Characterization of grapevine (Vitis vinifera L.) berry cuticle for its role in water stress response

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Abstract

This study aims at characterization of the grape berry cuticle (*Vitis vinifera* L.) subjected to two irrigation regimes: sustained deficit irrigation (SDI) and regulated deficit irrigation (RDI), belonging from two different sides of vine canopy (east and west). The analysis of the cuticular water permeance showed no impact of different treatments in the transpiration of intact berries. However, in de-waxed berries, permeance increased by 4-fold and RDIW showed significantly higher permeance suggesting the role of cuticular waxes in the control of berry water loss. TEM analysis indicates the effect of water deficit intensity and temperature on cuticle architecture by the increase of wax and pectin deposition. ATR-FTIR of berry cuticle, skin and cutin revealed the presence of methylene, ester, aromatic and polysaccharides groups. The mRNA expression pattern of seven cuticle related genes was analysed. Deficit irrigation impacts the expression of only four genes. The up-regulation of *VviLTP3* in RDIW suggests the synergetic effect of water and heat stresses in the stimulation of wax deposition at the berry surface to deal with the high transpiration demand of stressed berries. The up-regulation of *VviCYP716A15* and *VviCYP716A17*, involved in triterpenoid biosynthesis, in the most stressed berries pinpoint the possible role of triterpenoids in abiotic stress responses. Results herein presented are a starting point for the understanding of the biology of grape berry fruit cuticle and could be vital for future viticultural managements.

**Keywords:** Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR); heat stress; regulated deficit irrigation (RDI); sustained deficit irrigation (SDI); Transmission electron microscopy (TEM).
Resumo


**Palavras-chave:** Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR); stress do calor; rega deficitária regulada (RDI); rega deficitária sustentável (SDI); microscopia electrónica de transmissão (TEM).
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CER9</td>
<td>ECERIFERUM 9</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP716A15</td>
<td>Cytochrome P450 monooxygenases/hydroxylases, Homolog A15</td>
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<tr>
<td>CYP716A17</td>
<td>Cytochrome P450 monooxygenases/hydroxylases, Homolog A17</td>
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<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>ETc</td>
<td>Crop evapo-transpiration</td>
</tr>
<tr>
<td>FAE</td>
<td>Fatty acid elongase</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>FM</td>
<td>Full maturation</td>
</tr>
<tr>
<td>FW</td>
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<tr>
<td>GDSL</td>
<td>Glycine–aspartic acid–serine–leucine (GDSL) motif lipase/hydrolase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>KCS1</td>
<td>β-ketoacyl- CoA synthase, isoform 1</td>
</tr>
<tr>
<td>KCS6</td>
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<td>LTP3</td>
<td>Lipid transfer proteins 3</td>
</tr>
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<tr>
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<td>Messenger RNA</td>
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<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PM</td>
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<tr>
<td>PVPP</td>
<td>Polyvinyl polypyrrolidone</td>
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<td>qRT-PCR</td>
<td>Quantitative Real-time Polymerase Chain Reaction</td>
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<td>RDI</td>
<td>Regulated deficit irrigation</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>RNA</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase Polymerase Chain Reaction</td>
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<td>SDI</td>
<td>Sustained deficit irrigation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>Ssp</td>
<td>Subspecies</td>
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<tr>
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<td>Titrable Acidity</td>
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<tr>
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</tr>
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<td>Total soluble solids</td>
</tr>
<tr>
<td>V</td>
<td>Véraison</td>
</tr>
<tr>
<td>VLCFA</td>
<td>Very long chain fatty acid</td>
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1. Background and Introduction

The plants colonized the land around 450 million years ago, and have had to face the daunting task of acclimatizing to the new environmental conditions such as desiccation, extreme temperature conditions and exposure to UV radiations (Leliaert et al., 2011; Waters, 2003). This evolution of plants from mainly aquatic to terrestrial life style have necessitated the development of some physiological mechanism in plants such as development of complex cell walls for biomechanical support which are typical of modern land plants (Sørensen et al., 2011). However, among the several modification that happened in plants to adapt to the new terrestrial environment, the most critical adaptive trait would have been to develop the ability to retain water in otherwise highly desiccating external environment, considering the need and importance of water in the physiology of plant growth and development. Therefore, the capacity to synthesize, transport, deposit and maintain a hydrophobic layer or cuticle over the surface of their aerial organs was inarguably one of the most significant innovation occurred in the history of plant evolution. This idea is well supported by the both fossil evidence and ubiquity of cuticles is almost all plant from embryophytes to angiosperms (Budke et al., 2012).

Grapevine is one of the most important fruit species worldwide and ranks second in fruit production (FAO, 2007). More than 50 species of grapes are recognized but most commonly used for wine production and table grapes is Vitis vinifera L. Unlike most of the modern food, the wine’s attractions relies not on strong consistent flavors but upon a subtle array of shifting sensations which makes it charm difficult to define and where the role of grape berry skin comes into play since it contributes almost 90% to the wine quality (Bisson et al., 2002). Grape berry with its complex developmental process involving several metabolic changes and the highly active berry skin metabolism determines the final characteristics of the berry/wine. The skin or exocarp is formed of single layer of epidermal cells which are lined by cuticle. Cuticle serves as first barrier against the abiotic and biotic stresses (Domínguez et al., 2011a) and its main function in fruits are to minimize the water loss and limit the loss of substances from the internal tissues. In fact, several reports indicate the putative role of cuticle disruption in the berry weight loss (Rogiers et al., 2004) known as berry shrivel, observed at the last stages of berry maturation in some varieties. Apart from its protective role, cuticle wax composition deeply influences the efficacy of the fermentative process during wine production processes (Casado and Heredia, 2001; Orbán et al., 2009). In addition, changes in the cuticle composition and structure are being considered to be an integral part of the fruit ripening process (Saladié et al., 2007). Although grapevine is well adapted to the Mediterranean climate, the present prevailing environmental conditions and the predicted forecast due to the shift in the global environmental patterns, the effect on berry development and its quality had come into light. The response of berries under environmental stresses is a complex process and is well documented (Chaves et al., 2010; Schultz, 2003; Zarrouk et al., 2012). Such global environmental shift brought several regions around the world to the threshold of temperature and rainfall for optimum grapevine growth forcing the viticulturist to have increased dependence on the irrigation for grapevine cultivation, namely at the Mediterranean region (Chaves et al., 2010). However, the right balance between vegetative and reproductive growth is of paramount significance to the berry quality, and one way to address this key issue is the use of deficit irrigation.
practices (Chaves et al., 2010). Use of regulated deficit irrigation is one of the key strategies. However, sustained deficit irrigation is also used in vineyards in order to enhance the wine and berry qualities (McCarthy et al., 2002).

In spite of the importance of the cuticle under this scenario of global environmental shift, little is known about its function in grapevine. The majority of studies carried out on role of cuticle in plant has been limited to vegetative tissues, though in recent years there has been growing interest in biological role of cuticle in the fruits such as cherry and most intensely tomato fruits (Alkio et al., 2012; Isaacson et al., 2009; Leide et al., 2007; Shi et al., 2011; Yeats and Rose, 2013). However the tomato is a climacteric fruit where ethylene phytohormone plays a pivotal role in the ripening and fruit development in contrast to non-climacteric fruit such as grape berries where abscisic acid (ABA) phytohormone is reported to be significantly controlling the fruit ripening (Wheeler et al., 2009). Recent findings suggest the possible role of ABA in the biosynthesis of epicuticular waxes and cuticular water permeability, as well as in the development and composition of fruits waxy layer thereby enhancing the resistance to pathogens and pests (Curver et al., 2010; Romero et al., 2012). Several transcriptomic and proteomic studies have been reported on the effect of water deficit conditions on berry ripening and quality (Castellarin et al., 2007a; Deluc et al., 2009; Grimplet et al., 2009). Despite significant work being done by the grapevine research community in the last few years (Castellarin et al., 2007a,b; Deluc et al., 2009), there are still some knowledge gaps that exist pertaining to the role, cuticle plays in grape berry and the effect of water deficit on grape berry metabolism and berry cuticle in particular. Some insight into molecular genetics of cuticle biosynthesis had been reported from Arabidopsis (Kunst and Samuel, 2003; Samuel et al., 2008) and more recently from tomato fruit cuticle (Matas et al., 2011). To address some of these questions, the present “master thesis” was carried out for the morphological, metabolomic and molecular characterization of the cuticle under water deficit.
2. Review of Literature

2.1. Grapevine (Vitis vinifera L.)

Grapevine (Vitis spp) is one of the most cultivated fruit crops worldwide covering around 7.8 million hectares of land in 2011 and producing 67.5 million tons of berries (http://www.oiv.int/). More than 68% of the grape berries harvested are primarily used for wine production and the rest is used to provide the fresh table grapes (30%), raisins (2%) and minor products, such as jelly, grape juice, vinegar, ethanol, grape seed oil, tartaric acid, and fertilizers (Fasoli et al., 2012). Grape berries are known for their high content of antioxidants in form of polyphenols which have important health benefits and are valued in food, pharmaceutical and cosmetic industries. Each variety of grapevine is distinct in its own way and characterization of one variety will not be enough to give complete information about the entire species. Therefore it becomes essential to characterize each variety of grapevine separately in order to determine the overall characteristics of each of them. In addition, the same variety growing in different geographical locations can show different properties depending on the environmental conditions to which it is exposed to. Within the geographical region, the berry characteristics can show variability between different years as well (Genebra et al., 2014; Zarrouk et al., 2012, 2015).

2.1.1. Phenology of grapevine

Grapevines are woody perennial deciduous plants and their annual growth stages are quite similar to other such plants. Grape berries are non-climacteric fruit and a sink of water, minerals, micronutrients and primary metabolites such as sugar and amino acids. In addition, it is also the site for synthesis of the major determinants of the aroma, colour and flavour presents in the wine (Grimplet et al., 2007). The grape berry growth is characterized by a double sigmoid curve resulting from two consecutive stages of growth, separated by a slow phase or no growth as shown in Figure 1.

Stage I: This stage is characterized by berry formation which starts at bloom with rapid cell division. The berry expands in volume by accumulating lot of organic content which reach maximum during this stage, however different part of berries accumulate different acids and metabolites. For instance, skin and seeds begin to accumulate tannins, especially important for red wine imparting color and stability.

Stage II or lag phase: Seed embryos develop rapidly and berries attain almost half their size. 5 to 10 days after this stage, the cells continue to expand and accumulate acids and tannin reaching their maximum levels at véraison.

Stage III: This stage starts with berry softening and coloring with accumulation of soluble solids and is termed as véraison. The acid content begins to reduce and the berry size almost doubles. Seed tannins and some aroma also begin to decline. Post véraison, the significant increase in glucose and fructose occurs. Secondary metabolites, major determinant of wine such as anthocyanins for red wine continue to accumulate.
2.1.2. Role of plant phytohormone during grape berry ripening

Plant physiologist commonly use two terms: climacteric and non-climacteric in order to divide the fleshy fruits. Climacteric fruits are those that show increased ethylene evolution and respiration at the onset of ripening (Adams-Phillips et al., 2004). On the other hand, the control of ripening in non-climacteric fruits such as grapes where respiration rates do not exhibit large peaks at véraison is poorly understood. Even though the ethylene is not as important in ripening in non-climacteric fruit as it is in climacteric fruit, it has become evidently known that division between climacteric and non-climacteric fruit is less precise than once thought (Wheeler et al., 2009).

The control of grape berry ripening is a complex interplay between the various hormones. Lot of literature accounts for the role of ethylene or ethylene-releasing compounds that have been applied and produced increase in anthocyanin and/or sugar level (El-Kereamy et al., 2003; Tira-Umphon et al., 2007; Wheeler et al., 2009), making it apparent that ethylene does play role in the control of some genes expressed during non-climacteric fruit ripening as well (El-Kereamy et al., 2003; Tira-Umphon et al., 2007). Auxin (Jeong et al., 2004; Wheeler et al., 2009) and brassinosteroids (BR) (Symons et al., 2006) have also been reported to play a role in control of berry ripening. However, it is clear that ABA have a pivotal role in the control of grape berry ripening. Several studies showed that free ABA levels increase around véraison period (Gagne et al., 2006; Okamoto et al., 2004; Zarrour et al., 2012) and that ABA application can enhance both sugar and anthocyanin accumulation. Recent findings by Sun et al. (2010) implicated both ethylene and ABA to be likely responsible for the start of berry ripening through functional interaction and synergistic effect of ABA and ethylene combined. Some studies have been conducted which reveal that water stress cause production of signalling molecules namely ABA which helps plants to tolerate abiotic stresses (Seki et al., 2007). These ABA molecules could be involved in modulating the epicuticular wax biosynthesis in plants and also affecting the cuticle...
permeability, development and composition in fruit waxy layer (Curvers et al., 2010; Romero et al., 2010).

The berry ripening also depends on several environmental factors such as drought and temperature which thereby regulate the internal biosynthetic pathways under the control of these hormones. Recent findings by Zarrouk and co-authors (2012) have also suggested that water deficit induced changes in ABA level affect the ripening process and also the berry quality. However no reliable pattern of the effect of deficit irrigation on berry ripening process could be ascertained leaving this area of hormonal control and ABA in particular in berry ripening for further research (Zarrouk et al., 2015).

2.2. Water deficit and grapevine

Among the various problems associated to the viticulture, water scarcity is one of the most significant limitations to agriculture practices worldwide and to grape production in particular (Chaves et al., 2007). It is reported that more than 60 percent of major grape production areas worldwide receive precipitation below 700Lm$^{-2}$ (Flexas et al., 2010). Moreover, large fraction of vineyards located in these areas are subjected to intense seasonal drought that coincides with the grapevine growing seasons especially in the Mediterranean climate areas.

The areas of Mediterranean climates are particularly characterized by progressive soil water deficits and relatively higher leaf-to-air vapor pressure gradient along with high irradiance and temperatures which altogether result in the reduced yield and quality of grape fruit (Flexas et al., 2010). As reported by Chaves et al. (2007), the numbers of dry days have increased in Southern Europe and to go by recent climate prediction, viticulture areas subjected to water deficit limitation will further increase in near future forcing viticulturist to rely on the irrigation more. The impact of irrigation management will be most on the wine industry because several reports have established the controversial effect of excess irrigation on grape quality which eventually results in reduced quality through decrease in color and sugar content, and imbalanced acidity (Matthews et al., 1990, Medrano et al., 2003, Salón et al., 2005).

Although wine grape irrigation had been prohibited in many countries owing to the traditional believe that it reduces wine quality, the introduction of drip irrigation practices over the last few decades and the expansion of grape berry cultivation in many of the arid regions of the world (such as South Africa, Australia and some US states – California, Arizona etc), it has become obvious that the quality of wine can be maintained high even under different irrigation practice (Bravdo, 2001). The European authorities have also recently approved the irrigation of vineyards on slopes with steepness more than 30%. Vineyards in Portugal have also sought special permit from the national wine Institute (Bravdo, 2001). This has also been made possible due to the changing precipitation patterns in some of the semi arid Mediterranean region of the Southern Europe (Schultz, 2000) which has led the viticulturist of these regions to be more dependent on the irrigation in order to stabilize the yield and maintain or improve the berry and hence the wine quality.
Studies conducted by Bravdo (2001) suggested that wine quality can be maintained even under deficit irrigation regime, at the same time also enables to keep the soil water availability at optimum level thereby maintaining the sufficient vine water potential needed for different vegetative and reproductive stages. However, achieving high quality wine grape in irrigated vineyards is possible only with an appropriate balance between vegetative and reproductive development.

2.2.1. Sustained deficit irrigation in grapevine

The research on plant water stress had shown that response of plants to water deficits is very much dependent on the pattern of stress imposed (Dorenboos and Kassam, 1979). One such pattern of stress that has been frequently used is sustained deficit irrigation (SDI). In this, water deficit increases progressively as the season advances because of the combination of the uniform application of the reduced amount of water and corresponding depletion of the soil water reserve. This pattern therefore allows stress to develop slowly and helps plants to adapt to the water deficits gradually in soils with significant water storage capacity. Zarrouk and co-authors (2012) have reported the tissue specific influence of water deficit (RDI and SDI) in grape berry where they reported the differential accumulation of anthocyanin and sugar in seeds and skin, however this effect varied between the two years (2007 and 2008) under study (Zarrouk et al., 2015).

2.2.2. Regulated deficit irrigation in grapevine

For efficient irrigation management practices, the use of regulated deficit irrigation (RDI) serves as a promising practical solution for grapevine development that allows accurate control of water application rate and suitable timing. It was first used for grapevine by McCarthy et al. (2002). RDI consists in reducing the applied water at selected phenological stages which are less sensitive to water deficit thus subjecting the plant to the water stress in the controlled manner and would therefore be feasible water saving practice for arid areas with minimum impact on yield and fruit quality (Fereres and Soriano, 2007; Girona et al., 2006; McCarthy et al., 2002). In order to study the effect of water stress on berry development stages, Coombe and McCarthy (2000), based on their findings integrated their results in the form of graph as shown in Figure 2.

Figure 2: Curves showing vegetative and reproductive growth of grapevine (Vitis vinifera)(Coombe and McCarthy, 2000).
RDI helps to maintain the vine water status within the prescribed limits of deficit in relation to the maximum water potential required for different part of the developmental stages during the grapevine growth and helps to keep the balance between vegetative growth and reproductive growth as illustrated by Coombe and McCarthy, (2000) in the Figure 2. However timings and intensities of water deficit can be manipulated in order to achieve the desired objectives (Prichard et al., 2004).

