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Aleatico grapevine characterization: physiological and molecular responses to different water regimes

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To my parents

‘With tango shoes or work boots that is always me’

Lorenza
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ANTH</td>
<td>Anthocyanins</td>
</tr>
<tr>
<td>AOMT</td>
<td>Anthocyanins O-methyltransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CH$_3$CN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>CHL</td>
<td>Chlorophyll</td>
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<tr>
<td>CHLF</td>
<td>Chlorophyll fluorescence</td>
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<tr>
<td>CHS</td>
<td>Chalcone synthase</td>
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<tr>
<td>C$_t$</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CV</td>
<td>Cultivar</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>DFR</td>
<td>Dihydroflavonol 4-reductase</td>
</tr>
<tr>
<td>DHN1a</td>
<td>Dehydrin 1a</td>
</tr>
<tr>
<td>DOY</td>
<td>Day of year</td>
</tr>
<tr>
<td>DREB</td>
<td>Dehydration responsive element-binding protein</td>
</tr>
<tr>
<td>DWF1</td>
<td>Dwarf1</td>
</tr>
<tr>
<td>ET$_0$</td>
<td>Evapotranspiration</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>ETP</td>
<td>Potential evapotranspiration</td>
</tr>
<tr>
<td>FAOMT</td>
<td>Flavonol and Anthocyanin 3’,5’-O-methyltransferase</td>
</tr>
<tr>
<td>F3’H</td>
<td>Flavonoid 3’-hydroxylase</td>
</tr>
<tr>
<td>F3’5’H</td>
<td>Flavonoid 3’,5’-hydroxylase</td>
</tr>
<tr>
<td>FLAV</td>
<td>Flavonols</td>
</tr>
<tr>
<td>FLS1</td>
<td>Flavonol synthase 1</td>
</tr>
<tr>
<td>FRF</td>
<td>Far-red fluorescence</td>
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<tr>
<td>FW</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>G</td>
<td>Green</td>
</tr>
<tr>
<td>gs</td>
<td>Stomatal conductance</td>
</tr>
<tr>
<td>HCA</td>
<td>Hydroxycinnamic acids</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>HCOOH</td>
<td>Formic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>IR</td>
<td>Irrigated</td>
</tr>
<tr>
<td>Kc</td>
<td>Cultural coefficient</td>
</tr>
<tr>
<td>LDOX</td>
<td>Leucocyanidin oxygenase</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>MD</td>
<td>Midday</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MSA</td>
<td>Abscisic acid-, stress-, and ripening-induced (ASR) gene</td>
</tr>
<tr>
<td>Mx</td>
<td>Multiplex</td>
</tr>
<tr>
<td>MXK3</td>
<td>ABC transporter</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCED</td>
<td>9-cis-epoxycarotenoid dioxygenase</td>
</tr>
<tr>
<td>OMT</td>
<td>O-methyltransferase</td>
</tr>
<tr>
<td>P5CR</td>
<td>Pyrroline-5-carboxylate reductase</td>
</tr>
<tr>
<td>PIP2;1</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>Pn</td>
<td>Net photosynthesis</td>
</tr>
<tr>
<td>PRD</td>
<td>Partial Root Drying</td>
</tr>
<tr>
<td>PrDh</td>
<td>Proline dehydrogenase</td>
</tr>
<tr>
<td>qPCR-RT</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Red</td>
</tr>
<tr>
<td>RDI</td>
<td>Regulated Deficit Irrigation</td>
</tr>
<tr>
<td>RF</td>
<td>Red fluorescence</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROX</td>
<td>6-carboxy-X-rhodamine</td>
</tr>
<tr>
<td>RQI</td>
<td>RNA quality indicator</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SF</td>
<td>Sap Flow</td>
</tr>
<tr>
<td>STS</td>
<td>Stilbene synthase</td>
</tr>
<tr>
<td>UFGT</td>
<td>Flavonoid-3-O-glucosyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VIS</td>
<td>Visible</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>VPD</td>
<td>Vapour Pressure Deficit</td>
</tr>
<tr>
<td>VV</td>
<td>Vitis vinifera</td>
</tr>
<tr>
<td>WR</td>
<td>Water requirement</td>
</tr>
<tr>
<td>WS</td>
<td>Water stress</td>
</tr>
<tr>
<td>WUE</td>
<td>Water use efficiency</td>
</tr>
<tr>
<td>ZEP</td>
<td>Zeaxanthin epoxidase</td>
</tr>
<tr>
<td>Ψs</td>
<td>Stem water potential</td>
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Aleatico grapevine is a variety cultivated along Tuscany coasts and in Elba Island (Italy), from which a typical dessert wine ‘Aleatico passito’ is produced after partial post-harvest dehydration of berries. The research project was focused on this variety because the knowledge of the morphological traits and technological characteristics of Aleatico as well as the response of this grape variety to different environmental conditions and climatic changes, including reduced rainfall and water stress, is scarce. Therefore, to obtain a high quality wine that recently received the Denomination of Controlled and Guaranteed Origin and to be more competitive on the market, it is quite useful to conduct thorough studies on this variety and on its responses to different water regimes, especially in terms of secondary metabolites biosynthesis (phenolic compounds and flavours) during ripening.

Field trials were carried out, in 2008 and 2009, at ‘La Bulichella’ Winery (Suvereto, Livorno, Italy) in order to study physiological responses (midday stem water potential, gas exchanges and sap flow) and berry composition of non irrigated (WS) and irrigated (IR) Aleatico plants. The climatic trends for the 2008 and 2009 seasons showed that in the 2009 season relative humidity was higher (30-80%) in comparison with 2008 (10-40%). Global radiation in 2009 was also higher against to 2008. The air temperature frequently exceeded 26°C during 2008 season, while in 2009 this occurred and was concentrated during the second part of the season. Relative humidity and air temperature measured at the grape level did not markedly differ from those of the meteorological station. These climatic conditions influenced the midday stem water potential and the gas exchanges that reached lower values in 2009 than in 2008. In particular, in 2008 the photosynthetic activity and conductance of IR plants leaves increased during véraison and maintained higher values than in 2009, during which both parameters were decreasing, apart from the partial recovery due to water supply on August 15th.

In 2009 at harvest berry weight was reduced of about 20% in WS plants. An effect of similar magnitude was detected for skin weight, while seed weight was not affected. The sugar accumulation process resulted more pronounced in WS berries and this was paralleled by higher titratable acidity values both at véraison and harvest.
In 2009 the total phenolic content of the berries was influenced by water stress only at the end of the trial, with a reduction of the seeds phenolic compounds. The incidence of seeds on total phenolic content was higher than that of skins, confirming that this behaviour is a varietal characteristic.

Accumulation of anthocyanins (Anth) on whole wine grape bunches attached to the vine was studied using a non-destructive fluorescence-based sensor, extremely useful for a rapid and non-invasive determination of phenol compound-related parameters in the vineyard. The very same 50-60 bunches were monitored during the seasons at a weekly frequency from véraison to harvest. For each date of measurements, chlorophyll fluorescence signals under different excitation wavelengths were collected to derive Anth, flavonols (Flav) and chlorophyll (Chl) indices. The ANTH\textsubscript{R}, that is the Anth index based on a single fluorescence signal excited with red (R) light, and the FLAV index increased and decreased with time from véraison to harvest, respectively. The Chl index was monotonically decreasing, while the ANTH\textsubscript{RG}, based on two fluorescence signals excited with red (R) and green (G) light, followed a biphasic behavior increasing to a maximum at about complete véraison and then decreasing to harvest. All the indices suggested an early ripening process in 2009 compared to 2008, in agreement with other standard indicators such as véraison occurrence, technological maturity and berry development. Calibration of the fluorescence sensor was performed in 2008 by destructive HPLC analysis of phenolic compounds in berry skin extracts. Starting from complete véraison, the ANTH\textsubscript{RG} index was found to be fairly inversely correlated ($r^2 = 0.875$) to the Anth surface-based concentration (mg/cm\textsuperscript{2}) through an exponential function. On the contrary, the Flav index was uncorrelated to the Flav content, because of the interference of Anth on the fluorescence signals. The ANTH\textsubscript{RG} non-destructive index was able to detect differences in the Anth accumulation between seasons in accordance with the standard destructive analysis of Anth berry skin content. Water stress imposed in 2009 increased Anth accumulation in berries due to a reduction of berries in size but also to an increased Anth biosynthesis. This effect was observed by both destructive and ANTH\textsubscript{RG} non-destructive measurements.

In order to study at molecular level the expression of specific genes involved in the anthocyanin biosynthetic pathway and water stress-related responses in Aleatico berries, a research stage at the Wine Research Centre of the University of British Columbia (Vancouver, Canada) was carried out. The transcript accumulation of several putative
water stress-sensitive genes was preliminary analyzed by qRT-PCR to identify possible common biomarkers in leaves and in berries. Among these genes, Dehydrin1a showed significant changes in transcription in WS samples. Considering genes involved in the Anthocyanins pathway, the expression of UFGT (flavonoid 3-O-glucosyltransferase) and FLS1 (flavonol synthase) appeared to be up-regulated by WS. A similar response was also observed for two genes involved in the anthocyanin hydroxylation and methoxylation processes.

Taken together results indicate that the variety Aleatico appears to be tolerant to water stress condition and this information could be useful also for setting up targeted post-harvest dehydration strategies to produce dessert wines and to allow its cultivation in territories where irrigation is not available or saving water when the irrigation must be used in severe dry conditions.
1. INTRODUCTION

1.1 ALEATICO GRAPEVINE

Aleatico is a red-skinned variety cultivated mainly along the coastline of Tuscany and in the Elba Island (Italy) (Figures 1.1 and 1.2) for the production of a characteristic dessert wine (‘Aleatico dell’Elba Passito’), after partial post-harvest berry dehydration, that recently received the ‘Denomination of Controlled and Guaranteed Origin’ (DOCG).

This variety and the wine produced represent a strong link with the territory and, in the last recent years, growers carried out a substantial vineyards renewal mainly due to the productive cycle exhaustion (Scalabrelli et al., 2004).

In 1997 The Winegrowers Elba Association and ARSIA, supported a project of clonal selection having the objective to obtain the homologation of clones of Aleatico necessary for the production of certified plant material required for the new plantations, considered that at that time in Italy no homologated clones of Aleatico were available. According to the official methods, the work of genetic and health selection started with the objective to identify grapevine plants having grapes suitable for the dehydration process, characterized by valuable qualitative features, and free from the main viruses. The difficulties to find virus free plants in the Elba Island, suggested to extend the selection to others Tuscany provinces. Presumed clones that resulted free from viruses at the end of DAS- and TAS-ELISA health tests, were grafted in 1999 in the experimental vineyard planted on 1998 at the Acquabona farm (Portoferraio, Livorno, Italy). In this vineyard eight clones of Aleatico (clones ‘Entav’) homologated in France, supplied by the CIVAM of the Region Corsica were also grafted and planted for comparison. Results of this research activity pointed out that ‘Entav’ clones are genetically identical to the candidate clone of Aleatico of the Elba, while the candidate clones ‘Alchi 1’, ‘Alesca 59’ and ‘Alesca 60’ coming from the province of Grosseto, showed genetic diversity to the homologated clones of Aleatico ‘Entav 53’ (Scalabrelli et al., 2003). From this research, two candidate clones ‘Ale 102 and Ale 119 are now ready for the homologation (Scalabrelli et al., 2002), while, up to now, only three clones of Aleatico are registered to the National Catalogue of the Grapevine Varieties (I - AL-PA –1, I - VCR 438, I - ARSIAL-CRA 489).

Considering this limited amount of work carried out on Aleatico characterization, the knowledge of morphological traits and technological features of this variety as well as the
its response to different environmental conditions and climatic changes, including reduced rainfall and water stress, is scarce.

Aleatico cv. has large pentagonal and orbicular leaf, tri-lobed, smooth, of dark green face; it shoots and ripens quite early and it has a low fertility of first buds. Berries are medium-sized, discoid, very irregular in shape, blue vermilion, of a thick and with a heavy bloom skin. The cluster is medium-small, medium-compact, elongated loose with a single shoulder (Boselli et al., 2003). Both free and bound flavour compounds are abundant, mainly as terpenic compounds. Differently from Moscato varieties, Aleatico has small amounts of linalool, but higher quantity of geraniol following by nerol and citronellol. Aleatico is rich of phenolic compounds (~ 10 g/L) and the non-flavonoids poliphenols are highly represented (7.54 g/L). The cinnamic and benzoic acid, are usually present in small concentration and during fermentation the amount decreases even further because they are easily oxidized (Andrich et al., 2003).

During the post-harvest berries dehydration, phenolic compounds concentration decreases progressively if referred to dry weight but not when referred to fresh weight (concentration effect) and at same time the extractability increases. The candidate clones of Aleatico showed high variability on quality berries components and, in particular, the high phenolic profile variations suggest a clonal influence. A variable parameter is also represented by the contribution of skins and seeds in terms of total phenolic compounds content, even though the constantly higher incidence of seeds is a varietal characteristic (Scalabrelli et al., 2002).

The climate of Tuscan Coastal areas and Isles where Aleatico grapevine is grown are usually characterized by high temperature and low rainfall that can induce water stress conditions in vines. A moderate water deficit can lead to qualitative superior production in comparison to more favourable conditions with an optimal water supply (Düring et al. 1996, Wample and Smithyman 2002, Medrano et al. 2003, Fregoni 2005) but the response to drought is a varietal characteristic that has not been studied yet in cv. Aleatico.
**Figure 1.1.** Map of Italy; red circle highlights the main diffusion of Aleatico cv. in the Elba Island and along the coastline of Tuscany

**Figure 1.2.** Aleatico vineyards at Elba Island
1.2 WATER STRESS

Most of the world’s wine-producing regions experience seasonal drought and water deficits may become a limiting factor in wine production and quality (Chaves et al., 2007). Global warming is also affecting grapevine development, as indicated by changes in phenology and earlier harvests observed throughout the world (Jones and Davies, 2000; Webb et al., 2007), with some European regions coming closer to the thresholds of temperature and rainfall for optimum grapevine growth (Jones et al., 2005). In recent years, water deficit is also occurring in cool climate wine regions that exhibit special topography (van Leeuwen and Seguin, 2006; Zsöfi et al., 2009a). The frequency of extreme events such as heat waves or heavy rains is also predicted to increase, with negative effects on yield and quality of grapes. Sudden supra-optimal temperatures under conditions of water scarcity may lead to massive leaf shedding, with a consequent source-sink imbalance and incomplete berry maturation due to insufficient available carbohydrates (Chaves et al., 2007). These effects are unlikely to be uniform across varieties (Schultz, 2000; Jones et al., 2005). The constraints posed by climate change require adaptive management, namely irrigation to stabilize yield, maintaining or improving wine quality (Dry and Loveys, 1998; Medrano et al., 2003; Chaves et al., 2007) and other associated management techniques (e.g. soil cover) to minimize the effects of concentrated rainfall (Monteiro and Lopes, 2007; Schultz, 2007). The search for varieties adapted to growing seasons with altered length and displaying higher resilience to environmental stress is also critical to optimum berry ripening. An improvement in the productivity of water use is therefore required in vineyard management, with finely tuned deficit irrigation being able to fulfil that role.

Grapes are grown in a range of natural environments, but vine development and fruit composition are highly dependent on environmental conditions and particularly on vine water status (Jackson and Lombard 1993). Water stress may influence various physiological and developmental processes, including growth (cell division and expansion), photosynthesis (stomatal opening and enzyme-linked functions such as assimilation and respiration) as well as other metabolic and biochemical processes, prompting physiological modifications that will eventually have an impact on production and must quality (Lopez et al., 2007). Assessment and definition of the precise contribution of water stress to production losses and impaired quality are a major concern when evaluating crop water requirements (Tardaguila and Bertamini 1993).
1.2.1 VINE PHYSIOLOGY

The soil-plant-atmosphere system is characterized by a negative potential gradient due to the leaves transpiration and the environmental evaporative request. The water flow moves from less negative potential areas (soil, -0,03/-0,15 MPa) to more negatives (atmosphere -50/-120 MPa) meeting a series of resistances (Figure 1.3).

Under conditions of high irradiance and vapour pressure deficit (e.g. midday of clear summer days), water flow into grapevine leaves, as in many other species, is insufficient to compensate water losses through evapotranspiration, resulting in a midday to afternoon depression of leaf water potential (Schultz 2003; Chaves et al. 2007). As a consequence, midday to afternoon depression of stomatal conductance (gs) and net photosynthesis (Pn) has been reported in many cultivars, even under sufficient soil water availability (Gómez-del-Campo et al. 2004; Moutinho-Pereira et al. 2004).

The differences in the water-use efficiencies between grape cultivars are largely attributed to variation in stomatal conductance in response to water deficits (Bota et al. 2001, Schultz 2003, Soar et al. 2006), but also can be related to differences in the change of root hydraulic conductance and aquaporin (water channel) expression in response to water deficit (Vandeleur et al. 2008).

