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A comparative study on Glutathione S-transferase activities of the Needles of Two Pine species under Drought and Cold stresses

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Abstract

Total cytosolic Glutathione S-transferase (GST, EC 2.5.1.18) activities and the changes in the GSH pools of the needle samples from cold-resistant Pinus sylvestris and relatively drought resistant Pinus brutia species were screened for three seasons. Samples of spring season were accepted as reference for other two groups of sampling done in summer and winter; and, daily total precipitation, air humidity, minimum and maximum temperature values and freeze incidents were recorded. Results revealed the possible correlation between the dimensions of GSH pools and total GST activities of needle homogenates at cold and drought stresses. Those two species showed different behaviours for the same climatic conditions.

Keywords: *Pinus brutia*, *Pinus sylvestris*, GST, glutathione, cold, drought, stress.

Introduction

Pinus brutia, which is also known as red pine, is the dominant tree of forests located in the Mediterranean, the Aegean and the Marmara regions of Anatolian Peninsula. It has natural populations in the Mediterranean region: in Turkey, Italy, Greece, Cyprus, Syria, Lebanon, Jordan, Palestine, and the many islands of Aegean and Mediterranean, northern Iraq and over the north coast of Crimea[1]. Red pine is a coastal tree and when compared with other timber species found in the same region, it withstands more aridity and poor soils though it requires mild winters. As contrary, with a natural range from the Arctic Circle in Scandinavia to southern Spain and from western Scotland to the Okhotsk Sea in eastern Siberia, *P. sylvestris* (scots pine) is the most widely distributed conifer in the world[2] and it is also known as a cold-successive species adapted even to the harsh sub-arctic climate[3].

Glutathione S-transferases (GSTs) (GST, EC 2.5.1.18) have functions in detoxification of various xenobiotic compounds and oxygen radicals[4-6]. They catalyze the conjugation of glutathione (GSH) with many potentially dangerous compounds[7]. Their roles in those biochemical pathways make them useful markers in the detection of stress in plant metabolism. Under extreme environmental conditions like very high or very low temperature and drought, severe membrane damage occurs[8], which in turn, triggers a serious of transcriptional changes in the plant cells including an increase in the amount of GSH. For those plant cells, the production of GSH, which acts as an antioxidant by quenching reactive oxygen species to eliminate damaging peroxides, is a natural response[9].

The present work was designed to reveal the changes in the GST activities and GSH pools of pine needles with respect to environmental stress conditions. Author had the advantage to perform a comparison between the seasons and, cold and drought stresses for each of the species; and, moreover, an interspecies comparison for the same cases could be succeed for two pine species of completely different nature with respect to climatic needs.

Material and Methods

Pine needle samples: Pine needles were harvested from healthy pine trees of each of two species located at METU Forest (approximately, at the same region and elevation) at three different times: at the end of winter, at the end of spring and at the end of summer. For this purpose, 20 individuals of *Pinus brutia* and 20 individuals of *Pinus sylvestris* of same age were marked. Needles were collected from the tip parts of the branches having almost the same height with respect to ground and located all around the stem. Needle samples were wiped with wet paper towel to remove contaminants, and they were placed into special containers resistant to liquid nitrogen. Those samples were transported to the laboratory in liquid nitrogen tank and placed into -80^{o}C ultra freezer as soon as possible.

Preparation of needle cytosolic homogenates: As it was defined by Schröder and Berkau[10], pine needles were powdered in liquid nitrogen and 0.2 g of each sample is weighted into plastic tubes. Then, 2 ml of ice-cold homogenization buffer (0.1 M Tris HCl buffer, pH 7.8, containing 0.07% (v/v) 2-Mercaptoethanol, 5% (w/v) PVP-K 30, 2 mM of EDTA, 0.5% Nonidet P40, 3µg/ml of Pepstatin A) was added and vortexed well. Homogenization was applied by using Ultra-Turrax T-25 at 13500rpm, for 15 sec intervals of totally 4 times, in ice. Homogenates were transferred into eppendorf tubes and centrifuged at 12000g, at 4^{o}C, for 30 min. The supernatant were aliquoted and stored in -80^{o}C ultra freezer.

