GLUTATHIONE S-TRANSFERASE ACTIVITY AND GLUTATHIONE LEVELS IN DROUGHT STRESSED *PINUS BRUTIA* TEN. TREES GROWING IN ANKARA

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

GLUTATHIONE S-TRANSFERASE ACTIVITY AND GLUTATHIONE LEVELS IN DROUGHT STRESSED *PINUS BRUTIA* TEN. TREES GROWING IN ANKARA

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Turkish red pine is coastal tree and is a drought resistant pine that withstands more aridity and poor soils than most other timber species growing in the same climatic conditions. In Turkey, this species grows in southern and western Anatolia and is also found in the Marmara region.

Drought results in a water deficit in plant tissues, which, in turn, can lead to an imbalance in the redox poise of plant cells, and thus inducing oxidative stress in plants.

Resistance to conditions associated with oxidative-stress must, in part, rely on endogenous antioxidative defense mechanisms required to maintain cellular homeostasis. Glutathione is one of the major endogenous antioxidants in plants known to play an important role in plant defense mechanisms. Glutathione S-transferase (GST, EC 2.5.1.18) is a GSH dependent detoxifying enzyme in plants, which catalyzes the conjugation of GSH.

In this study, we investigated the changes in cytosolic glutathione S-transferase enzyme activity using CDNB as substrate and total thiol amount in *Pinus brutia* Ten., related to the drought stress during four months, June to September. The osmotic pressure in the needles was also determined as an indirect measure of drought condition. Together with the increase in the temperature values from June to July, GST enzyme activity increased from 15,78 ± 1,36 μmoles min^{-1} mg $protein^{-1}$ to 22,91 ± 1,99 μmoles min^{-1} mg $protein^{-1}$ which was statistically significant. However in August, GST activity had fallen to 16,54 ± 1,61 μmoles/min/mg protein, which may be because of a local rainfall at the beginning of the August in the sampling area. In September, GST activity significantly increased with respect to June, in accordance with high temperatures. The total thiol amount was not changed significantly during the sampling period. Although there were statistically significant changes in osmotic pressure in the needdles collected during the same sampling period, it did not exactly correlated to the changes in GST activity.

Keywords: *Pinus brutia*, drought stress, glutathione S-transferase, glutathione, total thiol

ÖZ

KURAKLIK BASKISI ALTINDAKİ, ANKARA'DA YETİŞEN *PINUS BRUTIA* TEN. AĞAÇLARINDA GLUTATYON S-TRANSFERAZ ENZİM AKTİVİTESİ VE GLUTATYON SEVİYELERİ

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Kızıl çam kıyı kesimlerde dağılım gösteren, aynı iklim koşullarında yetişen kereste ağaçlarına oranla fakir ve kurak topraklara daha iyi uyum sağlamış bir çam türüdür. Türkiye'de, Anadolu'nun batı ve güney kıyılarında, ve ayrıca Marmara Bölgesi'nde bulunur.

Su kısıntısıyla açığa çıkan kuraklık baskısı bitki hücrelerinde redoks dengesini bozar, ve böylece, oksidatif baskıyı tetikler.

Oksidatif baskı koşullarına karşı direnç hücre içi homeostasisi koruyan endojen antioksidan savunma mekanizmalarına bağlıdır. Glutatyon bitki savunma mekanizmalarında rol oynayan önemli endojen antioksidanlardan biridir. Glutatyon S-transferaz (GST, EC 2.5.1.18) glutatyon ile konjugasyonu gerçekleştiren, detoksifikasyon enzimlerinden biridir.

Bu çalışmada, Haziran Eylül arasında olmak üzere dört ay süresince gerçekleşen kuraklık baskısına bağlı olarak, *Pinus* brutia bitkisinde, CDNB substratı kullanılarak ölçülen sitosolik glutatyon S-transferaz aktivitesindeki ve toplam tiol miktarındaki değişimler araştırıldı. Kuraklık koşulunun dolaylı yoldan bir göstergesi

olarak iğnelerdeki ozmotik basınç değerleri de ölçüldü. Hazirandan Temmuza gerçekleşen sıcaklık artışı ile, GST enzim aktivitesi 15,78 ± 1,36 μmol dk^{-1} mg $protein^{-1}$'den 22,91 ± 1,99 μmol dk^{-1} mg $protein^{-1}$'e yükselmiştir. Bu artış istatistiksel olarak anlamlı bulunmuştur. Ancak, Ağustos'da, bu ayın başında, örnek toplanan bölgede gerçekleşen bölgesel yağışın yarattığı muhtemel etkiyle GST enzim aktivitesi 16,54 ± 1,61 μmol dk^{-1} mg $protein^{-1}$'e düşmüştür. Eylül'de, yüksek sıcaklığa bağlı olarak GST enzim aktivitesi Haziran ayına göre anlamlı şekilde yüksek bulunmuştur. Örnekleme periyodu boyunca, toplam tiol miktarında anlamlı bir değişim gözlenmemiştir. Aynı örnekleme periyodu içerisinde toplanan iğnelerde ölçülen ozmotik basınç değerleri arasında istatistiksel açıdan anlamlı farklılıklar gözlemlenmesine rağmen, GST aktivitesindeki değişimlerle doğrudan bir ilişki saptanmamıştır.

Anahtar Kelimeler: *Pinus brutia*, kuraklık baskısı, glutatyon S-transferaz, glutatyon, toplam tiol

To My Family...

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LIST OF ABBREVIATIONS

BSA Bovine Serum Albumin

CDNB 1-Chloro-2, 4-dinitrobeenzene

EDTA Ethylenediaminetetraacetic acid

GSH Reduced Glutathione

GSSG Oxidized Glutathione

GR Glutathione Reductase

GST Glutathione S-Transferase

GPX Glutathione peroxidases

H_2O_2 Hydrogen peroxide

NaOH Sodium hydroxide

NaCl Sodium chloride

PVP-K30 Polyvinylpyrrolidone K30

ROS Reactive oxygen species

Tris Tris (hydroxylmethyl) aminomethane

CHAPTER I

INTRODUCTION

1.1. Properties of the sample organism: *Pinus brutia* Ten

1.1.1. Taxonomy

Pinus brutia Ten (Turkish red pine) is a member of Division Gymnospermae, Class Coniferae, and Family Pinaceae, Genus Pinus L. Some botanists have indicated that *Pinus brutia* was a variety of Aleppo pine (*Pinus halepensis* Mill.) but the latest researches stated its classification as a separate species. Turkish red pine is coastal tree and is a drought resistant pine that withstands more aridity and poor soils than most other timber species growing in the same climatic conditions. Although it requires mild winters, some of its provenances can grow successfully, out of its natural habitat, on sites of drier and cooler climate (The Sylvicultyre of P. brutia in Turkey).

1.1.2. Morphology

The Turkish pine is a tree to 27-35 m, with a usually open crown of irregular branches. The bark on the lower trunk is thick, scaly, fissured, patterned red-brown and buff, and thin, flaky and orange-red higher in the crown. The shoots are slender, 3-7 mm thick, grey-buff, and rough with persistent small decurrent scale-leaf bases. The winter buds are ovoid-acute, with red-brown scales with long free tips revolute and fringed with white hairs. The adult leaves are retained for 1.5-2.5 years, with a persistent 1-1.5 cm sheath; on most trees they are in fascicles of two, and 10-18 cm long. They are bright green to yellow-green, slender, about 1mm thick, with serrulate margins, fine lines of stomata on both faces, and several marginal resin canals. The juvenile leaves are glaucous, 1.5-4 cm long, and continue to be grown for 2-4 years, mixed with the first adult foliage produced from 9 months from seed. The cones are erect to forward pointing on short stout stalks,

symmetrical, broad conic, (4-)6-10(-12) cm long, 4-5 cm broad when closed, green, ripening shiny red-brown in April two years after pollination. They open the same summer or 1-2 years later, to 5-8 cm broad, though the seeds are often not shed till winter rain softens the scales. The scales are short, broad, thick, woody, and very stiff; the apophysis is 10-15 x 15-20 mm, smoothly rounded, with a slight to moderate transverse ridge; the umbo is dorsal, flat to slightly raised, 5-7mm wide, and grey-buff. The seeds are grey-brown, 7-8 × 5mm with a broad, auricled 15-20 × 10 mm wing, yellow-buff streaked darker brown (Tenore, 2004)

1.1.3. Importance for Turkey

Pinus brutia shows a relatively restricted distribution, especially in the Mediterannean region. It is distributed to Italy, Greece, Turkey, Cyprus, Syria, Lebanon, Jordan, Palestine, and the many islands of Aegean and Mediterannean, northern Iraq and over the north coast of Crimea (The Sylviculture of P. brutia in Turkey).

In Turkey, this species grows in southern and western Anatolia and is also

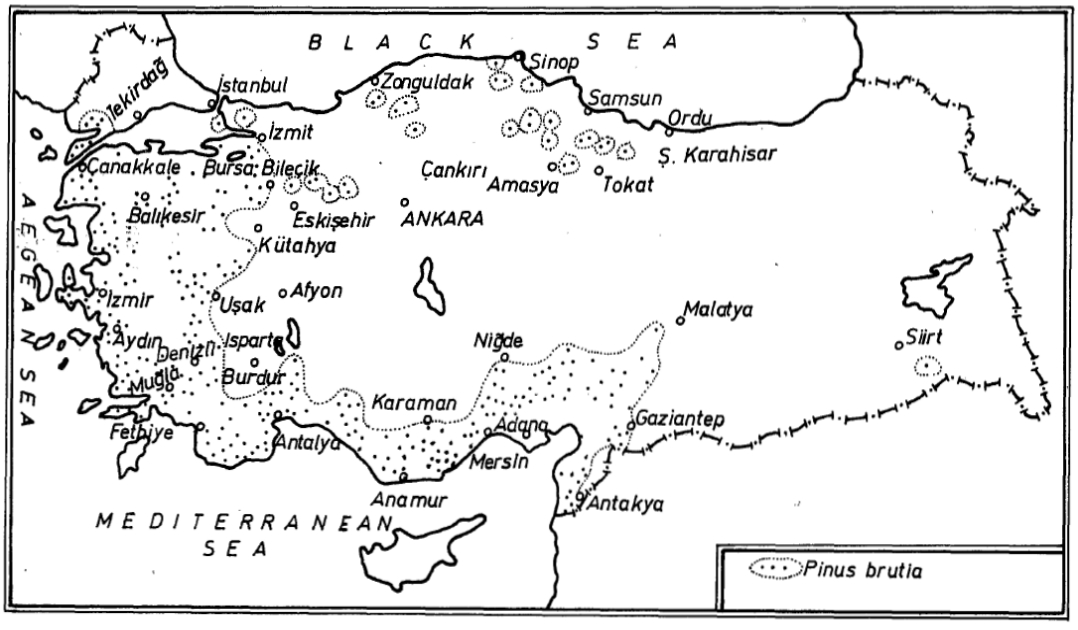

Figure 1.1. *Pinus brutia* distribution in Turkey (Gezer, A., CIHEAM)

found in the Marmara region. It is rarely found on the coastal areas of interior Black Sea region at the bottom of the drier and warmer valleys, too (Figure 1.1.).

The species grows from sea level to the elevation of 1300 meters in south; the upper limit reaches to some points 1650 meters in this region. However, it is not common to observe this tree above 900 meters in the west.

The climate throughout the growing range of *P. brutia* is predominantly mild during whole year and the annual precipitation is adequate, but it is poorly distributed. During the growing season, long period of drought may occur and the temperature may be very high. Because of the fact that the moisture is adequate, it partly compensates the effect of temperature. Frost is rarely seen. The botanical range also takes some cooler local areas of relatively high precipitation rates in southwest and interior parts of Taurus Mountains, in its boundaries within the limits of its range, *P. brutia* is found in localities which have precipitation as low as 412.8 mm annual average and only 128.4 mm average in its growing-season. Throughout the chief commercial range (Muğla and Antalya regions), the average annual precipitation is 1068.9 mm to 1220.9 mm, with 251.3 mm to 353.9 mm falling during the growing season (Gezer, A., CIHEAM).

For this species, the average annual temperature range is 12.5^{o}C to 22.9^{o}C. The maximum annual average is 18.1^{o}C to 22.7 ^{o}C and the minimum is 1.4 ^{o}C to 8.1 ^{o}C.

The species is a dominant forest tree in Turkey (Fig. 2), especially in Aegean forest region. It forms large pure forests after the maquis belt which covers large areas below 400 meters along the coast (Gezer, A., CIHEAM).

Pinus brutia Ten. is an economically important forest tree species for Turkey; its wood is an important export good as travers. In addition, Turkish red pine is used in local needs like in packing industry and in resin gum production (Aslan, 1994).

The factors affecting the management of *Pinus brutia* forests in Turkey are various: illegal cutting, entomological factors, forest fires, extensive grazing, diseases and etc. Among these factors, the most critical one is the fires.

Ağaç Türü	Alanı(ha)
Meşe	6,088,379
Kızılçam	4,167,524
Karaçam	3,302,650
Kayın	1,335,644
Ardıç	1,234,162
Sarıçam	1,018,475
Göknar	619,786
Sedir	336,341
Ladin	286,666
Kızılağaç	109,504
Kestan	99,433
Gürgen	99,300
Toplam	18,697,864

Kaynak : OGM-APK Bülteni (Kasım 2001)

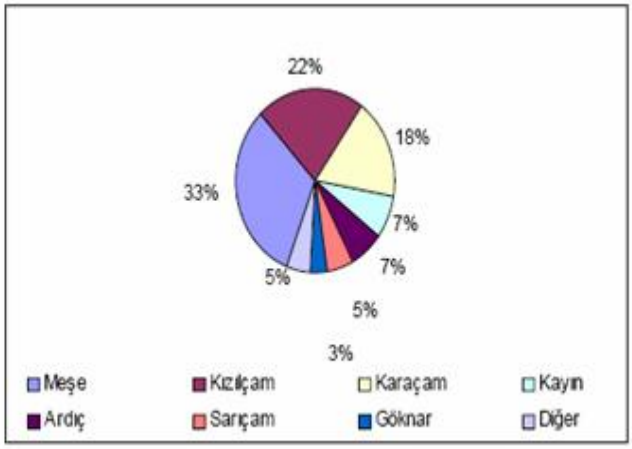

Figure 1.2. Distribution area percentages of forest trees in Turkey; *Pinus brutia* (Turkish red pine) covers 4,167,524 ha of total area (approximately 22% of all forests of Turkey) (Türkiye Çevre Atlası, 2004)

1.2 Stress Conditions and Their Effects on Plant Species

As a biological term, stress has more than one definition; so, it's better to apply the term stress in its more general sense as an "overpowering pressure of some adverse force or influence" that tends to inhibit normal systems from functioning. In general the use of the term stress should be restricted to the description of the influences rather than the responses (Shorter Technological Dictionary).

In many cases, if the effects of specific environmental factors are studied, it is appropriate to replace the general term stress by the appropriate quantitative measure (e.g. soil water content or water potential) together with an appropriate

measure of the plant response (e.g. growth rate, or the change in the specific activity of an enzyme).

Plant species are different from each other in the case of their optimal environments and their susceptibilities to particular stresses. According to some scientists working in this area, any environment is stressful if only it damages the plants and causes a qualitative change such as membrane damage or cell death, while others concludes that a stressful environment forces the plants to an increase in energy expenditure or decrease in the potential energy of the system (Jones et al, Plants Under Stress, Cambridge University Press).

