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STUDY OF GRAPEVINE ROOTSTOCK RESPONSE TO WATER STRESS

PhD student: Caramanico Leila

Tutor: Prof. Osvaldo Failla

Co-Tutor: Dott.ssa Gabriella De Lorenzis

Doctoral coordinator: Prof. Daniele Bassi

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Abstract

More than 80% of vineyards around the world use grafted plants: a scion of *Vitis vinifera* grafted onto a rootstock of single or interspecific hybrids of American *Vitis* species, resistant or partially resistant to Phylloxera (*Daktulosphaira vitifoliae* (Fitch, 1856)). The genetic variability of grapevine rootstocks plays a fundamental role in their adaptation to the environment (Serra et al., 2013). In the climate change scenario, predicting an increase of aridity in the near future (Dai, 2013), the more frequent and severe drought events may represent the major constrain for the future of viticulture (IPCC, 2018; Schultz, 2000). Therefore, the selection of new rootstocks able to cope with unfavourable environmental condition is a key asset, as well as a strategy to improve crop yield/vegetative growth balance on scion behaviour (Corso and Bonghi, 2014).

So far, the influence of rootstock on scion physiological performance during water stress has always aroused great interest. On the contrary, the scion impact on rootstock response is still less debated. Therefore, the effect of grafting on rootstock behaviour have been investigated. Phenotypical and large-scale whole transcriptome analyses on two genotypes, a drought-susceptible (101-14) and a drought-tolerant (1103 P), own-rooted and grafted with Cabernet Sauvignon, subjected to a gradual water shortage in semi-controlled environmental conditions have been performed. The ungrafted condition affected photosynthesis and transpiration, meaning the decisive role of scion in modulation of gas exchanges and in general in plant adaptation. Molecular evidence highlighted that the scion delays the stimulus perception and rootstock reactivity to drought.

Since 1985, the DiSAA research group operating at the University of Milan is carrying on a rootstock crossbreeding program which has led to the release of four genotypes: M1, M2, M3 and M4. They show from moderate to high tolerance to drought (M4 > M1 = M3 > M2). In order to characterize their performance during water stress, their physiological (gas exchanges and stem water potential) and transcriptome response (genes involved in ABA-synthesis and ABA-mediated responses to drought) under well-watered and water stress conditions were examined. The behaviour of M-rootstocks (M1, M2 and M3) was compared with that of other commercial genotypes largely used in viticulture, either tolerant (140 Ru, 41 B, 110 R, 1103 P), less tolerant (SO 4, K 5BB) and susceptible (420 A and Schwarzman). Discriminant analysis (DA) showed that when water availability starts to decrease, rootstocks firstly perceives the stress activating a transcriptome response, consequently physiological changes have been observed. It also demonstrated that the three M-rootstocks were clearly discriminated: M4 was grouped with the most tolerant genotypes while M3 with the less

tolerant or susceptible ones from a physiological standpoint, confirming their different attitude to tolerate water stress.

M4 has proven to be a promising rootstock due to its ability to adapt to drought conditions. Considering the constant great demand for vine planting materials, the obtainment of genetically homogeneous populations (i.e. clones) from elite individuals through micropropagation represents a rapid alternative to conventional multiplication. For this reason, an efficient high-throughput protocol for M4 *in vitro* propagation was set up. Its attitude to shooting, root development and callus proliferation was compared to that of other rootstocks largely used in viticulture (K5BB, 1103P, 101-14 and 3309C). Moreover, pro-embryogenic and embryogenic callus from bud explants were also produced, representing a cellular material manipulable with the genetic engineering techniques.

In water scarcity condition, among the mechanisms activated by M4, the great ability to scavenge ROS, related to the increased accumulation of stilbenes and flavonoids, may be such as to give it tolerance to the stress. In particular, the higher levels of *trans*-resveratrol were correlated with the up-regulation of some stilbene synthase genes, mainly VvSTS16, VvSTS18, VvSTS27 and VvSTS29. The over expression of these genes was linked to a structural variation in their promoter region. To confirm that VvSTSs genes may be considered putative factors of M4 better adaptation to water stress, a genome editing protocol based on the CRISPR/Cas9 system, aimed at knock-out the genes, was performed. For testing the gRNAs functionality, a transient assay on *in vitro* micropropagated plantlets of M4 and 101-14 was performed. The positive results obtained by this experiment will lead to the transformation of somatic embryos and regeneration of whole-edited plants using the vectors developed.

Summary

List of abbreviations and frequently used symbols

ABA: Abscisic Acid

ABA-GE: ABA-glucose ester

ANOVA: Analysis of Variance

Cas9 : CRISPR-associated system

Ci: Internal CO_2 Concentration

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

cv: cultivar

CYP: Cytochromes P450

DF: Discriminant Function

DNA: deoxyribonucleic acid

DSB: Double-Strand Break

E: Transpiration rate

Gs : Stomatal conductance

Hrs: hours

LA: Leaf area

Mb: Mega base

Mha: millions of hectares

Mhl: millions of hectolitres

Mt: millions of tons

Pn: Photosynthetic activity

PPFD: Photosynthetic Photon Flux Density

ppm: parts per million

qPCR: quantitative Real Time Polymerase Chain Reaction

RBOHs: Respiratory Burst Oxidase Homologs

RNA: ribonucleic acid

RNA-Seq: RNA sequencing

ROS: Reactive Oxygen Species

SGR: Stem Growth Rate

SSN: Sequence-Specific Nucleases

SSR: Single Sequence Repeats

SWC: Soil Water Content

SWP: Stem Water Potential

TALENs: Transcription activator-like effector nucleases

TFs: Transcription factors

Vpd: Vapor pressure deficit

VvSTS: Stilbene synthases genes

WS plants: Water-stressed plants

WUE_{int}: Intrinsic water use efficiency

WW: Well-watered plants

ZFNs: Zinc-finger nucleases

Ψleaf: leaf water potential

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Figure IV. 1 Electrophoretic analysis in 1.5% agarose gel of PCR products, referred to the samples reported in Table IV.2. Above, bands are related to M4 genotype; below they are related to 101-14 genotype. In the middle, the PCRBIO Ladder I (PB40 .11-01).

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Chapter I – General introduction

Grapevine (*Vitis* spp.) is one of the most cultivated and prized fruit crops around the world. The global surface under vines is 7.4 Mha with 5 countries representing the 50% of the world vineyards: Spain, China, France, Italy and Turkey. Grape yield is about 77.8 Mt, of which the 57% is represented by wine grape, 36% by table grape and 7% by dried grape. Global wine production amount to 292 Mhl and Italy is the first producer (54.8 Mhl), followed by France (48.6 Mhl) and Spain (44.4 Mhl). Vine is a crop of great economic interest in fact, during the decade 2008 – 2018, wine trade has increased progressively either as volume (from 90 Mhl to 108 Mhl) and value (from 20 to 31 billion EUR) (OIV, 2019).

The cultivation of domesticated grapes (*Vitis vinifera* subsp. *vinifera*) began 6000-8000 years ago, during the Neolithic Era, from the ancestor *Vitis vinifera* subsp. *sylvestris.* The primary domestication centre was the Near Est and the Northern Mesopotamia. From this area, cultivated *vinifera* spread to neighbouring regions following three main pathways. The first, towards Mesopotamia, East Mediterranean Basin, North Africa, Southern Balkans and Aegean Region (end of the 5th millennium BCE). Hence, toward Sicily and southern Italy, France, Spain and finally towards Central Europe (during the 1st millennium BCE), mainly through the rivers of Rhine, Rhone and Danube (Forni, 2012). The second path was the Central Asia (the 4th century BCE) and the third paths was China and Japan areas (near the 2nd century BCE) (Forni, 2012; McGovern, 2003). The Russian botanist Negrul (1946) proposed three main ecological or ecogeographical groups of varieties, also called *proles* (Latin proles=offspring), based on their region of origin: *occidentalis*, *pontica* and *orientalis*.

The major advantages of the domestication process were the switch from dioecy to self-pollinating hermaphroditism, an increase in seed and berry size and a higher sugar accumulation (This et al., 2006; Myles et al., 2011).

Through sexual reproduction by crossing (mainly interspecific hybrids) and perpetuation with vegetative propagation by cuttings, thousands of cultivars in use today have been obtained (Myles et al., 2011): they preserve the selected traits and high level of heterozygosity of grapevine. Somatic mutations added variability within cultivars and contributed to develop new phenotypes (Pelsy, 2010).

The first molecular phylogeny on plastid DNA markers revealed the monophyly of the genus *Vitis* and the presence of three clades: American, Asian and European, reflecting the geographical distribution of *Vitis* species. Within the Asian clade the genetic diversity is high while within North American and European ones is low. This mean that between cultivated grapevines and autochthonous accessions, in the evolution of the genus *Vitis,* there were hybridization (Tröndle et

al., 2010). The genetic variation of *vinifera* is huge, with polymorphisms that dates back tens of millions of years (Myles et al., 2010). Thus, an environmentally sustainable grape-growing industry could rely on access and use of genetic diversity to develop improved cultivars towards biotic and abiotic stresses, even considering the ongoing climate changes.

Grapevine genome was sequenced in 2007 by the French-Italian Public Consortium (Jaillon et al., 2007) and the Italian-American Collaboration (Velasco et al., 2007). It was the fourth genome released among flowering plants, the second for woody plants and the first one for fruit crops. The French Italian Consortium used as reference genotype the PN40024, a near homozygote, derived from inbreeding cycle of Pinot noir. The Italian American Collaboration opted for a clone of Pinot noir heterozygous.

Grapevine is diploid, with 19 chromosomes. The genome size is approximately 487 Mb and the last updated sequence currently available online is the 12X coverage of PN40024, that has become the reference genome for *Vitis vinifera*. Thanks to the genome sequence availability (Jaillon et al., 2007; Velasco et al., 2007) and the development of high-throughput analyses (i.e. microarray) and the next generation sequencing techniques (i.e. RNA sequencing) it is possible to study the entire grape transcriptome and gene expression during phenology phases and/or in response to biotic and abiotic stresses (Troggio et al., 2008).

I.2 Botanical classification

Grape is a liana from the *Vitacea* family. According to a phylogenetic analysis of the entire plastid genome, this family is the earliest diverging lineage of rosids (Jansen et al., 2006). It contains 17 genera comprising approximately 1000 species, mostly shrubs or woody tree-climbing, by means of their leaf-opposed tendrils, hence the name *Vitaceae* (from Latin *viere*= to attach). The genera *Muscadinia* (2n= 40 chromosomes) and *Vitis* (2n= 38 chromosomes) contain all cultivated grapes. The sister relationship between them was confirmed by molecular phylogenetic studies based either on the sequences of three chloroplast regions (Soejima and Wen, 2006) or nuclear GAI1 sequences (Wen et al., 2011). The principal characteristics of the vines belonging to these genera are simple leaves, simple or forked tendrils, generally unisexual flowers that are, either male (staminate) or female (pistillate), fused flower petals that separate at the base forming a calyptra or “cap”; soft and pulpy berry fruits. The two genera cannot be crossed easily for producing fertile hybrids because of the different number of chromosomes (Keller, 2015).

The genus *Muscadinia* is native from the southeastern United States to Mexico. It comprises three species: *M. munsoniana* (Small), *M. popenoei* (Fennell) and *M. rotundifolia* (Small), formerly *Vitis rotundifolia* (Michaux). This one has a huge genetic potential because coevolved with diseases and parasites originating in North America, which still represent a great threat to viticulture. They are the fungi powdery mildew and black rot, the slime mold downy mildew, the bacterium causing Pierce's disease, the aphid Phylloxera and the dagger nematode *Xiphinema index* (which transmits the grapevine fanleaf virus).

The genus *Vitis* occupies temperate and subtropical climate zones of the Northern Hemisphere (Mullins et al., 1992; Wan et al., 2008). It comprises by around 60-70 species (plus 30 fossil and 15 doubtful species) widespread especially throughout Asia (~40 species) and North America (~20 species), also divided into two main groups: the Eurasian group and the American one, whose dominant species greatly differ in their useful agronomic traits, making them attractive breeding partners (Alleweldt and Possingham, 1988; Keller, 2015). All the species within the genus *Vitis* are inter-fertile, meaning that they have a relatively recent common ancestor and can be grafted onto each other (Keller, 2015).

There are approximately 40 species in the Eurasian group, mostly confined to eastern Asia (Keller, 2015). The most well-known is *Vitis vinifera* (L.,753): the main and only species of agronomic interest. It comprises two forms: the wild form, *V. vinifera* subsp. s*ylvestris*, and the cultivated one, *V. vinifera* subsp. *vinifera* (or *sativa*). This one includes a huge number of cultivars, with 6000–10000 cultivars believed to exist in the world (Galet, 2000), from which derive the most famous wine grape varieties in the world: Chardonnay, Riesling, Pinot Noir, Syrah, Merlot and Cabernet Sauvignon (Galet, 1990). The old cultivar *Vitis* vinifera cv. Gouais blanc is the ancestor of a very large kin group of major wine grapes (Bowers et al., 1999; Hunt et al., 2010). Some others, Pinot blanc and Grenache, are mutations of the black cultivars (Kobayashi et al., 2004; Walker et al., 2007). The huge genetic variability available nowadays can be associated to crossing, vegetative propagation and mutations during the evolution of cultivated grapes. However most of the variety variability within *Vitis vinifera* comes from a close relationship generated by crosses among elite cultivars (Myles et al., 2011).

The American group contains between 8 and 34 species. Because most of them are resistant to North American grapevine diseases and pests, they are used in breeding program as crossing partners, or as rootstocks (Alleweldt and Possingham, 1988; This et al., 2006). All the species have specific native habitats mostly near permanent source of water (canyons, alluvial soil, along stream and moist woody areas) but also in dry and rocky zones (rocky hills, dry hillsides, dunes etc). This characteristic reflects the adaptation of each species to environmental conditions (Pongràcz, 1983; Morano and Walker,

1995; Padgett-Johnson et al., 2003). Among the American *Vitis* species there are: *V. aestivalis* (Michaux), *V. arizonica* (Engelmann), *V. berlandieri* (Planchon), *V. californica* (Bentham), *V. candicans* (Engelmann), *V. cinereal* (Engelmann), *V. cordifolia* (Michaux),, *V.doaniana* (Munson), *V. girdiana* (Munson), *V. labrusca* (Linné), *V. longyi* (Prince), *V. monticola* (Buckley), *V. riparia* (Michaux), *V. rupestris* (Scheele) (Galet, 1998; Pongràcz, 1983; Viala and Vermorel, 1901-1909). The naturalized populations of rootstocks have sexually reproduction and a distinct genetic pool with respect to *V. vinifera* ssp *sylvestris*. In some areas, they behave as invasive species (Arrigo and Arnold, 2007). With the travels of European explorers, American vine species were imported in Europe with their pest and diseases.

I.3 Rootstocks

Most of viticulture areas, where Phylloxera is one of the most serious threat for viticulture, the use of grafting technique is essential for grape survival. Phylloxera (*Daktulosphaira vitifoliae* Fitch, 1856), was accidentally imported in Europe from North America by steam ships began to cross the Atlantic Ocean (Gale, 2002). Since 1868, this pest spread firstly in the South of France and over the past 150 years to almost every major viticulture regions in the world, including North and South America, Asia, Europe, the Middle East, Africa and Australia (EPPO, 1990; Benheim et al., 2012). Around 1870's, Léopold Laliman and Gaston Bazille were the first ampelographers to observe that the roots of some American *Vitis* species were not damaged by the aphid (Pouget, 1990). North American *Vitis* species evolved resistance towards this pathogen, therefore they are used, as single or inter-specific hybrids, as rootstocks in order to neutralize the disease.

Nowadays, more than 80% of the vineyards around the world use grafted plants: a scion of *V. vinifera* grafted onto a rootstock that combine favourable characteristics of their parentage. Consequently the cultivated grapevine is a combination of two genomes: the aboveground organs (the scion) and the belowground organs (the rootstock) amongst which the grafting point is the interface (Ollat et al., 2015).

In addition to Phylloxera resistance, rootstocks contribute to the control of other soil borne pests (such as nematodes) and to different environmental condition (Whiting, 2005; Cordeau, 2002; Galet, 1998;). On this purpose, considerable researches have been done in relation to the effect of rootstocks on water uptake and on mineral nutrition (Bavaresco et al., 1991; Garcia et al., 2001; Zerihun and Treeby, 2002; Keller et al., 2001a, 2001b; Holzapfel and Treeby, 2007; Walker et al., 2010; Gong et al., 2011), on the adaptability to soil condition such as lime content, pH, salinity, iron deficiency,

water logging (Galet, 1998; Cordeau, 2002; Whiting, 2005). The interaction between scion and rootstocks has also a strong impact on shoot development and grape quality (Tandonnet et al., 2010; Ollat et al., 2003), bud fertility (Huglin, 1958; Benz et al., 2007), phenology (Pongràcz, 1983; Whiting, 2005), leaf area and canopy development (Paranychianakis et al., 2004; Clingeleffer and Emmanuelli, 2006; Koundouras et al., 2008), wood pruning weight (Ezzahouani and Williams, 2005; Stevens et al., 2008), and yield (Jones et al., 2009; Stevens et al., 2010). Affecting many fruit quality traits, such as carbohydrates (Ezzahouani and Williams, 1995), organic acids (Rühl et al., 1988; Garcia et al., 2001;), amino acids (Huang and Ough, 1989; Treeby et al., 1998) and secondary metabolites (Koundouras et al., 2009; Walker et al., 2000), rootstocks have also an intense economic impact. Consequently, their selection is based on many characteristics that must be considered in a breeding program.

I.3.1 Rootstock's genetic

The majority of the species used as parental are *V. berlandieri, V. riparia and V. rupestris* (Galet, 1998). Rootstock's genetic basis is extremely narrow because most of *V. vinifera* varieties (as many as 90%) are grafted to fewer than 10 rootstocks. Moreover, following the initial breeding efforts before 1900, the majority of them have not changed through the time (Galet, 1998; Huglin and Schneider, 1998). This represents a risk for viticulture if virulent strains of soil pests spread, including Phylloxera, and the resistance of the rootstock's breaks. Except for *M. rotundifolia*, rootstocks are not immune to Phylloxera or nematode but they are just tolerant, suffering less the disease caused by them with respect to *Vitis* vinifera. They may grow quite well with the pest, but they can also be symptomless carriers of virus diseases and, through vegetative propagation, infested material can further spread the infection in new viticulture regions (Mullins et al., 1992; Grzegorczyk and Walker, 1998; Huglin and Schneider, 1998; Keller, 2015).

The first rootstocks were pure form of *V. riparia* Michx (*V. riparia* cv. Gloire de Montpellier (syn. Portalis)) and of *V. rupestris* Scheele (*V. rupestris* cv. du Lot (syn. Saint George)). They were chosen for their capacity to root easily from woody cuttings and to graft well with *V. vinifera*. Later French breeders started to use them to develop hybrid rootstocks (Pongràcz, 1983; Viala and Ravaz, 1903; Riaz et al., 2019). The third species, *V. berlandieri* Planchon (syn. *V. cinerea* var. *helleri* (Bailey) M.O. Moore) was introduced to obtain lime-tolerant rootstocks, able to grow in European calcareous soils (Foëx, 1902; Viala and Ravaz, 1903; Riaz et al., 2019). Because it roots and grafts poorly, the hybridization with *V. riparia V. rupestris* or *V. vinifera* was required. Most of important rootstocks were developed by French breeders and nurseries Millardet and de Grasset in Bordeaux (i.e. Millardet

et Grasset 101-14), Couderc in Montpellier (i.e. Couderc 3309) and Richter in southern France (i.e., Richter 110). In Italy, Paulsen selected the rootstock Paulsen 1103 P and the sicilian Ruggeri developed Ruggeri 140 from the cross of *V. berlandieri* × *V. rupestris* (Bavaresco et al., 2015; Riaz et al., 2019). From *V. riparia* x *V rupestris*, in Moravia, Schwarzmann obtained a rootstock that bears his name. in 1896, a young Hungarian wine merchant, Zsigmond Teleki, used imported seed from the collection of Rességuier family in Alenya, France (Galet, 1998; Manty, 2005), initiation a breeding program with them. From the seedling population of *V. berlandieri* × *V. riparia* cross. Zsigmond Teleki, his son, Alexander Teleki, selected many widely used rootstocks (i.e. Teleki 5 C). Because these rootstocks combine Phylloxera resistance with lime tolerance, they served as base material for European breeding efforts, comprised those made by Kober in Austria (i.e. Kober 125 AA and Kober 5 BB), Cosmo of Conegliano (i.e. Cosmo 2 and Cosmo 10, Bavaresco et al. 2015) and Fuhr, director of the research station in Oppenheim, Germany (i.e., Selektion Oppenheim 4; Ruehl et al., 2015).

In sandy soils of California's Central Valley and in many parts of California's coastal countries, were Phylloxera was first discovered in the 1860s, the infestation is not severe enough/does not exist respectively to require the use of grafting. Nonetheless, the rootstock Rupestris du Lot was employed for its superior rooting and grafting properties (Riaz et al., 2019). Different other genotypes, deriving from the extensive trials performed by the University of Davis, were identified for their better performance in different environments across the State, among them 1103 P (*V. berlandieri* × *V. rupestris*) and 101-14 MGt (*V.riparia* × *V. rupestris*) are widely used (Riaz et al., 2019).

The rootstocks that are current used worldwide were bred nearly 100 years ago but the changing climate is prompting a new interest in their genetic improvement for facing the new viticulture challenges.

I.3.2 The recent M-rootstocks: the case of M4 tolerant to drought.

Since 1985 the DiSAA research group operating at the University of Milan is working on the selection of new rootstocks obtained from the cross of American *Vitis* species and, in some cases, with *V. vinifera*. Four rootstocks, named M (M1, M2, M3 and M4), were selected and registered at National Register of *Vitis* Varieties (G.U. N° 127 4/06/14) (Table I.1).

Accession name	Pedigree information	Main characteristics
M1	Historical pedigree (106-8 x Resseguier no. 4) not confirmed: compatible with Kober 5BB x Teleki 5C	Reduced vigour, high resistance to ferric chlorosis and salinity
M2	Historical pedigree (Teleki 8B x 333 E.M.) not confirmed: compatible 071 F1P95 (41 B x Teleki Stieler) UNIMI with Cosmo 10 x 140 Ruggeri	Medium vigour, good resistance to ferric chlorosis and medium resistance to salinity
M3	Historical pedigree (R 27 x Teleki 5C) partially confirmed: compatible with Kober 5BB x Teleki 5C	Reduced vigour, high efficiency in potassium absorption and low resistance to salinity
M4	Historical pedigree not confirmed, PO with 1103 Paulsen	Medium vigour, excellent resistance to drought and high resistance to salinity

Table I.1. Agronomic characteristics and pedigree information of the M-series rootstocks**.**

One of the innovations introduced by this breeding program was the reintroduction of *V. vinifera* as parental. This has permitted to broaden the relatively narrow genetic background of commercial rootstocks, due to the prolonged use of the same genotypes selected on the basis of a few phenotypic traits (rooting ability, Phylloxera resistance and scion-induced vigour) (Meggio et al., 2014). The discovery of new genotypes capable of dealing with unfavourable environmental conditions is a very important aspect in the expected climate scenario and for the future viticulture (Chaves et al., 2010; Schultz and Stoll, 2010).

The new M-rootstocks are distinguished by a reduced or medium vigour, particularly M1 and M3, associated with an average or above average productivity. M2 looks interesting for the ability to increase Mg and K uptake, while M3 stands out for its ability to absorption of Mn. This is an aspect particularly interesting for the influence on the advance of maturation and accumulation of anthocyanins and polyphenols associated with good sugar content. M2, M3, M4 favour greater sugary accumulations and M3 tends to maintain a lower pH compared to other rootstocks. M1 and M3 induce a higher capacity of accumulate polyphenols (anthocyanins and tannins) and M2 and M4 highlight significant capacities of resistance to water stress (Bavaresco et al., 2015).

The high tolerance of M4 rootstock towards water stress was characterized from a physiological, transcriptomic, metabolic and proteomic point of view (Meggio et al., 2014; Corso et al., 2015; Prinsi et al., 2018). In a study driven by Meggio et al. (2014), during water stress, M4 showed a typical anisohydric behaviour, decreasing progressively its Ψleaf (Lovisolo et al., 2010; Pou et al., 2012).

When water availability was very low (30% SWC), such to determine a photoinhibition for the susceptible genotype 101-14, M4 kept a higher stomatal conductance. This one started to decrease at Ψleaf less than -0.9 MPa (versus the -0.6 MPa of 101-14), suggesting that, despite the severe stress condition, M4 was able to maintain a partial stomatal aperture (Meggio et al., 2014). This rootstock was also able to recover photosynthetic activity after a period of water stress, suggesting a great capability to acclimatize to the changing water availability (Meggio et al., 2014). The reduction of transpiration showed by M4 in drought condition, was accompanied by a significant increase in leaf ABA, that was maintained high during the stress (Corso et al., 2015). This phenomenon was not linked to an up-regulation of ABA biosynthetic genes but rather to a down-regulation of the genes involved in its catabolism (Corso et al., 2015). They are two orthologues of the *Arabidopsis* cytochrome P450 genes (CYP706 and CYP707), which encode the major enzyme involved in ABA catabolism during dehydration. Water stress also induced the expression of JA- and GA-related genes in M4 roots that mediate many developmental processes and activate defence responses to biotic and abiotic stress in plants . The greater content of soluble sugars accumulated by M4 (Meggio et al., 2014), was congruent with the observation that a gene orthologous to an *Arabidopsis* sugar transporter protein (*AtSTP13/MSS1*) was highly induced in its stressed leaves (Corso et al., 2015). This gene, similarly to many other sucrose transporters, codes for a Suc/H+ symporter which could be potentially involved in the phloem loading and long-distance transport of soluble sugars from source organs to sinks, such as roots (Kühn and Grof, 2010). Corso et al. (2014) demonstrated that in M4 leaves, water stress induced the expression of many structural genes of the flavonoid pathway. Conversely, in roots, water-stressed plants accumulated transcripts and proteins corresponding to the stilbene synthases (STS), responsible of the biosynthesis of 3-hydroxy-*trans*-stilbene, better known as resveratrol. The higher content of resveratrol was observed in both genotypes (101-14 and M4) but in M4 water-stressed plants it resulted particularly abundant: the percentage of resveratrol, on the sum of resveratrol and piced, passed from 6% to 16%, while piceid fraction remained unaltered (Corso et al., 2015). *Trans*-resveratrol appears to have a great impact in scavenging the oxidative compounds related to various stresses (Waffo Teguo et al., 1998; Corso et al., 2015). At this purpose, eight VvSTS transcripts (VvSTS12, 13, 16, 17, 18, 24, 27, and 29) were found to be significantly up-regulated in M4 roots, whereas they were down-regulated in those of 101-14 after 2 days from water stress imposition. The tolerance displayed by M4 was, therefore, associated to the great capacity to scavenge ROS produced during water shortage, mainly conferred by a structural variations in the promoter regions of the genes involved in stilbene biosynthesis (Corso et al., 2015). The observation that VvSTSs genes were co-expressed with several WRKY TFs raised the question of whether these TFs

might be involved in the transcriptional regulation of the VvSTSs genes. An *in silico* search for putative *cis*-elements in the promoter regions of VvSTSs genes found them up-regulated in M4 roots, confirming that hypothesis (Corso et al., 2015).

In M4 water-stressed plants, the great ROS detoxification ability permits the lateral root development, resulting in higher water uptake capacity from the soil. At leaf level, the higher transpiration favours plant growth and photosynthesis (Corso et al., 2015). Fron the study of Corso et al. (2015), the putative genes considered the key factors of the M4 better adaptation to water stress have been identified. Among them, the VvSTSs genes were also included.

Prinsi et al. (2018) performed a proteomic analysis that strengthens the important role of M4 roots in water scarcity response. At a higher level of stress, they showed a great capacity to adjust osmolality, to preserve cell integrity, demonstrated by the higher content of proteins and ions, compared to the susceptible genotype 101-14. The positive metabolic response showed by M4 was considered potentially able to counteract the water stress effects. Firstly, M4 maintained a primary root elongation, which was linked to cell growth, and consequently to cell wall extensibility. Therefore this rootstock was able to maintain a certain water uptake. Some proteins involved in cell wall biosynthesis and expansion, a higher level of osmoprotective compounds (i.e. amino acids, raffinose, and some sugar alcohols) and proteins involved in cell wall loosening, were also found changed in abundance in M4. Water stress affected M4 roots in the increase of some compatible solutes, such as proline or many sugar alcohols, and some polyols (i.e. mannitol, inositol, galactinol, and erythritol). The authors interestingly observed that in M4 roots was found a higher content of two osmotin-like proteins which improve water stress tolerance (Barthakur et al., 2001; Kumar et al., 2017; Prinsi et al., 2018). A greater accumulation of enzymes involved in starch breakdown and sucrose synthesis, able to remedy to the lower photosynthetic activity, was also observed (Prinsi et al., 2018). According to Regier et al. (2009), the capability to maintain the photosynthetic rate and to improve the use of carbon skeletons in the roots, as demonstrated by M4 during the stress, is one the characteristic of tolerant rootstocks. Proteomic analysis revealed severe changes in mitochondrial functionality. Although some falls of the intermediates of the tricarboxylic acid (TCA) cycle occurred during the stress, M4 was able to maintain a good functionality of this cycle. This was evidenced by the greater accumulation of specific amino acids, such as valine, threonine, serine, proline, and methionine. Because drought negatively affects a large number of enzymes involved in amino acid metabolism, the increase of these compounds was also a consequence of protein degradation, associated with cell damage (Prinsi et al., 2018). It could represent a specific response evoked by the requirement for alternative substrates for respiration.

The main result showed by M4 was the increase of enzymes involved in the synthesis of flavonoids and stilbenes. This contributed to reinforce the conclusion that the tolerance of M4 to water stress was principally due to the ability to synthesize larger amounts of antioxidant compounds, particularly resveratrol. In addition, an increase of typical ROS scavenging enzymes, such as catalase and glutathione reductase have been found (Prinsi et al., 2018)

In synthesis, the greater tolerance of this genotype can be related to the activation of mechanisms for counteracting the oxidative stress that occurs in water stress conditions (Ober and Sharp, 2007; Miller et al., 2010; Das and Roychoudhury, 2014; Corso et al., 2015;Prinsi et al., 2018).

I.4 The stilbene synthase multigenic family

Plant stilbenes are a small group of phenylpropanoid, belonging to a minor class of these compounds involved in defence, often referred with the name of phytoalexins (from the Greek, meaning "warding off agents in plants"). They are low mass, lipophilic, antimicrobial compounds (Kuc, 1995; Purkayashta, 1995). Stilbenes biosynthesis in grapevine is spatially and developmentally regulated, and it is induced by many abiotic and biotic environmental factors (Vannozzi et al., 2018).

Stilbenes have been detected in at least 72 unrelated plant species, deriving from 12 different families (Fabaceae, Liliaceae, Moraceae, Myrtaceae, Papilionaceae, Pinaceae, and Poaceae). Despite the multiplicity of forms found in these different plants, most of stilbenes, comprised those produced in grapevine, derived from the basic unit *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene). From resveratrol modification more complex compounds were detected in grapevine, such as *cis*- and *trans*-piceid (Gatto et al., 2008; Waterhouse and Lamuela-Raventos, 1994), viniferins, pterostilbene and piceatannol (Langcake, 1981; Bavaresco et al., 2002).

Grapevine is one of the few species possessing stilbene biosynthetic genes for which the genome was completely sequenced. Forty-three VvSTS members were predicted with GAZE and JIGSAW tools in the 8.4 X coverage genome draft of the PN40024 genotype (French-Italian consortium) (Jaillon et al., 2007) while twenty-one members were predicted from the genome sequence of the PN ENTAV 115 genotype (IASMA) (Velasco et al., 2007). In the study of Vannozzi et al. (2012), 48 genes designated as VvSTS1 to VvSTS48 were identified through a search in the up-date version of the genome assembly of the grape PN40024 genotype (referred as 12X V1). They showed that at least 33 full-length coding genes, 8 pseudogenes and 7 sequences still unresolved were included in grape genome.

VvSTSs genes should derived from the same ancestral gene. Subsequent gene duplications and molecular divergences may have contributed to establish functionally distinct genes (Sparvoli et al., 1994; Vannozzi et al., 2012). VvSTS1-6 members are located in a region of 80 Kb on chr10, while VvSTS7-48 ones are within a region of 500 Kb on chr16. Based on the prediction tools (GAZE and JIGSAW), five sequences corresponding to the genes VvSTS11, VvSTS14, VvSTS34, VvSTS40 and VvSTS44 are found in genome regions which are not predicted to contain any gene.

Sequence alignment and phylogenetic tree analyses highlighted the existence of three VvSTS clades or groups (designated as A, B and C). Group A contains genes located on chr10 while group B and C included members placed on chr16 (Vannozzi et al., 2012) (Figure I.1).

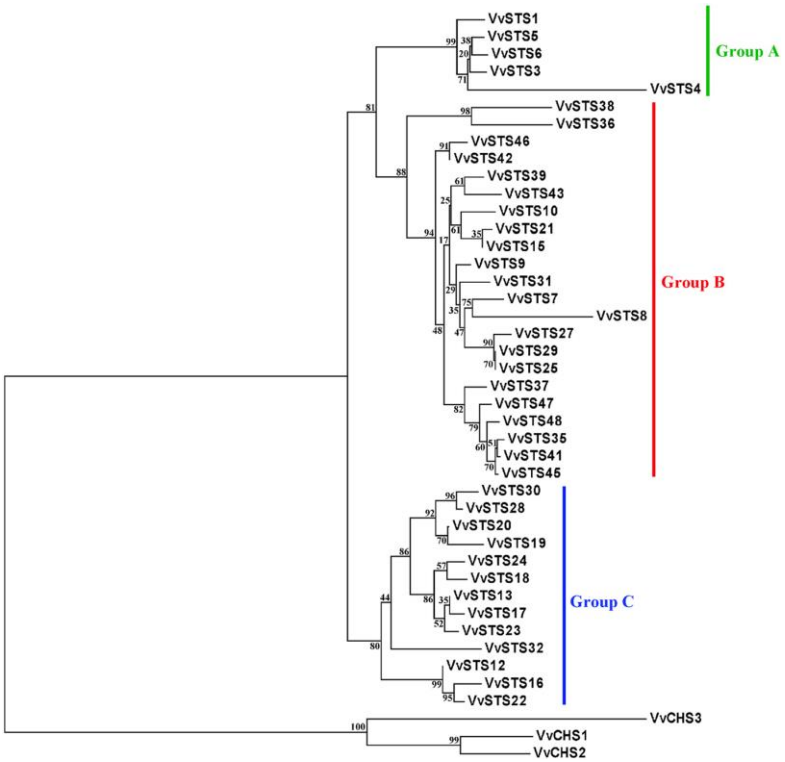


Figure I.1 Phylogenetic tree of predicted STS proteins in grapevine. Consensus phylogenetic tree generated after sequence alignment with MAFFT 6.0 using the neighbour-joining method. VvSTS gene members predicted to encode for a truncated ORF were not considered. Deduced protein for VvCHS1, VvCHS2 and VvCHS3 were also included in the analysis. Reliability of the predicted tree was tested using bootstrapping with 1000 replicates. Numbers at the forks indicate how often the group to the right appeared among bootstrap replicates. Different coloured bars indicate three main sub-groups designated as A, B and C (Vannozzi et al., 2012).

It has been demonstrated that the genes belong to the same phylogenetic cluster are linked not only by protein homology but also show similar transcriptional response (Vannozzi et al., 2012). The highest response to water stress treatments is showed by group B members, while member of group C exhibited a reduced response and of group A showed little or no transcriptional response. It also subsists a different quantitative response to the stress by different group members, revealed by a mRNA-seq analysis. It was observed that the transcript copy number of the gene VvSTS48 (group

B), at the peak of stress treatment, was found to be 15-50-fold higher than the levels of VvSTS16 and VvSTS6, belong to groups C and A respectively. On the contrary, UV-C treatment and downy mildew infection stimulate similarly and more rapidly the transcriptional response of the genes of group A (VvSTS6) and C (VvSTS16). This result suggests that the genes within groups A and C may be responding to different transcriptional signals (Vannozzi et al., 2012).

VvSTS genes from different groups show different timing in stress response, providing an explanation to the biphasic transcription observed by many authors (Wiese et al., 1994; Borie et al., 2004; Wang et al., 2010). According with Wiese et al. (1994), the biphasic nature of the VvSTS response indicates that this family can be divided in two groups: some expressed early with rapid degradation of mRNA and other expressed later providing more stable mRNA. The different patterns of transcriptional response between the VvSTS groups further suggest that these genes may be induced by different signalling pathways (i.e. by JA and ethylene signalling pathway) (Grimmig et al., 2003; Tassoni et al., 2005; Vezzulli et al., 2007; Belhadj et al., 2008; D'Onofrio et al., 2009) .

Stilbene synthase (STS) is the key enzyme of the synthesis of resveratrol (Schöppner and Kindl, 1984). STS belongs to the type III polyketide synthase super family: a class of enzymes which carry out iterative condensation reactions with malonyl-CoA (Jeandet et al., 2010). This family also comprises chalcone synthase (CHS) enzyme, which represents the archetypal one. STS is closely related to CHS but, in contrast with the ubiquitous CHS, is present only in stilbene producing plants. The catalytic activity of both STS and CHS enzymes is linked to a conserved cysteine residue, located in the central section of these proteins, which represents the binding site for the p-coumaroyl-CoA starting substrate (Lanz et al., 1991). STS and CHS in fact use the same substrates and catalyses the formation, in a single enzymatic reaction, of the same linear tetraketide intermediate (from p-coumaroyl-CoA and three malonyl-CoA). Because of the different cyclization, CHS leads to the formation of chalcones while STS to the production of stilbenes (Figure I.2) (Jeandet et al., 2010; Vannozzi et al., 2012). Between STS and CHS pathways it occurs a crosstalk, reflecting an antagonistic relationship since they share the same starting substrate. Indeed the tissues in which the expression of VvSTS genes is lower (i.e. stem, bud, young leaves, rachis at fruit set and developing berries), are characterized by a high constitutive expression of at least one of the three different VvCHS genes, involved in the accumulation of anthocyanins, tannins and flavonols. On the contrary, the expression of VvCHS is inhibited in tissues where the expression of VvSTS is more markedly induced (i.e. roots, senescing leaves, maturing rachides and berries) (Vannozzi et al., 2012).

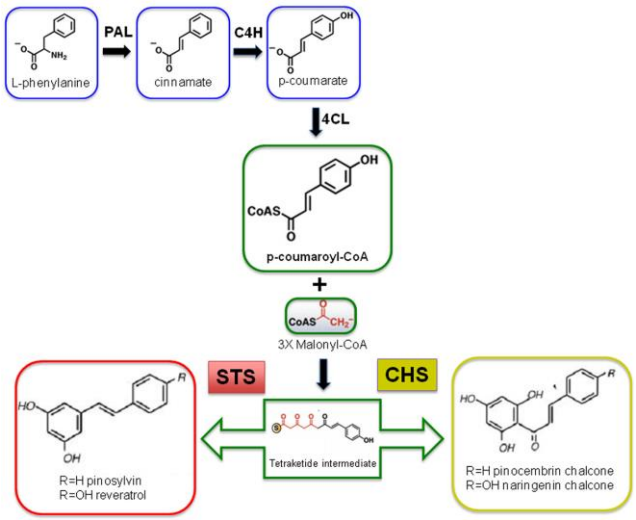


Figure I.2 General phenylpropanoid pathway and flavonoid and stilbene branching pathways. The enzymes shown in these pathways are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-cumaroyl: CoA-lyase; CHS, chalcone synthase; STS, stilbene synthase (Vannozzi et al., 2012).

In grapevine, stilbene biosynthesis is regulated by two transcription factors (TFs) R2R3-MYB (V-myb myeloblastosis viral oncogene homolog): known as MYB14 and MYB15 (Holl et al., 2013; Vannozzi et al., 2018). They are strongly co-expressed with certain VvSTS genes in different organs in response to biotic and abiotic stresses, including downy mildew (*Plasmopara viticola*) infection, mechanical wounding and exposure to UV-C irradiation. Their expression is also linked to the accumulation of *trans*-piceid in developing grape berries (Holl et al., 2013). A study performed by Wong et al. (2016) found that the two TF genes are closely similar to MYB13, suggesting that this one could be involved in the transcriptional regulation of at least some VvSTS genes in grapevine (Vannozzi et al., 2018).

In addition to MYBs, TFs belonging to WRKY family are found to be strictly involved in the regulation of VvSTS gene expression. WRKY TF family, in fact, is considered the most enriched families in terms of correlation frequencies with VvSTS genes, particularly WRKY03, WRKY24, WRKY43 and WRKY53 genes (Corso et al., 2015; Vannozzi et al., 2018).

Under normal condition, the expression of VvSTSs genes in grapevine tissues is very low, especially in the young ones. It increases with maturity and senescence (Vannozzi et al.,2012), as well as with dehydration events, a phenomenon which is also at the basis of berry maturation techniques for wine production (Zamboni et al., 2008). Their role in drought response is assuming increasingly interest (Winkel-Shirley, 2001; Vannozzi et al., 2012; Corso et al., 2015). Despite the reduction of ROS accumulation by stilbenes during abiotic stresses is ascertained, further investigations are required to

understand their role during a different types of abiotic stress (i.e. due to salinity, heat, cold or drought) (Huang et al., 2016).

I.3 Objectives of the thesis

Most of the vineyards around the world are characterized by seasonal drought, where atmospheric and edaphic water deficits, together with high temperature and irradiance, represent severe constraints on the regular growth cycle (Ollat et al., 2015). Despite grapevine is well adapted to arid and semi-arid environments, water stress can cause physiological changes, from mild until irreversible. In the climate change scenario, the more frequent and severe water shortages represent a substantial risk for viticulture (IPCC, 2018; Schultz, 2000).

This thesis wants to provide a contribution to the complex and well-debated study on grapevine rootstock response to water stress. It is organised into four main chapters, divided into sections which correspond to submitted articles or drafts in preparation. The chapters are arranged as follows:

Chapter II

Section 1: It is well-known that root system exerts a decisive role in plant drought adaptation. So far, most of the studies on grapevine response to water stress were focused on the role of rootstock as a factor of acclimation. However, the role of scion on rootstock drought reaction is still less discussed. In this section, we analysed the effect of grafting on rootstock behaviour during water shortage, both from physiological and transcriptome standpoints.

Section 2 Screening of available *Vitis* genetic diversity for new rootstock breeding programs has been proposed as a way from which the new viticulture challenges may arise. In 2014, four novel genotypes (M1, M2, M3, and M4), selected by the DiSAA research group operating at the University of Milan, have been registered in the National Register of Vine Varieties. Among them, M4 confirmed to be drought- tolerant while M1 and M3 are less tolerant. To characterize their behaviour during water stress, in this section, their physiological and transcriptomic responses were analysed, compared to those of other commercial genotypes widely used in viticulture.

