of the viticultural regions (black circles). Adapted from Hannah (*et al*., 2013).

According to some authors, by 2050 the growing areas suitable for grapevine could be drastically reduced in the principal wine-producing countries. On the contrary, other marginal regions (e.g. central and northern Europe), that will reach more favorable thermal conditions, are predicted to become important wine districts in the near future (Hannah *et al*., 2013; Cardell *et al*., 2019). Considering climate change in the European scenario, a shift

of the grapevine cultivation to higher latitudes and altitudes is expected, with a northward displacement of about 10 - 30 km every ten years (Palliotti *et al*., 2014). A strong modification of the current vineyard zones can be foreseen (Fig.11), with significant repercussions on natural habitats, water availability, territorial economy and human society (Fraga *et al*., 2012; Hannah *et al*., 2013).

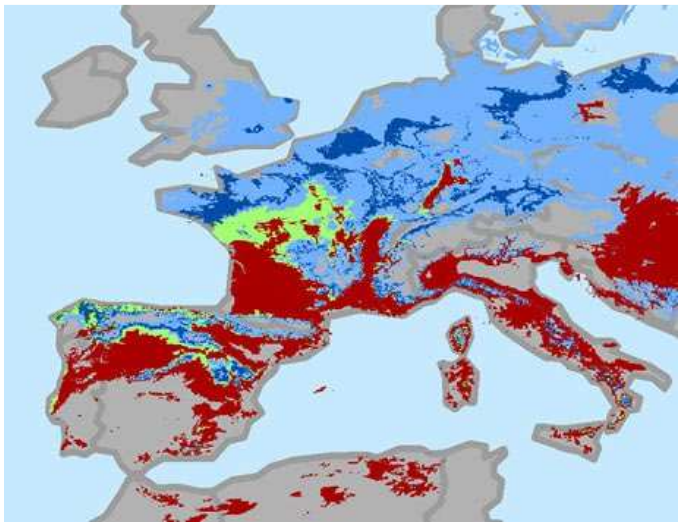

Fig.11 - A snapshot of European viticulture in 2050. Red: drought areas; green: suitable areas; blue: new potential areas. Adapted from Hannah (*et al*., 2013).

Given the importance of this topic, the effect of climate change is one of the most investigated subjects within the viticultural research at the present, with thousands of papers published in recent years.

Several alterations have already been detected in the context of the vegetative and productive development of grapevines worldwide. Some example of the main effects caused by climate change that can be counted are: shifting of the main phenological phases (Duchêne *et al*., 2010; Tomasi *et al*., 2011), advance in harvest dates (Seguin and De Cortazar, 2005; van Leeuwen and Darriet, 2016), reduction of photosynthetic efficiency or

permanent photoinhibition (Salazar-Parra *et al*., 2012), irregularity in yields (Schultz, 2000), unbalanced grape maturity (Sadras and Moran, 2012; Palliotti *et al*., 2014), berry shrivelling or sunburns (Rustioni *et al*., 2014), and increase in the pressure of fungal diseases (Salinari *et al*., 2006). Many of these events often occur in conjunction with extreme weather seasons, that are more and more frequent (Easterling *et al.*, 2000; Fraga *et al.*, 2012).

The grapevine is a perennial plant with a great ability to adapt to environmental constraints, and it has phenotypic plasticity that allows it to survive and carry forward fruit maturation even in limiting conditions (Lovisolo *et al*., 2010). In viticulture, the presence of moderate stress is desirable, in order to guarantee higher quality in grape chemical composition in red varieties, and to confer positive organoleptic characteristics to the wines produced (Peterlunger *et al*., 2005; van Leeuwen *et al*., 2009; Romero *et al*., 2013; Ferrandino and Lovisolo, 2014). Nonetheless, the vines that experience severe multiple summer stresses (e.g. excessive heat, water deficits, or high radiation levels) can suffer from serious damage to the whole plant metabolism (Fraga *et al*., 2012; Palliotti *et al*., 2014).

Focusing the attention on grapes, the major climate-related consequences given by higher temperatures and water shortage are: accelerated ripening, lower berry size, increased sugar accumulation (and consequently excessive alcohol levels in wines), drastic lowering of acidity resulting in arise of pH, decoupling of technological and phenolic maturity (especially for anthocyanin accumulation), decay of aroma profile and presence of atypical flavors. Therefore, grape quality can be strongly impaired (van Leeuwen and Destrac-Irvine, 2017).

In recent years, several adaptive strategies have been proposed and developed to mitigate the problem of global warming in viticulture. Various viticultural techniques can be adopted

by the winegrowers to reach complete maturity, keep grape composition balanced and maintain wine typicality.

Some management practices are easy to implement and readily applicable (also cumulatively), while others are long term solutions that require a complete redesign of the vineyard (Duchêne *et al*., 2014; van Leeuwen and Destrac-Irvine, 2017). Any change in the common practices to safeguard the quality of the grapes can have considerable costs and the farmers, that are more and more aware of the problem, are increasingly willing to make substantial investments.

Adaptive strategies can be divided into two main categories: modifications in viticultural techniques and modifications in plant materials. Some examples of the first category are: replacement of the traditional training systems; canopy management and manipulation, such as late-season leaf removal and severe canopy trimming; late winter pruning; sowing of grass covers; increase of soil organic matter; use of shading nets; irrigation of traditionally dry cultivation vineyards; application of innovative promising products, such as synthetic hormones, mineral sunscreen, antitranspirants, biostimulants (Schultz *et al*., 1998; Chaves *et al*., 2007; Böttcher *et al*., 2011; Lanari *et al*., 2013; Poni *et al.*, 2013; Palliotti *et al*., 2014; Filippetti *et al.*, 2015; van Leeuwen and Destrac-Irvine, 2017; Frioni *et al*., 2018; Gatti *et al.*, 2019).

The second category, instead, would cause much more drastic changes in wine style and quality but is certainly the most powerful tool to adapt vines to climate change and it is almost always environmentally friendly.

The hypothesis of substituting the traditional varieties used in a given territory with non-local later ripening cultivars is likely extreme, especially in European countries with

traditional appellations. Two more concrete solutions could be the introduction of new clones (selected to adapt to a changing environment) or, above all, the exploitation of rootstocks able to confer to the scion a higher drought and water deficit tolerance (Marguerit *et al*., 2012; Ollat *et al*., 2015).

In this regard, the rootstock is one of the key elements of a vineyard, whose role is sometimes overlooked. Considering that worldwide more than 80% of all vineyards grow vines grafted onto rootstocks (Ollat *et al*., 2015), this element is almost mandatory in the vineyard design to guarantee the development of healthy and productive plants. Rootstock choice should always be done wisely, and more attention should be given to the selection of new resistant hybrids, as well as to deepen the studies on rootstock-scion interaction, a genotype fusion that confers to the grapevine a higher resilience (Serra *et al*., 2014).

Rootstock as a tool against climate change:

As already mentioned, the rootstock not only gives resistance to phylloxera but also can affect some physiological parameters in the scion (Pavlousek, 2011).

Some rootstocks, thanks to intrinsic characteristics of their genotype, influence the resistance of the scion in case of environmental adversities and abiotic stresses, such as soil limestone, high salinity, stagnation, drought and frost (Corso and Bonghi, 2014; Warschefsky *et al*., 2016). Despite the great number of studies and hypotheses about the effect of the different types of rootstocks on plant's yield and grape quality, the molecular mechanisms that affect the metabolic processes in the scion remain largely unknown. It is therefore essential to deepen this issue to exploit rootstocks as key elements for climate change management in the future.

1.8 The rootstock-scion interaction

The rootstock acts as an interface between the soil ecosystem and the aerial portion of the grapevine (Ollat *et al*., 2017) and its role on the scion's physiology is a highly debated subject in the literature. According to some authors the rootstock modifies source-sink relations, influencing vine's performances (Di Filippo and Vila, 2011; Jones *et al.*, 2009; Marguerit *et al.*, 2012), whereas other studies suggest that the rootstock has minor effects on the physiological behavior of the scion, whose genotype is the main factor that concretely determines the shoot vegetative development and the characteristics of the grapes produced (Keller *et al*., 2012; Nuzzo and Matthews, 2006).

Cultivated grafted grapevines can be considered as composite plants, resulting from the union between two different genotypes, which interact together to create a final phenotype. A successful grafting requires the reconnection of the vascular system to allow the uptake and transport of water and nutrients from the roots to the above-ground portion of the future plant (Keller, 2010). The formation of a strong graft union implies a great reorganization at the transcriptomic level for both the scion and the rootstock portions. In fact, a massive reprogramming of gene expression enables the translation of several proteins involved in wound responses, cell wall synthesis, hormone signaling and secondary metabolism (Cookson *et al*., 2013; Melnyk *et al*., 2015). Grafting is perceived by the vine as a considerable trauma and it triggers the differential expression of a huge number of genes not only when performed, but it remains effective throughout the plant life (Cookson *et al*., 2014; Maré *et al*., 2016).

The complex rootstock-scion interplay occurs mainly through the exchange of small molecules, hormones and genetic materials across the graft junction. In fact, according to established scientific evidence, some macromolecule (mRNAs and mostly miRNAs) are mobile from the roots to the shoots *via* the phloem sap (Harada, 2010; Buhtz *et al*., 2010) and are the responsible elements for the influence given by the rootstock.

It is currently known that the rootstock can alter gene expression in the scion, especially in the presence of stress, diseases or limiting factors. Several transcriptome changes are related to the phenylpropanoid pathway genes, in particular to those responsible for stilbene and flavonoid biosynthesis, associated to pathogenesis response (Maré *et al.*, 2013; Corso *et al.*, 2015; Berdeja *et al*., 2015; Chitarra *et al*., 2017).

1.9 Aim of the research project

The research activity carried out is connected to the RINGO Project (Rootstock-scion INteraction in Grape: an Omics perspective), a bilateral project supported by the Ministries of Agriculture of Italy and Israel. The activity started in 2012, continued in the following vegetative seasons and was implemented and concluded within the Ph.D. course in Agricultural and Environmental Sciences – Cycle XXXII – University of Florence, with the theme "Study of Roostock-Scion Interactions in Grapevine".

This research work aimed to investigate how the rootstock influences gene expression and berry quality during some key phases of ripening. The project was set up in an experimental system of potted Pinot noir grapevines at CREA - Research Centre for Viticulture and Enology

(Arezzo – Italy), and included plants grafted on two rootstocks with opposite characteristics (1103 Paulsen and Mgt 101-14, respectively conferring high and low vigor to the shoot, and high and low tolerance to water stress) (Ollat *et al*., 2015; Palliotti *et al*. 2015), as well as not grafted plants.

During the first two growing seasons (2012 and 2013), the pot system was used to test the rootstock effects on the berry phenotype maintaining the same agronomic conditions and water supply for all the vines, using an "omic" approach. Transcriptome variations were evaluated on berry skins at two specific ripening points (veraison and maturity) by Next-Generation Sequencing, both on mRNA and small RNA fractions, comparing the expression profile of some miRNAs and genes correlated to secondary metabolism. Furthermore, chemical analyses on grape skins were performed to assess the accumulation and composition of phenolics.

Taking into account the deep-sequencing results previously obtained, during two following vegetative seasons comprised in the Ph.D. course (2017-2018) further experiments were set up using the same experimental pot system. The attention was focused on the role of some genes and miRNAs (already detected as differentially expressed between the two grafted root systems and not grafted plants) involved in secondary metabolism and its regulation during grape ripening, in conditions of optimal irrigation or water deficit. On the same samples, a parallel phenotyping activity was performed to verify the rootstocks influence vines' behavior and grape quality.

The research activity was conceived and designed to identify molecular determinants controlling rootstock-scion interaction, and therefore the rootstock-mediated effects on berry quality. The results could shed light on the role of rootstock during berry maturation,

even in case of water deficit, and give new insights for the genetic improvement of grapevine stress tolerance in a sustainable, non-transgenic way.

References

- Adams DO. Phenolics and Ripening in Grape Berries. American Journal of Enology and Viticulture. 2006;57:249-256.
- Amor S, Chalôns P, Aires V, Delmas D. Polyphenol Extracts from Red Wine and Grapevine: Potential Effects on Cancers. Diseases. 2018;6(4):106.
- Belli Kulhan J, Paim Pinto DL, Bertolini E, Fasoli M, Zenoni S, Tornielli GB, Pezzotti M, Meyers BC, Farina L, Pé EM, Mica E. miRVine: a micro RNA expression atlas of grapevine based on small RNA sequencing. BMC Genomics. 2015;16:393.
- Berdeja M, Nicolas P, Kappel C, Dai ZW, Hilbert G, Peccoux A, Lafontaine M, Ollat N, Gomès E, Delrot S. Water limitation and rootstock genotype interact to alter grape berry metabolism through transcriptome reprogramming. Horticulture Research. 2015;2:15012.
- Bogs J, Ebadi A, Mc David D, Robinson SP. Identification of the Flavonoid Hydroxylases from Grapevine and Their Regulation during Fruit Development. Plant Physiology. 2006;140:279-291.
- Boss PK, Davies C, Robinson SP. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol. 1996;111:1059-1066.
- Böttcher C, Harvey K, Forde CG, Boss PK, Davies C. Auxin treatment of pre-veraison grape (*Vitis vinifera* L.) berries both delays ripening and increases the synchronicity of sugar accumulation. Aust J Grape Wine Res. 2011;17:1-8.
- Buhtz A, Pieritz J, Springer F, Kehr J. Phloem small RNAs, nutrient stress responses and systemic mobility. BMC Plant Biol. 2010;10:64.
- Buono R, Vallariello G. Introduzione e diffusione della vite (*Vitis vinifera* L.) in Italia. Delpinoa. 2002;44:39-51.
- Cardell MF, Amengual A, Romero R. Future effects of climate change on the suitability of wine grape production across Europe. Reg Environ Change. 2019;1-12.
- Castellarin SD, Di Gaspero G, Marconi R, Nonis A, Peterlunger E, Paillard S, Adam-Blondon AF, Testolin R. Colour variation in red grapevines (Vitis vinifera L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. BMC Genomics. 2006;7:12.
- Cavallini E, Matus JT, Finezzo L, Zenoni S, Loyola R, Guzzo F, Schlechter R, Ageorges A, Arce-Johnson P, Tornielli GB. The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYBC2 repressors in grapevine. Plant Physiol. 2015;167:1448-1470.
- Chaves MM, Santos TP, Souza CR, Ortuno MF, Rodrigues ML, Lopes CM, Maroco JP, Pereira JS. Deficit irrigation in grapevine improves water-use efficiency while controlling vigour and production quality. Annals of Applied Biology. 2007;150: 237-252.
- Chitarra W, Perrone I, Avanzato CG, Minio A, Boccacci P, Santini D, Gilardi G, Siciliano I, Gullino ML, Delledonne M, Mannini F, Gambino G. Grapevine grafting: scion transcript profiling and defense-related metabolites induced by rootstocks. Frontiers in Plant Science. 2017;8:564.

- Chuck G, Candela H, Hake S. Big impact by small RNAs in plant development. Current Opinion in Plant Biology. 2009;12:81-86.
- Conde C, Silva P, Fontes N, Dias ACP, Tavares RM, Sousa MJ, Agasse A, Delrot S, Gerós H. Biochemical changes throughout grape berry development and fruit and wine quality. Food. 2007;1:1-22.
- Cookson SJ, Clemente Moreno MJ, Hevin C, Nyamba Mendome LZ, Delrot S, Trossat-Magnin C, Ollat N. Graft union formation in grapevine induces transcriptional changes related to cell wall modification, wounding, hormone signalling, and secondary metabolism. Journal of Experimental Botany. 2013;64:2997-3008.
- Cookson SJ, Clemente Moreno MJ, Hevin C, Nyamba Mendome LZ, Delrot S, Magnin N, Trossat-Magnin C, Ollat N. Heterografting with nonself rootstocks induces genes involved in stress responses at the graft interface when compared with autografted controls. Journal of Experimental Botany. 2014;65(9):2473-2481.
- Coombe BG, Mc Carthy MG. Dynamics of grape berry growth and physiology of ripening. Aust J Grape Wine Res. 2000;6:131-135.
- Coombe B. Ripening berries - a critical issue. Aus Vitic. 2001;5(2):28-34.
- Corso M, Bonghi C. Grapevine rootstock effects on abiotic stress tolerance. Plant Sci Today. 2014;1:108-113.
- Corso M, Vannozzi A, Maza E, Vitulo N, Meggio F, Pitacco A, Telatin A, D'Angelo M, Feltrin E, Negri AS, Prinsi B, Valle G, Ramina A, Bouzayen M, Bonghi C, Lucchin M. Comprehensive transcript profiling of two grapevine rootstock genotypes contrasting in drought susceptibility links the phenylpropanoid pathway to enhanced tolerance. J Exp Bot. 2015;66(19):5739-5752.
- Cosme F, Pinto T, Vilela A. Phenolic Compounds and Antioxidant Activity in Grape Juices: A Chemical and Sensory View. Beverages. 2018;4:22.
- Czemmel S, Heppel SC, Bogs J. R2R3-MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma. 2012;249(2):109-118.
- Dal Santo S, Zenoni S, Sandri M, De Lorenzis G, Magris G, De Paoli E, Di Gaspero G, Del Fabbro C, Morgante M, Brancadoro L, Grossi D, Fasoli M, Zuccolotto P, Tornielli GB, Pezzotti M. Grapevine field experiments reveal the contribution of genotype, the influence of environment and the effect of their interaction (G x E) on the berry transcriptome. Plant J. 2018;93:1143-1159.
- Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Mérillon JM, Hamdi S. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol. 2006;140:499-511.
- Deluc LG, Grimplet J, Wheatley MD, Tillett RL, Quilici DR, Osborne C, Schooley DA, Schlauch KA, Cushhman JC, Cramer GR. Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC Genomics. 2007;8:429.
- Di Filippo M, Vila H. Influence of different rootstocks on the vegetative and reproductive performance of *Vitis vinifera* L. Malbec under irrigated conditions. J Int Sci Vigne Vin. 2011;45(2):75-84.
- Downey MO, Harvey JS, Robinson SP. Synthesis of flavonols and expression of flavonol synthase genes in developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). Aust J Grape Wine Res. 2003;9:110-121.
- Duchêne E, Huard F, Dumas V, Schneider C, Merdinoglu D. The challenge of adapting grapevine varieties to climate change. Climate Research. 2010;41:193-204.
- Duchêne E, Huard F, Pieri P. Grapevine and climate change: what adaptation of plant material and training systems should we anticipate?. J Int Sci Vigne Vin - Special Laccave. 2014;61-69.
- Easterling DR, Meehl GA, Parmesan C, Changnon SA, Karl TR, Mearns LO. Climate extremes: observations, modeling, and impacts. Science. 2000;289(5487):2068-2074.

- Eitle MW, Cargnoni M, Acar A, Crespo Martinez S, Failla O, Kaul HP, Griessier M, Forneck A. Phylloxeration effects on the sink activity and assimilation rate in phylloxera (*Daktulosphaira vitifoliae* Fitch) infested grapevines (*Vitis* spp.). Acta Horticulturae. 2017;1188:291-298.
- Failla O. Botanica, morfologia e fisiologia. In: La Vite e il Vino. Coltura & Cultura - Bayer CropScience. Milano. 2007:2-45.
- Farkas O, Jakus J, Heberger K. Quantitative structure-antioxidant activity relationship of flavonoid compounds. Molecules. 2004;9:1079-1088.
- Fasoli M, Dal Santo S, Zenoni S, Tornielli GB, Farina L, Zamboni A, Porceddu A, Venturini L, Bicego M, Murino V, Ferrarini A, Delledonne M, Pezzotti M. The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. Plant Cell. 2012;24(9):3489-3505.
- Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Dal Santo S, Dokoozlian N, Pezzotti M, Tornielli GB. The timing and order of the molecular events that mark the onset of berry ripening in grapevine. Plant Physiol. 2018;178:1187-1206.
- Ferrandino A, Lovisolo C. Abiotic stress effects on grapevine (*Vitis vinifera* L.): Focus on abscisic acid-mediated consequences on secondary metabolism and berry quality. Environmental and Experimental Botany. 2014;103:138-147.
- Filippetti I, Movahed N, Allegro G, Valentini G, Pastore C, Colucci E, Intrieri C. Effect of post-veraison source limitation on the accumulation of sugar, anthocyanins and seed tannins in *Vitis vinifera* cv. Sangiovese berries. Australian Journal of Grape and Wine Research. 2015;21(1):90-100.
- Fournier-Level A, Hugueney P, Verriès C, This P, Ageorges A. Genetic mechanisms underlying the methylation level of anthocyanins in grape (*Vitis vinifera* L.). BMC Plant Biol. 2011;11:179-192.
- Fraga H, Malheiro C, Moutinho-Pereira J, Santos JA. An overview of climate change impacts on European viticulture. Food and Energy Security. 2012;1(2):94-110.
- Frioni T, Sabbatini P, Tombesi S, Norrie J, Poni S, Gatti M, Palliotti A. Effects of a biostimulant derived from the brown seaweed *Ascophyllum nodosum* on ripening dynamics and fruit quality of grapevines. Sci Hortic. 2018;232:97-106.
- Furiya T, Suzuki S, Sueta T, Takayanagi T. Molecular characterization of a bud sport of Pinot gris bearing white berries. Am J Enol Vitic. 2009;60:66-73.
- Galet P. Dictionnaire encylcopédique des cépages. Hachette, Paris. 2000.
- Gatti M, Garavani A, Krajecz K, Ughini V, Parisi MG, Frioni T, Poni S. Mechanical mid-shoot leaf removal on Ortrugo (*Vitis vinifera* L.) at pre- or mid-veraison alters fruit growth and maturation. Am J Enol Vitic. 2019;70(1):88-97.
- Gatto P, Vrhovsek U, Muth J, Segala C, Romualdi C, Fontana P, Pruefer D, Stefanini M, Moser C, Mattivi F, Velasco R. Ripening and genotype control stilbene accumulation in healthy grapes. J Agric Food Chem. 2008;56:11773-11785.
- Georgiev V, Ananga A, Tsolova V. Recent advances and uses of grape flavonoids as nutraceuticals. Nutrients. 2014;6:391-415.
- Goode J. Viticulture: fruity with a hint of drought. Nature. 2012;492(7429):351-353.
- Gomez C, Conejero G, Torregrosa L, Cheynier V, Terrier N, Ageorges A. *In vivo* grapevine anthocyanin transport involves vesicle-mediated trafficking and the contribution of anthoMATE transporters and GST. Plant J. 2011;67:960-970.
- Granett J, Omer AD, Pessereau P, Walker MA. Fungal infections of grapevines in phylloxera-infested vineyards. Vitis. 1998;37(1):39-42.
- Granett J, Walker MA, Kocsis L, Omer AD. Biology and management od grape phylloxera. Annu Rev Entomol. 2001;46:387-412.
- Guidoni S, Ferrandino A, Novello V. Effects of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. Am J Enol Vitic. 2008;1:22-29.

- Guillaumie S, Fouquet R, Kappel C, Camps C, Terrier N, Moncomble D, Dunlevy JD, Davies C, Boss PK, Delrot S. Transcriptional analysis of late ripening stages of grapevine berry. BMC Plant Biol. 2011;11:165.
- Hannah L, Roehrdanz PR, Ikegami M, Shepard AV, Shaw MR, Tabor G, Zhi L, Marquet PA, Hijmans RJ. Climate change, wine, and conservation. PNAS. 2013;110(17):6907-6912.
- Harada T. Grafting and RNA transport via phloem tissue in horticultural plants. Scientia Horticulturae. 2010;125:545-550.
- Harris JM, Kriedemann PE, Possingham JV. Anatomical aspects of grape berry development. Vitis. 1968;7:106-119.
- He F, Mu L, Yan GL, Liang NN, Pan QH, Wang J, Reeves MJ, Duan CQ. Biosynthesis of anthocyanins and their regulation in colored grapes. Molecules. 2010;15:9057-9091.
- Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot. 2011;62(8):2465-2483.
- IPCC - Intergovernmental Panel on Climate Change. An IPCC special report on the impacts of global warming of 1.5˚C above pre-industrial levels and related global greenhouse gas emission pathways. Geneva, Switzerland. 2018.
- Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, *et al*. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature. 2007;449:463-467.
- Jackson RS. Wine Science. Principles, Practice, Perception - Second edition, Academic Press. London, UK. 2000.
- Jeandet P, Delaunois B, Conreux A, Donnez D, Nuzzo V, Cordelier S, Clement C, Courot E. Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants. Bio Factors. 2010;36(5):331-341.
- Jeandet P, Clément C, Cordelier S. Regulation of resveratrol biosynthesis in grapevine: new approaches for disease resistance?. J Exp Bot. 2019;70:375-378.
- Jones GV, White MA, Cooper OR, Storchmann K. Climate change and global wine quality. Clim Change. 2005;73:319-343.
- Jones GV. Climate and terroir: impacts of climate variability and change on wine. Fine wine and terroir: the geoscience perspective. 2006;(9):1-14.
- Jones TH, Cullis BR, Clingelefer PR, Rühl EH. Effects of novel hybrid and traditional rootstocks on vigour and yield components of Shiraz grapevines. Australian Journal of Grape and Wine Research. 2009;15:284-292.
- Keller M. The science of grapevines. Anatomy and Physiology. Academic Press, Burlington. 2010.
- Keller M, Mills LJ, Harbertson JF. Rootstock effect on deficit-irrigated winegrapes in a dry climate: vigor, yield formation and fruit ripening. Am J Enol Vitic. 2012;63:29-39.
- Kennedy JA, Saucier C, Glories Y. Grape and wine phenolics: History and perspective. American Journal of Enology and Viticulture. 2006;57:239-248.
- Kobayashi S, Goto-Yamamoto N, Hirochika H. Retrotransposon-induced mutations in grape skin color. Science. 2004;304:982.
- Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, Gomès E, Li SH, Godoy F, Arce-Johnson P, Delrot S. Berry ripening: recently heard through the grapevine?. J Exp Bot. 2013;65:4543-4559.
- Kümpers BMC, Bishopp A. Plant grafting: making the right connections. Curr Biol. 2015;25(10):411-413.
- Lanari V, Lattanzi T, Borghesi L, Silvestroni O, Palliotti A. Post-veraison mechanical leaf removal delays berry ripening on Sangiovese and Montepulciano grapevines. Acta Hortic. 2013;978:327-333.
- Le Hénanff G, Profizi C, Courteaux B, Rabenoelina F, Gerard C, Clément C, Baillieul F, Cordelier S, Dhondt-Cordelier S. Grapevine NAC1 transcription factor as a convergent node in developmental

processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance. J Exp Bot. 2013;64:4877-4893.
- Lovisolo C, Perrone I, Carra A, Ferrandino A, Flexas J, Medrano H, Schubert A. Drought-induced changes in development and function of grapevine (*Vitis* spp.) organs and in their hydraulic and non-hydraulic interactions at the wholeplant level: a physiological and molecular update. Funct Plant Biol. 2010;37(2):98-116.
- Maré C, Aprile A, Roncaglia E, Tocci E, Corino LG, De Bellis G, Cattivelli L. Rootstock and soil induce transcriptome modulation of phenylpropanoid pathway in grape leaves. Journal of plants interactions. 2013;8(4):334-349.
- Maré C, Mica E, Cattivelli L. Molecular advances in rootstock-scion interaction in grapevine. Acta Horticulturae. 2016;1136:155-160.
- Marguerit E, Brendel O, Lebon E, van Leeuwen C, Ollat N. Rootstock control of scion transpiration and its acclimation to water deficit are controlled by different genes. New Phytol. 2012;194:416-429.
- Massonnet M, Fasoli M, Tornielli GB, Altieri M, Sandri M, Zuccolotto P, Paci P, Gardiman M, Zenoni S, Pezzotti M. Ripening transcriptomic program in red and white grapevine varieties correlates with berry skin anthocyanin accumulation. Plant Physiol. 2017;174:2376-2396.
- Mattivi F, Guzzon R, Vrhovsek U, Stefanini M, Velasco R. Metabolite profiling of Grape: Flavonols and Anthocyanins. J Agric Food Chem. 2006;54:7692-7702.
- Matus J, Aquea F, Arce-Johnson P. Analysis of the grape MYB R2R3 subfamily reveals expanded wine quality-related clades andconserved gene structure organization across *Vitis* and *Arabidopsis* genomes. BMC Plant Biol. 2008;8(1):83.
- Meggio F, Prinsi B, Negri AS, Di Lorenzo GS, Lucchini G, Pitacco P, Failla O, Scienza A, Cocucci C, Espen L. Biochemical and physiological responses of two grapevine rootstock genotypes to drought and salt treatments. Australian Journal of Grape and Wine Research. 2014;20:310-323.
- Melnyk CW, Schuster C, Leyser O, Meyerowitz EM. A developmental framework for graft formation and vascular reconnection in *Arabidopsis thaliana*. Curr Biol. 2015;25:1306-1318.
- Merli MC, Magnanini E, Gatti M, Pirez FJ, BuesaPueyo I, Intrigliolo DS, Poni S. Water stress improves whole-canopy water use efficiency and berry composition of cv: Sangiovese (*Vitis vinifera* L.) grapevines grafted on the new drought-tolerant rootstock M4. Agric Water Manage. 2016;169:106-114.
- Mica E, Piccolo V, Delledonne M, Ferrarini A, Pezzotti M, Casati C, Del Fabbro C, Valle G, Policriti A, Morgante M, Pesole G, Pé ME, Horner DS. Correction: High throughput approaches reveal splicing of primary microRNA transcripts and tissue specific expression of mature microRNAs in *Vitis vinifera*. BMC Genomics. 2010;11:109.
- Nesbitt A, Dorling S, Lovett A. A suitability model for viticulture in England and Wales: opportunities for investments, sector growth and increased climate resilience. Journal of land use science. 2018;13(4):414-438.
- Nuzzo V, Matthews MA. Response of fruit growth and ripening to crop level in dry-farmed Cabernet sauvignon on four rootstocks. Am J Enol Vitic. 2006;57(3):314-324.
- O.I.V. Statistical Report on World Vitiviniculture. http://www.oiv.int/public/medias/5479/oiv-en-bilan-2017.pdf. 2017.
- Ollat N, Diakou-Verdin P, Carde JP, Barrieu F, Gaudillère JP, Moing A. Grape berry development: a review. J Int Sci Vigne Vin. 2002;36:109-131.
- Ollat N, Peccoux A, Papura D, Esmenjaud D, Marguerit E, Tandonnet JP, Bordenave L, Cookson SJ, Barrieu F, Rossdeutsch L, Lecourt J, Lauvergeat V, Vivin P, Bert PF, Delrot S. Rootstocks as a component of adaptation to environment. In: Grapevine in a changing environment: a molecular and ecophysiological perspective. Eds Geros H, Chaves M, Medrano H, Delrot S. Wiley-Blackwell. Oxford, UK. 2015;68-108.

- Ollat N, Cookson SJ, Lauvergeat V, Marguerit E, Barrieu F, Gambetta G, Goutouly P, Tandonnet JP, Vivin P, Delrot S. Grapevines roots: the dark side. Acta Horticolturae. 2017;1188:213-226.
- Ordish G. The Great Wine Blight. Sidgwick & Jackson Limited. London. 1987.
- Pagliarani C, Vitali M, Ferrero M, Vitulo N, Incarbone M, Lovisolo C, Valle G, Schubert A. The accumulation of microRNAs differentially modulated by drought is affected by grafting in grapevine. Plant Physiol. 2017;173:2180-2195.
- Palliotti A, Tombesi S, Silvestroni O, Lanari V, Gatti M, Poni S. Changes in vineyard establishment and canopy management urged by earlier climate-related grape ripening: a review. Sci Hortic. 2014;178:43-54.
- Palliotti A, Poni S, Silvestroni O. La nuova viticoltura. Ed Agricole. Bologna, Italy. 2015.
- Pant BD, Buhtz A, Kehr J, Scheible WR. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. 2008;53:731-738.
- Pantaleo V, Vitali M, Boccacci P, Miozzi L, Cuozzo D, Chitarra W, Mannini F, Lovisolo C, Gambino G. Novel functional microRNAs from virus-free and infected *Vitis vinifera* plants under water stress. Nature - Scientific reports. 2016;6:20167.
- Pavlousek P. Evaluation of drought tolerance of new grapevine rootstock hybrids. J Environ Biol. 2011;32:543-549.
- Pelsy F. 2010. Molecular and cellular mechanisms of diversity within grapevine varieties. Heredity 104:331-340.
- Peterlunger E, Sivilotti P, Colussi V. Water stress increased polyphenolic quality in 'Merlot' grapes. Acta Hort. 2005;689:293-300.
- Poni S, Gatti M, Bernizzoni F, Civardi S, Bobeica N, Magnanini E, Palliotti A. Late leaf removal aimed at delaying ripening in cv. Sangiovese: Physiological assessment and vine performance. Australian Journal of Grape and Wine Research. 2013;19(3):378-387.
- Poni S, Gatti M, Palliotti A, Dai Z, Duchêne E, Truong TT, Ferrara G, Matarrese AMS, Gallotta A, Bellincontro A, Mencarelli F, Tombesi S. Grapevine quality: a multiple choice issue. Sci Hortic. 2018;234:445-462.
- Pouget R. Histoire de la Lutte Contre le Phylloxéra de la Vigne en France (1868-1895). Inst Natl Rech Agron. Paris. 1990.
- Powell S, Cooper PD, Forneck A. The biology, physiology and host-plant interactions of grape phylloxera *Daktulosphaira vitifoliae*. Adv Insect Physiol. 2013;45:159-218.
- Qiao Y, Zhang J, Zhang J, Ran A, Guo H, Wang D, Zhang J. Integrated RNA-seq and sRNA-seq analysis reveals miRNA effects on secondary metabolism in Solanum tuberosum L. Molecular genetics and genomics. 2017;292:37-52.
- Ribéreau-Gayon P. Recherches sur les anthocyannes des végétaux. Application au genre *Vitis*. Librairie general de l'enseignement. Paris. 1959.
- Rogers K, Chen X. Biogenesis, turnover and mode of actions of plant micro RNAs. Plant Cell. 2013;25:2383-2399.
- Romero P, Muñoz R, Del Amor F, Valdes E, Fernández JI, Martinez-Cutillas A. Regulated deficit irrigation based upon optimum water status improves phenolic composition in Monastrell grapes in wines. Agric Water Manag. 2013;121:85-101.
- Russell LM. *Daktulosphaira vitifoliae* (Fitch), the correct name of the grape phylloxera (Hemiptera: Homoptera: Phylloxeridae). J Wash Acad Sci. 1974;64:303-308.
- Rustioni L, Rocchi L, Guffanti E, Cola G, Failla O. Characterization of grape (*Vitis vinifera* L.) berry sunburn symptoms by reflectance. J Agric Food Chem. 2014;62:3043-3046.
- Sadras V, Moran M. Elevated temperature decouples anthocyanins and sugars in berries of Shiraz and Cabernet Franc. Aust J Grape Wine Res. 2012;18:115-122.
- Salazar-Parra C, Aguirreolea J, Sanchez-Diaz M, Irigoyen JJ, Morales F. Photosynthetic response of Tempranillo grapevine to climate change scenarios. Annals of Applied Biology. 2012;161:277-292.

- Salinari F, Giosué S, Tubiello FN, Rettori A, Rossi V, Spanna F, Rosenzweig C, Gullino ML. Downy mildew (*Plasmopara viticola*) epidemics on grapevine under climate change. Global Change Biology. 2006;12:1299-1307.
- Schultz H, Löhnertz O, Bettner W, Balo B, Linsenmeier A, Jähnisch A, Müller M, Gaubatz B, Varadi G. Is grape composition affected by current levels of UV-B radiation?. Vitis. 1998,37:191-192.
- Schultz H. Climate change and viticulture: a European perspective on climatology, carbon dioxide and UV-B effects. Aust J Grape Wine Res. 2000;6:2-12.
- Scienza A. Origine e Storia. In: La Vite e il Vino. Coltura & Cultura - Bayer CropScience. Milano. 2007:48-87.
- Seguin B, De Cortazar IG. Climate warming: consequences for viticulture and the notion of 'terroirs' in Europe. Acta Horticulturae. 2005;689:61-71.
- Serra I, Strever A, Myburgh PA, Deloire A. Review: the interaction between rootstocks and cultivars (*Vitis vinifera* L.) to enhance drought tolerance in grapevine. Aust J Grape Wine Res. 2014;20:1-14.
- Sharma D, Tiwari M, Pandey A, Bhatia C, Sharma A, Trivedi PK. MicroRNA858 is a potential regulator of phenylpropanoid pathway and plant development. Plant Physiology. 2016;171:944-959.
- Shimazaki M, Fujita K, Kobayashi H, Suzuki S. Pink-colored grape berry is the result of short insertion in intron of color regulatory gene. PLoS ONE. 2011;6:e21308.
- Skinkis P, Walton V, Kaiser C. Grape Phylloxera, Biology and Management in the Pacific Northwest. Oregon State University, Extension Service: Corvallis, OR, USA. 2009;1-23.
- Snopek L, Mlcek J, Sochorova L, Baron M, Hlavacova I, Jurikova T, Kizek R, Sedlackova E, Sochor J. Contribution of Red Wine Consumption to Human Health Protection. Molecules. 2018;23(7):1684.
- Solofoharivelo MC, Van der Walt AP, Stephan D, Burger JT, Murray SL. MicroRNAs in fruit trees: discovery, diversity and future research directions. Plant Biology. 2014;16:856-865.
- Teixeira A, Eiras-Dias J, Castellarin S, Geròs H. Berry Phenolics of Grapevine under Challenging Environments. Int J Mol Sci. 2013;14:18711-18739.
- Tomasi D, Jones GV, Giust M, Lovat L, Gaiotti F. Grapevine phenology and climate change: relationships and trends in the Veneto region of Italy for 1964-2009. American Journal of Enology and Viticulture. 2011;62(3):329-339.
- van Leeuwen C, Tregoat O, Choné X, Bois B, Pernet D, Gaud-illère JP. Vine water status is a key factor in grape ripening and vintage quality for red Bordeaux wine. How can it be assessed for vineyard management purposes?. Journal International des Sciences de la Vigne et du Vin. 2009;43:121-134.
- van Leeuwen C, Darriet P. The impact of Global Change on Viticulture and Wine Quality. Journal of Wine Economics. 2016;11(1):150-167.
- van Leeuwen C, Destrac-Irvine A. Modified grape composition under climate change conditions requires adaptations in the vineyard. Oeno ONE. 2017;51(2):147-154.
- Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, Pruss D *et al*. A high-quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One. 2007;12:e1326.
- Walker AR, Lee E, Bogs J, Mc David DAJ, Thomas MR, Robinson SP. White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. 2007;49:772-785.
- Wang C, Wang X, Kibet NK, Song C, Zhang C, Li X, Han J, Fang J. Deep sequencing of grapevine flower and berry short RNA library for discovery of novel microRNAs and validation of precise sequences of grapevine microRNAs deposited in miRBase. Physiologia Plantarum. 2011;143:64-81.
- Wang N, Zheng Y, Xin H, Fang L, Li S. Comprehensive analysis of NAC domain transcription factor gene family in *Vitis vinifera*. Plant Cell Rep. 2013;32:61-75.

- Warschefsky EJ, Klein LL, Frank MH, Chitwood DH, Londo JP, von Wettberg EJB, Miller AJ. Rootstocks: diversity, domestication, and impacts on shoot phenotypes. Trends Plant Sci. 2016;21:418-437.
- Waterhouse A. Wine Phenolics. Ann NY Acad Sci. 2002;957:21-36.
- Winkler AJ, Cook JA, Kliewer WM, Lider LA. General Viticulture, 2nd ed. University of California Press, Berkeley CA, USA. 1974.
- Yang Y, Mao L, Jittayasothorn Y, Kang Y, Jiao C, Fei Z, Zhong GY. Messenger RNA exchange between scions and rootstocks in grafted grapevines. BMC Genomics. 2015;15:251.
- Yu Y, Jia T, Chen X. The “how” and “where” of plant micro RNAs. New Phytol. 2017;216:1002-1017.
- Zhang B, Pan X, Cobb GP, Anderson TA. Plant microRNA: a small regulatory molecule with big impact. Dev Biol. 2006;289:3-16.
- Zhao D, Simon JE, Wu Q. A critical review on grape polyphenols for neuroprotection: Strategies to enhance bioefficacy. Critical Reviews in Food Science and Nutrition. 2019;7:1-29.
- Zavaglia C, Pecile M, Gardiman M, Bavaresco L. Production for propagating material of grapevine rootstocks in the EU and Italy. Acta Hortic. 2016;1136:57-62.

2. Research paper accepted for publication to

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Transcriptomic and biochemical investigations support the role of rootstock-scion interaction in grapevine berry quality

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Abstract

Background: In viticulture, rootstock genotype plays a critical role to improve scion physiology, berry quality and to adapt the grapevine (*Vitis vinifera* L.) to different environmental conditions. This study aimed at investigating the effect of two different rootstocks (1103 Paulsen - P - and Mgt 101-14 - M) in comparison with not grafted plants -

NGC - on the transcriptome (RNA-seq and small RNA-seq) and chemical composition of berry skin in *Pinot noir*, and exploring the influence of rootstock-scion interaction on grape quality. Berry samples were collected at veraison (T1) and maturation (T2), in two different years (2012 and 2013) to analyze the transcriptional and biochemical scenario regulating berry maturation.

Results: RNA-seq analysis highlighted that berry development was the strongest force influencing gene expression, then seasonal changes; the situation was slightly different concerning miRNAs whose expression was deeply conditioned by environmental factors. Data obtained comparing the M, P and NGC plants indicated that, at veraison, the transcriptomes of the berries skin are much less diverse among them than at maturity, suggesting a greater diversification at the transcriptional levels towards the end of the ripening process. In general, the genes identified as differentially expressed at T1 among the two grafted and the not grafted plants were linked to photosynthesis, putatively because of a delay in ripening of not grafted plants, while at T2 were mainly involved in the synthesis and transport of phenylpropanoids (e.g. flavonoids), cell wall loosening, and stress response, confirming that secondary metabolism is one of the pathways most subject to the modulation of gene expression during grape maturation. Grape phenotyping confirmed the transcriptomic results. The major differences in berry phenolic composition were detected between grafted and not grafted plants.

Conclusions: Transcriptomic and biochemical data demonstrate a stronger impact on berry maturation of 1103 Paulsen rootstock than Mgt 101-14 or not grafted plants on ripening processes related to the secondary metabolite accumulations in the skin tissue. Interestingly, the *MYB14* gene, involved in the feedback regulation of resveratrol

biosynthesis was up-regulated in 1103 Paulsen thus leading to a greater accumulation of stilbenes in mature berries.

Keywords:

Grapevine, *Vitis vinifera*, rootstock, RNA-seq, miRNA, transcriptomic, berry ripening, secondary metabolism.

