CHARACTERISATION OF GLUTATHIONE S-TRANSFERASE ACTIVITY IN TURKISH RED PINE (*Pinus brutia* Ten): VARIATION IN ENVIRONMENTALLY COLD STRESSED SEEDLINGS

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASE ACTIVITY IN TURKISH RED PINE (Pinus brutia, Ten.): VARIATION IN ENVIRONMENTALLY COLD STRESSED SEEDLINGS

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Plants can not escape from biotic and abiotic stress factors such as, extreme temperatures, high light intensity, drought, UV radiation, heavy metals, and pathogen attack. Plants have versatile defens systems against such stress conditions. In this study, the role of glutathione S-transferases (GSTs) in cold stress conditions were examined. Glutathione S-transferases are the enzymes that detoxify natural and exogenous toxic compounds by conjugation with glutathione. Glutathione, an endogenous tripeptide, is important as reducing agent, nucleophilic scavenger, and alleviate the chemical toxicity in the plants by the reaction of GSTs. Glutathione conjugates can be transported to the vacuoles or apoplast and are generally much less

toxic than the parent compounds. In plants there are four distinct families of the soluble GSTs, namely Phi (F), Type I; Zeta (Z), Type II; Tau (U), Type III; Theta (T), Type IV. By contrast with the mammalian families of GST, relatively little is known about the plant GST families. Up to date, there is not any study on GST isolation and characterization from Turkish red pine, in this respect, this study well play a frontier role the future research dealing with this topic.

In this study, some properties of Turkish red pine GST activity towards CDNB (1-chloro-2,4 dinitrobenzene) were examined. The average specific activity of Turkish red pine GST towards CDNB was found as 200±50 (Mean±SE, n= 18) nmole/min/mg cytosolic protein. GSTs in cytosol prepared from Turkish red pine needles retained its activity without loss for four weeks at -80°C. The rate of conjugation reactions were linear up to 0.8mg of Turkish red pine cytosolic protein and 0.4 mg cytosolic protein was routinely used. The Turkish red pine GST showed its maximum activity at pH 8.0 in 25 mM phosphate buffer and 42 ˚C. The measurements were carried out at room temperature (RT) of 25 °C. Turkish red pine GST seemed to be saturated at 1 mM CDNB and 1 mM GSH concentrations. The Vmax and Km values of Turkish red pine GST for CDNB was 416nmole/min/mg protein and 0,8 mM, respectively, and for GSH 106.4 nmole/min/mg protein and 0.10 mM, respectively. Turkish red pine cytosol was applied on DEAE-Sepharose fast flow column but almost no purification was achieved with respect GST activity. In order to examine the effects of cold stress on Turkish red pine GST activity, the GST activity was determined in 240 seedlings at –3°, 0° and 13 °C environmental temperatures. It was observed that GST activity was the highest at -3˚C and the lowest at 13˚C in both cold resistant and sensitive families with the exception of Yaylaalan and Çameli.

Key words: Turkish red pine , glutathione S-transferases, cold stress

ÖZ

KIZILÇAM BİTKİSİNDE GLUTATYON S-TRANSFERAZ ENZİMİNİN KARAKTERİZASYONU: SOĞUK STRESİ ALTINDAKİ KIZILÇAM FİDANLARINDA GLUTATYON S-TRANSFERAZ ENZİM AKTİVİTESİNDEKİ VARYASYONLAR

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Bitkiler; aşırı yüksek ve düşük sıcaklıklar, yüksek ışık yoğunluğu, kuraklık, UV ışını, ağır metaller ve patojen mikroorganizmalar gibi biyotik ve abiyotik stres etkenleriyle karşı karşıyadır. Bitkiler bu gibi stres durumlarına karşı birçok savunma sistemine sahiptir. Bu çalışma da, Glutatyon S-transferaz enziminin soğuk stres koşullarında Kizilcam bitkisindeki rolü araştırılmıştır. Glutatyon S-transferaz enzimi (GST), doğal ve dış kaynaklı toksik bileşikleri glutatyon ile konjügasyon oluşturarak detoksifiye eder. Endojen bir tripeptit olan glutatyon, bitkilerde indirgeyici ajan, nükleofilik çöpçü, ve GST enzimlerin reaksiyonu ile kimyasal toksisiteyi azaltıcı rollerinden dolayı önemlidir. GST enzimi, glutatyonun (GSH) birçok elektrofilik bileşikle konjügasyonunu katalize edebilen enzimdir. Glutatyon konjügeleri vakuollere veya apoplastlara taşınabilirler ve genellikle ana bileşiklerden daha az

toksiktirler. Bitkilerde çözünür GST enziminin bilinen dört ailesi vardır: Phi (F), Tip I; Zeta (Z), Tip II; Tau (U), Tip III; Theta (T), Tip IV. Memelilerdeki GST enzimine kıyasla, bitkilerdeki GST enzimi hakkında nispeten daha az bilgi vardır. Bugüne kadar Kızılçam bitkisinden GST izolasyonu ve karakterizasyonu hakkında hiçbir çalışma yapılmamıştır. Bu anlamda söz konusu çalışma gelecekte bu konudaki çalışmalara öncülük etmiş olacaktır.

Bu çalışmada, Kızılçam da GST enzimi CDNB substrat olarak kullanılarak incelendi. Kızılçam da bulunan GST aktivitesi 200 ± 50 (Mean ± SE, n = 18) nmol/dak/mg'dır. Kızılçam sitozolünde GST enzimi aktivitesinin -80˚C'de 4 hafta boyunca azalmadığı görüldü. Reaksiyon hızının 0.8mg proteine kadar doğru orantılı oluğu gözlendi ve rutin olarak 0.4 mg protein kullanıldı. Kızılçam GST enzimi en yüksek aktiviteyi pH 8.0, 42 ˚C'de verdiği saptandı. Kızılçam GST enziminin CDNB için Vmax ve Km değerleri sırayla 416 nmol/dak/mg ve 0,8 mM olarak, GSH için ise 106.4 nmol/dak/mg and 0.10 mM, olarak hesaplandı. Kızılçam sitozolü DEAE – Sepharose hızlı akışlı kolona uygulandı, fakat bir saflaştırma elde edilemedi. Soğuk stresin Kızılçam GST aktivitesi üzerindeki etkisini belirlemek için GST aktivitesi 240 fidanda, –3°, 0° and 13 °C çevresel sıcaklık koşullarında çalışılmıştır. Yaylaalan ve Çameli dışındaki tüm populasyonlarda -3 ^{o}C'de en yüksek, 13 ^{o}C'de ise en düşük GST aktivitesi gözlenmiştir.

Anahtar kelimeler: Kızılcam, glutatyon S-transferaz, soğuk stres

To my Dear Family

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CDNB	1-Chloro-2, 4-dinitrobeenzene
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
GPX	Glutathione peroxidases
GS-X pump	ATP-dependent glutathione S-conjugates export pump
H_2O_2	Hydrogen peroxide
NaOH	Sodium hydroxide
NaCl	Sodium chloride
PVP-K30	Polyvinylpyrrolidone K30
ROS	Reactive oxygen species
TEMED	N, N, N´, N´-tetramethylenediamine
Tris	Tris (hydroxylmethyl) aminomethane

CHAPTER I

INTRODUCTION

1.1 Biology and Taxonomy of Turkish red pine

Turkish red pine (Pinus brutia, Ten.) is a member of Division Gymnospermae, Class Coniferae, and Family Pinaceae, Genus Pinus L. According to some taxonomists, Turkish red pine has been regarded as a variety of *Pinus halepensis* Mill. the species hybridizes naturally with *Pinus halepensis.* In addition, chemical and physical analysis of the gum terpentine as genetic markers showed that Turkish red pine and *Pinus halepensis* were announced as two distinct species (Yaltırık and Boydak, 1993; Kandemir, 2002).

In fact, morphologically *Pinus halepensis* and Turkish red pine are different species. Turkish red pine is taller and straighter than the Aleppo pine (*Pinus halepensis* Mill.). The bark of it is thick, regularly and longitudinally deeply fissured, shoots are yellow-reddish, buds are oblong, 10-20 mm long. Needles are 12-18 cm long, and dark green. Flowers appear between March and May. Cones are usually 3-4 in a whorl, horizantally spreading, 6.5-9.5 cm long, almost sessile on a short stalk; apophysis upto 2 cm wide, with a totally flat umbo. Seeds are 7-9 cm long, darkly mottled. Turkish red pine has 10 isobranchial and 2 heterobrachial chromosomes, and these heterobrachials are 11th and 12th chromosomes (Vidakovic, 1991; Kandemir, 2002). It starts to produce seed between 7 and 10 years of age, 2 years later by comparing to Aleppo pine (Davis, 1965; Panetsos, 1981; Kandemir, 2002).

Turkish red pine exhibits considerable variation in its form and growth characteristics. It is an eastern Mediterranean species. Turkish red pine forest is climax vegetation of the Mediterranean region in Turkey. The species is also important for Turkish forestry and forest products since it has the ability to grow rapidly (with several flushes in a year). The annual shoot consists of one spring arising from a winter bud and one or more summer shoots (Lanner, 1976; Yıldırım, 1992; Kandemir, 2002).

1.2 Natural Distribution and Economic Importance of Turkish red pine in Turkey

Turkish red pine is the most widespread forest tree species in Turkey with a coverage area of 3,096,064 ha. This area corresponds 15% of the total forest lands (20.2 million ha) of Turkey. Natural distribution of Turkish red pine in Turkey covers the Mediterranean region, Aegean region and nortwestern part of Turkey (Kandemir, 2002).

Almost half of the Turkish red pine forests in Turkey are found in the Mediterranean region, mainly coastal areas (Neyişci, 1987; Kandemir, 2002). Because of wide range ecological requirements, species can grow in different climatic conditions and geological formations (Zohary, 1973; Arbez, 1974; Panethos, 1981; Kandemir, 2002). The Aegean Region has the second largest Turkish red pine distribution with 40% coverage. The rest of Turkish red pine forests in Turkey, which is about 10%, grow in the Marmara region, mainly in Gelibolu and Biga Peninsula. In addition, it is possible to find individuals and groups of Turkish red pine in the Black Sea region (Yenice-Zonguldak, Ayancık- Sinop, Erbaa, Taşova) and through the central Anatolia (west of Eskişehir, near Uşak and Denizli) region (Davis, 1965; Neyişci, 1987).

Turkish red pine can grow at different altitudes. It grows from sea level to 1300 m in stands and to 1500 m as individuals. It is possible to see between 800 to 900 m

in the Mediterranean region to the northern parts of Turkey, and from 600 to 700 m in the Marmara region (Neyişci, 1987).

1.3 Genetic Variation in Turkish red pine

Conifers are one of the most genetically variable groups of plants. The reasons of this high genetic variation are coming from wide rage of geographical distrubition, genetic length, population structure, pollination mechanism (mating system), seed dispersal (gene flow), stages of succession and fecundity features (El-Kassaby, 1991; Hamrick *et al*., 1992; Kandemir, 2002).

Most of the studies concerning Turkish red pine are related to natural regeneration and production (Alemdağ, 1962; Özdemir, 1977; Usta, 1991; Kandemir, 2002). In recent years, attention has been given to genetic variation in various traits and improvement of the species (Işık, 1986; Işık *et al*., 1987; Yıldırım, 1992; Kara *et al*., 1997; Kaya and Işık, 1997; Işık and Kara, 1997; Kandemir, 2002).

Genetic variation in seedling traits of Turkish red pine has first studied by Işık (1986). A total of 60 wind-pollinated families of Turkish red pine, grouped into six populations at different elevations in Southern Turkey, were raised in a nursery near Antalya, and assessed for 16 seed and seedling characteristics. The study concluded that subspecies brutia has locally adapted populations with a predominatly altitudinal variation pattern.

Işık and Kara (1997) studied altitudinal variation in Turkish red pine and its implication in genetic conservation and seed transfer in southern Turkey. The results of the study, based on growth and isoenzyme analyses, suggested that middle elevation populations (approximately between 400 and 900 m above sea level) growing in less stressful environments of Taurus Mountains perform better, represent higher total genetic diversity and have greater *adaptability* to high and low elevation sites than the populations from much lower and/or higher elevations.

The significant correlation between elevation and allele frequencies in certain enzyme systems (Kara *et al*., 1997), as well as between elevation and various morphological characteristics found in previous studies (Işık, 1986; Işık, 1993; Işık *et al*., 1987; Işık and Kara, 1997) showed that genetic variation between populations was clinical for height, and under strong genetic control, suggesting the existence of a combined selection pressure exerted by human activity and climatic factors associated with the sharp increase in altitude in the Taurus Mountains in the vicinity of Antalya.

According to Panetsos *et al*. (1998), factors such as geographic isolation, long-term negative selection due to needs in wood and resin, soil mosaic, climatic variability due to differences in altitude, as well as forest fires, are expected to have contributed to the species' present genetic structure. This suggests a certain plasticity and adaptability of Turkish red pine but also the existence of ecotypes adapted to different environments (Calamassi *et al*., 1988).

1.4 Stress Physiology

Stress can be defined as external factors, which cause disadvantageous influence on the plants. Throughout their life cycle, plants have to react to various threats coming from the outside environment. As plants are sessile, they have developed a broad range of strategies, collectively known as "defense" or "stress responses", to protect themselves against abiotic and biotic stresses (Wojtaszek, 1997). Abiotic stresses such as heat, chilling, drought, cold, salinity, fire etc. and biotic stresses like insect infestations play important roles by means of shaping life and determining the limits of adaptation and nearly all ecosystems are subject to periodic disturbances by natural or man caused events (Vogl, 1980; Grime, 1993).

Effects of stress on variation are related with the increased rates of mutation, recombination, and transposition. In addition, stress can increase the expression of variation at the phenotypic level by lowering thresholds for the expression of traits,

by influencing growth or metabolic flux, or by other processes (Figure 1) (Hoffman and Hercus, 2000).