Chapter III

Section 1: M4 is a promising rootstock for its ability to adapt to water shortage conditions, as well as to salt stress. Considering the great constant demand for vine planting materials and the widespread use of this rootstock by grape grower, the obtainment of genetically homogeneous populations (i.e. clones) from elite individuals through micropropagation represents a rapid alternative to conventional methods. This section aims to set-up and efficient and high-throughput protocol for micropropagation as well as for the obtainment of pro-embryogenic and embryogenic calluses to be manipulated through the CRISPR/Cas9 tool.

Chapter IV

Section 1: In water stress condition, the up-regulation of four VvSTS genes (VvSTS16, 18, 27 and 29) in M4 roots has led to think that they can be considered the putative factors of its better adaptation to drought. In order to confirm this hypothesis, a functional analysis of these genes has to be performed. In this section was reported the first step aimed at the identification of the VvSTS genes. We tried to develop an efficient CRISPR/Cas9 method for the knock-out of these ones. We have also described a transient assay, performed on M4 micropropagated plantlets, to test the gRNAs functionality.

I.4 Paper publishing and conference contribution

Posters

Caramanico L., De Lorenzis G., Rustioni L., Brancadoro L., Failla O. (2018) Caratterizzazione *in vitro* del portinnesto M4. Conavi, 2018, July 9-11th, Piacenza, Italy.

De Lorenzis G., Caramanico L., Olivares F., Prieto H., Scienza A., Pozzi C., Failla O., Brancadoro L. (2018). Geminivirus-mediated genome editing: è la tecnologia molecolare che aspettavamo? Conavi, 2018, July 9-11th, Piacenza, Italy.

De Lorenzis G., Caramanico L., Olivares F., Prieto H., Scienza A., Pozzi C., Failla O., Brancadoro L. (2018) Gemini-virus mediated genome editing. XII International Conference on Grapevine Breeding and Genetics, July 15-20th, Bordeaux, France.

Oral presentations

Caramanico Leila, De Lorenzis Gabriella, Brancadoro Lucio, Failla Osvaldo (2017). Grapevine regeneration and micropropagation protocol from shoots and leaf explants. Enoforum 2017, May 16-18th, Vicenza, Italy.

Caramanico Leila, De Lorenzis Gabriella, Brancadoro Lucio, Failla Osvaldo, Attilio Scienza (2018). Study of grapevine rootstocks behaviour towards water stress. VINITALY 2018, April 19th, Verona, Italy

Publications

Caramanico L.; Rustioni L.; De Lorenzis G.; 2017. Iron deficiency stimulates anthocyanin accumulation in grapevine apical leaves. Plant Physiology and Biochemistry, 119, 286-293.

Caramanico L.; Failla O.; Brancadoro L.; De Lorenzis G. Attitude of the new 'M4' grapevine rootstock to in vitro shooting, rooting and callogenesis from leaf and bud explants. SAJEV. Submitted.

Leila Caramanico, Daniele Grossi, Massimo Pindo, Erika Stefani, Alessandro Cestaro, Osvaldo Failla, Attilio Scienza, Lucio Brancadoro, Gabriella De Lorenzis. The readiness of grapevine rootstock to detect and respond to water stress is negatively affected by the scion. Scientific Reports. Submitted

Leila Caramanico, Gabriella De Lorenzis. 2019. Le tecniche di coltura *in vitro.* In: Vinifera - ripartiamo dalle radici. Il nuovo libro sui portinnesti. Ed Assoenologi. 512. 2019.

Chapter II – Rootstocks and water stress response

Water availability, together with carbon and mineral nutrients, represent one of the main resources that plants need to grow and produce fruits. Their scarcity causes sub-optimal growth conditions to which plants are exposed. Environmental stresses limit either the availability of one or several resources to the plant or its capacity to use these resources but the concept of stress is not absolute. It can be explained as the ability of the organism to adapt in a set of environments that makes it stressful or not (Keller, 2015). Most of vineyards around the world grow in regions characterised by seasonal drought, where grapevine undergoes either to a slow decrease in water availability during the growing season (edaphic water deficit) or short term water stress (atmospheric water deficit) (Chaves et al., 2003; Cramer, 2010; Lovisolo et al., 2010). Despite grape is well adapted to arid and semi-arid climates and traditionally non-irrigated, especially in Europe, the more frequent and severe water scarcity predicted in the near future, due to climate change, represents a risk for viticulture, particularly for fruit yield and quality. Lack of water, in conjunction with variable and unreliable water supply, is emerging as one of the biggest threats for viticulture.

Plants may react with different strategies to face water shortage: (*i*) escaping water stress (reducing phenological cycle); (*ii*) avoiding water stress (decreasing transpiration, increasing water uptake); (*iii*) maintaining growth under water stress through adaptative mechanisms; (*iv*) resisting to severe water depletion through survival mechanisms (Tardieu, 2005; Verslues and Juenger, 2011). Grapevines appear to primarily rely on drought avoidance mechanisms (Scienza, 1983; Chaves et al., 2010).

Verily, because mild water-deficit can enhance grape quality, agronomic practices may involve the induction of slight water shortages during the growing cycle of grapes (Chaves et al., 2010; Lovisolo et al., 2010). When drought events are repeated and prolonged in time, they can cause irreparable damage to plant.

The use of irrigation techniques, that could be a way to overcome water stress, does not represent a sustainable practice to counteract low water availability, either for legal limits, infrastructure problems (access to water), for the growing competition for water as a resource. Moreover, the requirement of irrigation for crop production may induce soil salinity which is another major abiotic stress (Rengasamy, 2006).

For all these reasons, the exploitation of the genetic variability of grapevine rootstocks seems to play a fundamental role in the adaptation to climate changes, especially to drought (Walker, 1992; Holl et al., 2013).

Most of American *Vitis* species cannot be employed alone as rootstock, due to their grafting incompatibility. However they can be used in breeding programs to obtain tolerant genotypes towards water scarcity (Duchêne and Schneider, 2005; Van Leeuwen et al., 2009). Especially from crosses between *V. berlandieri* and *V. rupestris*, genotypes better able to adapt to drought have been obtained. In order to understand the whole-plant response to water shortage condition, it is essential to perform studies on scion-rootstock interactions, which includes (i) the genetic trait of rootstock and scion; (ii) the mutual effect of scion and rootstock to cope with water stress; (iii) the anatomical structure and hydraulic architecture of rootstock genotypes. In the next two sections (II.1 and II.2) we investigated the effect of grafting on rootstock performance, as well as we performed a characterization of the M-rootstocks comparing them to other commercial genotypes, from physiological and transcriptome standpoints.

II.1 The readiness of grapevine rootstock to detect and respond to water stress is negatively affected by the scion

Leila Caramanico[1], Daniele Grossi[1], Massimo Pindo[2], Erika Stefani[2], Alessandro Cestaro[2], Osvaldo Failla[1], Attilio Scienza[1], Lucio Brancadoro[1], Gabriella De Lorenzis[1#]

[1] Università degli Studi di Milano, Dipartimento di Scienze Agrarie e Ambientali – Produzione, Territorio e Agroenergia (DISAA), via Celoria 2, 20133, Milano, Italy
[2] Fondazione E. Mach, Centro Ricerca e Innovazione, Via E. Mach 1, 38010 San Michele all'Adige (TN)

Abstract

In most of viticulture areas, *Vitis vinifera* is grafted onto a *Vitis x hybrids* rootstock. The interaction between scion and rootstock shows itself as resistance to biotic and abiotic factors, such as drought. In this work, the effect of grafting on rootstock performance under water limitation was studied on 101-14 and 1103 P plants, own-rooted and grafted with Cabernet Sauvignon, in two different water stress conditions. Gas exchange parameters and transcriptomic response, performed on leaf and root tissues, were evaluated. Under severe stress conditions, stomatal conductance decreased in all genotypes but it was followed by a reduction of photosynthesis and transpiration only in the ungrafted plants, meaning the decisive role of scion in the modulation of gas exchanges. The analysis of transcriptome showed that most of differentially expressed genes were detected in roots of own-rooted and grafted 1103 P plants. The different regulation of ABA-based signalling, ROS scavenging and osmolyte compound pathways revealed that 1103 P rootstock perceived readily the water scarcity and rapidly faced the stress. Molecular evidence highlighted how the scion delays the stimulus perception and rootstock reactivity to drought.

Keywords: gas exchanges; mRNA-Seq; rootstock/scion interaction; drought response; 101-14; 1103 P.

II.1.1 Introduction

Scarcity of water is a severe environmental constraint for plant productivity. Drought negatively affects the water transport from the soil through the plant into the atmosphere, in a soil-plant-atmosphere *continuum* interconnected by a film of water. Depending on the duration and intensity of

water deficit, a complex of responses can be displayed by plants (Bray, 1997). Water stress leads to cellular water loss, osmotic stress affecting cell division and elongation and modifying the growth of different organs (Serra et al., 2013). Under high level of tension, xylem embolism can be formed and hydraulic conductance can be drastically reduced (Lovisolo and Schubert, 1998; Shultz and Matthews, 1988). In order to mitigate these effects, one of the earliest response put in place by plants is stomatal closure, buffering the drop of xylem water potential and maintaining a favourable water balance (Tyree and Sperry, 1989; Tombesi et al., 2015). In grapevine, the major determinant inducing stomatal closure is abscisic acid (ABA). When soil dries out, water potential declines and roots produce a large quantity of ABA which, through xylem sap, reach guard cells. It induces an increase in reactive oxygen species (especially H_2O_2) that lead to a rise of calcium ions (Ca2+) in cytosol, stopping the influx and enhancing the efflux of potassium ions (K+) from guard cells (Allen et al., 2001) which lose their turgor. Stomata closure limits carbon gain and photosynthetic activity as well (Yordanov et al., 2000). Carbohydrate deficiency induces plant cells to consume starch reserves, normally allocated in woody tissues for spring resumption, in order to provide energy, sugars and derived metabolites to mitigate the water stress (Salleo et al., 2009). Sugars (i.e. sucrose and fructose), sugar alcohols (i.e. mannitol or glycerol) and amino acids (i.e. proline) are compatible solutes: small and hydrophilic compounds able to reduce cell osmotic potential permitting continued water uptake and helping plant tissues to maintain a higher water potential. They are also called osmoprotectans for their ability to preserve enzymes and membranes, stabilizing them, from osmotic stress (Keller, 2015). Osmotic stress triggers a secondary stress, that is oxidative stress (Apel and Hirt, 2004). This is due to the inability to use the energy provided by the photosynthesis because carbon fixation declines and light capture proceeds. The excess of energy interacts with oxygen, forming reactive oxygen species (ROS) (Keller, 2015). Compatible solutes, stress proteins, together with carotenoids and flavonoids contribute to the detoxification process by scavenging active oxygen species. For example, flavonoids can be oxidized and hence degraded by peroxidase, consuming H_2O_2 (Pérez et al., 2002).

Since the late of 19th century, *Vitis vinifera* is grafted onto rootstocks (Mullins et al., 1992) (non-*vinifera* species and hybrids) for avoiding the damage of Phylloxera, caused by the soilborne aphid *Daktulosphaira vitifoliae*. While this pest is able to attack *Vitis* vinifera roots leading to plant mortality, it is not capable to infest American *Vitis* species (Vorwerk and Forneck, 2006). The grafting is the result of interaction between two genotypes, scion and rootstock, at different levels. This interaction shows itself also resistance to other pathogens and to several abiotic factors such as drought and soil salinity, as well as influence on scion vigour, grape yield and composition (Mullins

et al., 1992; Keller, 2015). Scion depends on rootstock for water and mineral nutrient uptake, while rootstock relies on scion for photosynthetic assimilates (Kocsis et al., 2012).

Grapevine is predominantly cultivated in temperate climate areas and, although is relatively resistant under suboptimal water regimes and traditionally non-irrigated, a huge difference among genotypes in terms of physiological response to drought has been detected (Serra et al., 2013). Furthermore, the prediction of water scarcity in the near future is increasing the interest in understanding the drought tolerance that rootstocks can afford (Serra et al., 2013). On the basis of global climate scenarios, predicting an increase in aridity in the next future in some countries (Dai et al., 2013), the selection of rootstocks able to improve water use efficiency, plant growth capacity and scion adaptability represents an important strategy in facing the negative impacts caused by water deficiency (Marguerit et al., 2012; Corso et al., 2015). The study of the influence of rootstock genotype on scion physiological performance has always had great importance (Soar et al., 2006; Lovisolo et al.,2016; Marguerit et al., 2012) . Then again, the comprehension of the scion impact on rootstock performance towards water stress is still less debated. In this work, the effect of grafting on rootstock response to water stress was analysed. A phenotypical and a large-scale whole transcriptome analyses were performed for two genotypes, a drought-susceptible (101-14) and a drought-tolerant (1103 P), own-rooted and grafted with Cabernet Sauvignon, subjected to a gradually water shortage in semi-controlled environmental conditions. Physiological parameters, such as stem growth rate (SGR), gas exchanges and stem water potential (SWP), were detected. A transcriptome comparative approach between own-rooted and grafted plants of drought-susceptible and tolerant genotype, with regard to water stress, was performed.

II.1.2 Material and Methods

Plant material and growth conditions

The experiment was performed at the Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy (Milan) in environmental controlled conditions of a greenhouse equipped with supplementary light, with a 16-h light (PPFD, photosynthetic photon flux density, ~ 600 μmol photons $m^{-2}s^{-1}$) and 8-h dark photoperiod and a cooling system, with a range of temperature from 23°C to 28°C. Two-years-old rootstocks (101-14 and 1103 P), own-rooted or grafted with Cabernet Sauvignon, were grown in 4-L plastic pots filled with a sand–peat mixture (7:3 in volume) and supplemented with a layer of expanded clay aggregate on the bottom of pot. The vines were trained on 1 m stake and placed in a randomized complete block design. Thirty replicates per each rootstock, fifteen own-rooted and fifteen grafted with Cabernet Sauvignon, were monitored during

the experiment, for a total of 60 plants. During budding, the samples were maintained in WW conditions and monthly fertilized with 100 mL of a nutrient solution containing 0.54 g KNO_3, 0.084 g NH_4HPO_4, 0.42 g $MgSO_4$ and 0.01 g of a microelement mixture (OligoGreen, GREEN Italia, Canale d'Alba, Italy) in order to achieve a well-developed canopy (8^{th} and 9^{th} fully developed leaves).

Irrigation management and sampling

Two irrigation treatments were applied to the plants. For each rootstock own-rooted or grafted with Cabernet Sauvignon, nine plants were grown under WW conditions (80% SWC or T1) and six to WS conditions (50% SWC or T2 and 20% SWC or T3) . The WW plants were maintained at 80% SWC and water stress was applied by decreasing water availability up to T3. The SWC was calculated using the gravimetric method, according to the formula suggested by Gardner et al. (2001) as following:

$$SWC = (\text{fresh weight} - \text{dry weight})/\text{dry weight} \times 100$$

where fresh weight is referred to the soil weighed at field capacity and dry weight to the soil dried in an oven at 105°C for 48 hours.

Each pot containing one sample was weighed daily for a period of 10 days. When SWC reached the value of 50% and 20% (after 7 and 10 days respectively), water stressed plants were chosen for sampling (phenotyping and transcriptomics analyses). The plants maintained at 80% of SWC were watered every day, in order to restore the right field capacity and sampled together with the WS plants (Table II.1.1). For transcriptome analysis, whole root system and fully expanded leaves (i.e. from the fifth to the eighth node of primary shoot) were immediately sampled after the *in vivo* measurements of physiological parameters, frozen in liquid nitrogen and stored at −80°C.

Plant phenotyping

At each time point, SGR (Stem Growth Rate), gas exchanges and SWP (Stem Water Potential) measurements have been performed. Stem elongation, expressed as daily SGR (mm/day), was calculated by using a rule during the entire period of sampling and recorded at 50% and 20% SWC. For evaluating gas exchange parameters, two fully expanded leaves (8^{th} and 9^{th} leaf) per plant were selected. Pn (μmol CO_2 $m^{-2}s^{-1}$), Gs (mol H_2O $m^{-2}s^{-1}$), E (mmol H_2O $m^{-2}s^{-1}$), Ci (μmol CO_2 $m^{-2}s^{-1}$) and Vpd (KPa) were measured with CIRAS-2 portable photosynthesis system (PP Systems, Amesbury, MA, USA), equipped with PLC6 (U) cuvette 18 mm circular (2.5 cm^2 head plate), under constant saturating PPFD of 1500 μmol photons $m^{-2}s^{-1}$, CO_2 concentration of 300 μmol mol^{-1}, block temperature of 25°C and relative humidity between 60% and 70% allowing ~1.5 kPa of Vpd inside

the leaf chamber. Water use efficiency (WUE) was determined as the ratio of Pn to Gs, also termed intrinsic water use efficiency (WUE_{int}). The SWP (bar) was calculated using a Scholander-pressure chamber (Soil Moisture Equipment Corporation, Santa Barbara, CA), as suggested by Scholander et al. (1965). The same leaves, chosen for gas exchange measurements, were collected and placed in a plastic bag wrapped with an aluminium foil for 1 hr. Then, they were excised with a razor blade and placed in the chamber for measurement. The SWP values were measured within 30 seconds from leaf cutting by slowly pressurizing the chamber until sap emerged from the cut end of the petiole. Both gas exchanges and SWP measurements were taken between 11:00 am and 2:00 pm solar time.

Statistical analyses of phenotypical data

Statistical analyses were obtained by using SPSS statistical software (version PASW Statistics 24, SPSS, Inc. Chicago, IL). The significance of differences among the values of each parameter (gas exchanges and SWP), within each genotype own-rooted or grafted with Cabernet Sauvignon, was assessed by univariate analysis of variance ($p \leq 0.05$), followed by Duncan's test ($p \leq 0.05$).

RNA extraction

RNA was isolated from 120 tissue samples, 60 leaves and 60 roots, of 101-14 and 1103 P rootstocks, own-rooted and grafted with Cabernet Sauvignon, under controlled water conditions. RNA was extracted from 100 mg of tissue, ground with liquid nitrogen, using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Germany) commercial kit, according to manufacturer's instructions. RNA quantification was performed using Qubit® RNA HS Assay Kit by Qubit® 3.0 Fluorometer (Life Technologies, CA), the quality was checked both on an Agilent 2200 Tapestation (Agilent Technologies, CA), using the RNA ScreenTape (Agilent Technologies) for RNA integrity detection, and NanoDrop 8000 Spectrophotometer (Thermo Scientific, MA), for 260/230 and 260/280 ratio evaluation. A lithium-chloride (LiCl) treatment has been performed for those samples showing a 260/230 ratio lower than 1.8. Briefly, 7.5 M LiCl was added to the RNA solution up to a final concentration of 2.5 M, incubated at 4°C for 16 h and centrifuged at 13,000 g for 30 min at 4°C; the supernatant was removed and the pellet was washed with 500 µl of 70% ice-cold ethanol, centrifugated at 13,000 g for 30 min at 4°C and suspended in water.

Library construction and sequencing

One-hundred and twenty cDNA libraries were constructed starting from 1 µg of high-quality total RNA with KAPA Stranded mRNA-Seq Kit (Roche, Switzerland), according to manufacturer's

instructions. Each library was barcoded using SeqCap Adapter kit A and B (Roche NimbleGen, WI) and Tapestation 2200 (Agilent) was employed to confirm the final size of 250-280 bps. with High Sensitivity D1000 ScreenTape kit (Agilent). The libraries quantification was carried out by KAPA Library Quantification kit – Illumina (Roche, Switzerland), using LightCycler 480 (Roche,) and multiplexed random in 7 pools in an equimolar way. The sequencing was performed on Illumina HiSeq 2500 platform (Illumina, CA) with paired end runs of 2 × 50 bps. Illumina RTA v1.13 (Illumina) sequence analysis pipeline was used to check the base calling and the quality control.

Sequence annotation

The quality of raw reads was inspected by FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) software, after adapter removing. The reads showing low quality score were trimmed by Trimmomatic 0.36 software (Bolger et al., 2014) and mapped to the v1 prediction of grapevine PN40024 reference genome, retrieved from CRIBI Biotechnology Center (http://genomes.cribi.unipd.it/grape), using Bowtie2 (Langmead and Salzberg, 2012) tool with default parameters. The SAMtools (Li et al., 2009) software package was used to convert alignments from BAM (Binary Alignment/Map) format to SAM (Sequence Alignment/Map) format, to sort and indexed them. The number of reads aligned to each RefSeq mRNA was counted by *ad-hoc* python pipeline. Only transcripts with more than 5 reads were selected for statistical analysis.

Statistical analysis of differentially expressed genes (DEGs)

The count data were used to have an overview of similarities and dissimilarities among samples and to identify differentially expressed genes (DEGs), using DESeq2 (Love et al., 2014) R package. The similarities and dissimilarities among samples were highlighted by a heatmap analysis with hierarchical clustering and Principal Component Analysis (PCA), performed using *pheatmap* and *plotPCA* function of DESeq2 R package.

In order to evaluate the effects of treatment and time point, DEGs were discovered performing multifactor designs method of DESeq2 R package. Per each transcript, log2 fold change, *p*-value and adjusted *p*-value were evaluated and only those showing a false discovery rate (FDR)-adjusted *p*-value <0.05 were analysed.

The R package topGO version 2.26.0. (Alexa and Rahnenfuhrer, 2016) was used to estimate gene ontology (GO) enrichment. The analysis was performed on DEG lists with FDR-adjusted *p*-value <0.05 and gene2GO annotation file, where the terms of biological process ontology were included,

was provided by CRIBI Biotechnology Center (http://genomes.cribi.unipd.it/grape/). The analysis was performed based on a classical enrichment analysis by testing the over-representation of GO terms within the group of differentially expressed genes, using statistical Fisher's exact test. The top-50 significantly enriched GO IDs were recorded.

The overlaps among different DEG lists have been visualized by Venn Diagram using jvenn web-server (bioinfo.genotoul.fr/jvenn/example.html) (Bardou et al., 2014). The function *heatmap.2* implemented in *gplots* R package (Warnes et al., 2005) was used for the graphical representation of gene expression and hierarchical clustering.

Data Availability

The original sequencing datasets have been deposited in the European Nucleotide Archive (ENA) and the accession number is PRJEB32438.

II.1.3 Results

Phenotypical performance of the 101-14 and 1103 P rootstock during water stress.

The physiological response of 101-14 and 1103 P rootstocks, own-rooted and grafted with Cabernet Sauvignon, undergone to water shortage conditions (at 50% SWC and at 20% SWC), was analysed and compared to the well-watered (WW) plants maintained at 80% of SWC (Table II.1.1).

In terms of SGR (Supplementary Figure S1), both rootstocks, either own-rooted and grafted with Cabernet Sauvignon, did not record statistically significant differences with respect to WW plants at T2 (50% of SWC) and T3 (20% of SWC).

Rootstock	**Grafting**	**Treatment**	**T1[1]**	**T2[1]**	**T3[1]**
101-14	own-rooted	well-watered	80% SWC	80% SWC	80% SWC
		water stressed	80% SWC	50% SWC	20% SWC
	grafted with CS	well-watered	80% SWC	80% SWC	80% SWC
		stressed	80% SWC	50% SWC	20% SWC
1103 P	own-rooted	well-watered	80% SWC	80% SWC	80% SWC
		stressed	80% SWC	50% SWC	20% SWC
	grafted with CS	well-watered	80% SWC	80% SWC	80% SWC
		stressed	80% SWC	50% SWC	20% SWC

Table II.1.1 Experimental design of water stress experiment carried out on 101-14 and 1103 P grapevine rootstocks own-rooted and grafted with Cabernet Sauvignon (CS).

Table II.1.2 shows the effect of genotypes, own-rooted (101-14 and 1103 P) and grafted with Cabernet Sauvignon (101-14/CS and 1103 P/CS), on gas exchanges and SWP, at three levels of soil water content (80%, 50% and 20% of SWC). Mild level of water stress (50% SWC) was able to determine a sensitive reduction of the photosynthetic activity (Pn) in 101-14, furtherly decreased at 20% SWC. Pn also diminished in 1103 P at T3. At strongest water deprivation, for all the genotypes examined, a lowering of the stomata conductance (Gs) occurred, with 1103 P/CS which started to close the stomata since mild level of stress. The transpiration rate (E) underwent to a significant decrease in both own-rooted genotypes (101-14 and 1103 P) at both water stress levels (T2 and T3), as well as in 101-14/CS (only at T3) with respect to their control values maintained at 80% SWC. The amount of internal CO_2 concentration increased in 101-14 at T2 and it diminished significantly in 101/CS at T3. For all the genotypes studied, Vpd rose at T3, except for 101-14 whose value dropped at T2 and then increased again statistically significant. The genotype 101-14, either own-rooted or grafted with Cabernet Sauvignon, showed a significant drop of SWP at T2 while for 1103 P and 1103 P/CS it increased at T3 with respect to their control values (at 80% SWC). The WUE_{int} resulted significantly higher only for 101-14/CS and 1103 P when the stress was at T3 and T2 and T3 respectively.

Rootstock	SWC[1]	Pn[2]		Gs[3]		E[4]		Ci[5]		Vpd[6]		SWP[7]		WUE_{int} [8]	
	80%	9,1	a	207	a	4,1	a	181	a	23	a	4,5	a	50,7	a
101-14	50%	7,3	b	214	a	3,1	b	225	b	17	c	2,8	b	35,2	a
	20%	4,3	c	102	b	2,2	c	188	a	28	b	4,2	a	57,5	a
	80%	6,6	a	206	a	3,8	a	224	a	21,4	a	5,5	a	33,3	a
101-14/CS	50%	6,7	a	173	ab	2,8	ab	216	a	18,2	a	2,6	b	41	ab
	20%	5,1	a	114	b	2,4	b	182	b	25,9	b	5,7	a	58,4	b
	80%	6,4	a	266	a	4,4	a	208	a	19,8	a	3,5	a	26,2	a
1103 P	50%	8	a	203	ab	3,1	b	215	a	17,9	a	3	a	40	b
	20%	4,2	b	132	b	2,6	b	228	a	25,8	b	6,4	b	41,4	b
	80%	6,5	a	200	a	3,5	a	215	a	20,6	a	5,0	a	34	a
1103 P/CS	50%	5,5	a	135	b	2,4	a	216	a	19,8	a	3,5	a	41,5	a
	20%	4,2	a	97	b	2,3	a	217	a	28,4	b	5,7	b	42,4	a

[1] Soil water content; [2] Photosynthetic activity (µmol CO_2 $m^{-2}s^{-1}$); [3] Stomatal conductance (mol H_2O $m^{-2}s^{-1}$); [4] Transpiration (mmol H_2O $m^{-2}s^{-1}$); [5] Internal CO_2 concentration (µmol CO_2 $m^{-2}s^{-1}$); [6] Vapor pressure deficit (KPa); [7] Stem water potential (bar); [8] WUE_{intr} (Pn/Gs).

Table II.1.2. Effect of rootstocks on gas exchanges and stem water potential at 80%, 50% and 20% of SWC. Mean values reported. Significance of the differences are indicated by letters according to Duncan's test ($p < 0.05$).

Impact of water stress on root and leaf transcriptome

The whole transcriptome of roots and leaves of 101-14 and 1103 P grapevine rootstocks, own-rooted and grafted with Cabernet Sauvignon, were sequenced by NGS technology using HiSeq2000 platform. The trimmed reads were mapped on CRIBI PN40024 12X v2 grape reference transcriptome and the number of unique mapping reads per sample ranged from 45 to 80 million of reads, with an average of 57 million, and a percentage of 84% successfully mapped reads (ranging from 62 to 91% of reads).

Heatmap and hierarchical analysis clustered transcriptomes in two main groups (Supplementary Figure S2), according to the origin of plant material (roots or leaves). In root group, the highest correlation was highlighted among samples coming from the same genotype, independently of water condition and grafting. In leaf group, the two main clusters included WW and water stressed (WS) samples of own-rooted and grafted plants, respectively. Principal Component Analysis (PCA) showed a good correlation among three biological replicates (Supplementary Figure S3). The first two components of PCA accounted for 82% of total variance. Along the first component, the samples were split in two groups, one holding root samples and the other leaf samples. Only two samples (1103P: own-rooted:drought:roots:T3 and 1103P:own-rooted:well-watered:roots:T1) were grouped aside and were removed by the following analyses.

Table II.1.3 summarizes total DEGs (differentially expressed genes) in both 101-14 and 1103 P rootstocks subjected at three different water deprivation conditions. No statistically significant differences were observed among WW and water stressed (WS) samples at 80% of SWC (T1), for both genotypes and plant material. The time point showing the highest number of DEGs was T3. At T3, root DEGs were higher than leaf ones per each sample. 1103 P showed the highest number of DEGs in both own-rooted and grafted plants.

Genotype		Differentially expressed genes					
		T1		T2		T3	
		Roots	Leaves	Roots	Leaves	Roots	Leaves
				Own-rooted			
101-14	*Up*	-	-	46 (0.11)	282 (0.84)	3157 (8.21)	976 (2.50)
	Down	-	-	44 (0.10)	155 (0.38)	1900 (5.63)	831 (2.23)
	Total	-	-	90 (0.21)	437 (1.22)	5057 (13.84)	1807 (4.73)
1103 P	*Up*	-	-	114 (0.24)	1 (0.00)	3164 (8.32)	1463 (3.91)
	Down	-	-	98 (0.22)	-	2166 (6.25)	1341 (3.56)
	Total	-	-	212 (0.46)	1 (0.00)	5330 (14.57)	2804 (7.37)
				Grafted			
101-14	*Up*	-	-	42 (0.08)	56 (0.13)	2509 (6.32)	1195 (3.02)
	Down	-	-	72 (0.16)	84 (0.21)	1478 (4.27)	492 (1.35)
	Total	-	-	114 (0.25)	140 (0.34)	3987 (10.59)	1687 (4.37)
1103 P	*Up*	-	-	350 (0.82)	-	4162 (11.12)	1440 (3.93)
	Down	-	-	638 (1.50)	11 (0.02)	2978 (8.44)	846 (2.47)
	Total	-	-	988 (2.32)	11 (0.02)	7140 (19.56)	2286 (6.40)

Table II.1.3 Overview of differentially expressed genes (number and percentage in the brackets) detected in roots and leaves of two grapevine rootstocks (101-14 and 1103 P), own-rooted and grafted with Cabernet Sauvignon at three different water stress conditions. T1 = 80% of SWC (soil water content); T2 = 50% of SWC; T3 = 20% of SWC. "-" = No statistically significant differences were observed among well-watered and drought water stressed samples at 80% of SWC.

The GO enrichment assay was performed to identify the biological processes most affected by drought. The top-50 GO terms were grouped in five macro-categories (Figure II.1.1) and arranged based on the plant material (roots and leaves). The distribution of DEGs in each macro-category was similar between roots and leaves. The most enriched GO categories were "response to stimuli" for both plant material (around 60 and 55% for roots and leaves, respectively) and "cell wall" was the lowest (about 5%). The genes involved in primary metabolism appeared to be more affected in leaves in comparison to root samples (30 and 8%, respectively), while secondary metabolism was less represented in leaves than in root samples (5 and 17%, respectively).

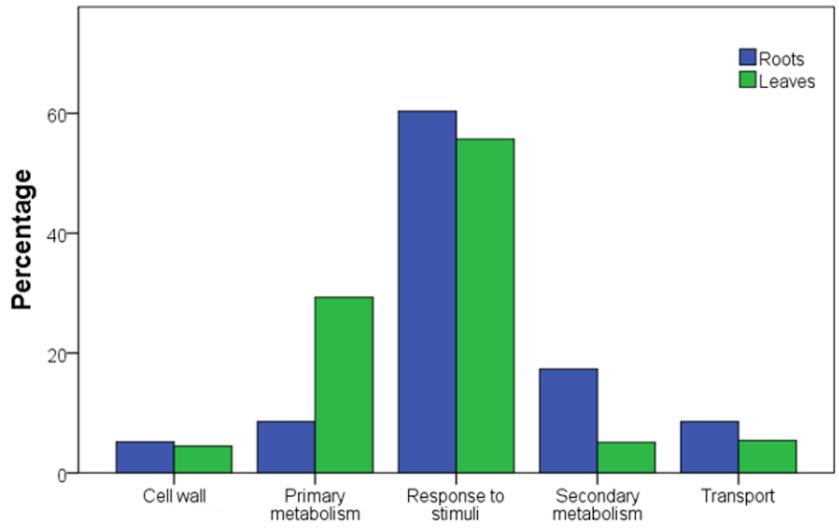


Figure II.1.1 Overview of ontology categories of DEGs in 101-14 and 1103 P roots and leaves of own-rooted and grafted plants under drought conditions. GO terms were grouped in five micro-categories: cell wall, primary metabolism, response to stimuli, secondary metabolism and transport

Genotype modulated specific root and leaf transcriptomic responses to drought

In order to identify the genes affected by water stress in both grapevine rootstocks, DEG lists were visualized by Venn diagrams. Only genes with a log2 fold change value higher than 2.0 and lower than -2.0 were retained. The number of DEGs was drastically reduced up to 0 in most of genotypes and plant material at T2. A reasonable number of DEGs was only retained for roots of 1103 P grafted with Cabernet Sauvignon. At T3, the highest number of shared DEGs was obtained among root samples (Figure II.1.2). One-hundred and twenty DEGs were found in common among roots of both own-rooted and grafted samples of both genotypes collected at T3 (Figure II.1.2A).

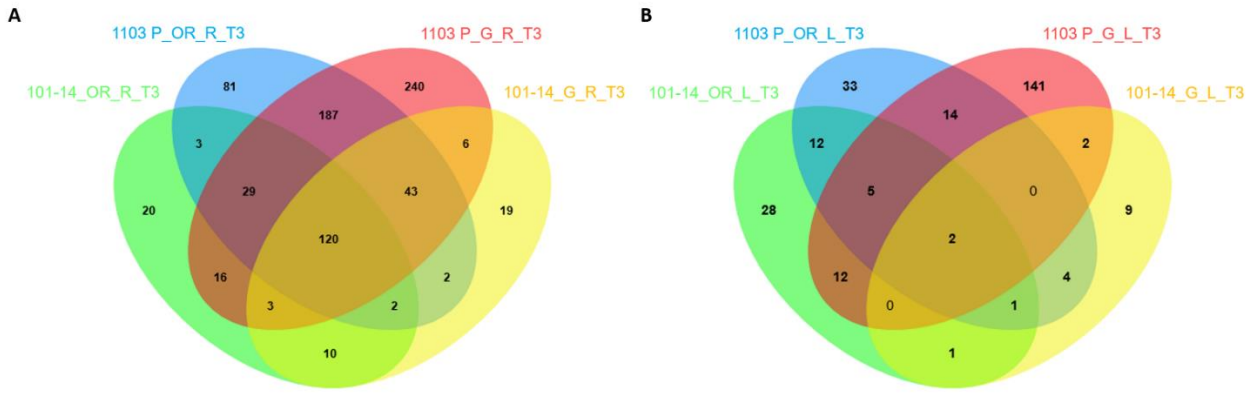


Figure II.1.2 Venn diagram illustrating DEGs by roots and leaves of two grapevine rootstocks (101-14 and 1103 P), own-rooted and grafted with Cabernet Sauvignon, under water stress (T3 = 20% of soil water content). Only the genes with a log2 fold change value higher than 2.0 and lower than -2.0 were viewed. A: DEGs in roots of 101-14 and 1103 P, own-rooted and grafted; B: DEGs in leaves of 101-14 and 1103 P, own-rooted and grafted. 101-14_OR_R_T3 = 101-14_own-rooted_roots_T3; 101-14_G_R_T3 = 101-14_grafted_roots_T3; 1103 P_OR_R_T3 = 1103 P_own-rooted_roots_T3; 1103 P_G_R_T3 = 1103 P_grafted_roots_T3; 101-14_OR_L_T3 = 101-14_own-rooted_leaves_T3; 101-14_G_L_T3 = 101-14_grafted_leaves_T3; 1103 P_OR_L_T3 = 1103 P_own-rooted_leaves_T3; 1103 P_G_L_T3 = 1103 P_grafted_leaves_T3.

The heatmap of this core number of DEGs did not identify divergent co-expression patterns affected neither by genotypes nor by grafting (Supplementary Figure S8 and Table S9). The response of 101-14 leaves to water deprivation was different from leaves of Cabernet Sauvignon grafted onto 101-14, as well as 1103 P leaves response in comparison to leaves of Cabernet Sauvignon grafted onto 1103 P (Figure II.1.2). Only four DEGs were shared between leaves of 101-14 and Cabernet Sauvignon grafted onto 101-14 and 21 DEGs between leaves of 1103 P and Cabernet Sauvignon grafted onto 1103 P. Unlike in root samples, only two shared DEGs were detected among own-rooted and grafted samples at leaf level.

Specific DEGs were identified per each genotype and grafting combination at both root and leaf level (Figure II.1.2). The samples showing the highest number of specific DEGs were roots of 1103 P grafted plants and leaves of Cabernet Sauvignon grafted onto 1103 P. Lists of the most specific up- and down-regulated genes and pathways are reported in Table II.1.4.

Rootstock	**Own-rooted**		**Grafted**	
	Up	Down	Up	Down
		Roots		
101-14	– root growth and development – volatile compounds biosynthesis – response to dehydration – ABA-regulated responses	– lignin biosynthesis – brassinosteroid biosynthesis – oxidative stress metabolism	– ROS production – transcription factors – biosynthesis of alkaloids	– pectinesterase inhibitor – extension protein
1103 P	– polyphenol biosynthesis – terpene biosynthesis – lignin degradation – zinc transporters	– ethylene-responsive transcription factors – MYB transcription factors	– cell wall construction – tonoplast intrinsic proteins – signal transduction	– transport and storage proteins
		Leaves		
101-14	– biosynthesis of volatile compounds – protein kinases – shikimate pathway	– heat shock protein – expansin-like protein	– heat shock proteins – cell wall construction	– stress-induced hydrophobic peptide
1103 P	– receptors or proteins regulated by hormones – lignin degradation – alkaloid biosynthetic – leaf growth – cell water retention – synthesis of cell-wall proteins	– heat shock proteins	– phenylpropanoid pathway – proteolytic enzymes – aquaporins – glucanases – auxin- and gibberellin-induced proteins – promotion of cell adhesion.	– redox-regulating protein

Table II.1.4 Specific differentially expressed genes and pathways in roots and leaves of 101-14 and 1103 P, own-rooted and grafted with Cabernet Sauvignon, under water deprivation (20% of soil water content).

II.1.4 Discussion

Rootstocks exert a decisive role in water stress response, being roots the first organ involved in perceiving the stress. Nevertheless, the level of water deficit (Soar et al., 2006), as well as, the rootstock/scion combination (Keller, 2015) can vary this response. Under drought condition, the modulation of gas exchanges (photosynthesis (Pn), CO_2 assimilation (Ci), stomatal conductance (Gs) and transpiration (E)) at leaf level is crucial for plant survival. The water losses through evapotranspiration are not adequately compensated by water supplies. This results in a decrease of stomata conductance and photosynthesis (Lovisolo et al., 2010). In the present study, own-rooted rootstocks (101-14 and 1103 P) significantly decrease their photosynthetic activity, with 101-14 affected since mild level of stress. On the contrary, grafted samples (101-14/CS and 1103 P/CS) were not affected by the stress, confirming the preponderant role exerted by scion in the regulation of gas exchanges. A similar trend was recorded for transpiration, with 101-14, 1103 P and 101-14/CS which decreased their water losses. By limiting transpiration through the regulation of stomatal conductance, water losses can be reduced. All the genotypes reduced their stomata conductance during the stress. In particular, 1103 P/CS appeared to be very sensitive to water deficit, starting to close the stomata at mild level of stress and maintaining the photosynthesis and the transpiration rates unchanged, thus showing a conservative behaviour. Stomata closure represents a drought-avoiding mechanism permitting to limit water losses and to adapt to the less water availability (Corso et al., 2015). Stomata controls either water losses and CO_2 assimilation which resulted changed only for the genotype 101-14, either own-rooted and grafted with Cabernet Sauvignon. In response to high level of Vpd of the atmosphere, plants reduce their stomatal conductance, limiting their ability to assimilate carbon (Romero et al., 2017). The intensification of Vpd is related with higher stomatal closure and photosynthesis reduction (Silva et al., 2013), due to stomatal (diffusion) and nonstomatal (mesophyll) limitations (Shibuya et al., 2017). Most plants show a nonlinear transpiration response to increasing Vpd (as 1103 P/CS), while some plants reduced their E while Vpd continues to increase (as 101-14, 101-14/CS and 1103 P), which has been referred to as an “apparent feedforward” response (Monteith, 1995). At mild level of stress, SWP decreased in 101-14, own-rooted and grafted with Cabernet Sauvignon, while in 1103 P it increased. The increase of SWP during drought is a natural consequence of the stress to enhance water uptake, coping with water deprivation (Serra et al., 2013). According with the ecological classification introduced by Jones (Jones, 1980), 1103 P behaved as a “pessimist” genotype, meaning that under drought condition it preserves its water status and utilizes future resources more conservatively (Dal Santo et al., 2016).

The improving of the WUE is an important issue under climate change. The technique of the root drying alters the balance between vegetative and reproductive growth and is related to some enhancement of fruit quality (Chaves et al., 2002). In our study, the WUE increased for 1103 P from the early stages of water stress and it maintains itself significantly high while the stress continued. The enhance of WUE in 101-14/CS occurred at the highest level of water deprivation. This confirm that the genetic variability of WUE in grapes (Flexas et al., 2010; Schultz and Stoll, 2010) is partly due to rootstocks (Iacono et al., 1998; Koundouras et al., 2008; Pou et al., 2008) and 1103 P is able to modulate the physiological response to drought since the early stages of water deprivation.

Root system is crucial for plant development because it anchors them to the substrate and supplies stem with nutrients and water, as well. Roots are the first organs in perceiving water availability and sending a signal to shoot and leaves to regulate shoot growth and water use (Comas et al., 2013). The most dominant GO terms (detection of stimuli) confirmed their universal involvement in sensing water deprivation and response to drought stress (Dong et al., 2014).

According to previous studies (De Zelicourt et al., 2016; Jones, 2016), the main steps for handling abiotic stresses are signal perception, signal transduction to other organs and expression of stress-inducible genes. In this work it was observed that the initiation of drought stress triggered a wide range of responses, which implies that there are many genes and mechanisms involved in drought response in *Vitis* spp., related to the metabolism of abscisic acid, sucrose and starch, secondary metabolites and amino acids.

Abscisic acid (ABA) plays a crucial role towards drought stress because when soil water potential declines, it acts as a messenger from roots to leaf guard cells, *via* xylem sap, inducing stomata closure and reducing transpiration (Hartung et al., 2002). In roots of own-rooted 1103 P, an up-regulation of the gene encoding for abscisate beta-glucosyltransferase (ABA-UGT) occurred, glycosylating ABA in ABA-glucosilester (ABA-GE). ABA-GE is a transport and storage form of ABA, which is critical for ABA homeostasis (Rattanakon et al., 2016). At the same time, the genes of ABA catabolism, such as ABA 8'-hydroxylases, able to convert the hormone in phaseic acid deactivating it irreversibly, resulted down regulated in own-rooted and grafted roots of 1103 P.

