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Response, Tolerance and Adaptation to Abiotic Stress of Olive, Grapevine and Chestnut in the Mediterranean Region: Role of Abscisic Acid, Nitric Oxide and MicroRNAs

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1. Introduction

Hot, dry summers and mild to cool, wet winters are the characters of the Mediterranean climate. Drought, extreme temperatures and extreme irradiation (UVs) often concomitantly - in some cases also together with salinity, significantly affect the growth, yield and quality of the Mediterranean crops.

Olive (*Olea europaea* L), grapevine (*Vitis vinifera* L) and sweet chestnut (*Castanea sativa*) are the most important woody crops in the Mediterranean among others. The olive tree and vineyard are familiar features of the Mediterranean landscape. In some mountain regions, these features are accompanied by the orchards of chestnut. Olive oil and wine are important products in that region. In some regions, such as Italy, Turkey, Spain, Portugal, and Greece, chestnut is one of the most important fruit products as well. Olive oil, grape and wine are a traditional icon of the Mediterranean diet. Enjoying the plentiful indigenous plant products, especially wine, olive oil and chestnut, is part of the Mediterranean civilization.

Olive oil is the main source of fat in the Mediterranean diet and one of those basic ingredients essential to life in the Mediterranean. It may also protect against heart disease, stroke, and certain cancers. The vine and wine are among the most important symbols of

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societies that have emerged around the shores of the Mediterranean (Stanislowski, 1970). In most Mediterranean countries such as Portugal, France, Italy, Greece and Spain—wine is more than just a beverage; it is an integral part of meals and an essential aspect of social gatherings.

The European chestnut species *C. sativa* has been cultivated in the Mediterranean region for both fruit and timber for dozens of centuries. Sweet chestnut provided staple food with nutritious and health properties for people in the Mediterranean for centuries especially in the mountains and used to be called the 'bread-tree' (Avanzato, 2009). Chestnuts are delicious and healthy foods, containing many highly valuable carbohydrates and phytochemicals, and no cholesterol and low fat. It is an ingredient in many traditional recipes.

Plant abiotic stresses and response of plants to these stresses have been extensively studied. In this chapter, we have summarized the recent advance in the response, tolerance and adaptation of these Mediterranean woody crops to the environmental stresses especially drought and extreme temperatures imposed by the typical Mediterranean climate, and the underlying mechanism. At molecular level, plants share some common pathways involved in different abiotic stress responses. Different forms of abiotic stresses may lead to similar responses in plants during the stress; likewise, different kinds of stresses have also been found to trigger responses in similar sets signalling molecules. The perception of stresses and the consequent adaptation by plants include physiological, molecular and biochemical changes in plants which largely depend on factors such as severity of stress, plant developmental stage and their genotype (Agarwal & Zhu, 2005). After the perception of a signal by plants, immediately there will be generated secondary signals which are normally nonprotein molecules, including membrane ion (K^+ and Ca^{2+}) flux, inositol phosphates (IPs), reactive oxygen species (ROS), and nitric oxide (NO). Each of these can activate plant mitogen-activated protein kinase (MAPK) and Ca^{2+} -dependant protein kinase (CDPK) and activation of protein phosphatases. These early events lead to hormone accumulation, particularly abscisic acid (ABA), salicylic acid (SA) and brassinosteroid hormonal, the synthesis of heat shock proteins, activation of antioxidant enzymes and synthesis of low molecular antioxidants and compatible solutes and membrane lipid peroxidation, followed by changes in transpiration, gas exchange, respiration, and growth, resulting in stress adaptation. MicroRNAs (miRNAs) also participate in stress adaptation response in plants. In this chapter we concentrate in the role of ABA, NO and miRNAs in the abiotic stress response and adaptation. Finally, the progress in genetic modification targeting improved abiotic stress tolerance of these plant species is reviewed.

2. Morpho-anatomical, physiological, and biochemical response and adaption

2.1 Olive capacity to withstand arid environments

Olive is a perennial, long-lived, evergreen tree of subtropical origin (Bongi & Palliotti, 1994) that, in the Mediterranean, flowers in mid-to-late spring. This adaptation allows olive to escape the deleterious effects of cold on flowering and fruit set but serves to increase reliance on a range of avoidance and tolerance mechanisms that maintain internal water status and metabolic activity during the hot, dry summers (Connor, 2005).

Olive tree is well known to be very resistant to drought (Bacelar et al., 2009; Bacelar et al., 2006; Connor, 2005; Fernández & Moreno, 1999; Giorio et al., 1999; Tognetti et al., 2004). Furthermore, it has been postulated that the minimum water requirement for olive is 200

mm year⁻¹ (Bongi & Palliotti, 1994). Abd-El-Rahman et al. (1966) measured the water content of olive leaves at saturation, finding a value, 1.59 g water g⁻¹ dry weight, extremely low compared with other species growing in the same environment (5.77 g water g⁻¹ dry weight for fig, 5.85 g water g⁻¹ dry weight for grape). There are many mechanisms by which it resists to more or less extended drought periods but some differences among olive cultivars have been observed concerning their ability for adaptation and production under drought conditions (Bacelar et al., 2004; Bosabalidis & Kofidis, 2002; Chartzoulakis et al., 1999b).

Olive culture has prospered under rainfed conditions in Mediterranean environments because the tree is capable of acceptable yield while subjected to the characteristic prolonged summer water shortage. Olive achieves this result with physiological, biochemical and morpho-anatomical responses that reduce water loss and maintain water uptake at high plant water status as drought commences (drought avoidance), and with others that tolerate dehydration at low plant water status as the drought deepens (drought tolerance) (Connor & Ferreres, 2005).

Olive leaves are well designed to control water loss. Morphological characteristics allow minimum radiation load and maximum heat exchange while the physiological responses of stomata to leaf water status and atmospheric humidity provide effective control of transpiration (Fernández et al., 1997; Loreto & Sharkey, 1990). Leaves minimise radiation load by small size, a dominantly vertical display (Mariscal et al., 2000) that is further aided by paraheliotropic movement under water stress (Natali et al., 1999) (Fig. 1A), a dense packing of the mesophyll layers (Bongi et al., 1987) and high reflectivity by a thick cuticle and epicuticular wax layers (Leon & Bukovac, 1978) (Fig. 1B). This combination of morphological features restricts temperature increase in leaves with small latent heat exchange when transpiration is restricted by stomatal closure.

Stomata are small and dense and occur only on the abaxial surface, under dense layers of peltate trichomes (or peltate scales) (Fig. 1C). The peltate trichomes reflect the sunlight and reduce the transpiration of the leaves.

An interesting characteristic in the anatomy of olive leaf is the presence of a complicated, dense network of filiform sclereids that are of idioblast nature (Karabourniotis et al., 1994) (Fig. 1D). This entangled network follows two major distribution patterns: the "subepidermal layer" consisting of the "T"-shaped sclereids extending between the adaxial epidermis and the palisade layer, and the branched the 'polymorphic sclereids that transverse the spongy mesophyll layers, producing a chaotic pattern. Sclereids act like synthetic optical fibres and, besides other functions, may contribute to the improvement of the light microenvironment within the mesophyll of the thick and compact sclerophyllous olive leaves (Karabourniotis et al., 1994).