2.2.3. Effect of water deficit on berry ripening/quality

Water deficit influences berry development and composition. Nevertheless its intensity and timing are two major factors that regulate the berry characteristics (Deluc et al., 2009). Additionally, each variety of grapevine shows different effect under water deficit which also depends on the specific tissues of the grape berries: skin, pulp, seeds (Deluc et al., 2009; Zarrouk et al., 2015). The fruit composition is largely dependent on the vine water status through an indirect effect on berry size which also affects the skin to pulp ratio (Kennedy et al., 2002). Various timings and intensities of the deficit can be used to achieve specific objectives (Prichard et al., 2004). In grapevines, controlled pre and post-véraison deficit irrigation have been reported to affect berry size, flavonoid and phenolic content, sugar and total acidity (Kennedy et al., 2002; Matthews et al., 1987; Prichard et al., 2004). Conflicting report about the effect of water stress conditions on anthocyanin accumulation in grape berries are available which many be reasoned due to the anthocyanin accumulation being dependent on several variable which overall determine the anthocyanin content in grape berries (Castellarin et al., 2007b; Downey et al., 2006; Dry et al., 1998; Kennedy et al., 2002; Zarrouk et al., 2012). However some studies have shown that water stress has relatively little to no effect on other phenols in berries (Downey et al., 2006; Genebra et al., 2014; Kennedy et al., 2002; Zarrouk et al., 2012).

Most of the findings corroborating the effect of water deficit on grape berry quality are descriptive with no proper reasoning behind this adaptive response occurring in the grapevine. This might led to conclude that the plant stress response is highly regulated complex phenomenon involving large number of molecular interactions with indefinite variables and factors that come into play which makes its almost unfeasible to study on an experimental scale.

2.3. Introduction to plant cuticle and its significance

Plant cuticle is an extra cellular hydrophobic layer that covers the aerial epidermis of all land plants. The cuticle is a physical structure that incorporates numerous functions of importance for plant life (Kerstiens, 1996). It is a non-living though highly multifunctional structure into which numerous functions have been integrated. This integration is sometimes not ideal as some physiological demands are in conflict with each other (Riederer and Muller, 2008).

Cuticle is synthesized by the epidermis which covers it and serves as first barrier against the abiotic and biotic stress which the plant has to undergo (Domínguez et al., 2011a). The main function attributed to the fruit cuticle are to minimize the water loss, limit the loss of substances from the internal tissues, serves as a protective barrier against the physical, chemical and biological attack and providing the mechanical framework to support the integrity of plant organ. However the fruit cuticle
has been largely disregarded in terms of its putative effect in modulating the development of fruit, specially the ripening and postharvest performance. In order to retain these functions, the structural integrity of cuticle through fruit development and expansion has to remain firm (Edelmann et al., 2005). These biophysical properties are however dependent to some extent upon the external conditions like temperature and relative humidity (Edelmann et al., 2005; Matas et al., 2005). Strength and rigidity of cuticle inversely varies with the increase of temperature and this influence of temperature is related to the relative humidity as water play an important role in plasticizing the plant cuticle and facilitating to withstand the external environment (Domínguez et al., 2011b). In this sense temperature and humidity are two important factors used in devising the storage strategies after the fruit is harvested (reviewed in Lara et al., 2014).

2.3.1. Role of cuticle as a barrier to water loss

After the vascular plants inhabited the dry land, they developed the special mechanism to protect against the desiccation with the help of cuticle. The main physiological attribute associated with its permeability to water basically lies in reducing the uncontrolled water loss adequately enough to allow higher plants to survive on land and it is quite evident that role of cuticle in helping the terrestrial plants to live is fulfilled (Burghardt and Riederer, 2006; Riederer and Schreiber, 2001). Schreiber and Reiderer (1996) have reported the cuticular permeability of plant species from different habitat and showed that fruit cuticles had the highest rate of cuticular transpiration probably because they have no stomata or their frequency is much lower compared to leaves hinting at the possible role, cuticle have as a transport barrier and how cuticular transpiration regulates fruit growth. They were also able to demonstrate that the cuticle thickness bears no direct relationship with the permeances but it is the wax that forms the effective transport barrier which was later supported by several studies (Riederer and Schreiber, 2001; Schreiber and Riederer, 1996). The question arises-than what is the physiological significance of cuticle when its serves as somewhat less than a perfect barrier to water and how much amount of water is lost through cuticle when stomata (if present) are still closed. The answer to these kinds of questions has been the inspiration for the lot of current paradigm of research which are done to investigate the physiology of cuticle and its role in stress mechanism especially in fruits such as grapevine subjected to water deficit conditions in arid and semi arid regions.

2.3.2. Cuticular Permeance

So far the research on cuticle permeance has been mostly restricted to fully developed leaves and fruits of dicotyledonous plant (Kerstiens 1996, 2006; Riederer and Schreiber, 2001). The possible relationship between cuticular waxes and permeance and the possible role of cutin in making cuticle the transport barrier have been assessed either by the comparison of plant surfaces intact with the ones that have had the waxes removed mechanically or altered (Jetter et al., 2000) or through analysis of waxes that are solvent extracted and reconstituted (Kirsch et al., 1997; Šantrůček et al., 2004; Schreiber and Schonherr, 1993).
The cuticle permeance study conducted by Leide et al. (2007) on the role of cuticular waxes in the transspirational water loss in the tomato fruit (Lycopersicon esculentum) ‘MicroTom’ and its lecer6 (mutant defective in a \( \beta \)-ketoacyl-coenzyme A synthase involved in very long chain fatty acid elongation) over the different developmental stages, showed that even though both wild type and mutant showed similar patterns of wax accumulation, they exhibited different water permeances. The lecer6 mutant was shown to exhibit about 3 to 8 fold increase in water loss per unit time and fruit surface compared to the wild type. However both the wild type and mutant immature green fruits showed similar water loss which showed that although deficiency in the LeCER6 \( \beta \)-ketoacyl-CoA synthase caused increased permeance in the mutant compared to wild type, but the major effect caused by this deficiency are apparently not displayed until the fruit entered the transition from fruit ripening category of immature green to mature green.

Several previous studies have been attempted to find out a correlation between the wax characteristics and transpirational barrier properties of the cuticle. However absence of any reliable astomatous plant model system so far had made the functional analysis of cuticle from plant with defined genetic modifications difficult and therefore all attempts to correlate water permeance with the qualitative or quantitative composition of cuticular waxes have proved futile (Riederer and Schreiber, 1995, 2001). Vogg et al. (2004) also followed the developmental stage of dwarf tomato ‘MicroTom’ and its lecer6 mutant in order to elucidate the quantitative and qualitative modification of cuticular wax composition during the fruit maturation and established that water permeability of cuticle in tomato fruits was largely dependent on the aliphatic component of intracuticular wax layer which was modified by the variable amount of triterpenoids during the various maturation stages.

Leide et al. (2007) were able to establish the negative correlation between the water permeance and wax coverage in tomato fruits. Just like the previous study (Riederer and Schreiber, 2001), findings of Leide et al. (2007) also indicated that cuticular wax quality rather than its quantity predominately affects the water barrier properties. However to the best of our knowledge no study on cuticular permeance of grape berry had been reported so far.

2.4. Cuticle Structure, Biosynthesis and Assembly

2.4.1. Composition and structure of cuticle

Plant cuticles are composite structures and are generally composed of a covalently linked macromolecular scaffold cutin and a variety of organic solvent-soluble lipids that together constitute waxes (Yeats and Rose, 2013). Plant cuticle is usually considered to be independent from the polysaccharide cell wall of epidermis lying underneath (Yeats and Rose, 2013) but the two structures are physically associated and have some overlapping function. It would not be therefore wrong to consider cuticle as a specialised lipid-modification of cell wall just as lignification is a common modification of secondary cell wall in plants (Yeats and Rose, 2013). The waxes are either deposited within the cutin matrix which are called intracuticular wax or accumulate on its surface as epicuticular wax crystals, or films. The epicuticular waxes confer distinct macroscopic surface properties to the
plant surface and are responsible for glossy appearance common to many leaves and fruits, while epicuticular wax crystals account for the dull, glaucous appearance of broccoli (Brassica oleracea) leaves and Arabidopsis (Arabidopsis thaliana) stems (Yeats and Rose, 2013) and serve as the interface of the plant and the outer external environment. These epicuticular waxes are highly hydrophobic in nature and are efficient barrier to transpirational loss (Bernard and Joubès, 2013). The structure and composition of cuticle is highly complex and can vary widely among plant species and within plant species in different organ and developmental stages. This is well illustrated in typical range of thickness (1-10 µm) and quantity (100-1000 µg cm⁻²) of deposited cuticle (Riederer and Muller, 2006). This protective barrier of cutin and cuticular waxes are assembled ultrastructurally in several layers as shown in Figure 3.

2.4.2. Biosynthesis and composition of cuticle components: waxes and cutin

Cuticle comprises mainly of waxes and cutin. Composition of waxes may differ substantially with species, ontogeny and environmental conditions (Jenks and Ashworth, 1999). In most of the cases, the majority of compounds constituting the cuticular wax are derived from very-long chain fatty acids (VLCFAs; C20-C34), including alkanes, aldehydes, primary and secondary alcohols, ketones and esters. Several lipophilic secondary metabolites such as pentacyclic triterpenoids, flavonoids, and tocopherols have also found to be substantial components (Jetter et al., 2006). Molecular biology underlying VLCFA-derived wax biosynthesis has made tremendous progress and to this end Arabidopsis has provided an excellent experimental model (reviewed in Bernard and Joubès, 2013).

Figure 3: Schematic of a plant surface showing the major structural features of the cuticle and underlying epidermal cell layer and representative model of cuticular wax secretion. ABC transporter, ATP binding cassette transporter; CER5, ECERIFERUM 5; FAE, fatty acid elongase; LTP, lipid transfer protein; WBC, WHITE-BROWN COMPLEX (Samuels et al., 2008).
Cutin is composed of interesterified hydroxyl fatty acids with lesser amounts of glycerol, phenylpropanoids, and dicarboxylic acids (Kolattukudy, 2001). Chemical breakdown of ester bonds using processes such as saponification readily releases these monomeric constituents, although in some species an additional lipidic polymer referred to as cutan remains recalcitrant to such treatments. Cutan is rich in ether and C-C bond but its structure remain unknown and restricted to relatively few species (Gupta et al., 2006). The hydroxy fatty acids of cutin typically comprised of ω-hydroxy fatty acids, usually with one or two additional mid chain hydroxyl groups or an epoxy group. Extensive studies have been done to identify the chemical composition of plant cutin as early as in 1970’s and 1980’s (Kolattukudy, 2001) before the composition of Arabidopsis cutin was determined (Bonaventure et al., 2004; Franke et al., 2005) and more recently in other fruits such as tomato fruits. This important model species contain atypical composition of cutin in stems and leaves, the dicarboxylic acid, implying that the predominant structural motif must be a copolymer with an unknown polyhydroxy compound, presumably glycerol (Pollard et al., 2008). Despite its atypical nature of cutin, Arabidopsis has proven to be an important model for deciphering the pathway of cutin biosynthesis.

Several studies have reported the composition and biosynthesis of cutin and cuticular waxes in detail which are summarized in recent reviews (e.g. Lara et al., 2015; Lee and Suh, 2015). However, majority of the work investigating cuticle biosynthesis and composition has been conducted on vegetative plant tissues and little information is available in relation to the fruits- both fleshy and non-fleshy fruits. The significant differences observed over the last 40 years in the study of cuticle and its components of different organs of the same plant, both cutin (Espelie et al., 1979; Järvinen et al., 2010; Marga et al., 2001) and waxes (Baker et al., 1975; Radler, 1965,1970) provided enough evidence that the assumption that fruit cuticle composition can be inferred from that of leaves and stems cannot be sustained. Therefore tomato fruit has been the excellent model for fleshy fruits and studied extensively recently. Tomato fruit cuticle provided valuable cuticle-related information as they are astomatous and comparatively thick which means it is possible to isolate considerable amount of "unperforated" material for biochemical, physical, or structural characterization (Martin and Rose, 2014). Albeit less intensively, other fruit cuticles corresponding to the crops of economic relevance have also been investigated and revealed significant differences across genotypes in their properties as the understanding of the functions and impact of this outer layer on quality, storage potential and shelf life of produce will require its characterization as a first step (Lara et al., 2015). The composition of cuticle component of some of the most common fruits has been summarized in Table 1. Detailed composition of cuticle and its components in grapevine is described below (section 2.4.3.).

2.4.3. Biosynthesis and composition of cuticle components in grapevine

Waxes from the grape berry cuticle mostly comprises of aldehydes, where both straight chain, even chain length type predominates. As early as 1965, it was reported in two separate studies that the surface waxes of ripe sultana grape variety consists largely of the triterpernoid oleanolic acid, with relatively lesser amounts of n-alcohols (C24, C26, and C28), n-aldehydes (largely C28 and C30), esters, free acids (mostly C24, C26, and C28) (Radler, 1965)
<table>
<thead>
<tr>
<th>Fruit</th>
<th>Ripening Type</th>
<th>Predominant wax components</th>
<th>Predominant cutin monomers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato ((Solanum lycopersicum))</td>
<td>Climacteric</td>
<td>n-hentriacontane ((C_{31})) triterpenoid alcohols ((\alpha-, \beta-, \delta)-amyrin)</td>
<td>(C_{16}) monomers ((10,16)-dihydroxy (C_{16:0}))</td>
<td>Isaacson et al. (2009); Leide et al. (2007); Petit et al. (2014); Shi et al. (2013); Yeats et al. (2012a)</td>
</tr>
<tr>
<td>Plum ((Prunus domestica))</td>
<td>Climacteric</td>
<td>n-nonacosane ((C_{29})) triterpenoid acids (oleanolic acid)</td>
<td>not reported</td>
<td>Ismail et al. (1977).</td>
</tr>
<tr>
<td>Peach ((Prunus persica))</td>
<td>Climacteric</td>
<td>n-tricosane ((C_{23})), n-pentacosane ((C_{25})) n-heptacosane ((C_{27})) triterpenoid acids (ursolic acid, oleanolic acid)</td>
<td>(C_{18}) monomers ((\omega)-hydroxy (C_{18:1}))</td>
<td>Belge et al. (2014a); Fernández et al. (2011)</td>
</tr>
<tr>
<td>Sweet cherry ((Prunus avium))</td>
<td>Nonclimacteric</td>
<td>n-nonacosane ((C_{29})) triterpenoid acids (ursolic acid)</td>
<td>cultivar-dependent ((\text{dihydroxy } C_{16:0} \text{ or dihydroxy } C_{18:x} \text{ reported mainly}))</td>
<td>Belge et al. (2014b); Peschel et al. (2007)</td>
</tr>
<tr>
<td>Strawberry ((Fragaria × ananassa))</td>
<td>Nonclimacteric</td>
<td>not reported</td>
<td>(C_{16}) monomers ((9(10),16)-dihydroxy (C_{16:0}))</td>
<td>Järvinen et al. (2010).</td>
</tr>
<tr>
<td>Olive ((Olea europaea))</td>
<td>Nonclimacteric</td>
<td>n-heptacosane ((C_{27})), triterpenoid acids (oleanolic acid)</td>
<td>not reported</td>
<td>Bianchi et al. (1992).</td>
</tr>
<tr>
<td>Grape ((Vitis vinifera))</td>
<td>Nonclimacteric</td>
<td>n-pentacosane ((C_{25})) triterpenoid acids (oleanolic acid), n-heptacosane ((C_{27})) n-nonacosane ((C_{29}))</td>
<td>not reported</td>
<td>Casado and Heredia (1999); Comménil et al. (1997); Pensec et al. (2014); Radler (1965,1970); Radler and Horn (1965)</td>
</tr>
</tbody>
</table>
and n-hydrocarbons (predominantly C25, C27, C29, and C31) (Radler, 1965, Radler, and Horn, 1965). Around 30% of oleanolic acid was also found in the cuticle waxes of ‘Palomino fino’ grapes (Casado and Heredia, 1999). However it was surprising that authors did not find any aldehydes and n-alkanes represented less than 5% of the total mass (Casado and Heredia, 1999). Oleanolic acid was also found to be dominant component in the cuticular waxes of ‘Pinot noir’ grapes along all phenological stages of berry development (Comménil et al., 1997). Oleanolic acid was also reported to be present in all eight different cultivars investigated in the recent study by Pensec et al. (2014) in which authors reported triterpenoid content to be as high as around 42% to 80% of the total cuticular waxes depending on the genotype. Major triterpenes such as oleanolic acid and β-sitosterol have significant importance for the success of wine fermentative processes and their health promoting properties have made them an ideal candidate for the study in the grape waxes and also to characterize the varieties based on their economical and pharmaceutical significance (Orbán et al., 2009; Szakiel et al., 2012).

Only minor quantitative differences were found in the cuticular wax fraction of the fruits of the European wild vine (Vitis vinifera ssp. sylvestris) and the cultivated vine (V. vinifera ssp. vinifera) suggesting their conservation during the evolution (Radler, 1970). Although significant literature and studies on the wax composition of the grape berry cuticle is available, data describing the composition of another important part of cuticle, the cutin, is still not available. However the evolution of total cutin amount during fruit maturation has been reported (Comménil et al., 1997). Considering the commercial importance of cutin with regard to its consumption both in the form of fresh table grapes and also wine making makes further studies addressing this issue more desirable.

2.5. Architecture of plant cuticle

Cuticle architectural organization can be discerned using a number of microscopic techniques such as scanning electron microscopy (SEM) which can reveal the elaborate and diverse morphologies of epicuticular wax crystals (Jeffree, 2006) or transmission electron microscopy (TEM) which helps to understand distinct patterning of interior layers of the cuticle, although this approach does not allow the visualization of wax structures. Cuticles vary considerably in their architectural design and, depending on the species and varieties, differ dramatically in their thickness from nanometer to the micrometer scale (Jeffree, 2006). Light microscopy can also be used on micrometer scale to elucidate the fine structure of cuticle and epidermal cell wall as reported by Yeats and Rose (2013). Histochemical staining coupled with confocal microscopy can further help to resolve the three-dimensional architecture of cuticle (Buda et al., 2009).