ABA signal transduction has been extensively studied at molecular level and a model of action utilizes RCAR (regulatory components of ABA receptors), comprising pyrabactin resistance 1 (PYR1)/ pyrabactin resistance-like (PYLs) receptors, type 2C protein phosphatases (PP2C) and sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2). PYR/PYL receptors work at the apex of a negative regulatory pathway to directly regulate PP2C, which in turn negatively regulates SnRK2 (Gonzalez-Guzman et al., 2012). Except in 101-14/CS leaves, the other samples showed an up-

regulation of PYR/PYLs and a down-regulation of PP2C. In addition, roots of own-rooted 1103 P showed a down regulation of SnRK2, as well. These results fitted well with the literature, confirming that the expression of ABA receptors increase in presence of endogenous ABA (Chan, 2012) The higher levels of ABA-GE, accompanied with the down-regulation of ABA catabolism genes, occurred in 1103 P roots of own-rooted samples, together with the over-expression of PYR/PYL receptors and the down-regulation of PP2C at leaf level, can explain the highest attitude of 1103 P to close the stomata in water stress condition more markedly than 101-14.

During periods of limited energy supply or enhanced energetic demands, plants can draw on carbohydrate reserves, specifically on starch (Krasensky and Jonak, 2012). Starch is produced in plastids from excess sugars during photosynthesis. Drought stress generally leads to a starch degradation that can be rapidly mobilized and converted in soluble sugars and other metabolites, that acting as osmoprotectants, support plant growth under stress and mitigate its negative effect (Krasensky and Jonak, 2012).

Roots of own-rooted 1103 P plants showed a down-regulation of starch synthesis, as well as its degradation through hydrolysis. On the opposite, the gene (NDP-glucose-starch glucosyltransferase) involved in amylose biosynthesis was up-regulated. This indicated that amylopectin synthesis was more severely affected than amylose synthesis, resulting in an increase of amylose to amylopectin ratio in the starch. Even though starch degradation appeared down-regulated as well as the conversion of maltose to glucose, fructose and sucrose, the synthesis of D-glucose (a compatible solute in response to drought (Turk and Smeekens, 1999), 1,3-beta-glucan and cellulose were positively regulated due to the up-regulation of beta-glucosidase, glucan endo-1,3-beta-D-glucosidase and endoglucanase genes.

Roots of own-rooted and grafted 1103 P plants showed an up-regulation of the genes involved in D-fructose-6-phosphate and D-glucose-6-phosphate biosynthesis, that are intermediate compounds of the PPP (pentose phosphate pathway). This one is the main plant glycometabolism pathway playing an important role in growth, development and physiological stresses. It leads to the synthesis of nicotinamide adenine dinucleotide phosphatase (NADPH) that, as a reducing agent, is involved for reductive biosynthesis of metabolic compounds, such as fatty acids, amino acids and intermediary metabolites (nucleotides) (Von Schaewen et al., 1995). One of the key enzymes is the glucose-6-phosphate dehydrogenase (G6PDH), involved in the regulation of the flux of carbon through the pathway. G6PDH genes respond to various environmental stresses including drought (Scharte et al., 2009).

At leaf level, leaves of 1103 P/CS plants showed an up-regulation of the gene encoding for beta-fructofuranosidase, able to hydrolyse the glycosidic bond of sucrose-6-phosphate in D-glucose-6-phosphate and D-fructose. They both are osmolytes, able to help cellular osmotic adjustments. Osmolytes may have also a significant effect at lower concentrations because for their additional roles as low-molecular-weight chaperons and ROS scavengers, as well as in the regulation of gene expression and metabolic processes (Kumar et al., 2017).

When ABA activates receptors, several enzymes, including RBOHs (respiratory burst oxidase homologs), can be activated. This activation causes a transient burst of ROS (reactive oxygen species) in guard cells, triggering a complex signalling cascade to close stomata (Watkins et al., 2017). Nevertheless, if ROS reach damaging levels within the cells, the deriving oxidative stress can cause irreversible modification of proteins, DNA and membranes (Mittler, 2002). In order to regulate ROS homeostasis and preventing oxidative stress, the flavonols and stilbenes synthesis is one of plant defence mechanisms, due to their antioxidant activities (Watkins et al., 2017). Only in leaves of own-rooted 1103 P and 101-14/CS samples, flavonoid synthesis was not altered by water limitation. Moreover, 1103 P roots showed an up-regulation of the genes related to resveratrol biosynthesis and its derivatives, such as pterostilbene. As members of stilbene family, they act as defence antioxidant compounds in plant protection mechanism against abiotic and biotic stresses (Corso et al., 2015; Vannozzi et al., 2017). Marè et al. (2012) also found that on sandy substrate a general up-regulation of phenylpropanoid metabolism in Pinot Noir grafted onto 1103 P occurred, unlike grafted on 101-14.

Isoprenoids and phenylpropanoids are secondary metabolites having the potential to complement the functional roles of antioxidant enzymes (Tattini et al., 2015) when a biotic or abiotic stress is in progress. Regarding isoprenoids synthesis, only roots of own-rooted 1103 P plants exhibited an up-regulation of mevalonate and non-mevalonate pathways, leading to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) synthesis, respectively. Both IPP and DMAPP, were converted into isoprene (by isoprene synthase enzyme) which was found to reduce photooxidative stress by quenching ROS/reactive nitrogen species (RNS) (Tattini et al., 2015). Similarly to isoprenoids, phenylpropanoids may support the action of antioxidant enzymes during severe stress conditions (Fini et al., 2011). In this work, roots of own-rooted 1103 P plants were the most sensitive organ to water stress in terms of phenylpropanoid pathway modulation.

The spectrum of amino acids can be altered by osmotic stress and water stress. Amino acids are compatible solutes, able to adjust osmotic potential when it decreases after the stress without interfering with cell metabolism (Rhodes et al., 1986). They play a significant role in detoxifying

ROS and heavy metals and regulating intracellular pH (Rhodes et al., 1986), as well. While 101-14 WS plants did not record amino acids modifications, 1103 P vines, own-rooted and grafted, showed appreciably changes. Particularly, in roots of own-rooted 1103 P plants, the genes encoding for enzymes involved in tryptophan, glutamic acid, l-glutamine and lysine syntheses were up-regulated. In 1103 P/CS, roots exhibited an up-regulation of genes related to l-glutamine biosynthesis. Like in Meggio et al. (2014) not appreciable changes in amino acid biosynthesis pathway were observed in leaves of both own-rooted and grafted plants.

The analysis of DEGs showed a different drought response in roots and leaves of own-rooted and grafted plants (Figure II.1.2). The grafting modulated specific genes in both roots and leaves of 101-14 and 1103 P plants. Roots and leaves of own-rooted and grafted 101-14 plants showed a balance of DEGs. By the opposite, roots and leaves of 1103 P/CS were more reactive than own-rooted plants, modulating about three and five times, respectively for roots and leaves, more the genes in grafting condition than in own-rooted one.

At root level, the grafting affected genes related to root growth, secondary metabolite biosynthesis and oxidative stress metabolism in 101-14 plants under water shortage. In 101-14/CS plants, the growth appeared to be inhibited due to the down-regulation of the genes involved in cell wall modification mechanism (pectinesterase inhibitor and extension protein) acting in cell elongation (Wormit and Usadel, 2018). In own-rooted plants, enzymes such as proline-rich glycoprotein, involved in the root growth and development in response to ABA accumulation (Tseng et al., 2013), were up-regulated. The grafting affected the secondary metabolites pathways, as well. In roots of grafted plants, 101-14 rootstock showed an up-regulation of the genes involved in the alkaloid production. While, the biosynthesis of volatile compounds was up-regulated in roots of 101-14 own-rooted plants. Alkaloid and volatile compounds together with other natural compounds have the role in dissipating the excess of energy and preventing the generation of toxic oxygen radicals (Yahyazadeh et al., 2018). Exposure to drought and salt stress caused an increased formation of reactive oxygen species and thus oxidative stress. The enzymes, such as 2OG-Fe oxygenase protein, that affects water transport modifying the composition and structure of leaf secondary cell walls (Fang et al., 2012) were up-regulated in roots of 101-14/CS plants. While in own-rooted 101-14 plants, glutathione *s*-transferase, proposed to afford protection under various stress conditions by detoxifying endogenous plant toxins that accumulate as a consequence of increased oxidative stress (Marrs, 1996), was down-regulated.

At leaf level, leaves of 101-14/CS plants showed an up-regulation of a gene encoding for xyloglucan endotransglucosylase protein, recognized as wall-modifying proteins, participating in multiple

physiological roles, such as drought (Campbell and Braam, 1999). While, leaves of 101-14 own-rooted plants showed an inhibition cell growth due to the down-regulation of enzymes such as expansin-like protein that are involved in the cell wall plasticity (Fukuda, 2014).

Regarding 1103 P, grafting affected at root level the secondary metabolite biosynthesis, lignin degradation, zinc transport, transcription factors, cell wall-related proteins, signal transduction, transport and storage. In roots of 1103 P/CS plants, the root system growth appeared increased due to the up-regulation of the genes encoding for the cell wall construction. Moreover, grafting influenced the retention and uptake of water into the cell due to the up-regulation of tonoplast intrinsic proteins (Li et al., 2016). After perceiving signals like drought, the receptor protein kinases on plasma membrane transmits the signal into the cells and initiate a series of signal transduction events by phosphorylation (Liu et al., 2000). This signal transduction appeared strongly activate by grafting in roots of 1103 P/CS plants. By the opposite, roots of own-rooted 1103 P plants showed an up-regulation of secondary metabolites, such as polyphenols, terpenes and lignin degradation, indicating that there was a shift in timing of stimulus perception and signalling cascades. Own-rooted 1103 P appeared to perceive the stress earlier than the grafted ones. This shift was less evident at leaf level, where an up-regulation of the genes encoding for downstream responses, such as aquaporins, biosynthesis of phenylpropanoids and alkaloid and the modulation of leaf growth occurred in both own-rooted and grafted conditions.

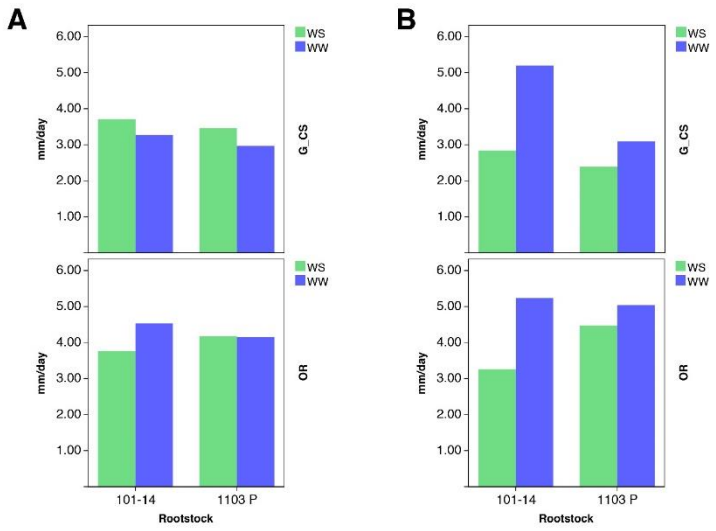
II.1.5 Conclusion

In conclusion, the rootstock has a preponderant role in perceiving drought and plant adaptation to the stress, being the roosts the first organ that feels water limitation. The scion reacts to the signal received by roots and can modulate the gas exchanges during drought. Plants display a variety of physiological and biochemical responses at cellular and whole-organism levels towards prevailing drought stress, making it a complex phenomenon. At physiological level, grafted plants seem to be less affected by water stress with 1103 P/CS showed a “pessimistic” behaviour: it conservatively preserves its water status under drought conditions in order to have resources in the future. Based on the transcriptomic evidence, 1103 P responded to water stress more actively than 101-14, especially at root level. 1103 P appeared readier than 101-14 to cope with the water scarcity, displaying an enhanced capacity to produce hormone water-stress signals (ABA), ROS scavenging compounds (flavonoids and stilbenes metabolites), as well as isoprenoids and phenylpropanoids, and osmolyte compounds. This readiness was affected by the grafting condition in terms of timing of stimulus perception and downstream

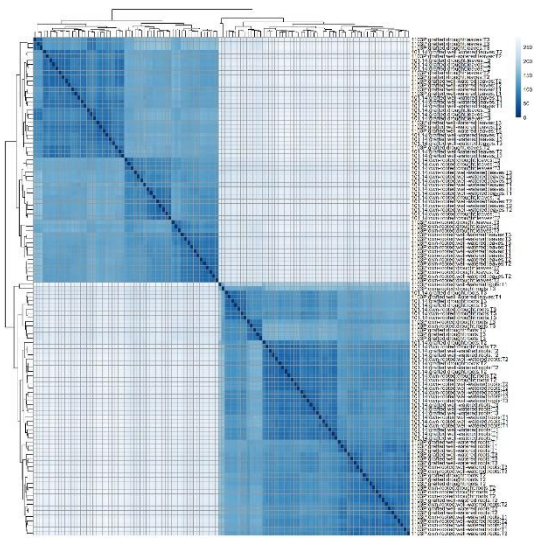
responses at the root level. The scion appeared to slow down the perception of water shortage in the rootstock.

II.1.6 Supplementary information

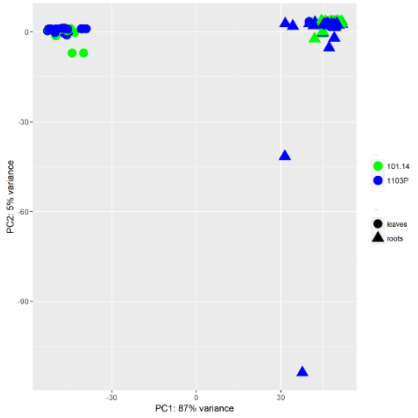
Supplementary Figure S1. Stem Growth Rate (SGR, mm/day) in 101-14 and 1103 P, water stressed (WS) and well-watered (WW) plants, own-rooted (OR) and grafted with Cabernet Sauvignon (G_CS). A: SGR during 50% of SWC. B: SGR during 20% of SWC.



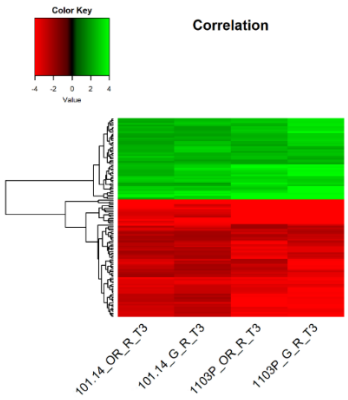
Supplementary Figure S2. Overall representation of changes in mRNA-Seq reads of roots and leaves of two grapevine rootstocks (101-14 and 1103 P), own-rooted and grafted with Cabernet Sauvignon, maintained at three different water conditions. T1 = 80% SWC (soil water content); T2 = 50% SWC; T3 = 20% SWC. Well-watered samples were kept at 80% SWC all the time.



Supplementary Figure S3. PCA (Principal Component Analysis) plot of mRNA-Seq read count table of roots and leaves of two grapevine rootstocks (101-14 and 1103 P), own-rooted and grafted with Cabernet Sauvignon, maintained at three different water conditions. T1 = 80% SWC (soil water content); T2 = 50% SWC; T3 = 20% SWC. Well-watered samples were kept at 80% SWC all the time. PC1 = Principal Component 1; PC2 = Principal Component 2.



Supplementary Figure S8. Heatmap of shared differentially expressed genes (DEGs) of 101.14 and 1103P grapevine rootstocks, own-rooted and grafted with Cabernet Sauvignon, in roots under water stress (T3 = 20% of soil water content). Green: upregulated genes; red: downregulated genes. 101.14_OR_R_T3 = 101.14_own-rooted_roots_T3; 101.14_G_R_T3 = 101.14_grafted_roots_T3; 1103P_OR_R_T3 = 1103P_own-rooted_roots_T3; 1103P_G_R_T3 = 1103P_grafted_roots_T3.



II.2 Physiological and transcriptional response of different M-rootstocks to water stress condition

II.2.1 Introduction

Water flows into the plant in a soil-plant-atmosphere *continuum* (Lazar, 2003). The whole water transport is influenced by anatomical structure of xylem vessels (Shao et al., 2008), hydraulic constraints (Steudle, 2000) and chemical signals (Schachtman and Goodger, 2008; Tombesi et al., 2015). When soil water availability decreases, one of the earliest response is stomatal closure, in order to maintain a favourable water balance, buffering the drop of xylem water potential and avoiding embolisms (Jones and Sutherland, 1991). The closure of guard cells determines a reduction of CO_2 assimilation and H_2O transpiration from leaves, consequently the photosynthetic activity decreases sharply (Medrano et al., 2015).

The key factor inducing stomatal closure is abscisic acid (ABA), an hormone mainly produced by roots and accumulated when soil dries out and water potential drops (Farquhar and Sharkey, 1982). This hormone, through the xylem sap, reaches guard cells enhancing the content of reactive oxygen species (ROS, especially H_2O_2). Stopping the influx and promoting the efflux of potassium ions (K+), it occurs a rise of calcium ions (Ca^{2+}) in cytosol and consequently cells lose their turgor. ABA is a carotenoid-derived compound whose synthesis is entrusted to a minor branch of the carotenoid pathway. The early steps of ABA biosynthesis are catalysed by zeaxanthin epoxidase (ZEP) and 9-cis-epoxycarotenoid dioxygenase (NCED) enzymes (Rock et al., 1991). VvZEP and VvNCED gene expressions are strongly induced by water stress (Qin and Zeevaart, 1999) and salt stress (Iuchi et al., 2001). ABA signalling pathway is mediated by three main components: i) ABA receptors (PYR/PYL/RCAR family of ABA receptors); ii) ABA-regulated Protein Phosphatase 2Cs (i.e. PP2CA); iii) ABA-regulated SNRK2 Protein Kinase (i.e. SnPK2) (Yoshida et al., 2002; Santiago et al., 2009). Without stimuli, ABA receptor is an unliganded form and the protein kinase is bound to the protein phosphatase. When ABA increases, it binds itself to ABA receptors (RCARs/PYR1/PYL) forming a complex that provides an active site for PP2C protein. The activated receptor binds to PP2C, frees SnPK2 which in turn is phosphorylated by another protein kinase. Multiple step phosphorylation of SnRK2 activates ABRB (ABRE-binding protein)/ ABF (ABRE-binding factor) which induce many ABA-responsive genes expression (Raghavendra et al., 2010).

Grapevine (*Vitis vinifera* L.) is one of the most cultivated and prized fruit crops around the world. In arid and semi-arid environments it undergoes to a slow decrease in water availability during the

growing season (Chaves et al., 2003). Although it is water stress adapted and traditionally non-irrigated, soil drying induces plants to display a multitude of physiological and biochemical changes, from minor until irreversible.

Eurasian viticulture, where Phylloxera (*Daktulosphaira vitifoliae* (Fitch)), accidentally imported in Europe from North America (Gale, 2002), is a severe threat for grapevine survival, is characterized by the use of *V. vinifera* varieties grafted onto a rootstock (*Vitis* spp.). North American *Vitis* species co-evolving with the pathogen, therefore they are utilized, as single or inter-specific hybrids, as rootstocks. Rootstocks also contribute to the control of other soil-borne pests such as nematodes as well as various abiotic constraints such as drought, salinity, limestone and mineral nutrition. They also modify whole plant development, biomass accumulation and repartition, and phenology (Ollat et al., 2015).

In grapevine, the expression of VvNCED1, VvNCED2 and VvZEP genes have been directly correlated with ABA accumulation, in response to water stress (Soar et al., 2006b; Speirs et al., 2013) and their expression was suggested as marker of ABA biosynthesis (Boneh et al., 2012). The expression of genes involved in ABA signalling pathway revealed that the genes coding for RCAR, SnRK and ABF are downregulated in drought conditions, while VvPP2C genes are generally up-regulated (Boneh et al., 2012; Haider et al., 2017).

Genetic variability of grapevine rootstocks undoubtedly plays a fundamental role in the adaptation of *vinifera* cultivation to climate changes, especially to water shortage (Walker, 1992; Serra et al., 2013). The Mediterranean basin is considered one of the most vulnerable regions of the world to climate change and with a high potential to deal with water scarcity and soil erosion in the next few years (Giorgi and Lionello, 2008; IPCC, 2018). Its climate is characterised by infrequent rainfall (less than 100 days per year) that is unevenly distributed over time (long periods of summer drought) and sometimes quite sparse (about 300 to 500 mm per year in some semi-arid regions). Most climate change scenarios for this area predict a decrease of rainfalls and higher temperatures. IPCC forecasts indicate a yearly temperature increase between 2 and 4°C and a decrease in rainfall between 4 and 30% by 2050 (IPCC, 2013). Due to their perennial status, grapevine will be highly vulnerable to environmental changes, representing a substantial risk for viticulture (Schultz, 2000).

In the context of global warming the exploitation of grapevine genetic diversity and the better understanding of plant response to environmental stresses represents the way from which the new viticulture challenges may arise (Vivier and Pretorius, 2002). Since 1985, the Department of Agricultural and Environmental Sciences (DiSAA) research group operating at the University of Milan is working on the selection of new rootstocks able to cope with abiotic stresses (Bianchi et al.,

2018). Some genotypes (series “M”: M1, M2, M3 and M4) were released in 2014 and registered in National Register of Vine Varieties (RNVV). All the genotypes show from moderate to high tolerance to drought (M4 > M1 = M3 > M2) (Porro et al., 2013; Meggio et al., 2014; Corso et al., 2015), M1 and M3 exhibit tolerance to iron-limited conditions (M1 >M3) (Porro et al., 2013; Vannozzi et al., 2017) and M2 and M4 display moderate resistance to salinity (Porro et al., 2013; Meggio et al., 2014). To get more insight into the response of these genotypes to drought, their physiological (gas exchanges and stem water potential) and transcriptomic performance (genes involved in ABA-synthesis and ABA-mediated responses to drought) have been evaluated under well-watered and water-stress conditions and compared with the response of other commercial genotypes largely used in viticulture, either tolerant (140 Ru, 41 B, 110 R, 1103 P), less tolerant (SO 4, K 5BB) and susceptible (420 A and Schwarzman).

II.2.2 Material and Methods

Plant material and growth conditions

The experiment was conducted in June 2017, under environmental controlled conditions of the greenhouse at the Department of Agricultural and Environmental Sciences (University of Milan). The greenhouse was equipped with supplementary light and a cooling system, with a 16-hr light [∼PPFD of 600 μmol of photons/(m^2 • s)] and 8 hr dark photoperiod and a range of temperature from 23 to 28°C. The experiment was carried out on eleven two-years old rootstocks, selected on their different behaviour in response to drought, from highly tolerant to susceptible (Table II.2.1). Nine replicates per rootstock, for a total of 99 plants, were monitored during the experiment. The vines were grown in 4-L plastic pots, trained on 1 m stake and placed in a randomized complete block design. The growth substrate was composed of 70% sand and 30% peat, supplemented with a layer of expanded clay aggregate on the bottom of the pot to avoid water flooding. During the phenological phase of budding, the samples were maintained in well-water conditions in order to develop a well-expanded canopy.

Rootstock	Pedigree	Behaviour in response to drought
M4	unknown x 1103 P	highly tolerant
110 R	unknown x *V. rupestris* cv. Du Lot	
1103 P	*V. berlandieri* cv. Resseguier nr. 2 x *V. rupestris* cv. Du Lot	
140 Ru	unknown x *V. rupestris* cv. Du Lot	
41 B	*V. vinifera* cv. Chasselas x *V. berlandieri* cv. Planchon	
K 5BB	*V. berlandieri* Resseguier nr. 2 x *V. riparia* cv. Gloire de Montpellier	less tolerant
M1	Kober 5BB x Teleki 5C (*V. berlandieri* cv. Planchon x *V. riparia*)	
M3	Kober 5BB x Teleki 5C	
SO 4	*V. berlandieri* cv. Resseguier nr. 2 x *V. riparia* cv. Gloire de Montpellier	
Schwarzmann	*V. riparia* x *V. rupestris*	susceptible
420 A	*V. berlandieri* x *V. riparia*	

Table II.2.1. List of 11 grapevine rootstocks subjected to water limitation, their pedigree (based on Migliaro et al. (2019)) and behaviour in response to drought.

Irrigation management and sampling

For each rootstock, three plants were maintained under well-watered conditions (80% of SWC), six of them were subjected to water stress (WS) conditions (50 and 20% SWC). The SWC was calculated using the gravimetric method, according to the formula suggested by Gardner et al. (2001):

$$SWC = (\text{fresh weight} - \text{dry weight})/\text{dry weight} \times 100$$

where fresh weight is referred to the soil weight at field capacity and dry weight to the soil dried in an oven at 105°C for 48 hours.

Each pot containing one sample was weighed daily for a period of 10 days. When SWC reached the value of 50% and 20%, water stressed plants were sampled for the physiological and transcriptomics analyses. The control plants, maintained at 80% of SWC, were watered every day, in order to restore the right field capacity and sampled together with the WS plants. For transcriptome analysis, whole root system and fully expanded leaves (i.e. from the fifth to the eighth node of primary shoot) were immediately sampled after the *in vivo* measurements of physiological parameters, frozen in liquid nitrogen and stored at −80°C.

Plant phenotyping

At each time point (T1, T2 and T3), gas exchange parameters and stem water potential (SWP) have been evaluated. Both measurements were carried out between 11:00 am and 1:00 pm solar time.

Two fully expanded leaves (8^{th} and 9^{th} leaf) per plant were selected to measure gas exchange indicators: Photosynthetic activity (Pn; μmol CO_2 $m^{-2}s^{-1}$), Stomatal conductance (Gs; mol H_2O $m^{-2}s^{-}$

^{1}), Transpiration (E; mmol H_2O $m^{-2}s^{-1}$) , Internal CO_2 Concentration (Ci; µmol CO_2 $m^{-2}s^{-1}$) and Vapor pressure deficit (Vpd; KPa). Gas exchanges were measured with a leaf portable photosynthesis system (CIRAS-2, PP Systems, Amesbury, MA, USA) equipped with PLC6 (U) cuvette 18 mm circular (2.5 cm^2 head plate), under constant saturating PPFD of 1500 µmol photons $m^{-2}s^{-1}$, CO_2 concentration of 300 µmol mol^{-1}, block temperature of 25°C and relative humidity between 60% and 70% allowing ~1.5 kPa of Vpd inside the leaf chamber.

As suggested by Scholander et al. (1965), SWP (bar) was calculated using the Scholander-pressure chamber (Soil Moisture Equipment Corporation, Santa Barbara, CA, USA). The same leaves used to evaluate gas exchanges were placed in a plastic bag wrapped with an aluminium foil for 1 hr. Subsequently, they were excised with a razor blade and put in the Scholander chamber for the analysis. The SWP value was recorded within 30 sec from the cutting of the leaf by slowly pressurizing the chamber until sap came out from the cut end of the petiole. Phenotypic data of the WS plants (at 50% or 20% SWC) were expressed as percentage of the WW plant values (at 80% of SWC) (treated value/control value x 100).

Gene expression analysis

Roots and leaves of each sample at 80%, 50% and 20% of SWC were collected, frozen with liquid nitrogen and kept at -80°C until RNA extraction. The total RNA was extracted from 100 mg of liquid nitrogen-ground tissue with Spectrum™ Plant Total RNA (Sigma-Aldrich, Germany) commercial kit, according to the manufacturer's instructions. To evaluate RNA quality, 260/230 and 260/280 ratios were checked *via* NanoDrop Spectrophotometer (Thermo Scientific, MA, USA). For those samples showing a 260/230 absorbance ratio lower than 1.8, a lithium-chloride treatment was carried out (as reported in De Lorenzis et al., 2016). RNA integrity was checked by electrophoresis on 1.5% agarose gel. RNA quantification was performed using Qubit® RNA HS Assay Kit by Qubit® 3.0 Fluorometer (Life Technologies, CA, USA).

Total RNA (200 ng) were used to synthetize cDNA employing 200 U of SuperScript® III Reverse Transcriptase (Thermo Fisher) and 50 µM oligo$(dT)_{20}$ primers in accordance with the manufacturer's instructions.

Six genes (three for root and three for leaf tissues; Table II.2.2) involved in tolerance response to drought were amplified *via* real-time RT-PCR. Ubiquitin (UBI; Fujita et al., 2007) and actin (ACT; Reid et al., 2006) were used as reference genes. RT-PCR was carried out in a 7300 Real Time PCR System (Applied Biosystems, CA, USA). For each reaction (20 µL), 200 nM of each primer, 2 µL of cDNA (1:100 dilution of the synthesis reaction), 1X SYBR Green Real-Time PCR Master Mix

(Thermo Fisher) and water up to 20 µL were added. Thermal cycling was pre-incubation at 95°C for 3 min, followed by 40 cycles of 94°C for 15 sec, 58°C for 20 sec and 72°C for 30 sec. For detecting non-specific amplifications in cDNA samples, a melting cycle with temperature ranging from 65 to 95°C was performed. Each real-time RT-PCR reaction was completed in triplicate. Ct (cycle threshold) values were normalized using the geometric mean of expression values of two reference genes. The expression of each gene in different genotypes and water conditions was calculated by comparing their $2^{-\Delta\Delta}$Ct values (Livak and Schmittgen, 2001).

Genes	Primer sequence (5'→ 3')	Reference	Tissue
VvNCED1	F: TGCAGAGGACGAGAGTGTAA R: AGCTACACCAAAAGCTACGA	Hayes et al. (2010)	roots
VvNCED2	F: ATGCTCAAACCGCCTCTGAT R: TCCCAAGCATTCCAGAGGTG	Lund et al. (2008)	
VvZEP	F: GGTAAGAAGGAAAGGTTGC R: CAATAGGAGTCCCTGATTTGATGC	Hayes et al. (2010)	
VvPP2C4	F: TGGGCTTTGGGATGTTATGT R: TGTGCAGGAGTCTCATCAGC	Boneh et al. (2011)	leaves
VvSnRK2.6	F: CACCAACCCACCTTGCTATT R: AAACGTGCCTCATCCTCACT	Boneh et al. (2012)	
VvABF2	F: GGCACCCAGGCTAGTTAA R: GCAGAGTACACGCTAGATTG	Rossdeutsch et al. (2016)	

Table II.2.2. List of genes amplificated *via* real-time RT-PCR in roots and leaves of 11 own-rooted grapevine rootstocks grown under water deprivation.

Statistical analysis

Statistical analyses were made by SPSS statistical software (version PASW Statistics 24, SPSS, Inc. Chicago, IL). Phenotypic and genetic data were used to perform a Discriminant Analysis (DA). The projections of the first two components were plotted on a 2D scatter plot. The variables mainly affecting DF1 (Discriminant Function) and DF2 were subjected to univariate analysis of variance ($p \leq 0.05$), followed by Bonferroni-corrected Fisher's protected least significant difference (LSD) test ($p \leq 0.05$), using the R package *agricolae* and LSD test function (www.r-project.org).

II.2.3 Results

The physiological response of rootstocks to drought

Gas exchanges and SWP were evaluated on 11 grapevine rootstocks under water shortage (50% and 20% of SWC) in order to compare the physiological response of M-rootstocks with respect to the commercial genotypes during drought. PnP (photosynthetic activity percentage), GsP (stomatal conductance percentage), EP (transpiration percentage), CiP (internal CO_2 concentration percentage), VpdP (vapor pressure deficit percentage) and SWPP (stem water potential percentage) data were

analysed with DA. At 50% of SWC, the first two DFs explained the 58.8% of the total variation, with DF1 and DF2 accounted for 35.7 and 23.1%, respectively (Supplementary Information S1). The variables SWPP and VpdP were the most significant within DF1, while PnP within DF2. At 20% of SWC, DF1 and DF2 explained the 82.3% of the variability, with 49.3 and 33% respectively (Supplementary Information S1). The variables mostly affecting the DFs were GsP and VpdP for DF1 and GsP and EP for DF2 (Supplementary Information S2). Physiological parameters at 50% of SWC did not markedly differentiate the rootstocks along the first two DFs (data not shown). On the opposite, significant discrimination among genotypes were observed at 20% of SWC (Figure II.2.1A). At the highest level of stress, DA separated M3 and M4 rootstocks along the first DF. M3 was grouped together with most genotypes with a less tolerant/susceptible behaviour towards water stress (K 5BB, SO 4 and Schwarzmann), while M4 was grouped together with most genotypes with a highly tolerant behaviour (110 R, 1103P and 140 Ru). M1 was placed in the middle between the two groups. Along the second DF, a clear differentiation among the genotypes was not detected. 1103 P rootstock appeared the most discriminated one.

Focusing on the variables (GsP, VpdP and EP) mostly affecting DFs at 20% of SWC, LSD test showed statistically significant values with respect to the well-watered values (80% of SWC) for most of the genotypes (Figure II.2.1B). Statistically significant GsP values were lower than 50% of control values in M4, 110 R, 1103P, 41 B, K 5BB, M3, SO 4 and Schwarzmann, ranging from 47% (41 B) to 14% (Schwarzmann). In M3 and M4, GsP values were around 45%. VpdP values increased in all the genotypes analysed and statistically significant values were detected for all the rootstocks, with few exceptions (41 B and Schwarzmann). The highest VpdP value was detected for 110 R (145%), followed by M4 (143%). The lowest values were detected for M1, M3 and SO 4 (117%). About EP, 140 Ru was the only genotype showing a statistically significant value higher than control (121 vs 100%), for the rest it was lower, with values ranging from 61 (M4) to 19% (Schwarzmann).

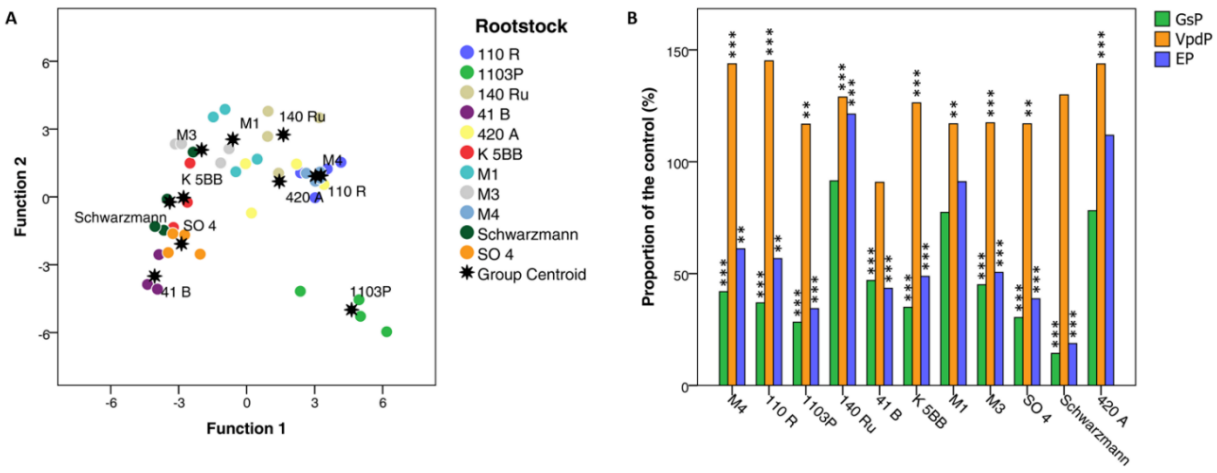


Figure II.2.1 A) First two functions of discriminant analysis (DA) for eleven grapevine rootstocks at 20% of soil water content (SWC). DA has been performed including gas exchanges (photosynthetic activity, stomatal conductance, transpiration, internal CO_2 Concentration and vapor pressure deficit) and stem water potential values, reported as percentual of the well-water condition (80% SWC). Each plant is shown as plot. B) Bar plots of variables mostly affecting the first two functions (DFs) of discriminant analysis (DA) for eleven grapevine rootstocks at 20% SWC. Gs (stomatal conductance) and Vpd (vapor pressure deficit) discriminated genotypes along the first DF, E (transpiration) along the second DF. Data are expressed as percentage of the well-watered plant (80% of SWC) values. Bars followed by '*' significantly differ from the well-watered value (100%) according to LSD test (**: *p*-value = 0.01; ***: *p*-value = 0.001).

The transcriptional response of rootstocks to drought

The expression level of six genes related to drought response was detected in roots (VvNCED1, VvNCED2, VvZEP) and leaves (VvPP2C4, VvSnRK2.6, VvABF2) of the all rootstocks growing under water limitation, *via* real-time RT-PCR. The behaviour of each rootstock to water conditions was evaluated comparing the $2^{-\Delta\Delta}$Ct values per each gene. Gene expression data were subjected to DA in order to identify the most discriminant level of stress among genotypes. At 50% of SWC, DF1 and DF2 contributed to 89% of the total variation (76 and 13%, respectively; Supplementary Information S3) (Figure II.2.2A). The variables VvPP2C4 and VvSnRK2.6 were the most significant within DF1, while VvZEP and VvABF2 within DF2 (Supplementary Information S4).

At 20% of SWC, DF1 and DF2 explained the 80% of the total variation, with DF1 accounting for 63 and DF2 for 17% (Supplementary Information S3). The variables mostly affecting the DFs were VvZEP and VvABF2 for DF1 and VvZEP and VvPP2C4 for DF2 (Supplementary Information S4). The 20% of SWC did not markedly differentiate the rootstocks along the first two DFs (data not shown). On the contrary, significant discrimination among genotypes were observed at 50% of SWC (Figure II.2.2A).

The genotypes were mainly separated along the first DF. M1 and SO 4 were the most discriminated along the both DFs. Among the M-rootstocks, M1 and M3 were separated each other, with M4 placed in between. Not a clear discrimination based on the attitude of rootstocks to drought was detected.

The variables VvPP2C4 and VvSnRK2.6 were the most significant within DF1, while VvZEP and VvABF2 within DF2. Among these variables, LSD test was applied to the genes showing a relative gene expression value higher than 1.5 (Figure II.2.2B). Gene expression values for ZEP were statistically significant only for M3 and SO4 rootstocks. Statistically significant values were obtained for VvPP2C4 in six out of 11 genotypes (110 R, 1103P, 140 Ru, 41 B, K 5BB and M3). SO 4 showed statistically significant values for relative gene expression of VvSnRK2.6. Four out of 11 genotypes (140 Ru, 41 B, K 5BB and 420 A) showed statistically significant values for VvABF2.

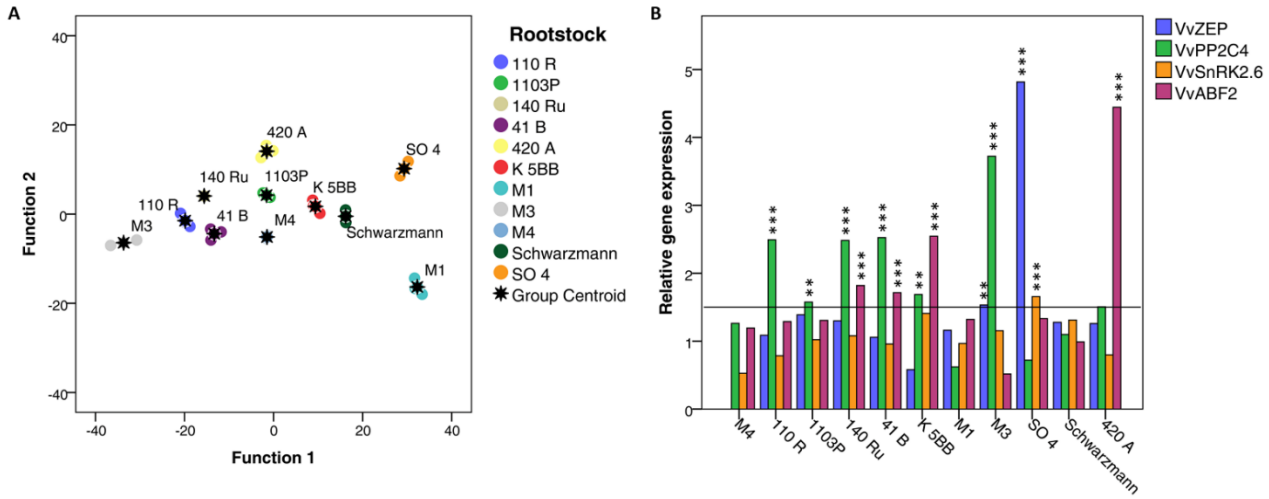


Figure II.2.2 A: First two functions of discriminant analysis (DA) for eleven grapevine rootstocks at 50% of soil water content (SWC). DA has been performed including gene expression values of genes involved in ABA-synthesis (VvNCED1, VvNCED2, VvZEP) and ABA-mediated responses (VvPP2C4, VvSnRK2.6, VvABF2) to drought, differentially expressed in respect to the well-water condition (80% SWC). Each plant is shown as plot. B**:** Bar plots of variables mostly affecting the first two functions (DFs) of discriminant analysis (DA) for eleven grapevine rootstocks at 50% of SWC. VvPP2C and VvSnRK2.6 discriminated genotypes along the first DF, VvZEP and VvABF2 along the second DF. The expression of each gene has been normalized using the geometric mean of expression values of two housekeeping genes (ubiquitin and actin). The relative gene expression has been determined based on the $2^{-\Delta\Delta Ct}$ method. The relative gene expressions of the well-watered plants (80% of SWC) reaching values around 1 are omitted. Bars followed by '*' significantly differ from the well-watered value (100%) according to LSD test (**: *p*-value = 0.01; ***: *p*-value = 0.001).

II.2.4 Discussion

It is well established that rootstock plays a fundamental role in water stress response. Nonetheless, genotypes do not react in the same way and they are classified as highly tolerant, less tolerant and susceptible to water deprivation (Flexas et al., 2009; Corso and Bonghi, 2014; Migliaro et al., 2019). The new M-rootstocks showed different reaction to water stress, going from high to moderate tolerance (M4 > M1 = M3 > M2). The understanding of rootstock performances during drought is useful for predicting the whole-plant behaviour in water scarcity condition, thus, to address the choice of the grape grower to the rootstock to be used. The aim of this study was to better characterize the

water stress response by the novel M-rootstocks (M1, M3 and M4), through a comparison with some commercial genotypes largely used in viticulture, with different capacity to face water stress.

Roots are the major interface between plant and soil and the organ that firstly perceive water availability. They are the main part of the plant involved in water stress signal perception, signal transduction and water stress-inducible gene expression that are the key steps for triggering a drought reaction.