ABA plays a vital role in grapevine water relations during osmotic stress (Cramer, 2010). Water deficit increases ABA concentrations in the xylem sap and leaves of grapevine and changes in stomatal conductance are well correlated with ABA concentrations of the xylem sap (Okamoto et al. 2004, Soar et al. 2004, Pou et al. 2008). ABA also influences hydraulic conductance (Hose et al. 2000), aquaporin gene expression (Tyerman et al. 2002, Kaldenhoff et al. 2008) and embolism repair (Lovisolo et al. 2008) in grapevines. Furthermore, there is significant variation in ABA concentrations between rootstocks originating from different Vitis species and which have an influence on scion (V. vinifera L. cv. Shiraz) photosynthesis and stomatal conductance (Soar et al. 2006).

Other chemical signals that may influence stomatal conductance during water deficits include malate, protons, cytokinins, and ABA conjugates (Schachtman and Goodger 2008). Peptides and proteins may also act as signals in the xylem (Aki et al. 2008). These signals are important players in plant adaptation to environmental stresses (Chavez et al., 2010). Since the mid-1980s evidence has been provided on the signalling role of compounds synthesized in drying roots of different species (including grapevines); they have been associated with leaf stomatal closure and/or inhibition of meristematic development.
(Loveys, 1984; Davies and Zhang, 1991). Although root-sourced chemical signalling is widely accepted, the identity and regulation of these signals is still under debate (Holbrook et al., 2002; Schachtmann and Goodger, 2008). Nevertheless, such knowledge has enabled us to manipulate responses to soil water availability in some crops, so that changes in shoot water status are minimized and performance under moderate stress is improved (Davies et al., 2002; Chaves and Oliveira, 2004).

Under mild to moderate water deficits stomata closure is among the early plant responses, restricting water loss and carbon assimilation (Chaves et al., 2003). Direct effects on photosynthetic metabolism (Lawlor and Tezara, 2009) and on the expression of a multitude of genes (Chaves et al., 2009) may also be present at early stages. Under long-standing water deficits acclimatization responses do occur, including those related to growth inhibition and to osmoregulation; these are key elements for the maintenance of plant water status and therefore plant carbon assimilation under water scarcity (Chavez et al., 2010). In grapevine, it has been reported for several varieties and different experimental conditions (greenhouse and field; short- and long-term) that photosynthesis is quite resistant to water stress (Flexas et al., 2002; Souza et al., 2003, 2005a; Chaves et al., 2007). Under low to moderate water availabilities occurring under deficit irrigation, maintenance of the activity of Calvin Cycle enzymes and of the maximum rates of carboxylation and electron transport has generally been observed (Souza et al., 2005a). However, when stress is intensified a decline in those parameters occurs, more markedly in electron transport (Maroco et al., 2002; Souza et al., 2005a), possibly a result of decreased ATP production. Lawlor and Tezara (2009) raised the hypothesis that reactive oxygen species produced under conditions of low \(CO_2\) and excess light might induce oxidative damage to chloroplastic ATPase. Under drought conditions, a close relationship was found between stomatal function and plant hydraulics (Sperry, 1986; Cochard et al., 2002; Sperry et al., 2002). Stomata keep water flow within safe limits preventing the plants from exceeding those limits at any particular water potential, therefore avoiding xylem embolism (Sperry et al., 2002). Higher stomata sensitivity to water deficits may compensate for higher vulnerability to cavitation under drought (Schultz, 2003). \textit{Vitis vinifera} shows high hydraulic conductivity in the main stem axis (Lovisolo et al., 2007). However, leaf hydraulic conductance can substantially constrain water transport, being a more important hydraulic bottleneck than the stem (Sack et al., 1993). It is also known that hydraulic conductance of roots and shoots influences stomatal regulation and plant transpiration.
The distribution of vessel sizes varies with variety and the larger sizes often result in higher sensitiveness to embolism under drought conditions (Chouzouri and Schultz, 2005). Leaf morpho-anatomy and related biochemistry (epicuticular wax composition, lipid composition, mesophyll thickness, etc.) may also play a role in explaining plant adaptation to water stress (Syvertsen et al., 1995; Boyer et al., 1997; Cameron et al., 2006). Differences among V. vinifera have been reported in these characteristics (Schultz, 1996; Moutinho-Pereira et al., 2007).

Grapevine is generally considered a ‘drought-avoiding’ species, with an efficient stomatal control over transpiration (Chaves et al., 1987; Schultz, 2003). However, some genotypes have shown a better control of stomata than others in response to water deficits and accordingly have been classified as isohydric (drought avoiders or ‘pessimistic’); the others, showing lower control over stomatal aperture under water stress, were considered anisohydric, with an ‘optimistic’ response (Schultz, 2003; Soar et al., 2006). Schultz (2003) considered ‘Grenache’ to be a nearly isohydric genotype showing a marked regulation of stomatal conductance to decreasing soil water, whereas ‘Syrah’ exhibited a response closer to an anisohydric type. However, contradictory reports appeared in the literature showing that the same variety could behave differently depending on experimental conditions (Lovisolo et al., 2010). Recent studies (Chaves et al., 2010) revealed differences between varieties ‘Touriga Nacional’, ‘Trincadeira’, ‘Aragonez’ (syn. ‘Tempranillo’), ‘Cabernet Sauvignon’ and ‘Syrah’, in the response of leaf stomatal conductance to deficit irrigation under field conditions. Stomatal conductance of ‘Touriga Nacional’ remained highest during the day (morning and afternoon) for similar leaf water potential, suggesting an anisohydric type of response. In contrast, ‘Syrah’ showed the lowest conductance of the five varieties, particularly at noon, therefore exhibiting a near-isohydric response, contrary to earlier reports (Schultz, 2003; Soar et al., 2006). A classification of grapevine varieties as strictly iso- or anisohydric may prove inappropriate. It seems plausible that stomatal responses to water deficits in a specific variety will vary according to the particular combination of the rootstock, the climate (VPD and temperature), and the intensity and duration of water deficits (Chaves et al., 2010). In fact, under prolonged water deficits more rigid cell walls may develop, leading to a larger decline in plant water potential at midday, characteristic of the anisohydric response. Moreover, osmotic adjustment may contribute to the maintenance of open stomata at lower
water potentials, by enabling an improved turgor in response to a slowly imposed water deficits. This combination of responses will interact with scion structural factors such as water conducting capacity of stems and petioles to dictate response to water deficits (Chaves et al., 2010).

According to Palliotti et al., (2009) the adaptive strategies include changes in root, shoot and leaf morphostructural and biochemical characteristics, canopy morphology and plant architecture. In grapevine the leaf age and position along the shoot and the genotype may influence these strategies. Vines of Montepulciano and Sangiovese field-grown under severe, multiple summer stresses showed morpho-biochemical and physiological behaviors which tended to optimize the whole-vine carbon gain. The cv. Sangiovese showed to be better adapted to drought conditions compared with Montepulciano (Palliotti et al., 2008) and the genetic background appears to have a crucial role in the adaptation to summer stresses and in the ability for CO₂ uptake and for accumulation of nonstructural carbohydrates into reserve organs (Palliotti and al., 2009).

![Diagram of water potential gradient in the soil-plant-atmosphere system. The water flow meets a series of resistance along the way.](image)

The question of when and how much water should be applied in a given environment and variety is still standing (Chaves et al., 2007) and it remains of considerable debate (Chaves et al., 2010). On the one hand, small water supplements may increase yield and maintain or
even improve berry quality (Matthews and Anderson, 1989; Santos et al., 2003, 2005). On the other hand, irrigation may promote excessive vegetative growth with a negative impact on berry pigments (colour) and sugar content, and therefore decrease wine quality (Bravdo et al., 1985; Dokoozlian and Kliewer, 1996).

With enhanced pressure on water resources, the increasing demand for vineyard irrigation will only be met if there is an improvement in the efficiency of water use (Davies et al., 2002; Chaves & Oliveira, 2004; Flexas et al., 2004; Cifre et al., 2005; Souza et al., 2005a). New approaches for irrigation management will have to reduce both water consumption and the detrimental environmental effects of current agricultural practices. This goal may be achieved in several ways, deficit drip irrigation being a widely used practice with the aim of saving water and simultaneously improving wine quality. Currently, the two most important irrigation tools, based on physiological knowledge of grapevine and other crops response to water stress, are regulated deficit irrigation (RDI) and partial root-zone drying (PRD). In RDI water input is removed or reduced for specific periods during the crop cycle, improving control of vegetative vigour, to optimise fruit size, fruitfulness and fruit quality (Chalmers et al., 1986; Alegre et al., 1999; Dry et al., 2001). RDI has been used successfully with several crops, reducing water use in crops, such as olive trees (Alegre et al., 1999; Goldhamer, 1999; Wahbi et al., 2005), peaches (Mitchell & Chalmers, 1982; Li et al., 1989; Boland et al., 1993), pears (Mitchell et al., 1989; Caspari et al., 1994; Marsal et al., 2002) and grapevines (Goodwin & Macrae, 1990; Battilani, 2000).

However, this technique needs control of water application, which is difficult to achieve in practice. Although deficit irrigation is already applied to vast regions worldwide in a more or less uncontrolled/unsophisticated way, the scientific knowledge underlying its optimal functioning is still needed.

PRD is a deficit irrigation strategy that has been shown to reduce vegetative growth in grapevines as measured by pruning weight, shoot growth rate and leaf area, without causing a significant change in fruit weight or sugar accumulation (Dry et al., 1996; Du Toit et al., 2003; Bindon et al., 2008a). For the measurement of acidity, however, a variable response has been obtained with PRD irrigation (Bindon et al., 2008b). Photosynthetic rates generally decline at lower pre-dawn water potentials than stomatal conductance, when grapevines are subjected to moderate water deficits. As a consequence, intrinsic water use efficiency (Pn/gs or WUEi) is usually higher in vines under deficit irrigation (mild to moderate water deficits) than under well-watered conditions (Chavez et
al., 2010). This is reflected in a lower water use and higher WUE by the crop, an important aim of deficit irrigation strategies in vineyards (Gaudillère et al., 2002; Chaves et al., 2004; Souza et al., 2005b). When analysing WUEi it is therefore important to study it throughout the day (Chavez et al., 2010). Field studies using ‘Moscatel’, ‘Castelão’ and ‘Aragonez’ (syn. ‘Tempranillo’) showed that deficit irrigation strategies (e.g. PRD and conventional DI, both at 50% ETc) promoted an increase in WUE, when compared with fully irrigated grapevines (100% ETc), both in the short term and the long term (Souza et al., 2005b). An increase in WUE and related water savings under deficit irrigation was also reported in studies carried out in different grapevine varieties and in different locations (Dry et al., 2000; Stoll et al., 2000; Loveys et al., 2004; Poni et al., 2007; Marsal et al., 2008).

In a number of early papers reporting on the use of irrigation as a tool to manipulate vegetative growth, deficit irrigation was typically associated with reduced yield (Matthews and Anderson 1989). However, more recent research has shown that the impact on yield depends on the strategy used to apply soil water deficit irrigation (Goodwin and Macre 1990; Dry 1997; McCarthy 1997; Loveys et al. 1998; Koundouras et al. 1999). In hot climates and in non-irrigated vineyards, shoot growth may be reduced, leading to more open canopies. However, the vines might suffer from water stress, resulting in a yield reduction. The main consideration when selecting a vineyard water-management regime must be quality (i.e. the desired enological characteristics in musts and wines). Regime choice is not easy, since quality is a subjective concept, and each grape variety has its own distinctive characteristics. Moreover, irrigation of grape vines affects vine physiology, which may affect yield and grape composition, both of which influence wine quality (Lopez et al., 2007).

Hence, a rational application of irrigation necessarily requires a clear understanding of the physiological responses of the vine to water stress (Cifre et al. 2005, Remorini et al. 2010) and a rapid monitoring of berry parameters. This is also of paramount importance for the characterisation of local varieties such as the cv. Aleatico studied in the present work.

Since irrigation criteria is based on vine water demand rather than relaying on weather and/or soil moisture measurements, irrigation scheduling can be managed in a precise manner using midday Ψs as a vine physiological indicator. The use of midday Ψs as a physiological index, demonstrated to be a suitable way to perform irrigation scheduling on grapevines under RDI, since it considers soil–plant–atmosphere factors. A mild water stress of down to -1.2 MPa, for the cv. Cabernet Sauvignon under RDI, showed to be the
most effective threshold to optimize soil water availability, irrigation scheduling, yield and grape quality (Acevedo et al., 2010).

Basing on midday $\Psi_s$ physiological index, a sensitivity ranking to water stress between different varieties was showed (Scalabrelli et al., 2011) identifying the Sangiovese, as a variety which significantly responds under drought conditions (Table 1.1).

<table>
<thead>
<tr>
<th>Physiological index</th>
<th>Cultivar</th>
<th>Coefficient</th>
<th>Sensitivity level</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD$\Psi_s$</td>
<td>Sangiovese</td>
<td>0.74</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td>Cabernet Sauvignon</td>
<td>0.23</td>
<td>low</td>
</tr>
<tr>
<td></td>
<td>Alicante</td>
<td>0.49</td>
<td>medium-high</td>
</tr>
<tr>
<td></td>
<td>Petit Verdot</td>
<td>0.68</td>
<td>medium-high</td>
</tr>
<tr>
<td></td>
<td>Syrah</td>
<td>0.67</td>
<td>medium-high</td>
</tr>
</tbody>
</table>

Table 1.1 Varieties ranking in response to water stress, based on midday stem water potential measurements. The arbitrary coefficient was calculated as ratio between the number of measures in which there were noted statistical differences on $\Psi_s$ ($p < 0.05$) in vines subjected to water restriction and the total of measurements made.

1.2.2 WATER RELATIONS AND GRAPE QUALITY

There are many abiotic stresses that significantly limit the distribution of grapes around the world. These stresses reduce crop yields, but only water deficit has been used in a positive way to enhance flavour and quality characteristics of the berries (Roby et al. 2004, Chapman et al. 2005). Several authors report that a moderate water deficit can lead to qualitative superior production in comparison to more favourable conditions with an optimal water supply (Düring et al. 1996, Wample and Smithyman 2002, Medrano et al. 2003, Fregoni 2005). In part, this effect is because of reduced shoot vigour and competition for carbon resources (a change in source to sink relationship) (Cramer, 2010). Berry size can also be reduced, concentrating flavours and colour by increasing the skin surface: berry mass ratio (the skin being a significant tissue for producing flavours, tannins and colour) (Cramer, 2010). In addition, there are fundamental biochemical changes in berries under water deficit that cause important metabolic changes that influence berry flavour and quality (Castellarin et al. 2007a, Deluc et al. 2009). Water deficit was also shown to enhance photoprotection mechanisms in berries (Deluc et al., 2009).
In vineyards under Mediterranean conditions it has been a common practice to manage the water deficit during the final phases of grape development (Williams & Matthews, 1990). However, in Australia, for example, the most common practice is to apply less water early in the season (McCarthy et al., 2000). Both of these practices have shown to benefit wine, in one case reducing the grape size by limiting available water and in the other one by limiting the potential for grape growth (Chaves et al., 2007). A key to improve winegrape quality in irrigated vineyards is to achieve an appropriate balance between vegetative and reproductive development (Chaves et al., 2007).

Grape berry is a non-climacteric fruit with a double sigmoid growth curve (Coombe, 1976) (Fig. 1.4). Stages I and III of growth are separated by a lag phase (stage II). During stage I, imported carbohydrates are used for seed development, cell proliferation and expansion, and synthesis of organic acids (Coombe, 1992). At this stage the berry is exclusively connected to the vine through the xylem, and the impact of water deficit on berry growth is thought to occur directly by changes in water import by the xylem, which possibly induces a decrease in mesocarp cell turgor (Thomas et al., 2006). There is consequently a reduction in the expansion of grape berries. However, it is also possible that the ABA synthesized under water stress limits cell division and consequently small berries are produced. The second hypothesis correlates well with the observed inhibition of grape development following water deficit at pre-veraison (Chaves et al., 2010). This leads to a cascade of events culminating in earlier grape ripening (e.g. accelerating sugar and anthocyanin accumulation and malic acid breakdown) (Castellarin et al., 2007a, b). The beginning of the second phase of berry growth (stage III), known as véraison, is characterized by softening and colouring of the berry and a size increase (Chaves et al., 2010). After véraison a reduction in berry size due to water deficit is probably the result of more than one mechanism (Thomas et al., 2006). At this stage, the berry’s connectivity to the vine is via the phloem (Thomas et al., 2006). Moreover, a reduction of berry size might be only indirectly caused by water stress, through a decrease in photosynthesis (Wang et al., 2003). Post-veraison water deficit increases the proportion of whole-berry fresh mass represented by seeds and skin (Roby and Matthews, 2004) and berries present ‘thicker skins’ at harvest probably due to a decrease in the activity of pectin methylesterase enzyme (Deytieux-Belleau et al., 2008), as was shown in water-stressed tomato cherry fruit (Barbagallo et al., 2008). This results in higher content of skin-based constituents (e.g. tannins and
anthocyanins) on a berry mass basis and as a consequence the must from those berries is much richer in skin derived extractives (Chatelet et al., 2008).