Background

Grapevine (*Vitis vinifera*) is one of the oldest and most economically important fruit crops and is well adapted to grow in a wide range of climatic conditions. It is a perennial plant mainly cultivated for wine production or fresh consumption, and as juice or raisins. In recent years, following the complete sequencing of its genome [1,2], has become a model plant for non-climateric fruit research.

An almost essential necessity in *Vitis vinifera* cultivation is the use of grafting on rootstock derived from American *Vitis* species, resistant to phylloxera (*Daktulosphaira vitifoliae* Fitch, a soil-dwelling aphid), that spread in Europe at the end of the 19th century and devastated a large portion of cultivated vineyards. Since its introduction, grafting represents the most used form of biological control against a pest [3]. During the selection of the different rootstock genotypes, several additional traits have been fixed by breeders to provide to the scion higher tolerance to environmental adversities and abiotic stresses, such as soil limestone, high salinity, stagnation, drought, and frost [4,5].

The rootstock acts as an interface between the scion and the soil ecosystem [6] and its role on the scion's physiology is a highly debated subject in the literature. According to some

authors the rootstock modifies source-sink relations, influencing vine's performances [7,8,9], whereas other studies suggest that the rootstock has a minor effect on the physiological behavior of the scion, whose genotype is the main factor that concretely determines the shoot vegetative development and the characteristics of the grapes produced [10,11].

The molecular processes governing rootstock-scion interaction remain largely unknown and deepening this topic is rather difficult because the grafting implies huge structural changes and hydraulic integration [12] through the reprogramming of gene expression and protein translation. Moreover, grafting is perceived as a considerable trauma by the plant that triggers some mechanisms of plant defense and stress response [13], such as the expression of genes involved in cell wall synthesis, hormone signaling and secondary metabolism [14]. According to recent discoveries, besides small molecules (such as water, ions, amino acids, and hormones), also some macromolecules (such as mRNAs, proteins, but most of all miRNAs) are mobile through the plant across the graft union [14,15]. It is currently known that the rootstock can alter gene expression of the scion, especially in the presence of stress, disease or limiting factors. Several transcriptome changes are related to the phenylpropanoid pathway genes, like those responsible for stilbene and flavonoid biosynthesis [13,17,18,19,20,21,22].

Stilbenes and flavonoids are secondary metabolites, both derived from the same precursor, the amino acid phenylalanine. These two classes of phenolic compounds are synthesized through the phenylpropanoid pathway, along which they share some initial steps [23]. Stilbenes are naturally present in grapes [24], and their synthesis increases in case of pathogen attack or at the onset of abiotic stresses. The main stilbene in grapes and wines is resveratrol, a molecule that is gaining attention for its nutraceutical and pharmacologic

properties [25,26]. Flavonoids are the most effective antioxidants in grapes and are located mainly in berry skins and as tannins in seeds, in considerable concentrations [23,27]. The flavonoid composition of grapes (anthocyanins, flavonols, and simple flavan-3-ols or proanthocyanidins) is essential for wine quality, having a great influence on the organoleptic characteristics and the aging aptitude. The accumulation of phenolic compounds in grapes can vary widely, depending on environmental conditions, nutrient availability, water status, canopy thickness and cluster exposure [28,29,30] and, according to some authors, there is also a possible influence of the rootstock genotype [14,19,20,31].

The transcriptional or post-transcriptional regulation of the structural genes involved in the phenylpropanoid biosynthetic pathway is controlled in plants at different levels by several mechanisms, such as transcription factors, for example MYB [32] or RNA interference, where miRNAs are key players [33,34]. In grapevine, R2R3-MYBs are by far the most important class of MYB that controls flavonoid and stilbene accumulations during ripening, at the different spatial-temporal level [27].

miRNAs are small non-coding RNAs (19-24 nt long), coded by specific MIR genes, that perform Post-Transcriptional Gene Silencing (PTGS), through a sequence-specific down-regulation of gene expression [35,36,37]. In recent years, some studies have revealed the central role of miRNAs in grapevine metabolism and development [35,37,38,39,40,41]. Grafting can alter miRNAs abundance in the scion, as their movement through the vascular system is coupled with stress signals, causing changes in the final phenotype [15,42].

This research aimed at investigating how different rootstocks influence gene expression and phenotype in berry skins, where secondary metabolites accumulate, to find out their actual effects on the quality of the grapes produced.

The project was set up in an experimental system of potted *Pinot noir* grapevines, that included plants grafted on two rootstocks with opposite characteristics (1103 Paulsen, highly vigorous and highly tolerant to drought, and Mgt 101-14, less vigorous and susceptible to drought) as well as not grafted plants. During two growing seasons, the pot system was used to test the rootstock effect maintaining the same agronomic conditions and water supply for all the vines. Gene expression was evaluated on berry skins at two specific time points (veraison and maturity) by Next-Generation Sequencing analyses, both on mRNA and small RNA fractions, comparing the expression profile of some miRNAs and target transcripts correlated to the secondary metabolism.

Alongside the genetic analysis, chemical analyses on grape skins were performed to assess the accumulation and composition of phenolic compounds, at the onset of ripening (veraison) and maturity.

Results

RNA-seq and reads mapping to grapevine genome

Total RNA was extracted from the berry skins of grafted on 1103 Paulsen (P), Mgt 101-14 (M) and not grafted control (NGC) grapevines, sampled at veraison (T1) and maturity (T2). Three biological replicates were considered for each stage/root system, for a total of 18 samples per year. The RNAseq libraries were sequenced with the Illumina HiSeq2000 platform, producing on average 25 million of reads (Additional file 1). Quality filtered reads were mapped to the *Vitis vinifera* 12x.25 reference genome. Pearson correlation coefficients within biological replicates were always above 0,97 (Additional file 2), indicating a high level of reproducibility.

Hierarchical Clustering analysis with rlog transformed data was used to evaluate sample correlation. Figure 1 clearly shows that the berry developmental stage was the strongest driving force: samples at T1 (veraison) were separated from samples at T2 (maturity), independently from the year of sampling. Considering each developmental group, it was possible to distinguish 2012 and 2013 samples, showing that the year effect on gene expression is also clear, especially in T2. At maturity, not grafted plants (NGC) were grouped together, divided by the grafted ones, both in 2012 and 2013. PCA (Additional file 3a) revealed again a clear distinction between samples at T1, and samples at T2, independently from the sampling season (2012 or 2013). Nonetheless, PCA performed on all the 36 samples did not plainly separate the samples based on the year nor the genotype, while PCA performed on season 2012 alone separated NGC from grafted samples, both at veraison and maturity (Additional file 3b).

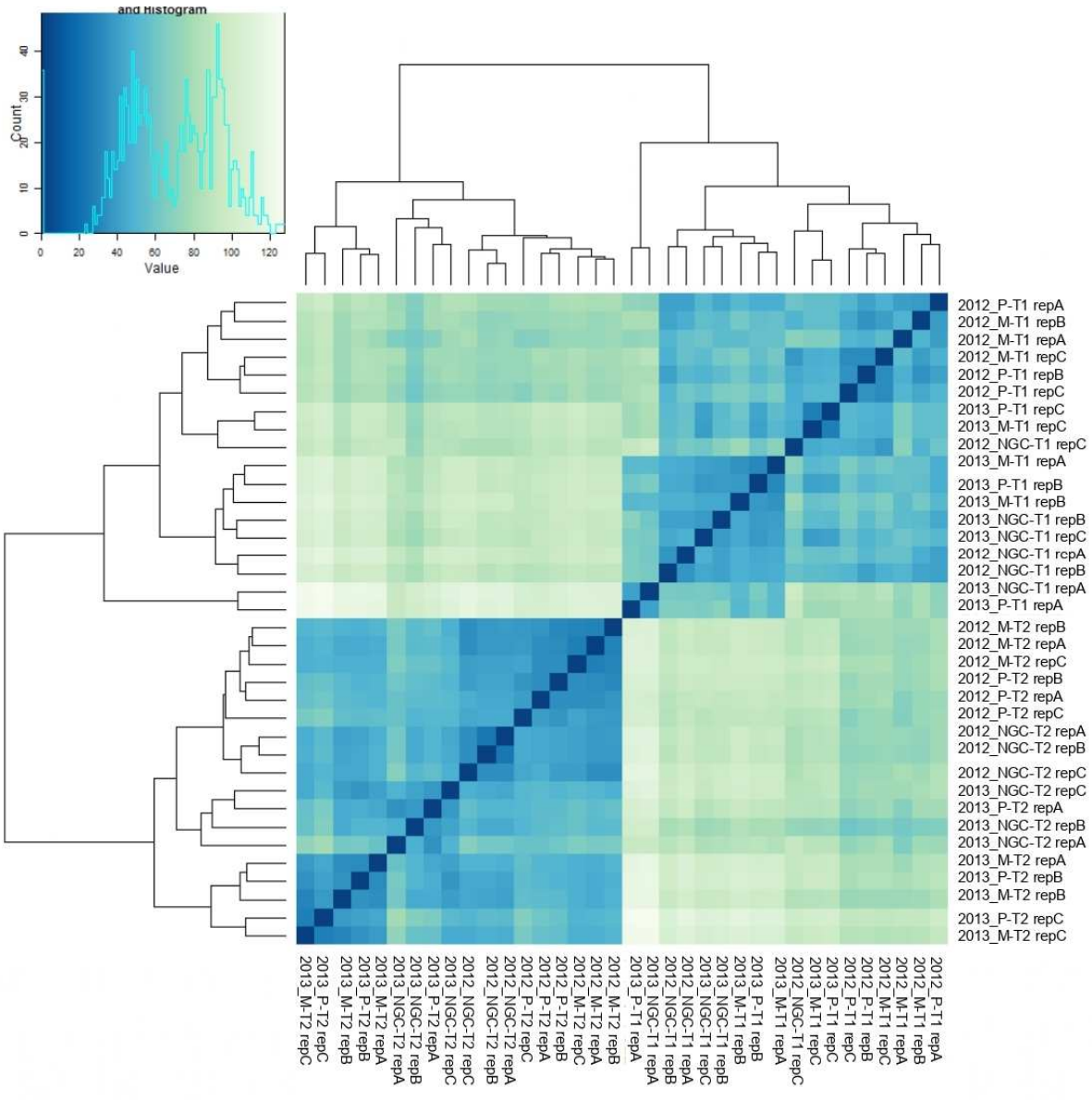

Fig.1: Hierarchical cluster analysis (HCA) of all samples sequenced in both seasons, by RNA-seq. Heatmaps reporting clustering of all samples were generated upon rlog-transformation of DESeq2-normalized expression data. Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; Replicate A, B, C. T1 = veraison; T2 = maturity.

Differential expression analyses

The R package DESeq2 was used to call differentially expressed genes (DEGs) for analyzing grapevine transcriptome data. Pairwise comparison between the grafted vines (M and P) and the not grafted (NGC), at the same developmental stage, were performed to evaluate the rootstock effects on berry skin transcriptome. DEGs were called setting a False Discovery Rate (FDR, Benjamini-Hochberg multiple test correction) threshold of 0.05.

The number of DEGs in the six comparisons, at the same developmental stage, for the year 2012 and 2013 (M-T1 *vs* NGC-T1; P-T1 *vs* NGC-T1; M-T1 *vs* P-T1; M-T2 *vs* NGC-T2; P-T2 *vs* NGC-T2; M-T2 *vs* P-T2) was highly variable ranging from zero to 2,247 (Fig.2 and Additional file 4). In general, for both years we can describe two major trends. First, comparing berry skins from plants with different rootstock/scion combinations we obtained low numbers of DEGs at T1, while at T2 the number of DEGs was higher, indicating stronger differences in the transcriptome towards the end of the ripening process. Second, M and P grafted plants were more similar to each other than to NGC plants, suggesting that the grafting per se has a significant impact on the transcriptome profile. Looking at differentially expressed genes and PCA performed on the 2012 or 2013 samples separately (Additional file 3b,c), it is clear that during 2013, the variability among samples is indeed not sufficient to perform additional analyses on DEGs, probably due to less stressful environmental conditions (for details see Methods and Additional file 5), even though the main trends of sample correlation and variability are confirmed in the two seasons. For this reason, subsequent analyses on DEGs were carried out only on the 2012 samples.

In 2012, among DEGs at T1, most genes are up-regulated in NGC when compared to M or P plants (77% and 71% respectively). At T2, the percentages are almost the opposite: 57% and

63% of DEGs are down-regulated in NGC compared to M or P plants, respectively. Comparing P with M, genes were mostly (66%) up-regulated in 1103 Paulsen. In general, the log_2 fold change was ranging between -4.8 and +3.2.

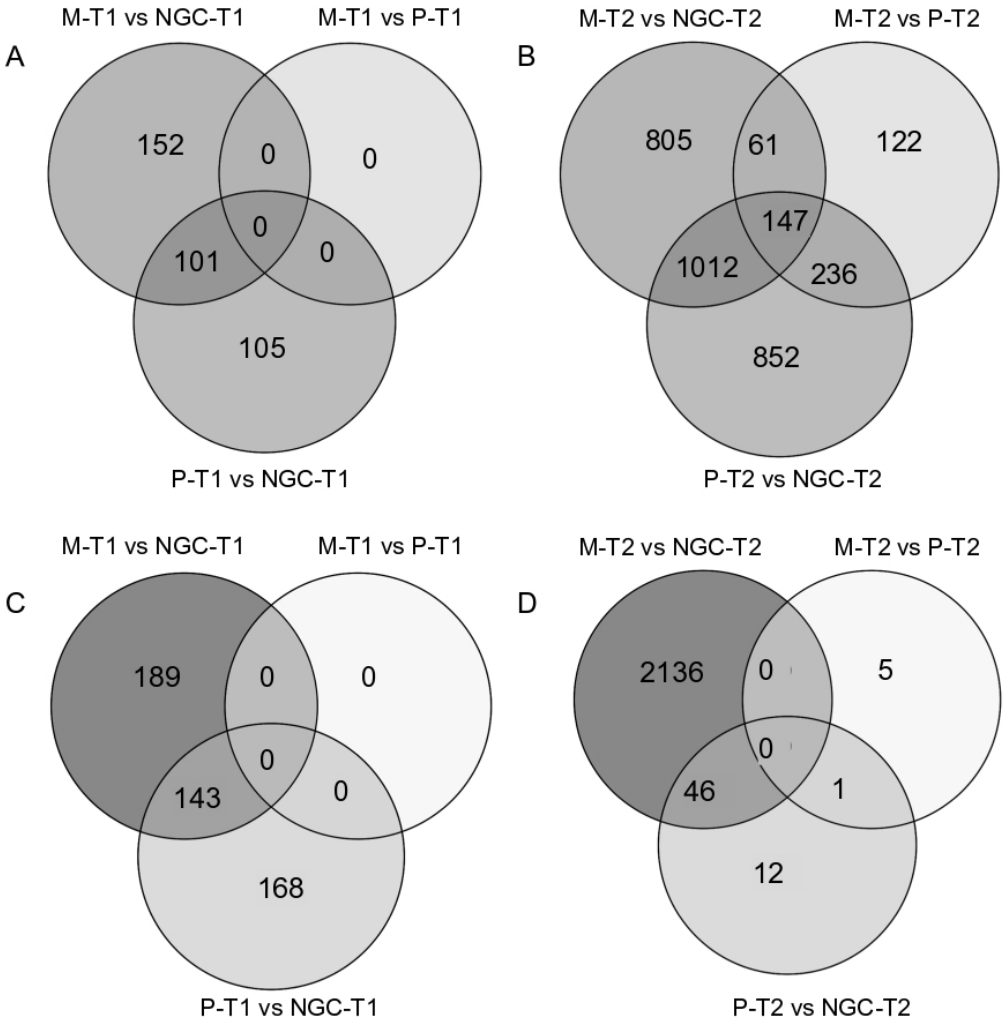

Fig.2: Venn diagrams of genes differentially expressed between the three root systems, at the same developmental stage, in 2012 (A, B) and 2013 season (C, D). DEGs were called setting the FDR threshold at 0.05. Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

To validate the RNA-seq data, we selected 10 genes (9 among the DEGs plus one not DE gene of special interest, namely *MYBC2-L3*) to be analyzed by qRT-PCR. All the genes chosen are specifically involved in key points of the phenylpropanoid pathway, as structural genes (*PAL - PHENYLALANINE AMMONIA LYASE*, 2 copies of *F3'H - FLAVONOID 3'-HYDROXYLASE*, *FLS - FLAVONOL SYNTHASE*, and *DFR - DIHYDROFLAVONOL-4-REDUCTASE*) or transcription factors belonging to MYB (*MYB14, MYB4R1,* and *MYBC2-L3*) and *NAC* (*NAC44*, and *NAC60*) gene families. qRT-PCR reactions were performed on M, P, and NGC samples, at two ripening points (T1 and T2) on three biological replicates, and the results were compared with the DESeq2 pairwise comparison outputs. The fold change values obtained by qRT-PCR confirmed those obtained by RNA-seq, validating the results and the technique (Tab.1 and Fig.3).

Tab.1: Comparision of transcripts fold changes detected by RNA-Seq and qRT-PCR for 9 selected genes in M (Mgt 101-14), P (Paulsen), NGC (not grafted control) at maturity (T2). For RNA-seq log2 fold change values were calculated by DESeq2; for qRT-PCR fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Gene Annotation	GeneID	Fold Change RNA-seq	Fold Change qRT-PCR	Fold Change RNA-seq	Fold Change qRT-PCR	Fold Change RNA-seq	Fold Change qRT-PCR
		M-T2	NGC-T2	M-T2	P-T2	P-T2	NGC-T2
NAC 60	VIT_208s0007g07670	-1,4	-1,0	-	-	-1,7	-1,3
NAC 44	VIT_206s0004g00020	-1,4	-1,4	0,7	0,7	-2,1	-2,1
F3'H	VIT_209s0002g01090	0,8	0,5	-	-	-	-
FLS	VIT_218s0001g03430	0,5	0,2	-	-	-	-
F3'H	VIT_217s0000g07200	-	-	-0,3	-0,4	-	-
MYB4R1	VIT_217s0000g02710	-	-	-1,1	-0,5	1,1	-0,1
MYB14	VIT_207s0005g03340	-	-	1,0	1,2	-0,9	-1,6
DFR	VIT_216s0039g02350	-	-	0,9	0,1	-0,7	-0,4
PAL	VIT_213s0019g04460	-	-	-	-	0,4	0,4

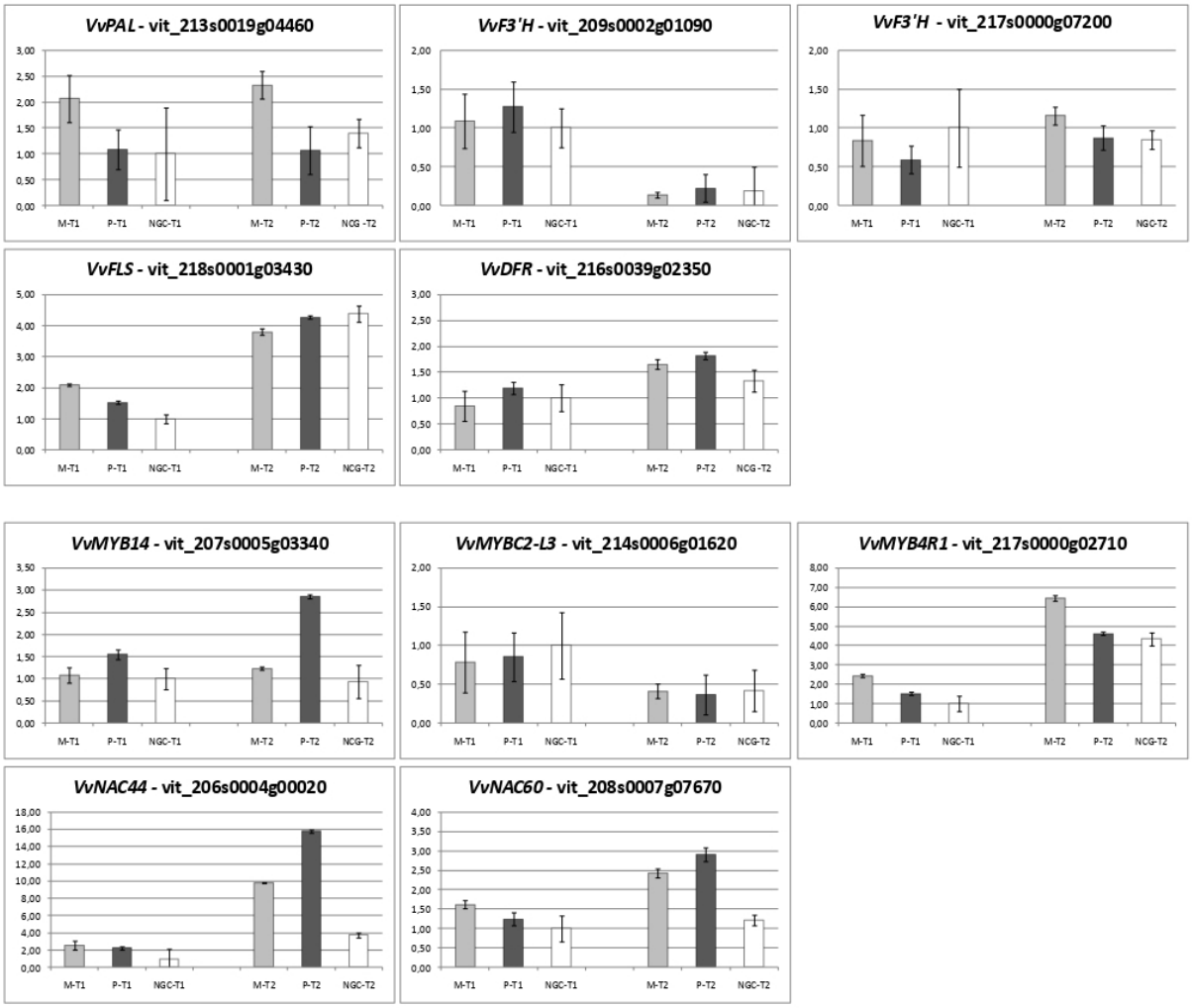

Fig.3: Expression profiles of the 10 selected genes coding for structural genes and transcription factors obtained by qRT-PCR, calculation from Ct value with the $2^{-\Delta\Delta Ct}$ method (the bars indicate the standard error). Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

Gene Ontology Enrichment

To gain insights into the main metabolic and signaling pathways involved in the considered comparisons, we conducted GO enrichment analysis.

Biological process enrichment analyses revealed that, at T1, there were 58 GO terms significantly over-represented in M *vs* NGC and 56 GO term in the comparison P *vs* NGC (Fig.4, and Fig.5). Of these, 42 were shared between the comparisons and were mainly

related to photosynthesis (such as GO:0015979, GO:0009773, GO:0009765, GO:0010207), plastid organization (GO:0009657), response to light stimulus (GO:0009637, GO:0010114, GO:0010218) and rRNA processing (GO:0006364). Besides, some GO terms were related to biotic and abiotic stress response (GO:0042742, GO:0009409, GO:0009607).

At T2, the number of GO terms enriched in the performed comparisons were more abundant than those at T1. We retrieved 203 and 168 GO terms (biological processes) when comparing M and P with NGC, respectively, and 49 GO terms comparing the M *vs* P plants. 34 GO terms were shared among the three comparisons, while 100 GO terms were shared only between M-T2 *vs* NGC-T2 and P-T2 *vs* NGC-T2. Among these, most were related to biotic or abiotic stresses response and hormonal regulation (i.e. GO:0010200, GO:0009611, GO:0009414, GO:0009651, GO:0042742, GO:0009867, GO:0009751), but more interestingly, we retrieved GO terms related to secondary metabolism/phenylpropanoid biosynthesis (GO:0009805, GO:0009809, GO:0009811, GO:0009813, GO:0043481) and cell wall biosynthesis (GO:0042546, GO:0009832, GO:0052546).

It is worth noting that 68 GO are specific to the M-T2 *vs* NGC-T2 comparison, and among them we recovered five biological processes referred to cell wall metabolism and modification (GO:0071555, GO:0042545, GO:0045490, GO:0046274, GO:0009831) and fruit ripening (GO:0009835), plus two related to cinnamic acid (GO:0009800) and alkaloid (GO:0009821) biosyntheses. No similar biological processes were present among GO terms specific to the M-T2 *vs* P-T2 comparison, while for the P-T2 *vs* NGC-T2 comparison we retrieved two GO related to pigment accumulation (GO:0046148, GO:0031537).

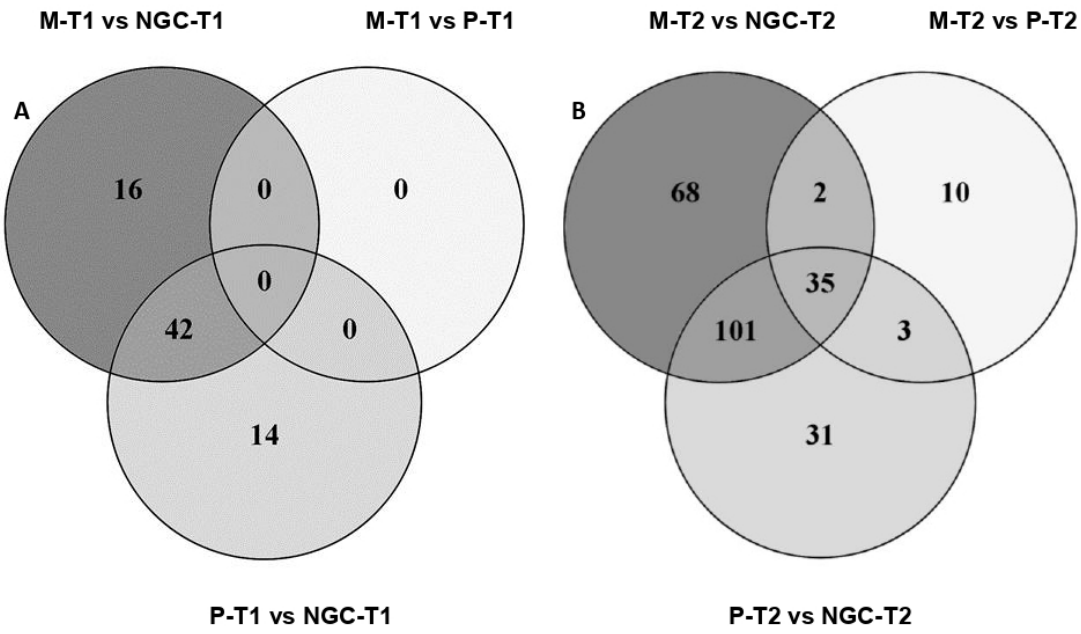

Fig.4: Venn diagrams of enriched GO terms (Biological Processes) in the three comparison considered at veraison - T1 (Panel A) and maturity - T2 (Panel B) in 2012 season. Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control.

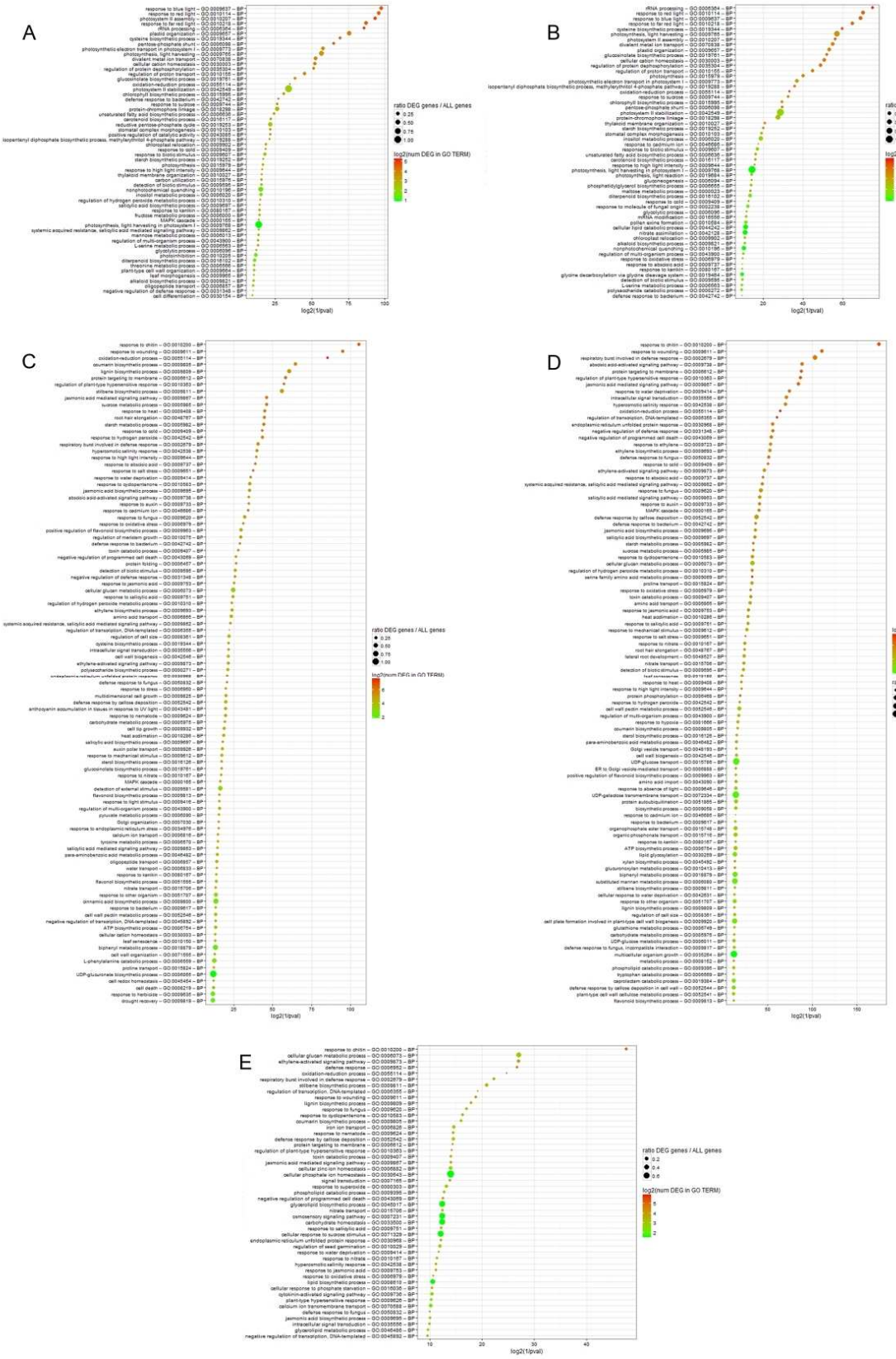
A
B
C
D
E
log2(1/pval)
ratio DEG genes / ALL genes
log2(num DEG in GO TERM)

Fig.5: GO enrichment for Biological Process (BP) domain in the comparison of the transcriptomes of grafted (M - Mgt 101-14 or P - 1103 Paulsen) and not grafted control (NGC) plants, at veraison (T1) or maturity (T2), in 2012 season. Panel A: GO enriched in the comparison M -T1 *vs* NGC – T1; Panel B: GO enriched in the comparison P-T1 *vs* NGC-T1; Panel C: GO enriched in the comparison M-T2 *vs* NGC-T2; Panel D: GO enriched in the comparison P-T2 *vs* NGC-T2; Panel E: GO enriched in the comparison M-T2 *vs* P-T2.
GO IDs and corresponding GO terms are as specified in the Y-axis. In order to place most significantly enriched GOs at the top on the Y-axis, GOs are sorted according to decreasing $\log_2(1/p\text{-value})$ on the X-axis. The absolute number of DEGs that matched the GO term ($\log_2$-transformed) is indicated by the color of each spot, whereas the size of each spot shows the ratio of DEGs versus all grapevine genes matching the same considered GO term.

MAPMAN analyses performed to evaluate metabolic pathways and cellular functions represented among differentially expressed genes confirmed the results obtained with Gene Ontology analyses (Fig.6). Most of the up-regulated genes at T1 in NGC plants were related to photosynthesis, secondary metabolism, transport, and protein synthesis and degradation. At T2, the number of modulated genes is, in general, higher than at T1 and the most represented classes are linked to secondary metabolism, lipids, and cell wall syntheses, with most of the genes up-regulated in P when compared with M and NGC. Also, transcription factors encoding genes and genes involved in protein degradation, modification, and signaling (receptor kinases and Ca^{2+} signalling) were modulated in T2 when comparing grafted and not grafted plants. Among transcription factors, the most represented families were MYB, bHLH, APETALA2/ERF, WRKY, Zinc-Finger, NAC, and some of them are well-known miRNA predicted targets (such as vvi-miR169d-CCAAT box binding factors, miR171e-GRAS, vvi-miR166a-HD-ZIP class III, vvi-miR858 and vvi-miR408-MYB, vvi-miR164-NAC). In detail, the P-T2 *vs* NGC-T2 comparison, showed the highest number of regulated TF, with 30 genes coding for MYB transcription factors (9 genes up-regulated in NGC and 21 in P) and 20 WRKY domain transcription factors up-regulated in P, except one.

When comparing directly the two grafted plants at T2, most of the genes belonging to secondary metabolism, transcription factors, protein synthesis/degradation, and signaling are more expressed in plants grafted on 1103 Paulsen (P) than those grafted on Mgt 101-14 (M).

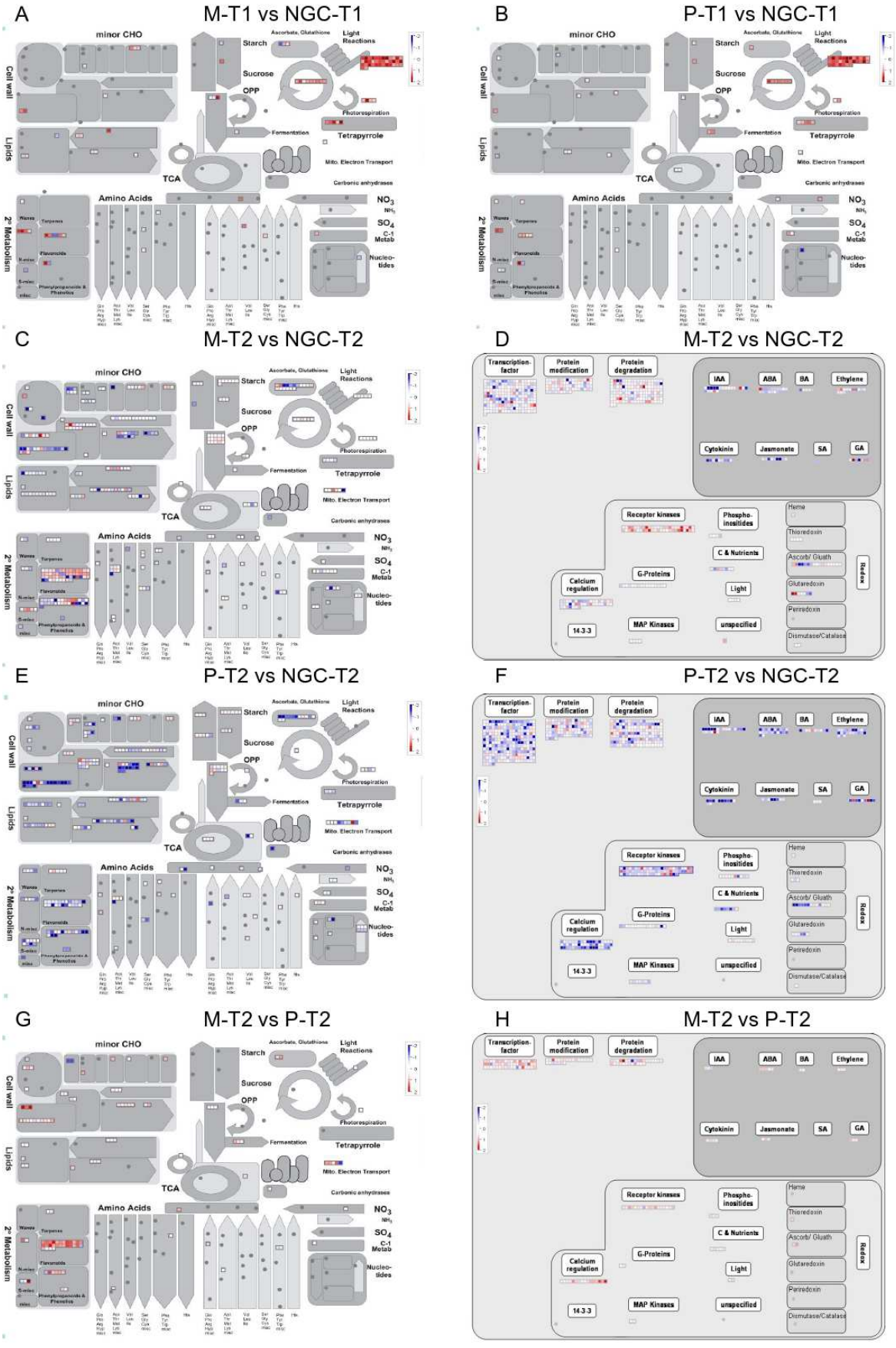
A
M-T1 vs NGC-T1
B
P-T1 vs NGC-T1
C
M-T2 vs NGC-T2
D
M-T2 vs NGC-T2
E
P-T2 vs NGC-T2
F
P-T2 vs NGC-T2
G
M-T2 vs P-T2
H
M-T2 vs P-T2
minor CHO
Starch
Sucrose
OPP
Fermentation
TCA
Ascorbate, Glutathione
Light Reactions
Photorespiration
Tetrapyrrole
Mito. Electron Transport
Carbonic anhydrases
Amino Acids
Cell wall
Lipids
2° Metabolism
Terpenes
Flavonoids
Phenylpropanoids & Phenolics
Nucleo-tides
C-1 Metab
Transcription-factor
Protein modification
Protein degradation
IAA
ABA
BA
Ethylene
Cytokinin
Jasmonate
SA
GA
Receptor kinases
Phospho-inositides
C & Nutrients
G-Proteins
Light
Calcium regulation
14-3-3
MAP Kinases
unspecified
Heme
Thioredoxin
Ascorb/ Gluath
Glutaredoxin
Peiredoxin
Dismutase/Catalase
Redox

Fig.6: Panel A, B, C, E, G: Differences in the expression of genes involved in the cellular metabolism (metabolism overview) in the comparison considered, visualized by MapMan. Each entity within a pathway is depicted by a color signal where red signifies genes with higher expression in the second sample compared to the first sample of the comparison (sample 1 *vs* sample 2), blue signifies genes with expression higher in the first sample of the comparison indicated on the graph. The intensity of the color indicates the level of expression. Scale bar displays log2 fold changes. Panel D, F, H: MapMan illustration depicting DEGs from the "Regulation" bins at maturity (T2). Log_2 fold changes are indicated as a gradient of blue (up-regulated in the first sample, as indicated on the graph) and red (up-regulated in the second sample, as indicated on the graph).

Small RNA Sequencing Statistics

We sequenced a total of 36 small RNA libraries, producing 226,104,103 raw redundant reads. After adapter trimming, we obtained 152,561,497 reads that were then reduced to 105,360,594 clean reads, ranging from 16 to 25 nt in length (Additional file 6).

Looking at size distribution of the libraries (Additional file 7) we observed distinct peaks at 21 and 24 nt, as expected for DICER derived products. The 21 nt peak is the highest in all libraries indicating a preponderance of miRNA-like molecules while when considering the number of unique, non-redundant reads, the 24 nt peak is the highest showing a large variety of the siRNA-like molecules. It is worth noting that the 24 nt peak is much higher in berries at veraison (M-T1, P-T1, NGC-T1) than in mature berries (M-T2, P-T2, NGC-T2).

miRNA identification

Clean and trimmed reads were used as input for miRNA identification and analyses, using CLC Bio Genomics Workbench software package. We performed a similarity search against miRNAs present in miRBase plus the user-defined dataset (see Materials). As a result, we identified 159 and 164 annotated MIR families, in 2012 and 2013 data respectively. All the 48 grapevine MIR families have been retrieved. Additionally, 98 and 107 precursors of the

137 in the user-defined grapevine miRNAs have been retrieved in sequencing data of 2012 and 2013, respectively.

Starting from the total reads mapping on the identified precursors, we performed PCA and Hierarchical Clustering analysis (Fig.7, and Fig.8) to monitor the quality of sample replicates and the overall similarity among samples evaluating sample clustering.

PCA performed on all the 36 samples together showed (Fig.7) a clear separation between samples of the two seasons, and among 2012 samples grafted and not grafted plants were clearly separated. Hierarchical Clustering (Fig.8) confirmed the distinction based first between season, then between grafted and not grafted plants and, in 2012, between T1 and T2.

