TRANSCRIPTIONAL AND PHYSIOLOGICAL RESPONSES OF BLACK POPLAR (*POPULUS NIGRA L.*) TO DROUGHT STRESS

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TRANSCRIPTIONAL AND PHYSIOLOGICAL RESPONSES OF BLACK POPLAR (*Populus nigra L.*) TO DROUGHT STRESS

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ABSTRACT

TRANSCRIPTIONAL AND PHYSIOLOGICAL RESPONSES TO DROUGHT STRESS IN *Populus nigra L*.

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In the current study, we investigated a number of drought related physio-biochemical processes and transcriptional comparisons at different stages of water availability to understand adaptation and response mechanisms of the black poplar (*Populus nigra L*.). Therefore, a well-watered, successive drought and post drought recovery periods were applied to black poplar clones in both field trial and greenhouse. According to their drought response, three adaptation strategies were identified in black poplar. One of the three genotypes was found to evolved a drought evading strategy (sensitive) in which their active portion of life generally takes place mostly during water abundant periods. Therefore, the clone could resist only mild drought stress. The sensitive clone was characterized with least leaf water potential (-12 $\pm$ 0.9 bars), complete defoliation and highest increase in antioxidant enzyme activities under progressive drought levels. On the contrary, another clones exhibited strong dehydration tolerance strategy (resistant) to even severe drought that the productivity of these resistant clones was much higher during post-drought recovery period. Although its leaf water potential values (-8 $\pm$ 0.7 bars) were significantly reduced, defoliation rate of this genotype was very low. Antioxidant enzyme results and hydrogen peroxidase content of the leaves of this genotype indicated lesser ROS production and stress situation as compared to the sensitive genotype. The last genotype in the study found to have drought avoidance strategy (moderate resistant). In this genotype, leaf water content did not decrease down to critical values (-4 $\pm$ 0.6 bars) as compared to other two genotypes. However, the lowest growth performances were recorded for this genotype during both field and greenhouse trials.

Among these genotypes, two clones named N.62.191 (Resistant) and N.03368.A (Sensitive) were selected for microarray based transcriptional profiling. Analysis of microarray data with principle component analysis indicated that 90% of variation in the experiment was due to clonal treatment differences. Hierarchical clustering of the genes that differentially

expressed during the experiment indicated that the transcriptional response occurred at severe drought level for the resistant clone while the same response was started much earlier (moderate drought) in sensitive one. One way ANOVA analysis was used to list all the genes differentially expressed (two fold at $P<0.05$) in all drought treatments for both genotypes. The analysis extracted 2453 and 5851 probe sets for the resistant and sensitive genotypes, respectively. Comparison of these probe sets identified genotype specific drought related genes.

In the current study, the sensitive genotype was characterized with severe defoliation and loss of leaf water content under drought stress. Therefore, the sensitive genotype specific genes were mostly annotated to leaf senescence and cell death. The highest expression of the genes such as NAC (JA), and ap2/erf transcription factors in the sensitive genotype indicated a potential role of Jasmonic Acid and Ethylen hormones controlling the leaf senescence. Expression of the genes involved in proteolysis, cell wall degradation and carbohydrate catabolism in the sensitive genotype were associated with drought induced sugar starvation and nutrient mobilization processes. Contrary to the sensitive genotype, the drought resistant black poplar genotypes did not defoliate until severe drought level. The maintenance and recovery of drought traded leaves of the resistant genotype attributed to enhanced synthesis of bark storage proteins during drought stress. These proteins were suggested to be remobilized under drought and re-watering period to be used as an energy source in the drought treated organs. The highest up regulation in chaperons such as Heat Shock Proteins and Dnaj Proteins mostly in the resistant genotype were also associated to drought tolerance. Conservation of energy metabolism in the resistant genotype was also suggested to be another important strategy to cope with drought stress. In the list of genotype specific genes highest up regulation was recorded in the probe sets annotated to the genes that have phosphorylase domain. These genes could be related with energy metabolism of the cells due to their roles in glycolysis and glycogenesis.

Key words; *Populus nigra*, black poplar, drought, sensitive, tolerant, microarray, gene profiling

ÖZ

Populus nigra L'da KURAKLIK STRESİNE KARŞI TRANSKRİPTOMİK VE FİZYOLOJİK TEPKİLER

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Bu çalışmada, karakavak (*Populus nigra L.*) türünde kuraklığa gösterilen tepki mekanizmalarını anlamak için farklı kuraklık seviyelerindeki fizyolojik, biyokimyasal ve gen ifade değişimleri gözlenmiştir. Bu amaçla, bol sulanan, kuraklık ve kuraklık sonrası sulanan olmak üzere üç ana su değişim evresi sera denemesinde bulunan karakavak klon kolleksiyonuna uygulanmıştır. Bu denemelerde gösterdikleri büyüme ve morfolojik özelliklere göre tüm kolleksiyon üç çeşit genotipe ayrılmıştır. Araştırılan genotiplerinden ilkinin kuraklıktan kaçma adaptasyonunu (hassas) geliştirdiği,bu nedenle aktif büyüme evresinin daha çok kuraklık öncesi sulanan dönemde gerçekleştiği kurak ve kurak sonrası dönemde ise büyümenin çok azaldığı tespit edilmiştir. Bu adaptasyon stratejisinde klonlar şiddetli yaprak dökümü,düşük yaprak su potansiyeli (12 $\pm$ 0.9 bar) ve yüksek antioksidant enzim aktivite özellikleri ile tanımlanmıştır. Buna karşılık, kuraklığa dayanıklılık stratejisi geliştiren ikinci karakavak klon grubu, hem sulanan hemde kurak evrelerinde en çok büyüyen klonları içermiştir. Sera denemesi sırasında kuraklığa bağlı yaprak dökme özelliği bu genotip için çok sınırlı kalmıştır sadece sararmalar meydana gelmiştir. Kuraklıkğa dayanıklı klonlar özellikle kuraklık sonrası sulanan evrede en yüksek büyüme performansını göstererek kuraklık öncesi dönemdeki durumlarına ulaşmışlardır. Kuraklık sırasında bu dayanıklı genotiplerin yaprak su potansiyeli (-8$\pm$ 0.7 bar) düşük seviyelere inmesine rağmen antioksidant ve hidrojen peroksit içeriği hassas klona göre çok az değişerek kuraklığın bu genotipte çok fazla stres yaratmadığını ortaya koymuştur. Karakavak klonlarının kuraklığa karşı gösterdiği son tepki mekanizması orta dereceli dayanıklılık mekanizması ile açıklanmıştır. Bu genotipte, yaprak su potansiyeli (-4$\pm$ 0.6) diğer iki genotipe göre çok az düşüş göstermesine rağmen büyüme performansları açısından en düşük değerler bu genotiplerde ölçülmüştür. Bu orta dereceli dayanıklılığa sahip bireylerde olgun yapraklar kuraklığın ilk evrelerinde dökülmeye başladığı böylece kuraklık sırasında suyun oluşan yeni yapraklarda tutularak kuraklığa karşı bir dayanıklılık sağladığı tespit edilmiştir.

Çalışılan bu genotiplerden hassas (N.03.368.A) ve dayanıklı (N.62.191.) karakavak klonları mikroarray temelli transkripsiyon profillemesi ve karşılaştırılması için seçilmiştir. Bu yolla kuraklığa karşı dayanıklılığın ve hassasiyetin genetik temeli ortaya çıkartılmaya çalışılmıştır. Yapılan analizlerde kuraklık esnasında genetik açıdan ilk tepkinin hassas klonda orta dereceli kuraklık seviyesinde verildiği gözlenmiştir. Ancak aynı tepki dayanıklı klonda ancak şiddetli kuraklık seviyelerinde ortaya çıkmıştır. Bu durum hassas klonda kuraklığın çok daha erken evrelerde etki etmeye başladığını göstermiştir. Mikroarray sonuçlarının karşılaştırılması sonucu toplam 2453 ve 5851 genin sırasıyla dayanıklı ve hassas bireyde kuraklık sırası ve sonrasında farklı ifade olduğu gösterilmiştir. Bu iki gen grubunun karşılaştırılması genotipe özgü kuraklık genlerinin belirlenmesini sağlamıştır.

Mikroarray verileri kuraklığa karşı hassas ve dayanıklı klondaki en önemli farkın, kuraklığa bağlı yaprak dökümünden kaynaklandığını göstermiştir. Hassas klon, kuraklık sırasında tüm yapraklarını dökerek dormanci periyoduna girmiştir. Bu nedenle hassas klonda yaprak dökümünü sağlayan etilen ve jasmonik asit genleri ile bu enzimlere bağlı NAC (JA), ve ap2/erf gibi transkripsiyon faktörlerinden sorumlu genlerin ifadelerinde kurak dönemde çok yüksek artışlar görülmüştür. Aynı genlerin dayanıklı klondaki değişimlerinin ise istastiki olarak anlamsız olduğu bulunmuştur. Bunun yanınında yine sadece hassas klonda proteaz, lipaz ve hücre duvarını parçalayan enzimlerden sorumlu genlerde çok yüksek artışlar olduğu görülmüştür. Bu durum yaprak dökümü sırasında bitkinin yaprak içerisindeki tüm nitrojen ve besin kaynaklarını parçalayarak kök ve gövdeye transfer etmek istemesi ile açıklanmıştır. Bu nedenle yine hassas genotipe özel artış gösteren ‘asparagine syntase’ gibi nitrojen transferinden sorumlu genlerin kuraklığa karşı hassas bireylerin belirlenmesinde marker olarak kullanılabileceği vurgulanmıştır. Hassas bireydeki yüksek yaprak dökümüne karşılık dayanıklı bireyde kuraklığa bağşı yaprak dökümü çok sınırlı kalmıştır. Yapılan transkriptomik analiz, bu karakavak klonundaki en yüksek ifadenin kabuk depo proteinlerinde olduğunu göstermiştir. Bu moleküllerin kuraklık sırasında yaprak tarafından sentezlenerek gövdeye gönderildiği böylece hem kuraklıkta eksilen besin kaynağının giderildiği hemde dormasi periyodu için hazırlığın yapıldığı tahmin edilmektedir. Bu aktif üretim nedeni ile kuraklığa dayanıklı karakavak klonu yaprağını dökmemiş, bunun yerine proteinlerin ve membran yapısının korunmasını sağlayan ‘heat shock’ proteinleri üretmiştir. Böylece yaprağın çok daha uzun süre bu depo proteinlerini üretebilmek için geç yaprak dökme özelliği göstediği düşünülmüştür. Bu depo proteinlerinin ayrıca kuraklık sonrası sulama döneminde tekrar yaprağa gönderilerek enerji kaynağı olarak kullanıldığı, böylece hem yaprağın hemde diğer organların tekrar sulama evresinde hızlı bir şekilde toparlanarak büyümeyi hızlandırdığı öngörülmüştür. Kabuk depo proteinlerinin ağaçlardaki kuraklık stresi açısından önemi ilk defa bu çalışma ile ortaya konmuştur.

Anahtar Kelimeler; *Populus nigra*, karakavak, kuraklık, hassasiyet, dayanıklılık, microarray, gen haritalaması

To my wife Zuhal and the meaning of our lives, our daughter İpek

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LIST OF ABBREVIATIONS

SWC: Soil Water Content

WWP: Well Watered Period

MDL: Mild Drought Stress

DAL: Drought Acclimation Level

MDL: Moderate Drought Level

SDL: Severe Drought Level

PDR: Post Drought Level

R:Resistant Black Poplar Clone (N.62.191)

S: Sensitive Black Poplar Clone (N.03.368.A)

ROS: Reactive Active Oxygen Species

APX: Ascorbate Peroxidase

GR: Glutathione Reductase

DHAR: Dehydroascorbate Reductase

H_2O_2: Hydrogen Peroxide

LWP: Predawn Leaf Water Potential

EL: Electrolyte Leakage

SEA: Single Enrichment Analysis

GO: Probe Set Gene Annotation

PCA: Principle Component Analysis

CHAPTER 1

GENERAL INTRODUCTION

1.1 Biology, Ecology and Distribution of *Populus Nigra* L.

Populus nigra L. (black poplar) is a member of the Salicaceae family and has a natural range from Europe to Siberia (Figure1.1). Black poplar constitutes local populations especially on alluvial soils by colonizing with their seeds, cuttings or root fragments. The reproductive age of the species is 10 to 15 years while the life of individual trees may be over 400 years. It is a dioecious species that male and female flowers clustered in pendulous catkins on different individuals (Figure1.1). Because of its wide geographic adaptation, fast growth abilities and immediate response to cultural practices, black poplar has been cultivated by farmers on private lands for centuries. The most widely cultivated black poplars in Turkey are *P. nigra var.italica* and *P. usbekistanica var. Afghanica*. These species are generally cultivated by farmers in fields, roadside and riparian plantations by using traditional vegetative propagation methods by farmers.

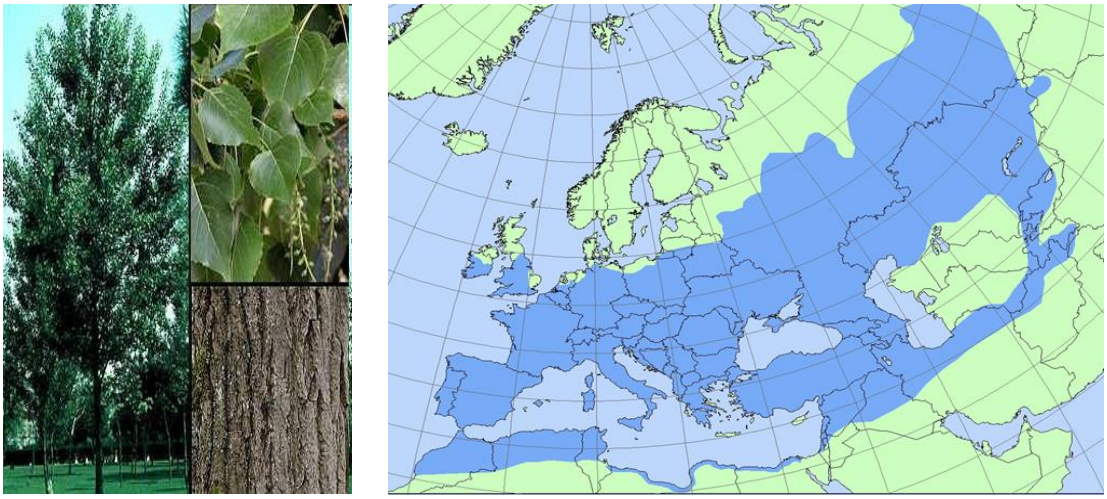


Figure 1.1. Phenotypic appearance of leaves, catkins and barks of *Populus nigra*. The map indicates natural distribution of the species (Broeck, 2003).

Furthermore, many gallery plantations established on the main river basins are under the control of governmental institutions. According to the latest studies, approximately 150,000 hectares of poplar plantation are standing in Turkey. Black poplar is comprised 47 % (70,000 ha) of these plantations supplying 57% of the annual poplar wood production, which is about 3.5 million m^3 (Işık and Toplu, 2004). Black poplar wood has been used as round wood for furniture, packaging, particleboard, plywood and matches industries (Toplu, 2005). Therefore, black poplar has an important contribution to both rural and national economy. In addition to its economic importance, black poplar has also played important roles in control of flooding and erosion in Turkey (Toplu, 2005).

1.2 Breeding Programme of Black Poplar in Turkey

Breeding and conservation programs of black poplar in Turkey were carried out under the framework of the European Forest Genetic Resources Program (EUFORGEN) for more than 40 years. In this framework, Poplar and Fast-Growing Forest Trees Research Institute initiated a countrywide conservation program including *in-situ* and *ex-situ* studies. *In situ* conservation studies have been carried out in the eastern Anatolia by including five natural populations of black poplar in the Melet, Kelkit, Munzur, Karasu and Pülümür rivers basins (Toplu 2005). With *ex situ* breeding and conservation programs of black poplar, effective efforts have been made to select 710 black poplar clones from natural populations and old plantations. Various clone trials have been established in different climatic regions of the country to assess growth performances and adaptabilities of these selected clones to different ecologic conditions. All these efforts have been resulted in development of five commercial black poplar clones (Gazi, Anadolu, Kocabey, Geyve and Behiçbey), which were registered by the International Poplar Commission. During the breeding of black poplar, major priority was given to selection of frost-resistant individuals (Toplu, 2005). However, black poplar is mostly grown in arid and semi-arid environments and is exposed to long drought periods. Therefore, adaptation of selected clones to water stress should be explored to increase productivity and survival of black poplar plantations.

1.3 Drought Stress and Its Effects on Plants

Drought or water-deficit is known to be the main abiotic stress factor reducing plant growth in all around the world. Climate models have predicted that destructive effects of this stress will become more severe and frequent on the agricultural and forest species due to the long-term effects of global warming. This situation indicated an urgent need to develop adaptive plant species for more prolonged drought conditions (Hamanishi and Campbell, 2011). Different from the agricultural species, trees are long-lived sessile organisms and are always compelled to prolonged drought stress over their lifetimes. Therefore, they have evolved many drought adaptation strategies which should be explored to understand the drought tolerance mechanisms of the trees (Wilkins et al., 2009; Anjum et al., 2010).

1.3.1 Plant Drought Stress: Effects, Responses and Mechanisms

The effects of drought stress on plants could be divided into three categories as: mechanical, metabolic, and oxidative effects. The mechanical effects of drought on plants observed when the soil water content decreased to a critical level. At that point, water leaves the plant cells initiating a decrease in cell volume, which is called as plasmolysis. In this condition, plasma membrane withdraws from the cell wall, remaining attached only at the plasmodesmata. The collapse of plasma membrane may cause induction of hydrolytic enzymes that are responsible for cellular autolysis of cytoplasm (Farooq et al., 2009). The metabolic effects of drought generally occur in the plasmolysis level. Water is a vital element for cellular metabolism, playing crucial role as a solvent for the cellular reactions and processes. The absence of the water in plant cells results in ion accumulation and changes in pH, inhibiting interaction of the proteins, disrupting membranes, enzyme inhibition and protein denaturation (Chaves et al., 2003). The first response of the plants to drought is stomatal closure to limit water loss. However, this closure also leads to a decrease in CO_2 uptake required for photosynthesis. These events result in a decrease in quantum efficiency and an excess of excitation energy at the reaction centres of the photosynthetic machinery. In this situation, $NADP^+$ becomes limited and ferredoxin selectively reduces oxygen instead of water, which causes formation of reactive oxygen species (ROS). These types of oxygen species are highly reactive that may lead to lipid peroxidation, fatty acid saturation, membrane damage, protein denaturation and nucleic acid degradation (Reddy et al., 2004; Sharma et al., 2012).Drought is also responsible for reduction in photosynthesis and growth on plants. As mentioned above when drought is sensed by plants, stomatas are closed to prevent water loss. This mechanism depends on Abscisic acid (ABA) activity which is synthesized in roots and transported to the guard cells of stomatas to initiate drought depended closure (Kim et al., 2010). At the mild drought level, this mechanism could be beneficial to keep turgor pressure and to prevent water loss. However, under prolonged drought conditions this situation, leads to carbon starvation and energy deficit in the plant cells. This drought induced energy deficit leads to autolysis of cellular sugar sources, proteins and lipids, which causes cell death, leaf senescence and reduction in growth eventually.

1.3.2 Adaptive Strategies of Plants against Drought Stress

Physiological responses of plants to drought could be divided into three adaptive strategies as: drought evading, avoidance and tolerance (Kozlowski and Pallardy, 2002; Blum, 2005). Evading strategy in plants simply relies on completing of their life cycle before physiological water deficits occur. In this mechanism, active portion of life generally takes place mostly during water abundant periods (Kozlowski and Pallardy, 2002). Therefore, the most important biomass production takes place in well-watered period. Plants that have this strategy immediately shed their leaves to enter into the dormancy period under drought stress. Therefore, drought evading plants can cope with only mild drought stress and are known as drought sensitive genotypes.

On the other hand, drought avoidance strategy depends on plants ability to maintain high leaf water status by limiting the water usage and transpiration under drought stress conditions (Touchette et al., 2007). The plants that have this strategy reduce stomatal conductance as a first response to limit water transpiration during drought stress. This response results in constant leaf water potential regardless of drought intensity. The high leaf water potential in the leaves of a plant under stress conditions generally associated with promotion in water homeostasis. It has been reported that to maintain this homeostasis, drought-avoided plants selectively shed the older leaves under water deficit to retain turgor and assimilation in the younger leaves (Blum and Arkin 1984). Blum (2005) reported that plants designed for this type of constitutive moderation of water use could not attain high yield potential. Therefore, these types of plants are characterized with having reduced plant size and leaf area, which are required for reduction in transpiration under drought stress.

Drought tolerance strategy is characterized with increases in tolerance with less reduction in above-ground growth in contrast to decrease in leaf water potential (Kramer and Boyer, 1995). Under drought stress, leaf abscission is very limited in these plants that it can be accepted as 'non-senescent' compared to other two adaptation strategies. Many reports provided evidence on the association between high rates of osmotic adjustment and drought tolerance strategy (Rood et al., 2000; Blum, 2005; McDowell et al., 2008). Osmotic adjustment comprises accumulation of solutes in plant cell vacuoles in response to drought. This accumulation causes an increase in osmotic potential of the plant cells, which initiate attraction of water into the cells to maintain turgor pressure. It is known that osmotic adjustment keeps growth of plants under drought stress conditions while the plant compensates transpiration demand by reducing its leaf water content (Kramer and Boyer, 1995; Blum, 2005).

1.3.3. Drought Stress Inducible Genes

Many molecular and microarray studies indicated that the drought inducible genes can be categorized into two classes as early (within seconds or minutes) and late responses (in hours, days or even weeks) (Huang et al., 2008; Parent et al., 2009). Early responsive genes are mostly related with recognition of the stress to provide initial protection, whereas later responding genes are involved in adaptation to prolonged drought stress conditions.

Loss of water from plant cell triggers many cellular signal transduction pathways responsible in drought tolerance. After recognition of drought by roots, the first response of plants to drought is known to be synthesizing of absisic acid (ABA) in the roots and transferring it to leaves to induce a range of stress adaptation responses including stomatal closure. In addition to this hormonal activation, many ABA independent drought-inducible genes are found to be responsible in signal transduction cascades between the initial signal of drought stress and the expression of specific genes (Yin et al., 2005; Xu et al., 2010).

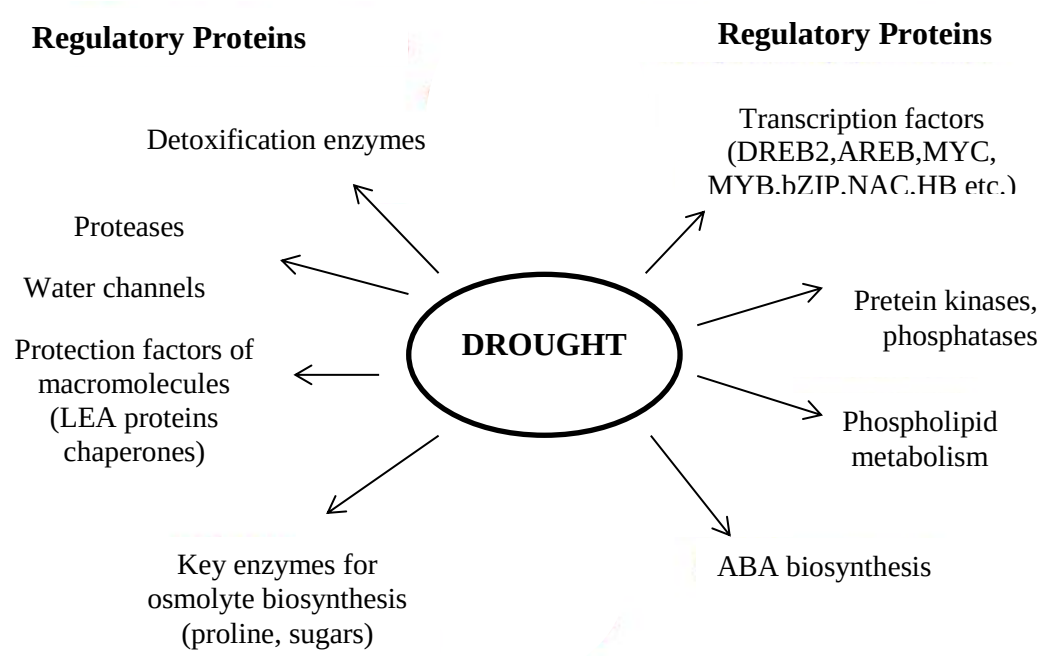


Figure 1. 2 Functional and regulatory drought stress-inducible genes in plant stress tolerance and response (Ramanjulu and Bartels, 2002).

Genes induced during drought stress are classified into two groups (Figure 1.2). The first group includes functional proteins that are probably involved in stress tolerance: water channel proteins, detoxification enzymes, osmoprotectants (sugars, proline, and glycine-betaine) and proteins that may protect macromolecules and membranes (late embryogenesis abundant (LEA) proteins, chaperons). The second group contains regulatory proteins which are involved in further regulation of signal transduction and gene expression that probably function in stress response: protein kinases, transcription factors and phospholipase C (PLC) (Ramanjulu and Bartels., 2002; Chaves et al., 2003; Bray, 2004).

1.4 Genomic Approaches to Reveal Drought Tolerance and Sensitivity

As stated in the previous section, plants modify their gene expression arrangements to adopt water limitations. With stress perception, a cascade of events including signal perception leads to changes in gene expression and protection responses at the cellular level. These transcriptional fluctuations cause either successful adaptations leading to tolerance or failure to adapt to the new environment, leading to sensitivity. However, improvement of tolerance to environmental stresses remained limited due to lack of information about complexity of stress signalling, adaptation processes and signal transduction for many plant species. Therefore, measurement of gene expression under a stress condition can explore many cellular processes, pathways, mechanisms and stress responses all of which can be used to select and to develop tolerant plant species.

Complete genome sequence information of some model plant species have yielded many tools to perform high-throughput, genome-wide screening of gene function. *A.thaliana* was the first plant species whose entire genome was sequenced. This information allowed application of many new technologies to analyse of many transcripts, proteins and metabolites of plant cells under various stress conditions. These advances have offered the opportunity to explore more complete set of plant genes, mechanisms and responses that can be integrated to create tolerance to abiotic stresses such as drought. *A.thaliana* is a small flowering plant with relatively short life cycle. Therefore, its genomic structure was not adequate to understand woody plants, which are long-lived sessile organisms that always compelled to withstand rapidly changing environments over their lifetimes (Wilkins et al., 2009; Anjum et al., 2010). Fortunately, after sequencing of the entire genome of *P. trichocarpa* in 2006, it is now possible to understand the complexity of stress response of trees on a large scale through genome-wide expression profiling using microarrays.