A further difficulty is that stress factors do not usually operate alone. Stress may have greater effect during certain phases of the plant's life cycle than others. Moreover, because of the complexity of biological responses makes it difficult to understand the cause and effect easily. To illustrate, the lack of soil humidity is an environmental stress that can cause a water deficit in certain cells of a plant. This cell water deficit may be regarded as a stress if it affects certain metabolic processes. This is why the term "water stress" is widely used although it does not conform to the favorite definition of the stress. Similarly, salinity or low temperature stresses may give rise to cellular water deficits that may be the immediate cause of many observed symptoms. In conclusion, it should be appropriate to consider different factors as stresses at different levels of organization for plant species (Jones et al, Plants Under Stress, Cambridge University Press).

There are several aspects of different environmental stress that either have common features or the plant responses or adaptations to those stresses may have common components or indicate general principles.

To predict the effect of stress on plants we need to know something about

(i) the temporal variation on stress
(ii) the plant's potential to acclimatize to stress
(iii) interaction between different stresses and the plant responses

Although some stress conditions such as water deficit, freezing and salinity may act through a common mechanism, in general, tress can be very dissimilar in nature. In other cases, different stresses may occur together; for example, high temperature and drought. Furthermore, different stresses can interact, both in their occurrence and in their effects. Because of this complexity, it is not so meaningful to pursue the theme of common features of different environmental stresses. On the other hand, there is substantial evidence that plant responses to very different stresses with some common features.

Against a range of environmental stresses, plants or other organisms are subjected to the altered synthesis of special proteins. Many of these "stress proteins" are highly conserved across a wide range of organisms and in some cases the similar proteins can be induced by different environmental stresses (Jones ***et. al.***).

1.2.1 Drought Stress Physiology

Water is a fundamentally important component of the metabolism of all living organisms, facilitating many vital biological reactions by being a solvent, a transport medium and evaporative coolant (Bohnert et al, 1995) so plant species developed diverse mechanisms (Fig 1.3.) to deal with the problem of drought.

One of the major consequences of drought stress is the loss of protoplasmic water leading to the increase in the concentrations of some ions. At high concentrations, these ions effectively inhibit metabolic functions (Hartung et al, 1998). Additionally, the concentration of protoplasmic constituents and the loss of water from the cell lead to the formation of what is termed as glassy state. In this state, whatever liquid is left in the cell has a very high viscosity, increase the chances of molecular interactions that can cause protein denaturation and membrane fusion (Hartung et al, 1998). Plants, thus, require the genetically encoded ability to ensure the maintenance of cellular turgor and metabolic functions (Sagadevan et al, 2002)

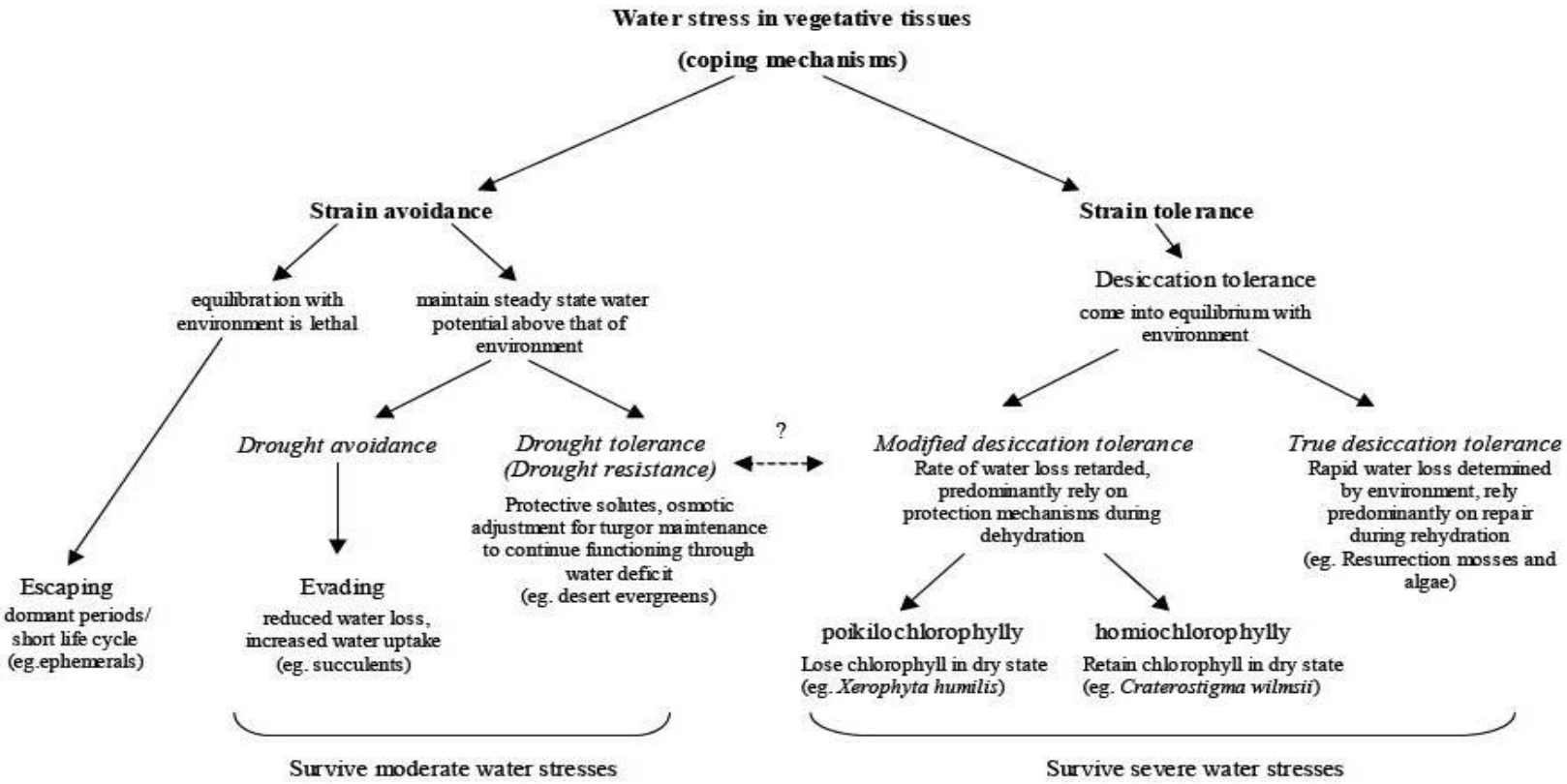

Figure 1.3. Classification of mechanisms performed by plants to cope with water stress (Sagadevan et al, 2002)

Drought results in a water deficit in plant tissues, which, in turn, can lead to an imbalance in the redox poise of plant cells, and thus inducing oxidative stress in plants (Noctor and Foyer 1998; Smirnoff 1998) like some other stress conditions (Fig 1.4.).

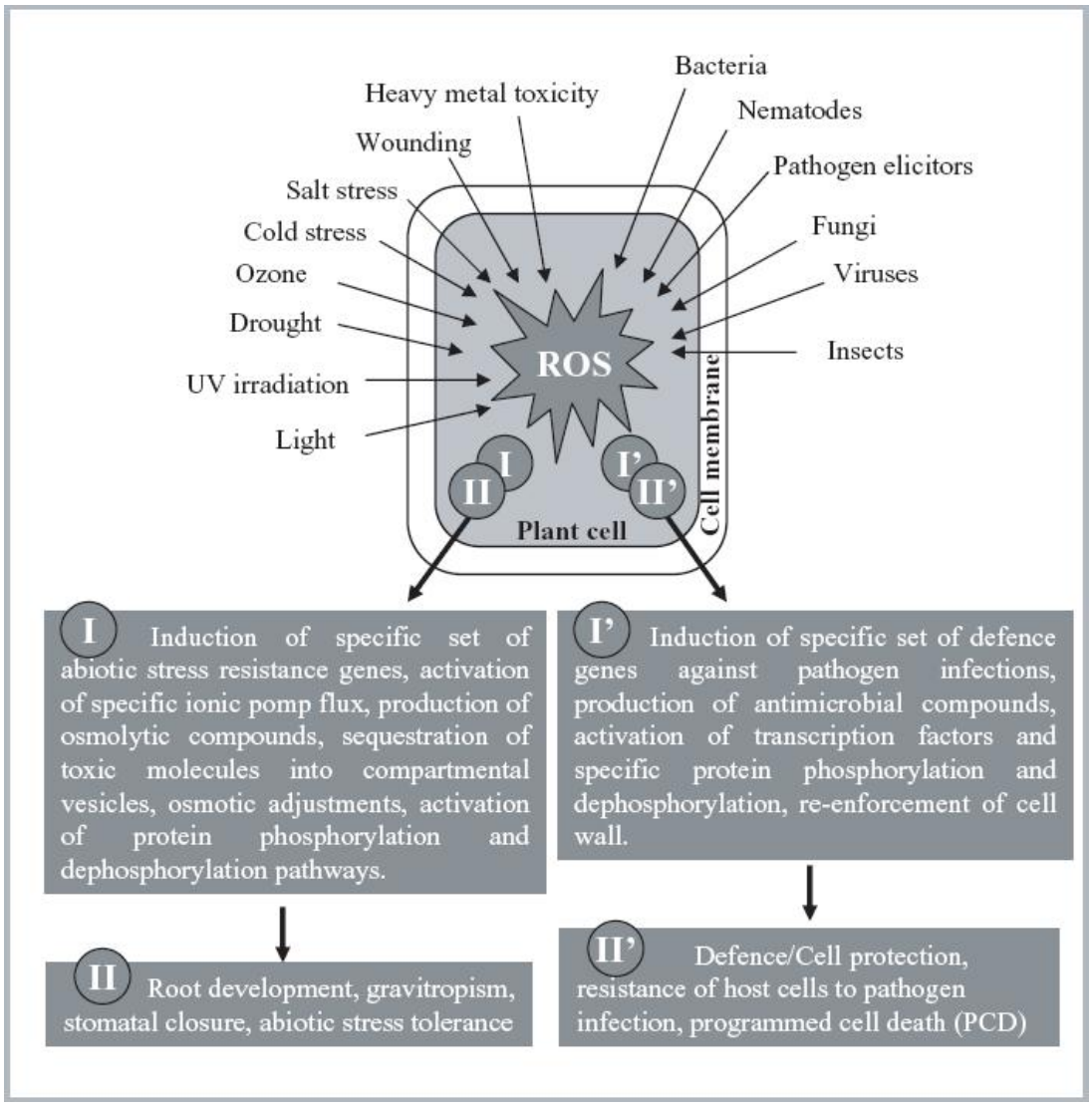

Figure 1.4. Involvement of reactive oxygen species (ROS) to cellular metabolic processes of plants under various environmental stress conditions (Kotchoni et al, 2006). I and II indicates the events generated by ROS in plant cells under abiotic stress conditions; I' and II' indicates the events generated by ROS in plant cells exposed to pathogens and pathogen-elicitors.

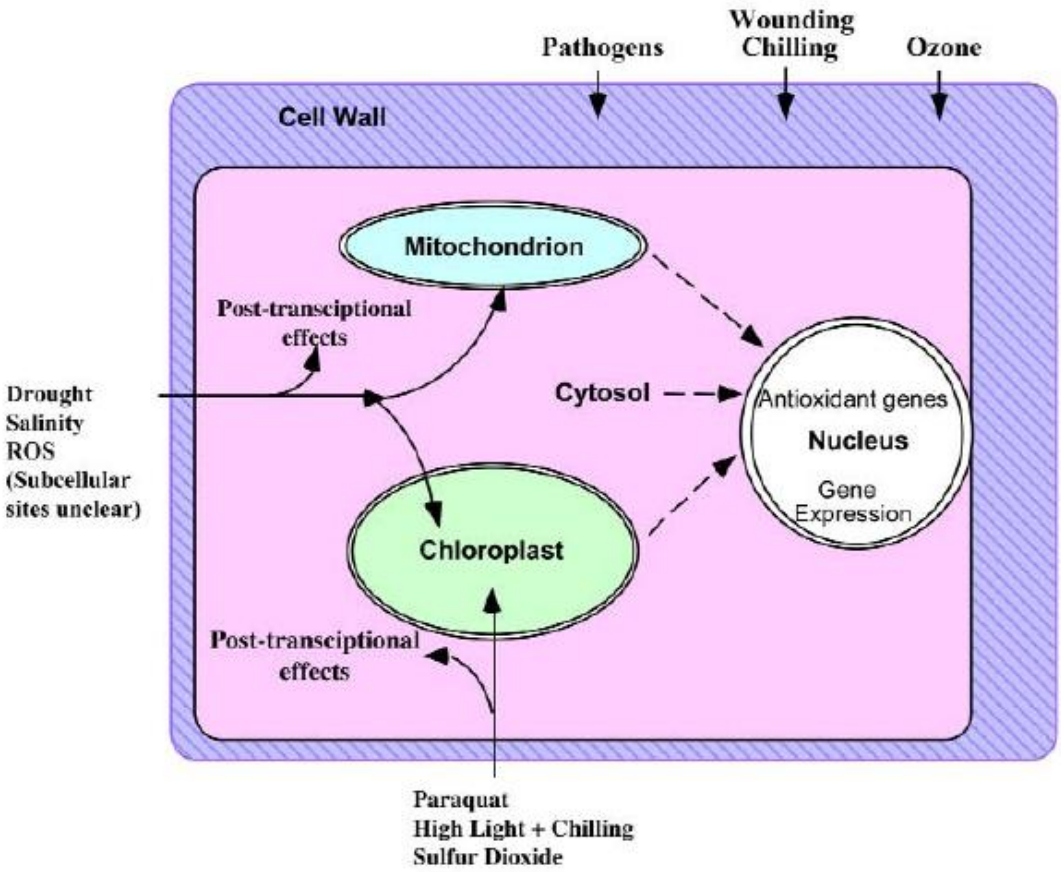

Figure 1.5. ROS arise throughout the cell (Ruth Grene, 2002)

The increased production of reactive oxygen species (ROS) within several subcellular compartments of the plant cell (Fig 1.5.) is a common aspect of harsh environmental conditions (Van Breusegem et al., 2001). ROS can be produced as by-products of regular cellular metabolism. However, under stress, their formation is usually aggravated. Under drought stress conditions, the weak sites of the plants are the organelles with highly oxidizing metabolic activities or with sustained electron flows such as chloroplasts and mitochondria; because, drought leads to the disruption of electron transport systems and thus under water deficit conditions the main sites of ROS production are those organelles (Toivonen and Vidaver, 1988; He et al., 1995).

Moreover, ROS (particularly superoxide and hydroxyl radicals) damage to the essential cellular components such as DNA, proteins and lipids. Among these,

the lipid subsistence of the cellular structures plays important roles in the integrity of the cell under drought conditions.

Along with proteins, lipids are the most abundant component of membranes and they play a role in the resistance of plant cells to environmental stresses (Kuiper, 1980; Suss and Yordanov, 1986). Strong water deficit leads to a disturbance of the association between membrane lipids and proteins as well as to a decrease in the enzyme activity and transport capacity of the bilayer (Caldwell and Whitman, 1987). Lipid peroxidation disrupts the membrane integrity of the plant cell. As a result, essential solutes leak out of organelles and from the cell, causing disruption in membrane function and metabolic imbalances.

DNA is the blueprint for both future form and function of the plant cells. Any damage to its integrity could mean that proteins that would have been essential for optimal function of the plant will not be synthesized. Similarly, denaturation of important proteins essential for biochemical reactions leads to the whole plant being negatively affected and unable to cope (Yordanov et al, 2003).