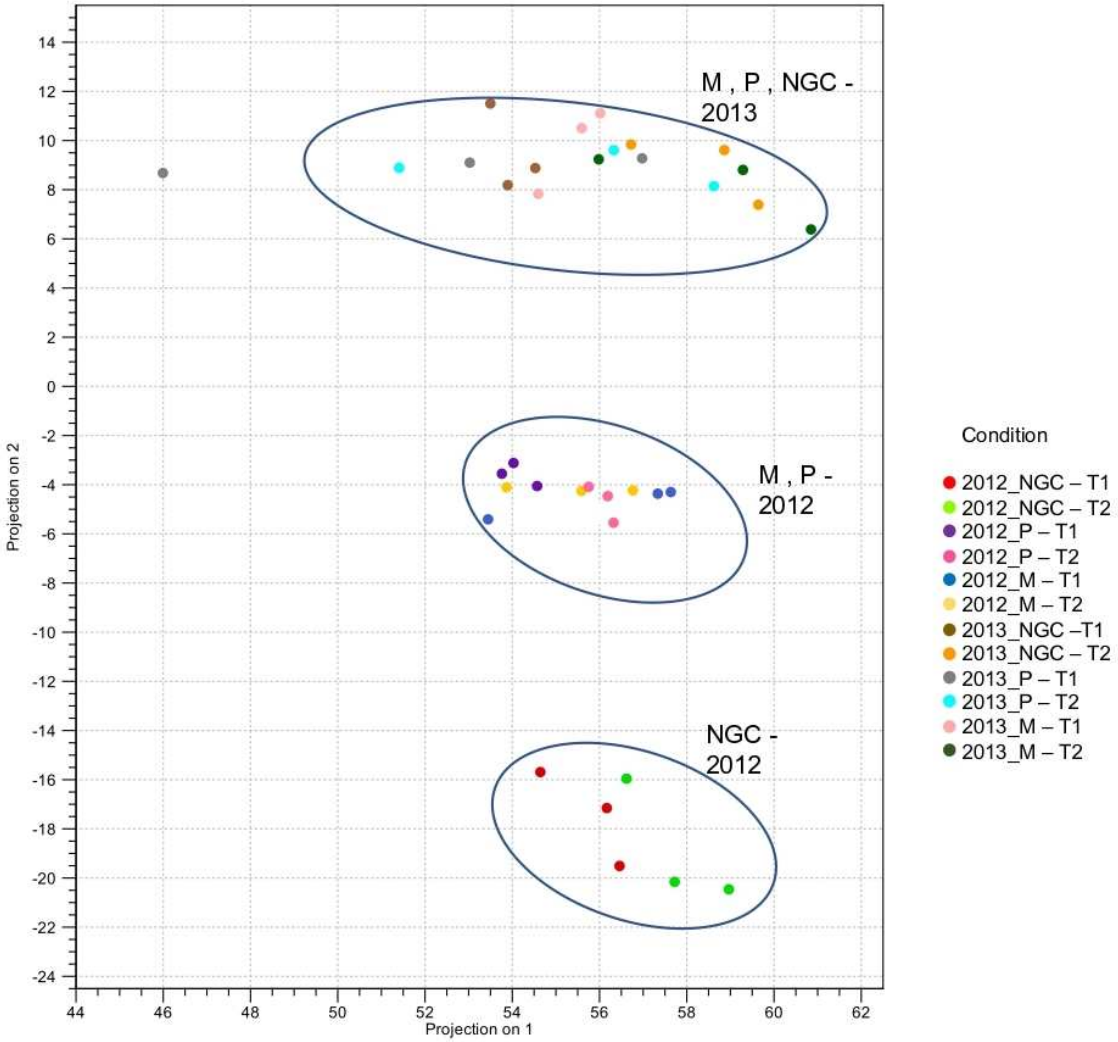

Fig.7: Principal component analysis (PCA) of all 36 samples in the small RNA-seq dataset (both seasons). The X-axis represents the first components and the Y-axis the second component. Each replicate of the same sample is associated with the same color and ovals indicate samples clustered together by year or by rootstock type (grafted or not grafted). Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

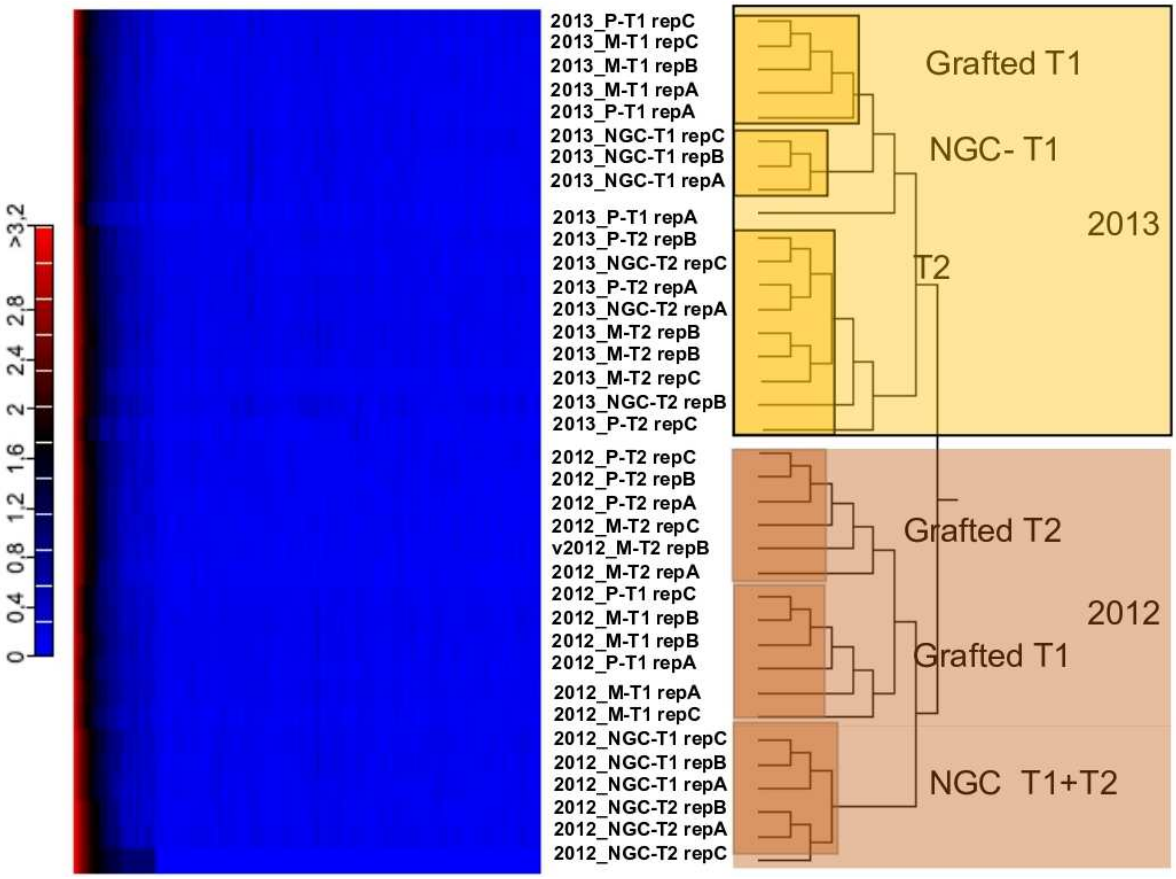

Fig.8: Hierarchical cluster analysis (HCA) of all 36 samples sequenced by small RNA-seq (both season). HC has been performed with normalized and log-transformed data, using 1-Pearson correlation as distance measure and Complete Linkage as linkage method.Sample names: Grafted: M = Mgt 101-14 and P = 1103 Paulsen; NGC = not grafted control; Replicate A, B, C. T1 = veraison; T2 = maturity.

Differential expression and target identification of DE miRNAs

Differential expression analysis of miRNA has been performed using CLC Bio software package, with all reads mapping to known plant miRNA precursors (miRBase Release 21 plus user-defined dataset - see Materials). We focused our attention, as for transcriptomic analyses, to the comparisons among grafted and not grafted plants, at the same developmental stage (M-T1 *vs* NGC-T1; P-T1 *vs* NGC-T1; M-T1 *vs* P-T1; M-T2 *vs* NGC-T2; P-T2 *vs* NGC-T2; M-T2 *vs* P-T2). The results of differential expression analyses (Fig.9, and Additional file 8) indicate that, as for mRNA-seq data, the less stressful 2013 season has drastically reduced the number of miRNAs differentially expressed. For this reason, only the 2012 season has been considered for further analyses. In general, the strongest differences arose when comparing grafted (either Mgt 101-14 or 1103 Paulsen) with not grafted control plants; most of the sequences were in common between the comparisons P-T1 *vs* NGC-T1 and M-T1 *vs* NGC-T1. Finally, almost all DE miRNAs are more expressed in not grafted plants than in grafted ones, at both veraison and maturity stages.

When comparing grafted plants directly (P-T1 *vs* M-T1 and P-T2 *vs* M-T2), only two or three sequences are differentially expressed at veraison and maturity, showing a minimal influence of different rootstocks on berry skin miRNAome.

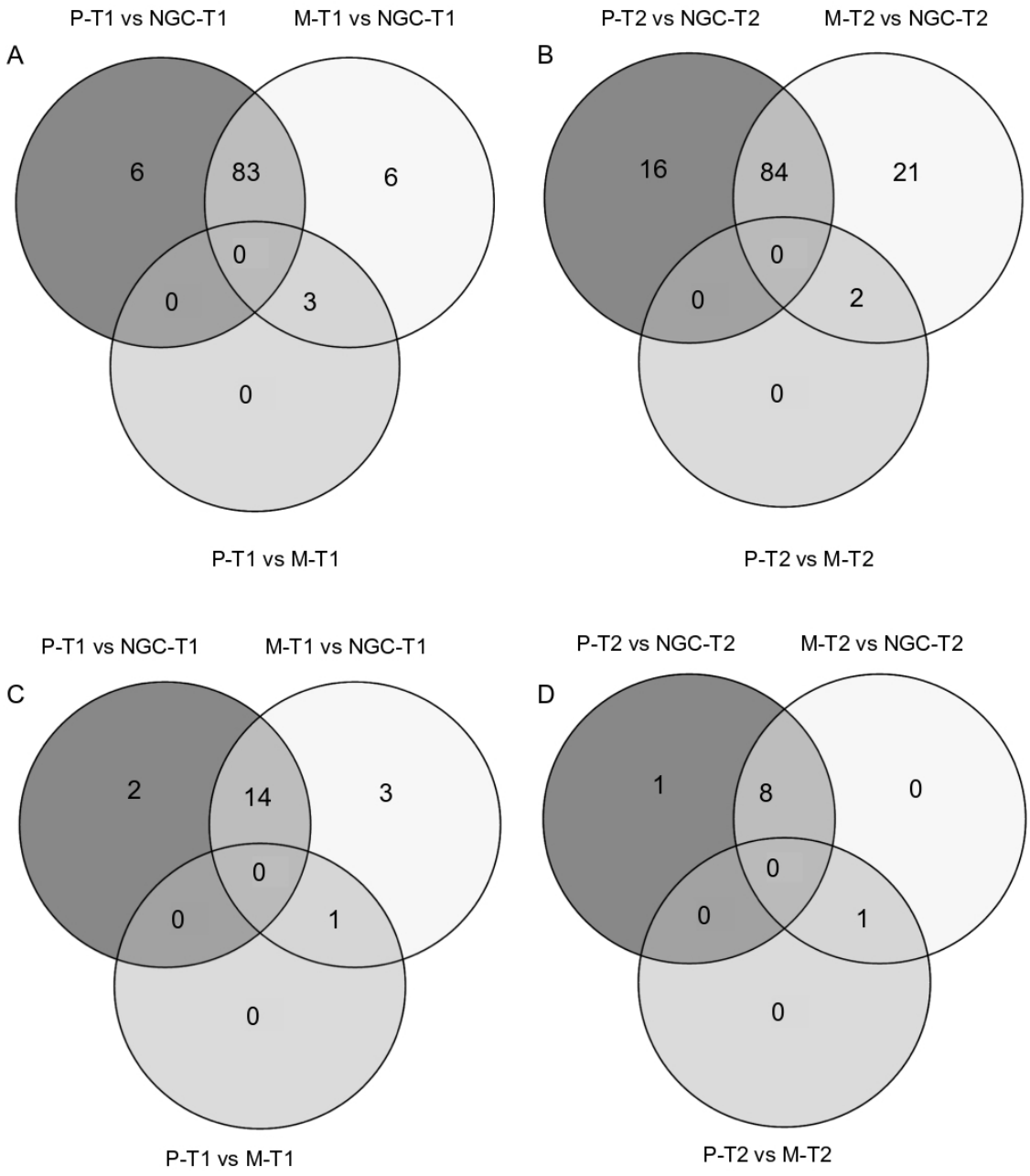

Fig.9: Venn diagram of differentially expressed miRNA sequences between the three root systems, at the same developmental stage, in 2012 (A, B) and 2013 season (C, D). DEGs were called setting the FDR threshold at 0.05. Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

On the whole, 98 and 123 sequences were differentially expressed at veraison and maturity, but it should be considered that more than one sequence may correspond to the same miRNA (isomiRNA), as indicated in Additional file 8. For each differentially expressed sequence, putative targets were identified *in silico* (Additional file 9).

Among the known miRNAs detected as differentially expressed between grafted and control plants, we found several miRNAs (such as miR482, miR535, miR396, miR3633, miR3632, miR3623, miR166 and miR159) regulating genes coding for disease resistance proteins and TMV-resistance protein, putatively reinforcing the evidence coming from mRNA-seq data showing the class of abiotic/biotic stress response gene as differentially expressed at both T1 and T2. Additionally, miR403, targeting AGO2 proteins (as validated in [43]), was found as differentially expressed. Apart from these, we focused our attention on those miRNAs with a putative function in secondary metabolism regulation, the main metabolic pathway that resulted as modulated between the samples after transcriptomic analyses.

To enrich the analyses, for each DE miRNA, we cross-checked the expression profile of putative predicted targets in our transcriptomic data, confirming, for some of them, the opposite expression trend (Additional file 10), and reinforcing the role of those miRNAs as negative regulators of expression.

For instance, miR156 expression was modulated between grafted and not grafted plants, at both veraison and maturity. miR156 was more expressed in control plants, and nearly not expressed in the grafted ones. In the comparison between 1103 Paulsen and not grafted plants at maturity, miR156 and its predicted target VIT_211s0065g00170 (*VvSPL10* - *Squamosa promoter-binding-like protein 12-like*) displayed an opposite expression profile. miR396 was more expressed in control plants than in grafted ones, both at veraison and

maturity, and, conversely, one of the GRF targeted by miR396 (VIT_215s0048g01740) was up-regulated in Mgt 101-14 and 1103 Paulsen at maturity.

As for miR858, not yet deposited in miRBase for grapevine, two sequence tags corresponding to ath-miR858a and ppe-miR858 were more expressed in not grafted plants than in grafted samples, at both veraison and maturity. These sequences are predicted to target 62 grapevine genes, of which 34 are R2R3-MYB transcription factors. Indeed, three *MYB* genes (VIT_218s0001g09850 - *MYB174*, VIT_218s0001g11170 - *MYB175* and VIT_205s0049g01010 - *MYB13*) were differentially expressed in the comparison M-T2 *vs* NGC-T2 and P-T2 *vs* NGC-T2, with an opposite profile compared to miR858, reinforcing target prediction.

Two grapevine specific miRNAs [41,44] were also differentially expressed and are predicted to target genes involved in the phenylpropanoid pathway. Grape_m-0721 expression was modulated between grafted and control plants at both developmental timings and showed higher expression in control plants. Both 5' and 3' mature sequences were DE and target, among others, anthocyanin 5-aromatic acyltransferase-like (VIT_213s0064g01165), a sterol oxidase (VIT_213s0019g02210) and an anthocyanidin 5,3-O-glucosyltransferase (VIT_216s0050g00240). Grape_m-1191 was DE at veraison between grafted and control plants. One of its targets is homologous to *TRANSPARENT TESTA 12* (*TT12* – accession VIT_212s0028g01160), responsible for flavonoids transport into the vacuole. For some of the miRNAs involved in the regulation of secondary metabolism previously described (miR858, Grape_m-1191, Grape_m-0721), and other entailed in stress response (miR395 and miR398), qRT-PCR was performed to validate the RNA-seq results (Additional file 11), but data were not confirmed, probably because of the presence of similar isomiR (one or two nt shorter)

more expressed, and with a similar expression level among all the samples, that primers were not able to distinguish.

Grape Phenolic Composition

In parallel with the transcriptomic investigations, chemical analyses were carried out by HPLC to assess the concentrations of phenolic compounds in berry skins, as these molecules play a determinant role for wine quality. The PCA (in Fig.10) was computed with the data of the chemical compounds from the 36 samples (M, P, NGC), in the two ripening points (T1 and T2), in both seasons considered (2012 and 2013).

The samples were more homogeneous at veraison, and they gather together on the right side of PC1 (group A), except for M-T1-12. In 2012, at T1, the grapes from the plants grafted on Mgt 101-14 likely had a higher degree of ripeness, and for this reason, resulted quite dissimilar in the graph. At T2, the samples were separated between 2012 (group B) and 2013 (group C). As previously seen, the climatic differences between the two years had a strong influence on the accumulation of secondary metabolites in berry skins at maturity.

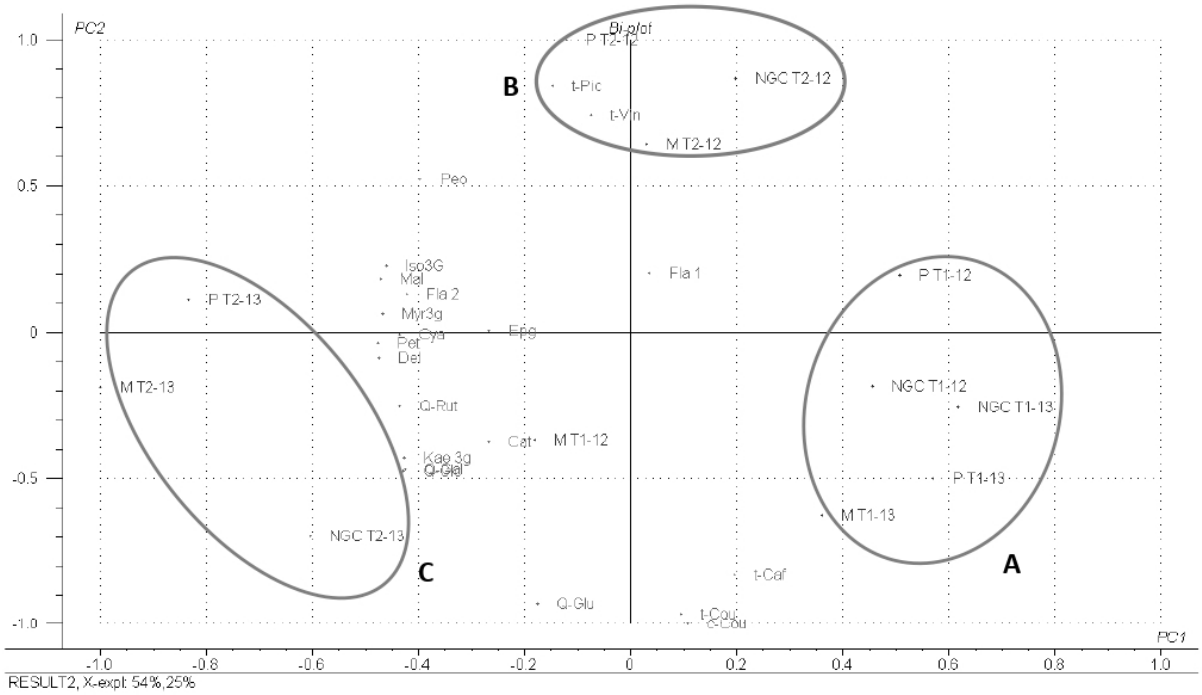

Fig.10: Principal Component Analysis (PCA) of the grape samples based on their chemical composition. Acronyms: EpC = Epigallocatechin; Cat = (+)-catechin; t-Caf = trans-caftaric acid; c-Cou = cis-coutaric acid; t-Cou = trans-coutaric acid; Fla 1 = unknown flavanol 1; Fla 2 = unknown flavanol 2; Myr3g = myricetin-3-O-glucoside; Q-Rut = quercetin-3-O-rutinoside; Q-Gal = quercetin-3-O-galactoside; Q-Glc = quercetin-3-O-glucuronide; Q-Glu = quercetin-3-O-glucoside; Kae3g = kaempferol-3-O-glucoside; Iso3G = isorhamnetin-3-O-glucoside; Del = delfinin; Cya = cyanin; Pet = petunin; Peo = peonin; Mal = malvin; t-Pic = trans-piceid; t-Vin = trans-ε-viniferin. Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity; 12 = 2012; 13 = 2013.

The PCA obtained with 2012 data (Fig.11), considering both phenolic compounds and gene expressions (qRT-PCR) gave interesting results. Again, the samples were separated in the two maturation stages. At T1 (right side of the graph), the major difference emerged between M and the other plants (P and NGC), which, as already said, was probably due to a different ripening degree at the sampling. In general, and according to the literature [23] at veraison (T1), flavonols, catechins, and hydroxycinnamoyl tartaric acids were the metabolites with the highest concentrations (data not shown). At T2 (left side of the graph),

the grafted plants were closer and more similar, compared to NGC, and the most present chemical compounds were the anthocyanins, with the prevalence of malvidin-3-glucoside and peonidin-3-glucoside (data not shown), as typical of *Pinot noir* grapes [45].

Considering gene positions in the PCA (Fig.11), interestingly, the genes more expressed at veraison were the early-acting copy of *F3'H* (VIT_209s0002g01090) and *MYBC2-L3* (VIT_214s0006g01620), a TF acting as repressor of anthocyanin synthesis in grapes. At maturity, *MYB14* (VIT_207s0005g03340), *MYB4R1* (VIT_217s0000g02710) and *NAC44* (VIT_206s0004g00020) genes are close to some stilbenes (*trans*-piceid and *trans*-ε-viniferin), confirming their key role in the biosynthesis of this class of compounds. The *PAL* (VIT_213s0019g04460) and *F3'H* (VIT_217s0000g07200) genes, being in the central part of the graph, had a similar expression level both at T1 and T2, reflecting a constitutive activity during berry maturation. Finally, the structural genes *DFR* (VIT_216s0039g02350), and *FLS* (VIT_218s0001g03430) and even *NAC60* (VIT_208s0007g07670), a TF involved in grape ripening and stress response, were more expressed at T2.

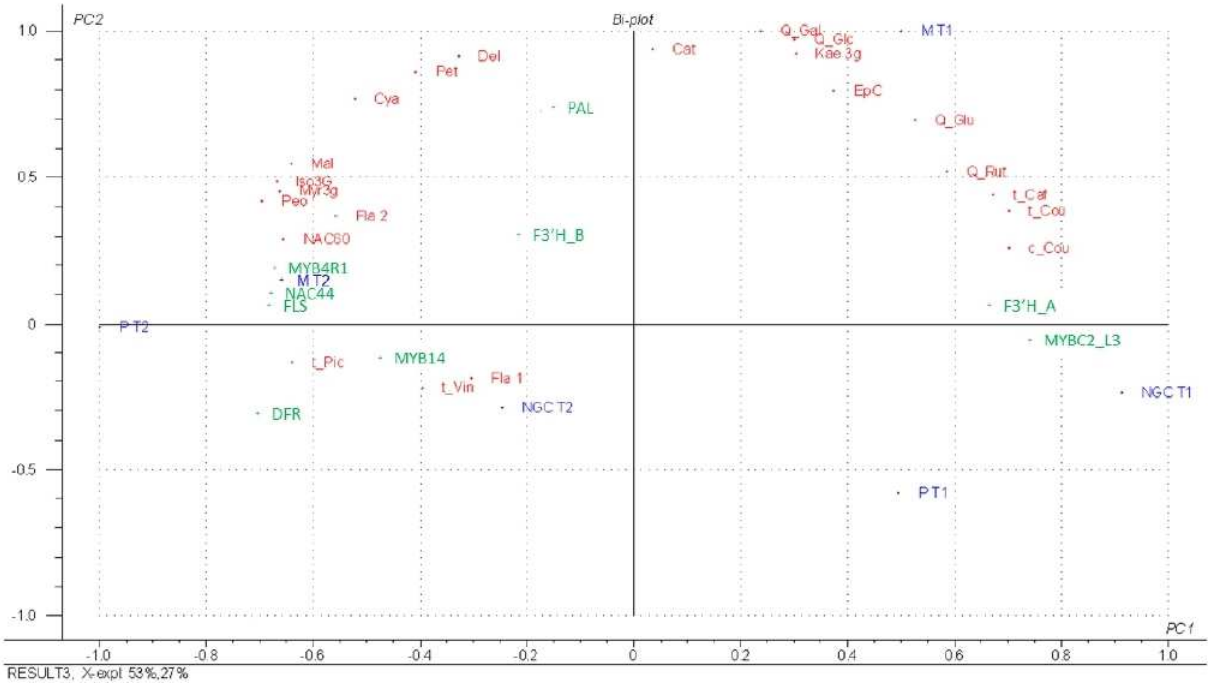

Fig.11: Principal Component Analysis (PCA) of 2012 samples based on both qRT-PCR results and chemical analyses. Acronyms of phenolic compounds (in red): EpC = Epigallocatechin; Cat = (+)-catechin; t-Caf = trans-caftaric acid; c-Cou = cis-coutaric acid; t-Cou = trans-coutaric acid; Fla 1 = unknown flavanol 1; Fla 2 = unknown flavanol 2; Myr3g = myricetin-3-O-glucoside; Q-Rut = quercetin-3-O-rutinoside; Q-Gal = quercetin-3-O-galactoside; Q-Glc = quercetin-3-O-glucuronide; Q-Glu = quercetin-3-O-glucoside; Kae3g = kaempferol-3-O-glucoside; Iso3G = isorhamnetin-3-O-glucoside; Del = delfinin; Cya = cyanin; Pet = petunin; Peo = peonin; Mal = malvin; t-Pic = trans-piceid; t-Vin = trans-ε-viniferin. List of genes (in green): PAL (VIT_213s0019g04460), F3'H_A (VIT_209s0002g01090), F3'H_B (VIT_217s0000g07200), DFR (VIT_216s0039g02350), FLS (VIT_218s0001g03430), MYBC2-L3 (VIT_214s0006g01620), MYB14 (VIT_207s0005g03340), MYB4R1 (VIT_217s0000g02710), NAC44 (VIT_206s0004g00020), NAC60 (VIT_208s0007g07670). Sample names (in blue): M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

Discussion

The present research on *Vitis vinifera* was conceived to get information about the influence of a rootstock on the final grape quality. Since it is practically mandatory to use a resistant rootstock to contrast phylloxera in most of the wine districts worldwide, the results obtained may be of interest, since they suggest an impact of rootstock on metabolite accumulation in the berries through modulation of gene expression. The work was carried out using an experimental pot system that was specifically designed to have a series of advantages over open field trials. It allowed to control many variables (for example irrigation, fertilization, pest control, environmental monitoring), with an adequate number of replicates and to obtain statistically significant results, although simulating the real conditions of a vineyard. The grapevines were grown in pots containing a known volume of soil with the same texture and characteristics, that was collected in a vineyard plot subjected to zoning, located in the Chianti Classico district (Tuscany - Italy). All the agronomical practices and the irrigation level were under control. The choice of the cultivar *Pinot noir* and clone ENTAV115 was accurate, considering that the genome sequence is of this cultivar is available [1,2], an important aspect, given the great varietal diversity within the *Vitis vinifera* species [46,47]. Moreover, the use of pots made it possible to insert not grafted plants as a control, that would not be feasible in a real vineyard, due to the looming presence of phylloxera.

Grafting influence is well perceivable at maturity when the developmental plan is already established

The analyses carried out allowed to perform an in-depth comparison of the transcriptomic scenario in developing berries between the grapevines grown on the three different root

systems. The characterization of the entire transcriptome of both mRNA and small RNA molecules in two crucial time points of ripening (veraison - T1, and maturation - T2) provided a global view of all the molecular processes occurring in berry skins and it contributed to explaining the possible effects given by the rootstock. The elaboration of deep sequencing results described the differential expression of genes and miRNAs between Mgt 101-14 (M), 1103 Paulsen (P), and not grafted plants (NGC).

According to Hierarchical Clustering and Principal Component analyses on the transcriptomic data (Fig.1, and Additional file 3a), the pivotal effect leading gene expression was the berry developmental program, while the year effect drove the differentiation of samples regardless of the rootstock genotype. On the other hand, referring to the analysis of miRNAome (Fig.7, and Fig.8), the samples were primarily divided by the year effect, suggesting that environmental signals strongly influenced miRNA expression [44,48,49].

Considering both the 2012 and 2013 results, the most noticeable differences regarding the differentially expressed genes and miRNAs emerged at maturity (T2), while less transcriptomic changes were detected at veraison (T1) (Fig.2, and Fig.9). Moreover, when comparing the samples at T2, the highest dissimilarity was detected between the grafted plants (P and M) and the not grafted control (NGC), with a similar trend in both years. Minor fluctuations in gene or miRNA expressions were highlighted comparing M and P. In general, among the two vegetative seasons considered, 2012 shows the most significant data, while in 2013, all samples had a more homogeneous behavior in terms of gene/miRNA expressions. It is important to note that the 2012 and 2013 summer periods were characterized by very different climatic conditions (Additional file 5), and this aspect certainly had an influence. As it happens in vineyard trials [50,51], even using a controlled

experimental system in open field, the environmental conditions have strong effects on gene expression. According to the weather data described in Methods, 2012 was considerably warmer than 2013, and there were strong temperature differences during grape maturation, particularly in the period between the two sampling points (T1 and T2).

It is well known that grapevine physiology and berry ripening are highly influenced by air temperature along the growing season [52] and evapotranspiration, that drives grapevine water status, increases in warmer climates enhancing the plants' water demand [53]. Moderate stress conditions (like those recorded in 2012) can be beneficial, favoring an optimal maturation and stimulating the accumulation of secondary metabolites in red grape varieties without significantly compromising yield [23,54,55]. In 2012, which was certainly more stressful then 2013 for the plants, greater differences emerged among the grape samples and the influence of the rootstock on the metabolic responses was more prominent. For the reasons described above, only 2012 results were considered for further analyses on DEGs.

Differentially expressed genes are mainly involved in secondary metabolism and its regulation

The differentially expressed genes and the enrichment analysis of their ontologies identified the major functional categories represented at T1 (M *vs* NGC and P *vs* NGC) as those related to photosynthesis, plastid organization, response to light stimulus, and only to a lesser extent rRNA processing, response to biotic and abiotic stress, and secondary metabolism (Fig.5a,b, and Fig.6a,b). Hence, at veraison, when berry skin color was shifting from green to purple, the photosynthetic activity decreased at a different rate between grafted and not

grafted plants: NGC showed a higher expression of photosynthetic genes when compared to M and P, suggesting a residual photosynthetic activity. At T2, with a number of DEGs higher than in T1, the main biological processes differentially regulated were hormonal changes, and the response to biotic or abiotic stresses, with most ontologies related to secondary metabolism, phenylpropanoid pathway, and cell wall biosynthesis (Fig.5c,d,e, and Fig.6c-h). In general, the genes related to these classes were more expressed in P than in NGC or M; when comparing NGC and M plants, the genes of secondary metabolism class were mostly up-regulated in NGC, while genes of other ontologies were predominantly up-regulated in M (Fig.6c-h). Flavonoid, anthocyanin, stilbene, and lignin biosynthesis together with cell wall modifications via pectin and cellulose metabolism are strictly related to berry ripening and softening and, hence, to grape quality. Taken together, these data show that berries grown on the 1103 Paulsen rootstock have a higher expression, at T2, of genes involved in secondary metabolism suggesting a stronger influence of 1103 Paulsen rootstock than Mgt 101-14 or not grafted control plants on ripening processes related to secondary metabolite accumulations in berries.

In addition, by comparing the grafted plants with NGC at T2, many genes coding for transcription factors and genes involved in protein degradation, modification, and signalling were clearly differentially regulated (Fig.6 c-h). The TFs differentially expressed belonged mainly to MYB, bHLH, and WRKY families, with MYB and bHLH as the key regulators of the phenylpropanoid pathway [56,57].

Forty-one genes belonging to MYB/bHLH families were mainly up-regulated in P-T2 compared to NGC-T2, reinforcing the hypothesis of a strong modulation effect coming from this rootstock genotype on the berry phenolic contents in the scion.

From the additional analyses carried out by qRT-PCR (Fig.3) among the structural genes that gave the most noteworthy confirming results, we can mention *VvDFR* (VIT_216s0039g02350). This gene codes for a DIHYDROFLAVONOL-4-REDUCTASE, that carries out the first step of anthocyanidins synthesis, converting dihydroflavonols into leucoanthocyanidins. The substrate of DFR is common with FLS (FLAVONOL SYNTHASE), and between the two enzymes, there is a dichotomy for the alternative synthesis of anthocyanidins and proanthocyanidins or flavonols [58]. According to the RNA-seq and the additional qRT-PCR data, *DFR* is not DE at veraison, while at maturity it is more expressed in P than in M and NGC. A higher expression of *DFR* at T2 in 1103 Paulsen suggests that this rootstock conferred a greater aptitude to the synthesis of anthocyanins in berry skins towards maturity.

Both copies of the *VvF3'H* genes (VIT_209s0002g01090 and VIT_217s0000g07200) present in the grapevine genome [59] were detected as DE. The enzyme encoded by these genes is the FLAVONOID 3'-HYDROXYLASE; it catalyzes the hydroxylation of dihydrokaempferol at the 3' position of the B-ring, leading to the respective flavonols, anthocyanidins, and proanthocyanidins [60]. F3'H is responsible for the bifurcation of the metabolic pathway of anthocyanins synthesis by competing with F3'5'H (FLAVONOID 3',5'-HYDROXYLASE) for substrate recruitment. F3’H and F3’5’H deliver disubstituted (3’,4’-hydroxylated) or trisubstituted (3’,4,5'-hydroxylated) anthocyanins, precursors of red or blue skin pigments, respectively. The prevalence of F3'H on F3'5'H leads to a greater accumulation of cyanidin, the precursor of peonidin to the detriment of the accumulation of delphinidin, precursor of malvidin and petunidin [45,59,61].

In qRT-PCR results (Fig.3), the transcript of VIT_209s0002g01090 showed a significant difference in gene expression between T1 and T2, in accordance with previous findings available in the literature. Several authors claimed that *VvF3'H* has a decreased expression level towards grapevine berry maturation [62], whereas F3'5'H has a major role at later stages [63].

The other copy of *F3'H* gene (VIT_217s0000g07200) had a different behavior; at maturity, it was more expressed in M when compared to P and NGC. According to these data, we can deduce that Mgt 101-14, a rootstock that is known to confer less tolerance to drought in the scion, stimulated a greater synthesis of disubstituted anthocyanins. Conversely, some vines tend to synthesize more trisubstituted anthocyanins in berry skins to cope with environmental stresses, for example in case of water deficit [29].

We further investigate some of the genes coding for transcription factors with a mainstream role in the regulation of secondary metabolism. The MYB transcription factors belong to MYB-bHLH-WD40 (MBW) protein complex, which finely controls the phenylpropanoid synthesis pathway in grapevine [64]; the MYB family includes several members, both positive or negative regulators, most of which have been largely characterized in grapevine, while for few of them the information available in the literature is sometimes scarce.

The *VvMYB14* gene (vit_07s0005g03340) is involved in the feedback regulation of resveratrol biosynthesis, a branch of the phenylpropanoid pathway that leads to stilbene accumulation [65]. Resveratrol has an important role in the dissipation of oxidative stress, and it has been shown that it is synthesized also in healthy grapevines, towards the end of maturation [24]. The UV-C rays stress during the ripening period, in fact, induces *VvMYB14* gene that, together with the transcription factor WRKY3, up-regulates the activity of the *VvSTS29* gene

(STILBENE SYNTASE), resulting in resveratrol accumulation. On the contrary, when resveratrol level increases, *VvMYB14* is down-regulated by *WRKY8* and the transcription of *VvSTS29* is blocked, preventing the accumulation of this metabolite [66].

The gene coding for MYB14 transcription factor was DE in the comparison between M-T2 *vs* P-T2 and P-T2 *vs* NGC-T2, both in the RNA-seq and qRT-PCR analysis. This gene was up-regulated in 1103 Paulsen, and the expression was almost doubled compared to M and NGC plants. These results suggest that the plants grafted on 1103 Paulsen have a greater predisposition to the synthesis of MYB14, which could induce a greater accumulation of resveratrol in mature berries. This hypothesis could explain the effect of greater tolerance to drought given by this commonly used rootstock and it is also supported by literature data. In a work published by Corso *et al*. [19], the transcript profiles of two rootstocks with opposite drought susceptibility was compared (Mgt 101-14, the same as the present work and M4, a new drought-tolerant rootstock); according to their findings, MYB genes (including *MYB14*) were found as DE between the root genotypes under water stress, both in leaves and roots. The results can be similar to ours, even dealing with different plant tissues; the MYB family was one of the most represented among the DE genes and had opposite expression kinetics between Mgt 101-14 and the drought-resistant rootstock M4, which has intrinsic characteristics very similar to 1103 Paulsen in stress tolerance.

The *MYBC2-L3* (vit_214s0006g01620) codes for a TF that acts as a transcriptional repressor in the anthocyanin synthesis [67]. In transgenic tobacco [68], *VvMYBC2-L3* represses the *DFR* gene and might induce the expression of *FLS*, although this latter hypothesis was not confirmed. According to DESeq2 output and qRT-PCRs confirmation, *MYBC2-L3* is not DE, neither at T1 nor at T2, but is listed among the predicted targets of vvi-miR858 (Additional

file 9). The expression of this repressor was higher at veraison, while it decreased at maturation. From these results, we can hypothesize that, at T1, the up-regulation of *MYBC2-L3* promoted the flavonols synthesis to the detriment of anthocyanins in grape berries, while at T2 the lower expression of *MYBC2-L3* repressor favored the accumulation of anthocyanins.

A total of 74 different *NAC* genes have already been described in grapevine [69], having different roles in plant development, fruit ripening and the response to biotic and abiotic stresses. The precise functions of each *NAC* gene are not yet completely clear, but it is known that they are often tissue-specific and can be activators or repressors, depending on the environmental conditions [70]. *VvNAC44* (vit_206s0004g00020) was one of the most interesting genes to be counted among the DEGs. It seems to be involved in berry ripening and stress response [71], in particular in the biosynthetic pathway of stilbenes; as a matter of fact, it was found as co-expressed with *VvSTS* genes [72]. According to our results, *VvNAC44* was differentially expressed in each of the three pairwise comparisons at T2; and the same trends were confirmed by qRT-PCR (Fig.3). This gene was more expressed in P and had lower expression in M, while not grafted control presented the lowest expression. Having little information available on the actual role of *VvNAC44*, it is not feasible to gain evidence of the differential expressions on the phenotype of the plants considered, but this gene is certainly to be taken into consideration for future insights.

Another *NAC* gene that gave interesting results is *VvNAC60* (vit_208s0007g07670). It is assumed that this gene is involved in grape ripening [73] and the response to different kind of stresses [74], and it can be up-regulated in the presence of elicitors [75]. *VvNAC60* was found as DE between the grafted plants (M and P) compared to NGC, both in RNA-seq and

qRT-PCR, suggesting that grafting had a role in the differential expression of this NAC-family transcription factor.

To further the analyses on the regulatory factors acting in the phenylpropanoid pathways, we analyzed the results coming from small RNA sequencing data to highlight their putative involvement in secondary metabolism. In this perspective, among the analyzed miRNAs, it is interesting to show the results of vvi-miR858, a microRNA that targets some MYB transcription factors involved in the control pattern of flavonoid synthesis. miR858, already identified in apple, peach, and Arabidopsis [76,77,78] is not deposited in miRBase for *Vitis vinifera*, but it has already been reported in previous works [39,79,80], and it is known to be one of the master regulators of *MYB* genes. Indeed, we predicted among its targets 34 R2R3-MYB transcription factors (Additional file 9). Within the putative targets, there are three *MYB* genes (VIT_218s0001g09850 - *MYB174*, VIT_218s0001g11170 - *MYB175* and VIT_205s0049g01010 - *MYB13*), that were identified as differentially expressed in the comparison M *vs* NGC and P *vs* NGC, with an opposite expression profile compared to miR858 (Additional file 10), reinforcing the idea that MYB transcription factors regulating secondary metabolism might be modulated by rootstock effect.

We designed primers on miR858-5', considering that the differential expressed sequence is 2bp shorter than the most abundant isomiR that is not differentially expressed, interestingly the DE sequence of miR858 has some peculiar *MYB* genes as targets that are not predicted to be targets of the most expressed sequence. Unfortunately, the results of qRT-PCR (Additional file 11) did not coincide with those of RNA-seq, putatively because it was impossible to distinguish among the DE and not DE isomiR of the miR858. An interesting fact, however, is the decrease in the expression of miR858 between the two sampling points. As

expected, at veraison miR858 is more expressed than maturity. This finding indicates that the translation of the mRNAs coding for the MYB TFs involved in the secondary metabolism was certainly more inhibited at T1 and favored at T2.

Grape phenotyping confirmed the transcriptomic results

The importance of secondary metabolites in grapevines' behavior and their response to grafting was underlined by the fact that a high number of identified DEGs were involved in different key points of the biosynthesis pathway of phenylpropanoids, mainly at T2. The phenotyping activity to assess the accumulation of phenolic compounds in berry skins was performed with specific HPLC analyses and the results (Fig.10, and Fig.11) reflect the trends reported in RNA-seq.

Above all, the chemical analyses confirmed that the main discriminating factor was grape ripening, and as expected, the differences were remarkable comparing grapes at veraison and maturity. Within each season, the grape samples from the three root systems were separately distributed between T1 and T2. As well known, the individual classes of phenolic compounds (i.e. flavonols, proanthocyanidins, anthocyanins) follow different accumulation curves in berry tissues during the ripening process [23,27]. Secondly, the samples were divided by the year effect, and separated between 2012 and 2013, to a lesser extent at veraison (when the grapes were more homogeneous), and with a greater relevance at maturity, confirming that in viticulture the environmental conditions have a strong influence and modulate the final content of secondary metabolites differently, in every single vintage.

Analyzing the differences at maturity only for the year 2012, where according to transcriptomics the most interesting results emerged, all the samples were distinguished,

with the major differences between the grafted plants and not grafted control, suggesting an effect given by both the rootstocks. The correlation between gene expression (qRT-PCR) and concentrations of phenolic compounds showed confirmation of the role of some TFs in stilbenes biosynthesis (MYB14, MYB4R1, and NAC44).

Conclusions

Although grafting has an essential role in viticulture, the molecular network behind the rootstock-scion interaction remains largely unknown, particularly concerning grape quality. We conducted a detailed analysis of the transcriptome coupled with chemical analyses on grapevine berry skins. Our data confirmed that rootstocks can determine important effects on grape phenotype during ripening, affecting the final berry quality. In general, grafting *per se* has a strong influence on berry skin transcriptome and chemical composition at maturity, NGC plants are well distinct by both grafted plants and the genes identified as differentially expressed at maturity were mainly involved in the synthesis of phenylpropanoids and the transport of flavonoids. Besides, the secondary metabolism was more significantly modulated during grape ripening in the plants grafted on 1103 Paulsen than in those grafted on Mgt 101-14. Interestingly, the plants grafted on 1103 Paulsen had a greater predisposition to the synthesis of MYB14 compared to Mgt 101-14, which could induce a greater accumulation of resveratrol in mature berries.

In light of the results obtained, we can conclude that rootstocks influenced the molecular mechanisms of berry development and grape quality. Considering the fundamental role of grafting in viticulture, the choice of the most suitable rootstock during vineyard design can be fundamental to deal with limiting environmental conditions and to preserve grape

characteristics and wine typicality, especially in the context of the actual climate change, which is causing a lot of concern among wine growers worldwide.