Determination of protein concentration: Total protein concentrations of samples were detected by Lowry Protein Assay[11]. It was modified for ELISA Plate Reader (Bio-Tek ELx808) system. Crystalline bovine serum albumin (BSA), in concentrations of 1, 2.5, 5, 10 µg/well, was preferred for the construction of standard curve. Samples were diluted as 1:24 and 1:49, and added into wells as triplicates with a final volume of 50 µl. Then, 200 µl of freshly prepared Lowry ACR (including 2% cupper sulfate ($CuSO_4.5H_2O$), 2% sodium-potassium tartarate, 2% (sodium hydroxide, sodium carbonate) in the ratio of 1:1:100) was added. 20 µl of Folin Cilcateu Phenol Solution was added after 10 min of incubation at room temperature. Absorbance values were measured at 650 nm at the end of 45 min of incubation. Protein concentration in each well was calculated by the software of the instrument (KCjunior™).

Determination of total thiol groups: More than 95% of the water soluble thiol (-SH) content of needles was occupied by GSH[12]; so the changes in thiol content could be considered as the changes in GSH pool[13].

The method defined by Sedlak and Lindsay[14] was used for the measurements of cytosolic total thiol amount of each sample after adjusting for ELISA plate reader system. This method is based on the reduction of 5`-dithiobis-(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups, to produce a characteristic yellow colour which gives its maximum absorbance at 412 nm.

10 µl of cytosol was added into 30 µl of 200 mM Tris Buffer, pH 8.2 containing 20 mM of EDTA. Then 20 µl of 2 mM DTNB and 140 µl of pure MeOH were added into each well. After a 30 min of incubation period at room temperature in dark, absorbance values were measured. Concentration values were calculated by the slope of standard curve.

GST enzyme activity assay: GST enzyme activity was determined spectrophotometrically by monitoring the thio-ether formation at 340 nm by using CDNB as the substrate, basically according to the method of Habig *et al.*[15]. The method was modified and optimized for ELISA plate reader system. Each reaction mixture contained 100 mM potassium phosphate buffer, pH: 7.8, 1.0 mM GSH, 1.0 mM CDNB and 3.5-6 mg/ml cytosolic homogenate in a final volume of 250 µl in 96 well plate. The reactions were started by the addition of enzyme into each well. Slopes of the best lines, which had been drown for each well separately by the software of the instrument, were used as the rate of reaction (dA/dt) and the further calculations were completed.

The results of determination of total thiol concentration and total cytosolic GST activity tests were evaluated statistically by the licensed software Minitab® 16. t-test of difference were applied for winter and summer samples against spring sample which was used as the reference group in all experiments. p values were calculated for the 95% confidence interval (CI) and any p value smaller than 0,05 pointed the statistically significant difference between the compared set of data. The dagger (†) in the column graphs indicates a difference in the 99.5% CI ($p<0.005$).

Results and Discussion

Climatic data for the sampling period clearly indicated the presence of all physical conditions for cold stress in winter season and drought and heat stresses for summer season as demonstrated in the figure 1. There existed freeze incidents between December and March very frequently and this severe cold stress continued even till April. However, the rest of spring season, especially May, was very favourable for both pine species in the cases of total precipitation and average temperatures. In summer, maximum temperature values were between 25-35^oC and total precipitation was almost zero especially in the second half of the season. In accordance with those high temperature values, decreased average daily air humidity created drought and heat stresses on the populations.

Pinus brutia and *Pinus sylvestris* showed similar patterns in the changes of total thiol content in the needle homogenates as shown in figure 2; levels were higher for winter and summer samples with respect to spring samples. GSH accumulation is observed in all stress conditions; not just because of cold stress, but also high temperature and drought stress[16], because it is involved in ascorbate-glutathione cycle and performs the quenching of ROS as an anti-oxidant to eliminate damaging peroxides[9]. According to these findings, the elevated GSH levels in winter and summer samples for both of the pine species might be a sign of the presence of high stress possibly created by cold and drought. The level of total thiol groups in the needle homogenates of scots pine decreased by 72% at spring season, and elevated twice at the end of summer season. Although such a dramatic increase occurred from spring to summer, GSH pool of the needles was smaller with respect to winter season. For red pine, this level reduced by 23.3% from winter to spring season, and increased by 71.9% at the end of summer as summarized in table 1. Unlike *P. sylvestris*, maximum GSH concentration in needle homogenates of *P.brutia* was measured for summer samples.