Resistance to conditions associated with oxidative-stress must, in part, rely on endogenous antioxidative defense mechanisms required to maintain cellular homeostasis. A highly efficient antioxidative defense system which has both non-enzymatic and enzymatic constituents is present in plant cells to counteract the toxicity of reactive oxygen species (Noctor and Foyer 1998, Polle 2001).

Glutathione is one of the major endogenous antioxidants in plants (Alscher 1989) known to play an important role in plant defense mechanisms. Glutathione functions as a substrate in antioxidative defense mechanisms by conjugating to toxic electrophilic compounds, scavenging free radicals, and reducing peroxides. Several important catalytic enzymes that utilize glutathione in defense mechanisms, such as glutathione S-transferase (GST) and glutathione reductase (GR) show differential patterns of activity in plant tissues exposed to environmental stress conditions such as; cold, drought and wounding treatments (James et al, 2004).

The tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine), and its structural analogues are the principal non-protein thiol compounds in plants. Following the explanation of its chemical structure, glutathione was soon identified as an antioxidant and later recognized as an essential component of antioxidative and detoxification systems in plant cells.

As it was mentioned before, glutathione (GSH) is a cellular protectant that is the major reservoir of non-protein reduced sulfur in plants (Noctor et al, 1998; Foyer et al, 2001). GSH is associated with stress resistance owing to its redox-active thiol group, and it is an important antioxidant responsible for the maintenance of antioxidative machinery of the cells under stress. In most tissues, GSH is predominantly present in its reduced form. The regeneration of GSH from oxidized glutathione (GSSG) is catalyzed by glutathione reductase (GR, E.C. 1.6.4.2) enzyme (Komives et al, 1998) GSH is oxidized by various free radicals and oxidants to glutathione disulfide (GSSG) (Fig 1.6.), while glutathione reductase (GR, EC 1.6.4.2) uses NADPH to reduce GSSG to GSH (Noctor et al, 1998).

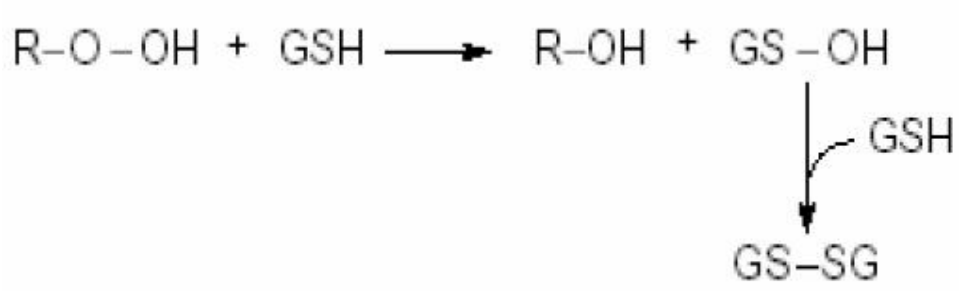

Figure 1.6. Reduction of an organic hydroperoxide to the monohydroxy alcohol by GSH conjugation (Edwards et al, 2000)

Most reactive oxygen species are reduced, directly, by GSH; and, by the activity of glutathione reductase, GSH scavenges hydrogen peroxide which is involved in the detoxification of lipid peroxides rather than hydrogen peroxide per se (Noctor et al, 1998).

Glutathione S-transferase (GST, EC 2.5.1.18) is another GSH dependent detoxifying enzyme in plants, which catalyzes the conjugation of GSH (Noctor et al, 1998) with many potentially dangerous compounds.

GSH is synthesized from glutamate, cysteine and glycine in two ATP-dependent reactions catalyzed by γ-glutamyl cysteine synthetase (γ-GCS; EC 6.3.2.2) and glutathione synthetase (GSHS; 6.3.2.3) (Noctor et al, 1998) (Fig 1.7.) GSH synthesis is regulated by cysteine (Cys) availability, feedback inhibition of γ-GCS by GSH, transcriptional control of γ-GCS and translational regulation of γ-GCS by the ratio of reduced to oxidized glutathione. In some cases, increases in the levels of GSH during stress responses have been correlated with an increase of γ-GCS activity (Kun-Ming Chen, 2004).

1.2.2 Plant GST Enzymes

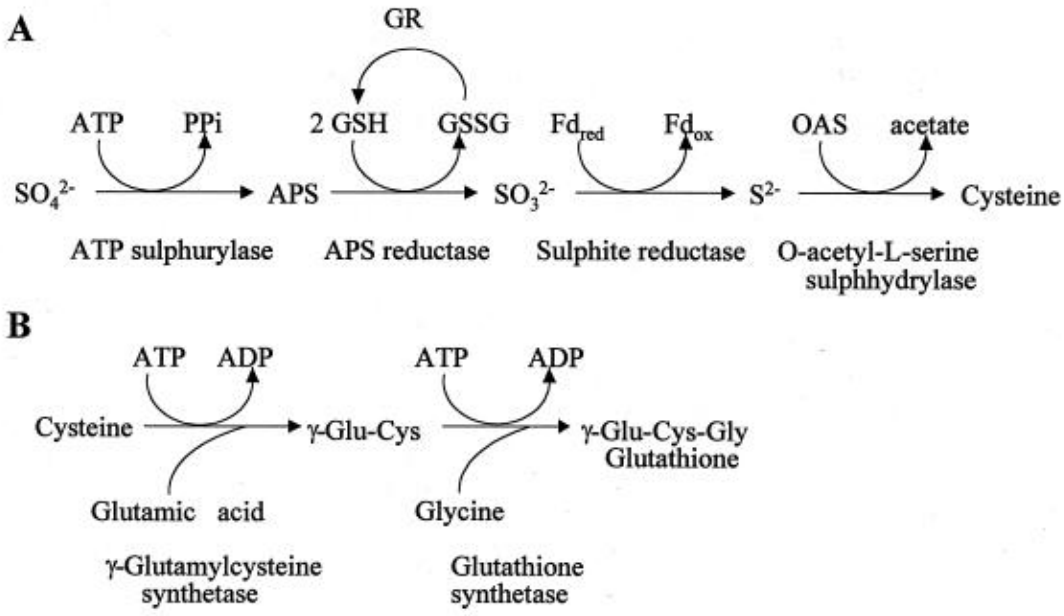

Figure 1.7. (A) Cysteine synthesis, (B) Glutathione synthesis (Kocsy et al, 2001)

Many defense strategies have evolved in organisms enabling them to deal with the threat of a broad spectrum of both foreign and endogenous cytotoxic and genotoxic compounds. Enzyme systems which perform the chemical detoxification

and elimination processes of these molecules are divided into three major but interrelated groups:

- Phase I enzymes, such as the cytochrome *P450* superfamily, activate chemicals by forming reactive functional groups (e.g. epoxides) in them.

- Phase II enzymes deactivate reactive chemicals by appending a hydrophilic moiety (e.g. glutathionyl, glucuronyl, or sulphuryl) to the functional group.

- Phase III enzymes mediate the cellular elimination of the inactive and water-soluble products of those chemical reactions (Dirr et al, 1994).

The glutathione S-transferases superfamily (EC 2.5.1.18) represents an integral part of the phase II detoxification mechanism. These intracellular proteins are found in most aerobic eukaryotes and prokaryotes, and by catalyzing the S-conjugation between the thiol group of glutathione and an electrophilic moiety in the hydrophobic and toxic substrate, they protect cells against chemical-induced toxicity and stress.

Glutathione S-transferases are perhaps the single most important family of enzymes involved in the metabolism of alkylating compounds (Dirr et al, 1994). The discovery of a catalytic activity for the addition of GSH to CDNB in cytosolic extracts of liver initiated three decades of increasing interest in the genetics and enzymology of the glutathione S-transferases (EC 2.5.1.18). The studies in this area resulted in the exposition of the three-dimensional structures of several cytosolic isoenzymes in the first half of this decade. The structural information has had a deep impact on our understanding of the catalytic mechanisms of the glutathione S-transferases, the evolution of the protein folding, and the molecular basis for their participation in the detoxification reactions (Armstrong, 1997).

The biological function and significance of the GSTs could be understood, if only the knowledge of where the enzymes came from, how they work, and how their expression is regulated are clearly solved (Armstrong, 1997). The glutathione S-transferases catalyze the general reaction shown below:

$$GSH + R\text{-}X \rightarrow R\text{-}SG + HX$$

Glutathione transferases are soluble proteins with typical molecular masses of around 50 kDa, and each is composed of two polypeptide subunits. Classically, they catalyze the transfer of the tripeptide glutathione to a cosubstrate (R-X) containing a reactive electrophilic center to form a polar S-glutathionylated reaction product (R-SG) (Dixon et al, 2000).

The presence of GST enzymes in plants was, first, recognized shortly afterwards in 1970. GST activity from maize was shown to be responsible for conjugating the chloro-S-triazine atrazine with GSH, thereby protecting the crop from injury by this herbicide (Marrs, 1996). From that time, GST and other proteins functioning in such situations have become a popular area of study. In addition to the dimeric soluble GSTs, other proteins have been identified as having a restricted ability to conjugate xenobiotics (foreign organic compounds) with GSH, notably the distantly related mitochondrial kappa GSTs and the trimeric microsomal GSTs of animals (Dixon et al, 1999).

1.2.2.1 General Structure of Plant GSTs

GSTs are products of gene superfamilies, each producing isozymes with broad substrate specificities. Despite the fact that they perform a similar reaction, GSTs share very little overall amino acid sequence identity, typically no more than 25–35%, though there are usually regions of much higher localized similarity within the N-terminus (Marrs, 1996).

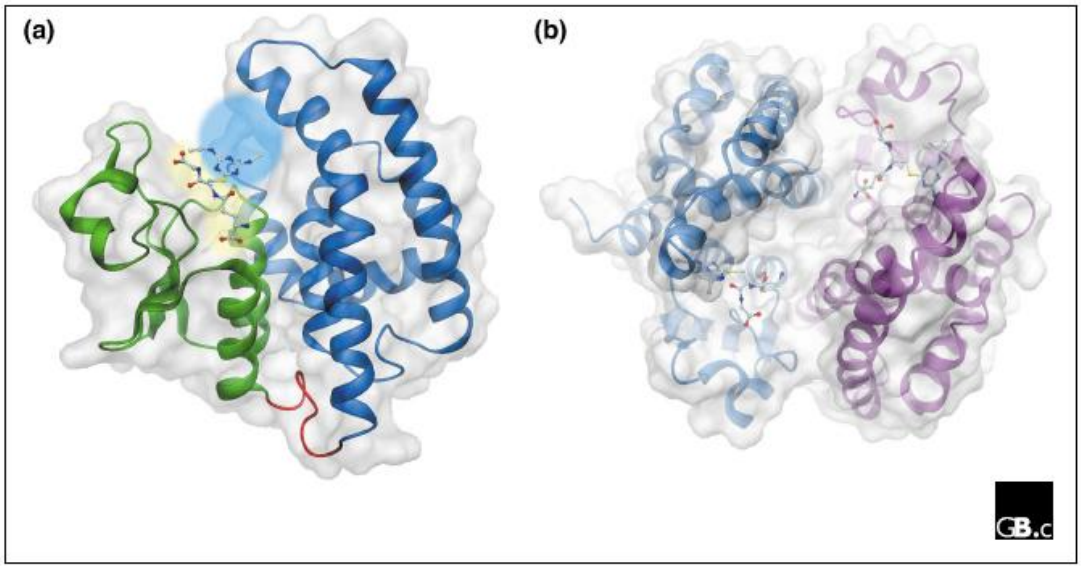

Figure 1.8. GST dimer structure and substrate binding (Dixon et al, 2002): **a)**Amino-terminal domain is in green, linker region is in red, carboxy terminal domain is in blue and the protein surface is in gray color.
b)Subunits are in blue and purple colors.

Glutathione S-transferases include two subunits existing as homo- or heterodimers each of which has a GSH binding site (G-site) located in the N-terminal domain, and an adjacent electrophilic substrate binding site (H-site) which will accommodate a diverse range of hydrophobic compounds; besides, they function independently (Abu-Hijleh, 1999). These subunits are characterized by the GST typical modular structure with spatially distinct domains (Sheehan et al, 2001). Domain I contains much of the G-site and it is also referred as the N-terminal domain. Because of its high specificity, only glutathione and its derivatives such as γ-glutamylcysteine and homoglutathione can bind to this site (Mannervik and Danielson, 1988). Domain II contains all of the H-site and it is also called the C terminal cosubstrate binding site. Unlike the G-site, Domain II conform a diverse range of hydrophobic compounds (Abu-Hijleh, 1999) with broad substrate specificity (Fig 1.8.). These substrates commonly have Michaelis reaction acceptor. Michelis acceptors are compounds that induce GSTs or that are recognized as substrates share a common chemical signal: carbon-carbon double bonds adjacent to an electron-withdrawing group. This feature, termed a “Michael acceptor”, is either contained naturally by GST substrates or acquired by Phase I metabolism.

Michael acceptors

$CH_2 = CH - Z$

$Z' - CH = CH - Z$
(quinone)

$R - C \equiv C - Z$
(acetylene)

Crystallography has shown that certain GSTs can bind an additional GSH molecule adjacent to the active site, although the functional significance of this secondary binding site has received little attention. In addition to the diversity of GST genes in each species, heterodimer formation could give rise to up to (1+2+3+…*n*) distinct heterodimers, where *n* is the number of GST genes of a particular class.

1.2.2.2 Functions of Plant GSTs under Stress Conditions

Plants and other organisms have developed mechanisms to defend themselves against herbivores, insects, pathogens, or chemical compounds that are harmful to their survival. These strategies have been classified in two distinct groups: constitutive and induced defences (Karam Decio).

Constitutive defenses provide natural protection while induced defenses are created by an external. One induced mechanism is activation of specific enzymes that inactivate chemical compounds in plants (Karam Decio). Glutathione S-transferase is a member of these "inducible" mechanisms.