Abbreviations

AGO: Argonaute; ath: *Arabidopsis thaliana*; bHLH: basic Helix-Loop-Helix; Ct: threshold Cycle; DE: Differentially Expressed; DEG: Differentially Expressed Gene; DFR: Dihydroflavonol-4-Reductase; D.O.C.G.: Denomination of Controlled and Guaranteed Origin; ERF: Ethylene Response Factor; F3'H: Flavonoid 3'-Hydroxylase; F3'5'H: Flavonoid 3',5'-Hydroxylase; FDR: False Discovery Rate; FLS: Flavonol Synthase; GDD: Growing Degree Days; GO: Gene Ontology; GRF: Growth Regulating Factor; HC/HCA: Hierarchical Clustering Analysis; HPLC: High-performance liquid chromatography; L.: *Linnaeus*; M: Mgt 101-14; MIR genes: genes coding for microRNA; miRNA/miR: microRNA; NAC: NAM-ATAF1,2-CUC2; NGC: not grafted control; nt: nucleotide; O.I.V.: International Organization of Vine and Wine; P: 1103 Paulsen; PAL: Phenylalanine Ammonia Lyase; PCA: Principal Component Analysis; ppe: *Prunus persica*; PTGS: Post Transcriptional Gene Silencing; qRT-PCR: quantitative Real Time PCR; siRNA: Short interfering RNA; SPL: Squamosa Promoter-binding-like; SSR: Simple Sequence Repeats; STS: Stilbene Syntase; T1: Time 1 , veraison; T2: Time 2, maturity; TF: Transcription Factor; TT: Transparent Testa; UBI: Ubiquitin; UV: ultraviolet; *vs*: *versus*; Vv/vvi: *Vitis vinifera.*

Methods

Plant materials

A pot system for grapevines monitoring was set up at CREA - Research Centre for Viticulture and Enology, in Arezzo (43°28'36'' N, 11°49'27'' E, Italy). It consists of plastic pots of 70 liters,

filled by a silty-clay texture soil (40% clay, 41% silt, 19% sand), with a volumetric soil water content of 34% at field capacity, collected from a real vineyard of the Chianti Classico D.O.C.G. district (Tuscany - Italy). The grapevines in the pots are 7-year-old *Pinot noir* plants, clone ENTAV 115 with two different rootstock combinations: 1103 Paulsen (P) *V. berlandieri* x *V. rupestris*, highly vigorous and known for its drought tolerance, and Mgt 101-14 (M) *V. riparia* x *V. rupestris*, less vigorous and less tolerant to drought; not grafted plants were used as control (NGC). The vines were trained on vertical shoot positioned trellis, with spur cordon pruning and an average of 10 buds per vine. The pots were positioned in an open field, spaced at the distance of 1 m within the row and 2,5 m between the rows, with orientation north to south, and were arranged in a randomized block design with 9 replicates for each root system. The plants were maintained in the same agronomic conditions: all the pots were fertilized before the beginning of the vegetative season with 40g of Nitrophoska (12N-12P-17K, EurochemAgro) and were abundantly irrigated by drip emitters during the summer period, with the same water regime.

In 2012 and 2013, grape samples for molecular analyses were collected at two ripening times: veraison (75% of colored berries, T1) and at maturity (maturity, T2). Berries (15 per plant, 3 plants per replicate) were randomly hand-picked at different positions of the clusters, dissected to separate skin tissues, stored in Falcon tubes and immediately frozen at -80°C for further processing. In total, the experiment entailed the collection of 18 berry samples (vines with three different root systems, two ripening times, three biological replicates) for each vegetative season considered.

At harvest, technological maturity was evaluated on musts according to O.I.V. official methods (O.I.V., 2009), confirming commercial ripeness; no significant differences emerged

between the grape samples (2012 average data: sugars 22.2° Brix, pH 3.6, total acidity 6.7g/L tartaric acid, berry weight 0.9 g; 2013 average data: sugars 20.8° Brix, pH 3.5, total acidity 6.8 g/L tartaric acid, berry weight 1.0 g).

Plant specimen

The plant material used belongs to *Vitis vinifera* species or hybrid species of *Vitis* commonly used in viticulture and freely available for cultivation or research activity. In particular, *Pinot noir* is officially registered in the Italian National Catalogue of Grape Varieties (identification code n°195 - admitted in 1970). The information is available at the following link: http://catalogoviti.politicheagricole.it/result.php?codice=195. *Pinot noir* clone ENTAV 115 is officially registered in the French National Catalogue of Grape Varieties (admitted in 1971). The information is available at the following link: http://plantgrape.plantnet-project.org/it/cepage/Pinot%20noir. The rootstock 1103 Paulsen is officially registered in the Italian National Catalogue of Grape Varieties (identification code n°625 - admitted in 1971). The information is available at the following link: http://catalogoviti.politicheagricole.it/result.php?codice=625. The rootstock Mgt 101-14 is officially registered in the Italian National Catalogue of Grape Varieties (identification code n°604 - admitted in 1971). The information is available at the following link: http://catalogoviti.politicheagricole.it/result.php?codice=604.

The plant materials employed in the experimental pot system was preventively genotyped using a set of nine SSR loci internationally recognized for grapevine identification (http://www.oiv.int/public/medias/6886/oiv-viti-609-2019-en.pdf). Furthermore, the

identity of the *Pinot noir* cultivar was confirmed by repeated ampelographic surveys on shoots, leaves, bunches, and berries.

Weather conditions

The climate data were recorded during the 2012 and 2013 vegetative seasons using a non-stop automated control unit (Ecotech GmbH, Germany) placed nearby the experimental pot system area. The following parameters were measured in the period comprised between April 1st and October 31st (conventionally considered the vegetative period for the grapevine): Daily Maximum Temperature (°C), Daily Average Temperature (°C), Daily Minimum Temperature (°C); Daily Average Rainfall (mm). The data collected were daily checked and processed for each year at the end of the season; Growing Degree Days (GDDs) and the Winkler Index were calculated on a 10° C basal temperature, according to [81], to get information about the sum of all the daily average temperature that influenced the plant growth during the season.

The data recorded during the years 2012 and 2013 are reported in Additional file 5. The climate trend was very different in the two vegetative seasons considered. This aspect is particularly evident in the period between the two sampling points, which occurred on similar dates, but the weather conditions were strongly dissimilar. In general, the 2012 season was much warmer, with 1450 GDDs accumulated in the period April 1st – August 22nd, while in 2013 (April 1st – August 23rd), the GDDs accumulation was 1276, with a substantial difference of 174 degrees. Considering the interval between veraison (T1) and maturity (T2) only, all the temperature values recorded were significantly higher in 2012 (Average T_{max} = 35.6° C; Average T_{avg} = 26.6° C; Average T_{min} = 16.2° C) than in 2013 (Average

T_{max} = 32.6° C; Average T_{avg} = 24.1° C; Average T_{min} = 15.1° C) and almost a third of the difference between the GDDs (347 in 2012 *vs* 296 in 2013) were accumulated between these two phenological phases corresponding to samplings.

Library preparation and sequencing

Total RNA extraction was performed using Plant RNA Isolation Reagent (PRIR – Life Technologies™) starting from 200 mg of ground berries skin tissue in 1 ml of reagent, followed by RNA Clean up and Concentration kit (NorgenBiotek Corp) according to manufacturers' protocols. Total RNA was then subjected to Dnase I treatment (DNA-free™ Kit, Applied Biosystems). The concentration and purity of total RNAs were evaluated using a spectrophotometer (DU640 Beckman) and a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and their integrity was assessed by an Agilent 2100 Bioanalyzer using an RNA 6000 Nano kit (Agilent Technologies), according to the manufacturer's instructions. All RNA samples were stored at -80°C for subsequent analyses.

Small RNA libraries were prepared using the TruSeq Small RNA Sample Preparation Kit (Illumina®), following all manufacturers' instructions. Thirty-six bar-coded small RNA libraries were constructed starting from 1 µg of total RNAs. The quality of each library was assessed using an Agilent DNA 1000 kit. Sequencing was performed using a 6-plex sequencing approach on an Illumina GAIIx platform.

mRNA seq libraries were prepared from the same total RNA (1 µg) extracted for small RNA ones, using TruSeq RNA sample preparation kit (Illumina), according to manufacturers' instructions. Libraries were quantified through qRT-PCR, as recommended by the protocol, and single-end sequenced for 100 bases on an Illumina Genome Analyzer (GAIIx).

Bioinformatics and statistical methods

miRNAs methods:

Identification and quantification of grapevine miRNAs have been carried out with the software CLC Bio Genomics Workbench (v.8, Qiagen). Raw redundant reads have been processed to trim the adapter and retaining reads between 16 and 25 nt long, then comparing with all plant species miRNAs deposited in miRBase v.21, and, additionally, with a set of 139 novel grapevine miRNAs (user-defined dataset) identified in our previous works [41,44]. Differentially expressed miRNAs were identified using the software CLC Bio Genomics Workbench using multiple comparison analysis, with all reads mapping (with 0 mismatches) to known plant miRNA precursors (miRBase plus user-defined dataset). For each library, ungrouped reads perfectly mapping to the miRNA precursors were considered as the input for the expression analysis.

Given the main focus of our work, we aimed at identifying miRNAs differentially expressed between the two grafted plants and among grafted and control plants, sampled at the same developmental stage. We performed the Empirical Analysis of digital gene expression (DGE), an implementation of the "Exact Test" present in the EdgeR Bioconductor package, as implemented in CLC Bio Genomics Workbench software. We estimated tagwise dispersion with multi-comparison unpaired test option, setting FDR-adjusted p-value ≤ 0.05. We classified the differentially expressed sequences based on the miRNA family they belong to, and on the correspondence to the mature 5' or 3' miRNA product or the position into the precursor stem-loop structure.

PCA and Hierarchical Clustering analyses have been performed within the software CLC Bio Genomics Workbench, using normalized (tag per 1 million TP1M) and transformed data

(log_{10} (n+1)), where n is the normalized value for each sequencing tag. Hierarchical Clustering analyses have been performed using 1-Pearson correlation as distance measure and Complete Linkage as linkage method.

All differentially expressed sequences have been used as input for psRNATarget software, in order to predict putative target sequences for each DE miRNA, from Grapevine transcript database originated from JGI - Phytozome v11 and Genoscope 12x assembly. Default settings have been used to run analyses, modifying HSP size for sequences shorter than 20 nt.

RNA-Seq, differentially expressed genes, GO enrichment and further methods

Raw reads (101 bases, single end; on average 25 million of reads for each sample, Additional file 1) were checked for adapters and contaminants via FastQC application [82]. Adapters and low-quality regions were filtered out by Cutadapt application [83]. Subsequently, TopHat version 2.0.12 and Bowtie2 [84] were implemented to map filtered reads to the grapevine genome sequence (*Vitis vinifera*; [1]; Vitis_vinifera.IGGP_12x.25). Read counts were generated from Bam alignment files with HTSeq software version 0.6.1 [85]. Data normalization and call of differentially expressed genes (DEGs) was implemented with DESeq2 version 1.2.8 Bioconductor (R) package [86] by setting fitting to local, and False Discovery Rate (FDR) threshold to 0.05 and enabling independent filtering. No fold change threshold was set.

GO enrichment analyses were conducted with the Goseq Bioconductor package. Goseq was specifically designed to minimize length-derived bias which may affect RNA-seq data [87]. Data preparation for Goseq analysis was as previously reported [88].

MapMan [89] figures were generated upon binning of *Vitis* cDNA sequences to MapMan bins by the Mercator application [90]. PCA of samples were based on R function prcomp from stats package as implemented in DESeq2 Bioconductor package.

qRT-PCR analyses of miRNAs and gene expression

miRNAs expression levels were evaluated by stem-loop Real-Time PCR (qRT-PCR); the primers (listed in Tab.2) were designed according to [91]. For reverse transcription, a stem-loop primer for each miRNA was used. Stem-loop reverse transcriptase primers consist of a selfed stem-loop sequence, in addition to a specific nucleotide extension at the 3' end, complementary to the last 6 nucleotides at the 3' end of each miRNA of interest.

The RT reactions were performed starting from 200 ng of DNase treated total RNA, using Superscript III (Invitrogen), according to the manufacturer's instructions. The reverse transcription products were amplified using a miRNA-specific forward primer and a reverse primer on the stem-loop adapter.

The Real-Time PCR reactions were set up in 25 µl using SYBR Green PCR Master Mix (Applied Biosystem). Three independent biological replicates were analyzed in triplicate, on a 7300 Real-Time PCR System (Life Technologies™) with the following conditions: 95° C for 10 minutes, followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute plus 1 cycle for dissociation curve. A poly-ubiquitin transcript (*VvUBI*) was always used as an internal standard. After the amplification, the 7300 Sequence Detection System Software was used to set the baseline and the threshold for each reaction. The relative quantification of each miRNA was calculated from the Ct value, using the $2^{-\Delta\Delta Ct}$ method.

To evaluate gene expression level, primers were designed in non-conserved coding regions (Tab.2) to avoid cross-amplification of genes belonging to multigenic families; primer efficiency was calculated using serial dilutions of berry skin cDNA. cDNA was produced from DNase-treated RNA using SuperScript II Reverse transcriptase kit (Invitrogen) according to manufacturer's instructions.

Before the setting up of qRT-PCR on the chosen genes, the efficiency of the 10 pairs of primers, previously designed (Tab.2) was tested with successful results on serial dilutions of berry skin cDNA. The Real-Time PCRs were performed in a final volume of 10 µl, with SsoAdvanced Universal SYBR® Green PCR Supermix (BioRad), considering three technical replicates for each sample. The plates were analyzed on a 7300 Real-Time PCR System (Life Technologies) with the following conditions: 95° C for 30 seconds, followed by 40 cycles of 95° C for 10 seconds and 60° C for 1 minute plus 1 cycle for primer dissociation. After the amplification, the 7300 Sequence Detection System Software was used to set the baseline and the threshold for each reaction. The relative quantification was calculated from average Ct value, using the $2^{-\Delta\Delta Ct}$ method, considering a poly-ubiquitin transcript (*VvUBI*) as an internal standard.

Chemical Analyses

The phenotyping activity was carried out on grape quality, in particular on the content of phenolic compounds in berry skins. The samples (15 berries per plant, 3 plants per replicate) were collected simultaneously for molecular and chemical analyses at veraison (T1) and maturity (T2). The skin tissues were separated and immediately ground into a powder using a mortar and liquid nitrogen, then were stored at -80°C in falcon tubes, until use.

Before analyzing, the berry skin powder was weighed and resuspended in 10 mL of methanol (ultra) gradient HPLC grade (JT Baker, USA) and 50 µl of Formic Acid 98% (PanreacApplichem, Spain). The solution was centrifuged at 3000 rpm for 10 minutes and then 2 mL of extract were pipetted into a syringe, filtered with Minisart RC 0.45 µm filters (Sartorius, Germany), and injected into HPLC glass vials. The analyses were performed using an Agilent 1100 Series HPLC, equipped with solvent degasser, quaternary pump and diode array detector and controlled by a PC running Agilent ChemStation for LC 3D System software (Agilent, USA). A Luna® Omega 5µm Polar C18 Column (Phenomenex, USA) was used to separate phenolic compounds, following the method of [92]. In total, the experiment comprised 18 berry samples (vines with three different root systems, two ripening times, three biological replicates) per each vegetative season considered, 2012 and 2013. Principal Component Analyses on the results were performed using Unscrambler (V10.3, CAMO Process AS, Norway).

Accession numbers and data repository

In the process of uploading all the data.

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Authors' contributions

AZ managed the experimental pot system and processed the climate data, collected berry samples, carried out real-time PCRs, and wrote the manuscript. CC prepared RNA-Seq libraries and wrote the manuscript. PB and PF carried out the bioinformatic analyses. LB interpreted the RNA-Seq data. NR and SP carried out chemical analyses and relative statistical analyses. AT performed RNA extractions. PS, LC, AF, MD, CC contributed to the experimental system setup, and conceived the research project. EM analyzed and interpreted the RNA-Seq data, contributed to the bioinformatic analyses, wrote the manuscript and coordinated the research activity.

All authors have read and revised the manuscript draft, and have given approval of the version to be published.

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References

1. Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, *et al.* The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature. 2007;449:463-467.
2. Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, Pruss D *et al.* A high-quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One. 2007;12:e1326.
3. Anwar SA, McKenry M, Ramming D. A search for more durable grape rootstock resistance to root-knot nematode. Am J Enol Vitic. 2002;53:19-23.
4. Corso M, Bonghi C. Grapevine rootstock effects on abiotic stress tolerance. Plant Sci Today. 2014;1:108-113.
5. Warschefsky EJ, Klein LL, Frank MH, Chitwood DH, Londo JP, von Wettberg EJB, *et al.* Rootstocks: diversity, domestication, and impacts on shoot phenotypes. Trends Plant Sci. 2016;21:418-437.
6. Ollat N, Cookson SJ, Lauvergeat V, Marguerit E, Barrieu F, Gambetta G, *et al.* Grapevines roots: the dark side. Acta Horticolturae. 2017;1188:213-226.
7. Di Filippo M, Vila H. Influence of different rootstocks on the vegetative and reproductive performance of *Vitis vinifera* L. Malbec under irrigated conditions. J Int Sci Vigne Vin. 2011;45(2):75-84.
8. Jones TH, Cullis BR, Clingelefer PR, Rühl EH. Effects of novel hybrid and traditional rootstocks on vigour and yield components of Shiraz grapevines. Australian Journal of Grape and Wine Research. 2009;15:284-292.
9. Marguerit E, Brendel O, Lebon E, van Leeuwen C, Ollat N. Rootstock control of scion transpiration and its acclimation to water deficit are controlled by different genes. New Phytol. 2012;194:416-429.
10. Keller M, Mills LJ, Harbertson JF. Rootstock effect on deficit-irrigated winegrapes in a dry climate: vigor, yield formation and fruit ripening. Am J Enol Vitic. 2012;63:29-39.
11. Nuzzo V, Matthews MA. Response of fruit growth and ripening to crop level in dry-farmed Cabernet sauvignon on four rootstocks. Am J Enol Vitic. 2006;57(3):314-324.
12. Pina A, Errea P. A review of new advances in mechanism of graft compatibility-incompatibility. Scientia Horticolturae. 2005;106:1-11.
13. Cookson SJ, Clemente Moreno MJ, Hevin C, Nyamba Mendome LZ, Delrot S, Magnin N, *et al.* Heterografting with nonself rootstocks induces genes involved in stress responses at the graft interface when compared with autografted controls. Journal of Experimental Botany. 2014;65(9):2473-2481.
14. Cookson SJ, Clemente Moreno MJ, Hevin C, Nyamba Mendome LZ, Delrot S, Trossat-Magnin C, Ollat N. Graft union formation in grapevine induces transcriptional changes related to cell wall modification, wounding, hormone signalling, and secondary metabolism. Journal of Experimental Botany. 2013;64:2997-3008.

15. Yang Y, Mao L, Jittayasothorn Y, Kang Y, Jiao C, Fei Z, Zhong GY. Messenger RNA exchange between scions and rootstocks in grafted grapevines. BMC Genomics. 2015;15:251.
16. Zhang L, Marguerit E, Rossdeutsch L, Ollat N, Gambetta GA. The influence of grapevine rootstocks on scion growth and drought resistance. Theor Exp Plant Physiol. 2016;28:143-157.
17. Pant BD, Buhtz A, Kehr J, Scheible WR. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. 2008;53:731-738.
18. Maré C, Aprile A, Roncaglia E, Tocci E, Corino LG, De Bellis G, Cattivelli L. Rootstock and soil induce transcriptome modulation of phenylpropanoid pathway in grape leaves. Journal of plants interactions. 2013;8(4):334-349.
19. Corso M, Vannozzi A, Maza E, Vitulo N, Meggio F, Pitacco A, *et al.* Comprehensive transcript profiling of two grapevine rootstock genotypes contrasting in drought susceptibility links the phenylpropanoid pathway to enhanced tolerance. J Exp Bot. 2015;66(19):5739-5752.
20. Berdeja M, Nicolas P, Kappel C, Dai ZW, Hilbert G, Peccoux A, *et al.* Water limitation and rootstock genotype interact to alter grape berry metabolism through transcriptome reprogramming. Horticulture Research. 2015;2:15012.
21. Maré C, Mica E, Cattivelli L. Molecular advances in rootstock-scion interaction in grapevine. Acta Horticulturae. 2016;1136:155-160.
22. Chitarra W, Perrone I, Avanzato CG, Minio A, Boccacci P, Santini D, Gilardi G, *et al.* Grapevine grafting: scion transcript profiling and defense-related metabolites induced by rootstocks. Frontiers in Plant Science. 2017;8:564.
23. Teixeira A, Eiras-Dias J, Castellarin S, Geròs H. Berry Phenolics of Grapevine under Challenging Environments. Int J Mol Sci. 2013;14:18711-18739.
24. Gatto P, Vrhovsek U, Muth J, Segala C, Romualdi C, Fontana P, *et al*. Ripening and genotype control stilbene accumulation in healthy grapes. J Agric Food Chem. 2008;56:11773-11785.
25. Snopek L, Mlcek J, Sochorova L, Baron M, Hlavacova I, Jurikova T, *et al.* Contribution of Red Wine Consumption to Human Health Protection. Molecules. 2018;23(7):1684.
26. Amor S, Chalôns P, Aires V, Delmas D. Polyphenol Extracts from Red Wine and Grapevine: Potential Effects on Cancers. Diseases. 2018;6(4):106.
27. Czemmel S, Heppel SC, Bogs J. R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma. 2012;249(2):109-118.
28. Downey MO, Dokoozlian NK, Krstic MP. Cultural practice and environmental impacts on the flavonoid composition of grapes and wines: A review of recent research. Am J EnolVitic. 2006;57: 257–268.
29. Castellarin SD, Matthews MA, Di Gaspero G, Gambetta GA. Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. Planta. 2007;227(1):101-112.
30. Matus JT, Loyola R, Vega A, Pena-Neira A, Bordeu E, Arce-Johnson P, Alcalde JA. Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. J Exp Bot. 2009;60:853-867.
31. Loureiro MD, Moreno-Sanz P, García A, Fernández O, Fernández N, Suárez B. Influence of rootstock on the performance of the Albarín Negro minority grapevine cultivar. Sci Hort. 2016;201:145-152.
32. Liu J, Osbourn A, Ma O. MYB Transcription Factors as Regulators of Phenylpropanoid Metabolism in Plants. Mol Plants. 2015;8(5):689-708.
33. Sharma D, Tiwari M, Pandey A, Bhatia C, Sharma A, Trivedi PK. MicroRNA858 is a potential regulator of phenylpropanoid pathway and plant development. Plant Physiology. 2016;171:944-959.

34. QiaoY, Zhang J, Zhang J, Ran A, Guo H, Wang D, Zhang J. Integrated RNA-seq and sRNA-seq analysis reveals miRNA effects on secondary metabolism in Solanum tuberosum L. Molecular genetics and genomics. 2017;292:37-52.
35. Chuck G, Candela H, Hake S. Big impact by small RNAs in plant development. Current Opinion in Plant Biology. 2009;12:81-86.
36. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. Biochimica et Biophysica Acta. 2010;1803:1231-1243.
37. Solofoharivelo MC, Van der Walt AP, Stephan D, Burger JT, Murray SL. MicroRNAs in fruit trees: discovery, diversity and future research directions. Plant Biology. 2014;16:856-865.
38. Mica E, Piccolo V, Delledonne M, Ferrarini A, Pezzotti M, Casati C, *et al.* Correction: High throughput approaches reveal splicing of primary microRNA transcripts and tissue specific expression of mature microRNAs in *Vitis vinifera*. BMC Genomics. 2010;11:109.
39. Pantaleo V, Vitali M, Boccacci P, Miozzi L, Cuozzo D, Chitarra W, *et al.* Novel functional microRNAs from virus-free and infected *Vitis vinifera* plants under water stress. Nature-Scientific report. 2016;6:20167.
40. Wang C, Wang X, Kibet NK, Song C, Zhang C, Li X, Han J, Fang J. Deep sequencing of grapevine flower and berry short RNA library for discovery of novel microRNAs and validation of precise sequences of grapevine microRNAs deposited in miRBase. Physiologia Plantarum. 2011;143:64-81.
41. Belli Kulhan J, Paim Pinto DL, Bertolini E, Fasoli M, Zenoni S, Tornielli GB, *et al.* miRVine: a micro RNA expression atlas of grapevine based on small RNA sequencing. BMC Genomics. 2015;16:393.
42. Pagliarani C, Vitali M, Ferrero M, Vitulo N, Incarbone M, Lovisolo C, *et al*. The accumulation of microRNAs differentially modulated by drought is affected by grafting in grapevine. Plant Physiol. 2017;173:2180-2195.
43. Allen E, Xie Z, Gustafson AM, Carrington JC. MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell. 2005;121:207-221.
44. Paim Pinto DL, Brancadoro L, Dal Santo S, De Lorenzis G, Pezzotti M, Meyers BC, *et al.* The influence of genotype and environment on small RNA profiles in grapevine berry. Frontiers in Plant Sciences. 2016;7:1459.
45. Mattivi F, Guzzon R, Vrhovsek U, Stefanini M, Velasco R. Metabolite profiling of Grape: Flavonols and Anthocyanins. J Agric Food Chem. 2006;54:7692-7702.
46. Venturini L, Ferrarini A, Zenoni S, Tornielli GB, Fasoli M, Santo SD, *et al.* De novo transcriptome characterization of *Vitis vinifera* cv. Corvina unveils varietal diversity. BMC Genomics. 2013;14:41.
47. Da Silva C, Molin AD, Ferrarina A, Boido E, Gaggero C, Delledonne M, Carrau F. The Tannat genome: unravelling its unique characteristics. Bio web of conferences. 2019;12:01016.
48. Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, SaitoK, Takahashi H, Dalmay T. Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J. 2009;57:313-321.
49. Zhu C, Ding Y, Liu H. MiR398 and plant stress responses. Physiologia Plantarum. 2011;143:1-9.
50. Jones GV, Davis RE. Climate Influences on Grapevine Phenology, Grape Composition, and Wine Production and Quality for Bordeaux, France. Am J Enol Vitic. 2000;51:249-261.
51. Dal Santo S, Zenoni S, Sandri M, De Lorenzis G, Magris G, De Paoli E, *et al.* Grapevine field experiments reveal the contribution of genotype, the influence of environment and the effect of their interaction (G x E) on the berry transcriptome. Plant J. 2018;93:1143-1159.
52. Fraga H, Malheiro C, Moutinho-Pereira J, Santos JA. An overview of climate chenge impacts on European viticulture. Food and Energy Security. 2013;1(2):94-110.

53. Keller M. The science of grapevines. Anatomy and Physiology. Academic Press, NY USA. 2010.
54. van Leeuwen C, Tregoat O, Choné X, Bois B, Pernet D, Gaud-illère JP. Vine water status is a key factor in grape ripening andvintage quality for red Bordeaux wine. How can it be assessed for vineyardmanagementpurposes?. Journal International des Sciences de la Vigne et du Vin. 2009;43:121-134.
55. Ferrandino A, Lovisolo C. Abiotic stress effects on grapevine (*Vitis vinifera* L.): Focus on abscisic acid-mediated consequences on secondary metabolism and berry quality. Environmental and Experimental Botany. 2014;103:138-147.
56. Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, *et al.* Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol. 2006;140:499-511.
57. Wang M, Vannozzi A, Wang G, Liang YH, Tornielli GB, Zenoni S, *et al.* Genome and transcriptome analysis of the grapevine *Vitis vinifera* L. WRKY gene family. Horticulture Research. 2014;1:16.
58. Luo P, Ning G, Wang Z, Shen Y, Jin H, Li P, *et al.* Disequilibrium of Flavonol Synthase and Dihydroflavonol-4-Reductase Expression Associated Tightly to White *vs*. Red Color Flower Formation in Plants. Frontiers in Plant Science. 2015;6:1257.
59. Falginella L, Castellarin S, Testolin R, Gambetta G, Morgante M, Di gaspero. Expansion and subfunctionalisation of flavonoid 3',5'-hydroxylases in the grapevine lineage. BMC Genomics. 2010;11:562.
60. Bogs J, Ebadi A, Mc David D, Robinson SP. Identification of the Flavonoid Hydroxylases from Grapevine and Their Regulation during Fruit Development. Plant Physiology. 2006;140:279-291.
61. Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, Gomès E, *et al.* Berry ripening: recently heard through the grapevine?. J Exp Bot. 2014;65:4543-4559.
62. Degu A, Hochberg U, Sikron N, Venturini L, Buson G, Ghan R, *et al.* Metabolite and transcript profiling of berry skin during fruit development elucidates differential regulation between Cabernet Sauvignon and Shiraz cultivars at branching points in the polyphenol pathway. BMC Plant Biol. 2014;14:188.
63. Castellarin SD, Di Gaspero G, Marconi R, Nonis A, Peterlunger E, Paillard S, *et al.* Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. BMC Genomics. 2006;7: 12.
64. Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot. 2011;62(8):2465-2483.
65. Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Santo SD, *et al.* Timing and order of the molecular events marking the onset of berry ripening in grapevine. Plant Physiol. 2018;178:1187-1206.
66. Jeandet P, Clément C, Cordelier S. Regulation of resveratrol biosynthesis in grapevine: new approaches for disease resistance?. J Exp Bot. 2019;70:375-378.
67. Cavallini E, Matus JT, Finezzo L, Zenoni S, Loyola R, Guzzo F, *et al.* The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYBC2 repressors in grapevine. Plant Physiol. 2015;167:1448-1470.
68. Zhu Z, Li G, Li L, Zhang Q, Han Z, Chen X, Li B. A R2R3-MYB Transcription Factor, VvMYBC2L2, Functions as a Transcriptional Repressor of Anthocyanin Biosynthesis in Grapevine (*Vitis vinifera* L.). Molecules. 2019;24:92.
69. Wang N, Zheng Y, Xin H, Fang L, Li S. Comprehensive analysis of NAC domain transcription factor gene family in *Vitis vinifera*. Plant Cell Rep. 2013;32:61-75.

70. Le Hénanff G, Profizi C, Courteaux B, Rabenoelina F, Gerard C, Clement C, *et al.* Grapevine NAC1 transcription factor as a convergent node in developmental processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance. J Exp Bot. 2013;64:4877-4893.
71. Suzuki M, Nakabayashi R, Ogata Y, Sakurai N, Tokimatsu T, Goto S, *et al.* Multiomics in Grape Berry Skin Revealed Specific induction of the Stilbene Synthetic Pathway by Ultraviolet-C irradiation. Plant Physiology. 2015;168:47-59.
72. Vannozzi A, Wong DCJ, Höll J, Hmamm I, Matus JT, Bogs J, *et al*. Combinatorial regulation of stilbene synthase genes by WRKY and MYB transcription factors in grapevine (*Vitis vinifera* L.).Plant Cell Physiol. 2018;59(5):1043-1059.
73. Ma Q, Yang J. Transcriptome profiling and identification of the functional genes involved in berry development and ripening in *Vitis vinifera*. Gene. 2019;680:84-96.
74. Sun X, Korir NK, Han J, Shangguan LF, Kayesh E, Leng XP, Fang JG. Characterization of grapevine microR164 and its target genes. Mol Biol Rep. 2012;39:9463-9472.
75. Almagro L, Carbonell-Bejerano P, Belchì-Navarro S, Bru R, Martìnez Zapater JM, Lijavetzky D, Pedreno MA. Dissecting the Transcriptional Response to Elicitors in *Vitis vinifera* Cells. Plos One. 2014;9(10):e109777.
76. Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Cumbie JS, *et al.* High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. PLoS One. 2007; 2(2):e219.
77. Xia R, Zhu H, An YQ, Beers EP, Liu Z. Apple miRNAs and tasiRNAs with novel regulatory networks. Genome Biol. 2012;13(6):R47.
78. Zhu H, Xia R, Zhao B, An YQ, Dardick CD, Callahan AM, Liu Z. Unique expression, processing regulation, and regulatory network of peach (Prunus persica) miRNAs. BMC Plant Biol. 2012;12:149.
79. Sunitha S, Loyola R, Alcalde JA, Arce-Johnson P, Matus T, Rock CD. The role of UV-B light on small RNA activity during grapevine berry development. G3 Genes Genomes Genet. 2019;9:769-787.
80. Tirumalai A, Swetha C, Nair A, Pandit A, Shivaprasad PV. miR828 and miR858 regulate *VvMYB14* to promote anthocyanin and flavonol accumulation in grapes. Journal of Experimental Botany. 2019; erz264.
81. Winkler AJ, Cook JA, Kliewer WM, Lider LA. General Viticulture. University of California Press, Berkeley, CA, USA. 1974.
82. Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc. 2010.
83. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17:10-12.
84. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg S. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013;14:R36.
85. Anders S, Pyl PT, Huber W. HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166-169.
86. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. GenomeBiol. 2014;15:550.
87. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 2010;11:R14.
88. Biselli C, Bagnaresi P, Cavalluzzo D, Urso S, Desiderio F, Orasen G, *et al*. Deep sequencing transcriptional fingerprinting of rice kernels for dissecting grain quality traits. BMC Genomics. 2015;16:1091.

89. Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, *et al.* Mapman: a User-Driven Tool To Display Genomics Data Sets Onto Diagrams of Metabolic Pathways and Other Biological Processes. Plant J. 2004;37:914-939.
90. Lohse M, Nagel A, Herter T, May P, Schroda M, Zrenner R, *et al.* Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. Plant Cell Environ. 2014;37:1250-1258.
91. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 2007;3:12.
92. Gomez-Alonso S, Garcia-Romero E, Hermosìn-Gutiérrez I. HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. Journal of Food Composition and Analysis. 2007;20:618-626.

Additional File Legends

Additional file 1: Raw reads and mapping statistics for RNA-seq libraries.

Additional file 2: Correlation coefficient among replicates and samples.

Additional file 3: Principal component analysis (PCA) of all samples in the RNA-seq dataset. The X-axis represents the first components and the Y-axis the second component. Panel A: PCA on all the 36 samples (both seasons) distinguishes between T1 and T2; Panel B: PCA performed only on 2012 samples is able to distinguish between T1 and T2, and between grafted (red circle) and not grafted (blue circle) plants; Panel C: PCA performed only on 2013 samples is able to distinguish between T1 and T2. The two components explain 84%, 90% and 87% of the total variance in the three panels, respectively. Each replicate of the same sample is associated with the same color and ovals indicate samples clustered together by developmental stage (T1 or T2) or by rootstock type (grafted or not grafted). Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; T1 = veraison; T2 = maturity.

Additional file 4: List of differentially expressed genes, indicating for each gene in each comparison FDR, Log2 Fold Change, expression level for each sample as the output of DESeq2 and Blast2GO field description.

Additional file 5: Weather conditions (April 1st - October 31st) of the two vegetative seasons considered. Rainfall = Daily Average Rainfall (mm); T max = Daily Maximum Temperature (°C); T avg = Daily Average Temperature (°C); T min = Daily Minimum Temperature (°C); DGGs = Growing Degree Days; DOY = Day of the Year. The grey arrow indicates the veraison sampling date (T1); the black arrow indicates the maturity sampling date (T2).

Additional file 6: Raw reads and trimming statistics for small RNA-seq libraries.

Additional file 7: Size distribution of sequencing reads, between 16 and 25 nt, for each sample sequenced by small RNA seq, in 2012 and 2013 season. For each year and each sample, it is reported the number of unique-different sequences, and the total number (redundant) of sequences of a given length.

Additional file 8: List of differentially expressed sequence tag, for small RNA seq in 2012 and 2013. For each sequence is given: length, reference miRNA and the reference species, the miRNA type (5' or 3', exact match or shifted) average normalized abundance, log2 Fold Change and FDR.

Additional file 9: Target predicted in silico (psRNA Target), for each differentially expressed sequence in small RNA seq data of 2012 season.

Additional file 10: List of differentially expressed miRNAs (as calculated by small RNAseq analysis) and their relative differentially expressed targets (as calculated by RNAseq data analysis). For each miRNA/target pair is reported the comparison considered, target id and putative function, miRNA name and sequence, and log2 fold change of the target and the miRNA. Only statistically significant DEG and DE miRNAs are reported.

Additional file 11: Expression profiles of the 5 selected miRNAs obtained by qRT-PCR, calculation from Ct value with the $2^{-\Delta\Delta Ct}$ method (the bars indicate the standard error).

Sample names: M = Mgt 101-14; P= 1103 Paulsen; NGC = not grafted control; T1= veraison; T2 = maturity.

Tab.2: List of forward, reverse and stem-loop reverse transcriptase primers used for qRT-PCR to test genes and miRNAs expression. Gene ID/miRNA sequences are specified.

Gene/miRNA	Gene ID/miRNA sequence	Forward Primer (5'3' Seq.)	Reverse Primer (5'3' Seq.)	Stem-loop reverse transcriptase Primer (5'3' Seq.)
PAL	VIT_213s0019g04460	CGCCAAACACAGCCACTCA	GCAGCTTTAGTACCAGTGTCTCCC	--
F3'H A	VIT_209s0002g01090	TCCTACCACCTCACCAACGC	CGAGAGGAGGATAAGAGCCACAGT	--
F3'H B	VIT_217s0000g07200	GCCTCCGTTGCTGCTCAGTT	CGTAGGGAGCGAACACCAGA	--
FLS	VIT_218s0001g03430	TTGATATCCCACGACACACCG	ATTGAGATCAGCACCAGAGGC	--
DFR	VIT_216s0039g02350	TGAGAAGGAGAAACATGCATGCCA	AGGTGACCCATTGCAACTTTCA	--
MYB14	VIT_207s0005g03340	CGGAGAGCCTTGGGTATGGA	TGCAGGGTGTAGTAATGTCGGA	--
MYBC2-L3	VIT_214s0006g01620	CTCACCATTGCCATTCCTGCT	AGGATTTGCGTCACCTTCCAC	--
MYB4R1	VIT_217s0000g02710	CCTCTCTCATTGAAGCCGCTC	GTTTCTGGATTGCACGGAGGA	--
NAC44	VIT_206s0004g00020	GGACGACTGGGTTCTTTGCC	CCATCGTCTTCAGCCACCTC	--
NAC60	VIT_208s0007g07670	ACGTTCGAGCATGGATGGG	CTTTGCGGGAGGTCTGACTG	--
UBI	VIT_219s0177g00040	AATGGTCAGTTGGCCCTACCT	TGGCTGAGACCCACAAAACC	--
miR395	CTGAAGTGTTTGGGGGAACTC	TGACGCTGAAGTGTTTGGGG	GTGCAGGGAGGGAGGT	GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACGAGTTC
miR398	TGTGTTCTCAGGTCGCCCCTG	TCGCTTGTGTTCTCAGGTCG	GTGCAGGGAGGGAGGT	GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACCAGGGG
miR858	CGTTGTCTGTTCGACCTTG	TCGCCCGTTGTCTGTTCG	GTGCAGGGTCCGAGGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGGT
Grape_m-0721	TTACCAACACCTCCCATTCC	TGCGGATTACCAACACCTCC	GAGCTGGGTCCGACGT	GTCGTATCCAGAGCTGGGTCCGACGTATTCGCTCTGGATACGACGGAATG
Grape_m-1191	GCTGAACAAGAGAGAACCT	GCGCGGCTGAACAAGAGA	GAGCTGGGTCCGACGT	GTCGTATCCAGAGCTGGGTCCGACGTATTCGCTCTGGATACGACAGGTTC

3. Research paper in preparation for publication to Agronomy - Special Issue "Tackling Grapevine Water Relations in a Global Warming Scenario"

BERRY QUALITY OF GRAPEVINE UNDER WATER STRESS AS AFFECTED BY ROOTSTOCK-SCION INTERACTIONS THROUGH GENE EXPRESSION REGULATION

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Abstract

Background: The grapevine is one of the fruit-crops most threatened by climate change. Despite the phenotypic plasticity that allows the adaptation to harsh environments, the vines that experience severe abiotic stresses can suffer from serious metabolic damages, with negative consequences on grape production and quality.

Grafting, which is commonly employed in most of the vineyards worldwide to confer tolerance to phylloxera, is an affordable adaptive strategy to mitigate climate change since the rootstock genotypes can influence the scion's behavior increasing vigor and resistance to drought.

This research work aimed to obtain information about the rootstock effects on grape quality in vines subjected to *pre*-veraison water deficit. The activity was set using potted Pinot noir

vines grafted onto two different rootstocks (Mgt 101-14 and 1103 Paulsen) and not grafted vines, as references.

Before, during and after the water stress trial, repeated measurements were carried out to assess the vines' water status, leaf gas exchanges, and photosynthetic efficiency. At harvest, productions, technological and phenolic maturities were detected on grape samples. Moreover, molecular analyses by qRT-PCR were executed on berry skins to assess the expression levels of ten genes and five miRNAs belonging to the phenylpropanoid pathway, that were already found as differentially expressed between the root systems in a previous deep-sequencing project, on the same experimental system, held under optimal irrigation.

Results: The presence of water stress, confirmed by the measurements on grapevines' physiology, did not affect the main production parameters, but caused significant alterations in grape technological maturity. The rootstock effect was not detected on primary metabolism. On the contrary, the accumulation of phenolic compounds in berries was altered both by water deficit and rootstock genotype. Finally, significant differences were identified in the expression of genes (structural or coding for transcription factors), and miRNAs between water-stressed and well-watered vines.

Conclusions: According to our results, the response to water stress can be modulated by the rootstock, which mainly acts by regulating the secondary metabolism in the scion and influencing final grape quality.

Keywords:

Vitis vinifera, rootstock, water stress, berry ripening, secondary metabolism, gene expression, miRNA

Introduction

In recent years, the effect of climate change is one of the most alarming issues in viticulture. It is expected to cause a steady global increase in air temperature and a shift in the rainfall pattern, which may favor the amplification of drought periods (IPCC, 2018). The main areas suited to grapevine cultivation are located in semi-arid climates, within specific latitude bands (Hannah *et al.*, 2013), characterized by hot temperatures and water deficit during the growing season. This kind of climate condition, if worsened, can cause serious damages in the vineyards, endangering the viticulture itself (Jones *et al.*, 2005; Duchêne *et al*., 2010).

Vitis vinifera, one of the most important fruit crops, is a perennial plant with great ability to adapt to environmental constraints, due to its phenotypic plasticity that allows to survive and carry forward grape maturation even in the presence of limiting factors (Lovisolo *et al.*, 2010; Keller, 2010). Nonetheless, the vines that experience severe multiple summer stresses (such as excessive heat and solar radiation, especially if concomitant with water shortage) can suffer from serious physiological and ripening problems. The major climate change-related consequences on grapes are accelerated ripening, reduced berry size, increased sugar accumulation (and consequently excessive alcohol levels in wines), drastic lowering of acidity resulting in arise of pH, delay and decoupling of technological and phenolic maturity (especially for anthocyanin accumulation), decay of aroma profile and presence of atypical flavors. Therefore, grape quality can be strongly impaired (van Leeuwen and Destrac-Irvine, 2017).

Among the recommended adaptive strategies against climate change, it is included the exploitation of some rootstocks able to confer to the scion a higher drought and water deficit tolerance (Pavlousek *et al.*, 2011; Marguerit *et al.*, 2012). The need for resistant

rootstock derived from American *Vitis* species arose in Europe at the end of the 19th century to get the tolerance to phylloxera (*Daktulosphaira vitifoliae Fitch*), a harmful pest that spread, seriously endangering the viticulture itself. Grafting is a phenomenally durable form of biological control, and since its introduction, only a few genotypes (as *Vitis* species or interspecific hybrids) are the most common rootstocks used worldwide (Ollat *et al.*, 2015). Besides overcoming phylloxera, the rootstocks were selected by breeders to provide to the scion further positive traits, modifying its vigor and phenology, but most of all increasing the tolerance to environmental stresses such as soil limestone, high salinity, stagnation, drought, and frost (Corso and Bonghi, 2014; Warschefsky *et al*., 2016).

