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Research Article [Araştırma Makalesi]

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Glutathione S-transferase activities and glutathione levels in needles of drought stressed Pinus Brutia Ten. trees

[Kuraklık baskısı altındaki Pinus brutia Ten. ağaçları iğne yapraklarında Glutatyon S-transferaz aktiviteleri ve glutatyon seviyeleri]

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ABSTRACT

Aim: Glutathione S-transferases (GST) take roles under stress conditions by the conjugation of Glutathione (GSH) to electrophilic substrates to increase their solubility and facilitating further metabolic processing. This is one of the main pathways functioning in stress resistance and, for dought stress, there is not yet a study on Pinus brutia Ten. which is a very important forest tree of Turkey.

Materials and Methods: The needles of 30 different individuals of Pinus brutia Ten. were collected three times in the season: at the begining, in the middle and at the end of summer (June-August). The total precipitation and the temperature of the region were surveyed during sampling period. The osmotic pressure values, total GST activities and the GSH pools of the needle samples were measured and statistically analysed.

Results: The osmotic pressure values showed 16% increase throughout the season. Moreover, total precipitation and temperature values demonstrated that the highest drought stress was observed at the end of sampling period. Approximately 80% of increase in total GST activity between the beginning and the end of summer probably indicated the response against drought; although there was no significant change in the GSH pool.

Conclusion: The presence of drought, elevated osmotic pressure and a satistically significant ($p<0.005$) increase in total GST activities in needle samples were detected. The lack of direct correlation between the GSH pool and total GST activities was explained by the rate of GSH biosynthesis and its redox cycling.

Key Words: Pinus brutia, drought stress, glutathione S-transferase, glutathione

Conflict of Interest: The authors declare that they do not have any conflict of interest

ÖZET

Amaç: Baskı koşullarında, Glutatyon S-transferazlar (GST), Glutatyon (GSH) ekleyerek elektroncul substratların çözünürlüğünü artırırlar ve daha ileri metabolik işlemlere imkan verirler. Bu, baskı koşullarına dirençte ana yolaklardan biridir. Türkiye'nin en önemli orman ağaçlarından biri olan Pinus brutia Ten. için kuraklık baskısının GST aktivitesine etkileri üzerine henüz bir çalışma bulunmamaktadır.

Gereç ve Yöntemler: Pinus brutia Ten. türünden 30 farklı ağacın ibre yaprakları yaz sezonu içerisinde 3 farklı zamanda ayrı ayrı toplanmıştır: yazın başında, ortasında ve sonunda. Örnekleme alanındaki toplam yağış miktarı ve sıcaklık, örnekleme süresi boyunca ölçülmüştür. İbre yapraklara ait ozmotik basınç değerleri, toplam GST aktiviteleri ve GSH havuzu ölçülmüş ve istatistiksel olarak analiz edilmiştir.

Bulgular: Ozmotik basınç değerleri sezon boyunca %16 artış göstermiştir. Sıcaklık ve toplam yağış miktarı ölçümleri de örnekleme alanında en şiddetli kuraklık baskısının örnekleme periyodunun sonunda gerçekleştiğini göstermiştir. GSH havuzunda belirgin bir değişim olmamasına rağmen, toplam GST aktivitesinde, kuraklık baskısına karşı muhtemel tepkiyi gösterir nitelikte %80 oranında bir artış oluşmuştur.

Sonuç: Örnekleme bölgesinde kuraklığın varlığı, ibre yapraklarda ozmotik basınç değerlerinin yükseldiği ve toplam GST aktivitesinde istatistiksel olarak anlamlı ($p<0.005$) bir artışın gerçekleştiği belirlenmiştir. GSH havuzu ile toplam GST aktivitesi arasında doğrudan bir bağıntının olmaması ise GSH biyosentezinin hızı ve redoks çevrimiyle açıklanmıştır.

Anahtar Kelimeler: Pinus brutia, kuraklık baskısı, glutatyon-s transferaz, glutatyon

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Introduction

Pinus brutia is a coastal tree and a drought resistant pine species that withstands more aridity and poor soils than most other timber species growing in the same climatic conditions. It shows a relatively restricted distribution, especially in the Mediterranean region having populations in Italy, Greece, Turkey, Cyprus, Syria, Lebanon, Jordan, Palestine, and the many islands of Aegean and Mediterranean, northern Iraq and over the north coast of Black Sea [1].

P. brutia is one of the two important forest tree species of Turkey and it forms the forest cover on the regions in South Marmara, West and South-Southwest coasts of Anatolian Peninsula. Because of the fact that the largest populations are located in Turkey, it is also called Turkish Red Pine.