Exposure of plants to unfavourable environmental conditions such as vicissitudes of temperature, water availability, air pollutants, or salt stress cause injury in cell membrane systems of plants. (Thomashow, 1990; Roxas *et al*., 2000; Hara *et al*., 2003). Two factors have been considered as causes of membrane damage under cold stress. The first is a correlation between chilling sensitivity and the degree of unsaturation of fatty acids. Chilling-sensitive plants possess a high degree of saturated fatty acids in phosphatidyl-glycerol in membranes. Since saturated fatty acids has a high melting point, membranes isolated from chilling sensitive plants can undergo a phase transition from the liquid crystalline phase to gel phase even at room temperature. The second factor causing cold injury by membrane damage is the decrease in membrane fluidity and loss of function produced by lipid peroxidation. Cold stress enhances production of reactive oxygen species (ROS) including single oxygen (O_2) , superoxide radicals (O_2^{-1}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) and peroxidized membranes. This causes the loss of unsaturated fatty acids, an increase in membrane rigidity due to the formation of covalent bonds among lipid radicals, a higher lipid-phase transition temperature and membrane degradation (Roxas *et al*., 2000; Hara *et al*., 2003).

To protect themselves against these toxic radicals, plants employ defense systems that include the enzymes such as superoxide dismutases, catalases, ascorbate peroxidases (APX), glutathione S-transferases and glutathione peroxidases (GPX) that catalyze the scavenging of reactive oxygen species (Roxas *et al*., 2000).

Responses of organisms to stress differ depending on their genetic compositions. In addition, the nature and intensity of response to a particular stress factor may vary considerably, depending upon the age, degree of adaptation, and seasonal activity of the species (Larcher, 1995).

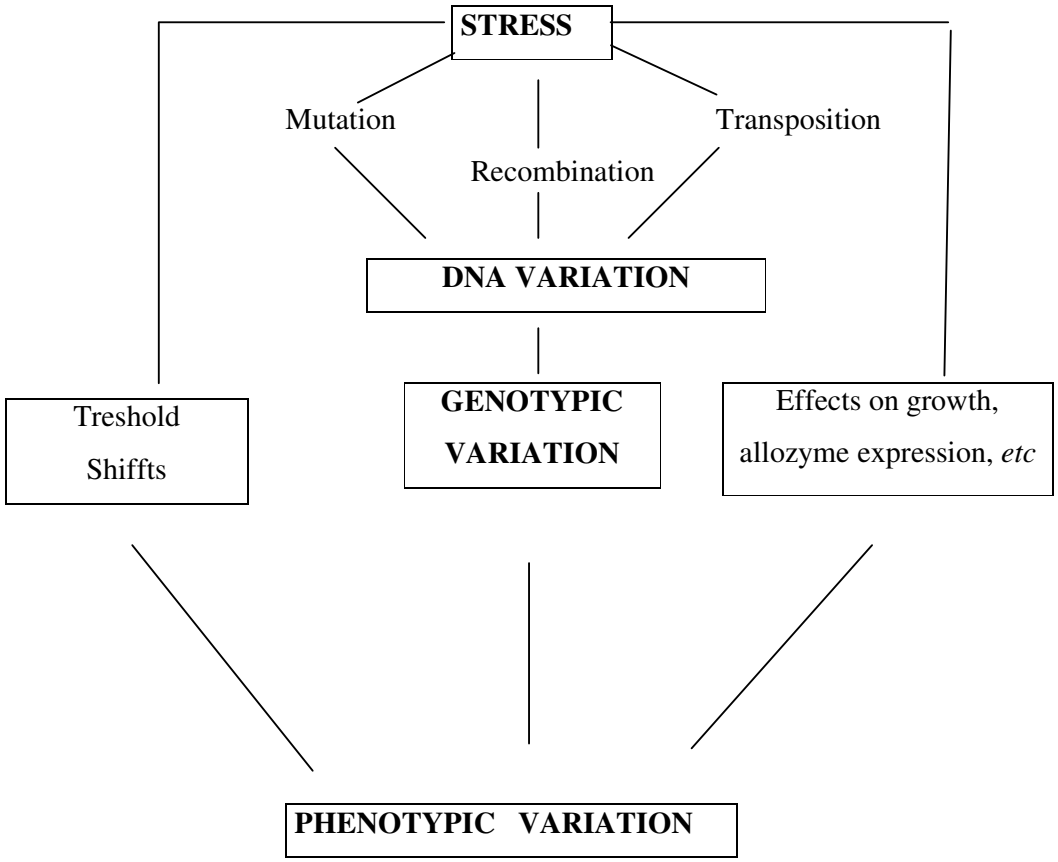

Figure 1. Effects of stress on genetic variation (Hoffman and Hercus, 2000).

1.5 Glutathione S-Transferases

Glutathione S-transferases are a super family of enzymes that conjugate reduced glutathione to a wide variety of compounds that are lipophilic and have an electrophilic center (Mannervik and Danielson, 1988; Coles and Ketterer, 1990). GST's are belonging to a family of phase II detoxification enzymes that catalyse the nucleophilic addition of glutathione to electrophilic centres in organic molecules (Marrs, 1996). This reaction yields a GSH conjugate that is often inactive, water soluble, and is usually less toxic than the parent compound (Droog *et al.*,1993).

Almost all of the known cytocolic GSTs are homo- or hetero- dimers of subunits with moleculer weights of 23-29 kDa (Droog, 1997).Each subunit has a GSH binding site (G-site) and a adjacent electrophilic substrate binding site (H-site) (Marrs, 1996).

Plant GSTs are typically divided into three types Droog *et al*.(1995) – type I, type II, and type III- based on a combination of sequence conservation, immunological cross reactivity, and intron/ exon structure of the gene. Type I GSTs have two introns, and many are induced, both transcriptionally and translationally, by environmental perturbations, such as dehydration, wounding, active oxygen, pathogen attack, or the hormones auxin and ethylene. Type II GSTs have nine introns; however, to date, the only reported sequence is from carnation. Type III GSTs have a single intron and are transcriptionally and translationally inducible by various phytohormones, pathogen attack, and heavy metals (Marrs, 1996; Droog *et al*., 1995; Alfenito *et al*., 1998). GST's are distributed in a wide range of organisms ranging from E.coli to mammals (Mannervik and Danielson, 1988). GSTs have been identified and characterized in insects, in bacteria and in many plants such as maize (Edwards and Owen, 1986; Rossini et al. 1996; Jablonkai and Hatzios, 1991; Scarponi et al., 1992; Jepson et al., 1994; Holt et al., 1995; Marrs et al. 1995; Hatton et al., 1996; Dixon et al., 1997; Marrs and Walbot, 1997), wheat (Jablonkai and Hatzios, 1991; Mauch and Dudler, 1993, Romano et al., 1993; Edwards and Cole, 1996; Riechers et al., 1996; Riechers et al., 1997), tobacco (Droog et al., 1995), dwarf pine (Schroder and Rennenberg, 1992), soybean (Ulmasov et al., 1995; Andrews et al., 1997), Arabidopsis thaliana (Reinemer et al. 1996), barley (Romano et al. 1993; Wolf et al. 1996), Setaria spp. (Wang and Dekker 1995), carnation (Meyer et al. 1991), potato (Hahn and Strittmatter 1994), chickpea (Hunatti and Ali 1990, 1991), sorghum (Gronwald et al. 1987; Dean et al. 1990), velvetleaf (Anderson and Gronwald 1991) and sugarcane (Singhal et al. 1991).According to Lamoureux and Rusness (1993) there are 33 plant species with GST activity, although in many cases the GSTs have not been purified (Marrs, 1996).

Distrubution of GST is ubiquitous and GST presumably evolved with GSH in aerobic organisms to protect the cells from oxidative damage and electrophilic

attack. Also, GSTs serve in the intracellular detoxification of mutagens, carcinogens and other toxic compounds (Mannervik and Danielson, 1988). The most well known role of plant GST is detoxification of nucleophilic xenobiotic compounds by conjugating with GSH (Sandermann,1992).

Multiple GST isozymes are present in most plants. However, the multiplicity of GST isoforms in most organisms indicates that these enzymes have a wide range of substrates. Multiple forms of GST are often found in a single tissue or cell type (Mannervik and Danielson, 1988). The multiple isozymes are thought to evolved to accommodate exposure to diverse substrates and ligands. Thus, various classes of GSTs possess overlapping but distinctive binding and substrate specificity (Mozer *et al*., 1983; Sandermann, 1992).

1.5.1 Structure of the Glutathione S-transferases

Glutathione S-transferases comprise two subunits existing as either homodimers or heterodimers where each subunit in the dimeric protein functions independently (Abu-Hijleh, 1999). Its subunit is characterized by the GST-typical modular structure with two spatially distinct domains (Sheenan *et al*., 2001). Domain I, the N-terminal domain, contains much of the G-site, whereas domain II which is a C terminal cosubstrate binding site; contains all of the H site, which will accommodate a diverse range of hydrophobic compounds (Abu-Hijleh, 1999; Edwards *et al*., 2000).

The specificity of the G-site is so high that only GSH and its closely related derivatives, such as homoglutathione, or γ-glutamylcysteine, can bind to it (Mannervik and Danielson, 1988). The binding of GSH involves ionic bonds and the catalytic mechanism of activation involves deprotonation the thiol group of GSH (Mannervik and Danielson, 1988).

A conserved tyrosine residue is located in the active site and is required for catalytic function (Amstrong, 1997). Unlike the G-site, the substrate specificity of

the H-site is broad. These substrates commonly have Michaelis reaction acceptor-carbon-carbon double bonds adjacent to an electron-withdrawing group.

X-ray crystallography has shown a similar three-dimensional topology for three phi-class GSTs from Arabidopsis and maize which share only 20% sequence identity in all three phi GSTs, the active site is situated on either side of a large, open cleft formed between the subunits, allowing access to large planar and spherical molecules (Figure 2). However, each active site interacts minimally with the adjacent subunit. Crystallography has also 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 (Abu-Hijleh, 1999; Edwards *et al*., 2000). Members of the GST family show the greatest sequence similarity in their GSH-binding domain, four highly conserved amino acids in this domain are a 'signature motif' for GSTs, but this motif is not sufficient to identify a GST.

The G-site Glu-Ser-Arg trio provides a notable distinction between type I and type III GSTs. In type I, the first position Glu can have the conservative substitution Asp, and Ser-Arg is found in all of the type I sequences. In the type III the first two residues are always Glu-Ser, and the Arg in the third position is rarely present, usually replaced by Leu. In the type III GSTs the totally conserved Arg-17 may substitute for the missing Arg in the G-site trio (McGonigle *et al*., 2000).

There is also a Trp residue that is conserve in all of the type I and type III GSTs, but is missing in the type II GSTs. This Trp is located in the region of the GST I structure that forms the interface between the two subunits of the dimer, but is not close enough contact to contribute to the hydrophobic interactions between the two subunits.

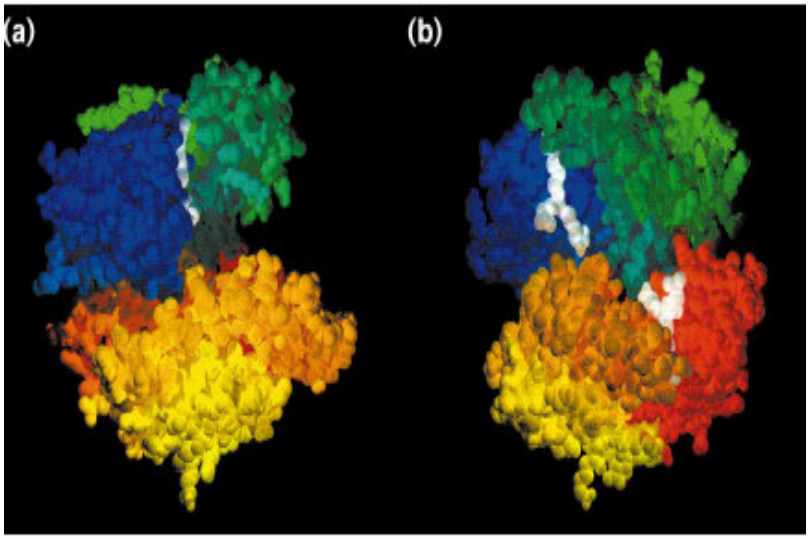

Figure 2. Two views of a space- filled model of the crystal structure of the Arabidopsis phi class glutathione S-transferase (GST) dimer AtGSTF2-2. (a) A side–on view to illustrate the large active- site cleft. (b) A view into the active sites. For both views, both subunits are visible: the lower subunit is shaded from red at the N-terminus to yellow at the C-terminus and the upper subunit is shaded from blue at the N-terminus to green at the C-terminus. Each of the two active sites is occupied by a molecule of S-hexylglutathione (white). The glutathione moiety is close to the dimer interface and interacts exclusively with the N-terminal domain of the GST. Whereas the S-hexyl moiety lies further from the dimer interface and interacts with both N-terminal and C-terminal domains of the protein (Edwards *et al*., 2000).

When all of the types III GSTs are compared, it is evident that there are four distinct segments of homology that are a strong feature of the type III GSTs. The four strongly conserved segments consist of S20-E38, K49-H68, E76-E86, and L101-W114. The regions of Type III strong homology correspond to distinct structural features in the model. The first of these is an α helix that begins with the active site Ser and ends with a turn and beginning of a β sheet. The second is the latter one-half of a 3_{10} helical segment, followed by a sharp turn and another β sheet strand. This region contains a flexible loop that is thought to be important in induced substrate fit in the active site. The third and fourth regions are two antiparallel α helix that appear to be arranged in a four–helix bundle with their counterparts on the other subunit of the dimer. The poorly conserved sequence between these segments is the linker segment between the N-terminal domain and the helix- rich C-terminal

domain. By contrast, the type I GSTs have several strongly conserved residues, but they are more widely distributed around the protein (McGonigle *et al*., 2000).

1.5.2 Classification of Plant GSTs

Mammalian cytosolic GSTs have been catalogued into five species independent gene classes according to the relationship between substrate recognation or antibody cross-reactivity. Those classes are Alpha, Mu, Pi (Mannervik *et al*., 1985), Sigma (Buetler and Eaton, 1992), and Theta. However, the nomenculature of plant GSTs is not unified as mammalian GSTs (Marrs, 1996).