Table-1
Total thiol concentrations in the needle homogenates of both species, in all sampling seasons

Sample	Total Thiol concentrations (µmole/g of tissue)	Standard Deviation
PB-Winter	68.78	±1.83
PB-Spring	52.75	±5.97
PB-Summer	90.71	±2.51
PS-Winter	72.12	±1.83
PS-Spring	20.03	±3.34
PS-Summer	40.07	±2.36

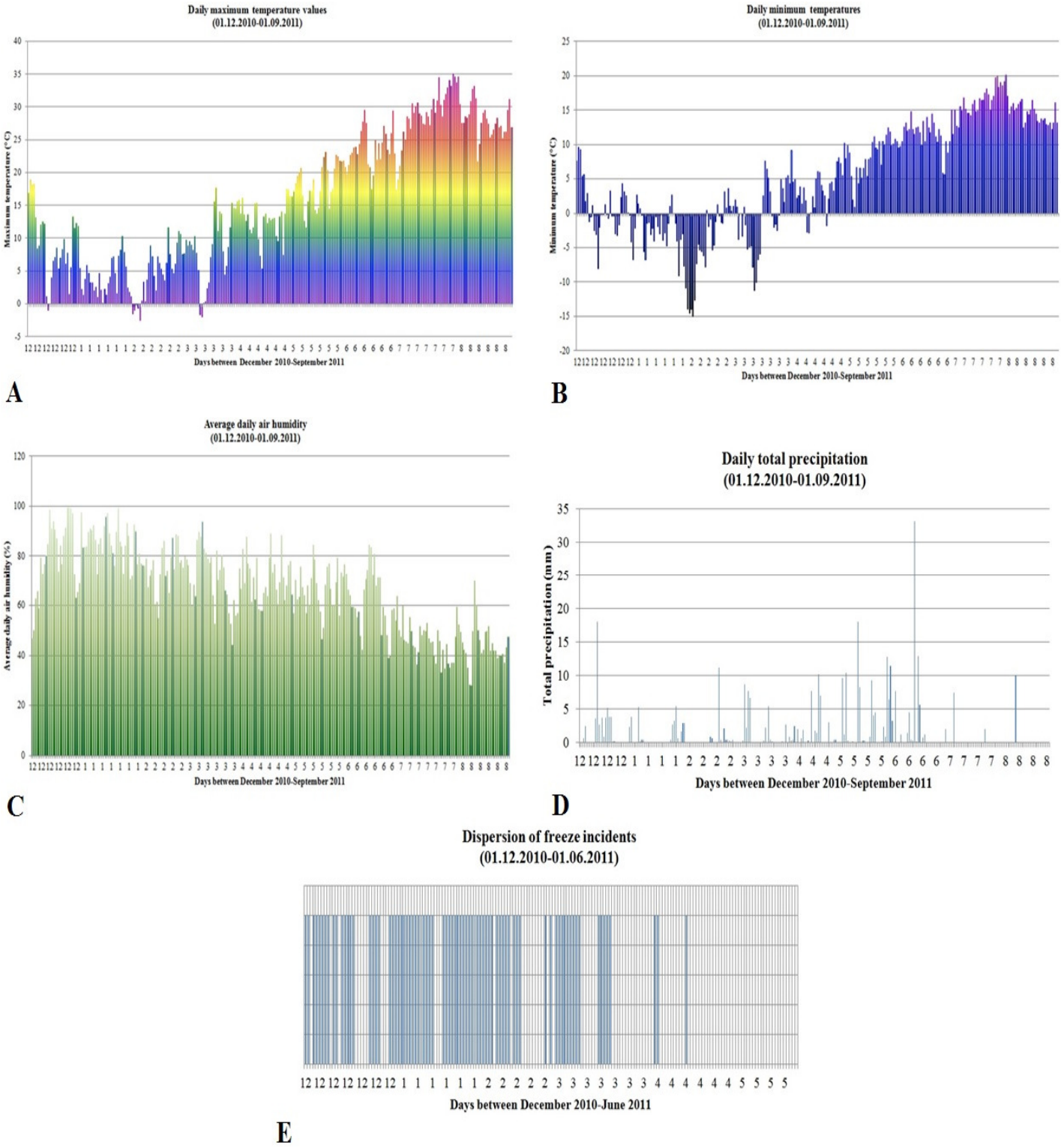

Figure-1

Meteorological data recorded for the sampling area: (A) Daily maximum temperature values, (B) Daily minimum temperature values, (C) Average daily air humidity values, (D) Average daily total precipitation, (E) The number and the dispersion of freeze incidents. All raw data are belonging to the period of 01.12.2010-01.09.2011 and kindly provided by Turkish State Meteorological Service (station number 17134)

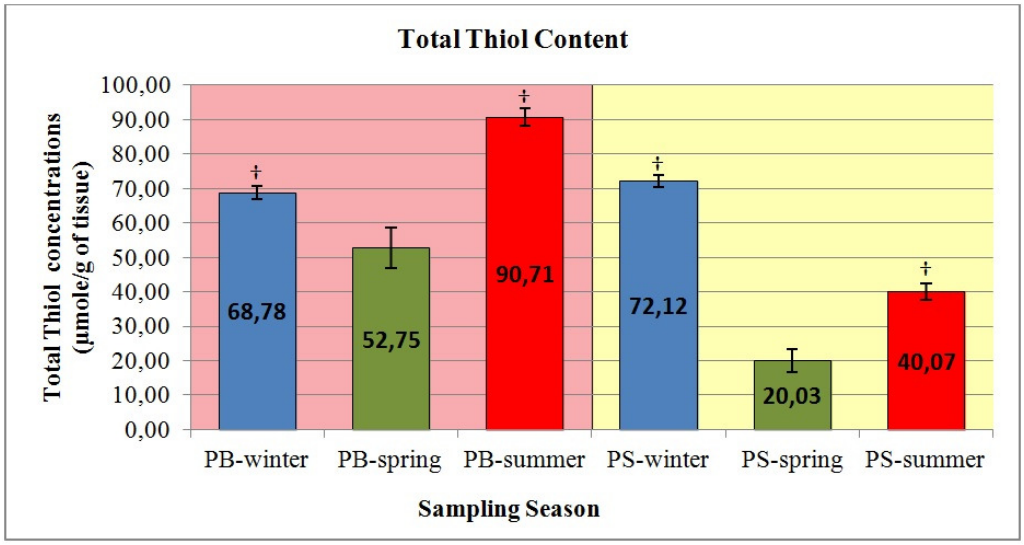

Figure-2

Total thiol amounts measured for needle homogenates of *Pinus brutia* (PB) and *Pinus sylvestris* (PS) (Colors of columns: green is for spring, scarlet is for summer and blue is for winter). Red area is for *P. brutia*, and yellow area is for *P. sylvestris*, and, standard deviation values are indicated as error bars. (†): Statistically different with respect to spring season, p<0.005

GSH pool of scots pine needles reached its maximum under cold stress; while red pine needles succeeded it at the end of summer. Those results were meaningful for a cold-successive pine species of *P.sylvestris* and drought-adapted *P.brutia* if the total GST activities were taken into consideration, too.

Although there wasn't a considerable change between winter and spring samples, the total specific activity values of GSTs in *P.brutia* showed a decreasing pattern from winter season to summer with an approximately 31.5% of difference. On the contrary, as it could be seen in figure 3, the specific activities of GSTs in the needle homogenates of *P.sylvestris* increased almost three times in summer.

Another interesting outcome of those measurements was that the levels of the specific activities of GSTs for scots pine were considerably low with respect to the one of red pine as indicated in table 2.