The rootstock act as an interface between the soil and the aerial portion of the plant (Ollat *et al.*, 2017), but the knowledge on the molecular networks regulating the rootstock-scion interaction mechanisms are still limited, particularly it is interesting to shed light on those pathways influencing grape quality under abiotic stress conditions (Koundouras *et al.*, 2009). The complex rootstock-scion interplay occurs mainly through the exchange of some macromolecules (mRNAs and, mostly, microRNAs) that are mobile from the roots to the shoots and are transferred *via* the phloem sap (Harada, 2010; Buhtz *et al.*, 2010). Moreover, it is currently known that the rootstock can alter gene expression in the scion, especially in the presence of stress, diseases or limiting factors. The major changes at the transcriptomic level concern several structural genes and transcription factors belonging to the phenylpropanoid pathway, responsible for flavonoid and stilbene biosyntheses (Maré *et al.*, 2013; Corso *et al.*, 2015). Flavonoids and stilbenes are two different classes of phenolic compounds that accumulate in berry, mainly in skin tissues, during the ripening phases. As secondary metabolites, they have multiple biological functions, protecting leaves and fruits

against UV photo-oxidative damage, acting as free radicals scavengers, and playing a role against biotic and abiotic stresses (Adams, 2006; Teixeira *et al*., 2013).

Given the risk of severe drought events in traditionally rain-fed wine districts, due to more and more frequent extreme weather seasons related to climate change (Palliotti *et al*., 2014a), this research aimed to study the effects of two rootstocks with opposite characteristics on the grapes produced by vines that have undergone *pre*-veraison water stress. The activity was set up in an experimental system of potted Pinot noir grapevines, which included vines grafted on 1103 Paulsen (drought-tolerant - P) and Mgt 101-14 (drought-sensitive - M), and not grafted vines (NGC). During the 2018 growing season, the pot system was used to test the rootstock-effect applying three different irrigation protocols (severe water deficit - WS-1, intermediate water deficit - WS-2, and well-watered control - WW) on plant physiology and grape quality. Moreover, the expression of ten genes and five miRNA involved in the phenylpropanoid pathway or stress response was tested in berry skins at maturity.

On this purpose, we chose as markers specific genes and miRNAs that we already described as differentially expressed among the three root systems, in a previous work on the same vines, but in conditions of equal water supply (Zombardo *et al*., under publication), to check any expression differences due to a water limitation period during fruit ripening.

In the actual global warming scenario, it is important to investigate the effects of rootstocks, especially on grape quality, in order to exploit their genetic variability for future vineyard management and for improving vine stress tolerance in a sustainable, non-transgenic way.

Methods

Plant materials

A pot system for grapevines monitoring was set at CREA - Research Centre for Viticulture and Enology, in Arezzo (43°28'36'' N, 11°49'27'' E, Italy). It consisted of 70 liters plastic pots, filled by a silty-clay texture soil (40% clay, 41% silt, 19% sand), with a volumetric soil water content of 34% at field capacity, collected from a real vineyard of the Chianti Classico D.O.C.G. district in Tuscany - Italy (Costantini, 2013). The research was made with 12-year-old Pinot noir potted vines, clone ENTAV 115 grafted onto two different rootstock: 1103 Paulsen (P) *V. berlandieri* x *V. rupestris*, highly vigorous and known for its drought tolerance, and Mgt 101-14 (M) *V. riparia* x *V. rupestris*, less vigorous and less tolerant to drought (Palliotti *et al.*, 2015); own-rooted vines were used as references (not grafted control - NGC). The vines were trained on upward vertical shoot positioned trellis, with spur cordon pruning and an average of 10 buds per vine. The pots were positioned in an outdoor area, spaced at the distance of 1 m within the row and 2,5 m between the rows, with orientation north to south and were arranged in a randomized block design with 9 replicates for each root system. The vines were maintained in the same agronomic conditions: all the pots were fertilized before the beginning of the vegetative season with 40 g of Nitrophoska (12N-12P-17K, Eurochem Agro, Italy), and pest management was scheduled with calendar sprays at 10-day intervals. The pots were painted in white to avoid root system overheating and during the summer the canopies were covered with nets against possible attacks by insects and birds. Irrigation was abundantly supplied (at field capacity) to each pot by automated drip emitters from the end of May to harvest (at 3-days intervals), except for the water stress trial period.

Weather conditions

The climate data were recorded during the 2018 vegetative season using a non-stop automated control unit (Ecotech GmbH, Germany) placed nearby the experimental pot system area. The following parameters in the period between April 1st and October 31st (conventionally considered the vegetative period for the grapevine) were measured: Daily Maximum Temperature (°C), Daily Average Temperature (°C), Daily Minimum Temperature (°C); Daily Average Rainfall (mm). The data collected were daily checked and processed at the end of the vegetative season. Growing Degree Days (GDDs) and the Winkler Index were calculated on a 10° C based temperature, according to Winkler (*et al.*, 1974), to get information about the sum of all the daily average temperature that influenced plant growth and grape maturation.

Irrigation protocols

During the 2018 vegetative season, a water stress trial was set on the experimental pot system to get information on the behavior of the three root systems (M, P, and NGC) in case of different water availability. The water stress trial was designedly held during berry growth phase I (Coombe and Mc Carthy, 2000), from cluster closure (BBCH 79) to veraison (BBCH 83). It started on July 4th (DOY 185) and ended on July 29th (DOY 210), when the vines reached full veraison. Following a protocol previously tested at CREA-VE (Puccioni *et al.*, 2016), three irrigation strategies were applied, using a calculated standard water supply apiece. The trial was split into two levels of water stress, a severe deficit (WS-1; 25% of field capacity) and an intermediate deficit (WS-2; 40% of field capacity), which provided for manual water supplies every three days, plus a control level (WW; 90% of field capacity) with

drip irrigation every three days. The actual water stress level in the vines was monitored by midday stem water potential (Ψ_{stem} - see below). During the execution of the water stress trial, the pot surfaces were coated with aluminum foils to oppose soil water evaporation and avoid possible rainfall infiltrations.

Measurements on grapevine physiology

Midday stem water potential, leaf gas exchanges, and chlorophyll fluorescence were measured before (DOY 184), during (DOY 194 and 208) and after the water stress trial (DOY 221, and 232, the latter point with the exception of chlorophyll fluorescence). These parameters were executed to evaluate the starting physiological conditions of the vines, to confirm the onset of water stress, verify the water status and the photosynthetic efficiency of each root system combination and, finally, to check if the recovery after water deprivation occurred. The measurements *per* vine replicate were carried out on three adult fully developed intact leaves grown between the 4th and the 10th node from the shoot base, chosen on the same side of the canopy; the same leaves were used to determine all the parameters in each measurement point.

The midday stem water potential (Ψ_{stem}) was assessed with a Model 670 pressure chamber (PMS Instruments Co., USA), according to Scholander (*et al.*, 1965). Leaf gas exchanges were detected using an infrared gas analyzer (Ciras 1 - PP Systems, USA) as net photosynthesis (Pn), transpiration rate (E), stomatal conductance (*gs*). Chlorophyll fluorescence was assessed using a Handy Peas chlorophyll fluorimeter (Hansatech, UK) as minimal fluorescence - dark (F0), maximum fluorescence - light (Fm); maximum quantum yield of photosystem II (Fv/Fm) was calculated as (Fm-F0)/Fm) (Bussotti *et al*., 2012).

Grape sampling for molecular analyses

Grape samples for molecular analyses were collected at harvest, on August 20th. The harvest date was set at the time when, according to random berry samplings, the grapes reached a minimum sugar content of 20° Brix. Berries (15 per vine) were randomly hand-picked at different positions of the clusters, dissected to separate skin tissues, frozen in liquid nitrogen, and stored in Falcon tubes at -80° C for further processing.

Only two irrigation protocols were considered for gene expression analyses: the most restrictive WS-1 as water stress (WS), and WW, as control. In total, the experiment entailed 18 berry samples: vines with three different root systems (Mgt 101-14 - M, 1103 Paulsen - P, and not grafted control - NGC), two irrigation protocols (water stress – WS, and well-watered - WW), and three vines as biological replicates.

RNA extraction

Total RNA extraction was performed using Plant RNA Isolation Reagent (PRIR – Life Technologies, USA) starting from 200 mg of ground berries skin tissue in 1 ml of reagent, followed by RNA Clean up and Concentration kit (NorgenBiotek Corp., Canada), according to manufacturers' protocols. Total RNA was then subjected to Dnase I treatment (DNA-free™ Kit, Applied Biosystems, USA). The concentration and purity of total RNAs were evaluated using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and their integrity was assessed by an Agilent 2100 Bioanalyzer using an RNA 6000 Nano kit (Agilent Technologies, USA), according to the manufacturer's instructions. All the samples were suitable to obtain meaningful gene expression data, confirmed by consistent RIN outputs (RNA Integrity Number ≥ 7). RNA samples were stored at -80° C for subsequent analyses.

qRT-PCR analyses

The qRT-PCR analyses were performed on ten genes and five miRNAs (Tab.1) already tested in a related experimental work on berry skins of grapes produced by Pinot noir grafted (on Mgt 101-14 and 1103 Paulsen) and not grafted vines (Zombardo *et al.*, under publication).

To evaluate gene expression level, primers were designed in non-conserved coding regions to avoid cross-amplification of genes belonging to multigenic families (Tab.1). cDNA was produced from 200 ng of DNase-treated RNA using the SuperScript II Reverse transcriptase kit (Invitrogen Corp., USA) according to the manufacturer's instructions. Before the setting up of qRT-PCR on the chosen genes, the efficiency of the 10 pairs of primers was tested with successful results on serial dilutions of berry skin cDNA. The Real-Time PCRs were performed in a final volume of 10 µl, with SsoAdvanced Universal SYBR® Green PCR Supermix (BioRad, USA), considering three technical replicates for each sample. The plates were analyzed on a 7300 Real-Time PCR System (Life Technologies, USA) with the following conditions: 95° C for 30 seconds, followed by 40 cycles of 95° C for 10 seconds and 60° C for 1 minute plus 1 cycle for primer dissociation.

miRNAs expression levels were assessed by stem-loop Real-Time PCR (qRT-PCR); the primers (listed in Tab.1) were designed according to Varkonyi-Gasic (*et al.*, 2007). For reverse transcription, a stem-loop primer for each miRNA was used. Stem-loop reverse transcriptase primers consist of a selfed stem-loop sequence, in addition to a specific nucleotide extension at the 3' end, complementary to the last 6 nucleotides at the 3' end of each miRNA of interest. The RT reactions were performed starting from 200 ng of DNase treated total RNA, using Superscript III (Invitrogen Corp., USA), according to the manufacturer's instructions. The reverse transcription products were amplified using a miRNA-specific forward primer

and a reverse primer on the stem-loop adapter. The Real-Time PCR reactions were set up in 25 µl using SYBR Green PCR Master Mix (Applied Biosystem, USA). The three independent biological replicates were analyzed in triplicate, on a 7300 Real-Time PCR System (Life Technologie, USA) with the following conditions: 95° C for 10 minutes, followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute plus 1 cycle for dissociation curve.

After the amplifications, a 7300 Sequence Detection System Software (Applied Biosystem, USA) was used to set the baseline and the threshold for each reaction. The relative quantification was calculated from average Ct values, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2011), considering a poly-ubiquitin transcript (*VvUBI*) as an internal standard. The well-watered protocol (WW) was used as a reference for each root system to highlight the effect of water stress.

Grape yields and technological maturity assessment

At the harvest, the grapes were manually hand-picked and production traits such as yield *per* vine (g), clusters *per* vine (number), average cluster weight (g) were quantified. From the clusters collected *per* each vine, 100 berries were randomly selected to assess technological maturity on must, measuring sugar content (°Brix), pH, and titratable acidity (g/L tartaric acid), according to O.I.V. official methods (O.I.V., 2009). Moreover, during the winter, the 1-year-old pruning wood was weighed (g) with a portable electronic scale.

Berry characteristics and phenolic compound contents

At the harvest, a pool of 30 berries was randomly selected on the clusters collected from each vine to assess phenolic maturity. Total phenolic compounds were assessed in berry skins and seeds, according to the method described by Di Stefano e Cravero (1991). Based on the preparation protocol of the total extracts, average berry weight (ABW), berry skin weight, seed number per berry and seed weight were determined.

Moreover, using the berry skins extracts obtained for the previous chemical analyses (Di Stefano e Cravero, 1991), the contents of phenolic compounds were assessed by HPLC following the method of Gomes-Alonso (*et al.*, 2007). In particular, the berry skin extracts were centrifuged at 3000 rpm for 10 minutes and then an aliquot of 10 mL was dried with a rotary evaporator (VV2000 Heidolp, Germany), and resuspended with 2mL of methanol (HPLC PLUS gradient grade - Carlo Erba Reagents, Italy), 1 mL of HPLC-grade water (Carlo Erba Reagents, Italy) and 0.3 mL of formic acid 98% (PanReac, Spain). Subsequently, the solution was pipetted into a syringe, filtered with Minisart RC 0.45 µm filters (Sartorius, Germany), and injected into HPLC glass vials. The analyses were performed using an 1100 Series HPLC (Agilent, USA), equipped with a solvent degasser, quaternary pump and diode array detector and controlled by a PC running Agilent ChemStation for LC 3D System software (Agilent, USA). A Luna® Omega 5µm Polar C18 Column (Phenomenex, USA) was employed to separate phenolic compounds. Commercial standards of phenolic compounds (Extrasynthese, France) were used as references to calculate individual retention times and to recognize absorption spectra. In total, the experiment comprised 27 berry samples (vines with three different root systems – M, P, and NGC; three irrigation protocols – WS-1, WS-2, WW; three vines as biological replicates).

Statistical analyses

Statistical analyses on the obtained results were done with the software Statgraphics (Statgraphics Technologies Inc., USA). The data were processed by a two-way (Root system x Water protocol) analysis of variance (ANOVA) and statistically significant differences were assumed for $P<0.05$. The mean values were then separated by the LSD post-hoc test ($P<0.05$), except for the mean values of anthocyanin profiles of berry skins that were separated by the Bonferroni test ($P<0.05$).

Results

Weather conditions

The climate data recorded in the experimental area are reported in Fig.1. In general, 2018 was a warm season, with 1457 GDDs accumulated in the period April 1st – August 20nd (harvest date). The sum of GDDs was very similar to the same time frame in 2012, the year when the transcriptomics pilot project was carried out on the experimental pot system (1450 GDDs - Zombardo *et al.*, under publication).

Considering the period of water stress trial (from July 4th – DOY 185, to July 29th – DOY 210), the temperature values recorded were the following: Average T_{max} = 32.8° C; Average T_{avg} = 24.1° C; Average T_{min} = 15.2° C); the maximum daily temperature breached 35° C on four days, with the highest temperature recorded (35.7° C) on July 14th (DOY 195). Few rain events were recorded with a limited amount of rainfall, for a total of 1.6 mm. The GDDs accumulated during the water stress trial were 367. Considering, on the other side, the period from full veraison to harvest (from July 30th – DOY 211, to August 20th – DOY 232), the

temperature values recorded were the following: Average T_{max} = 34.4° C; Average T_{avg} = 25.1° C; Average T_{min} = 16.7° C); the maximum daily temperature breached 35° C on ten days, with the highest temperature recorded (37.7° C, the highest of the whole vegetative season) on August 7th (DOY 219). The amount of rainfall was very scarce, with only 0.4 mm. The GDDs accumulated in this time frame were 319.

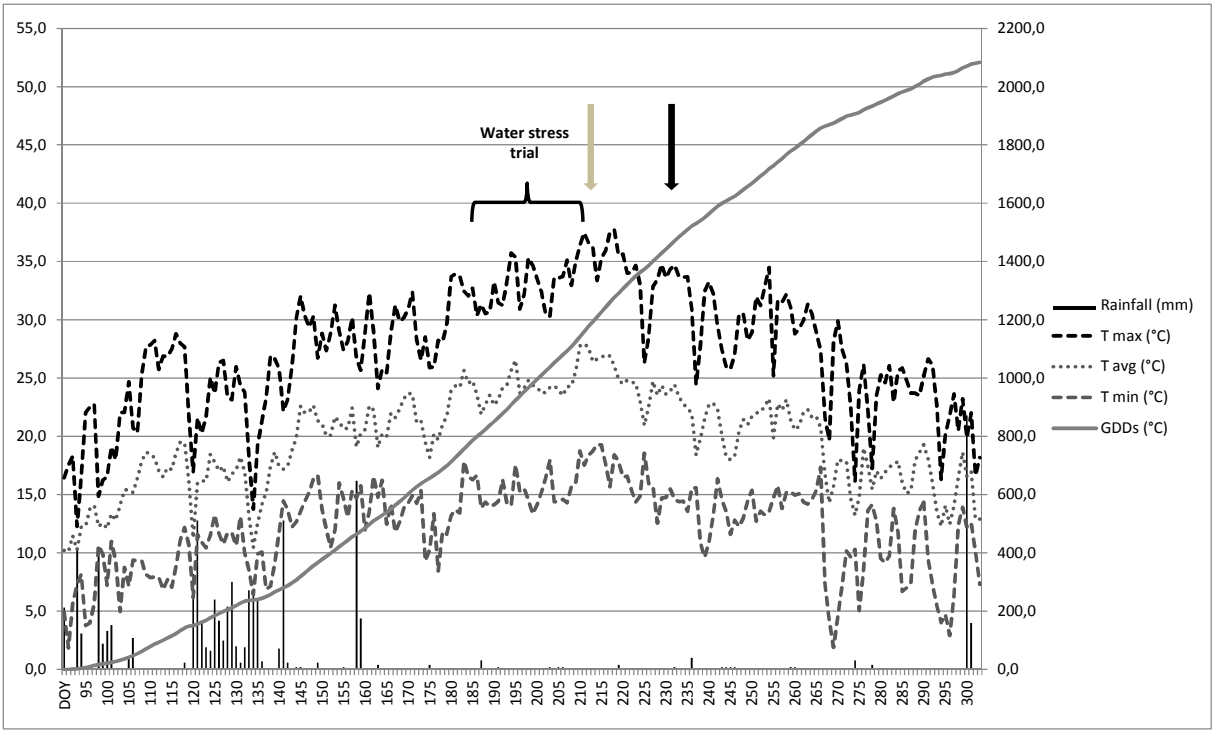

Fig.1. Weather conditions (April 1st - October 31st) of the 2018 vegetative season. Rainfall = Daily Average Rainfall (mm); T max = Daily Maximum Temperature (°C); T avg = Daily Average Temperature (°C); T min = Daily Minimum Temperature (°C); DGGs = Cumulative Growing Degree Days (base 10° C); DOY = Day of the Year. The grey arrow indicates the date of full veraison; the black arrow indicates the date of harvest. The black brace indicates the period of water stress trial.

Measurements on grapevine physiology

The measurement of midday stem water potential (Ψ_{stem}), leaf gas exchanges (*gs*, A, E), and chlorophyll fluorescence (Fv/Fm) executed before (DOY 184), during (DOY 194 and 208) and after (DOY 221 and 232, the latter with the exception of chlorophyll fluorescence) the application of the differentiated irrigation protocols confirmed the onset of water status alterations and a decrease in the photosynthetic performance in the vines subjected to both WS-1 and WS-2, while the vines with optimal irrigation (WW) had more uniform values and maintained their physiological leaf functionality (Fig.2, Fig.3, and Fig.4).

Midday stem water potential measurements were homogeneous among the vines before the application of the differentiated irrigation protocols (-0.69 MPa); during the water stress trial, Ψ_{stem} reached negative values, significantly different in the two reduced irrigation protocols and well-watered vines (-1.33 MPa in WS-1, -1.14 MPa in WS-2, and -0.94 MPa in WW at DOY 194; -1.48 MPa in WS-1, -1.43 MPa in WS-2, and -1.21 MPa in WW at DOY 208). Once irrigation to field capacity was resumed, the water status returned similar among the vines (-1.04 MPa at DOY 221, -0.86 at DOY 232), with more negative values compared to the date before the water stress trial (Fig.2A), probably due to the high temperatures of mid-August. In fact, between veraison and harvest, the daily maximum temperatures often exceeded 35° C (Fig.1). No significant differences were detected in vine water status among the three different root systems (M, P, NGC), in none of the measurement points. Therefore the rootstock did not have any influence on the Ψ_{stem} parameter (Fig.2B).

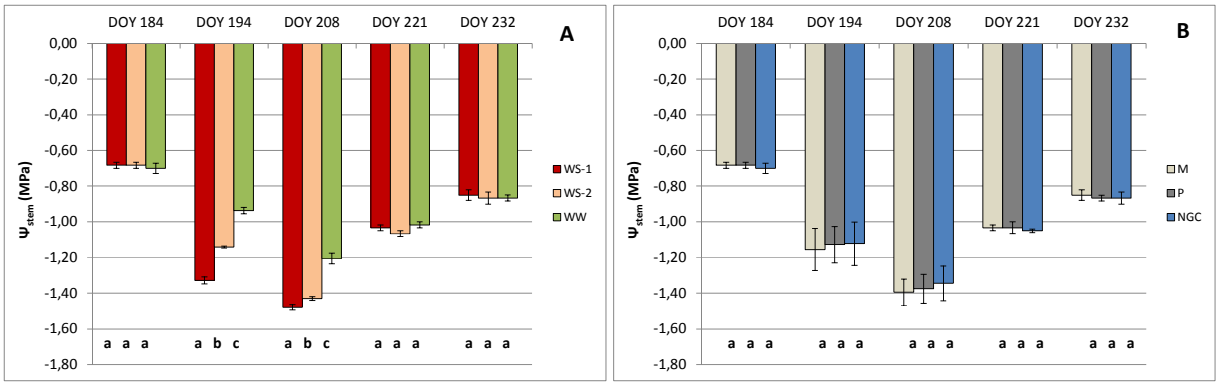

Fig.2. Leaf Water Potentials (Ψ_{stem}, MPa) in adult leaves of Pinot noir, subjected to three irrigations protocols (box A: WS-1 in red, WS-2 in light orange, and WW in green), in the three root systems (box B: Mgt 101-14 - M, in light grey, 1103 Paulsen - P, in dark grey, and not grafted vines - NGC, in blue). The measurements were conducted before (DOY 184), during (DOY 194 and DOY 208) and after (DOY 221 and DOY 232) the water stress trial. The bars indicate the standard error. Different letters indicate significant differences (LSD test, P<0.05).

Regarding leaf gas exchanges (Fig.3), all the vines had similar trends before starting the water stress trial (*gs* = 126 mol H_2O m^{-2} s^{-1}; A = 9.40 µmol CO_2 m^{-2} s^{-1}; E = 5.64 mmol H_2O m^{-2} s^{-1}), but during the water deficit, the vines with limited water supply suffered a sharp drop in stomatal conductance (*gs*: WS-1 = 68.89, WS-2 = 101.94; WW = 165.67 mol H_2O m^{-2} s^{-1} at DOY 194; WS-1 = 11.74, WS-2 = 27.78; WW = 48.50 mol H_2O m^{-2} s^{-1} at DOY 208), net photosynthesis (A: WS-1 = 1.93, WS-2 = 4.07; WW = 7.21 µmol CO_2 m^{-2} s^{-1} at DOY 194; WS-1 = 1.12, WS-2 = 3.51; WW = 4.69 µmol CO_2 m^{-2} s^{-1} at DOY 208), and transpiration (E: WS-1 = 1.78, WS-2 = 2.60; WW = 3.87 mmol H_2O m^{-2} s^{-1} at DOY 194; WS-1 = 0.37, WS-2 = 0.81; WW = 2.12 mol H_2O m^{-2} s^{-1} at DOY 208). Once abundant water was restored, WS-1 and WS-2 vines resumed their functionality, albeit, in some cases, at a lower level than *pre*-stress conditions (*gs* = 124 mol H_2O m^{-2} s^{-1}; A = 7.33 µmol CO_2 m^{-2} s^{-1}; E = 3.77 mmol H_2O m^{-2} s^{-1} at DOY 221; (*gs* = 98 mol H_2O m^{-2} s^{-1}; A = 7.36 µmol CO_2 m^{-2} s^{-1}; E = 3.41 mmol H_2O m^{-2} s^{-1} at DOY 232). The obtained results (Fig.3A-C) showed that the vines subjected to stress during the trial (especially at DOY 208), stimulated photosynthesis and lowered the rate of

transpiration to oppose the water deficit and keep the physiological functions of the leaf active. Moreover, from the values recorded at DOY 208, a stress condition appeared even for well-watered vines. Given that these measurements were made at the maximum distance from the previous irrigation, these results can be explained by the fact that in the days before the surveys the temperatures were particularly limiting (average T max over 35 degrees, and average T min over 24). Despite the low values of the physiological parameters (Ψ_{stem}, *gs,* A, E) in WW vines, significant differences persisted in comparison to the vines subjected to WS-1 and WS-2 irrigation protocols (Fig.3A-C).

The rootstocks had a statistically significant effect on gas exchanges only at the second measurement point, the first included in the water stress trial, namely at DOY 194 (*gs*: M = 111, P = 80; WW = 146 mol H_2O m^{-2} s^{-1}; A: M = 4.09, P = 3.07; NGC = 6.05 µmol CO_2 m^{-2} s^{-1}; E: M = 2.47; P = 1.88; NGC = 3.90). In general, not grafted vines showed better performances. Under optimal irrigation conditions, no significant differences emerged between grafted and not grafted vines (Fig.3D-F).

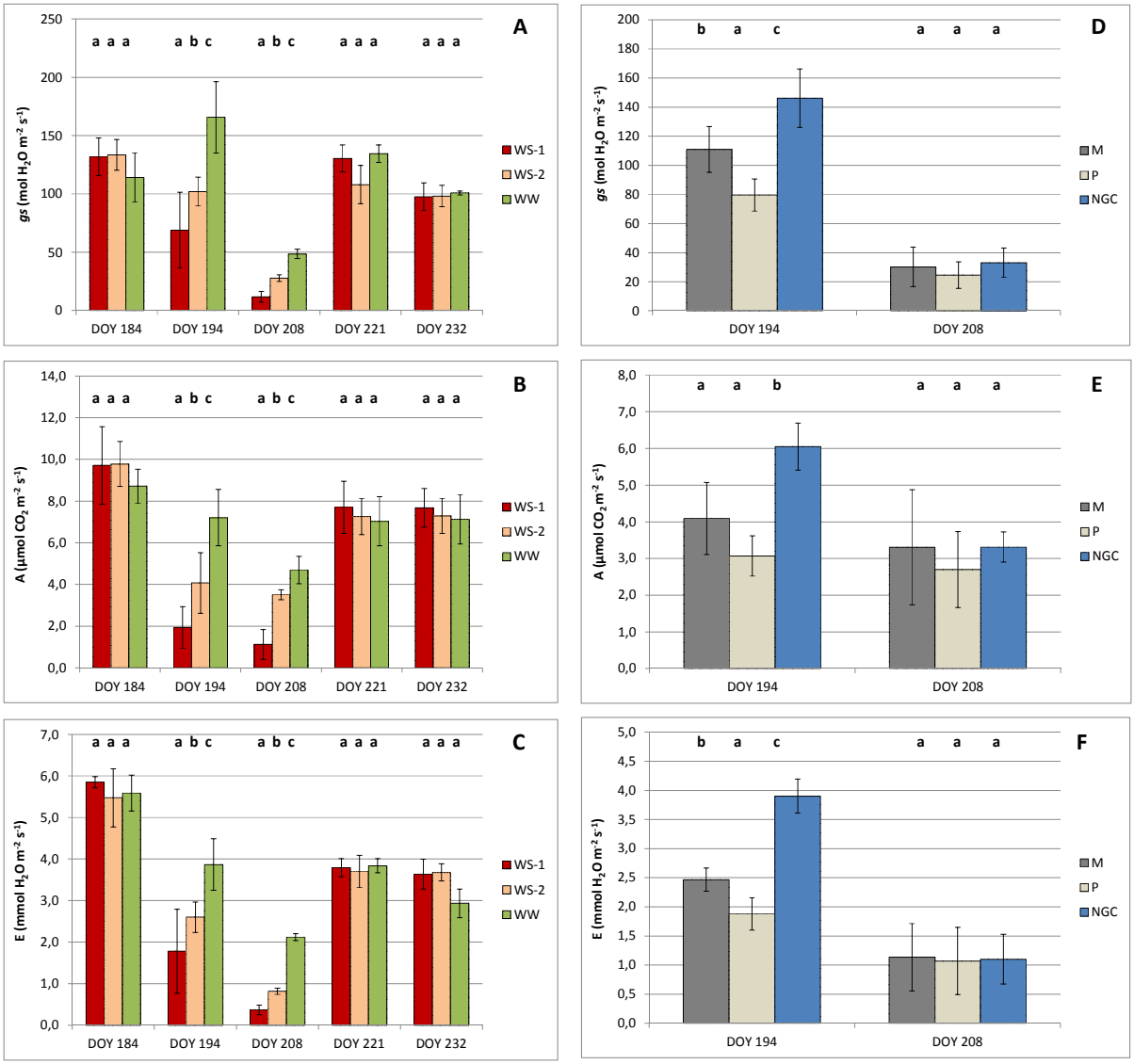

Fig.3. A-C: Stomatal conductance (*gs*), Net Assimilation (A), Leaf Transpiration (E), in adult leaves of Pinot noir, subjected to three irrigations protocols (WS-1 in red, WS-2 in light orange, and WW in green). The measurements were conducted before (DOY 184), during (DOY 194 and DOY 208) and after (DOY 221 and DOY 232) the water stress trial.
D-F: Stomatal conductance (*gs*), Net Assimilation (A), Leaf Transpiration (E), in adult leaves of Pinot noir grafted on Mgt 101-14 (M, in light grey), 1103 Paulsen (P, in dark grey) and not grafted vines (NGC, in blue). The measurements reported refer exclusively to the period of water stress trial (DOY 194 and DOY 208). The bars indicate the standard error. Different letters indicate significant differences (LSD test, P<0.05).

Chlorophyll fluorescence (Fig.4) was detected on the same leaves and in the same days as the other physiological parameters, but excluding the day of harvest (DOY 232).

At the starting conditions (DOY 184) the Fv/Fm parameter was statistically identical for all the vines (0.79). During the water stress trial, this value was significantly lower in the measurements performed on the vines with reduced water supply (WS-1 = 0.73, WS-2 = 0.73, WW = 0.80 at DOY 194; WS-1 = 0.65, WS-2 = 0.71, WW = 0.78 at DOY 208). Once the irrigation was restored, Fv/Fm returned similar among the vines (0.78), although slightly lower in the vines that suffered from water deficit conditions (Fig.4A).

About the root system, it did not determine any difference in chlorophyll fluorescence throughout the investigation period (Fig.4B).

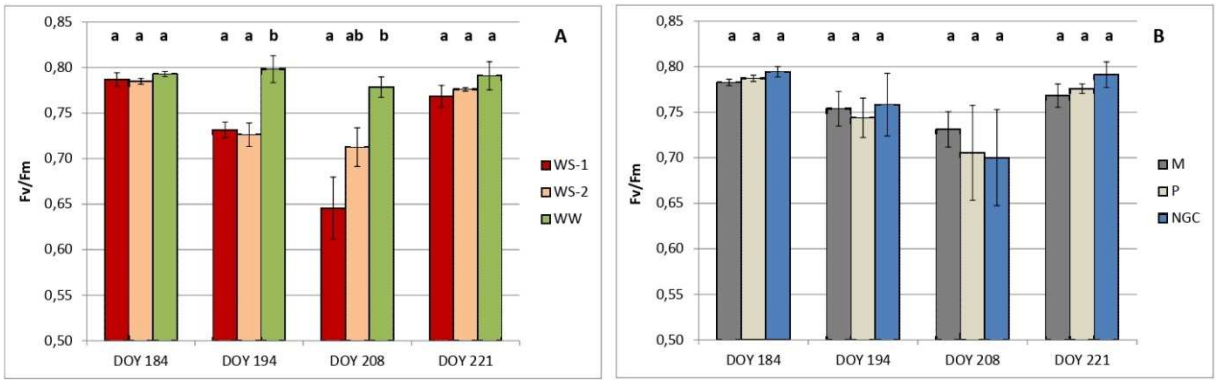

Fig.4. Chlorophyll fluorescence (Fv/Fm) in adult leaves of Pinot noir, subjected to three irrigations protocols (box A: WS-1 in red, WS-2 in light orange, and WW in green), in the three root systems (box B: Mgt 101-14 - M, in light grey, 1103 Paulsen - P, in dark grey, and not grafted vines - NGC, in blue). The measurements were conducted before (DOY 184), during (DOY 194 and DOY 208) and after (DOY 221) the water stress trial. The bars indicate the standard error. Different letters indicate significant differences (LSD test, $P<0.05$).

According to the two-way ANOVA on the results of all the measurements on grapevine physiology, the interaction between root system x water protocol did not have a statistically significant effect, confirming that the differences that emerged were caused by the individual factors.

Grape production and technological maturity assessment

At harvest, all the clusters had a similar, typical of Pinot noir variety shape: small, compact, and cylindrical. The berries had a medium size, spheroidal shape, homogeneous black skin (description according to O.I.V. ampelographic guidelines, 2009). The production surveys (yield *per* vine, clusters *per* vine, average cluster weight) were executed (Tab.2). Yield *per* vine was generally low, mainly due to the condition of pot growth. Although alterations caused by different irrigation protocols were expected (Ollat *et al.*, 2002), most of the production traits measured were similar among the treatments.

According to the results about technological maturity on musts, irrigation regimes significantly affected sugar content (°Brix), titratable acidity (g/L tartaric acid), and pH. In particular, the sugar content was significantly higher in WS-1 and WS-2 vines, as well as pH values (with significant differences also between the two levels of water stress); on the contrary, titratable acidity was significantly higher in WW vines compared to the vines subjected to water-stress. No significant differences emerged between the three root systems, therefore the rootstock effect was not detected on any considered parameters (Tab.2).

Finally, significant differences emerged in the pruning wood weight, due to both the rootstock, with significantly different values among the three root systems (with the highest weight in P, intermediate in M, and lowest in NGC) and the irrigation protocol, with significantly lower values in water-stressed vines compared to well-watered vines.

According to the two-way ANOVA, the interaction between root system x water protocol did not have a statistically significant effect, confirming that the differences that emerged were caused by the individual factors.

Tab.2. Production parameters and technological analyses at harvest on grapes of Pinot noir vines grafted on Mgt 101-14 (M), 1103 Paulsen (P) and not grafted vines (NGC), treated with three irrigations protocols (WS-1, WS-2, and WW). Data were subjected to two-way ANOVA: *, **, ***, ns indicate significant differences at P<0.05, 0.01, 0.001 or not significant. Within Root system and Water protocol, different letters indicate significant differences (LSD test, P<0.05).

	Yield *per* vine		Clusters *per* vine		Average cluster weight		pH		Titratable acidity		Sugars		Pruning wood weight	
	g		n		g				g/L tartaric acid		° Brix		g	
Root system														
M	1028	a	12,4	a	79,31	a	3,21	a	5,69	a	20,7	a	123,0	b
P	872	a	10,3	a	74,95	a	3,19	a	5,73	a	20,2	a	176,8	c
NGC	715	a	9,1	a	75,04	a	3,17	a	5,62	a	21,0	a	70,2	a
Water protocol														
WS-1	943	a	11,4	a	79,59	a	3,30	c	5,35	a	22,4	c	99,5	a
WS-2	768	a	10,2	a	72,81	a	3,20	b	5,32	a	21,0	b	97,0	a
WW	904	a	10,2	a	76,89	a	3,07	a	6,38	b	18,5	a	173,6	b
Root system	ns		ns		ns		ns		ns		ns		**	
Water protocol	ns		ns		ns		***		***		***		**	
A x B	ns		ns		ns		ns		ns		ns		ns	

Berry characteristics and phenolic compound contents

On berry samples collected at the harvest, some parameters related to berry characteristics were evaluated during the preparation of the total extracts, as recommended in the method by Di Stefano and Cravero (1991). Then, total polyphenols of grape skins and seeds and total skin anthocyanins were assessed, to get information about grape phenolic contents (Tab.3).

In this experiment, no differences emerged in average berry weight or berry diameter. On the contrary, water stress led to some differences in skin weights. In particular, WW vines had thinner berry skins, than ones under severe water stress (WS-1). The root system factor caused some differences at the seed level. Not grafted vines had a significantly higher number of seeds *per* berry, but the weight of the individual seeds was lower, especially compared to P vines. Conversely, the water protocols did not affect seed characteristics.

Significant differences arose regarding the accumulation of skin polyphenols, seed polyphenols, skin anthocyanins (expressed in mg/Kg of grapes) depending on both the irrigation protocol and rootstock.

The grapes subjected to water stress (WS-1 and WS-2, equally) showed a higher content of polyphenols in seeds and skins and also a higher content of anthocyanins in the skins (in this case, with the three irrigation protocols clearly separated). Similarly, the vines grafted on P accumulated a higher concentration of polyphenols in skins and seeds and anthocyanins in the skins.

According to the two-way ANOVA, the interaction between Root system x Water protocol did not have a statistically significant effect, confirming that the differences were caused by the individual factors considered.

Tab.3. Berry characteristics and phenolic compound contents at harvest on grapes of Pinot noir vines grafted on Mgt 101-14 (M), 1103 Paulsen (P) and not grafted vines (NGC), treated with three irrigations protocols (WS-1, WS-2, and WW). Data were subjected to two-way ANOVA: *, **, ***, ns indicate significant differences at P<0.05, 0.01, 0.001 or not significant. Within Root system and Water protocol, different letters indicate significant differences (LSD test, P<0.05).

	Average berry weight		Berry diameter		Berry skin weight		Seeds *per* berry		Average seed weight		Skin anthocyanins		Skin polyphenols		Seed polyphenols	
	g		cm		g		n		g		mg/Kg grapes		mg/Kg grapes		mg/Kg grapes	
Root system																
M	1,26	a	1,00	a	0,129	a	2,32	a	0,032	ab	871	a	1556	a	4117	a
P	1,20	a	0,99	a	0,128	a	2,19	a	0,034	b	1034	b	1753	b	4872	b
NGC	1,21	a	0,99	a	0,132	a	2,67	b	0,029	a	945	ab	1583	a	4188	a
Water Protocol																
WS-1	1,25	a	0,98	a	0,149	b	2,29	a	0,031	a	1146	c	1738	b	4782	b
WS-2	1,20	a	0,99	a	0,127	ab	2,36	a	0,033	a	1002	b	1798	b	4431	b
WW	1,23	a	1,00	a	0,110	a	2,53	a	0,031	a	703	a	1357	a	3963	a
Root system	ns		ns		ns		**		*		*		*		**	
Water protocol	ns		ns		*		ns		ns		***		***		**	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

Regarding the anthocyanins profiles, HPLC analyses confirmed the absence of acylated anthocyanins, as typical of Pinot noir grapes (Mattivi *et al.*, 2006).

Analyzing the anthocyanin composition of all the samples considered, some differences emerged in the percentage of the five individual anthocyanins contributing to the profile (Tab.4).

The content of trisubstituted or disubstituted anthocyanins was found to be significantly different due to both root system and irrigation protocol. In particular, the vines grafted on 1103 Paulsen had the highest content in trisubstituted anthocyanins due to a higher accumulation of malvidin, while the vines grafted on M101-14 and not grafted had higher values of disubstituted anthocyanins, due to a higher accumulation of cyanidin and peonidin. Consequently, the ratio between disubstituted and trisubstituted anthocyanins was, as well, significantly different.

Furthermore, also the amount of available water during the water stress trial affected the same parameters of anthocyanin profiles. The WS-1 vines had a higher content of disubstituted anthocyanins due to an increased accumulation of peonidin, while the WS-2 and WW vines were similar, with a higher content of trisubstituted anthocyanins, due to an increased accumulation of delphinidin, petunidin and malvidin (the latter only in WW vines).

Tab.4. Anthocyanin profiles of berry skins of Pinot noir vines grafted on Mgt 101-14 (M), 1103 Paulsen (P) and not grafted vines (NGC), treated with three irrigations protocols (WS-1, WS-2, and WW). Data were subjected to two-way ANOVA: *, **, ***, ns indicate significant differences at P<0.05, 0.01, 0.001 or not significant. Within Root system and Water protocol, different letters indicate significant differences (LSD test, P<0.05).

	Delphindin		Cyanidin		Petunidin		Peonidin		Malvidin		Trisubstituted anthocyanins		Disubstituted anthocyanins		Trisubstituted Disubstituted Ratio	
	%		%		%		%		%		%		%			
Root system																
M	4,47	b	2,21	b	6,25	b	29,15	b	57,93	a	68,64	a	31,36	b	2,21	a
P	3,69	a	1,43	a	5,56	a	23,96	a	65,36	b	74,61	b	25,39	a	3,19	b
NGC	3,77	a	2,03	b	5,56	a	28,71	b	59,94	a	69,26	a	30,74	b	2,41	a
Water Protocol																
WS-1	3,61	a	1,92	a	5,40	a	30,24	b	58,83	a	67,84	a	32,16	b	2,20	a
WS-2	4,15	b	1,85	a	6,03	b	26,16	a	61,81	ab	71,99	b	28,01	a	2,74	ab
WW	4,15	b	1,90	a	5,94	b	25,42	a	62,59	b	72,68	b	27,32	a	2,87	b
Root system	*		***		*		*		*		*		*		*	
Water protocol	*		ns		*		*		*		*		*		*	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

HPLC analyses on berry skins allowed to separate in chromatograms some phenolic compounds accumulated in the skins, which were detected at different wavelengths (Tab.5). Some differences emerged in the content of the catechins, due to the root system only; no difference was determined by the water supply. In particular, the vines grafted on rootstock M had the highest concentration of procyanidin B1 and epicatechin in their berry skins, and the lowest concentration was in not grafted vines (NGC).

The results confirmed the already known flavonol profile of Pinot noir, which is characterized by a high content of quercetin, followed by lower amounts of myricetin and minimum quantities of kaempferol and isorhamnetin (Mattivi *et al.,* 2006). No significant differences arose in the accumulation of flavonols in berry skins, neither due to the root system, nor the irrigation protocol.

Regarding the quantity of hydroxycinnamoyl tartaric acids (HCTA), the root system has determined some differences in the content of trans-caftaric acid and trans-fertaric acid;

here too, the highest concentrations were in the grapes coming from vines grafted on the M rootstock, and the lowest in not grafted vines (NGC).

