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ECO-PHYSIOLOGICAL CHARACTERIZATION OF NEW GRAPEVINE ROOTSTOCKS UNDER DROUGHT STRESS

Ph.D. student DANIELE GROSSI

Supervisor: Prof. ATTILIO SCIENZA

Doctoral Board Coordinator: Prof. PIERO ATTILIO BIANCO

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Extended Abstract

ECO-PHYSIOLOGICAL CHARACTERIZATION OF NEW GRAPEVINE ROOTSTOCKS UNDER DROUGHT STRESS

The objectives of grapevine rootstock breeding selections have undergone a continuous evolution over the years. From the first American vine species introduced to face the invasion of phylloxera and the mildews through Europe, recent breeding programmes aims to obtain plants which are also tolerant to biotic and abiotic stresses such as nematodes, drought and salt stress. Furthermore, the main present interest is on rootstocks that show good performance in different places and in favorable years, but that maintain a good efficiency in difficult conditions. The selection of grapevine rootstocks for resistance to drought conditions is particular important across the activities of modern breeding. Water stress tolerance but above all the water use efficiency (WUE) is becoming more and more important cause the variability of the environmental factors such as limited availability and irregular distribution of water resource. The achievement of the objectives of selection is closely linked to the efficiency and quality of characterization of the phenotype under stress conditions. Traditional phenotyping techniques, although consolidate and widespread, showed considerable limitations like time-consuming and destructive methods. Current technologies allow the development of new systems named high-throughput phenotyping techniques. Thermography, detecting heat patterns in the infrared-wavelength spectrum, is one of the techniques applied in viticulture to assess the plant water conditions.

In addition to phenomics techniques, the detection of changes at the molecular level related to the ability to modify the phenotype under stress also play key roles. The analysis of the changes in gene expression induced by water stress is part of this evolution and the analyses of the transcriptional regulation of some genes involved in the responses to water deficiency shown particular interests.

The present work aims to characterize the eco-physiological responses of new grapevine rootstocks under water stress in comparisons with the most widespread commercial rootstocks and other genotypes of *Vitis spp*. In particular the study focuses on the strategies in response to water stress and how these modifications can be transmitted to the scion by the rootstock.

The first goal achieved has been the validation of the methods used in high-throughput phenotyping. Thermography has proven a valuable tool in order to assess the water condition of the plant and its evolution during the experiments. The effects of water stress on the variation of stomatal conductance and the rate of growth of the plants have been confirmed allowing the acceleration in phenotyping. It was also possible classify the different behaviors in response to water stress conditions providing a database of phenotypic information to be associated with genotypic data. This point has been particularly important as support to genetic association studies (GWAS) aimed to develop molecular markers to assist and optimize future breeding programs of grapevine rootstock.

Another aspect observed is how the rootstock is able to influence some of the main responses to water stress and how these effects characterize the behavior of grafted variety. In particular several combinations of rootstock with the same scion have been compared: five of the most widespread commercial rootstocks and four new developed rootstocks has been tested under a dry down experiment under controlled greenhouse conditions.

Changes in the eco-physiological status of plants in response to different levels of water stress has been evaluated. The rootstocks have been able to influence the responses to water stress in terms of stomatal conductance (Gs), net photosynthesis (Pn) and stem growth rate (SGR). The modification of gene expression in the roots of the different rootstocks and in the leaves of the scions have also been determined. The differences were observed on transcripts involved in the phenylpropanoid biosynthesis and relative transcription factors involved in the regulation of this pathway, stilbene synthases pathway and on the expression of abscisic acid (ABA) related genes.

The analysis of transcriptional regulation of secondary metabolism has been considered as the main responses involved in the role of protection against oxidative stress induced by drought conditions.

In conclusion, the rootstock has determined a different response according to the genotype but also was able to develop different responses in the scion. This shows that the biosynthetic pathways of ABA, stilbene and flavonoids synthases involved in scion response to drought conditions can be controlled by the rootstock.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Grapevine Rootstocks

1.1.1 A new viticulture in Europe

In the second half of the 19th century the European viticulture was devastated by new grapevine diseases and pest infestations. In the 1845, in Margate, a small town in the south-west of England a gardener, Edward Tucker discovered a strange powder on the leaves of some vines grown in greenhouse (Ainsworth, 1976).

He sent a sample to reverend-phytopathologist Miles Joseph Berkeley for identification. Berkley considered this a new species of fungus and named it *Oidium Tuckerii* in honor of the gardener. In 1846 the same powder was find on the vines of the Palace of Versailles (Unwin, 1991).

The spread of the disease was particularly fast throughout Europe: in 1851 some infections were identify in southern France, Algeria, Greece, Hungary, Spain, Italy, Swiss and the effects of powdery mildew [*Uncinula necator* (Schwein) Burrill] reduced the yields vertically. Oidium (powdery mildew) showed up in 1846 and, after several years of insidious activity, triumphed in the general disaster of 1854 (Harry, 1996).

French production, falling from 45 million hectoliters in the 1840s to 29 million hectoliters in 1852 and 11 million hectoliters in 1854 (Lachiver, 1988).

Phylloxera (1864) was ably supported by invasions of downy mildew (1878) and black rot (1885). It does not seem that there were any devastating diseases before the invasion of oidium at mid-century (Harry, 1996).

This remarkable reduction of yields caused an intense activities in the search of remedy. In 1852, Grison, a gardener at the Palace of Versailles, suggested the application of a mixture composed by sulphur and lime subsequently named *Eau Grison* that show some success in the control of infestation (Unwin, 1991).

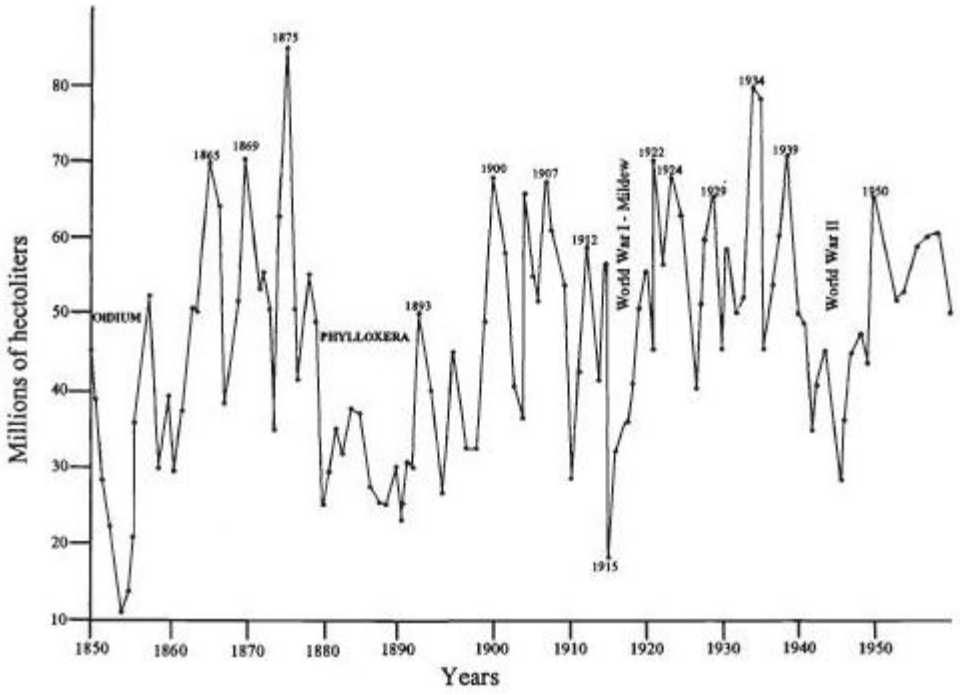

Figure 1: French wine production 1850-1956 (modified from Harry, 1996)

The same remedy was successfully use by Edward Tucker who first recorded powdery mildew (Ainsworth, 1976). By the early 1860s, however, another solution, the dusting of vines with fine sulphur was found to be successful by Henry Mares, and this became the standard form of treatment throughout Europe (Unwin, 1991).

Such careful treatment, however, could not be duplicated over large areas of vineyard, and, recognizing that certain American vines were resistant to attack by oidium, a number of producers began importing and cultivating American vines (Unwin, 1991).

In fact at the time of the appearance of mildew in France around 1850, many American vines present in many regions, were noted for their resistance. They had no symptoms then the French grape varieties planted side-by-side were severely affected (Pouget, 1990).

High resistance to mildew by American vines raised new interest by several amateurs: between 1858 and 1862, imports from the United States in the form of seeds, cuttings and rooted vines, have developed rapidly in France (Bordeaux, Gard, Alsace) and Europe (England, Italy, Germany, Switzerland, Portugal, etc). In 1863, Durieu de Maisonneuve, Director of the Botanical Garden of Bordeaux, received from the

America rooted vines and he sent a part of them to the Garden Botanical Dijon. In Bordeaux, Leo Laliman, viticulturist and experimentation, cultivated varieties of American vines since 1840. He received from Mr. Berchmans, Augusta (Georgia), Mr. Durand of Philadelphia and he played the role of nursery distributing this material to amateurs who established some important collections in the Bordeaux vineyards (Medoc, Graves, Saint Emilion, etc.) and other French vineyards (Pouget, 1990).

The massive introduction of American vines to Europe, which began in large part by the desire to control powdery mildew, inadvertently led to a crisis of much bigger proportions, a crisis that would have serious economic and social repercussions for the European wine producers (Figure 2).

In 1863 a sample of insects on a vine leaf from a greenhouse in Hammersmith, to the west of London, had been sent to Oxford University, where they were later identified by J.O. Westwood (1869) as the aphid Phylloxera (Unwin, 1991).

Imported varieties were often planted near indigenous grape varieties, in order to facilitate the study of their behavior and comparisons. Of course, it came to the idea of isolating the person imported to prevent the spread of any pest plant material. Phylloxera (*Daktulosphaira vitifoliae* Fitch) was obviously not known in France, even in America, where it was endemic. For this reason the danger for the vine cultivated *V. vinifera* was not suspected (Pouget, 1990).

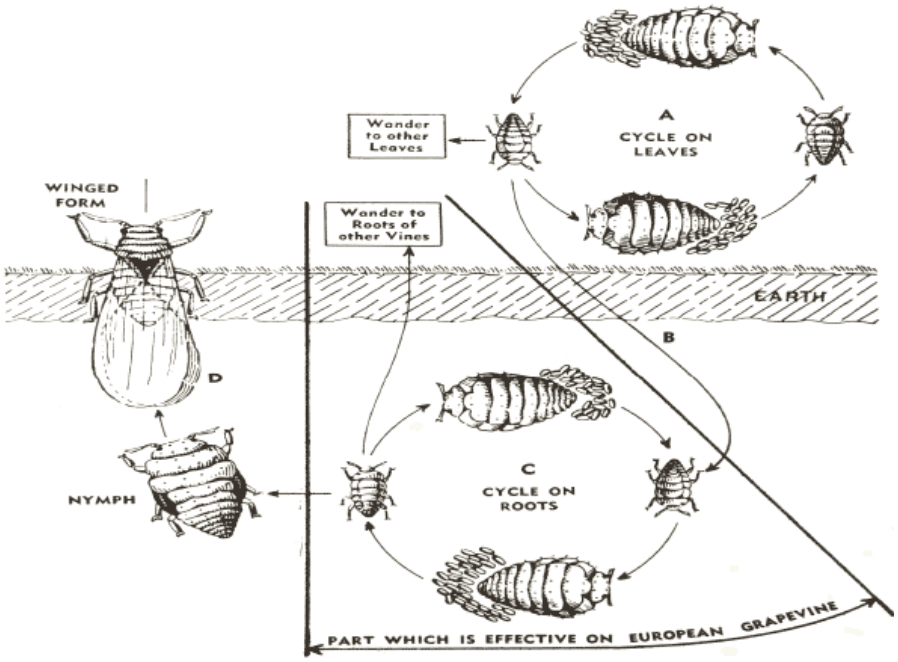

Figure 2: Cicle of Phylloxera (*Daktulosphaira vitifoliae* Fitch) on Vinifera and other species from www.phylloxera.com.au

The invasion of the vineyards by the Phylloxera insect has an important effect on rural social structure, traditional political arrangements and urban drinking habits. As describe by Harry (1996) wine and wheat, two key items in rural thought, were of great importance in politics.

There are several arguments for the explanation of the timing of the arrival of Phylloxera in Europe in the late 1850s and 1860s (Unwin, 1991).

Jules-Emiles Planchon (Montpellier University Professor, 1823-1888) who first identified Phylloxera in southern France, was of the opinion that the importation of a considerable number of rooted American vines had taken place into the region between 1858 and 1862, and that it was possibly this that had led to the infestation of Europe's vineyards (Unwin, 1991).

The presence was confirmed in:

- Portugal and Turkey by 1871
- Austria-Hungary by 1872
- Switzerland by 1873 or 1874
- Spain by 1875

- Italy by 1879, Valmadrera, Lecco (Maffi, 2010)
- Germany by 1881

The search for a cure for the damage inflicted by phylloxera was slow. The outbreak of the Franco-Prussian War in 1870, and the proclamation of the Third Republic in September of the same year took considerable attention away from a problem which at that time appeared to be confined to a few parts of France, and had yet seriously to affect the supplies of wine to the capital (Unwin, 1991).

After phylloxera hit France it was soon noticed that the vines of the sandy soils of the Mediterranean littoral did not succumb to the insect. Scientists explain the inability of the insect to attack the vines in sandy soils with the movement of water in the sand, especially near tidal waters, seems to destroy the larva and the eggs producers extended their holdings into soils having a clay content of less than 3 percent, the ideal soil having at least 60 percent siliceous rather than calcium sand (Harry, 1996).

Another successful form of protection that became obvious to growers strong in analogical reasoning was drowning the pest in flooded vineyards, a procedure called the Faucon system, named by the viticulturist Louis Faucon, owner of 21 hectares (ha) at Gravison (Bouches-du-Rhone). In 1875, after five years of flooding and fertilizing, Faucon produced 2480 hectoliters (hl) of wine compared with 925 hl in 1867, a pre-phylloxera year when the vines were not fertilized, and 45 hl in 1868, the first year of invasion. The duration of flooding the vineyard with 20 to 25 cm of water varied from 35 to 40 days in autumn to 45 to 50 days in the winter. Faucon obtained permission to take water from the canal des Alpines. Vines recovered after four years of flooding with heavy fertilization and produced high yields up to 100 hl per ha and more.

Other experiments have focused the attention on the use of chemicals.

The most successful chemical treatment for infected vines proved to be the application of carbon disulphide (CS_2) (Figure 1). This highly toxic and inflammable chemical had been found to be successful in the eradication of other pests during the 1850s, and despite early failures in the late 1860s subsequent experiments in the 1870s involving its injection into the soil around the roots of vines were effective in eradicating the phylloxera aphid (Ordish, 1987). However, the method was expensive, there was considerable concern about its influence on the taste of wine, its effectiveness varied

with soil type, and it only offered a temporary solution since it did nothing to prevent the subsequent reinfestation of the vines.

Figure 3: Advertisement on carbon disulphide from Pacific Rural Press, 1881

In 1870, in France, the Minister of Agriculture had offered a small prize of 20.000 francs for a remedy, but it was only four years later in July 1874 that the government as a whole became sufficiently concerned to offer a prize of 300.000 francs for the inventor of a cure.

In order to evaluate the proposed remedies the School of Agriculture at Montpellier set aside an infested vineyard, known as Las Sorres, where the Commission départementale de l'Hérault pour l'étude de la maladie de la vigne (1877) tested 317 of the 696 remedies submitted to them in the period before October 1876.

In only two experiments did the treated plots show any marked advantage over the control plots, and these involved the treatment of the vines with potassium sulphide in human urine and the application of sulphide with colza cake (Unwin, 1996).

Numerous other chemical treatments were tried (Mouillefert, 1876), but to little avail, and gradually during the 1870s those fighting phylloxera began to fall into two conflicting schools of thought. On the one hand were those still advocating the use of chemicals "the sulfuristes", and on the other those, following Laliman (1879, 1889), who supported the use of American vines called "the americanist" (Unwin, 1991).

The turning point in the fight against phylloxera came in 1881 at the International Phylloxera Congress held in Bordeaux (Fitz-James, 1889), when it was eventually accepted that the best solution was the grafting of French vine scions onto American rootstocks (Figure 4).

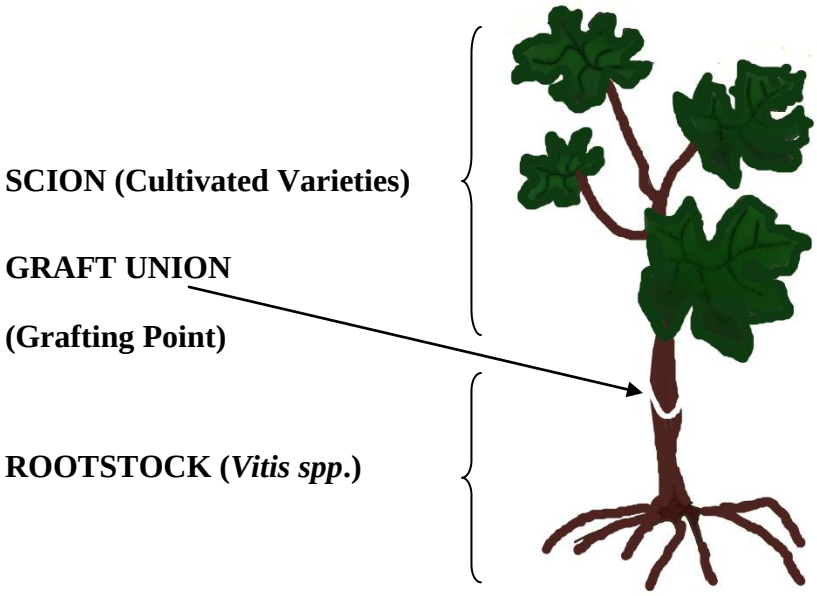

Figure 4: Grafted vine, a bi-member plant

The grafted vine, taller and more vigorous, resulted in increased production. Immediately before the plague of Phylloxera, from 1863 to 1875, average wine production in France was 56.9 million hl from a cultivated surface of 2.2 million ha: 25 hl per ha. In the period after the reconstitution of vineyards, from 1899 to 1909, annual average production was 55.5 million hl from the reduced area of 1.5 million hectares: 32 hl per hectare. In the decade from 1922 to 1931, the annual average was 56.6 million hl from 1.4 million ha: 39 hl per ha. The increase was not due solely to grafting. Changes in cultivation also counted, but there is no doubt that diseases of the vine completely changed growing practices (Harry, 1996).

Moreover, the widespread introduction of American vines brought with it yet another fungal parasite, downy mildew, which was first noted in France in 1878. By 1882 it had affected most of the major wine producing areas of the country, further contributing to the decline in yields initiated by phylloxera (Unwin, 1996). The discovery of a remedy was much more rapid, and following successful experiments by Millardet using copper sulphate sprays in the Gironde in 1883 and 1884, the use of this 'Bordeaux mixture' became universal by the end of the decade.

The indirect consequences of phylloxera include the introduction of the practice of grafting with an accentuation of vine vigor, sensitivity to fruit rots of grape and viruses. In addition, there was a simplification of ampelographic platform and the introduction of direct producer hybrids (Calò, 1992).

In fact in 1887 after a violent invasion of black rot in the Midi and the southwest of France grape growers turned from vines grafted with scion *Vitis vinifera* to direct-producing hybrid vines, which scientists had chosen out cause of their resistance to diseases (Harry, 1996).

Scientists at the University of Montpellier's school of agriculture advocated replacing dead vines with French plants or scions grafted on aphid-resistant American rootstocks while wine producers, in many southern departments, chose instead direct production (non-grafted) hybrids considering their superior resistance to diseases. A second major issue in the quarrel was wine quality: hybrid vines produced mediocre wines but were resistant to disease, whereas grafted vines produced good wines but were prone to disease and lived for only about 25 years or less (Harry, 1996).

In Figure 5 is represented the spread of the direct production hybrids in France from 1870 to 1990. In Europe, hybrids cover about 700000 ha (Harry, 1996).

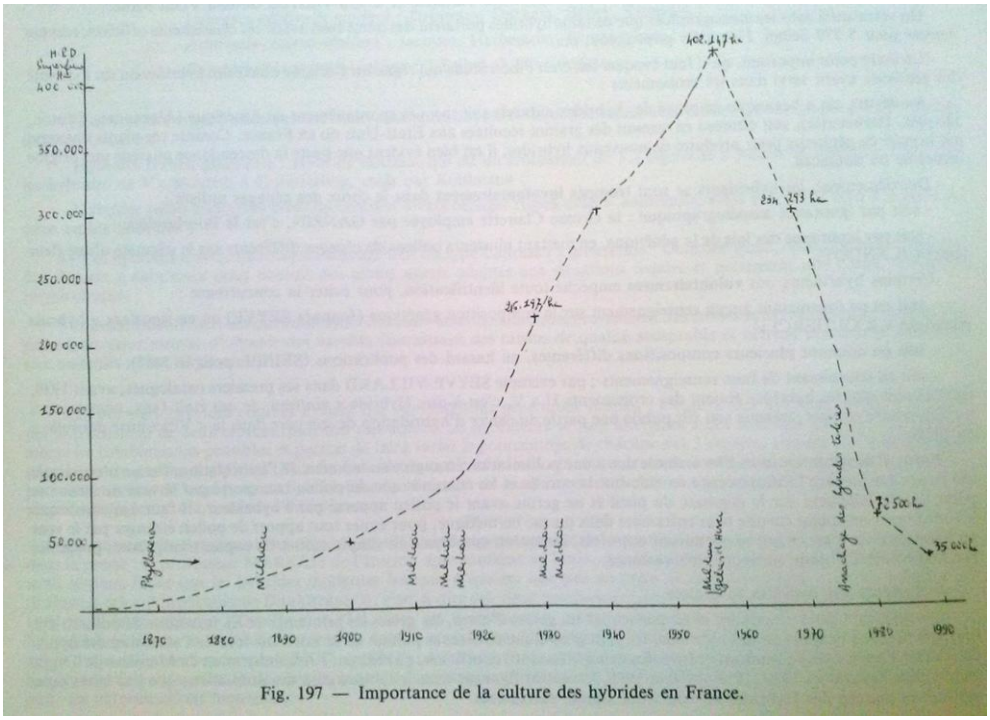

Fig. 197 — Importance de la culture des hybrides en France.

Figure 5: Importance of the direct-production hybrids in France (picture from Galet, 1988)

The scientific issue in the debate over direct-production hybrids was inseparable from social structure, for the peasantry and poorer producers were pro-hybrid and the producers of fine wines were pro-grafted. Nor can we separate the issue from its cultural context. Choice of vine depended on the consumer's acceptance or rejection of the taste of wine from two types of vine: the peasant palate could tolerate hybrid wine, while the bourgeois palate could accept only *vins de crue* (Harry, 1996).

The two great wars of the twentieth century gave a big boost to the hybrid, which required far less care and chemicals than the *V. vinifera* vine. In wartime, materials (copper sulfate and sulfur, especially), and agricultural labor were in short supply or, more usually, unavailable. After the First World War the resistant direct producer was more than ever the cheap vine of the future for the production of a drinkable wine (Harry, 1996).

The spread reached its peak in 1953 with 400000 ha and then decreases as a result of several regulatory actions (Galet, 1988).