The climate models guess approximately 15% decrease in rainfall and in total humidity which shows a possible increase in environmental stress on this pine species in its natural habitat. The supplement report of Intergovernmental Panel on Climate Change (IPCC) of year 1992 [2] presented the data, which was derived in a series of measurements in many different locations of Europe and Turkey, stated that the total soil humidity decreased by 25% with respect to the past. IPCC alerts Turkey for future climate change and probable consequences in its Fourth Assessment Report [3], because Turkey is located in the Eastern Mediterranean basin where countries are in the highest risk group. Ecological and meteorological models showed an overall reduction in summer moisture availability in response to rising concentrations of greenhouse gases [4]. In the report of the date 01.06.2009 [5], The World Bank underlined the fact that, in EuroAsia Region, Turkey is one of the three countries which would have the most extreme climatic changes such as heavy rain, freezing temperatures and drought till the end of this century.

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 [6,7]. Glutathione (GSH) is a cellular protectant that is the major reservoir of non-protein reduced sulfur in plants [8]. It 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. Glutathione S-transferase (GST, EC 2.5.1.18) is a GSH dependent detoxifying enzyme in plants, which catalyzes the conjugation of GSH with many potentially dangerous compounds. GSTs 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 co-substrate containing a reactive electrophilic center to form a polar S-glutathionylated reaction product [9].

The aim of this study was to demonstrate how the GSH pool and the activities of the GSTs in needles of Turkish red pine were changed with respect to increasing drought stress in nature. Therefore, this study would provide an access for the further projects explaining the roles of GSTs in overwhelming of pernicious effects of drought for a successive drought resistant pine species. Moreover, it might proclaim GSTs as important targets in researches aiming to protect natural forest cover of Turkey and the ecosystem of west and south-west of Anatolian peninsula against the increasing threat of drought by cultivating new generation saplings relatively more resistant to drought.

Materials and Methods

Plant Material

Pinus brutia needles were harvested from 30 different healthy individuals of a local population, in METU Campus, Yalincak area, three times in hot and dry summer season: at the beginning (i), at the middle (ii) and at the end of the period (iii). Sampling was performed in a way that the needles were collected all arround the tree, from the tips of the branches located almost the same level. By this way, authors collected spring needles of the same year; i.e, samples were younger than one year old. This was repeated twice to form two sets of sample for each individual; in other words, there were 180 samples of 30 trees for whole study.

Reagents and Chemicals

Folin-Ciaceltau Phenol Reagent (Sigma), potassium dihydrogen phosphate (Fluka, min. 99%), dipotassium hydrogen phosphate (Merck, extra pure), reduced glutathione (GSH) (Sigma, min 99%), Polyvinylpyrrolidone (PVP)-K30 (Fluka), hydroxymethyl aminomethane (Tris) (Sigma, min 99.9%), hydrochloric acid (Merck, 37%), octylphenoxypolyethoxyethanol (Nonidet P-40) (Sigma), Bovine serum albumin (BSA) (Sigma), ethanol absolute (Riedel-de Haën, %99.8), methanol absolute (Riedel-de Haën, %99.8), 1-chloro-2.4-dinitrobenzene (CDNB) (Sigma, min 98%), copper sulfate ($CuSO_4.5H_2O$) (Riedel-de Haën, extra pure), sodium-potassium tartrate (Fluka, min. 99%), sodium hydroxide (Merck), sodium carbonate (Riedel-de Haën, extra pure), 2-mercaptoethanol (Merck, min. 98%), Pepstatin (Fluka), 5`-dithiobis-(2-nitrobenzoic acid) (DTNB). All chemicals were analytical grade and were obtained from commercial sources at the highest purity available.

Sample Preparation

Crude extracts were prepared by the homogenization process modified from the one described by Schröder and Berkau [10] for spruce needles. Needles were pounded in ceramic mortar by freezing with liquid nitrogen and 0.2 g of fine powder was weighted into plastic tubes. 2 ml of homogenization buffer (0.1 M Tris HCl buffer, pH 7.8, containing 20 mM of 2-Mercaptoethanol, 5% PVP-K 30, 0.5% Nonidet P40 and 3 µg/ml of Pepstatin

A) was added to each tube and mixed well. The mixtures were homogenized in ice, at 13,500 rpm of Ultra-Turrax T25, in 15 min intervals for 1 min. The homogenates were centrifuged at 12,000 g for 45 min at 4°C. The pellets were discarded and the supernatants were used as the enzyme source.