In many parts of the world, drought started to be severe problem due to the unpredictable changes in weather conditions, which affect productivity and survival of forest trees. Therefore, investigation of the gene expression changes during severe drought conditions is essential for understanding of acclimation and tolerance mechanisms in trees. With the full genome sequence of *P. trichocarpa*, many studies applied transcriptional comparisons on poplar genotypes to examine molecular networks underlying complex nature of drought response in recent years (Street et al., 2006; Bogeat-Triboulot et al., 2007; Caruso et al., 2008; Wilkins et al., 2009; Cohen et al., 2010; Hamanishi et al., 2010; Yang et al., 2010). In these studies, different poplar species exposed to different water limitation methods and different tissues, developmental stages and platforms were selected to identify differential expression of the drought related genes in poplar species. However, the combination of drought related physiological, morphological or biochemical responses with gene expression profiling was not studied before. Furthermore, comparative transcriptional studies between resistant and sensitive poplar clones have not been performed on the response of poplar plants to prolonged drought levels and post drought recovery periods yet.

1.5 Aims of the Study

In this study, the first aim was to present an analysis of controlled drought stress and its effect on the physiology, morphology and biochemical responses of black poplar clones. For this purpose, investigation of relationships between antioxidant enzyme activities with physiological and morphological traits under successive drought and re-watering cycles were firstly intended. Then it was planned to identify the most resistant and sensitive black poplar genotypes according to these relationships. The major aim of this study was to perform microarray based gene profiling on the leaves of these genotypes to select the genes that likely contribute to the intra-specific variation of drought response in black poplar. The comparison of these two types of genotypes will reveal not only reliable drought markers, but also the divergences and similarities in transcriptional networks, highlighting candidate genes for future diversity screening in black poplar.

CHAPTER 2

PHYSIOLOGICAL, MORPHOLOGICAL AND BIOCHEMICAL RESPONSES OF *Populus nigra* CLONES TO DROUGHT STRESS AND POST-DROUGHT RECOVERY

2.1. INTRODUCTION

Black poplar (*Populus nigra* L.) is native to Anatolia and has a wide distribution in Turkey with its three subspecies *nigra, caudina* and *usbekistanica* (Stanton, 2009). Because of its wide geographic adaptation, fast growth ability and short rotation age, black poplar has been widely planted in rural areas of the country by the farmers. Its wood has been used as a construction material for many years. This species have been also used as an investment tool by the farmers in Turkey. With the birth of a baby, a poplar plantation is established in Anatolian culture and when the baby comes to age of ten, this plantation is cut and the money obtained from the wood is used for the child's future. These types of small poplar plantations have compensated important part of wood demand in Turkey. According to the latest studies, approximately 150,000 hectares of poplar plantations are standing in Turkey. Black poplar is comprised 47 % (70,000 ha) of the total poplar plantations, supplying 57% of the annual poplar wood production, which is about 3,5 million m^3 (Işık and Toplu, 2004). Black poplar wood has been used for furniture, packaging, particleboard, plywood and matches industries, thus making important contribution to national economy (Toplu, 2005).

Due to its importance in Turkey, *ex situ* breeding and conservation programs of black poplar were initiated in 1960s. With the program, effective efforts have been made to select 710 black poplar clones from natural populations and old plantations. Although, major priority was given to frost-resistance during the selection process (Toplu, 2005), black poplar clone-collection enabled selection of the clones for almost any environmental conditions. Various clone trials were established in different climatic regions of the country to assess growth performance and adaptability of clones under different ecologic conditions. All these efforts have been resulted in development of five commercial black poplar clones (Gazi, Anadolu, Kocabey, Geyve and Behiçbey) which were registered by the International Poplar Commission.

Black poplar plantations are mostly established in arid and semi-arid environments and are exposed to long drought periods with high water deficits in Turkey. Thus, investigation of adaptation strategies of black poplar clones to drought stress is essential to develop drought tolerant black poplar clones for maintain productivity of these plantations. Additionally, climate models predicts that destructive effects of drought stress will become more severe and frequent especially on forest species (Hamanishi and Campbell, 2011). This situation also indicates an

urgent needs to develop adaptive black poplar clones that can cope with more prolonged drought conditions.

Drought is one of the most important environmental limitation affecting photosynthesis, growth and survival of plants and involves in many sets of morphological, biochemical and molecular responses. The first response of plants to drought stress is known to be synthesis of the absisic acid (ABA), which induce a range of stress adaptation responses including stomatal closure (Apel and Hirt, 2004). As the drought stress severity increases, the stomatal closure lasts longer, which reduce CO_2 uptake and leads to decrease in net photosynthesis (Lei, 2008). Down regulation of especially photosystem II activity results in disruption on the generation and utilization of electrons, revealing potentially dangerous molecules known as reactive active oxygen species (ROS). These molecules attack many biological molecules resulting in DNA nicking, protein oxidation and lipid peroxidation in plant cells (Reddy et al., 2004). Although a series of regulatory mechanisms have evolved within the plant cell to limit the production of these toxic molecules, oxidative damage is a potential problem for plants especially under drought stress. Therefore, plants adopted many non-enzymatic (flavanones, anthocyanins, carotenoids and ascorbic acid (AA) and enzymatic [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and dehydroascorbate reductase (DHAR)] defence systems to keep the levels of ROS under control. In the plant defence systems most important role has generally attributed to the ascorbate–glutathione cycle antioxidant enzyme system which was comprised of SOD, APX, MDHAR, DHAR, and GR enzymes as shown in Figure 2.1 (Gill and Tuteja, 2010).

Drought dependent changes of these antioxidant enzymes in many poplars species except black poplar are well-documented in the literature. Up to now, researches generally dealt with comparing small number of clones or species to observe changes in these enzyme activities in different drought stress levels (Regier et al 2009; Guo et al., 2010; Anjum et al., 2010). Activity of those enzymes was found to be increased in all poplar species under drought stress conditions. However, there is no published information dealing with the changes of these enzymes in post drought recovery periods. Furthermore, there are no studies investigating the importance of ascorbate–glutathione enzymes associated to drought tolerance and/or susceptibility, and not much is known about the usage of those enzymes as a selection marker in plant-breeding programs.

In the current study, drought related changes in the physiological traits and antioxidant enzyme activities of black poplar clones were investigated to improve our understanding of their adaptive mechanisms to drought. These traits can be used in breeding, which aim to combine high productivity with enhanced drought tolerance. Therefore, the main objective of this work was to explore the relationship between antioxidant enzyme activities and physiological traits under successive drought and re-watering cycles. By this way, we aimed to understand impact of drought stress on black poplar and to find out adaptation strategies of the species that can be used in the breeding programmes.

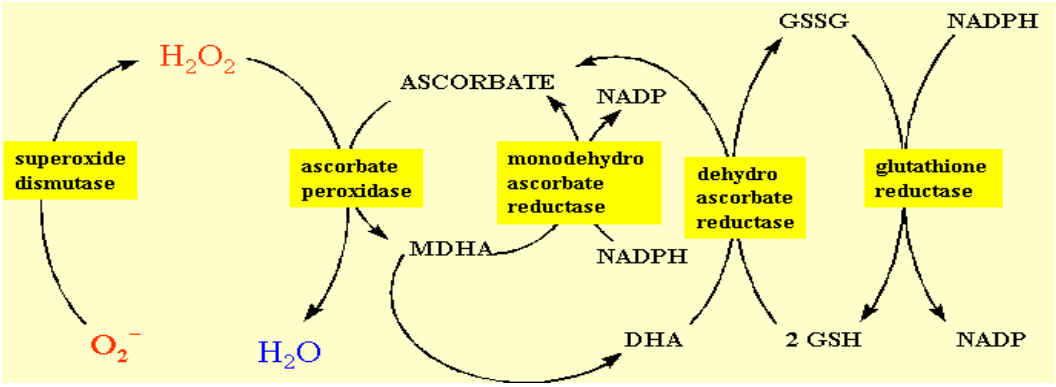


Figure 2.1 The redox cycling of ascorbate referred to as Halliwell-Asada pathway or Ascorbate-Glutathione Cycle (Asada, 1999).

2.2. MATERIALS AND METHODS

2.2.1. Field Trial and Selection of Clones Exhibiting Different Drought Response

As mentioned in the introduction part, during the breeding studies 710 black poplar clones were collected from all around Turkey and transferred to the nurseries found on different climatic regions of the country. The effects of drought on the black poplar clones and their adaptation strategies to this stress factor were firstly assessed in a field trial established with 300 black poplar clones collected from different parts of Turkey. During the tests of the field trial, growth performances and some other morphological traits such as leaf abscission and terminal shoot desiccation were recorded in the growing seasons of 2010 and 2011 including rainy (well-watered) and drought periods. The results of the field trial revealed significant genotypic variability, which facilitate identification of three genotypic forms in terms of clonal response to drought stress. The first genotypic form included N.62.191 and Gazi clones that exhibited the highest growth rate at the end of each growing season and lowest leaf abscission during the drought period. For the second genotype, we selected two clones named as N.03.368.A and N.91.083 which were found to have a high growth rate in rainy periods, whereas almost complete leaf abscission followed by subsequent necrosis and loss of especially the terminal shoots under drought conditions. Another form of drought adaptation was observed in the clones that showed the least growth performance in the field trial (N.03.368.1 and N.90.010). These black poplar clones started leaf abscission before drought period but they keep their leaves on their branches and continued growing throughout the drought season. By this way, we identified three different genotypes that could be classified as drought resistant (N.62.191 and Gazi), drought sensitive (N.03.368.A and N.91.083) and moderate drought resistant (N.03.368.1 and N.90.010).

2.2.2. Green House Trial and Leaf Sample Collection

For the present study, above mentioned six black poplar clones exhibiting different drought response in the field trial were selected and transferred to a greenhouse to apply drought stress treatment in much controlled environment and to carry out further physiological and biochemical analysis. For this purpose, fifteen-centimetre cuttings (60 cuttings/ clone) of the clones were firstly planted in 5 L plastic bags in 15 March 2012. After sprouting and growing for about 2 months, the best grown 20 seedlings per clone were selected and replanted into 20 L plastic pots filled with 3.5 kg homogenized soil (1 seedling per pot). The soil was a clay loam (pH: 7.8) with organic carbon and total nitrogen content of 3.2 and 1.4 mg g^{-1}, respectively. The plants were moved to a semi-controlled greenhouse at the Biology department of Middle East Technical University (METU/Ankara/Turkey). The day temperature of the greenhouse ranged between 25–31 °C with a night temperature range of 15–23 °C, and the relative humidity was measured between 50–65% from 16 March to1 August 2012. An overview about the experiment was represented in the figure 2.2.

After transferring the seedlings into pots, all the plants were subjected to a well-watered period (WWP) by weighing the pots every day and re-watering up to field capacity by replacing the amount of water transpired. Soil water content at field capacity was around 75% of a pot. This well watered period was accepted as control level in the experiment and carried out for three weeks. Progressive drought treatment started on 5 June 2012 by dividing each clone in to two groups: randomly selected 10 pots per clone was used for drought stress and the remaining 10 pots allocated as control by keeping their soil water content at field capacity. To enable acclimation of the clones to the drought stress, the selected seedlings (10 pots / clone) were subjected to gradual controlled water depletion. When the treatment was started, the pots were weighted every day and allowed transpire until the soil water content (SWC) decreased down to 50% (D1) of a pot. Then the pots were kept at that SWC (50%) for one week. The same procedure was applied when the SWC decreased down to, 35% (D2), 15% (D3) and 5% (D4). After maximum level of water stress (5% SWC) was reached, drought treated seedlings were re-watered again up to field capacity to understand post-drought recovery of the clones after a drought period.

2.2.3. Leaf Sample Collection, Measurement of Growth and Leaf Water Status

To determine various physiological and biochemical traits, the fully expanded leaves of the clones located on sixth or seventh nodes from the apex of stems were harvested for all different watering regimes mentioned above. All the collected samples were frozen immediately in liquid nitrogen and kept at -80^0C until use. Plant height and stem diameter of all the clones were measured weekly throughout the experiment. Drought dependent leaf abscision was another

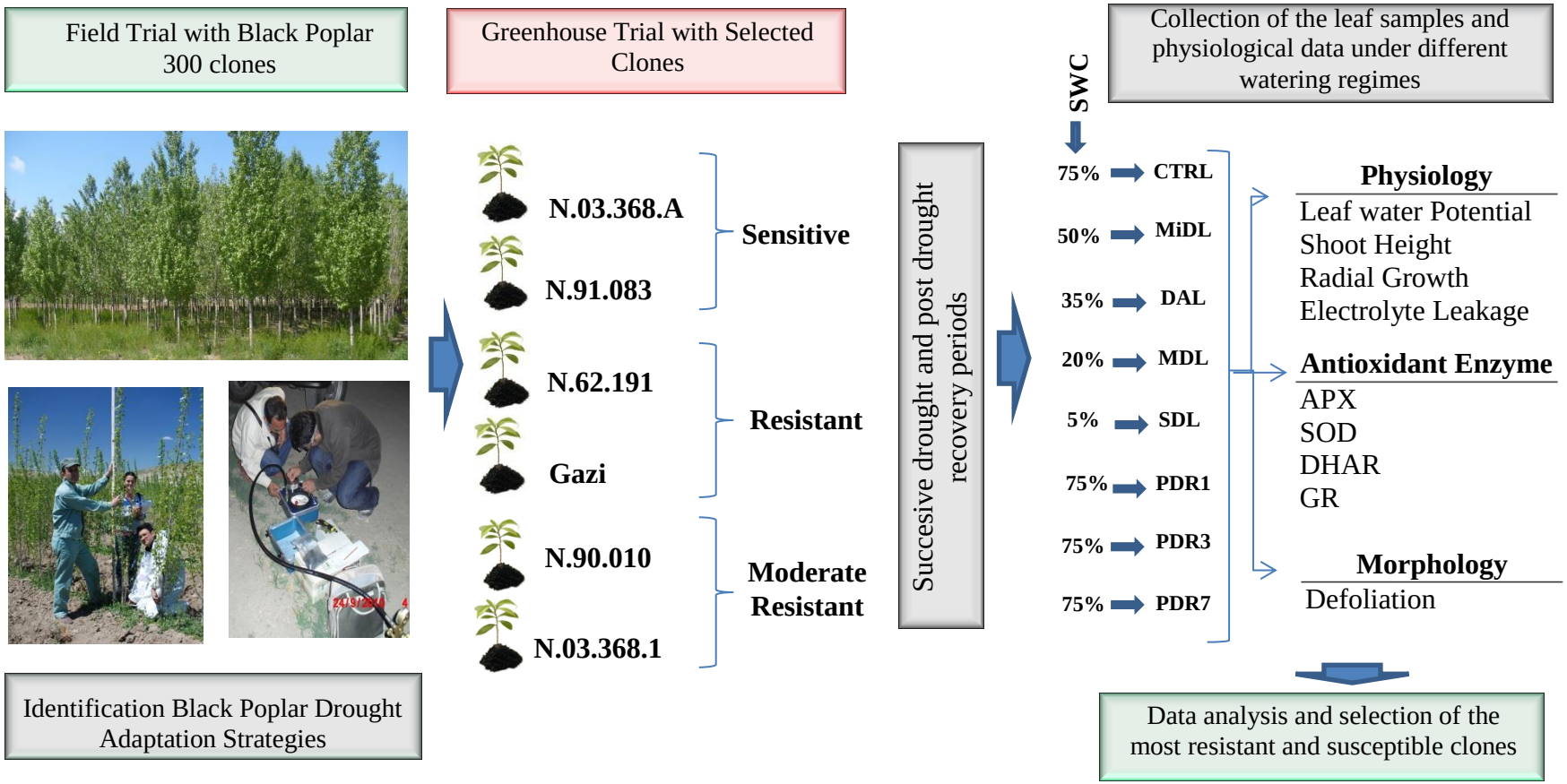


Figure 2.2 Overview of experimental strategies in the study presenting identification and testing of black poplar drought adaptation strategies. Six black poplar clones transferred from field to greenhouse trial according to their drought response. Each two clone represented one response as: Sensitive, Resistant and Moderate drought resistant. Successive drought were applied in 5 different soil water content (SWC) which are named as: well watered (CTRL), Mild drought level (MiDL), Drought Acclimation Level (DAL), Moderate drought level (MDL), severe drought level (SDL) and post drought recovery (PDR) periods.

important measurement and was observed weekly by counting the remaining leaves on the plants. Randomly selected five drought treated and five control seedlings of each clone were used to estimate predawn leaf water potential (LWP) for every three days throughout the experiment. The measurements of pre-dawn LWP were carried out at mid-nights with three fully expanded leaves collected from median segment of stem. Sholander pressure chamber (PMS Instrument Co., Corvallis, OR, USA) was used to measure LWP.

2.2.4. Measurement of Membrane Leakage

Membrane damage was estimated by measuring of electrolyte leakage (EL) from the leaf cells according to the method of Nanjo et al. (1999). EL estimation was done with the fully expanded leaves collected at midday. Then, the leaves were put into separate 150 ml falcon tubes containing 50 ml of 0.4 M mannitol and kept at room temperature with gentle shaking for 3 h. The electrical conductance were measured and recorded by using Mettler Toledo MPC 227 conductivity meter as *C1* (initial conductivity). Then the tubes containing the samples were put into boiling water for 10 minutes. After cooling the tubes down to room temperature, the second electrical conductance was measured and recorded as *C2* (total conductivity). The conductivity due to electrolyte leakage was expressed as the percentage of the initial conductivity over the total conductivity *[(C1/C2)** 100].

2.2.5. Antioxidant Enzyme Activity Measurements

All the leaf samples collected from the stress treated clones were grounded into powder with liquid nitrogen by using mortar and pestle. The powder was dispersed into several pre-cooled tubes and stored at -80 ^{o}C until use. Approximately 100 mg frozen leaf powder were homogenized with 600 µl 50 mm potassium phosphate buffer (pH 7.8) containing 2 mm ethylenediaminetetraacetic acid (EDTA), 2% polyvinylpyrrolidine (PVP-40) and 0.5% Triton X-100. The cell debris was removed by centrifugation at 15000 *g* for 20 min at 4 °C. The supernatant obtained from this procedure was used to measure all the enzyme activities. In order to define activity as nanomole of substrate consumed or product formed per minute per milligram of protein, the protein content was determined by the Bradford method (Bradford, 1976) using a commercial protein assay kit (*Thermo* Fisher Scientific, *Germany*).

Ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) activity procedures used in this study were taken from Murshed et al., (2008) and superoxide dismutase (SOD) activity was measured according to Giannopolitis and Reis (1977). All enzyme assays were performed with the 96 well UV- micro-plate at 25 ^{o}C by using Epoch Microplate Spectrophotometer (BioTek,France) equipped with an internal temperature incubator

for kinetic analysis. Samples were analysed in triplicate. Blank corrections for non-enzymatic reduction of the enzymes were carried out in the absence of the enzyme sample.

SOD activity was measured by its capability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in assay mixture (185µL) consisted of 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA solutions. The assay mixture was placed in each well of a 96-well plate and 10 µl enzyme containing total protein isolate were added to the wells. At last 1mM riboflavin (5µl) was added to each well. The reaction mixture was mixed by pipetting and placed under the light source illuminating 5000Lx for 15 minutes. The absorbance at 560 nm was determined by microplate spectrophotometer. In addition, a reaction mixture was placed in dark and used as a control. One unit of enzyme activity (U) was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction.

APX activity was measured by following the method adapted from Murshed et al., (2008). The reaction buffer (185 µl/well) consisting of 50 mM potassium phosphate buffer (pH 7.0) and 0.25 mM ascorbate (AsA) was dispensed into all microplate wells. Then 5µl of sample supernatant was placed in each microplate well by pipetting. The APX reaction was started by the addition of 5 µl of 200 mM H_2O_2 into wells by using an 8-channel pipette. The decrease in ascorbate concentration was recorded at 290 nm for 10 min at 25 C^o. Specific activity was calculated by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

The GSH-dependent **DHAR activity** was assayed by measuring the increase in the absorbance at 265 nm for 10 min. The DHAR reaction was initiated by addition of 5 µl 8 mM DHA (freshly prepared) into a final concentration of 0.25 ml solution, containing 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.1mM EDTA and 10 µl sample supernatant. Specific activity was calculated by using an extinction coefficient of 14 mM^{-1} cm^{-1}.

The **GR activity** was measured as NADPH oxidation at 340 nm in a reaction solution containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.25 mM NADPH and 10 µl enzyme containing sample supernatant. The GR reaction was started by the addition of 5µl of 20 mM glutathione disulphide (GSSG) to each well containing 185µl reaction solution. The specific activity was calculated by using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

The **H_2O_2 content** was estimated according to Brent and Bergmeyer (1974). About 250 µl supernatant obtained from the extraction step was added into 1 ml peroxidase reagent including 100 mM phosphate buffer (pH 7.0), 0.005 % (w/v) o-dionizidine and 40 µg/ml peroxidase. The solution was incubated at 30 ^{0}C for 10 min in a water bath and the reaction was stopped by the addition of 1N perchloric acid. Then the mixture was centrifuged at 5000 g for 5 min. Then the absorbance at 436nm was recorded. The content of H_2O_2 was estimated by using a peroxide standard.

2.2.6. Statistical Analysis

Throughout the experiment, data were regularly collected from six black poplar clones and used to identify the drought responses of black poplar to different soil water depletions. Statistical analyses of all the measured traits were done with the average values in the resistant (N.62.191/ Gazi), the moderate resistant (N.90.110/N.03.368.1) and the sensitive (N.03.368.A/N.91.083) genotypes by using Minitab (Minitab Release 13.1, Minitab, State College, PA) statistical package program. Two-way ANOVA was applied in order to evaluate the effect of different soil water depletion and different genotypes for each measured traits. In addition, 95% confidence intervals and the mean of each trait were provided in Appendix A to make comparison between genotypes and treatments.

2.3. RESULTS

2.3.1. Classification of Drought Stress Levels

The six black poplar clones exhibiting three different drought responses were investigated in the current study. Measurements, observations and leaf collections were done regularly when the SWC decreased from 75% (control:C) to 50% (D1), 35% (D2), 15% (D3) and 5%(D4) during the course of drought experiment. The same data and leaf collections were repeated in the first (PDR1), third (PDR3) and seventh (PDR7) day of the post-drought recovery period (PDR). At harvest D1, the growth parameters of all the clones were found to be negatively affected by the water depletion. Although the pots lost only 15% of the water in D1 period, 24% and 40% reductions in shoot and radial growth were recorded, respectively (Appendix A). The LWP and EL vales were not significantly different ($p<0.05$) in D1 level compared to well watered period. The APX, DHAR and GR activities indicated slight increase in D1 drought condition. Therefore, this period was defined as mild drought level (MiDL). On the other hand, the measured traits in D2 level were not significantly different from those at the D1 level. This could be explained by a drought acclimation level (DAL) of the plants to drought stress at D2 level. Most significant differences in all measured physiological and enzyme characters were observed at the D3 level. Furthermore, the clones were firstly differentiated morphologically at this level. Senescence symptoms such as yellowing and shedding of the leaves in especially sensitive clones were observed at D3 level indicating a moderate effect of drought on the plants (MDL). At D4, plants experienced very severe stress and shoot and diameter growth were stopped almost completely, and highest reduction in LWP was recorded at this level. This severe drought level (SDL) caused a limited leaf desiccation in the resistant genotypes, but it did a complete defoliation followed by subsequent necrosis in the seedlings of the sensitive genotypes. In the post-drought recovery (PDR) phase, there were clear clonal differences with respect to recovery ability. The resistant genotypes almost completely recovered themselves at the end of the three weeks of re-watering, whereas the effects of drought stress on especially the sensitive ones continued.

2.3.2. The Effects of Drought and Post-Drought Recovery on the Physiology of Black Poplar Clones:

2.3.2.1. Growth Performance of the Clones

In the field trial, the major differences concerning drought response between selected clones were related to the growth parameters. In the field test, at the end of three years the best height and diameter performances were observed in the resistant clones (N.62.191 and Gazi) during well watered and drought periods. On the other hand, sensitive clones such as N.03.368.A and N.91.083 exhibited high growth performances under well watered period, but great majority of ramets of these clones defoliated completely and lost their terminal shoots in drought periods. The moderate resistant N.03.368.1 and N.90.010 clones were the least grown individuals in the field trial during all periods. However, shoot desiccation or severe leaf defoliation was not observed in these clones under drought periods.

Similar growth performances of these clones were also observed in the greenhouse trial. In well watered period (WWP), the best growth performance was exhibited by the sensitive clone; N.03.368 (30 cm/week). Throughout the drought treatment general trend in growth parameters were very similar in all three genotypes (Figure 2.3). As it can be seen from Figure 2.3 and Appendix A, shoot elongation (height) and radial growth (diameter) of all the clones were significantly decreased when the plants were exposed to drought stress. The first response of the clones to drought was a sharp decline at MiDL and then a stable growth for one week with the acclimation of the clones to drought stress at DAL. However, when the drought stress was increased further, radial and shoot growths in all clones significantly reduced at MDL and stopped completely at SDL. Only in sensitive clones, terminal shoots of six ramets were desiccated under severe drought condition. The most discriminative drought response in terms of growth was recorded in the post-drought recovery period. At the end of the third re-watered week, the resistant clones recovered almost completely by increasing their growth performances compared to their control level (Figure 2.3). On the contrary, the ramets of the sensitive and the moderate resistant clones could not restore their growth throughout the recovery period.

2.3.2.2. Leaf Desiccation

The selected black poplar clones manifested contrasting leaf abscission responses under drought stress. The older leaves of the sensitive genotypes desiccated suddenly in response to drought with a reduction in soil water content from 75% to 35% (MDL). At the severe drought level all the leaves of these sensitive genotypes were almost completely defoliated (Figure 2.4/S). Until PDR period, just a few leaves were remained on the stems of the sensitive clones. All of these drought treated leaves could not recovered themselves and shedded in the re-watering period.