2.5.1. Ultrastructure of grape berry cuticle

Microscopic techniques have been widely used to study the structure of cuticle and more recently electron microscopic technique has also been employed. Casado and Heredia (2001) studied the ultrastructure of cuticle in grape berry during development using electron microscopy (SEM and TEM) in which they found that during the period of rapid expansion, the cuticle spread out over the grape berry and outer wax layer of 0.5 µm was identified. However, the final stage of berry growth was
characterized by the homogenous cuticle with a thickness of 3µm (Casado and Heredia, 2001). The studies on fruit cuticle has revealed that it is highly diverse but to date not much information could be obtained regarding the patterns of cuticular deposition and what are factors that regulate such unique architectural integrity. Nevertheless, cuticle structure at various scales certainly influences the properties such as water permeability or pathogen adhesion (Yeats et al., 2012a). Therefore investigation of such structure-function relationship would be an area worth exploring in the near future.

2.6. Spectroscopic characterization of grapevine cuticle and its components

Plant cuticle has biological and agricultural significance but its applied use as a source of organic compounds and its importance as plant biomass have recently begun to be investigated. In this regard, cuticle components such as waxes and cutin are potential alternative feedstock for aliphatic compounds found in plants (Tsubaki and Azuma, 2013). The lipid composition of cuticle is commonly determined by gas chromatography coupled with mass spectroscopy. However these techniques present limitations and the identification of the components is not always complete. It’s also not possible to distinguish some functional groups after depolymerization such as ester/carboxylic acid/carboxylate functional groups and non-degradable fractions cannot be analyzed (Pollard et al., 2008). Amorphous nature of plant cuticle also presents some limitations to the use of more traditional techniques like X-ray diffraction (Luque et al., 1995). Other spectroscopic measurement using $^{13}$C nuclear magnetic resonance provide limited information concerning molecular structure due to overlapping of broad spectral lines and, for quantitative measurements, long acquisition times are required (Serra et al., 2012). In order to overcome these bottlenecks, more recently infrared (IR) and Raman spectroscopic techniques have been used which are non-destructive and accessible techniques which have shown certain advantages in terms of chemical and structural analysis of plant cuticles: like identification of functional groups and conformations, determination of intra and intermolecular interactions of cuticle components with exogenous molecules and qualitative estimation of the cutin polymerization. While Raman spectroscopy presents some drawbacks such as fluorescence interference and overlapping signals, IR measurements are fast, easy and no interferences are produced by other mechanisms. However, sample preparation for IR spectroscopy presents some hindrance on sample thickness, uniformity and dilution to avoid saturation. Wide set of different modes of acquisition of IR techniques provide more important and complementary chemical information. One mode of acquisition is ATR-FTIR. ATR is a useful technique to obtain the IR spectrum of the surface of samples such as cuticle (Günzler and Gremlich, 2002; Heredia-Guerrero et al., 2014).

Several studies on characterization of plant cuticles by IR spectroscopy have provided significant amount of information on the nature of functional groups present in cuticle matrix and on the structural arrangement of the cuticular components (España et al., 2014; López-Casado et al., 2007; Ramírez et al., 1992). However it is remarkably important to note that despite the advances of IR and other spectroscopic techniques, the information acquired from them is very limited. It is also important to remark that most of the spectroscopic studies are focused on fruit cuticle of tomato. This bias can be
justified by the enhanced agronomic interest of these fruits and their high proportion of cuticle material and easy isolation of their cuticles (Heredia-Guerrero et al., 2014).

Although tomato fruit cuticle is considered as model system of fleshy climacteric fruit with well known composition, structure and properties, direct extrapolation of results to cuticles of the other species could be wrong (Heredia-Guerrero et al., 2014). It is therefore important to use the information from tomato cuticle and apply it to other non-climacteric fruit such as grape berries to get further insight into the cuticle system of other plant. However, small size and contamination by several interfering compounds makes the process of isolation of cuticle from grape berry, a very tedious job. However, the present thesis aims to present some basic insight into grapevine cuticle system using IR spectroscopy which could be a good starting point in the research of other non-climacteric fruit cuticle research. However in depth analysis of IR coupled with other techniques like microscopes (microspectroscopy) could help in better understanding of this heterodox and complex plant system.

2.7. Genes implicated in fruit cuticle biosynthesis

The majority of fundamental research to study the cuticle biosynthesis has been carried out in Arabidopsis (Pollard et al., 2008; Samuels et al., 2008; Schreiber, 2010) which is a dry fruit bearing species. However, using the information available from the Arabidopsis, homologous genes with identical predicted function have been identified in a large number of fleshy fruit species using the transcriptomics approaches (Albert et al., 2013; Alkio et al., 2012; Mintz-Oron et al., 2008). Nevertheless, the range of genes identified in the model plant Arabidopsis had been limited by its relatively small size and thin cuticle. Although the bioinformatics tool available for homology search are useful and could suggest the possible mechanistic conservation, however it does not lead to any possible discovery of the molecular pathways and processes that might be fruit specific. The fruit cuticle of other plant and fleshy fruits in particular are often more substantial compared to the Arabidopsis and therefore serve as a more excellent model for identifying new genes involved in formation and restructuring of cuticle. Despite tremendous efforts in the recent times, several key areas of cuticle biogenesis remain unclear. First, the molecular mechanism underlying the intracellular and extracellular transport of wax and cutin precursors remains poorly understood, although key ABC transporters for their export across the plasma membrane have been identified (Bird et al., 2007; Chen et al., 2011; Fabre et al., 2015; Pighin et al., 2004). The recent strides made in the field of genetics and advancement of latest molecular techniques had made tomato a most favourite model plant which made it possible to characterize the cuticle-related genes or its mutant. Several transgenic tomato lines have been produced to explore and confirm the cuticle-related gene function and are well documented in the available literature (Girard et al., 2012; Shi et al., 2013; Vogg et al., 2004; Yeats et al., 2012b).

Cuticle being the major interface between plant surface and the external environment makes it extremely important to study its properties and its role in grape berries for regulating the loss of water. Major components of the cuticle are cutin and cuticular waxes. Cutin is structural backbone of the cuticle, while cuticular wax is embedded in the cutin polymer as intracuticular wax and covers the cutin
matrix as distinct layer called epicuticular waxes (Kunst and Samuels, 2009; Samuels et al., 2008). The present thesis currently focuses on the cuticular wax component of the cuticle for which several genes have been identified in the literature. The precursors of the cuticular wax biosynthesis are very long chain of fatty acids (VLCFAs) which are generated by the de novo fatty acid biosynthesis in the plastid whereas fatty acid elongation occurs in the endoplasmic reticulum of the epidermal cells. The C16 or C18 acyl-CoAs serve as precursors for the biosynthesis of VLCFAs (C20-C34) by the addition of C2 units derived from malonyl-CoA. This formation of VLCFAs is usually carried out by the enzymes of fatty acid elongase (FAE) complex in the endoplasmic reticulum (Kunst and Samuels 2009; Li-Beisson et al., 2013). The FAE complex consists of β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and trans-2,3-enoyl-CoA reductase (ECR), which catalyze the sequential reactions: condensation, reduction, dehydration, and a second reduction, respectively (Kunst and Samuels 2009; Li-Beisson et al., 2013). The aliphatic components of the wax are derived from VLCFAs with the chain length of 24 to 34 carbons (Samuels et al., 2008) and biosynthesis requires a unique set of condensing enzymes: KCS1, CER6/KCS6/CUT1 and KCS9. (Kim et al., 2013; Todd et al., 1999). For the present master thesis, we decided to focus on KCS1 encoding for KCS1 enzyme involved in the C26, C28 and C30 elongation and CER6/KCS6/CUT1 (hereafter only referred to as KCS6) encoding for a major condensing enzyme responsible for wax precursors. Other classes of VLCFAs synthesis genes are also described, namely LACS (Lü et al., 2009; Weng et al., 2010) which are involved in the synthesis of VLCFAs required for pollen coat formation (Jessen et al., 2011). There are other cuticle related genes which affect the cuticle biosynthesis and transport which have been summarized in Table 2.

Wax formation involves various steps from its synthesis in plastids to its elongation in ER from where it is transported to the plasma membrane (PM). Beyond plasma membrane, the hydrophobic wax components have to traverse the hydrophilic cell wall, the function which is probably facilitated by the cell wall associated lipid transfer proteins (Bernard and Joubes, 2013; Kunst and Samuels, 2009). Two glycosyl-phosphatidylinositol (GPI)-anchored Lipid Transfer Proteins (LTPGs), LTPG1 and LTPG2, were shown to be required for wax export from the PM to the plant surface (DeBono et al., 2009; Kim et al., 2012) but the mechanism underlying their roles in wax export is still not clear. Several lipid-transfer proteins have been reported to modulate plant responses to environmental stress, namely biotic stress. Recent reports showed that wax carrier gene such as LTP3, positively regulated by MYB96 transcription factor (modulator of wax biosynthesis) is also significantly induced by drought stress and ABA application (Guo et al., 2013; Seo et al., 2011). Recent investigation by our group has identified specific lipid transfer protein (LTP3) in the proteome of grape berry cuticle (Zarrouk et al., unpublished data) suggesting its role in the VLCFAs transport in grape berries. LTPs are most important lipid transfer protein for the extrusion of wax beyond PM and study of its relative expression under water deficit conditions might unfold the adaptive response of grape berries under stress conditions and how cuticular wax load is modulated under stress conditions. Overview of wax synthesis is illustrated in Figure 4.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Family name</th>
<th>Affected Cuticular components</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCS1</td>
<td>β-Ketoacyl-coenzyme A synthase</td>
<td>Cuticular wax biosynthesis</td>
<td>Todd et al. (1999)</td>
</tr>
<tr>
<td>KCS6/CER6/CUT1</td>
<td>β-Ketoacyl-coenzyme A synthase</td>
<td>Cuticular wax biosynthesis</td>
<td>Hooker et al. (2002); Millar et al. (1999)</td>
</tr>
<tr>
<td>FATB</td>
<td>Fatty acyl-ACP thioesterase B</td>
<td>Cuticular wax biosynthesis</td>
<td>Bonaventure et al. (2003)</td>
</tr>
<tr>
<td>LACS1/CER8</td>
<td>Long chain acyl-CoA Synthetase</td>
<td>Cuticular wax biosynthesis</td>
<td>Lü et al. (2009), Weng et al. (2010)</td>
</tr>
<tr>
<td>LACS2</td>
<td>Long chain acyl-CoA Synthetase</td>
<td>Cuticular wax biosynthesis</td>
<td>Lü et al. (2009), Weng et al. (2010)</td>
</tr>
<tr>
<td>LCAS4</td>
<td>Long chain acyl-CoA Synthetase</td>
<td>Cuticular wax biosynthesis</td>
<td>Jessen et al. (2011)</td>
</tr>
<tr>
<td>KCS2/DAISY</td>
<td>β-Ketoacyl-coenzyme A synthase</td>
<td>Cuticular wax biosynthesis</td>
<td>Franke et al. (2009); Lee et al. (2009)</td>
</tr>
<tr>
<td>KCS9</td>
<td>β-Ketoacyl-coenzyme A synthase</td>
<td>Cuticular wax biosynthesis</td>
<td>Kim et al. (2013)</td>
</tr>
<tr>
<td>KCR1</td>
<td>β-Ketoacyl-coenzyme A reductase</td>
<td>Cuticular wax biosynthesis</td>
<td>Beaudoin et al. (2009)</td>
</tr>
<tr>
<td>CER2</td>
<td>BAHD acyltransferase</td>
<td>Cuticular wax biosynthesis</td>
<td>Haslam et al. (2012, 2015); Xia et al. (1996)</td>
</tr>
<tr>
<td>CER1</td>
<td>Alkane-forming pathway</td>
<td>Cuticular wax biosynthesis</td>
<td>Aarts et al. (1995); Bourdenx et al. (2011)</td>
</tr>
<tr>
<td>CER3/WAX2</td>
<td>VLC-Acyl-CoA reductase putative</td>
<td>Cuticular wax biosynthesis</td>
<td>Chen et al. (2003)</td>
</tr>
<tr>
<td>ABCG12/CER5</td>
<td>ABC transporter</td>
<td>Cuticular wax transport</td>
<td>Pighin et al. (2004)</td>
</tr>
<tr>
<td>LTPG1/LTPG2/LTPG3</td>
<td>GPI-anchored lipid transfer protein (LTPG)</td>
<td>Cuticular wax transport</td>
<td>DeBono et al. (2009); Kim et al. (2012)</td>
</tr>
<tr>
<td>LTP3</td>
<td>Lipid transfer protein</td>
<td>Cuticular wax transport</td>
<td>Guo et al. (2013); Seo et al. (2011)</td>
</tr>
<tr>
<td>CER9</td>
<td>Putative E3 ubiquitin ligase</td>
<td>Post-translational regulation of wax biosynthesis</td>
<td>Lü et al. (2012), Zhao et al. (2014)</td>
</tr>
<tr>
<td>GDSL/CD1</td>
<td>Cutin synthase/hydroxyacylglycerol Transesterase</td>
<td>Cutin and wax biosynthesis and epidermal patterning</td>
<td>Beisson and Ohlrogge (2012); Buxdorf et al. (2014); Shi et al. (2013); Yeats et al. (2012b)</td>
</tr>
<tr>
<td>WSD1</td>
<td>Wax synthase</td>
<td>Wax biosynthesis</td>
<td>Li et al. (2008).</td>
</tr>
</tbody>
</table>
The process of cutin polymerization from 2-monooacylglycerol units had eluded many scientists till date. A recent study of tomato cutin deficient (CD1) mutant which has limited amount of cutin and accumulates 2-monooacylglycerol at the fruit surface led to the identification of the member of glycine–aspartic acid–serine–leucine (GDSL) motif lipase/hydrolase superfamily that acts as the cutin synthase (Beisson and Ohlrogge 2012; Girard et al., 2012). This CD1 gene is expressed in the epidermal cells and the encoded GDSL protein is localized extracellularly within the cuticle which allows cutin polymerization (Yeats et al., 2012b). GDSL is an important gene for cutin synthesis, therefore we decided to study its relative expression in the grape berries and how its expression change along the phenological stages and under various treatment.

Plant cuticle biosynthesis, transport and deposition is a complex and highly regulated network of genetic interplay and is influenced by environmental stress but details of this regulation are not well understood (Bernard and Joubes, 2013; Nawrath et al., 2013). Apart from the network of transcription factors that regulate cuticle biosynthesis, namely WIN1/SHN1, MYB106 and MYB16 (Aharoni et al., 2004; Broun et al., 2004; Oshima et al., 2013), regulatory mechanism that do not involve direct transcriptional activation or repression by promoter binding have recently been discovered. One such example is the studies from CER9 which is the first described gene related to cuticle biosynthesis whose deficiency improves plant response to water deficit (Lü et al., 2012). Although the exact role
and mechanism of CER9 which caused these changes in still not clear, but recent finding by Zhao et al. (2014) reinstated the role of CER9 in cuticle biosynthesis and its role in drought tolerance.

Waxes of several fruits, including grape berries, have showed secondary metabolites presence, namely oleanolic acid as one of the major component (Casado and Heredia, 1999). Fukushima et al. (2011) have recently identified two homologs of Medicago truncatula CYP716A12 genes: CYP716A15 and CYP716A17 and showed their ability in terpenoids production in grapes such as oleanolic acid. CYP716A subfamily members are multifunctional oxidases which are involved in triterpenoid biosynthesis (Fukushima et al., 2011). They are Cytochrome P450 monooxygenases/hydroxylases (P450s), which are enzymes found in plants, human and bacteria and catalyze the oxidation of various substrates by oxygen and NAD(P)H (Nelson et al., 2004; Nelson, 1999). Plant P450s are involved in secondary metabolism and catalyze the wide variety of monooxygenation/hydroxylation reactions (Mizutani and Sato, 2011; Shibuya et al., 2006). Several other P450s have been identified in plants such as Soybean CYP93E1(β-amanin C-24 hydroxylase) which is involved in soyasaponin biosynthesis (Achnine et al., 2005). The CYP88D6 (β-amanin oxidase) was characterized in both Glycyrhiza glabra and Glycyrhiza uralensis. Glycyrhiza uralensis CYP93E3 was identified as β-amanin 24-hydroxylase and CYP72A154 was found to catalyze C30 oxidation of β-amanin (Seki et al., 2011). Fukushima et al. (2011) identified Medicago truncatula CYP716A12 as a multifunctional enzyme with β-amanin 28-oxidase, α-amanin 28-oxidase and lupeol 28-oxidase activities. Authors also identified homologs of CYP716A12 in grape (CYP716A15 and CYP716A17), indicating that the function of the CYP716A subfamily among plants is highly conserved. Grape berry skin and particularly cuticle is rich in terpenoids (oleanolic acid) requiring putative P450 genes (Seki et al., 2011; Yonekura-Sakakibara and Saito, 2009) for its productions (Yonekura-Sakakibara and Saito, 2009). These terpenoids have been reported to possess medicinal properties (Jennewein et al., 2001). The cyclic terpenoid such as oleanolic acid comprises up to 30% of grape berry cuticular waxes and are important for the wine industry and may determine the efficacy of the alcoholic fermentative process (Casado and Heredia, 2001; Orbán et al., 2009). Therefore we decided to study the expression pattern of these two genes (CYP716A15 and CYP716A17) to see the expression pattern of these genes under water stress.