It has been reported that olive leaves formed under water stress are more able to control transpiration, being smaller and thicker and having more dense and smaller stomata (Bosabalidis & Kofidis, 2002; Chartzoulakis et al., 1999b). However, Lo Gullo and Salleo (1988) observed that despite all this protection against water loss, leaves of the wild olive tree (*O. oleaster*) underwent a substantial water loss under conditions of water stress.

A drought avoidance response not displayed by olive is the development of a deep rooting system (Bongi & Palliotti, 1994). However, the extensive root system of olive tree seems to be designed for absorbing the water of the light and intermittent rainfall usual in its habitat (Fernández & Moreno, 1999). Most of the main roots grow more or less in parallel to the soil surface, and the highest root density is found close to the trunk, although the volume explored by the roots can easily extend beyond the canopy projection (Fernández & Moreno,

1999). This rooting habit is probably the result of sensitivity to hypoxia and may allow for efficient water absorption (Bongi & Palliotti, 1994; Fernández & Moreno, 1999). A high portion of the root is of small diameter, which also favours the absorption capacity. Absorption by olive roots is also enhanced by high potential gradients between roots and soil caused by osmotic adjustment (Fernández & Moreno, 1999).

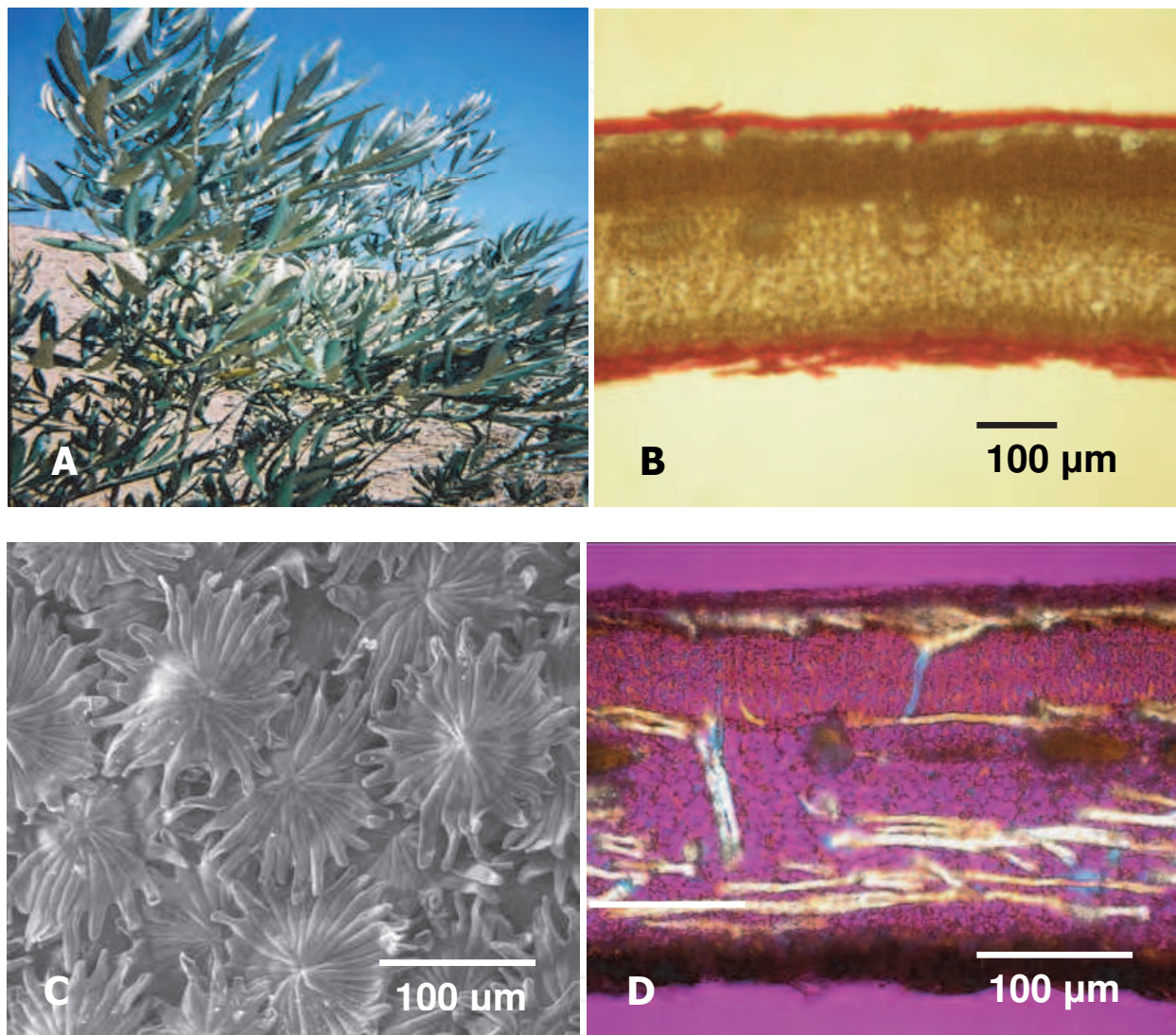


Fig. 1. Olive protections at leaf level against water loss and excessive irradiance. (A) Paraheliotropic movement under water stress; (B) Dense packing of the mesophyll layers and thick cuticle and epicuticular wax layers (optical micrograph); (C) Dense trichome layer of abaxial surface protecting the stomata (SEM micrograph); (D) Dense network of sclereids (optical micrograph, cross-polarized light).

The olive is a diffuse-porous tree having a dense wood with abundant fibers and little parenchyma (Fernández & Moreno, 1999). The large amount of fibers, which makes olive wood so hard, accounts for the low vessel lumina of the species in comparison with other diffuse-porous Mediterranean plants. Salleo et al. (1985) observed that the vessel lumina, when expressed as percentage of the total xylem cross-sectional area, was half that measured in other Mediterranean species such as *V. vinifera*. The low hydraulic conductivity

of olive xylem is a feature that seems to play an important role in the tree's water relations. Salleo and Lo Gullo (1993) observed losses of about 10% of hydraulic conductivity in 1-year-old twigs of young *O. oleaster* trees, when these became stressed, due to xylem cavitation. One consequence of this is that olive trees prevent excessive water loss on days of high water demand by closing their stomata soon after midmorning (Fernández et al., 1997).

During periods of water stress, olive tree typically experience reductions in transpiration, stomatal conductance and net photosynthesis (Giorio et al., 1999). Nevertheless, environmental and physiological factors do not affect H₂O and CO₂ exchange to the same extent, resulting in possible variations in water use efficiency in this species (Xiloyannis et al., 1988). Meanwhile, some differences in gas exchange responses to water stress between olive cultivars have been observed in previous experiments (Chartzoulakis et al., 1999a; Tognetti et al., 2002).

In moderate drought conditions, olive plants stop shoot growth but not photosynthetic activity and transpiration. This allows the continued production of assimilates as well as their accumulation in the various plant parts, in particular in the root system, creating a higher root/leaf ratio compared to well-watered plants (Xiloyannis et al., 1999).