1.1.2 Evolution of Rootstocks selection

The major reason to use rootstocks is in their resistance to some severe biotic problems such as phylloxera and nematodes (Sanjun, 2005). After the scientists recognized that phylloxera came from North America, they reasoned that if the wild grapes of North America grew in areas infested with phylloxera without damage, the roots of these wild vines must be, in some way, resistant to phylloxera.

Table 1: Classification of *Vitis* genus and their origin (picture from Unwin, 1991)

For this reason an extensive experimentation followed to identify which selections of North American grapes were suitable for use as rootstocks in European vineyards (Cousin, 2005).

In 1873 Planchon returned from his visit to the USA and recommended a number of vine varieties as suitable for direct production, as rootstock, or both. Unfortunately, among his recommendations were several varieties with high percentages of *V. labrusca* an American species from the cool north-eastern woods with parentage such as 'Concord' and 'Clinton'. These vines were rapidly shown to have three important faults: first, they couldn't tolerate the heat of southern France namely the 'region of the olive', secondly, they were not sufficiently phylloxera-resistant under French conditions and in the end, the wines were undrinkable (Gale, 2003).

In the two winters of 1872 and 1873, over 700000 cuttings of these *V. labrusca*-based vines were imported from St. Louis (Missouri) in the United States (Gale, 2003).

The first attempts, using *V. labrusca*-based varieties, failed due to insufficient resistance; the second, using *V. aestivales*-based varieties, failed due to unacceptably low takes.

Sahut in 1888 declared that grape growers, devastated once by the Phylloxera, were now destroyed again, once and for all, by the "Concord" disaster (Gale, 2003).

As new pure US wild species were discovered particularly *V. riparia* and then *V. rupestris* making their first appearance. First, orders were sent to Missouri and several other states, for cuttings taken from wild vines of the target species. Once in France, the cuttings would be rooted in place, there to be grafted during their second season. Take was highly variable, and resistance varied as well. Not all wild *V. riparia* vines were the same, and nor were *V. rupestris* (Gale, 2003).

A second wave of popularity developed around Millardet's idea of raising rootstock plants from pure US species seeds gathered in the wild (Gale, 2003). This idea was soon criticized, and rightly so, for the excessive variability in resistance that was found in the seedlings. In the end, Montpellier solved the problems by selecting from among its enormous collection of pure US species only those individual vines that were easy to graft, compatible with most French varieties, and highly resistant to Phylloxera. Best among the dozen or so that were eventually propagated and disseminated were 'Riparia

Gloire de Montpellier' and 'Rupestris du Lot', both of which still see widespread service worldwide.

In 1879 Millardet discovered the resistance of *V. rupestris* (Galet, 1988).

But in the beginning the American vines were introduced for the production of rootstocks for grafting without paying much attention to the relations between soil, climate, and plant. Soon the nonsuccess of the American plants became as important an issue as phylloxera (Harry, 1996).

The new grafted vines also proved to be much less tolerant to the limestone, and tended to develop chlorosis on soils with a high lime content. In some instances this led to a shift in vineyard location from areas of chalky hillslope to the deeper and more acidic soils of the plains (Unwin, 1991).

Gustave Foex studied the chlorosis suffered by the vines planted in calcareous soil (Harry, 1996) and Pierre Viala was to move the problem far along the road to solution after his visit in 1887 to the United States, where he looked for vines in soils similar to the killer soils in Cognac and Champagne country (Viala, 1889).

Viala spent from 5 June to 8 December 1888 in America (Figure 1). During the first period he visited New Jersey, Maryland, Virginia, North Carolina, New York and Ohio. Next he visited Tennessee, Missouri, The Indian Territory, California and Texas. He made important findings in Tennessee, Missouri and Texas (Gale, 2011).

He visit several nursery in these three state, in particular in Texas, where he visit the M.T.V. Munson who established and operated a thriving nursery in Denison, Texas. There he discovered, with the help of T.V. Munson, a huge area of chalky soils, similar to Charente Department (region of Cognac), extending from the panhandle in the north to the Pecos River and from the New Mexico border in the west to a region bounded north to south by Dallas, Austin and S. Antonio in the est (Gale, 2011). Luckily there were native vines like *V. berlandieri*, *V. cordifolia*, *V. cinerea*, *V. candicans*, *V. monticola* and numerous hybrids from resulting from the various crossing of these species (Gale, 2011).

His discoveries were *Vitis berlandieri*, *Vitis cinerea*, and *Vitis cordifolia*. Viala found, in Belton, Texas, the object of Charentais desire, *Vitis berlandieri*, flourishing in a soil where other vines succumbed to chlorosis (Harry, 1996).

V. berlandieri (also known as *V. aestivalis* and *V. monticola* Buckley) had first been revealed to the scientific world in 1834 by the Belgian-Swiss botanist J.-L. Berlandieri, who found it in Texas. Elevating this vine to the status of a new species in 1880, Planchon dedicated it to Mr. Berlandieri (Harry, 1996).

Viala returned to France the following year, and through him, the entire French wine industry began anew, by grafting the various well-tested French Vinifera fruit woods onto resistant American *Vitis labrusca* rootstock primarily mustang grape plants from Central Texas. Thomas Volney Munson was awarded the Chevalier dv Merite Agricole of the Legion of Honor on January 1889, for his significant part in saving the vineyards, and wineries, of France (Woodruff, 1998).

Figure 6: Une Mission Viticole en Amèrique (picture from Viala, 1889)

On his return to Montpellier, where he became Professor of Viticulture in 1886, Viala mounted a scientific assault on phylloxera. Planchon had pointed out that American vines had brought the disaster and could possibly save French viticulture as well. But the vines used were often diseased and hard to acclimate. Worst of all, they often produced grapes and wine that tasted foxy. Viala used his own two vineyards of Cournonterral and Laverune, which he had inherited from his parents, to carry out a series of experiments on resistance to major diseases in 400 varieties of vines grafted on

different rootstocks. Ten years of laboratory and field research provided the basis of certainty on which Viala proceeded to rebuild the vineyards of France (Harry, 1996).

V. berlandieri was considered the "life raft" revealed the disagreeable surprise of extreme difficult of rooting (Gale, 1943). For this reason, the activity was oriented towards the creation of hybrids with *V. berlandieri*.

Three principal breeders were the "accidental" creators of berlandieri hybrids: Foex, Couderc and Millardet. In particular Couderc and Millardet, in the early 1880s had quite purposefully crossed *V. berlandieri* and other species because of their strong interest in how the hybrids would perform in term of their phylloxera resistance (Gale 2011).

In 1888 Courdec and Millardet started testing berlandieri hybrids (Gale, 2011).

By the 1890s second-generation hybrid rootstocks, designed for a better match with the typical French soils (Figure 7).

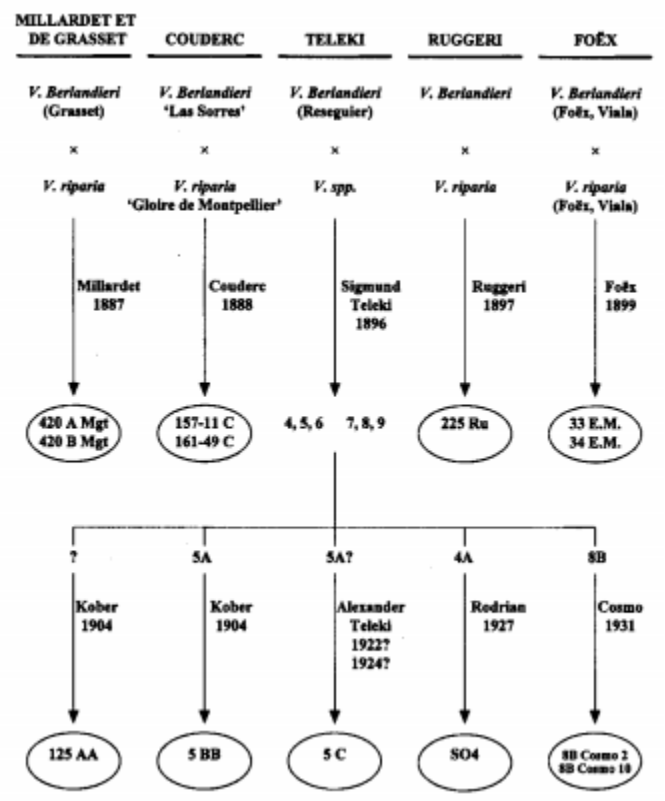

Figure 7: Pedigrees of V. berlandieri X V. riparia hybrids (Guerra and Meredith, 1995)

Although the Italian authorities imported French hybrid rootstocks, because of some unique indigenous terrain, success was not complete. Italian viticulturists, in particular Federico Paulsen in Sicily, had to develop their own special rootstock varieties (Gale, 2003). The Phylloxera infestation came to Sicily in the early 1880s, more than a decade after it arrived in France (Nesto and Di Savino, 2013).

Beyond resistance to phylloxera Sicily need rootstock that adapted well to particular characteristic of its dry salt-affected and high-active-lime soils (Nesto and Di Savino, 2013).

In the late nineteenth and the early decades of the next century Sicily became a prestigious laboratory, where an intense activity of breeding led to new rootstocks at present really spread.

In 1888 the Palermo Royal Nursery of American Vines was established with branches in Marsala, Milazzo, Catania, Caltagirone, Noto and Piazza Armerina. Federico Paulsen (1861-1943), an agricultural expert from Rome was put in charge. Between 1894 and 1897 he was to create one of the most important Sicilian rootstock (1896): 1103P (Nesto and Di Savino, 2013).

In 1894, Antonio Ruggeri from Messina working in the Ragusa area for Vittoria and Ragusa research facility became a series of hybridization (Nesto and Di Savino, 2013). In Vittoria he obtained the first of his most important hybrid: the berlandieri x rupestris du lot n. 42.

In 1896 he was transferred to Milazzo, where the Ministry of Agriculture gave him the direction of the Local Government Nursery and in 1897 another important Sicilian rootstock was selected: 140Ru (Nesto and Di Savino, 2013).

Other leading researchers worked in the field of rootstocks breeding obtaining several collections (Teleki Richter Kober selections) (Galet, 1988)

Breeders crossed *V. berlandieri* with *V. rupestris* and *V. riparia* and developed new families of rootstocks that combine adaptation to calcareous soils with ease of propagation (Cousin, 2005).

The three groups of rootstocks formed by the hybridization of these species are the most important in viticulture today (Cousin, 2005).

In Table 1 some of the important rootstocks most widespread in the world and some new rootstocks results of recent selections.

Rootstock	Parentage	Vigor conferred to scion	Pyllo xera Resis tance	Nema tode X. Index (Dag ger)	Resistance M. incognita (Root-Knot)	Soil Prefere nce	Drough t Toleran ce	Wet Feet	Active Lime Toleran ce	Salt Toler ance	Influence on Maturity	General Comments
Riparia Gloire	V.riparia	Low/ Moderate	High		Moderate	Deep/Fe rtile	Low	High	Low <6%		Early	
Saint George	V. rupestris	Very High	High		Susceptibl e but Tolerant	Deep, Uniform Loam	High	Low	14%	Mode rate	Late	Susceptible to oak root fungus. Suitable for deep, dry farmed sites. Tends to reduce fruit set on vigorous site
1616 Courderc	V. solonis x V. riparia	Low	Mode rate/ High		Moderate	Deep/Fe rtile		High	11%	Mode rate/ High	Early	
3309 Courderc	V.riparia x V. rupestris	Moderate/ High	High	Susce ptible	Susceptibl e	Deep Well Drained	Low	High	11%	Low Mode rate	Mid	
44-53 Malegue	V.Riparia x 144M	Moderate	Mode rate/ High	Mode rate	Susceptibl e	Loam/G ood Fertility	Moderat e	High	10%		Mid	Often suffers from Mg deficiency
101-14 Millardet et De Grasset	V.riparia x V. rupestris	Low/ Moderate	High		Moderate	Heavy Clay	Low/Mo derate	High	9%	Very Low	Early	More vigorous tham Riparia Gloire
Swarzmann	V.riparia x V. rupestris	Low/ Moderate	High	High	Some	Deep/Fe rtile	Low/ Moderat e		6-9%			
41B Millardet et De Grasset	V.berlandieri x V. Vinifera		Low	Susce ptible	Susceptibl e	Dry Lime	Low/Mo derate	Low	40%	Very Low	Early	
420A Millardet et De Grasset	V. berlandieri x V. riparia	Low	Mode rate		Moderate	Deep/Fe rtile	Low	Mode rate	20%	Low	Late	Siutable for high density plantings. Less vigorous than 5C and 5BB. Susceptible to potassium deficiency. High performance on Mg absorption.
Selection Oppenheim n°4	V. berlandieri x V. riparia	Moderate	High	High	Moderate	Clay	Low	High	18%	Low	Mid	Susceptible to magnesium deficiency and bunch stem necrosys
Kober 5BB	V. berlandieri x V. riparia	Moderate	High		Moderate	Clay	Low	High	20%	Very Low	Mid	Slightly more drought tolerant than 5C or 420A, yet less than 110R and St. George. Not recommended for site with standing water or a history of phytophtfora. Genetically identical to 5A.
5C Teleki	V. berlandieri x V. riparia	Moderate	High	High	Moderate High	Clay	Low	High	20%		Early	Similar to 5BB, more suitable for higher attitudes. Broad spectrum of nematode tolerance
1103 Paulsen	V. berlandieri x V. rupestris	High	High	Susce ptible	Moderate	Clay, Lime	High	High	18%	Mode tate	Late	Vigor is between 99R and 110R
RS-3	Ramsey x Schwarzmann	Low		High	High	Sandy		Low-Medi um		Medi um	Medium-High	RS-3 should not be over-irrigated. Fanleaf tolerant and broad nematode resistance.
RS-9	Ramsey x Schwarzmann	Medium		High	High			Low-Medi um		Medi um	Low	Suited for close planting, broad nematode resistence
Kingfisher	PC01126-29	V. champinii x V. rufotomentosa x Riparia Gloire	High		Resistant	High						
Matador	PC0188-151	101-14 Mgt x (V. mustangensis x V. rupestris)	High		Resistant	High						
Minotaur	PC0188-32	101-14 Mgt x (V. mustangensis x V. rupestris)	High		Resistant	High						

GRN-1	V. rupestris x muscadinia	Moderate/ High	Very High	Very High	Very High		Moderate	Tolerant	Low	Low	Moderate/ High	Highly resistance to ring, citrus and lesion nematodes
GRN-2	V. rufotomentosa x V. champinii	Low/ Moderate	Very High	Very High	Very High		Moderate	Moderate	Moderate	Moderate?	Low/ Moderate	Hightly resistance to lesion nematode and moderately resistant to citrus and ring nematode
GRN-3	V. rufotomentosa x V. champinii	Moderate+	Very High	Very High	Very High		Moderate/ High	Moderate	Moderate/ High	Moderate/ High?	Moderate+	Also resists citrus and lesion nematodes, but not ring
GRN-4	V. rufotomentosa x V. champinii	Moderate/ High	Very High	Very High	Very High		High	Moderate	Moderate/ High	Moderate/ High?	Moderate/ High	Also resists citrus and lesion nematodes, low to moderate ring resistance
GRN-5	V. champinii x V. berlandieri x V.riparia	High	Very High	Very High	Very High		High	Low/ Moderate	Moderate/ High	Moderate/ High?	High	Also resists citrus an lesion nematodes, moderate ring resistance, moderately difficult to propagate
110 Richter	V. berlandieri x V. rupestris	High	High		Moderate	Moderate Fertility	High	High	17%	Moderate	Late	Suitable for hill-side, dry-farmed sites can be overly vigorous on deep fertile soils.
140 Ruggeri	V. berlandieri x V. rupestris	Very High	High		Moderate	Sandy Moderate Fertility	Moderate/ High	Low		Low	Late	Tolerates a wide variety of soil
Freedom	1613 C x V. champinii	High	Moderate	Very High	High	Sandy Moderate Fertility	Moderate/ High	Low		Low	Late	Must use virus free scion material. More vigorous than Harmony, but less than Dog Ridge and Salt Creek.
Harmony	1613 C x V. champinii	High	Low	Susceptible	High	Sandy Moderate Fertility	Moderate/ High					More vigorous tham 1613C, less than Dog Ridge and Salt Creek.
Ramsey	V. champinii	Very High	Moderate	High	High	Light Sand Low Fertility	High	Moderate		High	Late	Tends to have Zn deficiency. Less vigorous than Dog Ridge. Reduced fruit set.
VR 039-16	V. Vinifera x V. rotundifolia	High	Low	Very High	Susceptible		Low				Late	Highly recommended for vineyard sites infested with grape fanleaf virus.
333 E.M.	Vitis Vinifera x Vitis Berlandieri	High	Moderate		Susceptible		High	Low	40%			well suited to shallow, dry and chalky soils
R27	Vitis berlandieri x Vitis riparia	High										
106/8	V.riparia x (V.cordifolia x V.rupestris)	Moderate	Moderate			Heavy Clay		High	Low			Well suited to clay soils but not calcareous and flooded in winter
M1	106/8xV.berlandieri	Low	High			Deep/Fertile			40%	Moderate/ High		high resistance to chlorosis induced by calcareous soils. high ability to accumulate anthocyanins and polyphenols
M2	Teleki 8Bx333 E.M.	Moderate	High			Deep/Fertile			15%			high efficiency in absorbing jointly both Mg and K
M3	R27xTeleki 5C	Low	High			Moderate Fertility			22%	Low		high efficiency in the absorption of K
M4	41BxV.berlandieri	Low	High			Moderate Fertility			22%	High		drought tolerant and high resistance to salinity

Table 2: Grapevine rootstocks guide (modified from vintagenurseries.com)

1.1.3 Biotic and abiotic stresses: selection of new grapevine rootstocks

Although several rootstocks are available (Galet, 1988), most of widely spread growing rootstocks are no more than ten varieties (http://catalogoviti.politicheagricole.it/).
This is related to a limited genetic background due to the fact that 90% of all rootstocks used around the world originated from less than ten different rootstock cultivars (Serra et al., 2013).
Phylloxera resistance was a principal component in the beginning rootstock selections. Currently the main goals of breeding are the adaptability to the environment conditions (related to the soil) and nurseries had to be able to easily root dormant cuttings of rootstock selections and cuttings needed to graft easily with *V. vinifera* scion varieties as well (Cousin, 2005).
E.U. is world leader on grapevine nursery industry with 546 million of grafted vine produced in 2012. Spain, France and Italy represent the 87% of the total nurseries hectares with 41 varieties grown (Zavaglia et al., 2014).
In Italy 39 rootstocks are allowed to growing and can be considered a wide availability and choice but 78% of the total surface is occupied by only 5 rootstock: 1103P, K5BB, SO4, 110R, 420A (NRVV, http://catalogoviti.politicheagricole.it/).
The increasing incidence of pest emergencies, represented by nematode, viruses or root rot and the consequences of climate change on water availability and the raising of salinity of the soils, reveals traditional rootstocks inadequate and imposed the develop of new genotypes with improved characters of resistance to biotic and abiotic stresses.
Is also necessary associate the ability to reduce energy inputs, such as fertilizer use, using the great variability of the different species of the genus *Vitis spp.* with selective absorption of some mineral elements, both to reduce the risk of deficiencies that to avoid the excesses that may in the case of nitrogen, favor the occurrence of fungal diseases botrytis in the first place.
The existing rootstocks have repeatedly demonstrated critical situation based on the recent demands of modern winemaking that sets the stage in the response to abiotic and biotic stresses. For example, some rootstocks widely spread in French viticulture as 161-49C and 420A are responsible of serious decay phenomena for the scion whose

causes have not been identified yet and do not find in other genotypes of valid substitute.

The combination of plant genomics, physiology and agronomy, as well as recently developed plant modeling techniques carried to a Second Green Revolution (Wollenweber, 2005).

The Second Green Revolution, combining biotechnology and traditional farming techniques focuses attention on sustainable agriculture based on the improving of the performance under environment limitations caused by biotic and abiotic stresses.

Following are listed the present aims of the main research groups who are working on rootstocks selection:

- Australia: available for commercial use from 2007, Merbein 5489, Merbein 5512 and Merbein 6262 are the three new rootstock available resistant to phylloxera, *Meloidogyne spp.* with high crop water use index and chloride and sodium exclusion (Clingeleffer, 2007).

- USA: the UCD-GRN rootstocks (GNR1, GNR2. GNR3. GNR4 and GNR5) were developed over a period of 15 years and available for commercial sales in 2010 and shown a resistance to nematode. In Figure 8 is shown the diagram of sequence of event, screening and testing that has resulted in the release of five rootstocks.

- Italy: at the Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DiSAA) of the University of Milan (UniMI) a new breeding project finalized to develop new rootstock selecting four new genotypes that show tolerance to water and salt stresses and ferric chlorosis (M series rootstocks: M1, M2, M3 and M4) (figure 9).

In detail, the genetic background of materials are: M1 - 106/8 [V.rip. x (V. cord. x V. rup.)] x V. berlandieri cv. Resseguier n. 4 – M2 - Teleki 8B (V.berl. x V.rip.) x 333 E.M. (V.vin. x V.berl.) – M3 - R 27 (V.berl. x V.rip.) x Teleki 5C (V.berl. x V.rip.) and M4 - 41 B (V.vin. x V.berl.) x V. berlandieri cv. Resseguier n.4.

- Australia: the CSIRO Division of Plant Industry has developed a breeding program with 55 novel inter- and intra-species hybrids. Three of these hybrids (2– Merbein 5489, 3–Merbein 5512 and 12–Merbein 6262) have recently been released for Australian viticultural industry (Jones, 2010).

