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# Abiotic Stress Tolerance in Plants with Emphasizing on Drought and Salinity Stresses in Walnut

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Additional information is available at the end of the chapter

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## 1. Introduction

Drought and salt stress, together with low temperature, are major problems for agriculture because these adverse environmental factors prevent plants from realizing their full genetic potential. Salt stress afflicts agriculture in many parts of the world, particularly irrigated lands [4]. Compared to salt stress, the problem of drought is even more pervasive and economically damaging [1; 3]. Temperature and precipitation are key determinants of climate. The Koppen Climate Classification System recognizes five major climatic types: A, Tropical Moist Climates; B, Dry Climates; C, Moist Mid-latitude Climates with Mild Winters; D, Moist Mid-latitude Climates with Cold Winters; and E, Polar Climates. The Dry Climates are easily recognized (a desert is after all a desert) but water-limited environments can be difficult to classify precisely [30]. Meigs [30] developed a widely used system for classifying water-limited environments based upon mean precipitation. Extremely arid lands have at least 12 consecutive months without rainfall, arid lands have less than 250 mm of annual rainfall, and semiarid lands have a mean annual precipitation of between 250 and 500 mm.

Drought stress signaling certainly merits separate treatment. Nevertheless, most studies on water stress signaling have focused on salt stress, primarily because plant responses to salt and drought are closely related and the mechanisms overlap. Salinity is detrimental to plant growth, causing nutritional constraints by decreasing uptake of phosphorus, potassium, nitrate and calcium, ion cytotoxicity and osmotic stress. Under salinity, ions like  $\text{Na}^+$  and  $\text{Cl}^-$  penetrate the hydration shells of proteins and interfere with the function of these proteins.

Uptake of abundantly available  $\text{Na}^+$  and  $\text{Cl}^-$  therefore, offers a comparatively cheap way to lower the tissue-osmotic potential. To avoid the risk of ion toxicity associated with this strategy,  $\text{Na}^+$  and  $\text{Cl}^-$  are generally compartmentalized in the vacuole and/or in less sensitive tissues [228]. In parallel, adjustment of the cytoplasmic compartment is achieved via production of

compatible osmolytes such as, proline, mannitol, sorbitol, and glycine betaine. The latter also acts as an antioxidant and thus detoxifies reactive oxygen species (ROS) [227]. Ionic toxicity, osmotic stress, and nutritional defects under salinity lead to metabolic imbalances and oxidative stress. From a practical point, salt stress can be imposed more easily and precisely in laboratory settings. Although the importance of salt and drought stress signaling was recognized long ago, few molecular components were known until recently.

Also, drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell [5-6]. Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins [7]. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways [8] and cellular responses, such as production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes [9-11]. Compatible solutes are small organic metabolites that are very soluble in water and are non-toxic at high concentrations. Therefore, breeding for drought and salinity stress tolerance in agronomy and horticultural crops (for food supply) and in forest trees (a central component of the global ecosystem) should be given high research priority. Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress related genes. These genes are involved in the whole sequence of stress responses, such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. Recently, research into the molecular mechanisms of stress responses has started to bear fruit and, in parallel, genetic modification of stress tolerance has also shown promising results that may ultimately apply to agriculturally and ecologically important plants [180].

Persian walnut (*Juglans regia* L.) is one of the most economically valuable tree species of northwest, northeast and central regions of Iran. Natural distribution of this species is quite sensitive to site water status [213]. Walnut trees need large amounts of water for optimum growth and productivity and are among the more sensitive plants to abiotic stresses [14]. The majority of walnut trees in the world are propagated by seed or by grafting onto seedling rootstocks [101]. Hence, there is huge genetic diversity among rootstock traits. For example, there are many old Persian walnut trees in Iran that have been planted on the banks of rivers. The survival of these trees for hundreds years may indicate possession of valuable stress resistance genes that help them cope with unfavorable environmental conditions [101].

Finding genetic resources tolerant to abiotic stress at different growth stages is important for such arid and semiarid regions. In studies of fruit trees, half-sib progeny of several species (for example, 'Serr' walnut, 'Texas' almond, 'Lovell' and 'Missouri' peach) have been used for producing rootstocks [180]. Because half-sibs are individuals that have one parent in common and differ in the other parent, the mean genotypic value of the group of half-sibs is by definition half the breeding value of the common parent. This reduces the number of seedlings of half-sib families needed as replicates for studying tolerance genes in rootstock breeding programs [213; 219].

The various parts of a walnut tree differ in their needs during the year for photosynthate for respiration, growth of new plant parts, and developing nut crop. Depending on the cultivar, heavy crop loads may adversely impact the following year's crop by reducing female flower initiation and the amount of stored carbohydrates. The processes associated with nut production appear to be under strong genetic control; thus annual heavy nut production will require selection of seedlings of walnut cultivars exhibiting multiple leaf layers to maximize photosynthetic production, tendencies toward lateral bearing, good resistance to anthracnose, and efficient use of photosynthesis for tree growth and nut production.

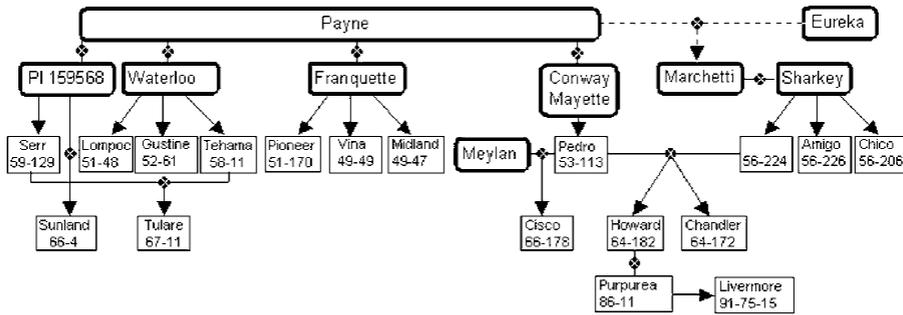
The genus *Juglans* consists of four sections. Three of these, Rhysocaryon (black walnuts native to the Americas), Cardiocaryon (Japanese, Manchurian and Chinese walnuts, including selections known as heartnuts) and Trachycaryon (the butternut of eastern North America), exhibit thick shells and non-dehiscent hulls [234]. The fourth section, *Juglans*, is comprised of a single species, *Juglans regia* L., distinguished by a dehiscent hull which separates from the shell at maturity [234]. *J. regia*, the Persian walnut, is native to central Asia and grows as a wild or semi-cultivated tree in a wide area from south-eastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. It has been cultivated for its nut crop for at least several thousand years and was probably introduced into European commerce and agriculture by the ancient Greeks. It was prized by the Romans as *Jovis glans* and was utilized in medieval Europe as an herbal medicine, particularly for brain and scalp ailments. Since its introduction into North America it has commonly been referred to as the English walnut to distinguish it from the American black walnut while the correct name is Persian walnut [15; 217].

Species of walnut are distributed in temperate and subtropical areas of the Northern Hemisphere, mainly in mountain forests. Walnuts are distributed in three separate regions, Mediterranean, East-Asian Himalayas, and North American. Walnut is deciduous, monoecious, and wind-pollinated. Walnut trees are sharply differentiated from other fruit trees by their size and vigor, tree height, and crown diameter, often reaching 30 m and trunk diameters as large as 2 m. Trees may have single trunks or be multi-stemmed [13].

The pedigree of major seedlings of walnut cultivars and advanced selections in the breeding program in California is shown in Figure 1.

Walnut (*Juglans spp.*) is generally very sensitive to specific ion toxicities [218]. but genetic variation in growth indices and morphological, physiological, biochemical and cellular responses to water stress, especially in germination and early growth of walnut seedlings, have been studied to some extent. There are clear economic incentives for identification of drought-adapted walnut genotypes that can be used successfully in extensive arid and semi-arid regions. Our preliminary work has identified some walnut seedlings that are very tolerant to drought and especially to salt stresses at the germination stage [192; 212]. Mechanisms of adaptation and tolerance in selected walnut rootstocks of walnut were also investigated.

To facilitate breeding for improved water use and drought resistance, a number of questions should be addressed: (1) What physiological traits contribute to efficient water use and high drought resistance and how do they interact with traits of rapid growth? (2) What is the range



**Figure 1.** Pedigree of major seedlings of walnut cultivars and advanced selections in the breeding at the California, Davis, [Courtesy Dandekar et al., 2004]

of variation among walnut varieties in critical traits setting the potential for breeding? (3) Can diagnostic tools be developed for identification of critical traits that could serve as selection tools in breeding programs? In this chapter we review and discuss the available literature and current knowledge regarding abiotic stress in walnut. Following we explain the morphological, physiological and molecular aspects of abiotic stress responses in plants emphasizing on walnut in three separate sections and summarize related research in each case.

## 2. Morphological responses to abiotic stresses

### 2.1. Soil-root interface and water absorption

The efficiency of any tree in terms of water relations depends on its ability to absorb water at a rate able to prevent internal water deficits during periods of high transpiration. Water supply to trees implies two major steps: absorption and transport of water (i.e. ascent of sap), both driven by transpiration. The efficiency of soil water absorption in trees depends on both spatial extension and density of their root system [18]. Spatial extension: water uptake by individual trees depends on fine root exchange surface, i.e., on their cumulated length or biomass. Both vertical root distribution and seasonal root growth dynamics depend closely on physical soil properties (mainly texture like clay content, bulk density, content of coarse elements etc.) and the physiological constraints on root survival and development (water table, oxygen supply, nutrients, aluminum or manganese toxicity, soil pH). Climate itself could also influence fine root dynamics [19]: data from a literature survey support the view that rainfall is one of the major environmental factors controlling fine root biomass [20].

Surprisingly, an exceptionally small fine root biomass was detected in a dry beech stand when compared to five other stands with higher rainfall [20]; this could be due to a large mortality of fine roots during peak drought. It is also well established under continental [21-22] or like mediterranean climates [21] that soil water uptake displays a gradual downward shift as the

soil dries out, and that a small fraction of total fine roots, growing deeper into the soil, ensures the overnight recovery of the soil to tree water potential equilibrium [22], and supports a fraction of tree transpiration during periods of stomatal closure. At least this small fraction of root systems enables survival of the trees by providing the unbearable amount of water. Most of the studies reported the occurrence of two periods of active root growth in fruit trees, namely during spring and early autumn. The two periods of slowest root growth occur during winter and summer, and coincide with lowest soil temperatures and with lowest soil moisture, respectively [21-22].

With its large canopy and expansive root system, the walnut tree (*Juglans regia*) has more specific soil and water requirements than most other temperate-zone fruit trees. However, its cultivation has been extended more recently to marginal lands with inappropriate soil quality and limited water resources, aggravating the problems of nutrition and irrigation management. A study conducted during 1999-2000 in a 13-year old walnut orchard established on a shallow flood plain with stratified sand and loam soil was undertaken to diagnose the main factors that adversely affect walnut growth and yield. Results showed that high boron (B) concentration in irrigation water (>300 ppm) was the main factor inhibiting tree growth, reducing expected yield by 94 % and adversely affecting nut quality. Shallow soil, light soil texture, and deficiency in micro and macro elements also adversely affect orchard establishment and lead to poor yield and low nut quality [180].

## 2.2. Strategies for water economy

Homoiohydric plants have evolved a hierarchy of protective mechanisms that maintain favorable protoplasmic water content or modify the deleterious effects of stress on cellular constituents. In contrast, poikilohydric plants are unable to control water loss to the environment with the result that cellular water content fluctuates in concert with external water availability. The prefixes homo- and poikilo- are widely used in terminology related to eukaryotic physiology. For clarity they are defined, by the Oxford English Dictionary (<http://dictionary.oed.com>), as 'of the same kind' and 'variegated', respectively. However, we maintain that no plants are homoiohydric in the strict definition of the term because plants are incapable of maintaining their water content at a fixed value. Plants cannot create water where none exists, and ultimately all plants are unable to control water loss to the environment [12].

In the dry season, plants with deep root systems are believed to take water from the deep soil layers, thereby avoiding or minimizing water stress [28]. However, detailed studies of soil water status, root distribution, water resource derivation and shoot water stress development under natural, varied moisture conditions during the same time period were lacking for walnut until 2011. Of interest is that deep water resources can compensate for drought in the air and upper soil layers. For example, *Juglans regia*, which has an extensive root system, has a wide distribution in the mountainous regions of northern, central and west Iran and northern China. This could show that the ecophysiological responses of the aboveground shoots of *J. regia* in response to drought in their natural habitat, as well as under controlled greenhouse conditions, have been well studied and the mechanisms underlying these shoot responses are well understood [16; 121]. Also, detailed studies on

the role of below-ground root structures of *J. regia* during the development of water stress in field environments and variation in soil water uptake and its effect on plant water status during dry and wet seasons have been published [17].

In recent years, hydrogen and oxygen isotopic application has contributed significantly to tracing and understanding below-ground processes [23-24]. During water transport between roots and shoots, the isotopic composition of xylem water remains unaltered from that of the soil [23]. Therefore, it is reasonable to analyze the branch xylem water to determine the water source [25]. Soil water is also a key factor in restoring forest ecosystems in arid and semi-arid zones [26], while the efficiency of soil water uptake by trees could be the ultimate determining factor in their productivity [26-27]. Therefore, knowledge of root distribution and mechanisms of soil water extraction and transport by trees is indispensable for successfully restoring ecosystems.

Walnut root growth differs in dry and wet seasons [17]. Mean root length in both the upper (0-30 cm) and deep (30-80 cm) soil layers shortened when the soil water content and relative humidity of the air were lower [17]. After rain events, re-watering, or irrigation, the total root length increased compared with dry periods [17]. The abundance of new roots significantly increased in both the upper and deep soil layers in response to the rain and rewatering events. The growth of new roots was greater in the upper soil profile than in the deep soil profile. Dead root length in the upper soil layer was significantly greater in the wet season than in the dry season, while no difference in dead roots has detected in the deep soil layer between the seasons and diameter of the roots did not significantly change by season [17]. Water supply to trees involves two major steps: absorption and transport of water (i.e. ascent of sap), both driven by transpiration. The efficiency of soil water absorption in trees depends on both spatial extension and density of their root system [18].

There is significant variation in the vertical distribution of roots among different walnut varieties [17; 212]. Roots are the most abundant at 10-30 cm depth, followed by 0-10 cm depth. Root biomass decreases with depth below 30 cm. Generally, most of the root surface area, root length density and root biomass were confined to the upper soil layers (0-30 cm), accounting for 61, 62.5 and 79% of the total root measurements from the 0-80 cm soil layers, respectively [17]. Walnut roots were mainly distributed in the upper soil layers at our study sites and likely in the whole region. Soil moisture was a key factor regulating root growth and water uptake efficiency of the roots [17; 212]. The shallow roots had reduced efficiency in water uptake in the dry season, and therefore *J. regia* was compelled to extract a greater ratio of water from the deep soil layers. However, the shift was not able to prevent water stress on the plants, which were characterized by increased pre-dawn branch xylem PLC, reduced pre-dawn leaf water potential and transpiration with soil drying [17]. In addition to serving as an indicator of water sources, changes in the stable-hydrogen isotope ( $\delta D$ ) values in walnut branch xylem water reflected plant water status and the severity of soil drought.