The list of genes involved in cuticle biosynthesis had been extensive topic of research in both model and non-model plant and is growing as more and more transcript are being studied. With more and more genome based techniques and the genetic resources available, it would be feasible to study the existing list of potential candidate genes that are associated with cuticle biosynthesis, transport and deposition and would shed more light on the dynamics of cuticle biology in near future (Martin and Rose, 2014). Understanding of cuticle biosynthetic pathways would bring us closer to an ability to selectively modify cuticle properties in order to enhance agricultural productivity in fruits such as grapevine. Nevertheless, in order to make such modification rationally will require an in-depth understanding of the complexity of cuticle function at molecular level, an area where far less progress has been made. To this end, further work aimed at understanding the ecophysiological aspects of cuticle and its adaptive response under environmental stress will provide a framework for
understanding the complex interaction of cuticle structure, composition and its functions (Yeats et al., 2012a; Yeats and Rose, 2013). These findings might reveal the cuticle complexities that underscore the fact that the cuticle is much more than just a preformed barrier to water loss (Yeats and Rose, 2013).
3. Research Objectives and Hypotheses

3.1. Research objectives:

Main objectives of this work are:

1. To characterize grape berry cuticle in terms of its morphology and biochemistry which may shed light on its stress tolerance and to evaluate the role of cuticle as a barrier to water loss.
2. Qualitative and quantitative estimation of cuticular metabolites during the course of berry development and along the ripening process.
3. To identify putative genes relating to the cuticle biosynthesis, transport and deposition.

3.2. Research hypotheses

This study is based on following hypotheses:

1. Water deficit affects the cuticle physiological properties in grape berries.
2. Grape berry skin and cuticle architecture are affected by water stress.
3. Spectroscopic techniques provide significant information about the cuticle and its macromolecular arrangement.
4. Water stress affect cuticle related gene expression in grape berries.
4. Materials and Methods

4.1. Field conditions and plant material

Grape berries were harvested from Aragonez (*Vitis vinifera*) variety grafted on Paulsen 1103 rootstock along the berry development (June to September of 2014) cultivated at a commercial vineyard (Herdade do Esporão) located at Reguengos de Monsarraz, Alentejo region.

Grapevines were subjected to two irrigation regimes:
- Sustained Deficit Irrigation (SDI) in which 20% of evapo-transpiration ($ET_c = 235$ mm) were supplied.
- Regulated Deficit Irrigation (RDI): in which 14% of $ET_c$ ($ET_c = 235$ mm), were supplied.

The general field lay out plan of the vineyard is represented in Figure 1 (Appendix I). To avoid ‘border effect’ only berries belonging from the grapevine of middle rows were sampled (columns marked in colors). Grape berries were randomly sampled from both sides (East and West) of the canopy subjected to two different irrigation regimes at four developmental stages:
- Pea size (PS): 7 weeks after anthesis (collected at 16.06.2014);
- Véraison (V): 9 weeks after anthesis (collected at 14.07.2014);
- Mid Ripening (MR): 11 weeks after anthesis (collected at 29.07.2014);
- Full maturation (FM): 13 weeks after anthesis (collected at 11.08.2014).

The berries sampled from different phenological stages have been shown in Figure 5. The pre-dawn water potential ($\Psi_{PD}$) and temperature of both side of the vine was monitored over the period of growth and maximum air and berry temperature on east and west sides of the vine canopy for SDI and RDI treatment has been shown in Appendix II and Appendix III respectively.

![Figure 5: Grape berries sampled from four different phenological stages: pea size(a), véraison(b), mid-ripening(c) and full maturation(d).](image)
For each of the sampling time, approximately 50 bunches representative of the vineyard were collected. Berries from these bunches was transported at 4°C to the laboratory. A first sub-sample of berries was then used to determine the fresh weight and juice was extracted to determine the total soluble solids (°Brix) and titrable acidity (TA). A second subsample was used for the cuticular permeance study and a third subsample was frozen in liquid nitrogen and stored at -80°C for gene expression analysis.

4.2. Characterization of berry

4.2.1. Determination of Total Soluble Solids (TSS) and Titrable Acidity (TA)

The grape berries were crushed using mortar and pestle and the grape juice of the crushed berries was used subsequently for TSS (°Brix) and titrable acidity determination. The concentration of TSS in the juice was quantified using a manual refractometer (ITREF 32, Instrutemp). The titrable acidity was assessed according to Office International de la Vigne et du Vin (OIV, 1990).

4.3. Characterization of cuticle

4.3.1. Isolation of Cuticle

Cuticles were enzymatically isolated from grape berry skins following the protocol described by Petracek and Bukovac (1995), with slight modifications. An aqueous sodium citrate buffer (50 mM, pH 3.7) with a mixture of fungal cellulase (4% w/v, Sigma, USA) and pectinase (4%, w/v, Sigma, USA) was used. 1 mM NaN₃ was added to prevent microbial growth. In order to facilitate enzyme penetration, vacuum was applied to the suspension of fruit cuticles and incubated with continuous agitation at 37°C for 10-14 days. Cuticular membranes were then manually separated from the epidermis, rinsed in distilled water and stored under dry conditions.

4.3.2. Isolation of cuticle components

Cuticular components such as waxes were removed by refluxing the isolated cuticle samples in chloroform:methanol (2:1, v/v) for 3 h at 50°C. Afterwards, the cuticles were thoroughly washed thrice in methanol. Cutin isolates were obtained after refluxing the de-waxed cuticles in a KOH (1%, w/v) methanolic solution at 35-40°C for 48 h in order to hydrolyze cutin matrix (López-Casado et al., 2007). After this time, residual material appeared as continuous and fragile films.

4.4. Determination of wax content

In order to determine the total wax content, three replicate composed by 10 to 15 berries each, were used. Berries were immersed in chloroform for 10 seconds and the extracts were left to dry overnight under hood. The wax content was estimated using the weight by precision (0.1 mg) balance (Radwag, Poland).
4.5. Determination of cuticular permeance

Cuticular water permeance for the grape berries was determined for each developmental stage and for each treatment (irrigation and sides of canopy) using the methodology of Leide et al. (2007). The surface of the berries from each treatment was determined using the vernier calipers as the average of vertical and horizontal diameters by assuming ellipsoidal shape of the berries. Three replicates of 10 berries per treatment were used (2 irrigation treatments x 2 sides of the canopy x 3 replicates). The attachment site of pedicel to the berry was sealed using the paraffin. The permeance of untreated berries and de-waxed berries was measured. To remove waxes, berries from each treatment were dipped in aqueous gum arabic solution (45% w/v, Sigma Aldrich) and then allowed to dry on filter paper at room temperature for approximately 1 hour. Afterwards, the gum arabic film was peeled off gently from the berries using a small spatula. The berries were then dipped in the chloroform solution for 10 s, making sure that the chloroform does not dissolve the paraffin from the attachment site of the pedicel. This technique allows the removal of most of the extra and intra epicuticular waxes (Buschhaus and Jetter, 2011).

The untreated and de-waxed berries were then placed in the closed bottle containing silica at 25°C and the amount of water transpired was measured using a balance with a precision of 0.1 mg (Radwag, Poland) against the time interval of 15 minutes. At least seven data points were recorded: 0-15 min-30 min-45 min-60 min-75 min-90 min.

The calculation of cuticular permeance is based on the equation calculated for tomato by Leide et al. (2007). The water flow rate of cuticle (F, g/s) for individual set of fruits were determined from the slope of a linear regression line fitted for the gravimetric data (Appendix IV). Coefficients of determination \( r^2 \) was also determined and it averaged \( r^2 = 1 \). Flux (\( J \) in \( \text{gm}^{-2} \text{s}^{-1} \)) was calculated by dividing \( F \) by the total berry surface area as determined previously.

\[
\text{Flux}(J) = \frac{\text{Flow rate}(F)}{\text{Surface Area}}
\]

(Eq. 1)

Under conditions of storage in the silica containing bottle, it was assumed that the external water vapour concentration was nearly zero. Vapour phase-based driving force is calculated using the difference between the outer water vapour condition and the internal vapour condition and is the established value considered for the present study which was found to be 23.07 g m\(^{-3}\). For calculating the water permeance (\( P \) in ms\(^{-1}\)), which was based on water vapour concentration, flux was divided by the vapour phase-based driving force (\( \Delta c \)) for transpiration.

4.6. Ultra-structure of cuticle using transmission electron microscopy

Grape berry skins were analysed using transmission electron microscopy (TEM) at Electron Microscopy Facility at the Instituto Gulbenkian de Ciência, Oeiras, Portugal. Skins were fixated in glutaraldehyde solution (2% v/v, with phosphate buffer 0.2 M and pH 7) fixation buffer and stored at 4°C. Small pieces of fixated skin were sliced into small pieces of about 1-2 mm and rinsed thoroughly.
in fresh buffer. Afterward, skins were post-fixed in osmium tetroxide (2% v/v, in phosphate buffer) for 4h at 4°C and then rinsed again thoroughly in phosphate buffer. Samples were dehydrated using acetone solution gradients: 40, 50, 60, 70, 95 and 100% with increasing times from 15 min to 1h 30 min for dehydration. Samples were then kept in a mixture of acetone and resin with 1:1 v/v concentration for 2h in vacuum followed by overnight storage at room temperature. Skins were then embedded in an araldite resin for 1h in vacuum and kept for polymerization during 72h at 60°C. Ultra-microtome (Reichert) with diamond knife was used to obtain transverse sections of approximately 70-80 nm. Sections were then observed at 100kV with TEM (Hitachi H-7650).

TEM images were analysed and processed using open source ImageJ software (http://rsb.info.nih.gov/ij). Average of 5-10 images with 10-15 measurement for each image for each of the parameters studied were considered for further analysis.

4.7. Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Infrared spectra of cuticle, skin, de-waxed cuticle and depolymerised cutin were obtained with an ATR accessory coupled to FTIR spectrometer (Bruker Optics, Ettlingen, Germany). All spectra were recorded in the 4000 to 600 cm\(^{-1}\) range with 4 cm\(^{-1}\) resolution and accumulating 128 scans. The cuticle, de-waxed cuticle and depolymerised cutin were used after isolation as described in section 4.3.1 and 4.3.2. For skin, the peels were removed, treated with chloroform for 1h, followed by 1:1 v/v mixture of chloroform and methanol for 1h followed by methanol for 1h and finally treated with chloroform for 1h.

The samples were then gently collocated on the spot of the ATR accessory and slowly pressed. The analysis depth, \(d_p\), of ATR-FTIR depends on several factors such as the wave number, \(\lambda\), the angle of incidence \(\theta\), the refractive index of the ATR crystal \((n_1)\), and the sample \((n_2)\), as indicated in the following equation:

\[
d_p = \frac{\lambda}{2\pi n_1 \sqrt{\sin^2 \theta - (n_2/n_1)^2}}
\]

(Eq. 2)

In our ATR-FTIR spectrometer, \(n_1 = 2.43\) and, considering the refractive index of the cuticle as 1.5 (Holloway, 1982), the penetration depth of ATR into the sample varies between 0.47 μm at 4000 cm\(^{-1}\) and 2.71 μm at 700 cm\(^{-1}\).

4.8. Gene expression analysis

4.8.1. Extraction of RNA from grape berry cuticle

RNA extraction was performed according to the protocol described by Reid et al. (2006) with modifications. Berry skins were removed using the sharp scalpel and were grounded to a fine powder in the liquid nitrogen using the mortar and pestle with PVPP (100mg for 1-2g tissue). Before the extraction was carried out, the extraction buffer (Appendix V) was pre-warmed at 65°C using the thermal mixer and 2% β-mercaptoethanol was added to the grounded tissue. The grounded powder
was then added to the pre-warmed extraction buffer (1ml/100-500mg tissue) and was shaken vigorously using the vortex mixer.

The mixture of skin material and extraction buffer was then incubated at 65°C for 10 minutes with continuous mixing. Mixtures were extracted twice with equal volume (1ml) of chloroform:isoamyl alcohol (24:1) and centrifuged at 13200 rpm for 10 minutes at 4°C. The aqueous layer obtained after centrifugation was transferred to the new tube and centrifuged again at 13200 rpm for 15 minutes at 4°C to remove any remaining insoluble material. To the supernatant, 0.1 vol. (100 µl) of sodium acetate (3M, pH 5.2) and 0.6 vol (600 µl) of isopropanol were added, mixed thoroughly and then incubated at -80°C for 30 minutes. Nucleic acids were pelleted along with any remaining carbohydrate using the centrifugation at 13200 rpm for 30 minutes at 4°C. The pellet was dissolved in 250 µl of TE (pH 7.5) and in order to selectively precipitate RNA, 0.3 vol (90 µl) of 8M lithium chloride (LiCl) was added and the sample was stored at 4°C for overnight. The following day, the RNA was obtained in the form of pellet by centrifugation at 13200 rpm for 30 minutes at 4°C and then washed with ice-cold 70% ethanol, air dried and dissolved in 100 µl of DEPC-treated water. Extracted RNA was evaluated for its concentration, 260/280nm and 260/230nm using Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

4.8.2. Purification of RNA

RNA was purified using the RNeasy Mini Kit protocol (Qiagen). Before following the purification protocol, DNase digestion was performed for which the DNase I stock solution was prepared by mixing 10 µl RDD buffer with 2.5 µl DNase I. To the 100 µl of extracted RNA sample, 12.5 µl of DNase I stock solution was added and allowed to stand at room temperature for 10 minutes. Then 350 µl of RTL buffer was added and mixed well, followed by 250 µl of ethanol (96-100%) and was mixed with pipetting. The content of the mixture were then transferred to the RNeasy mini spin column and centrifuged for 15s at 13200 rpm. The flow through was discarded and 500 µl of RPE buffer was added to the RNeasy mini spin column. After centrifuging it at 13200 rpm for 15s, the flow through was discarded and 500 µl of RPE buffer was added again to the RNeasy mini spin column. Now, it was centrifuged for 2 minutes at 13200 rpm and flow through was discarded and centrifugation was again repeated for another 1 minute at 13200 rpm. RNeasy mini spin column were then placed in the new 1.5 ml collection tube and 30 µl of RNase free water was added. Collection tube was then centrifuged for 13200 rpm for 1 minute in order to elute the RNA. Purity of the extracted RNA, its concentration, 260/280 nm and 260/230 nm ratio was again determined.

4.8.3. Optimization of RNA extraction protocol

The RNA extraction from grape berries is very difficult because of the presence of lot of phenol compounds which interfere with the extraction methodology. The content of these agents is highly dependent on the seasonal variation and prevailing weather conditions and therefore their content can vary from year to year. The grape berries used for the current year were extremely difficult to process
in order to isolate RNA and therefore the available extraction protocols were suitably modified in order to increase the purity and yield of RNA extracted.

In the first modification of the protocol described above, slight increase in the mixing time (15 minutes) was done in first step of mixing on thermal mixer. This was expected to facilitate the efficient mixing of powdered plant material with the extraction buffer and help in the better extraction yield. Subsequent modifications were made in the centrifugation step by increasing the centrifugation time by up to 5-10 minutes depending upon the steps. In order to have better yield, double precipitation with LiCl was attempted after precipitating the RNA pellet with LiCl for overnight. However the yield obtained were not as expected. Finally we precipitated the purified RNA (RNA purified by Qiagen Kit) using 33% LiCl for 90 minutes on ice and pelleting at 13200 rpm by centrifuging it for 30 minutes. The pellet was then re-washed with 70% ethanol, re-dried and finally re-dissolved in 30 µl RNAase free water and the concentration and purification ratios were estimated again using Nanodrop ND-2000 spectrophotometer. The RNA extracted was to be used for the cDNA synthesis using RT-PCR for which 1000ng of RNA would be required. In recalcitrant samples, RNA was additionally concentrated using the CentriVap vacuum concentrators (LABCONCO, Kansas City, USA). After concentration using CentriVap, RNA concentration and 260/280 nm ratio was again determined using a ND-2000 spectrophotometer. RNA integrity was evaluated by 1% (w/v) agarose gel electrophoresis.

4.8.4. DNA synthesis (RT-PCR)

First strand cDNA was synthesized using the Omniscript® Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The cDNA was prepared from 1000 ng of total extracted RNA. The reverse-transcription reaction mixture comprised the following components in final concentrations: buffer RT 1X, 0.5 mM of each dNTP, 1 µM oligo-dT primer, 0.5 units of RNase inhibitor, 0.2 units of Omniscript Reverse Transcriptase enzyme and template RNA calculated for 1000 ng to make 20 µL of final reaction volume. cDNA was synthesized at 37 °C for 60 min and the cDNA was stored at -80 °C.

4.8.5. Primer design

For PCR amplification, primers for VviKCS6, VviKCS1 and VviGDSL were designed based on the blast search (http://www.ncbi.nlm.nih.gov/) of these genes against the genome of the tomato (Lycopersicum esculentum) and for VviCER9, the primer was designed based on the blast search of this gene against the genome of the Arabidopsis thaliana. For VviLTP3, the primer was designed based on the cDNA sequence corresponding to the amino acid sequence obtained for the protein identified in berry cuticle proteome (Zarrouk et al., unpublished data). The primers of VviCYP716A15 and VviCYP716A17 were designed based on the genes identified in the Vitis genome (http://vitaceae.org). The primers for actin were retrieved from the literature (Reid et al., 2006). The primer details are represented in Table 3.
4.8.6. Quantitative RT-PCR

The cDNAs were diluted 1:50 with RNase free-water. Aliquots of the same cDNA sample were used with all primer sets for Quantitative Real-time (qRT) PCR. Each reaction was performed in 20 μl containing 250 nM of each gene-specific primers, 5μl of diluted (1:50) cDNA and 10ul of Power SYBR Green Master Mix (Bio-Rad).