The plant hormone abscisic acid mediates many physiological responses of plant to drought: avoidance as well as tolerance responses. It is produced in roots as well as in leaves (De Smet and Zhang, 2013). Levels in both parts increase upon exposure to drought and are accompanied by major changes in gene expression and physiological responses, such as stomatal closure. Our findings reveal that a moderate level of stress (50% SWC) is not able to discriminate rootstocks from a physiological point of view. For having appreciable differences among them they have to undergo to a more severe stress (such as 20% SWC). On the contrary, a SWC of 50% is enough for distinguishing rootstocks on the basis of their transcriptional response. Therefore, during water deprivation, roots firstly perceive the stress, activate the response mechanism modulating the expression of the genes involved in ABA biosynthesis and transduction. The stomatal closure, observed in almost all the rootstocks investigated, is a natural consequence of the accumulation of ABA at leaf level. The decrease of stomatal conductance, limits water losses through transpiration. M4 and M3 appeared to be very sensitive to water deficit, reducing their Gs with respect to their WW controls. In M3 and M4, Gs reduction is accompanied by a significant reduction of E. While in M1 neither Gs nor E were significantly reduced. All three M-rootstocks recorded higher Vpd during water stress with respect to their well-watered controls. This is particularly evident in M4 recording the highest Vpd. The capability to regulate stomatal aperture to counter the effect of soil water deficit and Vpd permits to reduce water fluctuations of tissues and maintains xylem integrity (Jones, 1998). According to the DA analysis, at 20% of SWC, M4 reacted as some other commercial genotypes classified as highly tolerant (110 R, 1103P and 140 Ru). M1 displayed an intermediate behaviour while M3 performance is comparable to that of the less-tolerant/susceptible ones (K 5BB, SO 4 and Schwarzman) (Figure II.2.1B). These results confirmed their different aptitude to tolerate water stress condition.

At mild level of stress, rootstocks react modulating the expression of the genes involved in ABA biosynthesis and signal transduction. The zeaxanthin epoxidase (ZEP) is the enzyme that catalyse the early steps of ABA biosynthesis, synthesized from cleaved xanthophylls. In this study, SO 4 was the only genotype whose roots significantly expressed VvZEP, involved in ABA biosynthesis (Figure II.2.2B).

A model of action of ABA signal transduction is represented by the RCAR (regulatory components of ABA receptors). It comprises pyrabactin resistance 1 (PYR1)/ pyrabactin resistance-like (PYLs) receptors, type 2C protein phosphatases (PP2C) and sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2). PP2C negatively regulates SnRK2s via dephosphorylation. When SnRK2 auto phosphorylates, then activate other transcription factors. In this study, a significant up-regulation of VvPP2C4 was observed in almost all genotypes, except for M4, M1, SO4, Schwarzmann and 420 A. M3 was that most of all recorded great level of PP2C transcript abundance. According with the literature, the enhanced activity of VvPP2C genes during drought stress suggests that it has a primary role in regulating ABA response (Chan, 2012). As reported by Rattanakon et al. (2016), PP2Cs increased transcript abundance to slowdown the activation of ABA signalling pathway that occurs from a rapid increase of the hormone itself.

Another key protein of ABA signal transduction is SNF1-related protein kinase 2 (SnRK2). It is strongly activated by ABA and has essential roles in the positive regulation of ABA signalling (Fujii et al., 2011; Mustilli et al., 2002; Yoshida et al., 2002). In the present work, only the genotype SO4 modulated positively the gene. Downstream targets of ABA signalling include several transcription factors, whose expression is activated in response to stress in ABA-dependent manner (Boneh et al., 2012). We analysed the expression of the ABRE-BINDING FACTOR2 (ABF 2), which regulate ABRE-dependent ABA signalling involved in drought stress tolerance. Among the M-rootstock, all three did not change ABF 2 modulation at mild level of stress. On the contrary, 140 Ru, 41 B, K 5BB and 420 A over-expressed the gene significantly.

II.2.5 Conclusion

M-rootstocks are promising genotypes, able to face with different abiotic stresses. Water scarcity represents one of the main constraints of the climate change events. Rootstocks do not react in the same way to drought and differences among genotypes have been observed. The differentiation of rootstocks to the reduced water availability was faster based on transcriptional response than based on physiological one. Mild level of stress was not able to discriminate genotypes on the basis of their phenotypic behaviour but at more severe water deficiency, genotypes react with different strategies (escaping, avoiding, maintaining growth under water stress through adaptative mechanisms; (*iv*) resisting through survival mechanisms). There are also plant that succumb to stress, showing photoinhibition and embolism. It is well-known that M4 is tolerant to drought (Corso et al., 2015; Meggio et al., 2014). At higher level of Vpd, it promptly closed the stomata, like other highly tolerant genotypes. On the contrary, from M3 and M1 genotypes a less marked response was observed, as

well as from the other less tolerant/susceptible genotypes. The closure of the stomata represents a way to preserve the water status under drought conditions in order to have resources in the future and to counteract the high levels of Vpd which otherwise would cause too large water leaks and risk of embolism. Based on the transcriptome evidence, even if M-rootstocks were clearly discriminated, only in M3 the higher expression of VvZEP determined a significant accumulation of ABA counteracted by the expression of VvPP2C which is a negative-regulator of ABA signalling transduction. The fact that M4 did not record statistically significant values of the variables mostly affecting the DFs (VvPP2C, VvSnRK2.6, VvABF2 involved in ABA mediated response and VvZEP ABA signal-transduction) lets to think that the closure of the stomata may be associated with a different catabolism of ABA.

II.2.6 Supplementary information

Supplementary Information S1. Eigenvalues of the discriminant functions of the phenotypic data

SWC	Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
20	1	11,411[a]	49,3	49,3	,959
	2	7,624[a]	33,0	82,3	,940
	3	1,601[a]	6,9	89,2	,785
	4	1,237[a]	5,3	94,6	,744
	5	1,126[a]	4,9	99,4	,728
	6	,127[a]	,6	100,0	,336
50	1	3,770[b]	35,7	35,7	,889
	2	2,439[b]	23,1	58,8	,842
	3	2,240[b]	21,2	80,0	,831
	4	1,833[b]	17,4	97,3	,804
	5	,177[b]	1,7	99,0	,388
	6	,103[b]	1,0	100,0	,306

Supplementary Information S2. Coefficients of the discriminant functions of the phenotypic data

SWC		Function					
		1	2	3	4	5	6
20	CiP	-1,530	-1,043	1,372	,476	-,343	,576
	EP	-1,228	-3,544	4,319	1,313	-3,043	-2,916
	GsP	2,235	4,508	-5,216	-,704	2,916	2,841
	PnP	-,382	,364	1,366	-,060	,452	-,080
	VdpP	1,603	1,816	-,649	-,855	,400	,567
	SWPPP	,940	-,551	,233	,087	,180	,059
50	CiP	,557	,493	,291	,476	-,878	,216
	EP	,178	,038	,646	,851	,448	-1,113
	GsP	-,211	,459	-,848	-,682	,882	1,264
	PnP	,259	-,709	,836	,384	-,074	,234
	VdpP	,725	,424	,700	-,379	,360	,173
	SWPPP	,852	-,199	-,511	,162	,051	-,061

Supplementary Information S3. Eigenvalues of the discriminant functions of the genetic data

SWC	Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
20,0	1	124,560[a]	63,3	63,3	,996
	2	32,864[a]	16,7	80,1	,985
	3	20,306[a]	10,3	90,4	,976
	4	10,373[a]	5,3	95,7	,955
	5	5,444[a]	2,8	98,4	,919
	6	3,091[a]	1,6	100,0	,869
50,0	1	509,998[b]	76,3	76,3	,999
	2	84,374[b]	12,6	88,9	,994
	3	40,630[b]	6,1	95,0	,988
	4	22,358[b]	3,3	98,3	,978
	5	10,107[b]	1,5	99,8	,954
	6	1,117[b]	,2	100,0	,726

Supplementary Information S4. Coefficients of the discriminant functions of the genetic data

SWC		Function					
		1	2	3	4	5	6
20,0	NCED1	,553	,524	1,036	,254	,644	,264
	NCED2	,479	-,622	,272	,636	-,966	,423
	ZEP	1,137	,765	-,647	-,045	,708	-,354
	PP2C4	,543	1,132	,432	,466	,039	-,528
	SnRK26	,062	,224	-,183	-,631	-,426	,688
	ABF2	-1,622	,044	-,590	,242	,109	,141
50,0	NCED1	,266	-,513	-,266	,480	-,298	,780
	NCED2	,814	-,879	-,016	,366	,433	-,419
	ZEP	-,503	1,086	,287	,644	-,477	-,016
	PP2C4	-1,715	-,155	,164	,254	,233	-,143
	SnRK26	1,327	,124	,678	-,557	,535	,295
	ABF2	,024	,926	-,627	,335	,356	,011

Chapter III - *In vitro* culture of grapevine rootstocks

By *in vitro* culture is meant a set of techniques that allow to regenerate and propagate plants starting from cells, tissues or organs in sterile conditions, on a culture medium and in controlled environment. They are methods widely used for large-scale plant multiplication. Micropropagated plants, complete of shoot and root systems, are uniform in development and homogeneous as genetic constitution, that is clones (Thorpe, 2007). *In vitro* culture finds its foundation in the principle of cell totipotency proposed by the Austrian plant physiologist, Gottlieb Haberlandt in 1902 . In vascular plants, the presence of two apical meristems (the shoot and root apical meristems), causes the embryo to evolve into an elongated bipolar structure. During the life cycle of the plant, meristems continuously originate new organs (stems, leaves, roots) that are added to those present in embryo. Because of this unlimited growth, plants are defined as organisms with continued embryogenesis or recurrent ontogenesis and any somatic cell can originate a new individual (Brink, 1962). *In vitro* the growth and multiplication of cells, isolated from specialized plant organs or tissues can be observed. Cells are able to de-differentiate and transform into amorphous cell masses, called calluses, which can be maintained indefinitely *in vitro* or induced to regenerate entire organs and/or organisms through the organogenesis process, behind appropriate stimuli. In order for this to happen, certain conditions must be met: (i) the identification of the best culture medium (in terms of nutrients, hormones and pH); (ii) the identification of the best culture environment (light, temperature, photoperiod); (iii) the maintenance of aseptic conditions.

III.1.1 Culture medium

Culture medium is the substrate from which the explant draws all that is necessary for its development. In 1962 Skoog, together with the student Murashige, established the macronutrients and micronutrients composition of the medium that bears their initials (MS) and that is one of the most employed for tissue culture (Murashige and Skoog, 1962). Many substrates have been formulated over time (i.e. MS (Murashige and Skoog, 1962), NN (Nitsch and Nitsch, 1969), WPM (McCown and Lloyd, 1981), Gamborg B5 (Gamborg et al., 1968)): all specific for diversified objectives (i.e. regeneration, transformation, micropropagation), different in composition but with the common aim of providing to the explant what is necessary for its growth, in an assimilable form. They generally consist of a mineral part, composed by macro and microelements, of an organic component (vitamins, amino acids and other nitrogen compounds) and sugar.

In tissue culture, phytohormones (or PGR - Plant Growth Regulator) are key substances because they regulate cell development and differentiation (Vondráková et al., 2016). The most used belong to the family of auxins and cytokinins. Their presence and concentration in the culture medium varies according to the research purposes and the species of interest. The first plant hormone, indol-acetic acetic acid (IAA), was identified by the physiologists Went and Thiman in 1937. IAA promotes cellular elongation through the so-called acid growth theory (Rayle and Cleland, 1970). Auxins not only promote cellular distension but also adventitious root formation. Auxin compounds mostly used in *in vitro* culture are α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), both synthetics, and 3-indolbutyric acid (IBA) which is natural (Velasquez et al., 2016).

In 1955 the Swedish botanist Folke K. Skoog isolated the first cytokinin (kinetin), demonstrating its function in inducing cell division in tobacco callus cultures. Cytokinins have a central role during the cell cycle and influence numerous developmental programs (Werner et al., 2001). In the tissue culture field, they stimulate the production of adventitious buds, the formation of somatic embryos, the development of axillary shoots (inhibiting apical dominance), while limiting the growth of the roots. The most used molecule is BAP (6-benzylaminopurine).

In 1957 Skoog and Miller were the first to study the mechanisms of chemical regulation that intervene in the development and formation of organs starting from tissues grown under aseptic conditions. High auxin/cytokinin ratios favour rhizogenesis while low ratios induce caulogenesis. When auxins and cytokinins are equivalents in the culture medium, callogenesis occurs in the absence of light.

Another class of hormones used in *in vitro* culture, although to a lesser extent not always necessary, is that of gibberellins. GA3 (gibberellic acid) is the most used gibberellin. It determines the extension of internodes, the development of meristems and buds and inhibits the formation of adventitious roots. Therefore, it is used above all in the shoot elongation phase.

III.1.2 Physical factors affecting *in vitro* culture: light, temperature, photoperiod

Once started a tissue culture procedure, explants are kept in growth chambers with constant light and temperature levels. As *in vivo*, even *in vitro* temperature controls plant growth, which is reflected on cell and organ development, enzymatic activity and therefore plant metabolism. The optimal range varies according to the species and is generally comprised between 18-25°C. The morphogenetic processes of cell cultures are influenced by light. The management of photoperiod and luminous intensity inside the growth chamber is fundamental to obtain a good proliferation and favour the

rhizogenesis process. Both vary on the basis of the species and different developmental stages. Generally, in the multiplication stage, the optimal luminous intensity is between 1000 and 3000 lux, while in the rooting phase it must be at least 3000 lux, allowing the shoots to gradually adapt to the external environment. The wavelengths in red and blue, corresponding to the absorption peaks of chlorophyll-a, and a photoperiod of 16 hours of light are preferred (Nguyen and Kozai, 1998).

III.1.3 Sterile environment for plant tissue culture

Operating in conditions of absolute sterility is one of the main characteristics of the *in vitro* culture process. To allow explant survival and proliferation, it is necessary to avoid microorganism development that would find optimal growth conditions in the culture medium. Possible sources of contamination can be: (i) the starting explants (parts of plant taken from *in vivo* mother plants); (ii) the work environment; (iii) the culture medium; (iv) the instruments used; (v) the operator. The explant sterility is obtained by immersing it in a solution of sodium hypochlorite (or ethanol, calcium hypochlorite or mercury chloride) while that of the culture medium, autoclaving it at 121°C for 20 minutes. The work environment is made aseptic by working under a laminar flow hood, where the flow guarantees the expulsion of any foreign microbe.

III.1.4 The phases of micropropagation

Micropropagation process is characterized by different phases:

1. **Mother plant selection and preparation** - The choice of the mother plant from which to take the explant is fundamental to ensure high-quality propagation cycle and plant production. The mother plant can be bred either in a controlled environment or in the field. It must comply with the varietal standard, be pathogen-free (in particular virus-free for fruit plants), vigorous and to have not suffered environmental stresses. The explant can be collected from plants in active development or in dormant stage, depending on the species, the phenological phase and culture method. It must also be reactive to the in vitro culture or, otherwise, be made such (for example, if the explant are dormant buds, these can be treated with the cold to remove dormancy).
2. **Organization of aseptic culture** – This phase is characterized by collection, sterilization and initiation of the explants to the *in vitro* culture operations. Sterile containers containing the culture medium rich in cytokinins are employed and different types of explants (i.e. buds, axillary buds, apical buds, microcuttings or vegetative apexes) can be used.

3. **Multiplication or proliferation** - In this phase, the shoots from the previous step are transferred to substrates which favour the rapid growth and activity of axillary and/or adventitious buds to sprout. These, in turn, can either be sent to the next rooting phase or be subjected to an elongation phase (in a medium rich in gibberellic acid) before the rooting induction or be multiplied again on a fresh substrate to obtain new ones (subculture). In this way, at the end of this phase, it is possible to produce a very large number of vegetative axes that grow up to the third or fourth subculture, then tends to stabilize.
4. **Rooting** - If the newly formed shoots are not used for a new cycle of multiplication and are well developed, they are transferred to a substrate rich in auxins to induce the formation of root primordia. When their root system is well-developed (of few centimetres in length), they are ready for the *ex vitro* acclimatization phase. There is also the possibility of simultaneously carrying out the rooting and acclimatization phase ("*ex-vitro*" rooting) by transferring the shoots in the soil directly or after an auxin solution immersion.
5. **Acclimatisation** - The plantlets with a root system able to ensure an *in vivo* rooting are transferred from the growth chamber to the greenhouse. In the beginning, high humidity levels (close to 100%), low light intensity and temperature similar to *in vitro* conditions are kept. After a few days, temperature and humidity are progressively brought to the greenhouse conditions. After 40-50 days, plants can be transferred in pots outside (in the shade), and then at full light.

III.1.5 The case of grapevine

The aptitude to be reproduced agamically makes grapevine suitable for *in vitro* culture techniques which permit to overcome the limits of traditional propagation systems, speeding up the multiplication of higher organisms identified through breeding programs and sanitary selections. They also solve the problem of recalcitrance to propagation, of recovering from virus-infected plants, of preserving germplasm and obtaining cellular material to be manipulated using genetic engineering techniques.

Grapevine *in vitro* culture dates back to 1944 when Morel selected a method to allow a dual culture of vine and downy mildew. This method was applied only around 1980 and calluses were replaced by micropropagated plants (Aldwinckle, 1980; Lee and Wicks, 1982; Klempka et al., 1984; Palys and Meredith, 1984; Barlass et al., 1986; Loubser and Meyer, 1990; Bavaresco and Walker, 1994; Forneck et al., 1996; Bouquet and Torregrosa, 2003). Over time, several explants and protocols were tested, but only in 1973 Hawker identified the long-term *in vitro* growth conditions. After the impulse

launched by Morel (1963), with the first propagation trial through meristem cultures, the multiplication techniques of economically important species, including grapevine, has assumed an increased interest (Murashige, 1974). Through micropropagation it is possible to obtain uniform, stable, distinguishable varieties with a high-agronomic value: all characteristics required by modern agriculture. These results can also be reached with traditional propagation methods (budding, layering, cuttings and grafting) which, however, need longer times and guarantee a lower production of plant material. The use of nodal buds for the multiplication of rootstocks is one of the most widely used tissue culture procedure. It involves the culture of a microcutting (or nodal segment with a bud) which, after sterilization, is started to *in vitro*, on a specific substrate, to sprout and root. By increasing the concentration of cytokinins in the medium it is possible to improve the efficiency proliferation of axillary buds by obtaining clusters of shoots without roots. Either the culture of nodal buds and the proliferation of axillary buds have the advantage of starting from a bud already differentiated *in vivo*, therefore only shoot lengthening and root differentiation are required for the complete development of the plantlets.

Grapevine is considered recalcitrant to tissue cultures, as well as mostly of woody perennials. The implementation of efficient protocols for different grapevine explants and cultivars is necessary, although several methods and techniques have been already tested (Martinelli & Gribaudo, 2009). Their optimisation has regarded the experiment, explants, variety and cultivar. This make the work time-consuming and technically demanding (Stander and Vivier, 2016). Moreover, consumer resistance against genetically modified tools, has determined setbacks for the development of grapevine tissue culture and transformation technologies (Terrier et *al.*, 2009, Lashbrooke et *al.*, 2013).

III.1.6 Why *in vitro* culture is advantageous?

The advantages deriving from *in vitro* culture techniques are manifolds. The main is the achievement, in small spaces and rapid time, of a large quantity of good quality plant material, genetically controlled (responding to the original varietal identity or "true to type"), pest-free and with a homogeneous agronomic behaviour. Moreover, the disease recovery, the overcoming of the recalcitrance of certain species to the ordinary propagation and the conservation of germplasm *ex situ* (the creation of a bank of genes) are further benefits given by tissue culture. Furthermore, in the field of genetic improvement, *in vitro* culture is useful for the manipulation of cellular material with the New Breeding Technologies (NBTs) (see chapter IV) and for the induction of somaclonal variability in somatic embryos. Somaclones can have a genetic basis deriving from various causes: karyotype

changes, chromosomal rearrangements, transposable elements, gene amplification/deletion, somatic crossing-over and chromatid exchanges. It has been observed that in the presence of high levels of PGR in the culture medium, the frequency of somaclonal mutations increases (Krishna et al., 2016). Obviously, if the final purpose is the reproduction of rare clones or genotypes resistant to biotic and abiotic agents or species selected by an elite, the somaclonal variation represents a strong limit; on the contrary, it is interesting for the genetic improvement of species.

III.4 Attitude of the new M4 grapevine rootstock to *in vitro* shooting, rooting and callogenesis from leaf and bud explants.

Leila Caramanico[1], Osvaldo Failla[1], Lucio Brancadoro[1], Gabriella De Lorenzis[1]

[1] Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio e Agroenergia, via Celoria 2, 20133, Milano, Italy

Abstract

The new grapevine rootstock M4 is resistant to drought stress, putting in place some physiological adaptations. With a view to a widespread use of the M4 rootstock in modern viticulture for tackling the climatic change scenario, the objective of this study is to set up an efficient high-throughput protocol for M4 *in vitro* propagation and callogenesis. The M4 attitude to shooting, root development and callus induction from leaf explant has been evaluated on Murashige and Skoog (MS) media (Murashige and Skoog 1962) added to a different type and concentration of auxins and citokynins, and it has been compared to that of other rootstocks largely used in viticulture (K 5BB, 1103 P, 101-14 and 3309C). In terms of microcutting development, M4 showed the best performance in the passage from the rooting medium containing 6-benzyl amino purine (BAP) and 3-indoleacetic acid (IBA) to the propagation substrate without hormones. In these media it also developed roots in equilibrium with the foliage. The higher concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl amino purine (BAP) in the two callogenesis media tested, resulted more favourable for M4 and, in general, for almost all genotypes. These results confirmed the decisive role of Plant Growth Regulators (PGRs) and of their concentrations in the *in vitro* culture techniques, strictly linked to the variety under study. The importance of the setting-up of a valid protocol of micropropagation lies in producing plant material, also grafted, in a short period of time and the obtainment of manipulable cellular material, in form of calli, is indispensable for the genetic improvement of plants through the New Breeding Techniques (NBTs).

Key words: *Vitis*, micropropagation, plant growth regulators, Murashige and Skoog.

III.4.1 Introduction

Eurasian viticulture is characterised by the grafting of *Vitis* vinifera species onto American *Vitis* rootstocks, which confer resistance to phyllossera (*Daktulosphaira vitifoliae* Fitch, 1856) pest and

affect yield and vigour of the scion. In light of ongoing climate change events, rootstocks are also assuming an increasingly decisive role to cope with abiotic stresses and the selection of ones better able to overcome to drought conditions, soil salinity and iron deficiency is becoming a crucial factor for a sustainable viticulture. At this purpose, since the 80s, the Department of Agricultural and Environmental Sciences of the University of Milan (Italy) is pursuing an intense breeding activity for developing grapevine rootstocks (Bianchi et al., 2018; Migliaro et al., 2019). Novel genotypes have been established, entering in the National Registry of Grape Varieties from the Italian Ministry of Agriculture since 2014, currently they are also employed in experimental trials in France (INRA - Institut National de la Recherche Agronomique, https://www6.bordeaux-aquitaine.inra.fr/egfv/Activites-scientifiques/Projets-en-cours) and Spain (UNIR - International University of La Rioja). One of these, named M4, was selected for its resistance to drought, maintaining a higher transpiration rate also upon water deficit condition (Meggio et al. 2014). This phenomenon is connected to a different abscisic acid's regulation (ABA catabolism), reflecting the transcriptome analyses driven by Corso et al. (2015). Moreover, upon drought conditions, M4 roots accumulate higher level of resveratrol related to a greater expression of VvSTSs genes (stilbenes synthase genes). In view of a widespread use of M4 rootstock and considering the constant great demand for vine planting materials, the obtainment of genetically homogeneous populations (i.e. clones) from elite individuals using *in vitro* culture method, or micropropagation, represents a rapid alternative to conventional plant multiplication (Diab et al., 2011). In fact it offers many advantages over conventional vegetative propagation, particularly: 1) the relatively short time and space required to produce a large number of plants starting from single explants; 2) the possibility to propagate species throughout the year, disregarding the seasonal cycle; 3) tissue cultured plants are generally free from fungal and bacterial diseases; 4) the multiplication rate is greatly increased (Debnath et al., 2006).

The main objectives of this study were: 1) to set up an efficient high-throughput protocol for M4 *in vitro* propagation and for its *in vitro* performance characterization, making a comparison between M4 and other rootstocks largely used in viticulture (K 5BB, 1103 P, 101-14 and 3309C); 2) the obtainment of pro-embryogenic and embryogenic calli from leaf tissues and bud explants.

III.4.2 Materials and methods

Plant material and explant preparation

The genotypes M4, K 5BB, 1103 P, 101-14 and 3309C were issued from the vineyard germplasm located at the Regional Research Station of Riccagioia, in Lombardy region (Italy). From field grown

rootstock plants, branches pruned in January were placed in pots containing water, in controlled environment conditions at 23 ±1°C and 16-8 hr of light and dark until shoot developed. The light source was provided by Valoya NS1 LED lights (Valoya Oy, Helsinki, Finland) which generate a mixture of ultraviolet (1%), blue (20%), green (39%), red (35%) and far-red (5%). Photosynthetic Photon Flux Density (PPFD) was 110 µmol m^{-2} s^{-1}. After approximately 30 days, shoots elongated (with about 10 nodes) and small leaves expanded (of 2 cm of diameter) were started to *in vitro* culture. They were washed thoroughly under tap water for 10 minutes, then surface-sterilized for 20 minutes in sodium hypochlorite (25%) solution and a few drops of Tween 20 detergent and rinsed three times in sterile distilled water. Working under a fume hood, shoots were cut into one-node segments (or microcuttings) while their leaves, deprived of petioles, were excised and dissected removing the edge of the lamina. Microcuttings and leaf discs were cultured in different media for micropropagation and callogenesis induction, respectively. Subsequently, M4 and 1103 P seedlings cultivated *in vitro* were part addressed to an acclimatization phase and part were chosen to provide axillary buds for pro-embryogenic and embryogenic development and, therefore, for the regeneration of seedlings from them.

Culture media and cultural conditions

All basal media for micropropagation and callus induction from leaves were composed of Murashige and Skoog (MS) salts and vitamins supplemented with 30 g/l sucrose and 8 g/l agar, adjusted to pH 5.8 and autoclaved at 121°C for 20 minutes. They differed mainly for hormone content, in order to choose the optimum concentration for each step of plant tissue culture cycle: shooting, rooting and callogenesis. Shooting and rooting media were plated in sterile boxes (150mm x 105mm x 85mm), while media for callus induction were plated in 90 mm petri dishes.

Shoot elongation and root formation

One-bud microcuttings of each genotype, obtained from field after surface sterilization, were placed for 10 days in two rooting media, R1 (with 0.1 mg/l IBA and 0.5 mg/l BAP) and R2 (1 mg/l IAA), at 23 ±1°C, 16-8 hr of photoperiod under Valoya AP67 LED lights (Valoya Oy, Helsinki, Finland) with ultraviolet (0%), blue (14%), green (16%), red (53%) and far-red (17%). The PPDF was of 110 µmol m^{-2} s^{-1}. After 10 days they were transferred in new boxes for adventitious shoot formation, testing two MS growth media: M1, hormone free, and M2 containing the auxin BAP (1 mg/l). They were incubated at 23 ±1°C, 16-8 hr of photoperiod under Valoya AP67 LED lights (Valoya Oy, Helsinki, Finland) with a PPDF of 110 µmol m^{-2} s^{-1} until new whole plantlets grew. The comparison

among rootstock attitudes to be *in vitro* propagated were evaluated as shoot growth, expressed as mean shoot length (cm), scored every 10 days for 8 weeks, and root development, using a scale from 1 to 6 (1= root not developed/revert to callus; 2= 1-3 cm; 3= 3-5 cm; 4 = 5-7 cm; 5= 7-9 cm; 6 = ≥ 9 cm) assessed when the samples were completely developed. Statistical analysis of both data was made considering the values of the last relief.

*Acclimatization of in vitro plantl*ets

Once plantlets achieved a well-development in both propagation media (M1 and M2), part of them were addressed to an *ex vitro* acclimatization phase. They were washed under tap water, in order to remove any agar residues from their roots and then they were put in vessels filled with a peat-based substrate (Vigor Plant). Initially, pots were placed in a mini-chamber in controlled environment, at saturate humidity, 23±1°C and LED lights (NS1, Valoya Oy, Helsinki, Finland) with PPDF 110 µmol m^{-2} s^{-1}. After a couple of days, gas exchanges were gradually allowed. The mini-chamber was moved to the experimental greenhouse of the University of Milan, characterized by a temperature range from 21°C to 28°C and a photoperiod of 16-8 hr of light and dark. The plants more adapted to survive *ex vitro* were then transferred into larger vessels suitable for development and were left to grow in the green-house before to be transferred in the field.

Callus induction from leaf explant

Callogenic competence of leaves was assessed on two ½ MS media (C1 and C2), both containing 1 mg/l BAP and different 2,4-D content (0.1 mg/l in C1 and 1 mg/l in C2), 7 g/l agar and 30 g/l sucrose (pH 5.8). Leaf blades were placed with their lower surface in contact with the medium, in petri dishes. Dishes were sealed with parafilm and kept at 23 ±1°C in the darkness until callus appeared. The capacity of leaves to produce calli pro-embryogenic were evaluated after 40 days, doing two sub-cultures, as responsive explant: the percentage (%) of the explant surface transformed in cream-colored and compact callus (from 0 % explant not turned into callus/necrotic to 100% explant entirely turned into callus).

Pro-embryogenic callus induction from axillary buds

Micropropagated plants of M4 and 1103 P were used to collect axillary buds, to obtain pro-embryogenic and embryogenic callus. Buds were removed by using a stereoscope (Wild Heerbrugg M420, 1,25x). They were either collected with their external protections (Close Bud (CB)) and without any protection (Open Bud (OB) represented by the only apical meristem). Pro-embryogenic

competence was tested on two media, BI and NB. The first one contained ½ MS basal salts, 1 ml/l B5 vitamins (1M), 5 ml/l Fe/EDTA (200X), 5 ml/l BAP (1 mM), 5 ml/l 2,4-D (1 mM), 5 ml/l NAA (1mM), 15 g/l sucrose, 1 g/l myo-inositol. The second one was composed by NN salts and vitamins (Nitsch and Nitsch, 1969), 1 ml/l BAP (1 mM) and 1 ml/l 2,4-D (1 mM). For both the pH was adjusted to 5.8, they were solidified by 8 g/l agar and they autoclaved at 121°C for 20 minutes. In each plate about 25 buds were inserted. For each genotype, two replicas of each medium and type of explant combinations (BI_OB; BI_CB; NB_OB; NB_CB) were provided. The plates were put in the dark, and incubated at 24 ±1°C. Once calluses developed, firstly they were sub-cultured twice in the same medium, and then they were transferred to the embryogenic medium. The ability of buds to produce callus was studied over a period of 60 days, divided in 4 main times of 15 days (T1, T2 , T3 and T4). The parameters evaluated were: i) the percentage (%) of explant surface turned into callus (1 = 0%; 2 = 25%; 3 = 50%; 4 = 75% and 5 = 100%; ii) the colour (1 = white; 2 = cream; 3 = brown/necrotic); iii) the consistency (1 = friable; 2 =compact; 3 = watery). The optimal were buds entirely transformed into cream-colored and compact callus.

*Embryogenic callus induction and plant*lets *regeneration*

Pro-embryogenic calluses developed on BI and NB media were transferred onto a somatic embryogenic substrate, named GISCA consisting of modified GS1CA (Franks et al., 1998). It was composed of ½ MS basal salts; 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 60 g/l sucrose; 10 ml/l NOA (1M); 1 ml/l BAP (1M); 20 ml/l IAA (1M) and 2.5 g/l of activate charcoal. The pH was adjusted up to 6.2 and the medium was sterilized at 121°C for 21 minutes, after the addition of agar (8 g/l). Once embryogenic calluses developed, they were transferred on a regeneration medium in order to test their aptitude to caulogenesis, rhizogenesis and organogenesis. Initially they were put on RM (Regeneration Medium) substrate, then on SH (Shooting) medium. Both solid media were composed of ½ MS basal salts, 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 30 g/l sucrose and the pH was adjusted to 5.7. The only difference between RM and SH was the quantity of the hormone BAP used, equal to 2.2 ml/l and 10 ml/l of BAP (1 mM), respectively.

Data analysis

The experiment was conducted using an average of 50 microcuttings (10 in each veg box) and 50 leaf (10 in each petri dishes) and bud explants (25 for each petri dishes) (either open or close) for each genotype/medium combination. For the evaluation of shoot elongation and root formation, as well as of callus induction from leaf explants, variance analysis of data was performed using ANOVA

program for statistical analysis. For post-hoc analysis, Duncan test was performed (IBM SPSS Statistics 24). The significant differences between means was determined at $p \leq 0.05$.

Data concerning the quality of pro-embryogenic calluses derived from bud explants, were analysed using R software (R Development Core Team, 2010). The analysis carried out concerned a non-parametric Kruskal-Wallis test. Different interactions were evaluated: i) the behaviour of each genotypes, M4 and 1103 P, in the two different media (BI and NB); ii) the total effect of the two culture media, BI and NB on the pool of samples; iii) the interaction between the type of bud (open or close) and culture media; iv) the trend of the parameters in different times. The significance of the averages was evaluated on the basis of a chi-squared test and at p-value≤ 0.05. Data were displayed in boxplots type graphs.

III.4.3 Results

Micropropagation. The *in vitro* multiplication protocol applied to the five grapevine rootstock genotypes showed different levels of success in the present study. The Figure III.2 displayed that the genotype (curve) and the medium (pendency of the curve) had a significant influence on the *in vitro* plantlets' development, either singly ($p \leq 0.005$ and $p \leq 0.000$, respectively) and interacting themselves, highlighted by the interpolation of the curves (supplementary information SI1). Among the four combinations (R1M1, R1M2, R2M1, R2M2) of rooting and multiplication media, R1M2 resulted significantly less efficient for the propagation than the other three and, among genotypes, K5BB recorded the minor development efficiency statistically significant (≈ 2.5 cm of elongation) (supplementary information SI1).

The switch from R1 (MS, IBA and BAP) to M1 (MS, free hormone) was the most efficient statistically especially for M4 microcutting elongation (≈ 3.3 cm), showing the best development capacity statistically significant, followed by 101-14, 3309C and 1103 P. On the opposite, K5BB plantlet growth was the lowest (≈ 2.2 cm) (supplementary information SI2). In R1M2 (MS + BAP) combination the genotype M4 was also performing, even if in a lesser degree than in the medium without plant growth regulators, M1 (highlighted by the decreasing pendency of the curve). This trend was comparable to that of 101-14, 3309C and K5BB samples among which there were not differences statistically significant. On the contrary, 1103 P shoot development increased in the multiplication medium M2 (MS+BAP), with respect to M1 (of about 0.5 cm), getting to the greatest result (supplementary information SI2). Basically, from the same rooting medium containing either auxin (IBA) and cytokinin (BAP) (R1 medium), M4, 101-14 and 3309C took advantage from the switch to the multiplication medium without hormone (M1), differently 1103 P and K5BB (M2).

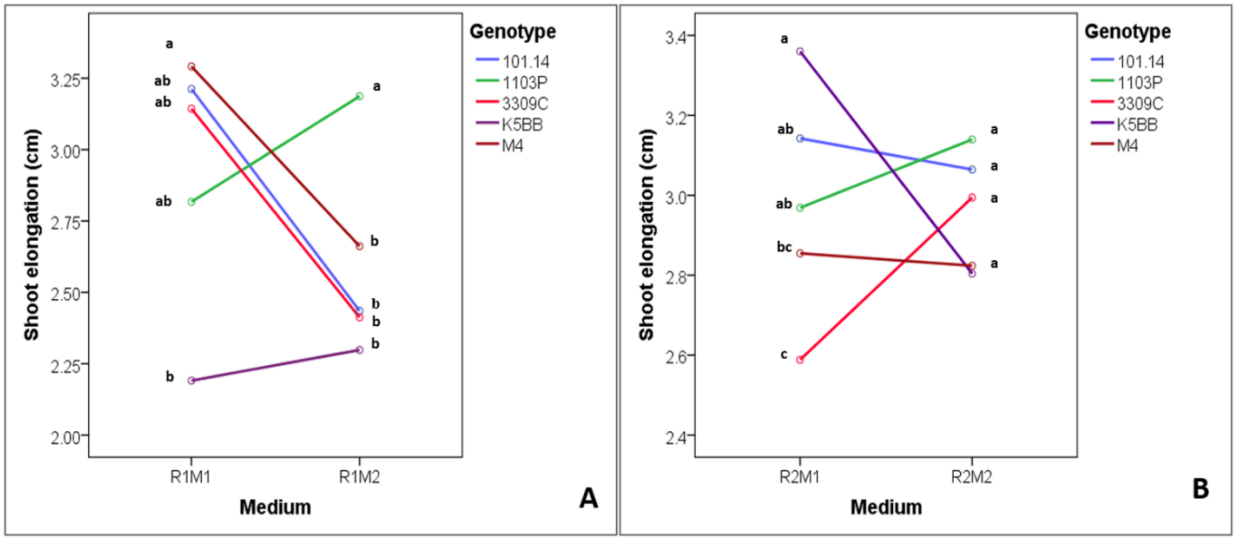


Figure III.2 Comparison of grapevine rootstock performances in term of shoot elongation, expressed as mean of shoot length (cm). A) Shoot elongation in media R1 combined with M1 or M2, at 23 ±1°C and 16-8 hr of photoperiod. B) Shoot elongation in media R2 combined with M1 or M2, at 23 ±1°C and 16-8 hr of photoperiod. Data were collected every 10 days for 8 weeks and the statistical analysis regarded the value of the last relief. Genotypes are represented by the lines; the medium are indicated by the pendency of the lines and their interaction is the interpolation among lines. Differences have been considered significant for P = 5%.

The Figure III.2B also explained that the interaction between media (R2 and M1 or M2) and genotype was also noteworthy (notable by the interpolation of the curves). The switch from R1 to M1 was disadvantageous for 3309C shoot elongation statistically significantly (≈ 2.6 cm), M4, 1103 P and 101-14 samples reacted with an intermediate growth (comprises between 2.8 and 3.1 cm) while K5BB reached the highest value (≈ 3.4 cm) (supplementary information SI3). In the M2 no significative differences among the genotypes under study were recorded (range from 2.8 to 3.1 cm).

The Figure III.3 is related to the root development of the five grapevine rootstocks studied, expressed in a scale from 1 to 6 referred to their length (cm). These data referred only to the combination R1M1 and R1M2 media because in R2M1 and R2M2 samples developed calli, inhibiting the growth of radicle (Figure III.1A).

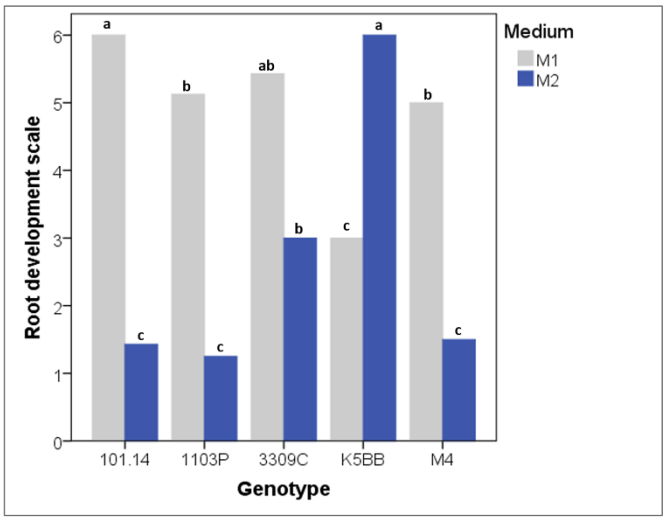


Figure III.3 Comparison of grapevine rootstock performances in term of root development, in the combination of media R1 (MS + 0.1 mg/l IBA and 0.5 mg/l BAP) with M1 (hormone free) and M2 (1 mg/l BAP). They were kept in box, at 23 ± 1°C and 16-8 hr of photoperiod under cool white fluorescent tube. The evaluation was made using a scale from 1 to 6, indicating differences in root length: 1=not developed/revert to callus; 2 =1-3 cm; 3= 3-5 cm; 4= 5-7 cm; 5= 7-9 cm; 6=≥9 cm. Differences have been considered significant for P = 5%.

There were evident differences among genotypes and media tested and their interaction was statistically significant. 3309C and K5BB roots were longer than the other genotypes that did not register differences (supplementary information SI4). The medium without hormone (M1) promoted rooting better than M2 (with cytokinin) and in the case of 101-14 and 3309C (≥10 cm) also root lignification (Figure III.1B) (supplementary information SI5). M4 formed long (7 cm) and not excessively woody roots, in balance with its foliar apparatus. Roots of K 5BB resulted significantly shorter (≈3.5 cm) than the other samples, on the contrary in the M2 this one recorded the highest value forming long taproots (≈10 cm). The M2 root medium was not appropriate for the rooting of 101-14, 1103 P and M4 producing radicles (≈ 3cm) that were about to turn into callus (Figure III**.**1C), instead, in M2, K 5BB formed roots more developed than the other genotypes (≈10 cm), while 3309C ones were on average developed (supplementary information SI5).

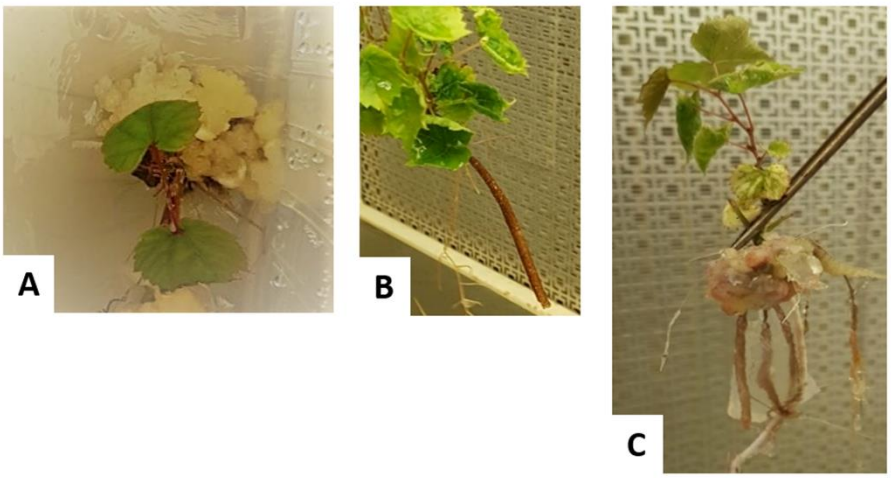


Figure III.2 Micropropagated plants of grapevine rootstocks in box containing MS basal media, after 8 weeks at 23±1°C and 16-8 hr of photoperiod under cool white fluorescent tube. **A)** Plantlet of 'K 5BB' in the combination media R2 (MS + 1 mg/l IAA) M2 (MS + 1 mg/l BAP) with callus at its base. **B)** Root lignified of '3309C' switched from R1 (MS IBA and BAP) in M2 (MS + 1 mg/l BAP). **C)** Roots of 'M4' in M2 (MS + 1 mg/l BAP) with callus at their apex.