Olive tolerates drought by maintaining turgor through osmotic adjustment and changes in cell wall elasticity (Connor, 2005). Active and passive osmotic adjustment plays an important role in maintaining cell turgor and leaf activities which depend on it (Xiloyannis et al., 1999). Mannitol and glucose play a major part in the osmotic adjustment of olive leaves (Cataldi et al., 2000). In addition, the osmotic adjustment observed in the root system allows maintenance of cell turgor, avoiding or delaying the separation of roots from soil particles (Xiloyannis et al., 1999). The accumulation of proline under drought stress in both leaves and roots of 2-year-old *O. europaea* (cv. Coratina) plants (Sofa et al., 2004b) also indicates a possible role of proline in drought tolerance.

Under field conditions, particularly in the Mediterranean regions, water stress is often accompanied by other environmental constraints, such as steep leaf-to-air water vapour gradients, and high irradiance and temperature (Osório et al., 2006). Measurements have revealed non-stomatal limitations to photosynthesis consistent with photoinhibition in olive leaves exposed to high irradiance (Angelopoulos et al., 1996). The synergic action of high irradiance level and water stress reduces the capacity of the photosynthetic systems to utilize incident radiation, leading to a higher degree of photodamage (Bacelar et al., 2007; Sofa et al., 2004a). The increase of malondialdehyde content and lipoxygenase activity, two markers of oxidative damage, observed by Sofa et al., (2004b) in both leaf and root tissues of olive plants during the progressive increment of drought stress, indicates that water deficit induces lipid peroxidation. This result suggests that higher activities of some antioxidant enzymes and non-enzymatic antioxidants are required for a better protection against oxidative stress related to water deficit.

2.2 Vine's response, tolerance and adaptation to abiotic stress

Most of the world Wine Regions, such as the Douro Region in Portugal, has a Mediterranean climate with a strong continental influence. In these regions the rainfall is mainly concentrated in the winter months and the springs and summers are characterized by exceedingly hot and dry. In these conditions grapevines are often subjected to periods of severe drought associated with strong light and high temperature (Chaves et al., 2002). Consequently, the vineyard experiences irreparable damage on physiology behaviour and

yield attributes. The implementation of cultural strategies, which aims a better adaptation to these conditions, is a major goal, especially in the current scenario of global climate change (IPCC 2007).

2.2.1 Abiotic stress response

Under summer stress and as the first limitation, the photosynthetic productivity is limited by the stomatal closure, either in response to a large decrease in leaf water potential or due to an increase in atmospheric vapour pressure deficit. Several studies undertaken in the Douro Region clearly have shown that grapevines growing under severe summer stress experience a significant decline in productivity, mostly owing to stomatal limitations to photosynthesis (Moutinho-Pereira et al., 2004).

Grapevine cultivars differ in the degree of control exerted by stomata under conditions of water limitation. While some varieties are genetically programmed to react to early signs of dryness in the air and/or soil, others may have greater difficulties in stomatal regulation (Moutinho-Pereira et al., 2007). For instance, under water stress conditions the water use efficiency and the correlation between net photosynthesis rate and stomatal conductance are significantly higher in 'Riesling' than that in 'Silvaner' (Düring, 1987). The ABA concentration, arriving from roots to leaves, is directly implicated in this behaviour (Correia et al., 1995). On the other hand, in grapevine the stomata response to ABA concentration is not uniform across the leaf surface. According to Düring (1992), this behaviour is related with the heterobaric anatomy (patchiness) of vine leaves, which makes the gas diffusion difficult in the intercellular spaces of the mesophyll and is responsible for non-uniform aperture of stomata over the leaf surface.

The photosynthetic apparatus is generally tolerant to water stress. However, if the imposition of dehydration of mesophyll cells is moderate but continued or severe but brief, a metabolic adjustment takes place through metabolic pathways, mainly related with RuBP regeneration and Rubisco activity (Medrano et al., 2002).

The structural integrity of chloroplasts and the photochemical reactions and electron transport chain do not seem to be much affected by low water potentials. Only the thickness of thylakoid lamellae seems to decrease (Chaves, 1991). In fact, Flexas et al. (1998) and Escalona et al. (1999) found that in its natural environment and under water stress the vine only developed a few signs of down-regulation of the photochemical activity. However, in Mediterranean climate, water stress is usually associated with many clear and hot days, which, in a synergistic action, leads to a significant down-regulation or photoinhibition of the photosynthetic apparatus (Osório et al., 1995). Under these conditions, the vineyard experiences irreparable damage. Frequently, some leaves display irreversible photoinhibition and chlorosis, followed by necrosis and leading to low grapevine water-use efficiency (Moutinho-Pereira et al., 2003). The air temperature increase will accelerate the grapevine phenology, leading to a reduction in the vegetative and reproductive period (Seguin & Cortazar, 2005).

2.2.2 Tolerance and adaptation

The ability of the vineyards to grow and produce satisfactorily in severe summer stress conditions depends on the development of morphological and physiological mechanisms, which allows them to retard the level of dehydration that is detrimental to cellular metabolism. In general, this is achieved through an improvement in water absorption by

roots and/or by reducing water loss. The formation of a deeper and dense root system by rootstock depends on the interaction of its genetic characteristics and is usually an effective strategy for grapevine to capture more water in periods of lower water availability (Palliotti et al., 2000). In this context, the selection of rootstock with these characteristics and a good soil preparation are one way to achieving these mitigation objectives.

One of the most widespread mechanisms to reduce grapevine water loss is achieved through lower vigor and/or partial senescence of leaves (Chaves, 1991). The increase in stomatal conductance mediated by the ABA concentration is another mechanism developed for the same purpose, especially in the periods of the day with deficits of higher vapor pressure (Iacono et al., 1998). The prevention of photoinhibition and overheating of the leaves in consequence of the lower leaf transpiration can also be undertaken by changing the leaf angle, e.g., from 53° to 80° (angle between the blade and petiole) (Smart, 1974).

The grapevine adaptations to dry and hot habitats seem to be strengthened by changes that occur at the level of vascular system, particularly the reduction in xylem section, which induce a significant decrease in hydraulic conductivity and thus minimize the susceptibility of these vessels to the phenomenon of cavitation (Lovisolo & Schubert, 1998; Schultz & Matthews, 1988).

The active accumulation of soluble sugars and other low molecular compounds is responsible for lower osmotic potential, allowing the cell turgor maintained as much as possible, with positive values. This process, known as osmotic adjustment, has been shown in vines gradually subjected to water stress, either in leaves (Düring, 1984) or in roots (Düring & Dry, 1995). Under prolonged drought, a decrease of 4 to 5 bars in osmotic potential, mainly more evident and rapid in young leaves than in adult leaves (Düring, 1984). The capacity for lowering the osmotic potential might be the dominant strategy for better restricting the leaf water losses in grapevines growing under water stress conditions (Patakas & Noitsakis, 1999).

3. Limitations of European chestnut growth at low latitudes

European chestnut (*Castanea sativa* Mill.) is characterized as a mesophilic species (Cortizo et al., 1996). Plants from this species are moderately thermophilic and well adapted to ecosystems with a yearly mean temperature ranging between 8 °C and 15 °C and monthly mean temperatures during 6 months over 10 °C. Unfortunately, nowadays, chestnut tree growth shows some constrains which might be partially attributed to the climatic alterations.