### 2.3. Excess water supply

On soils subject to flooding or with shallow restrictive layers, excess soil moisture can also be a problem. Excess soil moisture during the growing season leads to decreased oxygen in the

soil and death of roots needed to absorb adequate soil water during periods of high transpiration. On soils with restrictive layers in the walnut rooting zone, soil water accumulates above the restrictive layer leading to a perched water table during the dormant season. Walnut roots within the perched water table die from a lack of oxygen. If these roots are not replaced during the growing season, it results in a reduced capacity to absorb soil moisture during the following growing season, followed by stomatal closure from moisture stress and subsequent decreases in the rate of photosynthesis [14].

#### **2.4. Deficient water supply**

Insufficient available soil moisture causes stresses that can lead to wilting and premature defoliation under extreme conditions. Under less extreme conditions, the stomata close to decrease the rate of transpiration. When this occurs, carbon dioxide can no longer enter into the leaves through the stomata and photosynthesis decreases. If walnut orchards are not going to be irrigated, then soil depth and water holding capacity become very important during site selection for the walnut orchard. The water held within the rooting zone determines if adequate soil moisture is available during dry spells. In the central hardwood region, droughts usually occur in late summer when there is a high demand for photosynthesis to fill the developing nuts. Lack of adequate soil moisture in late summer can also affect the physiological condition of the tree and suppress the initiation of female flowers necessary for the following year's crop [58].

#### **2.5. Germination under abiotic stress conditions**

The germination percentage of walnut seeds of eighteen cultivars decreases significantly in response to decrease in (more negative) water potentials and increases of salinity level. Decreasing the water potential to  $-1.0$  MPa reduced the germination of all varieties to less than 50% and at  $-1.50$  MPa, the germination decreased to less than 25% [192]. The drought and salt stress treatments were unaffected by the size of seeds or seed weight and there was not a significant correlation between percent germination and either seed or kernel weight [212]. Seedlings of walnut cultivars showed differential responses to salt stress under greenhouse conditions. Increase in salinity levels decreased root and shoot length, diameter, and fresh and dry mass, especially those of shoots. Seedlings of 'Lara' and 'Chandler' were most and least affected by salt stress, respectively. The increase in salinity levels was accompanied by a substantial decrease in root RWC (relative water content) [212]. Seed germination rates were generally more rapid in control (no salt stress) than in salt containing media. The FGP (final germination percentage) values were significantly lower at higher salinity levels and there were also differences in FGP among species [212].

The mean germination time differed both among different treatments and cultivars and also a significant interaction was found between these two factors under salt stress condition [212]. For all the seedlings of walnut cultivars studied, the mean germination time was shorter in the control than in the other treatments [212]. There were also differences in the mean germination time among cultivars. At high salinity levels (200 and 250 mM), the average mean germination

time for 'Chandler' and 'Panegine20' was 2.3 to 5.2 d and 2.6 to 5.4 shorter, respectively, than those observed in the other cultivars [192; 212].

In the study of 18 walnut cultivars, the lengths of nuts were varied from 2.07 ( $\pm$  0.47) cm for 'Lara' to 4.08 ( $\pm$  0.78) cm for 'K72'. Seed size could be a factor affecting germination in stressed media [212]. Many studies have shown that various seed sizes and weights may behave differently in terms of germination under stress conditions [180; 212]. It is generally believed that large seed sizes have a higher propensity for germination in saline and dry media. However, some previous studies, found a negative relationship between seed size and germination capacity in *Trianthema triquetra* L. Within the range of seed sizes studied, we did not observe any significant differences in the germination response and analysis failed to show any relationship between percent germination and seed weight under both salt and drought stress.

## 2.6. Wilting

The amount of water lost before visible leaf wilting varies by species. Temporary wilting is the visible drooping of leaves during the day followed by rehydration and recovery during the night. During long periods of dry soil, temporary wilting grades into permanent wilting. Prolonged permanent wilting kills trees [14; 212; 220]. The relation between water loss from leaves and visible wilting is complicated by large differences among species in the amount of supporting tissues leaves contain. Leaves of black cherry (*Prunus*), dogwood (*Cornus*), birch (*Betula*), and basswood (*Tilia*) wilt readily. Leaf thickness and size do not prevent wilting. Rhododendrons are also extremely sensitive to drought with leaves that curl, then yellow and turn brown. By comparison, the leaves of holly and pine are supported with abundant sclerenchyma tissue (i.e. tough, strong tissue) and do not droop readily even after they lose considerable water.

## 2.7. Leaf shedding

In normal abscission, an organized leaf senescence process, which includes the loss of chlorophyll, precedes leaf shedding. With severe drought, leaves may be shed while still full of valuable materials [220]. For example, sycamore (*Platanus*) sheds some leaves, and buckeye (*Aesculus*) may shed all of its leaves, as drought continues. On the other hand, leaves of dogwood (*Cornus*) usually wilt and die rather than abscise. Many times these leaves are stunted [220]. Walnut is also known to shed leaves in response to drought [61]. Sometimes drought-caused leaf shedding may not occur until after rehydration. Abscission can be initiated by water stress but cannot be completed without adequate water to shear-off connections between cell walls. The oldest leaves are usually shed first [220].

Injury to foliage and defoliation are most apparent in portions of the crown that are in full sun. These leaves show drought associated signs of leaf rolling, folding, curling, and shedding. Over the past 20 years, our knowledge of the hydraulic architecture of trees has increased and some hypotheses have been raised to explain how trees might be designed hydraulically to help them cope with period of drought [220]. Hypotheses have generally invoked a mechanism

that permits plants to shed expendable distal components of its shoots while preserving other parts that represent years of carbon investment. Leaf shedding is a potentially cost-effective way for plants to deal with drought stress by a plant segmentation mechanism.

## 2.8. Growth inhibition

Growth of vegetative and reproductive tissues of walnut is constrained by cell initiation shortages, cell enlargement problems, and inefficient food supplies. Cell enlargement depends upon hydraulic pressure for expansion and is especially sensitive to water stress. Cell division in generating new cells is also decreased by drought.

## 2.9. Shoot growth

Internal water deficits in trees constrain the growth of shoots by influencing development of new shoot units (nodes and internodes). A period of drought has a carry-over effect in many species from the year of bud formation to the year of expansion of that bud into a shoot. Drought also has a short-term effect by inhibiting extension of shoots within any one year. The timing of leaf expansion is obviously later than that of shoot extension. If shoot extension finishes early, a summer drought may affect leaf expansion but not shoot extension [220].

Shoots of some trees elongate for only a few weeks in late spring. This growth form is called fixed or determinant growth. Other species elongate shoots over a period of several months which is called multiple flushing or continuous growths. A late July drought may not affect current-year shoot elongation in species with fixed growth, like oaks. Oak shoots expand only during the early part of the growing season [220]. A late July drought can inhibit expansion of shoots from multiple flushing species, like sycamore, which elongate shoots during much of the summer. Spring and summer droughts damage both types of trees. In the southern pines, late summer droughts will influence expansion of shoots in the upper crown to a greater extent than those in the lower crown [212; 220]. This is because the number of seasonal growth flushes varies with shoot location in the crown. Shoots in the upper crown normally exhibit more seasonal growth flushes than those in the lower crown. Buds of some lower branches may not open at all [220].

In walnut, the maximum decrease in shoot fresh weight was observed after 4 days of osmotic stress treatment [192; 212-213]. Response of half-sib families differed as the severity of water and salt stress increased. Under severe osmotic stress (-1.50 MPa), offspring of 'Panegine20' and 'Chandler' produced the greatest shoot fresh weight [212].

Available water, more than any other resource, determines the annual growth potential of individual trees. Variations in water availability account for up to 80% of the inter-annual variability in size increment in temperate stands. Tree water deficits dramatically reduce height and radial growth as well as bud production [168]. Abiotic stress experiments on one and two year old trees of promising walnut varieties showed the same trends [192; 212-213]. Twig growth patterns are affected during several years, as demonstrated by Fulton and Buchner [14] for Persian walnut in California. Recovery from the previous drought was still not complete when the next drought began, and induced even further

growth suppression. A similar reduction of twig growth over several years after drought was also seen in black walnut [214-215].

### 2.10. Cambial growth

Cambial growth slows or accelerates with rainfall. Cambial growth is constrained by water supply of both the current and previous year. Last year's annual growth ring of wood affects growth material supply on this year's growth [220]. This year's drought also will affect next year's cambial growth. Such a delayed effect is the result of drought impacts upon crown development, food production, and tree health. Drought will produce both rapid and delayed responses along the cambium [220]. Shoot thickness of seedlings of sensitive and semi-tolerant walnut genotypes decreased significantly in response to increased osmotic stress [212-213].

The stem of a woody plant comprises several different cell/tissue layers [222], from the periphery and inwards: the protective outer bark; the inner bark with the phloem responsible for sugar transport from leaves to roots; the vascular cambium responsible for growth of new phloem outwards and new xylem inwards; and the mature xylem responsible for water transport [222]. Transport occurs in conduits, comprising separate cell elements, interconnected by pores in their walls and/or series of cell elements forming vessels; all water conducting cell elements die after completion of secondary cell wall growth and are then filled with water. Zwieniecki et al. [37; 88] suggested that the interconnecting pores have a variable diameter, since pectin is present in the pores and acts as a hydrogel in response to variable ion concentration in the transported water.

### 2.11. Root growth

When roots are exposed to drought, the allocation of food to root growth may increase [220]. This provides more root absorptive area per unit area of foliage and increases the volume of soil colonized. Extended drought leads to root suberization to prevent water loss to the soil. Good water absorbing ability, coupled with a low transpiration rate for the amount of food produced (high water-use efficiency), allows trees a better chance to survive drought conditions [220]. The annual root system (absorbing roots) takes up a majority of the water in a tree. Annual roots are not the woody roots seen when a tree is dug. Large woody roots have bark. Any bark crack or damage is quickly sealed-off so little water flows through these areas. It is the young roots, the roots easily damaged by drought, which are the major absorbers of water and essential elements in a tree [220].

In a study on walnut, under drought and salt stress, root length and dry weights for the seedlings of many genotypes decreased significantly in response to increased osmotic stress levels. Albeit under high osmotic pressure due to drought or salt stress root length was greatest in the most tolerant varieties, 'Chandler' and 'Panegine20' [212-213]. Root dry weight of most genotypes decreased significantly in tolerant genotypes vs. non-tolerant ones. Tolerant genotypes ('Chandler' and 'Panegine20' and relatively 'Hartley'), had more or less similar trend in term of root length and dry weight and did not show significant differences at high  $\Psi_s$ .

Generally, the root component accounts for 20 to 90% of the total resistance (reciprocal of conductance) of the plant [38]. This variability largely reflects differences in the proportion of roots, their anatomy and the depths at which they grow [39; 41]. The resistance to water transport in roots is initially relatively high as water has to pass a complex anatomical structure before reaching the conduits of the xylem [40; 42].

The importance of roots for plant water relations increases with the onset of drought for several reasons. First, root growth is typically favored over leaf growth early on during drought, thus growth of the organ exploiting the most limiting resource is favored [43]. Second, under more severe conditions of drought, root layers may shrink or lateral roots may die from dehydration causing deteriorated contact with soil particles holding water, thus increasing the resistance of hydraulic water transport from soil to roots [44; 46]. Third, roots seem to be particularly prone to suffer cavitation of conduits. In many species, including poplar [45; 47], willows and walnut [212] roots are more vulnerable to xylem cavitation than shoots.

## **2.12. Root and shoot water content**

Tissue water content may be expressed in several ways, including the amount of water per unit dry or fresh weight and per unit weight of water at full hydration. Fresh weight seems to be the less accurate of them to measure tissue water content because is highly influenced by changes in tissue dry weight [213]. Sometimes decreases in tissue water content may be more important than decreases in water potential or pressure potential in terms of influencing growth.

The vast majority of land plants, including all major horticultural plants, would be classified as drought avoiders. Although vascular plants do produce specialized structures capable of withstanding severe stress (e.g. pollen, seeds and spores), few species can survive substantial loss of water from their vegetative tissues [34 -36]. Tolerance is the ability to withstand a particular environmental condition. Under water-limiting conditions, plants will experience a net loss of water to the environment and cells will dehydrate (i.e.  $\Psi_w$  and relative water contents, RWC, will decline). Land plants can be classified based upon how they respond to this water deficit. Drought-avoiding plants strive to maintain elevated  $\Psi_w$ . Drought-tolerant plants are able to tolerate extended periods of water deficit. However, both drought-avoiding and drought-tolerant plants will reach a 'permanent wilting point' where  $\Psi_w$  has declined to such a degree that the plant cannot recover upon rewatering.

Under stress condition, derangement in the leaf water potential and its components takes place [31]. It is reported that the water relation and transpirational parameters are closely correlated, and in the laboratory, where equipment to quantify plant water potential are not available, determination of the RWC is still a valid parameter to quantify the plant water status [32-33].

RWC is a measure of the relative cellular volume that shows the changes in cellular volume that could be affecting interactions between macromolecules and organelles. As a general rule, a RWC about 90-100% is related to closing of the stomata pore in the leaf and a reduction in the cellular expansion and growth. Contents of 80-90% are correlated with changes in the

composition of the tissues and some alterations in the relative rates of photosynthesis and respiration.

Under salt and drought stress, different seedlings of walnut cultivars show significant differences in RWC content. Semi-tolerant ('Hartley') and tolerant ('Chandler' and 'Panegine20') cultivars of walnut have moderate and high levels of TWC and RWC at osmotic stress level [212]. RWC below 80 % usually implies a water potential on the order of -1.5 MPa or less, and this would produce changes in the metabolism, reduced photosynthesis, increased respiration and increased proline and abscisic acid accumulation.

### 2.13. Root biomass

Walnut root growth differs significantly between the dry season and wet season. Mean root length in both the upper (0-30 cm) and deep (30-80 cm) soil layers was shortest in early July when the soil water content and air relative humidity were lower [17]. After rewatering events, the total root length in late August and early October increased by 128% and 179%, respectively, compared with that in early July [17]. The abundance of new roots significantly increased in both the upper and deep soil layers in response to the recovery events. The growth of new roots was greater in the upper soil profile than in the deep soil profile. Dead root length in the upper soil layer was significantly higher in the wet season than in the dry season, while no difference in dead roots was detected in the deep soil layer between the seasons. In walnut, the diameter of the roots did not significantly change by season [17]. The increase in osmotic drought level was accompanied by a substantial decrease in root relative water content and differences between genotypes at different osmotic levels were highly significant [213].

There was a significant variation in the vertical distribution of roots under stress condition (Table 1). Roots were the most abundant at 10-30 cm depth, followed by 0-10 cm depth. Root biomass decreased with depth below 30 cm. Generally, most of the root surface area, root length density and root biomass were confined to the upper soil layers (0-30 cm), and accounting for 60.9, 62.2 and 78.9% of the total root measurements from the 0-80 cm soil layers, respectively.

### 2.14. Leaf architecture and position

Annual heavy nut production will require selection of seedlings of walnut cultivars with multiple leaf layers to maximize photosynthetic production, tendencies toward lateral bearing, good resistance to anthracnose, and efficient use of photosynthates for tree growth and nut production [212].

Leaves are extraordinarily variable in form, longevity, venation architecture, and capacity for photosynthetic gas exchange. Much of this diversity is linked to water transport capacity [17]. The pathways through the leaf constitute a substantial ( $\geq 30\%$ ) part of the resistance to water flow through plants, and thus influence rates of transpiration and photosynthesis. Leaf hydraulic conductance ( $K_{leaf}$ ) varies more than 65-fold across species, reflecting differences in the anatomy of the petiole and the venation architecture, as well as pathways beyond the xylem through living tissues to sites of evaporation.