Finally, also the stilbenes content had significant differences in some compounds due to the water supply (resveratrol) or both the root system and water supply (trans-ε-viniferin). Resveratrol and trans-ε-viniferin were more accumulated in the vines subjected to severe water deficit. Moreover, a higher content of trans-ε-viniferin was found in M vines.

Tab.5. Phenolic compounds detected by HPLC in berry skins of Pinot noir vines grafted on Mgt 101-14 (M), 1103 Paulsen (P) and not grafted vines (NGC), treated with three irrigations protocols (WS-1, WS-2, and WW). Data were subjected to two-way ANOVA: *, **, ***, ns indicate significant differences at P<0.05, 0.01, 0.001 or not significant. Within Root system and Water protocol, different letters indicate significant differences (LSD test, P<0.05).

	Procynidin B1		Epigallocatechin		Catechin		Epicatechin		Quercetin		Myricetin		Kaempferol		Isorhamnetin	
	HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area	
Root system																
M	61,67	b	49,40	a	26,18	a	35,47	b	2261,67	a	1194,99	a	205,27	a	113,61	a
P	52,82	ab	42,51	a	33,65	a	25,67	a	1991,94	a	1055,96	a	177,42	a	111,89	a
NGC	44,53	a	37,69	a	23,70	a	25,57	a	2153,76	a	985,35	a	204,85	a	109,74	a
Water Protocol																
WS-1	54,53	a	41,31	a	21,37	a	32,57	a	1957,17	a	1079,94	a	212,03	a	118,30	a
WS-2	50,53	a	41,32	a	25,27	a	27,89	a	2065,51	a	1016,93	a	169,82	a	103,54	a
WW	53,97	a	46,97	a	36,88	a	26,24	a	2384,69	a	1139,42	a	205,69	a	113,41	a
Root system	*		ns		ns		*		ns		ns		ns		ns	
Water protocol	ns		ns		ns		ns		ns		ns		ns		ns	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

	Protocatechuic acid		Trans-caftaric acid		Cis-cutaric acid		Trans-cutaric acid		Trans-fertaric acid		Polydatin		Resveratrol		Trans-ε-viniferin	
	HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area		HPLC area	
Root system																
M	17,89	a	26,75	b	154,94	a	112,27	a	38,72	b	354,84	a	127,73	a	7,31	b
P	14,78	a	24,18	ab	112,91	a	94,27	a	28,89	ab	287,15	a	100,94	a	5,00	a
NGC	16,36	a	21,64	a	111,19	a	98,29	a	26,39	a	373,48	a	105,65	a	6,18	ab
Water Protocol																
WS-1	19,40	a	24,62	a	119,76	a	103,75	a	34,38	a	348,30	a	136,86	b	7,58	b
WS-2	16,10	a	23,69	a	124,01	a	98,39	a	28,42	a	320,27	a	85,96	a	5,15	a
WW	13,53	a	24,27	a	135,26	a	102,70	a	31,19	a	346,90	a	111,50	ab	5,76	ab
Root system	ns		*		ns		ns		*		ns		ns		*	
Water protocol	ns		ns		ns		ns		ns		ns		*		*	
A x B	ns		ns		ns		ns		ns		ns		ns		ns	

qRT-PCR analyses of gene and miRNA expressions

The molecular analyses included berry skin samples belonging to vines having three different root system combinations (M, P, NGC), subjected to two different irrigation levels only: the WS-1 protocol (severe water stress, indicated hereafter as WS), and well-watered vines (WW), as control.

qRT-PCR reactions were executed to assess the expression levels of specific genes and miRNAs and check for any transcript differences due to early water stress. The ten genes studied are all involved in some key points of the phenylpropanoid pathway, as structural genes (*PAL - PHENYLALANINE AMMONIA LYASE*, 2 copies of *F3'H - FLAVONOID 3'-HYDROXYLASE*, *FLS - FLAVONOL SYNTHASE*, and *DFR - DIHYDROFLAVONOL-4-REDUCTASE*) or genes coding for transcription factors belonging to MYB (*MYB14, MYB4R1,* and *MYBC2-L3*) and *NAC* (*NAC44*, and *NAC60*) gene families; the five miRNAs are involved in the regulation of secondary metabolism (miR858, Grape_m-1191, Grape_m-0721), or stress response (miR395 and miR398). The results of the qRT-PCR were elaborated with the $2^{-\Delta\Delta Ct}$ method, normalizing the samples with a poly-ubiquitin transcript (*VvUBI*) as an internal standard and well-watered protocol (WW) as a reference for each root system. The values obtained for each gene are shown in Fig.5 and for each miRNA in Fig.6.

The *VvPAL* gene (vit_13s0019g04460) encodes a *PHENYLALANINE AMMONIA-LYASE*, the enzyme responsible for the first step of the phenylpropanoid pathway. In particular, PAL catalyzes the deamination of phenylalanine to trans-cinnamic acid, the common substrate of the subsequent reactions (Boss *et al*., 1996). No differences were detected for this gene between water-stressed and irrigated grapevines (Fig.5A).

Both copies of the *VvF3'H* genes (VIT_209s0002g01090 and VIT_217s0000g07200) present in the grapevine genome (Falginella *et al.*, 2010) were analyzed. They encode for two isoforms of *FLAVONOID 3'-HYDROXYLASE*, an enzyme that performs the hydroxylation of dihydrokaempferol at the 3' position of the B-ring, leading to the respective flavonols, anthocyanidins, and proanthocyanidins (Bogs *et al.*, 2006). In particular, *F3'H* is responsible for the bifurcation of the metabolic pathway of anthocyanins synthesis by competing with *F3'5'H* (*FLAVONOID 3',5'-HYDROXYLASE*) for substrate recruitment; *F3'H* and *F3'5'H* deliver 3'OH or 3',5'OH products, namely trisubstituted or disubstituted anthocyanins, precursors of red and blue skin pigments, respectively. From our results, it emerged that the transcript profile of *F3'H A* (VIT_209s0002g01090) is strongly upregulated in WS vines of NGC, while in grafted vines (both M and P) no differences were detected between WW and WS (Fig.5B).

The other copy, *F3'H B* (VIT_217s0000g07200) was not detected as differentially expressed between WW and WS, this gene maintained a constant expression level, without variability among the vines considered (Fig.5C).

The *VvFLS* gene (vit_18s0001g03430) encodes a *FLAVONOL SYNTHASE*, an enzyme that catalyzes the formation of flavonol aglycons from dihydrokaempferol, dihydromyricetin, and dihydroquercetin (Downey *et al*., 2003). From the data obtained, no differences due to the different water supply emerged between the vines grafted on M rootstock, while the vines grafted on P and the not grafted vines showed an opposite behavior: in P berry skins, the presence of *FLS* transcript was almost doubled in WS compared to WW; in NGC, the expression was considerably higher in WW than in WS (Fig.5D).

The substrates of *FLS* (dihydroflavonols) are common to the enzyme DIHYDROFLAVONOL-4-REDUCTASE, coded by the *VvDFR* gene (vit_16s0039g02350). DFR carries out the first step of

anthocyanidin synthesis, converting dihydroflavonols into leucoanthocyanidins (Bogs *et al.*, 2006). The results obtained for *VvDFR* showed a significantly different gene expression in all the three root systems in WS *vs* WW vines. In particular, NGC vines showed an expression rate tripled compared to the control, while smaller differences emerged in the grafted (both M and P) vines (Fig.5E).

The *VvMYB14* gene, involved in the feedback regulation of resveratrol biosynthesis (Fasoli *et al.*, 2018), displayed a significant upregulation in the three root systems, with a higher expression level in NGC-WS and P-WS vines. On the contrary, *MYB14* in M is overexpressed under the WW protocol (Fig.5F).

The *VvMYBC2-L3* gene acts as a transcriptional repressor in the synthesis of anthocyanins and proanthocyanidins (Cavallini *et al.*, 2015). All the three root systems displayed a significant down-regulation of *MYBC2-L3* under water stress, with a lower expression decrease in grafted vines (Fig.5G).

Also *VvMYB4R1* gene codes for a transcription factor involved in stilbene biosynthesis, but few further details on its actual functions are known (Vezzulli *et al.*, 2019). *MYB4R1* expression was significantly upregulated in two cases: M-WS and NGC-WS vines, whereas there were no differences between WS and WW in P vines (Fig.5H).

The *VvNAC44* gene, involved in berry ripening and stress response (Suzuki *et al.*, 2015), was significantly differentially expressed in all the root system combinations, with strongly higher expression in WS (3-fold in M, 5-fold in P, and nearly 7-fold in NGC) compared to irrigated controls (Fig.5I).

Finally, the *VvNAC60* gene, considered a master regulator in the transition from unripe to ripe grape berries (Palumbo *et al*., 2014), was observed as up-regulated in P-WS, but above

all in NGC-WS vines, while no differences between Ws and WW were found in the M vines (Fig.5J).

Comparing the qRT-PCR results, the ten genes were generally more expressed in WS vines, except for MYBC2-L3, a transcription factor (repressor) that was down-regulated by water deficit. Hence, the application of early water stress caused lasting effects on grape quality, manifested by altered gene expression in berry skins at maturity.

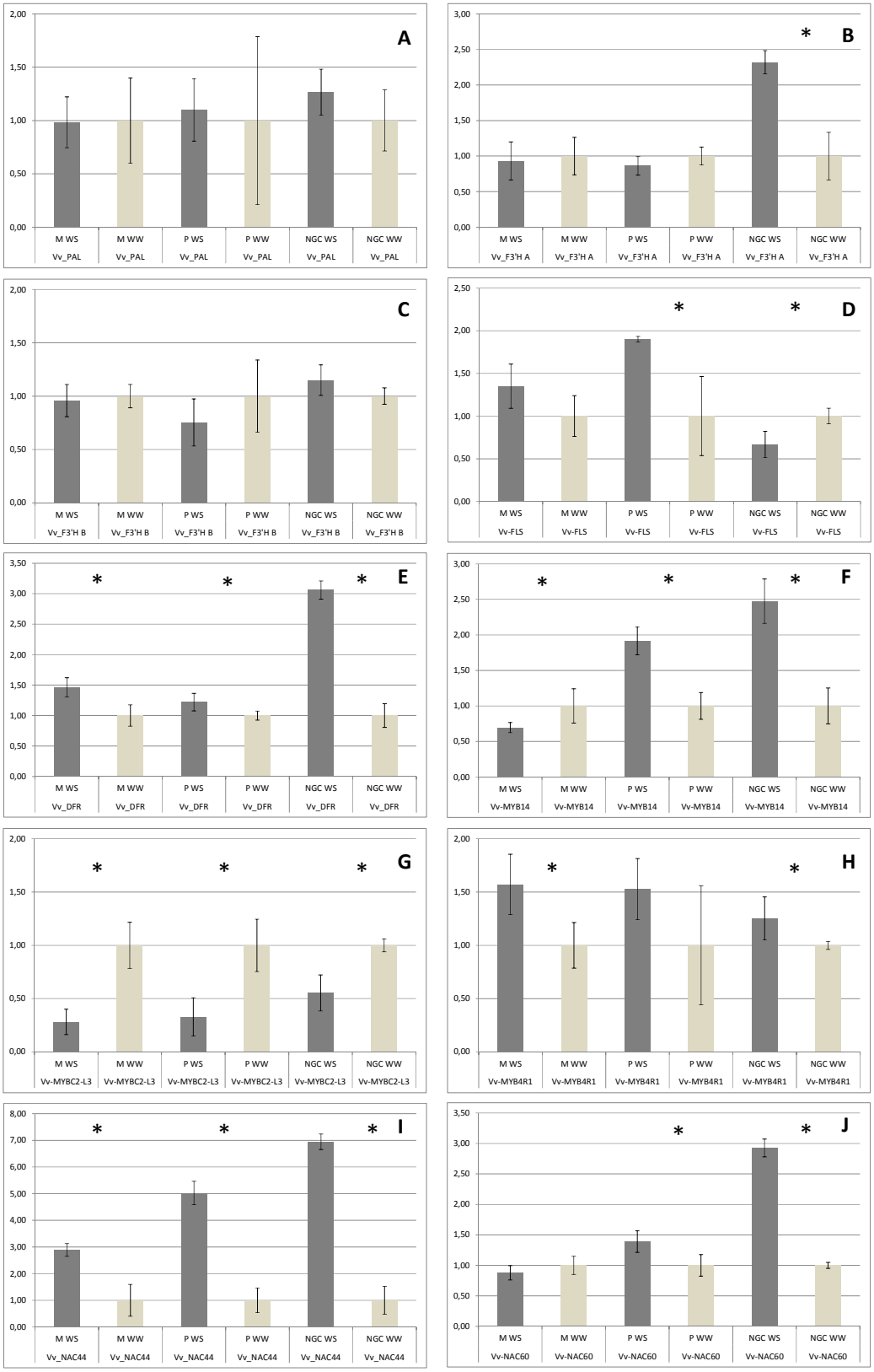

Fig.5. Expression profiles of the ten selected genes coding for structural genes and transcription factors obtained by qRT-PCR, calculation from Ct value with the $2^{-\Delta\Delta Ct}$ method (the bars indicate the standard error). Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; WS = water stress; WW = well-watered. Asterisks mean a significant difference (P<0.05).

miR395 is known to be involved in sulfate uptake (Kawashima *et al*., 2009). Although the sulfate metabolism is poorly understood in grapevine, several enzymes and flavonoid transporters are known to require sulfur, thus miR395 may have a role in the phenylpropanoid pathway (Tavares *et al*., 2013). miR395 was found as downregulated in M-WS vines and up-regulated in P-WS vines, showing an opposite behavior among the two rootstocks considered. In NGC no differences were detected between the two irrigation levels (Fig.6A).

miR398 plays an important role in the mechanisms of dissipation of oxidative stress (Jagadeeswaran *et al*., 2009). The mature sequence of miR398 targets a family of Cu/Zn superoxide dismutase genes that detoxify reactive oxygen species (Zhu *et al.*, 2011). According to the results, miR398 was differentially expressed only in P vines, with higher accumulation in WS protocol (Fig.6B).

miR858 is known to be one of the master regulators of *MYB* genes (Tirumalai *et al.*, 2019). It was down-regulated in both the grafted root systems (M and P), while no differences were found between WW and WS treatments in not grafted vines (Fig.6C).

Grape_m-1191 is a grapevine specific miRNA, and has some functional genes of secondary metabolism as targets (Paim Pinto *et al.*, 2016); one of them is homologous to transparent testa 12 (tt12), responsible for flavonoid transport into the vacuole in *Arabidopsis thaliana*. Grape_m-1191 was observed as down-regulated in NGC-WS vines only (Fig.6D).

Grape_m-0721 is another grapevine specific miRNA and has some functional genes of secondary metabolism as targets, such as *ANTHOCYANIN 5-AROMATIC ACYLTRANSFERASE-LIKE*, a *STEROL OXIDASE* and an *ANTHOCYANIDIN 5,3-O-GLUCOSYLTRANSFERASE* (Belli

Kulhan *et al*., 2015). Here too, only NGC vines showed down-regulation in case of water deficit (Fig.6E).

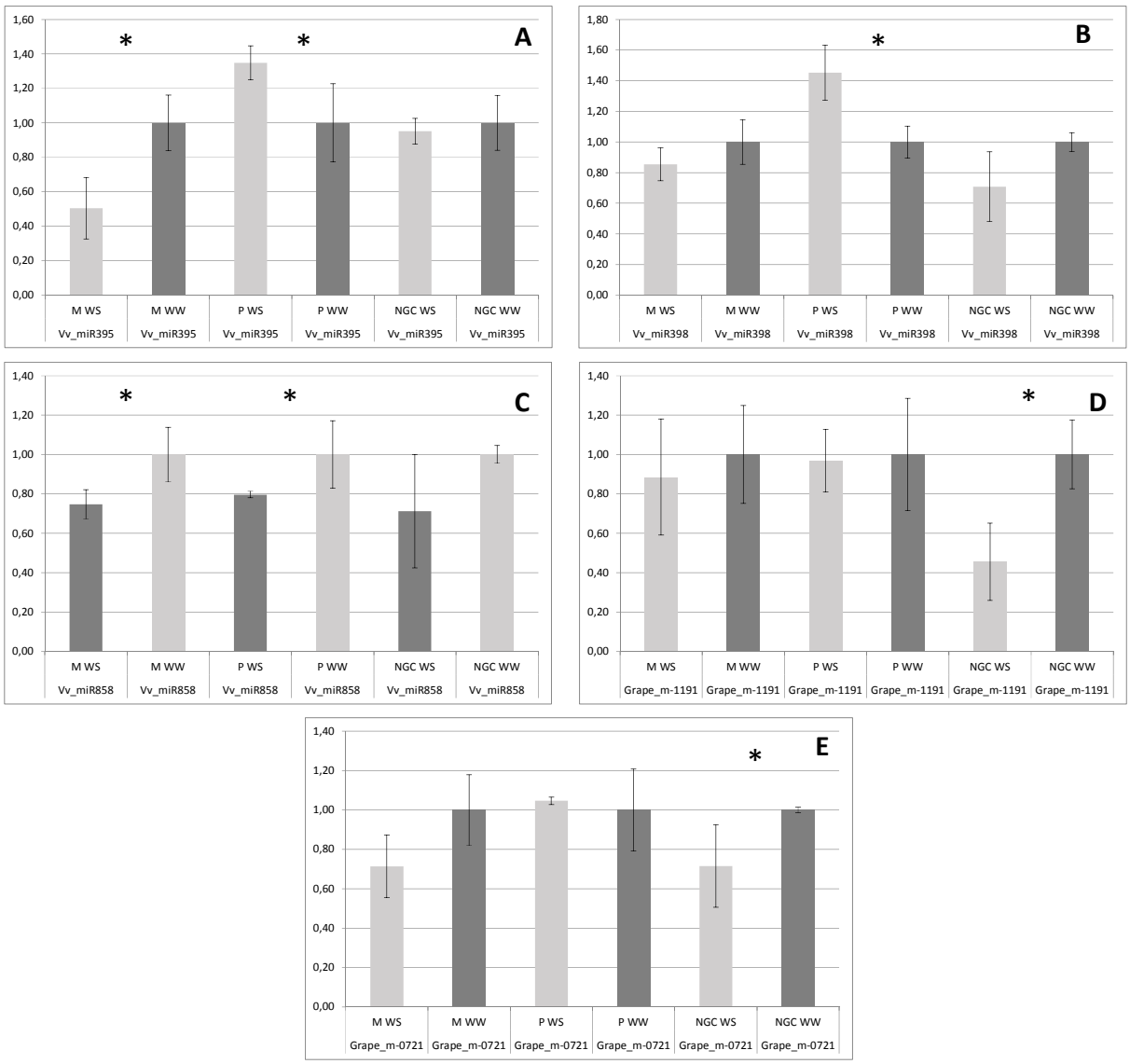

Fig.6. Expression profiles of the five selected miRNAs (miR395, miR398, miR858, Grape_m-0721, Grape_m-1191) obtained by qRT-PCR, calculation from Ct value with the $2^{-\Delta\Delta Ct}$ method (the bars indicate the standard error). Sample names: M = Mgt 101-14; P = 1103 Paulsen; NGC = not grafted control; WS = water stress; WW = well-watered. Asterisks mean a significant difference ($P<0.05$).

Discussion

The burden of climate change is a major concern among winemakers because the grapevine, despite its marked ability to adapt to environmental adversities, is one of the fruit crops most sensitive to drought conditions and water shortage (Jones *et al.*, 2005; Chaves *et al.*, 2010; Goode *et al*., 2012). According to reliable forecasts, climate change is expected to cause a steady global increase of air temperature (with warming up to 1.5° C between 2030 and 2052) and a strong modification in the precipitation pattern (IPCC, 2018). In viticulture, the presence of moderate stress is desirable to guarantee higher quality in the chemical composition of red grapes and confer positive organoleptic characteristics to the wines produced (Peterlunger *et al*., 2005; van Leeuwen *et al*., 2009; Ferrandino and Lovisolo, 2014). But if the environment becomes excessively limiting due to the combined effect of drought, high air temperature and high evaporative demand during summer, the vines can suffer from serious damage and grape yield and berry quality can be strongly affected (Fraga *et al*., 2012; Palliotti *et al*., 2014a). The rootstock, an element of mandatory use in vineyard design, is able to confer to the scion a higher drought and water deficit tolerance, and, for this reason, can actually be exploited for the mitigation of global warming (van Leeuwen and Darriet, 2016).

The present research work was conceived to investigate the role of two rootstocks, with opposite characteristics in terms of resistance to drought given to the scion, on berry quality in grapes that have undergone early water stress. The activity was carried out using an experimental system of potted Pinot noir vines that was carefully described in a previous paper (Zombardo *et al*., under publication). It was conceived to simulate the open field conditions. Using a controlled experimental system it was possible to keep control over

many external variables, primarily water availability, and improve the quality of the phenotyping activities. In view of gene expression analyses, the cultivar Pinot noir clone ENTAV115 was specifically chosen as the genome sequence is fully mapped (Jaillon *et al*., 2007; Velasco *et al*., 2007). Moreover, the use of pots made it possible to insert not grafted vines as a control, normally not employable in a commercial vineyard.

A water stress trial was set up to highlight the differences given by the two tested rootstocks (Mgt 101-14 or M and 1103 Paulsen or P) on the production and quality of the grapes, compared to not grafted vines (NGC). The application of three differentiated irrigation protocols (severe deficit or WS-1, intermediate deficit or WS-2, and well-watered or WW) lasted 25 days during July 2018, and it was specifically performed between cluster closure and veraison. According to many scientists (Ojeda *et al.*, 2001; Ollat *et al*., 2002; Keller, 2010), in fact, the onset of water shortage straddling the first phase of berry growth (phase I, or herbaceous phase) can have remarkable effects on berry size or on the accumulation of some primary and secondary metabolites (such as sugar contents, anthocyanins, resveratrol), compared to a stress occurred in post-veraison (phase II, or ripening phase). Moreover, recovery is unlikely to take place, even if the water returns abundantly available.

The physiological behavior of the grapevines confirmed the presence of water stress during the application of differentiated irrigation protocols

During the months of July and August, repeated measurements were carried out to assess the physiological status of each vine involved in the experimental system, to verify the presence of water shortage induced by the two different irrigation protocols (WS-1 and WS-

2) within the water stress trial period and to evaluate any possible differences between the root systems.

From the results obtained from water potentials, leaf gas exchanges, and chlorophyll fluorescence, all the vines were in similar conditions before the water stress trial. Subsequently, the vines that received lower water supplies showed a much more negative water potential than irrigated control (WW). Also, the values related to leaf gas exchanges and photosynthetic efficiency were considerably reduced, confirming that the water stress was induced as required by WS-1 and WS-2 protocols (Poni *et al*., 1993; Lovisolo *et al*., 2010; Palliotti *et al*., 2014b). Once abundant irrigation was restored, some values returned similar to *pre*-stress levels (Fv/Fm – Fig.4A), while others (e.g. Ψ_{stem}, *gs*, A, E) reached a lower threshold than the first measurement point (Fig.2A and Fig.3A-C), due to the more restrictive climatic conditions between the end of July and the first half of August, from veraison to maturity (Fig.1), and probably also due to the ageing of the leaves (Poni and Intrieri, 2001). According to the collected data, the rootstocks showed some significant effects on leaf gas exchanges only during the first phase of water deficit (as shown at DOY 194), while before and after the water stress trial no differences were detected between grafted (both M and P) and not grafted (NGC) vines (Fig.3D-F).

Root system and water supply did not affect grape production, but water deficit had a strong influence on the technological maturity

It is well known that water deficit affects grape production both in terms of yield and quality but, as already mentioned, fruit development and chemical composition can greatly vary depending on the phenological stage in which the stress occurs, its severity and the actual

duration (Chaves *et al.*, 2010). Moreover, the results in the literature are often contradictory and highlight dissimilar physiological responses depending on grapevine variety and cultivation environment (Castellarin *et al.*, 2011).

From the obtained results, no differences emerged in yield *per* vine, cluster number and weight, and berry size and diameter of the vines subjected to severe (WS-1) or intermediate (WS-2) water deficits (Tab.2). Therefore, it can be deduced that, in our experimental system, the grape production was affected neither by the irrigation protocol nor by the root system.

Simultaneously to grape total extracts preparation, it was noted that the vines that suffered severe water stress had a higher skin weight, while the number and weight of the seeds were determined by the type of root system only.

A parameter found to be significantly different was the pruning wood weight, which was influenced by both rootstock and irrigation protocol factors. The highest values, in particular, were obtained under optimal irrigation conditions (WW) and for the vines grafted on 1103 Paulsen, a rootstock that is known to confer greater vigor in the scion (Bauerle *et al.*, 2008).

As regards technological maturity, the different water supplies strongly conditioned the accumulation of sugars (°Brix), the titratable acidity (g/L tartaric acid) and the pH in the berries (Tab.2). The information regarding the effect of water stress on primary metabolism is often controversial (Castellarin *et al.*, 2011), but some studies on Pinot noir showing trends similar to our results are found in the literature (Girona *et al.*, 2006).

On the contrary, no influence given by the type of rootstock was highlighted on technological maturity parameters. Therefore, as previously seen on the same experimental system (Zombardo *et al.*, under publication), it is possible to confirm that grafting on Mgt 101-14 and 1103 P rootstocks did not affect the primary metabolism of Pinot noir potted

grapevines. The flattening of the results on primary metabolism can be explained by the condition of pot-growth, in which the vines were not as efficient as in the open field, and the differences given by the individual M and P genotypes could have been compressed. A controlled experimental system is important for the undisturbed study of the plant behavior but can cause suffering to adult vines, such as those considered.

Phenolic compounds accumulation was influenced in berries both by water supply and root system

From the analyses on total extracts, we found that the vines subjected to water stress (in both WS-1 and WS-2 protocols) accumulated more total polyphenols in the berry tissues considered (skin and seed), and had a higher concentration of total anthocyanins in the skins (Tab.3). Therefore, the vines that suffered from water deficit had the same productivity of well-watered vines but accumulated a greater quantity of secondary compounds in mature berries. These results are in agreement with many published papers demonstrating that water deficit significantly affects the accumulation of polyphenols in grapes, with skin anthocyanins that normally show higher sensitivity to a possible water shortage (Castellarin *et al*., 2007; Koundouras *et al.*, 2009; Corso *et al.*, 2015). Even the rootstock factor had a significant influence on these parameters. In fact, in 1103 Paulsen the accumulation of secondary metabolites in skins and seeds was greater than in the other two root systems.

The HPLC analyses on secondary metabolites in berry skins showed important differences in the accumulation of some compounds synthesized within the phenylpropanoid pathway. In particular, the anthocyanin profile was significantly altered by both root system and water status (Tab.4). Anthocyanins are the molecules responsible for the color of grapes and,

although each cultivar has a conserved and distinctive anthocyanin profile, it can vary considerably depending on the environmental conditions (Guidoni *et al*., 2008). The alternative accumulation of trisubstituted rather than disubstituted anthocyanins is due to a bifurcation in the anthocyanin synthesis pathway and is linked to the activity of F3'H and F3'5'H, enzymes that compete for common substrates and convey their reaction products to the synthesis of one or the other anthocyanin class (Castellarin *et al*., 2006). In enology, higher concentrations of trisubstituted anthocyanins in berry skins are desirable, especially in grapes lacking acylated anthocyanins, such as Pinot noir and Sangiovese (Mattivi *et al*., 2006), since malvidin-like anthocyanins confer greater color stability in red wines and better aging aptitude (De Freitas *et al*., 2017). In the present work, a higher accumulation of trisubstituted anthocyanins was favored by the WS-2 irrigation protocol (intermediate water stress), and in WW vines, as well as by the rootstock P (the most drought-tolerant).

The concentrations of other phenolic compounds detected were influenced by the root systems, such as catechins and HCTA, or by both root system and irrigation protocol, such as stilbenes (Tab.5).

About stilbenes, their concentration in grapes is generally low, but it can burst in the case of pathogen attacks or at the presence of limiting factors (Jeandet *et al*., 2010). In our results, the concentration of resveratrol (3,5,4’-trihydroxy-*trans*-stilbene), the main stilbene in grapes, and trans-ε-viniferin (a cyclic oligomer of resveratrol) was favored by the severe water deficit (WS-1), confirming that these molecules play a key role in the response to abiotic stress. Furthermore, trans-ε-viniferin was accumulated in higher quantities in berry skins of the vines grafted on M rootstock. The accumulation mechanisms of stilbenes (especially resveratrol) can be considered very interesting because these molecules have

attracted the attention of the scientific community for their pharmacologic properties in preventing human diseases (Pastor *et al*., 2019). Finally, the flavonol content was not conditioned during the growing season under consideration.

Early water stress modulated the expression of genes and miRNAs involved in secondary metabolism with lasting effects, still evident at maturity

Ten genes (structural genes or coding for transcription factors) and five miRNA involved in the phenylpropanoid biosynthesis pathway were selected as genetic determinants based on previous transcriptomics results, gathered on the same experimental pot system (Zombardo *et al.*, under publication). The genes and miRNAs were already detected as differentially expressed between vines having different root systems (M, P, NGC) in conditions of optimal irrigation. Therefore, the present research work was designed to verify if and how their expression profiles were further modulated in vines that have undergone an early water stress period, between cluster closure and veraison. Indeed, it is well-known that the rootstocks can influence the scion phenotype in the presence of stress or limiting factors altering gene expression (Berdeja *et al*., 2015; Chitarra *et al*., 2017), and this effect was confirmed by the chemical results that we obtained on grapes.

The comparison of gene and miRNA expression was carried out by qRT-PCR between vines subjected to severe stress (WS-1 only, renamed WS) and well-watered vines (WW), in each root system.

The results acquired on the ten genes studied showed that the period of *pre*-veraison water shortage still had an effect on gene expression in berry skins at maturity. Most genes studied resulted, in fact, differentially expressed in at least one of the three root systems (M, P, and

NGC). In particular, three out of five structural genes were DE (VvF3'H A, VvFLS, and VvDFR), while all five genes coding for transcription factors (VvMYB14, VvMYBC2-L3, VvMYB4R1, VvNAC44, and VvNAC60) were DE in at least two or all three root systems, with considerable differences due to the irrigation protocol (Fig.5A-J).

The obtained results underline the role of gene regulation within secondary metabolism as a plant response to mitigate water stress. The major evidence has emerged in the expression patterns of transcription factors, which were strongly modulated according to vine water status. Also in previous studies, the expression of MYB and NAC transcription factors was altered in the case of abiotic stress (Corso *et al.*, 2015). Besides, our results are in accordance with other findings that demonstrated that VvNAC genes display a stress-inducible up-regulation (Wang *et al.,* 2013). Also in other species, for example in rice (*Oryza sativa*) and soybean (*Glycine max*), the overexpression of NAC transcription factors can enhance drought resistance (Nakashima *et al.*, 2007; Tran *et al.*, 2009).

Some specific miRNAs, non-coding small RNAs (19-24 nucleotides long) that perform post-transcriptional gene silencing in plants (Chuck *et al*., 2009; Mica *et al*., 2010; Solofoharivelo *et al*., 2014), were included in this study since, in *Vitis vinifera*, it has been demonstrated that grafting can alter miRNAs abundance in the scion, causing changes in the final phenotype (Pagliarani *et al.*, 2017; Yang *et al.*, 2015).

The results showed that, in the comparison between WW and WS, each of the five miRNA considered (miR395 and miR398, miR858, Grape_m-1191, Grape_m-0721) resulted differentially expressed in at least one of the three root systems (Fig.6A-E).

miR395 is one of the first miRNAs whose role in the response to abiotic stresses was demonstrated, as it can be induced by environmental stimuli (Jones-Rhoades and Bartel,

2004). According to the literature, miR395 is significantly up-regulated in the presence of drought stress in *Oryza sativa* (Zhou *et al.*, 2010). In our data, miR395 had a higher expression in WS *vs* WW irrigation protocols only in the vines grafted on 1103 Paulsen and this behavior may be due to its marked characteristics as a drought-resistant rootstock.

miR398 is another negative regulator that participate to stress adaptation and it is usually down-regulated to dissipate oxidative stress in plant tissues (Sunkar *et al.*, 2006; Zhu *et al.*, 2011). In this trial, miR398 was differentially expressed in 1103 Paulsen only, with up-regulation in WS vines. In this case, it can be hypothesized that in 1103 Paulsen the accumulation of ROS was lower, being a rootstock more tolerant to water stress.

miR858 has several R2R3-MYB transcription factors as targets (Zombardo *et al.*, under publication), which are involved in the control pathway of flavonoid biosynthesis. According to the data obtained, miR858 is down-regulated in WS vines of both Mgt 101-114 and 1103 Paulsen, suggesting that the level of mRNAs coding for MYB TFs is up-regulated due to water stress in grafted vines only.

Grape_m-1191 and Grape_m-0721, two miRNAs involved in the transport and biosynthesis of flavonoids, had similar accumulation trends between WS *vs* WW, but not grafted vines showed differential expression, with down-regulation in the vines that suffered water deficit.

Despite the valuable results obtained in the analyses carried out on gene and miRNA expression and secondary metabolite content in berry skins, it was not possible to find a direct correlation between the phenolic compounds detected and the transcript level of the genes and miRNAs considered. This aspect can be explained by the fact that secondary metabolism has a multi-level post-transcriptional and post-translational control, and along

the complex phenylpropanoids pathway there is competition between substrates, the need for cofactors and protein complexes (etc.), with strong repercussions on the final quantities of chemical compounds (Pastore *et al.*, 2017). Furthermore, the presence of multigene families for the genes involved can give rise to different space-time expression profiles for members concerned, which may or may not be in line with the final quantity of the metabolite produced by the biosynthetic pathway (Kuhn *et al*., 2014).

Conclusions

By adopting an integrated biochemical and molecular approach, the research allowed testing the rootstock influence on grapevine productivity and fruit quality under water stress conditions.

Physiological measurements confirmed the presence of the *pre*-veraison water stress imposed, a condition that caused clear metabolic responses in the grapes produced by the vines that have undergone water limitation. According to the results obtained in our experimental system, the water-stressed vines remained productive but berry quality was altered in comparison to the vines grown with abundant water availability. In particular, water stress drove the carbon flux towards sugars and secondary metabolites accumulation. Similar behavior was also determined by 1103 Paulsen, a rootstock genotype that had a greater influence on the scion as a response to water deficit.

The evidence was supported by the differential gene and miRNA expression levels due to water stress, that was highlighted for structural genes belonging to the phenylpropanoid pathway, but most of all for the genes coding for transcription factors included in MYB and

NAC gene families. As expected, the major differences emerged between grafted and not grafted vines, as already found in the previous transcriptomic study (Zombardo *et al*., under publication). Indeed, grafting has determined remarkable transcript concentration changes in berry skins, and these results could be partially dependent on the miRNA-mediated post-transcriptional regulatory network.

The results that emerged from our study suggested a rootstock-dependent response in case of water stress, which impacted on gene expression and phenolic compounds accumulation in berries. However, further investigation is needed about rootstock-scion interaction, a genotype fusion that can confer to the grapevine a higher resilience to be exploited against the detrimental effects of climate change.

Tab.1: List of forward, reverse and stem-loop reverse transcriptase primers used for qRT-PCR to test genes and miRNAs expression. Gene ID/miRNA sequences are specified.

Gene/miRNA	Gene ID/miRNA sequence	Forward Primer (5'3' Seq.)	Reverse Primer (5'3' Seq.)	Stem-loop reverse transcriptase Primer (5'3' Seq.)
VvPAL	VIT_213s0019g04460	CGCCAAACACAGCCACTCA	GCAGCTTTAGTACCAGTGTCTCCC	--
VvF3'H A	VIT_209s0002g01090	TCCTACCACCTCACCAACGC	CGAGAGGAGGATAAGAGCCACAGT	--
VvF3'H B	VIT_217s0000g07200	GCCTCCGTTGCTGCTCAGTT	CGTAGGGAGCGAACACCAGA	--
VvFLS	VIT_218s0001g03430	TTGATATCCCACGACACACCG	ATTGAGATCAGCACCAGAGGC	--
VvDFR	VIT_216s0039g02350	TGAGAAGGAGAAACATGCATGCCA	AGGTGACCCATTGCAACTTTCA	--
VvMYB14	VIT_207s0005g03340	CGGAGAGCCTTGGGTATGGA	TGCAGGGTGTAGTAATGTCGGA	--
VvMYBC2-L3	VIT_214s0006g01620	CTCACCATTGCCATTCCTGCT	AGGATTTGCGTCACCTTCCAC	--
VvMYB4R1	VIT_217s0000g02710	CCTCTCTCATTGAAGCCGCTC	GTTTCTGGATTGCACGGAGGA	--
VvNAC44	VIT_206s0004g00020	GGACGACTGGGTTCTTTGCC	CCATCGTCTTCAGCCACCTC	--
VvNAC60	VIT_208s0007g07670	ACGTTCGAGCATGGATGGG	CTTTGCGGGAGGTCTGACTG	--
VvUBI	VIT_219s0177g00040	AATGGTCAGTTGGCCCTACCT	TGGCTGAGACCCACAAAACC	--
miR395	CTGAAGTGTTTGGGGGAACTC	TGACGCTGAAGTGTTTGGGG	GTGCAGGGAGGGAGGT	GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACGAGTTC
miR398	TGTGTTCTCAGGTCGCCCCTG	TCGCTTGTGTTCTCAGGTCG	GTGCAGGGAGGGAGGT	GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACCAGGGG
miR858	CGTTGTCTGTTCGACCTTG	TCGCCCGTTGTCTGTTCG	GTGCAGGGTCCGAGGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGGT
Grape_m-0721	TTACCAACACCTCCCATTCC	TGCGGATTACCAACACCTCC	GAGCTGGGTCCGACGT	GTCGTATCCAGAGCTGGGTCCGACGTATTCGCTCTGGATACGACGGAATG
Grape_m-1191	GCTGAACAAGAGAGAACCT	GCGCGGCTGAACAAGAGA	GAGCTGGGTCCGACGT	GTCGTATCCAGAGCTGGGTCCGACGTATTCGCTCTGGATACGACAGGTTC

References

1. Adams DO. Phenolics and Ripening in Grape Berries. American Journal of Enology and Viticulture. 2006;57:249-256.
2. Bauerle TL, Smart DR, Bauerle WL, Stockert C, Eissenstat DM. Root foraging in response to heterogeneous soil moisture in two grapevines that differ in potential growth rate. New Phytologist. 2008;179:857-866.
3. Belli Kulhan J, Paim Pinto DL, Bertolini E, Fasoli M, Zenoni S, Tornielli GB, *et al.* miRVine: a micro RNA expression atlas of grapevine based on small RNA sequencing. BMC Genomics. 2015;16:393.
4. Berdeja M, Nicolas P, Kappel C, Dai ZW, Hilbert G, Peccoux A, Lafontaine M, Ollat N, Gomès E, Delrot S. Water limitation and rootstock genotype interact to alter grape berry metabolism through transcriptome reprogramming. Horticulture Research. 2015;2:15012.
5. Bogs J, Ebadi A, Mc David D, Robinson SP. Identification of the Flavonoid Hydroxylases from Grapevine and Their Regulation during Fruit Development. Plant Physiology. 2006;140:279-291.
6. Boss PK, Davies C, Robinson SP. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol. 1996;111:1059-1066.
7. Buhtz A, Pieritz J, Springer F, Kehr J. Phloem small RNAs, nutrient stress responses and systemic mobility. BMC Plant Biol. 2010;10:64.
8. Bussotti F, Kalaji MH, Desotgiu R, Pollastrini M, Loboda T, Bosa K. Misurare la vitalità delle piante per mezzo della fluorescenza della clorofilla. Firenze University Press. 2012;Vol.137.
9. Castellarin SD, Di Gaspero G, Marconi R, Nonis A, Peterlunger E, Paillard S, Adam-Blondon AF, Testolin R. Colour variation in red grapevines (Vitis vinifera L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. BMC Genomics. 2006;7:12.
10. Castellarin SD, Matthews MA, Di Gaspero G, Gambetta GA. Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. Planta. 2007;227(1):101-112.
11. Castellarin SD, Bucchetti B, Falginella L, Peterlunger E. Influenza del deficit idrico sulla qualità delle uve: aspetti fisiologici e molecolari. Italus Hortus. 2011;14:63-79.
12. Cavallini E, Matus JT, Finezzo L, Zenoni S, Loyola R, Guzzo F, Schlechter R, Ageorges A, Arce-Johnson P, Tornielli GB. The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYBC2 repressors in grapevine. Plant Physiol. 2015;167:1448-1470.
13. Chaves MM, Zarrouk O, Francisco R, Costa JM, Santos T, Regalado AP, Rodrigues ML, Lopes CM. Grapevine under deficit irrigation: hints from physiological and molecular data. Ann Bot. 2010;105:661-676.
14. Chitarra W, Perrone I, Avanzato CG, Minio A, Boccacci P, Santini D, Gilardi G, Siciliano I, Gullino ML, Delledonne M, Mannini F, Gambino G. Grapevine grafting: scion transcript profiling and defense-related metabolites induced by rootstocks. Frontiers in Plant Science. 2017;8:564.
15. Chuck G, Candela H, Hake S. Big impact by small RNAs in plant development. Current Opinion in Plant Biology. 2009;12:81-86.
16. Coombe BG, Mc Carthy MG. Dynamics of grape berry growth and physiology of ripening. Aust J Grape Wine Res. 2000;6:131-135.
17. Corso M, Bonghi C. Grapevine rootstock effects on abiotic stress tolerance. Plant Sci Today. 2014;1:108-113.