The classification of plant GSTs depends on the amino acid sequence identity and conservation of exon: intron replacement (Droog *et al*., 1995; Marrs, 1996). A phylogenetic tree of plant GSTs is shown in Figure 3. According to these trees, almost all plant GSTs belongs to the theta class. Plant GSTs were further classified into four subgroups, according to the amino acid sequence identity and conservation of intron: exon placement. Droog *et al*.(1995) cataloged plant GST genes into three types, i.e., Type I, Type II, and Type III, and Marrs (1996) added an unclassified subgroup. A new nomenculature system be adopted for plant GST genes. Therefore, the classification of plant GSTs should be amended to include the following new classes:

Phi (F) ⟶ a plant –specific class replacing Type I
Zeta (Z) ⟶ replacing Type II
Tau (U) ⟶ a plant specific class replacing Type III
Theta(T) ⟶ replacing Type IV

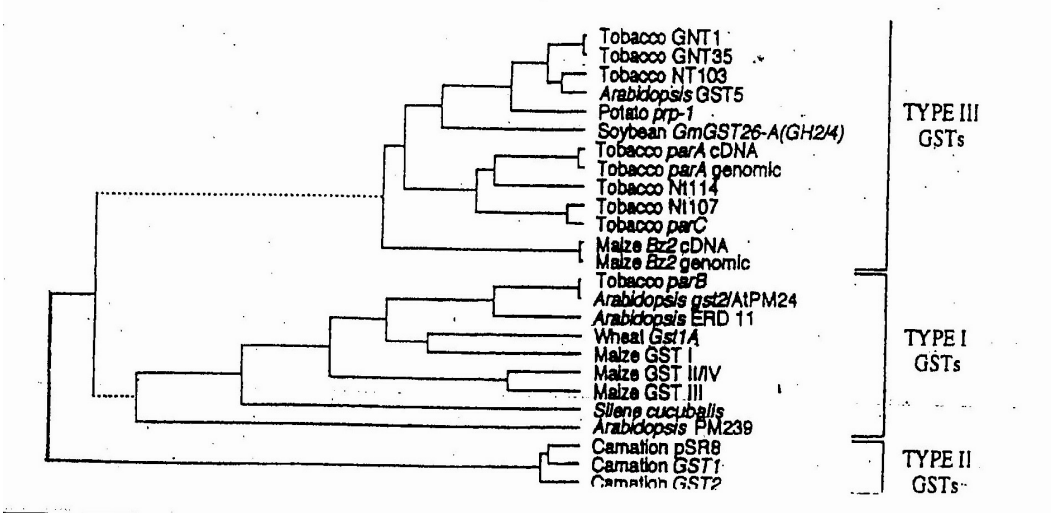

Figure 3. Phylogenetic tree of plant type I, II, and III GSTs. The tree was constructed using the DNASTAR sequence program (Marrs, 1996).

Type I

These GSTs (where the gene structure is known) contain three exons and two introns (Marrs, 1996; Karam, 1998). All of these enzymes have activity against 1-chloro-2,4-dinitrobenzene (CDNB) and the herbicide alachlor (2-chloro-N-(2,6-diethylphenyl)-N-(methoxy-methyl) acetamide. Some of type I GSTs have defensive and cellular protectant functions producing gene products in response to pathogen attack, wounding, senescence, and the resulting lipid peroxidation that accompanies these processes (Alfenito *et al.*, 1998). Other type I GSTs are induced in response to auxins and may serve a ligandin function toward indole acetic acid (IAA) (Marrs, 1996).

The Type I GSTs include enzymes from Arabidopsis, broccoli, Silene cucubalis, sugarcane, tobacco, and wheat. The well-characterized GSTs of maize belong to type I GSTs. This group includes the four distinct maize GSTs which are GST I, GST II, GST III, and GST IV (Karam, 1998) that differ with respect to subunit composition and substrate specificity with respect to herbicides (Marrs,

1996) such as alachlor, atrazine, or metolachlor (Karam, 1998). GST I and III are constitutively expressed, and GST II and IV are induced by herbicide safeners. Maize isomers I, III and IV are homo-dimers of 29, 26 and 27 kDa subunits, respectively and GST II is a hetero-dimer composed of 29 kDa GST I and 27 kDa GST IV subunits (Reinemer *et al*., 1996).

Type II

These GSTs contain ten exons and nine introns (Marrs, 1996; Karam, 1998) and represented only by GST1 (pSR8) and GST2 from carnation as ethylene- and senescense–related genes expressed in floral organs (Marrs, 1996; Karam, 1998). Carnations substrate specificity is unknown although there is speculation that they participate in lipid peroxidation (Karam, 1998). Significant amino acid sequence homology exists with type III GSTs, but the intron: exon patterns are characteristic of mammalian alpha class GSTs.

Type III

These GSTs (where the gene structure is known) contain two exons and one intron (Marrs, 1996; Karam, 1998). This subclass was originally identified as a set of homologous genes from a variety of species that were inducible by a range of different treatments–particularly auxin, but also ethylene, pathogen infection, heavy metals, and heat shock (Marrs, 1996). Type III consist of GmHsp26A or GHT2 in soybeans, prp1-1 (gene) also called Gst1 from potato, parA/Nt114, parC/Nt107, Nt103, and Nicotina plumbaginfolia msr1 (pLS216) from tobacco, bronze-2 from maize, and GST5 from Arabidopsis thaliana, first identified as a set of homologuos genes, is induced toward auxin, ethylene, pathogen infection, heavy metals, and heat shock (Karam, 1998).

Type IV (Unclassified GSTs)

Many plant GSTs have not yet been characterized (Marrs, 1996) Some GST enzyme activities fall into the "unclassified GST" group exists because their amino acid sequences are not yet known. For example; Sorghum GSTs 1-6, GST I, II, III, IV, from chickpea, soluble (37kD), soluble (47kD), and microsomal from pea which activity toward herbicide safeners and natural substrate cinnamic acid (Marrs, 1996), GST25 and GST26 from wheat which were strongly induced by cadmium, atrazine, paraquat, and alachlor but not by pathogen attack (Marrs, 1996), GST1 from Arabidopsis thaliana, GST I and GST II from Picea abies, Zea mays (function as 30 kD monomer) from maize, and Phaseolus vulgaris from French bean have been characterized, but because of the amino acid sequence still unavailable impeding grouping in classes I, II, and III (Marrs, 1996; Karam, 1998).

1.5.3 Functions of Glutathione S-Transferase

GSTs are known primarily as detoxification enzymes. They catalyze the conjugation of reduced glutathione with reactive electrophilic and hydrophobic molecules. In mammalian systems, increased levels of expression of several related GST isozymes are known to protect the cells from structurally diverse electrophiles that are present in our environment or are used in cancer chemotheraphy .

GSTs are present at every stage of plant development from early embryogenesis to senescence and in every tissue type examined (McGonigle, 2000). GSTs play a major role in the detoxification of herbicidal compounds. Detoxification in plants is a process that metabolizes foreign compounds. It can be seperated into two sequential process: chemical (modification) transformation and compartmentation. These two process are divided into three phases: phase I (activation), phase II (conjugation), phase III (internal compartmentation and storage processes) (Karam, 1998). Phase I usually involves hydrolysis catalysed by esterases and amidases or oxidation catalyzed by the cytochrome P-450 system. Phase II involves the deactivation synthesis of xenobiotic or a phase I-activated metabolite by covalent linkage to an endogenous hydrophilic molecule, such as glucose, malonate

or glutathione resulting in a nontoxic or less toxic compound. Phase II is catalyzed by glucosyl-, malonyl, or glutathione transferases. A very important conjugation in plant herbicide metabolism is the thiol reaction attack of the GSH to an eletrophilic substrate with a displacement of a nucleophile (Karam, 1998). These reactions usually are catalyzed by GSTs in maize with conjugation of glutathione and atrazine (Karam, 1998).

$$R\text{-}X + GST \longrightarrow R\text{-}SG + HX$$

In phase III, the inactive water soluble conjugates formed in phase II, are exported from the cytosol by membrane-located proteins, which initiate the compartmentalization and storage in the vacuole (soluble conjugates) or in the cell wall (insoluble conjugates) (Pickett and Lu, 1989; Rossini *et al*, 1996; Gronwald and Plaisance, 1998; Karam,1998).

In plants, which have no excretion system, soluble glutathione S- conjugates are stored in the vacuole. The glutathione S-conjugates of N-ethylmaleimide and of metalachlor were taken up by vacuoles by an ATPase biochemically identical to the GS-X pump, on the basis of inhibition by vanadate, ATP-dependence, and the recognition of numerous glutathione S-conjugates. Anthocyanin-GSH conjugates also appear to be transported into vacuoles by this pump. Thus, anthocyanins are an endogenous substrates for the GS-X pump (Marrs, 1996; Kocsy *et al*., 2001; Sheehan *et al.,* 2001).

GSTs are also involved in the synthesis of secondary products such as anthocyanins and cinnamic acid (Figure 4) (Marrs *et al*., 1995). Anthocyanins belong to a large group of compounds known as flavonoids. Most of these pigments are synthesized in the endoplasmic reticulum and are transported to the vacuoles. In maize, the bz-2 gene encodes a type III GSTs and performs the last genetically defined step in anthocyanin biosynthesis. This step is the conjugation of glutathione to cyanidin 3-glucoside (C3G). Glutathionated C3G is transported to the vacuole by a tonoplast Mg-ATP-requiring glutathione pump (GS-X). In bz-2 mutant plants,

cyanidin-3-glucoside accumulates in the cytoplasm leading to a bronze color, poor vigor, or even death in some plants. The protein encoded by bz-2 shows high similarity with plant GSTs and is active against on CDNB. Genetically, the comparable step in the petunia anthocyanin pathway is controlled by the Anthocyanin13 (An13) gene. An13 found to encode a type I plant GST. Bz2 and An13 have evolved independently from distinct types of GSTs, but each is regulated by the conserved transcriptional activators of the anthocyanin pathway (Alfenito et al., 1998). Anthocyanin biosynthesis may represent a common mechanism for the ability of plants to sequester structurally similar but functionally diverse molecules in the vacuole (Marrs *et al*., 1995; Holton and Cornish, 1995)

Cyanidin-3-glucoside is a candidate as a natural substrate for GSTs. Though weak, one of maize GSTs can use cyanidin-3-glucoside as substrate and may contribute to pale pink instead of bronze color in some mutant plants and the deep purple pigments in wild type Bz-2 plants (Marrs, 1996).

The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-CoA and p-coumaroyl-CoA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with p-coumaroyl-CoA to yield tetrahydroxychalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isomerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. Naringenin is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can subsequently be hydroxylated by flavonoid 3$^{'}$-hydoxylase (F3$^{'}$H) to produce dihydroquercetin (DHQ) (Holton and Cornish, 1995).

At least three enzymes are required for converting the colorless dihydroflavonols to anthocyanins. Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding red cyanidin pigments. Anthocyanidin 3-glucosides may be modified further in many species by glycosylation, methylation, and acylation. (Holton and Cornish, 1995).

Plants defend themselves against microbial infection or fungal attack is through the formation of phytoalexins at infection sites. Phytoalexins, which are absent in healthy plants, are hydrophobic compounds of low moleculer weight that are static or toxic to a broad range of microorganisms. Phytoalexin synthesis in response to fungal elicitors has been strongly inhibited by cinnamic acid, a precursor to phytoalexins. Glutathione S-cinnamoyl transferases (GCSTs) catalyze conjugation of cinnamic acid with GSH and have been hypothesized to remove inhibitory cinnamic acid during the initial steps of phytoalexin synthesis and to reduce accumulation of other toxic phenolic compounds produced under stress conditions (Edwards and Dixon, 1991; Dean *et al*., 1995; Marrs, 1996)

All GSTs are able to bind noncovalently a range of chemicals that are not substrates, including steroid and thyroid hormones, bile acids, bilirubin, heme, fatty acids, and penicilin (Abu-Hijleh, 1999).

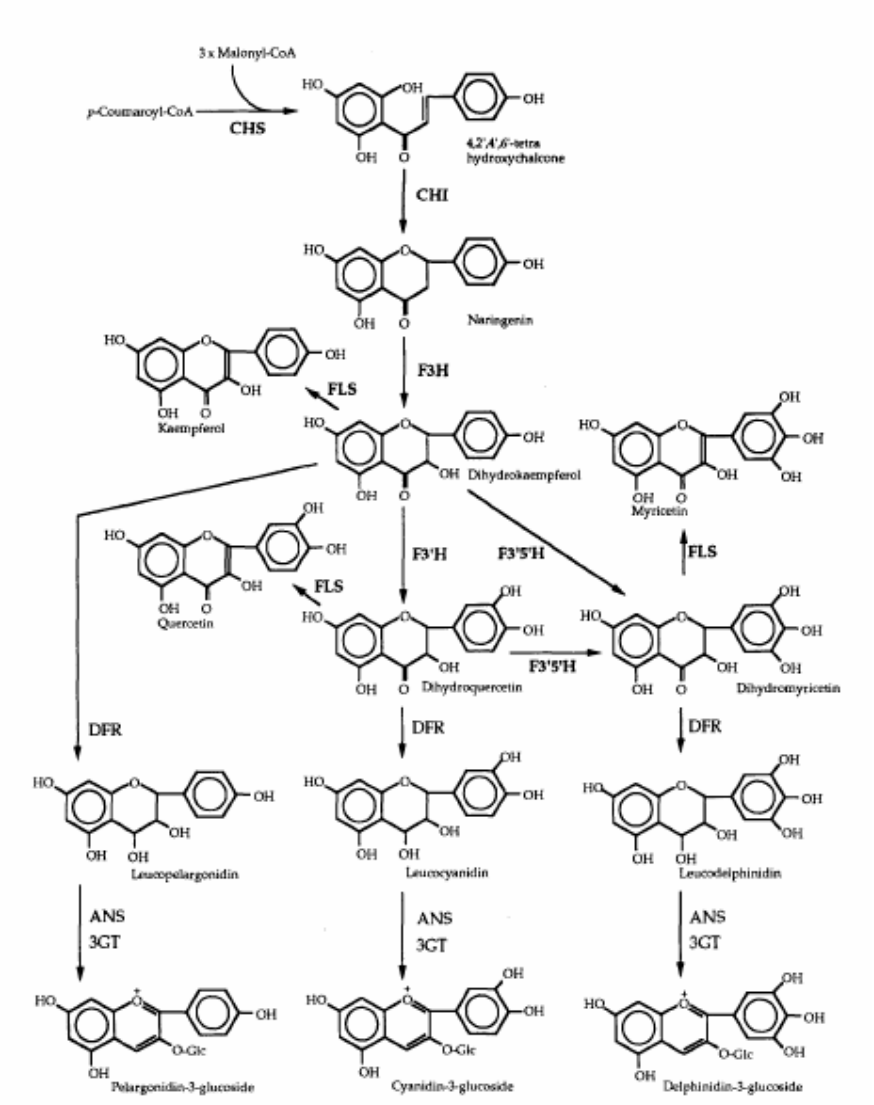

Figure 4. Anthocyanin Biosynthesis Pathway (Holton and Cornish, 1995)

GSTs may also function as a reversible ligand and it is with this function that they may play a role in hormonal regulation (McGonigle *et al*., 2000). In plants some GSTs apparently serve as carriers of the natural auxin indole 3-acetic acid (IAA). It is very likely that auxins from various sources can be perceived by cells either as true hormones, binding GSTs as a ligand and inducing expression of some GSTs and

other so-called genuine auxin-regulated genes, or as electrophilic xenobiotic substance to induce GST gene expression selectively.