In Europe, the chestnut is widespread. The Azores archipelago (25° - 31° W) is the most Occidental point for *C. sativa* and the Canary Islands is the most Southern point (27° - 29° N). Towards the north, chestnut fruit production reaches 52° N latitude to the south of the United Kingdom, northern Germany, Poland and Ukraine.

It is found at sea level in some littoral areas above 39° N latitude, as such the northern Iberian peninsula, north of Italy and Middle Eastern Greece due to sea influence. Below this latitude, still in the littoral areas, adequated climatic conditions for chestnut are found in higher altitudes, as it happens in Sierra Nevada (1500 m a.s.l., Granada, Southern Spain), Teide Mountain (2000 m a.s.l., Santa Cruz Tenerife, Canary Islands) or in Etna mountain (2000 m a.s.l., Sicily Island, Southern Italy). In the interior part of Europe, under continental climatic influence, chestnut only can grow above 500 m a.s.l. being the maximal altitude 1100 m a.s.l. in the highest mountains of Trás-os-Montes (Northeast of Portugal) or even to

1800 m in Caucasus Mountains, the former altitudes corresponding to the ancient orchards and the highest altitudes to the newest plantations (Gomes-Laranjo et al., 2005; Pereira-Lorenzo et al., 2010).

Below 600 m a.s.l. climate is hotter and dryer than the adequate conditions for chestnut, corresponding to a transition altitude, vineyards, olive tree and almond being now the main crops. Contrarily, above 1100 m a.s.l. climate is colder and wetter, and vegetative cycle is shorter than that needed for fruit production. So, typical climate is a continental temperate type, with mean annual values of sunlight and precipitation, 2400 to 2600 h and 600 to 1200 mm, with the total amount of temperature from lowest and highest altitude orchards ranging between 2800 °D and 3400 °D, respectively (Gomes-Laranjo et al., 2007). Degree-days (°D) represent the amount of heat required, between the lower and upper thresholds (where $T_0 = 6.0$ °C, see Fig. 2) for an organism to develop from one point to another in its life cycle (Cesaraccio et al., 2001; Zalom et al., 1983). For overall Portuguese varieties, half rate of photosynthesis (A_{50}) is found when temperature reaches 11°C (T_{50m}) and 38°C (T_{50M}), being the optimal value around 24°C (Gomes-Laranjo et al., 2007). In relation to limitant radiation intensities, results suggest that it is a dimlight species, since 75% of maximal photosynthetic rate is found at 900 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which corresponds almost at half full sunlight intensity, and A_{50} is at 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. So, European chestnut could be indicated to be included in restoration programmes for the European forest, since adult trees save most of light in the top of their canopies and only low intensity will attain soil level. Identical conclusions have been drawn by Joesting et al., (2009) in relation to American chestnut (*C. dentata* (Marsh.) Borkh) with the aim to restore chestnut populations in eastern deciduous forest from Appalachian mountains.

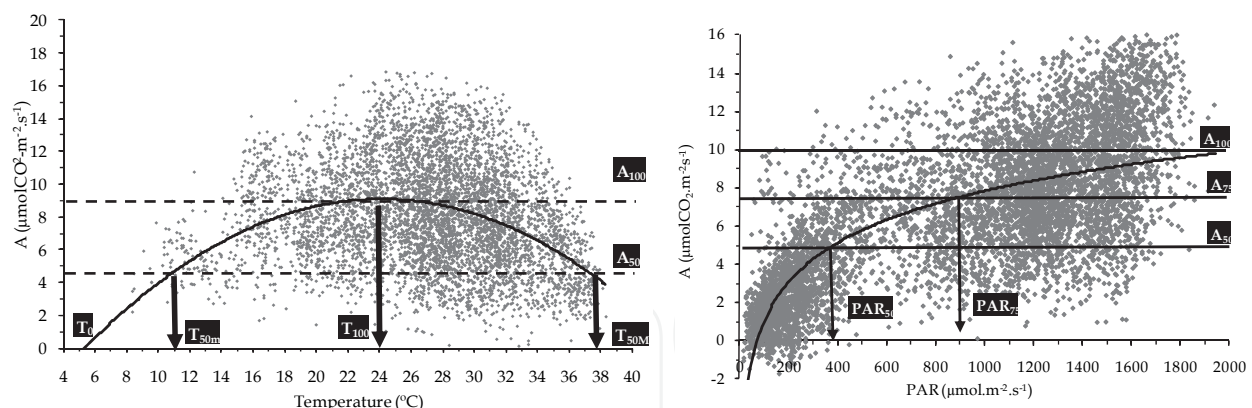


Fig. 2. Threshold temperature (left) and radiation (right) for photosynthesis rates in chestnut leaves. Study was done with 13 Portuguese varieties in Trás-os-Montes Region during 6 years. Concerning temperature study, T_0 represents the temperature value for vegetative zero growth, T_{50M} and T_{50m} the values that induce half rate (A_{50}) of the maximal photosynthesis (A_{100}). Values were obtained according to second polynomial curve, $y = -0.0253x^2 + 1.2349x - 6.2532$, $R^2 = 0.1094$. In relation to the radiation study, A_{100} , A_{75} and A_{50} , mean the maximal, 75% and half of the maximal value of photosynthesis rate, respectively, being PAR_{50} and PAR_{75} the respective values of photosynthetic active radiation (PAR). These values were calculated from logarithmic equation, $y = 2.9628\ln(x) - 12.62$, $R^2 = 0.5335$ ($n=8852$).

In the interior regions of Europe, where chestnut grows under continental climatic influence, altitude is decisive to define adequate climatic conditions. As the Fig. 3 shows, photosynthesis increases with altitude, and inversely with temperature. So, maximal rates can be found above 800 m a.s.l. where temperature down to 25 – 22 °C, the range of optimal temperature as has been referred above. In the lowest altitudes, photosynthesis rate decreases around 40%, indicating that chestnuts under these climatic conditions, nowadays start to suffer from abiotic stresses, mainly due to the heat stress. Regarding internal water balance, Martins et al., (2010) have demonstrated that adult trees can be saved from water stress, since they can continuously absorb water from deep soil layers and so preserving predawn leaf water potential in the range of -0.6 to -0.9 MPa.

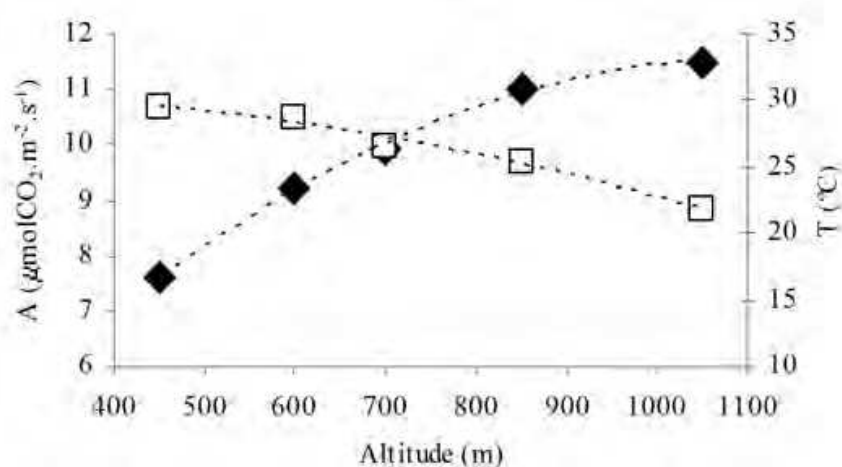


Fig. 3. Variation of mean daily photosynthesis rate (A, closed symbols) in leaves from chestnut (var. Judia) and air temperature (open symbols) as a function of altitude.