<table>
<thead>
<tr>
<th>Vitis Gene</th>
<th>Primer Sequence forward/reverse(5' → 3')</th>
<th>Amplicon length(bp)</th>
<th>Melting Temperature (Tm, °C)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>VviKCS1</td>
<td>GGAGGATGGTGCGGAAGATGT/TGGAACTGAACCGTCTC</td>
<td>20</td>
<td>56.4</td>
<td>XM_002264685.2</td>
</tr>
<tr>
<td></td>
<td>TGGAACTGAACCGTCTCCTC</td>
<td></td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>VviKCS6</td>
<td>GCCAAGTACGGTCTAGTCCCA/TTCTTCGTAGACGCATG</td>
<td>20</td>
<td>56.2</td>
<td>XM_002283694.3</td>
</tr>
<tr>
<td></td>
<td>TGGAACTGAACCGTCTCCTC</td>
<td></td>
<td>56.1</td>
<td></td>
</tr>
<tr>
<td>VviGDSL</td>
<td>GGAAACCCTAGCTGCTCTCT/GGTGCTTGACCTGCCATAAC</td>
<td>20</td>
<td>56.4</td>
<td>XM_002277667.2</td>
</tr>
<tr>
<td>VviCER9</td>
<td>TGGAGCATTTCATGCATCAGG/AGCACAAAGCCGTCTC</td>
<td>21</td>
<td>57.7</td>
<td>XM_010604926.1</td>
</tr>
<tr>
<td></td>
<td>TGGAGCATTTCATGCATCAGG/GGTGCTTGACCTGCCATAAC</td>
<td></td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>VviLTP3</td>
<td>GGTCCTGGAGAGTGATGATGGA/CTTACTGAGTGATGATGGA</td>
<td>21</td>
<td>55.8</td>
<td>Proteomic data in our lab</td>
</tr>
<tr>
<td></td>
<td>GGTCCTGGAGAGTGATGATGGA/CTTACTGAGTGATGATGGA</td>
<td></td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>VviCYP716A15</td>
<td>GATGCCACATGAATGGAATGGGAA/TGATAGGAAATGGGAAATGGCAGCA</td>
<td>24</td>
<td>54.4</td>
<td>NM_001281186.1</td>
</tr>
<tr>
<td>VviCYP716A17</td>
<td>TGAGAGCAAGGCTCAAAAAC/GCATCCATCCATCTCATCTC</td>
<td>21</td>
<td>55.8</td>
<td>NM_001281147.1</td>
</tr>
<tr>
<td></td>
<td>TGAGAGCAAGGCTCAAAAAC/GCATCCATCCATCTCATCTC</td>
<td></td>
<td>55.5</td>
<td></td>
</tr>
<tr>
<td>VviActin</td>
<td>CTTGCATCCCTCAGCACCTT/TCCTGTGGACAATGGATGGA</td>
<td>20</td>
<td>54.0</td>
<td>Reid et al.(2006)</td>
</tr>
<tr>
<td></td>
<td>CTTGCATCCCTCAGCACCTT/TCCTGTGGACAATGGATGGA</td>
<td></td>
<td>53.9</td>
<td></td>
</tr>
</tbody>
</table>

Thermal cycling conditions used for qPCR were: 95°C for 10 min followed by 95°C for 10s, 60°C for 10s and 72°C for 10s for the total of 40 cycles. qRT-PCR was performed in the iQ5 2.0 Standard Edition (Bio-Rad, Hercules, CA, USA), sequence detection system in a 96-well reaction plate. Dissociation curves were obtained by heating the amplicon from 55°C- 95°C and these curves for each amplicon were then analyzed to verify the specificity of each amplification reaction. No evidence for any primer-dimer or other non-specific product formation was detected for any of the primer pairs used. Each PCR cycle was run in triplicate within the same plate and the average of cycle threshold (Ct) values obtained from the technical replicates was obtained. Gene transcripts were quantified by comparing the Ct of the target gene with that of the actin as described below. Gene expression was expressed as mean and standard error calculated over the three biological replicates. The relative gene expression was determined using efficiency (E) method:

\[
E_{gene} = 10^{(-\frac{1}{1}\cdot \text{Ct}_{\text{actin}})} - 1
\]

(Eq. 3)
\[ E_{actin} = 10^{\left(\frac{1}{\text{slope}}\right)} - 1 \] (Eq. 4)

where \( E_{gene} \) is the efficiency of our target gene and \( E_{actin} \) is efficiency of actin (control). Slope is obtained from a semi-log regression line plot of \( C_t \) values vs. log of input nucleic acid.

4.9. Statistical analysis

All the experiments were performed in triplicate and the gene expression study was also conducted based on three biological and three technical replicates. All the results are expressed as mean± standard error (SE). Data were examined by analysis of variance (ANOVA) with SPSS software package 16.0 for Windows (SPSS Inc., Chicago, USA). When the t-test was significant, means were separated by Duncan’s multiple range tests (\( p \leq 0.05 \)).
5. Results

5.1. Grape berry characterization

The fresh weight (FW) and dry weight (DW) increased along the phenological stages from pea size stage to full maturation (Table 4). Significant differences could be observed between the irrigation treatments and two sides of canopy at véraison and full maturation stage. Total soluble solids (TSS, °Brix) increased along phenological stages as seen in Table 4. Significant differences were observed only at véraison and full maturation stage, in what concern total soluble solids content between irrigation treatments and cluster position within the canopy (e.g. east and west sides).

### Table 4: Effect of different irrigation regime on the Fresh (FW), Dry weight (DW), °Brix, Titrable acidity (TA) and pH for different phenological stages.

<table>
<thead>
<tr>
<th>Berry Properties</th>
<th>Irrigation Treatment</th>
<th>Pea Size</th>
<th>Véraison</th>
<th>Mid Ripening</th>
<th>Full Maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW (gram/berry)</td>
<td>East</td>
<td>0.76±0.02</td>
<td>1.35±0.07</td>
<td>1.81±0.08</td>
<td>2.01±0.02</td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>0.87±0.05</td>
<td>1.03±0.01</td>
<td>1.67±0.10</td>
<td>1.98±0.18</td>
</tr>
<tr>
<td></td>
<td>SDIE</td>
<td></td>
<td>1.09±0.04</td>
<td>1.79±0.07</td>
<td>1.78±0.10</td>
</tr>
<tr>
<td></td>
<td>RDIW</td>
<td></td>
<td>1.26±0.02</td>
<td>1.64±0.08</td>
<td>2.15±0.06</td>
</tr>
<tr>
<td></td>
<td>East</td>
<td>0.077±0.002</td>
<td>0.235±0.015</td>
<td>0.368±0.016</td>
<td>0.272±0.002</td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>0.089±0.004</td>
<td>0.172±0.003</td>
<td>0.327±0.02</td>
<td>0.271±0.024</td>
</tr>
<tr>
<td></td>
<td>SDIE</td>
<td></td>
<td>0.197±0.01</td>
<td>0.363±0.018</td>
<td>0.243±0.015</td>
</tr>
<tr>
<td></td>
<td>RDIW</td>
<td></td>
<td>0.217±0.004</td>
<td>0.321±0.017</td>
<td>0.303±0.011</td>
</tr>
<tr>
<td>°Brix</td>
<td>East</td>
<td>5.87±0.74</td>
<td>14.97±0.03</td>
<td>18.60±0.25</td>
<td>21.03±0.41</td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>6.10±0.80</td>
<td>12.73±0.37</td>
<td>17.53±0.47</td>
<td>20.47±0.29</td>
</tr>
<tr>
<td></td>
<td>SDIE</td>
<td></td>
<td>15.60±0.31</td>
<td>18.60±0.35</td>
<td>22.07±0.18</td>
</tr>
<tr>
<td></td>
<td>RDIW</td>
<td></td>
<td>13.27±0.18</td>
<td>18.13±0.07</td>
<td>22.73±0.18</td>
</tr>
<tr>
<td>Titrable Acidity (g L⁻¹)</td>
<td>East</td>
<td>16.45±0.05</td>
<td>8.03±0.04</td>
<td>5.18±0.13</td>
<td>5.43±0.16</td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>16.58±0.04</td>
<td>9.25±0.11</td>
<td>4.69±0.21</td>
<td>5.73±0.17</td>
</tr>
<tr>
<td></td>
<td>SDIE</td>
<td></td>
<td>7.38±0.44</td>
<td>4.73±0.06</td>
<td>4.73±0.17</td>
</tr>
<tr>
<td></td>
<td>RDIW</td>
<td></td>
<td>8.95±0.22</td>
<td>4.58±0.23</td>
<td>4.55±0.11</td>
</tr>
<tr>
<td>pH</td>
<td>East</td>
<td>2.72±0.01</td>
<td>3.07±0.01</td>
<td>3.47±0.05</td>
<td>3.41±0.04</td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>2.69±0.02</td>
<td>3.03±0.01</td>
<td>3.48±0.09</td>
<td>3.46±0.01</td>
</tr>
<tr>
<td></td>
<td>SDIE</td>
<td></td>
<td>3.10±0.02</td>
<td>3.55±0.08</td>
<td>3.51±0.01</td>
</tr>
<tr>
<td></td>
<td>RDIW</td>
<td></td>
<td>3.10±0.02</td>
<td>3.53±0.02</td>
<td>3.54±0.01</td>
</tr>
</tbody>
</table>

Titrable acidity (TA) expressed as g L⁻¹ of tartaric acid equivalent. SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. The values are represented as mean± SE. Different letters (a, b, c, ab, bc) indicate significant differences among treatments using Duncan’s test (p ≤ 0.05).

Titrable acidity (TA) result showed that the acidity remarkably decreased from the pea size stage to the full maturation stage (Table 4). No significant differences between the east and west side were observed at the pea size stage, however significant differences in acidity content between the different irrigation and berry side treatments were observed at véraison, mid-ripening and at full maturation stages. The pH of grape juice was virtually same and increased after véraison till mid ripening and
decreasing slightly up to the full maturation. Final juice pH did not differ by more than 0.06 pH units among the treatments. There was no apparent trend among treatment (Table 4).

5.2. Grape berry cuticle characterization

5.2.1. Quantitative changes in the wax content

Wax weight per unit surface was estimated in berries over the different developmental stages and is represented in Figure 6. It was found that maximum wax content was obtained at the pea size stage.

Figure 6: Wax content (µg.mm$^{-2}$) for different phenological stages. SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. PS- pea size; V- Véraison; MR- Mid Ripening; FM- Full Maturation. The values are represented as mean± SE.

At the pea size stage, no significant differences between the east and west side were observed. However it was observed a tendency of waxes to be higher at west side compared to east side.

Véraison stage showed a highly reduced wax content in both treatments as compared to pea size stage. In addition, RDI presented slightly lower content of waxes as compared with SDI, though the differences were not significant. SDI west had relatively lesser waxes compared to its east side and no differences in the wax content between east and west side were observed in RDI.

At mid ripening stage, the wax content increased as compared to véraison stage. No significant differences due to berry position were observed in SDI treatment. However RDI east had significantly higher wax content compared to the west side.

At full maturation, wax content of SDI east was higher when compared to the west side. However, the opposite trend was observed in RDI treatment with west side of the treatment having more wax content and the difference was quite significant between the east and west side of the RDI treatment.

5.2.2. Cuticular water permeability during grape berry fruit development

Cuticle permeance of the grape berry was determined during the different developmental stages both in intact and in de-waxed berries (Table 5).
Cuticle permeance of intact berries was maximal at pea size stage, and gradually decreased along berry development (Table 5). At pea size stage, the berries belonging from the west side showed increased permeance compared with berries belonging from the east side. However, the differences were not significant. At véraison, permeance decreased by up to 26% compared to the pea size stage. Significant differences between the irrigation type and canopy sides were observed. SDIE showed the lowest cuticle permeance. At mid ripening, although a tendency of higher permeance of berries belonging from the west side of canopy in the two irrigation treatments (SDI and RDI) were observed, no significant differences were recorded. At full maturation, no significant differences between the irrigation treatments or two sides of the canopy was recorded. Overall, the permeance decrease at maturation stage was about 34% compared to the pea size stage.

Table 5: Permeance for water (×10^-5 ms^-1) of untreated and chloroform treated de-waxed grape berry var. Aragonez for different phenological stages.

<table>
<thead>
<tr>
<th>Category</th>
<th>East</th>
<th>West</th>
<th>SDIE</th>
<th>SDIW</th>
<th>RDIE</th>
<th>RDIW</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>1.26±.06</td>
<td>1.36±0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>V</td>
<td>0.93±0.01^a</td>
<td>1.08±0.02^b</td>
<td>1.04±0.03^b</td>
<td>1.08±0.02^b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>0.85±0.03^a</td>
<td>0.93±0.01^a</td>
<td>0.79±0.02^a</td>
<td>0.9±0.09^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FM</td>
<td>0.67±0.03^a</td>
<td>0.76±0.08^a</td>
<td>0.67±0.02^a</td>
<td>0.62±0.02^a</td>
<td></td>
</tr>
<tr>
<td>Treated -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dewaxed</td>
<td>V</td>
<td>2.4±0.24^a</td>
<td>2.45±0.12^a</td>
<td>2.23±0.17^a</td>
<td>2.07±0.06^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FM</td>
<td>1.83±0.21^a</td>
<td>2.15±0.26^a</td>
<td>1.7±0.16^a</td>
<td>2.78±0.56^b</td>
<td></td>
</tr>
</tbody>
</table>

SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. PS- Pea size stage; V- Véraison; MR- Mid Ripening; FM- Full Maturation. Treated berries are with waxes removed using gum arabic+chloroform. The values are represented as mean± SE. Different letters (a, b) indicate significant differences among treatments using Duncan’s test (p ≤ 0.05).

In contrast with intact berries, the permeance of de-waxed berries increased along berry development when waxes were removed (Table 5). No differences were observed between east and west sides at pea size stage. The permeance of de-waxed berries increased by approximately 1.2-fold at this stage compared to the intact berries (Table 5). At véraison, the cuticle permeance was higher compared to the pea size. However no significant differences between the treatments were observed. The cuticle permeance of de-waxed berries increased approximately 2-fold as compared with the intact berries. At full maturation, no significant differences between the two sides were observed in SDI treatment. However in RDI, west side presented significantly higher permeance than the east side. At this stage, the permeance of de-waxed berries increased upto 4-fold than untreated berries.

5.2.3. Ultra-structure of grape berry cuticle using transmission electron microscopy (TEM)

The ultra-structure of grape berry cuticle examined during the different developmental stages and under the different treatments using TEM are shown in Figures 7-10. Right from the onset of pea size stage, the formation of cuticle is visible and is often seen with minute cuticular ridges. At pea size
stage (berries with diameter of 1.9 cm), the average thickness of the cuticle was about 2.7 µm. Cuticle thickness increased to around 3 µm at véraison (berries with diameter of 2.1 cm).

Figure 7: Transmission electron micrographs of transverse sections of grape berry cuticle at pea size (PS) East (a), West (b). EW- Epicuticular Waxes; CW- Cell wall; IC- inner cuticle; C- Cuticle; P- Pectin like material. Arrows indicate EW. Bar: 0.5 µm.
Figure 8: Transmission electron micrographs of transverse sections of grape berry cuticle at véraison (V)-SDIE (a), SDIW (b), RDIE (c), RDIW (d). EW- Epicuticular Waxes; CW- Cell wall; IC-inner cuticle; C- Cuticle; P- Pectin like material. Arrows indicate EW. Bar: 0.5 µm
Figure 9: Transmission electron micrographs of transverse sections of grape berry cuticle at mid-ripening (MR)- SDIE (a), SDIW (b), RDIE (c), RDIW (d). EW- Epicuticular Waxes; CW- Cell wall; IC-inner cuticle; C-Cuticle; P- Pectin like material. Arrows indicate EW. Bar: 0.5 µm
Figure 10: Transmission electron micrographs of transverse sections of grape berry cuticle at Full maturation (FM)- SDIE (a), SDIW (b), RDIE (c), RDIW (d). EW- Epicuticular Waxes; CW- Cell wall; IC- inner cuticle; C- Cuticle; P- Pectin like material. Arrows indicate EW. Bar: 0.5µm.
The outer most zone of the berry skin in the pea size stage is constituted by a convoluted and electron light amorphous layer with rugose appearance of variable thickness (0.3-0.4µm), corresponded to the epicuticular waxes. Beneath this wax layer in all the stages, cuticle and inner cuticle can be distinguished, though the differentiation might not be clearly visible. Cuticle is generally seen as an amorphous layer above the cell wall material. On the other hand inner cuticle includes all cuticle without the epicuticular wax layer as seen in Figure 7-10. Inner layer formed more than three-fourth of the cuticle. Inner cuticle layer present on the inner most side of the cuticle surface appears more electron dense and reticulate probably due to the incrustation formed due to the polysaccharides or other cell-wall materials including pectinaceous materials as seen in Figure 7-10. The details of the TEM analysis are presented in the Table 6. Five to ten images with 10-15 measurements for each TEM images of the berry skin have been analysed and differentiated into 5 different parameters to draw comparison between different phenological stages and between different treatment types. The parameters under study were: 1) Cuticle; 2) Epicuticular waxes; 3) Inner cuticle; 4) Cell wall; 5) Dark pectin like material. All these parameters have been clearly demarcated and showed in the Figure 7-10.

5.2.3.1. Cuticle

Cuticle increases from pea size to véraison stage and decreases thereafter (Table 6). At pea size stage, there were no significant differences between the two sides: east and west. The maximum cuticle thickness was observed at véraison and significant differences between the irrigation regimes and side of the canopy were observed (Table 6). The cuticle thickness decreased from véraison to the full maturation. Significant differences between the irrigation type and two sides of canopy were observed at mid ripening and full maturation with west side having significantly higher thickness compared to the east side.

5.2.3.2. Epicuticular waxes

Epicuticular waxes increased from pea size stage to full maturation stage (Figure 11). At pea size, no significant differences were observed between the two canopy sides. Contrasting with results of extractable soluble waxes referred above (section 5.2.1.), the thickness of epicuticular waxes increased from pea size to véraison. At véraison, SDIE showed the lowest epicuticular waxes thickness while RDIW showed the highest. At mid-ripening, epicuticular waxes increased and RDIW showed significantly higher thickness compared to all the other treatments. Trend continued at full maturation stage and the thickness increased with significant differences between irrigation type and two sides could be observed. In general the west side had higher epicuticular wax thickness than the east side, and RDIW had the highest thickness.
5.2.3.3. Inner cuticle

Inner cuticle showed similar trend of increase as the cuticle and thickness increased from pea size to véraison. However no significant differences were observed between the irrigation treatments and sides of the canopy, both at pea size and véraison. From véraison, the thickness of inner cuticle decreased to full maturation and significant differences were observed between the two irrigation treatments (SDI and RDI) and canopy sides. At full maturation, RDI in general showed the higher inner cuticle being RDIW the highest one (Table 6). Overall from the irrigation treatment, the thickness of inner cuticle on the west side was significantly higher than the east side.