Plant GSTs have secondary activities as glutathione peroxidases (GPOXs) and are able to protect cells from cytotoxicity by reducing organic hydroperoxides to their corresponding less toxic alcohols, the resulting sulfenic acid derivative of GSH then spontaneously forming a disulfide with another GSH molecule (Figure 5) (Marrs, 1996; Roxas *et al*., 1997; Dixon *et al*., 1998; Cummins *et al*., 1999; Edwards *et al*., 2000).

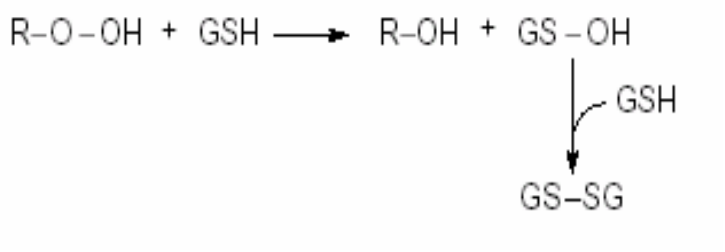

Figure 5. GST functioning as a glutathione peroxidase, using GSH to reduce an organic hydroperoxide (ROOH) the monohydroxy alcohol (ROH))(Edwards *et al*., 2000).

A further link between GSTs functioning as glutathione peroxidases and oxidative- stress tolerance was discovered in black grass. Herbicide- resistant weeds that are cross resistant to multiple classes of herbicides express a phi GST that is highly active glutathione peroxidase, this GST is barely detectable in herbicide-sensitive black grass.

GSTs are also essential for the isomerization of specific metabolites (Figure 6). The proposed mechanism involves the isomerization of the compound and finally the release of the isomer and GSH. In plants, the isomerase activity of GSTs has been demonstrated using thiadiazolidine herbicides; these are bioactivated by GST mediated isomerization to triazolidines, which are potent inhibitors of protoporphyrinogen oxidase. A likely mechanism for isomerization involves

nucleophilic attack at the carbonyl group by GSH, a process that is activated by the GST. Ring opening then allows rotation around the N-C bond, with the C=N double bond transferred to the C-S group; the nitrogen atom attacks the carbonyl group to reform the ring and eliminate the glutathione (Edwards *et al*., 2000).

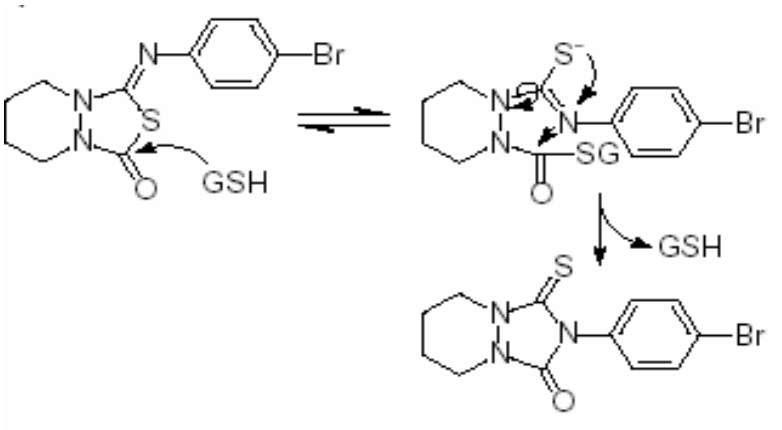

Figure 6. GST catalyzing the isomerization of a thiadiazolidine proherbicide to the phytotoxic triazolidine, showing the proposed reaction mechanism and the formation of the GSH-conjugated intermediate)(Edwards *et al*., 2000).

1.5.4. Role of Plant GSTs under stress

As plants are confined to the place where they grow, they have to develop a broad range of defence responses to cope with outside environment. Plants general defence reactions, such as wall reinforcement, phytoalexin production and accumulation of antimicrobial proteins, and their temporal and spatial regulation are the most decisive factors governing the outcome of the host-pathogen interactions. Initiation of a resistance response requires perception of signal molecules, either synthesized by the invading organism or released from plant cell walls. These signal molecules have collectively been termed 'elicitors', but only a few have been defined at the molecular level as oligosaccharides, (glyco)proteins, and glycopeptides. The elicitors may be specific for a particular plant host–microbe system or are very general molecules, e.g. components of cell walls of the invading microbe. When mixtures of elicitor molecules or purified elicitors have been used to induce defence

reactions in suspension-cultured plant cells, many novel aspects of the early defence response have been established. These include rapid and transient responses that occur mainly at the plant cell surface and are based on the activation of pre-existing components rather than involving the biosynthetic machinery of the cell. Among the reactions identified were: release of reactive oxygen species (ROS) termed the "oxidative burst", 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).

Most cells possess the ability to produce and detoxify ROS. In normal conditions ROS appear in cells as inevitable by-products formed as a result of successive one-electron reductions of molecular oxygen (O_2). Most cells have also acquired the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. In some cases, however, especially under stress conditions, these protective mechanisms are overridden by the rapid, transient production of huge amounts of ROS, namely the oxidative burst. ROS produced in the oxidative burst could serve not only as protectants against invading pathogen, but could also be the signals activating further plant defence reactions, including the HR (Hypersensitive response) of infected cells (Wojtaszek , 1997).

Reactive oxygen species (ROIs) can be induced by environmental stress, such as, high light intensity, heavy metal, ozone, pathogen attack, wounding (Allen, 1995). Plant GSTs protect plants from oxidative damage triggered by ROIs to lipid bilayers, DNA and proteins (Sandermann, 1992). In mammals, leukocytes and other phagocytic cells fight microbes, such as bacteria, and virus-infected cells by destroying them with nitrogen oxide, superoxide hypochlorite, and hydrogen peroxide, a mutagenic oxidizing agent. These oxidants help to protect animals from immediate death from infection. These oxidants can also cause oxidative damage to DNA. DNA damage can be a starting point to the carcinogenic processes. Plants have similar mechanisms to defend themselves from pathogen attack. GSTs have been implicated from oxidative damage. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) in the reactions catalyzed by glutathione

reductase and glutathione peroxidase. GSTs catalyze the nucleophilic attack of the sulfur atom of GSH on electrophilic groups in a second substrate (Marrs, 1996).

Glutathione is an important component of the ascorbate-glutathione cycle, which is involved in the regulation of hydrogen peroxide (H_2O_2) concentrations in plants. This cycle is present in the cytoplasm and chloroplast, and also in mitochondria and peroxisomes (Jimenez *et al*., 1997; Kocsy *et al*., 2001). Under non-stress conditions more than 90% of the total glutathione is in reduced form (GSH) and this reducing environment in the cells prevents the formation of intermoleculer disulphide bonds, thus helping to ensure the correct folding and appropriate activity of the proteins (Levitt, 1962; Aslund and Beckwith, 1999; Kocsy *et al*., 2001). A high level of GSH compaired to its oxidised form (GSSG) is maintained by Glutathione Reductase, which regenerates GSH from GSSG using NADPH as a reductant (Foyer *et al*., 1994; Kocsy *et al*., 2001)

Under stress conditions like chilling or cold acclimation, the ratio of GSH to GSSG decreases, because GSH is used for the reduction of excess H_2O_2 in the ascorbate-GSH cycle. The high level of H_2O_2 during chilling and cold acclimation derives mainly from the Mehler reaction, which proceeds at a higher rate at low temperatures because of the lower rate of CO_2 fixation. Besides the chloroplasts, mitochondria, are another source of H_2O_2, because in the respiratory electron transport chain. During H_2O_2 formation, O_2 is first reduced to the superoxide radical (O_2^-), which is further reduced to H_2O_2 by superoxide dismutase (Marrs, 1996).

Mehler reaction:

$H_2O + 2O_2 \longrightarrow 2\ O_2^- + 2H^+ + +1/2O_2$,

$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$

$H_2O_2 \longrightarrow H_2O + 1/2O_2$.

$H_2O + 2O_2 \longrightarrow H_2O + 2O_2$,

In order to prevent H_2O_2 accumulation to toxic levels during chilling or cold acclimation, an increase in GSH synthesis and/or GR activity is necessary to maintain a high GSH/ GSSG ratio. Changes in H_2O_2 concentration and GSH/GSSG ratio alter the redox state of the cells and may activate special defense mechanisms through a redox signalling chain. Low temperetaures activates redox signalling either by directly via changes in H_2O_2 concentration and GSH/GSSG ratio (unbroken lines) or indirectly by affecting ABA (Abscisic acid), Ca^{+2} or SA (Salicyclic acid) concentrations (dotted lines), which then alter the GSH/GSSG ratio. The involvement of unknown signal molecules (X) is also possible. Redox signalling ensures adaptation to low temperature stres by appropriate changes in gene expression (Figure 7) (Kocsy, 2001).

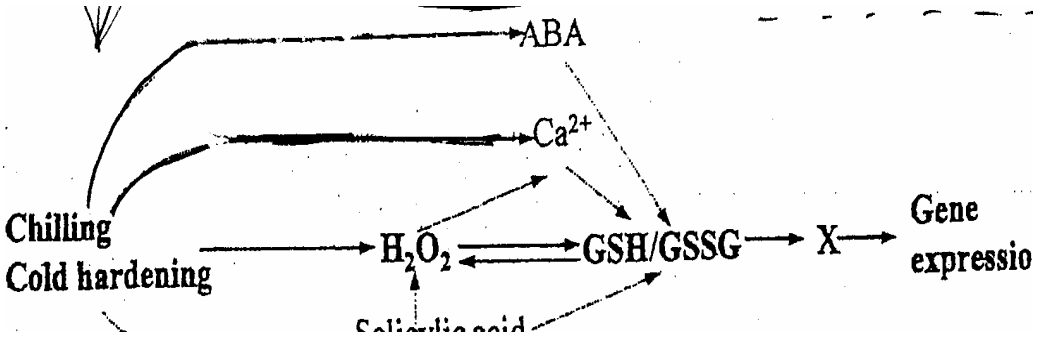

Figure 7. Possible involvement of GSH in redox signalling (Kocsy, 2001).

Generally the substrates for GSTs are Michael reaction acceptors that have a common chemical signature consisting of a carbon-carbon double bond adjacent to an electron-withdrawing group.

The isozymes have widely varying Km and Vmax values towards different xenobiotics and GSH. Thus, the contribution of each to the overall GST activity will depend on the xenobiotic used and the reaction conditions. Xenobiotics which have electrophilic sites, have centers for low electron density that can accept an electron pair to form a covalent bond (Coleman *et al*., 1997). Xenobiotics are also lipophilic

and hydrophobic. Therefore xenobiotics are in the lipid bilayer and makes difficult to solubilize in the cytoplasm and resulting detoxification.

A great number of herbicides have electrophilic sites that are subject to direct GSH conjugation (Lamoureux *et al*., 1991). GSTs catalyze herbicide-GSH conjugation that tends to make the herbicide water-soluble and non-toxic.

Plant GSTs first identified in 1970 in maize by their ability to catalyzing GSH conjugation with atrazine (Lamoureux *et al*., 1991). Since then, plant GST isozymes have been externally studied because of their importance in the detoxification of herbicides. Atrazine [(6-chloro-N-ethyl-N'-(1-methyethyl)-1,3,5,-triazine-2,4 diamine)] tolerant species (corn, sorghum, Sudan grass, sugarcane) contain high levels of GST activity while susceptible species (pea, oats, wheat, barley, and pigweed) have low GST activity (Frear and Swanson, 1970).

Tolerance of maize or sorghum to alachlor, metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide)] and EPTC (S-ethyldipropyl carbamothiate) sulfoxide has also been attributed to GST (Singhal *et al*., 1991).

The standard experimental assay for GST activity utilizes 1-chloro-2,4-dinitrobenzene (CDNB), a model substrate for most, but some GSTs show little activity with this compound (Figure 8) (Karam, 1998; Edwards *et al*., 1998). To date, at least 6 GST isoforms have been characterized in maize. GST I (29kD) and GST III (26kD) are constitutive homodimers with activity against CDNB and alachlor. GST II is a safener-induced heterodimer (27 and 29 kD) also active against CDNB and alachlor, and GSTIV is a safener- induced homodimer of 27 kD active against alachlor and metolachlor but not CDNB. The BZ-2 GST (26kD) is active against CDNB and the anthocyanin precursor cyanidin-3-glucoside. The sixth GST is a 30 kD monomer active against phenylpropanoids but not CDNB, metalachlor, or atrazine. Variations of individual GSTs between different cultivars can be responsible for differential herbicide tolerance (Marrs, 1996).

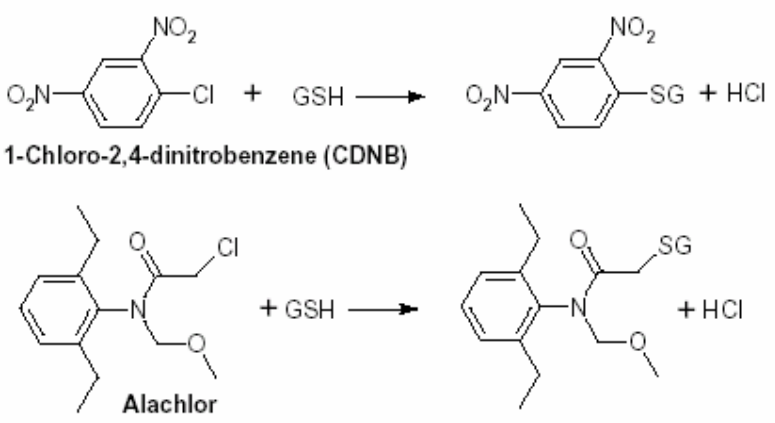

Figure 8. The classic detoxification reactions catalysed by GSTs with the model substrate 1-chloro-2, 4 dinitrobenzene and the herbicide alachlor, respectively in both cases, the glutathione substitution products are stable and the reaction irreversible)(Edwards *et al*., 2000).