Additionally, the European chestnut ecotypes coming from wet sites are more locally adapted and less plastic than those from dry sites and hence more vulnerable to the climate changes (Villani et al., 2010). Five gene pools have been determined in Europe: three in Greece, one on the northwestern coast of the Iberian Peninsula and a large gene pool covering the rest of the Mediterranean basin (Martin et al., 2010; Mattioni et al., 2008).

The existence of some adaptative variation among populations from extreme conditions is proposed (Fernández-López et al., 2005): populations from Greece initiate growth earlier followed by those from South Italy and South Spain, while ecotypes from north Spain and Italy initiate later. A significant genetic variation between north and south Iberian ecotypes has also been confirmed (Fernández-López et al., 2005). The expected global climate changes are a great challenge for forest tree breeders. Eriksson et al., (2005) established a xerothermic index to characterize each one of those ecotype's local origin and they found a negative correlation between it and plant growth at both 25°C and 32°C, but a positive correlation with carbon isotope discrimination, suggesting a large additive coefficient of variation for growth traits. This variation confers to the species good possibilities to respond genetically via natural or artificial selection to environmental change. Dinis et al., (2011) working with plants from the portuguese Judia variety, have also concluded that the morphological and phenological differences among ecotypes are not only related to the small genetic differences, but are simply phenotypic adaptations to different climatic conditions.

Lowest altitudes and so, highest temperatures, seem to induce sun characteristics in leaves, demonstrated by low chlorophyll amount (Chl), since thermoinhibition might speed light saturation of the photosynthetic process (Dinis et al., 2011). On the other side, leaves present high Chla/b and low Chl/Car ratios are consistent with their acquired tolerance to warm and sunny conditions (Gomes-Laranjo et al., 2006; Pearcy, 1998). Chla is the main photosystem I pigment, which is located in exposed thylakoid membranes, and carotenoids have the chlorophyll protection function against photoinhibition (Demmig-Adams & Adams, 1996). Moreover, increase in Chla/Chlb suggests higher proportion of stacking thylakoid membranes, which in turn might induce higher photosynthesis rates, if any stress factor imposes (Anderson et al., 1988).

Altitude (s.l.m)	Chltot mg.cm ⁻²	Chla/b	Chl/Car	Total fatty acid		PI	UI	Malonic aldehyde x10 ⁻⁴ (mM)	
				Saturated (%)	msaturated (%)			Control	ADP-Fe
1050	121.5 b	3.12 c	4.8 a	27.0	73.0	111.5	171.3	1.35	3.88
900	145.9 a	3.10 c	5.0 a	32.4	67.6	97.5	154.2	1.67	4.57
700	99.1 c	3.30 b	4.4 b	38.2	61.8	119.2	156.3	2.00	4.91
600	143.9 a	3.40 b	4.6 b	33.1	66.9	47.5	146.1	1.90	4.53
450	80.9 d	3.60 a	3.9 c	43.9	56.1	79.6	121.2	3.12	5.36

Table 1. Determination of photosynthetic pigment content (n=10), fatty acid composition (n=3) and malonic aldehyde (n=3) in chestnut chloroplast (var. Judia) (Gomes-Laranjo et al., 2005) isolated from leaves collected in the range of altitudes between 450 and 1050 m a.s.l

Altitude and consequently air temperature, also affects the thylakoid fatty acid composition (Table 1). In highest altitude locals, the unsaturation index is highest and inversely in the lowest ones that is the lowest. This adjustment is very important since hotness induces more fluidity in the membrane fatty acids and by this way, they must be more saturated in order to be more stable and consequently forming stable thylakoid membranes (Murata & Siegenthaler, 1998). In the Portuguese varieties, Judia, Longal and Aveleira, the most heat tolerant variety Aveleira has the lowest unsaturated fatty acid index (158.5) and viceversa Judia the least heat tolerant has the highest fatty acid index (175.1) (Gomes-Laranjo et al., 2006).

Additionally, decrease in thiobarbituric reactive species (MDA) and ADP-Fe peroxidation in the leaves suggest the lower in peroxidation susceptibility the higher altitude (Table1).

4. Abiotic stress signalling

4.1 The role of abscisic acid (ABA)

Here we briefly introduce some of the recent research on the regulation of ABA levels in the aforementioned Mediterranean plants. ABA signal transduction will not be discussed in detail since the studies related to these plants are still scarce or almost inexistent, which undoubtedly open new frontiers for future investigations.

ABA, a phytohormone that plays a fundamental role in abiotic stress adaptation, is a small sesquiterpenoid (C₁₅) that also plays important roles in plant growth and development, as well as in response and tolerance to dehydration. ABA is central in regulating the plant response to a variety of abiotic stressful conditions *e.g.*, drought, salt and osmotic stress (Marion-Poll & Leung, 2006). Environmental parameters are known to affect ABA and water status, which in turn affect physiological processes in plants (Kitsaki & Drossopoulos, 2005). ABA is related to both, long- and short-term responses of the plant to several environmental

stimuli. Environment parameters, such as dry soil conditions, temperature stress, relative atmospheric humidity, flooding, photoperiod, light intensity, salinity and wounding, as well as internal factors serving as developmental cues, affect ABA concentration in leaves and other plant organs. ABA regulates transpiration and water loss via stomatal closure (Rock et al., 2010). It is also noteworthy that nitric oxide (NO) functions as a second messenger in the ABA signaling pathway in guard cells (Acharya & Assmann, 2009).