Time	Depth (cm)	Surface area (cm <sup>2</sup> )	Average diameter (mm)	Root length density (cm/dm <sup>3</sup> )	Weight (g)
May	0-10	65.4±19.4 b	0.8± 0.2 b	466.3± 52.6 b	1.3± 0.4 ab
	10-20	103.0± 17.9 a	1.0± 0.2 a	541.7± 93.6 a	2.3± 1.1 a
	20-30	121.3± 11.6 a	1.0± 0.2 a	257.3± 26.5 c	1.8± 0.5 ab
	30-40	61.1± 16.5 b	0.8± 0.2 ab	201.9± 61.2 cd	1.2± 0.6 b
	40-50	54.0± 14.5 b	0.8± 0.1 b	179.0± 61.4 cd	0.8± 0.3 bc
	50-60	39.9± 3.8 c	0.7± 0.1 b	152.6± 24.8 d	0.6± 0.1 c
	60-70	23.7± 3.3 d	0.6± 0.1 bc	112.7± 27.4 de	0.3± 0.1 d
	70-80	13.5 ±1.8 e	0.4± 0.1 c	81.0 ±18.0 e	0.2 ± 0.1 d
October	0-10	82.1± 32.6 b	0.7± 0.2 c	539.9± 78.6 b	2.3± 0.7 ab
	10-20	142.6± 53.3 a	0.8± 0.2 bc	720.7± 82.5 a	2.9± 0.7 a
	20-30	131.8± 35.1 a	1.3± 0.4 a	408.7± 52.9 c	3.6± 1.3 a
	30-40	76.1± 20.4 b	1.0± 0.4 ab	344.9± 39.5 cd	2.0± 1.1 ab
	40-50	60.7± 15.9 bc	1.0± 0.3 b	230.2± 25.4 e	1.3± 0.7 b
	50-60	40.5± 12.0 c	0.8 ±0.2 bc	190.2± 31.4 e	0.7± 0.5 bc
	60-70	37.5 ±13.5 cd	0.9± 0.2 bc	171.3± 22.1 e	0.5± 0.3 c
	70-80	25.0± 9.3 d	0.8± 0.2 c	120.5± 20.9 f	0.2± 0.2 d

**Table 1.** Vertical root distribution in different soil layers. Roots from four different distances (50, 100, 150 and 200 cm) from the tree trunk in the same soil layer from each sampling location were pooled. Three sampling locations were used. Means and SD are shown (n= 3). Different letters refer to significant difference at the P ≤ 0.05 level within the same sampling time [Courtesy Sun et al., 2011].

Angle between main stem and lateral branches, lateral branches and petioles of leafs are the most suitable morphological markers for cultivar screening in walnut [213]. Under stress, the angle between the main stem and lateral branches and especially angle between lateral branches and petioles of leaves showed significant decreases [213].

Drought during the year of bud formation decreases the number of new leaves formed in the bud and the number of new stem segments (internodes) present. These phenomena were observed for several walnut varieties during the first and second years of growth [212]. Drought then influences the number of leaves, leaf surface area, and twig extension the following year when those buds expand [212]. Summer droughts can greatly reduce shoot

elongation in species that exhibit continuous growth or multiple flushing. Drought may not inhibit the first growth flush that usually occurs before peak drought intensity, but may decrease the number of nodes formed in the new bud that will then expand during the second (or third, etc.) flush of growth. If drought continues, all growth flushes will be affected [212].

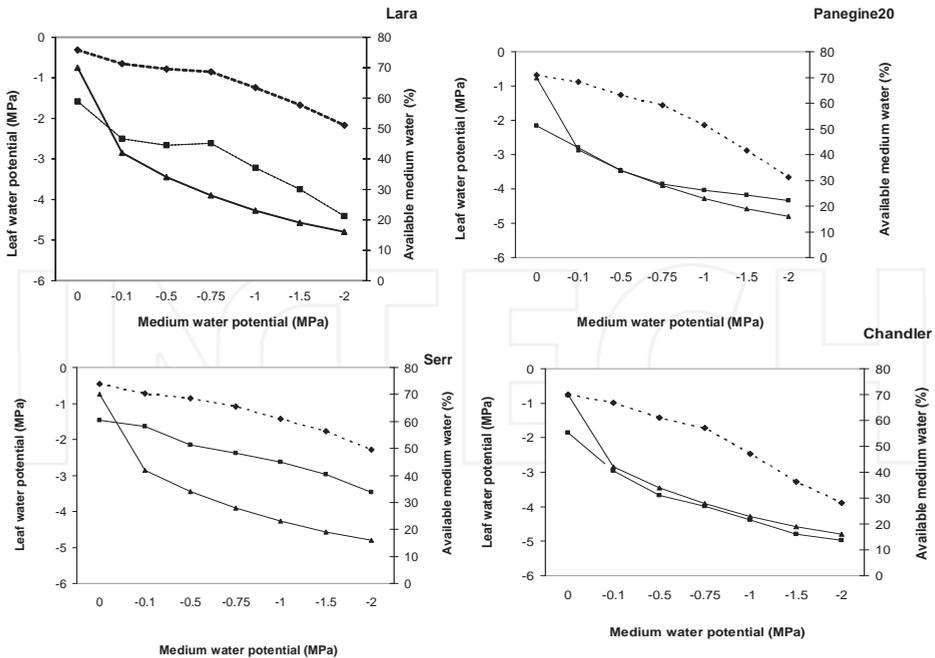
As a consequence, severe drought limits leaf area production by reducing the number and viability of leaf buds and thus the tree's ability to recover an efficient crown development after resuming normal water availability [212]. At the stand level, leaf area index may be reduced by as much as 2–3 the year following a severe drought [212–213], without any tree mortality, and the recovery of LAI to pre-drought levels may require several years. Leaf area index of walnut stands may also decrease after severe drought, due to an abnormal shedding of older leaves. Such a reduction in tree leaf area has also been reported from crown transparency observations, as used for tree vitality assessment in European forest condition monitoring and in walnut stands of Iran [169; 213]. When too much or too little water is applied repeatedly over the life of the orchard, it may be at the expense of overall productivity and orchard longevity [14].

### 3. Physiological responses to abiotic stresses:

#### 3.1. Plant water status

During stress by water deficit, the water status of the plants plays a key role in the activation of defense mechanisms. Contrasting results under the same experimental conditions can be related to difference in species, growth conditions, and stage of the plants [221]. Decline in relative water content in the walnut seedlings at different osmotic potentials was paralleled by a substantial decrease in water potential ( $\Psi_w$ ), especially in tolerant genotypes (Figure 2). Values of  $\Psi_w$  decreased during the day and subsequently recovered and re-equilibrated at night, showing a pattern of progressive decline during the drought treatment. During the last day (29<sup>th</sup> day) of the drought treatment,  $\Psi_w$  decreased in all plants subjected to drought stress. But in 'Panegine20' and 'Chandler' progeny, there was a quick reduction in  $\Psi_w$  from  $-1.8$  MPa in control plants to  $-4.9$  at  $-2.0$  MPa of osmotic treatments (Figure 2). So these genotypes have mechanisms (like ion homeostasis, osmotic regulation) to keep an osmotic potential gradient in leaf and stem tissues and are tolerant to osmotic stress [213].

The water status of a plant is a function of uptake (by roots) and loss (via stomata and cuticle) of water. Water status in walnut under stress conditions was investigated in several previous studies. Parker and Pallardy [214] demonstrated genetic variation in the drought response of leaf and root tissue water relations of seedlings of eight sources of black walnut (*Juglans nigra* L.) using the pressure-volume technique. Tissue water relations were characterized at three stages of a drying cycle during which well-watered plants were allowed to desiccate and then were re-irrigated. Sources varied both in the capacity for, and degree of, leaf and root osmotic adjustment, and in the mechanism by which it was achieved. A decrease in osmotic potential at the turgor loss point ( $\Psi_{\pi p}$ ) of  $0.4$  MPa was attributable to increased leaf tissue elasticity in seedlings of four sources, while seedlings of an Ontario source exhibited a  $0.7$ – $0.8$



**Figure 2.** Patterns of predawn leaf water potential (◆), midday leaf water potential (■) and soil available water (▲) measured in walnut seedlings during drought treatments [Lotfi et al., 2010].

MPa decline in  $\Psi_{\pi p}$  as a result of both increased solute content and increased leaf tissue elasticity. Seedlings of a New York source showed no detectable osmotic adjustment [214].

They concluded that in roots, decreased  $\Psi_{\pi o}$  (osmotic potential at full hydration) and  $\Psi_{\pi p}$  were observed under drought. Sources that exhibited significant leaf osmotic adjustment also generally showed a similar response in roots. Tissue elasticity and  $\Psi_{\pi o}$  of roots were higher than those of shoots, whereas  $\Psi_{\pi p}$  of the two organs was similar for most sources. Because of greater elasticity, roots exhibited a more gradual decline in turgor and total water potential than did leaves as tissue relative water content decreased [214].

Cochard et al. [29] focused their analysis on some of the endogenous physiological parameters likely to be altered during a water stress and reported in the literature to be associated with stomatal responses. These parameters are the  $\Psi_{soil}$  (soil water potential), the  $R_{soil}$  (soil resistance), the  $R_{root}$  (root hydraulic resistances), and the  $R_{shoot}$  (shoot resistance); all of these parameters are strongly correlated under natural drought conditions. The experiments were designed to alter  $R_{plant}$  in very different ways, which probably had a primary influence on different parts of the pathway.

Soil dehydration provoked mainly a drop in  $\Psi_{soil}$  and an increase of  $R_{soil}$ . The resistance of the interface between the soil and the root, probably increase during drought stress [87]. When  $R_{root}$  modified to the extent that the radial flow into the root xylem altered.  $R_{shoot}$  probably not

altered, because the level of xylem embolism remained low during these experiments. However, if the ionic composition of the sap changed dramatically as a result of the drought, then  $R_{\text{shoot}}$  may have varied [37]. Stem pressurization provoked only an increase in  $R_{\text{shoot}}$  when the pressure exceeded the point of embolism induction (about 2.0 MPa) [89]. If the air was propagated along the xylem flow path significantly beyond the injection point,  $R_{\text{root}}$  and  $R_{\text{leaf}}$  (leaf hydraulic resistances) may also have been altered. Therefore, combining the results of all these experiments, it is possible to determine whether  $g_s$  and  $E_{\text{plant}}$  (plant transpiration) were responding specifically to changes in  $\Psi_{\text{soil}}$ ,  $R_{\text{soil}}$ ,  $R_{\text{root}}$ , and/or  $R_{\text{shoot}}$  or not. Because air humidity, air temperature, and light intensity were maintained constant in many such experiments, leaf to air vapor deficits and leaf boundary layer conductance were also constant. Therefore, the  $g_s$  and  $E_{\text{plant}}$  patterns corresponded in drought stress. The relationship between  $g_s$  and hydraulic parameters are likely to depend on these environmental conditions, contrary to the relationships with  $E_{\text{plant}}$  [90].

The results showed that different experiments significantly reduced  $E_{\text{plant}}$  and  $g_s$ . Therefore, the response of  $g_s$  to  $\Psi_{\text{soil}}$ ,  $R_{\text{root}}$ ,  $R_{\text{soil}}$ , and  $R_{\text{shoot}}$  was neither specific nor exclusive. An alternative analysis of the problem is not to consider  $\Psi_{\text{soil}}$ ,  $R_{\text{root}}$ ,  $R_{\text{soil}}$ , and  $R_{\text{shoot}}$  individually but rather to examine their combined effect on  $P_{\text{rachis}}$  or  $\Psi_{\text{leaf}}$ . The relationship between  $P_{\text{rachis}}$ ,  $\Psi_{\text{soil}}$ ,  $R_{\text{root}}$ ,  $R_{\text{soil}}$ ,  $E_{\text{plant}}$ , and  $g_s$  under steady-state conditions is well described by the Ohm's law analogy [91]:

$$P_{\text{rachis}} = \Psi_{\text{soil}} - (R_{\text{soil}} + R_{\text{root}} + R_{\text{shoot}}) \cdot \text{SF plant} \cdot g_s \cdot D \quad (1)$$

where SF plant is the plant leaf area and D the air vapor pressure deficit, two parameters that remained constant during experiments. The gravity term and the xylem sap osmotic potential are assumed negligible in equation 1. A similar relationship is obtained with  $\Psi_{\text{leaf}}$  if we further include the leaf blade hydraulic resistance. The dependency of  $g_s$  or  $E_{\text{plant}}$  on  $P_{\text{rachis}}$  (water pressure in the leaf rachis xylem) and  $\Psi_{\text{leaf}}$  were similar whatever the experiments. This would suggest that,  $g_s$  were not correlated to changes in  $\Psi_{\text{soil}}$ ,  $R_{\text{soil}}$ ,  $R_{\text{root}}$ , or  $R_{\text{shoot}}$  per se but rather to  $P_{\text{rachis}}$  and/or  $\Psi_{\text{leaf}}$ . An identical relationship was obtained between  $E_{\text{plant}}$  and  $C_{\text{plant}}$  (defined as  $[R_{\text{soil}} + R_{\text{root}} + R_{\text{shoot}}]^{-1}$ ). These results are in agreement with the findings of Saliendra et al. [92], Sperry [93], and Hubbard et al. [94]. Many of the studies [29; 89; 92-94] concluded that combining different experimental procedures, stomata were not responding to changes in  $\Psi_{\text{soil}}$ ,  $R_{\text{soil}}$ ,  $R_{\text{root}}$ , or  $R_{\text{shoot}}$  per se but rather to their impact on  $P_{\text{rachis}}$  or  $\Psi_{\text{leaf}}$  [29].

Genetic variation in tissue water relations of black walnut under drought was studied in two consecutive years by Parker and Pallardy [214]. Black walnut seedlings of some sources studied in 1983 exhibited osmotic adjustment under drought in both leaves and roots. Significant variation among sources in root tissue elasticity was also evident before drought, but was not observed thereafter. Initial differences in osmotic potential at full saturation were not evident at the point of turgor loss [214].

Walnuts close stomata under high leaf-to-air vapor pressure deficit (VPD) or low leaf water potential ( $\Psi_l$ ) [61], preventing the stem water potential ( $\Psi_s$ ) from becoming lower than -1.4 MPa, when cavitation occurs in the xylem [17; 62]. Hence walnut has been defined as a "drought avoider" [61]. Daily course of  $\Psi_s$  and gas exchange was tested in previous studies

of Rosati et al. [121]. Stem water potential ( $\Psi_s$ ) decreased during the day and was lower in droughted than in control trees [121]. The lowest average  $\Psi_s$  values were  $-1.2$  MPa in droughted trees and  $-0.4$  MPa in control trees.

The decline in relative water content in Persian walnut seedlings at different osmotic potential was paralleled by a substantial decrease in water potential ( $\Psi_w$ ), especially in tolerant genotypes [213]. Values of  $\Psi_w$  decreased during the day and subsequently recovered and re-equilibrated at night, showing a pattern of progressive decline during the drought treatment. During the last day (29<sup>th</sup> day) of the drought treatment,  $\Psi_w$  decreased in all plants subjected to drought stress. But in 'Panegine20' and 'Chandler' progeny, there was a quick reduction in  $\Psi_w$  from  $-1.8$  MPa in control plants to  $-4.9$  at  $-2.0$  MPa of osmotic treatments [213].

### 3.2. Stomata responses to water stress

Foliar conductance to water vapor of mesophytes and crop plants often lie in the range of  $10$ - $20$   $\text{mm s}^{-1}$  under conditions in which stomata are largely open, and these figures fall to values near  $0.1$   $\text{mm s}^{-1}$  or lower-equivalent to the cuticular conductance—when stomata close [164-165]. In xerophytes and many trees, conductance under water stress can fall still lower to values approaching  $0.01$   $\text{mm s}^{-1}$ . Clearly, understanding the factors that control stomatal aperture will be crucial to future developments toward improving vegetative yields in the face of increasing pressure on water resources and arable land usage.