GST was, first, identified in corn over many years ago. The purpose of that research was to understand the ability of that plant to detoxify herbicides. However, the continuing studies on many other plants (wheat, tobacco, carnation, broccoli, soybean, dwarf pine, barley, sorghum, *Arabidopsis thaliana*, velvetleaf, sugarcane…etc) demonstrated that it has other important activities (Fig 1.9.); such as, in the cellular response to auxins, in normal metabolism of secondary products,

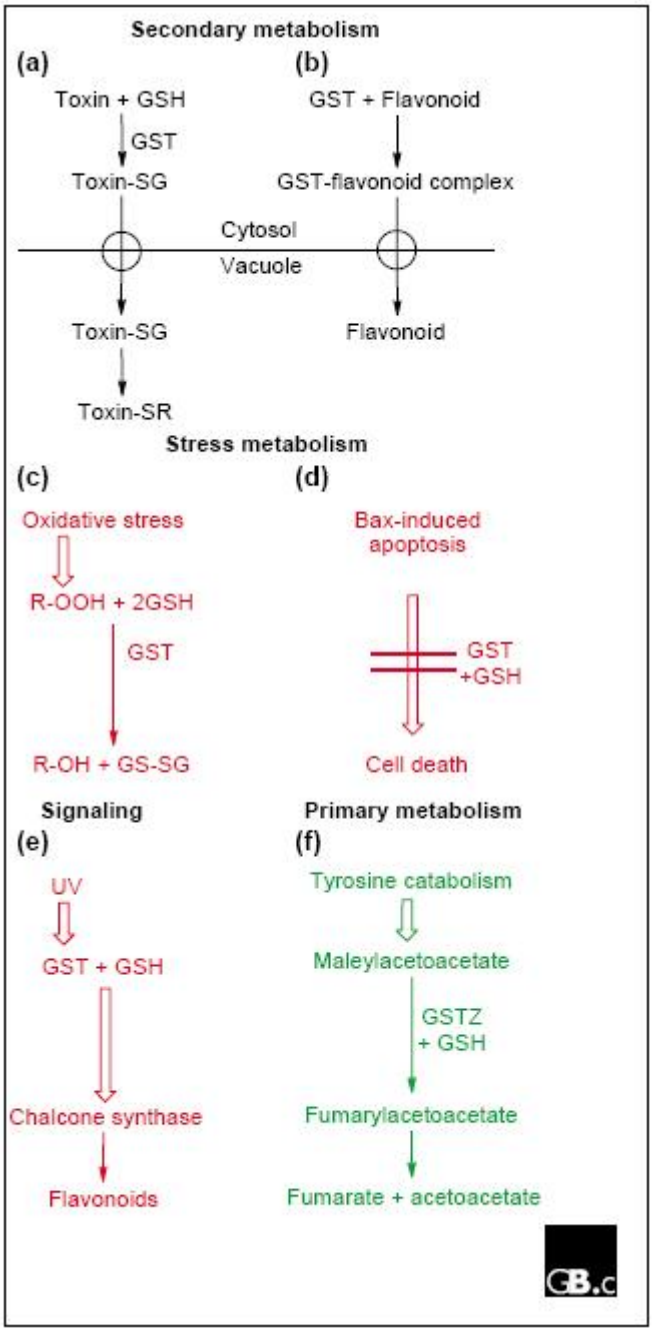

Figure 1.9. A general scheme of the known GST functions in plants (Dixon et al, 2002)

and in many stress responses including pathogen attack, heavy metal toxicity, oxidative stress because of some environmental factors.

The functions of GSTs can be combined under three subtitles:

1-They catalyse conjugation reactions with natural products similar to those observed with xenobiotics.

2-They function as binding and carrier proteins for phytochemicals between cellular compartments. GST enzymes function in targeting for transmembrane transport; in plants, many secondary metabolites are phytotoxic, even to the cells that produce them. As a result, targeting to the appropriate cellular localization, usually the vacuole, is crucial. After glutathionation, xenobiotic conjugates are rapidly imported into the vacuole before being further metabolized into a range of known sulphur-containing metabolites (Edwards et al, 2000).

3-And finally, they catalyse alternative GSH-dependent biotransformation reactions. They carry the responsibility of protecting tissues from oxidative damage that is initiated by hydroxyl radicals, in most of the times. GSTs conjugate GSH groups with endogenously produced electrophiles and detoxify them.

Its catalytic activity is based on the nucleophilic attack of the sulfur atom of glutathione to the electrophilic center of a wide variety of substrates. Substrate specificity of the G-site is high, with only GSH and structurally related molecules serving as substrates and it facilitates the formation of catalytically active thiolate anion of GSH.

The issues with respect to how these enzymes function can be framed by two general categories of questions:

i) how the enzyme recognizes and activates glutathione for nucleophilic attack

ii) how, or even if, the enzyme specifically recognizes electrophilic substrates (R-X). This division is not entirely arbitrary (Armstrong, 1997)

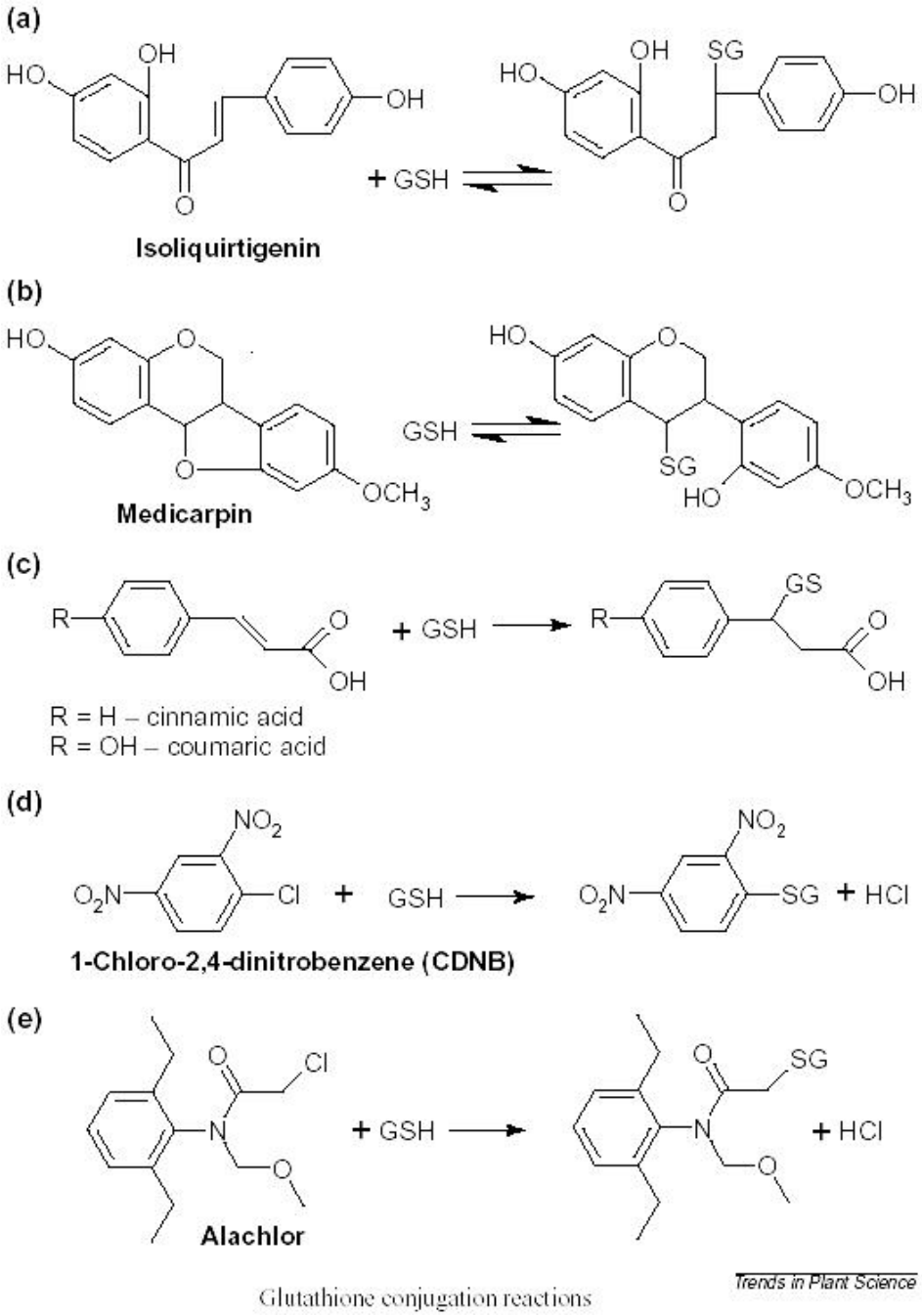

Figure 1.10. Glutathione conjugation reactions of some substrates (Edwards et al, 2000)

It should be stressed that these "detoxification enzymes" are remarkably variable in the recognition of substrates (Fig 1.10.). In plants, members of these

same enzyme families also play normal, defined roles in secondary metabolism and thus recognize and transport both natural and xenobiotic substrates (Marrs, 1996).

Together with its wide function area, there are many types of substrates that are reacted by GSTs: 1-chloro-2,4-dinitrobenzene (CDNB) is one of the "models" for most, "but not all", GSTs. When the enzyme conjugates with this substrate by chlorosubstitution, the result is a change in the absorbance of the compound at 340 nm. In plants, 4-hydroxynonenal, a toxic alkenal released following oxidative damage of membranes, is actively detoxified by phi GSTs from sorghum, and tau GSTs from wheat which have similar activities. Pathogen-inducible GSTs probably also detoxify exogenous natural products, though of exogenous origin, such as phytotoxins produced by competing plants or by invading microbial pathogens (Edwards, 2000).

Moreover, GSTs can work as ligandins: In addition to their catalytic function, GSTs serve as nonenzymatic carrier proteins (ligandins) involved in the intracellular transport of many compound especially in animal cells. It has been proposed that the ligandin function of GSTs prevents cytotoxic events that could result from the excessive accumulation of molecules at membranes or within cells (Marrs, 1996)

At DNA level, the gene family (*gst*s) responsible for the production of different subunits of the glutathione S-transferases becomes much more popular together with the advancement in proteomics and genomics. The combined results on the analysis of *gst* promoters suggest that there might be a common factor in the signaling transduction pathway from the initial recognition of the stimulus to the activation of gene expression for many of the plant *gst* genes (Droog, 1997). They, also, suggest that the activity of the encoded proteins is needed under many different conditions. The gene expression of at least a subset of *gst* genes is activated by the occurrence of oxidative stress, and the activity of the encoded proteins is needed to protect cells against oxidative damage (Droog 1995). An involvement of stress is not only indicated by the wide variety of substances and treatments leading to activation

of the *gst* genes but also by the high levels of inducers needed and the linearity in the dose-response curves. It is now well established that one of the first responses to pathogen infection is the occurrence of an oxidative burst: the release of reactive oxygen species; changes in exocellular pH and in membrane potentials, ion fluxes, changes in protein phosphorylation patterns and the oxidative immobilization of plant cell wall proteins (Wojtaszek, 1997).

1.2.2.3 Classification of GSTs

The first GST activity to be described in plants was a maize enzyme responsible for the detoxification of the herbicide atrazine (Frear and Swanson 1970) as it was mentioned before. Subsequently, numerous GST activities have been characterized which are involved in detoxification of several classes of chemicals in different plant species. These various GST activities show differences in their

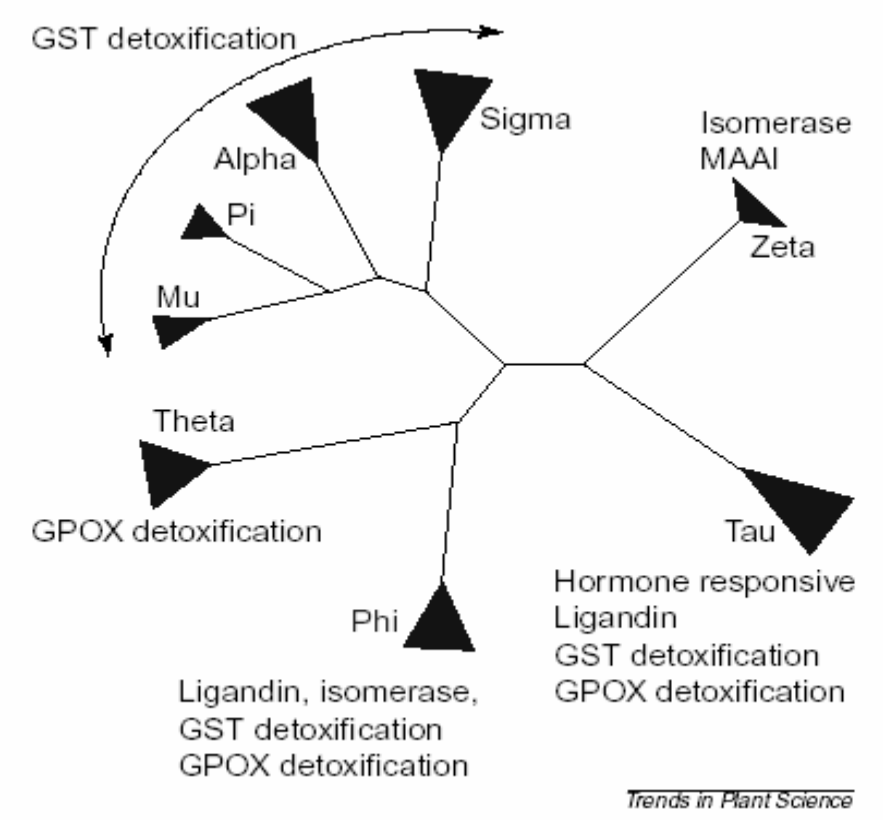

Figure 1.11. GST isozymes and their main functions (Edwards et al, 2000)

regulation and display distinct but sometimes overlapping substrate specificities. The variation in regulation and substrates also illustrates the versatility of the GST isozymes (Fig 1.11.). Moreover this indicates that studies on the regulation and activity of individual GST isozymes in one species need to be interpreted with caution.

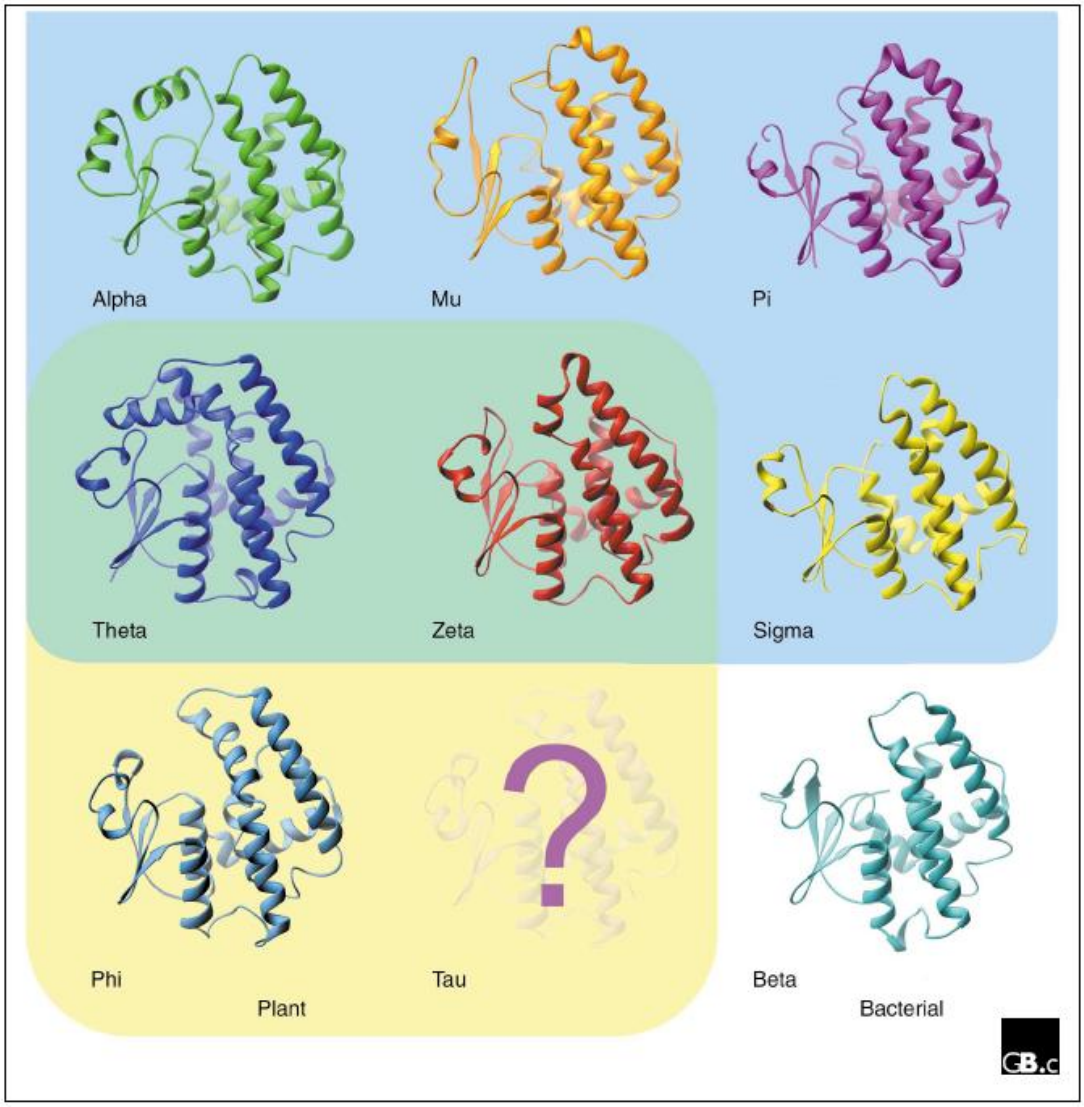

Figure 1.12. The structures of GST subunits (Dixon et al, 2002): Blue background indicates GSTs specific to mammals, yellow background indicates plant specific ones and white background shows bacterial-specific GSTs. Although there is little sequnce similarity, there is significant conservation in overall structure.