Cramer et al., (2007) have demonstrated that a large number of transcripts involved in ABA metabolism or responsive to ABA are increased with water deficit or salinity over 16 days, suggesting that ABA plays a critical role in grape abiotic stress responses. Water deficit induced increases in ABA concentrations in the xylem sap and leaves of grapevine and changes in stomatal conductance are well correlated with ABA concentrations of the xylem sap (Pou et al., 2008; Soar et al., 2004). ABA also influences hydraulic conductance, aquaporin gene expression and embolism repair in grapevines (Cramer, 2010; Lovisolo et al., 2010). It is hypothesised that in the isohydric grapevine cultivar Grenache, the drought-induced root ABA biosynthesis increases apoplastic concentration because of a concomitance of events: an increase in suberisation of apoplastic barriers causes a reduction in water conductivity which is not compensated by aquaporin-mediated water transport (Lovisolo et al., 2010). Kaldenhoff et al., (2008) suggest an ABA-aquaporin interaction in the repair of grapevine embolism and in the aquaporin activation during water stress. The root and shoot ABA-mediated responses to water stress conditions, or, more generally, to abiotic stresses, are relevant to vine yield and productivity (Lovisolo et al., 2010). As described earlier by Keller, (2005), water stress influences ABA accumulation at the root, shoot and leaf level, and also affects berry quality. However, a connection between ABA and berry quality (sugar composition during fruit development) has not yet been clarified. Grapevine is among the first plant species in which a direct role of ABA in stomatal closure is demonstrated (Lovisolo et al., 2010). In effect, in different grapevine genotypes, during the gradual imposition of soil water stress (non-irrigation) or partial root drying, negative correlations are often observed between stomatal conductance, and either xylem or leaf tissue ABA contents (Lovisolo et al., 2010). These authors have pointed that ABA synthesis in grapevine shoots and leaves increases in response to soil water stress, which implies that some other root-based biochemical signal may trigger this response. Also, further work is required to clearly understand the role of hydraulics on stomatal regulation in grapevine (Lovisolo et al., 2010), in spite of that it is leaf ABA and not whole-plant hydraulic conductivity that determines stomatal conductance. Nevertheless, the primary role of a root-to-shoot hydraulic signal is generally followed by an increased ABA biosynthesis in the shoot that regulates stomata and leaf growth (Chaves et al., 2010).

Vvrd22, a dehydration-responsive gene has been recently isolated and cloned from grapevine of the Cabernet Sauvignon variety (Hanana et al., 2008). It is constitutively expressed at a low level in all analyzed tissues, not only responsible for drought stress but also responsible for salt stress. ABA induces Vvrd22 expression, even at a low level.

Gene expression of the ABA and ethylene pathways is particularly increased by stress compared with other hormone pathways and is negatively correlated with stem water potentials (Cramer, 2010). Using transcript and metabolite profiling, Deluc et al., (2009) have shown that water deficit has significant impacts on the metabolism of grape berries. Water deficit affects the metabolism of ABA in the grapevine cultivars Cabernet Sauvignon and Chardonnay in different ways: it increases ABA concentrations in Cabernet Sauvignon

berries, but doesn't in Chardonnay berries. Effectively, Cramer (2010) has recently emphasized that many of the grapevine responses to osmotic stress appear to be transcriptionally regulated, but proteomic studies indicate that there are post-translational controls as well. Also, metabolite profiling has revealed that accumulation of amino acids and polyamines (PAs) is dependent on ABA production, suggesting the integration of ABA signaling to accumulation of protective molecules (Toumi et al., 2010). The ABA signaling pathway integrates PAs and amine oxidases (AOs) in order to regulate the generation of hydrogen peroxide (H_2O_2), which signals further stress responses of the programmed cell death (PCD) syndrome. ABA enhances PA accumulation in grapes and, at the same time, induces the PA oxidation pathway, thus originating secondary protective effects *e.g.*, the stomata closure. Furthermore, PA's catabolism caused by enhanced expression of AOs, which is induced by ABA, generated H_2O_2 which correlated with the levels of peroxidases and phenolics during vascular differentiation (Paschalidis et al., 2009).

The olive tree tends to acclimatize to prolonged hot-dry periods by reducing the level of ABA at the end of summer, in spite of the low water potential (Kitsaki & Drossopoulos, 2005). During winter periods, leaf ABA content remains low, while water potential values are at their highest level. Concerning ABA content, young olive leaves to be more sensitive to most environmental parameters than old ones. Significant differences in water-stress-induced ABA accumulation have been observed between two *O. europaea* cultivars, thus reflecting the degree of stress experienced (Guerfel et al., 2009). The drought tolerant cultivar 'Chemlali' accumulates lower levels of ABA in their leaves to regulate the stomatal control in response to water stress compared to the drought sensitive cultivar 'Chetoui', which accumulates ABA in large amounts. ABA in nutrient medium originates different olive carbohydrate spectra regarding abiotic stress type (salinity or low temperature) (Rejšková et al., 2007).

4.2 The role of nitric oxide (NO)

Reactive nitrogen species (RNS) are a family of reactive molecules derived from nitric oxide ($\bullet NO$; hereafter called NO). The major RNS in the plant cell is NO (Besson-Bard et al., 2008). NO has the ability to cross cell membranes and can thereby transmit signals to other cells. A biologically important reaction of NO is S-nitrosylation, converting thiol groups (including cysteine residues in proteins) in S-nitrosothiols (RSNO). NO has an important function in numerous cell signalling processes, regulating cell growth, the hypersensitive response, the closure of stomata, plant response to stressors such as drought, high or low temperature, salinity, heavy metals and oxidative stress (Besson-Bard et al., 2008), and also has defense functions (Neill et al., 2008).

Concerning the Mediterranean species, the studies about NO are very scarce. In olive plants, salinity produces a 40% reduction in leaf fresh weight, induces oxidative stress and a dramatical increase in proteins that undergo tyrosine nitration (Valderrama et al., 2007). The specific NOS activity in olive leaves is dependent on L-arginine, NADPH and calcium (Valderrama et al., 2007). Salt stress induces an increase in the L-arginine-dependent production of NO, total RSNO and several proteins that undergo tyrosine nitration, thus functioning as good markers of nitrosative stress. Additionally, the vascular tissues could play an important function in the redistribution of NO-derived forms during nitrosative stress and in signalling-related processes. NO are produced in the olive reproductive organs in a stage- and tissue- specific manner (Zafra et al., 2010).

4.3 The role of MicroRNAs

4.3.1 MicroRNAs are ubiquitous gene regulators at post-transcriptional levels

MicroRNAs (miRNAs) are a newly identified class of 21-24 nucleotide (nt) (predominantly 21 nt) in length, endogenous non-protein-coding short RNAs (sRNAs) in animals, plants and viruses. They are derived from 70- to 500-nt long single stranded primary transcripts (pri-miRNAs), which are transcribed from miRNA genes (*MIR* genes) by RNA polymerase II, by the action of RNase III-like enzymes DICER-LIKE1 (DCL1) or DCL4 (Liu et al., 2009). The mature miRNA is loaded to the RNA induced silencing complex (RISC) to guide the complex to the target mRNAs (containing a stretch of perfect or near perfect complementary sequence). miRNAs have been considered one of the most important regulatory molecules, which regulate gene expression at the post-transcriptional levels via targeting mRNAs for direct cleavage of mRNAs, repressing mRNA translation, or small RNA-directed transcriptional silencing (Jones-Rhoades et al., 2006). Recently, the identified number of conserved and non-conserved species-specific plant miRNAs is rising at an accelerated speed by the newly developed deep sequencing technologies. The latest information on plant miRNAs can be obtained from the miRBase database maintained by Sanger Institute (<http://microrna.sanger.ac.uk>) (Kozomara & Griffiths-Jones, 2011).

MicroRNAs play crucial roles in essential biological processes, including developmental timing, stem cell differentiation, signaling transduction, human disease, and cancer (Couzin, 2008). In plant, miRNAs play a pivotal role in many aspects, such as organ development, phase change, signal transduction, and response to environmental stress (Shukla et al., 2008; Zhang et al., 2007). Many miRNAs are expressed in a cell- or tissue-specific manner during development of organisms and may contribute to the establishment and/or maintenance of cellular identity (Makeyev & Maniatis, 2008).