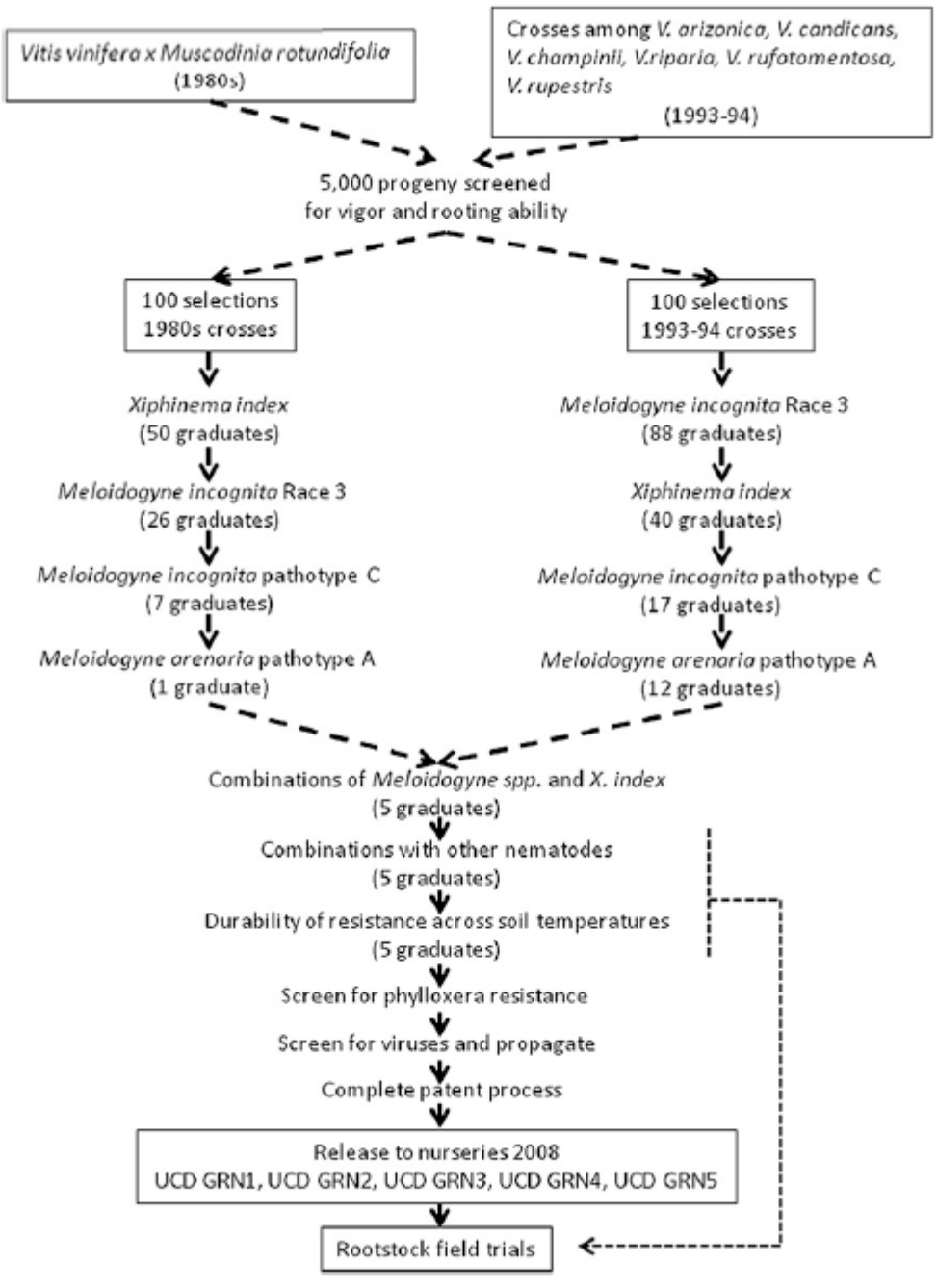

Figure 8: Protocol designed for the selection of GRNseries rootstocks (from Ferris et al., 2012)

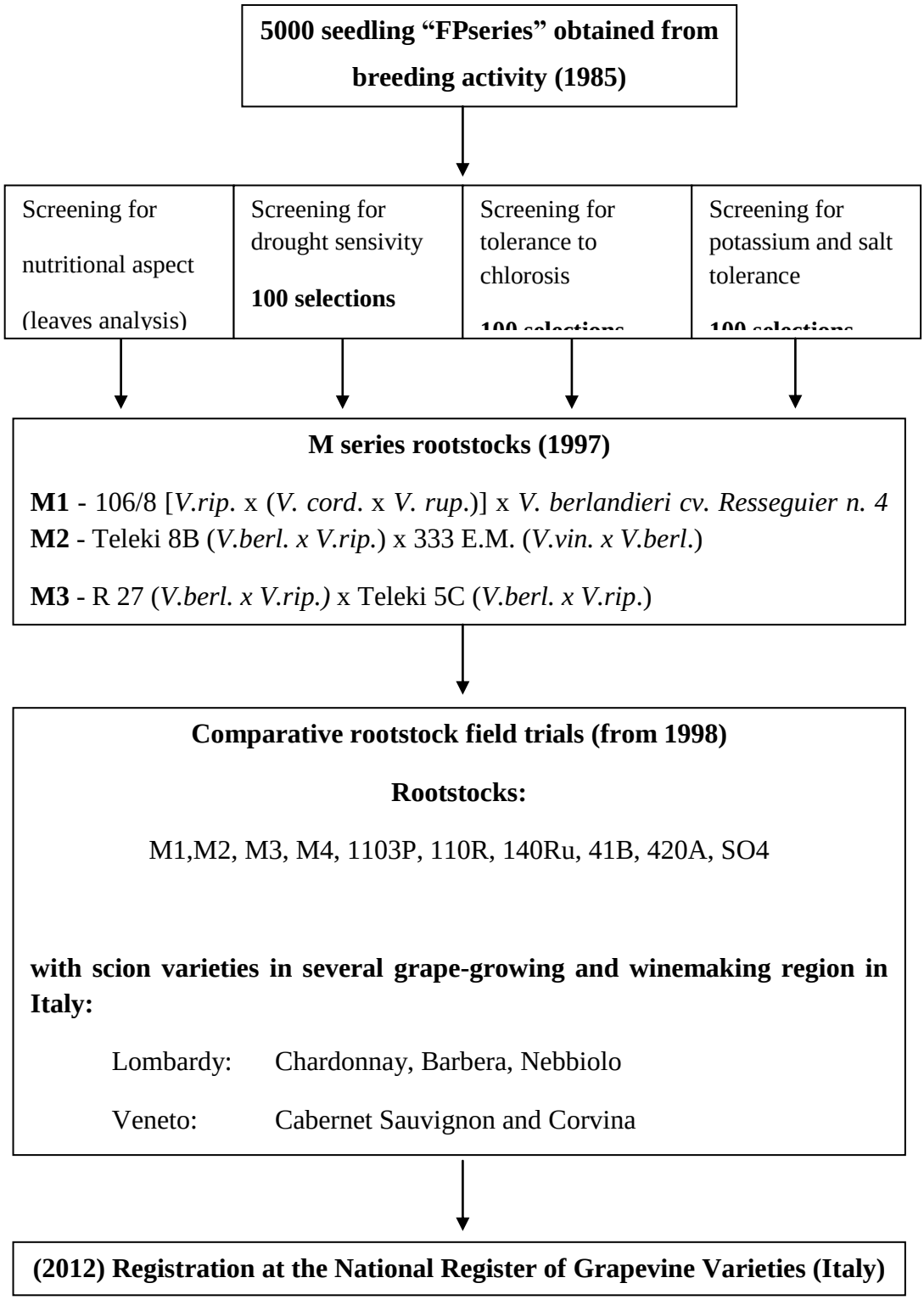

Figure 9: screening process of M series rootstocks

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1.2 Drought Stress in Viticulture

1.2.1 Effects of drought stress and different behaviors in grapevine

Abiotic stress continues to have a significant impact on plants based upon the percentage of land area affected and the number of scientific publications directed at various abiotic stresses (Cramer, 2011).

According with FAO World Soil Resources Report 2000, drought stress affects 64% of global land area and 16% of global rural land area.

Agriculture is a major user of water resources in many regions of the world. With increasing drought and a growing population, water will become an even scarcer commodity in the future (Chavez, 2003).

A large proportion of vineyards are located in regions with seasonal drought (e.g. Mediterranean-type climates) where soil and atmospheric water deficits, together with high temperatures, exert large constraints on yield and quality. The increasing demand for vineyard irrigation requires an improvement in the efficiency of water use (Chavez, 2003).

Plants can respond to drought stress using different ways: escape, avoidance and tolerance strategies. Drought escape is the ability of a plant to complete its life cycle before serious soil and plant water deficits occur (Shashidhar, 2013). Plants can also endure drought conditions by avoiding tissue dehydration, while maintaining tissue water potential as high as possible, or by tolerating low tissue water potential (Chaves, 2003).

Grapevine is an interest model plant to study because it has evolved different strategies to face with drought stress. In fact is possible to find the two drought tolerance mechanisms (with no drought escape) in the form of drought responses such as stomatal closure, decrease of cell growth and photosynthesis, activation of respiration, and accumulation of osmolytes and proteins (Tsegay, 2014) .

Isohydric represents a plant behavior in which leaf water potential is kept steady (regardless of soil water status) while anisohydric represents a plant behavior in which, under decreased water availability, leaf water potential decreases accordingly **(**Hochberg et al., 2012).

This classification is analogous to the physiological classification into isohydric and anisohydric plants and fundamentally linked to stomatal behavior (Shultz, 2003).

Isohydric species tend to have tighter control over stomatal aperture, with the result that fluctuations in leaf water potential in response to soil water deficit are minimized. Anisohydric species express less control over stomatal aperture resulting a substantial reductions of leaf idraulic potential with increasing soil water deficit (Soar et al. 2006).

In the literature is possible find a classification of grapevine varieties considering the response of the water potential to water deficit (iso or anisohydric), cultivated in soil or in pots (Chaves et al., 2010).

Recent studies confirmed that isohydric and anisohydric behaviours are linked to several environmental condition of growth (Lovisolo et al., 2010).

The same individuals can move from an isohydric-like behavior when transpiration is low to an anisohydric-like behavior with increasing water demand. For this reason is better talk about isohydric and anisohydric like behaviors as responses to drought stress (Figure 10).

At saturated light, under drought stress the decrease of stomatal conductance (Gs) with increase of vapour pressure deficit (VDP) (showed in figure 9 like LnD) is proportional to reference Gs for isohydric like behavior (Xeric line at figure n (A). It has been shown that reference stomatal conductance Gs_{ref} (Gs_{ref} = Gs at D= 1 KPa) and the sensitivity (Sens.) of the stomatal response to D are both a function of soil moisture and whole-plant hydraulic conductance (Kplant, Kleaf and Kroot) (Domenec et al., 2012).

Instead for anisohydric like behaviour Gs_{ref} and Sens to D can decrease as soil moisture increases and that the same individuals can switch from an anisohydric-like behavior when soil water moisture content is high to an isohydric-like behavior when soil water is low (Domenec et al., 2012). Thus, a combination of hydraulic and hormonal signal (ABA) in some species could be a mechanism allowing some species to switch from an isohydric to anisohydric behavior (Domenec et al., 2012).

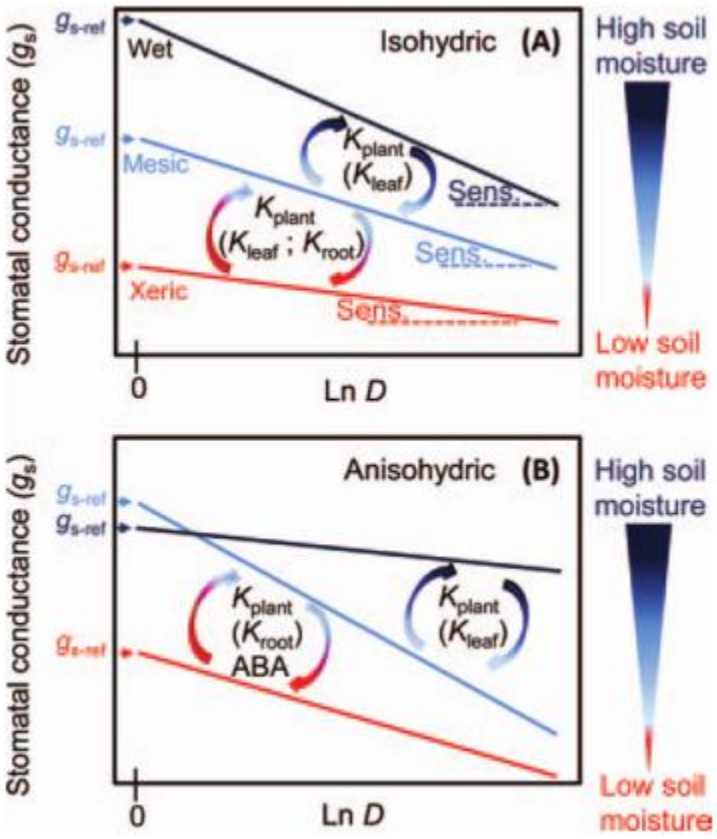

Figure 10: Isohydric and anisohydric behavior in different soil conditions (Xeric, Mesic and Wet) from Domec, J.C. and Johnson, D.M.,2012

Other important responses are the hydraulic-chemical signals from the roots considered the long-distance signaling of water deficits (Chaves et al., 2012).

Hydraulic responses are connected to hydraulic architecture of the plants (Shultz, 2003) which is based on three general qualitative properties: integration, compartmentation and redundancy as shown in figure 11 (Cruiziat et al., 2003).

Integration consider the vascular system like unique network where any which any root is more or less directly connected with any branch and not with a single one (Cruiziat et al., 2003).

Another characteristic is compartmentation of the conducting system, builds of tracheids and vessels, forms a kind of small compartment and the connections are ensured by pits (Cruiziat et al., 2003).

Redundancy consider the percentage of wall surface in common and if one element of a given track is blocked, water can pass along another parallel track (Cruiziat et al., 2003).

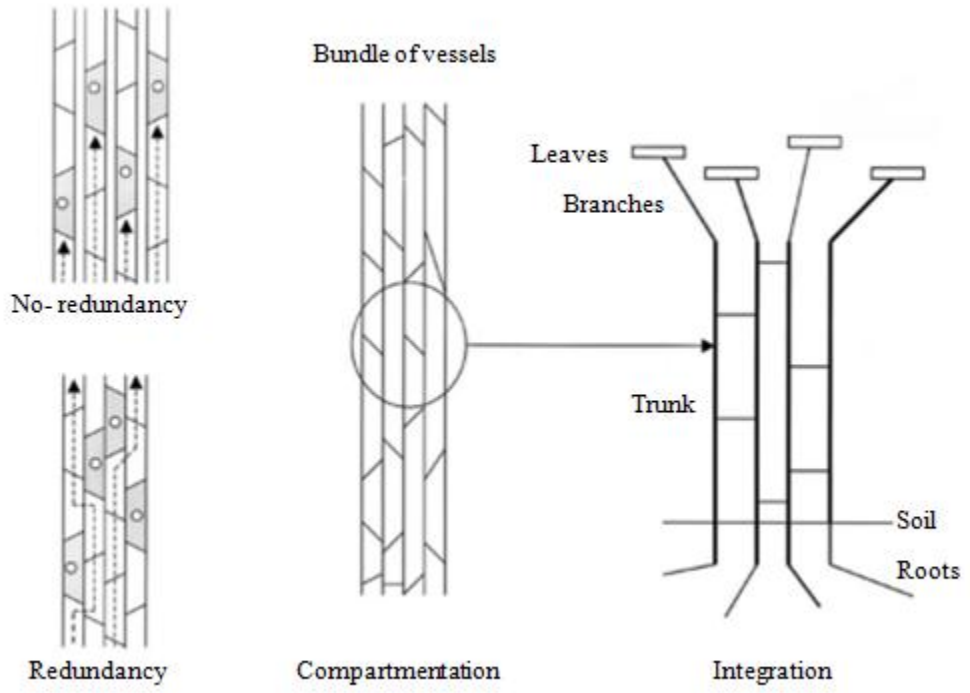

Figure 11: Idraulic architecture proprierties: Integration, Compartmentation and Redundancy (from Cruiziat et al., 2003)

Hydraulic architecture is also a complex interaction of three important component (Cruiziat et al., 2003): the first is based on the "electrical analogy" or Van den Honert model that is the earlier approach, described in 1948, to deal with using resistances, capacitances, water potentials, flow to explain water transfer through the soil-plant-atmosphere continuum (Steudle and Peterson, 1998).

The second is the cohesion-tension theory based on this important property of the water (cohesion) and the tensions generated in the xylem who permits a continuous water column from the leaves to the root apices and throughout all parts of the apoplast in every organ of the plant (Tyree, 1997).

The third is anatomy of xylem conducting system composed by tracheids and vessels and pits (Figure 12).

Tracheids are constituted by elongated specialized cells, with long size, thin and tapered; they have also an important role on the support.

Vessel elements are shorter, wider; perforated end walls; stack to form tubes; where the water flows freely.

The Pits represent the elements of conduction between the vessels, which play a major role in protecting the con-ducting system from entrance of air. Water travels through pits (Cummings B., 2005).

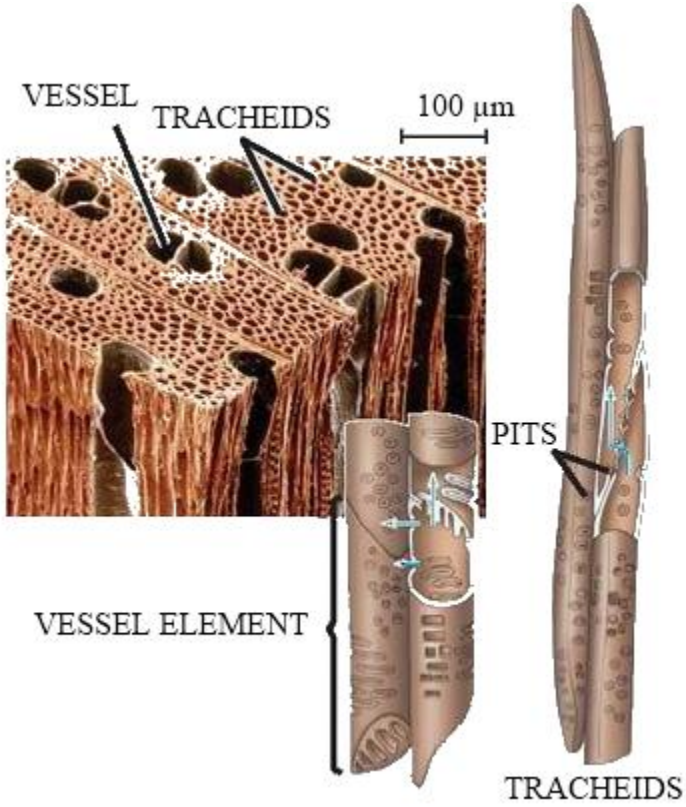

Figure 12: Xylem conducting system: xylem tracheids and vessel element (from Cummings B., 2005)

Under drought stress one of the first possible effect is xylem cavitation (embolism) which occur when a water critical tension is reached in the lumen of xylem vessels and pits in vessel walls allow the passage of air through them (Steudle, 2001).

At negative pressure the ascendant water show a metastable state of tension and pressure in xylem vessels is much smaller than the equilibrium water vapor pressure at the given temperature (Cruiziat et al., 2002).

This concept is better explained by the vulnerability curves (VC) (Cruiziat et al., 2002).

Another phenomena involve in the xylem embolism is the air seeding. In a dehydrated stem the air is pulled in the vessel through the pit membrane pores (Sperry et al. 1996). In correspondence of the pores an air-water meniscus is formed until the difference between gas pressure and xylem pressure forces holding it in that place (Sperry et al. 1996).

The resistance to cavitation is one of the most important parameter determining the drought resistance of a tree (Cruiziat et al., 2002).

According to Cruiziat (2002) the vulnerability to drought-induced embolism is due to the diameter of the pit pores and not by the diameter of the conduits. Recent studies shown that the vessel morphology is unrelated with xylem tolerance to drought in the elm genotype studied (Venturas et al., 2013).

Assuming that the basic process of embolism are air seeding and the metastable state of water under negative tension, is possible speculate that vessel size could play an important role on xylem cavitation for the following reason: larger vessels tend to have greater total pit area, which increases the probability that large pores occur in pits at the inter-vessel junction.

Large pores allow cavitation at a lesser negative water potential, hence resulting in a greater vulnerability to embolism (Choat et al., 2008).

This reminds of the traditional view that events of cavitation should occur more often in vessel members having a bigger volume than tracheids (Steudle, 2001).

Morfological architecture can affect the sensitivity of stomatal conductance to drought stress (Shultz, 2003; Wheeler et al., 2005; Hacke et al., 2006; Sperry et al., 2006).

Hydraulic signals are not the only responses to drought stress. During the early stage of drought stress chemical signals play also an important role on root to shoot signaling like the change of chemical composition of the xylem sap (pH), ABA, cytokinins, malate and a precursor of ethylene content (Serra et al., 2013; Tsegay et al., 2014).

Another effect of dehydration lead an increasing of the pH of the leaf apoplast is the reduction of H^+-ATPase activity. However, this mechanism was seemingly not

involved in the alkalinization of xylem sap of plants in drying soil. (Wilkinson and Davies, 1997).

The change on the pH of xylem sap might due to the low nitrate availability (Schachtman and Goodger, 2004). Under water stress nutrient availability is reduced leading an increasing of malate and reduction of the activity of nitrate reductase, causing changes in the pH with the alkalization of the xylem sap (Schachtman and Goodger, 2004).

This alkalization of the xylem sap promotes the dissociation of the undissociated form of ABA (ABAH) in ABA^-, building-up the ABA content in the apoplast at stomatal level where specific plasma-membrane-bound receptor like GCR2 (g-protein coupled receptor2) and intracellular receptors CHLH (the H subunit of the magnesium protoporphyrin-IX chelatase that is localized in the chloroplast), are present (Ferradino et al., 2009; Schachtman and Goodger, 2004).

An increasing of the abscisic acid in the concentrations in the apoplast lead to an efflux of potassium (K^+) and anions (A^+) alter guard cell turgor causing stomatal closure (Schachtman and Goodger, 2004).

Therefore under drought stress the production of ABA at root level and the subsequent transportation to the leaves is one of the main mechanism the plant uses to report on the water status of the soil but according to Schachtman (2008) some ABA synthesis within the leaves that may interact with this communication mechanism.

This studies confirm the important role of ABA on root to shoot chemical signals and different grapevine rootstocks have different tendency to generate these signals (Tsegay et al., 2014)

Other phytohormones involve at root to shoot signal are the cytokinins (CKs) especially because are synthesized mainly in the roots (Schachtman and Goodger, 2004).

Under drought stress the concentration level of two main cytokinins plant hormone like Zeatin (Z) and Zeatin ribose (Zr) in roots, shoot tips and buds decreased (Chaves M., 2003). In particular Stoll et al. (2000) found in grapevine under water stress a decreasing of 50% of CKs content.

1-aminocyclopropane-1-carboxylic (ACC) precursors of ethylene could also play an important role under drought stress and may play a role in decreased leaf growth like Voisin et al. (2006) found in maize (Schachtman and Goodger, 2004).

Another effect of drought stress is the change in morphology of the leaf especially on stomatal density. Serra et al. (2008) found that stomatal density (number of stomata per unit area) and size are affected by drought stress and the same scion grafted onto different rootstock showed different stomatal densities and sizes.

In particular the leaves of Pinotage grafted onto 140Ru presented lower stomatal density but bigger pore diameter than those grafted onto 110 Richter and 1103 Paulsen (Serra et al., 2008).

Abiotic stress conditions such as drought can also affect the responses at molecular level. Is widely recognized that drought stress can influence the gene expression and the transcriptional regulation in leaf and roots tissue (Soar et al., 2006; Gambetta et al., 2012)

After cell drought signaling, the responses diverge in different pathways according to the involvement or not of the abscisic acid. Considering the ABA-dependent pathway, the accumulation of ABA activates various stress-associated genes (Chaves, 2003).

In Arabidopsis, under water deficit, a key enzyme 9-cis-epoxycarotenoid dioxygenase (NCED3) is upregulated promoting ABA biosynthesis from carotenoids (Daszkowska-Golec and Szarejko, 2013).

In grapevine ABA abundance in water stressed tissues have been linked with the expression of one or more of the ABA biosynthetic genes, in particular the genes, *Vv*NCED1 and *Vv*NCED2, encoding the NCED enzyme and zeaxanthin epoxidase (*Vv*ZEP) encoding for ZEP enzime (Speirs et al., 2013 and Soar, 2004).

ABA biosynthesis pathway from C40 β-carotene is shown in figure 13.