At the same time, the guard cells that surround the stomatal pore have become a focus of attention in fundamental research. The ability of these cells to integrate both environmental and internal signals and their unique situation within the leaf tissue has provided a wealth of experimental access points to signal cascades that link membrane transport to stomatal control.

Stomata have a fundamental role in controlling two of the most important processes in vegetative plant physiology, photosynthesis and transpiration: they open to allow sufficient  $\text{CO}_2$  to enter the leaf for photosynthetic carbon fixation, and they close to reduce transpiration under conditions of water stress [192]. The mechanics of stomatal function are intimately connected with their morphology. On the other hand, as may be expected, estimates of the change in guard cell volume between the closed and open states of stomata vary between species because, even in one species, guard cell size can vary dependent on growth conditions and the age of the plant [192].

A study about stomatal density of leaf samples in different walnut varieties revealed that the shape and volume of stomata significantly differ among varieties [212]. Tolerant and semi-tolerant varieties had a small volume of guard cells and high stomatal density especially in the abaxial epidermis of leaves [212]. So these varieties have a high potential to maximize  $\text{CO}_2$  entry to the leaf for photosynthetic carbon fixation and they close quickly to reduce transpiration under conditions of abiotic stress [212].

### 3.3. Xylem embolism under abiotic stresses

A certain degree of water stress is generally experienced by plants irrespective of life cycle and habitat [57]. Particularly in trees, the decrease in water potential may be greater, since hydraulic

resistance increases through embolism in the xylem. The plant water content recovers at night, equalizing to the soil water potential and allowing the plant to reach its highest water potential just before dawn. Trees are even more sensitive to changes in atmospheric humidity [58-59], however, and stomates close as the vapor pressure deficit between the leaf and the air increases [57]. Hydraulic conductivity of the soil and root-soil contact is potentially important in limiting water flux to roots in drying soil [60]. The xylem water potential necessary to induce this cavitation varies widely among plants [48-49] and has been shown to correlate with the lowest xylem water potentials normally experienced under natural conditions [50]. Plants tend to control stomata such that the xylem water potential does not fall below cavitation inducing pressures [51-52]. As soil moisture or humidity declines, either transpiration is reduced or leaf-specific hydraulic conductivity is increased. In this way, plants balance the demand for transpirational water loss and carbon uptake by leaves with allocation to root absorption or stem-conducting tissue [53-54; 209-210]. There is only a modest negative relationship or trade-off between the hydraulic conductivity and the susceptibility to drought cavitation for the wild-land species that have been examined to date [55]. This may be because susceptibility to cavitation is more a function of vessel and tracheid pit anatomy than conduit size [56].

Walnuts close stomata under high leaf-to-air vapor pressure deficit (VPD<sub>l</sub>) or low leaf water potential ( $\Psi_l$ ) [61], preventing the stem water potential ( $\Psi_s$ ) from becoming lower than  $-1.4$  MPa, the point at which cavitation occurs in the xylem [29]. Many species have been found to operate very close to the point of embolism. Stomata controls both plant water losses and sap pressure and thus may actively control the risk of xylem embolism [63].

Many hypotheses have been raised about xylem embolism and cavitation in walnut.  $\Psi_{soil}$ ,  $\Psi_{root}$ ,  $\Psi_{shoot}$ , and  $\Psi_{soil}$  have been used to identify hydraulic parameters associated with stomatal regulation during water stress and test the hypothesis that stomata control embolism during water stress [29]. Clear hydraulic segmentation was reported in a few species like walnut trees (*Juglans regia*) [212-213]. In these species, petioles disconnect the leaves from the stem through massive cavitation during drought and avoid irreversible damage to perennial parts of the tree. Nevertheless, this is not a general trend; some species showing more vulnerable twigs than petioles. Fewer data are available for root vulnerability than for branches but roots were found to be less vulnerable. [47-48].

At elevated CO<sub>2</sub>, the decreased osmotic potential, symplasmic water fraction and rate of water transport, increased the modulus of elasticity and no changes in the formation of xylem embolism were found in tolerant walnut varieties [83; 213]. We postulate here that embolism and cavitation are important factors which influence the tracheid volume in stressed environments in walnut species [17].

#### 3.4. Leaf water potential and branch xylem embolism at pre-dawn

Predawn leaf water potential varies by season with a significant difference in pre-dawn embolism of walnut between dry and wet seasons. The pre-dawn embolism of walnut branches was found to be 23.20% and 26.60% on 2 July and 15 August, respectively, higher than the 17.56% and 16.25% observed on 27 August and 6 October, respectively (Figure 3b) [17]. As drought progressed, the water potential reached a minimum of  $-1.51$  MPa on 15 August. After

rain events, the water potential rapidly increased and was significantly higher than in the dry season (Figure 3). The embolism level increased with xylem  $\delta D$ . Similar analyses were performed between xylem  $\delta D$  and leaf pre-dawn water potential, leaf transpiration and photosynthesis. The former two parameters had significantly negative and linear correlations with xylem  $\delta D$ , while photosynthesis was not significantly correlated with xylem  $\delta D$  (Figure 4b–d) [17].

The daily sap flow varied significantly between seasons and was mainly determined by the daytime sap flow. Generally, in summer, even in early October, the flow was higher than in spring (17 April). On 15 August, at the point of lowest soil moisture, the daily sap flow was also restricted. The daily sap flow was significantly correlated to transpiration demand and also to mean air temperature. For all individuals, the sensors showed negligible night-time sap flow with lowest values on 15 August [17].

The leaf transpiration rate exhibited similar dynamics to the pre-dawn water potential in the growing season [17]. There was a significant difference in transpiration between the dry and wet seasons. During the dry season, the transpiration rates ranged as 0.9–1.6 mmol m<sup>-2</sup> s<sup>-1</sup>, significantly lower than the range of 2.1–2.5 mmol m<sup>-2</sup> s<sup>-1</sup> observed in the wet season. The assimilation rate did not completely follow the dynamic pattern of transpiration (Figure 4d). Photosynthetic rate was lowest on 15 August, when the soil moisture was lowest in the growing season; however, the highest photosynthetic rate occurred on 17 April, when the soil moisture was not highest [17] (Table 2).

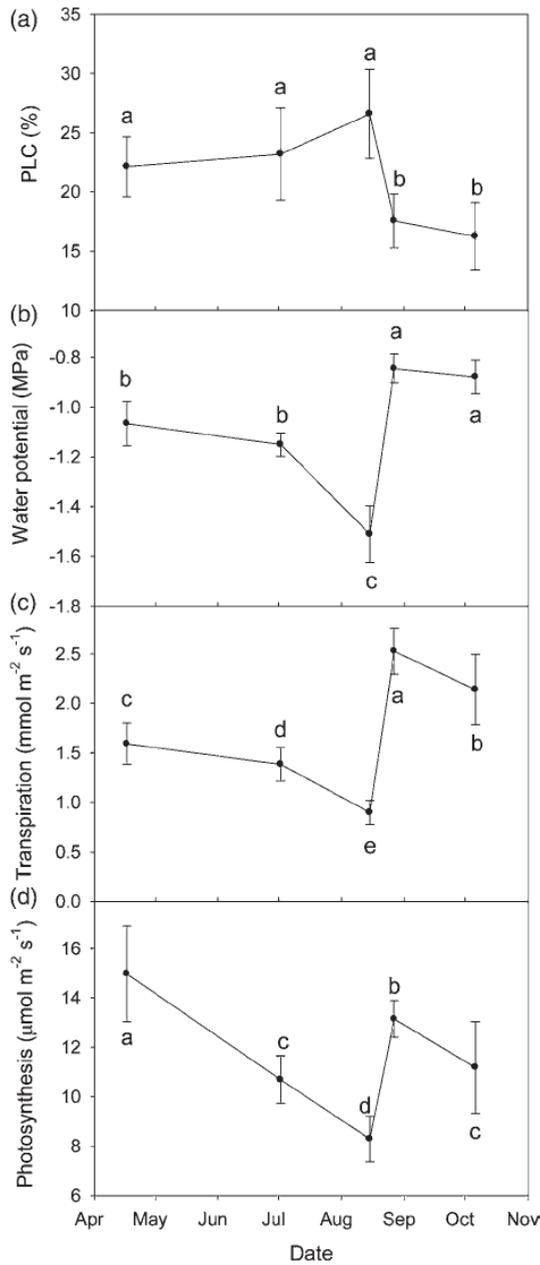
	Regression equation	R value	R <sup>2</sup> value	P value
Transpiration	Y = -1.770 + 0.398x	0.951	0.904	0.013
Air temperature	Y = -2.141 + 0.541x	0.894	0.800	0.041

**Table 2.** Relationships between the daily sap flow and transpiration and air temperature in walnut [Courtesy Sun et al., 2011].

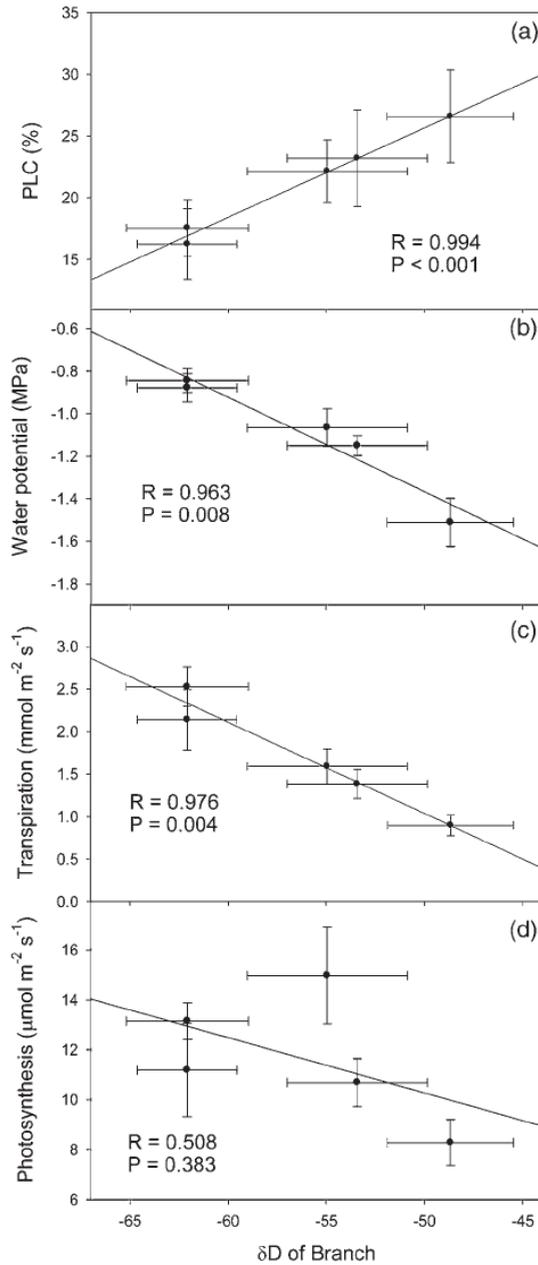
### 3.5. Vulnerability to cavitation

Cavitation occurs when negative sap pressure exceeds a threshold value defined by anatomical characteristics [62-65]. Many species have been found to operate very close to the point of embolism. Therefore, stomata control both plant water losses and sap pressure and, thus, may actively control the risk of xylem embolism [63-69].

Vulnerability curves (VCs) were constructed by plotting the changes in the percentage loss of xylem conductance (PLC) versus xylem pressure were demonstrated by Cochard et al at [29]. Significant differences were found between organs. Leaf rachises were the most vulnerable, roots the least vulnerable, and leaf veins and shoots intermediate. Turgor pressure ( $P_{leaf\ 0}$ ) at full turgor averaged  $0.93 \pm 0.06$  MPa ( $n = 5, \pm SE$ ) and the turgor loss point averaged  $-1.53 \pm 0.04$  MPa. When plants were continuously exposed to a constant and high light intensity for 1 week, a higher level of water stress was obtained.  $E_{plant}$  and  $g_s$  dropped close to zero whereas



**Figure 3.** Variations in (a) pre-dawn branch xylem PLC, (b) pre-dawn leaf water potential, (c) leaf transpiration and (d) assimilation over the growing season. Means and SD are shown ( $n = 6$ ). Different letters above the bars refer to significant difference at the  $P \leq 0.05$  level [Courtesy Sun et al., 2011].



**Figure 4.** a) Pre-dawn branch xylem PLC, (b) pre-dawn leaf water potential, (c) transpiration and (d) assimilation as a function of the  $\delta D$  values of branch xylem water. Linear Pearson's correlation was performed between these measurements. The error bars refer to SD [Courtesy Sun et al., 2011].

$P_{\text{rachis}}$  leveled at approximately  $-1.4\text{MPa}$ . At this point, the degree of xylem embolism in the leaf rachis was still less than 10 PLC [29].

The relationship between cavitation vulnerability and climate has been investigated in several tree species. Conifer seedlings originating from the most mesic populations were found to be the most susceptible to water-stress-induced cavitation [76]. Walnuts, native to dry zones, are less susceptible to drought-induced cavitation than species native to well-watered areas [75].

### 3.6. Evidence for a stomatal control of xylem embolism in walnut

Effect of stomatal closure is to maintain  $P_{\text{rachis}}$  above a threshold value around  $-1.4\text{MPa}$  and  $\Psi_{\text{leaf}}$  above approximately  $-1.6\text{MPa}$ . To further understand this behavior, it is necessary to identify a major physiological trait associated with a stomatal closure that would threaten plant integrity at lower  $P_{\text{rachis}}$  and/or  $\Psi_{\text{leaf}}$  values [74]. The answer to this question is obviously very complex, because many traits are probably involved and correlations between them probably exist [29; 213]. Cochard et al argues that, xylem cavitation is correlated with the stomatal closure [29; 70-73]. A physiological trait associated with a stomatal closure during water stress should meet at least the following three main conditions. First, its impairment should represent a serious threat to plant functioning. This results from the consideration that the reduced carbon gain, reduced growth, reduced reproductive success, etc. So the gain associated with the regulation should overcome the loss. Cavitation is a serious threat for plants because it impairs the xylem conductive capacity and may eventually lead to leaf desiccation and branch mortality [95]. Indeed, leaf desiccation was not observed in some studies as long as the xylem integrity was maintained. Leaf desiccation was noticed only when high levels of embolism were measured in the leaf petioles [29]. The gain associated with stomatal closure was thus the maintenance of leaf vitality, which largely overcomes the drawbacks cited above.

The second condition is that the impairment of the trait should be water deficit dependent because the effect of stomatal closure is precisely to prevent excessive leaf dehydration. The mechanism of water stress-induced cavitation has been well documented [138]. Air is sucked into the xylem lumens through pores in the pit wall when pressures in the sap exceed the maximum capillary pressures that can sustain the pores. Therefore, the likelihood of cavitation occurrence is directly determined by the degree of water deficit in the xylem, more precisely by  $P_{\text{rachis}}$ . The maintenance of leaf turgor above cell plasmolysis is another physiological trait that might also satisfy these first two conditions.