Determination of Protein Concentration

For the detection of total protein concentration in various samples, Lowry Protein Assay is used. Lowry Method [11] was adjusted for the measurement by ELISA Plate Reader (Bio-Tek ELx808) with crystalline BSA as the standard. Samples were diluted as 1:24 and 1:49, and added into the 96 well plates as triplicates as a final volume of 50 µl. Then, 200 µl of freshly prepared Lowry ACR (including 2% copper sulfate, 2% sodium-potassium tartarate, 2% (sodium hydroxide, sodium carbonate) in the ratio of 1:1:100) was added and mixed by pipetting. After 10 min of incubation at room temperature, Folin Cilcateu Phenol Solution (commercially available form was diluted 1:1) was added as 20 µl with immediate mixing. At the end of 45 min. incubation at room temperature, plates were read at 650 nm. Protein concentration in each well was calculated by the software of the instrument (KC Junior) after providing necessary dilution factors. Standard curve was prepared by using BSA solutions diluted as 1, 2.5, 5, 10 µg/well.

Enzyme Activity Assay

GST enzyme activity was determined spectrophotometrically by measuring the thio-ether formation in reaction media at 340 nm, as stated by the method of Habig et al [12] which used CDNB as substrate. This method was modified to ELISA Plate Reader system in a way that each reaction medium contained 100 mM potassium phosphate buffer at pH 7.8, 1 mM GSH, 1 mM CDNB and 3-8 mg/ml cytosolic extract in a final volume of 250µl. Samples were diluted 20 times by mixing with 10 mM phosphate buffer at pH 7.8 (in ice bucket) and added into reaction media as 12.5 µl just before the measurement which took place 10 min in the shortest possible interval, for each microtiter plate. Each reaction medium was prepared as triplicated wells of a 96 well-plate with its own triplicated blanks. Absorbance detections were done by using ELISA Plate Reader (Bio-Tek ELx808). One unit of Glutathione S-transferase was defined as the amount of enzyme which conjugated 1 µmol of CDNB with reduced glutathione per minute at 25°C.

Determination of Total Thiol Groups

Glutathione makes up more than 95 % of the water soluble thiol (-SH) content in needles of spruce [13]; so the changes in thiol content can be considered as changes in glutathione in reduced form (GSH) [14]. Cytosolic total thiol amount of each sample was determined by the method defined by Sedlak and Lindsay [15]. This method is based on the reduction of DTNB) by sulfhydryl groups, to produce a characteristic yellow color which gives its maximum absorbance at 412 nm. Each free –SH group reduces the DTNB into 1 molecule of 2-nitro-5-mercaptobenzoic acid that creates the yellow color. To construct the standard curve, GSH was used as the standard at the concentration range of 0.1-1.0 µM. 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 methanol were added into each well. After a 30 min of incubation period at room temperature (25°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, which transformed the absorbance values into "µmoles/min/mg protein" unit after researchers entered the numerical values for volumes and protein concentration.

Measurements of Osmotic Pressure Change

The osmotic pressure values of each needle sample were determined by the instrument Wescor's Vapor Pressure Osmometer, model VAPRO® 5520. In determination of the osmolality between different samples, the use of vapor pressure as a parameter assures researchers with a fast, easy and convenient way; so, this method is popular if the solvent is water [16,17]. For each tree, a single sample pool was prepared, that is, totally 30 measurements were done in every sampling time. Each cleaned sample was cut into small pieces by scissors and filled into a disposable plastic injector as soon as possible. By the help of a clamp, injector is pressed and the juice of the needles was collected in eppendorf tubes. Those tubes were centrifuged at 3,000 g and 10 µl of supernatant was applied onto special filter paper discs before placing into the measuring tray. Values were expressed with unit of mmol/kg.

Statistical Analysis

For the comparison of experimental data between sampling seasons, repeated measures ANOVA were used. In order to gain more information on the significant results after ANOVA analysis, as a post hoc comparison tool, Tukeys test was applied and the p values were checked. In this study, Analysis of Variance (ANOVA) and Tukeys test were performed by the MINITAB™ statistical Software Release 13 (BCIS Lab, St Cloud State University). All the other statistical values; such as mean, standard deviation, averages, and etc. were calculated by the same statistical software.