1.6 Scope of the Work

By contrast with the mammalian families of GST, relatively little is known about the plant GST families. Up to date, there have not any study of GST isolation and characterization of Turkish red pine, for this respect this study pay attention for being a leader study of the future research under this topic. In this study, we aimed to establish the optimum conditions for the GST activity in the Turkish red pine. For this purpose, the reaction conditions, such as enzyme amount, pH, and temperature of the reaction medium, substrate and cofactor concentrations were optimized. In the present study, polymorphic distribution of GST activity in Turkish red pine populations was determined and also cold stress effect on enzyme activity was examined in the same population.

CHAPTER II

MATERIALS AND METHODS

2.1. Plant Material

Five populations (3 over- exploited and 2 natural) were used in the study. These populations were sampled from the Southern Turkey for another study (Kandemir, 2002). Topographic properties of populations were given in Table 1.

Table 1. Topographic Description of studied Turkish red pine populations from the Southern Turkey (Kandemir, 2002).

Codes	Population Locations	Altitude (m)	Longitude (E)	Latitude (N)	Rainfall (mm/year)
1	Alanya (Natural)	350	31˚ 57´	36˚ 36´	1103
2	Yaylaalan (Over-exploited)	500	31˚ 30´	36˚ 57´	1050
3	Çalkaya (Over-exploited)	50	30˚ 50´	36˚ 55´	1060
4	Gölhisar (Over-exploited)	1100	29˚ 32´	37˚ 40´	634
5	Çameli (Natural)	800	29˚ 07´	37˚ 06´	1222

2.1.1 Detailed Description of Turkish Red Pine Populations

According to Kandemir (2002), The brief descriptions of studied five populations were given as;

1. Alanya-Kargı (Seed Stand): Low elevation, good Turkish red pine stand with dense understory maquis. Due to distance to the villages and the difficulty in reaching, no human disturbance is evident in this stand.

2. Manavgat-Yaylaalan (Degraded Seed Stand): It is close to Yaylaalan village by Manavgat, and consists of three fragmented stands seperated by roads. Due to closeness to villages, illegal cuttings, and overgrazing, the stands here are highly degraded.

3. Antalya-Çalkaya (Degraded Seed Stand): It is located between Çalkaya and Aksu villages. This population has been highly fragmented by the roads connecting villages to Alanya or Antalya. This population has also been highly degraded by anthropogenic factors due to nearby settlements.

4. Burdur-Gölhisar (Degraded Seed Stand): This is the most inland (from south to north) high elevation Turkish red pine stand. *Pinus nigra* stands also present, on higher elevations, *Pinus nigra* and Turkish red pine mixed forests are present on the hills while on the foot steps of mountains the sampled Turkish red pine seed stands relocated. Probably, the stand has been also over-exploited by nearby villagers.

5. Çameli-Göldağ (Seed Stand): It is located in the watershed of Esen Creek (Before Alcı village on the Acıpayam Road). The population is characterized with trees having conical crown and straight stem forms.

2.1.2 Experimental Design

Open pollinated seeds of parent trees (families) were sown in a forest nursery bed as a six seedling row plants of 240 families (6 populations × 40 families per populations) in spring of 1998 by Kandemir (2002), and families were randomly allocated to plot location in a complete block design with 3 replicates. Total of 4320 seedlings (3 × 6 × 40 × 6) were grown in Ankara Forest Nursery (Figure 9). After seeds are planted into plastic tubes, they were covered with wire mesh to prevent from bird damages during the early periods of growing in the nursery (Figure 10).

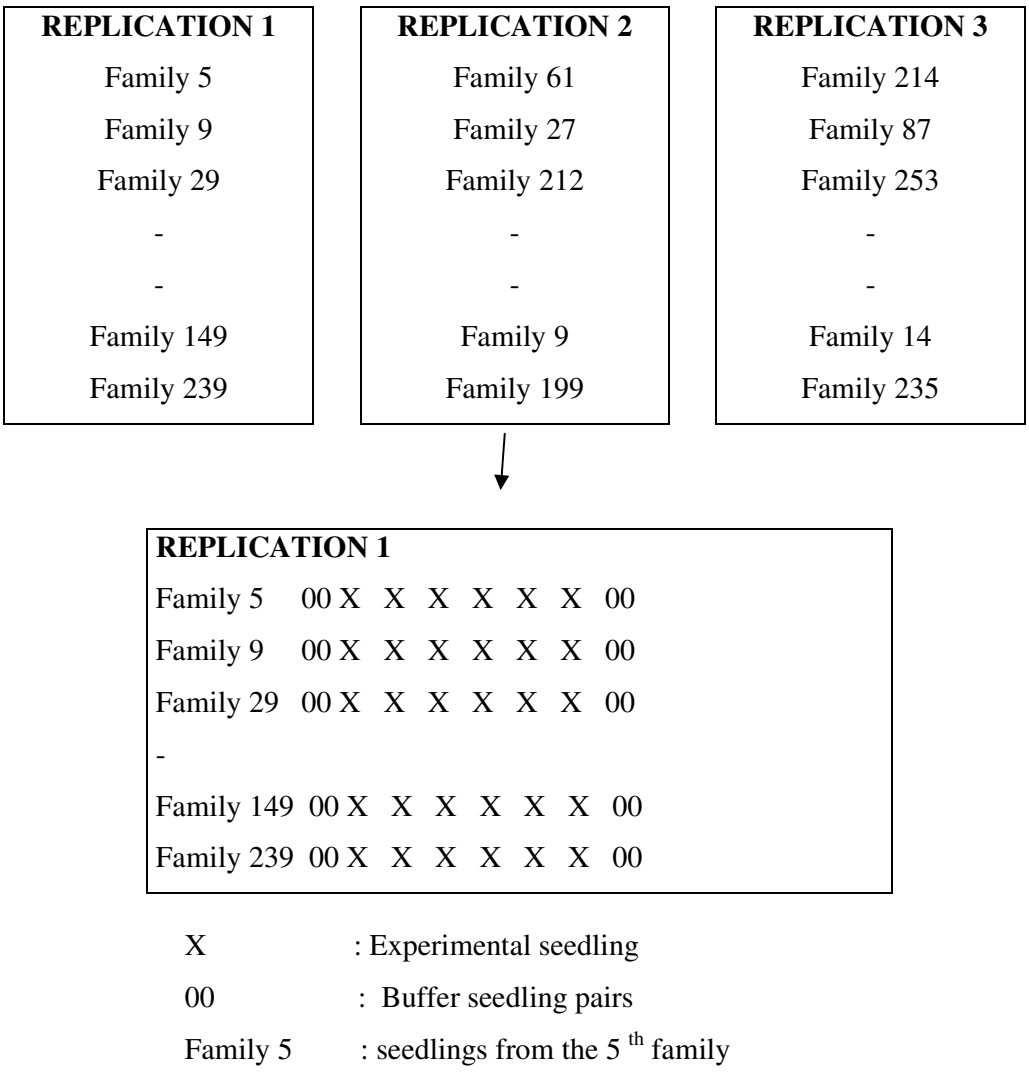

Figure 9. Nursery design and lay out of experimental seedlings (Kandemir, 2002)

Figure 10. A view of seedling in the Ankara Forest Nursery in May of 1999 (Kandemir, 2002).

In the present study, needles from Turkish red pine were randomly collected from a total of 40 families including both (R) and (S) types among 5 population from Replication 1 in Ankara Forest Nursery in Söğütözü, Turkey, in fall of 2001. Every family contain 6 seedlings, so 240 plants were sampled in total. The outline of sampling is given in Table 2. In order to examine the effects of cold stress at -3˚C, 0˚C, 13˚C, and needles from Replication 1 were harvested in the morning after a night at which air temperature was either -3˚C, 0˚C or 13˚C and needles were kept in liquid nitrogen during the harvesting. Temperature readings were obtained from the Turkish State Meteorological Department. The values are the values recorded in the closest meteorological station to Sogutozu. After reaching laboratory, they were kept at -80˚C in deep freezer until the day of homogenization.

Table 2. Sampling schemes in the present study.

Population	Cold Resistant Families		Cold Sensitive Families	
	Number of families	Number of seedlings	Number of families	Number of seedlings
1-Alanya (Natural) (350 m)	2	12	3	18
2-Yaylaalan (Over-exploited) (500m)	3	18	1	6
3-Çalkaya (50 m) (Over-exploited)	--	--	15	90
5-Gölhisar (1100m) (Over-exploited)	8	48	--	--
6-Çameli (Natural) (800m)	7	42	1	6
TOTAL	20	120	20	120

2.2 Chemicals

1-chloro-2,4 dinitrobenzene (CDNB), reduced glutathione (GSH), PVP-K30, ethylenediaminetetraacetic acid (EDTA), coomassie brilliant blue G, hydroxymethyl aminomethane (Tris), N,N,N´,N´-tetramethylenediamine (TEMED), Nonidet P-40, Methanol (CH_4O), Sodium Carbonate (Na_2CO_3), Acetonitrile were purchased from Sigma Chemical Company, St.Louis, MO, USA. Pepstatin A were obtained from Fluka Chemical Company, Neu-Ulm, and FRG. 2-Mercaptoethanol, dimethyl sulfoxide (DMSO) were from E.Merck, Darmstadt, Germany. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.3 Methods

2.3.1 Homogenization of cytosolic extracts of Turkish red pine

Crude enzyme extracts were prepared from Turkish red pine needles by a modification of the procedure described by Schröder and Berkau (1993) for spruce needles. Turkish red pine needles were collected in Ankara Forest Nursery in Söğütözü, Ankara, Turkey, in fall of 2001. For the optimization studies needles collected from mature trees (about 10 year old) from Yalıncak, Ankara. Needles are stored at -80˚C until the day of homogenization. 0.2 g pine needles cut into small pieces by scissors. After this process, small pieces were pulverized in liquid nitrogen in a porcelain mortar and added with 10 vol(w/v) of 0.1 M Tris-HCl buffer, pH 7.8, containing 20 mM 2-Mercaptoethanol, 5% PVP-K30, 2 mM EDTA, 0.5% Nonidet P40, 5 mM GSH, 3 µg/ml Pepstatin A. After homogenization for 1 min at 13 500 rpm with an ultrathorrax, the crude homogenate was centrifuged at 15 000 rpm (Hettich INC, USA) using 1112 rotor for 30 min, at 4°C. The pellet was discarded and supernatant (cytosol) was collected as GST enzyme source. GST activity and protein determination were either carried out immediately or cytosol was stored in small aliquots of 0.5 ml, at -80˚C in deep freezer until they are used. (Figure 11).

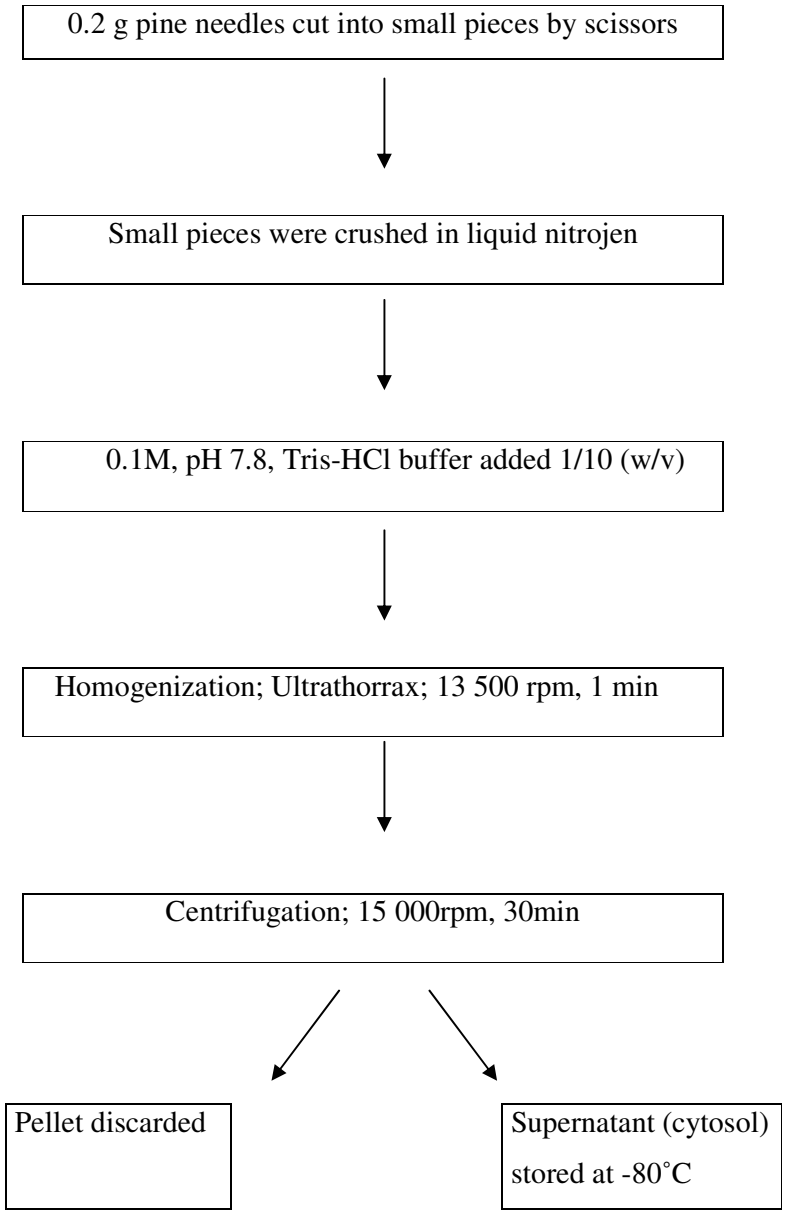

Figure 11. Outline of cytosol preparation from Turkish red pine

2.3.2 Protein Determination of cytosolic extracts by Bradford Method

The protein concentrations in the cytosol prepared from Turkish red pine needless were determined by the method of Bradford with crystalline bovine serum albumin (BSA) as a standard. Cytosol was taken into tubes and mixed with 5 ml Bradford reagent. This reagent includes; Coomassie Brilliant Blue G-25, 95% ethanol, 85% (w/v) phosphoric acid. All tubes mixed immediately with 8 seconds by vortex. Tubes were let to stand 10 min at room temperature. The intensity of colour developed in each tube was measured at 595 nm. A standard curve of l mg/ml BSA was also constructed and used to calculate the protein amounts in the cytosolic extracts (Bradford, 1976) (Figure 12).