Laboratory molecular cloning and computational prediction of miRNA genes based on the conservation of sequence and secondary structure are two methods of plant miRNA study. Historically, most plant miRNA genes have been discovered by one or both of the two methods (Meyers et al., 2008).

MicroRNA study concerning the Mediterranean species is rare and almost all from vine. Recently, the grapevine genome of a highly homozygous genotype (Jaillon et al., 2007) and of a heterozygous variety (Velasco et al., 2007) has been published by two independent groups, respectively. These genome data provide a solid support for the study of sRNA-based regulatory networks in grapevine. By a computational-based BLAST search of sequences using *Arabidopsis* miRNAs' genes as references Jaillon et al. identified 164 miRNA genes with a medium size of 103.5 bp and total of 0.002 Mb in the homozygous grape genome (2007); Velasco et al. (2007) identified 143 miRNA genes representing 28 families in the heterozygous genome. They predicted 28 conserved and non-conserved miRNAs in grapevine. A total of 81 potential miRNAs have been computationally predicted; the length of miRNA precursors in grapevine varies from 68 to 207 nucleotides, with an average of 117 ± 42 (Y.D. Lu et al., 2008).

4.3.2 Progress in the study of grapevine miRNA on abiotic stress

Recently, attention has been paid on the role of miRNA in plant abiotic stress mediation, indicating that miRNAs participate in regulating various abiotic stress response, such as drought (Kantar et al., 2011; Xu et al., 2010), salt (Ding et al., 2009), cold (Zhou et al., 2008), heat (S.F. Lu et al., 2008), (see reviews by Phillips et al., (2007) and Shukla et al., (2008)). In

plants, miRNAs target regulatory proteins such as transcription factors, suggesting that miRNAs are master regulators (Phillips et al., 2007; Shukla et al., 2008). Stress-induced or upregulated miRNAs target negative regulators of stress responses or positive regulators of processes that are inhibited by stresses (e.g., cell division and expansion). Alternatively, stress downregulated miRNAs could repress the expression of positive regulators and/or stress upregulated genes. The existence of stress-related elements in miRNA promoter regions provides further evidence supporting its role in abiotic stress (Liu et al., 2008).

For the experimental analysis of miRNAs and other sRNAs, the first and most important step is the isolation of high-quality total RNA. High-quality RNA extraction from grapevine and other Mediterranean woody plants is problematic due to the presence of polysaccharides, polyphenolics and other compounds that bind or co-precipitate with the RNA. A rapid and effective cetyltrimethylammonium bromide (CTAB)-based method for RNA extraction from different tissues of grapevine and other woody plants including olive and chestnut has been reported (Gambino et al., 2008). Eighteen miRNA were computationally predicted to be responsible for abiotic stress in grape (Wei, 2009). Here we will summarize the identified and validated miRNAs of grapevine on abiotic stress.

4.3.2.1 miR398 and oxidative stress

miR398 down-regulates two closely related Cu/Zn-Superoxide Dismutase genes: cytosolic CSD1 and plastidic CSD2 that can detoxify superoxide radicals (Sunkar et al., 2007; Sunkar et al., 2006). It is expressed in a spatial- and temporal-specific manner under normal growth conditions finely tuning the expression of CSD1 and CSD2 transcripts and in turn regulating the levels of superoxide or other ROS required for signalling. miR398 expression is down-regulated transcriptionally by oxidative stress, and this downregulation is important for posttranscriptional CSD1 and CSD2 mRNA accumulation and oxidative stress tolerance. Computational prediction reveals that the miR398 family is represented by three members (MIR398a, MIR398b, and MIR398c) in grapevine (Y.D. Lu et al., 2008) and these members have been recently validated by deep sequencing analysis (Mica et al., 2010; Pantaleo et al., 2010). Among them, miR398b is at least 100 fold higher expressed in root than either leaf or early inflorescences. Furthermore, transgenic *Arabidopsis* plants overexpressing a miR398-resistant form of CSD2 accumulate more CSD2 mRNA than plants overexpressing a regular CSD2 and are consequently much more tolerant to high light, heavy metals and other oxidative stressors (Sunkar et al., 2006), which strongly demonstrates the role of miR398 in plant abiotic stress.

4.3.2.2 miRNAs and water deficit stress

A few miRNAs have been identified to regulate plant drought stress. Two drought-induced miRNAs, miR169g and miR-393, have been validated by microarray analysis in rice plants upon drought stress (Zhao et al., 2007). miR169g is induced more prominent in roots than in shoots. Two ABA-independent dehydration-responsive elements (DREs) exist in the upstream of the promoter region of the MIR169g gene, supporting its role in plant water deficit (Zhao et al., 2007). miRNA169a/c are found to be drought downregulated in *Arabidopsis thaliana* (Li et al., 2008). miR393 is a plant miRNA thought to regulate expression of mRNAs encoding the F-box auxin receptor, including transport inhibitor response1 (TIR1), which in turn targets AUX/IAA proteins for proteolysis by SCF E3 ubiquitin ligases in an auxin-dependent manner and is necessary for auxin-induced growth processes. Thus, miR393-mediated inhibition of TIR1 would down-regulate auxin signalling and seedling growth under abiotic stress conditions and further relate to drought stress. In addition,

miR1867, miR474, miR398, miR1450, miR1881, miR894, miR156, and miR1432 are upregulated in *Triticum dicoccoides* under drought stress (Kantar et al., 2011). It is expected that miRNAs that shut down processes involved in normal metabolism and growth are upregulated during drought stress, in order to conserve water and protect the cell. One good example would be miR156, which downregulates transcription factors involved in development and flowering. miR169g and miR169c have been computationally identified (Y.D. Lu et al., 2008), and 25 miR169 members (miR169a-y) have been validated in grapevine (Mica et al., 2010). miR393 has also been predicated to be involved in signalling pathways by regulating transport TIR1 (Y.D. Lu et al., 2008). Experimental work has shown that miR393 is expressed at a higher level in inflorescences than in tendrils in grapevine (Pantaleo et al., 2010). miR156 has been computationally identified (Velasco et al., 2007) and cloned (Pantaleo et al., 2010) in vine, respectively. In another independent work, it is predicted that ath-miR156 and vvi-miR157 share the same target SPL (Y.D. Lu et al., 2008).

4.3.2.3 miRNAs and nutrient deficiency stress

MicroRNA399 regulates phosphate stress responses. Upon Pi starvation, increased miR399 expression represses the expression of ubiquitin-conjugating E2 enzyme (UBC24) and consequently the repression of Pi uptake is alleviated in Arabidopsis (Aung et al., 2006). Where in grapevine miR399 is predicated to target AF2 (Y.D. Lu et al., 2008). miRNA399 is more highly expressed in roots (Mica et al., 2010) and at a very low level in leaves (Pantaleo et al., 2010), which may explain the absence of cleaved targets.

MiR395 is involved in sulphur accumulation and allocation by targeting both ATP sulfurylases and the sulfate transporter AST68 (SULTR2;1). During sulfate limitation, expression of miR395 is significantly up-regulated (Liang et al., 2010). In grapevine miR395 is predicted to target mRNAs coding for ATP sulphurylases (Y.D. Lu et al., 2008). The expression of miR395 family is higher at leaf than at tendrils, inflorescences and berry (Mica et al., 2010); and that is higher at tendrils than in inflorescences (Pantaleo et al., 2010).