The third condition is that the impairment of the trait should have the same water deficit dependence as stomata. Stomata were completely closed in walnut trees when  $P_{\text{rachis}}$  reached about approximately  $-1.4\text{MPa}$  and  $\Psi_{\text{leaf}}$  about approximately  $-1.6\text{MPa}$ . The impairment of the trait associated with stomatal closure should therefore occur at comparable  $P_{\text{rachis}}$  or  $\Psi_{\text{leaf}}$  values. The leaf rachis was the most vulnerable organ along the sap pathway in the xylem and was also exposed to the lowest xylem pressure values. Leaf rachis is therefore the Achilles' heel of the walnut tree sap pathway. Segmentation in xylem vulnerability to cavitation has been demonstrated for several other species [29; 94]. A lot of variation exists between species, and occasionally the roots appear to be the most cavitation sensitive organs in the plant [96]. The dependencies of leaf rachis xylem embolism and transpiration on water deficit were very

similar. Stomata were completely closed at the incipience of xylem embolism in the leaf rachis. Variations of  $E_{\text{plant}}$  and leaf turgor pressure ( $P_{\text{leaf}}$ ) were concurrent with bulk  $\Psi_{\text{leaf}}$ . It is also clear from this graph that stomata were completely closed at the incipience of leaf cell plasmolysis (turgor loss point). The maintenance of xylem integrity and leaf turgor was closely associated with stomatal closure during water stress in walnut [29]. Stomatal closure was rather preemptive in avoiding cavitation. This behavior might be explained by the potential for “catastrophic xylem failure” [51]. There is a feedback between xylem conductance and xylem pressure during cavitation. Cavitation decreases xylem conductance, which in turn decreases xylem pressure and thus provokes more cavitation. Tyree and Sperry [51] and Jones and Sutherland [63] have computed that catastrophic xylem failure occurs at the expense of some xylem conductance and at a critical transpiration rate ( $E_{\text{crit}}$ ) only slightly greater than the actual maximum  $E$ . The hypothesis of a stomatal control of catastrophic xylem failure was evaluated with a hydraulic model of a walnut tree explicitly taking into account the feedback between xylem pressure and xylem conductance. Our simulations confirmed the results of Sperry et al [64] and Comstock and Sperry [65]. Transpiration was maximized ( $E_{\text{crit}}$ ) at the expense of all conductance in the distal leaf rachis segment.  $E_{\text{crit}}$  was therefore much higher than the actual  $E_{\text{plant}}$ . Using the same model, they have computed  $E_{\text{plant}}$  provoking 1% ( $E_{\text{IPLC}}$ ) and 10% ( $E_{\text{10PLC}}$ ) loss of rachis conductance. The onset of tree water loss regulation occurred when  $E_{\text{plant}}$  reached  $E_{\text{IPLC}}$  and  $E_{\text{plant}}$  tracked  $E_{\text{10PLC}}$  when plant conductance was further reduced. This model suggests that the risk of catastrophic xylem failure was not associated with the stomatal regulation in walnut.  $g_s$  was not maximized at the expense of all xylem conductance. Rather, xylem conductance was maximized at the expense of all  $g_s$ . To experimentally validate these computations, we have tried, without success, to feed stressed plants with fusicocine, a drug supposed to promote stomatal opening. The use of mutants lacking efficient stomatal regulation is probably a better way to test such hypotheses [66].

These experiments demonstrate that stomatal closure caused by soil drought or decreased air humidity can be partially or wholly reversed by root pressurization [29].

### 3.7. Recovery of conductivity after drought-induced embolism

Recovery from drought-induced embolism is rarely reported in trees when the xylem has experienced low water potentials. More often, the conductivity is restored only the following year by the formation of a new ring of functional xylem. For tree species generating positive xylem sap pressure in the roots during spring, like walnut, the recovery of conductivity is partially achieved by flushing embolised vessels with pressurized sap and full recovery of the transport ability occurs usually only after the new year ring has been developed [77]. Recovery of xylem conductivity after embolism can occur during spring due to xylem pressure generated by starch hydrolysis [78] or during transpiration, as has been reported for *Laurus nobilis* which is able to recover despite predawn leaf water potential remaining as low as  $-1$  MPa [81]. Similar refilling events have been reported for a range of species [79-80]. Nevertheless, the reality of such refilling of embolised vessels in transpiring trees is still a matter of debate and although several models have been proposed to explain it, there is a clear need for further research in this area [82]. Regardless of mechanism, embolism repair after drought remains a costly

process requiring metabolic energy to generate the necessary positive pressure. Cavitation avoidance is probably a much more efficient way to cope with reduced soil water, and stomatal control of transpiration probably plays a major role in this respect.

### 3.8. Biological lag effects

Drought and salt stress can also produce chronic symptoms such as shoot die-back, crown and root rot, tree decline and eventual death [14; 213]. In some seasons and in some field settings too much water is the result of uncontrollable natural phenomena such as excessive rainfall, high water table, and flooding. In other situations, too much water may be the result of water management decisions such as starting the irrigation season too soon, applying too much water per irrigation, irrigating too frequently, operating irrigation systems that apply water non-uniformly, or exposing sensitive parts of the tree such as the root crown to excessive water [14; 213]. Conversely, too little water may result from starting the irrigation season too late, applying too little water per irrigation, irrigating too infrequently, or operating irrigation systems that apply water non-uniformly. When too much or too little water is applied repeatedly over the life of the orchard, it may be at the expense of overall productivity and orchard longevity [14; 213].

In 1986, Dreyer and Mauget [22] tested immediate and delayed effects of summer drought on development of young walnut trees (*Juglans regia*). Two treatment periods were defined: in spring, after the first shoot growth flush, and at the end of summer, following complete cessation of shoot elongation. These treatments induced both immediate effects (halted growth, reduction of leaf area) and significant delayed effects appearing at resumption of watering. During summer, many normally quiescent buds resumed growth on trees submitted to drought after rewatering. Winter dormancy of buds was reduced by late summer drought. Unlike other trees, walnut trees showed no detectable residual effect on the timing of spring bud burst the following growing season.

### 3.9. Gas exchange

Light-saturated net CO<sub>2</sub> assimilation rate ( $A_{\max}$ ) and stomatal conductance ( $g_s$ ) are closely related in many species [85; 107-108]. However it is not clear whether the reduction in carbon fixation is due to closing of stomata or changes in leaf biochemistry. In walnut,  $A_{\max}$  decreases at high temperatures [109-110], but it is not clear whether temperature has a direct effect on photosynthesis, or just affects  $g_s$ . Another hypothesis is that  $A_{\max}$  and  $g_s$  are co-regulated under water stress [111-112]. While  $g_s$  is, at times, correlated with VPD<sub>l</sub> [113], an increasing body of literature suggests that  $g_s$  depends on leaf water status [72 -74; 84], possibly leaf or turgor pressure potentials [85-86]. Thus, while both water status and VPD<sub>l</sub> affect  $g_s$ , the mechanisms of such responses are not clear.

In an attempt to answer this question, Rosati et al. [121] studied diurnal changes in the water status and gas exchange of droughted [50% crop evapotranspiration (ETc)] and fully irrigated (100% ETc) walnut trees, over 2 d. Stem water potentials ( $\Psi_s$ ) ranged from -0.5 MPa in the morning to -1.2 MPa in the afternoon under drought, and from -0.1 MPa to -0.4 MPa under

full watering. Net CO<sub>2</sub> assimilation ( $A_{\max}$ ) ranged from 15  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the morning to 3  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the afternoon under drought, and from 25  $\mu\text{mol CO}_2$  in the morning to 10  $\mu\text{mol CO}_2 \text{ mm}^{-2} \text{ s}^{-1}$  in the afternoon under full watering. At these times, stomatal conductance ( $g_s$ ) varied from 0.2 to 0.02  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  and from 0.7 to 0.2  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ , respectively.

Drought reduced the internal CO<sub>2</sub> concentration ( $C_i$ ) by about 55  $\mu\text{mol mol}^{-1}$  on day 1, and by about 100  $\mu\text{mol mol}^{-1}$  on day 2 and increased leaf temperature ( $T_l$ ) by about 2–5 °C. The reductions in  $g_s$  and  $C_i$  with drought suggest that lower photosynthesis was associated with stomatal closure [121]. However, in each treatment,  $A_{\max}$  decreased during the day, while  $C_i$  was stable, suggesting that photosynthesis was also reduced by a direct effect of heat on leaf biochemistry. Both  $A_{\max}$  and  $g_s$  correlated with  $T_l$  and with the leaf-to-air vapor pressure deficit ( $\text{VPD}_l$ ), but with different relationships for droughted and control trees. However, when stomatal limitations to photosynthesis were accounted for (i.e., based on the assumption that under stomatal limitation photosynthesis is proportional to  $C_i$ ), a single relationship between  $A_{\max}$  and  $T_l$  described all the data ( $R^2 = 0.81$ ). Thus, photosynthesis was limited by both the closing of stomata under drought and by a direct effect of heat on leaf biochemistry. These results suggest that hot and dry weather reduces photosynthesis and potential productivity in walnut in the absence of a soil water deficit [121].

To test the hypothesis that  $A_{\max}$  was limited by both  $T_l$  and  $g_s$ , we corrected  $A_{\max}$  for the  $g_s$  (i.e.,  $C_i$ ) limitation and plotted the corrected  $A_{\max}$  ( $A_{\max\text{Corr}}$ ) against  $T_l$ . A single fit described all the data, suggesting that CO<sub>2</sub> assimilation responded directly to  $T_l$ , and that the rest of the variation in  $A_{\max}$  was due to additional  $g_s$  limitations (i.e., low  $C_i$ ), especially under drought. Given the close correlation between  $T_l$  and  $\text{VPD}_l$ ,  $A_{\max\text{Corr}}$  was also closely correlated with  $\text{VPD}_l$  [121]. Stomatal conductance is probably more related to  $\Psi_l$  [72-74; 84] and not  $\Psi_s$ , but these two parameters are closely related in droughted walnut [29]. If  $g_s$  was limited by water status at low  $\Psi_s$ , rather than by  $\text{VPD}_l$ , then it remains unclear why  $g_s$  was also closely related to  $\text{VPD}_l$  ( $R^2 = 0.85$ ) under drought (i.e., low  $\Psi_s$ ), although with a different relationship than for the controls [121]. This was probably due to the strong link between  $\Psi_s$  and  $\text{VPD}_l$  [180]. A strong relationship between  $\Psi_s$  and  $\text{VPD}_l$  or  $\text{VPD}$  (i.e., vapor pressure deficit in the air) has been found in several species, and is commonly used to explain variation in  $\Psi_s$  for fully-irrigated trees [106].

Also stomatal patterns of  $A$ ,  $g_s$ ,  $C_i$  and  $E$  were studied for irrigation treatments under salt stress conditions by Girona et al [215]. All of the traits studied were highly affected by salt stress. Gas exchange parameter seasonal patterns showed three groups of responses: A) less affected plants, B) moderately affected plants and C) highly affected plants [215].

### 3.10. Relationship between variation in water source partitioning and plant water status

Comparison of the  $\delta\text{D}$  values in plant stem water and soil water at different depths demonstrated that *J. regia* was compelled to take a higher ratio of water from the deep soil layers in the dry season. However, measurements of water relationships indicated that the larger water uptake from deep soil was not able to prevent water stress on the plants. Deep soil water resources may allow plants with deep root systems to survive in dry seasons [104]. Also, deep

soil water supplementation could maintain the hydraulic conductivity of roots in the nutrient-rich upper soil throughout the dry season [46], keeping roots ready to extract water when moisture becomes available in the upper soil. Otieno et al [105] found that *Quercus suber*, with a deep root system, took up most of its required water from the deep soil layers during drought to maintain good water status, but no growth was recorded during this time. Water in the upper soil layers, however, seemed to play a more important role in tree productivity. Values of  $\delta D$  trace the ratio of water sources, but not the absolute amount of water. Lower  $\delta D$  values suggest that xylem water has a higher ratio of water from the deep soil layers, but cannot be automatically translated into greater water uptake from the deep soil layers. Such a finding could also indicate reduced water uptake from the upper soil layers or a mixture of reduced water uptake from the upper layers and increased water uptake from the deep layers. Thermal dissipation probe transpiration measurements indicated that the daily sap flow decreased by around 30% on the driest day in comparison with 2 July and 27 August, suggesting that the highest xylem  $\delta D$  on 15 August would be mainly attributed to reduced water uptake from the upper soil layers. Additionally, the  $\delta D$  values in xylem water were significantly correlated with the shallow soil layers (0–20 and 20–40 cm depths), but not so significantly correlated with the deeper soil layers (40–60 cm depths) [17], suggesting that water uptake by walnut would tend to be mainly determined by water supply of the upper soil. During water transport between roots and shoots, the isotopic composition of water remains unaltered; therefore, it is reasonable to believe that water in sap flow was also mainly provided by the upper soil [17]. Many studies with stable isotopic hydrogen and oxygen on seasonal changes in water sources investigated the water source shift from upper to deep soil layers with decreasing precipitation, and the results sometimes imply that water uptake from deep soil, where water is available, could solve the drought problem. Deep water can help but not always enough to avoid serious stress.

Walnut roots were mainly distributed in the upper soil layers [212]. Soil moisture was a key factor regulating root growth and water uptake efficiency of the roots [17]. The shallow roots had reduced efficiency in water uptake in the dry season, and therefore *J. regia* was compelled to extract a greater ratio of water from the deep soil layers. However, the shift was not able to prevent water stress on the plants, which was characterized by increased pre-dawn branch xylem PLC, reduced pre-dawn leaf water potential and transpiration with soil drying. In addition to serving as an indicator of water sources, changes in the  $\delta D$  values in walnut branch xylem water reflected plant water status and the severity of soil drought [17].

In previous studies, comparison of the  $\delta D$  values of plant stem water and soil water at different depths revealed the existence of different water source partitioning patterns between different soil moisture conditions in a planted walnut forest for example in northern China [17]. The  $\delta D$  values showed that plants mainly used water from the upper soil in the wet season, while upper and deep soil water more or less equally contributed to plant xylem water in the dry season. The result is consistent with that of previous studies. McCole and Stern [102] reported a change in juniper water use from a predominantly deep water source during summer, when it was hot and dry, to a predominantly upper soil source during winter, when it was cool and wet. *Pinus edulis* and *Juniperus osteosperma* largely use monsoon precipitation during the

monsoon period, but use of this precipitation declines sharply with decreasing summer rain input [103]. No other water source was available for trees in this system. However, the roots might penetrate through the dense gravel layers and may be in contact with groundwater. Therefore, the influence of groundwater on xylem isotopic signature cannot be completely excluded, although Williams and Ehleringer [103] found that plants did not use groundwater in the pinyon-juniper ecosystem of the southwestern USA, a site similar to this study region. Nevertheless, it should be noted that the seasonal change of water resource partitioning was based on the two measured depths.

Rosati et al. [121] studied Kaolin applications to mitigate the negative effects of water and heat stress on walnut physiology and productivity. Kaolin applications were found to improve  $A_{\max}$  in apple but only under high temperature and vapor pressure difference [122]. Other authors found no effect or even a reduction in yield,  $A_{\max}$  or both [97-98; 122]. Little data are available for other tree species: kaolin improved  $A_{\max}$  and stomatal conductance ( $g_s$ ) in citrus at mid-day but not in the morning [99] and no effect was found on pecan [100].

$A_{\max}$  for walnut was highest in the early morning and decreased throughout the day, for both the water-stressed (S) and the well-irrigated (W) treatments [121].  $A_{\max}$  was always lower for the S treatments, especially in the afternoon. Kaolin application reduced  $A_{\max}$  (by up to 4 mmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) within each irrigation treatment, especially in the morning when  $A_{\max}$  was high, whereas in the afternoon this effect tended to disappear in the W treatment and disappeared completely in the S treatment [123; 125]. The average reduction in  $A_{\max}$  during the day was minor compared with the reduction due to water stress and was 1-4 mmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> in the S treatments and 2-4 mmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> in the W treatment [121].