18. Corso M, Vannozzi A, Maza E, Vitulo N, Meggio F, Pitacco A, Telatin A, D'Angelo M, Feltrin E, Negri AS, Prinsi B, Valle G, Ramina A, Bouzayen M, Bonghi C, Lucchin M. Comprehensive transcript profiling of two grapevine rootstock genotypes contrasting in drought susceptibility links the phenylpropanoid pathway to enhanced tolerance. J Exp Bot. 2015;66(19):5739-5752.
19. Costantini EAC. Oltre la zonazione – Beyond the zoning Tre anni di studio al Castello di Brolio. Edizioni Polistampa, Firenze. 2013.
20. De Freitas VAP, Fernandes A, Oliveira J, Teixeira N, Mateus N. A review of the current knowledge of red wine colour. OENO One. 2017;51(1).
21. Di Stefano R, Cravero MC. Metodi per lo studio dei polifenoli dell'uva. Rivista di Viticoltura e di enologia. 1991;2:37-45.
22. Downey MO, Harvey JS, Robinson SP. Synthesis of flavonols and expression of flavonol synthase genes in developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). Aust J Grape Wine Res. 2003;9:110-121.
23. Duchêne E, Huard F, Dumas V, Schneider C, Merdinoglu D. The challenge of adapting grapevine varieties to climate change. Climate Research. 2010;41:193-204.
24. Falginella L, Castellarin S, Testolin R, Gambetta G, Morgante M, Di gaspero. Expansion and subfunctionalisation of flavonoid 3',5'-hydroxylases in the grapevine lineage. BMC Genomics. 2010;11:562.
25. Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Santo SD, *et al*. Timing and order of the molecular events marking the onset of berry ripening in grapevine. Plant Physiol. 2018;178:1187-1206.
26. Ferrandino A, Lovisolo C. Abiotic stress effects on grapevine (*Vitis vinifera* L.): Focus on abscisic acid-mediated consequences on secondary metabolism and berry quality. Environmental and Experimental Botany. 2014;103:138-147.
27. Fraga H, Malheiro C, Moutinho-Pereira J, Santos JA. An overview of climate change impacts on European viticulture. Food and Energy Security. 2012;1(2):94-110.
28. Girona J, Mata M, del Campo J, Arbonés A, Bartra E, Marsal J. The use of midday leaf water potential for scheduling deficit irrigation in vineyards. Irrigation Science. 2006;24:115-127.
29. Gomez-Alonso S, Garcia-Romero E, Hermosìn-Gutiérrez I. HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. Journal of Food Composition and Analysis. 2007;20:618-626.
30. Goode J. Viticulture: fruity with a hint of drought. Nature. 2012;492(7429):351-353.
31. Guidoni S, Ferrandino A, Novello V. Effects of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. Am J Enol Vitic. 2008;1:22-29.
32. Hannah L, Roehrdanz PR, Ikegami M, Shepard AV, Shaw MR, Tabor G, Zhi L, Marquet PA, Hijmans RJ. Climate change, wine, and conservation. PNAS. 2013;110(17):6907-6912.
33. Harada T. Grafting and RNA transport via phloem tissue in horticultural plants. Scientia Horticulturae. 2010;125:545-550.
34. IPCC - Intergovernmental Panel on Climate Change. An IPCC special report on the impacts of global warming of 1.5˚C above *pre*-industrial levels andrelated global greenhouse gas emission pathways. Geneva, Switzerland. 2018.
35. Jagadeeswaran G, Saini A, Sunkar R. Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta. 2009;229:1009-1014.
36. Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, *et al.* The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature. 2007;449:463-467.

37. Jeandet P, Delaunois B, Conreux A, Donnez D, Nuzzo V, Cordelier S, Clement C, Courot E. Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants. Bio Factors. 2010;36(5):331-341.
38. Jones GV, White MA, Cooper OR, Storchmann K. Climate change and global wine quality. Clim Change. 2005;73:319-343.
39. Jones-Rhoades MW, Bartel DP. Computational identification of plant micro-RNAs and their targets, including a stress-induced miRNA. Mol Cell. 2004;14:787-799.
40. Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, SaitoK, Takahashi H, Dalmay T. Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J. 2009;57:313-321.
41. Keller M. The science of grapevines. Anatomy and Physiology. Academic Press, Burlington. 2010.
42. Koundouras S, Hatzidimitriou E, Karamolegkou M, Dimopoulou E, Kallithraka S, Tsialtas JT, Zioziou E, Nikolau N, Kotseridis Y. Irrigation and rootstock effects on the phenolic concentration and aroma potential of *Vitis vinifera* L. cv. Cabernet Sauvignon grapes. J Agric Food Chem. 2009;57:7805-7813.
43. Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, Gomès E, *et al.* Berry ripening: recently heard through the grapevine?. J Exp Bot. 2014;65:4543-4559.
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 2001;25:402-408.
45. Lovisolo C, Perrone I, Carra A, Ferrandino A, Flexas J, Medrano H, Schubert A. Drought-induced changes in development and function of grapevine (*Vitis* spp.) organs and in their hydraulic and non-hydraulic interactions at the whole-plant level: a physiological and molecular update. Funct Plant Biol. 2010;37(2):98-116.
46. Maré C, Aprile A, Roncaglia E, Tocci E, Corino LG, De Bellis G, Cattivelli L. Rootstock and soil induce transcriptome modulation of phenylpropanoid pathway in grape leaves. Journal of plants interactions. 2013;8(4):334-349.
47. Marguerit E, Brendel O, Lebon E, van Leeuwen C, Ollat N. Rootstock control of scion transpiration and its acclimation to water deficit are controlled by different genes. New Phytol. 2012;194:416-429.
48. Mattivi F, Guzzon R, Vrhovsek U, Stefanini M, Velasco R. Metabolite profiling of Grape: Flavonols and Anthocyanins. J Agric Food Chem. 2006;54:7692-7702.
49. Mica E, Piccolo V, Delledonne M, Ferrarini A, Pezzotti M, Casati C, Del Fabbro C, Valle G, Policriti A, Morgante M, Pesole G, Pè ME, Horner DS. Correction: High throughput approaches reveal splicing of primary microRNA transcripts and tissue specific expression of mature microRNAs in *Vitis vinifera*. BMC Genomics. 2010;11:109.
50. Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 2007;51(4):617-630.
51. O.I.V. Compendium of international methods of analysis of wines and musts. http://www.oiv.int. 2009.
52. Ojeda H, Deloire A, Carbonneau A. Influence of water deficits on grape berry growth. Vitis. 2001;40:141-145.
53. Ollat N, Diakou-Verdin P, Carde JP, Barrieu F, Gaudillère JP, Moing A. Grape berry development: a review. J Int Sci Vigne Vin. 2002;36:109-131.
54. Ollat N, Peccoux A, Papura D, Esmenjaud D, Marguerit E, Tandonnet JP, Bordenave L, Cookson SJ, Barrieu F, Rossdeutsch L, Lecourt J, Lauvergeat V, Vivin P, Bert PF, Delrot S. Rootstocks as a component of adaptation to environment. In: Grapevine in a changing

environment: a molecular and ecophysiological perspective. Eds Geros H, Chaves M, Medrano H, Delrot S. Wiley-Blackwell. Oxford, UK. 2015;68-108.
55. Ollat N, Cookson SJ, Lauvergeat V, Marguerit E, Barrieu F, Gambetta G, *et al*. Grapevines roots: the dark side. Acta Horticolturae. 2017;1188:213-226.
56. Pagliarani C, Vitali M, Ferrero M, Vitulo N, Incarbone M, Lovisolo C, Valle G, Schubert A. The accumulation of microRNAs differentially modulated by drought is affected by grafting in grapevine. Plant Physiol. 2017;173:2180-2195.
57. Paim Pinto DL, Brancadoro L, Dal Santo S, De Lorenzis G, Pezzotti M, Meyers BC, *et al.* The influence of genotype and environment on small RNA profiles in grapevine berry. Frontiers in Plant Sciences. 2016;7:1459.
58. Palliotti A, Tombesi S, Silvestroni O, Lanari V, Gatti M, Poni S. Changes in vineyard establishment and canopy management urged by earlier climate-related grape ripening: a review. Sci Hortic. 2014a;178:43-54.
59. Palliotti A, Tombesi S, Frioni T, Famiani F, Silvestroni O, Zamboni M, Poni S. Morpho-structural and physiological response of container-grown Sangiovese and Montepulciano cvv. (Vitis vinifera) to re-watering after a *pre*-veraison limiting water deficit. Functional Plant Biology. 2014b;41:634-647.
60. Palliotti A, Poni S, Silvestroni O. La nuova viticoltura. EdAgricole. Bologna, Italy. 2015.
61. Palumbo MC, Zenoni S, Fasoli M, Massonnet M, Farina L, Castiglione F, Pezzotti M, Paci P. Integrated Network Analysis Identifies Fight-Club Nodes as a Class of Hubs Encompassing Key Putative Switch Genes That Induce Major Transcriptome Reprogramming during Grapevine development. The Plant Cell. 2014;26:4617-4635.
62. Pastor RF, Restani P, Di Lorenzo C, Orgiu F, Teissedre PL, Stockley C, Ruf JC, Quini CI, Garcia Tejedor N, Gargantini R, Aruani C, Prieto S, Murgo M, Videla R, Penissi A, Iermoli RH. Resveratrol, human health and winemaking perspectives. Crit Rev Food Sci Nutr. 2019;59(8):1237-1255.
63. Pastore C, Dal Santo S, Zenoni S, Movahed N, Allegro G, Valentini G, Filippetti I, Tornielli GB. Whole plant temperature manipulation affects flavonoid metabolism and the transcriptome of grapevine berries. Front Plant Sci. 2017;8:929.
64. Pavlousek P. Evaluation of drought tolerance of new grapevine rootstock hybrids. J Environ Biol. 2011;32:543-549.
65. Peterlunger E, Sivilotti P, Colussi V. Water stress increased polyphenolic quality in 'Merlot' grapes. Acta Hort. 2005;689:293-300.
66. Poni S, Lakso AN, Turner JR, Melious RE. The effects of *pre*- and *post*- veraison water stress on growth and physiology of potted Pinot noir grapevines at varying crop levels. Vitis. 1993; 32:207-214.
67. Poni S, Intrieri C. Grapevine photosynthesis: effects linked to light radiation and leaf age. Adv Hort Sci. 2001; 15(1-4):5-15.
68. Puccioni S, Valentini P, Zombardo A, Leprini M, Perria R, Storchi P, Priori S, Costantini EAC, Salvi L, Cataldo E, Mattii GB. Acta Italus Hortus. 2016;19:95-96.
69. Scholander PF, Hammel HT, Bradstreet ED, Hemmingsen EA. Sap pressure in vascular plants. Science. 1965;148:339-46.
70. Solofoharivelo MC, Van der Walt AP, Stephan D, Burger JT, Murray SL. MicroRNAs in fruit trees: discovery, diversity and future research directions. Plant Biology. 2014;16:856-865.
71. Sunkar R, Kapoor A, Zhu JK. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell. 2006;18:2051-2065.
72. Suzuki M, Nakabayashi R, Ogata Y, Sakurai N, Tokimatsu T, Goto S, *et al.* Multiomics in Grape Berry Skin Revealed Specific induction of the Stilbene Synthetic Pathway by Ultraviolet-C irradiation. Plant Physiology. 2015;168:47-59.

73. Tavares S, Vesentini D, Fernandes JC, Ferreira RB, Laureano O, Ricardo-Da-Silva JM, Amancio A. Vitis vinifera secondary metabolism as affected by sulfate depletion: diagnosis through phenylpropanoid pathway genes and metabolites. Plant Physiol. Biochem. 2013;66:118-126.
74. Teixeira A, Eiras-Dias J, Castellarin S, Geròs H. Berry Phenolics of Grapevine under Challenging Environments. Int J Mol Sci. 2013;14:18711-18739.
75. Tirumalai A, Swetha C, Nair A, Pandit A, Shivaprasad PV. miR828 and miR858 regulate *VvMYB14* to promote anthocyanin and flavonol accumulation in grapes. Journal of Experimental Botany. 2019; erz264.
76. Tran LS, Quach TN, Guttikonda SK, Aldrich DL, Kumar R, Neelakandan A, Valliyodan B, Nguyen HT. Molecular characterization of stress-inducible GmNAC genes in soybean. Mol Genet Genomics. 2009;281(6):647-664.
77. van Leeuwen C, Tregoat O, Choné X, Bois B, Pernet D, Gaud-illère JP. Vine water status is a key factor in grape ripening and vintage quality for red Bordeaux wine. How can it be assessed for vineyard management purposes?. Journal International des Sciences de la Vigne et du Vin. 2009;43:121-134.
78. van Leeuwen C, Darriet P. The impact of Global Change on Viticulture and Wine Quality. Journal of Wine Economics. 2016;11(1):150-167.
79. van Leeuwen C, Destrac-Irvine A. Modified grape composition under climate change conditions requires adaptations in the vineyard. Oeno ONE. 2017;51(2):147-154.
80. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 2007;3:12.
81. Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, Pruss D *et al.* A high-quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One. 2007;12:e1326.
82. Vezzulli S, Malacarne G, Masuero D, Vecchione A, Dolzani C, Goremykin V, *et al*. The Rpv3-3 Haplotype and Stilbenoid Induction Mediate Downy Mildew Resistance in a Grapevine Interspecific Population. Front Plant Sci.2019;10:234.
83. Wang N, Zheng Y, Xin H, Fang L, Li S. Comprehensive analysis of NAC domain transcription factor gene family in *Vitis vinifera*. Plant Cell Rep. 2013;32:61-75.
84. Warschefsky EJ, Klein LL, Frank MH, Chitwood DH, Londo JP, von Wettberg EJB, Miller AJ. Rootstocks: diversity, domestication, and impacts on shoot phenotypes. Trends Plant Sci. 2016;21:418-437.
85. Winkler AJ, Cook JA, Kliewer WM, Lider LA. General Viticulture. University of California Press, Berkeley, CA, USA. 1974.
86. Yang Y, Mao L, Jittayasothorn Y, Kang Y, Jiao C, Fei Z, Zhong GY. Messenger RNA exchange between scions and rootstocks in grafted grapevines. BMC Genomics. 2015;15:251.
87. Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L. Genome-wide identification and analysis of drought-responsive microRNAs in Oryza sativa. J Exp Bot. 2010;61:4157-4168.
88. Zhu C, Ding Y, Liu H. MiR398 and plant stress responses. Physiologia Plantarum.2011;143:1-9.
89. Zombardo A, Crosatti C, Bagnaresi P, Bassolino L, Reshef N, Puccioni S, Faccioli P, Tafuri A, Delledonne M, Fait A, Storchi P, Cattivelli L, Mica E. Transcriptomic and biochemical investigations support the role of rootstock-scion interaction in grapevine berry quality. BMC Genomics. Under publication.

4. General conclusions

The Ph.D. research activity carried out made it possible to investigate in detail rootstock-scion interactions in grapevine, using an integrated molecular and biochemical approach. The attention was focused on the rootstock influence on secondary metabolism in grapes, its regulation, and the accumulation trends of phenolic compounds in berries.

The research project was developed using in an experimental system of potted Pinot noir grapevines, that included plants grafted on two rootstocks with opposite characteristics (1103 Paulsen, highly vigorous and highly tolerant to drought, and Mgt 101-14, less vigorous and susceptible to drought) as well as not grafted plants. The experimental pot system was specifically designed to have a series of advantages over open field trials. It allowed to control many variables (for example irrigation, fertilization, pest control, environmental monitoring), with an adequate number of replicates and to obtain statistically significant results, simulating the real conditions of a vineyard. The choice of the cultivar Pinot noir and clone ENTAV115 was accurate, considering that the genome sequence of this cultivar is fully mapped. Moreover, the use of pots made it possible to insert not grafted plants as a control, that would not be feasible in a real vineyard, due to the looming presence of phylloxera.

The research activity was developed within a previous project carried out using the same experimental system (RINGO - Rootstock-scion INteraction in Grape: an Omics perspective) that involved the evaluation of gene expression on berry skins at two key-points of ripening (veraison and maturity) by Next-Generation Sequencing analyses (both on mRNA and small RNA fractions) coupled with phenotyping on grape quality, during two consecutive growing seasons (2012 and 2013). Implementing and elaborating the data already collected, interesting results were obtained about the role of rootstock-scion interaction in grapevine berry quality.

The elaboration of the deep-sequencing outputs described the differential expression of genes and miRNAs between the root systems considered, at two different stages of grape maturation. From the obtained results, it is possible to state that the pivotal effect leading gene expression was the berry developmental program, while the year effect drove the sample differentiation regardless of the rootstock genotype; miRNA expression, instead, was primarily divided by the year effect, suggesting a strong influence of environmental signals. Grafting *per se* had a strong influence on berry skin transcriptome and chemical composition at maturity, and this effect was more evident in 2012, a considerably warmer vegetative season. In general, the major differences were detected in grafted (both M and P) compared to not grafted vines, both at the molecular and biochemical levels. The genes identified as differentially expressed at maturity, according to the enrichment analysis of their ontologies, were primarily involved in the biosynthetic pathway of phenylpropanoids, the transport of flavonoids and stress response. Besides, the secondary metabolism was more significantly modulated during grape ripening in the vines grafted on 1103 Paulsen than in those grafted on Mgt 101-14. Therefore, it is possible to argue that rootstocks, especially 1103 Paulsen, influenced the molecular mechanisms of berry development and grape quality.

During the growing season 2017, the vines comprised in the pot system were treated with the same agronomic management, under the same water regime of the previous transcriptomics study. An accurate phenotyping activity on vines phenology and leaf physiology, not performed before, was carried out alongside with chemical analyses on grapes, and qRT-PCR analyses on the expression level of some genes belonging to the phenylpropanoid pathway, both structural and coding for transcription factors were executed at veraison and maturity. In particular, nine specific genes were selected because

already detected as differentially expressed between the three root systems in the preceding deep-sequencing analyses.

The results allowed to deduce that the rootstocks used on Pinot Noir vines, namely 1103 Paulsen and M101-14, in optimal irrigation conditions and the absence of stress factors, did not cause alterations in the scion in terms of development, photosynthetic efficiency, and not even on grape production and technological maturation. The rootstocks, therefore, did not have direct effects on the vines' primary metabolism. On the contrary, some significant differences were found in the secondary metabolism of the grape skins, in particular in the anthocyanin profile, and in the accumulation of different classes of phenolic compounds (flavonols, flavan-3-ols, and stilbenes). Moreover, according to the molecular analyses carried out, the nine genes studied were differentially expressed between the three root systems, with more significant differences in the samples at maturity. It was not possible to cross molecular and chemical results to find a direct correspondence between secondary metabolites concentrations and gene expression levels, due to the complexity of the post-transcriptional and post-translational regulation of this pathway and to the presence of multigene families in grapevine genome.

During the growing season 2018, the experimental pot system was used to test the influence of the two rootstocks considered (1103 Paulsen and M101-14) on plant physiology and grape quality, in the event of early water limitation. For this purpose, a water stress trial was performed between the phenological stages of cluster closure and veraison, when three different irrigation protocols were applied: severe deficit (WS-1), intermediate deficit (WS-2), and well-watered vines (WW). The surveys executed during the 2018 vegetative season were the same as the previous year, an in-depth phenotyping work was carried out on vine

physiology and the chemical composition of grapes. Furthermore, at maturity, the expression levels of some genes and miRNAs belonging to the phenylpropanoid pathway or involved in stress response were detected. Here too, ten genes and five miRNA were selected because already detected as differentially expressed between the three root systems in the transcriptomic project.

The measurements on water status, leaf gas exchanges, and photosynthetic efficiency confirmed the presence of the *pre*-veraison water stress imposed, a condition that caused clear metabolic responses in the grapes produced by the vines that have undergone water shortage. According to the results, the water-stressed vines remain productive but berry quality was altered in comparison to the vines grown with abundant water availability. In particular, water stress drove the carbon flux towards the accumulation of sugars and secondary metabolites. Similar behavior was also determined by 1103 Paulsen, a rootstock genotype that had a greater influence on the scion as a response to water deficit. The evidence was supported by the differential gene and miRNA expression levels due to water stress, that was highlighted for structural genes belonging to the phenylpropanoid pathway, but most of all for the genes coding for transcription factors (*MYB* and *NAC* gene families). As expected, the major differences emerged between grafted and not grafted vines, as already found in the previous transcriptomic study. The results that emerged from the present study suggested a rootstock-dependent response in case of water stress, which impacted on gene expression and phenolic compounds accumulation in berries.

The research work carried out has allowed obtaining valuable information about rootstock-scion interaction in grapevine. The comparative evaluation of plants grafted on two rootstocks with opposite characteristics in terms of vigor and resistance to drought given to

the scion and not grafted plants, both in conditions of optimal irrigation or water stress, has enabled to highlight significative differences on grape secondary metabolism. The main effects concerned berry skins, the plant tissues containing most grape phenolic compounds, which are extremely important not only in the winemaking process but also as nutraceutical substances. Through the preliminary transcriptomic project and the subsequent qRT-PCR analyses, some genetic determinants (genes and miRNAs) were identified as involved in the metabolic responses due to rootstock-scion interaction, also participating in water stress response.

Despite the significance of this topic and the countless studies about it, the molecular network that regulates the interaction between rootstock and scion remains largely unknown, particularly concerning grape quality. This aspect is additionally complicated by the genetic variability that characterizes the genus *Vitis*. For these reasons, further investigation is needed about rootstock-scion interaction, a fusion of genotypes that can confer to the grapevine a higher resilience to be exploited against the detrimental effects of climate change.

Annex I. Research Poster

presented at Enoforum 2018 - Vicenza

EFFETTO DEL PORTINNESTO SUL METABOLISMO SECONDARIO DI UVE *PINOT NERO*

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Introduzione:

L'utilizzo dell'innesto è una pratica diffusa nella maggior parte delle aree vitivinicole mondiali quale unica soluzione efficace contro la fillossera, un afide terricolo fortemente dannoso per l'apparato radicale di *Vitis vinifera*. Il portinnesto, oltre a dare resistenza contro *Daktulosphaira vitifoliae,* può modulare lo sviluppo e l'adattabilità della vite all'ambiente, poiché è in grado di condizionare l'espressione genica nel nesto, soprattutto in presenza di stress o fattori limitanti. Le alterazioni che avvengono sono principalmente a carico dei geni coinvolti nel metabolismo secondario, in particolar modo quelli appartenenti al pathway di biosintesi dei flavonoidi e degli stilbeni. Nonostante la grande quantità di studi e ipotesi sull'interazione tra i bionti coinvolti, i network molecolari rimangono in gran parte poco conosciuti e si hanno scarse informazioni riguardo l'effetto del portinnesto sulla qualità finale delle uve.
Lo scopo del presente lavoro è stato quello di valutare l'influenza di due diversi portinnesti (aventi tra loro caratteristiche opposte per vigoria indotta e tolleranza alla siccità) sulle caratteristiche di uve *Pinot nero,* sia a livello fenotipico, che di espressione genica.

Materiali e metodi:

La ricerca è stata effettuata usando un sistema di vasi in plastica da 70 L contenenti piante di vite di 10 anni, cultivar *Pinot nero* (clone ENTAV 115) e terreno argillo-limoso (40% argilla, 41% limo, 19% sabbia). Sono state impiegate 3 tesi: viti franche di piede (FRA - come controllo) e viti innestate su 1103 Paulsen (PAU - *Vitis berlandieri* x *V. rupestris)* e su M101-14 (101 - *V. riparia* x *V. rupestris).* Le viti erano poste in file parallele all'aperto, allevate a controspalliera con potatura a cordone speronato e mantenute nelle stesse condizioni agronomiche, con identico abbondante apporto idrico. Sono state considerate 9 ripetizioni per tesi, seguendo uno schema di disposizione a blocchi randomizzati.
Durante la stagione vegetativa 2017 è stata monitorata la fenologia e valutato lo stato fisiologico delle piante. A vendemmia (fine agosto), è stata quantificata la produzione e analizzata la maturità tecnologica delle uve. Sugli acini è stata, poi, eseguita un'approfondita attività di fenotipizzazione, a livello del contenuto in metaboliti secondari delle bucce (sostanze fenoliche, tramite HPLC). Inoltre, sulle bucce di acini raccolti sia all'invaiatura che a maturità è stata svolta l'analisi di espressione di 10 geni (tramite qReal-Time PCR), tutti appartenenti al pathway dei fenilpropanoidi (5 geni strutturali e 5 geni codificanti per fattori di trascrizione), selezionati come differenzialmente espressi in uno studio di trascrittomica effettuato in precedenza sul medesimo sistema sperimentale.

Risultati:

Dai rilievi fenologici eseguiti non sono emerse differenze significative tra le tesi. Le misurazioni relative alla fisiologia delle viti hanno confermato che l'irrigazione fornita era adeguata per evitare l'insorgenza di stress idrico e danni da siccità, nonostante l'estate 2017 particolarmente calda e asciutta. In particolare, non sono state riscontrate differmità tra le tesi negli scambi gassosi e nell'efficienza fotosintetica. A vendemmia, anche la resa produttiva delle piante e la maturità tecnologica delle uve non avevano differenze significative.

Al contrario, sono emerse interessanti differenze nel profilo antocianico percentuale delle bucce (Fig.1). Le piante innestate su 1103 Paulsen avevano un contenuto di antociani disostituiti più alto, dovuto ad un maggiore accumulo di peonidina-3-glucoside; le piante innestate su M101-14 avevano valori intermedi, mentre le piante controllo avevano un maggior contenuto di antociani trisostituiti, dovuto ad un maggiore accumulo di malvidina-3-glucoside. Di conseguenza, anche il rapporto tra antociani disostituiti e trisostituiti è risultato significativamente diverso.

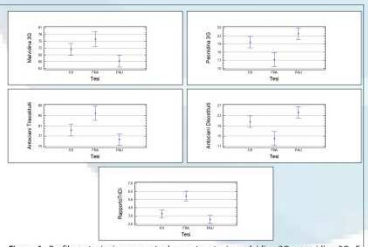

Figura 1. Profilo antocianico percentuale: contenuto in malvidina-3G, peonidina-3G, Σ antociani trisostituiti, Σ antociani disostituiti e rapporto tra antociani trisostituiti e disostituiti. Tesi: 101=M101-14; FRA= controllo; PAU=1103 Paulsen (LSD assunta per P<0,05).

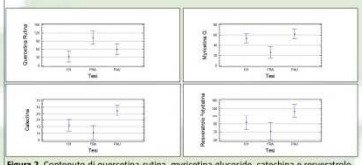

Figura 2. Contenuto di quercetina-rutina, myricetina-glucoside, catechina e resveratrolo-polydatina espresso come area HPLC. Tesi: 101=M101-14; FRA=controllo; PAU=1103 Paulsen (LSD assunta per P<0,05).

Anche nel contenuto in flavonoli sono emerse differenze significative. Le piante controllo avevano una quantità maggiore di quercetina-rutina, mentre una concentrazione inferiore è stata identificata nella tesi 1103 Paulsen e quella minima in M101-14. Inoltre, la presenza di miricetina-glucoside era significativamente diversa tra le tesi, più alta in 1103 Paulsen e M101-14 e più bassa nelle piante controllo. Riguardo i flavan-3-oli, l'unico metabolita presente in quantità significativamente diverse tra le tesi è stata la catechina, con un contenuto maggiore in 1103 Paulsen e inferiore in M101-14 e piante controllo. Infine, il resveratrolo-polydatina è stato rilevato in concentrazioni significativamente diverse: maggiore in 1103 Paulsen, mentre M101-14 e le viti non innestate ne avevano una quantità inferiore, simile tra loro (Fig.2).

L'analisi effettuata su alcuni geni chiave della via biosintetica dei fenilpropanoidi (5 strutturali, fila in alto, e 5 codificanti per fattori di trascrizione, fila in basso) ha dimostrato una considerevole variabilità di espressione (Fig.3).
Nella maggior parte dei casi, infatti, i geni selezionati sono stati convalidati come differenzialmente espressi tra le tesi nella stagione vegetativa 2017, con differenze più consistenti al momento della maturità.

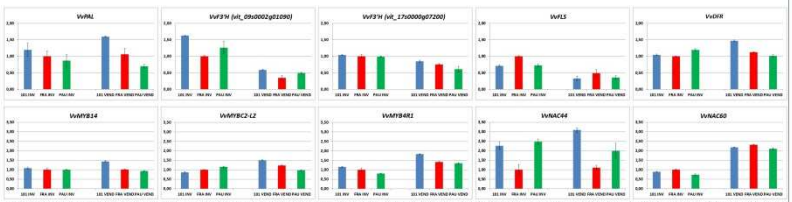

Figura 3. Espressione dei 10 geni monitorati. Analisi con qReal-Time PCR, calcolo da Ct value con il metodo $2^{-\Delta\Delta Ct}$ (le barre indicano l'errore standard). Tesi: 101=M101-14; FRA=controllo; PAU=1103 Paulsen; INV=invaiatura; VEND=maturità.

Conclusioni:

I risultati ottenuti nel presente lavoro permettono di dedurre che i portinnesti impiegati su uve *Pinot nero*, in condizioni ottimali di irrigazione e in assenza di fattori limitanti, non hanno avuto effetti sul metabolismo primario delle viti. Alcune importanti differenze, invece, sono state riscontrate a livello del metabolismo secondario, in particolare nell'accumulo di sostanze fenoliche nelle bucce. Le analisi molecolari hanno confermato che alcuni geni coinvolti nel pathway dei fenilpropanoidi sono differenzialmente espressi tra le tesi in due time-point della maturazione e possono influire sulla concentrazione finale di metaboliti. A causa della complessità di questa via biosintetica e dato l'alto numero di meccanismi post-trascrizionali e post-traduzionali coinvolti, però, non è stato possibile correlare direttamente i risultati ottenuti dalla fenotipizzazione con i livelli di espressione dei dieci geni studiati.

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Annex II. Extended Abstract

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INFLUENZA DEL PORTINNESTO SUL METABOLISMO SECONDARIO DI UVE *PINOT NERO*

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Introduzione

L'utilizzo dell'innesto è una pratica diffusa nella maggior parte delle aree vitivinicole mondiali quale unica soluzione efficace contro la fillossera, un afide terricolo (*Daktulosphaira vitifoliae*) che, con la sua diffusione capillare a partire dalla fine del XIX secolo, ha messo seriamente in pericolo la stessa coltivazione della vite. La fillossera risulta, infatti, fortemente dannosa per l'apparato radicale di *Vitis vinifera* e, in conseguenza ad un attacco, si formano sulle radici delle galle nodose che compromettono la funzione assorbente, portando ad un rapido deperimento della pianta. L'innesto, effettuato con porzioni di legno di *Vitis* americane (usate pure o come incroci, le quali hanno radici non sensibili in quanto sono co-evolute con l'insetto patogeno), non solo conferisce resistenza alla fillossera, ma può modulare l'adattabilità della pianta all'ambiente e la sua risposta a diversi tipi di stress abiotico (ad esempio siccità, salinità, ristagno idrico). L'effetto del portinnesto è un argomento molto dibattuto in letteratura: secondo alcuni autori modifica le relazioni source-sink della vite, influenzandone la fisiologia (Mattii *et al.*, 2005; Di Filippo & Vila, 2011), mentre secondo altri ha solo un effetto indiretto sullo sviluppo della pianta, poiché il genotipo del nesto e l'ambiente sono sempre fattori più determinanti (Keller *et al.*, 2012; Nuzzo & Matthews, 2006). Lo studio dell'interazione tra i bionti coinvolti è piuttosto complesso: esso implica una connessione idraulica e cambiamenti strutturali. Le viti innestate, quindi, devono essere considerate a tutti gli effetti come piante composte da due genotipi che interagiscono tra loro per creare il fenotipo finale. Alcuni ricercatori hanno recentemente scoperto che, oltre alle piccole molecole (come acqua, ioni, aminoacidi e ormoni), anche alcune macromolecole (soprattutto mRNA e microRNA) riescono ad attraversare il punto di innesto e si spostano per via floematica (Harada, 2010; Buhtz *et al.*, 2010; Yang *et al.*, 2015; Maré *et al.*, 2016). Il portinnesto, dunque, è in grado di condizionare l'espressione genica nell'intera pianta, soprattutto in presenza di stress o fattori limitanti e le alterazioni che avvengono sono principalmente a carico dei geni coinvolti nel metabolismo secondario, in particolar modo correlati alla biosintesi dei flavonoidi e degli stilbeni (Cookson *et al.*, 2014; Corso *et al.*, 2015; Chitarra *et al.*, 2017).

Nonostante il considerevole numero di ricerche effettuate sull'argomento, i network molecolari nesto-portinnesto rimangono in gran parte poco conosciuti e si hanno scarse informazioni riguardo il reale effetto dei diversi tipi di portinnesto sulla produzione e qualità dell'uva, ovvero gli aspetti di maggiore rilievo in viticoltura. Lo scopo della presente ricerca è stato quello di studiare l'influenza di due portinnesti con caratteristiche opposte tra loro (in termini di resistenza alla siccità e vigoria) sul fenotipo di uve *Pinot nero* e sul profilo di espressione di alcuni geni chiave del metabolismo dei composti fenolici. Essendo i portinnesti l'interfaccia tra l'ambiente e la pianta (Ollat *et al*., 2017), ottenere informazioni riguardo la composizione finale delle uve può costituire una sfida vantaggiosa, in prospettiva dell'adattamento ai cambiamenti climatici in atto e può essere particolarmente importante per il miglioramento genetico in viticoltura.

Materiali e metodi

Il sistema sperimentale:

Il lavoro di ricerca è stato effettuato utilizzando un sistema sperimentale di vasi in plastica da 70 L contenenti piante di vite di 10 anni, cultivar *Pinot nero* (clone ENTAV 115) e terreno argillo-limoso (40% argilla, 41% limo, 19% sabbia), proveniente da un vigneto appartenente alla DOCG Chianti Classico. Sono state impiegate 3 tesi: viti franche di piede (FRA - come controllo) e viti innestate su 1103 Paulsen (PAU - *Vitis berlandieri* x *V. rupestris)* e su M101-14 (101 - *V. riparia* x *V. rupestris)*. Le caratteristiche intrinseche dei portinnesti scelti sono elencate in Tab.1. Le viti erano poste in file parallele all'aperto, allevate a controspalliera con potatura a cordone speronato (10 gemme/pianta) e mantenute in condizioni agronomiche uniformi. L'irrigazione era fornita con un impianto a goccia, uguale per tutte le piante. Sono state considerate 9 ripetizioni per tesi, seguendo uno schema di disposizione a blocchi randomizzati.

Portinnesto	Resistenza alla fillossera	Tolleranza al calcare	Tolleranza al secco	Vigoria indotta	Tolleranza al ristagno	Tolleranza alla salinità
1103 Paulsen	Ottima	17%	Ottima	Ottima	Ottima/Buona	Buona
M101-14	Ottima	9%	Discreta/Mediocre	Discreta	Buona	Buona

Tab.1. Caratteristiche dei portinnesti utilizzati (da Palliotti et al., 2015).

Fenotipizzazione:

Durante la stagione vegetativa 2017 sono state monitorate le fasi fenologiche ed è stato valutato lo stato fisiologico delle piante, con l'obiettivo di verificare eventuali differenze tra le tre tesi. In particolare, sono state eseguiti i seguenti rilievi: osservazione visiva dello sviluppo degli organi della vite (germoglio, foglia, grappolo) durante le principali fasi di crescita (scala BBCH); misurazioni settimanali (nei mesi di luglio e agosto) dello status idrico e dell'efficienza fotosintetica: potenziale idrico come *midday stem water potential* (con camera di Scholander – Ecosearch), scambi gassosi fogliari (mediante IRGA-Ciras1 - PPSystem), misurazione della fluorescenza della clorofilla (con Handy-Pea Clorophyll Fluorimeter - Hansatech) e contenuto di clorofilla in unità SPAD (con Chlorophyll Meter SPAD-502 Plus - Konica Minolta).

Al momento della vendemmia, è stata quantificata la produzione per pianta (resa/ceppo, numero di grappoli, peso medio del grappolo, peso medio dell'acino). Sulle uve raccolte è stata valutata la maturità tecnologica, secondo i metodi ufficiali O.I.V. (O.I.V., 2009). Per ogni tesi e ripetizione sono stati raccolti campioni di acini per svolgere le analisi di maturità fenolica (polifenoli e antociani estraibili e totali), secondo il metodo di Di Stefano e Cravero (1991). Sugli estratti delle bucce è stato esaminato il contenuto di sostanze fenoliche tramite HPLC, seguendo il metodo di Gomez-Alonso (*et al.* 2007). Infine, durante l'inverno è stato pesato il legno di potatura. Le analisi statistiche su tutti i risultati ottenuti sono state elaborate con il software Statgraphics, utilizzando l'analisi della varianza (ANOVA). La least significant difference di Fisher (LSD) è stata assunta per $P<0,05$.

Analisi molecolari:

Per le analisi molecolari sono stati raccolti 15 acini per pianta, considerando 9 repliche biologiche per ogni tesi, all'invaiatura (75% delle bacche invaiate nel grappolo) e a piena maturità, per un totale di 18 campioni (3 tesi portinnesto - 2 tempi di maturazione - 3 repliche biologiche). Le bucce sono state immediatamente separate e congelate a -80°C. Tali tessuti sono stati poi macinati per estrarre l'RNA totale usando il Plant RNA Isolation Reagent (Life Technologies), seguito da purificazione con il kit RNA Clean-up and Concentration (Norgen Biotek Corp.).

Sulla base di un esperimento di trascrittomica (RNA-seq), condotto nella stagione vegetativa 2012 sullo stesso sistema sperimentale di viti *Pinot nero* allevate in vaso (mantenuto nelle medesime condizioni e sulle stesse tesi e ripetizioni) descritto da Mica (*et al.*, 2014), sono stati selezionati 9 geni (elencati in Tab. 2) che risultavano differenzialmente espressi tra le tesi.

Gene	Funzione	Riferimenti bibliografici
VvPAL	FENILALANINA AMMONIO LIASI - codifica per un enzima che promuove la deaminazione della L-fenilalanina ad acido cinnamico	Boss *et al.*, 1996
VvF3'H	FLAVONOIDE 3'-IDROSSILASI - codifica per un enzima che catalizza l'idrossilazione dei flavonoidi nella posizione 3' dell'anello B, portando ai rispettivi flavonoli idrossilati, proantocianidine e antociani	Bogs *et al.*, 2006 Castellarin *et al.*, 2006 Cui *et al.*, 2017
VvFLS	FLAVONOLO SINTASI - codifica per un enzima che catalizza la formazione di flavonoli agliconi a partire da diidroflavonoli	Downey *et al.*, 2003
VvDFR	DIIDROFLAVONOL-4-RIDUTTASI - codifica per un enzima che catalizza la conversione dei diidroflavonoli a leucoantocianine	Bogs *et al.*, 2006 Castellarin *et al.*, 2006
VvMYB14	Fattore di trascrizione - regolatore della famiglia genica codificante per la stilbene sintasi	Fasoli *et al.*, 2018 Jeandet *et al*, 2019
VvMYBC2-L3	Fattore di trascrizione - coinvolto nella biosintesi delle antocianine, ha attività di repressore	Cavallini *et al.*, 2015 Zhu *et al*, 2019
VvMYB4R1	Fattore di trascrizione - coinvolto nella sintesi degli stilbeni, come regolatore della trascrizione	Vezzulli *et al.*, 2019
VvNAC44	Fattore di trascrizione - coinvolto nella maturazione degli acini e nella risposta a stress, in particolare nell'accumulo di stilbeni	Suzuki *et al.*, 2015 Vannozzi *et al.*, 2018
VvNAC60	Fattore di trascrizione - coinvolto nella maturazione degli acini e nel metabolismo secondario, in particolare nell'accumulo di antociani	Palumbo *et al.*, 2014 Massonnet *et al.*, 2017

Tab.2. Elenco dei 9 geni differenzialmente espressi nel progetto pilota su Pinot nero del 2012, scelti per l'analisi molecolare.

I primer forward e reverse (elencati in Tab. 3) sono stati disegnati su regioni gene-specifiche, considerando che la maggior parte dei geni scelti appartengono a famiglie multigeniche con alto livello di omologia di sequenza. L'RNA totale, trattato con DNasi I (Applied Biosystem), è stato usato per sintetizzare cDNA a singolo filamento con il kit SuperScript II Reverse transcriptase (Invitrogen). Le Real-Time-PCR sono state eseguite in un volume finale di 10 µl, con SYBR® Green PCR Master Mix (Applied Biosystem), considerando tre repliche tecniche per ciascun campione. Le piastre sono state analizzate su 7300 Real-Time PCR System (Life Technologies) con le seguenti impostazioni: 1 ciclo di 95°C per 30 secondi, 40 cicli di 95°C per 10 secondi e 60°C per un minuto, un ciclo di dissociazione dei primer (per verificare i prodotti di PCR non specifici) di 95°C per 15 secondi, 60°C per 30 secondi e 95°C per 15 secondi.

La quantificazione relativa del trascritto di ciascun gene è stata calcolata dal Ct value, utilizzando il metodo del $2^{-\Delta\Delta Ct}$, usando un trascritto di poli-ubiquitina (*VvUBI*) come standard interno.

Gene	Gene ID	Primer Forward (Seq. 5'3')	Primer Reverse (Seq. 5'3')
VvPAL	vit_13s0019g04460	CGCCAAACACAGCCACTCA	GCAGCTTTAGTACCAGTGTCTCCC
VvF3'H	vit_17s0000g07200	GCCTCCGTTGCTGCTCAGTT	CGTAGGGAGCGAACACCAGA
VvFLS	vit_18s0001g03430	TTGATATCCCACGACACACCG	ATTGAGATCAGCACCAGAGGC
VvDFR	vit_16s0039g02350	TGAGAAGGAGAAACATGCATGCCA	AGGTGACCCATTGCAACTTTCA
VvMYB14	vit_07s0005g03340	CGGAGAGCCTTGGGTATGGA	TGCAGGGTGTAGTAATGTCGGA
VvMYBC2-L3	vit_214s0006g01620	CTCACCATTGCCATTCCTGCT	AGGATTTGCGTCACCTTCCAC
VvMYB4R1	vit_17s0000g02710	CCTCTCTCATTGAAGCCGCTC	GTTTCTGGATTGCACGGAGGA
VvNAC44	vit_06s0004g00020	GGACGACTGGGTTCTTTGCC	CCATCGTCTTCAGCCACCTC
VvNAC60	vit_08s0007g07670	ACGTTCGAGCATGGATGGG	CTTTGCGGGAGGTCTGACTG
VvUBI	vit_219s0177g00040	AATGGTCAGTTGGCCCTACCT	TGGCTGAGACCCACAAAACC

Tab.3. Elenco dei geni studiati, relativo Gene ID e sequenza dei primer forward e reverse utilizzati.

Risultati e discussione

Fenotipizzazione:

Dai rilievi fenologici eseguiti durante la stagione vegetativa 2017 non sono emerse differenze significative tra le tesi, lo sviluppo è stato regolare e omogeneo per ognuna delle viti. In particolare, le fasi di invaiatura al 75% e piena maturità (in corrispondenza dei campionamenti delle uve) sono stati raggiunti nelle medesime date, in tutte le piante.

Le misurazioni dello stato fisiologico hanno confermato che l'irrigazione a goccia fornita era adeguata per evitare l'insorgenza di stress idrico e danni da siccità nelle viti, nonostante l'estate particolarmente calda e asciutta. Nello specifico, il *midday stem water potential* è sempre rimasto sotto della soglia di -1 MPa ed i parametri relativi agli scambi gassosi (fotosintesi netta, traspirazione, conduttanza stomatica e *water use efficiency*) non sono mai risultati significativamente differenti tra le tesi. I dati di fluorescenza della clorofilla (F_0, Fm, Fv/Fm) sono stati omogenei tra le piante, in ciascun rilievo effettuato. Le misurazioni della clorofilla delle foglie (SPAD) hanno fornito valori stabili durante il periodo di indagine e le foglie adulte avevano un buon contenuto di pigmenti fotosintetici. Le viti afferenti ad ognuna delle tesi, dunque, sono risultate sane, dotate di buona funzionalità fogliare, fotosinteticamente attive ed efficienti.