Grape quality largely depends on sugar/acid balance at harvest (Chavez et al., 2010). Moderate water deficit promotes sugar accumulation either as a result of inhibiting lateral shoot growth, which induces a reallocation of carbohydrates to fruits, or as a direct effect of ABA signalling on fruit ripening (Coombe, 1989). Indeed, experimental evidence suggested activation of ABA-mediated uptake of hexose (Deluc et al., 2009). However, the mechanisms underlying accumulation of hexoses under water deficit have not been elucidated completely. The effects of water deficit on sugar content of grapevine berries are variety-dependent (Gaudillère et al., 2002). For example, no significant changes were observed in ‘Merlot’ sugar content under water deficits, while a significant increase in sugar content was observed in ‘Cabernet Sauvignon’ berries (Castellarin et al., 2007a, b). Similarly, Deluc et al. (2009) observed an increase in berry sugar content under water deficits in ‘Cabernet Sauvignon’ but not in ‘Chardonnay’. This may be explained either by differences in vigour, and therefore source/sink equilibrium, between varieties, or by different mechanisms underlying the response of grape berry development to water limitation according to the timing and intensity of water stress imposition (Chavez et al., 2010). Indeed, it was shown that water deficit has more effect on berry sugar accumulation when imposed before véraison (Keller, 2005; Keller et al., 2006). In most cases, no titratable acidity changes have been observed in the must from moderately water-stressed vines (Matthews and Anderson, 1989; Esteban et al., 1999). However, some studies report a reduction of titratable acidity due to deficit irrigation as compared with full irrigation (Sheltie, 2006; Santos et al., 2007). Malate/tartarate ratio is in general lower due to malate breakdown in vines with low water status (Matthews and Anderson, 1989).

The phenolic compounds concentration in berry depends, besides genetic factors, on specific metabolism (synthesis/degradation) and berry growth rate, both affected by cultural practices and environmental conditions, including vine water status (Kennedy et al. 2002, Ojeda et al. 2002, Downey et al. 2006, Castellarin et al. 2007a). Regulating grapevine water deficit is a powerful tool to manage the amount of these compounds and improve wine quality (Kennedy et al., 2002).
The effect of water deficit on the synthesis and concentration of phenolic compounds (flavan-3-ols, anthocyanins (Anth) and flavonols (Flav)) depends on the stress level and the berry phenological stage as observed in cv. Shiraz (Ojeda et al. 2002) and Cabernet Sauvignon (Kennedy et al. 2002). It was higher from anthesis to véraison under moderate water stress and from véraison to harvest under strong water stress. Flavan-3-ols biosynthesis decreases under first water deficit, proanthocyanidins and Anth increase only from véraison to harvest under strong water stress; each level of water stress increases the tannins polymerization degree.

Flavonoids (anthocyanins, flavonols and proanthocyanidins) and stilbenes, the most important phenolic compounds are mainly localized in exocarp and seed endocarp tissues (Chaves et al., 2010).

The reported increase in skin tannin and anthocyanin that accompanies water deficits seems to result from different sensitivity of berry tissues to water deficits, with the exocarp being less affected than the inner mesocarp (Roby et al., 2004).

Anth are synthesized via the flavonoid pathway in the berry skin of red grapevines from véraison (Chaves et al., 2010). The major anthocyanins synthesized are peonidin 3-O-b-glucoside and malvidin 3-O-b-glucoside, because methoxylation of delphinidin to produce its derivate petunidin rarely occurs (Castellarin et al., 2007b; Deluc et al., 2009) and water stress seems to have a greater impact on anthocyanin composition than on its total concentration (Chaves et al., 2010).

Flavonols act as co-pigments with anthocyanins and stabilize colour in young red wines play a fundamental role in grape quality (Boulton, 2001). Flavonol biosynthesis is closely related to that of anthocyanins (Jeong et al., 2006). However, in contrast to anthocyanins, a small number of flavonols were identified and available data were limited to a few grape varieties (Mattivi et al., 2006). The main flavonols reported in grape berries are quercetin-3-glucoside and quercetin-3-O-glucuronide (Downey et al., 2003). Deficit irrigation was reported to have a moderate effect on flavonol synthesis in red grapevines (Grimplet et al., 2007). In turn, the timing of water deficit does not change flavonol content (Kennedy et al., 2002). Mattivi et al. (2006) have suggested that anthocyanins and flavonols share the same biosynthetic enzymes. This may indicate that, like anthocyanins, changes to flavonol under water deficits may occur rather in composition than in accumulation (Chaves et al., 2010).

More recently, in a white grapevine (‘Chardonnay’), flavonol concentrations were reported to increase under water deficits, which was not the case in a red grapevine (‘Cabernet
Sauvignon’) in the same study (Deluc et al., 2009). This suggests a greater need for berry photoprotection in these varieties, as previously shown in apples with low levels of anthocyanins (Merzlyak et al., 2008).

Proanthocyanidins or condensed tannins are flavan-3-ol oligomers. They are important sensory components, providing wine with bitterness and astringency. However, little is known about proanthocyanidins (Dixon et al., 2005; Xie and Dixon, 2005) and a standardized measure of tannins has not yet been adopted (Downey et al., 2006). Besides, changes occurring in proanthocyanidins during grape development are complex, involving increases in the degree of polymerization, in the proportion of (–)epigallocatechin extension units, and in polymer-associated anthocyanins (Kennedy et al., 2002). Proanthocyanidins appear to be only slightly affected by water deficit (Downey et al., 2006) and the increases in skin tannin that accompany water deficits appear to result more from differential growth sensitivity of the inner mesocarp and the exocarp than from direct effects on phenolic biosynthesis (Roby et al., 2004). The effect of concentration of seed tannins on wine characteristics is not known (Matthews and Nuzzo, 2007). Moreover, few works have reported whether water status influences seed proanthocyanidin content. Two studies performed with the same variety (although in different environments) did not show any significant effects of water deficit on seed proanthocyanidins (Kennedy et al., 2000; Geny et al., 2003).

Stilbenes belong to the non-flavonoid class of phenolic compounds. Generally, stilbenes are considered as phytoalexins, and their formation in grape leaves was correlated with disease resistance (Chaves et al., 2010). Resveratrol is considered the most bioactive stilbene in grapevines (Bavaresco et al., 2008). In grape berries, resveratrol synthesis is catalysed by stilbene synthase (STS), which shares the same substrates used by chalcone synthase for flavonoid production (Versari et al., 2001). It accumulates mainly in the grape skin and seeds, and it has been found both in red and white grapes at a large range of concentrations, depending on biotic and abiotic conditions (Jimenez et al., 2007). Conflicting results have been found on the effects of water deficit on resveratrol synthesis (Chaves et al., 2010).

The aroma that builds up in grapes results from several compounds (terpenoids and their derivatives, esters, aldehydes and thiols) stored as non-volatile precursors mainly in exocarp vacuoles (Chaves et al., 2010). The influence of the irrigation strategy on grape
berry aromas has not received much research. However, two major studies suggest that deficit irrigation alters several sensory attributes of the wine as well as the concentration of carotenoids and their derivatives in berries, as compared with standard irrigation grapevines (Chapman et al., 2005; Bindon et al., 2007). Chapman et al. (2005) reported that water deficits led to wine with more fruity and less vegetal aromas than those from vines with high water status, in the variety ‘Cabernet Sauvignon’. Bindon et al. (2007) observed that deficit irrigation led to an increase in the concentration of hydrolytically released C13-norisoprenoids (b-damascenone, b-ionone and 1,1,6-trimethyl 1,2-dihydronaphthalene) in ‘Cabernet Sauvignon’ grape berries at harvest.

Figure 1.4. Berry development and ripening at 10-day intervals after flowering. (Illustration by Jordan Koutroumanidis, Winetitles, Kennedy et al., 2002)
1.2.3 MOLECULAR RESPONSES

Whereas physiological and biochemical data are numerous regarding the effect of water deficit, little is known about gene expression in grape berries exposed to water deficit and the timing of its imposition. The changes in the individual transcript abundance of many genes during long-term water stress study are similar to changes in short-term study (Tattersall et al. 2007); however, there were indications that a larger and more complex response in the acclimation process occurred with a gradual long-term stress.

A berry tissue analysis using global gene expression techniques indicated that water deficit affected the mRNA abundance of 13% of genes at grape maturity within the three tissues of the berry (skin, pulp and seeds), with the greatest changes located in the pulp and skin (Grimplet et al. 2007b). While the function of many of the genes differentially expressed within the seed and pulp remain to be elucidated, other genes over-represented in the skin were clearly associated with phenylpropanoid metabolism, ethylene, pathogenesis-related responses, energy metabolism and stress responses.

The responses to water stress include changes in hormone metabolism, particularly abscisic acid (ABA), photosynthesis, growth, transcription, protein synthesis, signalling and cellular defences.

Metabolic responses appear to be influenced by the cultivar and the colour of the grape (Deluc et al. 2009). Water deficit particularly affects ABA metabolism in Cabernet Sauvignon berries, but not in Chardonnay berries. ABA is known to enhance proline, sugar and anthocyanin accumulation in plants and the increased ABA concentration in Cabernet Sauvignon by water deficit was consistent with this hypothesis resulting in increased accumulation of these components relative to well watered controls. In Chardonnay, water deficit did not increase ABA concentration likewise sugar and proline concentration were not significantly different from the well-watered controls.

The stomatal conductance that in grapevine is one of the most sensitive index of plant water deficit is negatively correlated with ABA concentrations in the xylem sap and ABA concentrations in the leaves are correlated with the transcript abundance of VvNCED1 gene (Soar et al. 2004). Cramer et al. (2010) showed that the expression of NCED, the rate limiting enzyme for ABA biosynthesis, first increases in response to water deficit (Endo et al. 2008). Under water stress conditions changes in water potentials increases the expression of aquaporins that influence cell and root hydraulic conductivity (Vandeleur et al. 2008). Another consequence of water stress conditions is that plants need to dissipate
the excess of absorbed light energy or chlorophyll fluorescence for the prevention of photooxidative damage of the photosynthetic apparatus (Niyogi et al. 1998). Synthesis of xanthophyll pigments are needed at this point, so is likely that high levels of Zeaxanthin epoxidase and Violaxanthine de-epoxidase, is a response to this stress.

In water deficit plants the higher concentrations of glucose, malate and proline not only aid plants in osmotic adjustment, but also may help plants cope with reactive oxygen species detoxification and photoinhibition. As consequence, transporters for nitrate, nitrite, sulfate, proline, ATP, amino acids (proline) and organic acids exhibit greater expression patterns in response to water deficit (Cramer et al. 2007).

Transcripts of several transcription factors are also positively up-regulated by drought conditions as members of DREB family (1608315_at) (Castellarin et al., 2007b), which bind to a drought-responsive element in the promoter of drought-induced genes (Liu et al. 1998).

Specific proteins called Dehydrins are reactive to various dehydrating stress conditions such as cold, salinity and also drought and they can accumulate in vegetative tissues and in seeds at later stages of embryogenesis (Xiao et al., 2006). Zamboni et al. (2008) reported in the molecular results of a post-harvest withering grape experiment that DHN1a, a gene coding for dehydrin biosynthesis, had higher expression level in off-plant withered berries.

The response of anthocyanins to water deficit is irrelevant in white berry varieties as Chardonnay cv. because they cannot produce Anthocyanins for a multi-allelic mutation (Walker et al., 2007). In red varieties Anthocyanins are synthesized via the flavonoid pathway that harbour the wild-type VvmybA1 transcription factor for the expression of UFGT (Kobayashi et al., 2004). The encoded enzyme UFGT catalyses the glycosylation of unstable anthocyanidin aglycones into pigmented anthocyanins (Figure 1.5). Two primary anthocyanins (cyanidin and delphinidin) are synthesized in the cytosol of berry epidermal cells. Cyanidin has a B-ring di-hydroxylated at the 3’ and 4’ positions, whereas delphinidin has a tri-hydroxylated B-ring because of an additional hydroxyl group at the 5’ position. Flavonoid precursors are initially recruited from the phenylpropanoid pathway by a small family of chalcone synthases (CHS1, CHS2, CHS3) and enter the flavonoid pathway. Parallel pathways downstream of F3’H and F3’5’H (Bogs et al. 2006; Castellarin et al. 2006) produce either cyaniding or delphinidin. The 3’ position of cyanidin and delphinidin...
and sequentially the 5′ position of delphinidin can be methoxylated by OMT that generate peonidin, petunidin and malvidin, respectively.

Water deficit has been considered to enhance accumulation of anthocyanins, through the stimulation of anthocyanin hydroxylation, probably by up-regulating the gene encoding the enzyme F3′5′H (Mattivi et al., 2006; Castellarin et al., 2007b).

Genes coding for O-methyltransferase (OMT) were also up-regulated in berries from dehydrated plants in which anthocyanin composition enriched in more methoxylated derivatives such as malvidin and peonidin, the grape anthocyanins to which human gastric bilitranslocase displays the highest affinity (Castellarin et al., 2007b).

Gene regulation of the anthocyanin pathway was known to be affected also by the timing of imposition of water deficit (Castellarin et al., 2007a). Early imposition of water stress led to increased sugar accumulation, which accelerates anthocyanin synthesis (Castellarin et al., 2007b), probably due to ‘sucrose boxes’ in the promoters of LDOX and DFR genes (Gollop et al., 2001, 2002). Colour differences were the result of increased anthocyanin synthesis caused by water deficit applied either early or late in the season (Matthews and Anderson 1988, Castellarin et al. 2007a, Deluc et al. 2009). It was suggested that both ABA and sugar signalling might affect accelerated anthocyanin development.

Considering the brassinosteroid synthetic pathway which is implicated in hormonal control of ripening (Symons et al. 2006), the gene DWF1 was found up-regulated in WS vines throughout véraison (Castellarin et al., 2007b).

The induction in WS plants of structural and regulatory genes of the flavonoid pathway and of genes that trigger brassinosteroid hormonal onset of maturation suggested that the interrelationships between developmental and environmental signalling pathways were magnified by water deficit which actively promoted fruit maturation and, in this context, anthocyanin biosynthesis.

Transcriptomic analysis of genes encoding enzymes involved in the biosynthesis of volatile compounds revealed an increase in the transcript abundance of one terpenoid synthase, one carotenoid cleavage dioxygenase and several lipoxygenases under conditions of water deficits (Deluc et al., 2009). However, the correlation of enzyme transcript abundance with the reaction products they catalyse is not straightforward, given the complexity of gene regulation, enzyme activity modulation and differential expression of multigenic families (Chaves et al., 2010).
1.3 NEW OPTICAL SENSORS TO EVALUATE GRAPE QUALITY

An appropriate evaluation of grape maturity is fundamental for the production of high-quality wine (Conde et al. 2007, Kennedy et al., 2006). However, monitoring the phenolic content of grape berries is difficult because of its large spatial and temporal heterogeneity among the different vineyard plots (Bramley, 2005). Grape phenolic maturity is usually determined by destructive laboratory analysis (Harbertson et al., 2006; Di Stefano et al., 2008), which are time-consuming and require an accurate sampling approach to be representative of the vineyard block considered. The development of new portable optical sensors dedicated to the non-destructive assessment of Anth in grape clusters attached to the vines represents a useful complementary tool to evaluate phenolic maturity and better characterize grapevine varieties (Agati et al. 2009, Cerovic et al. 2009, Ben Ghozlen et al. 2010a,b).
These new optical techniques based on near infrared spectroscopy linked to chemometrics are emerging (Gishen et al., 2005). Estimation of skin anthocyanins (ANTH) through direct berry colour measurement without extraction has also been used (Carreño et al., 1995). More recently, a method was proposed to assess the skin content of phenolics. It is based on their screening of excitation of chlorophyll fluorescence (ChlF). The method is applicable to winegrape leaves (Kolb and Pfundel, 2005) and fruits (Kolb et al., 2003; Agati et al., 2007) using either UV light for flavonols (FLAV) or visible light for ANTH. A visible beam for which the epidermis is transparent is used as a reference (cf. Agati et al., 2007). Assessment of FLAV and ANTH has also been attempted in apple skin by devices originally developed for leaves (Hagen et al., 2006). Cerovic et al. (2008) used a new instrument Dualex ANTH in addition to the leaf-clip Dualex FLAV, and a new prototype hand-held optical sensor Multiplex. The latter stands for multiple excitation fluorescence sensor and was derived from a work on fluorescence lidars (Ounis et al., 2001). The validation of the use of fluorescence-base sensors was performed by Cerovic et al., (2008) on wine grapes known to have large contents of ANTH. Dualex measurements were performed on berry caps in order to calibrate the method on grapes. Multiplex measurements were performed on whole bunches to evaluate the potential use of fluorescence as signature of phenolic maturity.

The Multiplex technique is based on chlorophyll fluorescence detection and the Anth screening effect on the excitation light used for the measurement: the larger the Anth concentration in the berry skin, the lower the chlorophyll fluorescence signal (Figure 1.6). This method has been validated in the laboratory by others spectroscopic studies on winegrape berries (Agati et al. 2007) and whole bunches (Agati et al. 2008) and also, due to their insensitivity to ambient light, directly in the field.