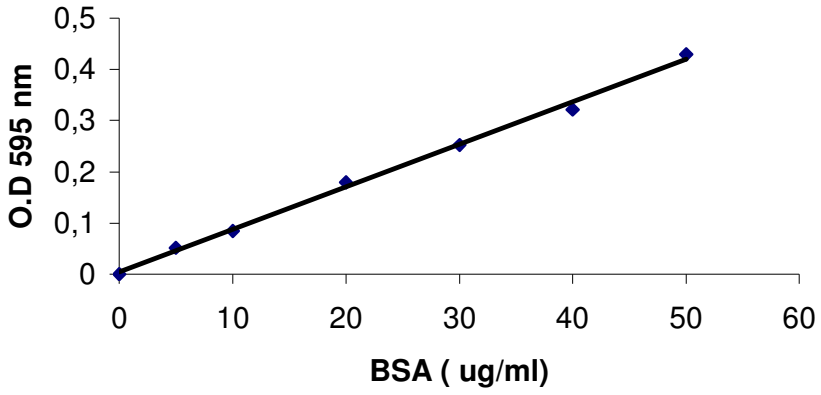

Figure 12. BSA Standard curve for protein determination (Bradford, 1976)

2.3.3 Determination of GST activity towards CDNB

GSTs activities were determined spectrophotometrically by monitoring the thioether formation at 340 nm using CDNB (1-chloro-2, 4 dinitrobenzene). The Turkish red pine needles cytosolic fractions were used as the enzyme source. All enzyme activity measurements were carried out at 25 ˚C using a spectrophotometer equipped with thermoregulated cell holder.

A typical reaction mixture included 25 mM phosphate buffer, pH 8.0, 1mM CDNB, 1 mM GSH, and 0.4 mg/ml Turkish red pine cytosolic protein in a final volume of 1 ml as shown in Table 3.

The reactions were started by the addition of substrate. Incubation mixtures without the enzyme source were used as blanks (nonenzymatic reactions), and concentrations of the formed conjugation products were determined from the slopes of initial reaction rates. The reaction rate was calculated using the ε values of CDNB as 0,0096 μM^{-1} cm^{-1} (Habig and Jakoby, 1981). The GSTs activities were expressed as unit/mg protein. One unit of enzyme activity is defined as the amount of enzyme that forms one nmole of product per minute under defined assay conditions.

Table 3. The constituents of the incubation mixture for GSTs enzyme assays with the CDNB .

Incubation Conditions	**Stock Concentration**	**Added amounts (µl)**	**Final Concentration in 1 ml cuvette**
Substrate (CDNB)	20mM	50	1mM
Combination solution • Buffer (pH 8.0) • GSH • H_2O	• 40mM • 50mM	900	• 25mM • 1mM
Enzyme source Cytosol (8mg/ml)		50	0.4 mg protein
Total		1000	

2.3.4 Ion-Exchange Column Chromotography on DEAE-Sepharose

The column (1.0 cm X 18 cm) packed with DEAE-Sepharose was equilibrated in the cold room with 25 mM Tris-HCl buffer, pH 8.0.The cytosol (5ml) prepared as described under the 'Materials and Methods' containing a total of 2,38 mg protein with 518 units of GSTs activity towards CDNB was applied to the column at a flow rate of 36ml/hour. Afterwards, the column was washed with the equilibration buffer at a flow rate of about 30ml/hour until no absorption of effluent at 280 nm was detected. The bound proteins were eluted from the column with a linear NaCl gradient (0-1.0 M) consisting of 100 ml of the equilibration buffer and 100 ml of the buffer containing 1.0 M NaCl.

The absorbance at 280 nm as well as GSTs activity against CDNB was measured in the fractions (3ml each) collected from the column. The gradient eluted fractions with the highest activity against CDNB used for HPLC analysis.

The DEAE-Sepharose ion-exchanger was regenerated in the column, without repacking, by washing with 2.0 M NaCl (about 2 bed volumes), to remove the bound substances, and then with 0.1 M NaOH in 0.5 M NaCl (about 2 bed volumes). The column was then washed extensively with distilled water (more then 10 bed volumes) and equilibrated again with the equilibration buffer. The resin is stored at 4˚C in 250 mM Tris-HCl buffer containing 20% ethanol as an antimicrobial agent.

2.4 Statistical Analysis

There were 720 seedlings (2 groups X 20 families X 6 seedlings/ family X 3 different temperatures) from which GST activities were determined. Also GST activity measurements were repeated 3 times. Therefore, the total sample size was 2160 (720 X 3 replicates). To determine the GST activity differences between groups (sensitive vs. resistant), populations within groups and families within populations,

analysis of variance was carried out using " proc ANOVA" procedure of SAS statistical packages. Also, GST activity means were calculated for groups and populations using the "proc mean" procedure of SAS statistical packages (SAS Inst, 1998).

CHAPTER III

RESULTS

3.1 The Turkish red pine cytosolic GST activity

Glutathione S-transferase activity was studied in Turkish red pine cytosol using CDNB as substrate. The optimum conditions for the maximum enzyme activity were established. GST activity was determined spectrophotometrically by quantifying the glutathione formation at 340 nm as described by Habig *et al.* (1974) and Schröder *et al.* (1990) using CDNB as a test substrate.

The specific activity of Turkish red pine GST against CDNB as substrate was determined under the optimum condition as 200±50 (Mean±SE, n= 18) nmole/min/mg cytosolic protein.

3.2 Characterization of Turkish red pine cytosolic GST activity

3.2.1 Effect of Enzyme Amount

The effect of protein concentration on enzyme activity was measured by changing the final protein concentration in the reaction mixture between 0.05 and 0.8 mg. The conjugated product formations were linear with protein amount upto 0.8 mg cytosolic protein in 1.0 ml reaction mixture (Figure 13). In order to obtain sufficient

quantity of product for the ease of determinations, 0,4 mg protein was routinely used throughout this study.

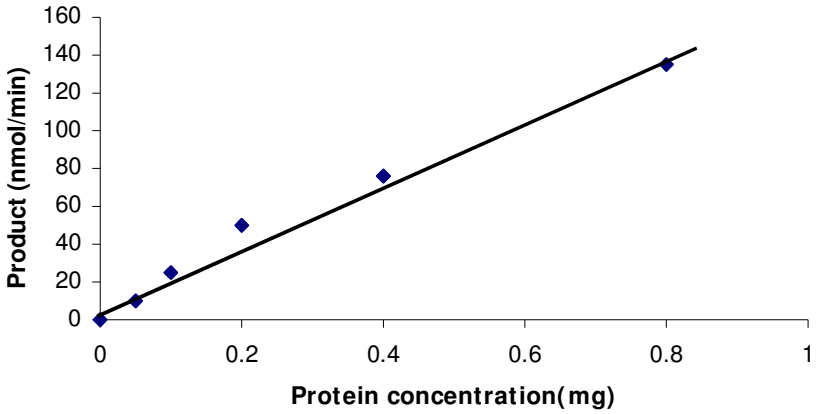

Figure 13. The effect of enzyme amount on GST activity. The reaction mixture was prepared 1 mM GSH, 1 mM CDNB, 25 mM phosphate buffer, pH 8, distiled water and the reaction was started by the addition of varying amounts of enzyme in a final volume of 1 ml. The amounts of thioester produced determined as described under 'Materials and Methods'. Each point was the mean of triplicate determinations.

3.2.2 Effect of pH on GST activity

The effects of pH, on the Turkish red pine GST activity is shown on Figure 14. The pH assays were carried out by using seven 40mM phosphate buffers (25mM in 1.0 ml reaction mixture) of pH values ranging between 6.2 and 8.2. The highest Turkish red pine GST activity was observed at pH 8.0. At higher pH values the velocity of thioester formation decreased. The points seen on the graph are means of 2 different sets of data and each point is the mean of triplicate determinations.

All the activity measurements were carried out as described under the‘Materials and Methods’. Reactions were carried out at 25˚C for 2 minutes. The enzyme activity was compared with its zero time blank determined separately at corresponding pH values. For the measurement of the other properties of GST, optimum pH value pH 8.0 was adopted.

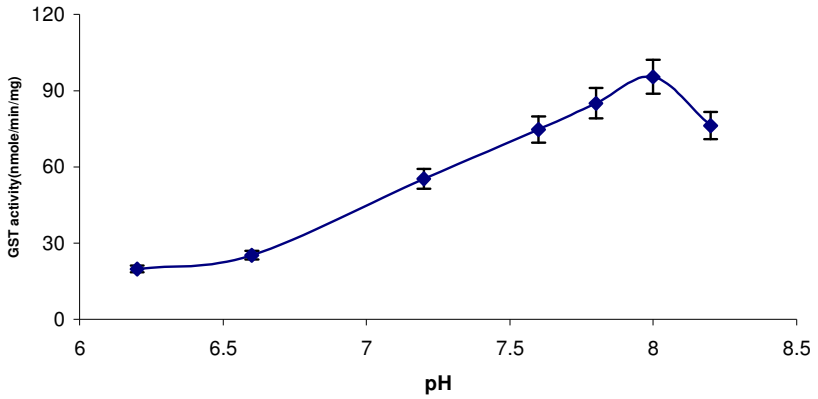

Figure 14. Effect of pH on cytosolic GST activity. Reaction mixture contained 1 mM GSH, 1 mM CDNB, 25 mM phosphate buffer and distile water. The reactions were carried out at 25° C for 2 minutes. Each point is the mean of three different sets of experiments done three independent experiments.

3.2.3 Effect of Temperature on GST activity

The effect of temperature on cytosolic GST activity was detected by following the reaction rate at various temperatures in the range of 4, 10, 17, 25, 30, 37, 42, 45, and 50°C. All of the constituents, except the enzyme source were incubated in the spectrophotometer cuvette at the desired temperature for 2 minutes prior to Turkish red pine cytosol addition. The reaction was initiated by the addition of cytosol and followed for 2 minutes at the same temperature. Figure 15 shows the temperature dependence of GST conjugation rate. The reaction rate was increased with increasing temperature up to 42°C. As the conjugation rate was 170,6

nmol/min/mg protein at 25°C, it was increased approximately 47% and reached 359 nmol/min/mg protein when reaction was carried out at 42°C. Turkish red pine cytosol as the GST source was kept at 0°C in ice bath during these measurements. GST activity determinations throughout this study were carried out at 25 °C for 2 minutes.

To determine the activation energy, *Ea*, for the conjugation of CDNB catalyzed by cytosol GST were determined by plotting, log V values, at the previously indicated temperatures, were plotted against 1/T ($°K^{-1}$) as shown in Figure 16 (Arrehenius plot).

The Arrhenius plot for cytosol GST catalysed reaction was linear up to 42°C indicating that a single enzyme catalyzes the conjugation of CDNB with glutathione. The activation energy, *Ea*, for cytosol CDNB was calculated as 13565 cal/mole.

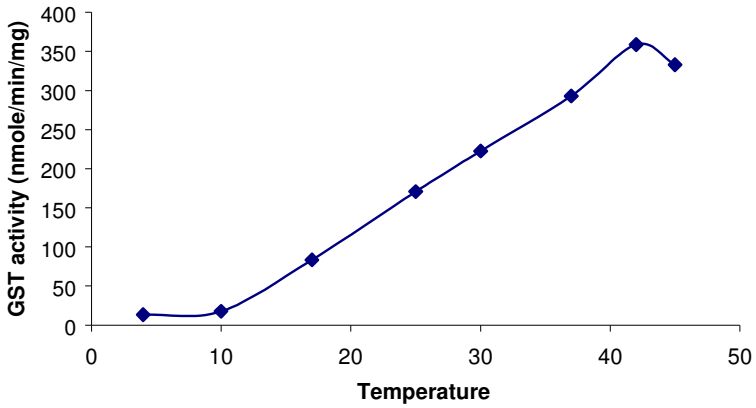

Figure 15. Effect of temperature on cytosolic GST activity. The reactions were carried out at indicated temperatures and the reaction mixture constituents were the same as described under the 'Materials and Methods'. The points were the means of three independent experiments.

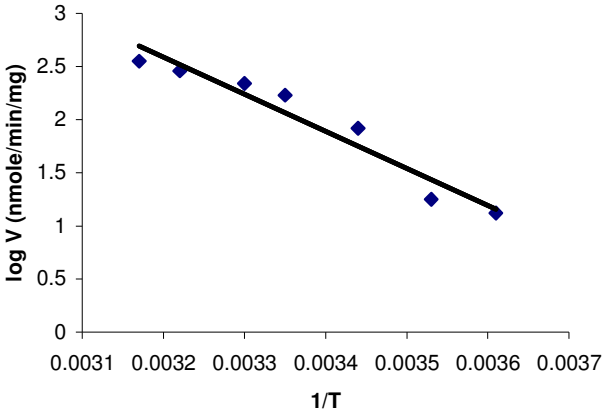

Figure 16. The Arrhenius plot for Turkish red pine GST catalyzed reactions. The slope, for calculating the activation energy value, was determined from linear scale. The conditions were identical to those described in the legend of Figure 17.

3.2.4 Effect of storage time on GST activity in Turkish red pine

In order to examine the Turkish red pine GST stability as a function of storage time, cytosol was stored in small aliquots in deep-freezer at –80˚C after preparation. The Turkish red pine GST activity was measured at various time points using a different aliquot of the same cytosol at a time as indicated under optimised conditions.

Figure 17 shows the effect of storage time on cytosolic GST activity. It has been found that Turkish red pine GST retained its activity without loss for four weeks.