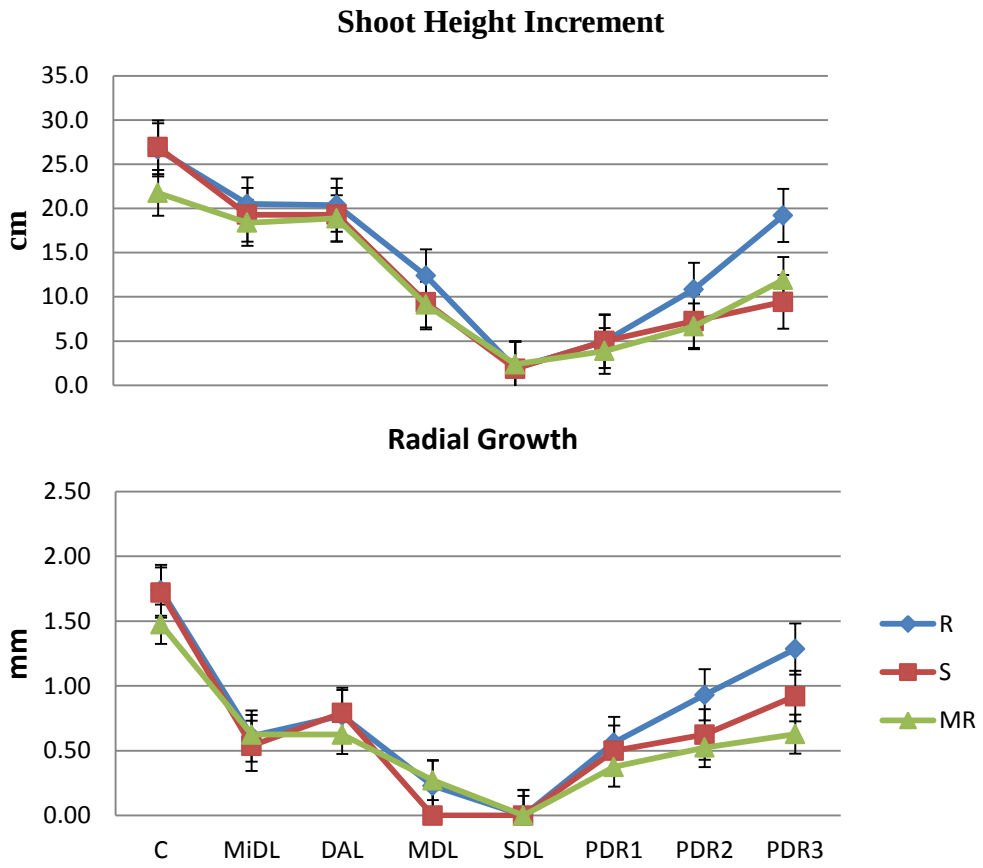
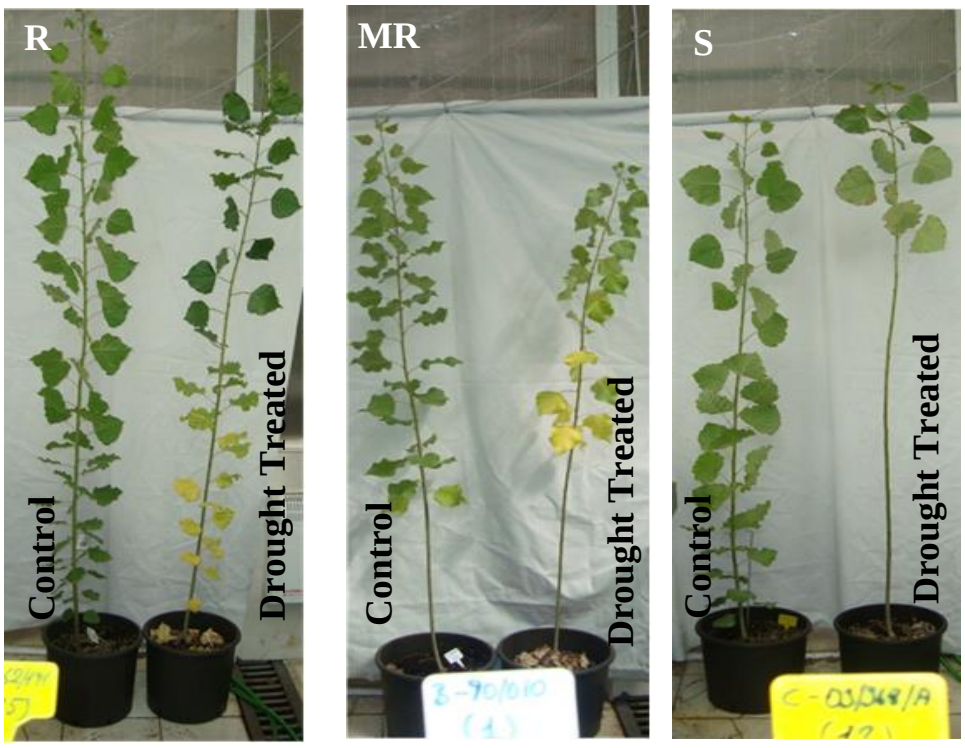


Figure 2.3 Shoot height and radial growth increments under different watering regimes in black poplar clones exhibiting three different drought response; Resistant (R): Sensitive (S) and Moderate resistant (MR).

Contrary to sensitive clones, leaves of the resistant clones formed only senescence symptoms such as yellowing and necrotic lesions but not exhibited severe leaf shedding after exposure to severe drought level. Drought dependent leaf abscission rate of the resistant genotypes could be considered as non-senescent as compared to the sensitive clones. Some of these flavescent leaves of the resistant genotypes recovered themselves and turned into green colour in the PDR period indicating recovery capacity of these clones after drought. In terms of leaf morphology, the moderate resistant clones possessed the smallest leaf area among the other two genotypes. Interestingly, leaf shedding of these moderate resistant clones started at the beginning of drought stress. The older leaves located on the lower parts of the stems were selectively shedded in both well watered and drought stress condition. Although defoliation rate increased especially under severe drought conditions, total leaf area of the moderate resistant clones was not significantly reduced as compared to their control ramets (Figure 2.4/MR). From this point of view, it could be argued that leaf shedding may serve as an important indicator of drought response in black poplar.



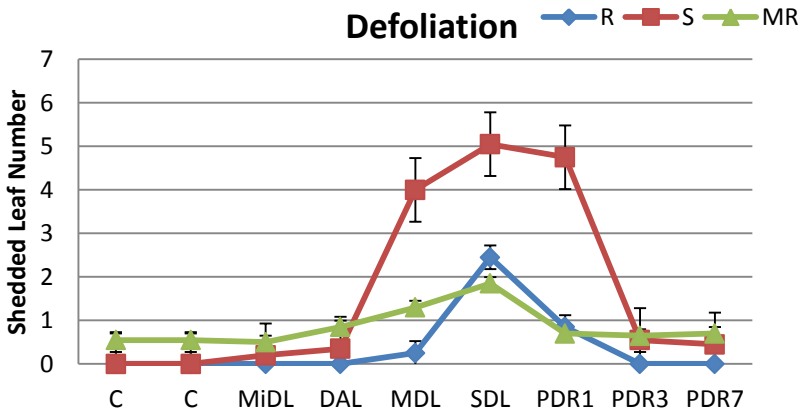


Figure 2.4 Upper figure indicates drought induced defoliation at severe drought level and the growth performances of three black poplar genotypes. The graph indicates the defoliation rate of the genotypes at different drought level and post drought recovery periods. Resistant (R): N.62.191, Sensitive (S) N.03.368.A and Moderate resistant (MR): N.90.010

2.3.2.3. Predawn leaf water potential (LWP)

Another important indicator of drought response among black poplar clones was pre-down leaf water potential (LWP). For the sensitive clones, LWP started to decrease in MiDL (Figure 2.5) and reached its maximum value at SDL (-12± 0.9 bars). On the other hand, the same parameter was not significantly decreased until the MDL for the other two genotypes. At SDL, LWP of the resistant genotype was also significantly decreased to -8 ±0.7 bars. The highest LWP values was recorded in the moderate resistant clones (-4 ± 0.6 bars) in the SDL, indicating highest leaf water content under drought among the studied genotypes. In the PDR period, LWP of all the clones were increased up to their control values.

2.3.2.4. Electrolyte Leakage (EL)

In the current study, electrolyte leakage (EL) was used to estimate cell membrane stability as an indicator of membrane senescence. The results in our research indicated that like in other measurements, electrolyte leakage in the leaves of the sensitive clones was much higher than it was found in other two clones throughout the stress treatment. The first statistically significant difference in EL was observed at MiDL and reached its maximum value (10%) at the severe drought conditions in the sensitive clones (Figure 2.5). For the other two clones, the significant difference was observed at MDL, but their maximum rate was two folds smaller than the sensitive one. The most important finding in this trait was recorded in post-drought recovery period. When the pots were re-watered, the resistant and the moderate resistant clones had electrolyte leakage values below the control level, indicating a recovery of the cell membrane in drought treated leaves of these genotypes. On the other hand, the electrolyte leakage was found to be higher in the sensitive genotypes compared to its control level at the PRD periods. This situation could be attributed to the on-going effects of drought on the sensitive clones even under re-watering periods.

2.3.3. Antioxidant Enzymes Activities in Black Poplar Clones under Different Watering Regimes

In the current study, the total SOD activity (Figure 2.6) exhibited highly different expression among the clones. Although other enzyme activities showed significant increase in MiDL, SOD activity remained at the control level in all clones until the soil water content (SWC) decreased to 35 % (DAL). The highest SOD activity for all the clones was observed in SDL. When the pots were re-watered in the experiment, SOD activity level decreased to control level at the 7th day of the post recovery period. In terms of clonal differences, the highest SOD activity in all watering regimes was observed in the sensitive clones (Figure 2.6).

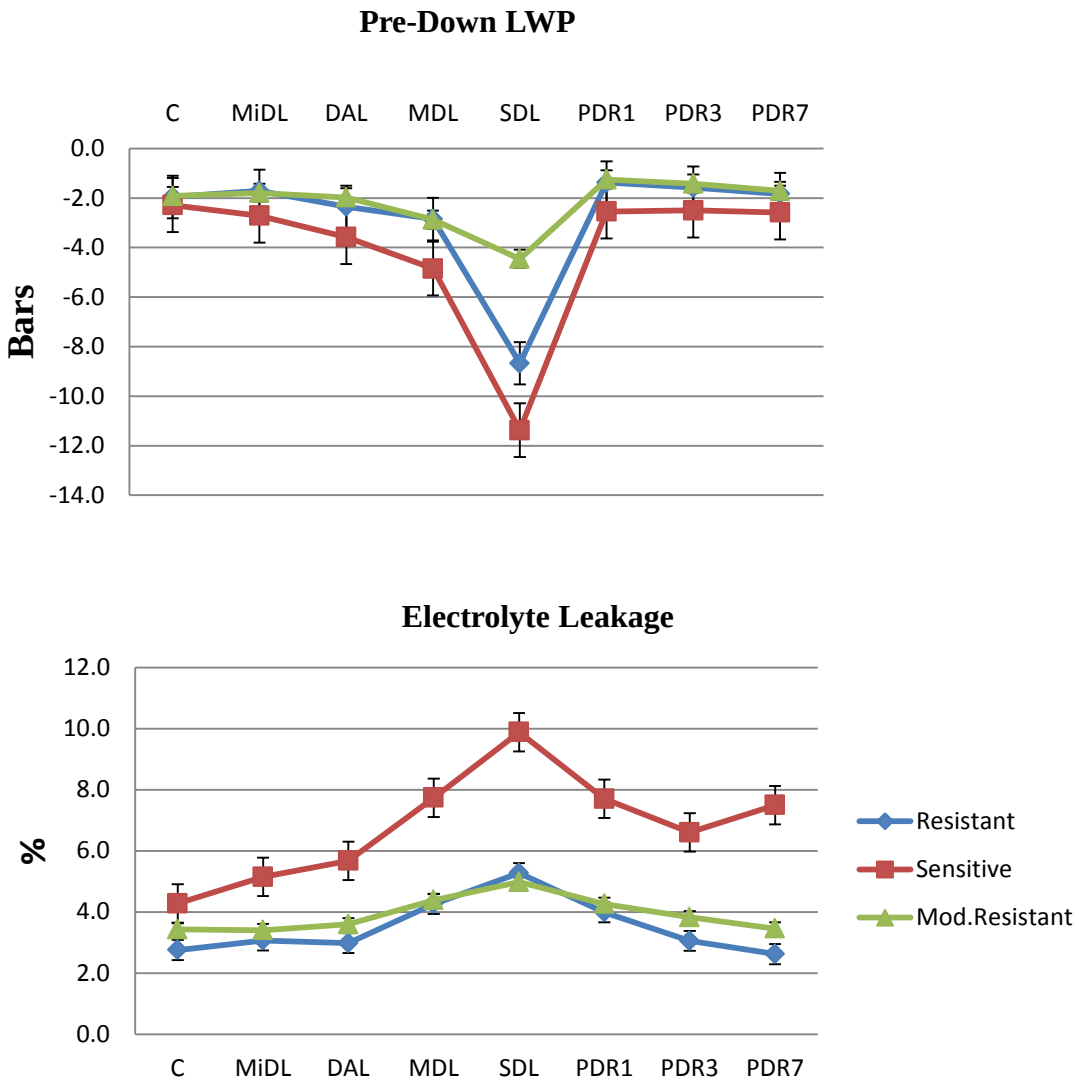


Figure 2.5 Pre-down leaf water potential and electrolyte leakage measurements of drought treated black poplar genotypes. (Green=MR: moderate drought resistant, Red=S:sensitive, Blue=R:Resistant. C: Well watered, MiDL: Mild Drought, DAL: Drought Acclimation SDL: Severe drought, PDR:Post drought recovery)

As shown in Figure 2.1, the basic activity of the SOD enzyme is to catalyse superoxide dismutation into less harmful molecules such as hydrogen peroxide (H_2O_2). By this way, it removes the risk of free radical formation. Therefore, H_2O_2 formation could be used as an indicator of stress level in the black poplar clones. In the experiment, H_2O_2 level indicated parallel changes with SOD activity in all watering regimes. As presented in figure 2.6, the H_2O_2 level exhibited statistically insignificant decrease in MiDL, but then started to increase until MDL. During the severe drought level, the H_2O_2 level decreased to control level in all the clones during the post drought recovery period. Regarding the clonal differences, parallel to the increase in SOD activity, the sensitive clones had the highest H_2O_2 production in drought stress period (Figure 2.6). Our results showed that the progressive loss of water from the leaf tissues caused an increase of H_2O_2 until MDL, whereas with abrupt increase in APX at the subsequent drought level (SDL) H_2O_2 content decreased (Figure 2.6).

In the current study, SOD activities and H_2O_2 changes throughout the stress treatment followed very compatible changes with APX activities which is responsible in converting H_2O_2 into water. Drought stress at MiDL caused a significant increase in APX activity in the leaves of all the clones. However, with acclimation of the clones to the drought stress condition, this increase was not apparent until SDL. The highest APX activities for all tested clones were recorded at SDL. During the re-watering period, APX activity decreased to control level in the all clones except the sensitive ones. Clonal comparison in terms of APX activity indicated that sensitive clones had much higher APX activity than the other two groups

Dehydroascorbate reductase (DHAR) catalyzes the reduction of dehydroascorbate to ascorbate and, thus, plays an important role in maintaining APX activity and ROS control. The first response of DHAR activity was an insignificant increase at beginning of the water deficit (MiDL). However, the increase in DHAR activity was not significant until the SDL. At this drought condition, DHAR activity reached its maximum level in all clones (Figure 2.7). Different than the other antioxidant enzyme activities DHAR activity was found to be higher in the resistant genotype in all watering regimes.

Glutathione reductase is the last enzyme in the ascorbate-glutathione cycle that catalyses the reduction of oxidized glutathione (GSSG) to its reduced form (GSH). The GR activity showed very similar trend with DHAR activity during the drought- stress treatments in the experiment. Although some fluctuations were observed among the clones during the treatment, the highest GR activity was recorded at SDL for all investigated clones (Figure 2.7). Changes in GR activity were found to be very similar in all genotypes. Therefore, in terms of clonal differences, GR activity was the least discriminative enzyme among the clones.

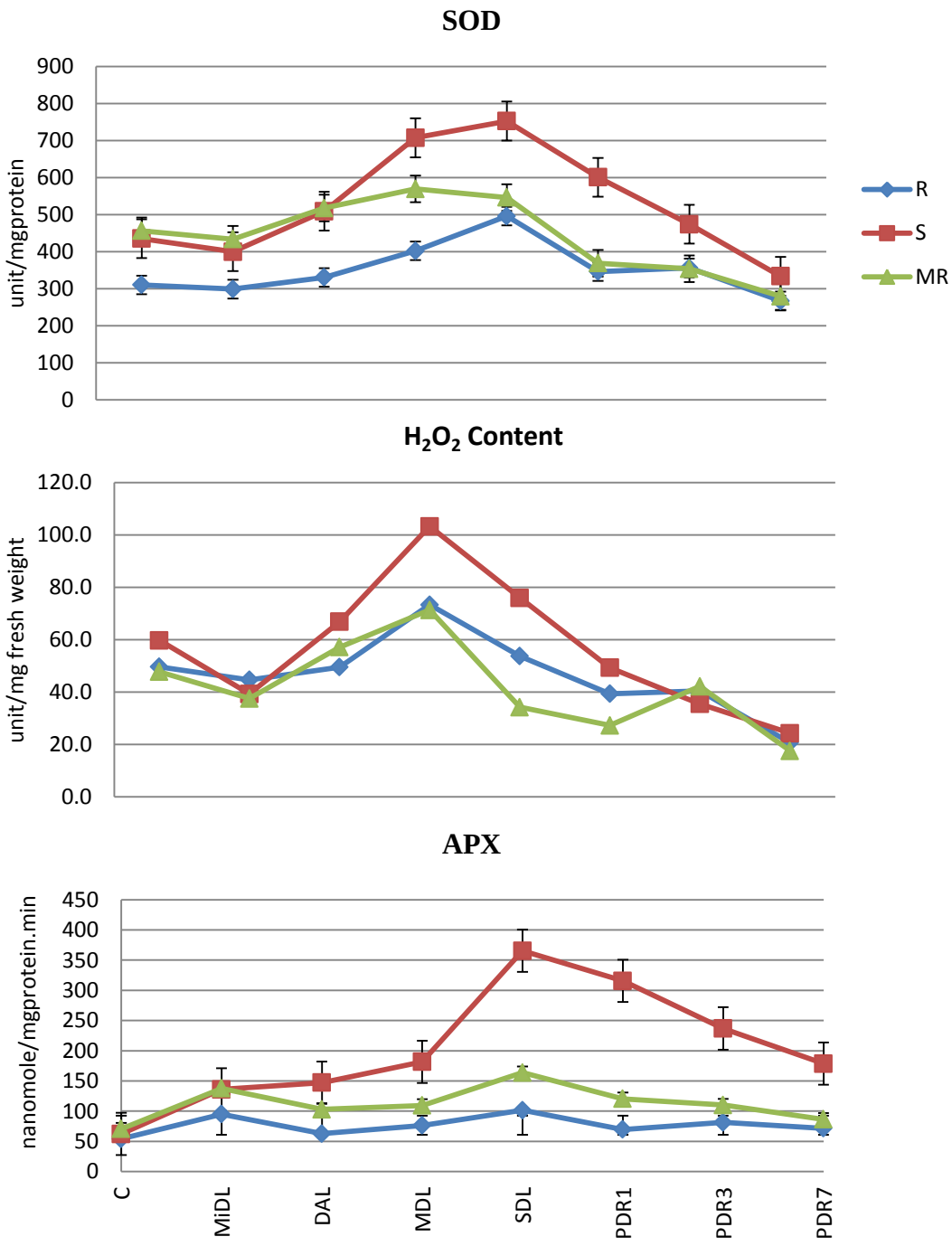


Figure 2.6 Changes in the SOD and APX antioxidant enzyme activities of black poplar genotypes throughout the drought treatment. Drought dependent H_2O_2 alteration was also represented. (Green=MR: moderate drought resistant, Red=S: Sensitive, Blue=R: Resistant. C: Well watered, MiDL: Mild Drought, DAL: Drought acclimation SDL: Severe drought, PDR: Post drought recovery)

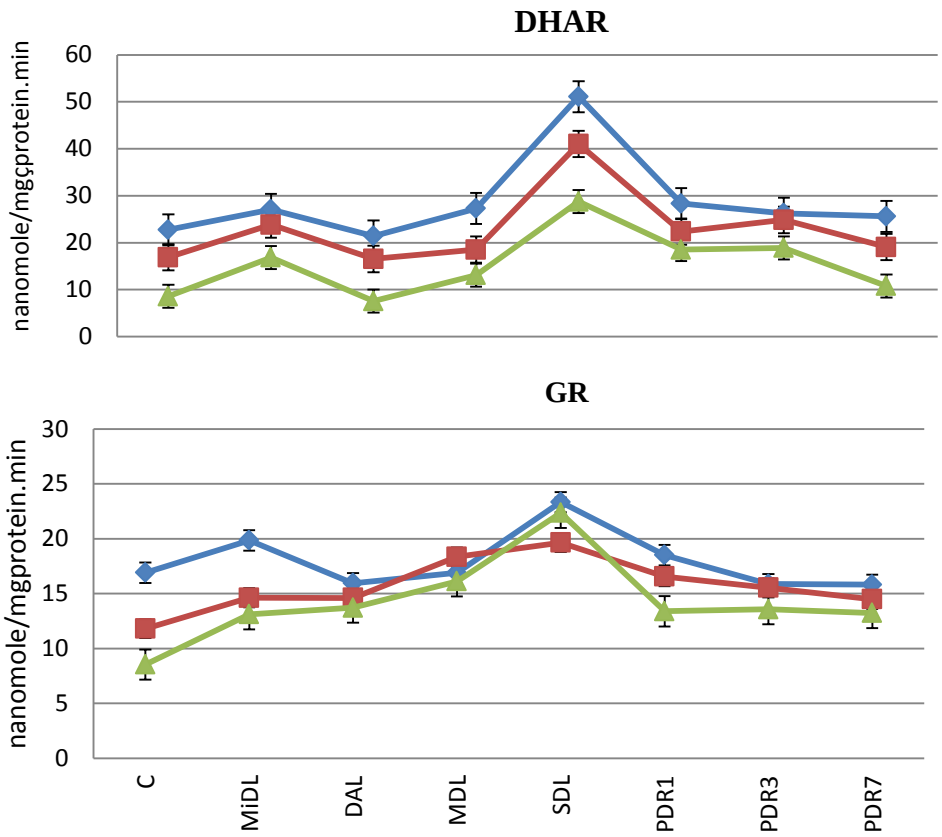


Figure 2.7 Changes in DHAR and GR antioxidant enzyme activities in the black poplar genotypes throughout the treatment (Green=MR: moderate drought resistant, Red=S: Sensitive, Blue=R: Resistant. C: Well watered, MiDL: Mild Drought, DAL: Drought acclimation SDL: Severe drought, PDR: Post drought recovery)

2.4. DISCUSSION

2.4.1. Physiological Analysis of Drought Adaptation Strategies in Black Poplar

In the current study, we investigated a number of drought related physio-biochemical processes at different stages of water availability to understand the adaptation mechanisms of black poplar species. Therefore, a well-watered, successive drought and post drought recovery periods were applied to three different black poplar genotypes which were identified according to their drought response in the field trial. The results of greenhouse trial confirmed the findings of the field test that black poplar clones gathered from all around Turkey evolved three contrasting adaptation strategies for survival and growth under limited water availability.

Among the investigated clones, the sensitive ones (N.03.368.A and N.91.083) in the current study were found to evolved a **drought evading adaptation strategy** (drought sensitive). In this strategy, active portion of plant life generally takes place mostly during water abundant periods (Kozlowski and Pallardy, 2002). Therefore, the most important biomass production takes place in well-watered period. The results of the current study corresponded well with this property of drought-evading strategy that two sensitive black poplar clones (N.03.368.A and N.91.083) exhibited the highest shoot elongation and radial growth under well watered conditions among the other genotypes. Fast growth of drought sensitive poplar genotypes under well watered period were also reported in previous studies (Tschaplinski et al., 1998; Yin et al., 2005; Regier et al., 2009; Yang et al., 2010). On the other hand, the growth rate of these sensitive clones declined more than four folds at MDL and stopped almost completely at the SDL. This dramatic effect of drought stress was also observed in post-drought recovery period that the growth rate of these clones could not reach their control level at the end of three weeks of re-watering period. Leaf shedding is known as a drought evading strategy to reduce leaf surface area and consequently the overall transpiration rate of a tree. (Kozlowski and Pallardy, 2002; Zapater et al., 2012; Jansson et al., 2010). In this study, the highest and the earliest leaf defoliations rates were observed at moderate drought level in only sensitive clones (Figure 2.4). At the severe drought conditions, the leaves of these sensitive clones shedded completely. Only a few leaves remained on the stems until post-drought recovery period, whereas these leaves could not recover themselves and shedded in the re-watering period. Physiological functions of the leaves, especially during drought period, mostly depend on cell membrane stability, which is crucial in sustaining of cell turgor pressure (Li et al., 2012). The results in our research indicated that EL increased more rapidly in the leaves of the sensitive clones than the resistant ones. During re-watering period, the EL values of the sensitive clones did not decreased to control level, which indicated ongoing membrane senescence in this clone. Prolonged reductions in leaf water potentials have been previously reported to result in xylem cavitation and embolism which cause subsequent hydraulic failure and branch sacrifice in many poplar species (Rood et al., 2000; Jansson et al., 2010; Klein et al.,2011). Among the investigated clones the highest reduction in leaf water potential was recorded in N.03.368.A (-13 ± 1.1 bars) clone under severe drought

conditions. The clone N.03.368.A exhibited an extreme form of stress sensitivity that terminal shoots of ramets of this clone desiccated at the severe drought level. The branch sacrificing was also observed in the ramets of the same clone in the field trial.

Among the investigated clones, two of them coded as N.03.368.1 and N.90.010 indicated physiological properties of **dehydration avoidance strategy (moderate resistant)**, which is defined as a plants' ability to maintain high leaf water status under the effect of drought conditions (Kozlowski and Pallardy, 2002; Touchette et al 2007). The strategy mostly relies on limiting the water usage and transpiration under drought stress (Klein et al 2012, Blum, 2005). The plants that have this strategy reduce stomal conductance as a first response to limit water transpiration during drought stress. This response results in constant leaf water potential regardless of drought intensity and reduction of efficient water usage (Rood et al., 2000; Blum, 2005; McDowell et al., 2008). Although we did not measure the stomatal conductance of the clones in the experiment, the highest pre-down leaf water potential (-4 ± 0.6 bars) during the severe drought stress indicated that N.03.368.1 and N.90.010 clones could have dehydration avoidance strategy. The high leaf water content in the leaves of a plant under stress conditions generally associated with promotion in water homeostasis. It has been reported that to maintain this homeostasis in dehydration avoided plants, older leaves are selectively killed under stress while the remaining young leaves retain high turgor (high LWP) (Blum and Arkin, 1984). In the present study, the leaves located at the lower parts of stem of N.03.368.1 and N.90.010 clones started to defoliate at the beginning of the drought stress treatment, which could be considered as preparation to drought period. However, the rate of defoliation was not increased during the drought period (Figure 2.4) and remained almost constant compared to control ramets of these drought avoided clones. Blum (2005) reported that plants designed for this type of constitutive moderation of water use could not attain high yield potential. Therefore, these types of plants are characterized with reduced plant size and leaf area, which are also useful in reduction of transpiration under drought stress (Kozlowski and Pallardy, 2002; Blum, 2005). The results of our experiment were also corresponded with these suggestions that these two clones had the least growth performances both in field and greenhouse trial (Appendix A, Figure 2.3). Li et al (2012) compared the EL of two white clover genotypes that had different leaf area. The results of the same study revealed that small-leafed genotypes maintained better membrane stability than the large-leafed ones. As stated earlier, during severe drought stress, the levels of EL increased significantly for all clones, but the EL of the N.03.368.1 and N.90.010 clones (small-leafed) was significantly lower than the drought evading clones (large-leafed).