5.2.3.4. Cell wall

Cell wall increased from pea size to véraison and decreased thereafter (Table 6). Differences between the irrigations (SDI and RDI) and canopy sides (East and West) were significant at all stages except at pea size. Since véraison onward, the berries belonging from the west side showed significantly higher cell wall thickness compared to the east side. Thickness of cell wall was significantly lesser in RDI compared to the SDI at mid ripening and full maturation.

5.2.3.5. Dark pectin like layer

A dark and amorphous layer was observed at cell wall and inner cuticle interface which was likely to be pectinaceous material. This pectin like dense layer increased in thickness from pea size to full maturation stage. At pea size, thickness of this dense layer showed no significant differences between the two sides. At véraison the pectinaceous material increased and was higher in SDIW in respect to SDIE and RDIW in respect to RDIE. Pectinaceous layer further increased at mid ripening but no significant differences were observed between all treatments. At full maturation, pectinaceous layer reached its maximum thickness, and SDIE showed the lowest thickness (Table 6).
Table 6: TEM analysis of the grape berry skin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pea Size</th>
<th>Véraison</th>
<th>Mid Ripening</th>
<th>Full Maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cuticle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>2.67±0.05 SDIE</td>
<td>2.87±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.72±0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.41±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>West</td>
<td>2.78±0.04 SDIW</td>
<td>3.00±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.92±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.83±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inner cuticle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>2.24±0.02 SDIE</td>
<td>2.42±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.08±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.86±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>West</td>
<td>2.43±0.03 SDIW</td>
<td>2.54±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.27±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.21±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell Wall</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>0.99±0.01 SDIE</td>
<td>1.01±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>West</td>
<td>1.04±0.02 SDIW</td>
<td>1.14±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99±0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.98±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inner cuticle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>0.55±0.02 SDIE</td>
<td>0.61±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>West</td>
<td>0.56±0.02 SDIW</td>
<td>0.69±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pectin like layer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>0.74±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.78±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>West</td>
<td>0.74±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.78±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. PS- pea size; V- Véraison; MR- Mid Ripening; FM- Full Maturation. The values (thickness in µm) are represented as mean± SE. Different letters (a, b, c, ab, bc) indicate significant differences among treatments using Duncan’s test (p ≤ 0.05).

5.2.4. Spectral characterization of cuticle, skin and cutin

The FTIR spectral analysis of isolated grape berry cuticle allowed the identification of several band characteristic of plant cuticle. Figure 12-14 shows the FT-IR spectrum between 4000 and 600 cm<sup>-1</sup> for an isolated grape berry cuticle, skin, and cutin respectively. The complex FTIR spectrum shows several absorption bands. The assignments for these bands were facilitated by previous data based on chemical composition of the cuticle and the study made on different isolated cuticular membranes (Espana et al., 2014; Luque et al., 1993; Ramírez et al., 1992).

5.2.4.1. Spectroscopic characterization of cuticle

The cuticle (both inside and outside) of grape berry showed broad medium intensity peak at 3300 cm<sup>-1</sup> and a weak band at approximately 2921 cm<sup>-1</sup> (Figure 12). The broad band is indicative of the hydroxyl group stretching vibrations where hydroxyl group is hydrogen bonded. Absorption bands at 2921 cm<sup>-1</sup> corresponded to asymmetrical vibrations of methylene (CH<sub>2</sub>) groups, ν<sub>a</sub>(CH<sub>2</sub>).

Weak IR absorption bands were also observed at around 3050 cm<sup>-1</sup> which corresponds to carbon-hydrogen stretching vibrations in aromatic constituents, namely the benzene derivative molecules. These bands were accompanied by the corresponding δ(CH<sub>2</sub>) bending vibrations at around 1380 and 761 cm<sup>-1</sup> that are potentially associated with hemicelluloses. These bands in general were ascribed to the aliphatic material present in the plant cuticle which are cutin, waxes and cutan.

Weak band around 1496 cm<sup>-1</sup> was assigned to aromatic stretching vibrations, ν(CC). A weak band at around 1728 cm<sup>-1</sup> corresponding to the carbon-oxygen stretching vibrations, ν(C=O) of ester groups.
accompanied by a single band at around 1166 cm\(^{-1}\) attributed to the asymmetrical (or symmetrical) C-O-C stretching ester vibrations. These bands were associated to the cutin matrix made by esterified bonds: i.e. the bond that links the different hydroxyl and epoxy fatty acids to form the cutin cross-linking (Holloway, 1982). These bands showed variable intensity depending on the treatment type and varied along the phenological stages with no significant variations as seen in Figure 12.

In addition to these important bands, other minor absorptions were also observed. Bands in the region of 1650-1500 cm\(^{-1}\) spectral region with variable intensity were related to the aromatic domain of the cuticle, mainly formed by phenolic compounds and C=C functional groups which confirms the presence of benzene derivative molecules. The strong band around 1600 cm\(^{-1}\) assigned to aromatic stretching vibration was generally found along all treatments and in all phenological stages (Figure 12). The presence of medium intensity band appears around 1436 cm\(^{-1}\) corresponding to an aromatic C-C stretching vibration is characteristic of the aromatic ring conjugated with unsaturated groups.

Some bands between 1300-1200 cm\(^{-1}\) assigned to the oxygen-hydrogen bending vibrations, \(\delta(OH)\) and these bands overlap to some degree with the neighbouring band at around 1218 cm\(^{-1}\) which is assigned to \(\nu(CC)\) vibration while band at around 1270 cm\(^{-1}\) is assigned to \(\delta(OH)\).

The \(\nu(C-O)\) of alcoholic group appear around 1090 cm\(^{-1}\). This band appeared as weak, narrow band. Band at around 830 cm\(^{-1}\) corresponded to the carbon-hydrogen and carbon-carbon out of plane bending vibrations. Some variations between the treatments were also observed. The general tendency of RDI having higher intensity of bands compared to the SDI with west side having stronger band absorption compared to the east side was observed. However, some minor deviation from the general trend was seen in pea size stage where two sides of canopy showed opposite trend. Also the two sides of cuticle showed differences in intensities of the absorption bands with outer side of cuticle had stronger band absorption compared to the inner side.

FT-IR spectra of de-waxed cuticle was also studied in order to see the differences between the intact cuticle and cuticles with the waxes removed. After wax removal, the intensity of bands associated with the C-H groups reduced (Figure 15).
Figure 12: Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) of inner and outer side of cuticle. Pea size (a); Véraison(b); Mid ripening(c); Full maturation(d). SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. Red- SDIE; Blue- SDIW; Green- RDIE; Black-RDI W. Continuous line shows inner side of the cuticle, shaded line is outer side of the cuticle. Arrows indicate important peaks: (a) 2925 cm$^{-1}$; (b) 1722 cm$^{-1}$; (c) 1602 cm$^{-1}$ (d) 1027 cm$^{-1}$. A1-A4 in legend of the FTIR spectra represents four phenological stages from Pea Size to Full Maturation respectively.
5.2.4.2. Spectroscopic characterization of skin

The cuticle of grape berry was also compared with the berry skin. The ATR-FTIR of berry skin showed that most of spectral properties of berry skin were similar to the cuticle with more intensity. The bands in the region of 1800 to 900 cm\(^{-1}\) are corresponding to the polysaccharide nature of the skin (Figure 13). The peak vibrations at around 1099 cm\(^{-1}\) and 1168 cm\(^{-1}\) were assigned to the \(\nu(C-O-C)\) stretching vibration corresponding to ester linkages while band at around 896 cm\(^{-1}\) was assigned to \(\gamma(C-H)\) bending(out of plane) corresponding to aromatic compounds. These bands may constitute pectin like substances observed as an extension of cell wall in TEM images. Bands had higher intensity on the west side compared to the east at pea size and \(\text{véraison}\), whereas east side of both irrigation treatments (SDI and RDI) tend to have higher intensity of these bands compared to the west side at mid-ripening and full maturation stages. Also no consistent pattern in the intensity could be observed in the difference between the irrigation treatments as well. Bands in the “fingerprint” region (600-1500 cm\(^{-1}\)) showed some spectral differences but these differences did not show any significant pattern of differences along the berry ripening.
Figure 13: Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) of skin of grape berries (*Vitis vinifera*, cv. Aragonez). Pea size (a); Véraison (b); Mid ripening (c); Full maturation (d). SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. Red- SDIE; Blue- SDIW; Green- RDIE; Black-RDI W. Spectra on the top is the enlarge view of the right panel in range of 900-1800 cm\(^{-1}\) characteristic of polysaccharides. A1-A4 in legend of the FTIR spectra represents four phenological stages from Pea Size to Full Maturation respectively.

The additional peak at around 1525 cm\(^{-1}\) was found in the skin samples which were not present in the cuticles. This peak was assigned to the stretching vibration, \(\nu(C-C)\) of aromatic compounds which constitute phenolic compounds. The intensity of this band increased along the berry ripening. It was also seen that intensity of this band was higher on east side of the two irrigation treatments compared to the west side. Also RDI had higher intensity of band at 1525 cm\(^{-1}\) compared to SDI (Figure 13).

5.2.4.3. Spectroscopic characterization of cutin

The IR spectrum of cutin is characterized by broad medium to strong intensity band at around 3250 cm\(^{-1}\) (Figure 14). This broad band is indicative of hydroxyl group stretching vibrations where hydroxyl group is hydrogen bonded. The depolymerised cutin showed a very weak band at around 2918 and 2848 cm\(^{-1}\). Alkaline hydrolysis leads to depolymerised cutin which showed shifted peak at around 1580 cm\(^{-1}\) which were generally found around 1600 cm\(^{-1}\) in the cuticle. Several weak peaks corresponding to \(\delta(CH_2)\) bending vibrations at around 1380 cm\(^{-1}\) in cuticle were missing (Figure 14).
Instead the methylene groups had three bending vibrations: wagging, twisting and rocking vibrations. In IR spectra, these modes were assigned to single strong peak at around 1380 cm\(^{-1}\) which was found in all phenological stages. The shape and relative intensities of the different peaks located at 1143, 1091 and 1006 cm\(^{-1}\) can be clearly assigned to the carboxylic acid derivatives with \(\nu\)(O-C) stretching vibrations and O-H deformations in secondary alcohols and define the infrared cellulose fingerprint. The characteristic shifted peak at 1580 cm\(^{-1}\) and the corresponding band present near 829 cm\(^{-1}\) and 702 cm\(^{-1}\) may be related to phenolic compounds and aromatic compounds present in the cutin (Figure 14).

The spectral comparison of cuticle, de-waxed cuticle, skin and depolymerised cutin is represented in Figure 15. De-waxed cuticle showed peak around 3300 cm\(^{-1}\) assigned to hydroxyl (OH) group which was of lesser intensity compared to the intact cuticle. However skin showed band vibration stronger than cuticle and de-waxed cuticle. Depolymerised cutin showed strong band intensity at around 2900 cm\(^{-1}\) assigned to methylene group which are exposed after depolymerisation of cutin.
5.2.5. Expression profiling of cuticle related genes

The mRNA expression levels of fatty acid elongase 3-ketoacyl-CoA synthase (VviKCS1), very-long-chain fatty acid β-ketoacyl-CoA synthase (VviKCS6), VviGDSL, Ubiquitin ligase (VvCER9), lipid transfer protein 3 (VviLTP3), multifunctional oxidases (VviCYP716A15, and VviCYP716A17) were determined by real time quantitative PCR during different phenological stages subjected to different treatment: two irrigation regimes and two sides of canopy. Transcripts for each of the studied genes showed different expression pattern under different treatment along the berry ripening.

5.2.5.1. Expression level of VviKCS1 gene in the cuticle of grape berry during different phenological stages

The expression of VviKCS1 was detected in the berry in all treatments since pea size except at the full maturation stage (Figure 16). The expression for VviKCS1 increased at véraison and decreased thereafter. No significant differences in the expression level were observed between the treatments (irrigation and cluster position) along the berry development.

![Figure 16: Expression of the gene VviKCS1 in grape berry cuticle of SDI and RDI on two sides of the canopy: East and West. Values presented are means ± SE (n ≥ 3). PS- Pea size (7 weeks after anthesis); V- Véraison(9 weeks after anthesis); MR- Mid Ripening(11 week after anthesis); FM- Full Maturation(13 weeks after anthesis).](image)

5.2.5.2. Expression level of VviKCS6 gene in the cuticle of grape berry during different phenological stages

The transcripts of VviKCS6 were detected in grape berry since pea size in all treatments and afterwards its expression level was maintained till mid-ripening and decreased at full maturation (Figure 17). No significant differences between irrigation regimes and canopy sides were observed.
5.2.5.3. Expression level of VviGDSL gene in the cuticle of grape berry during different phenological stages

The transcripts of VviGDSL were detected since pea size and the expression peaked at véraison and decreased thereafter till the full maturation as seen in Figure 18. Water deficit and side of canopy had no significant differences in the expression level of VviGDSL.

5.2.5.4. Expression level of VviCER9 gene in the cuticle of grape berry during different phenological stages

VviCER9 was expressed in all phenological stages except at full maturation (Figure 19). The VviCER9 attained its maximum expression at véraison. Significant differences were observed at pea size, being the west side up-regulated in relation to the east side. At mid ripening the VviCER9 was only expressed in RDI berries (east and west).
Figure 19: Expression of the gene VviCER9 in grape berry cuticle of SDI and RDI on two sides of the canopy: East and West. Values presented are means ± SE (n ≥ 3). PS- Pea size (7 weeks after anthesis); V- Véraison (9 weeks after anthesis); MR- Mid Ripening (11 weeks after anthesis); FM- Full Maturation (13 weeks after anthesis).

5.2.5.5. Expression level of VviLTP3 gene in the cuticle of grape berry during different phenological stages

Transcripts of VviLTP3 were detected in the grape berry cuticle since pea size. VviLTP3 showed peak of expression at Véraison with up-regulation in RDIW berries. VviLTP3 expression was downregulated thereafter till full maturation in all treatments (Figure 20). At full maturation, significant differences in the expression were observed where VviLTP3 showed higher expression under RDIW when compared to all the other treatments.

Figure 20: Expression of the gene VviLTP3 in grape berry cuticle of SDI and RDI on two sides of the canopy: East and West. Values presented are means ± SE (n ≥ 3). PS- Pea size (7 weeks after anthesis); V- Véraison (9 weeks after anthesis); MR- Mid Ripening (11 weeks after anthesis); FM- Full Maturation (13 weeks after anthesis).

5.2.5.6. Expression level of VviCYP716A15 gene in the cuticle of grape berry during different phenological stages

The transcripts of VviCYP716A15 were detected in the berry cuticle in all phenological stages (Figure 21). The expression of VviCYP716A15 peaked at Véraison and decreased thereafter. Significant differences between the two irrigation treatments and two sides of canopy were observed at Véraison and mid ripening.
Figure 21: Expression of the gene *VviCYP716A15* in grape berry cuticle of SDI and RDI on two sides of the canopy: East and West. Values presented are means ± SE (n ≥ 3). PS- Pea size (7 weeks after anthesis); V- Véraison (9 weeks after anthesis); MR- Mid Ripening (11 weeks after anthesis); FM- Full Maturation (13 weeks after anthesis).

5.2.5.7. Expression level of *VviCYP716A17* gene in the cuticle of grape berry during different phenological stages

*VviCYP716A17* was expressed in all phenological stages with maximum expression at *véraison* (Figure 22). No expression was seen in SDIE at full maturation stage. Both at *véraison* and full maturation stages *VviCYP716A17* was up-regulated in RDI berries.

Figure 22: Expression of the gene *VviCYP716A17* in grape berry cuticle of SDI and RDI on two sides of the canopy: East and West. Values presented are means ± SE (n ≥ 3). PS- Pea size (7 weeks after anthesis); V- Véraison (9 weeks after anthesis); MR- Mid Ripening (11 weeks after anthesis); FM- Full Maturation (13 weeks after anthesis).
6. Discussion

The present investigation was carried out in order to characterize the grapevine berry cuticle (*Vitis vinifera* cv. Aragonez) under water deficit conditions and analyse the component of berry cuticle such as waxes and the role they play in regulating the loss of water from the surface of the berries. Several previous studies have been conducted describing the effect of water deficit on berry quality and development (Reviewed by Chaves et al., 2010) but no studies have been reported regarding the role of cuticle in grape berries and the relation between the cuticle component and water stress remain elusive to-date. Cuticle studies have so far been limited to *Arabidopsis* and more recently to tomato fruits but this characterization of cuticle in grape berries would be the first such study in a non-climacteric fruit.

6.1. Water deficit affects berry qualities

The results show the increase in berry fresh and dry weight along the ripening. Water deficit conditions are already reported to interfere in the cell division process resulting in lesser fresh weight of berries (Thomas et al., 2006) which is in accordance with our data. The total soluble solids (TSS) are one of the most important grape wine qualities that are affected under water deficit conditions. Our results are in accordance with Deluc et al. (2009) and Okamoto et al. (2004) who also reported the increase in total soluble sugar in grape berries under deficit conditions. TSS and acidity showed more marked increase/decrease in the RDI at véraison stage which suggests that the onset of ripening in RDI berries is delayed in comparison to the SDI berries as reported by Zarrouk et al. (2012). The titrable acidity (TA) results obtained in the present study are also in accordance with the previous studies wherein several authors have reported that the TA decreases with the different phenological stages with maximum decrease reported at the onset of ripening (De la Hera-Orts et al., 2005; Sun et al., 2010). Several studies previously conducted have shown that reduced acidity content may be due to the additive result of a reduced synthesis of malic and tartaric acid during the berry development (Blouin and Guimberteau, 2000; Esteban et al., 1999; Souza et al., 2005). The higher temperature responsible for higher malic acid respiration rate due to more exposure to the sunlight may account for this effect. In our present study, berries belonging from the west side experienced higher temperature compared to the east side due to more hours of exposure to the sunlight (Appendix III), which affects the acidity content, being comparatively lower on the west side than the east one in both irrigation regimes at mid ripening and full maturation. RDI treatment shows always the lower acidity compared to the SDI. This is in accordance to several literature that have shown that increased water deficit had resulted in lower TA value as in the case of RDI where the water stress was more pronounced compared to the SDI (Deluc et al., 2009; Zarrouk et al., 2012).