The comparison of red grape to white grape varieties during a whole season revealed two fluorescence ratios as potential signatures of grape phenolic maturity (Cerovic et al., 2008). From this study, both types of optical sensors are considered useful for viticulture: the ‘‘leaf-clip’’ type Dualex is inherently more precise and can yield quantitative data on ANTH and FLAV in individual berries and these would be valuable for studies on environmental effects on grape maturation; the non-contact sensor Multiplex, on the other hand, has real potential for precision viticulture. Still, for very mature grapes an increase in green source power would be welcome (Cerovic et al., 2008).
1.4 AIM OF THE THESIS

It is acknowledged that the timing and intensity of the response to soil and atmospheric water deficits, namely in what concerns stomatal control, depends greatly on genotype (Chaves et al., 2010). This has profound implications in irrigation management, in particular the timing and amount of irrigation to optimize source–sink relationships, in order to achieve optimal fruit quality in each variety (Medrano et al., 2003; Chaves et al., 2007; Poni et al., 2007). *Vitis vinifera* L. is characterized by large genetic variability with several thousand varieties/varieties being cultivated worldwide (Alleweldt et al., 1990; Galet, 2000; Schultz, 2003). European countries like France, Spain or Portugal host a large number of native *V. vinifera* varieties. However, most of those genotypes remain uncharacterized, which limits their use for breeding, for example to increase WUE or improve berry quality traits.

In the present research, we report the results of two consecutive seasons trial aimed to characterize and improve the quality of Aleatico cv. under the Mediterranean climate condition of South of Tuscany.

As several authors report (Düring et al., 1996, Wample and Smithyman, 2002, Medrano et al., 2003, Fregoni, 2005), a moderate water deficit can result in higher quality production which effects may vary depending on different factors, including genotype (Remorini et
al., 2010). Considering that, Aleatico vines were subjected to different water regimes in order to study the physiological mechanisms and berry composition involved in the response to water stress.

Besides indicators and parameters such as stem water potential, gas exchanges and sap flow, the evolution of berry anthocyanin content was monitored in time using, in the field, a new optical portable sensor, Multiplex®2 (Agati et al., 2009) as index of phenolic maturity.

In order to study at molecular level the expression of specific genes linked to the anthocyanins pathway and to possibly identify other specific processes and potential biomarkers useful to monitor water stress conditions in Aleatico berries, a research stage was carried out at the Dr. Lund’s laboratory - Wine Research Centre of the University of British Columbia (Vancouver, Canada).

Although Canada is one of the youngest wine-producing regions in the world, the wine industry has grown rapidly over the most recent 10 years and in addition to producing the finest ice wines in the world, Canada has recently gained acclaim as a producer of high quality, award-winning wines, crafted from classic French and German V. vinifera varietals. Given the high economic value of grape production in numerous countries worldwide, increasing investments have recently been directed towards better understanding molecular mechanisms underlying ripening and berry flavour traits for improvement of the grape industries. In particular The Dr Lund’s research group studies regulatory and metabolic networks of gene expression during ripening in soft fruits for viticulture and human health applications. They are currently working to better understand the interaction of the grapevine genome, the vineyard environment, and viticultural practices in order to develop molecular-based tools to assist growers in management decision making in the vineyard each season and they are also using comparative biochemical genomics to advance the understanding of the antioxidant capacities imparted by vitamin C and anthocyanin flavonoids with the goal of developing molecular tools for marker assisted breeding in raspberry and engineering improved anthocyanin compositional chemistry in wine grapes.
2. MATERIALS AND METHODS

2.1 PLANT MATERIAL AND TREATMENTS

The experiment was conducted in two consecutive years (2008 and 2009) at the Bulichella farm, Suvereto (Livorno, Italy; 43°04'N, 10°41'E) on 3-year-old (in 2008) vines of grape (*Vitis vinifera*) cv Aleatico grafted on 110R, trained to 2–3 vertical shoots and spaced 0.6 × 2.0 m. Vines were planted in February 2006 in North-South oriented rows. For both trial periods, data from a nearby meteorological station were obtained from ARSIA, Tuscany Regional Agrometeorological Service. Portable data loggers (Tinytag TGU-4500, Gemini, Chichester, UK) were installed at the level of bunches and near the vineyard to monitor temperature and relative humidity. Evapotranspiration (ETo) was calculated by using the Blaney–Criddle’s theoretical method (Brouwer and Heibloem 1986); water requirement was calculated as WR= ETP*Kc – Rainfall. Two treatments were applied: non-irrigated (water stress, WS) and irrigated to prevent intense water stress (IR). The WS plants were also covered at soil level with plastic transparent sheets to avoid rain infiltration (Figure 2.1). In 2008, the experiment lasted from 17 June (day of the year, DOY, 169) to 5 September (DOY 249) when grapes were harvested at a °Brix of 27.0. Veraison occurred at the beginning of August, as determined by visual assessment of 10% berries colouring on 31 July (DOY 213). Water on IR plants was supplied on 28 July (DOY 210) and on 1 August (DOY 214) by a drip irrigation system applying 46.9 and 23.4 L water per plant, respectively. A main rain event (40 mm) occurred on 15 August (DOY 228). In 2009, vines were trained to horizontal cane. The experiment was carried out from 8 July (DOY 189) to 1 September (DOY 244) when grapes were harvested at a °Brix of 25.8. Veraison occurred at the end of July (10% of berries colouring on 22, July, DOY 203). Water on IR plants was supplied on 20 July (DOY 201) and on 15 August (DOY 227) applying 67.5 and 22.5 L of water per plant, respectively. A main rain event (20 mm) occurred on 4 August (DOY 216).
2.2 PHYSIOLOGICAL RESPONSE ANALYSES

From the beginning of the experiment, the water potential and gas exchange plant physiological indicators were used to evaluate vine performances and water deficiency status. Midday stem water potential (MD $\Psi_s$) was measured once a week using a Scholander pressure chamber (Technogas, Pisa, Italy) fired by a nitrogen cylinder on three non-transpiring leaves, from three different plants, that had been bagged with aluminium foil for 1 hour before measurements to balance leaf potential with xylem potential (McCutchan e Shackel, 1992). Leaves were fully expanded and exposed to the sunlight (Figure 2.2).

Photosynthetic rate (Pn) and stomatal conductance (gs) were measured once a week on 15 vines per treatment, one leaf per vine, using a portable gas exchange system (Li-Cor 6400, Li Cor Inc., Lincoln, NE, USA) from pre-véraison to complete ripening (Figure 2.3). The gs was calculated according to the LiCor 6400 manual. Measurements were performed in the morning from 09:00 to 10:30 h on fully expanded and sunlight exposed leaves. The instrument is based on an infrared reading technique not dispersive. Four gas analyzers, for $\text{CO}_2$ and water, are present. It is equipped with a 6 cm$^2$ Parkinson leaf chamber, a flow
pump to supply the outside air, a CO₂ mixer and an adjustable outside light source. It works with an open cycle and the system can measure different parameters using several sensors and the four reading cells. The outside air is filtered and corrected basing on values previously set by the operator, to provide a constant composition. All measurements were carried out using a PAR intensity of 1200 µmol m⁻² s⁻¹ to light the leaf. The instrument detects the variations of CO₂ concentration and water vapour that occur in the leaf chamber per time unit, because of the photosynthetic activity of CO₂ fixation and the gas emission by transpiration.

In 2008, cumulated sap flow (SF) was measured using the heat balance system (Steinberg et al., 1990). Sap flow sensors (Dinamax Inc., Houston, Texas) were applied on two selected plants per treatment and connected to a data logger Campbell CR7 (Campbell Scientific Inc., Logan, Utah). The measurements were taken at 15s intervals, their average stored every 30 min by Dymax software DGSF5.0 and at the end they were converted according to the plant leaf area. The energy was constantly provided by solar panels located close to the sensors so data was collected from the beginning to the end of the experiment. The sensors were installed at mid-day (maximum reduction of diameter dimension), the cortex was reduced and a G4 silicon product was applied between vegetal tissues and sensors, to assure a good sensors adherence to the trunks. Sensors were protected from the sunlight by aluminium foils to avoid the temperature gradients caused by the difference in the sun exposition (Figure 2.4).

On 2009 and 2010 season the bud fertility was determined to evaluate the possible effect of the different water regimes on flower differentiation. Pruning canes were weighed in winter on 36 plants for each treatment to determine shoot vigour.

In addition, after 2009 winter pruning, 13 canes per treatment were cut in single node from third to tenth node. Single cuttings were maintained in laboratory to induce budbreak, clusters were then counted to determine bud fertility along the cane (Figure 2.5).
Figure 2.2. Scholander pressure chamber used to measure the midday stem water potential (MD $\Psi_s$) of IR and WS plants.

Figure 2.3. Portable gas exchange system used to measure the photosynthetic rate (Pn) and stomatal conductance (gs) of IR and WS.
Figure 2.4. Heat balance system used to measured the cumulated sap flow (SF) in 2008 season on IR and WS plants. Sensors were protected from the sunlight by aluminium foils to avoid the temperature gradients caused by the difference in the sun exposition.

Figure 2.5 Single node cuttings of pruned shoots of Aleatico grapevine from third to tenth node after 2009 winter pruning.
2.3 THE FLUORIMETRIC SENSOR

To measure phenolic maturity, 60 (in 2008) and 50 (in 2009) different bunches distributed on four adjacent rows were marked and measured by the fluorimetric sensor (Figure 2.6) once a week from veraison to harvest, directly on the vines. The Multiplex 2 (Mx; FORCE-A, Orsay, France) was a handheld battery-operated optical sensor consisting of four excitation light-emitting diode (LED) sources in the UV-A (370 nm), blue (460 nm), green (516 nm) and red (637 nm) and three detection channels in the blue-green, red and far-red spectral regions. These two last detection bands at 680–690 nm (red fluorescence, RF) and 730–780 nm (far-red fluorescence, FRF), respectively, corresponded to the two emission peaks of chlorophyll (Cerovic et al. 1999). Being the LED sources pulsed and synchronized to detection, the sensor was insensitive to ambient light and could be used directly in the vineyard. The large detection area of the sensor (8-cm diameter) permitted to the signal to be acquired from a large area of each cluster. Acquisition time for a single bunch sample was 1s. The collected data were visible on a real-time display and stored on a secure digital card for further analysis. Different combinations of the red (RF) and far-red (FRF) fluorescence signals at the various excitation bands could be used as indices of different compounds, such as flavonols (Flav), anthocyanins (Anth), and chlorophyll (Chl). Considering the fluorescence signals RF_R and FRF_R, excited with red (R) light, and FRF_G and FRF_UV, excited with green (G) and ultraviolet (UV) radiation, respectively, we can define two ANTH indices:

\[
ANTH_{RG} = \log\left(\frac{RF_R}{RF_G}\right) \tag{1}
\]

and

\[
ANTH_R = \log\left(\frac{5000}{FRF_R}\right) \tag{2}
\]

the Flav index:

\[
FLAV = \log\left(\frac{FRF_R}{FRF_{UV}}\right) \tag{3}
\]

and the Chl index:

\[
CHL = \frac{FRF_R}{RF_R} \tag{4}
\]

The choice of these equations is based on previous spectroscopic studies (Cerovic et al. 2002, Agati et al. 2007) and on the optical properties of Chl and Flav. Briefly, the intensity of Chl fluorescence depends on the excitation light reaching the Chl layer inside the
berries. It is therefore reduced by Anth localized into the outer skin layers, which absorb part of the excitation light. The higher the Anth concentration, the lower the Chl fluorescence signal. The attenuation is also depending on the excitation wavelength, according to its overlapping with the absorption spectrum of Anth, and therefore it will be higher at the peak (at around 520 nm) than in the tail in the red of the Anth absorption band. The definition of the ANTH index given in Eqn 1, by comparing the Chl fluorescence intensity under G and R lights, represents a differential absorption measurement (in accordance with the Beer–Lambert’s law) that is proportional to the Anth content. The same above consideration applies in the case of Flav, with G light replaced by UV radiation, in order to explain the origin of the FLAV index defined by Eqn 3. The use of the single FRFR signal under R light in Eqn 2 relies on a sufficiently significant absorption of Anth at 637 nm. The 5000 value in Eqn 2 represented the full-scale value in mV of photodiodes. The ANTHRG and FLAV indices were corrected for differences in the R versus G and R versus UV LED light intensities, respectively, using a fluorescence standard film (FORCE-A) with known absorption properties. The CHL index (Eqn 4) is based on the partial reabsorption of RF, depending on the Chl concentration (Buschmann 2007), and on the absence of reabsorption on the FRF band. Consequently, the CHL index increases with the increase of Chl concentration.

Figure 2.6 The fluorimetric sensor Multiplex 2 used to measure phenolic maturity of grape bunches directly in field.
2.4 BERRY SAMPLING AND DESTRUCTIVE MEASUREMENTS

In order to calibrate the Mx sensor for the Anth and Flav content, for each date during the 2008 season, three different bunches per treatment were randomly sampled. Fourteen berries from the exposed side of each bunch were collected, measured by the Mx, weighed and frozen for subsequent extraction and high-performance liquid chromatography (HPLC) quantification of phenolic compounds. The skin from the upper half (flower scar face) of 14 berries was peeled off and ground under liquid nitrogen. The resulting skin powder was transferred to 3 mL of acidified extraction solvent (70% EtOH, 25% H2O, 5% formic acid), maintained under stirring in the dark for 1 h and then centrifuged for 3 min at 4100 × g. The pellet was re-extracted twice using the same procedure, and the final three pooled supernatants were adjusted precisely to 10 mL. The whole procedure was performed at room temperature (20–25°C). Extracted samples were stored at -20°C until HPLC analysis.

For both seasons, a set of 200 berries was randomly collected weekly from the beginning of veraison to harvest and processed for phenolic compounds extraction within the same day. For each sampling, 60 berries were randomly chosen, divided into three groups of 20 berries, which were used as triplicates, and processed according to the method of Di Stefano et al. (2008) slightly modified as follows. Berry skins of each replicate were manually separated from pulp and seeds, and skins and seed were separately weighed and extracted for 4 h at 25°C in 25 mL of a pH 3.2 tartaric buffer solution. This solution contained 12% (v/v) ethanol, 2 g/L sodium metabisulphite, 5 g/L tartaric acid and 22 mL/L NaOH 1 N. After grounding in a mortar and pestle, the extract was separated by centrifugation for 10 min at 3000 rpm. The pellet was re-suspended in 20 mL of buffer and centrifuged for 5 min. The final two pooled supernatants were adjusted precisely to 50 mL with the buffer solution. The skins extract was measured spectrophotometrically at 540 nm after dilution (1:20) with ethanol : water : HCl (70:30:1) and at 750 nm as the seeds extract in the following solution: 0.1 mL of the extract, 6 mL H2O, 1 mL Folin-Ciocalteu reactive, 4 mL 10% Sodium Carbonate (after 5 min) and H2O up to 20 mL. Anth were expressed as mg of equivalents of malvidin 3-O-glucoside and phenolic compounds as mg of equivalents of (+)-catechin. Remaining berries (140) were used to determine the concentration of total soluble solids (°Brix) by a digital refractometer (Model 53011, TR, Forli, Italy), the pH by a bench pH-meter (Hanna Instruments, Milano, Italy) and total acidity by titration with NaOH 0.1 N.
2.5 HPLC/DAD ANALYSIS

In 2008, HPLC/DAD analyses were performed on a HP 1100L liquid chromatograph equipped with a Diode Array Detector (DAD) detector and managed by an HP Chemstation software (Agilent Technologies, Palo Alto, CA, USA). Hydroxycinnamic acids and flavonoids were separated using a 4.6 × 250 mm Polaris E RP18 (5 µm) column (Varian, Darmstadt, Germany) operating at 27 ± 0.5°C. The eluent was H2O (adjusted to pH 3.2 by HCOOH)/CH3CN. A four-step linear gradient solvent system was used, starting from 100% H2O to 100% CH3CN during a 53-min period, at the flow rate of 0.8 mL/min as previously reported (Saracini et al. 2005). Anth were separated using a RP-80 C12 column (Phenomenex Synergi Max), 150 × 3 mm, 4 µ (Phenomenex, Torrance, CA, USA) operating at 27 ± 0.5°C. The eluent was H2O (adjusted to pH 2.0 by HCOOH/CH3CN). A four-step linear gradient solvent system, at the flow rate of 0.4 mL/min for 28 min, was used (Mulinacci et al. 2008).

2.6 HPLC/MS ANALYSIS

In 2008, HPLC/Mass Spectrometry (MS) analyses were performed using the same analytical conditions of HPLC/DAD analysis. In detail, the HPLC/DAD was interfaced with a HP 1100 MSD API electrospray (Agilent Technologies) operating in negative and positive ionization mode under the following conditions: nitrogen gas temperature 350°C, nitrogen flow rate 10 L/min, nebuliser pressure 30 psi, quadrupole temperature 30°C, capillary voltage 3500 V. The mass spectrometer operated at 120 eV of negative fragmentor for flavonoid and caffeic derivatives, and at 120 eV positive fragmentor for Anth.