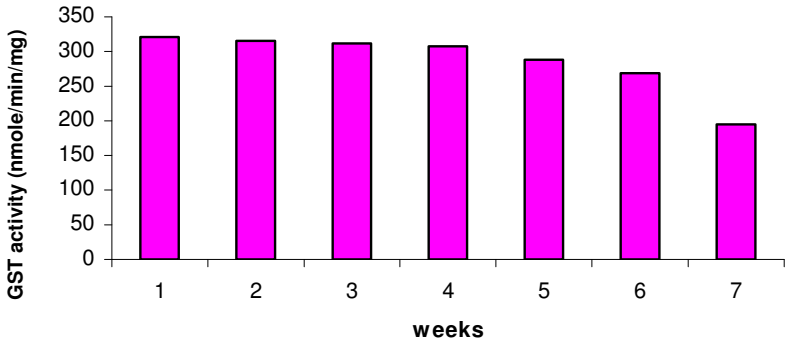

Figure 17. The effect of storage time on Turkish red pine cytosol GST activity.

3.2.5 Effect of GSH concentration on Turkish red pine GST activity

Figure 18 illustrates the substrate saturating curve for Turkish red pine GST with respect to GSH. The Turkish red pine GST activity seemed to be saturated by GSH at around 1 mM concentration. As the concentration was further increased, the Turkish red pine GST activity decreased indicating the presence of either product or substrate inhibition.

Figure 19 shows the Lineweaver–Burk plot for GSH derived from the substrate, GSH, saturation curve. The plot was linear suggesting a simple Michaelis-Menten kinetics for the conjugation of CDNB by Turkish red pine GST with respect to GSH. The Vmax and Km values for GSH as a substrate were calculated from the plot 106.4 nmoles/min/mg and 0.10 mM, respectively.

The GSH saturation curve for GST was also evaluated using Eadie-Scatchard and Hanes-Woolf plots. In each of them a linear plot was obtained in accordance with the Lineweaver-Burk plot. The Km and Vmax values of the enzyme for GSH calculated from Eadie-Scatchard and Hanes-Woolf plots were rather similar to the ones obtained from Lineweaver-Burk plot (Table 4).

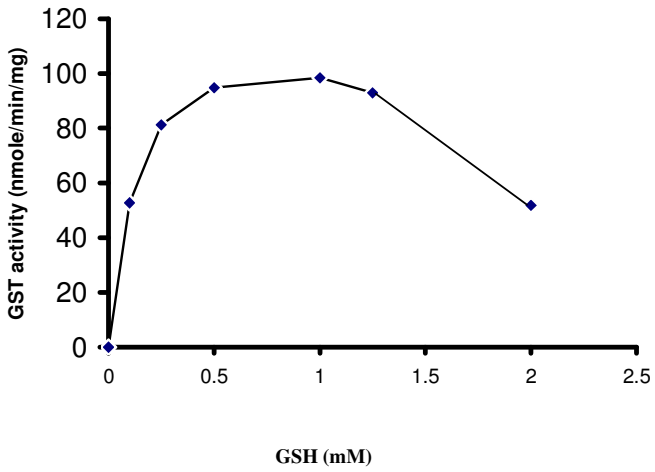

Figure 18. Effect of GSH concentration on Turkish red pine GST activity. The reaction medium contained varying concentrations of GSH in 1 mM CDNB, 25 mM Phosphate buffer, pH 8.0, and 0.4 mg cytosol as enzyme source in a final volume of 1ml. The reactions were carried out at 25 ℃ for 2 minutes. The points are means of three different data sets.

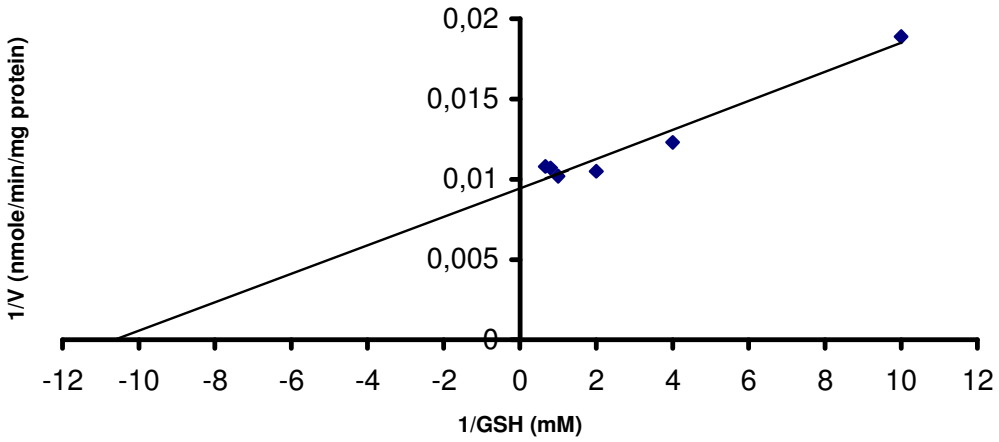

Figure 19. Lineweaver-Burk plot for GSH obtained with Turkish red pine GST activity at a saturating concentration CDNB, 1 mM. Other conditions were identical to those described in the legend of Figure 20.

Table 4. Kinetic parameters of Turkish red pine for GSH calculated from the saturation curve according to Lineweaver-Burk, Eadie-Scatchard and Hanes-Woolf models.

Method of calculation	Km	Vmax	R^2
Lineweaver-Burk	0.10mM	106.4nmole/min/mg	0.96
Eadie-Scathard	0.12mM	118,1nmole/min/mg	0,97
Hanes-Woolf	0,08mM	96,15nmole/min/mg	0,97

3.2.6 Effects of CDNB concentration on *Pinus brutia* cytosolic GST activity

Figure 20 shows the substrate saturating curve for cytosol GST with respect to CDNB at 25 ℃. The cytosol GST seemed to be saturated by CDNB at around 1 mM concentration.

Figure 21 illusrates the Lineweaver-Burk plot for CDNB that was linear suggesting a simple Michealis-Menten kinetics, obtained with cytosolic GST and derived from the previous substrate saturation curve (Figure 22), which was linear suggesting a simple. By the use of this plot, the Vmax and Km values for cytosolic GST with respect to CDNB as a test substrate were calculated as 416nmole/min/mg and 0,8 mM respectively.

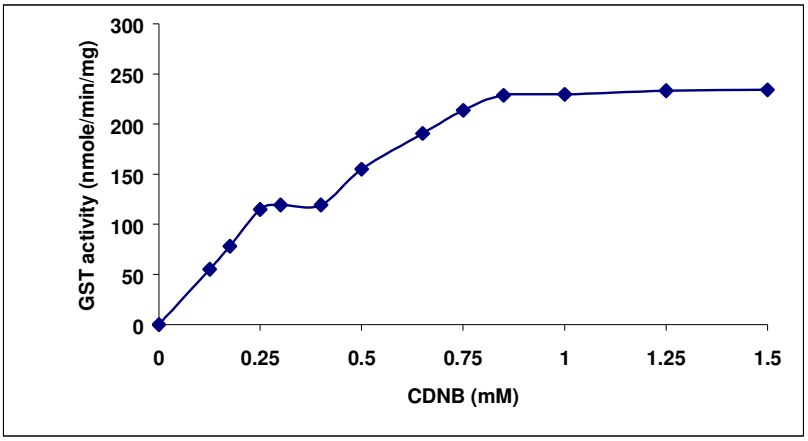

Figure 20. Substrate, CDNB, saturation curve for cytosolic GST. The reaction medium contained varying concentration of CDNB, 1 mM GSH, 25 mM phosphate buffer, pH 8.0, and 0.4mg enzyme containing cytosol. The reaction was carried out at 25 ° C for 2 minutes. The points are the mean of 3(4) sets of data.

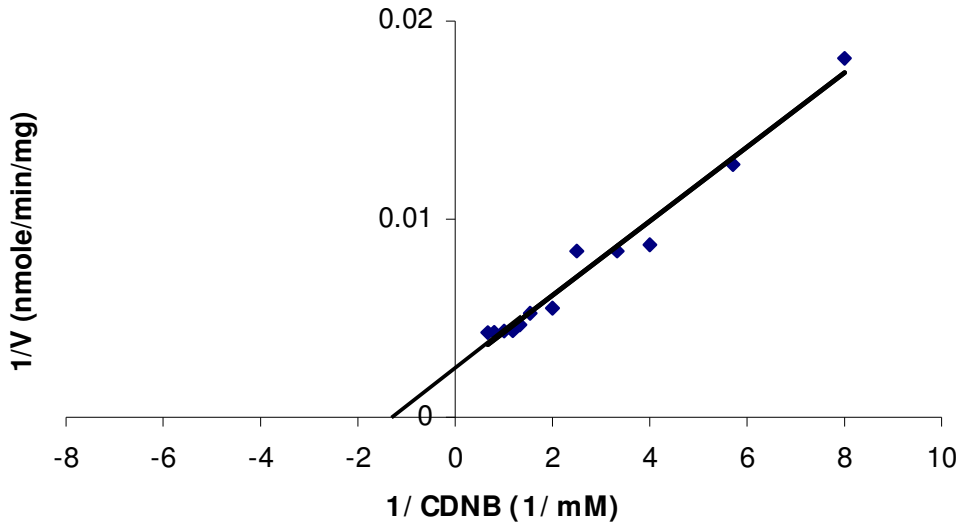

Figure 21. The Lineweaver-Burk plot for CDNB obtained with the cytosolic GST at a saturating concentration of GSH (1 mM). Other conditions were identical to those described in the legend of Figure 20.

Table 5. Kinetic parameters for CDNB conjugation by high activity exhibiting GST. The parameters were calculated from the CDNB saturation curve according to the Lineweaver-Burk, Eadie-Scatchard and Hanes-Woolf models.

Method of calculation	Km	Vmax	R^2
Lineweaver-Burk	0.8mM	416 nmole/min/mg	0.98
Eadie-Scathard	0.9mM	452 nmole/min/mg	0.95
Hanes-Woolf	0.75mM	435 nmole/min/mg	0.97

3.2.7 Variations of Pinus brutia GST activity under the stress conditions

The variation of *Pinus brutia* GST activity was investigated in 5 populations (2 natural and 3 over-exploited). *Pinus brutia* samples were collected from Ankara Forest Nursery in Söğütözü and homojenate were prepared as indicated under the 'Materials and Methods'. The enzyme activities were measured under the standard conditions, using the same amount of enzyme, 0.4 mg, and reactions were carried out as described under the 'Materials and Methods'.

Figure 22 summarizes the temperature course of GST activity in needles of *Pinus brutia* trees at the 3 different harvesting temperatures in fall of 2001. Table 6 shows the experimental means of GST activities at -3˚C, 0˚C, and 13˚C. When the *Pinus brutia* GST activity for the conjugation of CDNB was examined, it was observed that S3A (-3˚C) has the highest GST activity in all populations and S13A (13˚C) has the lowest GST activity in all populations except that population 1 (Alanya). Population 2 (Yaylaalan) showed the highest GST activity among all populations at -3˚C, 13 ˚C. Moreover, Population 5 (Gölhisar) showed the highest GST activity among all populations at 0˚C. The specific activities of populations at -3˚C, 0˚C, and 13˚C were measured in the ranges of 210±50 (Mean±SE, n= 720), 170±60 (Mean±SE, n= 720), and 130±40 (Mean±SE, n= 720) nmole/min/mg protein.

There was a difference in GST activity between over-exploited and natural populations. Natural populations exhibited generally lower GST activity than over-exploited populations at -3˚C, 0˚C. However, at the13˚C; the GST activity of natural populations was higher than in over-exploited populations.

Table 6. *Pinus brutia* cytosolic GSTs activity population means ± SE in seedlings exposed to different environmental cold temperatures.

Temp. (ºC)	**GST activity (nmol/min/mg protein) (Mean±SD)**				
	Over–exploited populations			**Natural Populations**	
	Yaylaalan	Çalkaya	Gölhisar	Alanya	Çameli
-3 (S-3A)	238,31±81,11	171,92±59,02	261,63±49,70	203,01±52,28	167,86±37,69
0 (S0A)	229,83±54,63	110,72±33,94	161,97±31,41	148,56±39,42	143,17±43,61
13 (S13A)	178,04±23,10	109,03±38,26	98,74 ±26,58	153,09±23,46	124,66±31,91

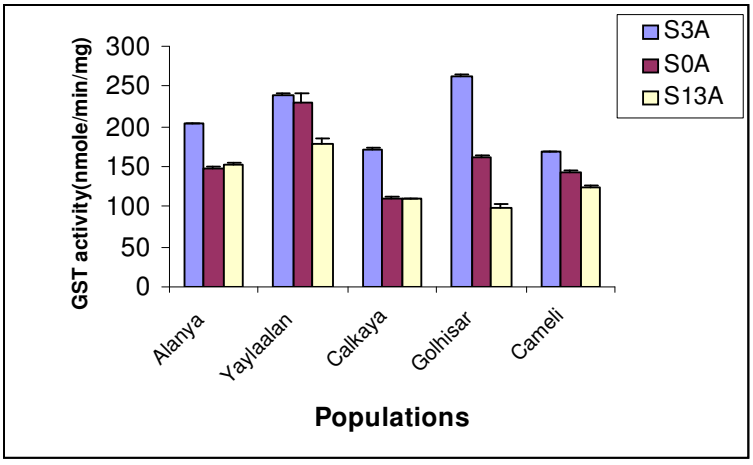

Figure 22. Experimental mean of GST activities under the cold stress conditions in all populations.

In addition to differences in total GST activity between over-exploited and natural populations, there were differences of the GST activity between resistant type (type I) and the sensitive (type II) of families. Sensitive type families showed lower GST activity than resistant type families at -3˚C and 0˚C. However, at the13˚C, the GST activity of sensitive type families was higher than resistant type families.

Figure 23 shows the GST activity variation in all resistant type population at -3˚C, 0˚C and 13˚C. The specific activities of populations were measured in the range of 210±50 (Mean±SE, n= 360), 180±60 (Mean±SE, n= 360), 120±50 (Mean±SE, n= 360) nmole/min/mg protein. S3A- GST activities (except that Yaylaalan) were greater than S0A-GST activities which were higher than S13A-GST activities. Gölhisar S3A-GST activity was the highest in all populations.