Table-2

The specific activity of GST enzyme values measured against the common substrate CDNB. Needle homogenates of both pine species in all three seasons were used as the enzyme source

Sample	Specific Activity (µmoles/min/mg protein)	Standard Deviation
PB-Winter	360,3	±18,44
PB-Spring	360,6	±13,46
PB-Summer	247,2	±10,39
PS-Winter	47,7	±2,97
PS-Spring	33,9	±6,48
PS-Summer	98,9	±2,16

Kawamura *et al*[17] and Kosmala *et al*[18] affirmed that the cold stress causes the accumulation of ROS in plant cells which in turn invites an elevation in the amount of scavenging enzymes especially functioning in ascorbate and glutathione metabolisms such as superoxide dismutase (SOD), ascorbate peroxidase (APX)[19], glutathione reductase (GR) and glutathione S-transferase (GST). Drought and heat generated by the climatic conditions of the summer season, also, generates an increase in the amount and specific activities of those enzymes, especially GSTs and GRs[4,20,21]. Specific activity values of GSTs measured for scots pine peaked at the end of summer season. This might be a result of its being in-tolerant to drought and heat stresses; because studies on *P. sylvestris* indicated a relatively high mortality in drought stress conditions[22-24]. Contrarily, the highest values of total GST activity were measured at winter time for brutia pine. *Pinus brutia* prefers Mediterranean climate which have mild winter conditions and hot summers. As a partially drought and heat resistant species, Turkish red pine was actually expected to have such high GST activities under cold stress.

GSH pools of the needle samples of both species were smaller at seasons when the total cytosolic GST activities peaked. For red pine, total thiol content measurements gave similar results for winter and spring seasons; and, total GST activities were almost the same. Meteorological data stated that the freezing temperatures and naturally the cold stress was effective till the middle of spring season which possibly caused the stress-resistance mechanisms of *P. brutia* to be highly active, including GSTs. A similar response was observed for scots pine: GSH pool reached its minimum value when the total GST activity was in its highest level at the end of hot and arid summer.

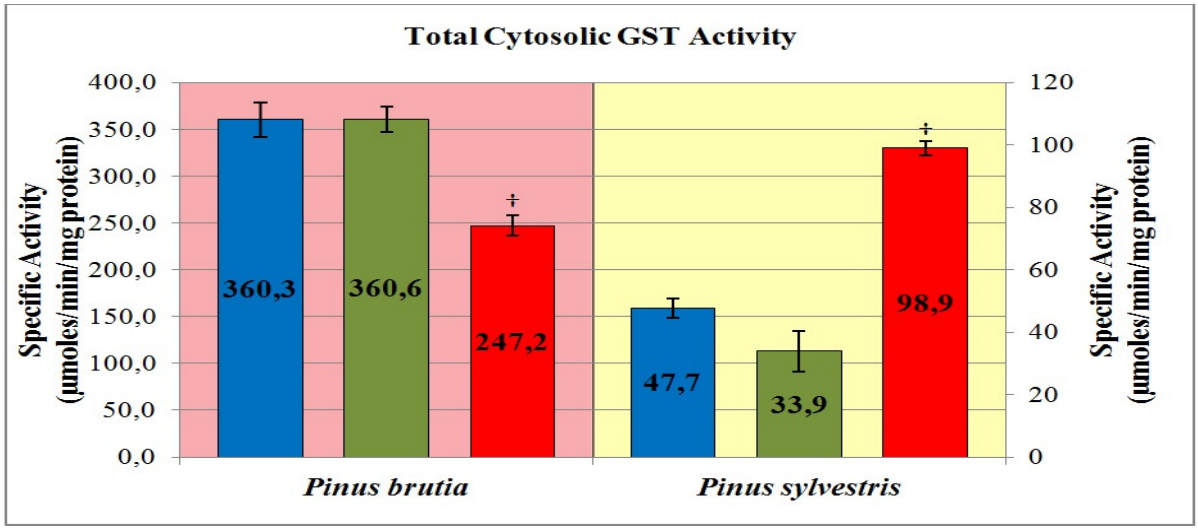

Figure-3

The specific activity of GST enzyme values of needle homogenates of both pine species in all three seasons (Colors of columns: green is for spring, scarlet is for summer and blue is for winter). Red area is for *P. brutia* (PB), and yellow area is for *P. sylvestris* (PS), and, standard deviation values are indicated as error bars. (†): Statistically different with respect to spring season, p<0.005

Conclusion

The changes in the GSH pool were purposeful with respect to the specific activity values of the total GST in both species: when the enzymatic activity rose, the pool was waned because of increased demand. Moreover, this relationship strengthens the proposition of higher oxidative stress in summer for *Pinus sylvestris* and in winter for *Pinus brutia*.

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