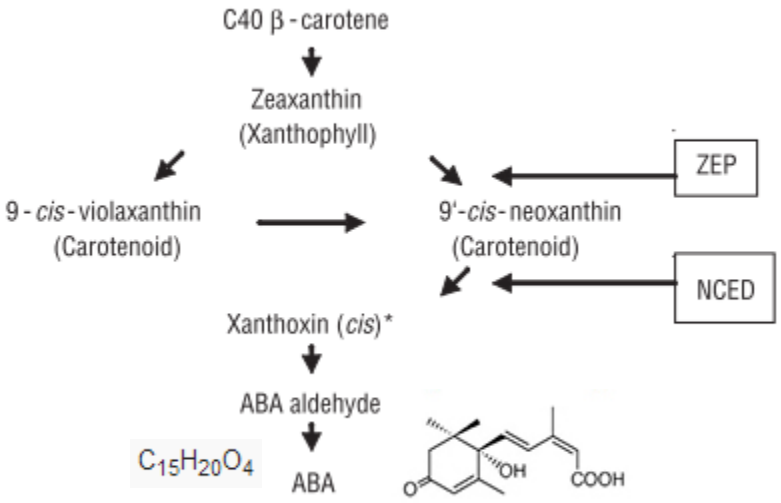

Figure 13: ABA biosynthesis pathway (from Soar, 2004)

Furthermore there are different responses in a short than in a long period of time.

Soar et al. (2006) studied NCED1 (*Vv*NCED1) in two grapevine variety detecting the gene expression in roots and leaves at variation in the atmospheric vapor pressure deficit (VPD).

They found that the *Vv*NCED1 was principally expressed in leaves than in roots at high level of VPD and in shorter period (Soar et al., 2006).

However, in a long period, the roots showed an high *Vv*NCED related gene expression negatively correlated with the amounts of irrigation being applied (Speirs et al., 2013).

As shown in Figure 14, ABA signaling pathway consists of three protein classes: the ABA receptors pyrabactin resistance (pyr)/regulatory component of aba receptor (rcar) (PYR/RCARS), the type 2C protein phosphatases (PP2Cs) and the SnRK2 kinases (Hubbard et al., 2012), (Boneh et al., 2012).

In well watered condition, ABA level is low and 2C-protein-phosphatase acts like negative regulators of ABA signaling prevents phosphorylation and activation of SnRK2s and downstream factors (DFs) (Park et al., 2009).

In stress condition, like water stress, the ABA receptor PYR/RCARs interact with PP2C promoted the interactions with the inhibition of phosphatase activity. This inhibition lead to an activation of SNF1-RELATED KINASE 2 (SnRK2) protein kinases, with the activation of transcription factors as basic/region leucine zipper (bZIP) which may activate the transcription of drought-related genes (Hauser et al., 2011).

Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression (Hubbard et al., 2010).

ABA regulatory genes and their expression are regulated mainly by two different families of bZIP transcription factors (TFs), ABI5 (seeds) and AREB/ABFs in the vegetative stage, in an ABA-responsive- element (ABRE) dependent manner (Boneh et al., 2012).

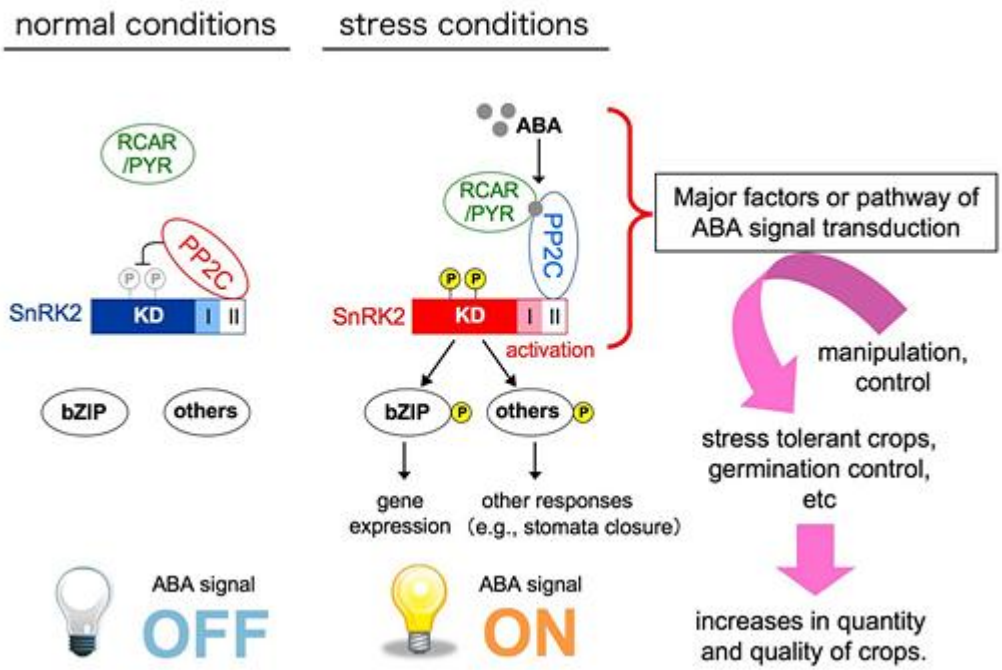

Figure 14: ABA signaling mechanism (ABA signal transduction) from http://www.riken.jp/en/pr/press/2009/20090922/

Among the responses ABA synthesis is important in regulating stomatal closure.

In Arabidopsis was found a ABC transporters (ABCG40) which is identified like ABA importer (Osakabe et al., 2013).

In the guard cells ABA acts like negative regulator by inhibiting SRK2E/OST1 kinase activity (Figure 15).

In Grapevine OST1 (OST1/SnRK2.6/SnRK2E) is known to be a positive regulator of ABA-dependent stomatal changes (Boneh et al., 2012). OST1 inhibits K^+ influx channels (KAT1), and activates anion channels like SLAC1.

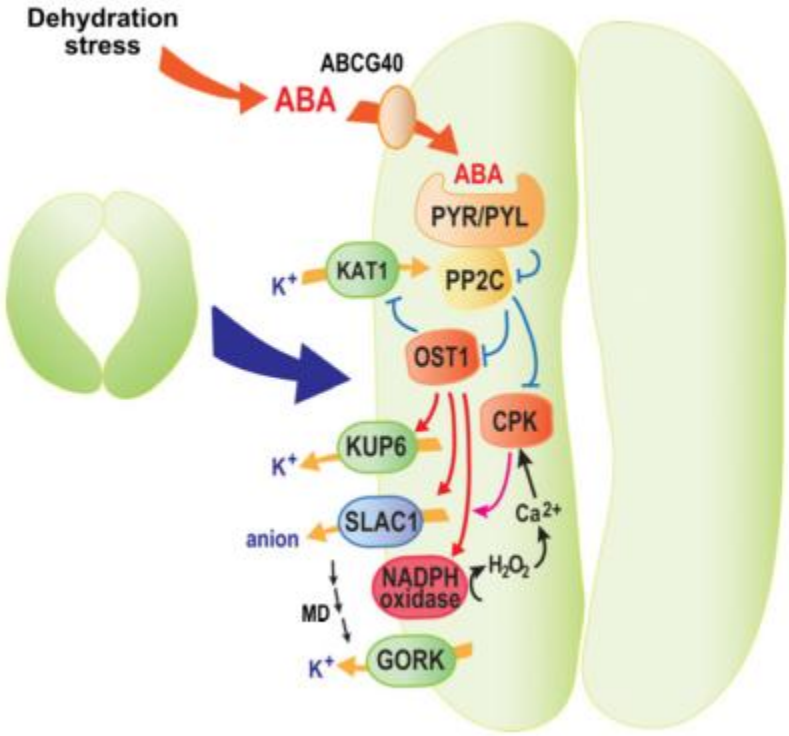

Figure 15: The signaling pathway and ion transport system involved in stomatal closure (from Osakabe et al.,2013)

All these responses to drought stress, especially the stomatal closure, allow an increasing of the WUE in grapevine. In fact, according to Chavez et al. (2010) intrinsic water use efficiency (Pn/gs or WUEintrinsic) is usually higher in vines under deficit irrigation (mild to moderate water deficits) than under well watered conditions.

Other response could involve specific proteins like aquaporins (Delrot et al., 2010).

Aquaporins are members of the major membrane intrinsic protein (MIP) family and are important on the transport across the cell-to-cell pathway (Vandeleur et al., 2009), (Gambetta et al., 2003). These proteins are classified in four sub-family: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins, and small basic intrinsic proteins (Vandeleur et al., 2009). Several genes that encoding for aquaporins were shown to be up-regulated in Arabidopsis in response to drought (Chaves et al., 2003)

In Grapevine has been detected a decrease in aquaporins expression between the grapevine root tips and older root portions (Gambetta et al., 2013).

Other studies in grapevine have also shown a down-regulation of some aquaporins expression under drought stress and an effect on the leaf hydraulic conductance in leaves (Pou et al., 2012).

Flavonoid metabolism is also involved in drought stress response. In figure n is shown the pathways and relative gene involved.

Perturbation in grapevine physiology associated to drought is may impact on flavonoid metabolism pathway. Drought often causes oxidative stress and an increase of flavonoids and phenolic acids in the leaves of several plants (Ramakrishna and Ravishankar, 2011), (Tattini et al., 2004).

In grapevine the flavonoid biosynthetic enzymes genes sensitive to endogenous and environmental stimuli connected to drought stress, and genes developmentally regulated in berry were studied (Castellarin et al., 2007).

Many flavonoid biosynthetic genes are induced under stress conditions and, accordingly, flavonoid levels increase during exposure to biotic and abiotic stresses, such as wounding, drought, metal toxicity and nutrient deprivation (Hernández et al., 2007). A common denominator in these environmental stress conditions is the production and accumulation of reactive oxygen species (ROS) (Hernández et al., 2007). Flavonoids have been suggested to act as antioxidants, protecting plants from oxidative stress in particular during exposure to biotic and abiotic stresses like drought stress (Hernández et al., 2007), (Ramakrishna and Ravishankar, 2011).

Another specific pathway involved in drought responses is the stilbenoids biosynthesis. The pathway of synthesis of stilbenes can be considered an alternative pathway to the biosynthesis of flavonoids (Hernández et al., 2007).

Stilbenoids are produced via the phenylalanine and the last step of which is catalyzed by the enzyme stilbene synthase, STS (figure 16).

Stilbene synthases are closely related to chalcone synthase, the key enzymes of the flavonoid pathway and they share the same substrates (Nopo-Olazabal et al., 2014).

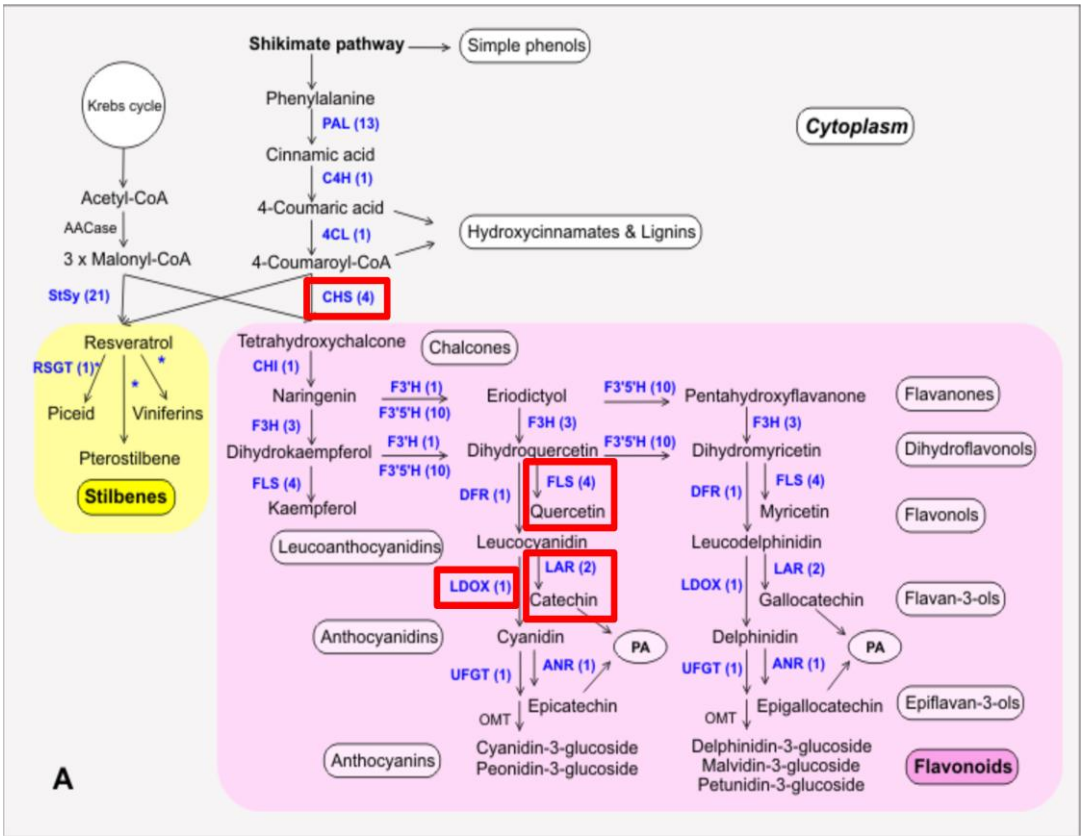

Figure 16: Phenylpropanoids biosynthesis (from Velasco et al., 2007)

Several transcription factors (TFs) are also involved in the regulation of flavonols, proanthocyanidins and anthocyanins pathways (Figure 17).

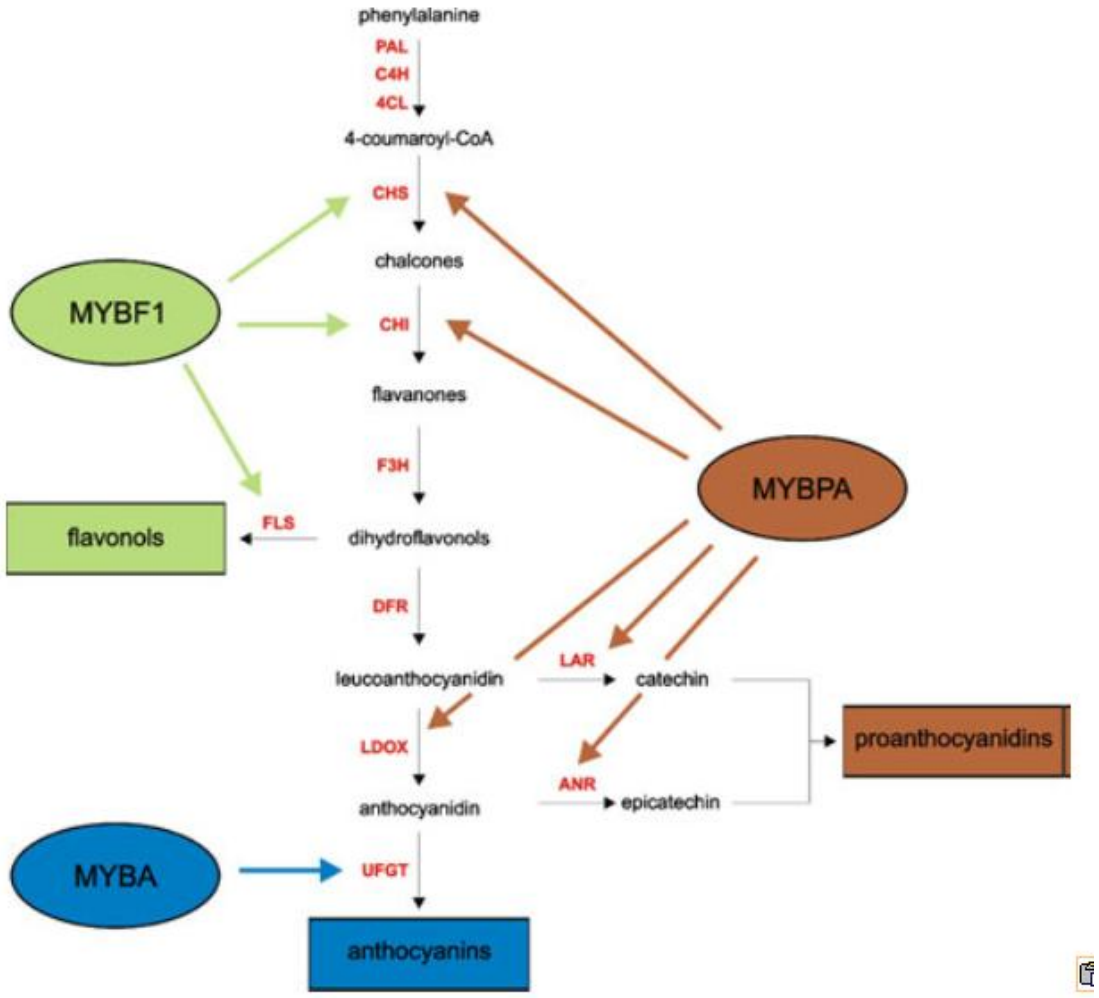

Figure 17: Transcription factors involved in the regulation of flavonols, proanthocyanidins and anthocyanins pathways (from Czemmel et al. (2012)

In particular VvMYBA TFs are involved in the regulation of the synthesis of anthocyanins, VvMYBPA TFs regulate different structural genes of the flavonoid pathway while VvMYB TFs seems involved in the synthesis of flavonols (Bogs et al., 2007).

In grapes, VvMYBPA appears capable of activating the both the early and late shared genes of the flavonoid pathway as well as the PA-specific genes encoding both ANR, LAR and CHS (Bogs et al., 2007).

In grapevine these transcription factors were studied with the flavonoid pathway responses during ripening of berries (Castellarin et al., 2007), (Bogs et al., 2007).

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1.3 Characterization of drought stress effects

1.3.1 Traditional Phenotyping

Among the methods to evaluate plant responses to drought stress, water potential (Ψ) is widely accepted as an indicator as a fundamental measure of plant water status (Hsiao, 1973).

Ψ is the algebraic sum of the component potentials arising from the effect of pressure (Ψp) of solutes (Ψs), and of matrix (Ψm):

$$\Psi = \Psi p + \Psi s + \Psi m$$

Considering that Ψm is very close to zero in well-watered leaves and fleshy tissue, in many species Ψm does not become significant numerically until much of the tissue water (e.g. 50%) is lost (Hsiao, 1973). So unless the tissue is badly dehydrated, the component potentials of concern in most cases are:

$$\Psi = \Psi p + \Psi s$$

Höfler diagram (figure 18) shows the interdependence between the potential and cell volume and all the component of water potential (Ψp and Ψs).

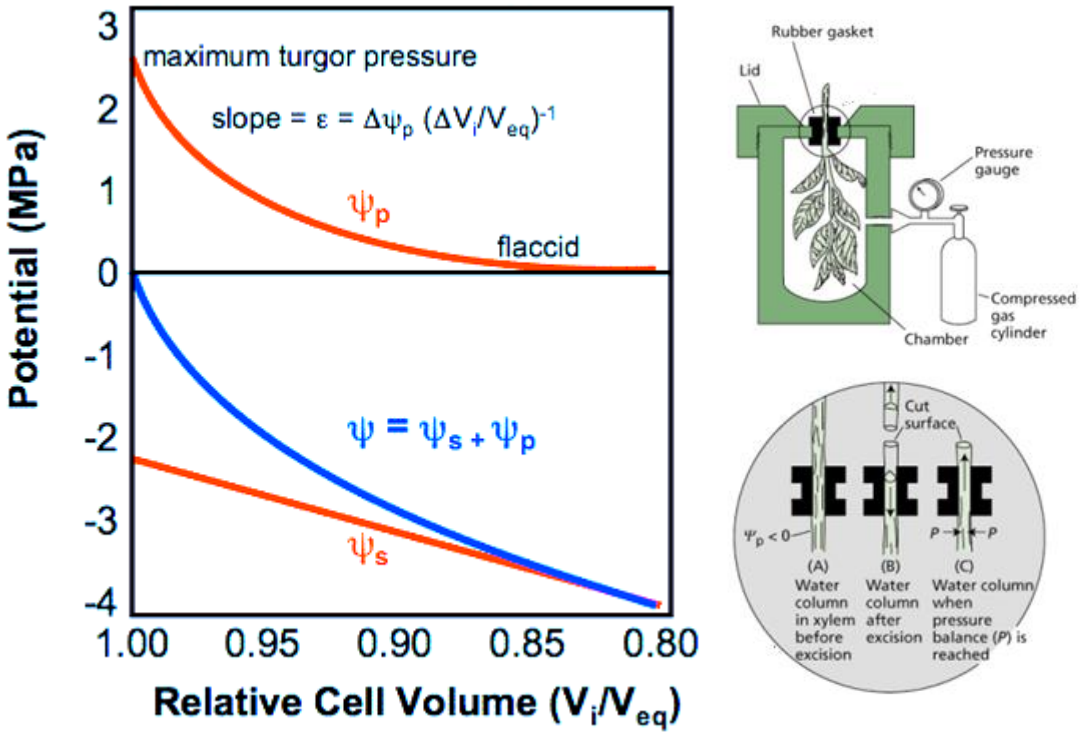

Figure 18: Pressure Chamber and Höfler diagram (from Koning, 1994 and Tiez, 2010)

Water potentials in vascular plants can be measured using a pressure chamber. Dixon was the first to use a pressure chamber to measure the water status of leaves but was popularized by Scholander an half century later (Turner, 1988).

These potentials are measured on plant organs, generally leaves. The pressure chamber technique can be used to measure:

– midday leaf water potential

– pre-dawn leaf water potential

– stem water potential

Leaf water potential can be used as approximates of the average of the concerning organ: in xylem tissue, the osmotic pressure component at apoplastic level is much lower, and therefore negligible, than the hydrostatic pressure and xylem is in close relation with most of the cells composing an organ. It is therefore acceptable to estimate the obtained hydrostatic pressure as the average equilibrium point between the apoplast (xylem) and the symplast of the organ (Delrot, 2010).

It is important to select the right moment of the day, being an inconstant value. For leafΨ and stemΨ 12.00 AM is a commonly accepted standard condition, corresponding with the time of maximal transpiration in the plant (Delrot, 2010).

Stem water potential is measured during the day on a leaf that is bagged with an opaque plastic bag at least 1 h prior to measurement (Delrot, 2010).

One hour after bagging is reached the balance between the leaf and the stem xylem potential.

Pre-dawn leaf water potential do not require to be bagged and it can also be assumed as being equal to the soil water potential.

There are other techniques to measure water potential using psychrometer and pressure probe (Tiez and Zeiger, 2010).

Tissue water content (percent of fresh weight) and fresh weight have also been used as indicators of water status (Hsiao, 1973). Another commonly used indicator of plant water status is relative water content, or RWC (Hsiao, 1973).

To measure relative water content, one leaf was sampled from one plant per plot (no plant was sampled twice). Then, immediately after cutting their blade, the leaves were wrapped in aluminum foil, put in a plastic bag and kept in a cool place. Fresh weight was determined two hours after cutting. Turgid weight was determined as follows: the leaves were held in distilled water at room temperature (approximately 20°C) for 16- 18 hours; then, they were quickly and carefully dried by tissue; next, their fresh weight was determined; and finally, their relative water content was calculated by the following equation:

$$RWC\ (\%) = [(W-DW) / (TW-DW)] \times 100$$

Where, W, TW and DW are sample fresh weight, sample turgid weight, sample dry weight respectively (Rahimi et al., 2011). Other indicators of plant water status are leaf thickness and stem diameter (Hsiao, 1973). Recent technologies have introduced innovative methods to measure the leaf thickness and there is generally a quite good overall correlation with leaf water potential (Zimmermann et al., 2013).

The measuring of stem diameter, in particular the trunk growth using linear dispenser transducer can reflect the effect of water stress (Escalona, 2002).