Figure 24 shows the GST activity variation in all sensitive type population at -3˚C, 0˚C and 13˚C. The specific activities of populations were measured in the range of 220± 60(Mean±SE, n= 18), 170±62 (Mean±SE, n= 18), 200±180 (Mean±SE, n= 18) nmole/min/mg protein.

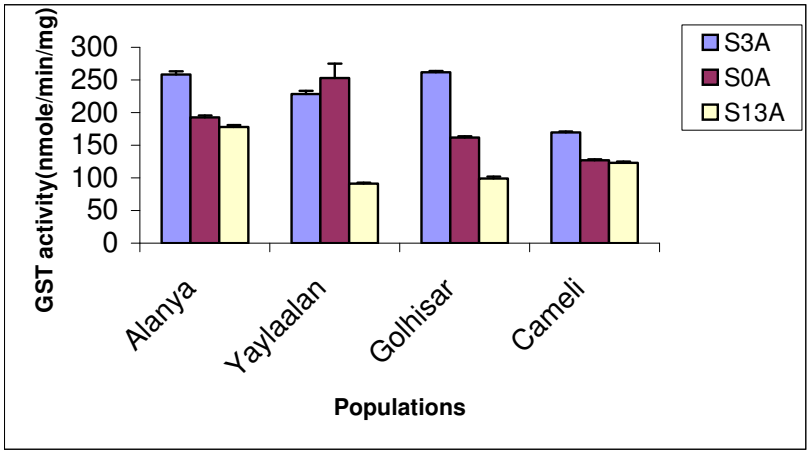

Figure 23. Experimental mean of GST activity when only cold type I (resistant) families included.

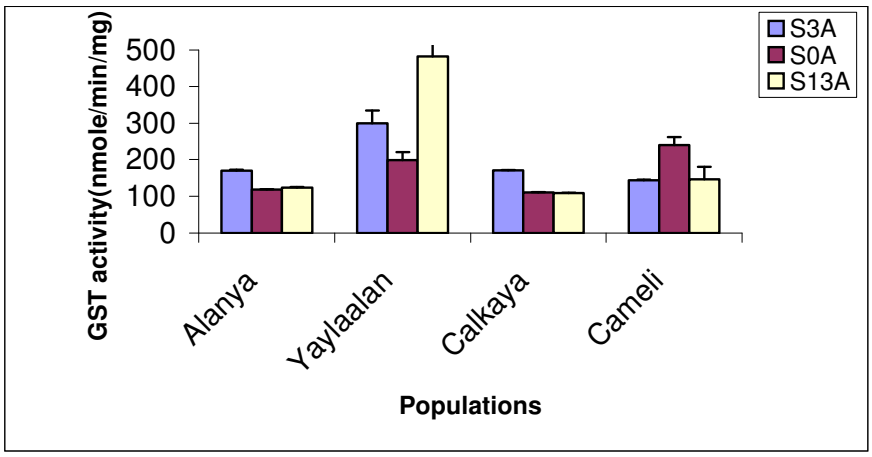

Figure 24. Experimental mean of GST activity when only cold type II (sensitive) families included.

3.2.8 Ion-Exchange Column Chromatography on DEAE-Sepharose

As described under the'Materials and Methods', the cytosol was applied to the DEAE- Sepharose Fast Flow column (1cm x18 cm). Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280 nm was detected. Then, the bound proteins were eluted from the column with a linear NaCl gradient consisting of 100 ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl.

GSTs activities against CDNB were measured in the fractions collected from the column. As it is clear from Figure 25**,** about 80 % of the total protein, applied to the column, with 5 % of the *Pinus brutia* GSTs active towards CDNB failed to bind the ion-exchange column and eluted with the other nonspecific proteins during sample application and washing. However, most of the *Pinus brutia* GSTs active towards CDNB bound tightly to the column and were eluted by the salt (NaCl) gradient varying between 0-1.0 M.

The gradient bound fractions with the highest activity against CDNB were combined in one fraction for further analysis. The combined fraction had a total of 0,4 mg

protein with 90 units of GST activity towards CDNB. According to these results, the ion exchange column provided about 1.04-fold purification with about 17 % recovery of the *Pinus brutia* GSTs activity against CDNB.

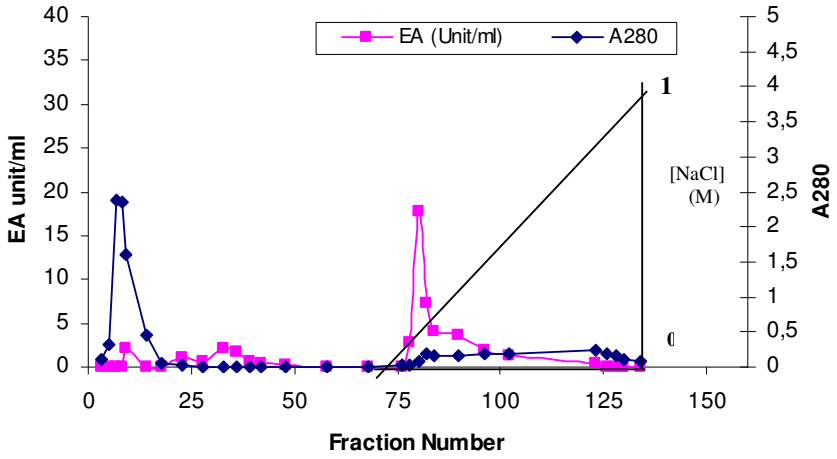

Figure 25. The Purification Profile of the *Pinus brutia* GSTs on the Ion-Exchange Chromotography column (1 cm X 18 cm) of DEAE Sepharose Fast Flow.

CHAPTER IV

DISCUSSION

Glutathione S-transferases are a family of enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on the electrophilic center of a variety of chemical compounds. These enzymes which have evolved together with GSH in the aerobic organisms are abundant and widely distributed in most forms of life, like animals, plants, insects, parasites, yeast, fungi, and bacteria. In almost all of the organisms in which it has been found, the GST activity comprises a number of isoenzymes with broad substrate specificities (Daniel, 1993).

In plants there are four known multigene classes of the soluble GSTs, namely Phi (F), Type I; Zeta (Z), Type II; Tau (U), Type III; Theta (T), Type IV. Some of type I GSTs have defensive and cellular protectant functions producing gene products in response to pathogen attack, wounding, senescence, and the resulting lipid peroxidation that accompanies these processes (Alfenito *et al.*, 1998). Other type I GSTs are induced in response to auxins and may serve a ligandin function toward Indole Acetic Acid (IAA) (Marrs, 1996). Type II GSTs are much closer to the mammalian zeta GSTs. In addition, significant amino acid sequence homology exists with type III GSTs. Type III was originally identified as a set of homologous genes from a variety of species that were inducible by a range of different treatments– particularly auxin, but also ethylene, pathogen infection, heavy metals, and heat shock (Marrs, 1996). Recently, a Type IV grouping was proposed for several *Arabidopsis* genes that are similar mammalian theta enzymes (Edwards *et al.*, 2000).

Glutathione S-transferases (GSTs) are the enzymes that detoxify natural and exogenous toxic compounds by conjugation with glutathione. Glutathione, an endogenous tripeptide, is important as reducing agent, nucleophilic scavenger, and alleviate the chemical toxicity in the plants by the reaction of GSTs. GSTs are detoxification enzymes capable of catalyzing the conjugation of glutathione (GSH) with a wide variety of electrophilic compounds, and have another role as glutathione peroxidases. Glutathione conjugates are can be transported to the vacuoles or apoplast and are generally much less toxic than the parent compounds. Oxygen radicals are also highly harmful to the cell components. Those toxic reactive oxygen species damage DNA, lipid layer, and proteins. Many GSTs can also act as glutathione peroxidases to scavenge toxic peroxides from cells. In addition, plant GSTs play a role in the cellular response to auxins and during the normal metabolism of plant secondary products like anthocyanins and cinnamic acid.

In our study, we aimed to show the presence of GSTs in Turkish red pine and to determine the optimum conditions for *Pinus brutia* GST activity measurements, and further compare the distribution of GST activity among the *Pinus brutia* populations. This study also included the effects of cold stress on GST activity in cold sensitive and cold resistant seedlings.

The characterization of GST activity was performed by using *Pinus brutia* cytosol as enzyme source and the effect of pH, temperature, substrate concentration and cofactor concentrations were determined and optimized.

The biochemical characterization of *Pinus brutia* GST was carried out with respect to the response of enzyme activity to varying pH and temperature. The reaction velocity was found to be pH dependent and decreased at low pH. The optimum working pH was chosen as pH 8.0 throughout this study. Since nonenzymatic conjugation of CDNB as substrate with GSH increases with increasing pH; at every pH studied, nonenzymatic reaction is also determined carefully and subtracted from the slope obtained in the presence of enzyme. The enzyme showed less than 30 % of its maximum activity below pH 6.5. The decline in the enzyme

activity below 7.8 and at 8.2 may be due to the formation of improper ionic forms of active site amino acid side chains involved in enzyme activity or active site topology.

When the temperature dependence of *Pinus brutia* GST was examined, it was observed that the enzyme activity of GST was increased by increasing temperature up to 42˚C. This may be explained by the folding proporties of GST. The working temperature was chosen as 25˚C. At this working temperature enzyme retained 50 % of its maximum activity.

The dependency of CDNB conjugation on cytosolic protein concentration was also determined. The product formation increased as a function of protein concentration up to about 800µg of *Pinus brutia* cytosolic protein. Throughout the study the reactions were followed for 2 minutes and a protein concentration of 0.4 mg protein was used.

The *Pinus brutia* GST was further characterized by examining the substrate concentration dependence. As shown in Figure 20, GST of *Pinus brutia* did not exhibit any substrate, CDNB, inhibition up to 1.5 mM. The enzyme seemed to be saturated by its substrate at almost 0.85 mM CDNB concentration. The apparent Km value was calculated as 0.8 nM and the apparent Vmax value was calculated as 416 nmoles/min/mg protein.

In the reaction catalyzed by *Pinus brutia* GSTs, the sulfur atom of GSH provides electrons for nucleophilic attack of CDNB. GSH is used as the first and essential substrate for all GSTs. In our study, the effect of GSH concentration on the enzyme activity is also determined. The rate of thioester formation is determined by varying the cofactor, GSH, concentration up to 2 mM; at higher concentrations a decrease in enzyme activity was observed indicating the presence of either substrate inhibition or product inhibition. Schroder and coworkers (1996), have shown that the kinetics of GSH conjugation with CDNB does not follow the Michaelis-Menten rule. The sigmoid curve in the GSH saturation and the evaluations according to Lineweaver-Burk, Eadie-Hofstee and Hanes have pointed a positive cooperative

effect in ligand binding to GST. As low GSH concentrations may not be sufficient to protect the active site against acetylation by CDNB, reduced velocities at low GSH concentrations could be attributed to the substrate inhibition (Schroder, 1996). However, Turkish red pine cytosolic GST exhibited typical Michaelis-Menten kinetics under the optimized conditions studied. The Vmax and Km values for *Pinus brutia* GST against GSH as cofactor were calculated as 106.4 nmol/min/mg protein and 0.1nM, respectively.

In order to study the distribution of this enzyme among *Pinus brutia,* GST activity against CDNB was measured in seedlings grown from the seeds collected from 3 over-exploited and 2 natural *Pinus brutia* populations. Over-exploited populations have been highly degraded by roads connecting villlages to the highways, illegal cuttings of trees, man caused forest fires, and overgrazing. These are also lower elevation populations by compairing to the natural ones. Natural populations are mostly inland populations.

In most of the samples, the GST activities towards the CDNB were higher in the over exploited populations than the natural populations. Figure 22 shows that the highest activity should have been measured at -3˚C, because, GST is one of the main enzymes that work against the harmful effects of cold and this might be because of the fact that the samples taken from areas very close to roads may be affected from the chemicals might create considerable fluctuations in enzyme activities. The average specific activity against CDNB was calculated as 224, 168, and 129 nmole/min/mg protein for over exploited populations at -3˚C, 0˚C, and 13˚C, respectively, and was calculated as 185, 146, and 139 nmole/min/mg protein for natural populations at -3˚C, 0˚C, and 13˚C, respectively (Figure 22 and Table 6). Two types of of *Pinus brutia* families were studied in this study, one of which resistant (Type I) and other is sensitive (Type II) to cold stress. Type I and Type II did not differ much from each other, however, GST activity towards CDNB is higher at -3˚C than that of 0˚C, and 13˚C (Figure 23, 24), although Yaylaalan and Çameli are exceptional.

Until relatively recently, there has been no protocol that allows the purification of active *Pinus brutia* GST. In this study, anion-exchange (DEAE-Sepharose fast flow), column was used to purify of *Pinus brutia* GST. Homogenate, prepared as described in the 'Materials and Methods', was loaded onto a DEAE-Sepharose column where the isozyme could bind to column. As detected from purification results, it seems that the GST activity of *Pinus brutia* decreased during the purification. Afterall, there is almost no purification practically on the DEAE-Sepharose column.

Since this work is the first study carried out in *Pinus brutia* regarding the GSTs, we have no means of comparing our results with the ones in the literature. In future, a further examination and purification of GST isozymes in *Pinus brutia* will clarify the isozyme composition in *Pinus brutia* and the specific role of isozyme(s) in cold defence.

CHAPTER V

CONCLUSION

- In this study, the existance of GST enzyme activity in *Pinus brutia* has been shown for the first time in the literature and it is characterized by using CDNB as a test substrate. The specific activity of *Pinus brutia* cytosolic GST was found to be (200±50, n= 18) nmole/min/mg cytosolic protein among the individual trees sampled.
- The Vmax and Km values of GST for CDNB as substrate were calculated as 416 nM/min and 0,8 nM respectively, and for GSH as 106.4nM/min and 0,1 nM respectively.
- When the pH and temperature dependence of GST were examined, *Pinus brutia* GST showed maximum activity at pH 8,0 and at temperature of 42 ˚C.
- The GST activity was measured among *Pinus brutia* populations by using optimum conditions (1mM CDNB, 1mM GSH, 40 mM Phosphate buffer, pH 8.0, and 25˚C).
- There is an increase in glutathione S-transferase activity under the cold stress conditions.

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