2.7 QUANTITATIVE ANALYSES

In 2008, the identification of individual phenolics was carried out by means of HPLC retention times, and both UV/Vis and MS spectra. Quantification of the single phenolic compounds was directly performed by HPLC/DAD using a five-point regression curve built with the available standards. Curves with a coefficient of determination r² > 0.998 were considered. In particular, hydroxycinnamic acids amounts were calculated at 330 nm using caffeic acid as reference. Quercetin and kaempferol glycosides were calibrated at
350 nm using quercetin 3-O-rutinoside and kaempferol 3-O-rutinoside, respectively. Finally, Anth glycosides were calibrated at 520 nm using malvidin 3-O-glucoside (oenin) as reference. The molecular weight correction has been applied, if necessary. Compound concentrations were expressed as mg/g of skin fresh weight or as µg per cm² of berry surface.

In 2009, the Anth extracts, obtained by Di Stefano et al. (2008) method, were also used to identify the skins Anth profile by HPLC quantification after purification of the extracts on a 300 mg C18 cartridge. A syringe connected with the 300 mg C18 cartridge was activated by using 2 mL of methanol and 2 ml of H₂SO₄ 0,01 N. Afterwards 2 mL of H₂SO₄ 0,1 N and 0,5 mL of the Anth extracts were charged in the syringe. Samples were eluted on the cartridge, washed with 2 mL of H₂SO₄ 0,01 N and the Anth were eluted with 2 mL of methanol, collecting the liquid phase in a 50 mL distillation ball, starting from the first coloured drop. The solvent was vacuum evaporated, recovered by 1 mL methanol-formic acid- water solution (50:10:40), filtered on a 0.2 µ membrane and injected in the following conditions:

- chromatograph Agilent 1100,
- pre-column ODS Hypersil 20 x 2,1 C18 (5 µm),
- column ODS Hypersil 200 x 2,1 mm (5 µm),
- solvent A: formic acid 10% in water,
- solvent B: formic acid 10%, methanol 50% in water,
- flow: 0,225 mL,
- wavelenght: 520 nm,
- injected volume: 20 µl,
- linear gradient from 65% of A to 45% of A in 25 minutes,
- from 45% of A to 40% of A in 20 minutes,
- from 40% of A to 5% of A in 25 minutes,
- from 5% of A to 1% of A in 5 minutes,
- from 1% of A to 65 % of A in 3 minutes,
- time of balance: 5 minutes.

The picks area and the percentage of every Anth in the extracts were determined by integration of the chromatogram. Compounds concentration was expressed as mg/g of
skin fresh weight, by using as reference (100%) the total Anth skins content (mg/g) obtained by Di Stefano et al. (2008) method.

2.8 GENE EXPRESSION ANALYSES

For each sampling date during the 2009 season, 20 homogeneous berries randomly detached from the exposed side of three different bunches per thesis were collected, frozen in liquid nitrogen and stored at -80°C.

Total RNA was extracted from berry pericarp using Spectrum Plant Total Rna Kit (Sigma-Aldrich) and double-strand cDNA was synthesized using Quantitec® reverse transcription kit (Quiagen) starting from 2 µg of RNA and finally quantified at the Dr. Lund's laboratory -Wine Research Center (Vancouver, Canada) by spectrophotometer NanoDrop (Thermo Scientific).

Preliminary qRT-PCR was performed at the Wine Research Centre of the University of British Columbia (Vancouver, CA) with a 7500 System Software V2.0.1 (Applied Biosystems) by using Dynamo HS SYBR Green qPCR kit (New England Biolabs). Each reaction (20 µl) contained 2 µl [5µM] of each primer, 4 µl of diluted cDNA (20 µg/mL), 10 µl of Master mix [2x], 0.25 µl of ROX ref [50x], 1.25 µl RNAse free water. Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles, followed by a melting curve stage of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60 °C for 15 s. Each cDNA sample was analysed at a dilution of 1:100 of the original cDNA and run in triplicate. Gene transcripts were quantified upon normalization to Ubiquitin E (UbqE), by comparing the cycle threshold (Ct) of the target gene with that of UbqE. Ubiquitin E was chosen because it showed the smallest standard deviation in the expression of WS biological replicates from different sampling dates compared with other eight putative reference genes: poliubiquitin (Ubq-10), elongation factor 1 (EF1), Tubulin alpha (Tub-alpha), ubiquitin L40 (L40), malate dehydrogenase (MDH), Sand, Actin, glucose-6-phosphate dehydrogenase (Gapdh) (Tab. 2.1). Gene expression was determined on one biological replicate for 3 sampling dates of 2009 season in order to analyse, in a preliminary way, a large number of genes putatively sensitive to water stress and/or linked to processes related to berry quality. Based on these preliminary results a further set of gene expression analyses (qRT-PCR) have been performed at the Life Science Institute Transcriptomic Lab of the Scuola Superiore
Sant'Anna (Pisa, Italy) adding new key-genes of the flavonoid pathway and using a 7300 System Software (Applied Biosystems) and the iTAQ SYBR Supremix w/ROX (Bio Rad srl) at the same thermal cycling conditions. Each reaction (15 µl) contained 1.5 µl [5µM] of each primer, 6 µl of cDNA (5 µg/mL), 7.5 µl of Master mix. Each cDNA sample was run in duplicate. Gene transcripts were quantified upon normalization to Ubiquitin E (UbqE) and Ubiquitin-conjugating factor (UbqCF) by using GeNorm VBA applet for Microsoft Excel. Gene expression was determined on 3 biological replicates for each sampling date (with the exception for IR first sampling date that, for technical problems, was analyzed in duplicate) and the cDNAs was obtained after re-extraction of RNA from the same samples stored at -80°C. Quantity and quality of RNA of all Aleatico samples was assessed by using the Automated Electrophoresis Station (Biorad Experion™). The results showed values between 8.2 and 9.5 of RQI scale indicating a good quality for all samples (Table 2.2) as confirmed by the virtual gel (Figure 2.7).

Primers pairs for UbqCF were retrieved from literature (Castellarin et al., 2007b), UbqE, NCED1, NCED2, Zeaxanthin epoxidase, MXK3, DREB, Dehydrin 1a, Pyrroline reductase, Proline from the Dr. Lund's laboratory, FAOMT (Lucker et al., 2010), F3'H, F3’5’H (Gutha et al., 2010), AOMT (Hugueney et al., 2009), UFGT, FLS1, MSA, PIP2;1, DWF1 were newly designed on the original DNA sequences to amplify ~100 bp gene fragments by using the software Primer Express (Applied Biosystems) (Tab. 2.3).

<table>
<thead>
<tr>
<th>Reference Gene</th>
<th>Code</th>
<th>C&lt;sub&gt;t&lt;/sub&gt; mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin E</td>
<td>UbqE</td>
<td>25.45</td>
<td>0.893</td>
</tr>
<tr>
<td>Poliubiquitin</td>
<td>Ubq-10</td>
<td>25.42</td>
<td>1.726</td>
</tr>
<tr>
<td>elongation factor 1 alpha</td>
<td>EF1</td>
<td>28.93</td>
<td>1.385</td>
</tr>
<tr>
<td>Tubulin alpha</td>
<td>Tub-alpha</td>
<td>29.37</td>
<td>2.150</td>
</tr>
<tr>
<td>Ubiquitin L40</td>
<td>L40</td>
<td>26.57</td>
<td>1.516</td>
</tr>
<tr>
<td>malate dehydrogenase</td>
<td>MDH</td>
<td>26.92</td>
<td>0.948</td>
</tr>
<tr>
<td>Sand</td>
<td>Sand</td>
<td>29.03</td>
<td>1.584</td>
</tr>
<tr>
<td>Actin</td>
<td>Actin</td>
<td>24.14</td>
<td>1.275</td>
</tr>
<tr>
<td>glucose-6-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>27.18</td>
<td>1.016</td>
</tr>
</tbody>
</table>

Table 2.1 Comparison between eight putative reference genes: each Ct value is the mean of Ct of WS biological replicates from different sampling dates. Ubiquitin E resulted to be the most stable reference gene because it showed the smallest standard deviation.
Table 2.2. RNAs quality of 2009 Aleatico samples measured by RQI scale: the RNA quality indicator (RQI) automatically assigns a numerical quality rating for eukaryotic total RNA samples to quickly judge the integrity of the total RNA sample (Experion Software, Bio Rad srl). A specialised algorithm compares three regions of an electrophoretic profile of RNA samples to a series of standardised degraded RNA samples. The RQI method returns a number between 10 (intact RNA) and one (highly degraded RNA) for each eukaryotic RNA sample run on an Experion RNA Stdsens or Highsens analysis chip. It gives a consistent evaluation of RNA quality and is a convenient quantifier in determining the extent of degradation of RNA samples.
Figure 2.7. Virtual gel of 2009 Aleatico RNAs samples (Experion Software for automated electrophoresis, Bio Rad srl)
Table 2.3. List of all primer sequences used in qRT-PCR

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Code</th>
<th>Sequence</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid 3-O-glucosyltransferase</td>
<td>UFGT</td>
<td>X75968</td>
<td>TTTGCAAGCCGCTTGTGTGGA</td>
<td>GCCACCTTTCTCTTTGGA</td>
<td>105</td>
</tr>
<tr>
<td>Aquaporin</td>
<td>MLP2</td>
<td>D0284568</td>
<td>TGGTCTGGCCACCTGCTAATC</td>
<td>CCAAGGCGCTCTCTTATGTTGA</td>
<td>96</td>
</tr>
<tr>
<td>Flavonol synthase</td>
<td>FLSI</td>
<td>AY257730</td>
<td>TCTTGTAGCCTGGCAAAAGAGGA</td>
<td>GCCCTCAGTCTCTTTGGA</td>
<td>101</td>
</tr>
<tr>
<td>Dwarf-1</td>
<td>DWF1</td>
<td>CF332599</td>
<td>CCCTGGCAGGAAGAGATTGCT</td>
<td>TGCCTGCTCAGGAAAGTGG</td>
<td>93</td>
</tr>
<tr>
<td>Abscisic acid-, stress- and ripening-induced (AS) gene</td>
<td>vMA</td>
<td>AF818588</td>
<td>CAGAGCAAGGCGCTGAGGA</td>
<td>ACCATCGTGAGCTAGCTTAG</td>
<td>91</td>
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<tr>
<td>Drought responsive element-binding protein</td>
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<td>GCCTTAATTAGTAAGCCCCGCT</td>
<td>105</td>
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<tr>
<td>ABC transporter</td>
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<td>CGGTCCTCCAAATGGAACCT</td>
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<td></td>
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<tr>
<td>3-dS-epoxycarotenoid dioxygenase 1</td>
<td>NCE1</td>
<td>XM_002277611.1</td>
<td>GGACGACCTTGCCCCTAACA</td>
<td>ATCATTTGTGAACCGAGCTGAC</td>
<td>96</td>
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<tr>
<td>3-dS-epoxycarotenoid dioxygenase 2</td>
<td>NCE2</td>
<td>AV332814</td>
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<td>TTCCCTCGTGGAGCAATCTC</td>
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<tr>
<td>Prolin oxidase/dehydrogenase</td>
<td>PIDh</td>
<td>XM_002282733.1</td>
<td>GTTGTGAATAAAAAGGCCTG</td>
<td>TACTGAGATGAGCAACAGG</td>
<td>95</td>
</tr>
<tr>
<td>Pyrroline-5-carboxylate reductase</td>
<td>P5CR</td>
<td>XM_002277607.1</td>
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<td>CCGTCTCCGGAATACCCGCT</td>
<td>91</td>
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<tr>
<td>Zeaxanthin epoxidase</td>
<td>ZEP</td>
<td>XM_00222507.1</td>
<td>AGTACGTTTGGCCTGGTCCCA</td>
<td>CTTTTGAAAGGCTGGGCCC</td>
<td>95</td>
</tr>
<tr>
<td>Flavonoid 3'-hydroxylase</td>
<td>FSH</td>
<td>GUS55355</td>
<td>ATCTGGACCCGCTGAAATG</td>
<td>AGCGGTATCCGTACACTG</td>
<td>156</td>
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<td>Flavonoid 3', 5'-hydroxylase</td>
<td>FSH1</td>
<td>GUS55357</td>
<td>AGAGTTGTAGCTGGTTTAAATCAAGAGAT</td>
<td>AGAGGAGTGTCTGTTAATGTAATGTA</td>
<td>156</td>
</tr>
<tr>
<td>Anthocyanin O-methyltransferase</td>
<td>ADMT</td>
<td>FJ426186</td>
<td>CTCTTGGGATCCTGTGA</td>
<td>CCCCCAACCAGTGCTGCCACA</td>
<td>159</td>
</tr>
<tr>
<td>Flavonol and Anthocyanin 3', 5'</td>
<td>FAOM</td>
<td>HM142924</td>
<td>TGGTCCATAGAGCGGCTG</td>
<td>CTTGGGATCTGGGTAATCAGAGG</td>
<td>103</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Reference Gene</th>
<th>Code</th>
<th>Sequence</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin-conjugating enzyme</td>
<td>UbqE</td>
<td>EC553322</td>
<td>GAAGGTTCAGCGGGATTGGA</td>
<td>GCCCTGACCTTACCATCTTAAG</td>
<td>75</td>
</tr>
<tr>
<td>Ubiquitin-conjugating factor</td>
<td>UbqC</td>
<td>CF203457</td>
<td>CTTGACTGCCTGCTGGGAGG</td>
<td>AAGCCAGGAAGGAGAACCT</td>
<td>154</td>
</tr>
<tr>
<td>Polylubrinin</td>
<td>Ubq10</td>
<td>CB152599</td>
<td>CAAATGCTGGGAGCCGAAA</td>
<td>TACACTCCAGTGTCGCTG</td>
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</tr>
<tr>
<td>elongation factor 1 alpha</td>
<td>EFL</td>
<td>CB1577511</td>
<td>GGCTCTGGCAGATGTCTTG</td>
<td>AGCTGCTGCTGCCCTTGTGG</td>
<td>83</td>
</tr>
<tr>
<td>Tubulin alpha</td>
<td>Tub-alpha</td>
<td>EC530899</td>
<td>CACGCGACATCTCCAGAGGCTT</td>
<td>GTCTCGGCAGAAGTCCATA</td>
<td>119</td>
</tr>
<tr>
<td>Ubiquitin L1</td>
<td>L1D</td>
<td>EC525411</td>
<td>CATAAACATTTGGGCAGTAGA</td>
<td>TGGTGATATTGAGGAGCTTCC</td>
<td>80</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH</td>
<td>EC521711</td>
<td>GACGAGATGATGAGCTAGGAA</td>
<td>GTCAAAACAGACTGCTGGAA</td>
<td>82</td>
</tr>
<tr>
<td>Sand</td>
<td>Sand</td>
<td>CF405449</td>
<td>CAAATCACCTTGCTCCCATTGAGA</td>
<td>GCATTGAATGCTGAGATAGA</td>
<td>76</td>
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<tr>
<td>Actin</td>
<td>Actin</td>
<td>EC596944</td>
<td>CTCTGGCTCGGCAGAGGCTCTT</td>
<td>TGGTGACAGTAGTGTTGA</td>
<td>82</td>
</tr>
<tr>
<td>glucose-6-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>CB973347</td>
<td>TTTCTGTTGAGGGCTATTTCA</td>
<td>CCACAGACCTCCATCGGTGACA</td>
<td>70</td>
</tr>
</tbody>
</table>

2.9 STATISTICAL ANALYSIS

Statistical analysis was carried out with SigmaPlot for Windows Version 11.0 (Systat Software, Inc., Erkrath, Germany) and statistical differences between treatments were analysed by one-way analysis of variance (ANOVA). Results are reported as means ± standard deviation (SD) or means ± standard error (SE).
3. RESULTS

3.1 PHENOLIC COMPOUND CHARACTERIZATION OF ALEATICO BERRIES

The main anthocyanosides detected in Aleatico berry skin were the 3-O-glucosides followed by the 3-O-p-cumaroylglucosides and 3-O-acetylglucosides; malvidin caffeate is present in low amounts. Among the 3-O-p-cumaroylglucosides the most abundant were malvidin and peonidin and, among the 3-O-acetylglucosides, malvidin is the predominant; peonidin, petunidin, delphinidin and cyanidin 3-O-acetylglucosides, and petunidin, delphinidin and cyanidin 3-O-p-cumaroylglucosides were also presents but in lower amounts (Table 3.1). Malvidin is the main Anth in Aleatico berries either as 3-O-glucoside, 3-O-acetylglucoside and 3-O-p-cumaroylglucoside.

Among Flavonols (Flav), quercetin and kaempferol glycosides were detected, with quercetin 3-O-glucuronide, quercetin 3-O-rutinoside, quercetin 3-O-glucoside as the main compounds. Kaempferol 3-O-glucuronide, kaempferol 3-O-rutinoside, and kaempferol 3-O-glucoside were present as minor compounds. Among hydroxycinnamic acids (HCA), caffeoyl-tartaric acid and p-cumaroyl tartaric acid were identified (data not shown).