As stated above, dehydration avoided plants reduced above-ground productivity due to the decrease in water use efficiency. On the other hand, **dehydration tolerance (resistant)** is characterized with increases in drought tolerance with less reduction in above-ground growth in contrast to decrease in leaf water potential (Kramer and Boyer, 1995). In this respect, it is possible to conclude that the clones coded as N.62.191 and Gazi in this study could have dehydration tolerance mechanism to cope with drought stress. The growth rate of both clones exhibited the highest performance in the field and greenhouse trials in all watering regimes

(Appendix A). Although, water potential of these resistant (-8 ± 0.7 bars) clones found to be low as compared to drought avoided clones, leaf abscission was very limited in these clones that it can be accepted as 'non-senescent' compared to other two adaptations. Only flavescent leaves were observed at the severe stress level, which were recovered themselves in PDR and turned into green. Although reduction in LWP in these tolerant genotypes was close to drought evaded clones , electrolyte leakage results of tolerant clones revealed strong membrane stability under severe drought conditions. Despite high drought tolerance, lower leaf water potential was generally associated with osmotic adjustment in many reports (Kramer and Boyer, 1995; Babu et al, 1999; Blum, 2005). Osmotic adjustment comprises accumulation of solutes in the plant cell vacuoles in response to drought. This accumulation results in reduction of osmotic potential of the plant cells, which initiate attraction of water into the cells and maintain turgor pressure. This mechanism sustains higher leaf water content in the cells at low LWP. Although we did not measure the accumulation of one of these types of solutes, high drought tolerance with lower LWP may depend on osmotic adjustment in N.62.191 and Gazi clones.

2.4.2. Biochemical analysis of drought adaptations of black poplar clones

One of the most important effects of drought on plants is known to be increased levels of ROS (Wang et al., 2003; Sofo et al., 2005; Anjum et al., 2010). It has also been reported that increase in ROS, such as H_2O_2 level, generate signals in the cell to trigger the defence response during the drought stress. The up-regulation of antioxidant enzymes found in the ascorbate–glutathione cycle is the first response in the plant cell to scavenge the excess of ROS (Asada, 1999). This increased response in antioxidant level against drought stress was well documented for many poplar species (Yang et al., 2009; Regier et al., 2009, Guo et al., 2010; Anjum et al., 2010). Therefore, in the current study, the effects of water deficit on black poplar clones were also explored at the antioxidant enzyme activity level. Our results showed that the activities of antioxidant enzymes involved in the ascorbate–glutathione cycle increased as the severity of drought stress increased. During the post-drought recovery period for almost all clones, ascorbate–glutathione system was down regulated to control levels in re-watered plants which is likely due to a reduced need for ROS removal.

Although there was similar antioxidant enzyme regulation in response to drought in all studied black poplar clones, the degree of enzyme activity levels significantly differed among them. Our results showed that the progressive loss of water from leaf tissues caused an increase of H_2O_2 until MDL, whereas at the subsequent point (SDL) with abrupt increase in APX, the level of H_2O_2 started to decrease (Figure 2.6). The highest values of H_2O_2 content were recorded for the drought evading (sensitive) clones during the drought treatment (Figure 2.6). As stated in the previous section, these were the most adversely affected clones in the drought treatment in terms of growth, leaf abscission and electrolyte leakages which are highly related with increased level of ROS in the plants. Therefore, increase in the H_2O_2 level could be meaningful for these clones because H_2O_2 level mostly depend on existence of ROS and conversion of these ROS into H_2O_2

by SOD (Asada, 1999; Sharma et al., 2012). Investigation of SOD activity along the drought treatment demonstrated this clear linkage between H_2O_2 and SOD enzyme activity that the highest increase of SOD activity was also observed in drought- evaded (sensitive) clones in all watering regimes (Figure 2.6). In fact, increased activity of SOD is often correlated with increased tolerance of the plant against environmental stresses. Even, it was suggested that SOD could be used as a selection criterion for screening of drought-resistant plants (Sharma et al., 2012; Regier et al., 2009; Yang et al., 2010). However, the high expression of SOD in the drought sensitive black poplars in the current study could be explained by the increased need of ROS removal from the leaves of these clones. Under drought conditions, activity of APX also increased to a greater extent in the drought- evaded clones than in dehydration-tolerant or avoided clones. The APX has a central function in ascorbate-glutathione cycle and a crucial role in the management of ROS during many stressful conditions. The basic activity of the APX is to reduce H_2O_2 into water (Sharma et al., 2012). In the current study, APX activities exhibited very compatible variations with SOD and H_2O_2 changes throughout the stress treatment. The rate of APX increase for the sensitive black poplar genotypes was three folds higher than it was in the other clones. This seemed to be matched with the suggestion of increased need of ROS removal in leaves of the drought sensitive genotypes. Although, GR and DHAR activity levels were also higher in drought-avoided clones, clonal discrimination capacity of these enzymes was less than the APX and SOD.

Interestingly increase in the antioxidant enzyme activity is always correlated with the drought tolerance in many poplar species in the literature (Guerrier et al., 2000; Edjolo et al., 2001, Lei et al., 2006). However, our results suggested that the relationship between antioxidant enzyme activities and drought tolerance exhibited a reverse situation in black poplar. Relatively lower antioxidant enzyme activity in the resistant black poplar clones could be associated with the high plant water status or osmotic adjustment. Drought tolerance is generally defined as the plant capacity to maintain high plant water status or cellular hydration under the effect of drought. Hence, by this mechanism the plant avoids being stressed because plant functions are relatively unexposed to tissue dehydration (Kramer and Boyer 1995; Kozlowski and Pallardy, 2002; Blum, 2005). In the current study the highest LWP values were recorded in dehydration avoided and tolerant clones in severe drought level. Therefore, clones that have these adaptation strategies may not need to increase their antioxidant enzyme activities due to the low level of ROS in the leaf tissues.

As it was discussed previously, the sensitive genotypes seem to have a drought evading strategy which is characterized with almost complete leaf abscission to decrease water transpiration and to pass drought period in dormant state. On the other hand, leaf abscission was limited in dehydration avoided and tolerant genotypes as compared to drought evaded clones. Therefore, highest increase in H_2O_2 level and antioxidant enzyme activities could be also related with leaf abscission signalling. Sakamoto et al, (2008) concluded that environmental stresses such as drought could be associated with excessive production of ROS in the leaf cells. Subsequent activation of abscission signalling by these ROS molecules may be a general mechanism in

stress-induced leaf abscission. Before a leaf is shed, the plant should translocate most of the leaf's nutrients so that they are not wasted. Therefore, before complete remobilization of these nutrients, plants leaf should control production and destructive effects of ROSs with increased activity of antioxidants to prevent cell from premature death and delayed senescence (Woo et al., 2004). Drought depended increase in activities of SOD and APX enzymes in the sensitive genotype could be related with delayed senescence for nutrient transfer from leaves to other sink organs.

2.4.3. Usage of Drought Adaptation Strategies For Wood Production

In recent years, considerable progresses have been made in conservation and breeding of black poplar in Turkey. Breeding studies accomplished by collection of best individuals from natural range of the black poplar species by selection of clones based on their adaptability and productivity in various test sites. The results of drought treatment both in field and greenhouse trial indicated that black poplar clones could be grouped in three adaptation strategies as drought evading, dehydration avoidance and dehydration tolerance for survival and growth under drought stress.

In the collection, a few clones were found to have drought evading adaptations. These clones can withstand only mild drought conditions. However, this strategy contributes to fast growth rates if there is available water resources. Therefore, these types of clones are very suitable for gallery plantations (Toplu, 2005). When the total length of the river basin (100 000 km) of Turkey is considered, gallery plantations that are established with these types of clones would be very beneficial in terms of wood production.

The clones with dehydration tolerance strategy combine high productivity with drought tolerance. These types of clones regarding to their growth performances comprised upper 10% of all clones in the black poplar clone bank. They adapted to conditions where drought periods are prolonged. Therefore, the clones that selected from this group could be used in industrial plantations for wood and biomass production in especially arid and semi-arid zones of Turkey. Among the five commercial black poplar clones in Turkey, four of them (Gazi , Kocabey, Geyve, Behiçbey) included in this group. However, there are many clones in the collection that the productivity and drought tolerance properties are much better than these commercial ones.

The last adaptation strategy in black poplar collection was dehydration avoidance mechanism, which is associated with high capacity for drought tolerance, lower growth rates. The great majority of the black poplar collection (70%) fell into this group. Due to their slow growth rates, the usage of these types of clones in the wood production plantations is not recommended. However, breeding studies require high genetic diversity to select the best individuals against other type of biotic and abiotic environmental stresses. Therefore, these drought avoided clones should be kept in black poplar collection to increase genetic diversity for future breeding programmes.

CHAPTER 3

MICROARRAY BASED GENE EXPRESSION PROFILING OF TWO CONTRASTING *Populus nigra* CLONES UNDER WELL WATERED, DROUGHT AND POST DROUGHT RECOVERY PERIODS

3.1. INTRODUCTION

Drought is one of the most important environmental fluctuations affecting productivity and survival of plant species including trees. It was reported that alterations in global climate and increase in drought periods strongly influenced forest distribution and survival in recent years (Hamanishi and Campbell, 2011). Trees are long-lived sessile organisms and are always compelled to withstand rapidly changing environments over their lifetimes. Therefore, they evolved many physiological adaptation strategies against drought stress (Wilkins et al., 2009; Anjum et al., 2010). Identification of the genetic basis of these adaptations is crucial for understanding of the basic mechanisms in drought tolerance. In this respect, due to their wide geographic adaptation, poplar species are one of the most suitable plant species to investigate the genetic architecture of trees and their drought responses (Nanjo et al., 2004; Street et al., 2006). Especially after sequencing the whole genome of *Populus trichocarpa* (Tuskan et al., 2006), poplar was accepted as a model organism that offer opportunities to develop many genomic tools such as high density arrays or whole-genome microarrays which could be used for drought induced transcriptional remodelling (Taylor 2002). Several recent studies on poplar genotypes were conducted to examine the molecular networks underlying the complex nature of drought response (Street et al., 2006; Bogeat-Triboulot et al., 2007; Caruso et al., 2008; Wilkins et al., 2009; Cohen et al., 2010; Hamanishi et al., 2010; Yang et al., 2010). In these studies, different poplar species were exposed to different water limitations and different tissues, developmental stages and platforms were selected to identify differential expression of the drought related genes. However, comparative transcriptional studies between drought tolerant and sensitive poplar genotypes were not performed during advanced drought stress conditions and post-drought recovery periods yet. In many parts of the world, drought started to be a severe problem in many arid and semi-arid zones due to global warming, which affect productivity and survival of forest trees. Therefore, investigation of the gene expression changes especially during severe drought conditions is essential for identification of acclimation and tolerance mechanisms in trees.

For understanding of trees response to drought, we selected black poplar species (*Populus nigra* L.) due to its wide geographic adaptation and fast growth abilities. Furthermore, black poplar is

one of the most important commercially cultivated tree species in Turkey. Wood production from these black poplar plantations compensates important part of wood demand in the country (Yıldırım et al., 2011). Therefore, breeding studies for this species have been started and carried out for more than 40 years in Turkey (Toplu, 2005).

Selection of the drought resistant genotypes was one of the major objectives for the black poplar breeding programme. Therefore, we firstly carried out an analysis of controlled drought stress and its effect on the physiology, morphology and biochemical response in two black poplar genotypes which were found to have contrasting response to drought stress (sensitive and resistant). Then, a microarray based gene profiling on the leaves of these genotypes was conducted to select the genes that might likely contribute to intra-specific variation in drought response. Drought dependent transcriptional comparison of these two genotypes will reveal not only reliable drought markers, but also the divergences and similarities in transcriptional networks, highlighting candidate genes for future diversity screening in black poplar breeding studies.

3.2. MATERIALS AND METHODS

3.2.1. Plant Materials and Experimental Design

Due to its importance in Turkey, *ex situ* breeding and conservation programs of black poplar were initiated in 1960s. With the program, effective efforts have been made to select more than 700 black poplar clones from their natural populations and old plantations in Turkey. Black poplar clone collection enabled selection of the clones for almost any environmental conditions, including drought stress. The assessment of this black poplar collection in terms of drought stress was firstly made with a field trial established with 300 black poplar clones which were selected according to their natural habitats. During the tests of field trial, growth performances and some other morphological traits such as leaf abscission and terminal shoot desiccation were recorded in the growing seasons of 2010 and 2011 including rainy (well-watered) and drought periods. The results of the field trial revealed significant genetic variability, which facilitated identification of several genotypes according to their drought response. The first genotypic form included a clone named as N.62.191, which exhibited the highest growth rate at the end of each growing seasons and lowest leaf abscission during the drought periods. For the second genotype, we selected another clone named as N.03.368.A. This clone was found to have high growth rate in rainy periods, whereas almost complete leaf abscission on whole branches fallowed by subsequent necrosis and loss of especially terminal shoots under drought conditions. In the current study, N.62.191 and N.03.368.A were defined as drought resistant and sensitive genotypes, respectively (Figure 3.1).

Above mentioned two black poplar clones were transferred to the greenhouse to test their drought response and to carry out further physiological, biochemical and microarray based transcriptional comparisons. For this purpose, fifteen-centimeter cuttings (60 cuttings/ clone) of the clones were firstly planted in 5 L plastic bags in 15 March 2012. After sprouting and growing for about 2 months, the best grown 20 seedlings per clone were selected and replanted into 20 L plastic pots filled with 3.5 kg homogenized soil (1 seedling per pot). The soil was a clay loam (pH 7.8) with organic carbon and total nitrogen content of 3.2 and 1.4 mg g^{-1}, respectively. The plants were moved to a semi-controlled greenhouse at the Biology department of Middle East Technical University (Ankara/Turkey). The day temperature of the greenhouse ranged between 25–31 °C with a night temperature range of 15–23 °C. Relative humidity was measured between 50–65% throughout the experiment. An overview of the experimental design was represented in the Figure 3.1.

After transferring the seedlings into pots, all plants were subjected to a well-watered period (WWP) by weighting the pots every day and re-watering up to field capacity by replacing the amount of water transpired. Soil water content at well watered condition was around 75% of a pot. This well watered period was accepted as control level in the experiment and carried out for three weeks. Progressive drought treatment was started on 5 June 2012 by dividing each clone in two groups: randomly selected 10 pots per clone were used for drought stress and the remaining 10 pots were allocated as control by keeping soil water content at the field capacity. To enable acclimation of the plants to the drought stress, selected seedlings (10 pots / clone) were subjected to gradual controlled water depletion. Therefore, when the drought treatment was started, the pots were weighted and led to transpiration of the water until the soil water content (SWC) decreased down to 50% (D1) of a pot. Then the pots were kept at that SWC for one week. The same procedure was applied in subsequent weeks when the SWC decreased down to, 35% (D2), 15% (D3) and 5% (D4). After reaching the maximum level of water stress (5% SWC), drought-stressed plants were watered again up to field capacity to understand post-drought recovery of the clones after a drought period.

3.2.2. Physiological and Biochemical Measurements

To test the effects of drought stress on the selected clones various physiological and biochemical traits were intended to be measured in the study. Therefore, the fully expanded leaves from six or seven nodes from the apex of stems were harvested from all the plants at all different watering regimes mentioned above. In the experiment the leaves were collected two times in well watered period, then in the five different drought level and three post drought re-watering points. All the collected leaf samples were frozen immediately in liquid nitrogen and kept at -80^0C until use. Plant shoot increment and radial growth of all the plants were measured weekly throughout the experiment. Leaf defoliation was another important measurement that observed weekly by counting the leaves on the plants. Randomly selected five drought treated and five control

seedlings of each clone were used to estimate predawn leaf water potential (LWP) for every three days. The measurements of pre-dawn LWP were carried out at mid-nights with three fully expanded leaves collected from median segment of the stem. Sholander pressure chamber (PMS Instrument Co., Corvallis, OR, USA) was used to measure LWP. Membrane damage in the leaf cells of the clones was estimated by measuring of electrolytes leaked from the leaves according to the method of Nanjo et al. (1999).

One of the most important effects of drought on plant cells is production of reactive active oxygen species (ROS), which may cause DNA nicking, protein oxidation and lipid peroxidation (Reddy et al., 2004). Plants adopted enzymatic defence systems to keep the levels of ROS under control. Most important role in plant defence system was generally attributed to ascorbate–glutathione antioxidant enzyme cycle which includes ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) and superoxide dismutase (SOD) enzymes. Therefore, in the current study the effects of drought and biochemical responses were followed by measurements of these antioxidant enzyme activities on the collected leaves from both genotypes throughout the experiment. The antioxidant enzyme activities of APX, GR and DHAR were quantified as described in Murshed et al., (2008). SOD activity was measured according to Giannopolitis and Reis (1977). In addition to these antioxidant enzymes, hydrogen peroxide content in the collected leaves was also determined according to Brent and Bergmeyer (1974). All these assays were performed within a 96 well UV- micro-plate at 25 ^{o}C by using Epoch Microplate Spectrophotometer (BioTek,France) equipped with an internal temperature incubator for kinetic analysis.

3.2.3. Microarray Experimental Design

In this study, Affymetrix poplar GeneChips were used to assess genome-wide expression during different watering regimes of two black poplar clones. The GeneChips contains 61251 probe-sets representing more than 56000 transcripts derived from fully sequenced *P. trichocarpa* genome and the other 13 *Populus* species (Affymetrix). To follow drought dependent gene expression, the leaves collected for physiological and biochemical measurements throughout the experiment were also used for microarray analysis. As stated before, the leaves were collected at ten different watering regimes during the experiment. Among them, we identified four important points that drought have significant effect on the clones. The leaves collected in well-watered period (CRTL) was accepted as control point to compare transcriptional changes in moderate drought level (MDL), severe drought level (SDL) and post-drought recover period (PDR). Therefore, RNA isolation was conducted separately from the leaves that collected from the six ramets for each clone at these watering regimes. For each watering regime three GeneChips were used as technical replicates. Therefore, the total RNA isolated from the leaves of six seedlings were assigned into three biological replicates each of which included equal total RNA of the same two ramets. By this way, totally 24 Affymetrix poplar GeneChips (2 genotypes × 4 treatments × 3 replicates) were used for transcriptional analysis (Figure 3.1)

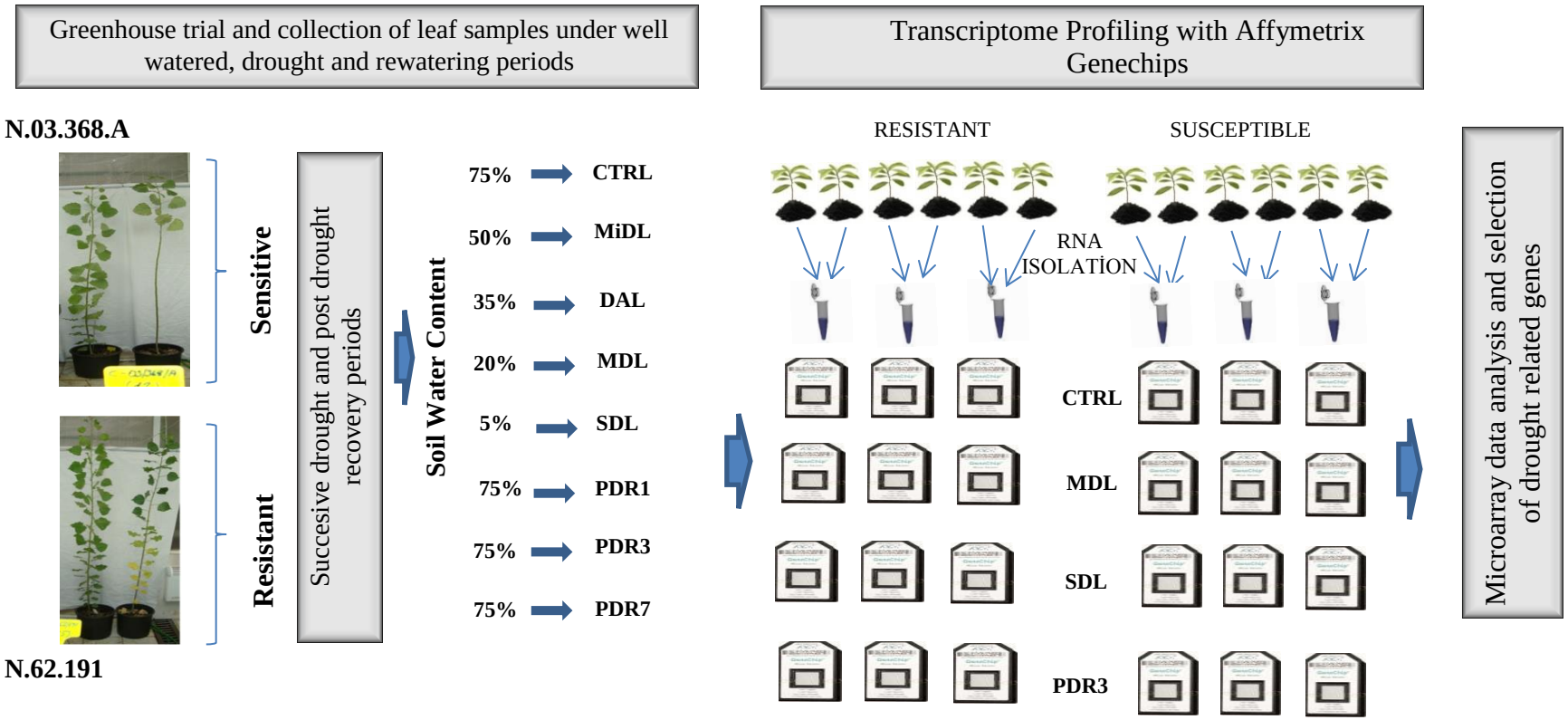


Figure 3.1 Overview of experimental strategies and microarray design in this study. Two black poplar clones transferred from field to greenhouse trial according to their drought response. Six different watering regimes were applied to the clones based on the soil water content of the pots. These watering regimes were well watered period (CTRL), mild drought level (MiDL), drought acclimation level (DAL), moderate drought level (MDL), severe drought level (SDL) and post drought recovery (PDR) periods. Microarray experiment conducted with 24 Affymetrix GeneChips at four different watering regimes (CTRL,MDL,SDL,PDR3) for two genotypes.

3.2.3.1. RNA Extraction

RNA extraction was performed according to Chang et al. (1993) with minor modifications as described in Le-Provost et al (2007). Before isolation, RNAse decontamination of all equipment was achieved by immersion them into diethylpyrocarbonate (*DEPC*) treated water. All the solutions used in RNA isolation was also prepared with DEPC-treated and autoclaved water. The leaves were firstly grounded into a fine powder with liquid nitrogen by using mortar and pestle. For RNA isolation, previously heated 1mL extraction buffer containing 2% CTAB, 2% polyvinylpyrrolidone (PVP), 100mM TRIS-HCL (pH 8.0), 25mM EDTA, 2.0 M NaCl and 0.5 g/l spermidine was added in 100-200 mg leaf samples. After adding 20 µL of β-mercaptoethanol, the mixture was vortexed and incubated in a waterbath at 65^{o}C for 10 min. Following the purification of RNA with Chloroform-isoamyl-alcohol (CIA) 24:1, LiCl (8M) precipitation was applied with an overnight incubation. Then, the solution was centrifuged at 9000g at 4°C for 20 min to precipitate the pellet, which was re-suspended with 500 µL of SSTE buffer. For final precipitation, 100 µL of NaCl (5M) and 250 µL of cold absolute ethanol were added and incubated at -80°C for 30 min. After centrifugation, two step ethanol washings were applied. The ultimate pellet was re-suspended in 30 µL of DEPC-water. Finally, the RNA clean-up was performed with RNeasy extraction kit (Qiagen) by following the manufacturer's instructions. RNA concentration was determined using a Nanodrop spectrophotometer (Nanodrop Tech., Wilmington, USA). The ratio of OD at 260/280 nm was used to assess the concentration and the purity of RNA samples. If the ratio of OD_{260}/OD_{280} was larger than 2.0, the RNA extract was accepted as pure and the samples that have lower values than that ratio was repeated. Quality and integrity of the total RNA were checked running extracted RNA samples with 1% agarose gel electrophesis. The samples that have no smearing below the 18s and 28s rRNA species' discrete bands demonstrated the integrity and quality of the RNA extracts (Figure 3.2).

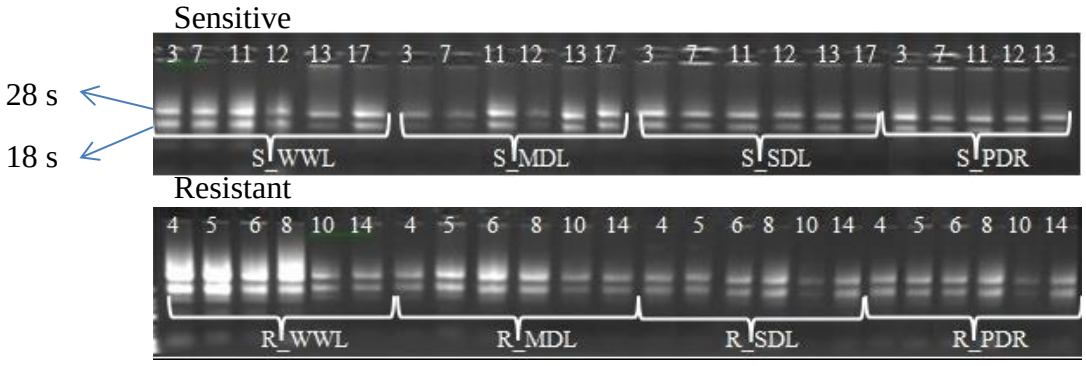


Figure 3.2 Degradation of the RNA samples was controlled by 1% agorose gel electrophoresis. The numbers indicated the codes for the selected 6 ramets of each genotype. R: Resistant, S: Sensitive, WWL: well watered level (control), MDL: Moderate drought level, SDL: Severe drought level and PDR: Post drought recovery period

3.2.3.2. Microarray Hybridization

Target preparation and loading onto GeneChip Arrays contain many steps that can be divided into three categories; reverse transcription, labeling and hybridization. In the current study, 10 µg of total pooled RNA was used to follow these steps according to the manufacturer's protocols ((Affymetrix GeneChip Expression Analysis Technical Manual http:// www.affymetrix.com). Briefly, total RNA isolated from the leaf tissues was firstly reverse transcribed using T7 promoter-oligo (dT) primers in the synthesis of single-stranded and then double-stranded cDNAs. This cDNA, which carries a transcriptional start site for T7 RNA polymerase, was column-purified and then used as template to incorporate biotin labelled nucleotides into a newly synthesized RNA molecule during in vitro transcription (IVT) step (labelling step). Fifteen µg of the biotin-labelled cRNA was fragmented into 35–200 bases strands as described in protocol. Hybridization was carried out at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640). The arrays were washed and then stained (SAPE, Streptavidin-phycoerythrin) with an Affymetrix Fluidics Station 450 followed by scanning with a GeneChip Scanner 3000. All the microarray experimental steps were summarized in Figure 3.3.