In addition, several UV stress responding miRNAs and their target genes have been computationally predicted in grapevine (Wei et al., 2008).

5. Genetic modifications targeting improved plant abiotic stress

Several plant breeding approaches will likely be needed to improve the abiotic stress tolerance and maintain optimum yield levels of the Mediterranean crops in field conditions. The main method of crop improvement continues to be the conventional plant breeding through sexual hybridization, sometimes combined with classical cytogenetic techniques (Roy et al., 2011). Conventional breeding and marker assisted selection are being used to develop cultivars more tolerant to abiotic stress. However, these methods are time and resource consuming and germplasm dependent. On the other hand, improvement of stress tolerance by genetic engineering overcomes the bottlenecks of plant breeding methods.

Recently, efforts have been devoted to identifying potential target genes for use in genetic engineering for crop abiotic stress tolerance (Cushman & Bohnert, 2000). These include specific heat shock proteins, ion transporters, water transporters (aquaporins), as well as signalling components, such as, MAP kinases, Ca²⁺-dependent protein kinases, transcription factors, like, DREB, CBF and Myb, and enzymes of plant hormone metabolism (Cushman & Bohnert, 2000; Mittler & Blumwald, 2010).

The use of mutation techniques in *Arabidopsis* to obtain knock out and up-regulated mutants, and the elucidation of stress defence mechanisms in yeast and humans, where these mechanisms are highly conserved in eukaryotes, has also made a major contribution (Cassells & Doyle, 2003). Publication of the genome draft sequence of two grapevine genotypes (Jaillon et al., 2007; Velasco et al., 2007) offers new perspectives on genomic research in grapevine as well as in other tree species (Gambino et al., 2009).

Plant response to drought stress is quite complex, and is associated with a large number of physiological and biochemical changes. Some of those changes, such as osmotic stress adjustment, ABA accumulation, and root morphology, are known to be controlled by multiple genes (Khan et al., 2009; Lilley et al., 1996). One promising genetic path is the mapping of quantitative trait loci (QTL) that relate performance and yield to abiotic stress factors (Collins et al., 2008). Recently, QTLs for downy mildew resistance have been localized in the genetic linkage maps of two interspecific grape crosses (Moreira et al., 2011). Besides *Agrobacterium*-mediated gene transformation, another advantageous transformation method is the particle bombardment, and indeed the only one available for many species (Altpeter et al., 2005). Although there is widespread belief that particle bombardment generates large, multi-copy loci prone to instability and silencing, refinements of the technology to produce clean transgene loci have demonstrated clearly that this is not the case, and that particle bombardment has many advantages for the production of commercial transgenic plants that perform well in the field and comply with all relevant regulatory processes (Altpeter et al., 2005). Protocol for olive somatic embryos genetic transformation by particle bombardment has been recently established (Perez-Barranco et al., 2009).

The induction of stress tolerance through engineering for over-expression of transcriptional factor genes is emerging as an attractive proposition (Yang et al., 2009). Transcription factors are regulatory proteins that modulate gene expression through sequence-specific DNA binding and/or protein-protein interactions. They are capable of acting as switches of the regulatory cascade by activating or repressing transcription of target genes. In plants, the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE) binding protein 1 (CBF/DREB1) transcription factors control an important pathway for increased freezing and drought tolerance. Three CBF/DREB1-like genes, CBF 1-3, have been cloned from both freezing-tolerant wild grape (*V. riparia*) and freezing-sensitive cultivated grape (*V. vinifera*) (Xiao et al., 2008). The transgenic grapevine over-expressing DREB1b has been proved to have a significantly improved resistance to cold stress (Jin et al., 2009).

The grapevine WRKY transcription factor has 66% and 58% identity at the DNA and amino acid sequence levels, respectively, with *Arabidopsis* AtWRKY11 genes, and has been therefore designated VvWRKY11 (Liu et al., 2011). Transgenic *Arabidopsis* seedlings over-expressing VvWRKY11 show higher tolerance to water stress induced by mannitol than wild-type plants. It is expected that a rapid progress in the development of stress tolerance genotypes in grapevines will be achieved in the near future, because there are large genetic resources for grapevine and there are a large number of high-throughput genomic tools available to conduct functional genomic analyses (Cramer, 2010).

cDNAs from *O. europaea* related to the aquaporin (AQP) gene family have been isolated and characterized (Secchi et al., 2007). The transcript levels of each AQP gene diminished strongly in plants submitted to drought. The down-regulation of AQP genes may result in reduced membrane water permeability and may limit loss of cellular water during periods of water stress (Secchi et al., 2007).

High temperature tolerance has been genetically engineered in plants mainly by over-expressing the heat shock protein (HSP) genes or indirectly by altering levels of heat shock transcription factor proteins (Singh & Grover, 2008). HSP70 has been cloned and characterized from olive tree (Drosopoulou et al., 2009). The presence and organization of many typical binding sites for the Heat Shock and GAGA factors in the sequence suggest that the promoter of this gene is highly heat-inducible and could be used for conditional expression in transformation systems (Drosopoulou et al., 2009).

Modern plant breeding involves novel technical approaches, and gene transfer is undoubtedly a powerful tool. However, genetic engineering does not always result in efficient transgene expression. Several cases have been reported, where transgene copy number does not correlate with the level of transgene expression (Gelvin, 2003).

6. Conclusions and future prospects

Olive tree and grapevine have evolved fine adaptation mechanism to drought, heat and high irradiation at morphological, anatomical, physiological and biochemical levels. Low altitude (consequently high air temperature) restricts the distribution of chestnut in Europe. An abiotic stress may initiate multiple signaling pathways in Mediterranean plants. Because ABA is involved in abiotic stress signaling, revealing how ABA is perceived certainly will help reveal how stress signals are sensed. The study of the newly identified signal molecules NO and miRNA on the Mediterranean crops is just emerging. The related data concerning chestnut is still absent. The elucidation of the interaction and crosstalk among NO, ROS and ABA, and their relation with miRNAs in regulating plant abiotic stress will give a novel panorama of the abiotic stress signaling networks. Introducing the most important genes involved in tolerance to the various abiotic stresses into sensitive Mediterranean species will allow the coordinated expression of these genes to improve abiotic stress tolerance.

7. References

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Changing environmental condition and global population demands understanding the plant responses to hostile environment. Significant progress has been made over the past few decades through amalgamation of molecular breeding with non-conventional breeding. Understanding the cellular and molecular mechanisms to stress tolerance has received considerable scientific scrutiny because of the uniqueness of such processes to plant biology, and also its importance in the campaign “Freedom From Hunger”. The main intention of this publication is to provide a state-of-the-art and up-to-date knowledge of recent developments in understanding of plant responses to major abiotic stresses, limitations and the current status of crop improvement. A better insight will help in taking a multidisciplinary approach to address the issues affecting plant development and performance under adverse conditions. I trust this book will act as a platform to excel in the field of stress biology.

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