A large number of GSTs and GST-like sequences have been cloned from a variety of plants, and in order to make sense of this plethora of genes, a classification system was set up, at first, with just three classes, theta, tau and zeta (Droog, 1997). As our understanding of GST gene families in plants and animals has expanded, this original classification system has had to be refined (Dixon et al, 2000).

Plants contain a diverse range of GSTs that can metabolize pesticides and pollutants, but unlike their counterparts in animals it is unlikely that these enzymes evolved primarily to detoxify foreign compounds, generating questions concerning their functions in plant metabolism (Edwards et al, 2000). On the basis of sequence comparisons, substrate specificities, sensitivities to inhibitors, N-terminal amino acid sequences, exon-intron comparisons and immunological cross reactivity (Qing-Vin Zeng, 2005), plant GSTs can be divided into four classes: phi, zeta, tau, and theta (Fig 1.12.) (Edwards et al, 2000).

The phi and tau classes are unique to plants and are relatively well characterized, being encoded by large gene families in all plant species studied to date (Edwards et al, 2000, Marrs, 1996). In all three phi GSTs, the active sites are situated on either side of a large, open cleft formed between the subunits, allowing access to large planar and spherical molecules. However, each active site interacts minimally with the adjacent subunit (Droog, 1997).

In contrast, the theta and zeta GSTs are less well represented in plants and the presence of homologues in animals and fungi suggests common essential functions in all eukaryotes (Hayes et al, 1999).

Another classification type was created according to the type of detoxification of electrophilic compounds. The last classification is based on amino acid sequence identity and conservation of intron:exon placement and not on substrate recognition or antibody cross-reactivity, features that are far more difficult to characterize (Fig 1.13.). For some of the genes in each subgroup, the intron:exon

structure has not been determined either because a cDNA was cloned or because the gene was only partially sequenced, and thus placement in the group has been by amino acid sequence alone (Marrs, 1996).

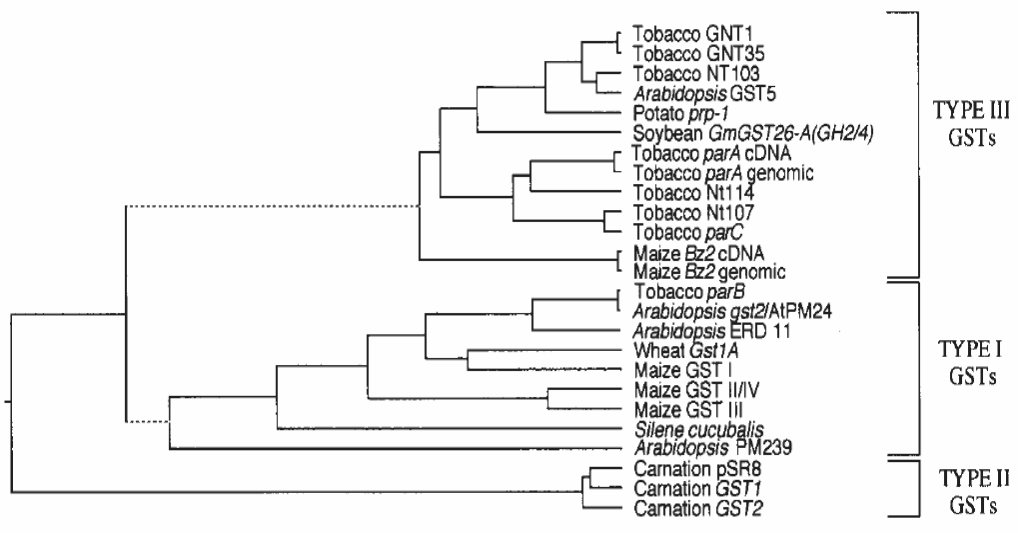

Figure 1.13. A sample phylogenetic tree of plant GSTs. The classification is based on amino acid sequence identity and conservation of intron:exon placement

Type-I GSTs contains 3 exons and 2 introns. Some of them are responsible for the production of proteins in response to "pathogen attacks, wounding senescence, and the resulting lipid peroxidation that accompanies these processes" and some others are induced in response to auxins and they may serve a ligandin function toward IAA.

Type-II GSTs contain 10 exons and 9 introns (but only in carnation). Their amino acid sequences are very similar with the ones of Type-III GSTs, but the intron:exon patterns are special to mammalian alpha class GSTs.

Type-III GSTs have just 2 exons and one intron and by the applications of many stress agents, this subclass was determined as a set of homologous genes before. "The genes have alternatively been called multiple stimulus response (*msr*) genes, auxin-related genes or the auxin regulated gene (ARG) subgroup.

Types I and III GSTs show less then 50% sequence divergence and have now been placed in separate classes. Moreover, a Type IV grouping was proposed for several *Arabidopsis* genes that are similar to classical mammalian theta enzymes.

On the other hand, although these are very effective and useful classifications, and although in some species, individual GSTs have been characterized, still amino acid sequences are not yet available. This situation prevents the formation of a better classification system for these enzymes.

Phylogenetic analysis would suggest that all soluble GSTs have arisen from an ancient progenitor gene. Zeta and theta GSTs are found in both animals and plants, but the tau and phi classes are plant-specific. Searches for GST-like sequences in the Arabidopsis genome identify a further two genes related to soluble GSTs. These genes have clearly evolved from a GST progenitor and are known to be co-induced with phi and tau GSTs in cereals following exposure to herbicide safeners, chemicals that increase tolerance to herbicides (McGonigle et al, 2000). Some scientists studying on this subject term those GST-like genes lambda GSTs. (Dixon et al, 2000).

From the size and sequence diversity within the GST superfamily in plants, it is clear that there is scope for considerable functional diversification. It is also probable that several GSTs have overlapping functions, effectively leading to some redundancy. Although genome information is not yet available in the public domain for adequate number of plants, analyses of large-scale EST projects in major crops provides valuable additional information on the relative diversity of the GST gene family (Dixon et al, 2000).

1.3. Aim of the Study

Although it became a popular area of study, still, there is no so much research on the plant GST enzymes. The number of studies on the Turkish red pine (*Pinus brutia* Ten.) is even less up to date. This species is a very important forest tree, both biologically and economically, for Turkey. In this study, we aimed to determine the changes in GST activity in this species under drought stress conditions to enlighten the role of GST enzymes under stress conditions. Moreover, we intended to understand the relationship between the changes in cytosolic GST activities and total thiol amount values of *Pinus brutia* Ten. needles. For these purposes, we labeled 30 different individuals in METU Yalıncak in two opposite directions (East-West) and collected samples in four months. After preparing cytosolic homogenates for each month's samples, we planned to measure the GST enzyme activity and GSH in total thiol amount. The results are correlated to drought stress conditions. Drought stress is determined by measuring osmotic pressure in the needles. Monthly weather temperature and precipitation records are also obtained from State Weather Forecast Department.

CHAPTER II

MATERIALS AND METHODS

2.1. Plant Material

30 different *Pinus brutia* TEN. individuals located in METU-Yalıncak area were used in this study. The sampling area was mapped (Fig. 2.1) and these individuals were labeled on their two different braches in opposite directions; east

Figure 2.1. Sampling Area; in METU-Yalıncak

and west. At the end of four months of the season (June, July, August and September of 2005), needles were collected from those the same branches at the same time interval of the day. For each individual, 2 sets of samples were collected from the branches of east (A) and west (B) sides for enzyme activity assay and total thiol determination and one set was collected from all around the tree for the osmotic pressure measurement for each month. As a result, totally 240 samples were collected for GST activity and total thiol group determinations. Samples were delivered to the deep freezer (-80^{o}C) in cold cycle and kept there till the homogenization.

2.2. Chemicals

1-chloro-2.4-dinitrobenzene (CDNB), reduced glutathione (GSH), PVP-K30, ethylenediaminetetraacetic acid (EDTA), hydroxymethyl aminomethane (Tris), Nonidet P-40, Methanol (CH_4O), Bovine serum albumin (BSA), ethanol absolute (%99.8), Folin-Ciaceltau Phenol Reagent, cupper sulfate ($CuSO_4.5H_2O$), sodium-potassium tartrate, sodium hydroxide, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, 2-mercaptoethanol, reduced glutathione (GSH), Pepstatin, hydrochloric acid, 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB).

All chemicals were analytical grade and were obtained from commercial sources at the highest purity available.

2.3. Methods

2.3.1. Osmotic Pressure Determination

The osmotic pressure values of each needle sample were determined by the Wescor's Vapor Pressure Osmometer, model VAPRO®. Apart from the samples collected for GST enzyme activity and total thiol amount determination assays, from all around the branches, at approximately the same height with respect to the soil level, needles were collected to use in the determination of osmotic pressure level. These samples were stored at -20^{o}C, before measurement. In the day of

measurement, each sample was cut into small pieces by a scissors and filled into a plastic injector. By the help of a clamp, injector is pressed and the juice of the needles was handled. 10 µl of needle juice is applied onto a piece of filter paper (provided commercially) then the instrument was started to measure. Values were expressed with unit of mmol/kg.

2.3.2 Preparation of cytosol from red pine needles

Crude extracts, whichwere used as enzyme source, were prepared from Turkish red pine needles by a modification of the procedure described by Schröder and Berkau (1993) for spruce needles. Approximately 1 g of needles were cut into small pieces and crushed (pulvarized) in liquid nitrogen by mortar and pestle. Powdered needles were kept in special plastic containers, marked and stored in freezer at -80^{o}C. At the time of homogenization, 0.2 g of powder were measured into plastic tubes with 2 ml (10 vol (w/v)) of 0.1 M Tris HCl buffer, pH 7.8, containing 20mM of 2-Mercaptoethanol, 5% PVP-K 30, 2 mM of EDTA, 0.5% Nonidet P40, 5mM GSH, 3µg/ml of Pepstatin A Then, the mixture was homogenized for 1 min as 15 sec. intervals for four times (15 sec x 4 times) by Ultra-Turrax T25 at 13500 rpm in ice. The crude homogenate was centrifuged at 12000 g (Eppendorf Centrifuge 5810 R with the rotor of Eppendorf F34-6-38) for 30 min at 4^{o}C. The pellet was discarded and the supernatant, which was the cytosol, was used as the sample. After pooling the supernatant was stored in small aliquots at -80^{o}C in deep freezer until used for assay.

2.3.3. Protein Determination of Cytosol by Micro-Lowry Assay

The protein amount in the *Pinus brutia* TEN. needles were determined by the Lowry Method (1951) adjusted to the measurement by ELISA Plate Reader (Bio-Tek ELx808) with crystalline bovine serum albumin (BSA) as the standard. Samples were diluted as 1/25 and 1/50 and applied to the 96 well plate as triples as a volume of 50 µl. Then, 200 µl of Lowry ACR (including 2% cupper sulfate ($CuSO_4.5H_2O$), 2% sodium-potassium tartarate, 2% (sodium hydroxide, sodium carbonate) in the

ratio of 1:1:100) was added and mixed. After 10 min of incubation at room temperature, Folin Cilcateu Phenol Solution (commercially available form was diluted 1/2) was added as 20 µl with immediate mixing. At the end of a 45 min. incubation, plate was read at 650 nm. Protein concentration in each well calculated by the software of the instrument (KCJunior) after providing necessary dilution factors. Standard curve was prepared by using BSA solutions diluted as 1, 2.5, 5, 10 µg/well (Fig. 2.2.).

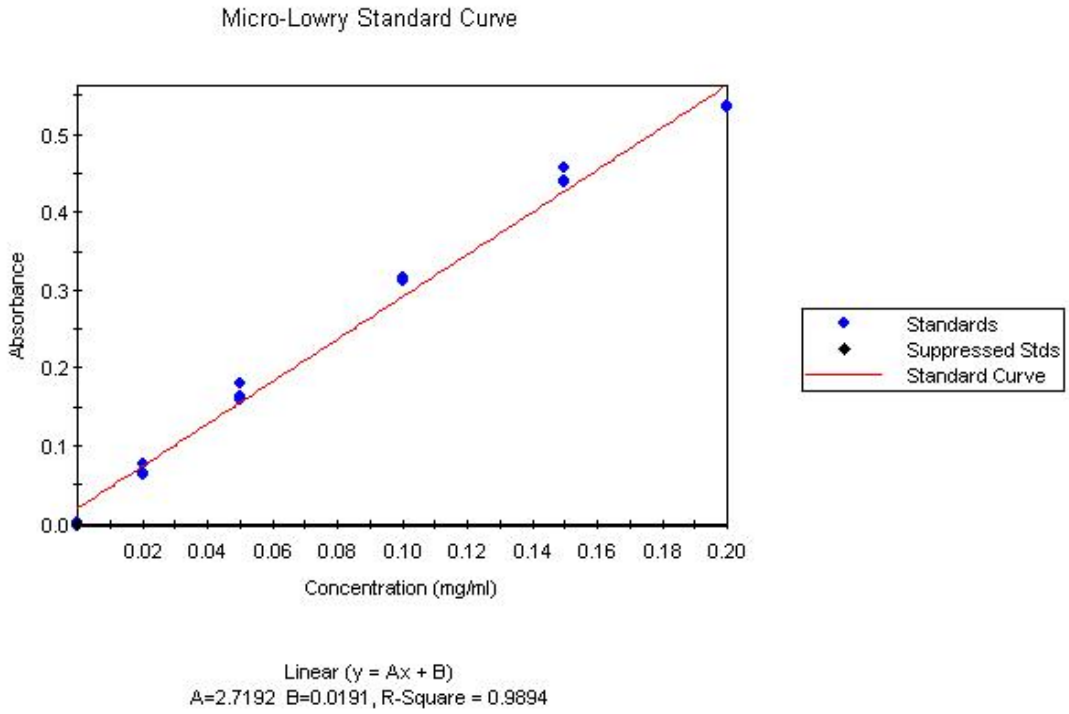

Figure 2.2. Standard curve for the determination of protein amount by micro-lowry procedure provided by the Software KC Junior

2.3.4. Determination of GST Enzyme Activity towards CDNB

GST enzyme activity was determined spectrophotometrically by monitoring the thio-ether formation at 340 nm using CDNB as substrate basicly according to the method of Habig et al (1974) as modified previously (Boyoglu 2004, Oztetik 2005).