6.2. Water stress affects cuticular wax accumulation and cuticular permeability

The analysis of wax content per unit surface area suggests that maximum wax accumulation occurred at the early stages of growth and decreases at véraison. Nonetheless it is important to keep in mind that véraison stage is characterized by rapid enlargement of berries which is probably not accompanied by similar rate of wax deposition. At mid-ripening stage, the wax content increases as
compared with *véraison* in all treatments but decreases at full maturation. These results imply the possibility of a new wax biosynthesis after *véraison*. However, the reason for a less wax content at full maturation remains unclear. The inconsistency in the wax deposition along development had also been reported previously by Yamamura and Naito (1983) who reported an increase of wax content in Delaware berry variety during early pre-*véraison* stages that remained constant thereafter. In contrast, Radler (1965) reported no change in the total waxes (1 μg mm⁻²) during berry growth. In Pinot Noir berries, wax content increased across berry development (Comménil *et al.*, 1997). Our results for Aragonez suggest that maximum wax synthesis occurs during early berry development stages and rate of synthesis/deposition reduces in the late stages. The contrasting reports on the amount of wax per unit surface area could be the consequence of variability in wax extraction methods which interferes with the solubility of the waxes in the solvent used. Corroborating these hypotheses, the ultrastructure analysis using TEM revealed that thickness of wax layer increased along berry development and significant effect of irrigation and side of canopy treatments was observed (Figure 11). It is suggested that nature of waxes is changing along berry development which is reflected by the variation in their solubility in chloroform.

The relation between the wax accumulation and transpirational barrier properties of the grape berry cuticle had been studied for the first time to the best of our knowledge. Several previous studies on the grape berries have reported the relationship between the berry quality and metabolite accumulation under water deficit and high temperature (Deluc *et al.*, 2009; Intrigliolo and Castel, 2010; Zarrouk *et al.*, 2012). However no study so far had reported the relationship between the wax characteristics and the transpirational barrier properties of the cuticle in grape berry, although some information about this topic had been coming from other climacteric species such as tomato fruit (Leide *et al.*, 2007). In the present findings, the increase of permeance rate along developmental stages of de-waxed berries contrast with the decrease of permeance rate along ripening of untreated berries. The cuticular permeance of untreated berries ranged between 1.26±0.06 x 10⁻⁵ ms⁻¹ at pea size stage to 0.62±0.02 x 10⁻⁵ ms⁻¹ at full maturation stage. For de-waxed berries, permeance was higher and it ranged from 1.46±0.08 x 10⁻⁵ ms⁻¹ at pea size stage to 2.78±0.56 x 10⁻⁵ ms⁻¹ at full maturation stage. For untreated berries, the west side of both SDI and RDI treatments showed highest water loss by transpiration. This suggests that berries from west side of both irrigation treatments experiencing higher temperature, tend to transpire more as compared with east side. Moreover, the absence of differences in the transpirational rate between sides in intact berries compared with de-waxed ones suggests the importance of waxes as effective barrier to water loss (Leide *et al.*, 2007; Schreiber and Riederer, 1996) and also to overcome with the heat stress condition. Water stress conditions causes closure of leaf stomata which may lead to increase in internal temperature of plant and in consequence of berry inducing heat stress (Zarrouk *et al.*, 2012). Results of TEM images, supports this hypothesis. In fact epicuticular wax thickness was higher on the west side berries than the east side. In addition, at full maturation stage, berries belonging from RDW had lower permeance compared to the RDIW. This result suggests the additive effect of deficit irrigation to heat stress in what concern the wax synthesis and/or composition to improve the berry water loss protection as reported in *Arabidopsis* leaves by Kosma *et al.* (2009). Taking into account only data of soluble waxes, our results suggest a negative
relation between the wax content (Figure 6) and permeance level (Table 5) as described for the tomato fruits by Leide et al. (2007). Our study may also suggest that both wax compositions as well as wax quantity are important for the transpirational barrier properties of cuticle in grape berries.

6.3. Water deficit affects the architecture of berry cuticle

The result of TEM analysis showed that grape berries undergoes several modifications during ripening. Additionally, several differences due to the irrigation treatment and the sides of the canopy were observed. These changes in the berry skin and cuticle in particular, might be an adaptive response of the fruit surface under environmental stress that includes both water and heat stresses. The cuticle is present in all phenological stages, in accordance with previous studies (Casado and Heredia, 2001; Considine and Knox, 1979). The authors reported a continuous synthesis and degradation of this lipophilic material and its physiological role as a mechanical support. However, in contrast with the results herein presented, an increase in cuticle thickness was reported along berry development (Casado and Heredia, 2001). Nonetheless, Comménil et al. (1997) studied the ultrastructure of berry cuticle and found that the cuticle thickness decreases between véraison and full maturation, which corroborate our TEM analysis. Our results show a slight increase in thickness from pea size to véraison stage and a decrease thereafter. This inversion in the cuticle thickness trend could be associated with the berry growth curve (Figure 1). Development of the berry occurs in two distinct phases of growth (Considine and Knox, 1979); first between anthesis upto véraison and second between véraison till it attains maturity. The second phase is marked by an extensive growth of fruits associated with the increase in plasticity of fruit. Increase in cuticle thickness at véraison had also been reported by Rajaei (1987) but these authors did not observe any variations of whole cuticle until fruit maturation. As mentioned earlier, previous studies (Considine and Knox, 1979) have reported identical cuticle thickness throughout the life of berry growth which is not the case in our present study. Cuticle is largely heterogeneous and dynamic structure with several distinct layers. Therefore it is not appropriate to study the entire cuticle membrane as a single entity without differentiating it into its different layers. Most importantly, each grape variety present distinct genetic makeup which could also present different physiology and adaptive response under stress and this could not be ruled out as a possible reason for the differences observed between the different studies done on grape berries (Comménil et al., 1997). Thickness of cell wall increased from pea size to véraison but decreased slightly between véraison and full maturation. This can largely be attributed to the rapid synthesis of cell wall material and polysaccharides during the early stages of berry development necessary for berry growth in size, volume and firmness. However along the berry growth and after the véraison, most of cell wall material is synthesized and cellular machinery utilizes its energy to synthesize material necessary to withstand environmental conditions like abiotic stress. It is therefore possible that most of the resources used for cell wall deposition might be diverted to the synthesis of epicuticular waxes which are first line of defence against water loss and possible pathogen attack. Interestingly, recent studies also reveal that pectins are important for maintaining water status by preventing transpirational loss under drought stress (Leucci et al., 2008; review by Le Gall et al., 2015 and Tenhaken 2014). Therefore much of the cell wall material might be utilized
towards fat material and pectin like material deposited on the surface of middle lamella of cell wall (Guzmán et al., 2014) as observed in our TEM images. Moreover, the overlapping layers between pectinaceous material and cell wall were not accounted for calculating the thickness of the cell wall.

6.4. Water stress enhanced the deposition of epicuticular waxes and other protecting materials (likely pectinaceous)

In the present study, epicuticular waxes have been reported to increase along the phenological stages. Waxes were much abundant at the west side compared to the east side which suggest that plant adapt to the high heat stress resulting due to the higher temperature on the west side (Appendix III) by synthesizing more waxes which also helps prevent excessive water loss. This increase in waxes is similar to what had been reported previously by Casado and Heredia (2001). Our results from the TEM analysis are contradictory to the wax obtained from chloroform extraction. This might be due to the fact that chloroform extraction of wax only yields surface waxes while TEM is the entire wax load on the cuticle surface which also include intracuticular waxes. Additionally it may suggest that chloroform solubilisation technique only yields a group of lipids and that the nature of waxes changed along the berry ripening.

Our TEM images revealed presence of dark and apparently amorphous layer stemming from the middle lamella of the cell wall (i.e., likely pectinaceous) into the cuticle as reported by Guzmán et al. (2014). Our personal communication with Dr. Dylan Kosma (University of Nevada) and Dr. Victoria Fernández (Technical University of Madrid), experts in cuticle and plant lipid polymers helped us to ascertain that this dark layer (Figure 7–10) is probably related to pectins which may have role in regulating water loss. TEM reveals that pectinaceous material deposition probably begins at the early stages of berry development and that their maximum thickness is attained at full maturation. The pectins are polysaccharide incrustations in the cell wall. Their high deposition along the phenological stages and more towards the west side support the enhanced adaptive mechanism of grape berries to withstand the water stress conditions by regulating the water stress as supported by some of the recent studies speculating the role of pectins in regulating the loss of water under stress conditions (Leucci et al., 2008; Review by Le Gall et al., 2015 and Tenhaken, 2014). However, further studies would be required to confirm the deposition of pectins on the cell wall in grape berry cuticle and how they regulate the water loss at molecular level.

6.5. FTIR study of berry cuticle, skin and cutin

The IR study showed that bulk of the cuticular membrane isolated is primarily aliphatic in nature. Nevertheless, the cuticular membrane contains detectable amount of aromatic compounds which corroborate previous data reporting the composition of grape berry cuticle with 30% of oleanolic acid (Casado and Heredia, 1999).

The intensity of methylene group (CH$_2$) is variable in the cuticle and it tends to increase slightly from pea size stage to véraison and upto the full maturation. This might be suggested due to the polymerization of cuticle components and more synthesis of waxes and cutin rich in this ubiquitous
structural component. However these weak bands at around 2900 cm$^{-1}$ also suggest that cuticle in grape berry is highly complex structure with cross linked aliphatic structure not easily detected through FTIR.

Weak bands at around 1728 cm$^{-1}$ corresponding to the esters are generally seen at same intensity across all phenological stages showing that esterification of cutin occurs as early as the pea size stage, followed by a marginal increase at the véraison. These results are well in consistent with the expression of VviGDSL gene required for the cutin synthesis which showed constitutive expression of VviGDSL along all stages with no significant differences between the irrigation regime and two canopy sides.

It is important to note here that grape berry cuticle is mostly comprised of waxes (upto 40%)(Casado and Heredia, 1999) and not much of the cutin. This is contrary to the tomato fruit whose cuticle comprises more than 80% of cutin and upto 5% of cuticular waxes (Ramírez et al., 1992).

Spectral differences between the two irrigation treatment (RDI and SDI) could be attributed to higher accumulation of waxes in the RDI compared to SDI. Two sides of cuticle also showed variation with outer side of the cuticle having stronger bands mainly due to the wax components more on the outer side than on the inner side. The spectral differences between the irrigation treatment and between inside and outside of the cuticle can further be attributed to the adaptive response of the berries under water stress conditions and as berries mature, they respond in better way with more synthesis of the wax and triterpenoid compounds on water stressed side. Absence of this trend at pea size stage could be due to a marginal wax layer formation at early stage of the berries growth. The results of FTIR might further substantiate the TEM analysis which showed wax deposition increased along the phenological stages with RDI having significantly higher thickness compared to the SDI. Similarly west side showed higher accumulation of wax compared to the east side.

The IR spectra showed characteristic absorbance of the hydroxyl group(-OH) around 3300 cm$^{-1}$ and absorption around 1026 cm$^{-1}$ corresponding to C-O-C vibrations which are characteristics of oleanane triterpenoid saponin (i.e. oleanolic acid) abundant in grape berry. These oleanane-type triterpenoid saponins are characterized by the C=O infrared absorbance due to oleanolic acid/ester but the ester linkages were not sufficiently detected by FTIR in our studies which might suggest that oleanane-type triterpenoids could be monodesmosidic saponins as detected in Entada leptostachya and Rapanea rhododendroides but these observations need further clarifications(Kareru et al., 2008). The peak intensity corresponding to these triterpenoids increased along the phenological stages which might suggest that amount of oleanolic increased from pea size stage to full maturation stage. The peak intensity corresponding to these triterpenoids increased along the phenological stages which might suggest that amount of oleanolic increased from pea size stage to full maturation stage which is in accordance with transcriptional data of both isoforms VviCYP716A15 and VviCYP716A17 responsible for oleanolic acid synthesis. Spectral differences in two sides of the cuticle related to these compounds could be attributed to the adaptive response of the berries under water stress conditions along ripening process. The increase of signal of triterpenoid in west side (more stressed side)
suggests the role of these compounds in the heat stress response. This trend disappeared at full maturation, and is in accordance with transcriptional data, where VviCYP716A15 and VviCYP716A17 were down-regulated at this stage. da Luz (2006) who studied leaf of Olea europea found oleanolic acid with the characteristic vibration at 1688, 1029 and 996 cm⁻¹.

TEM analysis showed dark layer (likely pectinaceous) deposited on the cell wall along the phenological stages and increased from pea size stage to full maturation stage. The cellulose and pectin bands exist in the region of 1800 to 900 cm⁻¹ (Chen et al., 1997; Wellner et al., 1998). The peak vibrations at around 896 cm⁻¹, 1099 cm⁻¹, 1168 cm⁻¹ could therefore be assigned to the pectin substances in skin. 896, 953, 1017, 1047, 1100, 1144 cm⁻¹ have been assigned to pectin polysaccharides in plant cell wall (Kacuráková et al., 2000). da Luz, (2006) and Wilson et al. (2000) assigned band at 1008 cm⁻¹ to the polygalacturonic acid, which is a variety of pectin. FTIR study of the berry skin showed that the intensity of the bands corresponding to the pectin substances showed variable intensity along the phenological stages. The presence of polysaccharides such as cellulose may be due to the partial molecular shield of the cellulose, presented in the high degree of crystallinity giving rise to the rest of the cuticular material (Villena et al., 2000).

Additional band around 1525 cm⁻¹ showed the presence of phenolic compounds in berry skin that intensity is differentially modulated by the side of canopy than by irrigation. This result suggests that irrigation did not have significant influence on phenolic compounds. However, higher temperature on west side tends to decrease the phenolic compounds as seen by the reduced intensity of bands on the west side. High temperature tends to impair the phenolic biosynthesis and accumulation (Zarrouk et al., 2012; Zarrouk et al., unpublished data) and slight variation in the intensity of peaks on two sides could be surmised due to the temperature variation on two sides.

Also the intensity of the bands increased along the phenological stages, consistent with the fact that mature berries accumulate higher phenolic compounds compared to the young berries.

The isolated berry cuticle was depolymerized in a KOH/Methanol(1%, w/v) solution, which yielded cuticular pieces with infrared absorptions as shown in Figure 14. The weak ester bonds have been broken and the corresponding hydroxyl fatty acids and some phenolics linked by ester bonds have been removed as seen by the absence of peak at around 1730 cm⁻¹. As seen in the spectra (Figure 14), the rest of the cuticular matrix is characterized by only three main chemical characteristics: a highly aliphatic composition, the aromatic domain and the presence of polysaccharides. The bands around 1500-1700 cm⁻¹ have significant contribution to this spectrum and appeared as a consequence of a putative hydrolysis of ester groups of the cutin (Heredia-Guerrero et al., 2014). The depolymerised grape berry cuticle spectra is similar to spectra of tomato cuticle after depolymerization with characteristic “fingerprint” infrared region (1000 cm⁻¹) as reported by López-Casado et al. (2007) and Villena et al. (2000).

It is also note worthy that the rate of depolymerization showed the tendency of decreasing from pea size to full maturation stage as observed by the intensities of the compounds identified in the spectral
peaks. However, the decline was not consistent and did not show any significant pattern explaining the irrigation type or the two sides of the canopy. However, this result supports the fragility of berry cuticle at early stages of growth that could easily degrade compared to the firm cuticle at full maturation. This also may explain the high rate of water loss by transpiration at the early stages of berry growth.

This behavior of depolymerized cuticular material is a clear demonstration of the complex nature of the plant cuticle showing that complete depolymerization cannot be achieved because of the special molecular arrangement of the different components of the cuticle (Villena et al., 2000). However, it is important to note that method employed for cuticular depolymerization also affect the cuticular component as analysed by Villena et al. (2000).

ATR-FTIR spectra of cuticle, skin and cutin made it possible to virtually approximate the contribution of various components present in each one of them and suggest the potential for using spectral information to analyze the cuticle, skin and cutin. However this technique of IR spectra has several limitations that need to be addressed for the full potential of its application to be realized. Firstly, when analyzing the complex biological sample like plant tissue, it is required to have a more complete ATR spectral database of pure compounds which will help to ensure the alignment of plant tissue spectra to the pure compounds for better identification. Another potential problem is the limited ATR penetration depth (c. 0.30–0.55 μm) in the upper part of spectrum (c. 2000–4000 cm\(^{-1}\)) which can limit the detection of certain compounds having spectral properties in this range. For instance, aliphatic fatty acids common in plant waxes (Bianchi, 1995) are rarely detected in the ATR spectra which display characteristic broad OH bands in the 2500-3500 cm\(^{-1}\). Wax thickness and distribution pattern is another important factor contributing to the ATR spectral differences between the irrigation treatments and two sides of the canopy. When surface waxes are relatively thin as in early stages of berry growth, the spectra are more strongly influenced by materials from the inner tissue layers such as polysaccharides and cutin to some extent. Conversely, when the surface waxes are thick (eg, in mid ripening and full maturation stage), spectra display weaker cutin and polysaccharides feature reflecting the interplay between the ATR-penetration depth and near-surface composition of plant tissue. This might further explain the anomaly of skin samples of grape berry at mid ripening and full maturation with no consistent pattern of band intensity between the irrigation treatment and two sides of the canopy. Also changes in the intensity of the band region in 900-1800 cm\(^{-1}\) could be related to variety of factors, none of which is currently well-understood. The possible factors may include the formation of new compounds, changes in crystallinity (van Soest et al., 1995), differences in the hydrogen bonding, anomeric or positional linkage differences (Kacuráková and Wilson, 2001) and differences in microfibril orientation caused by cell elongation (Wilson et al., 2000) or some changes in molecular environment. Thus using the in situ FT-IR spectroscopy could be useful means of describing the relationships between the different components of the plant cuticle and particularly grape berry considering the significance of this fruit in wine industry and its adaptive response under environmental conditions. This IR spectroscopic data could provide the future starting point in order to study the cuticle from different varieties of grape berries and further detailed study regarding how their
cuticle components could be vital for its environmental acclimatization would be worth exploring in future.