Also in this study, intercellular CO<sub>2</sub> concentration ( $C_i$ ) was greatly reduced with water stress in walnut while the irrigated walnut and the almond trees had similar  $C_i$  values [121]. Kaolin application increased  $C_i$  in all cases except for two out of five measurements in the S treatment in walnut. The average daily increase in  $C_i$  with kaolin was 28 mmol mol<sup>-1</sup> in the S and 19 mmol mol<sup>-1</sup> in the W treatments for walnut and 10 mmol mol<sup>-1</sup> for almond [121]. As a result they concluded that Kaolin application reduced leaf temperature ( $T_l$ ) and leaf to- air vapor pressure difference ( $VPD_l$ ), but not sufficiently to compensate for the increase in  $T_l$  and  $VPD_l$  with water stress in walnut. The kaolin-induced reduction in  $T_l$  and  $VPD_l$  did not mitigate the adverse effects of heat and water stress on  $A_{\max}$ . Kaolin application did not affect  $g_s$  and  $Y_s$ . The prevailing effect of kaolin application appeared to be the shading of the leaves and the consequent, albeit minor, reduction of  $A_{\max}$ , except at very low  $A_{\max}$  [121].

### 3.11. Delayed consequences of drought

Irreversible drought-induced damage leads to organ dysfunction, but it seldom results in direct and immediate tree decline and mortality. Drought induces short term physiological disorders like decreased carbon and nutrient assimilation, and sometimes even a breakdown of the photosynthetic machinery itself. These tissues have to be repaired before normal processes can resume. The tree must allocate existing stored reserves among the demands for repair, maintenance, growth and defense. As a consequence, tree ring width or leaf area is frequently smaller during several years following a severe drought [166-167]. Moreover,

physiological disorders increase tree vulnerability to secondary stresses like insect damage, frost or another drought [168].

## 4. Molecular responses to abiotic stresses:

### 4.1. Mineral composition and Ion homeostasis under abiotic stress

Perhaps the most significant change in plant electrophysiological studies, beginning about 25 years ago, was a shift in focus from more basic electrical and biophysical properties of plant membranes to pursuing an understanding of the physiological and cell biological functions of individual plant ion channel types [114]. In the 1990s, ion channels were characterized as targets of upstream signal transduction mechanisms, and in the later 1990s powerful combined molecular genetics, patch clamp, and plant physiological response analyses further manifested the importance of ion channels for many biological and stress responses of plants [114]. Essential metals and ions in the intracellular and intraorganellar spaces of plant cells contribute to the activities of regulatory proteins, signal transduction, and to the maintenance of turgor pressure, osmoregulation, toxic metal chelation, and membrane potential control. A large number of studies on mineral nutrition have sustained the profitable cultivation of plant growth and development and provided important knowledge on mechanisms of mineral absorption from soils [114]. Lotfi et al. [180] tested the mineral composition of promising walnut varieties under both salt and drought stress. Their results showed that differences in the range of sodium accumulation were minimal as compared with other minerals at different salt and drought stress levels. In control plants, the average sodium content ranged from 0.34 to 1.82 mg g<sup>-1</sup> dry weight (DW), whereas the shoots of the sensitive cultivars (Lara, Vina, and Serr) had significantly higher sodium contents than other cultivars [212].

In salt-treated plants, the average sodium content was higher than in control plants (nearly twice) and ranged from 0.52 to 7.92 mg g<sup>-1</sup> DW [212], and the Chandler seedlings had significantly less sodium content than the others. Sodium levels in roots were higher than in the shoots in almost all varieties, especially in the tolerant and semi-tolerant varieties [212]. In contrast, the increase in sodium content was more evident in shoots of sensitive and semi-sensitive varieties.

Results of mineral composition analysis showed that the calcium and potassium accumulations were increased by the increase in salt and drought stress levels, especially in shoots of semi-sensitive and tolerant cultivars [212]. Also, variations of magnesium accumulation in root and shoot samples were significant at all stress levels and were dependent on cultivar [212].

Several classes of Ca<sup>2+</sup> permeable channels have been characterized in the plasma membrane of plant cells, including depolarization-activated Ca<sup>2+</sup> channels [139; 140] and hyper polarization-activated Ca<sup>2+</sup> influx channels [114]. In general, plant Ca<sup>2+</sup> channels are not entirely Ca<sup>2+</sup> selective but also show permeability to other cations [166]. However, the genes encoding plasma membrane Ca<sup>2+</sup> channels remain less well-clarified. Two gene families are likely to provide possible candidates. One family includes 20 genes in the Arabidopsis genome and

encodes homologs to “ionotropic” glutamate receptors, which encode receptor ion channels in animal systems [115].

Calcium ions act as a second messenger in intracellular signal transduction during ABA signaling [132]. In-flow of calcium ions into the cytosol from the vacuole and extracellular space increases the cytosolic concentration of calcium ions in ABA-treated guard cells. The level of calcium ions oscillates at intervals of several minutes. This increase in calcium concentration is not observed in the ABA insensitive mutant's *abi1* and *abi2* [133]. Calcium ions suppress inward potassium channels and activate inward anion channels; thereby playing a central role in stomatal closure [134]. Also, the active oxygen species formed activate the calcium ion channel to increase the cytosolic concentration of calcium ions.

Uptake and distribution of sodium ions within the root is to a large extent connected with the effects of potassium, since  $\text{Na}^+$  efflux in root cortex cells is stimulated by  $\text{K}^+$  influx, which is related to the  $\text{K}/\text{Na}$  root selectivity [135]. The presence of potassium (and calcium) ions has been shown to decrease  $\text{Na}^+$  influx into plant cells (e.g. [136]). Potassium promotes cell elongation and maintains osmoregulation. Potassium promotes photosynthetic rate and controls the rate of transport of photosynthates from source to sink. Potassium is also essential for protein synthesis and activates nearly 45 enzymes involved in various metabolic processes [222].

We observed differential responses in the uptake of sodium and in the pattern of germination in seedlings of walnut cultivars which could account for the differences in response to salinity. The ability to maintain low sodium concentration in leaves and in growing shoots is crucial for plant growth in saline media. The salt tolerance in species that exclude salts is achieved by changes between sodium and calcium ions, rather than changes in osmotic potential, since adsorption of calcium ions on membranes of root cells leads to reduced penetration of monovalent cations [124]. This was demonstrated for wheat where inhibition of non-directional  $\text{Na}^+$  influx occurred following the addition of external  $\text{Ca}^{2+}$  [137]. Involvement of both  $\text{Ca}^{2+}$  sensitive and  $\text{Ca}^{2+}$  insensitive pathways (regulated mainly by non-selective cation channels) in the control of  $\text{Na}^+$  entry into the root has been proposed [138]. When sodium accumulates in the cytoplasm of shoot or leaf cells, it can lead to tissue necrosis and leaf abscission; thus, the photosynthetic apparatus is impaired and plant growth is hindered. The accumulation of sodium in shoots was significantly different in the three salt tolerance classes, but they presented distinct responses to the increasing concentration of  $\text{NaCl}$ . Similarly, Sixto et al [116] observed differences in leaf sodium content among *P. alba* cultivars from different Spanish origins subjected to salt stress. Possibly the halfsib seedlings of ‘Chandler’, which accumulated significantly less sodium in shoots, has mechanisms for sodium exclusion at the root level, which reduces sodium uptake and its translocation to the shoot tissues. Mechanisms for sodium exclusion in roots are well studied in *P. euphratica* [117] which is the most salt-tolerant poplar species. In *P. alba*, the ability to maintain lower sodium content in leaves has also been associated with less severe symptoms of salinity stress [116]. Our results confirm a negative relationship between sodium accumulation in the shoots and its effects on shoot growth in ‘Chandler’. The negative effect of long-term salt stress on shoot growth of ‘Lara’ is probably more due to sodium toxicity than to osmotic effect. The excess sodium can be both

actively accumulated in the vacuole or be excreted into apoplast. Sodium compartmentation in the vacuole is an adaptation mechanism typical of halophytes [118]. Ottow et al. [118] observed that *P. euphratica* could tolerate increasing sodium concentration by apoplastic accumulation of salt in the leaves' cell wall regions but not in the vacuole. A similar mechanism for apoplastic localization of sodium could operate in *P. alba* and accounts for the different behavior observed among the cultivars studied. These hypotheses need to be tested by further studies to determine the exact site of sodium localization at histological, cellular, and subcellular levels.

The results of our previous study suggest that seedlings of different walnut cultivars differ in tolerance to salinity and drought stress. Results demonstrated variability in germination ability and seedling growth in saline and drought habitats, implying that it might be possible to select walnut seedlings for salt and drought tolerance in germination stage [180; 212-213]. Salinity treatments caused a net  $K^+$  uptake, which is likely to be the result of osmotic adjustment in tolerant cultivars. Net  $Na^+$  uptake by sensitive cultivars was noticeably higher than in tolerant cultivars. Interestingly, in control plants, the sodium content in shoots of cultivars that belong to the sensitive groups was significantly higher than in the shoots of the other half-sib progeny. This suggests a constitutive ability of these cultivars to accumulate more sodium in the leaves. This feature could contribute to osmotic adjustment in response to salinity or drought as has also been observed in *P. euphratica* plants exposed to salt stress, in which the osmotic adjustment was mainly resulted from sodium accumulation [118]. In the tolerant and semi-tolerant groups, roots had higher potassium contents than shoots. This could reflect differences in the membrane transport properties of cells in different stress-tolerant groups [119]. The amount of calcium accumulation was increased by increase in salinity stress levels, especially in shoots of tolerant and semi-sensitive cultivars. Calcium is an essential plant nutrient that is required for its structural roles like in membrane integrity, as a counterion for inorganic and organic anions in the vacuole, as an intracellular messenger in the cytosol, and as an enzyme activator [120]. In conclusion, different strategies for adaptation to salinity or drought have been observed in seedlings of walnut cultivars with different climatic origins when grown in a greenhouse trial. Thus, a different genetic basis underlies the different behaviors observed under salt and drought stress. The degree of variation in salinity and drought tolerance in these cultivars could be linked to their different abilities in sodium exclusion at the root level or to different regulation of ion transport across shoot cell membranes. Our results suggest that the cultivars Chandler and Panegine20 could also be suitable models to be used for the study of the physiology and genetics of abiotic stress tolerance in walnut [212].

The higher content of seed nutrients is of vital importance for germination, but salinity and drought suppresses their role in the metabolism of seeds and the production of seedlings [144]. During germination of walnut seeds, a higher content of potassium, calcium, phosphorus, and nitrogen was partitioned into the plumule and radicle as a strategy of tolerance to salinity [213]. Guerrier [145] attributed the reduced salt tolerance of tomato to its inability to accumulate and transport lower amounts of calcium and potassium. The SOS pathway (salt overly sensitive) is triggered by a transient increase of cytosolic  $Ca^{2+}$  as a first effect of salt stress. The increase in  $Ca^{2+}$  concentration is sensed by a calcium binding protein (SOS3) [212].

#### 4.2. Seed germination and ion homeostasis under abiotic stress

The initial events in stem propagule germination may differ in some respects those of seeds but bud activation, elongation, and establishment events are similar. Germination of sugar cane sets (stem cuttings) exhibited significant reduction in the rate and percentage of germination due to NaCl damage [150]. These plants had an enhanced content of Na<sup>+</sup> and Cl<sup>-</sup>, a concomitantly reduced content of potassium, calcium, nitrogen, and phosphorus and reduced elongation and dry matter of seedlings.

Citrus rootstocks used to raise plantlets had a negative correlation of Cl<sup>-</sup> with certain nutrients [146]. Resting buds of salt-stressed poplar plant, grown *in vitro*, did not accumulate glycine-betaine and proline and thus had reduced growth of seedlings [2]. Similarly, tubers of hydrilla showed signs of salt damage and reduced germination [147-148]. There remains a shortage of information particularly about the salt tolerance of propagules during germination.

Exposure of seeds or seedlings to salinity results in the influx of ions with the imbibition of water, which exerts an adverse effect on the growth of embryo [141; 143]. This may lead to a marked decrease in the internal potassium concentration [143], a vital nutrient for protein synthesis and plant growth [149]. Seedlings exposed to salinity are highly prone to excessive ions, sometimes leading to their death shortly after emergence [142; 150]. The ability of plants to cope with ion toxicity is principally related to the greater transport of ions to shoot [143-144]. Grasses show a strategy of salt tolerance by storing toxic ions in the mesocotyl up to a certain limit [151-152]. This has significance in that the epicotyls and hypocotyl avoid ion toxicity, thus ensuring better growth [141].

#### 4.3. Regulation of Na<sup>+</sup> homeostasis in roots and shoots in tolerant walnut varieties

The fine-tuned control of net ion accumulation in the shoot involves precise *in planta* coordination between mechanisms that are intrinsically cellular with those that are operational at the intercellular, tissue or organ level [125, 157]. Several processes are involved, including the regulation of Na<sup>+</sup> transport into the shoot, preferential Na<sup>+</sup> accumulation into the shoot cells that are metabolically not very active and the reduction of Na<sup>+</sup> content in the shoot by recirculation through the phloem back to the root [125-126].

Ions loaded into the root xylem are transported to the shoot largely by mass flow, driven by the size of the transpirational sink [124-127]. A control response is to lower transpiration by a reduction in stomata aperture; however, this is only effective as a short-term response because plants need to maintain water status, carbon fixation and solute transport [157]. Controlling ion load into the root xylem restricts accumulation in the shoot to a level where cells in this organ can be effective ion repositories by vacuolar compartmentalization [125, 157]. In our studies, tolerant walnut varieties showed such trends under both salt and drought stress [213]. Endodermal cells constitute a major control point in radial ion transport from the soil solution to the root xylem since the Casparian strip is an impermeable barrier to apoplastic solute movement [128]. However, bypass systems that function through 'leaks' in the Casparian strip barrier or movement through areas of the root where the specialized endodermal cells are not fully developed may be additional major entry points [129-130]. Regardless, vacuolar com-

partmentalization in cells that form the interconnected network between the soil solution and the root xylem progressively lowers the content of ions that are entering the transpirational stream. It is presumed that NHX-like cation/H<sup>+</sup> transporters have a major function in this process [131; 157].

#### 4.4. Osmotic homeostasis: Compatible osmolytes

Osmotic balance in the cytoplasm is achieved by the accumulation of organic solutes that do not inhibit metabolic processes, called compatible osmolytes. These are sugars (mainly sucrose and fructose), sugars alcohols (glycerol, methylated inositols), complex sugars (trehalose, raffinose and fructans), ions (K<sup>+</sup>), charged metabolites (glycine betaine) and amino acids such as proline [156; 157]. The function of the compatible solutes is not limited to osmotic balance. Compatible solutes are typically hydrophilic, and may be able to replace water at the surface of proteins or membranes, thus acting as low molecular weight chaperones [157]. These solutes also function to protect cellular structures through scavenging ROS [6; 10; 157]. Salt tolerance requires that compatible solutes accumulate in the cytosol and organelles where these function in osmotic adjustment and osmoprotection [187]. With exceptions like K<sup>+</sup>, most compatible osmolytes are organic solutes. Genes that encode enzymes that catalyze the biosynthesis of compatible solutes enhance salt and/or drought tolerance in gain-of-function strategies [155].