Results

The Changes in Osmotic Pressure throughout the Sampling Period

For 30 individuals, average osmotic pressure values and standard deviations (SD) were calculated. As summarized in Table 1, the measurements showed an increase in osmotic pressure which possibly strengthens the reasoning for the presence of drought stress during the sampling

time. At the beginning of the sampling period (i) it was measured as 929.4 ± 50.2 mmol/kg and approximately 5% and 16% increases were detected in the middle (ii) and at the end of summer season (iii), respectively.

Table 1. The changes in the average osmotic pressure values measured for pine needle samples of first (i), second (ii) and third (iii) sampling time.

Sampling Time	Average Osmotic Pressure (mmol/kg) ± SD
i	929.4 ± 50,2 (n = 30)
ii	974.8 ± 49,1 (n = 30) *
iii	1078.6 ± 27,9 (n = 30) *,**

(*) Statistically different with respect to (i), p<0.005
(**) Statistically different with respect to (ii), p<0.005

These results were all consistent with the temperature and total precipitation records of the region as showed in Figure 1 and Figure 2. The records provided by Turkish State Meteorological Service (station number 17134) demonstrated an increase in temperature and a dramatic decrease in total precipitation during the summer season which might cause an increasing drought effect.

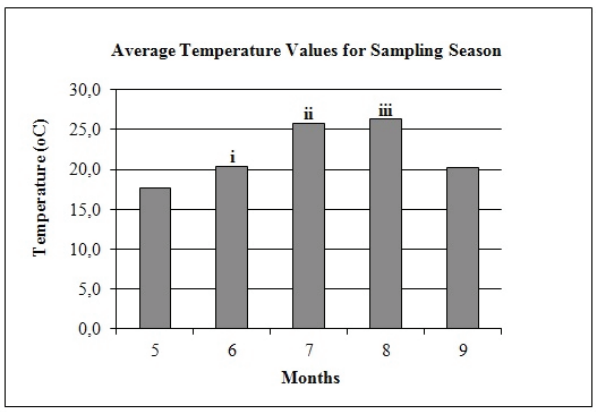

Figure 1. The changes in the average temperature values at sampling area for sampling season. (i), (ii) and (iii) stand for first, second and last sampling time, respectively.

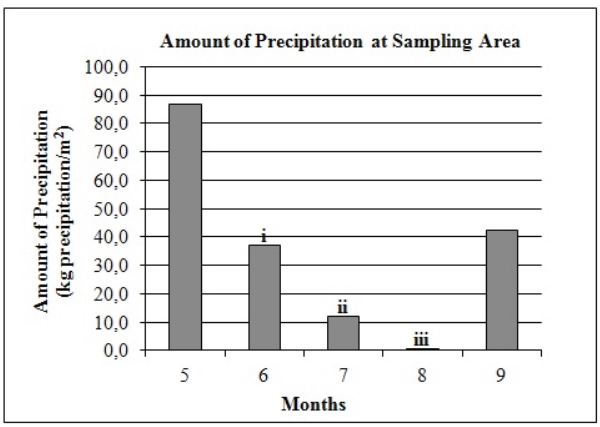

Figure 2. The changes in total precipitation at sampling area. (i), (ii) and (iii) stand for first, second and last sampling time, respectively.

The meteorological data pointed approximately 70% of decrease in the amount of precipitation till the middle of summer, and there was almost no rainfall at the second half of the season. The average temperatures reached its highest value at the middle of summer and continued till the end of season which means that the overall drought created by the lack of rainfall and high temperature values increased from the early summer to the end of August.

Total Glutathione S-Transferase Activity Changes in Cytosolic Extracts

Slope values of the changes in absorbance with respect to time (dA/dt), which were measured for 3 reaction wells in accordance with their 3 blank wells, were recorded for each sample couples. By this way, authors used the averages of two independent sets of measurements for each of 30 individuals after calculating the specific activities, in each sampling time. The final results were calculated as the averages of those 30 specific activity values with standard deviations as shown in Table 2.

Table 2. Total GST activities measured by using CDNB as the substrate. (i), (ii) and (iii) stand for first, second and last sampling time, respectively.

Sampling Time	Average Specific Activities (µmoles/min/mg protein) ± SD
i	15.78 ± 1.36 (n = 30)
ii	22.91 ± 1.99 (n = 30)*
iii	28.37 ± 2.38 (n = 30)*,**

(*) Statistically different with respect to (i), p<0.005
(**) Statistically different with respect to (ii), p<0.005

The results of the second sampling (ii) and the last one (iii) were compared to the first sampling (i) considering that at the beginning of the summer season, drought stress was in its minimum values after a relatively rainy spring season under mild temperatures, so, it might be used as the reference. GST activities of (ii) and (iii) showed statistically significant differences with respect to the values of (i). The comparisons were done by repeated measures ANOVA. In addition, there are statistically significant differences between GST activity values of (ii) and (iii) when they were compared and tested by Tukeys test. After the application of ANOVA for the 95% and 99.5% confidence interval between the results of each sampling time as couples, the p values were calculated. Any p value smaller than 0.005 was accepted as a statistically significant difference between the compared set of data.