In this study, the previous procedure was adjusted for Elisa plate reader. As shown in Table 1 , each reaction mixture contained 100 mM potassium phosphate buffer, pH:7.8, 1.0 mM GSH, 1.0 mM CDNB and 3.5-6 mg/ml ctosolic protein in a final volume of 250 µl in 96 well plate.

The reactions were started by the addition of enzyme into each well. The plate placed into Elisa Plate Reader adjusted at 25^{o}C. After machine mixing, reading is started automatically at every 20 seconds for 10 minutes.The blank wells contained all the constituents except cytosolas enzyme source. Instead, phosphate buffer was added to complete the volume to 250 µl.

Table 2.1. Costituents of cytosolic GST enzyme activity medium

Component	**Concentration**
Combination Buffer	
* Potassium Phosphate Buffer	100 mM (pH: 7.8)
* GSH	1.1 mM
CDNB	20 mM
Sample (Enzyme)	8.8-1.5 µg/well

For the blank mixture, all the components except the sample, instead of which 12.5 µl of 0.1 M, pH 7.8 phosphate buffer was added, were poured into the wells. Slopes of the best lines drown for each well separately by the software of the instrument were used as the rate of reaction (dA/dt) and the further calculations were completed.

$$\frac{dA/dt}{\varepsilon\ (mM^{-1})} \times \frac{0{,}250\ ml}{1000\ ml} \times \frac{1000}{12.5} \times DF \times \frac{1}{mg\ prot./ml}$$

The GST activities were expressed as unit/mg protein where one unit of an enzyme is defined as the amount of enzyme producing 1 µmole of product in one minute under defined reaction conditions.

2.3.5. Total Thiol Group Determination

Cytosolic total thiol amount of each sample was determined by the method defined by Sedlak and Lindsay (1968). This method is based on the reduction of 5`-dithiobis-(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups, to produce a characteristic yellow color which gives its maximum absorbance at 412 nm (Fig 9). Each free –SH group reduces the DTNB into 1 molecule of 2-nitro-5-mercaptobenzoic acid that creates the yellow color.

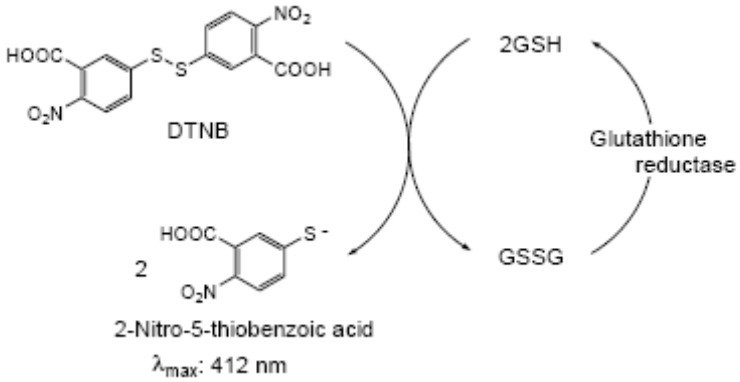

Figure 2.3. The reduction of 5`-dithiobis-(2-nitrobenzoic acid) (DTNB) by GSH

To construct the standard curve, GSH was used as the standard at the concentration range of 0.1-1.0 μM instead of cytosol.

By an adaptation to the ELISA microplate reader, 10 μl of cytosol was added into 30 μl of 200 mM Tris Buffer, pH 8.2 containing 20 mM of EDTA. Then 10 μl of DTNB (2 mM) and 150 μl of MeOH were added into each well. After a 30 min of incubation period at room temperature (25^{o}C), absorbance values were measured in Elisa microplate reader at 405 nm. and by the slope of the standard curve, concentration values were calculated by the Software KC Junior provided by the instrument.

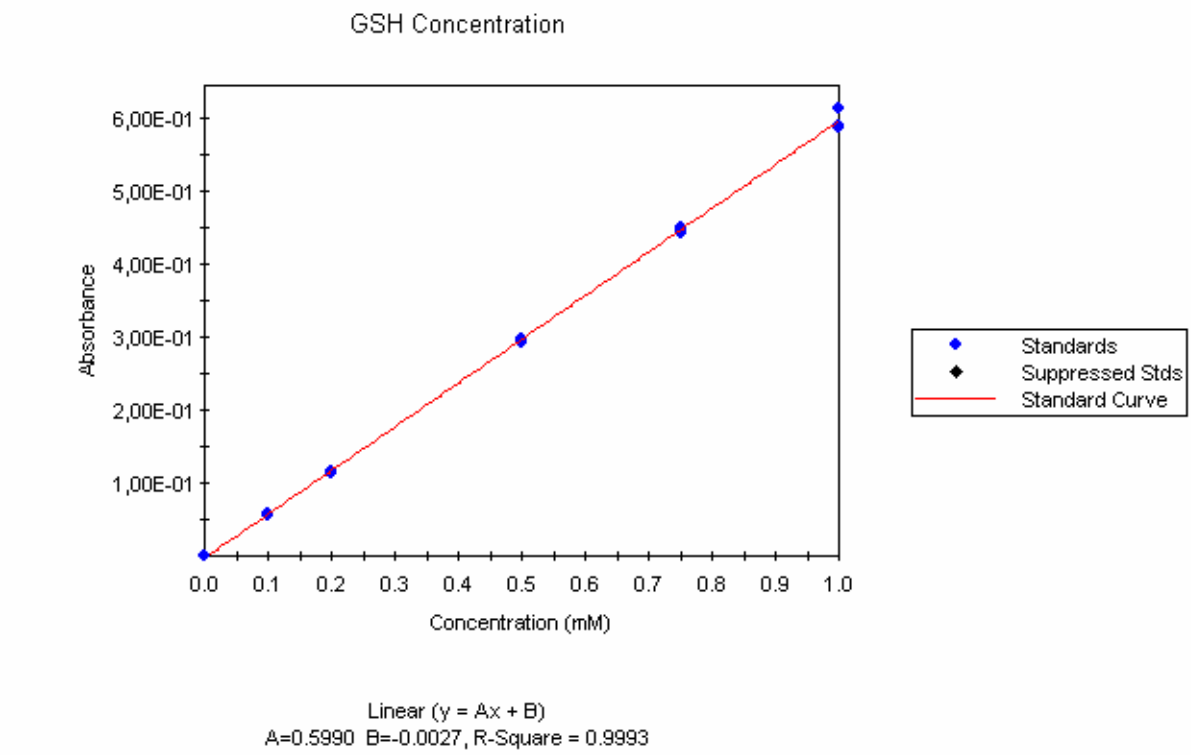

Figure 2.4. Standard curve for the determination of total thiole amount provided by the Software KC Junior

2.3.6. Statistical Analysis

In this study, Analysis of Varience (ANOVA), the t-test for the differences for the cytosolic GST activity results and the total thiol amount results were performed by the MINITAB^{TM} statistical Software Release 13,0 (BCIS Lab, St Cloud State University). All the other statistical values; such as mean, standard error of mean, averages, and etc. were calculated by the same statistical software.

CHAPTER III

RESULTS

3.1. Osmotic Pressure Change during the Sampling Period

The osmotic pressure values of each needle sample were determined by the instrument Wescor's Vapor Pressure Osmometer, model VAPRO® and the results obtained were summarized in the Table 3.1 and shown in the Figure 3.1.

Table 3.1. The average osmotic pressure values measured from the juice of freshly collected needles of *Pinus brutia* during drought season of 2005.

Months	Average Osmotic Pressure (mmol/kg) ± SE
June	929,4 ± 9,2 (n = 30)
July	974,8 ± 9,0 (n = 30) *
August	1078,6 ± 5,1 (n = 30) *
September	947,3 ± 11,0 (n = 30)

* Statistically different with respect to June, $p<0.05$

The data were subjected to statistical analysis using MINITAB™ two sample t-test of difference. Every month's data were compared to June, assuming the June samples might be considered as the control (Table 3.1.). There were statistically significant differences between the values obtained for the osmotic pressure values of July and August; but not for the one of September.

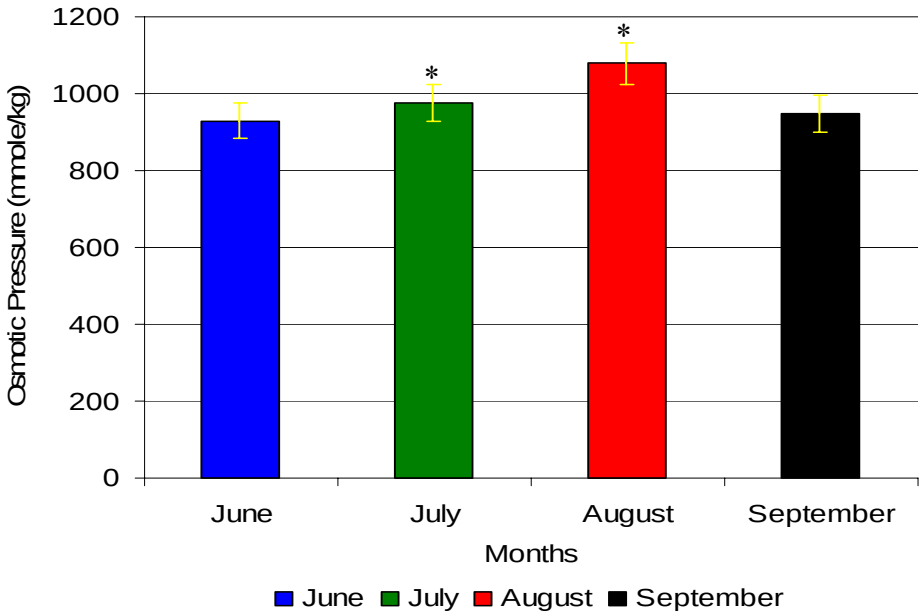

Figure 3.1. The average osmotic pressure values of *Pinus brutia* needles of 30 individuals each of which was measured in triplicates (samples were collected in 2005).

* Statistically different with respect to June, $p<0.05$

3.2 Cytosolic GST Activity Change Under Stress Conditions

Glutathione S-transferase activity was studied in *Pinus brutia* Ten. cytosolic extract using CDNB as the substrate. GST activity was determined spectrophotometrically the method described by Habig *et al.* (1974). The specific activity values of GST enzyme for the cytosolic extract of each sample were measured and the results for the each month were presented in Table 3.2.

Table 3.2. The average cytosolic glutathione S-transferase activities against CDNB prepared from *Pinus brutia* needles collected during drought season of 2005

Samples	**Average Specific Activities (µmoles/min/mg protein) ± SE**
June	15,78 ± 1,36 (n = 30)
July	22,91 ± 1,99 (n = 30)*,**
August	16,54 ± 1,61 (n = 30)
September	28,37 ± 2,38 (n = 30)*,***

(*): Statistically different with respect to June, $p<0.005$
(**): Statistically different with respect to August, $p<0.05$
(***): Statistically different with respect to August, $p<0.005$

All measurements were performed under optimum conditions defined by Boyoglu, S. (2003) and the specific activity values of each individual tree were calculated. Average cytosolic GST activities of July and September samples shows statistically significant differences with respect to the values of June (Fig 3.2.). The comparisons were done by two way ANOVA. Each month was compared to June considering June values as control since trees were not under stress in June after a rainy Spring season under mild temperatures (Appendix A).

In addition, there are statistically significant differences between all the average cytosolic GST activity values when they were compared pairwise by the two way ANOVA; except the one between June and August samples.

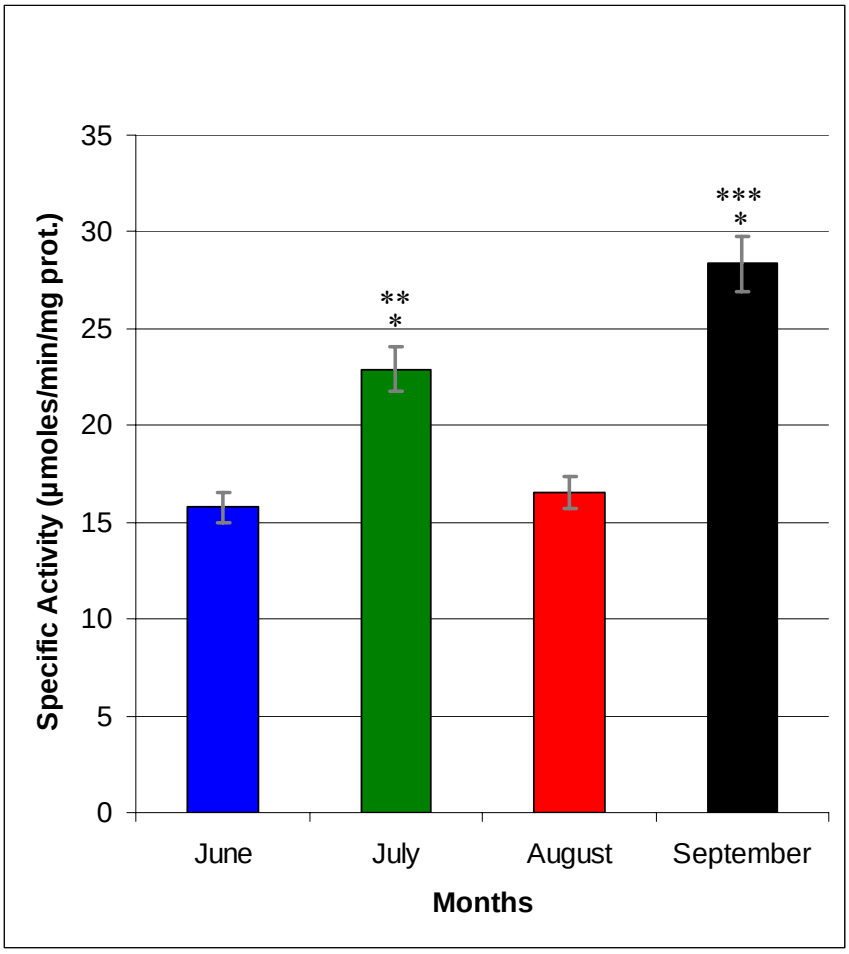

Figure 3.2. The average specific activity values for cytosolic GSTs (measured against the common substrate CDNB) of *Pinus brutia* (in 2005) obtained from the needles of 30 individuals (in two different sides: east & west) each of which was measured in triplicates.

(*): Statistically different with respect to June, $p<0.005$
(**): Statistically different with respect to August, $p<0.05$
(***): Statistically different with respect to August, $p<0.005$

After the application of t-test of difference for the 95% confidence interval between the cytosolic GST activity results of each month as couples, the p values were calculated. Any p value of t-test of difference smaller than 0,05 means a statistically significant difference between the compared set of data.

3.2 Cytosolic Total Thiol Change Under Stress Conditions

Total thiol group amounts of cytosolic extracts were measured by the method defined by Sedlak and Lindsay (1968) adapted for the ELISA plate reader and the results were expressed as the μmole / g of pulvarized tissue (needles). The data measured for the average total thiol amounts were shown in the Figure 3.3. and summarized as the Table 3.3.

Table 3.3. The average cytosolic glutathione S-transferase activities against CDNB prepared from *Pinus brutia* needles collected during drought season of 2005.

