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# Relation to Altitude Adaptation and Antioxidant Defence System in Five Shrubs and Trees Species From Middle Taurus Mountains

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## ABSTRACT

Changes of antioxidative defence systems were investigated as depend on altitude in the leaves of five shrubs and trees species from Middle Taurus Mountains. Leaves of angiosperm species *Quercus coccifera, Styrax officinalis, Pistacia terebinthus, Rosa canina,* and a gymnosperm species *Juniperus excelsa* collected from different sites between 400-1500 m. Leaf water contents were lower at high altitudes in all species, except *J. excelsa*. The contents of chlorophyll a and b increased in leaves of angiosperm species but decrease in leaves of gymnosperm, *J.excelsa,* from high altitude. Total ascorbate contents decreased in *S. officinalis* and *R. canina* from high altitude, but increased in the other species. α-Tocopherol contents were higher in all of the samples from high altitude. Superoxide dismutase (SOD), especially Mn-SOD activities were increased in samples from higer altitude except for *R. canina*. Glutathione reductase (GR) activities were increased in leaves of *Q. coccifera*, *S. officinalis* and *R. canina* from high altitude. These results suggested that angiosperm and gymnosperm species can give different biochemical responses to oxidative stress increasing depend on altitude. The higher levels of antioxidant compounds and antioxidant enzyme activities required for adaptation to high altitudes of angiosperm species.

**Key Words:** Ascorbate, Chlorophyll, Glutathione reductase, Superoxide dismutase, Tocopherol

## INTRODUCTION

Trees and shrubs at high altitudes exposed to higher climatic stress have lower growth rates than those at lower altitudes [1]. Tree line exist because of a thermal threshold for growth and development of trees. Others stress factors such as drought, high light intensity and nutrient deficiency were considered as regional modulators of the tree line [2]. At high altitudes trees and shrubs have developed different physiological and biochemical strategies such as high antioxidant content increased carotenoids / chlorophyll ratio, low water content and higher soluble protein and proline contents [3].

Environmental stress combinations (low temperature-drought-light intensity) present at high altitudes may cause an increased production of reactive oxygene species (ROS) and thereby increase the risk of oxidative damage [1]. High light damage is minimised by dissipation of excess excitation energy mediated by carotenoids in particular the pigments of xanthophyll cycle [4]. Since the functioning of the xanthophyll cycle depends on ascorbate, whose redox state is in turn maintained by the action of glutathione. α-Tocopherol acts as a chain-breaking antioxidant in cell membranes lipid peroxidation and can be regenerated by reduced ascorbate and glutathione [5]. Ascorbate also has the capacity to directly eliminate several different ROS including singlet oxygen, superoxide and hydroxyle radicals [6]. Other components of antioxidative defence systems are enzymes such as catalase, superoxide dismutase, ascorbate peroxidase, dehydroascorbate

**Table l.** Water contents, dry weight ratios and chlorophyll a, b contents and a/b ratio in leaves of five shrubs species from different altitude. Data represent the means and ± standard deviation (**P<0.01)

| Species | Altitude (m) | Water Content (%) | Dry Weight (%) | Chl-a (µg g$^{-1}$ FW) | Chl-b (µg g$^{-1}$ FW) | Chl a/b |
|---|---|---|---|---|---|---|
| *Quercus coccifera* | 1000 | 47.8 | 52 ± 5.7 | 394 ± 25 | 131 ± 22 | 3.0 |
| | 1500 | 35.0 | 65 ± 6.8 | 758 ± 128 | 249 ± 39 | 3.0 |
| *Styrax officinalis* | 1000 | 64.0 | 36 ± 2.9 | 521 ± 37 | 160 ± 9 | 3.2 |
| | 1500 | 52.9 | 47 ± 4.4 | 670 ± 71 | 232 ± 43 | 2.9 |
| *Pistacia terebinthus* | 400 | 51.1 | 49 ± 5.0 | 357 ± 44 | 128 ± 12 | 2.8 |
| | 1500 | 36.8 | 63 ± 7.3 | 719 ± 76 | 223 ± 34 | 3.2 |
| *Rosa canina* | 1000 | 64.1 | 36 ± 3.9 | 381 ± 27 | 119 ± 7 | 3.2 |
| | 1500 | 63.6 | 36 ± 2.6 | 786 ± 119 | 223 ± 42 | 3.6 |
| *Juniperus excelsa* | 1000 | 41.6 | 58 ± 6.1 | 310 ± 42 | 107 ± 9 | 2.9 |
| | 1500 | 42.8 | 57 ± 4.7 | 231 ± 28 | 79 ± 13 | 2.9 |
| *Statistics* | | | *Species*** *Altitude*** *Sp X alt*** *N=5* | *Species*** *Altitude*** *Sp X alt*** *N=3* | *Species*** *Altitude*** *Sp X alt*** *N=3* | *Species*** *Sp X alt*** |

reductase and glutathione reductase [7]. The antioxidative defence mechanism protects the unsaturated membrane lipids, nucleic acids, enzymes and other cellular structures from the harmful effects of ROS [8, 9].