The Variations in Cytosolic GSH Pools of Needle Samples

As an important oxidative stress marker and the substrate of GSTs in the cell, GSH pool is very important though highly inconstant; so, the amount of this mol-

ecule was measured for the samples to evaluate a possible correlation with the increasing GST activity during whole season. The same samples used in activity measurements were subjected to total thiol assay and, as it is denoted in Table 3, averaged values denoted approximatey 7% increase in the middle of the season and, then, there existed approximately 5% decrease concluding a value close to the one measured at the beginning of the summer.

Table 3. Average total thiol amounts which were used as the indicator of total GSH in pine needle cells. First, second and last sampling time was denoted by (i), (ii) and (iii), respectively.

Sampling Time	Average Total Thiol Amounts (μmoles / g of pulvarized needles) ± SD
i	74.90 ± 2.42 (n = 30)
ii	80.02 ± 3.20 (n = 30) *
iii	76.50 ± 2.84 (n = 30) **,***

(*) Statistically different with respect to (i), $p<0.005$
(**) Statistically different with respect to (i), $p<0.05$
(***) Statistically different with respect to (ii), $p<0.005$

Discussion

Drought and heat generated by the climatic conditions of the summer season generates an increase in the amount and specific activities of some enzymes, especially GSTs and Glutathione reductases (GRs) (EC 1.8.1.7) [18-20]. Meteorological data put the presence of possible environmental stress conditions forth as an inducer of the total GST activity. During all season, those climatic conditions continued to end with higher GST activities, as expected [21-25]. From the beginning of the summer season till the middle, together with an increase in temperature, there was a decrease in precipitation, possibly causing lowered soil humidity. As anticipated with the increase in the temperature and decrease in soil humidity, GST enzyme activity exhibited statistically significant increase from 15.78 ± 1.36 μmoles min^{-1} mg $protein^{-1}$ to 22.91 ± 1.99 μmoles min^{-1} mg $protein^{-1}$. This elevation in the specific activity values of GST continued to the end of the season at which, it reached 28.37 ± 2.38 μmoles min^{-1} mg $protein^{-1}$ indicating approximately 80% overall increase. When it is compared with the elevated temperature and reduced humidity, all this scheme of change from the start to the conclusion is highly logical.

The osmotic pressure measurements were in accordance with expectations, too. By the decreasing water availability in the soil, and increased transpiration demand under heat and drought stresses, pine needle cells lost water which probably caused an elevation in the osmotic pressure. As in some other studies [26-28], Wescor's Vapor Pressure Osmometer, model VAPRO® determined statistically significant changes between the samples indicating a possible water stress scaling up till the end of hot and arid summer.

GSH accumulation is observed in all stress conditions including drought stress [8], because it is involved in ascorbate-glutathione cycle and performs the quenching of reactive oxygen species (ROS) as an anti-oxidant to eliminate damaging peroxides [29]. This molecule has many other functions in plant cells including even the induction of the transcription of gst genes [19] Under environmental stress conditions, plants raise the concentration of GSH in especially foliar tissues as a part of their acclimation strategy via antioxidative defense systems [30]. However, Chen et al [31] concluded that the higher ratio of reduced to oxidized GSH or, glutathione / glutathione disulfide (GSH/GSSG), the rate of GSH biosynthesis and the capacity of its redox cycling, rather than GSH accumulation, might be essential for drought resistance in plants; so, one could/couldn't measure elevated glutathione levels, or even decreased levels might be detected. This means that, even if there observed little change in GSH amount during summer period, the GSH/GSSG redox state might be changed towards being more oxidized, more reduced, or not at all. Whether statistically significant or not, the undulation in the levels of GSH might be a simple consequence of pine trees. As a possible signal for longer-term acclimation process to stress conditions, pine trees show a slight decrease in the GSH/GSSG ratio as a short-term response against mild drought, but when the stress became more intense, degradation and further oxidation of glutathione was observed, too [32].

In conclusion, increased drought stress indicated by meteorological data and osmotic pressure measurements possibly caused an elevation in cytosolic total GST activities throughout the summer season although GSH pool didn't response in the same magnitute.

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

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