Total amounts of the three classes of phenolic compounds, HCA, Flav, and Anth, were calculated as the sum of concentrations expressed as mg/g of skin FW of the compounds identified for each class. The time course of HCA, Flav and Anth concentration (mg/g of skin FW) throughout berry development is shown in Figure 3.1A. Anth started to increase at DOY 213 (onset of veraison). Flav increased over the two first sampling dates and then remained somewhat constant. If expressed in terms of content per berry (Figure 3.1B), Flav followed a two-step increase separated by a lag phase; in fact, they increased up to the onset of veraison and then increased again towards the end of the developmental cycle. The rise in Anth biosynthesis appeared to be shifted in time with respect to Flav and the concentration of HCA remained almost unchanged during the investigated period.
### Tab 3.1 Anth profile in Aleatico grapevine during 2008 season.

Values are the mean (± sd) of three replicates for each date and expressed as percentage of total Anth content; values in bold mean higher incidence of the specific category; ±sd = standard deviation, DOY = day of the year.

<table>
<thead>
<tr>
<th>Compound</th>
<th>06 Aug/DOY 219</th>
<th>12 Aug/DOY 225</th>
<th>20 Aug/DOY 233</th>
<th>27 Aug/DOY 240</th>
<th>05 Sept/DOY 249</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delfinidin 3-O-gluc.</td>
<td><strong>5.05 ± 0.1</strong></td>
<td>5.33 ± 0.2</td>
<td><strong>6.21 ± 0.2</strong></td>
<td><strong>5.11 ± 0.4</strong></td>
<td>5.38 ± 0.2</td>
</tr>
<tr>
<td>Cyanidin 3-O-gluc.</td>
<td>3.83 ± 0.2</td>
<td>2.02 ± 0.3</td>
<td>1.53 ± 0.3</td>
<td>1.41 ± 0.4</td>
<td><strong>2.88 ± 0.7</strong></td>
</tr>
<tr>
<td>Petunidin 3-O-gluc.</td>
<td>5.67 ± 0.2</td>
<td>6.99 ± 1.3</td>
<td>6.83 ± 0.7</td>
<td>5.64 ± 0.4</td>
<td><strong>7.13 ± 1.7</strong></td>
</tr>
<tr>
<td>Peonidin 3-O-gluc.</td>
<td>23.39 ± 1.4</td>
<td>12.86 ± 1.5</td>
<td>10.70 ± 0.0</td>
<td>10.09 ± 0.2</td>
<td>14.06 ± 1.4</td>
</tr>
<tr>
<td>Malvidin 3-O-gluc.</td>
<td>39.25 ± 0.2</td>
<td>49.78 ± 3.5</td>
<td>48.32 ± 0.4</td>
<td>54.98 ± 2.3</td>
<td>52.34 ± 2.1</td>
</tr>
<tr>
<td>Anth 3-O-glucosides</td>
<td>77.19 ± 0.2</td>
<td>76.97 ± 0.2</td>
<td>73.60 ± 0.3</td>
<td>77.22 ± 0.1</td>
<td>81.79 ± 0.1</td>
</tr>
<tr>
<td>Delfinidin 3-O-p-cum.</td>
<td>1.07 ± 0.2</td>
<td>1.21 ± 0.0</td>
<td>1.34 ± 0.1</td>
<td>1.09 ± 0.0</td>
<td>1.00 ± 0.0</td>
</tr>
<tr>
<td>Cyanidin 3-O-p-cum.</td>
<td>0.43 ± 0.2</td>
<td>0.38 ± 0.2</td>
<td>0.27 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.25 ± 0.1</td>
</tr>
<tr>
<td>Petunidin 3-O-p-cum.</td>
<td>0.81 ± 0.3</td>
<td>0.88 ± 0.5</td>
<td>0.88 ± 0.1</td>
<td>1.02 ± 0.1</td>
<td>0.61 ± 0.1</td>
</tr>
<tr>
<td>Peonidin 3-O-p-cum.</td>
<td>4.09 ± 0.9</td>
<td>2.15 ± 0.6</td>
<td>2.00 ± 0.3</td>
<td>2.18 ± 0.6</td>
<td>1.72 ± 1.2</td>
</tr>
<tr>
<td>Malvidin 3-O-p-cum.</td>
<td>6.99 ± 0.1</td>
<td>8.80 ± 0.1</td>
<td>9.79 ± 0.2</td>
<td>13.23 ± 0.4</td>
<td>9.27 ± 0.2</td>
</tr>
<tr>
<td>Anth 3-O-p-cumaroy/glucosides</td>
<td>13.40 ± 0.2</td>
<td>13.43 ± 0.3</td>
<td>14.28 ± 0.3</td>
<td>17.78 ± 0.0</td>
<td><strong>12.85 ± 0.1</strong></td>
</tr>
<tr>
<td>Delfinidin 3-O-acetylgluc.</td>
<td>0.51 ± 0.2</td>
<td>0.50 ± 0.2</td>
<td>1.16 ± 0.1</td>
<td>0.24 ± 0.0</td>
<td>0.30 ± 0.1</td>
</tr>
<tr>
<td>Cyanidin 3-O-acetylgluc.</td>
<td>0.18 ± 0.1</td>
<td>0.28 ± 0.2</td>
<td>0.09 ± 0.1</td>
<td>0.00 ± 0.1</td>
<td>0.00 ± 0.1</td>
</tr>
<tr>
<td>Petunidin 3-O-acetylgluc.</td>
<td>0.62 ± 0.4</td>
<td>0.61 ± 0.5</td>
<td>1.06 ± 0.3</td>
<td>0.29 ± 0.3</td>
<td><strong>0.28 ± 0.5</strong></td>
</tr>
<tr>
<td>Peonidin 3-O-acetylgluc.</td>
<td>2.56 ± 0.6</td>
<td>1.50 ± 0.9</td>
<td>1.23 ± 0.8</td>
<td>0.65 ± 1.6</td>
<td>0.79 ± 1.9</td>
</tr>
<tr>
<td>Malvidin 3-O-acetylgluc.</td>
<td><strong>4.85 ± 0.0</strong></td>
<td><strong>5.73 ± 0.0</strong></td>
<td><strong>7.96 ± 0.0</strong></td>
<td><strong>3.37 ± 0.0</strong></td>
<td><strong>3.65 ± 0.0</strong></td>
</tr>
<tr>
<td>Anth 3-O-acetylglucosides</td>
<td>8.72 ± 1.5</td>
<td>8.63 ± 3.3</td>
<td>11.50 ± 0.8</td>
<td>4.55 ± 2.9</td>
<td>5.02 ± 3.3</td>
</tr>
<tr>
<td>Malvidin caffeate</td>
<td>0.68 ± 1.7</td>
<td>0.97 ± 1.3</td>
<td>0.61 ± 0.7</td>
<td>0.44 ± 0.8</td>
<td>0.33 ± 1.3</td>
</tr>
<tr>
<td>tot.%</td>
<td>99.99 ± 0.5</td>
<td>100.00 ± 1.6</td>
<td>99.99 ± 1.2</td>
<td>99.99 ± 2.4</td>
<td>100.00 ± 2.2</td>
</tr>
</tbody>
</table>

05 Sept/DOY 249
Figure 3.1. Time course of the hydroxycinnamic acid (HCA), flavonols (Flav) and anthocyanins (Anth) content determined by HPLC/ DAD analysis in Aleatico berry skin extracts (2008 trial). The arrow indicates the beginning of veraison (10% of berries colouring). Values are the mean (± SD) of three bunch samples (14 half-berries per bunch) for each date and expressed as mg/g of skin fresh weight (A) or as mg per single berry (B). DOY = day of the year.

3.2 MULTIPLEX INDEX CALIBRATION

When comparing the non-destructive ANTH indices with the related Anth contents from the destructive 2008 HPLC analysis, the best correlation was obtained between the compound concentration expressed on a surface basis and the ANTH$_{RG}$ index (Figure 3.2A). Here, only samples from complete veraison to harvest were considered. The
ANTH\textsubscript{RG} index was inversely correlated with the Anth surface concentration. The best fitting of data was obtained with an exponential function:

\[
\text{ANTH}_\text{RG} = 0.27 + 1.253 \cdot \exp(-\text{Anth}/97.94) \quad (5)
\]

giving a coefficient of determination, \(r^2\), of 0.875 (Figure 3.2A). Yet, a linear regression (\(\text{ANTH}_\text{RG} = 1.114 - 0.00371 \cdot \text{Anth}\)) can be appropriate as well (\(r^2 = 0.856\)). In Figure 3.2B, the concentrations of Flav expressed as \(\mu g\) per \(cm^2\) of berry surface are compared with the Multiplex non-destructive FLAV index. In this case, the FLAV index was completely uncorrelated to the Flav berry skin content. Our analysis show that while the ANTH\textsubscript{RG} index can be used to predict the Anth content in grape skin, the FLAV index cannot be considered a good proxy of Flav content, as explained below.
Figure 3.2. Relationship between the ANTHRG (A) and FLAV (B) indices measured in lab by the Multiplex sensor and the surface-based concentration in berry skin of Anth and Flav, respectively, as determined by HPLC.

3.3 COMPARISON OF 2008 AND 2009 SEASONS

The climatic parameters of the 2008 and 2009 seasons in the area where the experiments were carried out were recorded. The global irradiance was higher (25% as average) in 2009 in comparison with 2008. Relative humidity was similar for the two seasons, ranging between 40 and 80%. The air temperature frequently exceeded 26°C in 2008 (Figure
3.3A), while in 2009, this occurred and was concentrated mainly during the second part of the season (Figure 3.3.B). ETo and precipitation for the two experimental periods were comparable, with an average of 125 mm of rain during the pre-veraison phase of both seasons (Figure 3.4). Relative humidity and air temperature recorded by the Tinytag datalogger (located in the vineyard) did not markedly differ from those collected by the meteorological station (data not shown).

The vapour pressure deficit calculated in both years revealed higher values on 2009 compared to 2008 in the earlier months of the season and during ripening, which occurred in August and early September (Figure 3.5 A). The water requirement on 2009, was higher during the initial months of the season, and during the second and third decades of August which was the main period of ripening, if compared to the same period of 2008 (Figure 3.5 B).

The climatic conditions and water supply affected the midday stem water potential (Figure 3.6A), and the gas exchange parameters (Figure 3.6B and C) that reached lower values in 2009 than in 2008, comparing the irrigated vines. In particular, the 2008 photosynthetic activity (Figure 3.6B) and conductance of leaves (Figure 3.6C) increased during veraison and maintained higher values than those detected in 2009 (IR treatment). During 2009, both parameters were decreasing, apart from the partial recovery due to the water supply on DOY 227. These data indicate that the control vines experienced more pronounced stress conditions in 2009 than in the 2008 season.

This may be the reason for the differences in berry development and Anth concentration observed at harvest between the two seasons (Table 3.1). The Anth concentration in 2009 expressed as mg/kg of berries were 8% higher than that of 2008. The difference reached 26% when Anth were expressed as mg/g of skin, because of the marked difference in skin fresh weight. The time course of technological maturity, as indicated by the maturity index (°Brix • pH; Van Rooyen et al. 1984) reported in Figure 3.6A, as well as the evolution of berry fresh weight (Figure 3.7B) and the onset of veraison clearly indicated that a delayed berry ripening process occurred in 2008.

Such evidence was confirmed by the results of the non-destructive in-field measurements using the Mx optical sensor. The time course of the Anth indices derived by the Mx measurements in situ showed that, in both seasons, the ANTHR index increased exponentially starting from veraison (Figure 3.8A). On the other hand, the ANTHRG showed a biphasic behaviour, increasing during the initial accumulation of Anth, reaching
a peak at full veraison (100% of berries colouring) and decreasing thereafter until harvest (Figure 3.8B). The FLAV index decreased with time from veraison to harvest (Figure 3.8C), while the CHL index was linearly decreasing over the whole ripening period (Figure 3.8D). By comparing the time course of the 2008 and 2009 Mx indices, it is evident that berry ripening resulted anticipated in 2009, in agreement with phenological data (onset of veraison) and harvesting dates for the two seasons. The higher values in ANTHR$_{RG}$ recorded at harvest for 2008 with respect to 2009 suggested that, in the latter season, a more pronounced accumulation of these pigments occurred. This was confirmed by the destructive analysis of berry samples as shown in Table 3.2.

![Figure 3.3](image)

**Figure 3.3** Climatic parameters for the 2008 (A) and 2009 (B) seasons. Data were obtained from the Tuscany Regional Agro-meteorological Service.
Figure 3.4. Rainfall and ETo data for the 2008 (A) and 2009 (B) seasons obtained from the Tuscany Regional Agrometeorological Service and calculated according to the Blaney–Criddle’s method (Brouwer and Heibloem 1986).
Figure 3.5 Vapour pressure deficit, VPD (A) and water requirement (B) during 2008 and 2009 seasons. Data refer to the irrigated treatment (IR) and have been collected with a 15-day interval.
Figure 3.6 Changes in midday stem water potential (A), photosynthetic rate (B) and stomatal conductance (C) of Aleatico plants during the 2008 and 2009 seasons. Data refer to the irrigated vine (IR) treatment. Arrows in (A) show the onset of veraison. Water potential values are the average of three replicates. Gas exchange data are the average of 15 leaf samples measured between 9:00 and 10:30 a.m. Error bars represent the standard deviations.
Table 3.2 Aleatico whole berry and berry skin weights, and anthocyanin (Anth) concentration (referred to berry FW and skin FW) at harvest for the 2008 and 2009 seasons for irrigated vines (IR) treatment. Values with different letters are significantly different at $P < 0.05$. Data are the average (±SD) of three replicates. Anth are expressed as weight of malvidin 3-O-glucoside equivalents.

<table>
<thead>
<tr>
<th></th>
<th>Season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Berry weight (g FW)</td>
<td>1.382 ± 0.021$^a$</td>
</tr>
<tr>
<td>Berry skin weight (g FW)</td>
<td>0.253 ± 0.030$^a$</td>
</tr>
<tr>
<td>Anth (mg/g skin FW)</td>
<td>2.962 ± 0.248$^b$</td>
</tr>
<tr>
<td>Anth (mg/kg)</td>
<td>498 ± 3$^b$</td>
</tr>
</tbody>
</table>

Figure 3.7 Time course of the maturity index (A) and mean berry fresh weight (B) of Aleatico berries for the 2008 and 2009 seasons. Data refer to the irrigated vine (IR) treatment. Arrows in (A) show the onset of veraison. Values are the average of three replicates; error bars in (B) represent the standard deviations.
Figure 3.8 Time course of the anthocyanin (A, B), flavonol (C) and chlorophyll (D) indices measured in situ on Aleatico clusters by the Multiplex sensor. Arrows in (B, D) show the onset of veraison. Values are the average (±SD) over 60 and 50 bunches for the 2008 and 2009 season, respectively. See text for definition of ANTHR, ANTHRG, FLAV and CHL indices.

3.4 WATER DEFICIT EFFECTS

3.4.1 2008 SEASON

3.4.1.1 Physiological effects

The water status of plants was monitored by proper physiological indicators. Values of sap flow (Figure 3.8) and mid-day stem water potential (Figure 3.9A) decreased in both treatments until first irrigation was applied. Then, values started differentiating, SF and MD $\Psi_s$ of irrigated plants increasing at intermediate values and then decreasing again until
the next watering event. In the non-irrigated (WS) plants these parameters decreased progressively reaching minimum values at the end of the experiment.

Diurnal values of Pn (Figure 3.9B) and gs (Figure 3.9C) decreased in WS plants throughout the considered period and in IR plants only at the beginning of August in correspondence of the decrease of vegetative activity (data not shown).

The time course of the Anth index (Figure 3.9D) derived by the Mx measurements in situ increased exponentially starting from veraison for both treatments. The Anth accumulation for the WS treatment was significantly higher just at the end of the ripening. This pattern is confirmed by the Anth concentration evaluated spectrophotometrically in lab (Figure 3.10A, B and C) on berries that had similar berry and skin weights for both treatments (Figure 3.10D).

The evolution of total extractable phenolic compounds expressed as mg per berry showed significant difference between treatments only at DOY 233 (Figure 3.11), and this appears to be determined by the difference in phenolic compound concentration present in seeds rather than in skins (Figure 3.12 A and B).

Soluble solids, total acidity and pH values were not significantly affected. At ripening they resulted similar in both treatments (average values: 25°brix, 4.9 g/L total acidity, 3.70 pH).