This study investigated the antioxidant defense capacity in leaf samples from different altitudes of five shrubs and trees species, to test the hypothesis that leaves at high altitudes have greater antioxidant defense capacity than these at lower altitudes. For this purpose five shrubs and trees species selected that grow in Mediterranean Region from 400 to 1500 m.a.s.(meters above sea level) were analysed chlorophyll content and four different elements of their antioxidative defence system.

## MATERIALS AND METHODS

### Plant Materials

For each of the five species studied, four angiosperms and one gymnosperm, we chose low and high elevation populations within the range from 400 to 1500 m.a.s. between Mersin and Anamur in the Middle Taurus Mountains, in southern Turkey as follows. Angiospermae [*Quercus coccifera* L. (l000 and 1500 m.a.s., *Fagaceae), Styrax officinalis* L. (1000 and 1500 m.a.s., *Styracaceae), Pistacia terebinthus* L. (400 and 1500 m.a.s., *Anacardiaceae), Rosa canina* L. (1000 and 1500 m.a.s., *Rosaceae)*], and a shrups species from Gymnospermae [*Juniperus excelsa* L. (1000 and 1500 m.a.s, *Cupressaceae)*]. The species determined by reference to Davis [10].

Climate in the area is generally mediterranean, with hot summers and mild winters (mean annual rainfall is 611.4 - 1032.3 mm and mean annual temperature is 10.4 -18.8°C). Tectonic pressure has given rise to rich karstic formations with deep caves and canyons. Soil types in the research area is terrarosa and brawn soil with sandy and clay textures. The soil pH is generally alkali 7.1-9.0 and contained 5-43 % lime [11, 12].

Leaf samples were collected from at least three different plants within each population at same elevations and carried in a portable freezer (-20°C). Sampling was carried out in June 2004 arround midday. The intact leaves of samples were carefully separated and each material was kept in nylon bags and stored at -70°C. Necrotic or chlorotic leaves were not used for analyses. Leaf water content and dry matter content were determined as the difference between fresh weight and weight after drying at 110°C for 24 hours in an oven.

### Chlorophyll

Chlorophyll extraction from fresh leaf material was carried out with 80% acetone (buffered to pH 7.8 with phosphate buffer). The chlorophyll a, chlorophyll b and total chlorophyll measurements were carried out using a Cecil 5000 spectrophotometer. Chlorophyll contents were calculated according to Porra *et al.* [13] and chlorophyll a/b ratios were determined.

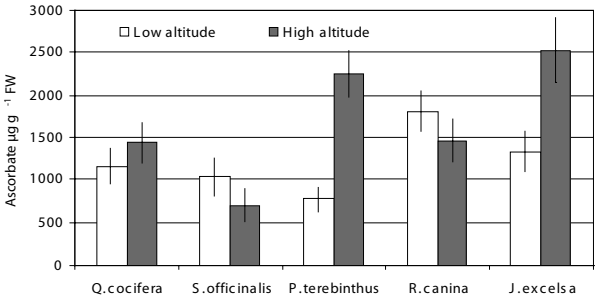


**Figure l.** Ascorbate contents in leaves of five shrubs species from different altitude. Statistics: altitude P = 0.000**, species P = 0.001**, altitude X species P = 0.004**, **significant at P < 0.01.

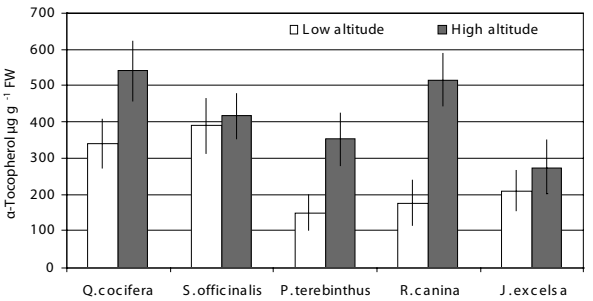


**Figure 2.** a-Tocopherol contents in leaves of five shrubs species from different altitude. Statistics: altitude P = 0.000**, species P = 0.000**, altitude X species P = 0.001**, **significant at P < 0.01.