1.3.2 High Throughput Phenotyping (Applicability of non-invasive and no destructive phenotyping tools)

Using novel "omics" approaches including genomics, epigenomics, transcriptomics, proteomics and metabolomics, scientists are more and more able to elucidate genes and mechanisms able to regulate major plant traits (Salekdeh et al., 2009).

The advances of these technologies are giving a considerable amount of data for marker assisted selection (MAS) of parents and progeny in early generations (Salekdeh et al., 2009).

For these reasons there is a pressing need for a searchable phenotypic database linking gene sequence to plant structure, development, composition and performance, all measured in a clearly defined environment (Furbank, 2011).

Almost all the phenotyping techniques, considered in the previous chapter used to check the water conditions of the plants, are invasive, destructive and take a long time for the measurements.

The applications of non-invasive phenotyping tools has become more common in laboratories in the commercial sector (Furbank, 2011).

These technologies fall into the circle of the plant phenomics.

Plant phenomics is the study of plant growth, performance and composition and could be described as simply 'high-throughput plant physiology' (Furbank, 2011).

To receive the full benefit of the available genomic information, plant phenomics, which integrates technologies such as photonics, biology, computers, and robotics, will permit the functional characterization of genes (Yang, 2013) .

The most frequently investigated phenotypic traits include root morphology, leaf characteristics, biomass, yield-related traits, photosynthetic efficiency, and abiotic stress response (Yang, 2013).

Several tools are included in the phenotyping: 2D, 3D digital images, infrared-hyperspectral imaging, 3D structural tomography and functional imaging (Yang, 2013).

The application of these technologies consider a different part of electromagnetic spectrum and can monitor several traits (Table 3).

Applications of the current photonics-based techniques in rice, wheat or barley

Optical technique	Cost	Trait	Species	Reference
Visible light imaging	Low	Shoot biomass	Barley	[19••]
		Yield traits	Rice	[20••]
		Panicle traits	Rice	[24]
		Root architecture	Rice	[25,26]
Near-infrared imaging	Medium	Leaf area index	Rice	[30–32]
Far-infrared imaging	Medium	Shoot or leaf temperature	Barley, wheat	[55]
		Insect infestation of grain	Wheat	[75]
Hyperspectral imaging	Medium	Leaf health status	Rice	[33,59]
		Leaf health status	Wheat	[61]
		Leaf growth	Rice	[34]
		Panicle health status	Rice	[35]
		Grain quality	Wheat	[36]
Fluorescence imaging	Medium	Photosynthetic performance	Multivarieties	[42]
		Leaf health status	Wheat	[61]
X-ray digital radiography	Medium	Grain quality	Wheat	[72–74]
X-ray computed tomography	Medium	Tillers	Rice	[38••]

Table 3: Trait observed at different wavelength of the electromagnetic spectrum (Yang, 2013)

In fact, every portion of the electromagnetic spectrum (Figure 19) can provide several information about the structure and physiological conditions of the plant (Figure 20).

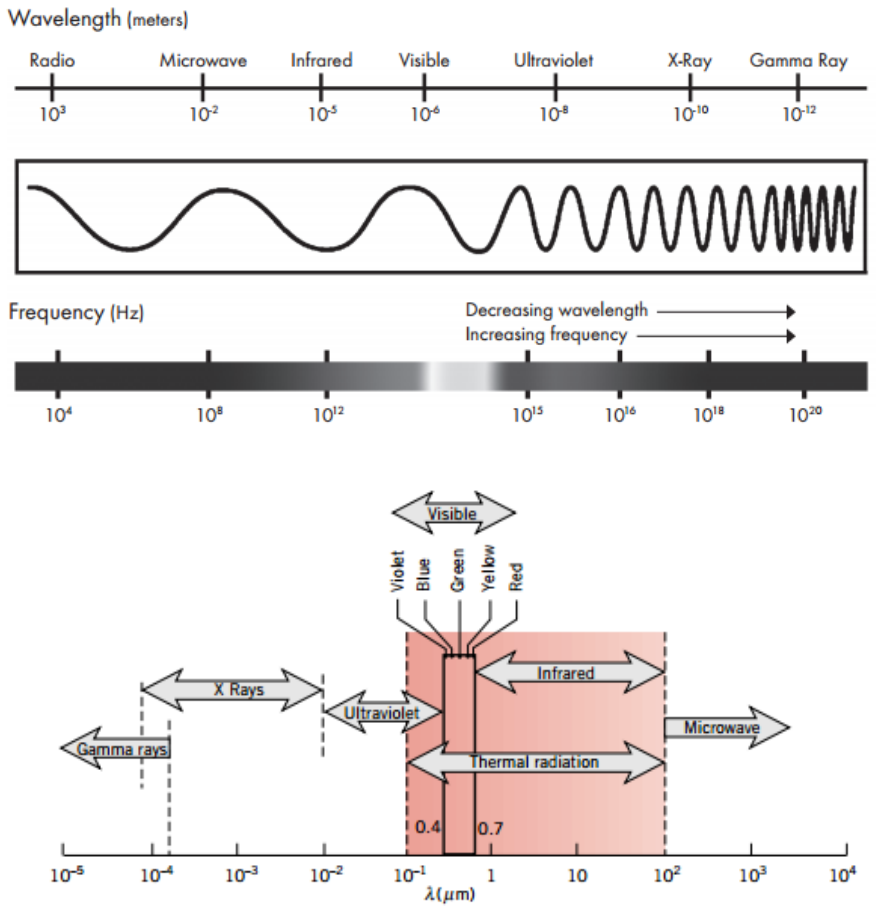

Figure 19: Electromagnetic spectrum and different wavelength http://www.WesternReservePublicMedia.org and Incopera et al., 2007

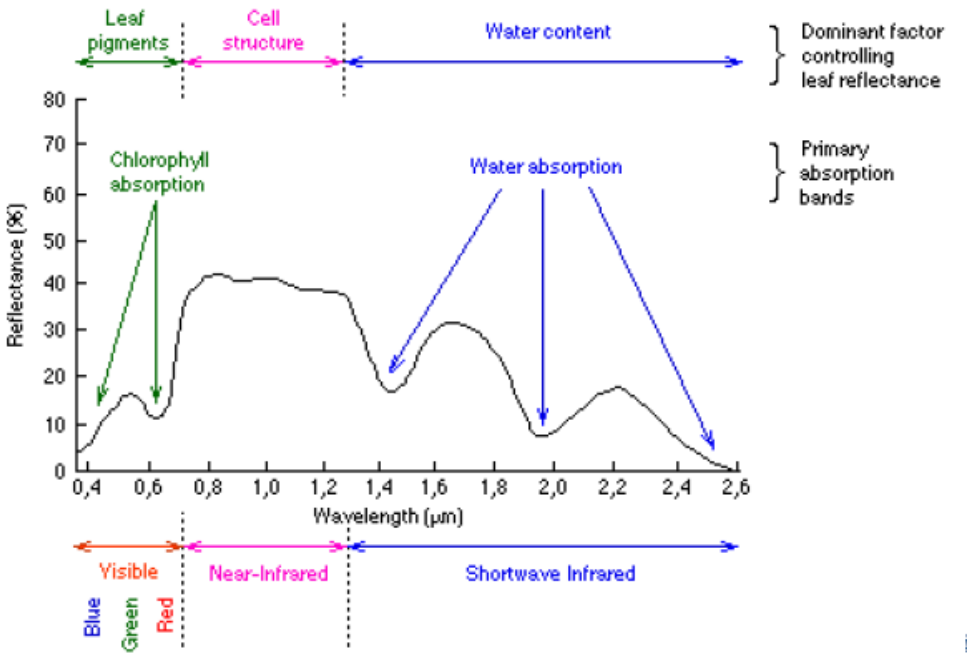

Figure 20: Theoretical spectral response of vegetation (from Boschetti, 2006)

According to Bass et al. (2001) all these measures are based on several properties of the targets monitored:

- Reflectance: the amount of flux reflected by a surface, normalized by the amount of flux incident on it.
- Transmittance: the amount of flux transmitted by a surface, normalized by the amount of flux incident on it.
- Absorptance: the fraction of incident flux that is absorbed (any flux not reflected or transmitted is absorbed).

In recent years the analysis of the responses at infrared radiation spectrum portion showed a significant increase.

1.1.3. Infrared thermography (IRT)

Thermography is included in the remote sensing measurements such as visible imaging, near-IR and thermal IR imaging, chlorophyll a fluorescence imaging, multispectral imaging and luminescence imaging (Costa et al., 2013).

The application of this technology is based on the detect and measure the radiation and put in relation with the surface temperature of an object.

Radiation is the movement of heat that occurs as radiant energy (electromagnetic waves) moves without a direct medium of transfer (American Technical Publishers, 2009).

Up to now the term "infrared radiation" or division of IR radiation is not standardized (Chrzanowski, 2005). Here the classification based on limits of spectral bands of commonly used infrared detectors and relative wavelength range:

- near infrared (NIR): 0.78 μm – 1 μm
- short wave infrared (SWIR): 1 μm – 3 μm
- mid wave infrared (MWIR): 3 μm – 6 μm
- long wave infrared (LWIR): 6 μm – 15 μm
- very long wave infrared (VLWIR): 15 μm – 1000 μm

There are three modes of heat transfer: conduction, convection and radiation and heat transfer by radiation between 0.78 to about 1000 μm of the electromagnetic spectrum (Kaplan, 2007).

At this spectrum portion (thermal radiation) another process is determinant for correct measurements: the emittance.

The emittance is the ratio of the radiance of an object or surface to the radiance of a blackbody (planckian radiator) at the same temperature (Bass et all, 2001).

To confirm this there are two important radiation law: Planck's radiation law consider that every object at a temperature above absolute zero (0 Kelvin) emits electromagnetic radiation in the IR region of the spectrum and Stefan Boltzmann law consider that the amount of infrared radiation emitted by an object depends on its emissivity (ε) and absolute temperature.

Emissivity is the fraction of blackbody emittance at a given wavelength emitted by a material (Campbel and Norman, 1998).

$$\varepsilon = M/Mb$$

where M = radiant emittance (W/m^2) power emitted from a surface of the body

Mb = radiant emittance (W/m^2) power emitted from a surface of a black body

Blackbody has emissivity ε = 1 (perfect emitter) than gray bodies ε is between 0 and 1 (Table 4).

Surface	Emissivity	Surface	Emissivity
maize leaf	0.94	human skin	0.98
tobacco leaf	0.97	snowshoe hare	0.99
bean leaf	0.94	caribou	1.00
cotton leaf	0.96	gray wolf	0.99
sugar cane leaf	0.99	gray squirrel	0.99
poplar leaf	0.98	window glass	0.90–0.95
cactus	0.98	concrete	0.88–0.93
polished chrome	0.05	soil	0.93–0.96
bright aluminum foil	0.06	water	0.96

Table 4: Several surfaces emissivity (from Campbell and Norman, 1998)

Of course there are to consider the radiation emitted by the atmosphere and the radiation emitted by the object surrounding and reflected by the object's surface but most thermal cameras take automatically the object's temperature once the emittance and background radiation has been input (Costa et al., 2013).

This is important because at the same temperature there could be a variation on thermal radiation emitted like confirmed by Leslie's cube (Leybold Didactic GMBH).

Infrared thermography is based on particular instruments defined thermal camera. After the first prototypes (Lisowska-Lis et al., 2011), the develop of devices which provide to record the electromagnetic energy in the thermal region started during the 1960's (Cracknel and Hayes, 1990).

There are several terms used as synonyms of the term "thermal camera" (figure n): thermal imager, thermograph, thermovision, thermal imaging systems, infrared imaging radiometer, infrared imaging system (IIS), thermal viewer, thermal video system, infrared camera, thermal imaging device (figure 21) (Chrzanowski, 2005).

By definition thermal camera is an infrared system enabling creation of two dimensional image of temperature distribution on the surface of the observed objects using thermal radiation emitted by these objects (Chrzanowski, 2005).

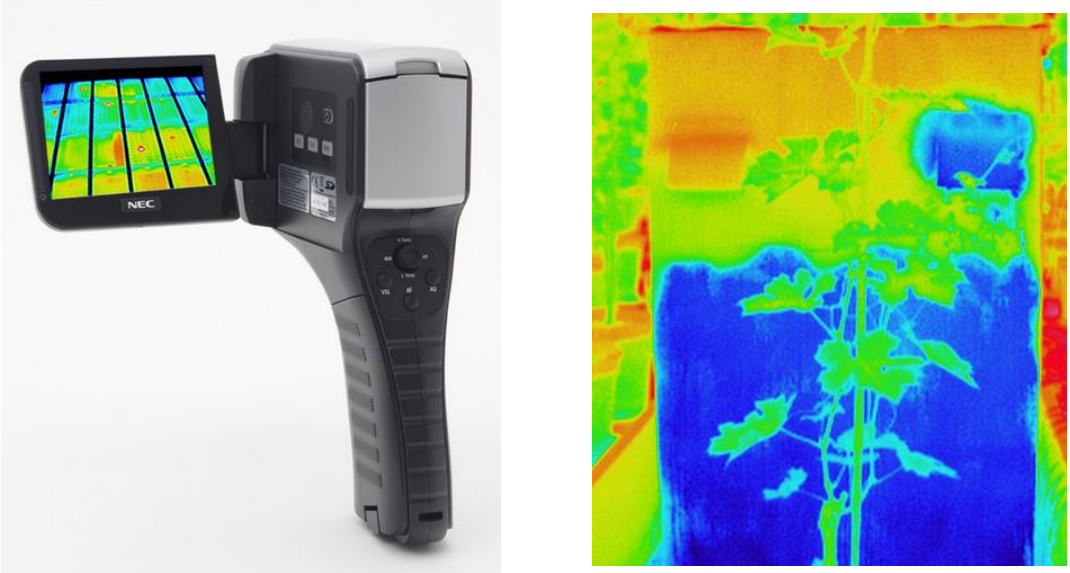

Figure 21: Thermal camera and thermal image of a young vine

A general understanding of how thermal imaging systems operatet is extremely important for a thermographer to work within the limitations of the equipment (American Technical Publishers, 2009). The infrared radiation is projected by the optical devices of the camera on a detector causing a reaction, usually a variation of voltage or electrical resistance that is read by the electronics in the thermal imaging system. The signal produced by the camera is converted into an electronic image, or thermal image, on a display screen. A thermogram is an image of a target prepared electronically on a display in which different color tones correspond to the distribution of the infrared radiation onto the target surface. In this simple process, the user of the tool is able to see the thermal image that corresponds to the energy radiating from the surface of the target (American Technical Publishers, 2009).

The detector usually measure radiation in the 3.5 – 5.0 µm and 8.0 - 14 µm (Cracknel and Hayes, 1990). The wavelength considered is short wave infrared (MWIR) camera, a long wave infrared (LWIR).

Other thermal cameras detects at SWIR capturing wavelengths between 1-3 µm (Hinesa et al., 2004). Selective use is made of these different wavelength regions depending on the target temperature.

The most common commercial thermal cameras work in the range of the radiation emitted between 8 µm to 14 µm falling in the long wave infrared range (LWIR).

Use of thermal camera need specific software for image processing and to facilitate analysis and report writing (American Technical Publishers, 2009).

1.1.1 Thermography in viticulture

The application of thermography techniques in viticulture started in the beginning of new millennium (Jones et al. 2002).

Several approaches have been employed according to the goals of the experiments: thermal images from above the canopy, sometimes hundred meters far to the object in open field (remote sensing) and thermal images taken laterally, from some meters to centimeters to the canopy (proximal sensing) in open field or controlled environmental condition (Jones et al. 2002, Jones et al. 2009, Zia et al., 2009).

One of the main goal of the application o thermography is the detection of the water status of the plants and has been also proposed for pathogen detection (Fuentes, 2012).

Actual growth rate and and stomatal closure are considered the most sensitive plants responses to drought (Jones, 1999).

Some thermal indices were developed to better understand the link between thermal effect on the leaves and stomatal conductance.

Ig and I3 (Jones, 1999), CWSI and CWSImodified (CWSIm) (Idso et al. 1981, Jackson et al., 1981, Idso et al. 1982).

Ig and I3 are correlated with leaf stomatal regulation and described in the following formulae:

$$Ig = (Tdry - Tleaf)/(Tleaf - Twet)$$

$$I3 = (Tleaf - Twet)/(Tdry - Tleaf)$$

CWSI and CWSIm are more linked to the conditions of plant water stress:

$$CWSI = (Tplant - Twet)/(Tdry - Twet)$$

$$CWSIm = (Tdry - Tplant)/(Tdry - Twet)$$

Tleaf or Tplant are the temperature of the canopy of interest, Tdry and Twet are the temperature of references surfaces related to very stressed canopy with closed stomata and well-irrigated canopy with maximum conductance, non-stressed.

In the first studies were developed natural reference using vaseline coated leaves as T dry reference and water sprayed leaves as Twet (figure n a, b)

In subsequent years a lot of effort where put on the develop of artificial reference surfaces as shown in figure 22 (Meron et al., 2003 and Costa et al., 2013).

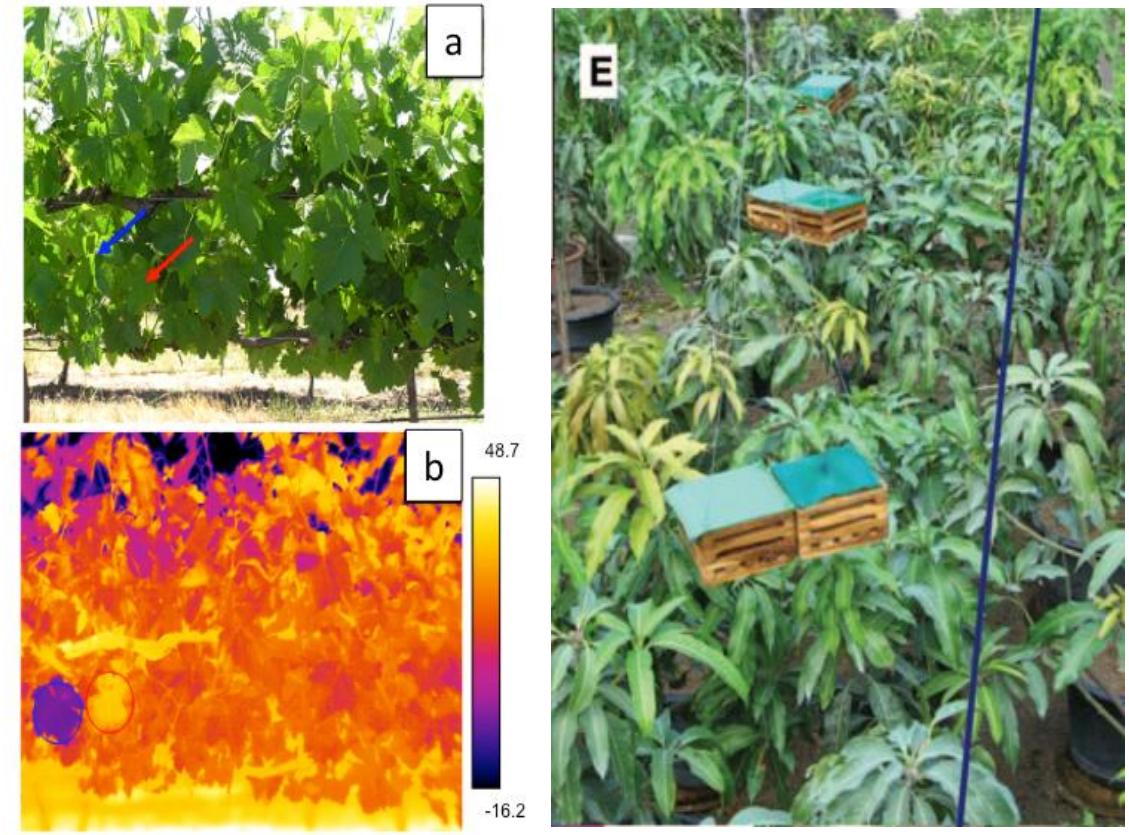

Figure 22: application of natural references (a,b) and artificial references (e) (from Fuentes et al., 2012 and Costa et al., 2013)

Other studies focus on the validation of thermal indices for status water identification in grapevine (Pou el al., 2014).

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CHAPTER 2.

EVALUATION OF DROUGHT STRESS RESPONSES IN GENUS *VITIS*: VALIDATION AND USE OF THERMOGRAPHY TO DISSECT GRAPEVINE ROOTSTOCKS RESPONSES TO DROUGHT STRESS

D. Grossi, G. Simone Di Lorenzo, L. Brancadoro, O. Failla and A. Scienza
Department of Agricultural and Environmental Sciences, Landscape, Agroenergy (DISAA), University of Milan, Italy

F. Emanuelli and M.S. Grando
Department of Genomics and Biology of Fruit Crops, Research Innovation Centre, Fondazione Edmund Mach (FEM), San Michele all' Adige, Italy

Keywords: Rootstock, Drought stress, High-throughput-phenotyping, Thermal indices, Stomatal conductance, Stem growth

This work has been submitted at the First International Roots Symposium (Rauscedo, October 2014) and has been reviewed for publication in Acta Horticulturae ISHS.

Abstract

The tolerance to drought stress is particular important among the aims of grapevine rootstocks breeding. The quality of phenotyping is crucial to achieve the targets of selection. The application high-throughput phenotyping techniques like infrared thermography has been widely spread over the last few years, allowing the study of plant-environment interactions through the development of specific algorithms based on canopy temperatures. In the framework of larger and long lasting programs, the evaluation and characterization of the new rootstock selections performance and the development-validation of non-destructive phenotyping techniques, have been carried out also to provide data for genetic association studies (GWAS). In 2012, 96 genotypes of Vitis spp., including a genus

core-collection, commercial rootstock and four new rootstocks (M series), were monitored during a dry down experiment in semi-controlled conditions. The physiological responses to water deficit were evaluated over 30 days considering Leaf Stomatal Conductance (gs), Leaf Temperature (Tl), Leaf and Stems Growth, through the use of Steady State Porometer (Licor Li-1600) and Thermal Camera (InfRec). Data analysis consisted in the elaboration of 5742 thermal imagines using the software InfraRecAnalyzer to obtain the thermal indices correlated to stomatal conductance. At the same time the growth of the plants (stems and leaves) were monitored. During the experiment the procedure for reducing the time for phenotyping has been validated. Combining the stomatal conductance and the growth data, all genotypes were classified by different physiological characteristics and behaviors under drought stress. The application of thermography allows to speed the phenotyping activities and make possible the screening of large number of genotypes. The use of this tool requires special attention during calibration phase.

2.1 INTRODUCTION

Breeding programs in grapevine rootstocks have evolved from aspects of resistance to phylloxera, to affinity of grafting and adaptability to calcareous soils. The main present interest is on rootstocks that show good performance in different places and in favorable years, but that maintain a good efficiency in difficult conditions.

Vitis vinifera L. cultivation is traditionally non-irrigated (especially in Europe) and spread widely across dry and semi-dry ecosystems (Lovisolo et al., 2010). For this reason, in recent years, the tolerance to drought stress is one of the most important aim of breeding and selection in grapevine rootstocks (Clingeleffer and Smith, 2011; Jones, 2012). The achievement of the objectives of selection is closely linked to the efficiency and quality of characterization of the phenotype under stress conditions.

Current technologies allow to provide significant information on the physiological conditions of the plants by the application of non-destructive methods and high-throughput techniques increasing the precision of phenotyping (Furbank and Mark, 2011). Thermography has been widely used over the last few years, allowing the study

of plant-environment interactions through the development of specific algorithms based on leaf temperature (Costa et al., 2012).