6.6. Water stress affects cuticle related gene expression

Seven genes were analysed. However, three genes were not considered to be differentially expressed under abiotic stress. All genes had similar expression profile with expression level maximum at *véraison* followed by decrease in abundance of transcript.

No significant effects of water and heat stress were recorded for *VviKCS1*, *VviKCS6* and *VviGDSL*, though these genes were slightly over expressed at *véraison*. In contrast, *VviCER9*, *VviLTP3*, showed differential expression at mid ripening and full maturation respectively whereas *VviCYP716A15* showed differential expression at *véraison* and mid ripening and *VviCYP716A17* showed differential expression at *véraison*, and full maturation.

*VviKCS1* and *VviKCS6* catalyses the elongation for the very long chain fatty acids (VLCFA) and are considered pivotal for the epicuticular wax synthesis. The trend of expression of both *VviKCS1* and *VviKCS6*, shows an up-regulation from pea size stage to *véraison* followed by a down regulation. This high expression level of both these genes at the early stages supports the wax deposition at the berry surface as fruit grows in size. Interestingly the west side tends to show a higher expression level which corroborates TEM results as well as cuticular permeance data. The higher temperature on west side of canopy coupled with the more stressed irrigation (e.g. RDI) probably induces an up-regulation of wax biosynthesis in order to prevent damages of berry water loss. In addition, the up-regulation of these VLCFA elongation transcripts may account for an urgent demand for covering large area by waxes in berry since *véraison* stage is characterized by a rapid berry enlargement. As the berry ripening process progresses, the expression level of *VviKCS1* and *VviKCS6* decreases gradually till full maturation, where *VviKCS1* was not expressed. *VviKCS1* gene had been reported to exhibit redundancy and overlap in the function of its elongase activities which might suggest that its function in the final stage of berry development could be shared by some other wax biosynthetic enzymes (Todd *et al.*, 1999). Our finding are similar to those reported by Hooker *et al.* (2002) showing the positive impact of environmental factors such as water deficit on *KCS6* transcript expression level, since the expression of *VviKCS6* was stimulated by RDI as compared to SDI at *véraison* and mid ripening.

*VviGDSL* gene which encodes for esterases/acylhydrolases commonly called GDSL-lipases has a crucial role in cutin deposition- an important component of cuticle (Girard *et al.*, 2012). The cuticle is composed by cutin and waxes which together constitute the important function of cuticle to prevent water loss. However exact contribution of each of these two components to cuticle is still unknown. The highest expression of *VviGDSL* gene was observed at pea size stage and gradually declines suggesting the role of *VviGDSL* in the rapid cutin deposition at the initial stages of berry development. The decline of *VviGDSL* expression level after *véraison* is also reflected in the TEM results which showed that thickness of inner cuticle or cutin decreased after *véraison* due to reduced cutin
deposition. The waxes which are probably more effective barrier to water loss in grape berry may not catch up the pace of the berry development in the early stages, the function of which is initially taken over by cutin. However as the berry development progresses, the role of cutin is shared by waxes and therefore the expression level of VviGDSL decreases from véraison to mid ripening up to the full maturation. Importantly, the function of cutin in water loss may not necessarily be dependent on cutin content as is reported by Isaacson et al. (2009). This might suggest the fact the VviGDSL expression was more on east side than on the west side. It is possible that both waxes and cutin having a synchronized function in controlling water loss. GDSL like proteins have become attractive research subjects because of their multifunctional properties but our understanding of plant GDSL enzymes is still very limited (Chepyshko et al., 2012). It is reported that GDSL family from Vitis vinifera alone consist of 96 members (Volokita et al., 2011) some of which might be functioning in grape berry cuticle regulating the cutin deposition. However further investigations are needed for better understanding of the cutin synthesis and deposition in grape berry.

VviCER9 is the first described gene related to cuticle biosynthesis whose deficiency improves plant response to water deficit and water use efficiency (Lü et al., 2012). Our results of VviCER9 expression showed a peak of expression at véraison that decreased thereafter. It is interesting to note that VviCER9 expression was only detected in RDI berries at midripening, while it was not able to detect any expression in SDI berries. The role attributed in Arabidopsis of CER9 as a negative regulator of ABA biosynthesis (Zhao et al., 2014) seems not to be verified in grape berries. Findings from our lab (Zarrouk et al., unpublished data) showed that ABA peaked at véraison (Appendix VI) contradicting with the role of VviCER9 (Zhao et al., 2014). These results may suggest the presence of other VviCER9 isoforms (Vitis genome) that could possibly be expressed differentially in the grape berry leading to the control of water stress in berries. The expression of VviCER9 only in water stressed berries at mid-ripening may also support the role of VviCER9 in water stress sensing (Zhao et al., 2014). However this is preliminary study of VviCER9 in grape berries and further understanding of its mechanism is still open to further research.

Preliminary results of berry cuticle proteome allow the identification of LTP3 protein in berry cuticle (Zarrouk et al., unpublished data). The expression of VviLTP3 peaked at véraison and decreased thereafter. LTP3 has been implicated in increasing tolerance to drought stress in several reports (Guo et al., 2013; Seo et al., 2011) which suggests the implication of VviLTP3 in water stress resistance in grape berries. Lipid transfer protein are able to bind lipids and act as lipid carrier between intracellular organelles and deliver the wax components during the assembly of cuticle (De Bono et al., 2009; Sterk et al., 1991; Yeats and Rose, 2008) from cytosol to the cell membrane or cell wall to form cuticular wax and thus protecting the plants against adverse environmental conditions such as water and heat stresses (Guo et al., 2013). High expression of VviLTP3 at véraison might suggest the increase of the rate of deposition of waxes as berry size increased. The up-regulation of VviLTP3 in RDIW berries corroborate results of TEM analysis and support the role of VviLTP3 in wax deposition.
The role of *VviCYP716A15* and *VviCYP716A17* in oleanolic acid biosynthesis has been reported in the grape berries (Fukushima *et al.*, 2011). Triterpenic compounds such as oleanolic acid are mainly distributed in cuticular wax of fruit (or leaves) and the content varies among different cultivars of fruit (Casado and Heredia, 1999; Szakiel *et al.*, 2012). The putative substrate by which *VviCYP716A15* and *VviCYP716A17* synthesize oleanolic acid is β-amyrin (Pollier and Goossens, 2012), synthesis of which is up-regulated under water stress conditions in olives (Martinelli *et al.*, 2012). This corroborates our results showing an up-regulation of *VviCYP716A15* and *VviCYP716A17* in RDI berries compared to the SDI ones. However, the effect of water stress on the expression of these two genes is more in earlier stages of berry development as reported in olive development by Martinelli *et al.* (2012). The distribution and seasonal changes in the content of triterpenoid such as oleanolic acid showed dynamic variation during growing season, being high in early growth and decreasing with fruit maturity as reported by Szakiel *et al.* (2011) consistent with the expression profile of these two genes with more expression at early stages and decreasing thereafter till full maturation in our present study. Expression of these two genes was found to be significantly higher on the west side compared to the east side. This might be attributed to its role in heat stress response and regulating the wax biosynthesis. More wax synthesis on west side need more synthesis of its components and one of them is oleanolic acid (Casado and Heredia, 1999). Therefore, high wax deposition on west side might corroborate with high expression of these two genes on west side in order to synthesize more triterpenoid content of waxes.

Although some genes showed no significant effect, similar effect of water stress on cuticle related gene expression in pine has been reported by Le Provost *et al.* (2013). It is also important to note that all genes peaked at *véraison* and decrease thereafter, suggesting the importance of early stages of berry growth in the cuticle biosynthesis and deposition. This hypothesis is supported by study conducted in barley where Richardson *et al.* (2005) showed that wax deposition occur independent of developmental stages.
7. Conclusion

The present work is carried out to characterize the grape berry cuticle for its role in water stress response. The initial findings from this investigation showed that berry qualities are affected by water deficit conditions as reported previously (Zarrouk et al., 2012). Preventing the water loss is essentially the main role of cuticle for which cuticular waxes play a pivotal role in limiting transpirational water loss from berry surface. For this purpose, the epicuticular wax load of the berry was studied. The soluble cuticular waxes decreased from pea size to full maturation. On the contrary, the TEM analysis revealed that the thickness of epicuticular waxes increased along the phenological stages with significant differences between the irrigation treatments (SDI and RDI) and two sides of the canopy (east and west). This variation in quantitative estimation of wax content and TEM analysis could be clearly attributed to the variation in the solubility of waxes in chloroform solution. It is also necessary to point out that TEM analysis was considered to be more accurate determination of epicuticular wax compared to the quantitative estimation of wax which was laced with significant variation due to the solubility and berry position within cluster or sub-cluster. Further in order to assess the role of these surface waxes, cuticle permeability to water loss was studied. The cuticle permeance of the berries with the wax removed showed significantly higher permeance and the findings were in accordance with permeance data available from some of the similar studies carried out in tomato fruit (Leide et al., 2007). De-waxed berries at RDIW of full maturation stage showed significantly much higher water loss compared to the intact berries further established the role of waxes as an effective barrier to water loss under water and heat stress. Our quantitative estimation and TEM analysis of waxes may help to conclude that quality as well quantity are together responsible for regulating the water loss in grape berries. It is expected that the quality and quantity may trade-off as an effective means to regulate water loss under water stress and heat stress. However several previous studies (Leide et al., 2007; Riederer and Schreiber, 2001) have proved that quality of waxes are more important for regulating the water loss rather than quantity, which may not completely hold true for the grape varieties growing under current environment of high temperature and water stressed conditions. This balance between quality and quantity of waxes in grape berries needs further study to better understand effective role of waxes as a barrier to water loss.

TEM was found to be excellent technique to study the architecture of cuticle and it showed that cuticle properties changes under water stress conditions. TEM study also revealed the deposition of some dark amorphous material on the surface of cell wall (likely to be pectinaceous) as reported by Guzmán et al. (2014) which increased along the phenological stages together with epicuticular waxes and significantly highest at west side of RDI treatments, suggesting that the waxes together with these pectin like protecting material might be an adaptive strategy of berries under water stress conditions as pectins are recently pointed out for drought tolerance potential (Leucci et al., 2008; Review by Le Gall et al., 2015 and Tenhaken, 2014). FTIR analysis of the berry skin though revealed characteristic band in “fingerprint” region corresponding to the polysaccharide nature of the skin but further study would be required such as quantitative estimation of these protecting material and how they change along the phenological stages and how water stress affects their deposition. Further in order to study
the cuticle and its components, spectroscopic characterization of isolated grape berry cuticle, skin and cutin was carried out using ATR-FTIR which provided useful insight about the functional groups present in the cuticular matrix and their structural role and their molecular arrangement. The weak peak at around 2925 cm\(^{-1}\) assigned to methylene group showed that they are the significant structural units in the cutin. However we found weak band corresponding to ester bond near 1735 cm\(^{-1}\) which showed that grape berries contain lesser amount of cutin compared to the model tomato fruit which consist of almost 80% cutin. Spectral shift in the peaks further authenticated the cutin depolymerization as seen in Figure 14. Cuticle biosynthesis, deposition and transport is an area that is still under intensive research and several model and non-model plants have been studied in order to understand the biochemical pathway for cuticle biosynthesis and how they are transcriptionally regulated. We identified seven cuticle related gene: VviKCS1, VviKCS6, VviGDSL, VviCER9, VviLTP3, VviCYP716A15 and VviCYP716A17, out of which four genes (VviCER9, VviLTP3, VviCYP716A15 and VviCYP716A17) were found to be differentially expressed under water deficit and heat stress conditions. The expression pattern of these genes studied could be promising expression candidate genes for further forward genetics studies. They will provide stepping stone to further work on the stress adaptation of grape berries for which relatively little is known yet.

It is to the best of our knowledge that the in-depth characterization of grape berry cuticle had been carried out. Cuticle permeance and spectral characterization for grape berry have been reported for the first time with some similarity observed with the tomato fruit. This study could provide base for future cuticle related research in other non-model plants having economical and agronomic significance. As far as grape berries are concerned, these are still preliminary findings which provide sufficient evidence that water deficit could be an effective viticulture practice that can be used for vineyard without compromising on the berry quality and yield.
8. Future Prospects

The present study dealt with cuticle characterization of grape berries and could be useful platform for further investigating the cuticular properties in other non-model plants. Most of the recent finding about the cuticular research had been limited to tomato fruit, so this study would provide novel insights into the cuticular role in non-climacteric fruits such as grape berries. However present work still leaves many questions unanswered which need to be addressed so as to have better understanding of the physiology of cuticle and how it modulates the stress response:

1. Even though the present study deals with spectroscopic (FTIR) characterization of cuticle, it is possible to further use this technique in order to study the interaction between the cuticle and external environment and application of models to study the diffusion of molecules through cuticle surface would be an interesting topic. Other advanced techniques such as X-Ray diffraction and Nuclear Magnetic Resonance would provide the structural information of this intact natural biopolymer.

2. The following study also opens up the possibility of studying the biomechanical properties of berry cuticle which will help to evaluate how cuticle responds to changes in the external temperature or humidity. Technique such as Atomic Force Microscopy would provide molecular basis to understand the physical properties of this unique polymer.

3. Although gene expression pattern revealed the putative role of some of the important genes involved in cuticle synthesis, transport and deposition, it would be desirable to explore entire transcriptome of the grape berry cuticle and study the differential expression of genes involved under stress conditions.

4. Furthermore, preliminary studies described herein shed light on the cuticular proteins by identifying one of the important proteins (LTP3), but identification and characterization of cuticular proteome during berry ripening would be an area worth exploring in future.

5. Since all grape varieties present different physiological and metabolic behaviour depending on the environmental conditions they grow under, it could form an interesting piece of work to characterize other varieties in order to decipher cuticular differences at the genotype level that would facilitate better selection of variety with better adaptive trait.

6. Our TEM analysis revealed the deposition of pectinaceous material on cell wall increasing along berry development and positively related to stress degree. This suggests its protectant role against excessive water loss. It is important to ascertain that dense material stemming from cell wall is actually pectin in nature and FTIR supported the presence of pectinaceous material. However, this study falls short of qualitative and quantitative estimation of pectin material and their changes along the phenological stages under water stress conditions which requires further study.
References


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Figure 1: General Field Plan of Vineyard
Appendix II

The pre-dawn water potential data are kindly provided by “Agriciencia”, Portugal, WIP5 partners of Innovine Project.

Figure 2: Plant water status of vines subjected to two different irrigation regimes (SDI and RDI). Water status is expressed as pre-dawn water potential ($\Psi_{pd}$).
Appendix III

The temperature data are kindly provided by “Agriciencia”, Portugal, WIP5 partners of Innovine Project.

Figure 3A: Maximum air and berry temperature on east and west sides of the vine canopy for SDI Treatment.

Figure 3B: Maximum air and berry temperature on east and west side of the vine canopy for RDI treatment.
Appendix IV

The cuticular water permeance gravimetric data

Figure 4A: Gravimetric data of cuticular water permeance of untreated(a-b) and de-waxed(c-d) grape berries from pea size stage. East (‘a’ and ‘c’); West (‘b’ and ‘d’).
Figure 4B: Gravimetric data of cuticular water permeance of untreated (a-d) and de-waxed (e-h) grape berries from véraison. SDIE ('a' and 'e'); SDIW ('b' and 'f'); RDIE ('c' and 'g'); RDIW ('d' and 'h').
Figure 4C: Gravimetric data of cuticular water permeance of untreated grape berries from mid ripening stage SDIE (a), SDIW (b), RDIE (c), RDIW (d).
Figure 4D: Gravimetric data of cuticular water permeance of untreated (a-d) and de-waxed (e-h) grape berries from full maturation. SDIE ('a' and 'e'); SDIW ('b' and 'f'); RDIE ('c' and 'g'); RDIW ('d' and 'h').
Appendix V

RNA extraction Buffer

DEPC H$_2$O (0.1M) ➔ 100mL H$_2$O + 100uL DEPC (4°C)

Tris-HCl ➔ Stock 1M in 100mL ➔ 12.11g Tris Base in 80mL MilliQ H$_2$O + 4.2mL HCl complete to 100mL 0.3M; MW

EDTA ➔ Stock 0.5M in 50mL ➔ 9.306g and complete to 50mL with DEPC H$_2$O 0.025M; MW 372.24

NaCl ➔ Stock 5M in 100mL ➔ 29.22g and complete to 100mL with DEPC H$_2$O 2M; MW 58.44

Extraction Buffer (100 mL)

- 30mL Tris HCl 1M
- 5 mL EDTA 0.5M
- 40 NaCl 5M
- 0.2g CTAB
- 0.2g PVP
- 0.05 Spermidine (4°C)
  ➔ Complete to 100 mL with DEPC H$_2$O

TE (25 mL)

10mM Tris (277uL) + 1mM EDTA (25uL) ➔ complete to 25mL with DEPC H$_2$O

NaOAc ➔ Stock 3M in 25mL ➔ 6.15g complete to 25mL with DEPC H$_2$O 3M; MW 82.03
Figure 6: Changes in the ABA concentration in cuticle of *Vitis vinifera* cv. Aragonez. SDI: Sustained Deficit Irrigation; RDI: Regulated Deficit Irrigation. E- East; W- West. PS- Pea size; V- *Véraison*; MR- Mid Ripening; FM- Full Maturation. The values are represented as mean± SE.