Months	**Average Total Thiol Amounts (μmoles / g of pulvarized needles) ± SE**
June	74,90 ± 2,42 (n = 30)
July	80,02 ± 3,20 (n = 30)
August	78,29 ± 3,19 (n = 30)
September	76,50 ± 2,84 (n = 30)

The data were subjected to statistical analysis using MINITAB^{TM} statistical Software Release 13,0 (BCIS Lab, St Cloud State University) two sample t-test of difference. The values found for the samples collected in July, August and September were compared to one collected in June, assuming the June samples were the control. There were no statistically significant differences between the values obtained for the total thiol group amounts of the cytosolic extracts (Fig 3.3.).

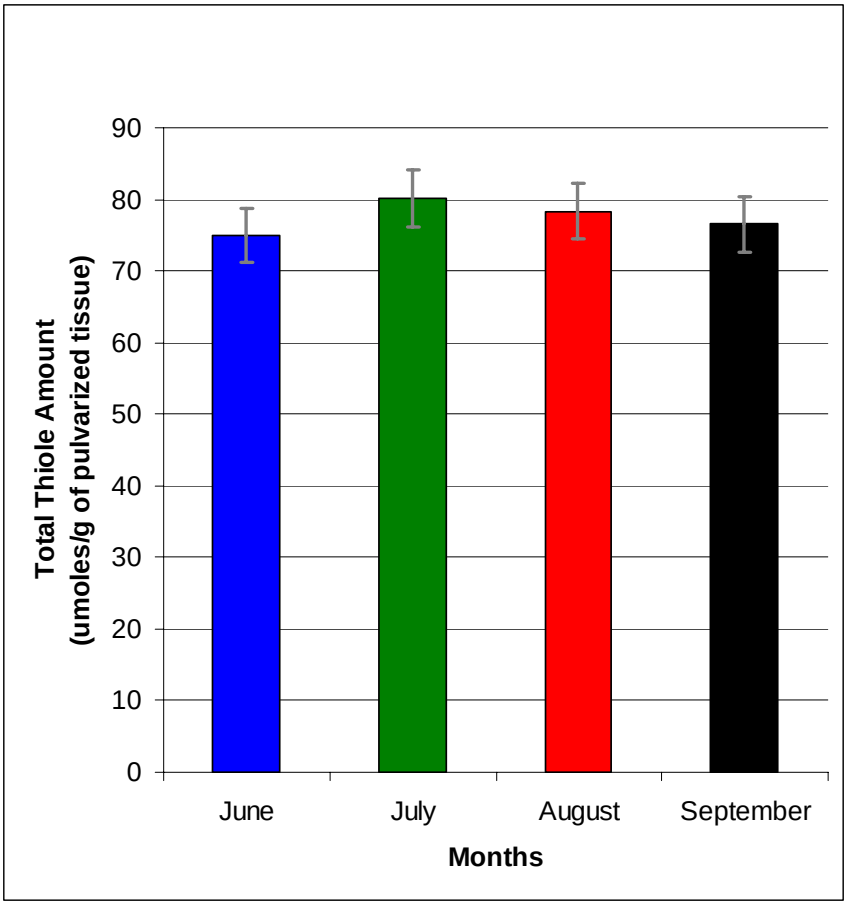

Figure 3.3. The average total thiol amount values for cytosolic extracts of *Pinus brutia* obtained from the needles of 30 individuals (in two different directions: east & west) each of which was measured in triplicates (and collected from METU-Yalıncak in 2005 summer season).

CHAPTER IV

DISCUSSION

Plants and many other organisms have developed mechanisms to defend themselves against many factors such as environmental stress conditions (high and low temperature values, salinity, drought... etc.), herbivores, insects, pathogens and some chemical compounds. Drought stress is a natural result of water deficit in the soil representing a decrease in the humidity of the environment. Any abrupt drought may cause irreversible injuries in the membrane structure of the plant cells; however, gradual imposition of stress, may not lead to a significant damage. Instead, it enables the plant to resist to a more intense stress effect (James et al, 2004). One of the major consequences of drought stress is the loss of protoplasmic water. This causes an increase in the concentrations of some ions in the cytosol and some organelles. At high concentrations of these ions, some important biochemical pathways are inhibited (Hartung et al, 1998) and there exists a change or an imbalance in the redox poise which, in turn, leads to the induction of oxidative stress (Noctor and Foyer, 1998; Smirnoff, 1998) causing the increased production of reactive oxygen species. Glutathione is one of the major endogenous antioxidants in plants (Alscher, 1989). Plants have several important catalytic enzymes which utilize glutathione in defense mechanisms. Glutathione S-transferase (GST) is one those enzymes and it shows differential patterns of activity in plant tissues exposed to cold, drought and wounding treatments (Davis and Swanson, 2001) (James et al, 2004).

In our study, we aimed to investigate the changes in cytosolic glutathione S-transferase enzyme activity measured against the substrate CDNB and to discuss the possible correlation between the GSH amount (calculated in the total thiol of cytosol) in the extracts of *P. brutia* needles collected over four month of drought season. The biochemical characterization of *P. brutia* GST was already performed previously (Boyoglu, 2004, Öztetik, 2005). In this study, previously determined

optimum conditions were applied in the measurement of cytosolic GST activity of the pine needles.

From June to July, together with the increase in the temperature values (Appendices B and C) there was a decreased precipitation (Appendix A), possibly decreasing soil humidity. As anticipated with the increase in the temperature and decrease in soil humidity, GST enzyme activity exhibited statistically significant increase from15,78 ± 1,36 μmoles min^{-1} mg $protein^{-}1$ to 22,91 ± 1,99 μmoles min^{-1} mg $protein^{-1}$, from June to July. However in August, GST activity had fallen to 16,54 ± 1,61 μmoles min^{-1} mg $protein^{-1}$. Although weather forecast reports did not record any precipitation in August, there was a local heavy rain several days before sampling. This might be the reason why GST activity measured in samples collected in August did not exhibit any statistically significant change with respect to June samples. This may explain the drop in GST activity in August. In such a case, one may expect a decrease in osmotic pressure. However, the osmotic pressure measurements were not in accordance with expectations. We couldn't determine a correlation between the osmotic pressure changes over the period with respect to the cytosolic GST enzyme activity change. That might be due to the fact that osmotic pressure measurements were affected by the weather temperature significantly; it changes quickly due to evaporation through the needles during the day. So, although heavy rain may not be reflected in osmotic pressure measurements, it seemed it influenced the GST activity (Figure 4.1.). In September, high temperature continued to provide an increasing drought stress which is correlated statistically significant high GST activity with respect to June.

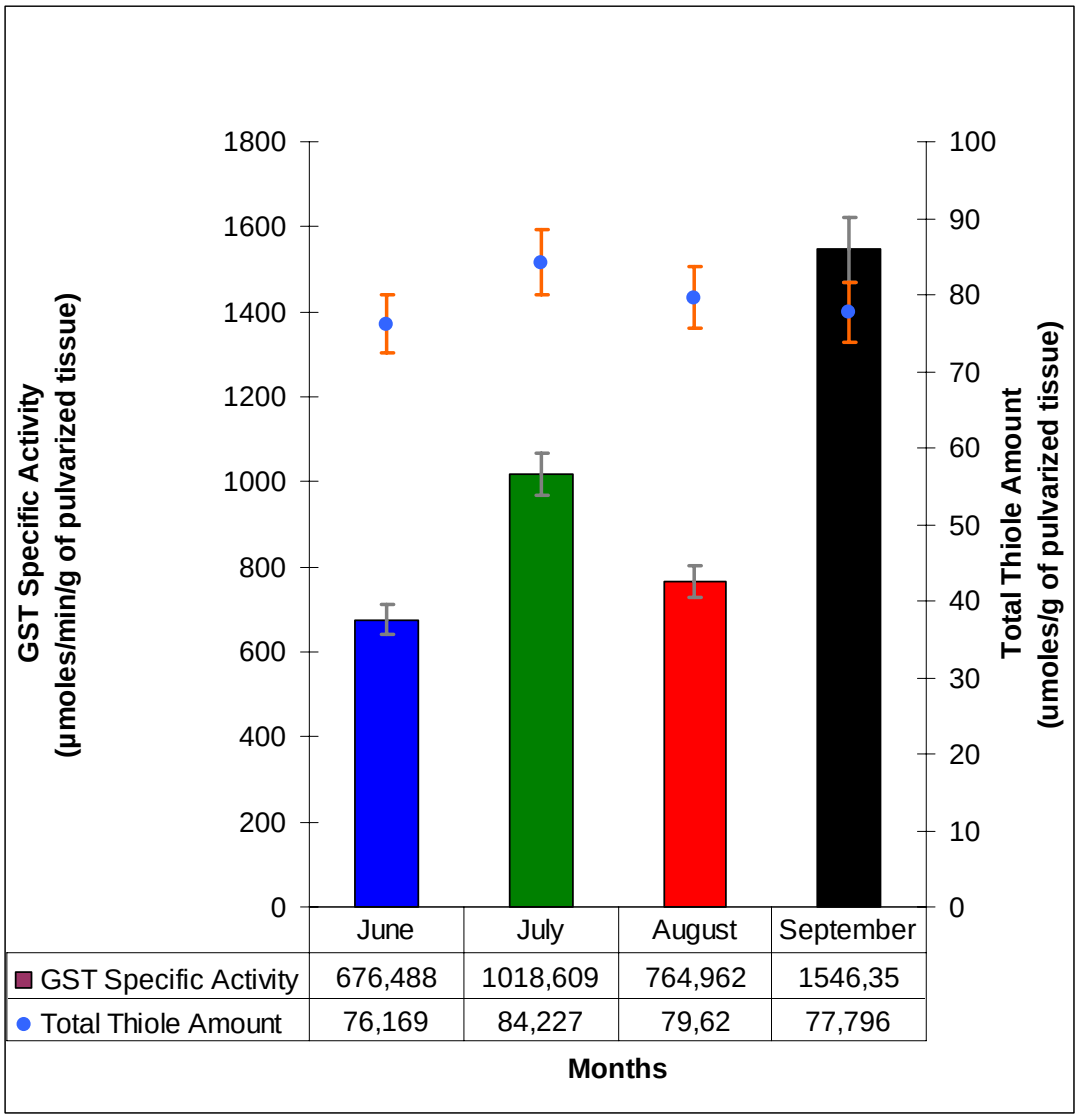

Figure 4.1. The average cytosolic GST specific activity and total thiole amount values for cytosolic extracts of *Pinus brutia* obtained from the needles of 30 individuals (in two different directions: east & west) each of which was measured in triplicates.

Our findings regarding the GST enzyme activity in response to drought stress were in accordance with the previous studies in literature on the effects of environmental stress on plants. Boyoglu (2004) stated that there was a statistically significant increase in the GST enzyme activity values in the cytosolic extracts of *P. brutia* under cold stress conditions in which, there exist oxidative stress. Mittova et al (2002) found that under salt-dependent oxidative stress, cultivated tomato species

showed higher GST and GPX activities. In another study, Dhindsa (1991) determined an increase in the GST activity during slow drying of moss (*Tortula ruralis*). Ágnes Gallé (2005) stated that, under drought stress conditions, for wheat cultivars (*Triticum aestivum* L. cv. Kobomugi and cv. Plainsman), higher GST enzyme activities were measued. However, Chen et al (2004) claimed that for the same species (Triticum aestivum L.), gradual drought stress under field conditions yielded lower GST and GP activity.

Glutathione metabolism and GSH pool are related to the responses of plants to a wide range of stress conditions. First observations of higher glutathione concentrations in plants (especially in foliar parts) exposed to environmental stresses were interpreted as an acclimation, which strengthens the antioxidative defence systems (Esterbauer and Grill, 1978). In the literature, some contradictory observations were reported regarding the GSH change under stress conditions. Chen et al (2004) stated that glutathione levels may or may not increase or may even decrease upon stress exposure. Indeed, in his study, GSH levels decreased in droughed plants (Chen et al, 2004). He suggested that glutathione might be largely consumed for regulating many important metabolic processes (Chen et al, 2004).

There are extensive reviews in literature on the response of glutathione to environmental stress impacts (Grill et al., 2001; Noctor and Foyer, 1998; Polle, 1997). Even if there observed no change in GSH amount, the GSH/GSSG redox state may change towards being more oxidized, more reduced, or not at all (Tausz et al, 2004). Severe drought at low relative water contents caused an oxidation of the glutathione pool in barley leaves (Smirnoff, 1993). Short-term responses to mild drought in pine trees also included a slight decrease in the GSH/GSSG ratio, which was suggested as a possible signal for longer-term acclimation processes. Changes in the glutathione system were only followed for two days in that study (Tausz et al., 2001). In a recent study on apple trees which were subjected to progressive drought, results showed that the GSH/GSSG ratio decreased significantly after 10 days, followed by an increase in total glutathione concentrations after 15 days. Later on,

when the stress became more intense, degradation and further oxidation of glutathione was observed (Tausz et al, 2004).

Chen et al (2004) concluded that under gradual stress conditions, the two enzymes (GST and GP) might not be essential for protecting plants from oxidative damages, and the depletion of GSH might be responsible for the decreases in activities. They stated that, GR, which is responsible for GSH regeneration from GSSG, increased in droughted plants. As a result of those changes, Chen *et al* concluded that the higher ratio of GSH/GSSH, the rate of GSH biosynthesis and the capacity of its redox cycling, rather than GSH accumulation, might be essential for drought resistance in plants. (Chen et al, 2004)

In our study, it was not obvious to what extent the changes in GST activity influenced the GSH amount, since changes in GSH amount during the sampling period was not significant. First of all, in this study, total GSH was not determined, but total thiol was determined. Actually there were some changes recorded in total thiol amount but they were far from being statistically significant. Secondly, there may be changes in the GSH/GSSG ratio which were not measured in our experiments. Nevertheless, the possibility of induction of GSH production in the cell to meet the requirements of increased GST activity may not be ruled out.

This study exemplified the effect of drought stress on the cytosolic GSH amount and GST activities of *Pinus brutia* . As a pioneer study, this research aimed to realize the effect of drought stress on the GST enzyme activity in *P. brutia* Ten. which was not studied before in this aspect. From this perspective, measurement of GSH and GST activity as stress markers will remain an important part in assessing the stress response of plants, but only if included more parameters such as, GSSG, GSH reductase and GSH peroxidase.

CHAPTER V

CONCLUSION

- The GST activity was measured among *Pinus brutia* Ten by using optimum conditions defined in previous studies (Boyoglu, 2004, Oztetik, 2005). The average specific content of GST in red pine needles, considering normal conditions in June, is 16,740 ± 9,57 μmoles/min/mg protein (n = 59).
- The average cytosolic total thiol amount values were determined in Turkish red pine needles as 76,17 ± 17,48 μmoles / g of pulvarized sample (n = 59), as an indicator of the GSH amount change.
- There is an increase in the cytosolic GST activity under drought stress conditions.
- There is a decrease in cytosolic total thiol activity (and possibly in GSH amount) under drought stress conditions, with continuing higher cytosolic GST activities.