Callus induction from leaf explants. The attitude to produce calli from leaf explants per each rootstock differed among the genotypes and the media and there was a significant effect of their interaction (supplementary information SI6). The genotype 1103 P showed the best attitude statistically significant to callogenesis, followed by K 5BB. 3309C and M4 callogenesis was intermediate significant statistically. On the opposite 101-14 was the lowest responsive. The higher quantity of the hormone 2,4-D in the C2 medium better promoted the dedifferentiation of leaf tissues forming callus masses (Figure III.4) (supplementary information SI7). In this medium 1103 P and 3309C samples resulted particularly predisposed to callogenesis, with quite all the leaf tissue (> 80%) reverting to an earlier developmental stage after 40 days, appearing as cream-colored and compact callus. M4 presented an intermediate behaviour between K 5BB and 101-14 with the 50% of the explant tissues regressed to callus. In the medium C1, after 40 days, calluses were not completely developed, especially 101-14explants showing a value of 20% of cell masses dedifferentiation. In C1 M4 recorded again an intermediate attitude to develop callus (≈ 50%) but still minor than in C2 (Figure III.4).

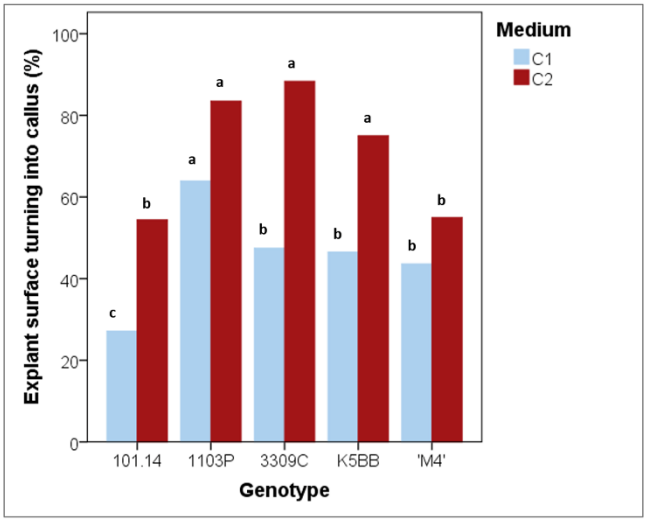


Figure III.4 Callogenic performance of five grapevine rootstocks, compared in two media that differed for hormone concentration, C1 (1/2 MS + 0.1 mg/l 2,4-D and 1 mg/l BAP) and C2 (1/2 MS + 1 mg/l 2,4-D and 1 mg/l BAP). Explants turning into callus were evaluated as the percentage of the leaf explant surface transformed in dedifferentiated cells (from 0 to 100%), after 40 days of culturing at 23±1°C in the darkness. Differences have been considered significant for P = 5%.

Pro-embryogenesis induction from bud explants. The ability to produce pro-embryogenic callus from bud explants was firstly analysed by a visual analysis. This one showed that BI medium, with compared to NB, was more efficient to generate high-quality calluses, in terms of cell surface turned into callus, colour (cream was optimal) and consistency (compact was optimal) (Figure III.5). Furthermore, on BI, calluses grew faster, developing a month earlier than those grown on NB and maintaining optimal characteristics over time (Figure III.5A). In contrast, calluses developed on NB medium showed signs of necrosis (Figure III.5 B).

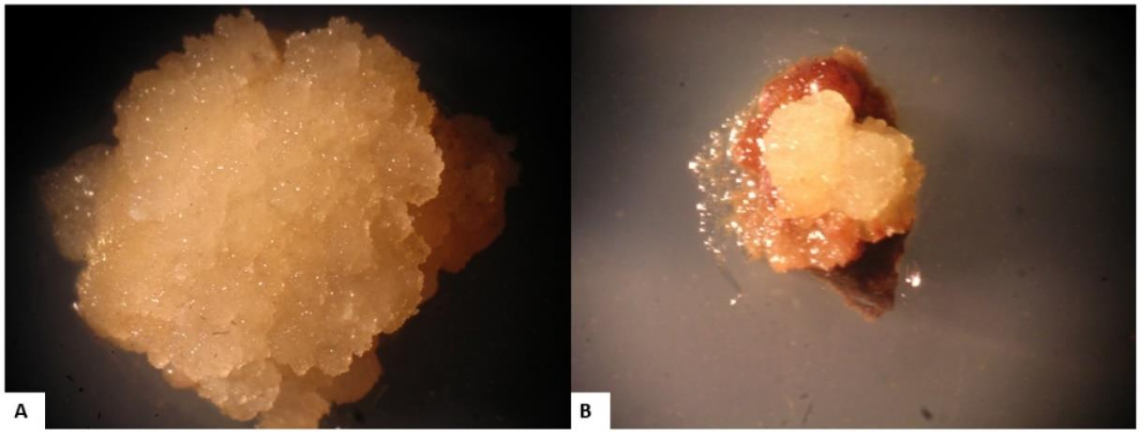


Figure III.5 Calluses of M4 genotype in petri dishes at 24±1°C in the darkness after 3 months from the first. **A)** M4 callus on BI medium (½ MS basal salts, 1 ml/l B5 vitamins. 5 ml/l Fe/EDTA, 5 ml/l BAP (1 mM), 5 ml/l 2,4-D (1 mM), 5 ml/l NAA (1mM), 15 g/l sucrose, 1 g/l myo-inositol; pH 5.8) which is totally developed, cream coloured and compact. **B)** M4 callus in NB (NN salts and vitamins, 1 ml/l BAP (1 mM) and 1 ml/l 2,4-D (1 mM); pH 5.8) medium which is necrotic and smaller in size.

Once pro-embryogenic, cream-colored and compact calluses developed, they were moved to an embryogenic medium, called GISCA. Calluses from BI transferred to GISCA developed, after about 2 months, embryogenic masses (Figure III.6), while calluses from NB to GISCA did not survive.

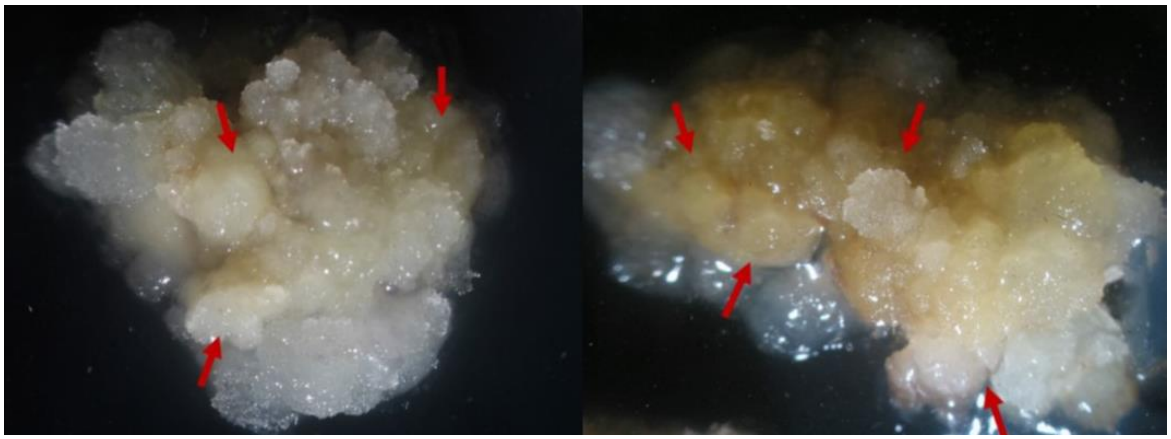


Figure III.6 M4 calluses on GISCA embryogenic medium (½ MS basal salts; 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 60 g/l sucrose; 10 ml/l NOA (1M); 1 ml/l BAP (1M); 20 ml/l IAA (1M); 2.5 g/l of activate charcoal; pH 6.2) after 5 months, at 24±1°C in the darkness. The red arrows indicate the embryogenic masses in development.

Genotype x medium interaction. Regarding M4 genotype, buds grown on BI medium (Figure III.7A) produced larger calluses than those on NB (with a diameter equal to 10 mm in BI against 5 mm in NB). In terms of colour (Figure III.8A) and consistency (Figure III.9A), in both BI and NB, the average of these values was close to 2 (considered optimal). Regarding 1103 P genotype, the interaction between genotype and medium was not statistically significant in terms of % of explant surface turned into callus (Figure III.7B) and consistency (Figure III.9B). As for the colour (Figure III.8B), in BI the value was significantly lower with compared to that recorded in NB medium. For BI medium, in fact, it was closer to 3.0 (tending to brown), while for NB it was closer to 2.

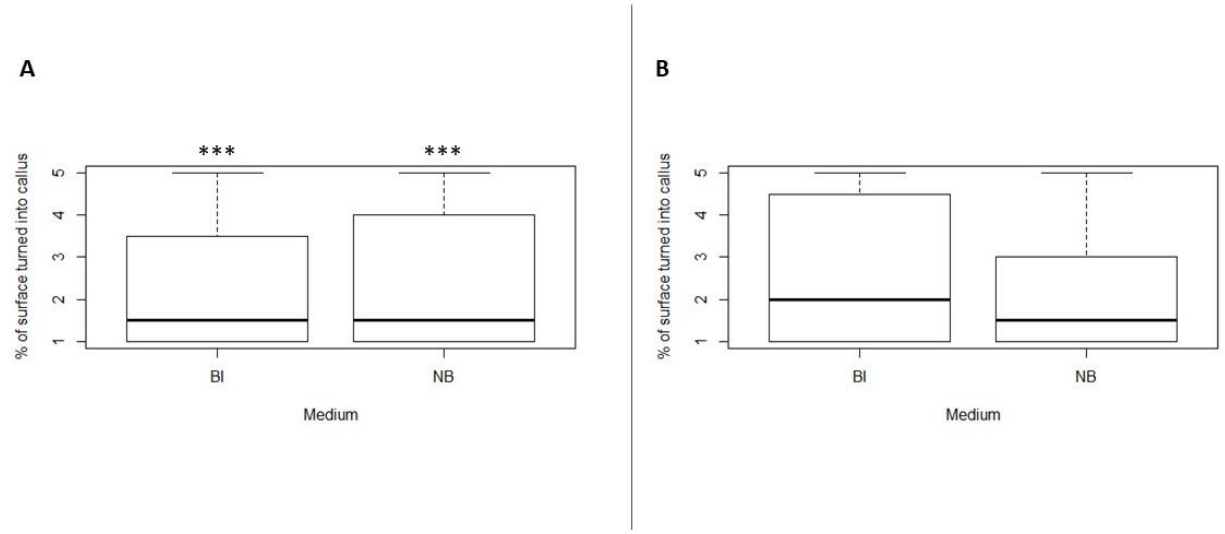


Figure III.7 Boxplot related to the parameter % of surface turned into callus in the comparison between M4 and 1103 P. The ordinate shows the scale % of surface turned into callus: 1 = 0%, 2 = 25%, 3 = 50%, 4 = 75%, 5 = 100%. The abscissa refers to culture medium tested, BI and NB. For each genotype, M4 (A) and 1103 P (B), the parameter was evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

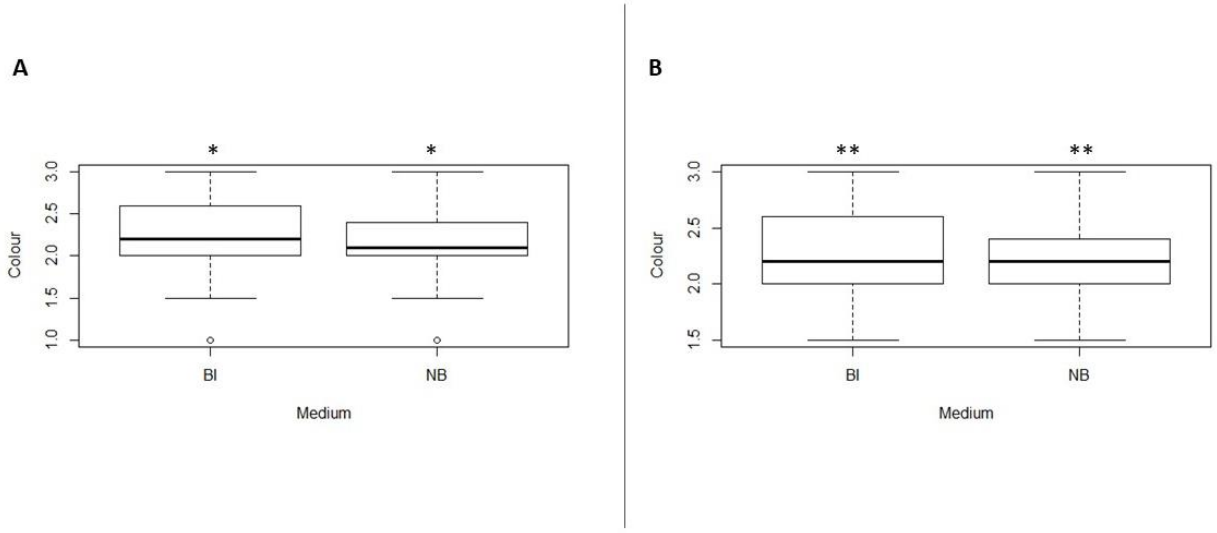


Figure III.8 Boxplots related to the parameter colour of callous in the comparison between M4 and 1103 P. The ordinate shows the scale of evaluation: 1=white, 2=cream, 3=brown (necrosis). The abscissa refers to culture medium tested, BI and NB. For each genotype, M4 (A) and 1103 P (B), the parameter was evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

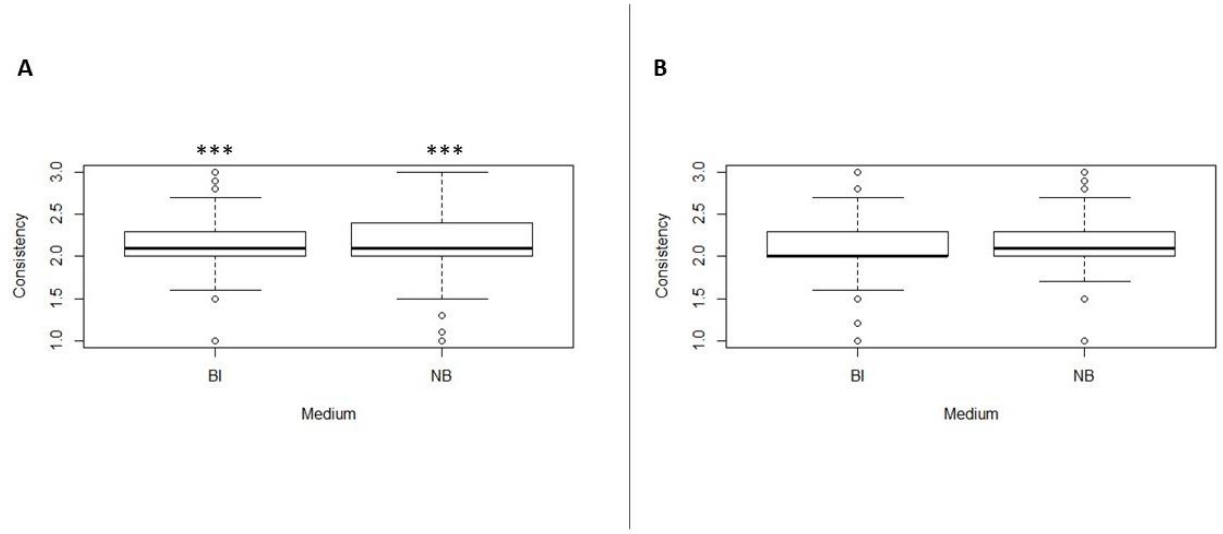


Figure III.9 Boxplots related to the parameter consistency of callous in the comparison between M4 and 1103 P. The ordinate shows the scale of evaluation: 1=friable, 2=compact, 3=watery. The abscissa refers to the culture medium tested, BI and NB. For each genotype, M4 (A) and 1103 P (B), the parameter was evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

Comparison between BI vs NB medium. Without considering the genotype effect, the comparison between BI and NB highlighted that the first one was the most efficient in terms of callus formation (Figure III.10). Bud explants grown on BI developed larger calluses masses, with an average of the % of the explant surface turned into callus equal to 25%. On the contrary, on NB the value ranged from 10 to 15% in average (Figure III.10). No statistically significant differences were recorded by the two media regarding the colour (Figure III.11) and consistency (Figure III.12) values.

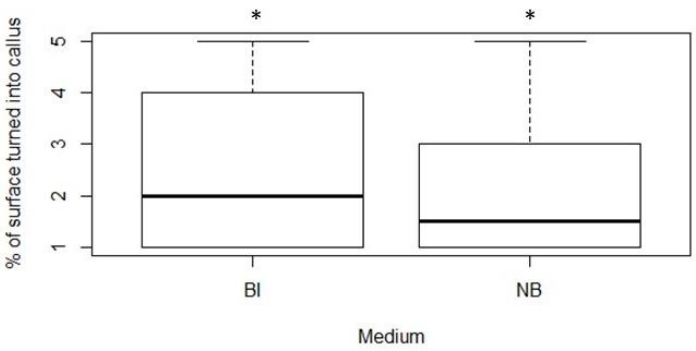


Figure III.10 Boxplots related to the parameter % of surface turned into callus in the comparison between BI and NB. The ordinate shows the scale of evaluation: 1 = 0%, 2 = 25%, 3 = 50%, 4 = 75%, 5 = 100%. The abscissa refers to the culture medium tested, BI and NB. The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

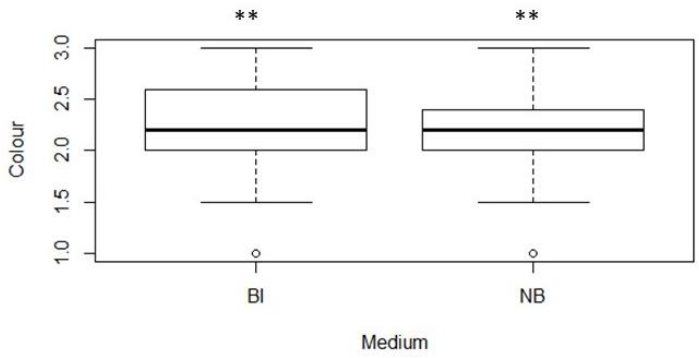


Figure III.11 Boxplots related to the parameter colour of callus in the comparison between BI and NB. The ordinate shows the scale of evaluation: 1=white, 2=cream, 3=brown (necrosis). The abscissa refers to the culture medium tested, BI and NB. The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

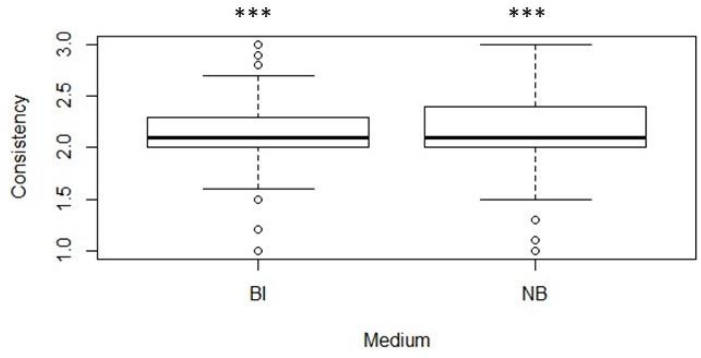


Figure III.12 Boxplots related to the parameter consistency of callus in the comparison between BI and NB. The ordinate shows the scale of evaluation: 1=friable, 2=compact, 3=watery. The abscissa refers to the culture medium tested, BI and NB. The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

Type of explant x medium. The interaction between the type of explant (open or close bud) and the medium revealed significantly differences in terms of % of explant surface turned into callus (Figure III.13). Close buds were more performing in BI while Open buds (O) in NB, reaching values close to 25%. On the contrary, their counterparties did not develop almost at all (about 0%) (Figure III.13). Smaller differences were recorded for the colour (Figure III.14) parameter, tending to 2 (optimal colour) for Close buds (C) and to 3 in the case of open buds (O). The same result was obtained in terms of consistency (Figure III.15).

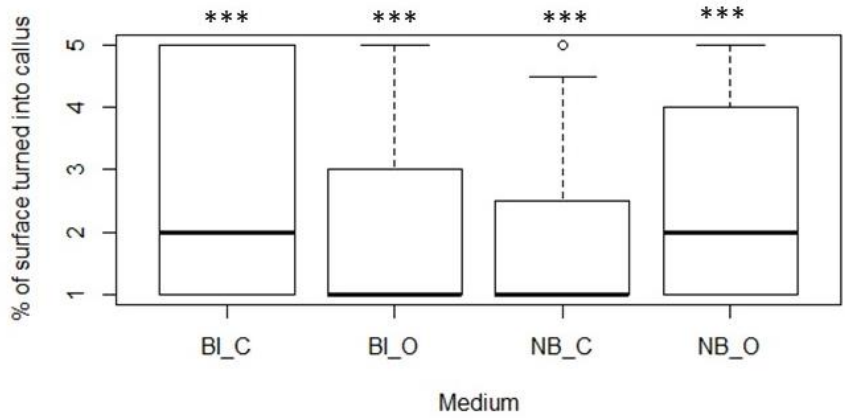


Figure III.13 Boxplots related to the parameter % of surface turned into callus in the comparison between Open bud (O) and Close bud (C) in the culture medium BI and NB. The ordinate shows the scale of evaluation: 1 = 0%, 2 = 25%, 3 = 50%, 4 = 75%, 5 = 100%. The abscissa refers to the 4 combinations evaluated (BI_C; BI_O; NB_C; NB_O). The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

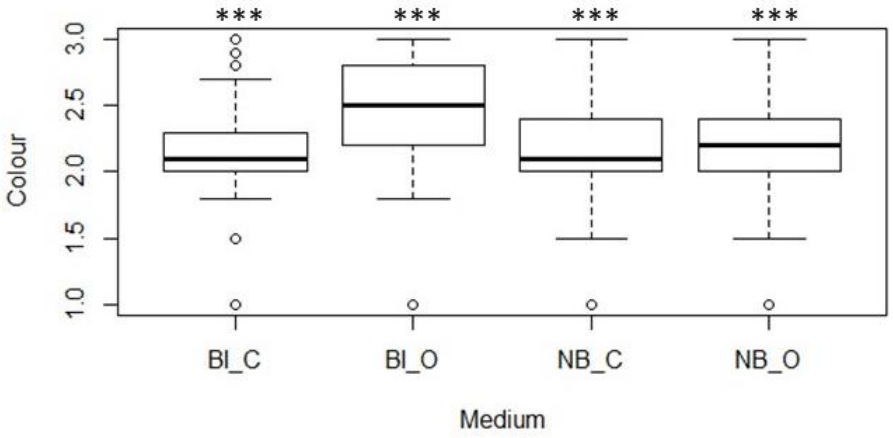


Figure III.14 . Boxplots related to the parameter colour of callus in the comparison between Open bud (O) and Close bud (C) in the culture medium BI and NB. The ordinate shows the scale of evaluation: 1=white, 2=cream, 3=brown (necrosis). The abscissa refers to the 4 combinations evaluated (BI_C; BI_O; NB_C; NB_O). The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

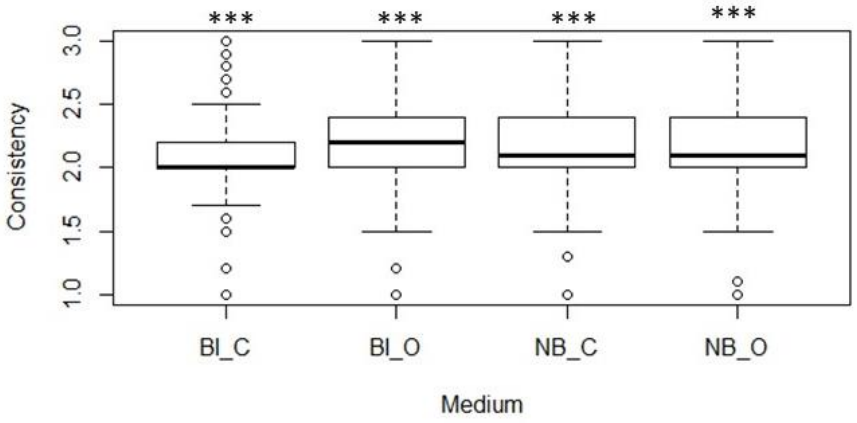


Figure III.15 Boxplots related to the parameter consistency of callus in the comparison between Open bud (O) and Close bud (C) in the culture medium BI and NB. The ordinate shows the scale of evaluation: 1=friable, 2=compact, 3=watery. The abscissa refers to the 4 combinations evaluated (BI_C; BI_O; NB_C; NB_O). The comparison was made involving all the samples, evaluated in 4 timing after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

Analysis of percentage (%) of surface turned into callus, colour and consistency over time. The trend over time of the growth of calluses (Figure III.16), and their quality, in terms of colour (Figure III.17) and consistency (Figure III.18) was evaluated every 10 days for two months. The highest statistically significant development rate was recorded between T3 and T4, in which the % of surface turned into callus increased from 25% to about 75% (respectively the 2.0 and 4.0 values of the scale used) (Figure III.16). No statistically significant differences resulted from the evaluation of colour (Figure III.17) and consistency (Figure III.18).

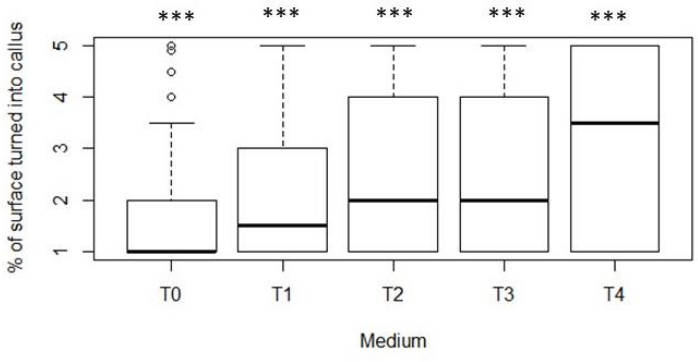


Figure III.16 Boxplots related to the parameter % of surface turned into callus over the time. The ordinate shows the scale of evaluation: 1 = 0%, 2 = 25%, 3 = 50%, 4 = 75%, 5 = 100%. The abscissa refers to the timing of evaluation, after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The comparison was made involving all the samples. The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

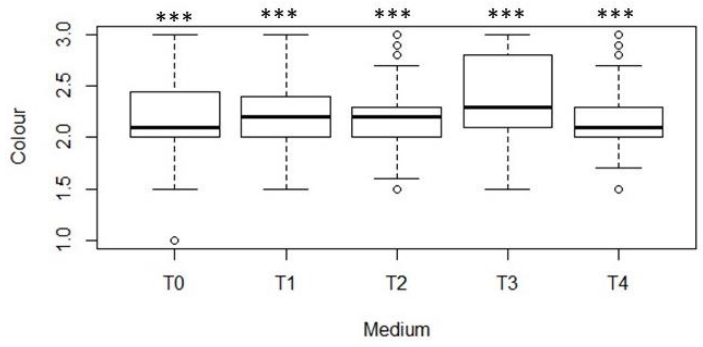


Figure III.17 Boxplots related to the parameter colour of callus over the time. The ordinate shows the scale of evaluation: 1=white, 2=cream, 3=brown (necrosis). The abscissa refers to the timing of evaluation after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The comparison was made involving all the samples. The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

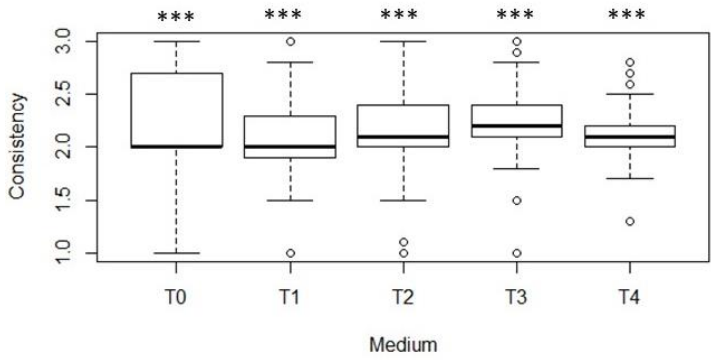


Figure III.18 Boxplots related to the parameter consistency of callus over the time. The ordinate shows the scale of evaluation: 1=friable, 2=compact, 3=watery. The abscissa refers to the timing of evaluation after the start of PEM induction: 30 days (T1), 40 days (T2), 50 days (T3), 60 days (T4). The comparison was made involving all the samples. The asterisks indicate the significance based on the Chi-square test: *: p-value <0.05, **: p-value <0.01, ***: p-value <0.001.

Embryogenesis process and callus regeneration. Pro-embryogenic masses were transferred onto GISCA medium to stimulate the embryogenesis process. As shown in Figure III.19A, GISCA was highly performing for embryogenic induction that occurred in a period of 2 months. Meanwhile, some calluses produced both root primordia and well-differentiated roots, characterized by the presence of root hairs (Figure III.19B). Proceeding with the transfers of the embryogenic calluses onto the regeneration substrate (RM), it was possible to observe the proliferation of new vegetative tissues (Figure III.19C). They were transferred onto SH medium, and after 1 month a more marked growth occurred, as shown by Figure III.19D. The above has demonstrated the applicability and efficiency of the regeneration protocol of plants for somatic callogenesis and embryogenesis, as regards the genotypes considered in the experiment.

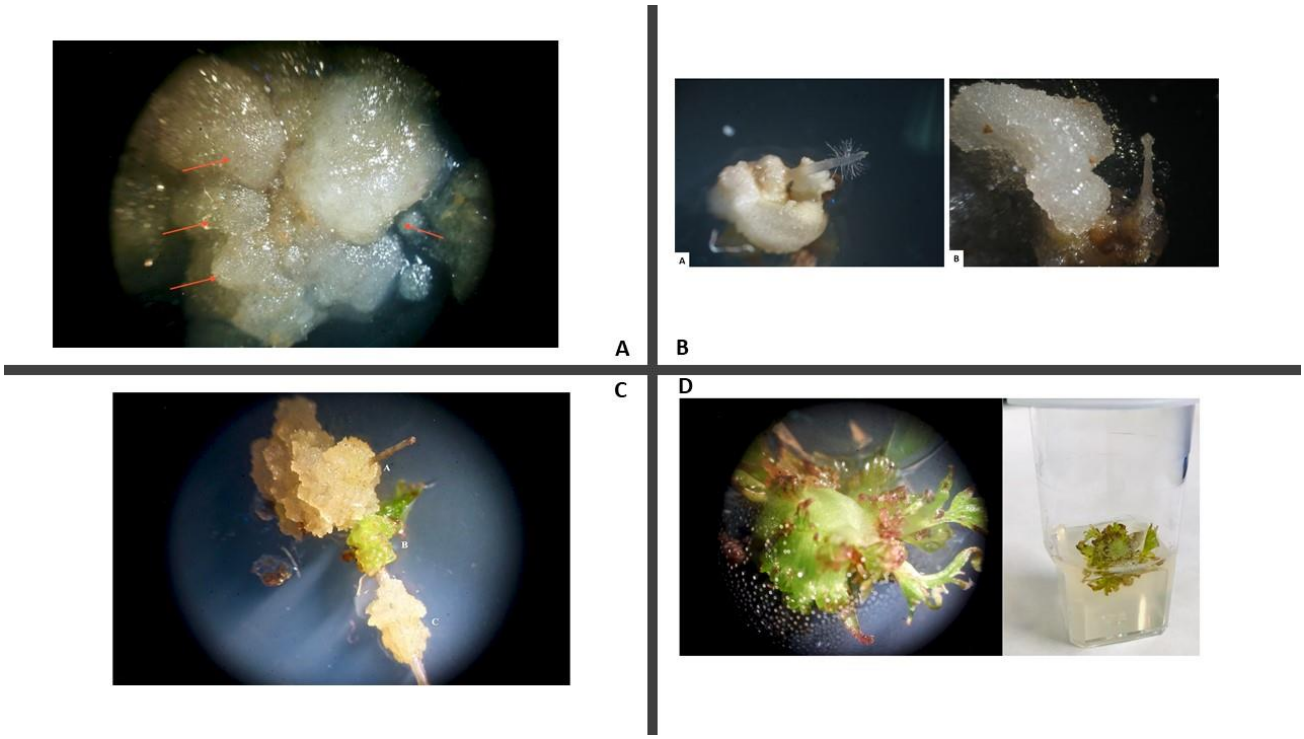


Figure III.19 Somatic embryogenesis and regeneration from somatic embryos. **A)** M4 somatic embryos in development, indicated by the red arrows, onto GISCA medium (½ MS basal salts; 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 60 g/l sucrose; 10 ml/l NOA (1M); 1 ml/l BAP (1M); 20 ml/l IAA (1M); 2.5 g/l of activate; pH 6.2) at 24±1°C in the darkness. **B)** M4 somatic embryos developing roots hair onto GISCA medium. **C)** M4 embryogenic callus transferred onto RM regeneration medium (½ MS basal salts, 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 2.2 ml/l BAP (1mM); 30 g/l sucrose and pH 5.7), starting to produce green tissues. **D)** Organogenesis of M4. Fully formed leaf primordia onto SH medium (½ MS basal salts, 1 ml/l B5 vitamins (1M); 5 ml Fe/EDTA (200 mM); 10 ml/l BAP (1mM); 30 g/l sucrose and pH 5.7).

III.4.4 Discussion

The degree of success of an *in vitro* culture trial depends by many factors. The main ones affecting plant micropropagation and callogenesis are culture medium (especially PGRs content), genotype as well as environmental condition, explant type and its developmental stages (Dhekney et al., 2009). In this study the effect of growth regulators and genotype have been studied. Shoot elongation per each sample changed based on the genotype, with K 5BB notable less responsive than the others. M4 recorded the best performance in the passage from the root inducing medium containing either auxin and cytokinin (IBA and BAP) to the multiplication ones without hormone (M1) (with almost the 85% of nodal segments developed shoots from axillary buds). The lowest development was registered in the media combination R1M2, resulting in general the least performing for all rootstocks studied, except for 1103 P (with almost . The combinations R2M1 and R2M2 put M4 in an intermediate position with respect to the other rootstocks and, in this combination, no significant differences between the two-multiplication media (M1 and M2) on M4 development occurred (in both cases it reaches 2.8 cm of elongation). The different *in vitro* responses of each genotypes examined can be

associated to the set of endogenous hormones, specific of every species, that make them ready to be micropropagated, but also to the exogenous ones characterising the culture medium (Gaj, 2004). In fact, the PGR composition and concentration of a substrate can induce either callogenesis or shoot development. Auxin and citokynins are key factors in the determination of *in vitro* culture attitude, because they strongly participate in cell cycle regulation and cell division (Francis and Sorrell, 2001; Gaj, 2004). In this study, R1M2 resulted the lowest performing for the shoot growth, maybe due to the final excessive content of BAP contained either in R1 and M2. In fact, even if BAP is the most effective cytokinin for inducing shoot development in *Vitis* spp (Lee and Wetzstein, 1988; Heloir et al., 1997), higher levels of this hormone (> 10 µM) can produce stem fasciated, short and depress the shoot number (Hicks et al., 1986; Banilas and Korkas, 2007). Moreover the occurrence of hyperhydricity (vitrification) was also observed (Alizadeh et al., 2010). So, as reported by Mhatre et al. (2000), it is a good practice to use a level of BAP up to 5 µM in order that shoots grow normally and within a month reach an appropriate size for rooting.

The data regarding root development was related only to the combinations between root inducing medium R1 (containing BAP and IBA) and multiplications media (M1, free-hormone, and M2-containing BAP). Samples that were moved from R2, containing IAA, to the multiplication media did not form roots but cellular masses, or calli. This can be due to the high content of the auxin in the substrate confirming the results obtained by Melyan et al. (2015). Indeed they tested several MS media with different content of IAA and found that at the concentration of 1 mg/l of this auxin, samples of *Vitis* spp showed the lowest root length that in most cases turned in calli. Except for K 5BB, which again showed an inverse behaviour with respect to the other rootstocks, the best performing combination was R1and M1, without hormones. In fact, although spontaneous rooting can occur in medium lacking IBA, it has been demonstrated that the addition of the auxin (at 5 µM) facilitate this process (Banilas and Korkas, 2007). According to Heloir et al. (1997), IBA is a suitable auxin, while other types of auxin (i.e. NAA- l-naphthaleneacetic acid) may led to callus formation (as well as IAA).

The callogenesis competence of the rootstocks tested resulted strictly linked to the genotype and the substrate composition. In fact, even if the same auxin and cytokinin were used in both C1 and C2 (2,4-D and BAP), their concentration plays a decisive role on the expression of callogenesis competence per each genotype. In this study, the medium containing the higher quantity of 2,4-D (C2) was more performing than C1, for all genotypes. After 8 weeks, in fact, leaf explants appeared almost all turned in compact-cream coloured calli (covering from 60% to 80% of the explant surface). According with the literature, for *Vitis vinifera* (cv. Chardonnay), the concentration ratio between

2,4-D/BAP should be of 2/1 to obtain embryogenic calli (Maillot et al., 2016). In this trial concerning hybrids deriving from the cross of American *Vitis*, the medium C2 leaded to satisfactory values, even if the ratio between the two hormones was 1/1. Auxin is the most important hormone in regulating embryogenesis *in vitro* (Cooke et al., 1993) and 2,4-D itself is believed to act as a stressor, by reprogramming somatic cells towards an embryonic state (Gaj, 2004; Karami and Saidi, 2010; Maillot et al., 2016). The culture of explants in medium containing 2,4-D increases the endogenous auxin level (IAA) in the responsive explant, establishing an auxin gradient during the induction of the callus, necessary for initiating bilateral symmetry of embryogenesis (Schiavone and Cooke, 1987; Jiménez et al., 2005). Anyway, higher concentration of auxin and cytokinin (> 9 µM) can lead to the browning of explant, suggesting phytotoxicity (Maillot et al., 2016).

Embryogenesis is a process which depends on the interaction of several factors such as genotype, explant source and culture medium. For this reason each *Vitis* species need of a specific embryogenesis protocol (López-Pérez et al., 2005). In our study we tested two genotypes, M4 and 1103 P, in two pro-embryogenesis media, by using two types of bud explants (OB and CB). We found that M4 genotype differentiated from 1103 P only for producing, in the medium BI, larger callus masses with respect to 1103 P. This can be associated to the higher content of hormones in the media but also to the association of the two auxin, 2,4-D and NAA that induce cell proliferation (Ikeuchi et al., 2013). Concerning callus colour and consistency, no differences were found for both genotypes and media. In fact, these variables tended to 2 (which was the optimal value). This mean that either M4 and 1103 P are performing genotype and that BI and NB are suitable for producing high-quality calluses. Without considering the genotype effect, the higher concentration of auxins and cytokinin contained in BI, promoted the proliferation of callus even it did not affect the quality. Regarding the type of explant, the absence of external bud protections (OB) was advantaged from the lower hormone content in the medium in terms of % of surface turned into callus. On the contrary, buds with perule (CB) were promoted by the higher one BI. It suggests that, higher is the differentiation degree of the tissues (i.e. the presence of protections) higher should be the content of hormones to promote cell de-differentiation and proliferation. The time factor affected the % of callus produced over the time, differently from colour and consistency. This was a sign that while callus mass increased, the quality was kept over time. After 40 days from the first explant culture, calluses tended to brown colour (values closer to 3.0, indicative of necrosis) and to watery consistency. Browning was due to the accumulation of polyphenols in the growth medium. This phenomenon causes stunted growth, lower regeneration rate and can lead to the necrosis of tissue up to the entire plant (Jones and Saxena, 2013).

The medium for the obtainment of somatic embryos gave positive result, confirming their validity extensively tested (Franks et al., 1998; Cadavid-Labrada et al., 2008).

III.4.5 Conclusion

M4 is considered a promising rootstock for its capacity to resist to drought and salt stress (Meggio et al. 2014, Corso et al. 2015). Therefore, with a view to a spread use of this rootstock, a large production of M4 plantlets is increasingly required. Micropropagation is considered an efficient method of rapid mass propagation and regeneration through organogenesis and embryogenesis (Stamp et al. 1990) but, for having success, a reliable and high-throughput protocol must be set-up. The results of the present study suggest that M4 can be easily micropropagated, in fact, its development in terms of shooting and rooting occurred in all four media combinations tested. Particularly, the switch from the rooting medium containing citokynins and auxins (R1) to the multiplication medium without hormones (M1) resulted the best performing for the obtainment of a substantial quantities of M4 plantlets, developed in height and with a root system in equilibrium with the foliar apparatus. Nevertheless, the growth of M4 plantlets was promoted also by the medium containing BAP (M2) even if it was not favourable to the root formation. Instead, the switch from R1, containing IAA, to the multiplication media M1 and M2 did not allow root apparatus development.

Callogenesis from leaf explants can be successfully induced by 2,4-D and BAP hormones in the medium. All genotypes benefited of the higher content of 2,4-D (1 mg/l) and specifically for M4, the complete de-differentiation of leaf tissues can take more than 40 days.

The higher content of 2,4-D, in combination with NAA and BAP in the medium BI favoured pro-embryogenic masses development. The transfer to the medium GISCA, which is a modification of the well-established GS1CA, favoured embryogenesis process.

In conclusion, the setting up of a valid protocol for rootstock *in vitro* multiplication allows to produce more plant material, also grafted, in shorter times with less labour and lower costs (Stamp et al., 1990), in respect to the traditional method of propagation. For vine rootstocks, the composition and concentration of hormones in the culture medium play a key role in the propagation of microcuttings and in the obtaining of calluses, closely influenced by the genotype x substrate interaction. Therefore the best cultural condition must be identified. In addition, an efficient method of callogenesis from tissue explants, going hand in hand with the boost that the genetic improvement of plants through the New Breeding Techniques (NBTs) is assuming, take advantage of cellular material genetically manipulable, such as embryogenic calli, passing through *in vitro* callogenesis and embryogenesis.

III.4.6 Supplementary information

Supplementary information 1. **Table A**. Analysis of variance of the effect on shoot elongation of genotype, medium and genotype x medium. **Table B**. Effect of all genotype on shoot elongation. **Table C**. Effect of the medium on shoot elongation.

Table A

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	82.918[a]	19	4.364	6.705	.000
Intercept	5168.077	1	5168.077	7939.622	.000
Genotype	9.634	4	2.408	3.700	.005
Medium	18.019	3	6.006	9.227	.000
Genotype * Medium	37.037	12	3.086	4.742	.000
Error	499.908	768	.651		
Total	7234.870	788			
Corrected Total	582.827	787			

Table B

Genotype	N	Subset	
		1	2
K 5BB	113	2.479	
3309	163		2.899
M4	151		2.920
101-14	182		3.016
1103 P	179		3.056
Sig.		1.000	.122

Table C

Medium	N	Subset	
		1	2
R1M2	224	2.632	
R2M1	148		2.943
R2M2	177		3.000
R1M1	239		3.069
Sig.		1.000	.154

Supplementary information 2. Effect of the interaction genotype x medium on shoot elongation in R1M1 (**Table A**) and R1M2 (**Table B**).

Table A

Genotype	N	Subset		
		1	2	3
K 5BB	22	2.191		
1103 P	29		2.817	
3309	60		3.143	3.143
101-14	81		3.212	3.212
M4	47			3.291
Sig.		1.000	.085	.523

Table B

Genotype	N	Subset	
		1	2
K 5BB	57	2.298	
3309	24	2.413	
101-14	37	2.435	
M4	52	2.662	
1103 P	54		3.187
Sig.		.066	1.000

Supplementary information 3. Effect of the interaction genotype x medium on shoot elongation in R2M1 (**Table A**) and R2M2 (**Table B**).