Proline occurs widely in higher plants, and normally accumulates to large quantities in response to environmental stresses [205]. In addition to osmotic adjustment, it is involved in prevention of protein denaturation and preservation of enzyme structure and activity [187]. Most of research on proline as an osmoregulatory compound has been carried out on the vegetative parts of the plants. Little attention has been paid to the reproductive organs, especially seeds. Recently, information has been published on osmotic adjustment of seeds under stress conditions. Salt stress increased proline accumulation in the cotyledons and roots of germinating ground-nut seeds [162]. Proline accumulated in the endosperm and radicles of germinating barley seeds with increasing NaCl concentrations in the growing media [163-164]. This proline probably originated from the degradation of stored protein in the endosperm. Walnut seeds average 15-25 g protein per 100 g of kernel and the proline content of seeds varies with genotype, ranging from 1100 to 1500 mg/100g kernel. A high amount of proline was detected in embryonic axis and leaves [181]. Our previous study revealed that the amount of proline in seeds of different genotypes of walnut, especially in semi-tolerant and tolerant genotypes, is high [212]. Even at the beginning of a drought period, the machinery for proline accumulation was most activated in the tolerant genotypes 'Chandler' and 'Panegine20' of walnut [180]. These initial differences in proline content, observed among the genotypes at day zero, prior to application of WI, and notably high in 'Panegine20' and 'Chandler', could be due to the natural adaptation to abiotic stress of the germplasm from which these genotypes were derived. Proline content of both 'Chandler' and 'Panegine20' were elevated and similar to each other early in the drought period, but at the end the proline content of 'Panegine20' was higher than that of 'Chandler' [180]. Proline appears to be a major osmotic regulator in 'Panegine20' and 'Chandler' under drought stress. Also, our previous study demonstrated

that in 'Panegine20', contrary to 'Chandler', "ion osmosis" is another important osmotic regulator under drought and salt stress [212].

Proline content increases significantly in relation to the severity of drought stress, in particular in roots of tolerant walnut genotypes [180]. In two and three years old walnut seedlings proline content of both roots and shoots was elevated initially and increased significantly with length of drought stress in tolerant genotypes [180]. During 16 days of water stress, root proline content increased 1.48 fold in 'Panegine20' and 1.38 fold in 'Chandler' seedlings [180]. Similarly, leaf proline content increased 2.07 times in 'Panegine20' and 1.50 times in 'Chandler' seedlings compared to the control plants [180]. The increase in proline content was greater in 'Panegine20' than in 'Chandler' and greater in roots than in shoots [180].

#### 4.5. Total soluble sugars and starch variation under abiotic stress

Salinity and drought cause the accumulation of soluble sugars, free proline, and soluble proteins [141; 154]. Parida and Das [177] reported that lower osmotic potential allows leaves to withstand a greater evaporative demand without loss of turgor. This requires an increase in osmotica, either by the uptake of soil solutes or by the synthesis of metabolically compatible solutes [138]. These findings appear to apply to olives since Tattini et al. [179] showed a correlation between leaf glucose and increasing levels of salinity in the root zone.

Drought and salt stress significantly increased the total soluble sugar content of roots and leaves only in 'Panegine20' and 'Chandler' varieties [180]. Leaf soluble sugar content increased 1.39 times in 'Panegine20' and 1.59 times in 'Chandler' compared to the controls. The increase in sugar concentration may result from the degradation of starch [202]. Soluble sugar content was elevated initially and increased progressively in drought stressed tissues of the tolerant genotypes. Sugars may act directly as osmotica or may protect specific macromolecules and thereby contribute to the stabilization of membrane structures [197]. In general, soluble sugar content tends to be maintained in the leaves of drought-stressed plants even though rates of carbon assimilation are partially reduced. In this study, observed increases in soluble sugar concentration coincided with decreases in starch content as the water potential dropped.

Metabolites may prove to be beneficial to germination, first by reducing osmotic inhibition and second by providing substrates for the growth of embryonic tissues [150; 153]. Imposition of different polyethylene glycol treatments on promising genotypes of walnut seedlings significantly increased total soluble sugar content [180]. Compared to the control, a drastic increase was observed in shoots and roots. Root content soluble sugar increased 1.65 times in 'Panegine20' progeny and 1.70 times in 'Chandler', and shoot soluble sugar content increased 1.73 times in 'Panegine20' and 1.60 times in 'Chandler' relative to control plants. Starch content significantly decreased in roots and shoots of both genotypes. Total starch content of roots decreased 49.46% in 'Panegine20' and 38.18% in 'Chandler'. This decrease was 52.79% in 'Panegine20' and 47.42% in 'Chandler' relative to the control plants [180].

Ability of LEA proteins to act synergistically with non-reducing sugars to form a glassy matrix, and thus confer drought protection, is an attractive hypothesis [170]. This hypothesis is supported by the abundance of LEA proteins and reducing sugars in desiccation-tolerant plant

tissues [171]. Several factors appear necessary to confer desiccation tolerance. Evidence implicates the accumulation of soluble sugars, especially sucrose and raffinose family oligosaccharides [172-173]. However, such sugars have also been detected in immature desiccation-intolerant embryos of maize and wheat [174]. Other factors, such as heat-stable late embryogenesis abundant proteins, may be involved [175], but some of these have been identified in recalcitrant (desiccation-intolerant) seeds [176]. Hence, examining the drought response of desiccation tolerant and intolerant seeds fails to provide conclusive evidence of a role in desiccation tolerance for either soluble sugars or heat-stable proteins. Soluble sugars and heat-stable proteins were equally likely (or unlikely) to be involved in the development of seed quality [178].

#### 4.6. Chlorophyll pigments and photosynthetic activity under abiotic stress

It is clear from numerous similar studies of water and salt relations that turgor maintenance alone does not assure continued leaf expansion [196]. It may be that photosynthetic capacity is insufficient to provide carbon for both wall synthesis and “turgor-driven cell expansion”. Or it may be that some higher level controls operate to limit expansion in spite of the available potential [221]. The Chl a and Chl b contents as well as the photosynthetic electron transport rate in leaves of stressed ‘Lara’ and ‘Serr’ seedlings decreased significantly at all drought and salt periods tested, but stressed ‘Panegine20’ and ‘Chandler’ seedlings did not differ significantly from the controls in regards to these traits at any time during the applied stress. The decreases were more apparent with longer drought exposure time [180]. The Chl a/b ratios remained constant in all cases and there were no significant differences observed within genotypes [180].

The stability of chlorophyll content and chlorophyll a/b ratio in ‘Panegine20’ and ‘Chandler’ seedlings suggests that the pigment apparatus is comparatively resistant to dehydration in these tolerant walnut cultivars. Drought and salt stress can directly or indirectly reduce the photochemical efficiency of PS2 due to either inefficient energy transfer from the light-harvesting complex to the reaction centre, or to inability of the reaction centre to accept photons as a result of structural alterations in the PS2 complex [201; 210]. The results obtained indicate that abiotic stress like drought and salt affects both the light-harvesting complex and the reaction centre of PS2. Also Rosati et al [121] revealed that Kaolin application in walnut under water stress did not affect dark respiration rate, nor Amax2500, but significantly reduced Amax2000/Amax2500 and apparent quantum yield, while compensation point was significantly increased. The modeled leaf photosynthetic response to PAR was different for the kaolin-coated and the control leaves [121]. Assuming that only 63% of the PAR incident on the kaolin-coated leaves actually reached the leaf surface, the modeled curves for the two treatments overlapped perfectly at any PAR [121].

Drought reduces photosynthesis in walnut (*Juglans regia* L.), but it is not known whether this is mainly due to the closure of stomata, or to possible effects on leaf biochemistry. In an attempt to answer this question, Rosati et al [121] studied diurnal changes in the water status and gas exchange in droughted [50% crop evapotranspiration (ETc)] and fully irrigated (100% ETc) walnut trees, over 2 d. They resulted that stem water potential ( $\Psi_s$ ) ranged from -0.5 MPa in

the morning to  $-1.2$  MPa in the afternoon under drought, and from  $-0.1$  MPa to  $-0.4$  MPa under full watering. Net  $\text{CO}_2$  assimilation ( $A_{\text{max}}$ ) ranged from  $15 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the morning to  $3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the afternoon under drought, and from  $25 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the morning to  $10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the afternoon under full watering. At these times, stomatal conductance ( $g_s$ ) varied from  $0.2$  to  $0.02 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  and from  $0.7$  to  $0.2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ , respectively. Drought reduced the internal  $\text{CO}_2$  concentration ( $C_i$ ) by about  $55 \mu\text{mol mol}^{-1}$  on  $\text{day}^{-1}$ , and by about  $100 \mu\text{mol mol}^{-1}$  on  $\text{day}^{-2}$  and increased leaf temperature (TL) by about  $2$ – $5^\circ\text{C}$ . The reductions in  $g_s$  and  $C_i$  with drought suggest that lower photosynthesis was associated with stomatal closure. However, in each treatment,  $A_{\text{max}}$  decreased during the day, while  $C_i$  was stable, suggesting that photosynthesis was also reduced by a direct effect of heat on leaf biochemistry. Both  $A_{\text{max}}$  and  $g_s$  correlated with TL and with the leaf-to-air vapor pressure deficit (VPD), but with different relationships for droughted and control trees. However, when stomatal limitations to photosynthesis were accounted for (i.e. based on the assumption that, under stomatal limitation, photosynthesis is proportional to  $C_i$ ) a single relationship between  $A_{\text{max}}$  and TL described all the data ( $R_2 = 0.81$ ). Thus, photosynthesis was limited by the closing of stomata under drought, and by a direct effect of heat on leaf biochemistry. These results suggest that hot and dry weather reduces photosynthesis and potential productivity in walnut in the absence of soil water deficit.

Under normal physiological conditions, electron transport is directed toward sequential and fully coordinated reduction of intermediate electron acceptors PS2 and PS1. However, drought and high temperature can provoke a state of hyper-reduction in the electron transport chain, enhancing generation of superoxide radicals as has been shown in cotton [183-184] and rice [186]. Theoretically, high photosynthetic efficiency can increase water-use efficiency as more carbon is assimilated per unit water transpired. In walnuts, a positive correlation was reported between photosynthesis and stomatal conductance—an important determinant of water use efficiency [121; 223]. The effect of salinity stress on the photosynthetic enzyme activities is postulated to be a secondary effect mediated by the reduced  $\text{CO}_2$  partial pressure in the leaves caused by the stomatal closure [224]. The present review also reveals that in all the walnuts grown in non-saline and desiccated soils, an increased rate of assimilation is coupled with increased stomatal conductance [180].

#### 4.7. Total phenols and PPO activity under abiotic stress condition

Walnut nuts have high amount of phenolic compounds. Walnut kernels are rich in oils composed of unsaturated fatty acids, such as linoleic and oleic acid, and are susceptible to oxidation. However the content of  $\alpha$ -tocopherol, an antioxidant, is lower in walnut than in other nuts such as almonds, hazelnuts, peanuts [212]. This implies that the nut contains antioxidants inhibiting lipid auto-oxidation. Recently, a walnut extract containing ellagic acid, gallic acid, and flavonoids was reported to inhibit the oxidation of human plasma and low density lipoproteins (LDL) in-vitro [158]. Although the presence of ellagic acid suggests the occurrence of its bound forms, ellagitannins, there are some reports on the tannin constituents of walnut [233-235]. Muir et al. [235] demonstrate that a shikimate pathway enzyme, SDH (shikimate dehydrogenase), is directly responsible for GA [gallic acid]

production in both plants and bacteria when shikimic acid (SA) or 3-DHS were used as substrates and NADP<sup>+</sup> as a cofactor. Finally, they showed that purified *E. coli* and *J. regia* SDH produced GA in-vitro. Also, they proposed that the C-terminal, AroE/SDH domain of the plant enzyme is the region of the protein responsible for GA production [235]. Because of the importance of GA as an antioxidant in plants, controlling its production and accumulation in plants could significantly increase the nutritional value and tolerance of walnut for abiotic stresses. Further expression studies using fragments of the walnut gene(s) will be performed to verify the activity of each individual domain in GA production [235]. Anderson [158] examined antioxidative tannins and related polyphenols in foods and nuts, isolating 16 polyphenolic constituents including three new hydrolysable tannins, along with adenosine and adenine, from commercial walnuts. Under abiotic stress, the profile of total phenols and PPO activity was similar in both roots and leaves of all genotype seedlings subjected to salt and drought stress, but amounts of phenolics and levels of PPO activity were higher in leaves than in roots [213]. A significant increase (25.3% and 38.4%) in total phenolic concentration was observed within 20 d of water deficit treatment in leaves of both 'Chandler' and 'Panegine20' [213] in contrast to a small and not significant increase in total phenolic concentration in seedlings of some varieties, especially in root tissues [213]. The reverse pattern was observed for PPO activity, with 'Chandler' and 'Panegine20' showing a slight decline in root and leaves while PPO activity in 'Lara' and 'Serr' increased sharply during drought in both tissues. Among the antioxidative enzymes analyzed, PPO was the only one clearly down-regulated under WI conditions. 'Chandler' and 'Panegine20' leaves showed a marked decline in PPO activity during water deficit stress but in roots PPO activity decrease were less sharp [213]. A significant increase in PPO activity in water stressed leaves of 'Lara' and 'Serr' (112% and 76%) was apparent after 7 d of drought [213]. In these varieties, PPO activity linearly increased until the  $\Psi_w$  was -1.84 MPa or more (during the 7th d of drought period) and then remained at a similar level [213]. The antioxidant properties of plant phenolic compounds are well-documented [206]. These are synthesized de novo [207] and can influence auxin metabolism, membrane permeability, respiration, oxidative phosphorylation, and protein synthesis [199] and their activity also has been related to the occurrence of physiological injury [208]. The changes in phenolic production and PPO activity observed in drought-stressed walnut seedlings show that some varieties, namely 'Lara' and 'Serr', are more sensitive to drought than the tolerant varieties 'Panegine20' and 'Chandler' [213].

#### 4.8. Effects of salt and drought on Malondialdehyde (MDA) content

Earlier, Jouve et al. [225] found that the endogenous level of MDA did not vary in control and in the salt stressed aspen (*Populus tremula* L.) plants. This indicates that the level of lipid peroxidation was similar in stressed and non-stressed plants. Likewise, MDA concentration changed with increasing salt concentration in the shoots of tolerant walnut varieties, decreased slightly at 100mM, while increased at 200 and 250mM salt stress which suggests that walnut shoots are better protected from oxidative damage under salt stress [213]. Changes in the MDA content of leaf tissues subjected to drought and salt were well documented by Lotfi [213]. Application of drought for 20 d caused a linear increase in the

MDA content of 'Lara' and 'Serr' seedlings with the MDA content peaking at  $\sim 143 \text{ nmol g}^{-1}$  FW on the 16th d. in comparison with control plants at  $\sim 67 \text{ nmol g}^{-1}$  FW, a trend similar to PPO. Seedlings of 'Panegine20' and 'Chandler' showed significant decreases in MDA content under the same conditions [213]. Under most oxidative conditions, malondialdehyde (MDA) is a product too often considered as a marker of peroxidative damage. It is important to interpret such measurements with caution, since there are a lot of drawbacks linked to the thiobarbituric acid (TBA) test for MDA determination [160-161]. MDA is produced when polyunsaturated fatty acids in cell membranes undergo peroxidation. The results reported here show that accumulation of MDA was higher in seedlings of sensitive varieties, especially in 'Lara'. The lower levels of MDA observed in 'Panegine20' and 'Chandler' suggests that less membrane damage occurs in droughted seedlings of these varieties, contributing to their tolerance [213].