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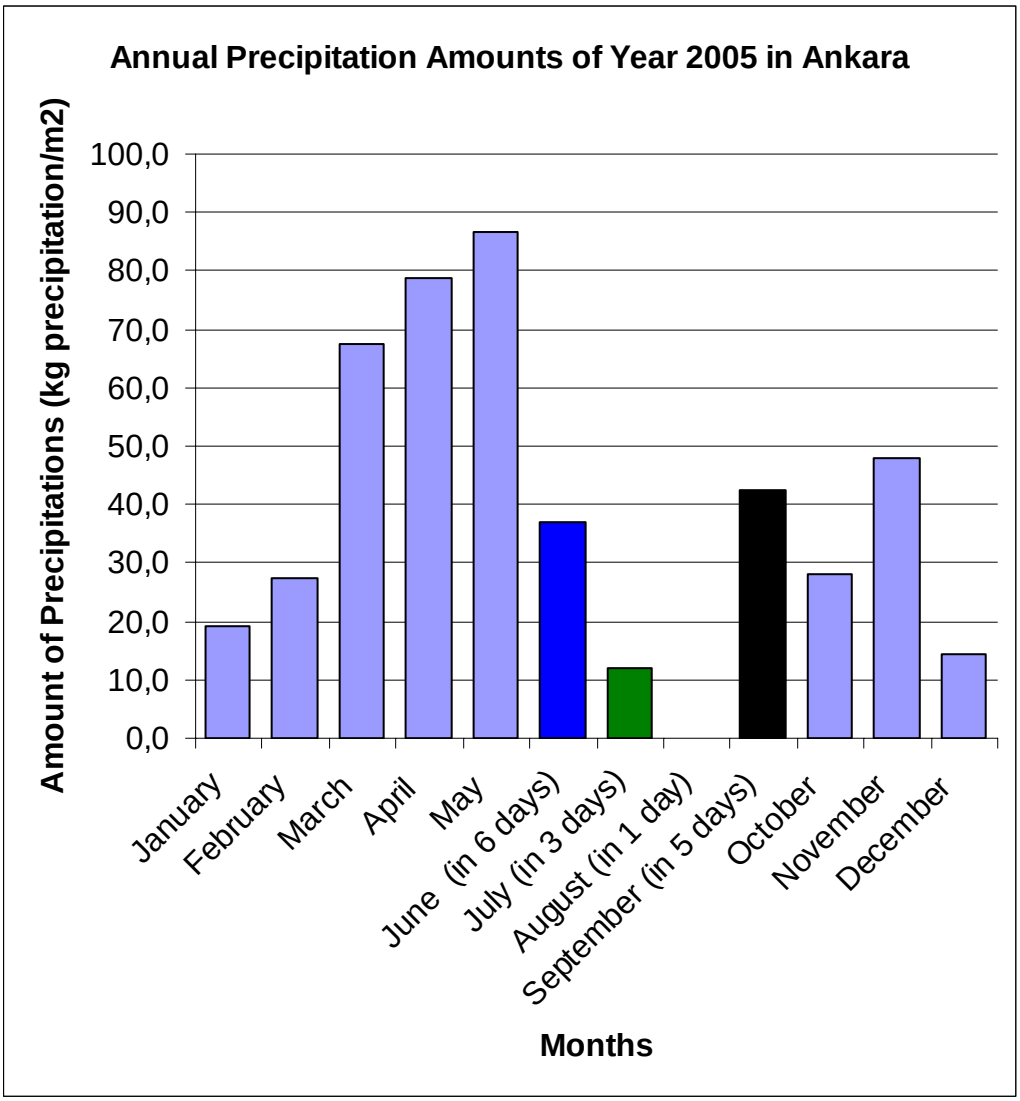
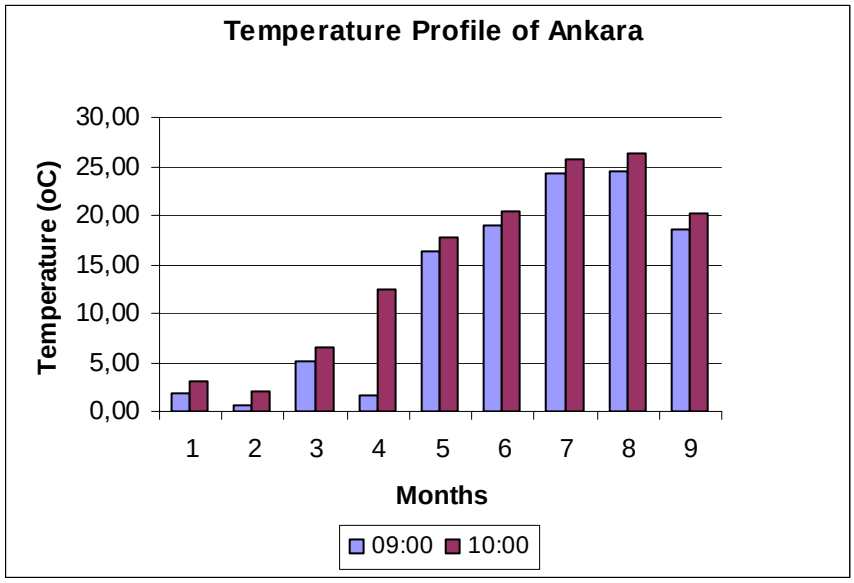
APPENDIX A: Annual precipitation change of Ankara in 2005; measured as the total amount of precipitation for each month of the year. Daily precipitation amounts were summed and given as the value of the month. The number of days of the sampling months in which precipitation occurred was indicated in the graph. (T.C. Çevre ve Orman Bakanlığı, Devlet Meteoroloji İşleri Genel Müdürlüğü, Elektronik Bilgi İşlem Müdürlüğü, Ankara. The values were taken in Keçiören, Kalaba Station)

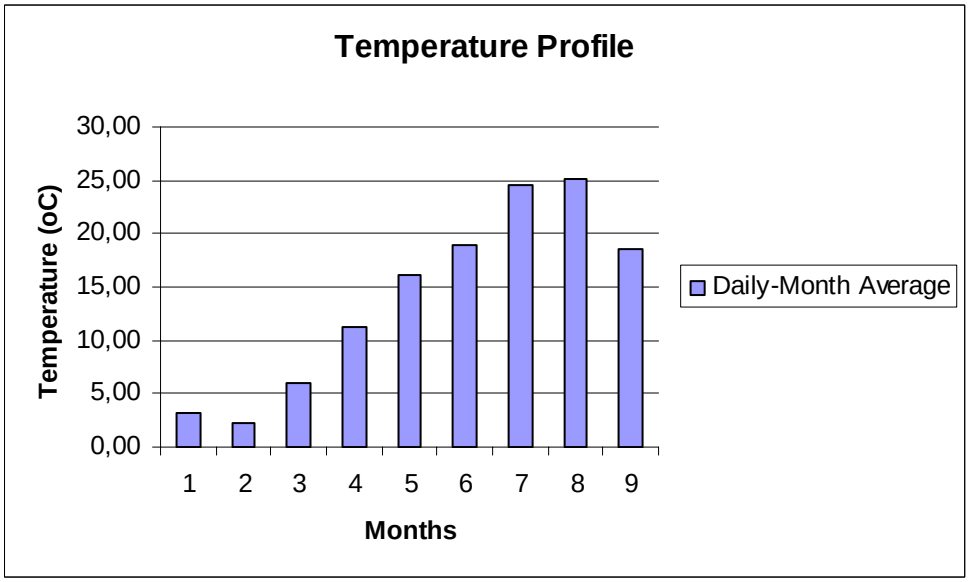


APPENDIX B:

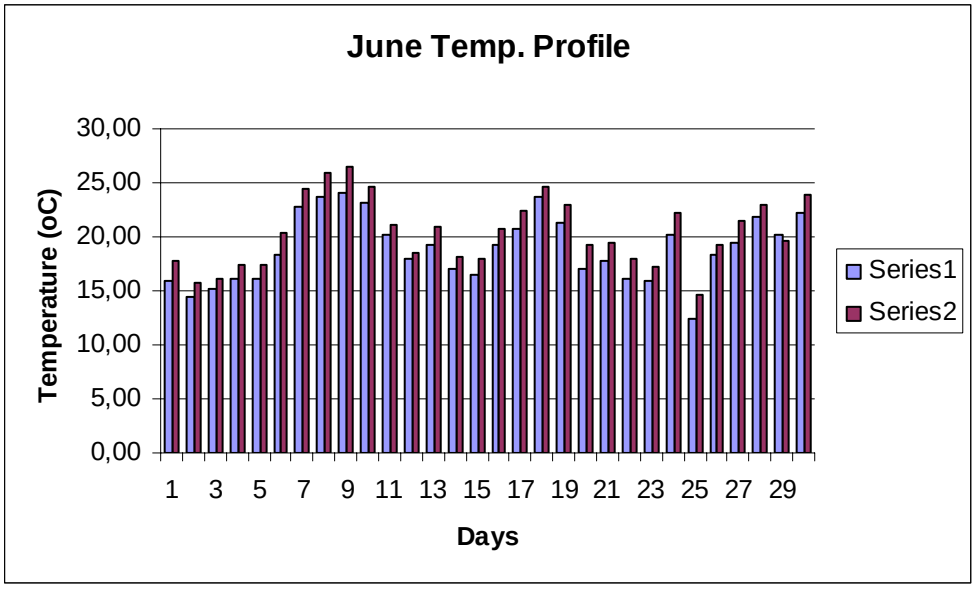
i) Temperature profile of Ankara in first nine months of 2005. Values were measured at 09:00 and 10:00 am, daily, and the averages were calculated for each month.

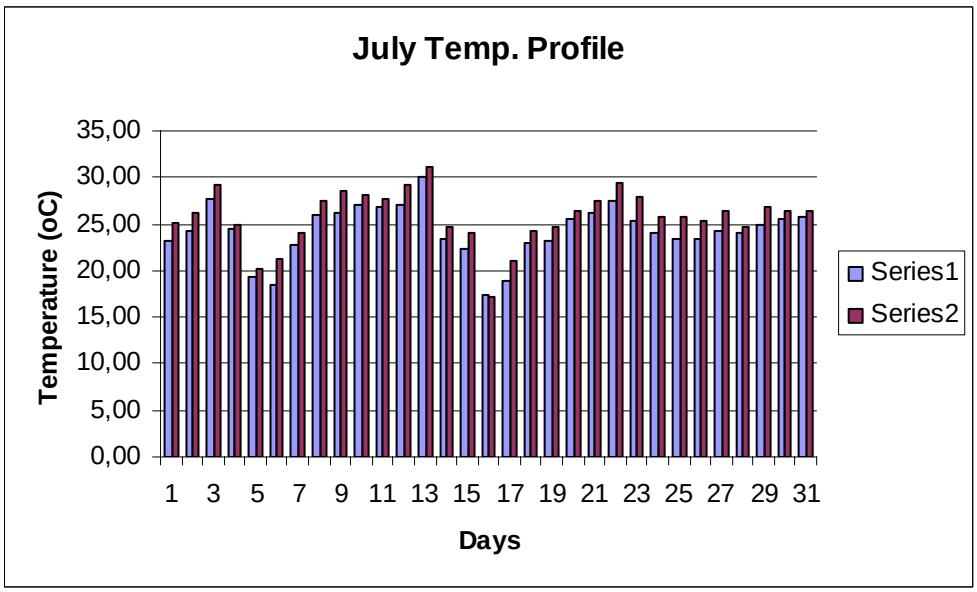


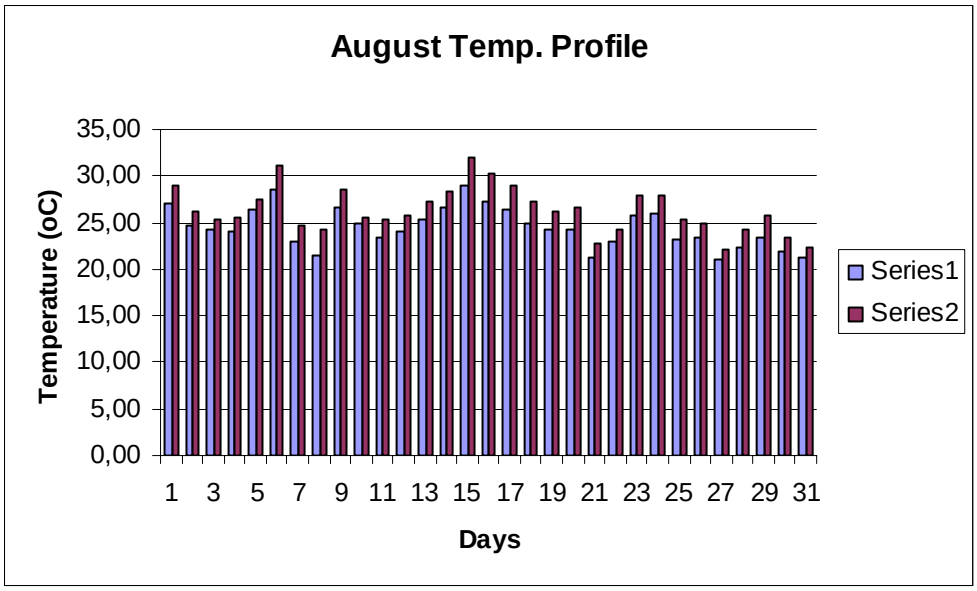
ii) Temperature profile of Ankara in first nine months of 2005. Values were calculated as the average of the daily averages. (T.C. Çevre ve Orman Bakanlığı, Devlet Meteoroloji İşleri Genel Müdürlüğü, Elektronik Bilgi İşlem Müdürlüğü, Ankara. The values were taken in Keçiören, Kalaba Station)

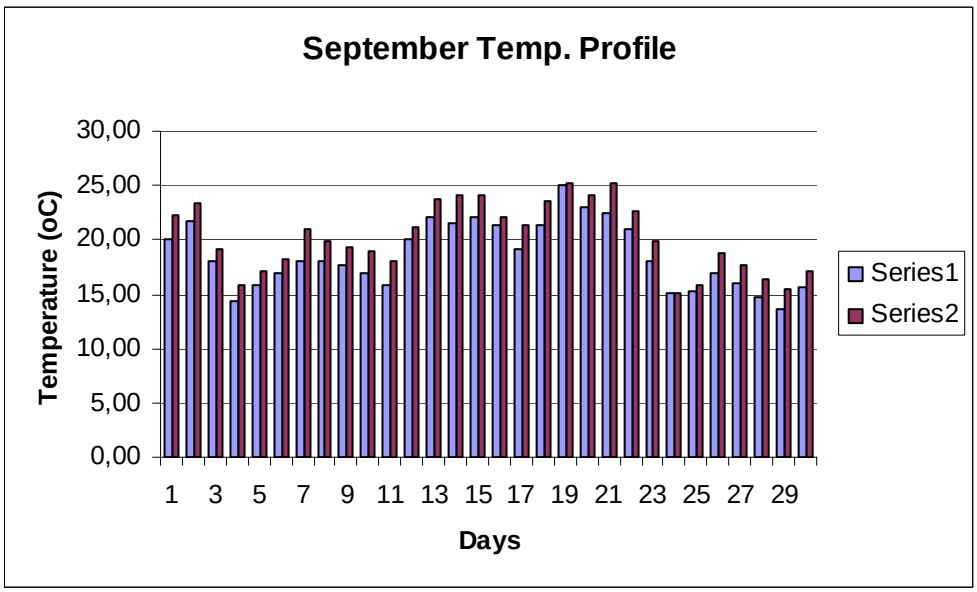


APPENDIX C: Daily temperature changes of four months (June, July, August and September) in which the sample collections were performed. Series 1 indicates the temperature values measured at 09:00 am; and Series 2 indicates the ones measured at 10:00 am. (T.C. Çevre ve Orman Bakanlığı, Devlet Meteoroloji İşleri Genel Müdürlüğü, Elektronik Bilgi İşlem Müdürlüğü, Ankara. The values were taken in Keçiören, Kalaba Station)






August Temp. Profile
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Temperature (oC)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
Days
Series1
Series2


September Temp. Profile
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Temperature (oC)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Days
Series1
Series2