Table A

Genotype	N	Subset		
		1	2	3
3309	26	2.588		
M4	31	2.855	2.855	
1103 P	48	2.969	2.969	2.969
101-14	33		3.142	3.142
K 5BB	10			3.360
Sig.		.108	.226	.098

Table B

Genotype	N	Subset
		1
K 5BB	24	2.804
M4	21	2.824
3309	53	2.994
101-14	31	3.065
1103 P	48	3.140
Sig.		.058

Supplementary information 4. **Table A**. Analysis of variance of the effect on root development of genotype, medium and genotype x medium. **Table B**. Effect of all genotype on root development.

Table A

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	235.446[a]	9	26.161	56.609	.000
Intercept	956.736	1	956.736	2070.273	.000
Genotype	18.358	4	4.590	9.931	.000
Medium	86.951	1	86.951	188.151	.000
Genotype * Medium	121.703	4	30.426	65.838	.000
Error	26.804	58	.462		
Total	1218.500	68			
Corrected Total	262.250	67			

Table B

Genotype	N	Subset	
		1	2
1103P	16	3.188	
M4	12	3.250	
101.14	13	3.538	
3309C	14		4.214
K 5BB	13		4.615
Sig.		.212	.131

Supplementary information 5. Effect of the interaction genotype x medium on root development in R1M1 (**Table A**) and R1M2 (**Table B**).

Table A

Genotype	N	Subset		
		1	2	3
K 5BB	6	3.000		
M4	6		5.000	
1103P	8		5.125	
3309C	7		5.429	5.429
10114	6			6.000
Sig.		1.000	.313	.157

Table B

Genotype	N	Subset		
		1	2	3
1103P	8	1.250		
101.14	7	1.429		
M4	6	1.500		
3309C	7		3.000	
K 5BB	7			6.000
Sig.		.506	1.000	1.000

Supplementary information 6. **Table A**. Analysis of variance of the effect of the genotype, medium and genotype x medium on callus induction. **Table B**. Effect of each genotype on callus induction.

Table A

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	119920.832[a]	9	13324.537	27.931	.000
Intercept	875520.544	1	875520.544	1835.289	.000
Genotype	52300.883	4	13075.221	27.409	.000
Medium	41659.846	1	41659.846	87.328	.000
Genotype * Medium	5673.803	4	1418.451	2.973	.019
Error	181278.142	380	477.048		
Total	1248400.000	390			
Corrected Total	301198.974	389			

Table B

Genotype	N	Subset			
		1	2	3	4
101.14	136	33.971			
M4	62		46.935		
3309C	82		53.415	53.415	
K 5BB	36			59.167	
1103P	74				70
Sig.		1.000	.092	.134	1.000

Supplementary information 7. Effect of the interaction genotype x medium on callus induction in C1 (**Table A**) and C2 (**Table B**).

Table A

Genotype	N	Subset		
		1	2	3
101.14	102	27.157		
M4	44		43.636	
K 5BB	20		46.500	
3309C	70		47.429	
1103P	51			63.922
Sig.		1.000	.418	1.000

Table B

Genotype	N	Subset	
		1	2
101.14	34	54.412	
M4	18	55.000	
K 5BB	16		75.000
1103P	23		83.478
3309C	12		88.333
Sig.		.945	.145

Chapter IV – The New Breeding Technologies (NBTs): the CRISPR/Cas9 system.

Genetic improvement of plant resistance to biotic and abiotic stresses is a continuous challenge for plant research and breeding. To date, for producing genetic changes in plant genomes, multiple and sophisticated techniques have been used. They range from point mutations to integration of chromosomal fragments from wild relatives into elite varieties (Langner et al., 2018). A long list of plant varieties genetically improved is available. Mostly deriving from natural mutations, wide crosses, hybridization and random mutagenesis using physical (X or γ radiation), chemical (ethyl methane sulfonate (EMS)) and biological factors (e.g., transposon). However, by conventional forward genetics approaches, the recovery of knock-out lines for a specific gene is time consuming. Moreover, random mutations in the mutant population can occur making it challenging to identify the desired genetic change (Langner et al., 2018). The advent of molecular techniques and of recombinant DNA technology has represented a big step forward for plant genetic improvement. They have led to the development of transgenic crops, also defined Genetically Modified Organisms (GMOs), obtained through the delivery of foreign DNA via transfer DNA (T-DNA) from *Agrobacterium tumefaciens* into plant genomes. However, this approach has been extensively criticized since the insertion of the transgene in the genome, takes place in random positions along with recombinant sequences of other species (e.g. bacteria and viruses) which can guide the expression of the inserted gene. This may potentially have an undesirable effect on targeted and non-targeted organisms as well as it could interrupt the function of other important genes.

Over the past 15 years, a set of techniques, collectively named New Plant Breeding Techniques (NPBTs) have been developed. This comprises approximately 20 approaches, including cisgenesis, intragenesis, RNA-dependent methylation, agroinfiltration, RNA interference (RNAi), reverse breeding, grafting on GM rootstock, oligo-directed mutagenesis (ODM) and SSNs (Lusser and Davies, 2013). A study driven by Lusser and co-workers (2012) demonstrated that plants produced by some of these NPBTs are at an advanced stage of development and some of them have already been commercialized in the United States and Canada.

IV.1 Genome editing

Genome editing technologies permits to create genetic modification and to modulate the function of DNA sequences in their endogenous genome (Langner et al., 2018). Being applicable to various

organisms, they represent a revolutionary plant research tool for genetic improvement (Barrangou and Horvath, 2017; Langner et al., 2018).

For introducing modifications in plant genome, genome editing makes use of Sequence Specific Nucleases (SSNs). SSNs are programmable proteins that can bind to DNA at the level of a target sequence and induce a double-strand break (DSB) on the site. So far, four major classes of DNA-binding proteins have been utilized: meganucleases (Smith et al., 2006; Pâques and Duchateau, 2007), ZFNs (Maeder et al., 2008), TALENs (Bogdanove and Voytas, 2011; Miller et al., 2011) and several molecules derived from CRISPR-Cas endonucleases (Jinek et al., 2012, 2013; Shmakov et al., 2015; Abudayyeh et al., 2016, 2017; Zetsche et al., 2017). The first generation (ZFN-, TALEN-, and meganuclease-based) are protein-dependent DNA cleavage while CRISPR-Cas endonucleases are programmable RNA-guided DNA or RNA cleavage systems (Langner et al., 2018).

Depending on the type of native repair pathway followed to fix the induced DNA break, three types of results can be generated: SSN-1, SSN-2, SSN-3 (Schaart et al., 2016). The SSN-1 originates from the nonhomologous end-joining repair mechanism (NHEJ), in which the break ends of DNA are ligated without a homologous template. This determine the introduction of small errors such as insertions or deletions (indels) of nucleotides. If it occurs into protein-coding sequences, it causes frameshift mutations that can interrupt prematurely the DNA translation (Schaart et al., 2016), effectively creating a gene knock-out. Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs (Cristea et al., 2013; Maresca et al., 2013; Bortesi and Fischer, 2015). On the other end, in the presence of a DNA repair template, DNA break may be repaired by homologous recombination (HR) mechanism. This mechanism can be exploited to achieve precise gene modifications or gene insertions (Bortesi and Fischer, 2015). Based on the type of DNA repair template, the HR results can be small changes after repair (SSN-2) or precise insertion of a larger DNA insert (SSN-3). With SSN-2 it is possible to introduce small substitutions in a DNA sequence while with SSN-3, a complete new gene (Schaart et al., 2016). Once the mutations have been introduced, the nucleases are counter-selected in the final individual. In genome editing, the SSN1 are the most used modifications in order to generate the gene knock-out; while SSN-2 and SSN-3 up to now poorly efficient (Schaart et al., 2016). Among the SSNs that allow genome modification, the CRISPR/Cas9 system has quickly become the principal one for plant genome modification, due to its ease of use, versatility and cleavage ability even in methylated loci (Belhaj et al., 2013; Hsu et al., 2013; Bortesi and Fischer, 2015).

IV.2 The CRISPR / Cas9 system

CRISPR/Cas9 system is one of the mechanisms used by prokaryotes to prevent viral infection and block plasmid transfer (Westra et al., 2012). Many bacteria and most archaea, in fact, harbour RNA-guided adaptive immune systems encoded by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and accompanying CRISPR-associated (Cas) proteins (Jiang and Doudna, 2015). CRISPR repeats were initially discovered in 1980s in *Escherichia coli* but their function was confirmed by Barrangou et al. (2007) who demonstrated that *Streptococcus thermophilus* could acquire resistance against a bacteriophage by integrating a genome fragment of an infectious virus as spacers between two adjacent repeats at the proximal end of a CRISPR locus (Bortesi and Fischer, 2015). The CRISPR arrays, including the spacer, are transcribed when encounter invasive DNA. Transcripts are then processed into small interfering CRISPR RNAs (crRNAs), of approximately 40 nt in length. This one combines with transactivating CRISPR RNAs (trascrRNAs) forming RNA complex. The crRNA/trascrRNA hybrid (sgRNA) acts as a guide for the endonuclease Cas9, which cleaves the invading nucleic acid.

Based on this model of action, CRISPR/Cas9 technology makes use of two components: the Cas9 and a single guide RNA (sgRNA). Cas9 is a monomeric endonuclease, bi-lobed, with a large globular recognition (REC) lobe and a small nuclease (NUC). This one comprises two nuclease domains and a domain that binds to PAM (Protospacer Adjacent Motif). The customizable gRNA is a noncoding RNA with two parts: the protospacer-containing RNA (crRNA) and the *trans*-activating crRNA (tracrRNA) (Barrangou and Horvath, 2017). The Cas9-sgRNA forms a complex. sgRNA drives the Cas9 endonuclease to cleave both DNA strands. A prerequisite for cleavage is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ (Gasiunas et al., 2012; Jinek et al., 2012). Following the double-strand break (DSB), the genome is repaired by DNA-DSB repair mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR) (See previous section IV.1).

The CRISPR/Cas9 genome editing system is much easier than other technologies because only a short guide of RNA needs to be fine-tuned and, because of its versatility, it can be applied to target important genes, introducing single point mutations (deletion, insertion or base changes). Ideally, by customizing a simple sgRNA it would be possible to modify any gene of interest. However, there is a limitation of this system because the sgRNA sometimes binds with DNA sequences containing mismatches, causing unwanted mutations in off-target sites (Fu et al., 2013). To overcome this problem, however, researchers have developed modifications to the system, in order to increase their specificity and reduce off-target (Schaeffer and Nakata, 2015).

Evidences that the CRISPR/Cas9 system could be transferred was discovered in 2011, when Siksnys and co-workers performed transplantation of the CRISPR type II locus from *Streptococcus thermophilus* to *Escherichia coli*. They demonstrated that this one is able to reconstitute CRISPR interference in different bacterial strains (Sapranauskas et al., 2011). In the following year, it has been demonstrated that a purified Cas9 from *Streptococcus thermophilus* or *Streptococcus pyogenes* can be driven by crRNAs to cleave target DNA *in vitro* (Gasiunas et al., 2012; Jinek et al., 2012). Furthermore, a single guide RNA (sgRNA) can be developed by fusing a crRNA of the target sequence to a tracrRNA, facilitating the cleavage by Cas9 in *in vitro* condition (Jinek et al., 2012).
The genome editing technologies are the tool of choice to study different biological systems (i.e. performing functional genomic studies) and for altering plants, creating varieties with desirable traits. It appears that these technologies will continue to rapidly advance our understanding of metabolic processes and holds the promise to revolutionize the field of plant biotechnology (Schaeffer and Nakata, 2015).

IV.3 A genome editing protocol mediated by geminivirus

Since 2016, all genome editing in grapevine has used conventional T-DNAs for expressing the editing gRNA and Cas9 mRNA (Malnoy et al., 2016; Nakajima et al., 2017; Wang et al., 2017). By using the mechanism of replication of viral vectors (Baltes et al., 2014), improvements in gene editing tools have been achieved. For this purpose, the single-stranded DNA (ssDNA) replicons from geminiviruses proved to be particularly efficient (Baltes et al., 2014). Geminiviruses are plant viruses belonging to the *Geminiviridae* family. They have small genomes (2.5–5.5 kb) which replicate by rolling circle replication (RCR) in plant-host nucleus. Through a double-stranded replication intermediate, that also serves as a template for transcribing the viral open reading frames, this process occurs (Hanley-Bowdoin et al., 2013). Bean yellow dwarf virus (BeYDV) is the smallest member in the family. By generating disarmed versions referred to as “LSL” (LIR-SIR-LIR; Mor et al., 2003) or “deconstructed virus” vectors (Gleba et al., 2004), it is possible to obtain a molecular tool to improve gene expression (Miccono et al., 2018). Virus replication machinery consists of three main elements, retained in the LSL vectors. They allow that virus replication by RCR can be emulated, permitting the transcriptional activation of the included expression cassettes. Two of these elements act in *cis*: LIR (a long intergenic region) and SIR (a short intergenic region) (Morris et al., 1992). The LIR contains a bidirectional promoter that drives transcription of the virion sense genes (V1, movement protein; V2, coat protein) and the complimentary sense genes (C1, RepA; C1/C2 with

spliced intron, Rep protein). It also includes a stem-loop structure that is the site of nicking by Rep protein, permitting the initiation of the RCR of the plus-strand (Sanz-Burgos and Gutierrez, 1998). The SIR contains polyadenylation signals for the protein-coding genes (Chen et al., 2011). The third element corresponds to the virus replication initiator protein (Rep), which acts in *trans* (Liu et al., 2017) and therefore must either be expressed by the viral replicon itself or be externally provided (Sanz-Burgos and Gutierrez, 1998; Mor et al., 2003b). In gene transfer experiments, Rep's nicking function acting on the LIR components, arranged in tandem in the delivered plasmid, activates the LSL vectors. It results in the replicative release of the recombinant viral DNA cloned between them (Mor et al., 2003b). Since the released DNA replicates episomally in the nucleus, it leads to the efficient expression of the encoded genes (Čermák et al., 2015).

In the study performed by Miccono et al. (2018), a BeYDV-derived replicons was used in a somatic embryogenesis-based gene transfer system in grapevines to generate edited non-transgenic individuals. Their results validate the DNA replicon technology for CRISPR/Cas9 genome editing in fruit crops.

On the basis of their work, by using a geminivirus vector provided by them, we followed the same protocol targeted to the knock-out of the VvSTSs genes (VvSTS 16, 18, 27 and 29) (see section I.4), considered the putative factors of the M4 better adaptation to water stress. The identification of the VvSTSs genes represents a starting point for functional analysis to be performed in the future.

To this end, two geminivirus vectors containing two gRNAs cassette have been developed: one, to knock-out all the four genes of interest; one, specific for the genes VvSTS27 resulting the highest expressed in M4 during water stress. Because the high homology among the VvSTSs genes, which shows numerous paralogous zones, not only at the level of coding regions, but also in non-coding regions (Vannozzi et al., 2012), the nucleic sequences of each one was divided in three or four fragments, with overlapping ends of 200 bp. For each fragment, a couple of primers was designed. Due to the impossibility to isolate them, the gRNAs were designed on the grapevine reference genome (PN40024). A transient assay to test the gRNAs functionality was performed on *in vitro* plants of M4 and 101-14, drought-tolerant and drought-susceptible respectively. It will be followed by the transformation of somatic embryos of both genotypes, from which whole-transformed plants will be regenerated (as described in the section III.4).

IV.3.1 Materials and Methods

Plant material

Plantlets of M4 and 101-14 deriving from *in vitro* micropropagated population (see section III.4) were used for the extraction of DNA. They were maintained in MS (Murashige and Skoog, 1962) medium without hormone, in controlled condition, at 23 ±1°C and 16-8 hr of photoperiod under cool white florescent tube.

Isolation and Identification VvSTS genes sequences

Nucleospin® Plant II extraction kit (Macherey-Nagel) was used to extract the DNA from 100 mg of grinded leaf tissues, following the manufacture's protocol. The extracted total genomic DNA was quantified by Qubit® DNA HS Assay Kit by Qubit® 3.0 Fluorometer (Thermo Fisher, Life Technologies, Carlsbad, CA) and its quality was checked by NanoDrop Spectrophotometer (Thermo Scientific), to evaluate the 260/230 and 260/280 ratios.

Primer design

The primers selected for the amplification of the candidate genes (VvSTS 16,18,27 and 29) were designed on the 12X V1 coverage assembly of the PN40024 genotype. Because the high homologies among the genes, each sequence was divided in three (VvSTS16, VvSTS18) or four parts (VvSTS27, VvSTS29) covering a maximum of 900 bp with overlapping ends of 200 bp. Initially, primers were designed using Primer3Plus program (https://primer3plus.com/), in conjunction with NCBI primer BLAST software (www.ncbi.nlm.nih.gov). To improve their specificity, they were then designed manually (Table IV.1), in correspondence of the few mismatches resulting from the alignment of the candidate gene's sequences (performed with ClustalW software, https://www.genome.jp/tools-bin/clustalw). The quality of the primers was checked on Net Primer software (www.premierbiosoft.com/netprimer).

VvSTS	ID Fragment	Primer Forward	Primer Reverse	Bp sequence covered
VvSTS 16	VvSTS16-I	TTCGAGTCAAAGAGGGTAAGC	AGCTCAAGGAACCAGTTTGAT	727
	VvSTS16-II	CCAGTCTGATTTCGCTGATT	GGAGATCAAAGTTGGCACATTA	869
	VvSTS16-III	GAACCGCTAAGGATCTTGCTG	TTTAATTTAAGGTCTCCAAGGAAA	816
VvSTS 18	VvSTS18-IV	CTAGCCCAGCATGTGATAAATATG	TCAACATCGTTCAAGTGAGTGT	744
	VvSTS18-V	AAGAAGTTCAACCGCATCTGT	GGTCAAAAGCCTTAGTCAAACAC	898
	VvSTS18-VI	TGGGTCAGCAGCTGTAATCA	TGAAAAAGTCCTCCAACAACG	690
VvSTS 27	VvSTS27-VII	CAGGAGGCTGGAAAAGTCCT	CTGGTAGACACAGTGGTCGG	445
	VvSTS27-VIII	CTTTCAAGCCAACTCCAAGC	CTTGATCATGGATTTGTCACCTAT	630
	VvSTS27-IX	GAGCTGTTCTTTGAATCATGTCTC	GGTGAGCAATCCAAAATAACG	865
	VvSTS27-X	CCACTTTAATTTCTGAAAACATTG	GCTCACCCAGAGGGTCTAACA	500
VvSTS 29	VvSTS29-XI	TTCTCAAGCCAACTCCAAACTT	CTTAATTATAGCTGGCAATGCGA	514
	VvSTS29-XII	ATTGGCACAGCTACCCCTG	CTTGATCATGGATTTGTCACTGTA	487
	VvSTS29-XIII	GCTGTTCTTTGAATCATGTCGC	GGGTGAGCAATCCAAAATAAG	885
	VvSTS29-XIV	TGTGCCCACTTTAATTTCTGAG	TGAGCTCACCAAGAGGGTCTAAT	670

Table IV.1 List of the primers used to amplify the STS genes whose sequences were divided in three (VvSTS 16,18) or four (VvSTS29) fragments. In the first column is reported the name of the gene. In the second column the fragment to which they refer to. In the third and fourth ones the primers sequences (5'-->3'). In the fifth column is reported the length of the fragment amplified by the couple of primers.

Sequences amplification

PCR amplification of each sample (Table IV.2), was performed using the genomic DNA as template and the couple of primers reported in Table IV.1. The amplification followed the manufacturer specification of GoTaq® G2 DNA Polymerase (Promega). Amplification cycle consisted of a pre-incubation cycle at 95°C for 2 min; denaturation at 95°C for 30 sec, annealing at 53–58°C for 30 sec and extension at 72°C for 1 min and 30 sec for 30 cycles; final extension at 72°C for 10 min. Each reaction was performed in triplicate of 50 µl. To verify the success of PCR, DNA bands were resolved by electrophoresis in 1.5% agarose gel. Their size was determined with a DNA marker PCRBIO Ladder I (MEDICAlliance). Each band was purified using Wizard® SV Gel and PCR Clean Up System (Promega) and sequenced with high-throughput applied biosystems 3730xl sequencer (Macrogen Europe). The first sequencing attempt failed because of the resulting "dirty" chromatograms with excessive noise, so a cloning was performed.

ID Sample	Genotype	ID Fragment
1.1	M4	*Vv*STS16-I
1.2	M4	*Vv*STS16-II
1.3	M4	*Vv*STS16-III
1.4	M4	*Vv*STS18-IV
1.5	M4	*Vv*STS18-V
1.6	M4	*Vv*STS18-VI
1.7	M4	*Vv*STS27-VII
1.8	M4	*Vv*STS27-VIII
1.9	M4	*Vv*STS27-IX
1.10	M4	*Vv*STS27-X
1.11	M4	*Vv*STS29-XI
1.12	M4	*Vv*STS29-XII
1.13	M4	*Vv*STS29-XIII
1.14	M4	*Vv*STS29-XIV
2.1	101-14	*Vv*STS16-I
2.2	101-14	*Vv*STS16-II
2.3	101-14	*Vv*STS16-III
2.4	101-14	*Vv*STS18-IV
2.5	101-14	*Vv*STS18-V
2.6	101-14	*Vv*STS18-VI
2.7	101-14	*Vv*STS27-VII
2.8	101-14	*Vv*STS27-VIII
2.9	101-14	*Vv*STS27-IX
2.10	101-14	*Vv*STS27-X
2.11	101-14	*Vv*STS29-XI
2.12	101-14	*Vv*STS29-XII
2.13	101-14	*Vv*STS29-XIII
2.14	101-14	*Vv*STS29-XIV

Table IV.2 Summary of the samples. Each ID sample is referred to a genotype (M4 or 101-14) and to a specific fragment

Cloning and E. coli competent cells transformation

For cloning, the plasmid pCBC_DT1T2 was used. It was digested with the enzyme Sal I at 37°C for 1 hr and inactivated at 65°C for 20 min. The sticky ends caused by the cutting were made blunt through a reaction with T4-DNA Polymerase and the addition of dNTPS (10mM). To make the plasmid ends T-overhanging, dTTPs were added in a reaction at 70°C for 1 hr and 30 min. To favour the ligation between T-plasmid and the PCR product, this one was made A-overhanging through the addition of dATPs. The ligation reaction consisted of an incubation cycle (at 16°C for 16 hr).

Once ligated was obtained, *E. coli* competent cells (NEB® Stable Competent *E. coli* C304OH) were transformed with it following the high efficiency transformation protocol provided from the manufacturer. *E. coli* cells were plated onto a selective media containing chloramphenicol. Once colonies grew (after 1-2 days at 37°C), 5 colonies were pick-up with sterile pipette tip to perform a colony-PCR and determine the presence or absence of insert DNA in plasmid construct. For this purpose, the same primers for amplifying the genes were used. The colony-PCR products were screened through the electrophoresis run and the positive clones (plasmid with the insert) were cultured in selective LB liquid solution overnight. Clones were purified with kit Pure Yeald^{TM} Plasmid Miniprep System (Promega) and sequenced.

Sequencing of the candidate genes

Purified cloned were sequenced through applied biosystems 3730xl sequencer (Macrogen Europe) after being prepared according to the instruction provided from the company.

Design of the gRNAs on grapevine reference genome

Because the high homology among the genes of interest, the attempt to isolate them following the previous cloning procedure resulted inefficient.

For this reason, gRNAs were designed on the grapevine reference genome (PN40024), using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/). The gRNAs selected were those with less off-targets, within introns (or intergenic regions) and with a %GC content greater than 45% (Table IV.3). It was decided to design two vectors: the first, containing two gRNAs in common among the four candidate genes (called V_STS1); the second containing two gRNAs, specifics for VvSTS27 gene (named V_STS27) which was the mostly expressed in M4 during water stress (Corso et al., 2015).

Geminivirus Vector construction

Plasmid (pCBC-DT1T2) was digested by the restriction enzyme Bas I, at 37°C for 1 hr in order to linearize it. The gRNAs were assembled into the vector, through the subsequent PCR reaction.

ID Primer	Primer Sequence 5'→3'
gRNA_VvSTS_1_DT2-R0	AACAAGAAATGCTTGAGGAGCACAATCTCTTAGTCGACTCTAC
gRNA_VvSTS_1_DT2-BsR	ATTATTGGTCTCGAAACAAGAAATGCTTGAGGAGCACAA
gRNA_VvSTS_2_DT1-BsF	ATATATGGTCTCGATTGAGTGATGTTGTACCATCAAGTT
gRNA_VvSTS_2_DT1-F0	TGAGTGATGTTGTACCATCAAGTTTTAGAGCTAGAAATAGC
gRNA_VvSTS27_2_DT2-R0	AACTGCTGGCCCTCTCCCCCTTCAATCTCTTAGTCGACTCTAC
gRNA_VvSTS27_2_DT2-BsR	AATTAAGGTCRCGAAACTGCTGGCCCTCTCCCCCTTCAA
gRNA_VvSTS27_2_DT1-BsF	ATATATGGTCTCGATTGTATTTGGATGAGATGAGAGTT
gRNA_VvSTS27_2_DT1-F0	TGTATTTTGGATGAGATGAGAGTTTTAGAGCTAGAAATAGC

Table IV.3 List of primers used for the production of the gRNAs obtained from the amplification of the PCBC_DT1T2 plasmid

The linearized vector pCBC-DT1T (100 ng) and the gRNAs DT2-R0 (1µM), DT2-BsR (20µM), DT1-BsF (20µM), DT1-F0 (1µM) (Table IV.3) were inserted into 50 µL of PCR reaction. The PCR cycle was performed following the manufacturer specifications relative to the Pfu DNA Taq-Polymerase (Promega). The reaction cycle was made by a pre-incubation at 95°C for 1 min; denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min, for 35 cycles; final extension at 72°C for 7 min. The PCR product was electrophoresed and purified from agarose gel with Wizard® SV Gel PCR Clean Up System (Promega).

The selected gRNAs were cloned into pGMV-U vector (developed by Miccono et al. (2018)) through a Golden Gate reaction as described by Xing et al. (2014). This one permits to assembly into a vector backbone multiple fragments using the simultaneous activities of a single Type IIS restriction enzyme, in our case Bsa I enzyme, and T4 DNA ligase. The Golden Gate reaction was performed with the following incubation cycles: 30 cycles of digestion/ligation at 37°C for 10 min and 16°C for 10 min, respectively; a final digestion at 55°C for 5 min; and a denaturation round at 80°C for 10 min. In order to verify the success of the Golden Gate Reaction, a PCR reaction using the Pfu DNA Taq-Polymerase (Promega) was performed using the Golden Gate product as template (1:10) and the primers P-hsegmv-S2 and P- gmv-A1 (10 mM) (Table IV.4). The amplification cycle was: 95°C x 1 min; 95°C x 30 sec, 60°C x 30 sec, 72°C x 1 min for 30 cycles; 72°C x 5 min. The PCR product was resolved in 1% agarose gel.

ID Primer	**Primer Sequence 5'→3'**
hsegmv-S2	TCA AAA GTC CCA CAT CGC TTA GA
gmv-A1	TGA AGT ACA CTC GGT CAA GCT

Table IV.4 List of the primers used to check the ligation of pGMV-U with gRNAs purified

Cloning of the Geminivirus Vector with E. coli competent cells.

The ligated pGMV-U-gSTSs vector was inserted into *E. coli* competent cells (NEB® Stable Competent *E. coli* C304OH) and selected for on 100 mg/l kanamycin LB medium following the manufacturer instructions. Cells were left to grow overnight at 37°C. The recombinant *E. coli* clones positive for pGMV-U-gSTSs (pGMV-U-gSTS1 and pGMV-U-gSTS27) were screened by a colony-PCR. Firstly, ten colonies were picked up with a sterile tip and inserted into 10 µl of ddH_2O. They were denaturated at 95°C for 10 min, of which 1 µl was used to perform the PCR reaction. The PCR

reaction comprised the Pfu DNA Taq-Polymerase (Promega) and the primers P-hsegmv-S2 and P-gmv-A1 (10 mM) (Table IV.4). The amplification cycle was: 95°C x 1 min; 95°C x 30 sec, 60°C x 30 sec, 72°C x 1 min for 30 cycles; 72°C x 5 min. Amplifications were resolved in 1% agarose gel. Positive clones were purified with Pure Yeald™ Plasmid Miniprep System (Promega – Cat. #A1222) kit, according to the manufactures' instruction.

Agrobacterium tumefaciens transformation and transient assay for gRNA functionality.

Plasmid extraction from assembled pGMV-U-gSTSs *E.coli* clones was electroporated into AGL1 *Agrobacterium tumefaciens* STR3006 (LifeScience), as described by (Tapia et al., 2009). The clones were plated onto 100 mg/l kanamycin LB medium. The transformation of *A. tumefaciens* with the plasmid vector was checked by a PCR reaction, as described by the section before, followed by an electrophoresis run.

Agrobacterium clones harbouring pGMV-U-gSTSs vector were grown overnight in 5 ml of LB, at 28°C with shaking at 200 rpm, following a modified protocol from Zottini et al. (2008). Aliquots of *Agrobacterium* liquid culture were diluted in 15 ml of fresh infiltration medium (50 mM MES, 2 mM Na_3PO_4, 0.5% glucose, 100 µM acetosyringone, pH 5.6) adjusting the OD_{600} to 0.2. The *Agrobacterium* infiltration solution was then incubated at 25°C for 2 hr in the dark with gentle agitation (60 rpm). Plant infiltrations were performed by placing 1.5 mL of the *Agrobacterium* infiltration solution (using 3-mL needleless syringes) onto the abaxial face of small-sized leaves. Each leaf received 15 infiltrations. The plants were kept under standard growth conditions, and leaf samples were collected between 10 and 14 days for genomic DNA extraction.

Genomic DNA extraction

Genomic DNA was extracted from the leaves (100 mg/leaf) using the Nucleospin® Plant II extraction kit (Macherey-Nagel), following the manufacturer instructions. The genomic DNA extracts were additionally treated with RNAase A (50 mg/mL) for 30 min at 37°C.

Target editing identification

For the identification of edited individuals, a pair of primers adjacent to the target region were used and reported in Table IV.4. The genomic DNA (25 ng) was used to perform the PCR reaction using Pfu DNA Taq-Polymerase (Promega) and the PCR product was resolved in 1% agarose gel.

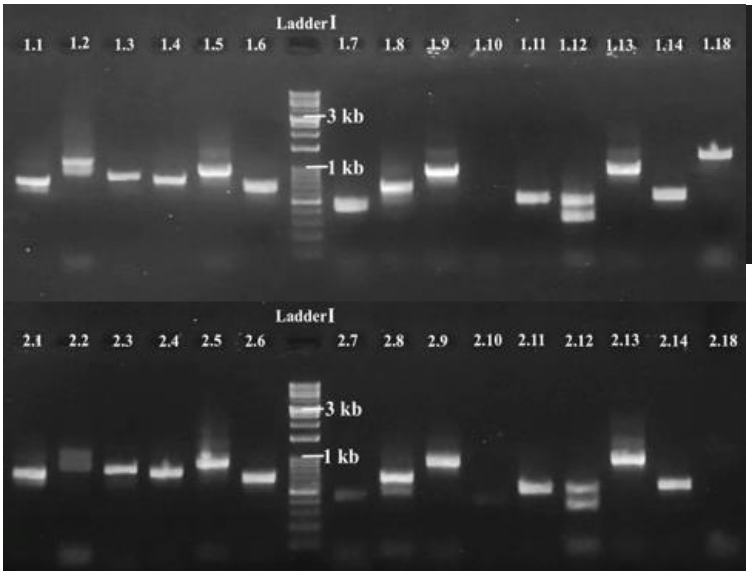


Figure IV. 1 Electrophoretic analysis in 1.5% agarose gel of PCR products, referred to the samples reported in Table IV.2. Above, bands are related to M4 genotype; below they are related to 101-14 genotype. In the middle, the PCRBIO Ladder I (PB40 .11-01).

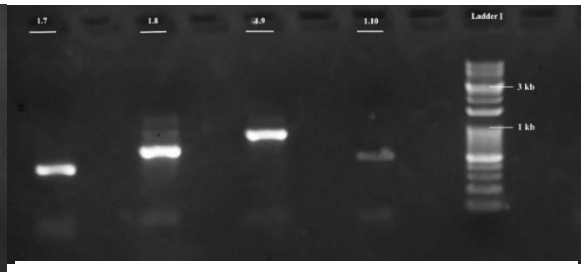


Figure IV. 2 Electrophoretic analysis in 1.5%, agarose gel 1.5% of 1.10 sample (on the right) obtained by decreasing the melting temperature of 1°C.

IV.3.2 Results and Discussion

In order to identify the nucleic acid sequence of each gene of interest (*Vv*STS16, 18, 27 and 29) for both M4 and 101-14 genotypes, several attempts of amplification and sequencing were performed testing the combination of primers reported in Table IV.1. The result of the first amplification round is shown in Figure IV.1.

All the samples were amplified, except 1.10 (referred to the genotype M4 and to the fragment VvSTS27-X (Table IV.2)). By decreasing of 1°C the annealing temperature used for PCR, it was possible to obtain also the amplification of this sample (Figure IV.2). Each agarose band was excised, purified and sequenced. However the first sequencing attempt gave negative results, because of too much baseline noises on the chromatograms (peaks uninterpretable) or failed reactions (as shown in the example Figure IV.3). This prevented the possibility to design the gRNAs onto the nucleic sequences obtained from the sequencing.

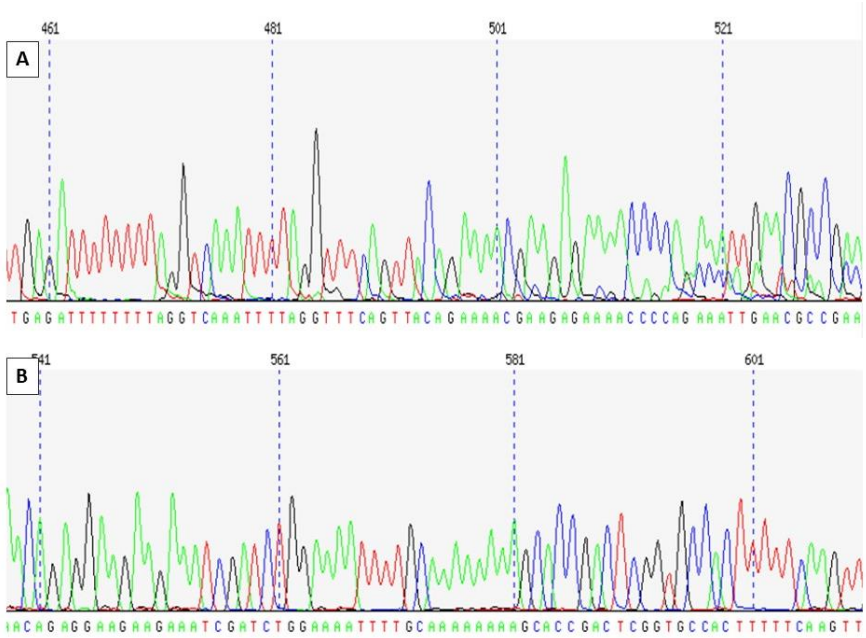


Figure IV.3 Chromatogram of the sample 2.14 A) and 2.13 B) obtained by Sanger sequencing of PCR purified products. The presence of baseline noise makes it impossible to determine the real nucleotide associated to each peak.

The obtainment of low-quality sequences can be due to the high homology subsisting among the genes of the STS family (as detailed in the section I.4). In grapevine, the STS multigenic family comprises 48 genes that share hundreds base pairs in the intragenic region (Vannozzi et al., 2012). To date, the works carried out have shown the complexity found in isolating the individual genes belonging to the STS family. This difficulty is such as to induce, in most cases, to use primers to identify candidate genes, specific for groups of two or three genes (Vannozzi et al., 2012; Chialva et al., 2018). Figure IV.4 shows a partial result of the colony PCR (only referred to the M4 genotype).

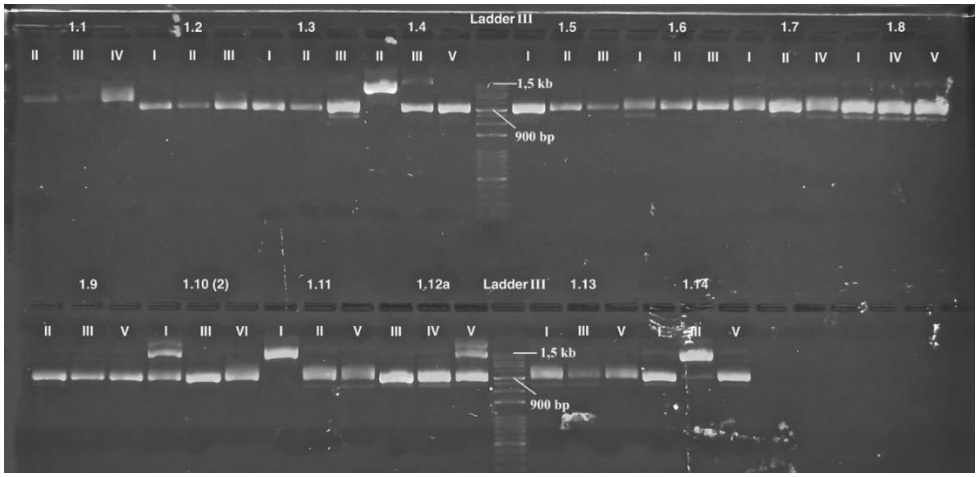


Figure IV.4 Electrophoretic analysis in 1.5% agarose gel of the colony-PCR products. The Arabic numerals refer to the ID samples reported in Table IV.2. The Roman numeral refers to the colony obtained from the cloning. For each sample, three selected colonies were analysed.

Positive clones were purified and sequenced. Also in this case, the identification of the nucleic sequences resulted unfinished, due to the dirty signal of the chromatograms obtained. In some cases, despite the improvement in the quality of the chromatograms (Figure IV.5), the presence of mismatches resulting from the alignment (obtained with ClustalW) with the reference sequence (Figure IV.6) did not allow to identify the exact nucleic sequences.

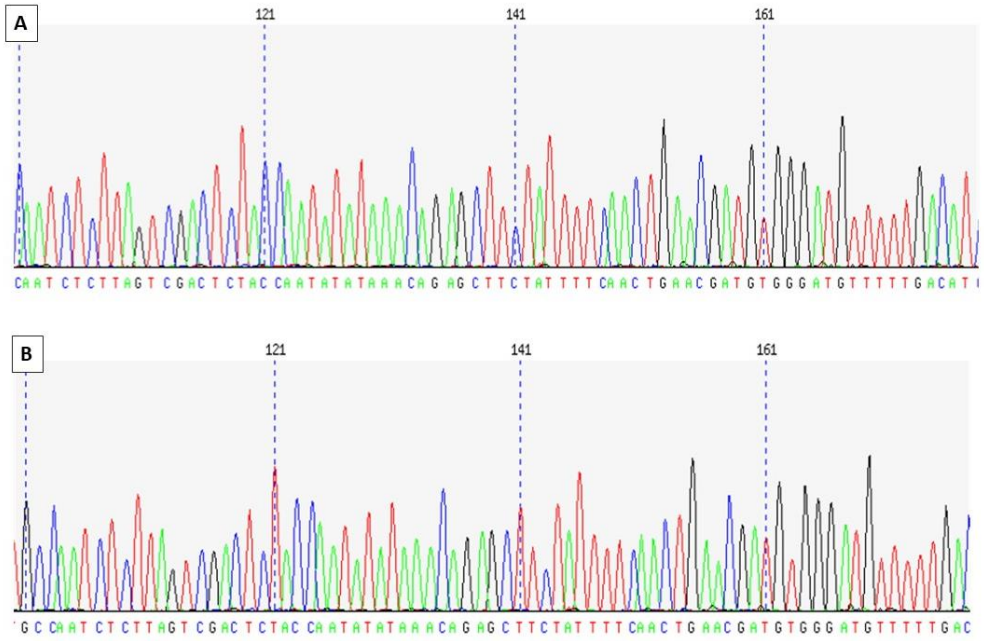


Figure IV.5 Chromatogram of the sample 1.8 A) and 2.8 B) obtained by Sanger sequencing of colony-PCR purified products. Each peak is described by a colour relative to the corresponding nucleotide. Its area and eight represent its quality. The real peaks are easy to call.

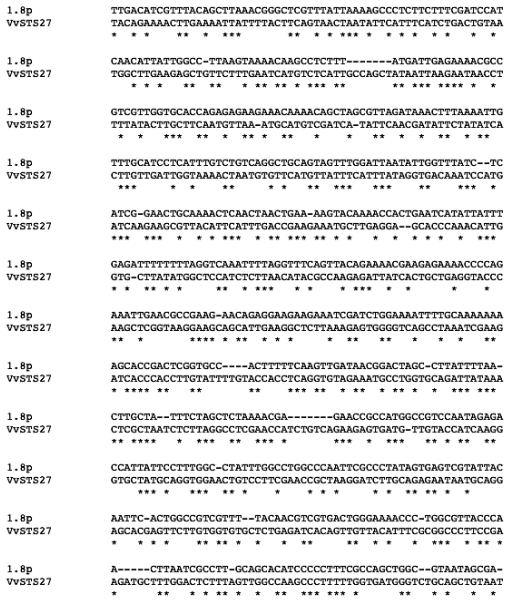


Figure IV.6 Result of multiple sequence alignment by ClustalW. The first line refers to the sequence obtained by Sanger sequencing referred to 1.8 sample. The second line refers to the corresponding sequence on the reference genome (PN40024). The asterisks (*) indicate a nucleotide correspondence between the two sequences. The dash (-) indicates a gap in the sequence due to deletions, insertions or missed reading.

In grapevine, VvSTSs genes are organised in multigenic family composed of 48 members, designated as VvSTS1 to VvSTS48. It includes at least 33 full-length coding genes, 8 pseudogenes and 7 sequences that remain to be resolved (Vannozzi et al., 2012). Few genes (VvSTS1-VvSTS6) are on the chr10, within an 80Kb region. The others (VvSTS7-VvSTS48) are located on chr16, in a region of 500 Kb. It shows numerous paralogue zones, either in coding or non-coding regions. It suggests multiple level of tandem and segmental duplication (Vannozzi et al., 2012). An analysis on the genome architecture of PN40024 line and its high-identity duplication content, showed that 85 Mb out of the 487 Mb comprising the grapevine genome is duplicated (Giannuzzi et al., 2011), and that chr16, containing the majority of VvSTS members, showing the highest percentage (25.08%) of segmental duplication among the assembled non-random chromosomes (Vannozzi et al., 2012). The high homology among the genes of STS multigenic family, did not allow to isolate them through their amplification and cloning. Even though the sequences were divided in three/four fragments in order to be amplified from specific primers manually designed (in correspondence of the few mismatches), it resulted impossible to isolate the genes of interest through their amplification. Probably, from the same PCR reaction, more than one sequence (not detectable through electrophoresis analysis) was amplified, highlighted by the baseline noise of the chromatograms. This could be associated to the low specificity of the primers. Even when cloning was performed and high-quality chromatograms

were obtained, the lack of correspondence between the sequence of the sample and the reference one, may be linked to the amplification of different genes.