In particular, several indices were developed: Ig and I3 (Jones, 1999), CWSI and CWSImodified (CWSIm) (Idso et al. 1981, Jackson et al., 1981, Idso et al. 1982).

Ig and I3 are correlated with leaf stomatal regulation and described in the following formulae:

$$Ig = (Tdry - Tleaf)/(Tleaf - Twet)$$

$$I3 = (Tleaf - Twet)/(Tdry - Tleaf)$$

CWSI and CWSIm are more linked to the conditions of plant water stress:

$$CWSI = (Tplant - Twet)/(Tdry - Twet)$$

$$CWSIm = (Tdry - Tplant)/(Tdry - Twet)$$

Tleaf or Tplant are the temperature of the canopy of interest, Tdry and Twet are the temperature of artificial references surfaces related to very stressed canopy with closed stomata and well-irrigated canopy with maximum conductance, non-stressed.

Following the new rootstock selection programs conducted by the Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DISAA) of the University of Milan, the study of the strategies in response to water stress within the Vitis genus is particularly interesting.

Specific objectives are the evaluation of the variability introduced by breeding programs and development-validation of non-destructive phenotyping techniques developed to provide data for genetic association studies.

2.2 MATERIALS AND METHODS

The experiment was established in a poly-tunnel providing semi-controlled conditions at University of Milan greenhouses facility (CeTAS, Tavazzano, Lodi) during July to August 2012.

96 genotypes of Vitis spp., including a genus core-collection designed at Edmund Mach Foundation-S. Michele all' Adige-TN, four new rootstocks (M series) and five commercial rootstocks were monitored during a dry down experiment. For each genotype six one-year own root cuttings were growth in pots containing a substrate composed by sand and peat in the proportions of 80% and 20% respectively.

According to Gardner et al. (2001), the soil water content (SWC) was measured by thermo-gravimetric method: after watering and subsequent excess water draining, the substrate was weighted at field capacity, oven-dried for 48 h at 105 °C until there is no weight loss and then re-weighed.

Subsequently the SWC was determined for the substrate contained in each pot using the formula:

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

In the beginning of the experiment all six biological replicates were maintained around 90% of the SWC. After 7 days three plants were subjected to water stress (WS) than three well-watered (WW) control plants were maintained at 90%. In WS treatment water deficit was gradually established to reach firstly a moderate stable water deficit (50% SWC for 7 days), then more severe and stable water deficit (30% SWC for 7 days) and finally a recovery stage to 90% of SWC. In Figure 23 is shown the irrigation management of both treatments. All plants were weighed every morning before irrigation and daily water amount was obtained from the weight differences between weight of the pot and relative weight at established SWC.

The physiological responses to water deficit were evaluated over 30 days considering Leaf Stomatal Conductance (Gs), Net Photosynthesis (Pn), Leaf Temperature (Tl), through the use of Steady State Porometer (Licor Li-1600), Thermal Camera (InfRec) and Portable Photosynthesis System (CIRAS-2) respectively. All the experimental measurements were performed at midday between 12:00 and 14:00.

Data analysis consisted in the elaboration of 5742 thermal images using the software InfraRecAnalyzer to obtaining the canopy temperature considering six sun-exposed mature leaves per vine and relative thermal indices using dry and wet reference

temperatures. At the same time the growth of the plants were monitored measuring the shoot growth rate and the leaf width and length growth rate.

Regression coefficients, correlations and classification (hierarchical cluster analysis) were obtained using IBM SPSS statistics 21.

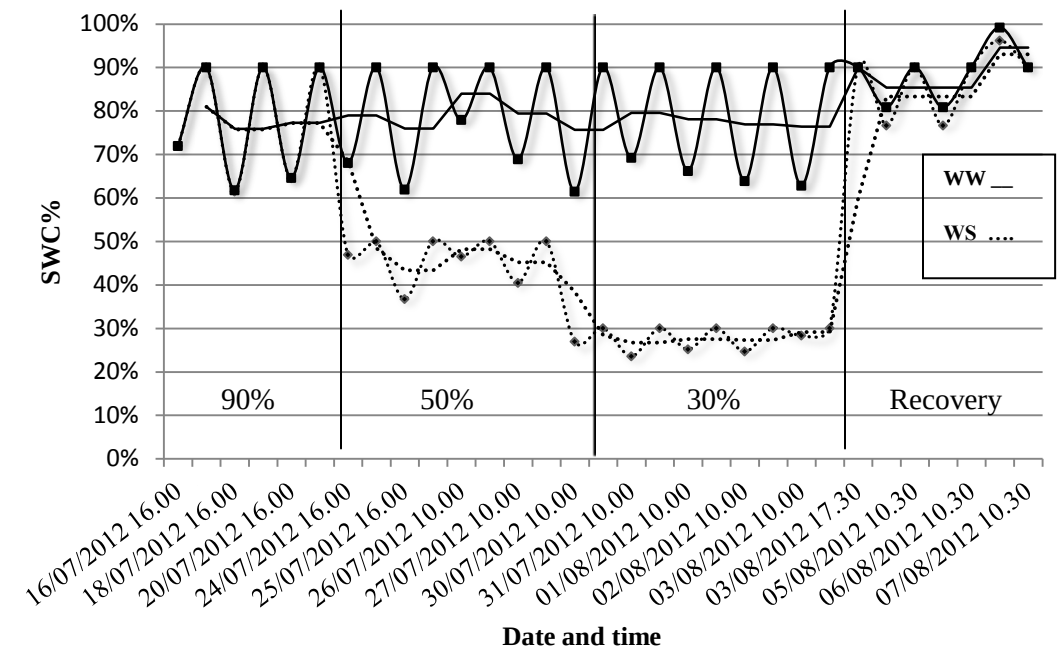


Figure 23: Irrigation management during the experiment

2.3 RESULTS AND DISCUSSION

To properly understand the meaning of the thermal indices, the results of a thermal analysis is reported in Fig. 24. Where the temperatures of wet and dry reference were 36 °C and 28 °C respectively and the variation of the temperature of the leaves are considered. Ig and CWSIm are proportional to stomatal conductance, and decrease following the stomata closure while I3 and CWSI are proportional to stomatal resistance arising following the stomata are closing.

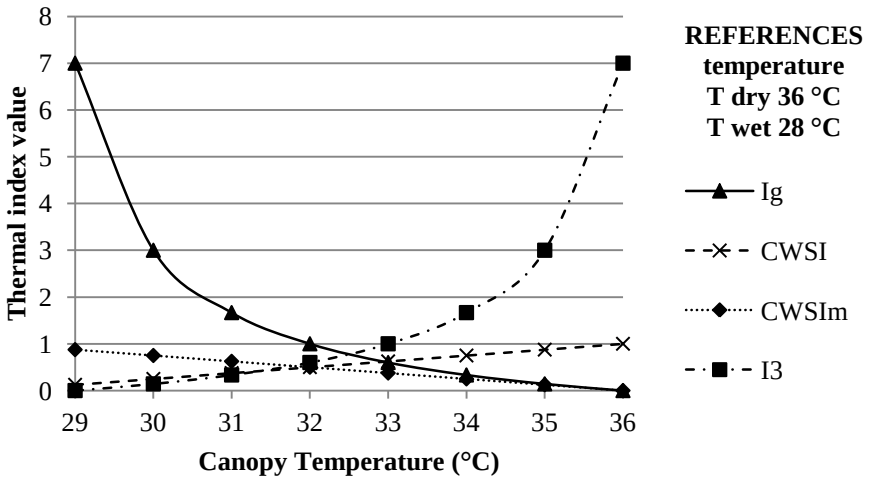
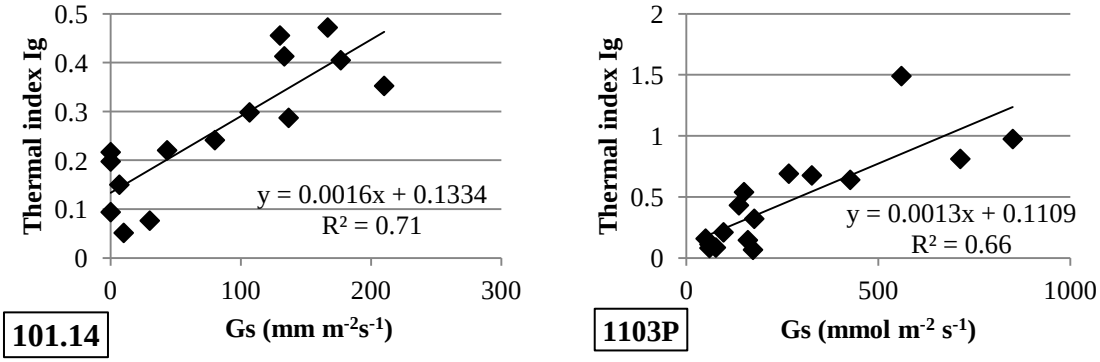


Figure 24: Simulation of thermal index values considering given reference temperatures

In Figure 25 the correlation between thermal index Ig resulting from the imaging elaboration and stomatal conductance Gs, measured using the porometer Licor LI-1600 of several genotypes are shown. According to the bibliography a good and significant correlation between leaf stomatal conductance and thermal indices were obtained. All the values, obtained from the calculations of the thermal index Ig, resulted included between 0 to 2.7 for all 96 genotypes.



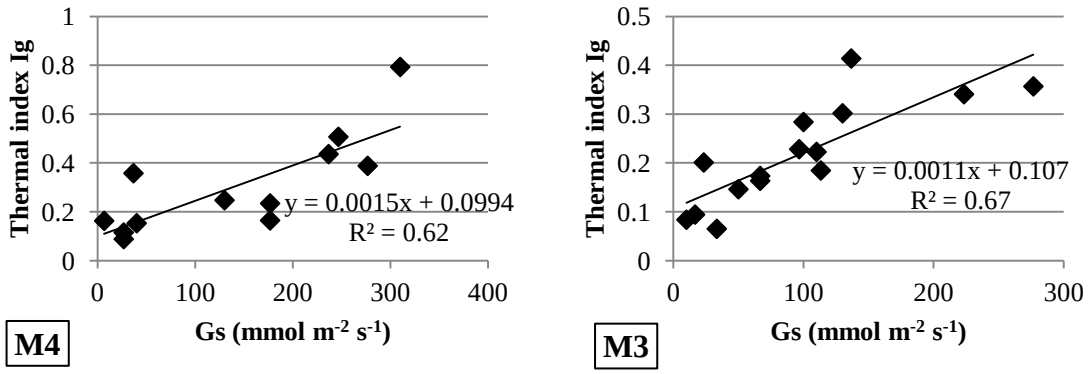
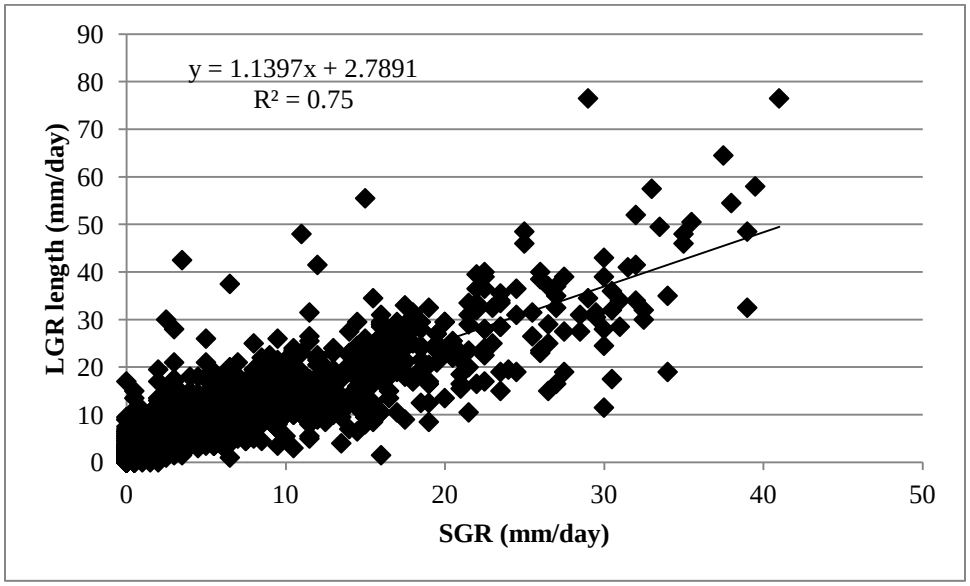


Figure 25: Correlation between Gs and Ig in several commercial rootstocks

Analyzing the leaves and shoot growth, a high relationship among plant daily growth based on stem length, leaf length and leaf width measures were observed (Fig. 26).



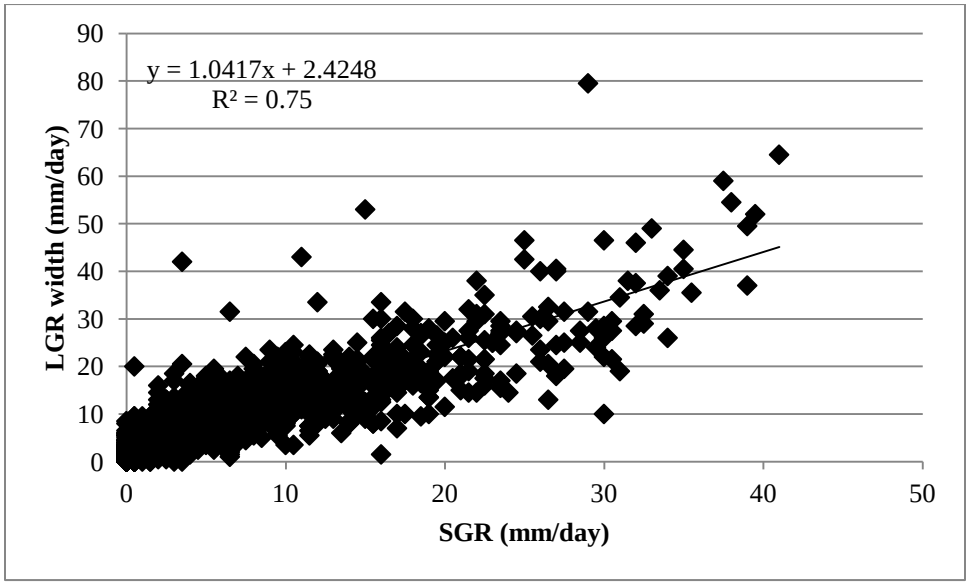
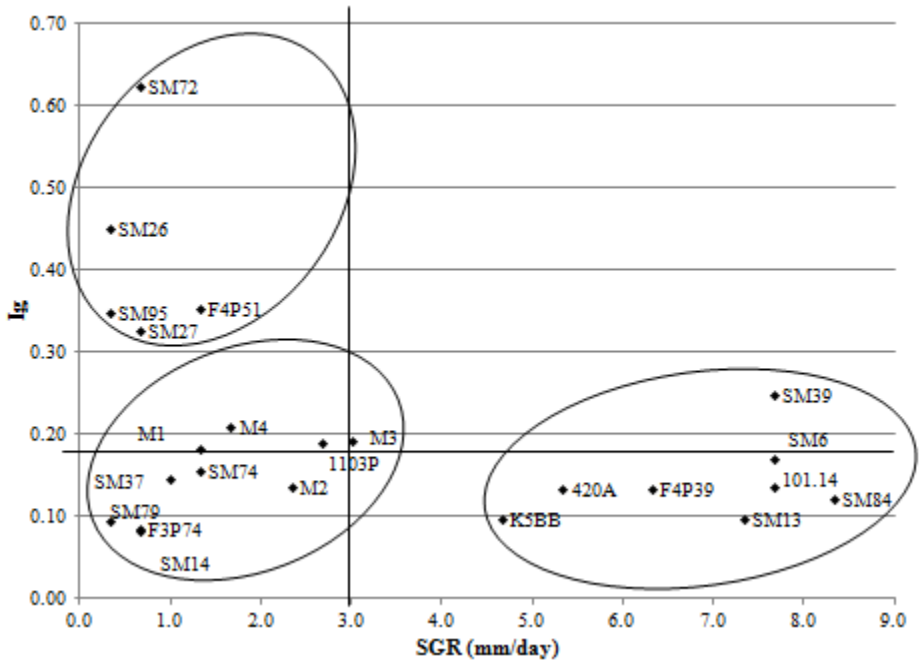


Figure 26: Correlations between leaf width-length growth rate (LGR) and stem growth rate (SGR)

Combining the stomatal conductance and the growth data, all genotypes were classified using a hierarchical clustering analysis based on average link, determining different physiological characteristics and behaviors under drought stress (Fig. 27).



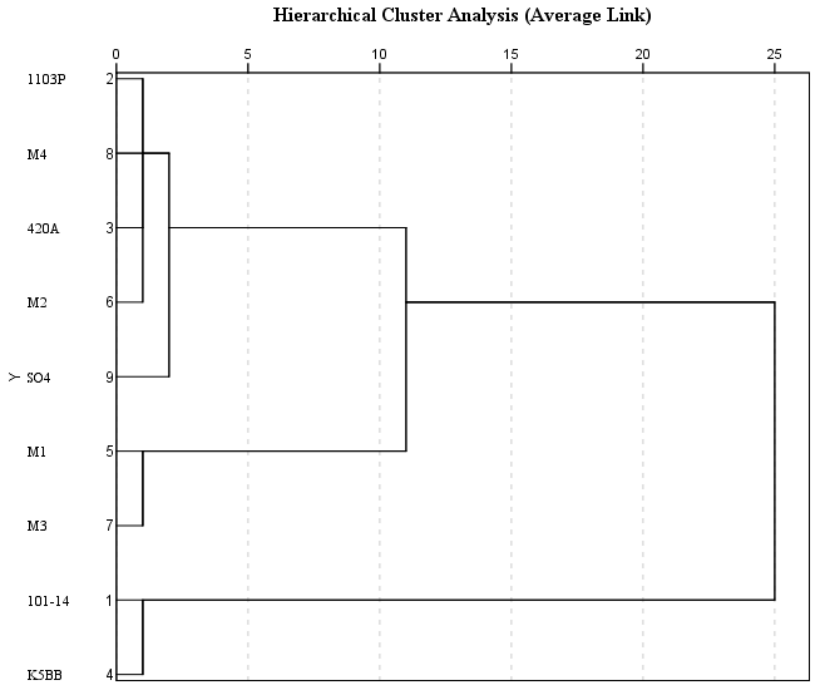


Figure 27: Classification of 22 selected Vitis spp. genotypes at 30% of SWC (hierarchical clustering analysis – average link)

2.4 CONCLUSION

Thermal imaging is an useful tool in the assessment of the effects on stomatal conductance conditions reflecting the water status of the plant. The use of this tool requires special attention during calibration phase especially when changes in environmental conditions related to temperature, wind and incident radiation occur. An appropriate validation of the measurements obtained with thermal camera and stomatal conductance measured on a pool of selected plants is recommended. The application of thermography allows to speed the phenotyping activities and make possible the screening of large number of genotypes.

Combining thermography with the measure of plants growth the information on responses to water stress may be more detailed.

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METHODS TO DISSECT GRAPEVINE ROOTSTOCKS RESPONSES TO DROUGHT STRESS

D. Grossi[1], F. Emanuelli[1,2], G. Simone Di Lorenzo[1], L. Brancadoro[1], O. Failla[1], M.S. Grando [2], A. Scienza[1]
[1]Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DISAA), University of Milan, Via G. Celoria, 2, 20133, Milano, Italy
[2]Department of Genomics and Biology of Fruit Crops, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010, San Michele all' Adige, Italy

INTRODUCTION

The tolerance to drought stress is particular important among the aims of grapevine rootstocks breeding. The quality of phenotyping is crucial to achieve the targets of selection. The application high-throughput phenotyping techniques like infrared thermography has been widely spread over the last few years, allowing the study of plant-environment interactions through the development of specific algorithms based on canopy temperatures. In the framework of larger and long lasting programs the evaluation and characterization of the new rootstock selections performance and the development-validation of non-destructive phenotyping techniques have been carried out also to provide data for genetic association studies (GWAS).

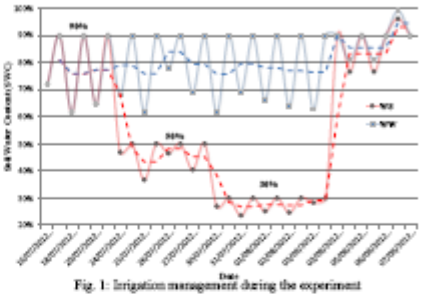


Fig. 1: Irrigation management during the experiment

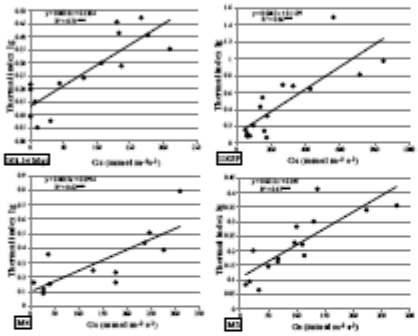


Fig. 2: Correlation between stomatal index Ig and stomatal conductance Gs

MATERIALS AND METHODS

In 2012, 96 genotypes of *Vitis spp.*, including a genus core-collection, commercial rootstock and four new rootstocks (M series), were monitored during a dry down experiment in semi-controlled conditions (Fig. 1). The physiological responses to water deficit were evaluated over 30 days considering Leaf Stomatal Conductance (Gs), Leaf Temperature (Tleaf), Leaf and Stems Growth, through the use of Steady State Porometer (Licor Li-1600) and Thermal Camera (InfRec). Data analysis consisted in the elaboration of 5742 thermal imaging (Fig . 3) using the software InfraRecAnalyzer to obtain the thermal indices correlated to stomatal conductance:

$$Ig = (Tdry - Tleaf) / (Tleaf - Twet)$$

$$CWSI = (Tdry - Tleaf) / (Tdry - Twet)$$

At the same time the growth of the plants (stems and leaves) were monitored

RESULTS AND DISCUSSION

During the experiment the procedure developed for reducing the time needed for phenotyping has been possible validated (Fig 2). Combining the stomatal conductance and the growth data, all genotypes were classified by different physiological characteristics and behaviors under drought stress (Fig. 4).

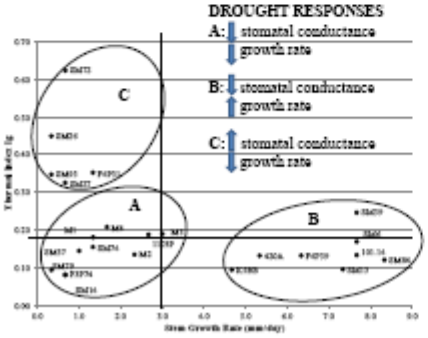


Figure 4: Classification of 22 selected genotypes at 30% of SWC (Hierarchical clustering analysis - Average Link)

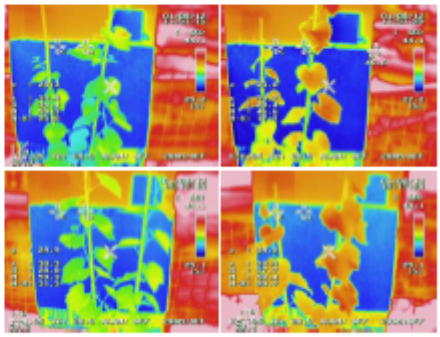


Fig. 3: Thermal imaging at 90% (left) and 30% (right) of SWC including leaves temperature and artificial reference

CONCLUSIONS

The application of thermography allows to speed the phenotyping activities and make possible the screening of large number of genotypes. The use of this tool requires special attention during calibration phase considering environmental effect like air temperature, radiation, humidity and wind speed on leaves temperature.