![Figure 3.8](image)

**Figure 3.8** Seasonal cumulated sap flow (SF) on irrigated (IR) and non-irrigated (WS) plants during 2008 season. Values are the average of two selected plants per treatment. Arrows indicate a rain event (thin line) and water supply (thick lines). Bars represent the standard errors.
Figure 3.9. Time course of midday stem water potential (A), photosynthetic rate (B), stomatal conductance (C) and the ANTH$_{RG}$ index (D) measured in situ by the Multiplex sensor for irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2008 season. IR data are the same presented in Figures 3 and 5B for water potential and gas exchange parameters and ANTH$_{RG}$, respectively. Up arrow in D indicate the onset of veraison; arrows in A indicate a rain event (thin line) and water supply (thick lines). Water potential values are the average of three replicates. Gas exchange data are the average of 15 leaf samples measured between 9:00 and 10:30 a.m. ANTH$_{RG}$ values are the average of 60 bunches. Error bars represent the standard deviations. Values in D with different letters indicate significance at $P < 0.05$ and no letters = not significant.
Figure 3.10. Time course of total anthocyanins concentration measured in lab and expressed as mg/g of skin fresh weight (A), mg/kg of berries (B), mg per berry (C) of irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2008 season. Whole berry and skin weights are also shown in panel (D). Arrows in (C, D) indicate the onset of veraison. Each data is the average of three replicates. Error bars represent the standard deviations; values with different letters indicate significance at $P < 0.05$; no letters = not significant.
Figure 3.11. Time course of total phenolic compounds measured in lab and expressed as mg per berry from irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2008 season. Arrow indicates the onset of veraison. Each point is the average of three replicates. Error bars represent the standard deviations; values with different letters indicate significance at $P < 0.05$; no letters = not significant.
Figure 3.12  Total phenolic compounds concentration in skins (A) and seeds (B) of berry from irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2008 season. Arrow indicates the onset of veraison. Each data is the average of three replicates. Error bars represent the standard deviations; values with different letters indicate significance at $P < 0.05$; no letters = not significant.
3.4.2 2009 SEASON

3.4.2.1 Physiological effects

The vine physiological response to the two irrigation regimes is represented by the temporal evolution of the midday stem water potential (Figure 3.13A), photosynthetic rate (Figure 3.13B) and stomatal conductance (Figure 3.13C). These physiological indicators revealed that the water stress in WS plants was already intense at veraison and increased thereafter. WS conditions induced a decrease of stem water potential to values lower than -1.4 MPa (Figure 3.13A), whereas under IR treatment, these levels were registered only for short periods. Values of Pn (Figure 3.13B) and gs (Figure 3.13C) were markedly different between the two applied water regimes during the whole season.

The ANTHRG index measured in the field was used to compare the effect of vine water status on Anth accumulation in berries during the 2009 season. This index showed that the evolution of ANTHRG was similar for IR and WS vines (Figure 3.13D); however, starting from DOY 230, the index values for stressed plants were significantly ($P < 0.05$) lower than those of plants under IR, indicating a higher accumulation of Anth under water-stress condition. This difference in the Anth content between the two samples was increasing with ripening ($P = 0.015$ at DOY 237 and $P < 0.001$ at DOY 244). The higher content of Anth in bunches of the WS vines as compared with IR samples was confirmed by the destructive analysis (Figure 3.14). The Anth concentration in WS samples started to increase more markedly than in IR berries after DOY 216 (Figure 3.14A, B, C). The difference between the two treatments increased with time and then partially attenuated towards harvest.

Total phenolic compound concentration per berry showed a significant difference between treatments only at harvest where IR samples displayed higher values than those detected in WS (Figure 3.15) and this might be due to the total phenolic compounds content present in the seeds (Figure 3.16B). The trend of total phenolic compound concentration in skin increased reaching a maximum at DOY 230 when the value in WS samples was significantly higher than that detected in IR (Figure 3.16A) and remained constant up to the harvest, differently from that observed in the seeds which decreased during ripening (Figure 3.16B).

The sugar accumulation process resulted more pronounced in WS berries and this was paralleled by higher titratable acidity values (Figure 3.17). At the end of the experiment all
technological parameters were higher in WS than in IR, including the pH values (3.70 in WS vs 3.54 in IR).

The bud fertility recorded in the field, expressed as bunches/shoot, was higher in 2010 than in 2009 and was not significantly affected by treatments, although WS plants showed slightly higher values than those of IR in both years (Figure 3.18A).

Additional observations were also made on bud fertility along the pruning canes sampled in winter 2010. The fertility gradient (Figure 3.18B) determined in laboratory on one node cuttings from positions 3-10, tended to increase from basal nodes to the median positions. The bud fertility was not significantly affected by water stress except at the eighth node level where it was found a decrease.

In 2009 shoot vigour was practically unaffected by treatments as the mean shoot weight was 77.83 gr for WS and 77.21 gr for IR; WS plants had an average number of 4.4 shoots, IR plants of 4.9 shoots.

**Figure 3.13** Time course of midday stem water potential (A), photosynthetic rate (B), stomatal conductance (C) and the ANTHRG index (D) measured *in situ* by the Multiplex sensor for irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2009 season. IR data are the same presented in Figures 3 and 5B for water potential and gas
exchange parameters and $\text{ANTH}_{RG}$, respectively. Arrows in (C, D) indicate the onset of veraison. Water potential values are the average of three replicates. Gas exchange data come from the average of 15 leaf samples measured between 9:00 and 10:30 a.m. $\text{ANTH}_{RG}$ values are the average of 50 bunches. Error bars represent the standard deviations. Arrows in (A) indicate a rain event (thin line) and water supply (thick line). Values with different letters in D indicate significance at $P < 0.05$; ns = not significant.

**Figure 3.14.** Time course of total anthocyanins measured in lab and expressed as mg/g of skin fresh weight (A), mg/kg of berries (B), mg per berry (C) and of berry and skin weight (D) of irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2009 season. Arrows in (C, D) indicate the onset of veraison. Each point represents the average of three replicates. Error bars represent the standard deviations; values with different letters indicate significance at $P < 0.05$; ns = not significant.
Figure 3.15. Time course of total phenolic compounds measured in lab and expressed as mg per berry of irrigated (IR, circles) and non-irrigated (WS, triangles) plants during the 2009 season. Arrow indicates the onset of veraison. Each point is the average of three replicates. Error bars represent the standard errors; values with different letters indicate significance at $P < 0.05$; no letters = not significant.
Figure 3.16. Total phenolic compounds contribution from skins (A) and seeds (B) per berry of irrigated (IR, circles) and non-irrigated (WS, triangles) plants measured in lab during the 2009 season. Arrows indicate the onset of veraison. Each point is the average of three replicates. Error bars represent the standard deviations; values with different letters indicate significance at $P < 0.05$; no letters = not significant.
Fig. 3.17. Technological parameters during 2009 season for irrigated (IR) and non-irrigated (WS) plants (s.s. = soluble solids, t.a. = titratable acidity). Arrow indicates the onset of veraison.
Figure 3.18. Aleatico fertility (expressed as number of bunches per node) in irrigated (IR) and non-irrigated (WS) plants. A) Comparison between 2009 and 2010 seasons; B) fertility gradient along the cane in 2010 determined in laboratory on single node cuttings (number of cluster per node from third to tenth node). Error bars represent the standard errors.

3.4.2.2 Biomolecular results

The research stage at Dr. Lund's laboratory -Wine Research Center of the University of British Columbia (Vancouver, Canada)- started with a preliminary expression analysis of target genes previously selected in that lab as putative drought biomarkers in leaves and recognized to be expressed in fruit tissues, too (Grimplet et al., 2007; Pilati et al., 2007; Castellarin et al., 2007a; Deluc et al., 2007; Zamboni et al., 2010). In addition, other genes known to be affected by water deficit and/or related to berry quality processes were selected, new primers were designed and their expression level evaluated in berry samples collected from IR and WS Aleatico plants in 2009.
Among genes preliminarily selected, P5CR, DHN1a, MXK3, (Fig 3.19A, B, G, respectively) showed different expression patterns during the last stages of berry development. The expression of DHN1a showed a decreasing trend, whereas the opposite was observed for MXK3 and P5CR. For all these three genes, a putatively higher expression in the WS thesis was detected in at least two sampling dates. Considering the five remaining genes preliminarily analyzed, NCED2 and DREB expression showed a decreasing trend in IR samples with a reduced specific transcript accumulation in two WS samples (Fig. 3.19D and F). No clear expression trends or no marked differences between IR and WS samples were detected considering the remaining selected genes.

The expression of FLS1 and UFGT genes (Fig. 3.20 A and B), both involved in the flavonoid pathway, resulted to be, in general, higher in WS treatment than in IR. In particular, FLS1 was markedly up-regulated by water stress in the last two sampling dates up to the harvest. UFGT expression displayed highest expression in both treatments on 18 August (DOY 230) and then decreased. WS condition induced an up-regulation in correspondence of the highest expression level. DWF1, MSA and PIP2;1 genes (Fig. 3.20 C, D and E) displayed decreasing expression trends during the last stages of berry development but no marked difference was observed between IR and WS samples.
Figure 3.19. Expression analysis of target genes (previously selected as putative drought biomarkers in leaves) in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season. Pyrroline-5-carboxylate reductase (P5CR) (A), Dehydrin 1a (DHN1a) (B), 9-cis-epoxycarotenoid dioxygenase 1 (NCED1) (C), 9-cis-epoxycarotenoid dioxygenase 2 (NCED2) (D), Zeaxanthin epoxidase (ZEP) (E), Dehydration responsive element-binding protein (DREB) (F), ABC transporter (MXK3) (G), Proline (PrDh) (H). Each point represents one biological replicate.
Figure 3.20. Expression analysis of selected genes (known to be affected by water deficit and/or related to berry quality processes in grapes) in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season. Flavonol synthase (FLS1) in A, Flavonoid 3-O-glucosyltransferase (UFGT) in B, Dwarf1 (DWF1) in C, Abscisic acid-, stress-, and ripening-induced (ASR) gene (MSA) in D, Aquaporin (PIP2;1) in E. Each point represents one biological replicate.

Based on the above described preliminary data on gene expression, specific genes have been selected and their expression analyzed more in detail in WS and IR berry samples from veraison stage till complete ripening. Considering the flavonoid pathway, the expression of FLS1 increased constantly in WS up to the end of the experiment,
differently from IR samples where specific transcript reached the highest accumulation on 25 August (DOY 237) and then decreased. Hence, at harvest FLS1 resulted markedly up-regulated in WS samples showing a significant difference with control berries. (Figure 3.21). The trend of UFGT gene expression (Fig. 3.22 A) shows that the highest expression levels occur when Anthocyanin content reaches the plateau (Fig. 3.22 B), with a significant difference between IR and WS samples 2 weeks before harvest. Delphinidin-3-glucoside resulted to be more abundant than Cyanidin-3-glucoside and appeared to be more influenced by WS (Fig. 3.22 C, D).

F3’H and F3’5’H are key-genes in the flavonoid pathway determining respectively the biosynthesis of di-substituted and three-substituted Anth. The expression of F3’5’H (Fig. 3.23 A) was higher than that of F3’H (Fig. 3.23 B), promoting, consequently, a higher biosynthesis of 3’4’5’-OH than of 3’4’-OH Anth (Fig.3.23 C, D). The highest expression of F3’H and F3’5’H in WS treatment corresponded to the time of the highest accumulation of Anth (Fig. 3.22B).

AOMT is a gene showing a preference for 3’5’ methylation, while FAOMT, identified at the Wine research Centre - Univ. Bristish Columbia, encodes for a methyltransferase that resulted to be highly specific for delphinidin-3-glucoside and consequently for the biosynthesis of malvidin and petunidin (Lucker, 2010). AOMT showed the highest expression at the onset of ripening whereas FAOMT expression data indicated the presence of one week shift to reach the highest values in transcript accumulation (Fig. 3.24 A). No significant effects of the two water regimes were observed on the expression of these genes responsible for Anth methoxylation, but the methoxylated Anth content of WS treatment was significantly higher than that observed in IR treatment. Considering methylated Anth, Malvidin-3-glucoside appeared to be the most abundant in Aleatico berries (Fig. 3.24 B), followed by peonidin-3-glucoside (Fig. 3.24 C) and petunidin-3-glucoside (Fig. 3.24 D).
Figure 3.21. Expression of FLS1 gene in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season. Bars represent ±SE, values with different letters indicate significant difference at $P < 0.05$. No letters = not significant.
Figure 3.22. Expression of UFGT gene in berries collected from irrigated (IR, circles) and non-irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season (A), time course evolution of total Anth (B), delfinidin-3-glucoside (C) and cyanidin-3-glucoside (D) concentration in berry skin. Total Anth (B) data are the same presented in Figure 3.14A for the last fruit developmental stages. Bars represent ±SE (A), ±SD (B, C, D); values with different letters indicate significant difference at $P < 0.05$. No letters = not significant.
Figure 3.23. Expression of F3’5’H gene (A) and F3’H gene (B) in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season, and time course of 3’4’5’-OH anth (C) and 3’4’-OH anth (D) concentration in berry skin. Bars represent ±SE (A, B), ±SD (C, D); values with different letters indicate significant difference at $P < 0.05$. No letters = not significant
Figure 3.24. Expression of FAOMT and AOMT genes in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season (A), and time course of malvidin-3-monoglucoside (B), peonidin-3-monoglucoside (C) and petunidin-3-monoglucoside (D) concentration in berry skin. Bars represent ±SE (A), ±SD (B, C, D); values with different letters indicate significant difference at $P < 0.05$. no letters = not significant

DHN1a showed significant differences between treatments throughout the considered sampling period, being WS samples characterized, in general, by higher transcript accumulations (Fig 3.25 A). The ABC transporter MXK3 did not show significant differences between WS and IR samples, but despite a high biological variability, it resulted expressed throughout the last berry developmental stages with higher transcript accumulation trend in WS samples (Fig 3.25 B).
Figure 3.25. Expression of Dehydrin 1a (A) and MXK3 (B) genes in berries collected from irrigated (IR, circles) and non irrigated (WS, triangles) Aleatico plants during the last developmental stages in 2009 season. Bars represent ±SE, values with different letters indicate significant difference at $P < 0.05$. No letters = not significant.
4. DISCUSSION

For several years the Anth profile has been used for a taxonomic purpose and for the varietal traceability of high quality red wines (Mattivi et al., 1990; Castia et al., 1992; Valenti et al., 1993; Bucelli et al., 1998; Gomez-Ariza et al., 2006; Baer et al., 2008; Kallithraka et al., 2009). This is very important and relevant also for Aléatico grapevine that recently received the ‘Denomination of Controlled and Guaranteed Origin (DOCG)’ that is the top ranking in the Italian wine ranking system. As reported in a previous study (Boselli et al., 2003), the most abundant anthocyanosides detected in Aleatico berry skin are the monoglucoside Anth due mainly to the malvidin 3-O-glucosides followed by petunidin 3-O-glucosides. These compounds can increase the wine quality more than the others Anth because they have a higher red and blue colour intensity. The high percentage contribution of malvidin is common in several varieties as Cabernet Sauvignon (González-Neves et al., 2006), Cabernet Franc, Carmenère, Grenache, Malbec, Sagrantino, Teroldego (Storchi et al., 2008), Nerello Mascalese and Corinto Nero (Nicolosi et al., 2007), Syrah (Huguney et al., 2009), but the entire profile can differ very much among varieties. The highest similarity is found with Cabernet Sauvignon, Grenache and Syrah (Storchi et al., 2008) that show malvidin as the main pigment followed by peonidin, delphinidin and petunidin in similar quantity, and cyanidin only in small amount as in Aleatico Anth profile. The 3-O-acetylglucosides are scarce whereas the 3-O-p-cumaroylglucosides are well represented. Anthocyanin accumulation (in particular if referred to mg/berry) follows a bi-phasic pattern: the initial high rates detected immediately after the inception of veraison and lasting about 20-25 days are followed by slower rates of accumulation. In 2008, the highest Anth concentration was reached 10-15 days before harvest, and is considered an information of great importance for the management of field operations (harvesting, in particular) and vinification as it concerns the timing of phenol maturation. Water deficit was shown to accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis, especially the anthocyanin pathway as measured by the increase in total anthocyanins (Castellarin et al., 2007a). Our results on Aleatico grapes are consistent with this study; in 2009 trial water deficit increased the accumulation of the five major anthocyanins relative to berries from IR vines (Figs 3.22 and 3.24). In fact, water stress conditions in general induced a magnification of these accumulating processes.
resulting in a significant increase in total anthocyanin concentration at harvest. This appears due to an increased expression of UFGT in correspondence of 2 weeks before harvest (Figure 3.22 A and B): this stage can be considered the period of the highest rate for Anth biosynthesis in Aleatico grapes with our experimental conditions (corresponding also to the maximum of the solar radiation).

Castellarin et al., (2007a) showed a constant effect of UFGT up-regulation by water stress throughout the last berry developmental stage: in our experiment this effect was less evident, probably because, in 2009, the IR treatment reached a moderate water stress before the water supply. Similar results and pattern of UFGT expression, induced by WS, were reported by Deluc et al. (2009) in a transcriptomic approach on Cabernet Sauvignon berries. Considering different Anth categories glycosylated by UFGT, delphinidin-3-glucoside showed a more pronounced increment induced by WS than cyanidin-3-glucoside (Figure 3.22 C and D), thus confirming the hypothesis that water stress affects Anth composition more than total concentration (Bindon et al., 2010; Chaves et al., 2010).