### Total ascorbic acid (TAA)

Frozen leaf material (0.5 g) was homogenized with Ultra Turrax (Homogenizer, IKA T25S1) for 30 s in 6 mL of 0.1 M cooled sodium acetate buffer, pH=3. The homogenate was centrifuged for 5 min at 4°C and 7000xg. The supernatant was filtered through a cellulose nitrate filter (Sartorius pore size 0.45 µm) and stored at -70°C. Total ascorbic acid was determined through a reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). Reaction mixture was incubated with 50 mM DTT at 25°C for 20 min and on ice for 40 min. For the chromatographic separation a Cecil 1200 HPLC system controlled by a personal computer and Data Control software was used. TAA was separated on a RP C18 column (250 X 4.6 mm) using a solvent of 0.1 M sodium acetate buffer, pH=5 with an isocratic flow of 1.2 mL $min^{-1}$. The elutes were monitored by an UV detector at 264 nm [14].

### α-*Tocopherol*

Frozen leaf material (0.5 g) was homogenized at 4°C for 30 s in 8 mL ethanol containing 0.1 g insoluble polyvinyl polypyrolidone (PVP) and 0.2 g $Na_2SO_4$. The homogenate was centrifuged for 5 min at 4°C and 5000Xg. The supernatant was filtered through a cellulose acetate filter, and stored at -70°C [14]. α-Tocopherol was separated at room temperature on a RP-C18 column (250 X 4.6 mm) using solvents A [95% methanol + 5% water (v/v)] and B [95% methanol + 5% ethyl acetate (v/v)] with a flow rate of l .2 mL $min^{-1}$. The gradient

elution started at 100% A and 0% B, changed to 90% A and 10% B within l min and finished with 100% A and 0% B in 15 min. Elutes were monitored by an UV detector at 292 nm. For this chromatographic separation a Cecil 1200-HPLC system, controlled by a personal computer including Cecil Data Control software was used. Amounts of α-tocopherol were calculated from a standard curve prepared with tocopherol acetate.

**Enzyme extraction**

Frozen leaf material (0.5 g) was homogenized in 6 mL 0.1 M potassium phosphate extraction buffer (pH 7, containing 100 mg insoluble PVP and 0.1 mM EDTA) with Ultra Turrax. The homogenate was centrifuged for 5 min at 6000Xg and 4°C. The supernatant was filtered through a Whatman GF/A glass fiber disc with a vacuum filtration system and stored at -70°C [15].

**Superoxide dismutase (SOD EC l. 15. l. l)**

SOD activity was determined according to Beyer and Fridovich [16]. The reaction mixture (3 mL) contained potassium phosphate buffer (pH 8, 0.025% Triton X-100 and 0.1 mM EDTA), enzyme extract, 12 mM L-methionine, 75 µM nitroblue tetrazolium chloride (NBT) and 2 µM riboflavin. The reaction mixture was kept under flourescent light for ten minutes at 25°C. One SOD unit was described as the amount of enzyme where the NBT reduction ratio was 50%. NBT reduction ratios were measured with a spectrophotometer adjusted to 550 nm. Activity of Mn SOD was measured after addition of potassium cyanide (2mM) to the assay solution. Activity of Cu/Zn SOD and Fe SOD activity was calculated by subtracting Mn SOD activity from the total SOD activity. Fe SOD activity was not seperately determined.

**Glutathione reductase (GR, EC l. 6. 4. 2)**

GR activity was assayed at 25 ºC in a 3 mL reaction volume containing 1.5 mL potassium phosphate buffer (0.1 M, pH=7), 150 µL GSSG (20 mM), 200 µL enzyme extract, l mL bidistilled water and 150 µL $NADPH_2$ (2 mM, dissolved in Tris-HCl buffer, pH=7). GR activity was measured according to Carlberg and Mannervik [17] by following the oxidation of $NADPH_2$ spectrophotometrically at 340 nm.

**Statistics**

Individual samples were analysed at least triplicate. Data were indicated as arithmetic means and standart deviations (± SD) in table and figures. The significance levels of differences between plant samples from high and low altitude, species and species - altitude interactions were analysed by ANOVA. Results of statistics analyses were shown with P values and significance levels in figure legends.

## RESULTS

The water content of the five shrubs and trees species significantly different measured related to altitude. In three angiosperm species that grown higher altitude were measured low water contents and high dry weight percentage. In *R. canina* and *J. excelsa* that grown at different altitudes, no change of water contents and dry weight percentages were determined (Table 1).