Funding Support: Progetto AGER-SERRES, grant 2010-2015

Figure 28: Poster presented at the 1st international Symposium on Grapevine Roots. 16th-17th October 2014, Rauscedo (PN) Italy

CHAPTER 3

PHYSIOLOGICAL AND MOLECULAR DROUGHT STRESS RESPONSES OF Cv. CABERNET SAUVIGNON (VITIS VINIFERA L.) GRAPEVINE GRAFTED ONTO NINE COMMERCIAL ROOTSTOCKS

3.1 INTRODUCTION

Drought stress is considered one of the most severe environmental stresses and the major limiting factor on plant productivity (Ramakrishna and Ravishankar, 2011).

Plants responses to drought stress vary depending on the severity of stress and the stage of drought progression (Kim et al., 2012). The drought tolerance can be associated with water use efficiency, stomatal conductance, plant hydraulic conductance, embolism repair, rooting depth and leaf dehydration tolerance (Hopper et al., 2014)

Is widely recognized that under mild to moderate drought stress the early response is the stomatal closure (Chaves et al., 2010).

The genus *Vitis spp.* is particularly interesting to study these responses because is possible find species with different behaviors and these responses are related to the leaf water potential.

Isohydric represents a plant behavior where at a given soil water status the leaf water potential is kept steady, whereas anisohydric represents a plant behavior where, under decreased water availability, decreases accordingly **(**Hochberg et al., 2012).

The regulation of stomata conductance (Gs) play a determinant role on these responses (Tsegay et al., 2014).

In the beginning these strategies were considered genotype specific (Chaves et al., 2010). Recent studies show that iso/anisohydric behaviors are influenced by the environmental conditions that plants are subjected (Lovisolo et al. 2010). For example isohydric or anisohydric behavior might depend to the soil condition water of the year (Lovisolo et al. 2010).Therefore, the term near iso/anisohydric is often used to describe different genotypes (Hopper et al., 2014).

Rootstocks could play an important role on the water deficit responses, controlling scion transpiration (Marguerit et al. 2012).

The stomatal closure can also affect the water use efficiency of the plants (Pou et al., 2008).

WUE can be expressed in several ways (Medrano et al., 2012; Padgett-Johnson et al., 2003):

- intrinsic leaf water use efficiency (WUE_{intr}) as the ratio between net photosynthesis (Pn) and stomatal conductance (Gs),
- instantaneous leaf water use efficiency (WUE_{ist})
- total plant water use efficiency (WUE_{plant}) biomass gain (grams) as a function of water use (litres)

However, plant WUE should not be a solely target for breeders, but it should be considered beside yield and grape quality (Medrano et al., 2012).

Another important response to drought results low photosynthesis and diminished shoot growth. Water stress predisposes the leaves to a depression of CO_2 assimilation and a to photohynibition cause an inactivation of primary photochemistry of the photosystem II reaction centre (During, 1988). Shoot growth rate (leaves and stem) are influenced by drought as well (Jones, 2012).

Tolerance to this abiotic stress is a complex phenomenon, comprising a number of physiological and biochemical processes at both cellular and whole organism levels. One of the main cellular events occurring during water deficit is extensive modification of gene expression resulting in a strict control of all the physiological and biochemical responses to the stress (Rampino et al., 2006).

Many authors focus their studies on the molecular aspect of drought stress physiology pathway of ABA, stilbene and flavonoid synthase (Hauser et al., 2011; Parage et al., 2012; Nopo-Olazabal et al., 2014).

Among the main genes associated with ABA biosynthesis there are **NCED1 and NCED2.** Nced1 and Nced2 are two genes encoding for 9-cis epoxycarotenoid dioxygenase enzymes. The changes in abundance of the Nced1 and Nced2 mRNAs lead to the ABA accumulation that, in grapevine, is attributed to high mRNA expression level of VvNCED1 (Boneh et al., 2012).

Another important gene involved in ABA signalling and ABA-mediated stomatal closure is protein kinases (**OST1**).

OST1(open stomata1) protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Gene involved in OST1 is a known positive regulator of ABA-dependent stomatal movements.

The protein, OST1, displays dominant kinase activity during drought stress response and is able to activate NADPH oxidase (Sirichandra et al., 2009). Mutants in OST1 showed a wilty phenotype in water deficit con- ditions because of the impairment of stomatal closure and ROS production (Mustilli et al., 2002; Yoshida et al., 2006).

Water deficit can play a role on the regulatation of flavonoid biosynthesis (Castellarin et al., 2007).

About the quantitative analysis of gene expression in **flavonoid metabolism** particular important are CHS, FLS, LAR, LDOX (Velasco et al. 2007).

A small family of **CHS** chalcone synthases (CHS1, CHS2, CHS3) flavonoid precursors are initially recruited from the phenylpropanoid pathway entering in the flavonoid pathway. Phylogenetic analysis associates these three genes with previously characterized plant CHS from 89% to 94% of identity at the protein level (Parage et al., 2012). In grapevine these genes are therefore likely to encode bona fide CHS proteins and have been named VvCHS1 to VvCHS3 (Parage et al., 2012).

Another family **FLS** is a family of genes (FLS1-5FLS) involved the encoding the enzyme flavonol synthase (FLS). These genes encode the biosynthetic enzyme converting dihydroflavonols to flavonols.

In grapevine VvFLS1 is recognized like an important gene in the studies on the expression of flavonoid pathway during the berries development (Downey et al.,2003).

Expression of the gene encoding **LDOX** leucoanthocyanidin dioxygenase, which is required for synthesis of epicatechin and anthocyanins.

Leucoanthocyanidin reductase (LAR) it is not known what regulates expression of LAR in PA synthesis in other plants. In grape berries, the first committed steps in PA biosyn- thesis are catalyzed by LAR and ANR by converting anthocyanidins to flavan-3-ols such as catechin and epicatechin, respectively (Bogs et al. 2007).

There is considerable interest in grape PAs because of their importance for the color and taste of wine and their antioxidant capacity (Bogs et al., 2007).

General scheme of flavonoid pathway and relative genes involved is shown in figure 29.

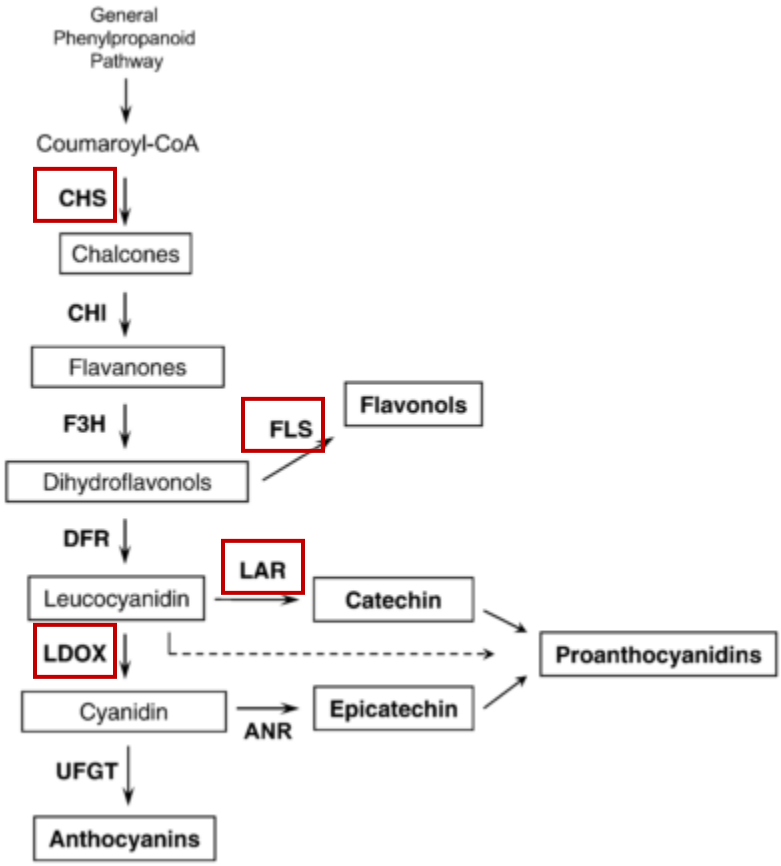


Figure 29: General scheme of flavonoid pathway and relative genes involved (from Bogs et al., 2007)

Another important role on the defense mechanisms in plants are Stilbene synthases (STSs)

The grapevine STS genes encode 392-amino acid proteins sharing a high level of conservation (Parage et al, 2012).

Many of the genes considered above are control mediated by transcription factors **transcription factors** that can also be involved in the responses to water deficit (Bogs et al., 2007). Candidate genes for the transcription factors that are known to regulate FLS activity are **MYBPA.**

VvMYBPA1 is able to induce promoters of both early and late flavonoid biosyn- thetic genes (Bogs et al., 2007).

This study aims to characterize the international variety Cabernet Suvignon grafted onto 5 widespread commercial rootstocks and 4 new rootstocks (Mseries) newly developed.

The specific objective is better understand how they differ in response to drought stress. The characterization was focused on both physiological and molecular aspects.

3.2 MATERIALS AND METHODS

Plant material and growth conditions

The experiment was performed under greenhouse environmental controlled conditions at the Department of Agricultural and Environmental Sciences, Production, Landscape, Agroenergy (DISAA) of University of Milan. Commercial two-years old vines of Cabernet-Sauvignon grafted onto 9 rootstocks (table 5) were grown in 4-L plastic pots.

Rootstock	Parentage
1103P	[*V. berlandieri x V. rupestris*]
140Ru	[*V. berlandieri x V. rupestris*]
K5BB	[*V. berlandieri x V. riparia*]
SO4	[*V. berlandieri x V. riparia*]
420A	[*V. berlandieri x V. riparia*]
M1	[*V.riparia* x (*V. cordifolia* x *V. rupestris*)] *x* [*V. berlandieri*]
M2	[*V. berlandieri x V. riparia*] x [*V.vinifera x V. berlandieri*]
M3	[*V. berlandieri x V. riparia*] x [*V. berlandieri x V. riparia*]
M4	[*V. vinifera x V. berlandieri*] x [*V. berlandieri*]

Table 5: backgrounds of the rootstocks examined in this experiment

Ten replicates per rootstock-scion combination were monitored during the experiment. The vines were trained on 1 m stakes and placed in a randomized complete block design (RCBD). The growth substrate was composed of 80% sand, 20% peat and supplemented with a layer of expanded clay aggregate on the bottom of the pot finalized to avoid water flooding.

In the beginning all the plants were maintained in well water conditions achieving a proper size of the canopy (10th fully developed leaf). The plants were grown under a constant established photoperiod with 16 hours day, 8 hours night. The greenhouse temperature were kept between 23 °C to 29 °C.

Irrigation management

Two irrigation treatments were applied (Figure 30). For each rootstock-scion combination 4 replicates were maintained in well water condition (WW) and the other 6 replicates were subjected to an increasing water stress (WS).

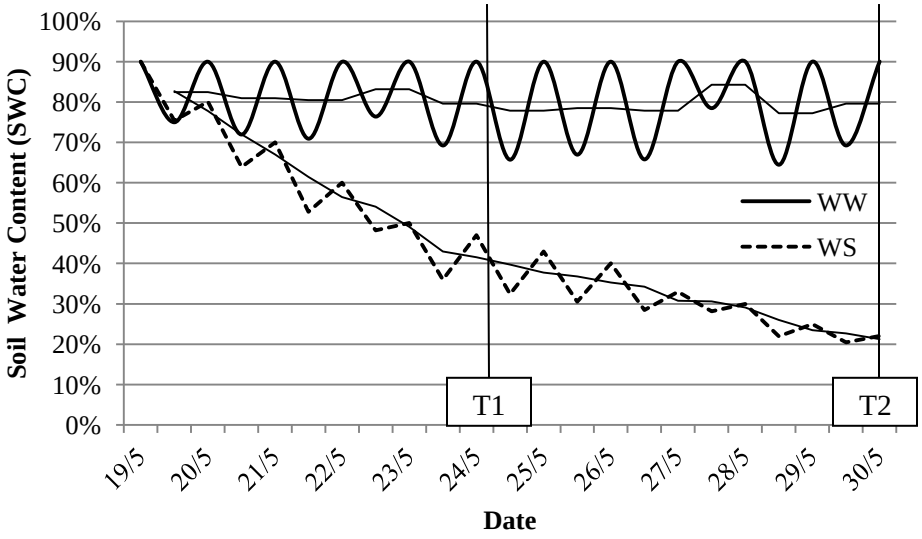


Figure 30: Water management and sampling timing T1 and T2

Irrigation managing were carried out with gravimetric method: Soil Water Capacity (SWC) were measured and management of nutrition water by weighing each pot being restored the level of field capacity (FC) or pot capacity (PC) desired, 90% for WW and a progressive drought stress.

The SWC was calculated according to Gardner et al. (2001) as:

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

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

Plants phenotyping

For all plants fully exposed leaves were selected for gas exchange measurements at the 8th node counting from the base of the shoot. Gas exchange measurements as Photosynthetic activity (Pn), Stomatal conductance (Gs), Evapotranspiration (E), Internal CO_2 Concentration (Ci) and Vapor Pressure Deficit (VPD) were performed

with leaf photosynthesis system (CIRAS-2, PP Systems, Amesbury, MA, USA) equipped with PLC6 (U) cuvette 18 mm circular (2.5 cm^2 head plate).

All measurements were taken between 10 and 14 h with control point as photosynthetic photon flux set at 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a CO_2 concentration at 300 $\mu mol\ mol^{-1}$.

The cuvette was set considering the stomata 100% at the abaxial side of the leaf.

Stems growth expressed as daily Stem Growth Rate (SGR) was monitored as well.

Stem Water Potential was measured at sampling timing (T1 and T2) using pressure chamber (Scholander chamber). The same 8th leaf used in the gas exchange measurements was selected, placed in a plastic bag wrap in aluminum foil. After 1 hour the leaf was excised with a razor blade and placed in the chamber for the measurement. The SWP was measured within 30 sec after of cutting the leaf by slowly pressurizing the chamber until sap emerged from the cut end of the petiole.

Sampling and samples

During the experiment at T1 (40% of FC) and T2 (22% FC) samples of roots and leaves in two control plants (WW) and three water stressed plants (WS) were collected.

The samples were frozen immediately in liquid nitrogen and stored at −80°C for the analysis of transcript. Fresh weight of roots and leaves per plant were also recorded.

RNA extraction and quality

Total RNA was extracted from 100 mg of the tissue powder using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and residual genomic DNA was removed by performing on-column DNase I digestion with On-Column DNase I Digest Set (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer instructions..

Total RNA extracted quality (A260/A280 and A260/A230 ratio) was checked using a micro-spectrophotometer Nanodrop ND-2000 spectrophotometer (Thermo ScientificNanodrop).

cDNA synthesis

For quantitative real-time PCR analysis (qPCR), cDNA was synthesized using 1µg RNA, 1 µl Oligo-dT and H_2O up to 13 µl. After briefly mix and spin down the combined components were incubate at 65 °C for 10 minutes then chill on ice. At the

same time a master mix (7 µl per cDNA reaction) was prepared by adding 4µl of 5X RT Buffer, 0.5 Protector RNase Inibitor, 2 µl of dNTPs (dATP, dCTP, dGTP and dTTP) and 0.5 µl RT Enzime. After incubation for 30 minutes at 55 °C, the RT enzyme was inactivated at 85 °C for 5 minutes. All the activity was carried out the Transcriptor First Strand cDNA synthesis kit (ROCHE). Subsequently, the cDNA was stored at –20 °C until further analysis.

Real-time quantitative PCR (qRT PCR)

Quantitative analysis of gene expression were performed by quantitative RT-PCR (qRT-PCR).

qRT-PCR was carried out in triplicate on two biological replicates for each sample with StepOne Plus Real-Time PCR System (Applied Biosystems) by using specific primers. Primers selected for gene of interest (GOI) and relative primer pairs (for for Real Time PCR are described in supplemental Table 6. Ubiquitin (*VvUbi1*) mRNAs was used as internal standards.

Gene	Primer pairs (forward-reverse)	General comments
VvUbi1	Forward;5′-GTGGTATTATTGAGCCATCCTT-3′ Reverse;5′-AACCTCCAATCCAGTCATCTAC-3′	housekeeping gene (reference gene, RG) Downey et al., 2003
VvNCED1	Forward; 5′-TGCAGAGGACGAGAGTGTAA-3′ Reverse; 5′-AGCTACACCAAAAGCTACGA-3′	Vitis homologues of NCED, VvNCED involve in ABA biosynthesis
OST1	Forward;5′GAAGAACCTCCCTGCAGACCTCATGG-3′ Reverse;5′CCATCCGTCATGTAGCTATTAAGGCCAT-3’	positive regulator of ABA-dependent stomatal movements
VvCHS2	Forward; 5′-TCTGAGCGAGTATGGGAACA-3′ Reverse; 5′-AGGGTAGCTGCGTAGGTTGG-3′	
VvFLS1	Forward;5′-CAGGGCTTGCAGGTTTTTAG-3′ Reverse: 5′-GGGTCTTCTCCTTGTTCACG-3′	Downey et al., 2003
VvLDOX	Forward;5′CGAGGATCCGTTTGCTTCCATCCCAATCTCACT-3′ Reverse:5′TGTCTCGAGAAATATCACTGATCTACTTGTTTTCC-3′	
VvLAR	Forward;5′CGAGGATCCTCGGAATAATTTCAT	

	AGGGCTTT-3′ Reverse;5′ATACTCGAGTCTGATGATGCTTCT TCTCTACTACTC-3′	
VvDFR	Forward;5′ATGTCATCAATGCCTCCAAGCCTC ATAA-3′ Reverse;5′GCAGAGGTCATCCAGGTGAACAA ATTG -3′	
VvSTS29	Forward;5′ TCCAACTTGTTTCAGCAGCGCA -3′ Reverse;5′TGAAAGGTGAGACCCACTTCACGT -3′	
VvMYBPA	Forward; 5′-AGATCAACTGGTTATGCTTGCT-3′ Reverse;5′-AACACAAATGTACATCGCACAC-3′	
Table 6: Genes of interest (GOI) and reference gene (RG), specific primers used for amplification of genes in quantitative real time PCR and general comments		

Reactions were carried out under the following conditions: 95 °C/30 s (1 cycle); 95°C/15 s, 58°C/20 s; 72°C/15 s (40 cycles), using the StepOne™ Real-Time PCR System (Life Technologies) 96 wells. All the experiments were performed with three biological replicates and three technical replicates.

Calculations of relative expression

Data were acquired, elaborated, and exported with the StepOne Software version 2.1 (Applied Biosystems), whereas all the final calculations were carried out with the automated Excel spreadsheet Q-Gene designed by Simon et al. (2003) using the modifications of the delta cycle threshold method suggested by Pfaffl et al. (2001). Gene expression values were normalized to the housekeeping gene UbiCF (Ubiquitin Conjugating Factor; CF203457) already used by Castellarin et al. (2007) and reported as arbitrary units of mean normalized expression, using equation 2 of Q-Gene.

Data Analysis

Data were analyzed via analysis of variance (ANOVA) and mean separations were determined using Duncan's multiple range test (DMRT). According to Van Peer et al. (2011) relative expression levels and fold changes were log2 transformed for further data-analysis. Gene expression data are expressed as $\log_2$ of fold change. Positive values show up-regulation and negative values indicate down-regulation. Genes with absolute $\log_2$ fold changes >1 are considered significant.

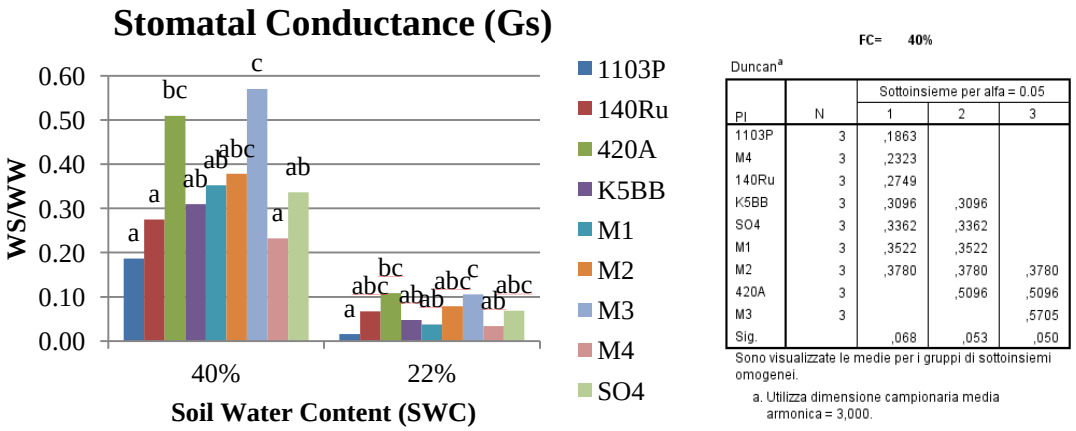
3.3 RESULTS AND DISCUSSION

Physiological responses

Stomatal conductance (Gs) responds to the soil water content (SWC) in all rootstock-scion combinations especially starting from 70% (data not shown). In control plants Gs follow the environmental condition of the greenhouse clearly subjected to the weather daily condition (data not shown). In plants subjected to water stress, Gs tended to decrease with significantly difference among rootstock starting from 60% of SWC.

To normalize the effect of the environment all measurement were considered compared to the control plants.

Relative value (WS/WW) of stomatal conductance showed a different behavior among the rootstocks. At 40% of SWC 1103P and M4 resulted the genotypes with low level of stomatal conductance respect to the control treatment whereas M3 and 420A keep an high level of Gs. At 22% the differences among the rootstock decrease but M3 and 420A keep the Gs around 10% of the control (Figure 31).



FC= 40%

Duncan[a]

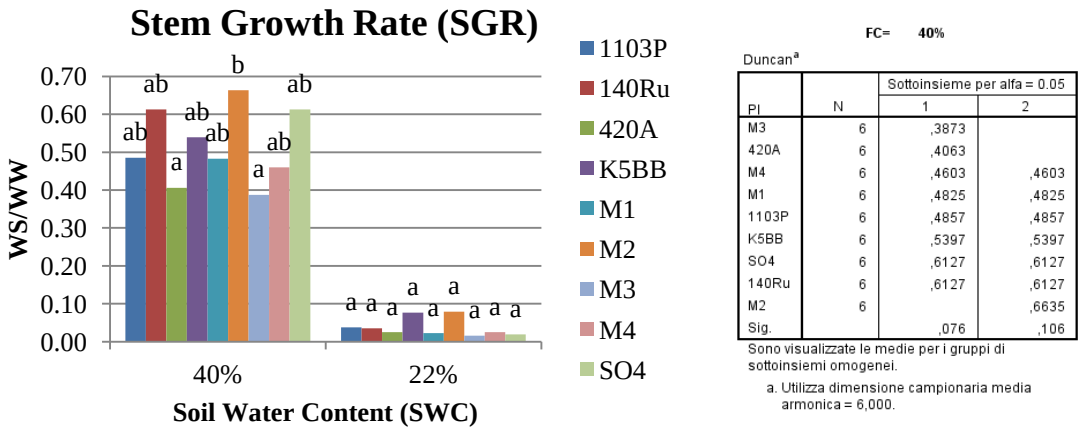
PI	N	Sottoinsieme per alfa = 0.05		
		1	2	3
1103P	3	,1863		
M4	3	,2323		
140Ru	3	,2749		
K5BB	3	,3096	,3096	
SO4	3	,3362	,3362	
M1	3	,3522	,3522	
M2	3	,3780	,3780	,3780
420A	3		,5096	,5096
M3	3			,5705
Sig.		,068	,053	,050

Sono visualizzate le medie per i gruppi di sottoinsiemi omogenei.

a. Utilizza dimensione campionaria media armonica = 3,000.