3.2.3.4. Microarray Data Analysis

3.2.3.4.1. Data processing; Normalization, Filtering and Clustering

Statistical analyses of microarray data were performed using the Genespring GX 12.5 (Agilent Technologies, Santa Clara CA) software programme. Firstly, Robust Multiarray Analysis (RMA) algorithm integrated into this software was used for data processing and normalization. After RMA, the initial probeset filtering procedure was applied according to the percentile of the raw signal intensities. The filtering process was applied on RMA normalized data to produce a detection call for each probe set. These detection calls ("present" or "absent") were used to apply an initial filtering step. For a given array, any probe set with signal intensity below 20% was labelled as "absent" and discarded from analysis. Statistical analyses were conducted with this normalized data and remaining "present" probe sets.

After the normalization and filtering procedures, moderate t-test was applied between MDL vs. CTRL, SDL vs. CTRL and PDR vs. CTRL to identify subsets of genes differentially expressed in different watering regimes in the both black poplar genotypes. Differential expression of a gene was determined based on the p values and fold changes: A gene was declared as differentially expressed if the *P-value* was smaller than 0.05 and fold change is larger than at least $\pm$ 2 folds. Then these differentially regulated genes in three comparisons were compared between two black poplar genotypes to identify candidate genes responsible in especially drought tolerance.

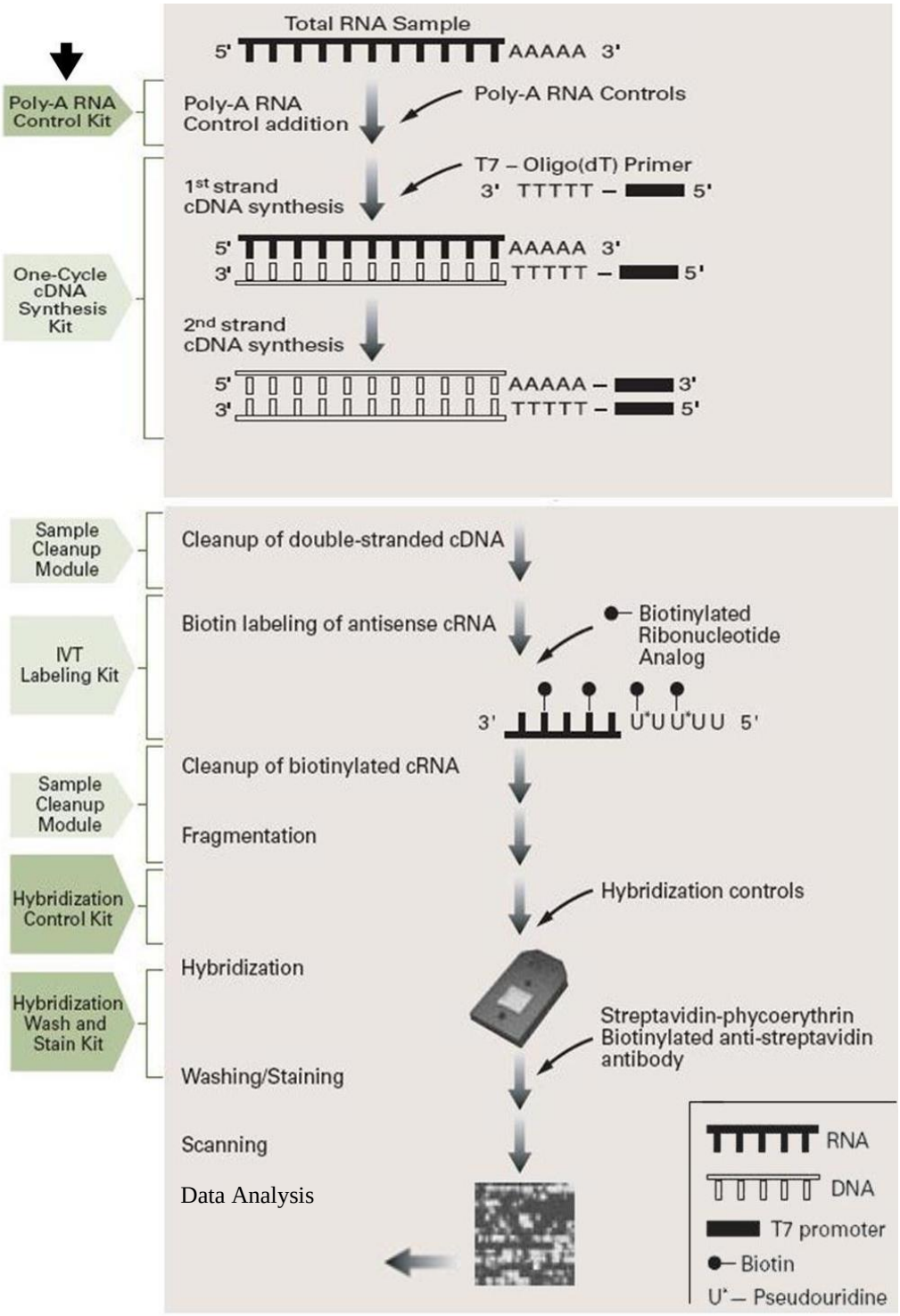


Figure 3.3 Microarray experimental process used in gene expression profiling of two black poplar genotypes under different watering regimes. (GeneChip Expression Analysis Manual, Affymetrix).

3.2.3.4.2. Principle Component Analysis (PCA)

Measuring the expression level of thousands of genes in different genotypes under many drought conditions increases the complexity of the microarray data. This exponential complexity becomes impossible to make a visual inspection of the relationship between genes and conditions in a simple plot. Therefore, the dimensionality of the microarray data should be reduced into 2 or 3 to visualize trends or relations. Principal Components Analysis (PCA) is most widely used method that reduces data dimensionality of the data by performing a covariance analysis between factors. In the current study, PCA was carried out to explain the variation of the dataset by reducing dimensionality of the data into genotype and stress treatment.

3.2.3.4.3. Single Enrichment Analysis (SEA) and Probe Set Gene Annotation (GO)

Extracting important biological information from huge microarray data is a major challenge in the transcriptome studies. Fortunately, with increase in the usage of microarray technology, many tools were developed for genome-wide RNA expression analysis. The best way to study microarray data is classification of the genes that share common biological, functional or cellular regulation. An overview of putative functional groups involved in drought responses was obtained through single enrichment analysis (SEA) option of AgriGo website (http://bioinfo.cau.edu.cn/ agriGO). The enrichment analysis of each up and down regulated genes was performed independently by using the *Populus* Affymetrix Genome array as a background list. We computed enrichment scores for GO terms based upon set of entered gene list and use enrichment scores and FDR corrected p-values ($p< 0.1$) to filter the biological, functional and cellular regulations.

Genes that showed differential regulation under drought treatment in the experiment were also annotated by using web-based queries (www.plexdb.com). Gene annotation analysis depends on sequence similarity to a known protein or EST in another organism provides useful and suggestive information to be used in selection of the drought related genes. The best homology implied a true orthologous in the gene annotations. Therefore, the highest homology values (e values) were taken into consideration to be sure about function of a gene. In the current study, mostly *Arabidopsis* gene model was used as an annotation template to describe poplar genes.

3.2.4. Microarray Validation

Microarray expression profiling is required confirmation by another independent gene expression profiling method. Real-time PCR is the most widely used method of choice to confirm microarray data. Four genes exhibiting differential expression patterns across treatments

in the microarray results were selected for validation by two-step RT-PCR (Appendix B). The ACTIN-1 and ACTIN-5 genes were used as references in RT-PCR validation procedure. The results of microarray experiment for selected probe-sets and their primers with amplicon sizes were given in the Appendix B. Although, specificity of the primers was tested by the BlastN analysis in NCBI, traditional PCR and subsequent gel electrophoresis were also applied to validate functionality of the primers and to see there was no non-specific amplification of any other genes. The primer pairs that produced only the desired amplicons were selected to be used in RT-PCR. Total RNA used for microarray analysis was also used as a starting material for RT-PCR. After synthesizing single stranded cDNA, a real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen). Then fluorescence measurements were completed via Rotor-Gene Q (Quigen) real-time cycler based on the instructions of the manufacturer. RT-PCR reactions were carried out with three biological replicates like in microarray analysis for each genotype in each treatment. Primer concentration was 0.5 μM. The amplification programs in thermal cycler were as follows: one cycle of 10 min at 95 °C for initial denaturation and *Taq* activation, following 35 cycles of 30 s at 95 °C for denaturation, 30 s at 55 °C for annealing and 30 s at 72 °C for final extension. After amplification, a melting curve cycle was performed with starting temperature at 50 °C with 5 °C increment steps, and ended with a temperature of 90 °C. The absence of non-specific PCR products (artefacts and primer dimers) was checked by analysis of the melting curve. When non-specific products were detected, the annealing temperature and primer concentration were adjusted to optimize the specificity of amplifications. Quantitative calculations were performed with C_T values of target and reference genes and their corresponding standard curves. The relative expression values in target genes were normalized by dividing them with corresponding reference genes. The results of the RT-PCR reactions were compared with the microarray results for validation purposes.

3.3. RESULTS

3.3.1. Physiological and Biological Responses of Black Poplar Clones to Drought

Among the investigated clones, N.03.368.A was found to have a drought-evading strategy, which was accepted as drought sensitive clone in the study. In this strategy, active portion of growth generally takes place mostly during water abundant periods. Therefore, the most important biomass production takes place in well-watered period. The results of the current study exhibited that sensitive clone has a higher shoot elongation and radial growth under well watered conditions. However, the growth rate of this sensitive clone declined more than four folds at MDL and stopped almost completely in SDL. This dramatic effect of drought stress was also observed in PDR period that the growth rate of the sensitive genotype could not reach their control level at the end of three weeks of re-watering periods. Leaf shedding is known as a drought evading strategy to reduce leaf surface area and consequently the overall transpiration

rate of a tree**.** In the current study, the highest and the earliest defoliation were occurred suddenly in the sensitive clone at MDL (Figure 3.4). Physiological functions of the leaves, especially during drought period, mostly depend on cell membrane stability, which is crucial in sustaining of cell turgor pressure**.** In this study, electrolyte leakage was used as a reliable indicator of membrane stability and senescence. The results from the current study indicated that EL increased more rapidly in the leaves of the sensitive genotype. The EL values of the same clone did not decrease to control level in PDR, which revealed on-going membrane senescence in that period. The LWP measurements indicated the lowest predawn LWP (-12 bar ± 1.2) in N.03.368.A revealing loss of great portion of water content of the seedlings (Figure 3.4). Prolonged reductions in leaf water potentials have been previously reported to result in xylem cavitation and embolism which cause subsequent hydraulic failure and branch sacrifice in many poplar species. Especially the sensitive genotype exhibited an extreme form of stress symptoms that terminal shoots of 30% of the ramets desiccated at SDL point in greenhouse trial. The branch sacrifice was also observed in some members of the same clone in the field trial.

On the contrary, the clone N.62.191 was found to have dehydration tolerance or resistance strategy to cope with drought stress. This strategy was characterized with increased drought tolerance with less reduction in above-ground growth in under decreased LWP. The growth rate of N.62.191 exhibited highest performance in the field and greenhouse trials in all watering regimes. Although, LWP values of the resistant genotype decreased to (-8 ± 0.7 bars) critical values at the severe drought level, no branch sacrifice or severe leaf desiccation was observed for this clone in both field and greenhouse trial. Under drought stress, leaf abscission was very limited in the resistant clone that it can be accepted as 'non-senescent' compared to the sensitive one. Only yellowing of the leaves was observed in the lower parts of seedling under SDL. Interestingly, some of these flavescent leaves recovered themselves and turn into green colour in the PDR period. Despite the reduction in LWP, electrolyte leakage results of these clones revealed strong membrane stability under severe drought conditions.

Measurement of antioxidant enzyme activities and H_2O_2 in the leaves of selected genotypes enabled us to understand stress level of selected clones under different watering regimes (Figure 3.4). The H_2O_2 level of the sensitive genotype was found to be always higher than the resistant one. This finding revealed that the level of ROS produced in the leaves of the sensitive genotype was much higher than the resistant genotype. Under the stress conditions, increase in some components such as hydrogen peroxide stimulates especially antioxidant and other ROS scavenging systems. In this study, the GR and DHAR activities of both genotypes increased significantly under especially severe drought stress conditions. However, this increase in enzyme activities found to be not significant among genotypes. In ROS scavenging system SOD and APX are the main enzymes that the former converts ROS into H_2O_2 and the latter catalyse the reduction of H_2O_2 into water. Especially SOD and APX enzyme activities were found to be highly activated in the leaves of the sensitive clone compared to the resistant one (Figure 3.4). This finding attributed to the increased level of reactive oxygen species in especially sensitive genotype due to drought stress.

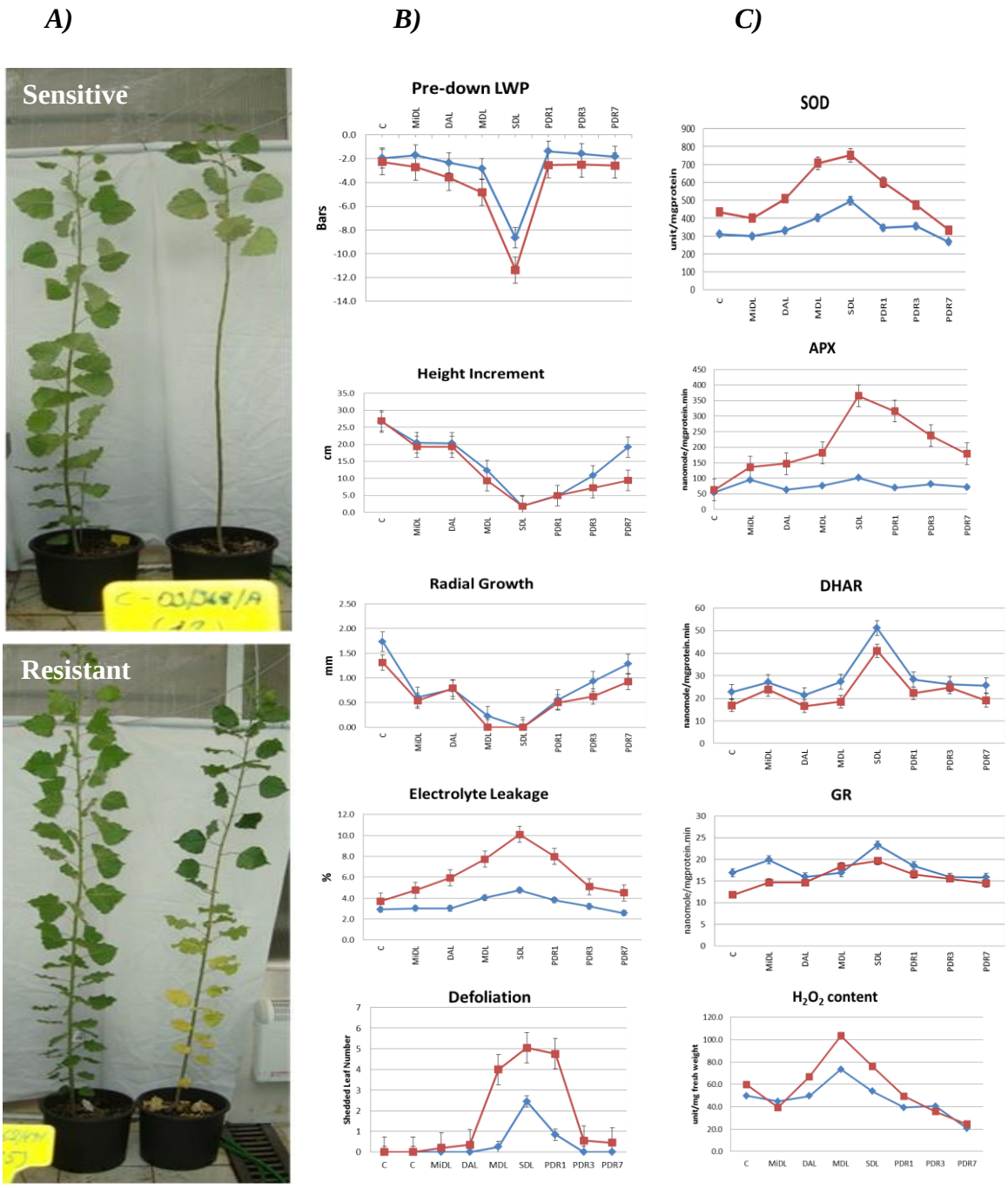


Figure 3.4 Effects of drought stress on black poplar genotypes exhibiting contrasting drought response. Morphological observations (A) were made on the defoliation rate of the resistant (N.62.191) and the sensitive (N.03.368.A) genotypes at severe drought level. Physiological (B) and Antioxidant enzyme activity (C) changes along with drought stress were also represented. Blue and red colour in the graphs represented the resistant and the sensitive genotypes, respectively. (C: Well watered, MiDL: Mild Drought, DAL: Drought Acclimation SDL: Severe drought, PDR: Post drought recovery)

3.3.2. Expression Profiling of Drought Related Genes in Black Poplar

Plant response to drought stress is a multigenic process that controlled by many types of molecular mechanisms. Therefore, expression profiling of a species is crucial to understand all these mechanisms. In the current study, transcriptome changes in response to drought stress was conducted using the leaf tissues of two black poplar genotypes exhibiting contrasting response to drought stress. Above mentioned results of morphological biochemical and physiological measurements indicated that N.62.191 (resistant) black poplar genotype was more tolerant to drought than N.03.368.A clone (sensitive).

3.3.2.1 Principal Component Analysis of Microarray Data

In the analyses of microarray data, PCA was used to understand the main sources of variation in the experiment. When all the arrays were represented as data points on a PCA scatter plot, biological replicates of each condition clustered together (Figure 3.5-I/II) as expected. The arrays belonging to the resistant and the sensitive black poplar clones were completely separated on the x axis (Figure 3.5/I) revealing that the genotype differences comprised the main source of variation (73%). Treatment factor was another important source of variation (27.3) represented along y axis. CTRL, MDL, SDL and PDR treatments of each clone well isolated from each other in the PCA scatter plot (Figure 3.5/II). All the PCA results indicated that the main sources of gene expression variance were due to the clonal differences and drought treatments.

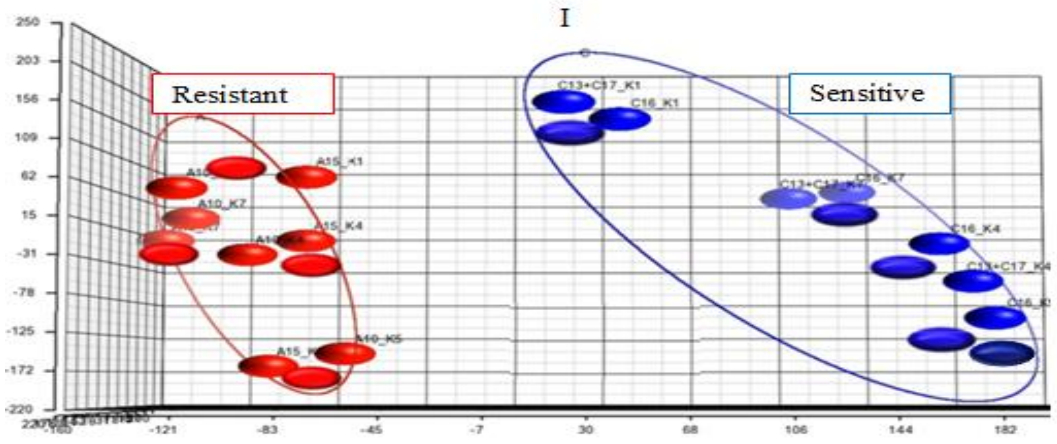


Figure 3.5/I Principle component analysis (PCA) of all microarray hybridizations conducted for two black poplar genotypes. On the scatter plot, the red and blue dots on the scatter plot represented the genechips used in drought related expression profiling of the resistant and the sensitive genotypes, respectively.

II

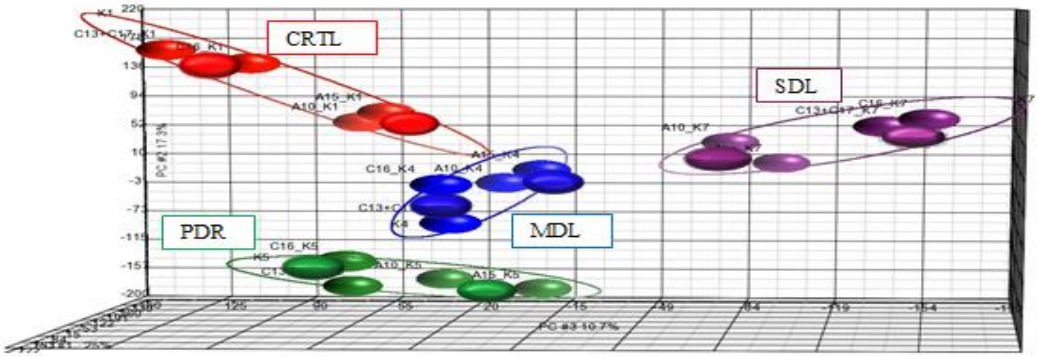


Figure 3.5/II Principle component analysis (PCA) of all microarray hybridizations conducted in four different watering regimes. On the scatter plot, each different colour represented one treatment; Red: Well Watered Period (CTRL), Blue: Moderate Drought Level (MDL), Purple: Severe Drought Level (SDL) And Green: Post Drought Recovery (PDR).

3.3.2.2 Determination of Drought Regulated Genes in Resistant Genotype

In this study, gene selection analysis with moderate t-test in the resistant black poplar genotype revealed totally 2453 probe sets differentially expressed as a consequence of three comparisons: MDL vs. CTRL, SDL vs. CTRL and PDR vs. CTRL. The comparison of drought treated leaf samples between MDL and CTRL yielded 430 differentially expressed probe sets in the resistant genotype. As the severity of the stress level progressed on the leaves of the same genotype, the number of significantly expressed genes was increased up to 1952 probe sets in SDL. Under re-watering period 919 drought related genes were found to be differentially expressed in PDR compared to CRTL in the resistant genotype. Among the differentially expressed probe sets, 52, 1379 and 447 genes were found to be only differentiated significantly under MDL, SDL and PDR, respectively. In addition, among the significantly altered genes, 246 genes were identified to be common in all treatments (Figure 3.6).

Up or down regulation of the genes differentially expressed throughout the experiment was exhibited another drought response in the study. Under MDL, the transcriptional response of the resistant genotype to drought was in a down regulated direction of the genes. The number of down regulated genes (367) were approximately fourfold higher than up regulated (93 genes) ones under MDL in the resistant genotype (Table 3.1). The numbers of up regulated genes (839 genes) under SDL increased and closed to down regulated genes (1036 genes) in the same genotype. During the post drought recovery, the up and down-regulated genes were 252 and 667, respectively. From these results it could be concluded that the basic transcriptional response of the resistant genotype to drought occurred at late stage (severe) of drought conditions.

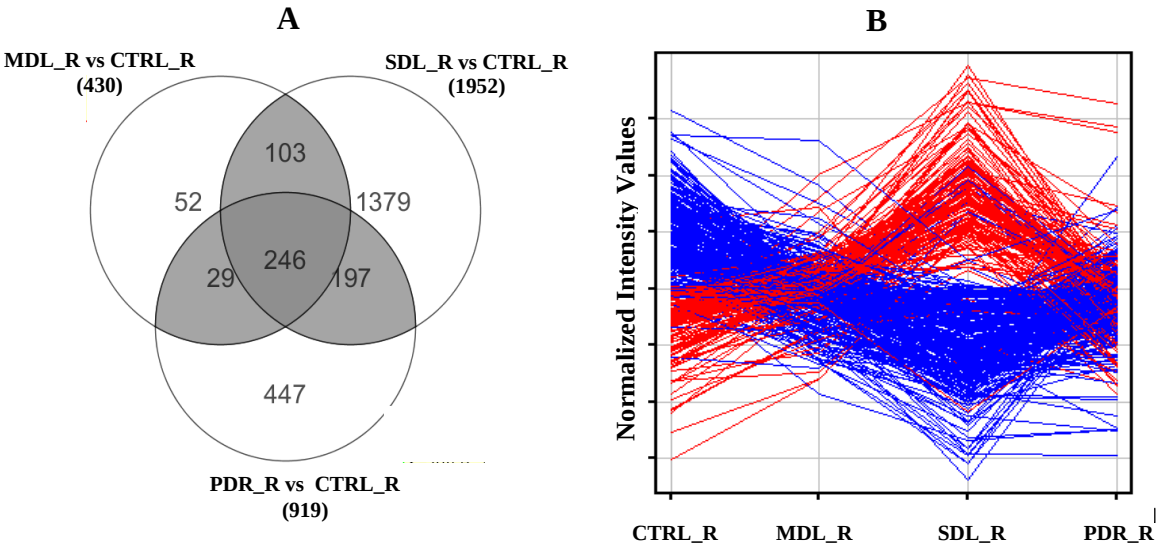


Figure 3.6 Number and expression pattern of differentially regulated genes (*P<0.05* and ± 2 fold) in the resistant genotype (N.62.191) under drought and post drought recovery periods. Venn diagram (A) exhibited the number of the genes regulated in each watering level. The graph indicated general trend in the up and down regulations of drought related genes under different watering regimes. (R: Resistant, CTRL: Control (well watered) MDL: Moderate Drought Level, SDL: Severe Drought level, PDR: Post Drought Recovery Period).

Table 3.1. Probe sets differentially regulated in the leaf tissues of the resistant (N.62.191) black poplar genotype under drought stress. Up and down regulations were assigned as relative to control conditions.