The maintenance of duplicate genes involved in secondary metabolism, as well as involved in the response to exogenous stimuli, appears to be more and more frequently (Casneuf et al., 2006; Hanada et al., 2008; Keeling et al., 2008). The preservation of extra copies should provide a foundation for consolidation and refinement of established functions, particularly in secondary metabolism, and guarantee a gene reservoir for adaptive evolution (Chapman et al., 2006; Ha et al., 2007; Ober and Sharp, 2007). The fact that the majority of plant do not possess VvSTS gene(s) may suggest that the production of stilbenes did not confer an evolutionary advantage or, on the other hand, that the majority of species suffer for the production of compounds such as resveratrol, that, although related to benefits at low range of concentrations, is phytotoxic to plant cells at higher concentrations (Chang et al., 2011). On the contrary, grapevine keeps a large STS-gene reservoir maybe for the important role exerted by stilbenes, particularly by resveratrol, as suggested by Corso et al. (2015) in the case of M4 rootstock.

Given these results, the gRNAs to be inserted into the vector for the knock-out of VvSTSs candidate genes, were designed on grapevine reference sequence (PN40024). For this purpose, it was decided to design two vectors: the first, containing two gRNAs in common between the four candidate genes (called V_STS1); the second, containing two specific gRNAs for the VvSTS27 gene (named V_STS27) which was the mostly expressed in M4 during water stress (Corso et al., 2015). Figure IV.7 shows that the insertion of the gRNAs, into the plasmid (pCBC_DT1T2), occurred correctly.

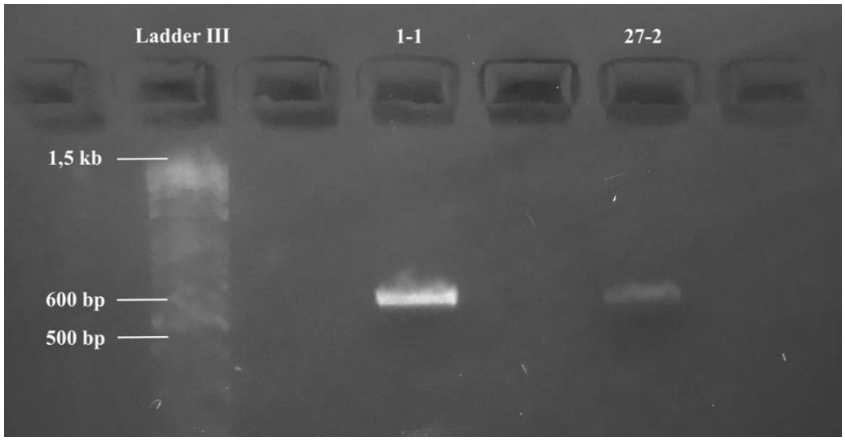


Figure IV.7 Electrophoretic analysis in 1.5% agarose gel of the gRNAs produced by PCR. The image shows the V_STS1 (on the left) and the V_STS27 (on the right) of the expected size, equal to 600 bp.

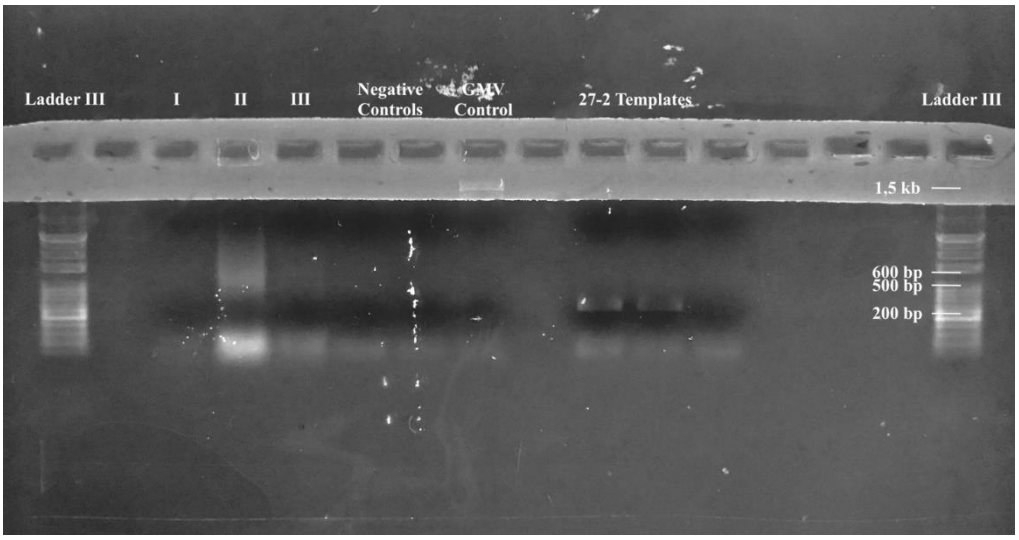


Figure IV.8 Electrophoretic analysis in 1.5% agarose gel of Golden Gate products. The bands refer to the pGMV-U-STS27 vector. The expected length is about 400-500 bp. PCRBIO Ladder III (PB40.13-01).

For checking if the ligation into pGMV-U occurred correctly, a PCR was performed. The electrophoresis analysis on agarose gel showed the expected length of the band, as shown by Figure IV.8.

A few numbers of *E. coli* colonies cloned with Geminivirus vectors (pGMV-U-gSTS1 and pGMV-U-gSTS27) grew on the selective medium (LB+kanamycin). Only three clones (I, II and III) transformed with pGMV-U-gSTS27 resulted positive to the colony-PCR reaction (Figure IV.9). Few clones transformed with pGMV-U-gSTS1 resulted positive to the colony-PCR reaction (figure not shown).

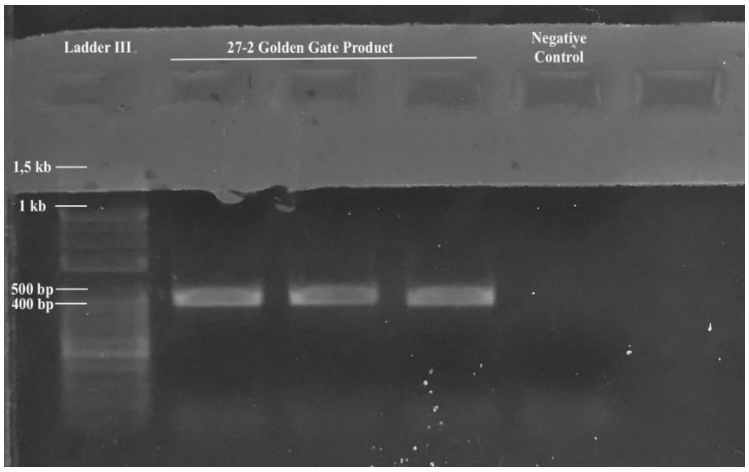


Figure IV.9 Electrophoretic analysis in 1% agarose gel of colony-PCR. The bands below the 200 bp line represent the primers. PCRBIO Ladder III (PB40.13-01).

All the pGMV-U-gSTS27 and pGMV-U-gSTS21 purified vectors were used to transform *A. tumefaciens* through electroporation. The growth of *A. tumefaciens* clones on LB selective substrate resulted positive. *Agrobacterium* clones harbouring pGMV-U-gSTS27 and pGMV-U-gSTS1 were used for testing gRNAs functionality through a transient assay.

The amplification of the target locus from M4 DNA, extracted from transient transformed leaf tissues, showed smaller bands than that showed by the wild-type DNA (Figure IV.10). This result confirmed that the modification of the genes for both designed constructs occurred and that the vector is suitable for grapevine editing.

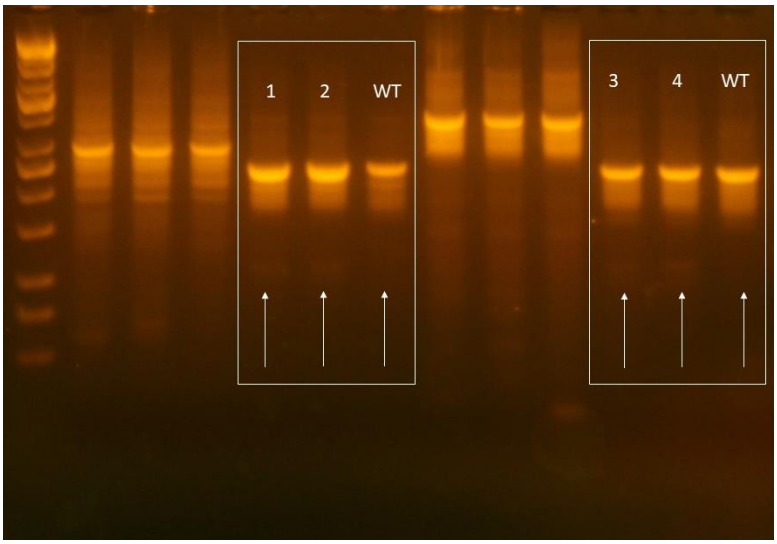


Figure IV.10 Amplification of the target locus in M4's leaf samples mutated, trough agro-infiltration. The edited fragments are indicated by the arrows. WT: M4 wild-type. 1, 2, 3, 4: M4 transformed.

IV.4 Conclusion

Grapevine is one of the few species possessing genes encoding for stilbene synthases enzymes. Apart from *Vitacea* family, these compounds have been detected in at least 72 unrelated species belong to different genera (31) and families (12). Most plant-stilbenes are derivatives of the basic unit *trans*-resveratrol (3,5,4'-trihydroxy-transstilbene). In addition to resveratrol, in grapevine, more complex compounds derived from its modification are also detected (such as *cis*- and *trans*-piceid, viniferins and piceatannol). It is the species that most of all contains the largest number of members comprising in the STS multigenic family, also present in extra copies in the genome (Vannozzi et al., 2012). Probably this can be linked to the important role assumed by resveratrol in the adaptative evolution. It has been shown that in roots of M4, during water stress, an accumulation of resveratrol occurs, due

to the up-regulation of mainly four genes (VvSTS16, 18, 27 and 29). In order to identify them, through a CRISPR/Cas9 approach aimed at their knock-out, we tried to isolate their nucleic sequences on which to design the gRNAs necessary for the gene editing. Given the high homology among VvSTS genes, the identification of their exact sequences resulted impossible to obtain, maybe due to the low-specificity of the primers.

Concerning the CRISPR/Cas9 trial we performed, we applied a method based on the use of DNA replicons able to express the CRISPR/Cas9 editing system and a paired-gRNAs strategy, as described by Miccono et al. (2018). One of the advantages provided by this system is that it permits the (re)generation of stably edited plant, without the necessity to integrate an exogen DNA permanently. On the contrary, the use of non-viral origin vector, carrying the CRISPR / Cas9 complex:gRNA(s), leads to the insertion of exogenous DNA in the host cells (represented by the Cas9), which makes their genome and of the whole organism unstable. This occurs because the elimination of the Cas9 happens only through plant crossing. This trial wants to confirm that the protocol described by Miccono et al. (2018) is efficient and highly-throughput. It is a great advantage for species as grapevine, for which the genetic improvement program developed up to now aim to preserve the genetic variability of the autochthonous germplasm.

General conclusions

The predicted changes in the climatic scenario represent a threat to the future of viticulture. One of the main constraints related to global warming will be the scarcity of water availability in many grape-growing countries. Even if grapevine is well adapted to arid and semi-arid environments, scarcity of water can affect negatively grapevine physiology, grape biochemistry and wine quality (Schultz, 2000; Mira de Orduña, 2010). The improvement of water management in vineyard and water utilization by plants are strategies to face drought. For this purpose, the choice of plant material is a key issue (Ollat et al., 2011). So far, the mechanisms of water stress adaptation focused on the role of rootstock have always aroused great interest. Less frequently the influence of scion on rootstock water stress reaction has been studied. In Chapter II (section II.1) the effect of grafting on rootstock behaviour during water shortage, both from physiological and transcriptome standpoints, was analysed. At this purpose, two rootstock genotypes, a susceptible (101-14) and a tolerant (1103 P) to water stress, own-rooted or grafted with Cabernet Sauvignon, were subjected to two water stress levels: mild (50% SWC) and severe (20% SWC). Their phenotypic and genetic responses were compared to those of their controls, maintained at 80% SWC during the entire experiment. It was demonstrated that rootstock has a preponderant role in perceiving drought, being roots in contact with soil water availability. At the same time scion is able to modulate gas exchanges during the stress. 1103 P/CS showed a pessimistic behaviour preserving its water status under drought conditions in order to have resources in the future. Based on the transcriptome evidence, 1103 P reacted to water stress more actively than 101-14, especially at root level, displaying an enhanced capacity to produce hormone water-stress signals (ABA), ROS scavenging compounds (flavonoids and stilbenes metabolites), as well as isoprenoids and phenylpropanoids, and osmolyte compounds. The scion appeared to delay the perception of water shortage by rootstock and 1103 P showed a higher reactiveness than 101-14 during water deficiency. Rootstock genotypes do not react in the same way to water shortage and they are classified as highly tolerant, less tolerant and susceptible (Flexas et al., 2009; Corso and Bonghi, 2014; Migliaro et al., 2019). The novel M-rootstocks, developed by DiSAA research group operating at Milan University, showed different behaviour, from highly to mildly tolerant (M4 > M1 = M3 > M2). The understand of rootstock performance during drought is useful for predicting the whole-plant behaviour in water scarcity condition, thus, to address the choice of the grape grower to the best rootstock to be used. The M-rootstocks (M1, M3, and M4), which demonstrated to have good characteristics with respect to different abiotic stresses, were characterized in terms of water stress response through a comparison with some commercial genotypes largely used in viticulture, with different capacity to face the stress (Chapter II, section II.2). Since the mild level

of stress (50% SWC) a transcriptional reaction such that differentiate the sampes under study occurred, while for having physiological discrimination, more severe water stress needed. Among M-rootstocks, M4 confirmed to be drought-tolerant while M1 and M3 were less tolerant to the scarcity of water.

The aptitude of M4 rootstock to face water stress was demonstrated by several authors (Meggio et al., 2014; Corso et al., 2015; Prinsi et al., 2018). Among the putative genes of M4 better adaptation to water stress (Corso et al., 2016), four VvSTS genes (VvSTS16, 18, 27 and 29), responsible for the higher accumulation of resveratrol in M4 roots were studied. Chapter IV (section IV.3) reports the first steps of a CRISPR/Cas9 approach aimed at the knock-out of them. Due to the high homology among the VvSTS genes, their exact nucleic sequence was impossible to obtain. Nonetheless, using the grapevine reference genome (PN40024) to design the gRNAs, it was developed a vector based on the use of DNA replicons able to express the CRISPR/Cas9 editing system and a paired-gRNAs strategy, as described by Miccono et al. (2018). A transient assay, performed on M4 micropropagated plantlets, to check the gRNAs functionality was also described. The assay was made on micropropagated M4 plantlets, obtained by *in vitro* culture techniques. Micropropagation is considered an efficient method of rapid mass propagation and regeneration through organogenesis and embryogenesis (Stamp et al. 1990). For having success, a reliable and high-throughput protocol must be set-up (Chapter III). For this purpose, different micropropagation media for M4 propagation and *in vitro* characterization were compared. Moreover, pro-embryogenic and embryogenic calli from leaf tissues and bud explants were obtained, comparing different protocols for type and quantity of hormones. This goes hand in hand with the boost that the genetic improvement of plants through the New Breeding Techniques (NBTs) is assuming, making use of cellular material genetically manipulable, such as embryogenic calluses. The breeding of new rootstock genotypes better adapted to the climate fluctuations, either with crossbreeding programs or with the genetic improvement, may be considered long-term strategies able to face with the climate changes.

References

Aldwinckle, H.S., 1980. In vitro techniques for studying obligate pathogens of Vitis., in: Proceed. 3rd Intern. Symp. Grape Breeding. Davis (California), pp. 87–91.

Alexa, A., Rahnenfuhrer, J.., 2016. topGO: Enrichment Analysis for Gene Ontology. R package version 2.24.0.

Alizadeh, M., Singh, S.K., Patel, V., 2010. Comparative performance of in vitro multiplication in four grape (Vitis spp.) rootstock genotypes. Int. J. Plant Prod. 4, 1735–6814.

Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E., Schroeder, J.I., 2001. A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 411, 1053–1057. https://doi.org/10.1038/35082575

Alleweldt, G., Possingham, J. V, 1988. Progress in grapevine breeding. Theor. Appl. Genet. 75, 669–673. https://doi.org/10.1007/BF00265585

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373– 399. https://doi.org/10.1146/annurev.arplant.55.031903.141701

Arrigo, N., Arnold, C., 2007. Naturalised Vitis rootstocks in Europe and consequences to native wild grapevine. PLoS One 2 e521. https://doi.org/http://dx.doi.org/10.1371/journal.pone.0000521.

Baltes, N., Gil-Humanes, J., Čermák, T., Atkins, P., Voytas, D., 2014. DNA replicons for plant genome engineering. Plant Cell 26, 151–163. https://doi.org/10.1105/tpc.113.119792

Banilas, G., Korkas, E., 2007. Rapid micro-propagation of grapevine (cv. Agiorgitiko) through lateral bud development. E-J. Sci. Tech. 2, 31–38.

Bardou, P., Mariette, J., Escudié, F., Djemiel, C., Klopp, C., 2014. Jvenn: An interactive Venn diagram viewer. BMC Bioinformatics 15, 1–11. https://doi.org/10.1186/1471-2105-15-293

Barlass, M., Miller, R., Antcliff, A.J., 1986. Development of methods for screening grapevines for resistance to infection by downy mildew. I. Dual culture in vitro. 37, 61-66.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science (80-.). 315, 1709 LP – 1712. https://doi.org/10.1126/science.1138140

Barrangou, R., Horvath, P., 2017. A decade of discovery: CRISPR functions and applications. Nat. Microbiol. 2, 1–9. https://doi.org/10.1038/nmicrobiol.2017.92

Barthakur, S., Babu, V., Bansal, K.C., 2001. Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. J. Plant Biochem. Biotechnol. 10, 31–37. https://doi.org/10.1007/BF03263103

Bavaresco, L., Fregoni, M., Fraschini, P., 1991. Investigations on iron uptake and reduction by excised roots of different grapevine rootstocks and a V. vinifera cultivar. Plant Soil 130, 109–113.

Bavaresco, L., Gardiman, M., Brancadoro, L., Espen, L., Failla, O., Scienza, A., Vezzulli, S., Zulini, L., Velasco, R., Stefanini, M., Di Gaspero, G., 2015. Grapevine breeding programs in Italy., in: Reynolds, A. (Ed.), Grapevine Breeding Programs for the Wine Industry. Elsevier, Cambridge, pp. 135–155.

Bavaresco, L., Walker, M.A., 1994. Techniques for successfully establishing Xiphinema index in dual culture with grape.No Title. Am. J. Enol. Vitic 45, 273–277.

Bavaresco, L.B., Regoni, M.F., Revisan, M.T., Attivi, F.M., Rhovsek, U. V, Alchetti, R.F., 2002. The occurrence of the stilbene piceatannol in grapes 41, 3–6.

Belhadj, A., Telef, N., Cluset, S., Bouscaut, J., Corio-Costet, M., Merillon, J., 2008. Ethephon elicits protection against Erisyphe necator in grapevine. J Agric Food Chem 56, 5781–5787.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Nekrasov, V., 2013. Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9, 39. https://doi.org/10.1186/1746-4811-9-39

Benheim, D., Rochfort, S., Robertson, E., Potter, I.D., Powell, K.S., 2012. Grape phylloxera (Daktulosphaira vitifoliae) - A review of potential detection and alternative management options. Ann. Appl. Biol. 161, 91–115. https://doi.org/10.1111/j.1744-7348.2012.00561.x

Benz, M.J., Anderson, M.M., Williams, M.A., Wolpert, J.A., 2007. Viticultural performance of three Malbec clones on two rootstocks in Oakville, Napa valley, California. Am. J. Enol. Vitic 58, 262–267.

Bianchi, D., Grossi, D., Tincani, D.T.G., Simone Di Lorenzo, G., Brancadoro, L., Rustioni, L., 2018. Multi-parameter characterization of water stress tolerance in Vitis hybrids for new rootstock selection. Plant Physiol. Biochem. https://doi.org/10.1016/j.plaphy.2018.09.018

Bogdanove, A.J., Voytas, D.F., 2011. TAL Effectors: Customizable Proteins for DNA Targeting. Science (80-.). 333, 1843 LP – 1846. https://doi.org/10.1126/science.1204094

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Boneh, U., Biton, I., Zheng, C., Schwartz, A., Ben-Ari, G., 2012. Characterization of potential ABA receptors in Vitis vinifera. Plant Cell Rep. 31, 311–321. https://doi.org/10.1007/s00299-011-1166-z

Borie, B., Jeandet, P., Parize, A., Bessis, R., Adrian, M., 2004. Resveratrol and stilbene synthase mRNA production in grapevine leaves treated with biotic and abiotic phytoalexin elicitors. Amer J Enol Vitic 55, 60–64.

Bortesi, L., Fischer, R., 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 33, 41–52.

Bouquet, A., Torregrosa, L., 2003. Micropropagation of the grapevine (Vitis spp.), in: Jain, S.M., Ishii, K. (Eds.), Micropropagation of Woody Trees and Fruits. Springer, Dordrecht, pp. 319–352.

Bowers, J., Boursiquot, J.M., This, P., Chu, K., Johansson, H., Meredith, C., 1999. Historical genetics: the parentage of Chardonnay, Gamay, and other wine grapes of northeastern France. Science (80-.). 285, 1562–1565.

Bray, E.A., 1997. Plant responses to water deficit. Trends Plant Sci. 2, 48–54. https://doi.org/10.1016/S1360-1385(97)82562-9

Brink, R.., 1962. Phase Change in Higher Plants and Somatic Cell Heredity. Q. Rev. Biol. 37, 1–22. https://doi.org/10.1086/403567

Cadavid-Labrada, A., Medina, C., Martinelli, L., Arce-Jhonson, P., 2008. Somatic embryogenesis and efficient regeneration of Vitis vinifera L . ' Carménère ' plants. Vitis 47, 73–74.

Campbell, P., Braam, J., 1999. Diversity of Genes , Enzymes and Potential Wall-Modifying Functions. Trends Plant Sci. 4, 361–366. https://doi.org/10.1016/S1360-1385(99)01468-5

Casneuf, T., De Bodt, S., Raes, J., Maere, S., Van de Peer, Y., 2006. Nonrandom divergence of gene expression following gene and genome duplications in the flowering plant Arabidopsis thaliana. Genome Biol. 7, R13–R13. https://doi.org/10.1186/gb-2006-7-2-r13

Čermák, T., Baltes, N., Čegan, R., Zhang, Y., Voytas, D., 2015. High-frequency, precise modification of the tomato genome. Genome Biol. 16, 232–245. https://doi.org/10.1186/s13059-015-0796-9

Chan, Z., 2012. Expression profiling of ABA pathway transcripts indicates crosstalk between abiotic and biotic stress responses in Arabidopsis. Genomics 100, 110–115.

Chang, X., Heene, E., Qiao, F., Nick, P., 2011. The phytoalexin resveratrol regulates the initiation of hypersensitive cell death in Vitis cell. PLoS One 6, e26405–e26405. https://doi.org/10.1371/journal.pone.0026405

Chapman, B.A., Bowers, J.E., Feltus, F.A., Paterson, A.H., 2006. Buffering of crucial functions by paleologous duplicated genes may contribute cyclicality to angiosperm genome duplication. Proc. Natl. Acad. Sci. U. S. A. 103, 2730–2735. https://doi.org/10.1073/pnas.0507782103

Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant responses to drought — from genes to the whole plant. Funct. Plant Biol. 30, 239. https://doi.org/10.1071/fp02076

Chaves, M.M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osorio, M.L., Carvalho, I., Faria, T., Pinheiro, C., 2002. How Plants Cope with Water Stress in the Field . Photosynthesis and Growth. Ann. Bot. 89, 907–916. https://doi.org/10.1093/aob/mcf105

Chaves, M.M., Zarrouk, O., Francisco, R., Costa, J.M., Santos, T., Regalado, A.P., Rodrigues, M.L., Lopes, C.M., 2010. Grapevine under deficit irrigation: hints from physiological and molecular data. Ann. Bot. 105, 661–676. https://doi.org/10.1093/aob/mcq030

Chen, Q., He, J., Phoolcharoen, W., Mason, H.S., 2011. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. Hum. Vaccin. 7, 331–338. https://doi.org/10.4161/hv.7.3.14262

Chialva, C., Muñoz, C., M, M., Eichler, E., Calderón, L., Prieto, H., Lijavetzky, D., 2018. Differential expression patterns within the grapevine stilbene synthase gene family revealed through their regulatory regions. Plant Mol Biol Rep. https://doi.org/10.1007/s11105-018-1073-3

Clingeleffer, P.R., Emmanuelli, D.R., 2006. An assessment of rootstocks for Sunmuscat (Vitis vinifera L.): a new drying variety. Aust. J. Grape Wine Res. 12, 135–140.

Comas, L.H., Becker, S.R., Cruz, V.M. V., Byrne, P.F., Dierig, D.A., 2013. Root traits contributing to plant productivity under drought. Front. Plant Sci. 4, 1–16. https://doi.org/10.3389/fpls.2013.00442

Cooke, T., Racusen, R., Cohen, J., 1993. The role of auxin in plant embryogenesis. Plant Cell 5, 1494–1495.

Cordeau, J., 2002. Création d'un vignoble, in Greffage de la Vigne et Porte-greffes. Élimination des Maladies à Virus, Édition Fé. ed. Bordeaux, France.

Corso, M., Bonghi, C., 2014. Grapevine rootstock effects on abiotic stress tolerance. Plant Sci. Today 1, 108–113. https://doi.org/10.14719/pst.2014.1.3.64

Corso, M., Vannozzi, A., Maza, E., Vitulo, N., Meggio, F., Pitacco, A., Telatin, A., D'Angelo, M., Feltrin, E., Negri, A.S., 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. J. Exp. Bot. 66, 5739–5752. https://doi.org/10.1093/jxb/erv274

Cramer, G.R., 2010. Abiotic stress and plant responses from the whole vine. Aust. J. Grape Wine Res. 16, 86–93. https://doi.org/10.1111/j.1755-0238.2009.00058.x

Cristea, S., Freyvert, Y., Santiago, Y., Holmes, M.C., Urnov, F.D., Gregory, P.D., Cost, G.J., 2013. In vivo cleavage of transgene donors promotes nuclease-mediated targeted integration. Biotechnol. Bioeng. 110, 871–880. https://doi.org/10.1002/bit.24733

D'Onofrio, C., Cox, A., Davies, C., Boss, P., 2009. Induction of secondary metabolism in grape cell cultures by jasmonates. Funct Plant Biol 36, 323–338.

Dai, A., 2013. Increasing drought under global warming in observations and models. Nat. Clim. Chang. 3, 52–58. https://doi.org/10.1038/nclimate1633

Dai, Z.W., Léon, C., Feil, R., Lunn, J.E., Delrot, S., Gomès, E., 2013. Metabolic profiling reveals coordinated switches in primary carbohydrate metabolism in grape berry (Vitis vinifera L.), a non-climacteric fleshy fruit. J. Exp. Bot. 64, 1345–1355. https://doi.org/10.1093/jxb/ers396

Dal Santo, S., Palliotti, A., Zenoni, S., Tornielli, G.B., Fasoli, M., Paci, P., Tombesi, S., Frioni, T., Silvestroni, O., Bellincontro, A., Onofrio, C., Matarese, F., Gatti, M., Poni, S., Pezzotti, M., 2016. Distinct transcriptome responses to water limitation in isohydric and anisohydric grapevine cultivars. BMC Genomics 17, 1–19. https://doi.org/10.1186/s12864-016-3136-x

Das, K., Roychoudhury, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2, 1–13. https://doi.org/10.3389/fenvs.2014.00053

De Lorenzis, G., Rustioni, L., Parisi, S.G., Zoli, F., Brancadoro, L., 2016. Anthocyanin biosynthesis during berry development in corvina grape. Sci. Hortic. (Amsterdam). 212, 74–80.

De Smet, I., Zhang, H., 2013. Abscisic acid in root growth and development., in: Eshel A. & Beeckman T. (Ed.), Plant

Roots : The Hidden Half. CRC Press., Baton Rouge, FL, USA, pp. 16.1–16.13.

De Zelicourt, A., Colcombet, J., Hirt, H., 2016. The Role of MAPK Modules and ABA during Abiotic Stress Signaling. Trends Plant Sci. 21, 677–685. https://doi.org/10.1016/j.tplants.2016.04.004

Debnath, M., Malik, C.P., Bisen, P.S., 2006. Micropropagation : A Tool for the Production of High Quality Plant-based Micropropagation : A Tool for the Production of High Quality Plant-based Medicines. Curr. Pharm. Biotechnol. 7, 33–49. https://doi.org/10.2174/138920106775789638

Dhekney, S.., Li, Z.T., Zimmerman, T.W., Gray, D.J., 2009. Factors influencing genetic and plant regeneration of Vitis. Amer.transformation J. Enol. Viticult. 60, 285–292.

Diab, A.., Khalil, S.., Ismail, R.., 2011. Regeneration and Micropropagation of Grapevine (Vitis vinifera L .) through Shoot Tips and Axillary Buds regeneration and micropropagation of grapevine (vitis vinifera l .) through shoot tips. Int. J. Adv. Biotechnol. Res. 2, 484–491.

Dong, Y., Fan, G., Deng, M., Xu, E., Zhao, Z., 2014. Genome-wide expression profiling of the transcriptomes of four Paulownia tomentosa accessions in response to drought. Genomics 104, 295–305. https://doi.org/10.1016/j.ygeno.2014.08.008

Duchêne, E., Schneider, C., 2005. Grapevine and climatic changes: a glance at the situation in Alsace. Agron. Sustain. Dev. 25, 93–99.

EPPO, 1990. Data sheets on quarantine pests: Viteus vitifoliae.

Ezzahouani, A., Williams, L.E., 2005. Performance of Dattier de Beyrouth and Alphonse Lavallée grapevines on eight rootstocks under dry-land conditions. J. Int. Sci. Vigne Vin 39, 91–94.

Ezzahouani, A., Williams, L.E., 1995. The influence of rootstock on leaf water potential, yield, and berry composition of Ruby Seedless grapevines. Am. J. Enol. Vitic. 46, 559–563.

Fang, L., Zhao, F., Cong, Y., Sang, X., Du, Q., Wang, D., Li, Y., Ling, Y., Yang, Z., He, G., 2012. Rolling-leaf14 is a 2OG-Fe (II) oxygenase family protein that modulates rice leaf rolling by affecting secondary cell wall formation in leaves. Plant Biotechnol. J. 10, 524–532. https://doi.org/10.1111/j.1467-7652.2012.00679.x

Farquhar, G.D., Sharkey, T.D., 1982. Stomatal conductance and photosynthesis. Ann. Rev. Plant Biol. 61, 561–591.

Fini, A., Brunetti, C., Ferdinando, M. Di, Ferrini, F., Tattini, M., 2011. Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. Plant Signal. Behav. 6, 709–711. https://doi.org/10.4161/psb.6.5.15069

Flexas, J., Barón, M., Bota, J., Ducruet, J.-M., Gallé, A., Galmés, J., Jiménez, M., Pou, A., Ribas-Carbó, M., Sajnani, C., Tomàs, M., Medrano, H., 2009. Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted Vitis hybrid Richter-110 (V. berlandieri×V. rupestris). J. Exp. Bot. 60, 2361–2377. https://doi.org/10.1093/jxb/erp069

Flexas, J., Galmés, J., Gallé, A., et al., 2010. Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. Aust. J. Grape Wine Res. 16, 106–121.

Foëx, G., 1902. Manual of Modern Viticulture: Reconstitution with American Vines. Robert. S. Brain, Government Printer, Melbourne, Australia.

Forneck, A., Walker, M.A., Merkt, N., 1996. Aseptic dual culture of grape (Vilis spp.) and grape phylloxera (Daktulosphaira vitifoliae Fitch). Vitis 35, 95–97.

Forni, G., 2012. The origin of "Old World" viticulture, in: Maghradze, D., Rustioni, L., Scienza, A., Turok, J., Failla, O. (Eds.), Caucasus and Northern Black Sea Region Ampelography. Julius-Kühn-Institut, Siebeldingen, Germany, pp. 27–38.

Francis, D., Sorrell, D., 2001. The interference between the cell cycle and plant growth regulators: a mini review. Plant Growth Regul. 33, 1–12.

Franks, T., He, D.G., Thomas, M., 1998. Regeneration of transgenic Vitis vinifera L. Sultana plants: Genotypic and phenotypic analysis. Mol Breed. 4, 321–333.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. 31, 822–826. https://doi.org/10.1038/nbt.2623

Fujii, H., Verslues, P.E., Zhu, J.K., 2011. Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses in vivo. Proc. Natl. Acad. Sci. U. S. A. 108, 1717–1722. https://doi.org/10.1073/pnas.1018367108

Fujita, A., Soma, N., Goto-yamamoto, N., Mizuno, A., Kiso, K., Hashizume, K., 2007. Effect of Shading on Proanthocyanidin Biosynthesis in the Grape Berry 76, 112–119.

Fukuda, H., 2014. Plant Cell Wall Patterning and Cell Shape, Plant Cell Wall Patterning and Cell Shape. Wiley Blackwell, Hoboken. https://doi.org/10.1002/9781118647363

Gaj, M.D., 2004. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to Arabidopsis thaliana (L.). Heynh. Plant Growth Regul. 43, 27–47.

Gale, G., 2002. Saving the vine from Phylloxera : a never-ending battle, in: Sandler and R. Pindler (Ed.), Wine: A Scientifc Exploration. Taylor and Francis, London, pp. 70–91. https://doi.org/10.1201/9780203361382.ch4

Galet, P., 2000. Dictionnaire encyclopédique des cépages. Paris, France.

Galet, P., 1998. Grape varieties and rootstock Varieties. Oenoplurimedia, Chaintré, France.

Galet, P., 1990. Cépages et vignobles de France. Tome2: L'ampélographie Française, 2nd ed. Imprimerie Déhan, Montpellier, France.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158.

Garcia, M., Ibrahim, H., Gallego, P., Puig, P., 2001. Effect of Three Rootstocks on Grapevine (Vilis vinifera L .) cv . Negrette, Grown Hydroponically . II . Acidity of Musts and Wines. S. Afr. J. Enol. Vitic 22, 101–103.

Gardner, C.M.K., Robinson, D., Blyth, K., Cooper, J.D., 2001. Soil Water Content, in: Smith, K.A., Mullins, C.E. (Eds.), Soil and Environmental Analysis: Physical Methods. Marcel Dekker Inc., New York,NY, pp. 13–20.

Gasiunas, G., Barrangou, R., Horvath, P., Siksnys, V., 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl. Acad. Sci. U. S. A. 109, 2579–2586. https://doi.org/10.1073/pnas.1208507109

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. https://doi.org/10.1021/jf8017707

Giannuzzi, G., D'Addabbo, P., Gasparro, M., Martinelli, M., Carelli, F., Antonacci, D., Ventura, M., 2011. Analysis of high-identity segmental duplications in the grapevine genome. BMC Genomics 12, 436.

Giorgi, F., Lionello, P., 2008. Climate change projections for the Mediterranean region. Glob. Planet. Change 63, 90–104.

Gleba, Y., Marillonnet, S., Klimyuk, V., 2004. Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. Curr Opin Plant Biol 7, 182–188.

Gong, H., Blackmore, D., Clingeleffer, P., Sykes, S., Jha, D., Tester, M., Walker, R., 2011. Contrast in chloride exclusion between two grapevine genotypes and its variation in their hybrid progeny. J. Exp. Bot. 62, 989–999. https://doi.org/10.1093/jxb/erq326

Gonzalez-Guzman, M., Pizzio, G.A., Antoni, R., Vera-Sirera, F., Merilo, E., Bassel, G.W., Fernández, Maria A Holdsworth, M.J., Perez-Amador, Miguel Angel Kollist, H., Rodriguez, P.L., 2012. Arabidopsis PYR/PYL/RCAR Receptors Play a Major Role in Quantitative Regulation of Stomatal Aperture and Transcriptional Response to Abscisic Acid. Plant Cell 24, 2483–2496. https://doi.org/10.1105/tpc.112.098574

Grimmig, B., Gonzalez-Perez, MN Leubner-Metzger, G Vogeli-Lange, R., Meins, F., Hain, R., Penuelas, J., Heidenreich, B., Langebar-Tels, C., Ernst, D., Sandermann, H., 2003. Ozone-induced gene expression occurs via ethylene-depedent and -independent signalling. Plant Mol Biol 51, 599–607.

Grzegorczyk, W., Walker, M.A., 1998. Evaluating resistance to grape Phylloxera in Vitis species with an in vitro dual culture assay. Am. J. Enol. Vitic. 49, 17–22.

Ha, M., Li, W.-H., Chen, Z.J., 2007. External factors accelerate expression divergence between duplicate genes. Trends Genet. 23, 162–166. https://doi.org/10.1016/j.tig.2007.02.005

Haberlandt, G., 1902. Culturversuehe mit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. Math.-Naturwiss. Kl. Abt.

Haider, M.S., Zhang, Cheng, Kurjogi, M.M., Pervaiz, T., Zheng, T., Zhang, Chaobo, Lide, C., Shangguan, L., Fang, J., 2017. Insights into grapevine defense response against drought as revealed by biochemical, physiological and RNA-Seq analysis. Sci. Rep. 7, 13134. https://doi.org/10.1038/s41598-017-13464-3

Hanada, K., Zou, C., Lehti-Shiu, M.D., Shinozaki, K., Shiu, S.-H., 2008. Importance of lineage-specific expansion of

plant tandem duplicates in the adaptive response to environmental stimuli. Plant Physiol. 148, 993–1003. https://doi.org/10.1104/pp.108.122457

Hanley-Bowdoin, L., Bejarano, E., Robertson, D., Mansoor, S., 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. Nat. Rev Microbiol 11, 777–788.

Hartung, W., Sauter, A., Hose, E., 2002. Abscisic acid in the xylem: Where does it come from, where does it go to? J. Exp. Bot. 53, 27–32. https://doi.org/10.1093/jxb/53.366.27

Hawker, J.S., Downton, W.J.S., Wiskich, D., Mullins, M.G., 1973. Callus and cell culture from grape berries. HortScience 8, 398–399.

Heloir, M., Fournioux, J., Oziol, L., Bessis, R., 1997. An improved procedure for the propagation in vitro of grapevine (Vitis vinifera cv. Pinot noir) using axillary-bud microcuttings. Plant Cell Tissue Organ Cult. 49, 223–225.

Hicks, G., Dorey, M., Chiarot, M., Murray, I., 1986. Propagation in vitro of “Michurinetz” grapes. Proc. N.S. Inst. Sci 36, 83–89.

Holl, J., Vannozzi, A., Czemmel, S., D’Onofrio, C., Walker, A.R., Rausch, T., Lucchin, M., Boss, P.K., Dry, I.B., Bogs, J., 2013. The R2R3-MYB Transcription Factors MYB14 and MYB15 Regulate Stilbene Biosynthesis in Vitis vinifera. Plant Cell 25, 4135–4149. https://doi.org/10.1105/tpc.113.117127

Holzapfel, B.P., Treeby, M.T., 2007. Effects of timing and rate of N supply on leaf nitrogen status, grape yield and juice composition from Shiraz grapevines grafted to one of three different rootstocks. Aust. J. Grape Wine Res. 13, 14–22. https://doi.org/10.1111/j.1755-0238.2007.tb00067.x

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G., Zhang, F., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827.

Huang, L., Zhang, S., Singer, S.D., Yin, X., Yang, J., Wang, Y., Wang, X., 2016. Expression of the Grape VqSTS21 Gene in Arabidopsis Confers Resistance to Osmotic Stress and Biotrophic Pathogens but Not Botrytis cinerea. Front. Plant Sci. 7, 1379. https://doi.org/10.3389/fpls.2016.01379

Huang, Z., Ough, C.S., 1989. Effect of vineyard locations, varieties, and rootstocks on the juice amino acid composition of several cultivars. Am. J. Enol. Vitic. 40, 135–139.

Huglin, P., 1958. Recherche sur les bourgeons de la vigne. Initiation florale et développement végétatif. Ann. Amélior. Plantes 8, 113–272.

Huglin, P., Schneider, C., 1998. Biologie et Écologie de la Vigne. Paris.

Hunt, H.V., Lawes, M.C., Bower, M.A. et al., 2010. A banned variety was the mother of several major wine grapes. A. Biol. Lett. 6, 367–369.

Iacono, F., Buccella, A., Peterlunger, E., 1998. Water stress and rootstock influence on leaf gas exchange of grafted and ungrafted grapevines. Sci. Hortic. (Amsterdam). https://doi.org/10.1016/S0304-4238(98)00113-7

Ikeuchi, M., Sugimoto, K., Iwase, A., 2013. Plant callus: mechanisms of induction and repression. Plant Cell 25, 3159–3173. https://doi.org/10.1105/tpc.113.116053

IPCC, 2018. Global warming of 1.5°C, in: Summary for Policymarkerskers. pp. 1–26.

IPCC, 2013. Climate Change 2013: The Physical Science Basis – Summary for Policymakers.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., 2001. Regulation of drought tolerance by gene manipulation of 9- cis -epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis 27, 325–333.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., et al., 2007. The rapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449, 463–467.

Jansen, R.K., Kaittanis, C., Saski, C., Lee, B., Tomkins, J., Alverson, A.J., Daniell, H., 2006. Phylogenetic analyses of Vitis (Vitaceae) based on complete chloroplast genome sequences : effects of taxon sampling and phylogenetic methods on resolving relationships among rosids 14, 1–14. https://doi.org/10.1186/1471-2148-6-32

Jeandet, P., Delaunois, B., Conreux, A., Donnez, D., Nuzzo, V., Cordelier, S., Clément, C., Courot, E., 2010. Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants. BioFactors 36, 331–341. https://doi.org/10.1002/biof.108

Jiang, F., Doudna, J.A., 2015. The Structural Biology of CRISPR-Cas Systems 100–111.

Jiménez, V., Guevara, E., Herrera, J., Bangerth, F., 2005. Evolution of endogenous hormone concentration in embryogenic cultures of carrot during early expression of somatic embryogenesis. Plant Cell Rep 23, 567–572.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science (80-.). 337, 816 LP – 821. https://doi.org/10.1126/science.1225829

Jones, A.M., 2016. A new look at stress: Abscisic acid patterns and dynamics at high-resolution. New Phytol. 210, 38–44. https://doi.org/10.1111/nph.13552

Jones, H., Sutherland, R., 1991. Stomatal control of xylem embolism. Plant, Cell Environ. 14, 607–612.