Al momento della raccolta, avvenuta a piena maturità nell'ultima settimana di agosto, dalla quantificazione della resa per pianta non sono apparse differenze significative tra le tesi a livello produttivo. Tutti i parametri valutati (resa/ceppo, numero di grappoli, peso medio del grappolo, peso medio dell'acino) sono risultati simili tra le tesi. La produzione delle piante è stata piuttosto contenuta (in media inferiore a 500 g), principalmente a causa della condizione di crescita in vaso. Tutti i grappoli avevano una forma simile, quella tipica della varietà *Pinot nero*: piccoli,

compatti e cilindrici. Le bacche avevano dimensioni medie, forma sferoidale, buccia nera con pigmentazione omogenea e contenevano in media 2-3 semi (descrizione secondo le linee guida ampelografiche, O.I.V., 2009). Anche a livello di maturità tecnologica delle uve non sono emerse differenze tra le tre tesi considerate e l'effetto del portinnesto non è stato rilevato su nessun parametro considerato. La maturità delle bacche era pienamente raggiunta in tutte le tesi: il livello medio di zuccheri nei mosti (attorno a 20° Brix) indicava un buon accumulo finale e l'acidità totale (circa 7 g/L di acido tartarico) ed il pH (circa 3,35) avevano valori appropriati per uve adatte alla vinificazione. Le due tesi di portinnesto hanno dato risultati simili tra loro e simili alle piante franche di piede, usate come controllo. I risultati ottenuti nel presente lavoro permettono, quindi, di dedurre che i portinnesti adoperati, in condizioni ottimali di irrigazione e in assenza di fattori limitanti, non hanno avuto effetto sul metabolismo primario delle viti.
Per quanto riguarda la maturità fenolica, dalle analisi sugli estratti delle uve (bucce e semi) non sono state riscontrate differenze significative tra le tesi, relativamente alla quantità (mg/kg uva) di antociani e polifenoli, sia estraibili che totali.

Al contrario, sono emerse interessanti differenze a livello di profilo antocianico percentuale delle bucce, che, come è noto, nel *Pinot nero* è caratterizzato dalla prevalenza di malvidina, seguita dalla peonidina e dalla totale assenza di antociani acilati (Mattivi *et al.*, 2006). Dalle analisi HPLC effettuate, si sono confermate le caratteristiche del profilo, ma è stato evidenziato tra le tesi un diverso accumulo percentuale delle 5 singole antocianine. Le piante innestate su 1103 Paulsen (PAU) avevano un contenuto più alto di antociani disostituiti, dovuto ad un maggiore accumulo di peonidina-3-glucoside, le piante innestate su M101-14 (101) avevano valori intermedi, mentre le piante controllo (FRA) avevano un maggior contenuto di antociani trisostituiti, dovuto ad un maggiore accumulo di malvidina-3-glucoside. Di conseguenza, anche il rapporto tra antociani disostituiti e trisostituiti è risultato significativamente diverso (Fig.1). La porzione relativa tra due tipi di antocianine determina la colorazione delle bucce, variabile tra rosso, viola e blu. Tale caratteristica è ritenuta molto importante in enologia, poiché può influenzare la qualità dei vini ottenuti, in termini di intensità colorante e tonalità, nonché la loro attitudine all'invecchiamento. Il profilo antocianico è un carattere determinato geneticamente ed è tipico in ciascuna varietà, ma può subire lievi alterazioni, anche significative, in questo caso dovute all'influenza del portinnesto, come ipotizzato anche da alcuni autori (Fasoli *et al.*, 2018).

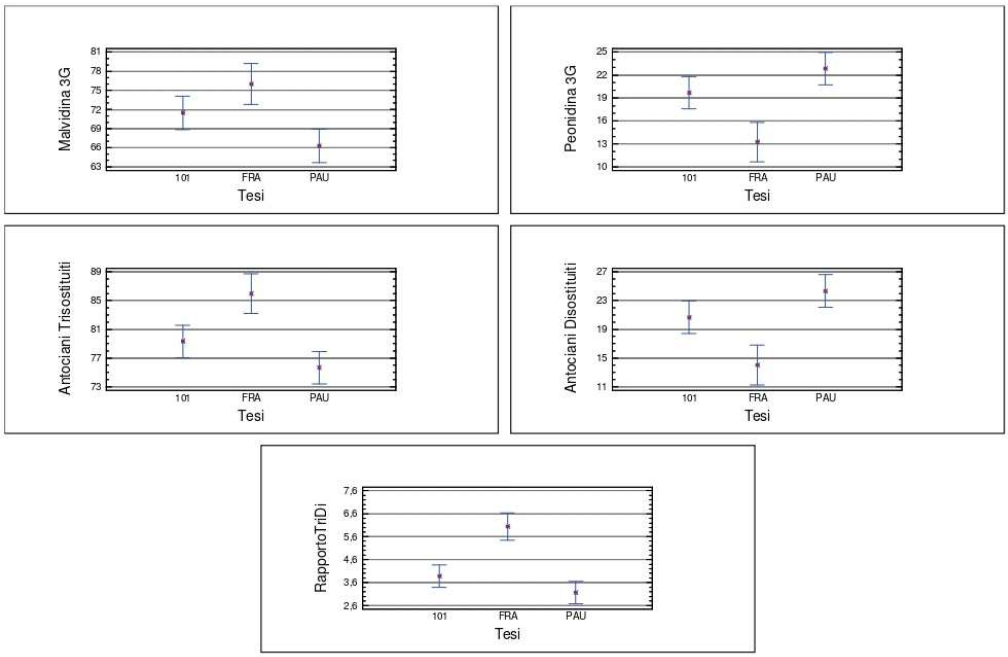

Fig.1. Profilo antocianico percentuale: contenuto in malvidina-3-glucoside, peonidina-3-glucosidde, Σ antociani trisostituiti, Σ antociani disostituiti e rapporto antociani trisostituiti/disostituiti. Tesi: 101=M101-14; PAU=1103 Paulsen; FRA=controllo (LSD assunta per P<0,05).

Per quanto riguarda il contenuto in flavonoli, una classe di composti che si trova sempre in forma glicosilata ed è presente nello strato più superficiale della buccia degli acini, in *Pinot nero* il profilo è caratterizzato da un alto contenuto di quercetina, seguito da una minore quantità di miricetina e da concentrazioni minime di kaempferolo e isoramnetina (Mattivi *et al.*, 2006). Le analisi effettuate hanno confermato il profilo già noto e, tra le tesi, sono emerse differenze significative nell'accumulo di alcuni flavonoli. Le piante controllo (FRA) avevano una quantità maggiore di rutina (o rutoside, un glicoside della quercetina), mentre un contenuto minore è stato identificato nella tesi 1103 Paulsen (PAU) e quello minimo in M101-14 (101). Inoltre, presenza di miricetina-glucoside era significativamente diversa tra le tesi, più alta in 1103 Paulsen e M101-14 e più bassa nelle piante controllo.

Riguardo i flavan-3-oli, che hanno solitamente alte concentrazioni in *Pinot nero*, l'unico metabolita presente in quantità significativamente diverse tra le tesi è stata la catechina, con un contenuto maggiore in 1103 Paulsen e inferiore in M101-14 e piante controllo.
Infine, il resveratrolo come la polidatina (o piceide, un glucoside stilbenoide, il principale derivato del resveratrolo nell'uva) è stato rilevato in concentrazioni significativamente diverse: maggiore nelle tesi innestate su 1103 Paulsen, mentre M101-14 e le viti non innestate ne avevano una quantità inferiore, più simile tra loro. Tutti i risultati sono mostrati in Figura 2.

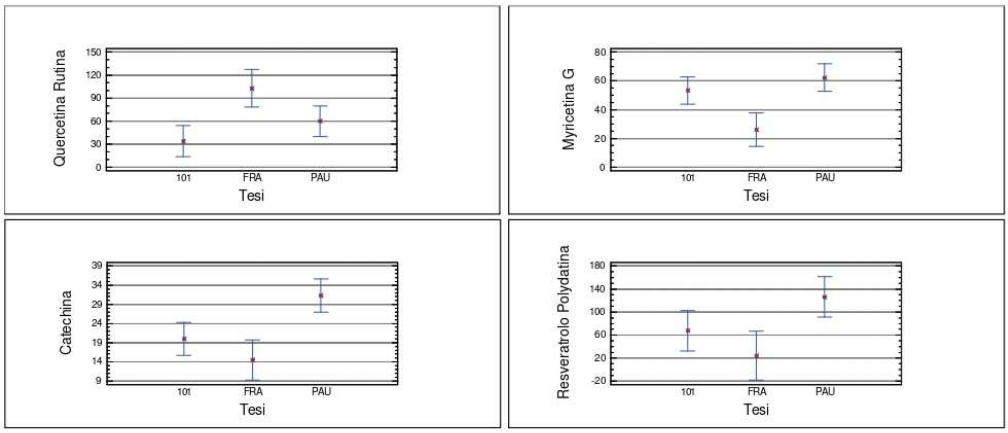

Fig.2. Contenuto di quercetina-rutina, miricetina-glucoside, catechina e resveratrolo-polidatina, espressi come area HPLC. Tesi: 101=M101-14; PAU=1103 Paulsen; FRA=controllo (LSD assunta per P<0,05).

Durante la stagione invernale tra 2017 e 2018 è stato effettuato un rilievo del peso di potatura per ogni pianta e, come ci si aspettava, le viti innestate su 1103 Paulsen (PAU) avevano una quantità significativamente più alta rispetto alle tesi innestate su M101-14 (101) e controllo (FRA) (Fig.3); questo risultato supporta l'ipotesi comunemente nota che questo portinnesto induce nel nesto un maggiore vigore vegetativo, probabilmente a causa della struttura del sistema radicale con ampio angolo geotropico.

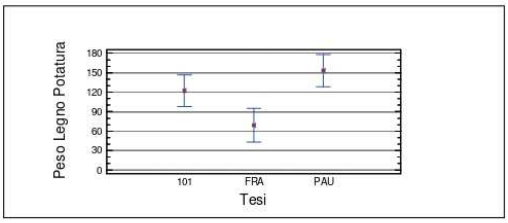

Fig.3. Peso del legno di potatura (g). Tesi: 101=M101-14; PAU=1103 Paulsen; FRA=controllo (LSD assunta per P<0,05).

Analisi molecolari:

In seguito all'analisi trascrittomica (RNA-seq), eseguita sullo stesso sistema sperimentale nel 2012, come progetto pilota (medesime tesi e ripetizioni, tenute in condizioni simili e medesimi tessuti campionati), è emerso che i geni maggiormente modulati tra le 3 tesi a maturazione appartengono al metabolismo secondario, ed in particolare alla via biosintetica dei fenilpropanoidi. Di questi, sono stati selezionati 9 geni differenzialmente espressi tra le 3 tesi, per valutare la variabilità nelle quantità di trascritto nelle bucce, in 2 precise fasi fenologiche, ovvero invaiatura (al 75%) e piena maturità nell'anno 2017. I ruoli dei 9 geni selezionati sono i seguenti: 4 sono geni funzionali, coinvolti in diversi punti chiave della sintesi di differenti sostanze fenoliche e 5 geni codificano per fattori di trascrizione (appartenenti alle famiglie MYB

e NAC), responsabili della regolazione trascrizionale di geni strutturali. Le analisi di espressione genica sono state effettuate tramite qRT-PCR e i risultati sono stati normalizzati con il metodo del $2^{-\Delta\Delta Ct}$. In generale, i 9 geni analizzati risultano essere differenzialmente espressi tra le tesi, con differenze più consistenti nei campioni a piena maturità, rispetto all'invaiatura. A causa della complessità della via biosintetica dei fenilpropanoidi e dato l'alto numero di meccanismi post-trascrizionali e post-traduzionali coinvolti nella regolazione del pathway molecolare, non è facile correlare direttamente i risultati ottenuti nell'attività di fenotipizzazione con i livelli di espressione dei 9 geni presi in esame. Si deve, inoltre, considerare che molti di questi geni, come già anticipato, sono membri di famiglie multigeniche, e non è quindi ovvio che siano direttamente ed univocamente responsabili del fenotipo osservato. È particolarmente difficile, infatti, trovare una corrispondenza diretta tra le concentrazioni finali dei metaboliti e la quantità di un trascritto dei geni coinvolti negli step intermedi di sintesi, soprattutto in un pathway complesso come quello esaminato. Un chiaro esempio è dato dal gene *VvPAL*, di cui è difficile avere riscontri fenotipici, poiché l'attività del relativo enzima ha un complesso controllo multi-livello (Zhang & Liu, 2015). Con i risultati ottenuti si è potuta confrontare, invece, la coerenza con alcune informazioni già presenti in letteratura, soprattutto riguardo le differenze di espressione genica tra invaiatura e maturazione.

Il gene *VvPAL* codifica per la FENILALANINA AMMONIO-LIASI, l'enzima responsabile del primo step del pathway di biosintesi dei fenilpropanoidi, in cui dalla fenilalanina viene sintetizzato l'acido cinnamico, il substrato comune delle reazioni successive. L'espressione di *VvPAL* aumenta in modo graduale durante la maturazione (Gatto et al., 2008), anche sotto lo stimolo dei raggi UV-B, che up-regolando alcuni geni tra cui *PAL*, innescano la sintesi di composti UV-assorbenti come i flavonoidi (Teixeira *et al.*, 2013). Dai risultati ottenuti, l'espressione del gene all'invaiatura è stata simile tra le tesi. Nel controllo la quantità di trascritto ha subito un aumento a maturità (come da letteratura), mentre in M-101-14 e in 1103P ha avuto un trend di espressione superiore nel primo stadio di campionamento ed è rimasto pressoché stabile o più basso nel successivo campionamento. Il gene *VvF3'H* codifica per la FLAVONOIDE 3'-IDROSSILASI, un enzima responsabile dell'idrossilazione in posizione 3' dell'anello B dei flavonoidi naringenina e di-idrokampferolo, che porta alla successiva sintesi di flavonoli, proantocianidine e antociani disostituiti. Secondo lo studio di Castellarin (*et al.*, 2006), condotto sulle variazioni di colore nelle uve a bacca nera, in *Merlot* gli antociani derivati dalla cianidina (disostituiti) sono sintetizzati in misura maggiore nella prima fase dell'invaiatura. Tale risultato è in linea con quelli ottenuti nel presente studio, in quanto la quantità di trascritto del gene *VvF3'H* è maggiore al momento dell'invaiatura, rispetto alla piena maturità. A maturità il gene *VvF3'H* è più espresso nel controllo e meno espresso nella tesi PAU. L'enzima F3'H compete con F3'5'H (codificato dal relativo gene, appartenente ad una famiglia multigenica compresa nel genoma di *Vitis vinifera*) per l'utilizzo di substrati comuni, biforcando, di fatto, il

pathway di sintesi dei flavonoidi. Secondo alcuni autori, la prevalenza di F3'H su F3'5'H porta ad avere un maggiore accumulo di cianidina, il precursore della peonidina, in sfavore dell'accumulo di delfinidina, precursore di malvidina e petunidina (Kuhn *et al.*, 2014). Nei risultati ottenuti in questo studio non c'è linearità diretta tra le concentrazioni dei metaboliti e il livello di espressione dei geni. Il gene *VvFLS* codifica per la FLAVONOLO SINTASI, un enzima che catalizza la formazione di flavonoli agliconi a partire da di-idrokampferolo, di-idromiricetina e di-idroquercetina. Durante la prima fase della crescita, nelle bucce si accumulano maggiormente flavonoli e proantocianidine (Bogs *et al.*, 2006) e secondo Ali (*et al.*, 2011) i trascritti della FLAVONOLO SINTASI diminuiscono progressivamente durante la maturazione nelle bucce di *Cabernet sauvignon*. Questa tesi si può considerare supportata dai risultati ottenuti, vista la maggiore espressione genica di *VvFLS* all'invaiatura e una minore alla maturità. Inoltre, l'espressione nei due time point è stata più alta nel controllo e potrebbe essere correlata con la maggiore concentrazione di rutina nelle bucce. I substrati di FLS (i di-idroflavonoli) sono comuni anche all'enzima DFR, che svolge il primo step di sintesi delle antocianidine, ovvero converte i di-idroflavonoli in leucoantocianidine. Secondo Castellarin (*et al.*, 2006), nella fase di post-invaiatura, l'accumulo di proantocianidine nelle bucce diminuisce in modo drastico. I valori ottenuti per l'espressione del gene *VvDFR*, che resta similmente alta in entrambi i time point di campionamento, possono far ipotizzare che all'invaiatura che i substrati intermedi vengano convertiti preferibilmente in proantocianidine, mentre a maturità il metabolismo sia a favore della sintesi di antociani.

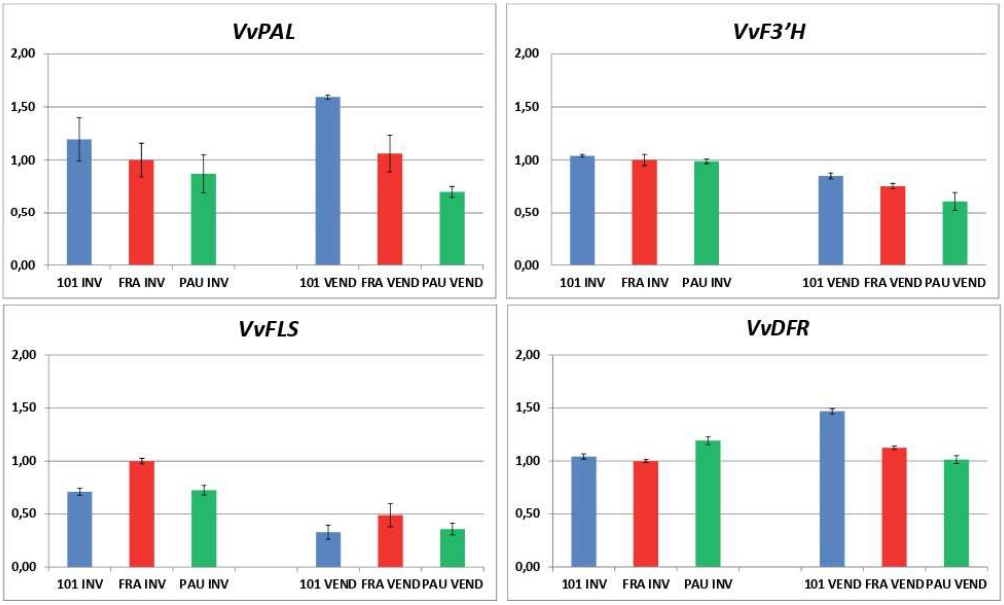

Fig.4. Espressione dei 4 geni strutturali. Analisi con qReal-Time PCR, calcolo da Ct value con il metodo del $2^{-\Delta\Delta Ct}$ (le barre indicano l'errore standard). Tesi: 101=M101-14; PAU=1103 Paulsen; FRA=controllo; INV=invaiatura; VEND=maturità.

I fattori di trascrizione MYB appartengono al complesso proteico MBW (insieme a bHLH e WD40), che controlla, a diversi livelli, il pathway di sintesi dei fenilpropanoidi. Le proteine MYB hanno una struttura conservata, caratterizzata da uno specifico dominio N-terminale capace di legare il DNA, che è costituito da 52 amminoacidi e si ripete 1, 2 o 3 volte (R1, R2, R3). La famiglia MYB comprende numerosissimi membri, tutti regolatori della trascrizione (sia positivi che negativi), coinvolti nella via biosintetica di flavonoli, antocianine, proantocianidine e stilbeni. Sul ruolo dei diversi geni MYB sono state effettuate e sono tutt'ora in atto numerose ricerche, ma le informazioni che si trovano in letteratura sono, a volte, discordanti.

Del gene *VvMYB14* si sa che è coinvolto nella regolazione a feedback della sintesi del resveratrolo, un ramo del pathway dei fenilpropanoidi (Fasoli *et al.*, 2018; Jeandet *et al.*, 2019). Il resveratrolo è una molecola che ha importante ruolo nella dissipazione dello stress ossidativo, e si è visto che viene accumulato anche negli acini di viti sane e non stressate verso la fine della maturazione (Gatto *et al.*, 2008). La produzione del fattore di trascrizione MYB14 è stimolata dallo stress indotto dai raggi UV-C, il quale, insieme ad un altro TF (WRKY3), induce l'attività del gene *STS29* (uno dei geni della famiglia genica codificante per l'enzima STILBENE SINTASI). In presenza di resveratrolo, *MYB14* è down-regolato dal TF WRKY8 che blocca, di conseguenza, l'attività di *STS29*, facendo scendere la concentrazione di resveratrolo. I risultati mostrano per *VvMYB14* un'espressione simile tra le tesi all'invaiatura, a maturità risulta più alta la quantità di trascritto in M101-14.

Il gene *VvMYBC2-L3* è coinvolto nella biosintesi delle antocianine. Si sa che agisce come repressore trascrizionale nella sintesi degli antociani (Cavallini *et al.*, 2015). Secondo Zhu (*et al.*, 2019) l'espressione di *VvMYBC2-L3* in petali di tabacco transgenico reprime l'espressione del gene *VvDFR* ed induce l'espressione di *VvFLS*. Ma si tratta soltanto di un'ipotesi, in quanto la regolazione negativa della sintesi degli antociani nelle piante resta poco conosciuta. Secondo i risultati, all'invaiatura l'espressione è maggiore nella tesi 1103 Paulsen, intermedia per il controllo e più bassa in M101-14; l'andamento risulta esattamente opposto a maturità, maggiore in M101-14, intermedio nel controllo e più basso in 1103 Paulsen.

Il gene *VvMYB4R1* è coinvolto nella sintesi degli stilbeni, ma si conoscono pochi ulteriori dettagli sulla sua attività funzionale (Vezzulli *et al.*, 2019). L'espressione di *VvMYB4R1* è minore nello stadio di invaiatura e sale in tutte le tesi al momento della maturità; la quantità di trascritto è più alta in entrambi i casi per M101-14.

I geni appartenenti alla famiglia *NAC* sono fattori di trascrizione specifici delle piante. Hanno un dominio NAM conservato al N-terminale per il DNA-binding e i membri, 79 in vite secondo Wang

(*et al.* 2013), hanno diversi ruoli nei processi di sviluppo della pianta e nella maturazione dei frutti, nonché nella risposta agli stress biotici ed abiotici. Le precise funzioni non sono state individuate per tutti, si sa soltanto che spesso sono tessuto-specifici e possono essere attivatori o repressori, anche secondo l'ambiente circostante (Le Hénanff *et al.*, 2013).
Il gene *VvNAC44* è coinvolto nella maturazione degli acini e nella risposta a stress (Suzuki *et al.*, 2015), in particolare nella sintesi di stilbeni. È molto espresso nelle tesi innestate rispetto al controllo, sia all'invaiatura che a maturità, con differenze notevoli, soprattutto nel secondo tempo di campionamento.

Anche *VvNAC60* è coinvolto nella regolazione della maturazione degli acini e nella risposta a stress (Sun *et al.*, 2012). Il gene ha un livello di espressione molto diverso nei due tempi, con differenze minime, ma significative tra le tesi. Il controllo ha sempre un livello di espressione più alto.

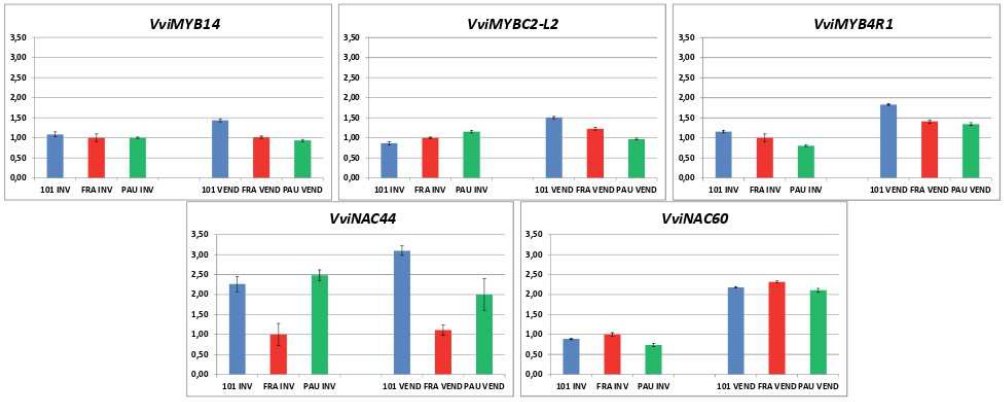

Fig.5. Espressione dei 5 geni codificanti per fattori di trascrizione. Analisi con qReal-Time PCR, calcolo da Ct value con il metodo del $2^{-\Delta\Delta Ct}$ (le barre indicano l'errore standard). Tesi: 101=M101-14; PAU=1103 Paulsen; FRA=non innestato; INV=invaiatura; VEND=maturità.

Conclusioni

Lo scopo di questo lavoro di ricerca è stato quello di studiare l'influenza del portinnesto sulla qualità dell'uva, in viti mantenute in condizioni agronomiche identiche e con medesimo abbondante apporto idrico. L'utilizzo di un sistema sperimentale di piante in vaso ha permesso di gestire in modo uniforme tutte le tesi. I risultati ottenuti consentono di dedurre che i portinnesti impiegati, ovvero 1103 Paulsen e M101-14, su uve *Pinot nero*, in condizioni ottimali di irrigazione ed in assenza di fattori limitanti, non hanno causato alterazioni nel nesto in termini di stadio fenologico di sviluppo, efficienza fotosintetica, produzione di uva e relativa maturazione tecnologica. Tali portinnesi, dunque, non hanno avuto effetti diretti sul metabolismo primario delle viti. Come ci si aspettava, l'unico parametro rilevato come significativamente

differente è stato il peso del legno per la tesi 1103 Paulsen, un portinnesto che notoriamente conferisce maggiore vigoria nel nesto.

Alcune differenze importanti, invece, sono state riscontrate a livello del metabolismo secondario delle uve, in particolare nelle bucce. Le 3 tesi hanno accumulato concentrazioni significativamente diverse di alcune molecole sintetizzate nell'ambito del pathway dei fenilpropanoidi, ovvero nel profilo antocianico percentuale (malvidina e peonidina), nei flavonoli (quercetina-rutina e miricetina-glucoside), nei flavan-3-oli (catechina) e negli stilbeni (resveratrolo-polidatina).

Anche le analisi molecolari, effettuate per avere informazioni riguardo il livello di regolazione dell'espressione di alcuni precisi geni strutturali o fattori di trascrizione, hanno dato risultati interessanti. I geni, selezionati a partire da uno studio precedente di trascrittomica, sono differenzialmente espressi tra le tesi nella stagione vegetativa 2017. Non è stata riscontrata una corrispondenza diretta tra le concentrazioni dei metaboliti secondari e la quantità di trascritto dei 9 geni considerati. Come si può ritrovare in molti studi sul medesimo argomento, non è, in effetti, possibile stabilire delle relazioni univoche riguardo l'accumulo di sostanze fenoliche, un processo complesso, ricco di interconnessioni (Pastore *et al.*, 2017). La concentrazione delle molecole sintetizzate in un tessuto ed in un determinato momento può non avere corrispondenza con il livello di espressione di un gene. Ciò viene spiegato dal fatto che nell'ambito del metabolismo secondario insorgono diversi livelli di regolazione post-trascrizionale, post-traduzionale, competizione tra gli enzimi per i substrati intermedi, necessità di cofattori nelle reazioni, che si ripercuotono sulla quantità finale di flavonoli, antocianine, proantocianidine e stilbeni. Inoltre, la presenza di famiglie multigeniche per i geni coinvolti nella biosintesi dei metaboliti secondari può dare origine a diversi profili di espressione spazio-temporali per i vari membri della stessa famiglia, che possono, o meno, essere in linea con la quantità finale del metabolita prodotto dalla via biosintetica (Kunh *et al.*, 2014).

L'effetto dei portinnesti sul fenotipo delle viti, ma soprattutto delle uve, è un argomento molto attuale, che stimola forte interesse nel mondo della ricerca. Anche nei lavori più recenti, e nonostante l'impiego di metodi di analisi avanzati e la disponibilità di tecnologie *high-throughput*, si sottolinea sempre che la complessità del network molecolare dello sviluppo della pianta e della maturazione dei frutti non permette di chiarimenti definitivi. La situazione è ancora più complicata in una coltura complessa come quella della vite, che ha una grande variabilità genetica data dalle numerosissime varietà che comprende e che esprime notevoli differenze nei frutti, anche all'interno di un singolo grappolo. Lo studio presentato avrà un seguito con la ripetizione delle ricerche sul medesimo sistema sperimentale, aggiungendo la variabile stress

idrico, per verificare se in condizioni di carenza di acqua viene ulteriormente modulata la risposta nel nesto, sotto l'influenza dei due diversi portinnesti considerati (1103 Paulsen e M101-14). Il portinnesto è un componente chiave che media l'effetto dell'ambiente sulla qualità delle uve (Ollat *et al.*, 2017) e comprendere alcuni meccanismi metabolici può risultare fondamentale per sfruttare questo elemento nell'ambito dei cambiamenti climatici e del miglioramento genetico in viticoltura.

Bibliografia

- Ali MB, Howard S, Chen S, Wang Y, Yu O, Kovacs LG, Qiu W (2011). Berry skin development in Norton grape: Distinct patterns of transcriptional regulation and flavonoid biosynthesis. *BMC Plant Biology*, 11: 7.
- Bogs J, Ebadi A, McDavid D, Robinson SP (2006). Identification of the Flavonoid Hydroxylases from Grapevine and Their Regulation during Fruit Development. *Plant Physiology*, 140: 279-291.
- Boss PK, Davies C, Robinson SP (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 Physiol*, 111: 1059-1066.
- Buhtz A, Pieritz J, Springer F, Kehr J (2010). Phloem small RNAs, nutrient stress responses and systemic mobility". *BMC Plant Biol*, 10: 64.
- Castellarin SD, Di Gaspero G, Marconi R, Nonis A, Peterlunger E, Paillard S, Adam-Blondon AF, Testolin R (2006). Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3′-hydroxylase, flavonoid 3′,5′-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. *BMC Genomics*, 7: 12.
- Cavallini E, Matus JT, Finezzo L, Zenoni S, Loyola R, Guzzo F, Schlechter R, Ageorges A, Arce-Johnson P, Tornielli GB (2015). The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYB C2 repressors in grapevine. *Plant Physiol*, 167: 1448-1470.
- Chitarra W, Perrone I, Avanzato CG, Minio A, Boccacci P, Santini D, Gilardi G, Siciliano I, Gullino ML, Delledonne M, Mannini F, Gambino G (2017). Grapevine grafting: scion transcript profiling and defense-related metabolites induced by rootstocks. *Front Plant Sci*, 8: 564.
- Cookson SJ, Clemente Moreno MJ, Hevin C, Nyamba Mendome LZ, Delrot S, Magnin N, Trossat-Magnin C, Ollat N (2014). Heterografting with nonself rootstocks induces genes involved in stress responses at the graft interface when compared with autografted controls. *J Exp Bot*, 65: 2473-2481.
- Corso M, Vannozzi A, Maza E, Vitulo N, Meggio F, Pitacco A, Telatin A, D'Angelo M, Feltrin E, Negri AS, Prinsi B, Valle G, Ramina A, Bouzayen M, Bonghi C, Lucchin M (2015). Comprehensive transcript profiling of two grapevine rootstock genotypes contrasting in drought susceptibility links the phenylpropanoid pathway to enhanced tolerance. *Journal of Experimental Botany*, 66: 5739-5752.
- Cui ZH, Bi WL, Hao XY, Li PM, Duan Y, Walker MA, Xu Y, Wang QC (2017). Drought Stress Enhances Up-Regulation of Anthocyanin Biosynthesis in Grapevine leafroll-associated virus 3- Infected in vitro Grapevine (Vitis vinifera) Leaves. *Plant Disease*, 101: 1606-1615.
- Di Fillippo M, Vila H (2011). Influence of different rootstocks on the vegetative and reproductive performance of *Vitis vinifera* L. Malbec under irrigated conditions. *J Int Sci Vigne Vin*, 45: 75-84.
- Di Stefano R, Cravero MC (1991). Metodi per lo studio dei polifenoli dell'uva. *Rivista di Viticoltura e di enologia*, 2: 37-45.
- Downey MO, Harvey JS, Robinson SP (2003). Synthesis of flavonols and expression of flavonol synthase genes in developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Aust J Grape Wine Res* 9: 110-121.
- Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Santo SD, Dokoozlian N, Pezzotti M, Tornielli GB (2018). Timing and order of the molecular events marking the onset of berry ripening in grapevine. *Plant Physiol*, 178: 1187-1206.
- Gatto P, Vrhovsek U, Muth J, Segala C, Romualdi C, Fontana P, Pruefer D, Stefanini M, Moser C, Mattivi F, Velasco R (2008). Ripening and genotype control stilbene accumulation in healthy grapes. *J. Agric. Food Chem,* 56: 11773-11785.
- Gomez-Alonso S, Garcıa-Romero E, Hermosìn-Gutiérrez I (2007). HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. *Journal of Food Composition and Analysis*, 20: 618-626.
- Harada T (2010). Grafting and RNA transport via phloem tissue in horticultural plants. *Scientia Horticulturae*, 125: 545-550.
- Jeandet P, Clément C, Cordelier S (2019). Regulation of resveratrol biosynthesis in grapevine: new approaches for disease resistance?. *J Exp Bot*, 70: 375-378.
- Keller M, Mills LJ, Harbertson JF (2012). Rootstock effect on deficit-irrigated winegrapes in a dry climate: vigor, yield formation and fruit ripening. *Am J Enol Vitic*, 63: 29-39.

- Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, Gomès E, Li SH, Godoy F, Arce-Johnson P, Delrot S (2014). Berry ripening: recently heard through the grapevine?. *J Exp Bot*, 65: 4543-4559.
- Le Hénanff G, Profizi C, Courteaux B, Rabenoelina F, Gerard C, Clement C, Baillieul F, Cordelier S, Dhondt-Cordelier S (2013). Grapevine NAC1 transcription factor as a convergent node in developmental processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance. *J Exp Bot* 64: 4877-4893.
- Maré C, Mica E, Cattivelli L (2016). Molecular advances in rootstock-scion interaction in grapevine. *Acta Horticulturae*, 1136: 155-160.
- Massonnet M, Fasoli M, Tornielli GB, Altieri M, Sandri M, Zuccolotto P, Paci P, Gardiman M, Zenoni S, Pezzotti M (2017). Ripening Transcriptomic Program in Red and White Grapevine Varieties Correlates with Berry Skin Anthocyanin Accumulation. *Plant Physiology*, 174: 2376-2396.
- Mattii GB, Orlandini S, Calabrese CE (2005). Analysis of grapevine vegeto-productive responses to plant density and rootstock. *Int. GESCO - Viticulture Congress*, 14, Geisenheim, Germany, vol. 2: 629-634.
- Mattivi F, Guzzon R, Vrhovsek U, Stefanini M, Velasco R (2006). Metabolite profiling of Grape: Flavonols and Anthocyanins. *J Agric Food Chem*, 54: 7692-7702.
- Mica E, Faccioli P, Tononi P, Delledonne M, Storchi P, Zombardo A, Morreale G, Vaňková R, Dobrev PI, Mocali S, Bernardo L, Giusti L, Costantini E, Priori S, Crosatti C, Reshef N, Or E, Fait A, Cattivelli L (2014). Rootstock-scion interaction in grapevine: An omics perspective. *Proceedings of the 58th Italian Society of Agricultural Genetics*, poster communication 1.33.
- Nuzzo V, Matthews MA (2006). Response of fruit growth and ripening to crop level in dry-farmed Cabernet sauvignon on four rootstocks. *Am J Enol Vitic*, 57: 314-324.
- O.I.V. (2009). Compendium of international methods of analysis of wines and musts. http://www.oiv.int.
- O.I.V. (2009). 2nd edition of the O.I.V. descriptor list for grape varieties and Vitis species. http://www.oiv.int.
- Ollat N, Cookson SJ, Lauvergeat V, Marguerit E, Barrieu F, Gambetta G, Goutouly JP, Tandonnet JP, Vivin P, Delrot S (2017). Grapevines roots: the dark side. Acta Horticolturae, 1188: 213-226.
- Palliotti A, Poni S, Silvestroni O (2015). La nuova viticoltura. Edagricole, Bologna, Italy.
- Palumbo MC, Zenoni S, Fasoli M, Massonnet M, Farina L, Castiglione F, Pezzotti M, Paci P (2014). Integrated Network Analysis Identifies Fight-Club Nodes as a Class of Hubs Encompassing Key Putative Switch Genes That Induce Major Transcriptome Reprogramming during Grapevine development. *The Plant Cell*, 26: 4617-4635.
- Pastore C, Dal Santo S, Zenoni S, Movahed N, Allegro G, Valentini G, Filippetti I, Tornielli GB (2017). Whole Plant Temperature Manipulation Affects Flavonoid Metabolism and the Transcriptome of Grapevine Berries. Front Plant Sci, 8: 929.
- Sun X, Korir NK, Han J, Shangguan LF, Kayesh E, Leng XP, Fang JG (2012). Characterization of grapevine microR164 and its target genes. *Mol Biol Rep,* 39: 9463-9472.
- Suzuki M, Nakabayashi R, Ogata Y, Sakurai N, Tokimatsu T, Goto S, Suzuki M, Jasinski M, Martinoia E, Otagaki S, Matsumoto S, Saito K, Shiratake K (2015). Multiomics in Grape Berry Skin Revealed Specific induction of the Stilbene Synthetic Pathway by Ultraviolet-C irradiation. *Plant Physiology*, 168: 47-59.
- Teixeira A, Eiras-Dias J, Castellarin S, Geròs H (2013). Berry Phenolics of Grapevine under Challenging Environments. *Int J Mol Sci*, 14: 18711-18739.
- Vannozzi A, Wong DCJ, Holl J, Hmmam I, Matus JT, Bogs J, Ziegler T, Dry I, Barcaccia G, Lucchin M (2018). Combinational Regulation of Stilbene Synthase Genes dy WRKY and MYB Transcription Factors in Grapevine (Vitis vinifera L.). *Plant Cell Physiol*, 59: 1043-1059.
- Vezzulli S, Malacarne G, Masuero D, Vecchione A, Dolzani C, Goremykin V, Mehari ZH, Banchi E, Velasco R, Stefanini M, Vrhovsek U, Zulini L, Franceschi P, Moser C (2019). The *Rpv3-3* Haplotype and Stilbenoid Induction Mediate Downy Mildew Resistance in a Grapevine Interspecific Population. *Front Plant Sci,* 10: 234.
- Wang N, Zheng Y, Xin H, Fang L, Li S (2013). Comprehensive analysis of NAC domain transcription factor gene family in *Vitis vinifera*. *Plant Cell Rep*, 32: 61-75.
- Yang Y, Mao L, Jittayasothorn Y, Kang Y, Jiao C, Fei Z, Zhong GY (2015). Messenger RNA exchange between scions and rootstocks in grafted grapevines. *BMC Genomics*, 15: 251.
- Zhang XB, Liu CJ (2015). Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. *Mol Plant*, 8: 17-27.
- Zhu Z, Li G, Li L, Zhang Q, Han Z, Chen X, Li B (2019). A R2R3-MYB Transcription Factor, *VvMYBC2L2*, Functions as a Transcriptional Repressor of Anthocyanin Biosynthesis in Grapevine (*Vitis vinifera* L.). *Molecules*, 24: 92.

Riassunto:
In gran parte dei distretti vinicoli mondiali, le viti coltivate devono essere necessariamente innestate su radici di Vitis americane per superare il problema della fillossera, una grave malattia esplosa in Europa alla fine del 1800. I portinnesti rappresentano una forma molto efficace di controllo biologico contro il parassita del suolo Daktulosphaira vitifoliae e, secondo molti autori, influenzano la fisiologia del nesto, l'adattabilità della pianta all'ambiente e la sua capacità di reagire agli stress abiotici. L'interazione tra i bionti è complessa ed implica cambiamenti strutturali, integrazione idraulica tra le parti innestate, scambio di ormoni e materiali genetici attraverso il punto di innesto. Nonostante la grande quantità di studi e ipotesi sull'effetto dei diversi portinnesti sulla resa della pianta e sulla qualità dell'uva prodotta, i network molecolari che regolano questa interazione rimangono in gran parte poco conosciuti.
Lo scopo del presente lavoro è stato quello di valutare l'influenza di due diverse combinazioni di portinnesto (1103 Paulsen e M101-14, opposti tra loro per vigore e tolleranza alla siccità) e viti non innestate (usate come controllo) sul metabolismo secondario di uve Pinot nero, sia a livello biochimico, che a livello di espressione genica. Le ricerche sono state effettuate in un sistema sperimentale di viti in vaso di 10 anni, allevate a controspalliera con potatura a cordone speronato; tutte le piante sono state irrigate in modo abbondante, con quantità di acqua identica per ognuna delle tre tesi.
I risultati hanno permesso di evidenziare considerevoli differenze, sia nella sintesi di alcune classi di flavonoidi che nell'espressione di alcuni geni (strutturali e regolatori) appartenenti al pathway dei fenilpropanoidi.

Abstract:
Most of the grapevines cultivated in the world are grafted in order to overcome the problem of phylloxera, a serious disease that exploded in Europe at the end of 1800. The rootstocks represent not only a very effective form of biological control against the soil-borne pest Daktulosphaira vitifoliae, but also an important way to influence the physiology of the scion, the adaptability to the environment and its response to different kind of abiotic stresses. The rootstock-scion interaction is complex because it implies structural changes, hydraulic integration, exchange of hormones and genetic materials through the graft junction. Despite the great amount of studies and hypotheses about the effect of the different type of rootstocks on plant's yield and grape quality, the molecular networks governing this interaction remain largely unknown.
The aim of this work is to evaluate the influence of two different rootstock combinations (1103 Paulsen and M101-14, mutually opposite in vigor and drought tolerance) and not grafted plants (as control) on the secondary metabolism of Pinot Noir grapes, both biochemically and in terms of gene expression. The project was set up on potted 10-year-old grapevines, trained on vertical shot positioned trellis and spur cordon pruned; all the plants were irrigated abundantly, with the same amount of water.
Taken together, our results suggest that different rootstocks have a direct effect on vines' phenotype and quality, in particular in the synthesis of some flavonoids and in the expression of some genes (both structural and regulatory), belonging to the phenylpropanoid pathway.

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Arrivare ad oggi, ultimo giorno dell'ultimo anno di Dottorato è una grande soddisfazione, soprattutto riguardando indietro a tutto il lavoro svolto. Infondo, non è una conclusione, ma solo l'inizio di nuove sfide e nuovi traguardi da raggiungere.