Transcriptional comparisons	Differential regulation of drought related genes (*P<0.05* and ± 2 fold)		
	UP	DOWN	TOTAL
MDL vs. CTRL_R	93	367	430
SDL vs. CTRL_R	839	1036	1875
PDR vs.CTRL_R	252	667	919

3.3.2.4 Drought Regulated Genes in Sensitive Black Poplar Genotype

Total number of differentially expressed genes in three comparisons (MDL vs. CTRL, SDL vs. CTRL and PDR vs. CTRL) of the sensitive genotype was found to be two folds greater (5851 genes) than the resistant clone (2453 genes). As stated in the physiological response part, the MDL was the most important stress level distinguishing both genotypes in terms of their drought response. A pairwise transcriptional comparison between MDL and CTRL clearly confirmed this differential drought responses between clones. In contrast to 430 differentially altered genes at MDL for the resistant genotype, drought had a greater impact on the transcriptome of the sensitive clone at the same level exhibiting 3016 differentially expressed genes (Figure 3.7). Out of these 3016 differently regulated genes in the sensitive clone, 1478 and 1538 genes were found to be up and down regulated, respectively (Table 3.2). These results highlighted that the response of the sensitive genotype to drought stress started much earlier than the resistant one. Most of the genes regulated in MDL increased their regulation level at SDL in the sensitive genotype (Figure 3.7). Under SDL, totally 4748 probe sets differentially expressed. Out of these genes expressed at SDL, 1943 and 2446 ones found to be up and down regulated, respectively (Table 3.2). During the PDR period, out of 2333 differentially expressed genes, 830 were expressed only in PDR period which was two fold higher than the resistant genotype.

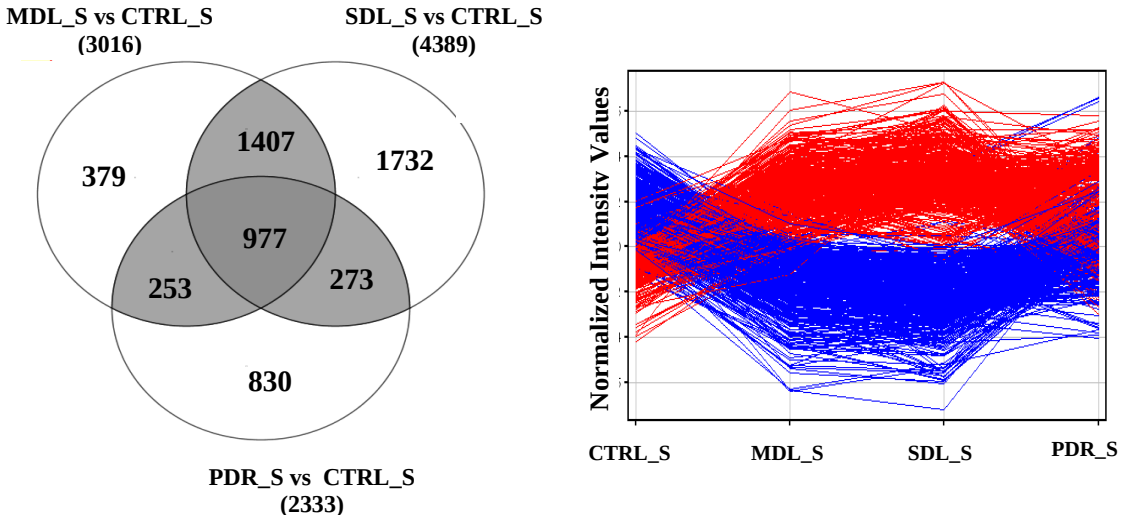


Figure 3.7 Number and expression pattern of differentially regulated genes (*P<0.05* and ± 2 fold) in the sensitive genotype (N.03.368.A). Venn diagram (A) exhibited the number of the genes regulated in each watering level. The graph indicated general trend in the up and down regulations of drought related genes under different watering regimes. (R: Resistant, CTRL: Control (well watered) MDL: Moderate Drought Level, SDL: Severe Drought level, PDR: Post Drought Recovery Period).

Table 3.2 Probe sets differentially regulated in the leaf tissues of the sensitive (S) (N.03.368.A) black poplar genotype under different watering regimes. Up and down regulations in moderate drought level (MDL), severe drought level (SDL) and post drought recovery period (PDR)were assigned relative to control conditions (well watered /CTRL).

Abbreviation (Treatments vs. Control_ Sensitive)	Differential Regulation of Drought Related Genes ($p<0.05$ and $\pm$ 2 fold)		
	UP	DOWN	TOTAL
MDL vs. CTRL _S	1478	1538	3016
SDL vs. CTRL _ S	1943	2446	4388
PDR vs. CTRL _S	1331	1002	2333

3.3.2.5. Comparative Singular Enrichment Analysis (SEA) of Drought-Dependent Up and Down Regulated Genes in Both Genotypes

To gain an insight into the biological, functional and cellular processes that occur in the black poplar under conditions of water deficit and post drought recovery, we utilized the SEA for classification of differentially expressed genes in the leaf tissues. Then these groups were compared to extract genotype specific drought related groups and genes. SEA was conducted independently on the up and down regulated genes that differentially expressed in MDL, SDL and PDR conditions. In the manuscript, the first five biological and functional processes that have the highest *P* values were represented. All the other groups were given in supplementary excel file 1 provided on CD.

3.3.2.5.1. SEA of Regulated Genes in Both Genotypes at MDL

In the resistant clone, SEA of differentially expressed genes at MDL revealed four biological processes that three of them (response to stress, stimulus and chemical stimulus) were common to both genotypes. On the other hand, 'protein folding' biological process and two functional annotations (Unfolded Protein Binding, Protein Binding) were grouped only in the resistant genotype under MDL condition. The genes that fell into this protein related groups were found to be annotated to heat shock proteins and chaperons.

As mentioned earlier, most significant morphological, biochemical and physiological differences were started to occur at MDL (35% soil water content) within the genotypes. Leaf desiccation and abscission were the main discriminative index between genotypes under MDL. At this condition sudden leaf senescence was observed only in the sensitive clone. Therefore, the SEA of differentially expressed genes under MDL was identified leaf abscission related groups in especially sensitive genotype. SEA of up regulated genes in MDL for the sensitive clone indicated five biological, two functional and two cellular annotation groups (Table 3.3). The SEA clustered two sensitive genotype specific groups named as 'Response To Abscisic Acid Stimulus' and 'Nitrogen Compound Catabolic Process'. Most of the drought related response in plants is known to begin with an abscisic acid (ABA) regulation. ABA induced signalling of stomatal closure and leaf abscission at the early and late stage of drought, respectively. Therefore ABA related annotation in MDL condition revealed this hormonal control in the sensitive genotype. Most of the differentially expressed genes at MDL in the sensitive genotype were grouped into 'Nitrogen Compound Metabolic Process' which is associated with protein degradation.

The SEA of down regulated genes of sensitive genotype identified many repressed categories, almost all of which related with growth, photosynthesis and respiration indicating a complete collapse in leaf cell energy metabolism (Table 3.3). On the other hand, down regulated genes of resistant genotype related mostly with growth and cell wall related carbohydrate metabolism. Decrease in growth and biomass production is known to be the first response of the plants against drought stress. In plants, leaf size and growth is a result of cell division and cell expansion both of which mostly depend on cell wall modification. In the current study, down regulated genes of the resistant clone at MDL, mostly enriched in clusters involved in the synthesis of cytoskeleton (cell wall polysaccharide metabolic process, cell wall macromolecule metabolic process) indicating this decrease in leaf growth.

3.3.2.5.2. SEA of Differentially Regulated Genes in Both Genotypes at SDL

The SEA revealed many common clusters at the SDL. From these clusters, "response to heat", "response to stress" and "protein folding" were the most enriched terms for both genotypes. Furthermore, the important parts of the enriched groups were also related with light and oxidative stress all of which highly associated with the drought stress in plants. The most important difference between clones was appeared on the sensitive genotype specific "cellular nitrogen compound catabolic process" group that is related with protein degradation and leaf senescence (Table 3.4). Enrichment analysis of down regulated genes in SDL did not differentiate much from MDL for the sensitive clone. As it can be expected, the reduction in the fold changes of the genes responsible in growth, photosynthesis and respiration were became more severe at the SDL for this sensitive clone. For the resistant genotype, the most statistically significant repression at the SDL and MDL was observed in the genes encoding microtubules, cytoskeleton elements, and cell wall biosynthetic enzymes all of which related with cell growth and cell wall synthesis of the leaf tissues.

Table 3.3 SEA of up and down regulated genes at moderate drought level (MDL) in the resistant (R) and the sensitive (S) black poplar genotypes. In the table 'Term' indicated functional and biological processes. False discovery rate (FDR) was used to test the significance level (*p<0.1*)

SEA of Sensitive black poplar clone at MDL (N.03.368.A)			**SEA of Resistant (R) black poplar at MDL (N.62.191)**		
S_MDL_UP			**R_MDL_UP**		
Term	**Number of genes**	**FDR**	**Term**	**Number of genes**	**FDR**
Biological Process					
Nitrogen Compound Catabolic Process	94	0.000	Protein Folding	9	0.000
Response To Stress	24	0.004	Response To Stress	20	0.000
Response To Stimulus	27	0.014	Response To Stimulus	24	0.002
Abscisic Acid Mediated Signalling	12	0.015	Response To Chemical Stim.	12	0.082
Response To Chemical Stimulus	121	0.055			
Functional Process					
Acyl Groups Transferase Activity	36	0.071	Unfolded Protein Binding	5	0.003
Oxidoreductase Activity	16	0.071	Protein Binding	19	0.098
Pectinesterase Activity	5	0.099			
S_MDL_DOWN			**R_MDL_DOWN**		
Biological process					
Photosynthesis	76	0.000	Polysaccharide metabolic pro.	25	0.000
Nitrogen metabolic process	122	0.000	Lipid localization		0.000
Photosynthesis, light reaction	37	0.000	Cell wall polysaccharide met.	5	0.000
Generation of energy	94	0.000	Cellular glucan metabolisim	19	0.000
Electron transport	110	0.000	Glucan metabolic process	19	0.000
Functional process					
Tetrapyrrole binding	52	0.000	Udp-glycosyltransferase act.	13	0.000
Oxidoreductase activity	171	0.000	Sucrose synthase activity	5	0.000
NADH dehydrogenase	17	0.000	Hexosyl groups transferase	15	0.000
Iron ion binding	68	0.000	Glycosyl groups transferase	15	0.000
Heme binding	38	0.000	Carboxy-lyase activity	6	0.000

Table 3.4 Functional grouping of the up and down regulated genes in severe drought level (SDL) in the resistant (R) and the sensitive (S) genotypes

SEA of Sensitive (S) black poplar clone (N.03.368.A)			SEA of Resistant (R) black poplar (N.62.191)		
S_SDL_UP			**R_SDL_UP**		
Term	**Number of genes**	**FDR**	**Term**	**Number of genes**	**FDR**
Biological process					
Response to heat	32	0.000	Response to heat	24	0.000
Nitrogen compound catabolism	106	0.000	Protein folding	6	0.000
Response to high light intensity	17	0.000	Response to abiotic stimulus	84	0.000
Response to inorganic substance	29	0.000	Response to high light intensity	16	0.000
Protein folding	40	0.000	Response to stress	133	0.000
Response to light intensity	18	0.002	Response to light intensity	17	0.000
Functional process					
Unfolded protein binding	25	0.001	Heat shock protein binding	19	0.000
Heat shock protein binding	22	0.012	Galactosidase activity	6	0.000
Acyl groups transferase activity	41	0.06	Unfolded protein binding	19	0.000
Sequence-specific DNA binding	31	0.065	Sequence-specific DNA bind	26	0.001
			Protein binding	179	0.002
S_SDL_DOWN			**R_SDL_DOWN**		
Biological process					
Nitrogen compound metabolism	242	0.000	Nitrogen compound metabolism	146	0.000
Photosynthesis	81	0.000	Secondary metabolic process	84	0.000
Generation metabolite and energy	122	0.000	Polysaccharide metabolic process	72	0.000
Electron transport	147	0.000	Phenylpropanoid metabolic pro.	40	0.000
Ketone metabolism	307	0.000	Aromatic compound biosynthesis	53	0.000
Functional process					
Oxidoreductase activity	272	0.000	Oxidoreductase activity	176	0.000
Cofactor binding	115	0.000	Sucrose-phosphate synthase acti	7	0.000
Coenzyme binding	78	0.000	Hexosyl groups transferase acti	47	0.000
Tetrapyrrole binding	63	0.000	Tetrapyrrole binding	40	0.000
Nadh dehydrogenase	21	0.000	Catalytic activity	702	0.000

3.3.2.5.3. SEA of Differentially Regulated Genes in Both Genotypes at PDR

The microarray experiment for PDR period was conducted with the leaves collected from drought treated seedlings of the clones at the third day of re-watering. The growth of the clones during the PDR period was one of the main discriminative index between genotypes. At the end of the three weeks of re-watering period, the resistant clone completely recovered itself in terms of growth traits and reached its control level. During SDL condition, most of the leaves located at the lower parts of stems turned into yellow colour, but they did not desiccate completely in the resistant clone. Interestingly, most of these flavescent leaves of the resistant clone turned into green colour at the end of re-watering period, indicating active production of chlorophyll molecule in the leaves. The enrichment analysis of up regulated genes in PDR definitely confirmed these physiological observations of the resistant clone. The SEA of up regulated genes in the resistant genotype at PDR clustered into many groups such as 'chlorophyll metabolic process, tetrapyrroles and porphyrin biosynthesis' which are related with active production of the chlorophyll in the lead cells. In addition, clusters including the genes responsible in plastid and chloroplast organizations were also up regulated in PDR period for the resistant clone, highlighting a reactivation of photosynthesis in the leaf cells.

During the PDR period, growth of the sensitive clone was much slower compared to the resistant clone due to the desiccation in the leaves and terminal shoots of the seedlings under drought conditions. As it was mentioned before, the leaf desiccation was so severe in the sensitive genotype that almost all the leaves were desiccated under drought stress period. Until re-watering period only a few leaves stayed on the stems of the sensitive genotype, whereas they could not recovered themselves in this period and continued to defoliate. All the morphological observations indicated that sensitive black poplar clone cannot resist to especially prolonged drought stress. When the severe drought occurred, this sensitive clone completely shut down itself to enter into a dormancy period. This situation could explain lesser growth performance of the sensitive clone compared to the resistant genotype in PDR period. It was realized that resistant clone keep their leaves on the seedlings as much as possible under drought stress. If the resistant clone regains the water from the environment, it immediately starts to synthesize chlorophyll to increase photosynthesis and sugar production which is required for cell wall synthesis and growth. On the other hand, to grow under re-watering period, the sensitive genotype should start from cellular division to produce cell and tissues. Enrichment analysis of up regulated genes of the sensitive genotype at PDR period clearly pointed out this suggestion that many clusters of SEA found to be related with cell division and mitosis (Table 3.5).

Table 3.5 Functional grouping of the genes up regulated in post drought recovery period (PDR) in the resistant (R) and the sensitive (S) genotypes

SEA of Sensitive (S) black poplar clone (N.03.368.A)			SEA of Resistant (R) black poplar (N.62.191)		
S_PDR_UP			**R_PDR_UP**		
Term	**Number of genes**	**FDR**	**Term**	**Number of genes**	**FDR**
Biological Process					
Ribosome biogenesis	87	0.000	Chlorophyll metabolic process	10	0.0002
Cellular component biogenesis	127	0.000	Tetrapyrrole biosynthetic process	9	0.0002
Translation	105	0.000	Nitrogen compound metabolism	29	0.0002
Nuclear division	14	0.000	Porphyrin metabolic process	10	0.0002
DNA replication initiation	9	0.000	Pigment biosynthetic process	11	0.0002
Functional process					
Structural constituent of ribosome	73	0.000	NO significant enrichment		
Structural molecule activity	84	0.000			
Unfolded protein binding	27	0.000			
Chaperone binding	7	0.000			
Rrna binding	18	0.000			

3.3.2.6. Genotype Specific Drought Induced Genes

One of the major aims of this study was to find drought regulated or related genes in black poplar to understand the genetic basis of drought adaptation strategies in trees. The genes that differentially expressed between the resistant and the sensitive black poplar genotypes will enable us to reach that purpose and to develop genetic markers which can be used in selection of drought tolerant trees in breeding programmes. In this study, to find the differentially and co-expressed genes between the resistant and the sensitive black poplar genotypes, we firstly listed all differentially expressed genes (two fold and $P<0.05$) that were extracted by comparisons of MDL vs. CRTL, SDL vs. CTRL and PDR vs. CTRL for both genotypes. As it was mentioned before, these comparisons were extracted totally 2453 and 5851 differentially expressed genes to be significantly altered in the resistant and the sensitive genotypes, respectively. All these genes were clustered in a Venn diagram to find the differentially and co-expressed genes between genotypes (Figure 3.8). Among all these gens, 1572 of them found to be co-expressed in both genotypes. The remaining 881 and 4278 genes were expressed in only the resistant and the sensitive genotypes, respectively (Figure 3.8). All these genes, gene annotations and fold changes were represented in the supplementary excel file 2 provided in the CD.

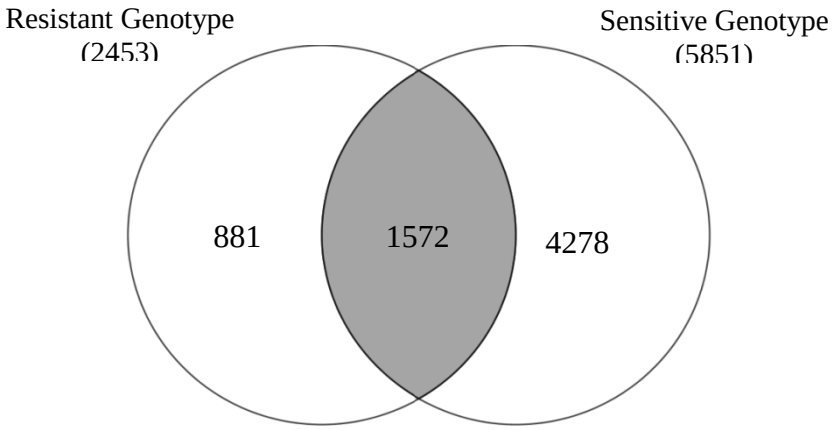


Figure 3.8 Differential and co-expressed genes between the resistant and the sensitive black poplar genotypes under drought and post drought recovery periods.

Due to large numbers of genotype specific genes, we extracted most strongly regulated genes that have fold change higher than ±30. Then a hierarchical clustering analysis (HCA) was applied to these selected genes to classify them into several functional groups. HCA results were represented in Figure 3.9. Although clusters created by HCA indicated similar results with SEA of the differentially expressed genes, it gave a summary about the drought dependent transcriptomic regulation in the sensitive and the resistant black poplar genotype.

As mentioned before, the sensitive genotype has a drought evading strategy which is characterized with complete leaf abscission under drought to enter into dormancy period. Leaf abscission is under the control of hormones and their corresponding transcription factors and requires enhanced activity of many hydrolysing enzymes that degrade cell walls, membranes and proteins. All these abscission related gene expressions were clearly appeared on the clusters of highest up regulated genes of the sensitive genotypes. Nitrogen remobilization from the abscised leaves to the sink organs was another event that occurs during defoliation. The highest induction of Asparagine Synthase genes in the sensitive genotype was associated to this remobilazation. The first response of the plants to drought is known to be reduction in growth parameters. Therefore, strong down regulation of the genes related with photosynthesis and cell wall synthesis (phenylpropanoids synthesis) could be related with reduction in growth.

On the contrary to the sensitive genotype, the resistant black poplar clone has a strong tolerance to drought that is characterized with delayed leaf senescence under drought and fast growth abilty especially in post drought recovery period. HCA results of the study indicated many interesting the resistant specific groups that could be used to identify genes functional in drought tolerance of the trees. All these groups and their corresponding genes will be discussed on the subsequent discussion part.

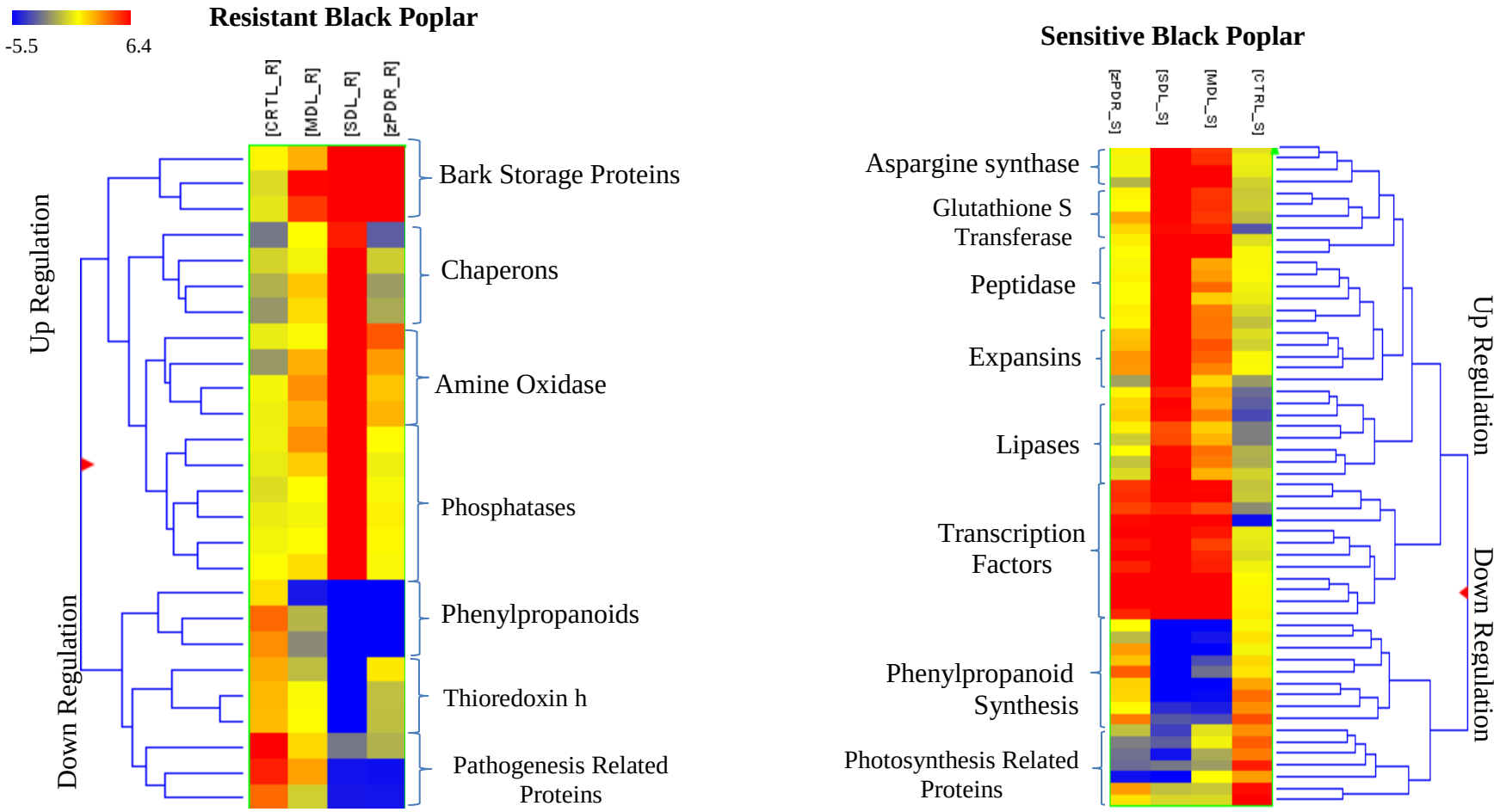


Figure 3.9 The groups of highest up and down regulated genes (> ± 30 fold change) only in the resistant and the sensitive black poplar genotypes. The list was sorted in terms of the fold changes in different watering level. (Ctrl: control (well watered), SDL; severe drought level, MDL moderate drought level and PDR; post drought recovery periods)

3.3.3. Validation of Microarray Results by Real-Time RT-PCR

After selecting the genotype specific genes, the microarray based gene expression profiles were validated by two-step real-time reverse transcriptase PCR (RT-PCR). Especially the genes that are highly expressed in the one genotype but not in the other were selected for RT-PCR validation. Total RNA samples used for microarray hybridizations were also used as a starting material for real-time RT-PCR analysis. The log transformed relative expression values were compared with those obtained by microarray analysis and results were represented in Figure 3.10. Expression profiles obtained from the microarray analysis showed very strong correlation with the results of real time RT-PCR (Figure 3.10). This analysis confirmed validity of expression profiles obtained from microarray data.

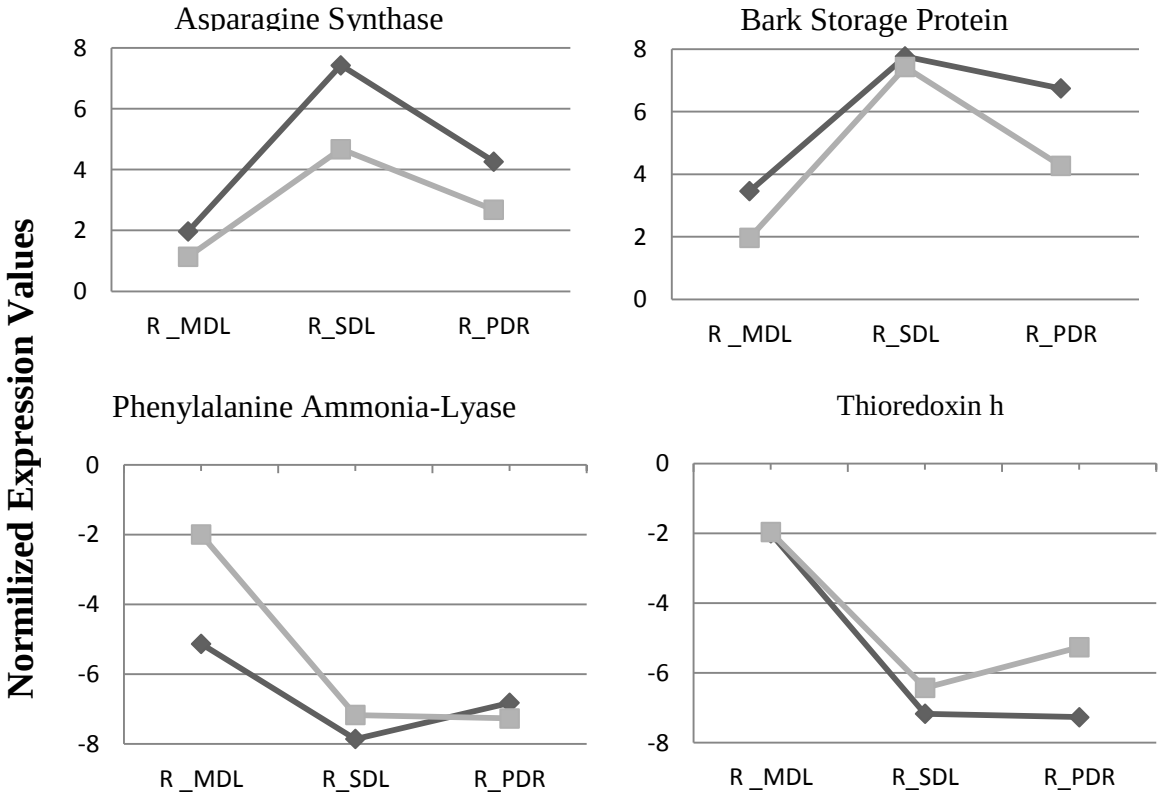


Figure 3.10 Comparison of microarray expression profiles of selected probe sets with expression values obtained from RT-PCR analyses. Black lines represent expression data from microarray and grey lines represent expression values from RT-PCR. (S: Sensitive, R:Resistant, MDL: Moderate Drought Level, SDL: Severe Drought level, PDR: Post Drought Recovery Period).