Figure 31: Relative level of stomatal conductance at 40% of SWC and 20% of SWC

Moving on growth responses, expressed like stem growth rate (SGR) as mm/day, M3 and 420A showed a slow down level of development whereas M2 keeps to grow around 60% of the control (Figure n). At severe drought stress the differences among rootstock decrease and no significant difference were observed (Figure 32).



FC= 40%

Duncan[a]

PI	N	Sottoinsieme per alfa = 0.05	
		1	2
M3	6	,3873	
420A	6	,4063	
M4	6	,4603	,4603
M1	6	,4825	,4825
1103P	6	,4857	,4857
K5BB	6	,5397	,5397
SO4	6	,6127	,6127
140Ru	6	,6127	,6127
M2	6		,6635
Sig.		,076	,106

Sono visualizzate le medie per i gruppi di sottoinsiemi omogenei.

a. Utilizza dimensione campionaria media armonica = 6,000.

Figure 32: Relative level of growth under moderate water stress (40% of SWC) and high level of water stress (20% of SWC)

Net Photosynthesis and WUE

In figure 5 is shown the net photosynthesis (Pn) levels at 40% and 20% of SWC for each thesis control (T) and stressed (S). Significant differences were detected between plants under different irrigation regimes. In particular, at 40% of SWC, the well water reference (T) 140Ru, 420 A, M1, M2,M3 and M4 plants under stress shown a reduction of Pn. At 22% of SWC these differences are more accentuated and all genotypes under drought stress reduced the photosynthetic activity (Figure 33).

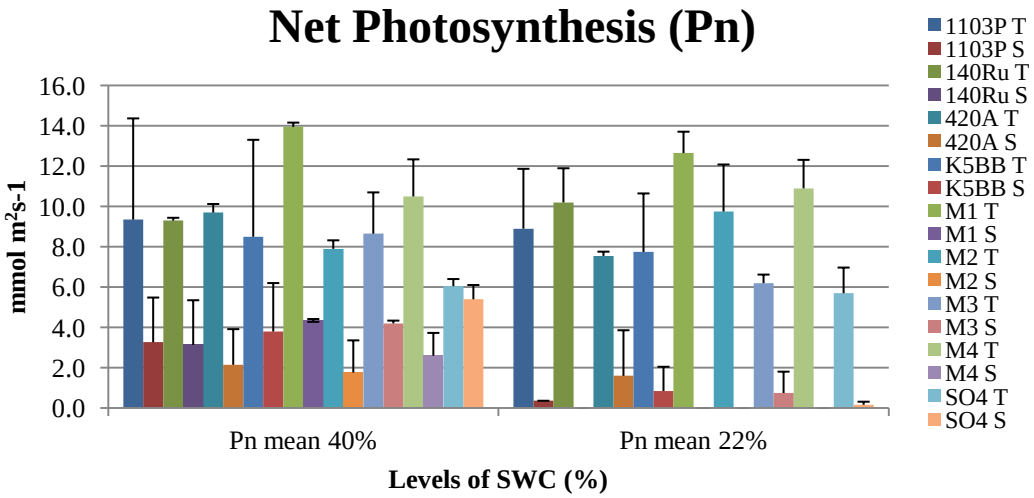


Figure 33: Net Photosynthesis at 40% and at 20% of SWC

About the instantaneous water use efficiency (istWUE) of the plants under well watered conditions have registered different behaviors: 140Ru, 420A, M2, M3 and M4 have shown a high levels compared to 1103P, K5BB, M1 and SO4.

Under moderate drought stress (40% of SWC) 1103P, 140Ru, M1, M3 and SO4 have performed better than 420A, K5BB, M2 and M4 (Figure 34).

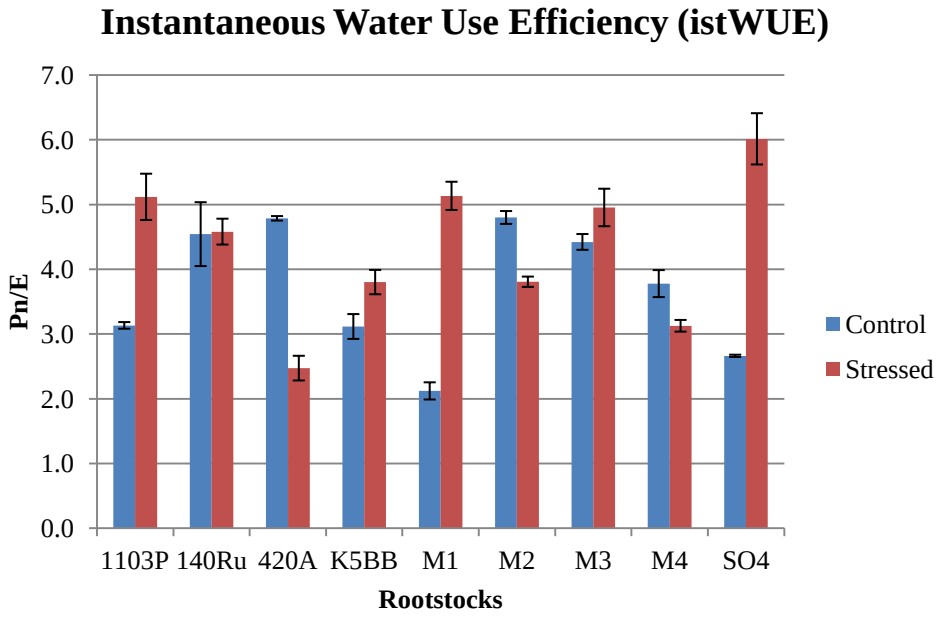


Figure 34: Instantaneous water use efficiency (istWUE) at 40% of SWC

Classification

In figure 35 is reported the classification result of combined measures of growth (SGR) and stomatal conductance (Gs) in all combinations at 40% of SWC.

After Hierarchical Clustering Analysis based on the Average Link four groups has been possible obtain.

M3 and 420 have performed keeping an high level of stomatal conductance

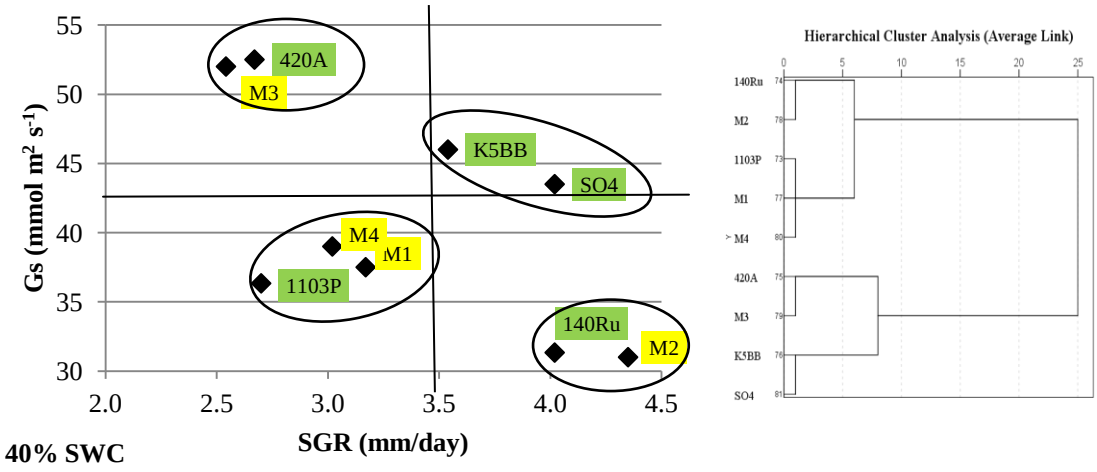


Figure 35: Classification by stomatal conductance (Gs) and shoot growth rate (SGR)

Molecular responses

The total RNA extracted from leaves and roots harvested at T1 (40% of soil water content) shown a good quality in both treatments (WW and WS) (data not shown).

At 20% of SWC (T2) the quality of leaves and roots total RNA of the plants subjected to drought stress was of lower quality and not suitable for the expression analysis (data not shown). This aspect is probably related to high presence of protein, phenol or other contaminants.

The results of the qRT-PCR analysis of samples which originated from plants harvested at the first sampling time (T1) were in agreement with the phenome responses.

The relative expression of ABA-related genes responds to the severity of drought stress. Consudering the relative expression of *NCED1* in leaves (figure 36) rootstock M4 and K5BB exhibited high levels of relative expression, while 1103, SO4 and 420A levels were low. In roots M3 and 420A had the highest levels of relative expression.

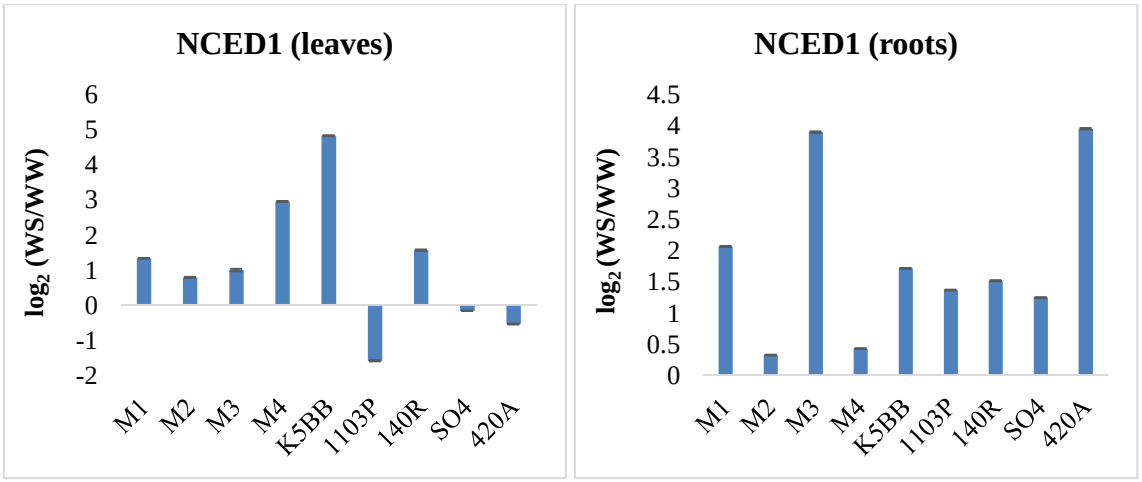


Figure 36: Histograms showing relative expression ratios (log_2transformed) of genes *NCED* in leaves (a) and roots (b)

Some positive feedback was found between physiological variables and *OST1* transcript relative expression level. The stomatal conductance level (Gs) of Cabernet Sauvignon grafted onto the rootstocks M3 and 420A and the relative transcript abundance of *OST1* have been found (Figure 37).

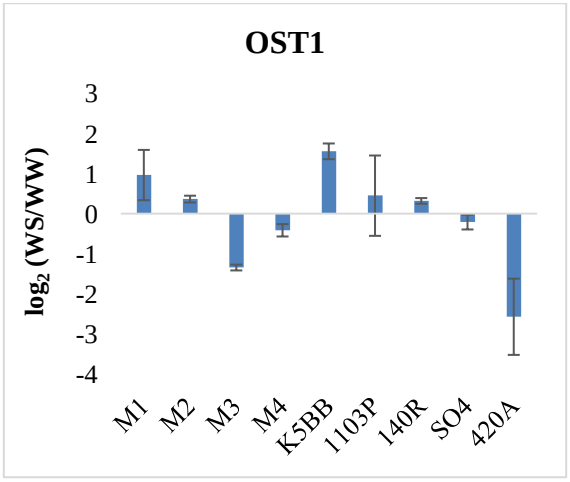


Figure 37: Relative gene expression of OST1 at 40 % of soil water content

The main interesting results have been found on the quantitative analysis of gene expression in **flavonoid metabolism**. In leaf tissues CHS2, FLS1, LAR, LDOX and DFR displayed similar level of expression patterns. In particular M1, M4 and K5BB shown an up-regulation of CHS LAR, LDOX and DFR. The only exception is

represented by M1 FLS1 where no difference has been observed with the control plants (figure 38). For M2, 140Ru and SO4 expressions of CHS2 gene was not significantly different from expression in the control leaf tissues.

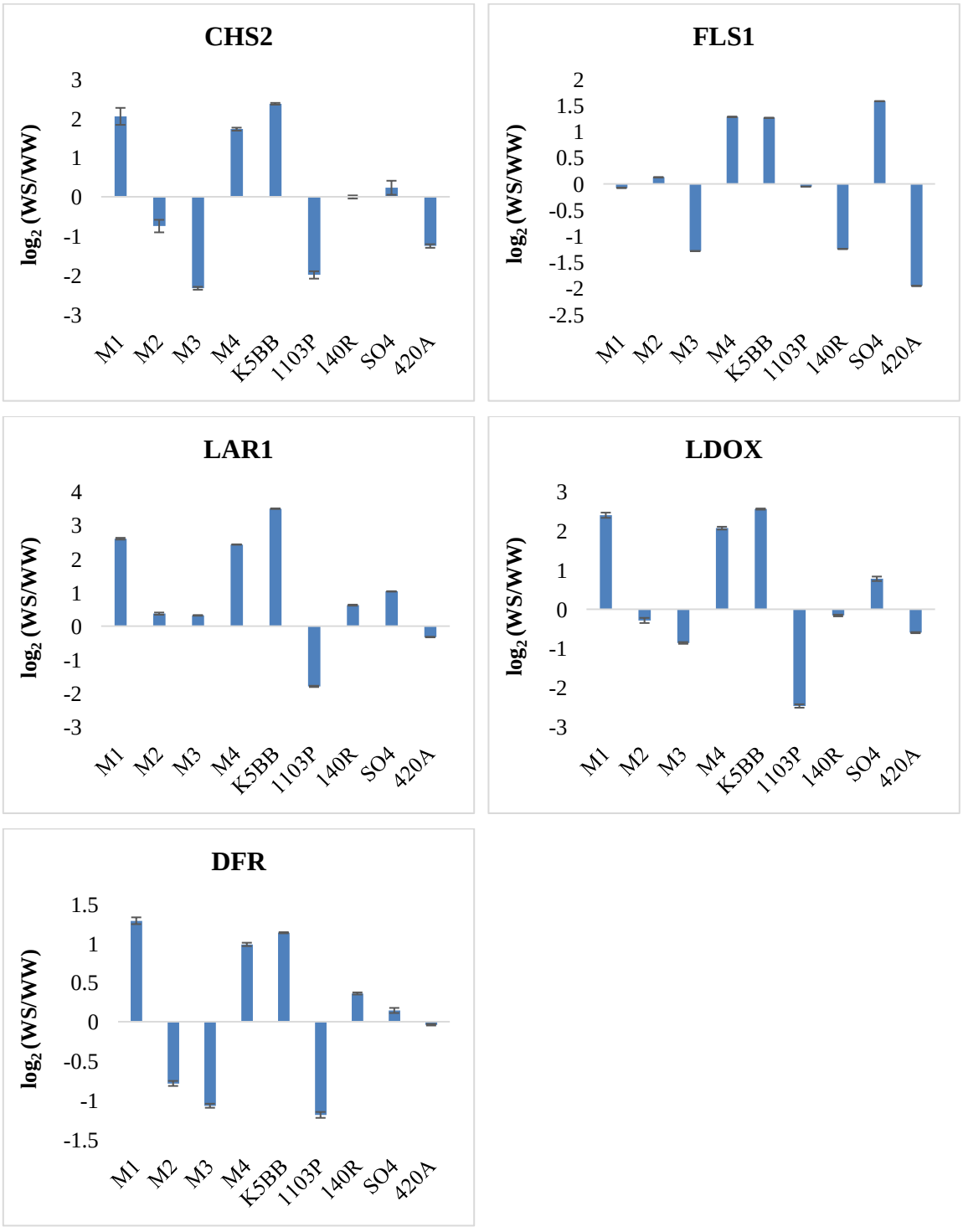


Figure 38: Expression patterns of gene expression involved in **flavonoid metabolism** in response to drought stress (leaves sampled at 40% of SWC)

The expression **transcription factors MYBPAs** in leaves shown similar responses (figure 39).

M1, M4, K5BB showed an up-regulation of both genes instead M3 and 420A displayed a down-regulation.

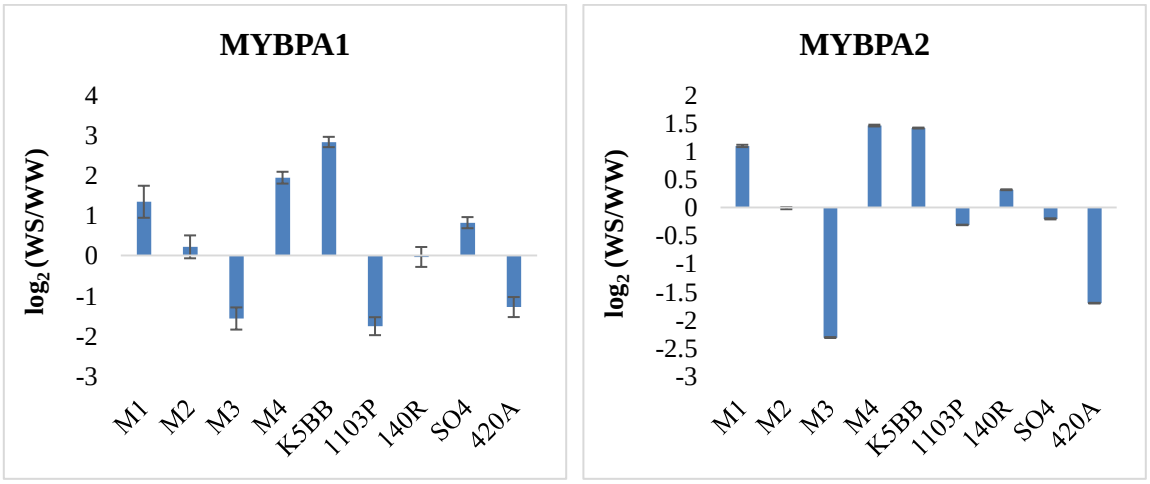


Figure 39: Transcription factors involved in the proanthocyanidin synthesis

MYBPAs are involved in stress responses and these transcription factors have been found to regulate the proanthocyanidin synthesis.

The expression of the **stilbene synthase** biosynthesis-related genes in roots STS29 (figure n) displayed a significant down-regulation in M1, M3, 1103P, SO4 and 420A.

In M2, M4, K5BB and 140 Ru the responses are similar to relative plants under well watered conditions.

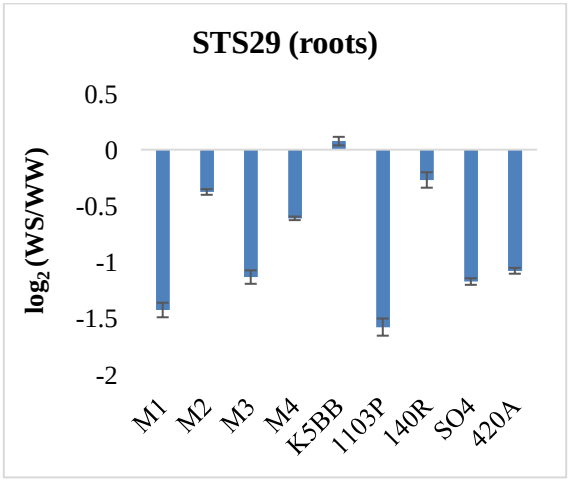


Figure 40: Relative gene expression of STS29 at 40 % of soil water content

3.4 CONCLUSIONS

This study revealed some fundamental aspects of rootstock-scion interactions and how drought stress can affect the responses of grapevine. The main evidence is that the scion-rootstock combination has a significant effect on different responses to water stress at physiological and molecular level.

In particular it was shown that roots to shoots signals can lead to an up-regulation or down-regulation depending on the scion-rootstock combination. This shows how the physiology pathway of ABA, stilbene and flavonoid synthase are involved in drought and osmotic stress tolerance and are controlled by rootstock.

The main aspect observed is how the rootstock is able to influence some of the main responses to water stress and how these effects characterize the behavior of grafted variety. In this experiment, several combinations of rootstock with Cabernet Sauvignon as scion have been compared. In particular, five of the most widespread commercial rootstocks and four new developed rootstocks has been tested under a drought (WS) experiment under controlled greenhouse conditions. The WS was imposed gradually by decreasing the water-availability in pots from 80% to a minimum level of 22% of field capacity, whereas WW plants, used as controls, were grown in pots with a water-availability equal to 80% of field capacity.

Along the experimental period, as physiological trait, stomatal conductance (Gs), net photosynthesis (Pn) and stem growth rate (SGR) were measured on fully expanded leaves immediately before sampling using Ciras portable photosynthesis system (CIRAS-2, PP Systems, Amesbury, MA, USA).

The CS/rootstocks combinations highlighted differential physiological responses to water stress in terms of Gs, Pn and SGR.

The expression of several genes involved in ABA and secondary metabolism was captured by real-time PCR on both leaves (scion) and roots (rootstock). As for secondary metabolism, was measured the expression of stilbenes (i.e. VvSTS29) and phenylpropanoid (i.e. VvCHS2, VvFLS1, VvLDOX, VvLAR, VvDFR, VvMYPA) – related genes in roots and leaves, respectively. Moreover, was evaluated the expression of ABA genes involved in biosynthesis (VvNCED) and signal transduction (VvOST1) pathways.

The analysis of transcriptional regulation of secondary metabolism has been considered as the main responses involved in the role of protection against oxidative stress induced by drought conditions.

Indeed, Stilbenes and flavonoids have ROS scavenging activity that protects against oxidative damage and controls ROS levels, which is mandatory for plant survival in the presence of abiotic stresses (Brunetti et al., 2013). It has been suggested that resveratrol (stilbenes) and flavonoids, whose biosynthetic genes are induced in some CS/rootstock leaves under WS, act as antioxidants in plant response to oxidative stresses (Ramakrishna and Ravishankar, 2011; Brunetti et al., 2013; Tillett et al., 2011; Stuart and Robb, 2013). Indeed, they protect against oxidative stress due to an excess of excitation energy in the chloroplast by absorbing solar wavelengths (Agati et al., 2012) and environmental perturbations (Hernández et al., 2009; Agati et al., 2012; Brunetti et al., 2013). In addition, flavonoids are capable of quenching H_2O_2 and other free-radicals, thus protecting the chloroplast membrane from oxidative damage by stabilizing membranes containing non-bilayer lipids (Agati et al., 2012).

The data support the hypothesis that in addition to the activation of "primary mechanisms" of ROS scavenging, drought-tolerant Vitis species also induce "secondary mechanisms" leading to the biosynthesis of other types of secondary compounds in roots and leaves. In this regard, similarly to other stress-tolerant grapevine genotypes, these rootstocks may have a greater capacity to induce a control ROS homeostasis in the aerial part of the plant (CS), and prevent oxidative damage than susceptible genotypes.

In conclusion, the rootstock has determined a different response according to the genotype but also was able to develop different responses in the scion. This shows that the biosynthetic pathways of ABA, stilbene and flavonoids synthases involved in scion response to drought conditions can be controlled by the rootstock.

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