The relative proportion of the two types of Anth (cyanidin/delphinidin) is largely under genetic control and typical for each cultivar (Castellarin et al., 2006). F3’H and F3’5’H are key genes in the biosynthesis of such types of Anth. In grapes, a higher expression of F3’5’H than F3’H has been reported by Mattivi et al., (2006) and Castellarin et al., (2007b) and our results confirm this condition (Figure 3.23 A and B): results show a higher concentration of tri-substituted than that of di-substituted Anth (Figure 3.23 C and D).

Castellarin et al., (2007b) reported that in Merlot the expression pattern of F3’5’H gene shows a decreasing trend from veraison to harvest, whereas F3’H has a less variability. Our results confirm these findings and also confirm that WS induces a marked increment of F3’5’H expression in correspondence of the highest rate of anth biosynthesis, as concluded in the same paper by Castellarin et al., (2007b). WS promoted the conversion of hydroxylated anthocyanins (cyanidin and delphinidin) into all their methoxylated derivatives (peonidin, petunidin and malvidin) (Fig. 3.24). This behaviour is slightly different from that observed in Merlot (Castellarin et al., 2007b) where petunidin (methoxylated at 3’ position) did not increase, differently from malvidine and peonidin, under drought conditions. The expression pattern of the two studied OMT genes (FAOMT and AOMT , Fig. 3.24A) is consistent with these observations. AOMT was characterised by Hugueney et al., (2009) in Syrah and Nebbiolo cv. that have a different Anth profile.
(malvidin dominant and peonidin dominant, respectively) and it showed a preference for 3’5’ methoxylolation. Aleatico resulted to have an Anth profile (Figure 3.24B) more similar to Syrah than Nebbiolo.

FAOMT was identified at the Wine research Centre and it codes for a methyltransferase that resulted to be highly specific for delphinidin-3-glucoside and consequently for the biosynthesis of malvidin and petunidin (Lücker et al., 2010). In several *V. vinifera* cultivar, OMT genes have the maximum expression at the onset of the ripening (Castellarin and Di Gaspero, 2007; Lund et al., 2008; Lücker et al., 2009) as our FAOMT results on Aleatico cv. confirmed (Figure 3.24A).

The Anth concentration in WS samples during 2009 season started to increase more markedly than in IR berries after around two weeks from véraison. The divergence increased with time and partially attenuated towards harvest. Since both total berry and skin weights in stressed plants were significantly lower than in watered plants, it is clear that a concentration (dilution) effect was present in the Anth values referred to g of skin or kg of berries. However, when Anth were expressed on a per berry basis, there was still a marked difference in the Anth content in favour of the WS samples, by up to 36%. On the other hand, the mean increase of Anth concentration, expressed in mg/kg of berries, was about 57% from DOY 223 to harvest in the WS samples, which cannot be accounted for the 21% mean reduction of berry mass induced by water deficit. Analogously, the 45% average higher Anth content, expressed as mg per skin fresh weight, in WS versus IR treatments cannot be explained by the 16% average difference in skin berry weight.

Plants responded to the higher water deficit applied during 2009 season in a similar way to those observed when water deficit was imposed to Cabernet Sauvignon vines (Castellarin et al., 2007a). The attenuation in the difference of Anth concentration between the two treatments, observed at the end of the trial, can be explained in terms of evolution of the ripening process. Maturity was accelerated by WS, as reported in other works (Castellarin et al., 2007a); therefore, WS berries reached the highest Anth content earlier than IR samples. Furthermore, the marked stress conditions imposed (MD Ψ < -1.8 MPa) could result in an increased structural complexity of phenolic compounds in the berry (i.e. higher degree of polymerization), leading to a lower Anth extractability (Sivilotti et al., 2005).

Concerning other phenol compound categories, in more than 60 red grapes varieties, the main flavonol appeared to be quercetin (mean = 43.99%), followed by myricetin (36.81%),
kaempferol (6.43%), laricitrin (5.65%), isorhamnetin (3.89%), and syringetin (3.22%) (Mattivi et al., 2006). The characterization of Aleatico has been implemented through the description and quantification of the different flavonol categories. These analyses showed that this variety is characterized by the presence of quercetin 3-O-glucuronide, quercetin 3-O-rutinoside, and quercetin 3-O-glucoside as the main compounds. The rise in Flav biosynthesis appeared to be shifted in time with respect to Anth: this could be due to the competition for the common substrate, dihydroflavonols, between flavonol synthase and dihydroflavonol 4-reductase (Davies et al. 2003, Jaakola et al., 2004). FLS1 is the key step in the flavonols biosynthesis. The expression increased at the onset of the ripening but its trend during ripening has a varietal influence: in Chardonnay it increases very fast (steadily towards harvest) at veraison stage, in Syrah increases slowly with a noticeable increase three weeks post-veraison (Downey et al., 2003), while in Merlot slightly increases from veraison to harvest as reported in Castellarin et al., (2007b). Similar expression trends have been observed in Aleatico (Fig. 3.21) where WS conditions resulted in an increased transcript accumulation at the end of the considered period (similarly to what was observed in Chardonnay and Merlot), whereas in control grapes (IR) a decrease was observed in correspondence of harvest. This expression pattern mirrors that of Flav concentration measured in 2008 season for IR treatment.

These molecular data, coupled with physiological indicators, are extremely useful and important for a better characterization of Aleatico responses to water deficit conditions. When the response of this variety was compared to that of other varieties, it was found that Aleatico had a similar behaviour to Cabernet Sauvignon while the sensitivity to water stress appeared to be higher in Alicante, Petit Verdot, Syrah, and Sangiovese (Scalabrelli et al., 2011).

During 2008 season the water stress in non-irrigated plants resulted to be only moderate and occurring primarily in correspondence of the last berry developmental stage. This may explain why differences on shoot growth, lateral shoot growth, and leaf area have not been observed. As a normal event occurring in most vineyards of Tuscany (Scalabrelli, personal communications), it is likely that growth stopped at time of veraison. In 2009 the water stress in non-irrigated plants resulted to be more intense and starting from pre-veraison. WS conditions determined a decrease of stem water potential which dropped to values lower than -1.4 MPa (Figure 3.13A), whereas in IR treatment these levels were registered only for short periods. Diurnal values of Pn and gs appeared markedly affected by the
applied water regimes during both seasons confirming a higher sensitivity of these physiological indicators to water deficit (Flexas et al., 2002; Maroco et al., 2002; Cuevas et al., 2006). In 2008 they decreased in non-irrigated plants throughout the experiment and in irrigated plants only at the beginning of August in correspondence to the end of vegetative activity (data not shown); in 2009 significant differences of \( P_n \) and \( g_s \) between the two treatments throughout the season were observed.

The water deficit of both seasons did not affect the plant fertility as reported by other authors (Alaa Al-Joumaly, 2003) although in 2010 a decrease in bud fertility was observed at the eighth node level (Figure 3.18 B), and this could be due to the stress conditions of the previous year.

As a consequence of the moderate water deficit occurred in the 2008 season, soluble solids, total acidity and pH values of berries were not significantly affected, whereas in the 2009 season WS conditions influenced the technological parameters resulting in a higher concentration of soluble solids and titratable acidity at harvest (Figure 3.17).

As observed in our 2008 trial, other authors demonstrated that no changes in titratable acidity (TA) are present in the must from moderately water-stressed vines (Matthews and Anderson, 1989; Esteban et al., 1999), while some papers reported a reduction of TA due to deficit irrigation (Sheltie, 2006; Santos et al., 2007); Malate/tartarate ratio is in general lower due to malate breakdown in vines with low water status (Matthews and Anderson, 1989). However, Matthew et al. (1988) and Ferreyra et al. (2004) found an increase of this parameter under water stress conditions. Consequently, the increase of titratable acidity on Aleatico WS plants can be due to a concentration effect confirmed by the significant decrease in berry weight (Figure 3.14D). The effects of water deficit on sugar content of grapevine berries are variety-dependent (Gaudillère et al., 2002). For example, no significant changes were observed in Merlot sugar content under water deficits, while a significant increase in sugar content was observed in Cabernet Sauvignon berries (Castellarin et al. 2007a, b). Similarly, Deluc et al. (2009) observed an increase in berry sugar content under water deficits in Cabernet Sauvignon but not in Chardonnay.

Water stress could be considered a useful instrument to improve quality of dessert Aleatico wines. The drought conditions imposed during the 2009 season favoured the decrease of phenolic compounds from seeds at the end of the trial when a strong water stress occurred (MD \( \Psi_s < -1.8 \) MPa). As reported in literature (Scalabrelli et al., 2004) Aleatico cv. is
characterized by a high quantity of tannins in seeds compared to those extracted from the skins. Moreover seed astringency and bitter taste are very frequent during ripening (Scalabrelli, personal communication) so phenolic maturity could be critical to obtain a dessert wine from Aleatico. The typical wine produced by post-harvest dehydration of Aleatico berries is sweet, hence an adequate amount of sugars can balance the tannic component making the astringency less pronounced. The water stress could have a positive effect on accelerating the degeneration/evolution of tannins in seeds (Roby et al., 2009), or promoting their polymerization as reported by Sivilotti et al., (2005) although more detailed studies on phenolic compounds should be carried out to corroborate these hypotheses.

In order to better manage water deficit conditions and understand plant reaction to this (and other) kind of stress, the elucidation of metabolic processes, particularly those affected by drought, and the identification of bio-markers can be extremely useful. Among the genes studied for this purpose, DHN1a resulted one of the most sensitive to water stress in shoot tips (Cramer et al., 2007) DHN1a encodes for proteins that accumulate in vegetative tissues to protect plants from different stresses (Xiao, 2006). These proteins are regulated by ABA and their role and mechanism of stress adaptation is not clear yet. DHN1a could be involved also in the berry ripening regulation (Pilati et al., 2007). DHN1a is considered a sensitive drought biomarker in shoot tips (Cramer, 2007) and leaves (Xiao, 2006) where the expression is reported to be increased during the vegetative growth, whereas on our trials in berries the expression decreased during the last developmental stages with a significant up regulation caused by WS. The effects of drought on DHN1a was studied only post-harvest where the expression increases when berries undergo partial dehydration (Zamboni et al., 2008). Considering the significant differences observed between treatments starting from the post-veraison stage (Figure 3.25B), this gene appears to be a good candidate as a biomarker for the detection of stress conditions in the berries.

MXK3 is an ABC transporter (Grimplet et al., 2007); ABC is a generic protein family associated with membranes having function of ions, water, sugars and also anthocyanins transport (Deluc et al., 2007). If marked increases in the gene expression had been detected in shoot tips after 16 days of water stress (Cramer et al., 2007), no significant difference in terms of transcript accumulation had been observed in berries (Figure 3.25C) even though an increasing trend was observed in the post-veraison stage. In addition to specific genes of
the phenylpropanoid biosynthetic pathways, our results indicate that other genes can be used to describe and monitor water stress conditions in the grape berries. Together with the identification of biomarkers, the development and practical application of portable and non-destructive instruments to detect compositional changes of grape berries is strategic for a modern viticulture. In this context, the Multiplex®2 sensor resulted to be a very useful instrument as preliminarily described by Agati et al., (2009).

The time was anticipated in 2009 (Figure 3.7), in agreement with phenological data (onset of veraison) and harvesting dates for the two seasons, and that the vines experienced more pronounced general stress conditions in 2009 than in the 2008 season.

With regards to Mx indices trends, the biphasic behaviour of ANTHRG can be explained by considering the difference in the absorption properties of Anth at the two excitation wavelengths in the green (516 nm) and in the red (637 nm). With increasing Anth concentration, i.e. with increasing DOY, both FRFG and FRFR signals will decrease, because of an increased screening effect on Chl, but the latter with a lower rate because of the lower absorptivity of Anth in the red. Applying the logarithmic function of Eqn 1 to calculate the time course of ANTHRG, according to the Beer–Lambert’s law, results in the difference of two components that increase exponentially with time but with different rate constants (see also Ben Ghozlen et al., 2010a). Consequently, an initial increase in the index is followed by a decrease. Once the maximum has been reached, ANTHRG is expected to be inversely correlated to the Anth content.

The sharp decrease of FLAV starting at DOY 213 (Figure 3.7C) is in contrast with the time course of Flav resulting from the HPLC analysis (Figure 3.1). This was not unexpected because of the absence of correlation between the Mx FLAV index and the Flav concentration (Figure 3.2B). It is because of the accumulation of Anth, which largely affects the FRFR signal, while the FRFUV was changed slightly. Consequently, according to their definition (Eqn 1 and 2), the FLAV index becomes similar to the inverse of the ANTHR index (FLAV ∝-ANTHR), as seen in Figure 3.7 (C and A). The same decrease with time in FLAV was previously observed in cvs. Pinot Noir and Pinot Meunier, while an increase in FLAV with time was found for the Anth-free cv. Chardonnay (Ben Ghozlen et al. 2010b).

Our analysis shows that while the ANTHRG index can be used to predict the Anth content in grape skin, the FLAV index cannot be considered a good proxy of Flav content.
The ANTH$_{RG}$ index measured in the field was used to compare the effect of the different vine water status of WS and IR treatments on Anth accumulation in berries during the 2008 and 2009 seasons. The accumulation of Anth determined by the ANTH$_{RG}$ index increased exponentially for both treatments, during both years (Figure 3.9D and Figure 3.13D) starting from veraison that corresponds to the initial phase of Anth biosynthesis (Figure 3.10 A, B, C and Figure 3.14 A, B, C).

As reported in literature, the Mx sensor has real potential for precision viticulture. Still, for very mature grapes an increase in green source power would be welcome (Cerovic et al., 2008).
5. CONCLUSIONS

The variety ‘Aleatico’ appears to be tolerant to water stress conditions and this information could be useful also for setting up targeted post-harvest dehydration strategies to produce dessert wines. However, the effects of the same and other (more intense) water stress must be studied in different seasons and on vines of different age.

The Anth profile was defined, identifying Malvidin as the predominant compound. The Aleatico anthocianic profile was defined showing high percentage of glycosylated Anth, with Malvidin and Petunidin as the main compounds; the 3-O-acetylglucosides are scarce whereas the 3-O-p-cumaroylglucosides are well represented. Among Flav, quercetin 3-O-glucuronide, quercetin 3-O-rutinoside and quercetin 3-O-glucoside are the most represented. Flav biosynthesis appears related to the plant capacity to respond to climatic stress (Remorini et al, 2008) and the remarkable amount of Flav found in the Aleatico variety suggests the possibility that this variety is able to respond better to environmental stress.

The water deficit applied had positive effects on quantity and quality of Anth and our results confirm previous observations that, in different varieties, WS not only increases Anth accumulation in berries as a result of a reduced berry size and a stimulation of Anth biosynthesis, but also has an influence on the Anth composition.

Considering biomolecular results, and as a future prospective, thorough studies about putative drought biomarkers in berries should be performed considering our results on Dehydrin 1a.

More in-depth and wider studies of varieties in response to environmental stresses are instrumental to the understanding of grapevine adaptation to more arid climates. Further knowledge of berry development, including the timing of accumulation of various berry components, and their dependence on water availability, is critical for an optimal choice of irrigation strategy. In this context, an additional interesting result is represented by the validation of a new important innovative technology for viticulture, the Mx sensor, a
portable non destructive instrument to measure Anth content directly in the vine, quickly and with a high level of precision.

The sensor evaluation of the large spatial and temporal heterogeneity in Anth accumulation can be useful as a support parameter in the harvest date decision or for vineyard zoning of phenolic maturity. It represents a rapid and non-invasive tool to compare the effect of different vineyard managements, soil and environmental factors on grape phenolic maturity. Using an appropriate calibration curve for the Mx indices, obtained by comparison between non-destructive measurements and destructive Anth analyses on the largest range of Anth concentrations, the Anth content, expressed as g/kg of grape, in different vineyard blocks can be predicted. The sensor cannot provide, at the moment, indices of Flav content in red winegrape because of the covering effect of Anth. However, it can be applied to white grapes where the FLAV index dynamics may represent a new useful parameter to follow maturity (Lenk et al., 2007). The capacity to retrieve non invasively at the same time, multiple parameters is an attractive advantage of the Mx sensor. Beside the information on the Anth content, the degradation of Chl can be followed simultaneously on the same samples. The Chl fluorescence decrease during grape berry ripening was proved to be inversely correlated to sugar concentration (Kolb et al., 2006, Lenk et al., 2007). Therefore, the Mx can also supply a rapid assessment of sugar content. Indeed, a first good correlation between the CHL Mx index and total soluble sugar in the cultivars Pinot Noir, Pinot Meunier and Chardonnay has been found (Ben Ghozlen et al., 2010a). Optical non-destructive sensors, such as that used in the present work, appear to be well suited as tools for integration in grapevine management practices facing the influence of global climate change on fruit quality and production (Keller, 2010). The cv. Aleatico proved to be tolerant to WS conditions, as preliminarily observed in potted plants (unpublished data), allowing its cultivation in territories where irrigation is not available or saving water when the irrigation must be used in severe dry conditions.
6. REFERENCES


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