3.4. DISCUSSION

The results on physiological, biochemical and morphological observations clearly indicated the contrasting response of two black poplar clones to drought stress. As it was explained previously, the sensitive black poplar clone was characterized with complete defoliation, severe membrane senescence and higher antioxidant enzyme activities under drought periods. On the other hand, the resistant genotype displayed only flavescent leaf formation, lower membrane senescence and antioxidant enzyme activities under drought stress. This resistant clone exhibited better growth performance under re-watering period. Therefore, comparison of gene expression profiling of both genotypes expected to display important genes and mechanisms that can be used to understand drought tolerance in black poplar and to develop reliable gene specific drought markers.

3.4.1. Transcriptional Profiling of Drought in the Sensitive Black Poplar Genotype

3.4.1.1 Ethylene Regulation Might Be the Main Factor in Leaf Defoliation of the Sensitive Genotype

As mentioned in physiological analysis part, the sensitive genotype was characterized with severe defoliation rate under the MDL and SDL. This situation was also confirmed by the genes that were highly up regulated in the sensitive genotype. Gene expression during senescence has been reported to be under the control of a complex combination of signalling pathways (Buchanan-Wollaston et al., 2005). Among these pathways, hormone stimulus was shown to be one of the main factors.

Many molecular and genomic studies indicated that Abscisic Acid hormone (ABA) plays a key role in plant adaptation to abiotic stress conditions including drought stress. It has been known for a long time that ABA accumulation is essential for controlling significant set of drought related genes (Shinozaki and Yamaguchi-Shinozaki, 2007; Huang et al., 2008; Wasilewska et al ., 2008; Xu et al 2010). In the current study, gene annotation results of differentially expressed genes indicated 41, 28 and 30 probe sets that were annotated to WKRY, MYB and bZIP transcription factors all of which are regulated by ABA accumulation in the leaves (Supplementary excel file 3/ sheet 1-2). Although their gene expression levels increased at MDL in the sensitive genotype, under SDL condition mode of regulation (up or down) between two black poplar genotypes was found to be similar. However, one of the WKRY genes strongly induced only in the sensitive genotype (161 fold Table 3.6). Recent studies indicated that some WKRY transcription factors were highly correlated with leaf senescence (Chen et al., 2012). Therefore, severe defoliation rate of the sensitive genotype under drought stress could be depend on these types of transcription factors.

Another important hormonal control of leaf defoliation depends on synergistic changes of auxin and ethylene. Under normal defoliation, the flux of auxin to the abscission zone is reduced, thus making this zone sensitive to ethylene which is the main responsible hormone in leaf shedding (Grbic and Bleecker,1995) Also, the jasmonic acid (JA) pathway is known to be involved in senescence. The JA treatment induces premature senescence and various senescence-enhanced genes in Arabidopsis (He et al., 2002). In terms of gene expression under drought stress, the most significant differences between the resistant and the sensitive black poplar genotypes were observed in the genes that functional in ethylene and JA production as well as ethylene dependent transcriptional factors. In the current study, the highest expression of Jasmonic acid related transcriptional factors (NAC) and ethylene response factors (AP2/ERF) in only the sensitive genotype indicated the potential role of JA and Ethylene in the control of leaf senescence (Table 3.6). In the current study, 53 AP2/ERF transcription factors were significantly up regulated only in the sensitive genotype. (Supplementary excel file 3/ sheet 3-7). The expression levels of these transcription factors were insignificant in the resistant genotype Furthermore, sensitive genotype specific induction of 1-aminocyclopropane-1-carboxylate (ACC) gene (40 fold in SDL), which is the key intermediate molecule in the conversion of methionine to ethylene, clearly support control of ethylene in the drought induced leaf abscission of the sensitive genotype. The same gene was found to be down regulated more than 5 fold in the resistant genotype at SDL period.

3.4.1.2 Nitrogen and Nutrient Remobilizations Might Be the Key Event Regulating Differential Gene Expression in the Sensitive Genotype

During the leaf senescence, plants should absorb most of the leaf's nutrients so that they are not wasted before a leaf is shed. Masclaux-Daubresse et al., (2010) pointed that Rubisco accounts for 50% of the total soluble protein content in the leaves of C3 plants and 20% in C4 plants providing an enormous source of nitrogen that plants can tap to supplement the nutrition of growing organs such as new leaves and seeds. Therefore, almost all the proteins stored on these types of molecules and also carbohydrates found in cell walls should be degraded and remobilized from abscised leaves into sink organs. This event requires strong up regulation of proteases, lipases and cellulases.

In the current study, among the differentially expressed genes, 170 probe sets were annotated to endo- and exo-peptidases (Supplementary excel file3/ sheet 8). Among these genes 70 were highly up regulated especially in the sensitive genotype indicating strong degradation of proteins in this black poplar genotype. Another indicator of protein degradation in the sensitive genotype was represented by the excessive expression of Asparagine Synthase (ASN) gene at SDL condition. Degradation of the cellular proteins in the cells leads to production of large quantity of ammonia. Due to its toxic effects on the plants ammonia is converted into asparagine and used as a remobilization intermediate in abscised leaves (Gaufichon et al 2010).

Table 3.6 Sensitive genotype (S) specific genes that have highest up and down regulation under severe drought conditions (SDL). All the probe sets were significantly differentiated in the sensitive genotype (*P<0.05*) while their alterations in the resistant (R) genotype was insignificant. Negative value indicates down regulation.

Probeset ID	**Gene Annotation**	**Fold (S-SDL vs. C)**	**Fold (R-SDL vs. C)**
PtpAffx.2311.1.S1_s_at	Asparagine Synthase	**229**	6.4
PtpAffx.218414.1.S1_at	Endopeptidase Activity	**188**	1.6
Ptp.6836.1.S1_at	Wrky Dna Binding Protein	**161**	10
Ptp.2657.1.S1_s_at	Beta-Galactosidase Precursor	**150**	2.6
PtpAffx.8983.1.S1_at	Glutathione S-Transferase.	**115**	-1.1
PtpAffx.10075.2.S1_at	NAC3	**73.9**	10
PtpAffx.8983.1.S1_s_at	Ap2/Erf Transcription Factor	**63.8**	1.7
PtpAffx.209096.1.S1_at	Galactinol Synthase	**62.4**	5.3
PtpAffx.1463.1.S1_at	Dessication-Related Protein	**62.1**	10
Ptp.3735.1.S1_s_at	Endopeptidase Activity	**60.7**	1.2
PtpAffx.75787.1.A1_s_at	Ap2/Erf Transcription Factor	**56.0**	4.1
PtpAffx.53192.1.S1_at	Endopeptidase Activity	**55.6**	2.2
PtpAffx.30938.1.A1_s_at	Regulation of Dna Transcription	**52.3**	2.0
PtpAffx.17914.2.A1_a_at	Expansin	**48.6**	1.0
Ptp.118.1.S1_at	Aminocyclopropane Carboxylate	**39.7**	1.1
PtpAffx.225012.1.S1_at	Flavonol 3-O-Glucosyltransferase	**-21.0**	-1.6
Ptp.2627.1.A1_x_at	Dna Polymerase II Gamma	**-22.1**	-3.0
Ptp.6711.1.S1_s_at	Chalcone Synthase	**-23.0**	-3.2
PtpAffx.22318.1.S1_at	Chlorophyll A/B-Binding Protein	**-23.0**	-6.1
Ptp.5264.1.S1_s_at	Myo-Inositol-1-Phosphate Synt	**-29.6**	-2.3
PtpAffx.204221.1.S1_at	Myb-Related Protein	**-31.0**	-10
Ptp.4267.1.S1_at	Acid Phosphatase Activity	**-34.0**	-2.5
PtpAffx.140781.1.A1_at	Isopentenyltransferase	**-49**	-1.3
Ptp.6057.1.S1_at	Anthocyanidin Synthase	**-39.4**	-1.4
PtpAffx.162261.1.S1_s_a	Acid Phosphatase Activity	**-42.5**	-3.8
Ptp.2165.1.S1_at	Chlorophyll-A/B Binding Protein	**-46.9**	-5.0
PtpAffx.7896.3.S1_a_at	Chalcone Synthase	**-55.5**	-2.4
PtpAffx.6733.4.S1_s_at	Carbonic Anhydrase,	**-56.0**	-8.5
Ptp.4730.1.A1_s_at	Phenylalanine Ammonia-Lyase	**-66.1**	-7.0
Ptp.845.1.S1_at	Nutrient Reservoir Activity	**-78**	-1.1

S: Sensitive, R: Resistant, CTRL:control (well watered) SDL: Severe Drought level

Transcriptional profiling of this study identified two Asparagine Synthase (Asn) genes (Table 3.6) that were highly induced (229 and 6-fold) in only the sensitive genotype. This result highlighted the enhanced degradation of proteins and nutrient remobilization from abscisied leaves to the sink organs in the sensitive genotype. Previous studies also indicated strong correlation between protein degradation and remobilization in defoliated leaves drought stress (Tassi et al., 1992; James et al., 1993; Moriyasu and Ohsumi 1996). From these results, it is highly possible to correlate ASN genes with drought sensitivity which can be used as a genetic marker to identify drought sensitive genotypes in the black poplar breeding programme.

Under drought stress electrolyte leakage of the sensitive genotype was found to be always higher than the resistant genotype. This leakage in electrolytes always associated with membrane senescence or degradation under drought conditions. This finding could also be associated to the enhanced activity of lipases and lipid degradation. Transcriptome and gene annotation analysis of drought treated black poplar genotypes identified 143 differentially expressed genes involved in fatty acid and lipid metabolism (Supplementary excel file 3/ sheet 9). Transcriptional analysis revealed insignificant increase or significant down regulations in the expression of genes involved in lipid synthesis in the resistant genotype. On the other hand, the identified transcripts that have a function in fatty acid degradation such as 3-Ketoacyl-CoA Thiolase and Acyl-CoA Oxidase, were highly induced in the sensitive genotype. Strong induction of the glutathione S-transferases (GSTs) genes in only sensitive genotype could be also attributed to detoxification of lipid peroxidation in abscised leaves of the sensitive genotype. Similar relationships between leaf senescence and increased gene expression of lipases, fatty acids and GST enzymes were also reported in Buchanan-Wollaston et al,. (2005).

In the current study, another important significant difference between two genotypes was observed in the expression level of cell wall modification proteins. These proteins included Expansins, Xyloglucan Endotransglucosylase/Hydrolases (XTHs), Endo-B-1, 4-Glucanases (EGAses) and Pectin Methylesterases (PMEs) (Sasidharan et al., 2012). The expansins are important regulators of cell division in plants. They are believed to be stimulating cell wall extension by distracting interactions between hemicelluloses and cellulose. Therefore, they have a function in cell division, thus having a significant role in root, shoot elongation and abscission (Cosgrove, 2000). Transcriptomic comparison and gene annotation results identified ten differentially expressed Expansin genes in the black poplar genotypes (Supplementary excel file 3/sheet 10). The expression levels of eight genes were started to be up-regulated at MDL point, continued to increase in SDL and reached their highest values at PDR in the sensitive genotype. In contrast, differential expression level of these genes in the resistant genotype was found to be small or non-significant. However, two Expansin genes were recorded to strongly down regulated in the resistant clone despite their up regulation in the sensitive genotype (Table 3.6). Up regulation of Expansins under drought was mostly observed in resurrected plants and associated with higher flexibility of the cell wall for shrinkage of the cells without loss of cellular integrity under water deficit (Sasidharan et al., 2012). However, in the present study, up regulation of Expansins was highly associated with drought induced leaf abscission. Among the other cell wall related genes, EGAases exhibited similar down regulation in both genotypes.

Gene annotation results identified 20 and 32 probe sets for XTHs and PMEs, respectively (Supplementary excel file 3/sheet 10). Almost all these probe sets were down regulated in the resistant genotype, whereas 9 XTHs and 20 PMEs were up regulated in the sensitive genotype. Similar drought induced degradation of xyloglucans and enhanced activity of cell wall degrading enzymes were also reported in previous studies (Sasidharan et al 2012, Scheible 2004).

3.4.1.3 Differential Expression Level of Antioxidant Enzymes Were Similar In Both Genotypes

As it was mentioned in the result section, the sensitive genotype thought to have a drought evading strategy which is characterized with almost complete leaf abscission to decrease water transpiration area and to escape from drought period in a dormant state. Therefore, highest increase in H_2O_2 level and antioxidant enzyme activities could be related with leaf abscission signalling. Sakamoto, (2008) concluded that environmental stresses such as drought could be associated with excessive production of reactive oxygen species (ROS) in the leaf cells and subsequent activation of abscission signalling. Before a leaf is abscised, nutrient and nitrogen remobilization should be completed. Therefore, before complete remobilization, production of ROS and their destructive effects should be controlled with enhanced activity of antioxidants to prevent cell from premature death (Woo et al., 2004). Drought depended higher activities in especially SOD and APX enzymes in the sensitive genotype could be related with delayed senescence for nutrient transfer from leaves to the other sink organs. On the other hand, leaf abscission was very limited in the resistant genotype, indicating lesser production of ROS and lesser need of their removal under drought stress.

The gene expression levels of all enzymes found in ascorbate glutathione cycle except DHAR were also significantly altered due to drought stress. The transcriptional comparison of the genes functional in APX, GR and SOD production were found to significantly up regulated in both genotypes. Furthermore, their transcriptional changes were found to be similar to their activity levels. The gene responsible in DHAR production was not included into differentially expressed gene list. Most probably drought induced changes in DHAR expression level was not significant so that it could not pass the filtering process of microarray data (± 2 fold and $p< 0.05$). Catalase is another important antioxidant enzyme converting hydrogen peroxide into water. The expression level of catalase gene was found to be increased 4.5 and 6.2 fold in the resistant and the sensitive genotypes in SDL that exhibited the highest up regulation among other antioxidant enzymes.

In the current study, two genes encoding alternative oxidases (Flavin-Containing Amine Oxidase (AOX)) were strongly up regulated only in the drought treated leaves of the resistant genotype. The role of the AOX enzymes is thought to limit ROS formation by reducing the activity of the electron transport chain, thereby reducing oxidative stress in the mitochondria (Lim et al., 2006). Therefore, higher expression of such types of oxidoreductase genes could be attributed to strong tolerance of resistant the black poplar genotypes to drought stress.

3.4.2. Transcriptional Profiling of Drought Tolerant Genotype

The most discriminative trait between the drought resistant and sensitive black poplar clones was drought induced leaf abscission in this study. Resistant black poplar clone did not shed their leaves until severe drought level. After this period only yellowing was observed in the leaves of that genotype. In terms of drought induced defoliation, resistant genotype could be defined as non-senescent, as compared to the sensitive genotype. During the re-watering period the colour of flavescent leaves turned into green colour indicating active production of chlorophyll and regaining of photosynthetic capacity. Therefore, the genes that are related with drought tolerance in black poplar clones were expected to be related with leaf maintenance and leaf recovery.

3.4.2.1. Production of Bark Storage Proteins under Drought Stress Could Be Related With Delayed Leaf Senescence and Drought Tolerance

As stated before, leaves of the sensitive genotype strongly desiccated under drought stress, indicating degradation of proteins, lipids and carbohydrates for remobilization of these molecules into stem or roots. In terms of nitrogen remobilization, the resistant genotype was found to have a different strategy that could be highly related with delayed or non-senescent properties of resistant genotype. Microarray gene profiling of the resistant genotype identified three genes that were differentially expressed more than 100 fold only in the resistant genotype (Table 3.7, Figure 3.9). These three genes were annotated to bark storage proteins (BSPs) which are important molecules in seasonal nitrogen and carbon trade-off between storage and mobilization. This means that instead of complete degradation of leaf cells for nutrient and nitrogen remobilization, resistant genotypes produced some storage proteins that can be used during drought and re-watering periods. Therefore, we suggested that the resistant black poplar genotype maintained the leaves to keep production of these storage molecules as long as possible under drought period. Under drought period, without degradation of important cellular proteins, membranes or cell wall molecules, the activity of the leaves regained easily by just synthesizing chlorophyll molecules to regaining photosynthetic activities. Furthermore, BSPs that stored during drought period could be used as an energy source in re-watering period. This could be the explanation for the fast recovery ability of the resistant clones under post drought recovery period. Therefore, we strongly recommended the usage of these storage molecules as a marker to select drought tolerant genotypes. The mobilization of these storage proteins has already been related to drought response in oak (Chantuma et al., 2009). In the leaves of *Populus* species under water stress, Plomion et al., (2006) and Durand et al., (2011) observed an accumulation of BSPs suggesting that the mobilization of nitrogen and carbohydrates stored in BSP may allow the cambium to remain active in spite of drastic limitation of photosynthesis and the consequent shortage of photosynthesis. In addition to increase in BSPs, a reduction of expression (50 fold down regulation) of the genes related with thioredoxin h production in only the resistant

genotype (Figure 3.9, Table 3.7) was also attributed to enhancement of the BSP content under drought stress. Thioredoxin h was investigated in several plant systems, including seed, phloem and pollen. Thioredoxin h was suggested to be reducing the major seed storage proteins, thereby enhancing their susceptibility to proteases (Gelhaye et al., 2004). Therefore, significant reduction of these genes under drought conditions and up regulation (5 fold up regulation) of these molecules under post drought recovery periods supported to their roles in BSPs production and degradations.

3.4.2.2 Heat Shock Proteins Could Be the Main Responsible Molecules in Drought Tolerance of Black Poplar

During the transpiration process, excess heat is also removed from the leaves by water vapour removal. Less transpiration due to stomata closure means low cooling of the leaves and less gas exchange and transportation of nutrients (McDowell et al., 2008). However, under drought conditions, ABA dependent closure of stomata inhibits both transpiration and evaporative cooling of the leaves. Drought dependent heat stress affects the metabolism and structure of plants, especially cell membranes and many basic physiological processes such as photosynthesis and respiration (Wahid et al., 2007). Under increased heat stress, protecting the cellular membranes and enzymes especially functioning in photosynthesis could be the key point for drought tolerance. Therefore, the induction and synthesis of heat-shock proteins (HSPs) under drought conditions are common phenomena in many plant species (Vásquez-Robinet et al., 2010). The HSPs play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, the HSPs aid in transporting proteins across membranes within the cell (Borges and Ramos 2005, Walter and Buchner 2002). Therefore, regulation of the HSPs like proteins functioning in protein folding and/or unfolding is crucial for cell survival under drought stress. Transcriptional comparison in the current study identified more than 60 probe sets related with heat shock proteins and chaperones (Supplementary excel file 3/sheet 11). Although all these genes were up regulated in both genotypes, fold changes of these genes were much higher in the resistant genotype (Figure 3.9 and Table 3.7). Additionally, three small heat shock proteins (sHSPs) and three chaperone 60 (Cpn 60) were significantly induced only in the resistant genotype. It has been reported that the sHSPs are often the first line of defence in the cell when proteins begin to miss fold (Waters, 2013). Cpn60 is also known as the RuBisCO subunit binding protein that have function in the assembly of RuBisCO in normal and stress conditions (Lindquist and Craig, 1988). All these results revealed that the drought tolerance of the resistant genotype mostly depends on the activities of the HSPs to maintain cellular mechanisms such as photosynthesis, respiration and water relations under drought stress.

Table 3.7 Resistant (R) specific 30 differentially expressed probe sets that showed highest up and down regulation under severe drought level (SDL). All the probe sets were significantly differentiated in resistant genotype (*P<0.05*) while their alterations in the sensitive (S) genotype was insignificant. Negative value indicates down regulation.

Probeset ID	**Gene Annotation**	**Fold (R-SDL vs. C)**	**Fold (S-SDL vs. C)**
PtpAffx.249.39.A1_x_at	Bark storage protein	**217**	-1.2
PtpAffx.59631.1.S1_s_at	17.6 Kda Heat Shock Protein	**172**	-1.1
PtpAffx.249.37.S1_x_at	Bark storage protein	**122**	1.2
PtpAffx.249.1.S1_x_at	Bark storage protein	**95.2**	1.1
PtpAffx.69240.1.A1_s_at	Flavin-Containing Amine Oxi	**82.7**	-3.2
PtpAffx.46615.1.S1_at	Heat Shock Protein 17	**58.3**	1.3
Ptp.4187.1.S1_at	Heat Shock Protein 17	**50.7**	-1.3
PtpAffx.95224.1.A1_at	Purple Acid Phosphatase	**50.0**	2.8
Ptp.2176.1.S1_at	Dnaj Protein	**37.9**	-2.8
Ptp.1442.1.S1_x_at	Fructose 1 phosphate aldolase	**30.4**	-1.3
PtpAffx.69240.1.A1_at	Glutathione S-Transferase	**24.6**	-3.3
PtpAffx.22667.1.A1_at	Beta-Galactosidase Acti	**17.7**	1.1
PtpAffx.4533.1.S1_at	Phosphoglycerate Kinase	**16.0**	-1.4
Ptp.7112.1.S1_s_at	Phosphoglycerate Kinase	**13.0**	-1.3
PtpAffx.216099.1.S1_at	Glycine-Rich Protein	**10.2**	1.3
PtpAffx.224932.1.S1_a_a	Terpene Synthase	**-18.6**	-1.2
PtpAffx.3539.1.S1_a_at	Terpene Synthase	**-18.7**	-1.5
PtpAffx.209542.1.S1_at	Hydrolase Ester Bonds	**-19.3**	1.5
PtpAffx.101017.1.A1_at	Terpene Synthase Activity	**-21.4**	-4.2
PtpAffx.209038.1.S1_s_a	Xylosidase	**-26.2**	-1.1
Ptp.2909.1.A1_s_at	Limonene Cyclase	**-27.6**	-5.4
PtpAffx.120846.1.A1_s_	Expansin	**-28.7**	-7.1
Ptp.7001.1.S1_at	Endochitinase	**-34.4**	-8.8
PtpAffx.88104.1.A1_at	Glutaredoxin	**-36.6**	-13
PtpAffx.44523.1.S1_a_at	Thioredoxin	**-54.5**	-1.2
Ptp.1264.1.S1_s_at	Thioredoxin	**-55.4**	-1.5
PtpAffx.9509.1.A1_s_at	Response To Karrikin	**-62.7**	-13
PtpAffx.1770.3.S1_s_at	Pathogenesis-Related Proteins	**-144**	-11
Ptp.6838.2.S1_a_at	Thaumatin-Like Protein	**-203**	2.5
PtpAffx.40283.1.S1_at	Putative Aldose Epimeras	**-232**	-17

A group of proteins called late embryogenesis abundant (LEA) like proteins were also reported to be synthesized during water stress. These LEA-like proteins are highly hydrophilic, glycine-rich and highly soluble. They have been found to be regulated by ABA (Xiong and Zhu, 2002). The LEA-like proteins are thought to act as chaperones, protecting enzymatic activities (Reyes et al., 2005), preventing misfolding, and denaturation of important proteins (Xiong and Zhu, 2002). In the current study, 15 probe sets were annotated to LEA-like proteins (Supplementary excel file3/sheet 11). Similar to the HSPs expressions, all LEA proteins were highly expressed in the resistant genotype.

McDowell et al (2008) suggested that under water deficit, for preventing stomatal closure can cause constraints on CO_2 diffusion which leads to reduction in photosynthesis thus decrease carbohydrate production. However, respiratory consumption of stored carbohydrates continued during the drought to maintain plant metabolism end energy consumption even if the growth is completely inhibited. Under prolonged drought conditions ongoing demand for carbohydrates under reduced photosynthetic rate results in depletion of the carbohydrate thus reserves leading to starvations of the plants. Therefore, there should be some genes responsible for maintaining energy metabolism in the resistant black poplar genotype. Transcriptional comparison in the current study revealed some genes that have phosphatase domains such as fructose Bisphosphate Aldolase and Phosphoglycerate Kinase which were highly induced especially in the resistant black poplar genotype (supplementary excel file 3/sheet 12). Due to their roles in glycolysis, up regulation of these genes attributed to energy maintenance in the leaves of the resistant genotype under drought stress.

CHAPTER 4

GENERAL DISCUSSION

Drought stress is known to be one of the most important constraints leading declines of forest area globally. Alterations in global climate and precipitation regimes strongly influence forest distribution and survival in recent years. Trees are long-lived sessile organisms and are always compelled to withstand rapidly to changing environments over their lifetimes. Therefore, they evolved many physiological adaptation strategies against drought stress. On the other hand, identification of drought adaptation strategies and understanding the effects of drought stress on especially tree species is difficult. Molecular and genetic mechanisms providing tolerance to drought are not revealed completely in tree species. In this respect, due to their fast growth, wide geographic adaptation and full sequenced genome, poplar species are one of the most suitable plant species to investigate the drought related genetic architecture of trees and their drought tolerance.

Black poplar is an economically important tree species in Turkey. With its breeding program, selection of many clones of the species from all around Turkey created a wide clonal collection, which enables researchers to select genotypes suitable for any type of stress condition. Therefore, black poplar (*Populus nigra L.*) species was selected to investigate drought adaptation strategies of the trees in the present study. For this purpose, first drought assessment was done in a field trail established with 300 black poplar clones. According to morphological and physiological measurements we identified three different drought adaptation strategies to drought stress in black poplar. Two clones per adaptation strategy was transferred to greenhouse to investigate relationships between these strategies with physiological, morphological and biochemical (antioxidant enzyme activities) traits. According to the results taken from both field and greenhouse trial these adaptation strategies defined as drought evading, dehydration tolerance and dehydration avoidance.

Drought evading strategy could also be defined as drought sensitivity. The clones that have evading strategy characterized with fast growth under well watered conditions. However, when they encountered with drought stress, the radial and shoot growth stopped, electrolyte leakage from leaf cells increased and almost all the leaves were desiccated at the end of the drought stress. Antioxidant enzyme activities and hydrogen peroxide contents were found to be always higher in these sensitive clones indicating higher production of reactive oxygen species and increased need of removal from these oxygen species.