Jones, H.G., 1980. Interaction and integration of adaptive responses to water stress: the implications of an unpredictable environment, in: Turner, N., Kramer, P. (Eds.), Adaptation of Plants to Water and High Temperature Stress. Wiley-Interscience, New York, pp. 353–365.

Jones, M.A.P., Saxena, P.K., 2013. Inhibition of Phenylpropanoid Biosynthesis in Artemisia annua L .: A Novel Approach to Reduce Oxidative Browning in Plant Tissue Culture 8, 1–13. https://doi.org/10.1371/journal.pone.0076802

Jones, T.H., Cullis, B.R., Clingeleffer, P.R., Rühl, E.H., 2009. Effects of novel hybrid and traditional rootstocks on vigour and yield components of Shiraz grapevines. Aust. J. Grape Wine Res. 15, 284–292.

Karami, O., Saidi, A., 2010. The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol Biol

Rep. 37, 2493–2507.

Keeling, C.I., Weisshaar, S., Lin, R.P.C., Bohlmann, J., 2008. Functional plasticity of paralogous diterpene synthases involved in conifer defense. Proc. Natl. Acad. Sci. U. S. A. 105, 1085–1090. https://doi.org/10.1073/pnas.0709466105

Keller, M., 2015. Environmental costraints and stress physiology, in: Academic Press (Ed.), The Science of Grapevines Anatomy and Physiology. Washington, USA, pp. 268–329.

Keller, M., Kummer, M., Vasconcelos, M.C., 2001a. Soil nitrogen utilisation for growth and gas exchange by grapevines in response to nitrogen supply and rootstock. Aust. J. Grape Wine Res. 7, 2–11. https://doi.org/10.1111/j.1755-0238.2001.tb00187.x

Keller, M., Kummer, M., Vasconcelos, M.C., 2001b. Reproductive growth of grapevines in response to nitrogen supply and rootstock. Aust. J. Grape Wine Res. 7, 12–18. https://doi.org/10.1111/j.1755-0238.2001.tb00188.x

Klempka, K.., Meredith, E.P., Sail, M.A., 1984. Dual culture of grape powdery mildew (Uncinula necator Burr.) on its host (Vitis vinifera L.). Am. J. Enol. Vitic 35, 170–174.

Kobayashi, S., Goto-Yamamoto, N., Hirochika, H., 2004. Retrotransposon-induced mutations in grape skin color. Science (80-.). 304, 982.

Kocsis, L., Tarczal, E., Kàllay, M., 2012. The effect of rootstocks on the productivity and fruit composition of Vitis vinifera L. ‘Cabernet Sauvignon’ and ‘Kékfrankos.’ Acta Hortic. 931, 403–412.

Koundouras, S., Hatzidimitriou, E. Karamolegkou, M., Al., E., 2009. Irrigation and rootstock effects on the phenolic concentration and aroma potential of Vitis vinifera L. cv. Cabernet Sauvignon grapes. J. Agric. Food Chem. 57, 7805–7813.

Koundouras, S., Tsialtas, I., Zioziou, E., Nikolaou, N., 2008. Rootstock effects on the adaptive strategies of grapevine (Vitis vinifera L. cv. Cabernet-Sauvignon) under contrasting water status: Leaf physiological and structural responses. Agric. Ecosyst. Environ. 128, 86–96. https://doi.org/10.1016/j.agee.2008.05.006

Krasensky, J., Jonak, C., 2012. Drought , salt , and temperature stress-induced metabolic rearrangements and regulatory networks. J. Exp. Bot. 63, 1593–1608. https://doi.org/10.1093/jxb/err460.Drought

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., Sadh, R.K., 2016. Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6, 54. https://doi.org/10.1007/s13205-016-0389-7

Kuc, J., 1995. Phytoalexins, Stress Metabolism, and Disease Resistance in Plants. Annu. Rev. Phytopathol. 33, 275–297. https://doi.org/10.1146/annurev.py.33.090195.001423

Kühn, C., Grof, C., 2010. Sucrose transporters of higher plants. Curr. Opin. Plant Biol. 13, 287–297.

Kumar, D., Al Hassan, M., Naranjo, M.A., Agrawal, V., Boscaiu, M., Vicente, O., 2017. Effects of salinity and drought on growth, ionic relations, compatible solutes and activation of antioxidant systems in oleander (Nerium oleander L.). PLoS ONES ONE 12, 1–22. https://doi.org/10.1371/journal.pone.0185017

Langcake, P., 1981. Disease resistance of Vitis spp. and the production of stress metabolites resveratrol, e-viniferin, α-viniferin and pterostilbene. Physiol Plant Pathol 18, 213–226.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9, 357–359. https://doi.org/10.1038/nmeth.1923

Langner, T., Kamoun, S., Belhaj, K., 2018. CRISPR Crops: Plant Genome Editing Toward Disease Resistance. Annu. Rev. Phytopathol. 56, 479–512. https://doi.org/10.1146/annurev-phyto-080417-050158

Lanz, T., Tropf, S., Marners, F., Schroder, J., Schroder, G., 1991. The Role of Cysteines in Polyketide Synthases 266, 9971–9976.

Lazar, T., 2003. Taiz, L. and Zeiger, E. Plant physiology. 3rd edn. Ann. Bot. 91, 750–751. https://doi.org/10.1093/aob/mcg079

Lee, N., Wetzstein, H., 1988. Protoplast isolation and callus production from leaves of tissue-cultured Vitis spp. Plant Cell Rep. 7, 531–534.

Lee, T., Wicks, T., 1982. Dual culture of Plasmopara viticola and grapevine and its application to systemic fungicide evaluation. Plant Dis. 66, 308–310.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 1000 Genome Project Data Processing Subgroup, 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, R., Wang, J., Li, S., Zhang, L., Qi, C., Weeda, S., Zhao, B., Ren, S., Guo, Y.D., 2016. Plasma membrane intrinsic proteins SlPIP2;1, SlPIP2;7 and SlPIP2;5 conferring enhanced drought stress tolerance in Tomato. Sci. Rep. 6, 1–15. https://doi.org/10.1038/srep31814

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K., Chen, L., 2017. CRISPR-P 2.0: An improved CRISPR/Cas9 tool for genome editing in plants. Mol Plant 10, 530–532.

Liu, Q., Zhang, Y., Shouyi, C., 2000. Plant protein kinase genes induced by drought, high salt and cold stresses. Chinese Sci. Bull. 45, 1153–1157. https://doi.org/10.1007/BF02886068

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lopez-Péerez, A.J., Carreno, J., Martinez-Cutillas, A., Dabauza, M., 2005. High embryogenic ability and plant regeneration of table grapevine cultivars (Vitis vinifera L .) induced by activated charcoal 44, 79–85.

Loubser, J.T., Meyer, A.J., 1990. Dual cultures of Meloidogynejavanica and grapevine rootstocks on artificial media. S. Afr. J. Enol. Vitic 11, 42–45.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–23. https://doi.org/10.1186/s13059-014-0550-8

Lovisolo, C., Lavoie-Lamoureux, A., Tramontini, S., Ferrandino, A., 2016. Grapevine adaptations to water stress: new

perspectives about soil/plant interactions. Theor. Exp. Plant Physiol. 28, 53–66. https://doi.org/10.1007/s40626-016-0057-7

Lovisolo, C., Perrone, I., Carra, A., Ferrandino, A., Flexas, J., Medrano, H., Schubert, A., 2010. 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. https://doi.org/10.1071/FP09191

Lovisolo, C., Schubert, A., 1998. Effects of water stress on vessel size and xylem hydraulic conductivity in Vitis vinifera L. J. Exp. Bot. 49, 693–700. https://doi.org/10.1093/jexbot/49.321.693

Lusser, M., Davies, H. V., 2013. Comparative regulatory approaches for groups of new plant breeding techniques. N. Biotechnol. 30, 437–446. https://doi.org/10.1016/j.nbt.2013.02.004

Lusser, M., Parisi, C., Plan, D., Rodríguez-cerezo, E., 2012. Deployment of new biotechnologies in plant breeding. Nat. Biotechnol. 30, 231–239. https://doi.org/10.1515/bchm2.1963.333.1.127

Maeder, M.L., Thibodeau-beganny, S., Osiak, A., Wright, D.A., 2008. Rapid " Open-Source " Engineering of Customized Zinc-Finger Nucleases for Highly Efficient Gene Modification. Mol. Cell 31.

Maillot, P., Deglène-Benbrahim, L., Walter, B., 2016. Efficient somatic embryogenesis from meristematic explants in grapevine (Vitis vinifera L.) cv. Chardonnay: an improved protocol. Trees 30, 1377–1387.

Malnoy, M., Viola, R., Jung, M.-H., Koo, O.-J., Kim, S., Kim, J.-S., Velasco, R., Nagamangala Kanchiswamy, C., 2016. DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins. Front. Plant Sci. 7, 1904. https://doi.org/10.3389/fpls.2016.01904

Manty, F., 2005. Hintergrunde zur Entstehung der Bezeichnungen der Unterlagenselektionen von Sigmund Teleki und Franz Kober. Deutsches Weinbau-Jahrbuch 2006 57, 159–164.

Maresca, M., Lin, V.G., Guo, N., Yang, Y., 2013. Obligate ligation-gated recombination (ObLiGaRe): Custom-designed nuclease-mediated targeted integration through nonhomologous end joining. Genome Res. 23, 539–546. https://doi.org/10.1101/gr.145441.112

Marguerit, E., Brendel, O., Lebon, E., Van Leeuwen, C., Ollat, N., 2012. Rootstock control of scion transpiration and its acclimation to water deficit are controlled by different genes. New Phytol. 194, 416–429. https://doi.org/10.1111/j.1469-8137.2012.04059.x

Marrs, K.A., 1996. The Functions and Regulation of Glutathione S-Transferases in Plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–158. https://doi.org/10.1146/annurev.arplant.47.1.127

McCown, B.H., Lloyd, G., 1981. Woody Plant Medium (WPM)—A Mineral Nutrient Formulation for Microculture of Woody Plant Species. HortScience 16, 453–453.

McGovern, P.E., 2003. Ancient wine. The Search for the Origins of Viniculture. Princeton University Press.

Medrano, H., Tomás, M., Martorell, S., Flexas, J., Hernández, E., Rosselló, J., Pou, A., Escalona, J.M., Bota, J., 2015. From leaf to whole-plant water use efficiency (WUE) in complex canopies: Limitations of leaf WUE as a

selection target. Crop J. 3, 220–228. https://doi.org/10.1016/j.cj.2015.04.002

Meggio, F., Prinsi, B., Negri, A.S., Simone Di Lorenzo, G., Lucchini, G., Pitacco, A., Failla, O., Scienza, A., Cocucci, M., Espen, L., 2014. Biochemical and physiological responses of two grapevine rootstock genotypes to drought and salt treatments. Aust. J. Grape Wine Res. 20, 310–323. https://doi.org/10.1111/ajgw.12071

Melyan, G., Sahakyan, A., Harutyunyan, A., 2015. Micropropagation of grapevine (Vitis vinifera L.) seedless cultivar "Parvana" through lateral bud development. Vitis 54, 253–255.

Mhatre, M., Salunkhe, C., Rao, P., 2000. Micropropagation of Vitis vinifera L.: towards an improved protocol. Sci. Hort. 84, 357–363.

Miccono, M., Madrid, G., Aguirre, Carlos Olivares, Felipe Olmedo, B., Mora, R., Sánchez, E., Quiroz, D., Prieto, H., 2018. DNA replicon-mediated genome editing in grapevine using CRISPR/Cas9 and a paired gRNA strategy, in: Plant Biology 2018.

Migliaro, D., De Lorenzis, G., Simone Di Lorenzo, G., De Nardi, B., Gardiman, M., Failla, O., Brancadoro, L., Crespan, M., 2019. Grapevine non-vinifera genetic diversity assessed by SSR markers as a starting-point for new rootstock breeding programs. Am. J. Enol. Vitic. in press.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R., 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell Environ. 33, 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., Dulay, G.P., Hua, K.L., Ankoudinova, I., Cost, G.J., Urnov, F.D., Zhang, H.S., Holmes, M.C., Zhang, L., Gregory, P.D., Rebar, E.J., 2011. A TALE nuclease architecture for efficient genome editing. Nat. Biotechnol. 29, 143–148. https://doi.org/10.1038/nbt.1755

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410. https://doi.org/10.1016/S1360-1385(02)02312-9

Monteith, L., 1995. A reinterpretation of stomatal responses to humidity. Plant Cell Env. 18, 357–364. https://doi.org/doi: 10.1111/j.1365-3040.1995.tb00371.x

Mor, T., Moon, T., Palmer, K., Mason, H., 2003a. Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol Bioeng 81, 430–437.

Mor, T., Moon, T., Palmer, K., Mason, H., 2003b. Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol Bioeng 81, 430–437.

Morano, L.D., Walker, M.A., 1995. Soils and Plant Communities Associated with Three Vitis Species. Am. Midl. Nat. 134, 254–263. https://doi.org/10.2307/2426296

Morel, G., 1963. La culture in vitro du meristeme apical de certaines orchidees. C.R. Acad. Sci. Paris 256, 4955–4957.

Morel, G., 1944. Le développement du mildiou sur des tissus de vigne cultives in vitro. C. R. Hebd Seances Académie des Sci. 218, 50–52.

Morris, B., Richardson, K., Haley, A., Zhan, X., Thomas, J., 1992. The nucleotide sequence of the infectious cloned DNA component of Tobacco yellow dwarf virus reveals features of geminiviruses infecting monocotyledonous plants. Virology 187, 633–642.

Mullins, M., Bouquet, A., Williams, L.E., 1992. Biology of the Grapevine. Cambridge University Press, Great Britain.

Murashige, T., 1974. Plant propagation through tissue cultures. Annu. Rev. Plant. Physiol. 25, 135–166.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Mustilli, A.-C., Merlot, S., Vavasseur, A., Fenzi, F., Giraudat, J., 2002. Arabidopsis OST1 Protein Kinase Mediates the Regulation of Stomatal Aperture by Abscisic Acid and Acts Upstream of Reactive Oxygen Species Production. Plant Cell 14, 3089 LP – 3099. https://doi.org/10.1105/tpc.007906

Myles, S., Boyko, A.R., Owens, C.L., Brown, P.J., Grassi, F., Aradhya, M.K., 2011. Genetic structure and domestication history of the grape 108, 3530–3535. https://doi.org/10.1073/pnas.1009363108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1009363108

Myles, S., Chia, J., Hurwitz, B., Simon, C., Zhong, G.Y., Ware, D., 2010. Rapid Genomic Characterization of the Genus Vitis. PLoS One 5, e8219. https://doi.org/10.1371/journal.pone.0008219

Nakajima, I., Ban, Y., Azuma, A., Onoue, N., Moriguchi, T., Yamamoto, T., Toki, S., Endo, M., 2017. CRISPR/Cas9-mediated targeted mutagenesis in grape. PLoS One 12. https://doi.org/10.1371/journal.pone.0177966

Negrul, A.M., 1946. Origin of cultivated grapevine and its classification, in: Frolov-Bagree, A.M. (Ed.), Ampelography of the Soviet Union. “Pishchepromizdat,” Moscow, pp. 159–216.

Nguyen, Q.., Kozai, T., 1998. Environmental Effects on the Growth of Plantlets in Micropropagation. Environ. Control Biol. 36, 59–75.

Nitsch, J.P., Nitsch, C., 1969. Haploid Plants from Pollen Grains. Science (80-.). 163, 85 LP – 87. https://doi.org/10.1126/science.163.3862.85

Ober, E.S., Sharp, R.E., 2007. Regulation of root growth responses to water deficit, in: Jenks, M.A., Hasegawa, P.M., Jain, S.M. (Eds.), Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops. Springer Netherlands, Dordrecht, pp. 33–53. https://doi.org/10.1007/978-1-4020-5578-2_2

OIV, 2019. 2019 Statistical Report on World Vitiviniculture.

Ollat, N., Peccoux, A., Papura, D., Esmenjaud, D., Marguerit, E., Tandonnet, J.P., Bordenave, L., Cookson, S.J., Barrieu, F., Rossdeutsch, L., Lecourt, J., Lauvergeat, V., Vivin, P., Bert, P.F., Delrot, S., 2015. Rootstocks as a component of adaptation to environment, in: Gerós, H.V., Chaves, M.M., Gil, H.M., Delrot, S. (Eds.), Grapevine in a Changing Environment: A Molecular and Ecophysiological Perspective. Chichester, UK, pp. 68–94. https://doi.org/10.1002/9781118735985.ch4

Ollat, N., Tandonnet, J.P., Bordenave, L., et al., 2003. Vigour conferred by rootstock: hypotheses and direction for research. Bull. OIV 76, 581–595.

Padgett-Johnson, M., Williams, L.E., Walker, M.A., 2003. Vine Water Relations, Gas Exchange, and Vegetative Growth of Seventeen Vitis Species Grown under Irrigated and Nonirrigated Conditions in California. J. Am. Soc. Hortic. Sci. 128, 269–276. https://doi.org/10.21273/jashs.128.2.0269

Palys, lM., Meredith, C., 1984. Dual cultures of grape (Vitis sp.) and the lesion nematode (Pratylenchus vulnus) for studies of nematode tolerance. Plant Dis. 68, 1058–1059.

Pâques, F., Duchateau, P., 2007. Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. Curr. Gene Ther. 7, 49–66.

Paranychianakis, N.V., Aggelides, S., Angelakis, A.N., 2004. Influence of rootstock, irrigation level and recycled water on growth and yield of Soultanina grapevines. Agric. Water Manag. 69, 13–27.

Pelsy, F., 2010. Molecular and cellular mechanisms of diversity within grapevine varieties. Heredity (Edinb). 104, 331–340.

Pérez, F., Villegas, D., Mejia, N., 2002. Ascorbic acid and flavonoid-peroxidase reaction as a detoxifying system of H2O2 in grapevine leaves. Phytochemistry 60, 573–580. https://doi.org/10.1016/S0031-9422(02)00146-2

Pongràcz, D.P., 1983. Rootstocks for Grape-Vines. Cape Town, South Africa.

Porro, D., Pedò, S., Bertoldi, D., Bortolotti, L., Failla, O., Zamboni, M., 2013. Evaluation of New Rootstocks for Grapevine: Nutritional Aspects. Acta Hortic. 109–115. https://doi.org/10.17660/ActaHortic.2013.984.9

Pou, A., Flexas, J., del Mar Alsina, M., et al., 2008. Adjustments of water use efficiency by stomatal regulation during drought and recovery in the drought-adapted Vitis hybrid Richter-110 (V. berlandieri × V. rupestris). Physiol. Plant. 134, 313–323.

Pou, A., Medrano, H., Tomàs, M., Martorell, S., Ribas-Carbó, M., Flexas, J., 2012. Anisohydric behaviour in grapevines results in better performance under moderate water stress and recovery than isohydric behavior. Plant Soil 359, 335–349.

Pouget, R., 1990. Histoire de la lutte contre le phylloxéra de la vigne en France 157.

Prinsi, B., Negri, A.S., Failla, O., Scienza, A., Espen, L., 2018. Root proteomic and metabolic analyses reveal specific responses to drought stress in differently tolerant grapevine rootstocks. BMC Plant Biol. 18, 1–28. https://doi.org/10.1186/s12870-018-1343-0

Purkayashta, R., 1995. Progress in phytoalexin research during the past 50 years, in: Daniel, M, Purkayashta, R. (Eds.), In Handbook of Phytoalexin Metabolism and Action. Marcel Dekker Inc., New York, pp. 1–39.

Qin, X., Zeevaart, J.A., 1999. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc. Natl. Acad. Sci. U. S. A. 96, 15354–15361. https://doi.org/10.1073/pnas.96.26.15354

R Development Core Team, 2010. R: A language and environment for statistical computing.

Raghavendra, A.S., Gonugunta, V.K., Christmann, A., Grill, E., 2010. ABA perception and signalling. Trends Plant Sci.

15, 395–401. https://doi.org/10.1016/j.tplants.2010.04.006

Rattanakon, S., Ghan, R., Gambetta, G.A., Deluc, L.G., Schlauch, K.A., Cramer, G.R., 2016. Abscisic acid transcriptomic signaling varies with grapevine organ. BMC Plant Biol. 16, 1–16. https://doi.org/10.1186/s12870-016-0763-y

Rayle, D.L., Cleland, R., 1970. Enhancement of wall loosening and elongation by acid solutions. Plant Physiol 46, 250–253.

Regier, N., Streb, S., Cocozza, C., Schaub, M., Cherubini, P., Zeeman, S.C., Frey, B., 2009. Drought tolerance of two black poplar (Populus nigra L.) clones: Contribution of carbohydrates and oxidative stress defence. Plant, Cell Environ. 32, 1724–1736. https://doi.org/10.1111/j.1365-3040.2009.02030.x

Reid, K.E., Olsson, N., Schlosser, J., Peng, F., Lund, S.T., 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol. 6, 27. https://doi.org/10.1186/1471-2229-6-27

Rengasamy, P., 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57, 1017–1023.

Rhodes, D., Handa, S., Bressan, R.A., 1986. Metabolic Changes Associated with Adaptation of Plant Cells to Water Stress. Plant Physiol. 82, 890–903.

Riaz, S., Pap, D., Uretsky, J., Laucou, V., Michel, J., László, B., 2019. Genetic diversity and parentage analysis of grape rootstocks. Theor. Appl. Genet. https://doi.org/10.1007/s00122-019-03320-5

Rock, C.D., Heath, T.G., Gage, D.A., Zeevaart, J.A., 1991. Abscisic alcohol is an intermediate in abscisic Acid biosynthesis in a shunt pathway from abscisic aldehyde. Plant Physiol. 97, 670–676. https://doi.org/10.1104/pp.97.2.670

Romero, A.P., Alarcón, A., Valbuena, R.I., Galeano, C.H., 2017. Physiological Assessment of Water Stress in Potato Using Spectral Information 8. https://doi.org/10.3389/fpls.2017.01608

Ruehl, E., Schmid, J., Eibach, R., Töpfer, R., 2015. Grapevine breeding programs in Germany, in: Reynolds, A. (Ed.), Grapevine Breeding Programs for the Wine Industry. Woodhead Publishing, Oxford, pp. 77–101.

Rühl, E.H., Clingeleffer, P.R., Nicholas, P.R., et al., 1988. Effect of rootstocks on berry weight and pH, mineral content and organic acid concentrations of grape juice of some wine varieties. Aust. J. Exp. Agric. 28, 119–125.

Salleo, S., Trifilò, P., Esposito, S., Andrea, N., Lo Gullo, M., 2009. Starch-to-sugar conversion in wood parenchyma of field-growing Laurus nobilis plants: a component of the signal pathway for embolism repair? Funct. Plant Biol. 36, 815–825. https://doi.org/10.1071/FP09103

Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S., Marquez, J., Cutler, S., Rodriguez, P., 2009. Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. Plant J. 60, 575–588. https://doi.org/10.1111/j.1365-313X.2009.03981.x.

Sanz-Burgos, A., Gutierrez, C., 1998. Organization of the cis-acting element required for wheat dwarf geminivirus DNA replication and visualization of a rep protein-DNA complex. Virology 243, 119–129.

Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., Siksnys, V., 2011. The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res. 39, 9275–9282. https://doi.org/10.1093/nar/gkr606

Schaart, J.G., van de Wiel, C.C.M., Lotz, L.A.P., Smulders, M.J.M., 2016. Opportunities for Products of New Plant Breeding Techniques. Trends Plant Sci. 21, 438–449. https://doi.org/10.1016/j.tplants.2015.11.006

Schachtman, D.P., Goodger, J.Q.D., 2008. Chemical root to shoot signaling under drought. Trends Plant Sci. 13, 281–287. https://doi.org/10.1016/j.tplants.2008.04.003

Schaeffer, S.M., Nakata, P.A., 2015. CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field. Plant Sci. 240, 130–142. https://doi.org/10.1016/j.plantsci.2015.09.011

Scharte, J., Schon, H., Tjaden, Z., Weis, E., von Schaewen, A., 2009. Isoenzyme replacement of glucose-6-phosphate dehydrogenase in the cytosol improves stress tolerance in plants. Proc. Natl. Acad. Sci. 106, 8061–8066. https://doi.org/10.1073/pnas.0812902106

Schiavone, F.., Cooke, T.., 1987. Unusual patterns of somatic embryogenesis in the domesticated carrot: developmental effects of exogenous auxins and auxin transport inhibitors. Cell Differ. 21, 53–62.

Scholander, P.F., Bradstreet, E.D., Hemmingsen, E.A., Hammel, H.T., 1965. Sap Pressure in Vascular Plants: Negative hydrostatic pressure can be measured in plants. Science (80-.). 148, 339–346. https://doi.org/10.1126/science.148.3668.339

Schöppner, A., Kindl, H., 1984. Purification and properties of a stilbene synthase induced cell suspension of peanut. J Biol Chem 259, 6806–6811.

Schultz, H.R., 2000. Climate change and viticulture: a European perspective on climatology, carbon dioxide and UV-B effects. Aust. J. Grape Wine Res. 6, 2–12.

Schultz, H.R., Stoll, M., 2010. Some critical issues in environmental physiology of grapevines: future challenges and current limitations. Aust. J. Grape Wine Res. 16, 4–24. https://doi.org/10.1111/j.1755-0238.2009.00074.x

Scienza, A., 1983. Adattamento genetico della vite allo stress idrico. Vignevini 10, 27–39.

Serra, I., Strever, A., Myburgh, P.A., Deloire, A., 2013. Review: The interaction between rootstocks and cultivars (Vitis vinifera L.) to enhance drought tolerance in grapevine. Aust. J. Grape Wine Res. 20, 1–14. https://doi.org/10.1111/ajgw.12054

Shao, H.B., Chu, L.Y., Shao, M.A., Abdul Jaleel, C., Hong-Mei, M., 2008. Higher plant antioxidants and redox signaling under environmental stresses. Comp. Rend. Biol. 331, 433–441.

Shibuya, T., Kano, K., Endo, R., Kitaya, Y., 2017. Photosynthetic Properties and Response to Drought in Cucumber Seedlings Acclimatized to Different Vapor-pressure-deficit Levels. Hort. J. 86, 334–339. https://doi.org/10.2503/hortj.MI-154

Shultz, H.R., Matthews, M.A., 1988. Vegetative Growth Distribution during Water Deficits in Vitis vinifera L. Aust. J. Plant Physiol. 15, 641–656.

Silva, M.D.A., Jifon, J.L., Moura, C., Jadoski, J., Alberto, J., 2013. Photosynthetic Capacity and Water Use Efficiency in Sugarcane Genotypes Subject to Water Deficit During Early Growth Phase. Braz. Arch. Biol. Techn 56, 735–748.

Skoog, F., 1955. Growth factors, polarity and morphogenesis. Année biol 31, 1–11.

Skoog, F., Miller, C.., 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11, 118–131.

Smith, J., Grizot, S., Arnould, S., Duclert, A., Epinat, J.-C., Chames, P., Prieto, J., Redondo, P., Blanco, F.J., Bravo, J., Montoya, G., Pâques, F., Duchateau, P., 2006. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nucleic Acids Res. 34, e149–e149. https://doi.org/10.1093/nar/gkl720

Soar, C.J., Dry, P.R., Loveys, B.R., 2006a. Scion photosynthesis and leaf gas exchange in Vitis vinifera L. cv. Shiraz: Mediation of rootstock effects via xylem sap ABA. Aust. J. Grape Wine Res. 12, 82–96. https://doi.org/10.1111/j.1755-0238.2006.tb00047.x

Soar, C.J., Speirs, J., Maffei, S.M., Penrose, A.B., Mc Carthy, M.G., 2006b. Grape vine varieties Shiraz and Grenache differ in their stomatal response to VPD: apparent links with ABA physiology and gene expression in leaf tissue. Aust. J. Grape Wine Res. 12, 2–12.

Soejima, A., Wen, J., 2006. Phylogenetic analysis of the grape family (Vitaceae) based on three chloroplast markers. Am. J. Bot. 93, 278– 287.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., Tonelli, C., 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (Vitis vinifera L.). Plant Mol. Biol. 24, 743–755. https://doi.org/10.1007/BF00029856

Speirs, J., Binney, A., Collins, M., Edwards, E., Loveys, B., 2013. Expression of ABA synthesis and metabolism genes under different irrigation strategies and atmospheric VPDs is associated with stomatal conductance in grapevine (Vitis vinifera L. cv Cabernet Sauvignon). J. Exp. Bot. 64, 1907–1916. https://doi.org/10.1093/jxb/ert052

Stamp, J.A., Colby, S.., Meredith, C., 1990. Direct shoot organogenesis and plant regeneration from leaves of grape (Vitis spp.) rootstock genotypes. Plant Cell Tiss Org Cult. 22, 127–133.

Stander, C., Vivier, M., 2016. Tissue cultures and viticultural applications. Vitic. Res. Winetech Tech.

Steudle, E., 2000. Water uptake by roots: effects of water deficit. J. Exp. Bot. 51, 1531–1542. https://doi.org/10.1093/jexbot/51.350.1531

Stevens, R.M., Pech, J.M., Gibberd, M.R., et al., 2010. Reduced irrigation and rootstock effects on vegetative growth, yield and its components, and leaf physiological responses of Shiraz. Aust. J. Grape Wine Res. 16, 413–425.

Stevens, R.M., Pech, J.M., Gibberd, M.R., et al., 2008. Effect of reduced irrigation on growth, yield, ripening rates and water relations of Chardonnay vines grafted to five rootstocks. Aust. J. Grape Wine Res. 14, 177–190.

Tandonnet, J.P., Cookson, S.J., Vivin, P., Ollat, N., 2010. Scion genotype controls biomass allocation and root development in grafted grapevine. Aust. J. Grape Wine Res. 16, 290–300. https://doi.org/10.1111/j.1755-

0238.2009.00090.x

Tapia, E., Sequeida, Á., Castro, Á., Montes, C., Zamora, P., López, R., Acevedo F, Prieto, H., 2009. Development of grapevine somatic embryogenesis using an air-lift bioreactor as an efficient tool in the generation of transgenic plants. J Biotechnol 139, 95–101. https://doi.org/10.1016/j.jbiotec.2008.09.009.

Tardieu, F., 2005. Plant tolerance to water deficit: physical limits and possibilities for progress. Comptes Rendus Geosci. 337, 57–67.

Tassoni, A., Fornale, S., Franceschetti, M., Musiani, F., Michael, A., Perry, B., Bagni, N., 2005. Jasmonates and Na-orthovanadate promote resveratrol production in Vitis vinifera cv. Barbera cell cultures. New Phytol 166, 895–905.

Tattini, M., Loreto, F., Fini, A., Guidi, L., Brunetti, C., Velikova, V., Gori, A., Ferrini, F., 2015. Isoprenoids and phenylpropanoids are part of the antioxidant defense orchestrated daily by drought-stressed Platanus 9 acerifolia plants during Mediterranean summers. New Phytol. 207, 613–626. https://doi.org/10.1111/nph.13380

Tergeo Unione Italiana Vini, 2016. I nuovi portinnesti M. Corr. Vinic. 31, 2015.

This, P., Lacombe, T., Thomas, M.R., 2006. Historical origins and genetic diversity of wine grapes. Trends Genet. 22, 511–519.

Thorpe, T., 2007. History of Plant Tissue Culture: Current Status and Opportunities. Mol Biotechnol. 37, 169–80.

Tombesi, S., Nardini, A., Frioni, T., Soccolini, M., Zadra, C., Farinelli, D., Poni, S., Palliotti, A., 2015. Stomatal closure is induced by hydraulic signals and maintained by ABA in drought-stressed grapevine. Sci. Rep. 5, 1–12. https://doi.org/10.1038/srep12449

Treeby, M.T., Holzapfel, B.P., Walker, R.R., Nicholas, P.., 1998. Profiles of free amino acids in grapes of grafted Chardonnay grapevines. Aust. J. Grape Wine Res. 4, 121–126.

Troggio, M., Vezzulli, S., Pindo, M., Malacarne, G., Fontana, P., Moreira, F.M., Costantini, L., Grando, M.S., Viola, R., Velasco, R., 2008. Beyond the Genome , Opportunities for a Modern Viticulture : A Research Overview ASEV Honorary Research Lecture 2007. Am. J. Enol. Vitic. 59, 117–127.

Tröndle, D., Schröder, S., Kassemeyer, H., Kiefer, C., Koch, M.A., Nick, P., 2010. MOLECULAR PHYLOGENY OF THE GENUS VITIS (VITACEAE) BASED ON PLASTID MARKERS 1 97, 1168–1178. https://doi.org/10.3732/ajb.0900218

Tseng, I.-C., Hong, C.-Y., Yu, S.-M., Ho, T.-H.D., 2013. Abscisic Acid- and Stress-Induced Highly Proline-Rich Glycoproteins Regulate Root Growth in Rice. Plant Physiol. 163, 118–134. https://doi.org/10.1104/pp.113.217547

Turk, S., Smeekens, S., 1999. Genetic modification of plant carbohydrate metabolism, in: Chopra, V., Malik, V., Bhat, S. (Eds.), Applied Plant Biotechnology. Science Publishers Inc, Enfield, pp. 71–100.

Tyree, M.T., Sperry, J.S., 1989. Vulnerability of xylem to cavitation and embolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 19–38.

Van Leeuwen, C., Trégoat, O., Choné, X., Bois, B., Pernet, D., Gaudillère, J.-P., 2009. 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? OENO One 43. https://doi.org/10.20870/oeno-one.2009.43.3.798

Vannozzi, A., Donnini, S., Vigani, G., Corso, M., Valle, G., Vitulo, N., Bonghi, C., Zocchi, G., Lucchin, M., 2017. Transcriptional Characterization of a Widely-Used Grapevine Rootstock Genotype under Different Iron-Limited Conditions. Front. Plant Sci. 7, 1–17. https://doi.org/10.3389/fpls.2016.01994

Vannozzi, A., Dry, I.B., Fasoli, M., Zenoni, S., Lucchin, M., 2012. Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and abiotic stresses. BMC Plant Biol. 12, 1. https://doi.org/10.1186/1471-2229-12-130

Vannozzi, A., Wong, D.C.J., Höll, J., Hmmam, I., Matus, J.T., Bogs, J., Ziegler, T., Dry, I., Barcaccia, G., Lucchin, M., 2018. Combinatorial Regulation of Stilbene Synthase Genes by WRKY and MYB Transcription Factors in Grapevine (Vitis vinifera L.). Plant Cell Physiol. 59, 1043–1059. https://doi.org/10.1093/pcp/pcy045

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D.A., Cestaro, A., Pruss, D., Al., E., 2007. A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One 2, E1236.

Velasquez, S.M., Barbez, E., Kleine-Vehn, J., Estevez, J.M., 2016. Auxin and Cellular Elongation. Plant Physiol. 170, 1206 LP – 1215. https://doi.org/10.1104/pp.15.01863

Verslues, P.E., Juenger, T.., 2011. Drought, metabolites, and Arabidopsis natural variation: a promising combination for understanding adaptation to water-limited environments. Curr. Opin. Plant Biol 14, 240–245.

Vezzulli, S., Civardi, S., Ferrari, F., Bavaresco, L., 2007. Methyl jasmonate treatment as a trigger of resveratrol synthesis in cultivated grapevine. Am J Enol Vitic 58, 530–533.

Viala, P., Ravaz, L., 1903. American vines (resistant stock): their adaptation, culture, grafting and propagation. Press of Freygang-Leary Company, San Francisco.

Viala, P., Vermorel, V., 1901. Ampélographie. Tomes I-VII, Masson, Paris.

Vivier, M.A., Pretorius, I.S., 2002. Genetically tailored grapevines for the wine industry. Trends Biotechnol. 20, 472–478.

Von Schaewen, A., Langenkamper, G., Graeve, K., Wenderoth, I., Scheibe, R., 1995. Molecular Characterization of the Plastidic Glucose-6-Phosphate Dehydrogenase from Potato in Comparison to its Cytosolic Counterpart. Plant Physiol. 109, 1327–1335. https://doi.org/10.1104/pp.109.4.1327

Vondráková, Z., Krajňáková, J., Fischerová, L., Vágner, M., Eliášová, K., 2016. Physiology and role of plant growth regulators in somatic embryogenesis, in: Park, Y.-S., Bonga, J., Moon, H.-K. (Eds.), Vegetative Propagation of Forest Trees. National Institute of Forest Science, pp. 123–169.

Vorwerk, S., Forneck, A., 2006. Reproductive mode of grape phylloxera (Daktulosphaira vitifoliae, Homoptera: Phylloxeridae) in Europe: molecular evidence for predominantly asexual populations and a lack of gene flow between them. Genome 49, 678–687. https://doi.org/10.1139/g06-028

Waffo Teguo, P., Fauconneau, B., Deffieux, G., Huguet, F., Vercauteren, J., Mérillon, J.-M., 1998. Isolation, Identification, and Antioxidant Activity of Three Stilbene Glucosides Newly Extracted from Vitis vinifera Cell Cultures. J. Nat. Prod. 61, 655–657. https://doi.org/10.1021/np9704819

Walker, A.R., Lee, E., Bogs, J., et al., 2007. White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. 49, 772–785.

Walker, M.A., 1992. Future directions for rootstock breeding, in: Wolpert, J.A., Walker, M.A., Weber, E. (Eds.), Rootstock Seminar: A Worldwide Perspective. American Society for Enology and Viticulture, Davis, USA., pp. 60–68.

Walker, R.R., Blackmore, D.H., Clingeleffer, P.R., 2010. Impact of rootstock on yield and ion concentrations in petioles, juice and wine of Shiraz and Chardonnay in different viticultural environments with different irrigation water salinity. Aust. J. Grape Wine Res. 16, 243–257. https://doi.org/10.1111/j.1755-0238.2009.00081.x

Walker, R.R., Read, P.E., Blackmore, D.H., 2000. Rootstock and salinity effects on rates of berry maturation, ion accumulation and colour development in Shiraz grapes. Aust. J. Grape Wine Res. 6, 227–239.

Wan, Y., Schwaninger, H., Li, D., Simon, C.J., Wang, Y., He, P., 2008. The eco-geographic distribution of wild grape germplasm in China. Vitis 47, 77–80.

Wang, W., Tang, K., Yang, H.R., Wen, P.F., Zhang, P., Wang, H.L., Huang, W.D., 2010. Distribution of resveratrol and stilbene synthase in young grape plants (Vitis vinifera L. cv. Cabernet Sauvignon) and the effect of UV-C on its accumulation. Plant Physiol. Biochem. 48, 142–152. https://doi.org/10.1016/j.plaphy.2009.12.002

Wang, Xianhang, Tu, M., Wang, D., Liu, J., Li, Y., Li, Z., Wang, Y., Wang, Xiping, 2017. CRISPR / Cas9-mediated efficient targeted mutagenesis in grape in the first generation 1–12. https://doi.org/10.1111/pbi.12832

Warnes, G.R., Bolker, B., Lumley, T., 2005. gplots: Various R programming tools for plotting data.

Waterhouse, A., Lamuela-Raventos, R., 1994. The occurrence of piceid, a stilbene glucoside, in grape berries. Phytochemistry 37, 571–573.

Watkins, J., Chapman, J.M., Muday, G.K., 2017. Abscisic acid-induced reactive oxygen species are modulated by flavonols to control stomata aperture. Plant Physiol. 175, 1807–1825. https://doi.org/10.1104/pp.17.01010

Wen, J., Nie, Z.-L., Soejima, A., Meng, Y., 2011. Phylogeny of Vitaceae based on the nuclear GAI1 gene sequences. Can. J. Bot. 85. https://doi.org/10.1139/B07-071

Went, F.W., Thiman, K.V., 1937. Phytohormones. MacMiNan.

Werner, T., Motyka, V., Strnad, M., Schmülling, T., 2001. Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. U. S. A. 98, 10487–10492. https://doi.org/10.1073/pnas.171304098

Westra, E.R., Swarts, D.C., Staals, R.H.J., Jore, M.M., Brouns, S.J.J., van der Oost, J., 2012. The CRISPRs, They Are A-Changin': How Prokaryotes Generate Adaptive Immunity. Annu. Rev. Genet. 46, 311–339. https://doi.org/10.1146/annurev-genet-110711-155447

Whiting, J.R., 2005. Grapevine rootstocks, in: Dry, P.R., Coombe, B.G. (Eds.), Viticulture, Volume 1: Resources, 2nd Edition. Winetitles Pty Ltd, Ashford, Australia, pp. 167–188.

Wiese, W., Vornam, B., Krause, E., Kindl, H., 1994. Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. Plant Mol Biol 26, 667–677.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493. https://doi.org/10.1104/pp.126.2.485

Wong, D.C.J., Schlechter, R., Vannozzi, A., Höll, J., Hmmam, I., Bogs, J., Tornielli, G.B., Castellarin, S.D., Matus, J.T., 2016. A systems-oriented analysis of the grapevine R2R3-MYB transcription factor family uncovers new insights into the regulation of stilbene accumulation. DNA Res. 23, 451–466. https://doi.org/10.1093/dnares/dsw028

Wormit, A., Usadel, B., 2018. The Multifaceted Role of Pectin Methylesterase Inhibitors (PMEIs). Int. J. Mol. Sci. 19, 2878. https://doi.org/10.3390/ijms19102878

Xing, H., Dong, L., Wang, Z., Zhang, H., Han, C., Liu, B., Wang, X., Chen, Q., 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol. 14, 327. https://doi.org/10.1186/s12870-014-0327-y

Yahyazadeh, M., Meinen, R., Hänsch, R., Abouzeid, S., Selmar, D., 2018. Impact of drought and salt stress on the biosynthesis of alkaloids in Chelidonium majus L. Phytochemistry 152, 204–212. https://doi.org/10.1016/j.phytochem.2018.05.007

Yordanov, I., VelikoVa, V., Tsonev, T., 2000. Plant Responses to Drought, Acclimation, and Stress Tolerance. Photosynthetica 38, 171–186.

Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., Ecker, J.R., Shinozaki, K., 2002. ABA-Activated SnRK2 Protein Kinase is Required for Dehydration Stress Signaling in Arabidopsis. Plant Cell Physiol. 43, 1473–1483. https://doi.org/10.1093/pcp/pcf188

Zamboni, A., Minoia, L., Ferrarini, A., Tornielli, G.B., Zago, E., Delledonne, M., Pezzotti, M., 2008. Molecular analysis of post-harvest withering in grape by AFLP transcriptional profiling. J. Exp. Bot. 59, 4145–4159. https://doi.org/10.1093/jxb/ern256

Zerihun, A., Treeby, M.T., 2002. Biomass distribution and nitrate assimilation in response to N supply for Vitis vinifera L. cv. Cabernet Sauvignon on five Vitis rootstock genotypes. Aust. J. Grape Wine Res. 8, 157–162. https://doi.org/10.1111/j.1755-0238.2002.tb00251.x

Zottini, M., Barizza, E., Costa, A., Formentin, E., Ruberti, C., Carimi, F., Lo Schiavo, F., 2008. Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. Plant Cell Rep 27, 845–853. https://doi.org/10.1007/s00299-008-0510-4