#### 4.9. Effects of drought and salt on peroxidase (PAO) activity

PAO activity peaked in leaf tissues of walnut 'Lara' and 'Serr' seedlings on 5<sup>th</sup> day of WI (24.56 and 19.78 mmol guaiacol/mg protein/min) simultaneously with increasing of PPO activity in these varieties [213]. 'Panegine20' and 'Chandler' seedlings showed insignificant increases in PAO activity [213]. In our study, the generation of ROS was tightly linked in sensitive genotypes to catabolism of PAs by PAO and decreased PAO activity coincided with accumulation of proline. PAO activity in drought tolerant seedlings under water stress was significantly lower than in sensitive seedlings [213], likely accounting for the higher accumulation of PAs in tolerant seedlings. The function of PAO is oxidation of Spermidine (Spd) to pyrroline, 1,3-diamine propane (DAP) and  $\text{H}_2\text{O}_2$ , and spermine (Spm) to aminopropylpyrroline, DAP and  $\text{H}_2\text{O}_2$  [182, 200]. Enhanced  $\text{H}_2\text{O}_2$  production as a result of PAO activity may be important in signal transduction leading to programmed cell death (PCD) and in expression of defense genes involved in responses to drought tolerance [185]. Low PAO activity in the tested tolerant cultivars, and probable resulting polyamine accumulation, likely reflect a protective response to abiotic stress.

Our results also show that low PAO activity and subsequent accumulation of endogenous PAs increased the activity of peroxidase (POD) and catalase (CAT), along with proline production in 'Panegine20' and 'Chandler' seedlings under WI [213]. Our results are in agreement with the results reported by Seki et al. [188]. DAO and PAO are also considered to be important controllers of the ABA signaling pathway involved in stomatal regulation [213]. In drought tolerant cultivars a decreases in PAO activity was observed relative to sensitive ones, indicating the ABA signaling pathway integrates PA, DAO and PAO activity in regulating  $\text{H}_2\text{O}_2$  production [191]. The high activity of antioxidants observed in this study in roots and shoots of 'Panegine20' and 'Chandler' seedlings suggests these may convey drought tolerance that can be a first step to protecting the plant leaves. The maintenance of root and leaf PA concentrations, along with low PAO activity, suggest that a balance between their biosynthesis and oxidation cannot be excluded as a further specific feature of 'Panegine20' and 'Chandler' two-phase responses under drought conditions. Further studies are needed to determine the specific PAs present in these cultivars.

#### 4.10. Effects of salt and drought on LOX activity

Both enzymatic and non-enzymatic lipid peroxidation have been previously implicated in ROS perception. Oxylipins resulting from enzymatic oxidation via lipoxygenases (LOX) might function in leaf senescence [159]. LOX activity in leaves of sensitive genotypes increased markedly by the 5<sup>th</sup> d of WI and then continued to rise slightly. Also under salt stress conditions the same trends were observed for walnut seedlings. Leaves were the most affected by water deficit, showing a four-fold increase in LOX activity over control seedlings [213]. LOX activities in root tissues were 1.7 and 1.6 times the control values at the maximum drought stress [213]. LOX activity of controls did not change significantly during the full 20 d of WI. There were no significant increases in LOX activity in seedlings of 'Panegine20' and 'Chandler' [213]. LOXs are a family of enzymes that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides (LOOHs) which are involved in responses to stresses [190]. Plant LOXs may be involved in growth and developmental control processes through the biosynthesis of regulatory molecules and volatile compounds [198]. The high degree of lipid peroxidation observed could produce lipid derivatives acting as secondary messengers capable of activating some drought stress associated genes by means of specific transcription factors, triggering plant responses to desiccation [212]. Increase in LOX activity can be due to an increased amount of enzymatic protein [204]. However, in this study a lower amount of the enzymatic protein was found in drought-stressed seedlings of 'Panegine20' and 'Chandler' than in controls [213].

#### 4.11. Effects of salt and drought on antioxidant defense systems

A recent comprehensive study revealed that both salt and drought stresses led to down-regulation of some photosynthetic genes, although most of the changes were small, possibly reflecting the mild stress imposed. Compared to drought, salt stress affected more genes and more intensely, possibly reflecting the combined effects of dehydration and osmotic stress under salt-imposed conditions [194]. Desingh and Kanagaraj [226] pointed out that photosynthetic rate and RuBP carboxylase activity decreased with increasing salinity but some antioxidative enzymes significantly increased. An important consequence of salt stress is the excessive generation of reactive oxygen species (ROS) such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radicals ( $OH^\cdot$ ), particularly in the chloroplast and mitochondria [195].

In plant cells, ROS are generated in high amounts by both constitutive and inducible routes, but under normal situations, the redox balance of the cell is maintained via the constitutive action of a wide range of antioxidant mechanisms that have evolved to remove ROS [194]. ROS are produced during photosynthesis and respiration, as by-products of metabolism, or via dedicated enzymes. Cells are equipped with a range of efficient antioxidant mechanisms to remove ROS. Changes in the cellular redox balance result from exposure to various abiotic and biotic stresses, with induction of both ROS generation and removal mechanisms. Enzymatic ROS scavenging mechanisms in plants include SOD (superoxide dismutase), present in many cellular compartments; catalase, located in peroxisomes; and the ubiquitous ascorbate-glutathione cycle. SOD catalyses the dismutation of superoxide to  $H_2O_2$ , and is thus one of the

primary mediators of  $H_2O_2$  production from intracellular sources of superoxide. Unlike most organisms, plants have multiple forms of the different types of SODs encoded by multiple genes [216]. According to our previous study, SOD activity in water-stressed 'Panegine20' walnuts increased 58% and 29% relative to controls in leaves and roots respectively. In 'Chandler' seedlings this increase was 51% and 33%, respectively [213]. In 'Serr' seedlings, the decline was 54% and 42% in the different tissues, respectively and in 'Lara' walnuts; the decline was 67% and 53% in leaves and roots. For all cultivars, the increase in SOD activity under WI conditions in roots was less than those recorded in leaves [213].

Increasing SOD activity induces a higher tolerance to oxidative stress under salt or drought stress [205]. Metallo enzyme SOD, which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress, is the most effective intracellular enzymatic antioxidant [184]. This enzyme provides the first line of defense against the toxic effects of elevated levels of ROS. Yang et al. [193] found that under drought conditions and high light SOD activity increased significantly relative to low light.

The patterns of SOD, CAT, POD and APX activities were roughly parallel in all the tissues examined, showing a significant increase under salt and drought-treatment conditions in tolerant walnut varieties [213] but at different levels among genotypes and plant tissues. 'Panegine20' and 'Chandler' seedlings of walnut showed the highest levels of antioxidative enzyme activity. The increases in APX and CAT activities in 'Panegine20' seedlings were significant in leaves and roots tissue and activity was greater in leaves than roots. In 'Chandler' seedlings, the increase in APX activity under WI was significant only in leaves [213]. In 'Panegine20' and 'Chandler' seedlings APX activity increased more than SOD or CAT activity under WI conditions [213]. Activities of SOD, CAT and APX peaked at the 7<sup>th</sup> d of WI, but POD activity climaxed on the 5<sup>th</sup> d and was higher in 'Panegine20' than 'Chandler' [213].

Abiotic stresses, such as drought stress, cause molecular damage to plant cells, either directly or indirectly, through the formation of AOS. In this study, the plants exposed to abiotic stress showed a significant increase in CAT, APX, SOD and POD activity. MDA is regarded as a biomarker of lipid peroxidation and stress-induced damage to the plasmalemma and organelle membranes [189]. In this study, the amount of MDA in tolerant varieties decreased with increasing drought stress. CATs are tetrameric heme-containing enzymes with the ability to directly convert  $H_2O_2$  into  $H_2O$  and  $O_2$  and are indispensable for ROS detoxification under stress conditions [204]. APX is thought to play an essential role in scavenging ROS and protecting cells by scavenging  $H_2O_2$  in water-water and ASH-GSH cycles and utilizing ASH as the electron donor.

APX has a higher affinity for  $H_2O_2$  ( $\mu M$  range) than CAT and POD (mM range) and may have a more crucial role in the management of ROS during stress. As expected, the activities of all these enzymes changed significantly in walnut seedlings under water stress. The observed greater increase in APX activity in leaves of water-stressed plants than in roots could be due to localization of APX in chloroplasts. The significant increase in APX activity seen in leaves could be a mechanism developed by walnut trees for protection of chloroplasts, which under stress conditions develop sustained electron flows and are the main producers and targets of ROS action [195]. The increase in CAT activity in leaves of water-stressed plants may be an

adaptation aimed at scavenging photo respiratory  $H_2O_2$  produced during drought stress [203]. The reduced PPO activity in stressed walnut seedlings could be a response to increase the abundance of antioxidative phenols. PPO could also be involved, through proteolytic action, in removing proteins damaged by oxidative stress effects [211].

The increased POD, APX and CAT activities observed in the more drought and salt-tolerant 'Panegine20' and 'Chandler' seedlings, relative to 'Lara' and 'Serr' seedlings, underline the effectiveness of 'Panegine20' and 'Chandler' antioxidative enzyme systems in protecting the cellular apparatus under water deficit conditions. Furthermore, the higher proline accumulation observed in 'Panegine20' and 'Chandler' seedlings under WI was accompanied by higher activities of SOD, APX, POD and CAT. These results suggest that proline accumulation could activate the antioxidative defense mechanism in walnut trees as has been suggested by Yang et al. [193] in salt-stressed soybean plants.

In conclusion, genotypic differences were observed among walnut seedlings in leaf water status, photosynthetic performance, pigment content, proline accumulation and antioxidative enzyme activity. The close relationship observed between photosynthetic rate ( $P_n$ ) and proline content points to an important role of this osmolyte in the maintenance of photosynthetic activity and therefore in drought tolerance. These literature reviews show that differences in SOD, APX, POD, PPO, LOX, PAO and CAT activities among walnut genotypes could be attributed to differences in the mechanisms underlying oxidative stress injury and subsequent tolerance to abiotic stress. Varietal differences in pigment content could be related to differences in antioxidative enzyme activity. Notably, the 'Panegine20' and 'Chandler' seedlings, which exhibit higher drought tolerance, also showed higher antioxidative enzyme activity than other walnut seedlings. Seed of the later cultivars should be considered high-risk for planting in dry areas. In addition, these results show that seedling genotypes with the higher photosynthetic activity ('Panegine20' and 'Chandler') also had higher proline content and antioxidative enzyme activity. This supports an interaction between proline and the antioxidative defense system as suggested by Yang et al [193]. To verify this hypothesis, we suggest further studies focusing on the effects of exogenous application of proline and paraquat on the activities of protective enzymes in walnut trees would be of interest.

#### **4.12. Biotechnology and abiotic stress engineering in walnut**

Breeding for drought and salinity tolerance in crop plants should be given high priority in plant biotechnology programs. Molecular control mechanisms for abiotic stress tolerances are based on the activation and regulation of specific stress-related genes. These genes are involved in the whole sequence of stress responses such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. A major objective of walnut rootstock breeding is vigour, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to quickly establish a full-sized bearing canopy. Other objectives include resistance to diseases and pests, most notably *Phytophthora*, nematodes and crown gall, and tolerance of soil-related problems including waterlogging, salt accumulation and cold. There is interest in controlling tree size but not at the cost of vigour. In walnut, breeding for abiotic stress tolerance or resistance has been limited

at best. One of the first attempts is transformation of somatic embryos of Persian walnut with a gene isolated from a cyanobacter. This gene controls expression of flavodoxin. The role of flavodoxin in response to salinity and osmotic conditions is known [229].

Ferredoxins are very ancient proteins widely used by anaerobic organisms for many metabolic pathways. Ferredoxin (Fd) is up-regulated by light, indicating that under autotrophic growth, Fd is the normal electron carrier [230]. As a replacement for Fd, Flavedoxin gene (fld) is induced under various environmental sources of stress including oxidative stress in enterobacteria and salinity stress in cyanobacteria [231]. Results showed that transgenic plantlets of walnut harboring the fld gene clearly grow better at 200 mM NaCl than the non-transgenic controls. The control plants did not produce any callus and turned brown and died after 10 days, while transgenic lines showed no brown symptoms, produced callus, and continued their growth for up to 45 days on 200 mM NaCl [229]. Compared to salt stress, the decrease in evaluated parameters of transgenic and non-transgenic SEs caused by PEG-induced stress was relatively lower. At the 1.5% PEG, the number of cotyledonary embryos was significantly increased in both transgenic and non-transgenic somatic embryos (SEs) [229]. With increasing concentrations of PEG in culture medium to 5% and 10%, significant differences between transgenic and non-transgenic SEs for most of the evaluated parameters were observed. The results showed that transformants reduced stress in both salt and osmotic stress conditions and the degree of response was greater to salt than to PEG. Over-expression of the fld gene in transgenic lines of Persian walnut partially decreases some of the hostile effects of salinity stress. Production of callus and new shoots by transgenic plants expressing this gene and grown on stress-inducing media is in agreement with previous reports in tobacco [232]. All findings reported show clearly that expression of cyanobacterial proteins can be a powerful tool to enhance the stress tolerance of some plants.

## 5. Conclusions and perspectives

- Cavitation avoidance is a likely physiological function associated with stomatal regulation during abiotic stress in walnut. This suggests that stomata are responding to leaf water status as determined by transpiration rate and plant hydraulics and that  $P_{\text{rachis}}$  might be the physiological parameter regulated by stomatal closure during water stress, which would have the effect of preventing extensive developments of cavitation during water stress.
- Hydraulic segmentation for walnut trees (*Juglans regia*) by petioles displaying a large vulnerability to abiotic stresses in sensitive genotypes. This phenomenon disconnects leaves through massive cavitation during stress and avoids irreversible damage to perennial parts of the tree.
- Photosynthesis is limited by stomatal closing during drought and by direct effects of heat on leaf biochemistry. This suggests that hot and dry weather reduces photosynthesis and potential productivity in walnut even in the absence of soil water deficit. But, some promising varieties show the sufficient net assimilation rate and photosynthesis under abiotic stress conditions.

- Walnut roots are mainly distributed in the upper soil layers. Soil moisture is a key factor regulating root growth and water uptake efficiency of the roots. The shallow roots lose efficiency in water uptake during the dry season and the shift to uptake by deeper roots does not fully compensate for the loss of uptake by shallow roots and is not able to prevent water stress, which is characterized by increased percentage loss of xylem conductance (PLC) in pre-dawn, reduced pre-dawn leaf water potential and transpiration during abiotic stresses.
- Understanding the ability of genotypes to absorb essential elements is indicative of their ability to withstand stress.
- Differences in antioxidative enzymes (such as SOD, APX, POD, PPO, LOX, PAO and CAT) activities among walnut genotypes could be attributed to differences in the mechanisms underlying oxidative stress injury and subsequent tolerance to abiotic stress.
- Higher proline accumulation observed in tolerant seedlings of walnut to osmotic stresses was accompanied by higher activities of antioxidative enzymes (e.g. SOD, APX, POD and CAT). These results suggest that proline accumulation could activate the antioxidative defense mechanism in walnut trees.
- The degree of stress tolerance found in seedlings of some walnut varieties has been characterized at various stages of growth. Identified stress-tolerant genotypes are candidates for further studies under longer periods of drought and field studies to determine their suitability for areas with adverse environmental conditions, and eventually for use as drought-tolerant rootstocks.
- Application of biotechnology tools for increasing tolerance to abiotic stresses in walnut is underway. Some promising results have been reported under in-vitro conditions.

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