Black poplar clones that have dehydration tolerance could also be considered as the drought resistant genotypes. Although the growth of these clones almost completely stopped under drought stress, leaf desiccation and shedding was very limited in these clones. Only flavescent leaf formation was observed most of which turn into green colour during post drought recovery period. The growth of these clones under re-watering period was much better than the drought sensitive clones. Antioxidant enzyme activities were not increased as much as drought sensitive clones indicating lower effects of drought on these dehydration tolerant genotypes. Therefore, this strategy defined with protection of the leaves during the drought stress and fast growth under re-watering period.

Dehydration avoidance was another black poplar strategy that was defined also as moderate drought resistance. The most important mechanism in this strategy was maintaining high leaf water status even under severe drought. The clones that have this strategy selectively shedded older leaves under stress to keep high turgor (high LWP) in the younger leaves. These dehydration avoided clones also have decreased water use efficiency (*i.e*, slow growth and smaller leaf size) to reduce transpiration rate under drought. Antioxidant enzyme activities and hydrogen peroxide content of dehydration avoided clones increased under the drought stress. However, their activity increase stay below drought sensitive clones like dehydration tolerant ones. This situation explained with the limited effects of drought on the drought avoided clones, due to the high leaf water content.

After identification of drought adaptation strategies in black poplar clone collection, two clones having dehydration tolerance (N.62.191-Resistant) and drought evading strategy (N.03.368.A-Sensitive) were selected for microarray based transcriptional profiling to explore genetic basis of drought tolerance and sensitivity in black poplar. The leaf samples collected for physiological and biochemical analysis during well-watered period (Control=CTRL) Moderate Drought Level (MDL), Severe Drought Level (SDL) and post drought recovery period (PDR) were used for microarray studies.

In the current study, the sensitive genotype was characterized with severe defoliation and loss of leaf water content under drought stress. Therefore, the sensitive genotype specific genes were mostly annotated to leaf senescence and cell death. The highest expression of the genes such as NAC3, and ap2/erf transcription factors in the sensitive genotype indicated a potential role of Jasmonic Acid and Ethylen hormones in controlling the leaf senescence. Expression of genes involved in proteolysis (Endopeptidase), cell wall degradation (Expansin) and carbohydrate catabolism (beta-galactosidase) in the sensitive genotype associated with nutrient mobilization processes.

Contrary to the sensitive genotype, the drought resistant black poplar genotypes protected its leaves from destructive effects of drought. To inhibit leaf senescence, the resistant genotype should reduce its growth to increase water use efficiency and to use produced sugar to feed

energy metabolism. Therefore major decrease in Terpenoid Synthase and Limonene Cyclase could be associated with decrease in growth due to their combination with phenylpropanoids. Contrary to the sensitive genotype, cell wall modification related genes such as Expansin and Xylosidase were significantly down regulated to inhibit both growth and cell wall degradation. The resistant genotype specific up regulation in bark storage proteins were suggested to be major factor in black poplar drought tolerance. These molecules were suggested to be responsible in delayed senescence and fast recovery ability of the resistant black poplar genotypes. The highest up regulation in chaperons such as Heat Shock Proteins and Dnaj Proteins only in the resistant genotype were also associated with the drought tolerance of black poplar. Chaperons required for protection of cell membrane and protein-protein interactions which are required for folding and assisting in the establishment of proper protein conformation. Another important strategy to cope with drought stress is regulation of energy metabolism due to the decrease in photosynthesis. Transcriptional profiling of tolerant genotype indicated that the highest up regulated probe sets were Fructose Bisphosphate Aldolase and Phosphoglycerate Kinase that have phosphorylase domain which could be functional in glycolysis/glycogenesis.

CHAPTER 5

GENERAL CONCLUSION

Due to its economic and ecologic importance, considerable progress have been scheved in conservation and breeding of black poplar in Turkey. Breeding studies accomplished by collection of best individuals from natural range of the black poplar species by selection of clones based on their adaptability and productivity in various test sites. Drought is known to be the major abiotic stress factor limiting plant production and development. Worldwide water shortage and ongoing climate change with global warming make improvement of drought resistance plant species crucially important. In the current study, to improve drought tolerance in black poplar, we investigated a number of drought related physio-biochemical processes at different stages of water availability to understand the adaptation mechanisms of the specie. The results of drought treatment both in field and greenhouse trials indicated that black poplar clones could be grouped in three adaptation strategies as drought evading (sensitive), dehydration avoidance (moderate resistant) and dehydration tolerance (resistant) for survival and growth under drought stress.

In the collection, the sensitive genotypes were found to comprise very small portion. There were a few clones found to have drought- evaded adaptations. These clones were found to enter into dormancy period under drought stress by complete defoliation. Therefore, they can withstand only mild drought conditions in short periods. However, this strategy contributes to fast growth rates under well watered periods. Therefore, these types of clones are very suitable for gallery plantations, which can be established in river basins.

The clones with dehydration tolerance strategy combine high productivity with drought tolerance. These types of clones comprised 10% of the black poplar clone collection. They were adapted to conditions where drought periods are prolonged. Therefore, the clones that selected from this group could be used in industrial plantations for wood and biomass production in especially arid and semi-arid zones of Turkey. Among the five commercial black poplar clones in Turkey, four of them (Gazi , Kocabey, Geyve, Behiçbey) included in this group. However, there are many clones in the collection that the productivity and drought tolerance properties are much better than these commercial ones.

The last adaptation strategy in black poplar collection was dehydration avoidance mechanism, which is associated with high capacity for drought tolerance. However, the clones that fell into

this group exhibited slow growth rates. The great majority of the black poplar clones (70%) were found in this group. Due to their slow growth rates, the usage of these types of clones in the plantations is not recommended. However, existence of these moderate drought clones enhances genetic diversity of breeding programme that enables researchers to select genotypes that can resist any other biotic or abiotic stress factors.

Transcriptional comparison of drought tolerant and the sensitive black poplar genotypes indicated many genes that can be used as a marker to select drought tolerant and sensitive genotypes. Results of microarray data enabled us to make a suggestion about what is happening in the resistant and the sensitive black poplar genotypes under drought stress.

The most significant differences between these two genotypes were observed in the leaf abscission trait under drought stress and growth performances under post drought recovery period. Sensitive black poplar indicated severe desiccation rate at the early stage of drought (MDL) and complete defoliation at severe drought level. This genotype was suggested to have drought evading strategy which is characterized with complete defoliation under drought stress to enter into dormancy period as much as possible. By this way, sensitive genotype tried to protect itself from destructive effects of drought stress. Therefore, transcriptional profiling of the sensitive genotype revealed many sets of genes related with leaf abscission. The most significant up regulation in the sensitive genotype was observed in the production ethylene and jasmonic acid hormones which are responsible in regulation of many transcription factors. The activity of these transcription factors were suggested to induce many proteases, lipases and cell wall degrading enzymes all of which are functional in leaf senescence. During the leaf senescence enhanced degradation of proteins is generally increases ammonia content in the leaf cells. Highest up regulation in asparagine synthase (ASN) genes in only sensitive genotype enabled us to make a suggestion that the ammonia molecule was converted into asparagine in drought treated leaves and used for nitrogen remobilization from the abscised leaves to the sink organs in the sensitive genotype.

In fact, nitrogen remobilization from sheded leaves is a known phenomenon in especially perennial plants like poplars. However, in the current study, the resistant black poplar genotype exhibited different nitrogen remobilization strategy than the sensitive genotype. As it was mentioned before, drought resistant genotype exhibited very limited leaf abscission even under severe drought level. The leaves of the resistant genotype turn into yellow colour under prolonged drought conditions but not defoliated completely. In re-watering period most of these flavescent leaves regained their green colour. The growth of resistant genotype was very fast in re-watering period that it completely recovered itself in a very short time. Transcriptional profiling of the resistant genotype exhibited completely different genetic regulation. Instead of induction in the ethylene and their corresponding transcription factors, many chaperons genes such as heat shock proteins and LEA proteins were stimulated at the early stage of the drought (MDL) in the leaves of resistant genotype. We suggested that these chaperons protected cellular proteins functional in photosynthesis, respiration, translation and many other metabolic systems.

Similar to asparagine synthase gene in sensitive genotype, drought resistant black poplar induced bark storage protein genes which are functional in nitrogen remobilization in drought treated leaves. However, different than the asparagine synthase, these bark storage proteins does not require protein degradation and ammonia formation. It is known that these storage proteins were synthesized in defoliated leaves in autumn, then remobilized and stored in the cells located under the bark tissue. During spring, these proteins were transferred to the buds after dormancy break to be used as nitrogen and nutrient source in newly produced leaves. From this information, we can suggest that under drought conditions, the resistant genotype synthesized bark storage proteins in the leaves and remobilized them in to the stem. Accumulation of BSP in the stem allowed the cambium to remain active in spite of drastic limitation of photosynthesis and the consequent shortage of carbohydrates. Therefore, xylem cavitation or drought dependent terminal bud desiccation, which were seen in the sensitive genotype, were not observed in resistant genotype. With the active cambium and not abscised leaves, the resistant genotype regained its growth performances easily under the re-watering period. During the post drought recovery period, BSPs were suggested to be remobilized into the leaves to be used as nitrogen and nutrient source during the synthesis of chlorophyll. The function of the bark storage proteins in drought tolerance was firstly indicated in this study. However, regulation of BSPs and their accumulation or remobilization should be followed in the stem cells to be sure about their functions in drought tolerance.

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APPENDIX A

TWO WAY ANOVA RESULTS PHYSIOLOGICAL MEASUREMENTS.

The mean of each trait and the 95% confidence intervals were also represented to compare statically changes of the trait between genotypes and treatments. MR, R and S represent moderate resistant, resistant and sensitive black poplar clones, respectively. The drought treatments were separated according to soil water content (SWC) of the pots and the effects on the plants. C: control (SWC:75%), D1: Mild drought level (SWC:50%), D2: drought acclimation level (SWC:35%), D3: Moderate drought level (SWC:20%), D4: Severe drought level (SWC:5%) RW1: Rewatered period first day. RW2: Re-watered period third day. RW3: re-watered period 7th day

Two-way ANOVA: LWP versus Genotype, Treatment

```
Analysis of Variance for Leaf water potential(LWP)
Source        DF        SS        MS         F        P
Genotype       2   175.752    87.876   114.27    0.000
Treatment      7  1185.235   169.319   220.18    0.000
Interaction   14   198.792    14.199    18.46    0.000
Error        264   203.016     0.769
Total        287  1762.795

                      Individual 95% CI
Genotype        Mean  -+---------+---------+---------+---------+
MR            -2.174                                    (--*--)
R             -2.796                          (--*--)
S             -4.052    (-*--)
                      -+---------+---------+---------+---------+
                   -4.200    -3.600    -3.000    -2.400    -1.800

                      Individual 95% CI
Treatment       Mean  ---+---------+---------+---------+--------
C              -2.05                                    (-*)
D1             -2.07                                    (-*)
D2             -2.63                                 (-*)
D3             -3.53                              (*-)
D4             -8.18    (*-)
RW1            -1.72                                     (*-)
RW2            -1.83                                    (-*)
RW3            -2.04                                    (-*)
                      ---+---------+---------+---------+--------
                     -8.00     -6.00     -4.00     -2.00
```

Two-way ANOVA: Radial Growth versus Genotype, Treatment

```
Analysis of Variance for Diameter
Source        DF        SS        MS        F        P
Genotype       2  0.039032  0.019516    20.08    0.000
Treatment      7  0.912066  0.130295   134.03    0.000
Interaction   14  0.059911  0.004279     4.40    0.000
Error        456  0.443301  0.000972
Total        479  1.454310

                      Individual 95% CI
Genotype        Mean  ------+---------+---------+---------+-----
MR            0.0566    (-----*-----)
R             0.0764                          (------*-----)
S             0.0580     (-----*------)
                      ------+---------+---------+---------+-----
                       0.0560    0.0640    0.0720    0.0800

                      Individual 95% CI
Treatment       Mean  --+---------+---------+---------+---------
C             0.1506                                        (-*-)
D1            0.0601                  (-*-)
D2            0.0731                     (-*-)
D3            0.0156       (-*-)
D4            0.0000    (-*-)
RW1           0.0473               (-*-)
RW2           0.0694                  (-*-)
RW3           0.0932                       (-*-)
                      --+---------+---------+---------+---------
                    0.0000    0.0400    0.0800    0.1200
```

Two-way ANOVA: Stem Height versus Genotype_1, Treatment_1

```
Analysis of Variance for Stem Height
Source        DF        SS        MS        F        P
Genotype       2    735.40    367.70    80.56    0.000
Treatment      7  26548.23   3792.60   830.90    0.000
Interaction   14    955.32     68.24    14.95    0.000
Error        456   2081.38      4.56
Total        479  30320.34

                      Individual 95% CI
Genotype        Mean  --------+---------+---------+---------+---
MR             11.61    (--*--)
R              14.56                                (---*--)
S              12.48            (---*--)
                      --------+---------+---------+---------+---
                          12.00     13.00     14.00     15.00

                      Individual 95% CI
Treatment       Mean  --------+---------+---------+---------+---
C              25.10                                         (*)
D1             19.30                                   (*)
D2             19.42                                   (*)
D3             10.27                  (*)
D4              2.11    (*)
RW1             4.57        (*)
RW2             8.80                (*)
```

```
RW3            13.49                        (*)
                       --------+---------+---------+---------+---
                             6.00      12.00     18.00     24.00
```

Two-way ANOVA: Electrolyte Leakage versus Genotype, Treatment

```
Analysis of Variance for EL
Source        DF        SS        MS        F        P
Genotype       2    293.75    146.88   143.89    0.000
Treatment      7    224.02     32.00    31.35    0.000
Interaction   14     90.44      6.46     6.33    0.000
Error        168    171.49      1.02
Total        191    779.71

                      Individual 95% CI
Genotype        Mean  -+---------+---------+---------+---------+
MR              3.77        (--*--)
R               3.42     (--*--)
S               6.20                                    (---*--)
                      -+---------+---------+---------+---------+
                    3.20      4.00      4.80      5.60      6.40

                      Individual 95% CI
Treatment       Mean  -+---------+---------+---------+---------+
C               3.31  (---*---)
D1              3.67      (---*---)
D2              4.10          (---*---)
D3              5.26                    (---*---)
D4              6.60                              (---*---)
Rw1             5.33                    (---*---)
RW2             4.03          (---*---)
RW3             3.41     (---*---)
                      -+---------+---------+---------+---------+
                    3.00      4.00      5.00      6.00      7.00
```

APPENDIX B

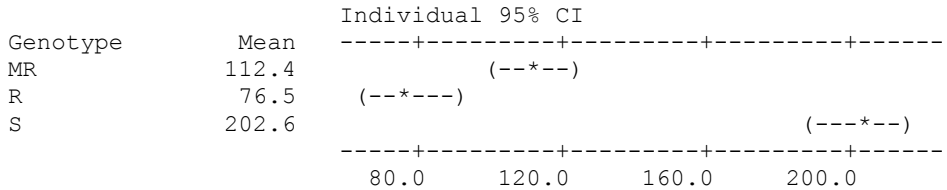
TWO WAY ANOVA RESULTS OF ANTIOXIDANT ENZYME ACTIVITIES AND HYDROGEN PEROXIDE CONTENT

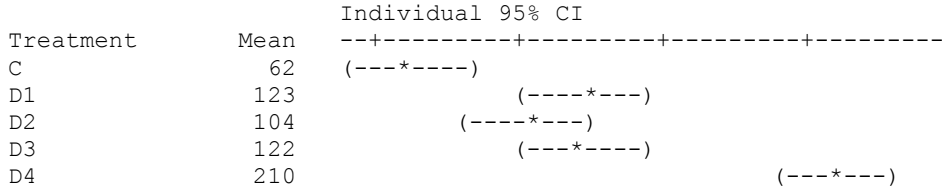
The mean of each trait and the 95% confidence intervals were represented to compare statically changes of the trait between genotypes and treatments. MR, R and S represent moderate resistant, resistant and sensitive black poplar clones, respectively. The drought treatments were separated according to soil water content (SWC) of the pots and the effects on the plants. C: control (SWC:75%), D1: Mild drought level (SWC:50%), D2: drought acclimation level (SWC:35%), D3: Moderate drought level (SWC:20%), D4: Severe drought level (SWC:5%) RW1: Re-watered period first day. RW2: Re-watered period third day. RW3: re-watered period 7^{th} day

---

Two-way ANOVA: Ascorbate Peroxidase (APX) versus Genotype, Treatment

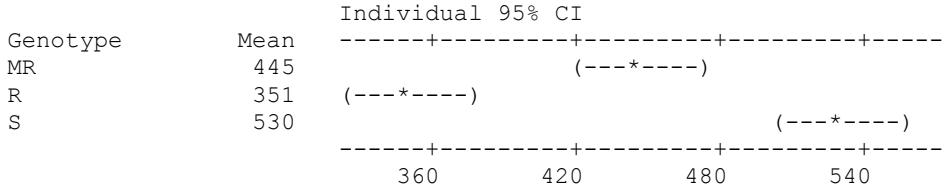
Source	DF	SS	MS	F	P
Genotype	2	540110	270055	89.01	0.000
Treatment	7	329319	47046	15.51	0.000
Interaction	14	279207	19943	6.57	0.000
Error	168	509719	3034		
Total	191	1658355			

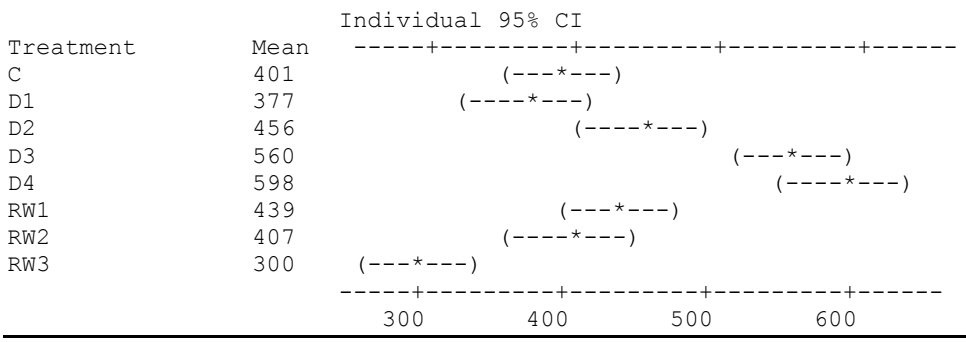




Two-way ANOVA: Superoxide dismutase (SOD) versus Genotype, Treatment

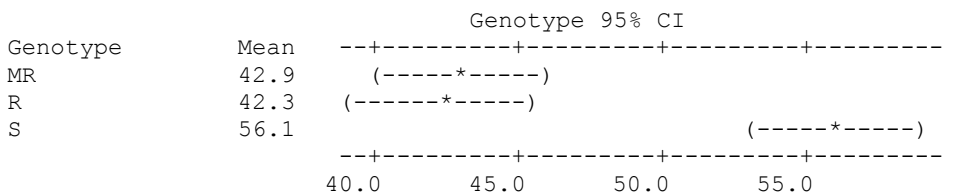
Source	DF	SS	MS	F	P
Genotype	2	1030454	515227	44.21	0.000
Treatment	7	1575888	225127	19.32	0.000
Interaction	14	416088	29721	2.55	0.002
Error	168	1958094	11655		
Total	191	4980523			

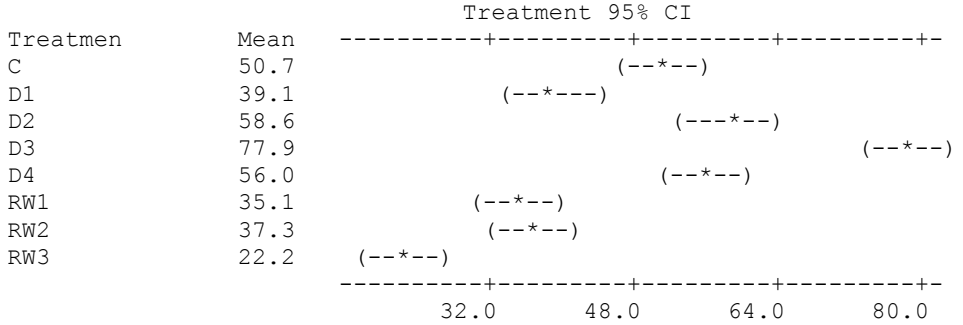




Two-way ANOVA:Hydrogen Peroxide (H_2O_2) Content vs. Genotype, Treatment

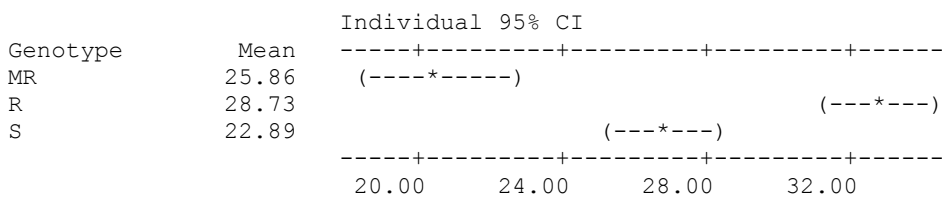
Source	DF	SS	MS	F	P
Genotype	2	7742	3871	24.63	0.000
Treatment	7	50365	7195	45.77	0.000
Interaction	14	3029	216	1.38	0.170
Error	168	26407	157		
Total	191	87542			

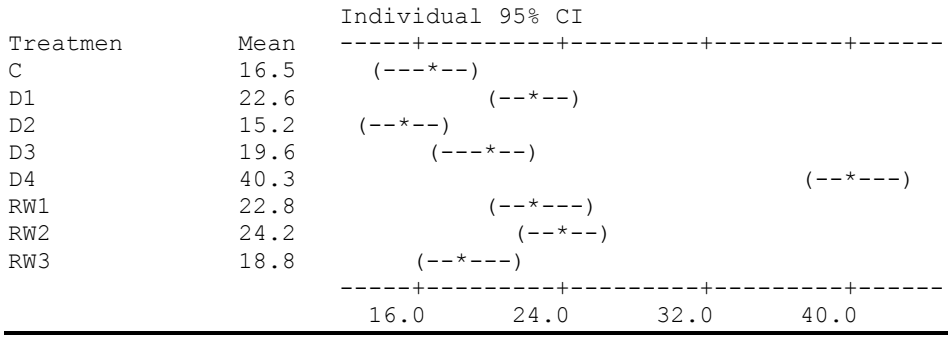




Two-way ANOVA: Dehydroascorbate reductase (DHAR) vs. Genotype, Treatment

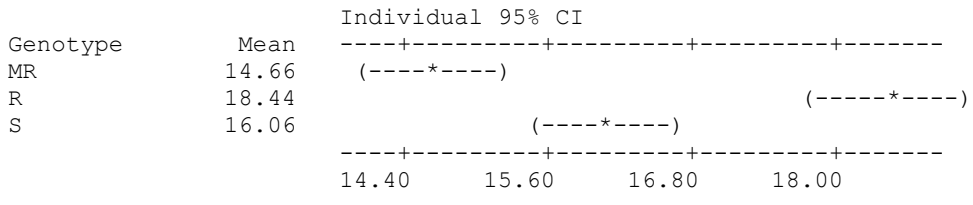
Source	DF	SS	MS	F	P
Genotype	2	5311.8	2655.9	65.52	0.000
Treatmen	7	10335.8	1476.5	36.43	0.000
Interaction	14	804.6	57.5	1.42	0.150
Error	168	6809.9	40.5		
Total	191	23262.0			

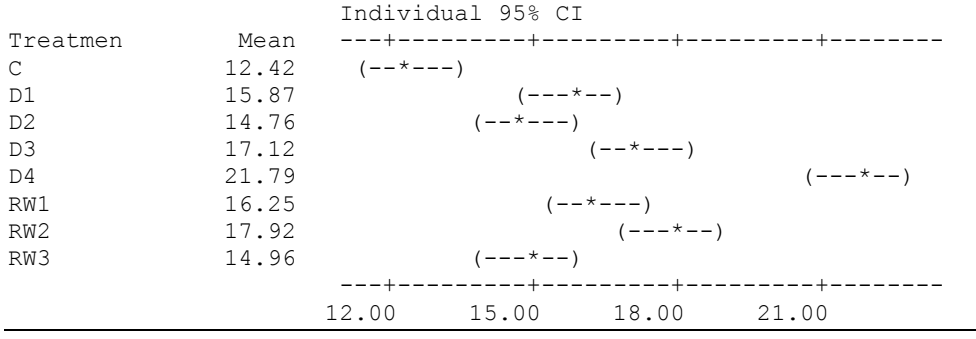




Two-way ANOVA: Glutathionine Reductase (GR) versus Genotype, Treatment

Source	DF	SS	MS	F	P
Genotype	2	465.39	232.69	35.61	0.000
Treatmen	7	1266.52	180.93	27.69	0.000
Interaction	14	325.83	23.27	3.56	0.000
Error	168	1097.89	6.54		
Total	191	3155.63			





APPENDIX C

SELECTED PROBE SETS FOR RT-PCR VALIDATION.

Table C.1. The probe sets were selected according to their up or down regulated fold changes (FC) in resistant (R) and sensitive (S) genotypes in Moderate Drought Level (MDL), Severe Drought Level (SDL) and Post Drought Recovery (PDR) period. The primer pairs for each probe and amplification length of PCR products were also represented.

	Probe Set ID	MDL (FC)	SDL (FC)	PDR (FC)	Forward (5'-3') primer	Reverse (5'-3') primer	Amplicon Size
Resistant N.62.191	**Up Regulated**						
	Bark Storage Protein	13.4	217	106.7	tgatgcaaaaactgccgata	aagtcgtggcgtaggaattg	194
	Down Regulated						
	Thioredoxin h	-32.0	-77	-7.3	ctgtgactctggggtttggt	ttagtcgcggaaatacatcg	106
Sensitive N.03.368.A	**Up regulated**						
	Asparagine synthase	4.5	229	1.0	gttagtgcttcgggagttgc	cacacgacttcaaaggacca	179
	Down Regulated						
	PhenylalanineAmmonia-Lyase	-51	-47	-1.6	cttggagctttgggatgtgt	aatgcggtaaccctcaacag	211

APPENDİX D

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PUBLICATIONS

Yildirim, K., Öztürk, H., Şiklar , S., Özgür, D.B., and Kaya, Z. 2011.Strong Genetic Control of High Wood Specific Gravity in Young Progenies of Pinus brutia:Potential of Early Selection for Industrial Plantations. Silvae Genetica 60 (6):249-258.

Yildirim, K., Öztürk, H., Şiklar , S., Özgür, D.B., and Kaya, Z. 2011.Strong Genetic Control of High Wood Specific Gravity in Young Progenies of Pinus brutia:Potential of Early Selection for Industrial Plantations. Silvae Genetica 60 (6):249-258.