Chlorophyll a and b contents were greater at high altitude in all species except *J. excelsa.* Increase of total chlorophyll contents was calculated in *R. canina* 102%, *P. terebinthus* 94%, *Q. coccifera* 91% and *S. officinalis* 32%. Although different chlorophyll a/b ratio among investigated species, these differentiation were not related to altitude (Table 1).

Ascorbate concentrations were high found in *Q. coccifera, P. terebinthus* and *J. Excelsa* from higher altitudes, in *S. officinalis* and *R. canina* from lower altitudes *J. excelsa* showed the highest concentration of ascorbate and the *P. terebinthus* has second highest ascorbate concentration (Figure 1). In all shrubs and trees species subjected to present study, α-tocopherol contents increased in samples from higher altitude. These increases reached to 300% in *R. canina* while 10% in *S. officinalis* (Figure 2).

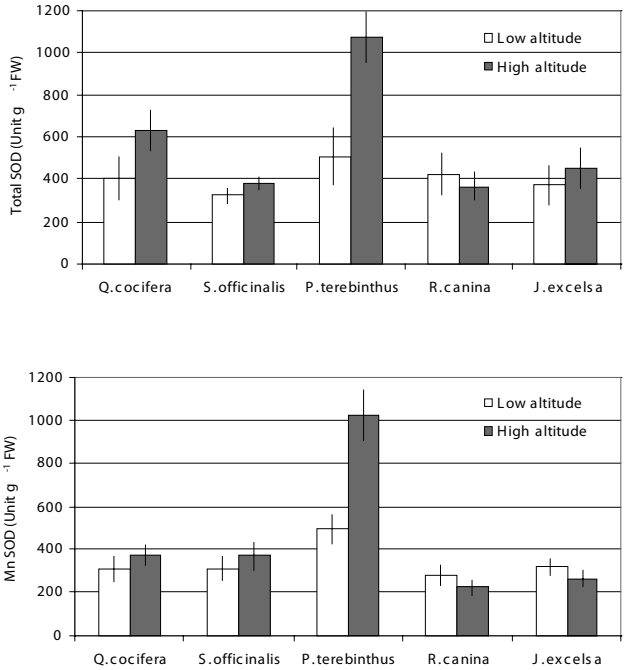


**Figure 3.** Total Superoxide dismutase (including Cu/Zn SOD, FeSOD and Mn SOD) and Mn SOD activities in leaves of five shrubs species from different altitude. Statistics for total SOD: altitude P=0.005**, species P=0.014*, altitude X species P=0.001**. Statistics for MnSOD: altitude P=0.005**, species P=0.023*, altitude X species P=0.044*, **significant at P < 0.01 and * significant at P < 0.05.

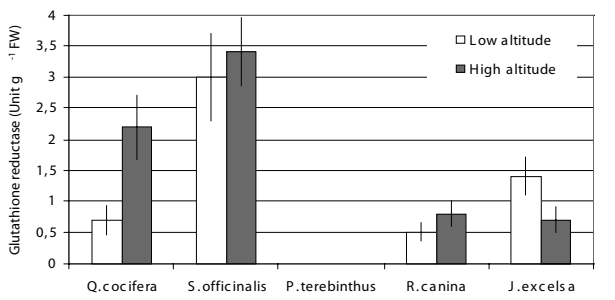


**Figure 4.** Glutathione reductase (GR) activities in leaves of five shrubs species from different altitude. Statistics: altitude P = 0.000**, species P = 0.000**, altitude X species P = 0.000**, * significant at P < 0.01.

SOD enzyme activities increased with altitude in four species but did not found increase in *R. canina.* In leaf samples of *P. terebinthus* from 1500 m was observed the high SOD activity. These high SOD activities resulted from increasing the Mn SOD activity rather than Cu/Zn SOD activity. Cu/Zn SOD activity was found significantly high in only *J. excelsa* species. Mn SOD activity was lower measured in leaf samples from higher altitude of *J. excelsa* and *R. canina* (Figure 3).

GR activities increased with altitude in *Q. coccifera, S. officinalis* and *R. canina,* while decreased in *J. excelsa.* In *P. terebinthus,* GR activity could not determined in samples both from 1000 m and 1400 m. GR activities of *S. officinalis* samples were found higher than other species (Figure 4).

## DISCUSSION

The high mountain vegetation consists of plants which adapt to low temperature and high irradiance conditions. These adaptations are accompanied by rapid metabolic changes and biochemical rearrangements which include antioxidant compounds and antioxidant enzymes. Although antioxidant defense is very important under high mountain conditions, some alpine plants possess other morphological and physiological adaptations that allow their survival at high altitudes even with low antioxidant capacity [3, 18]. In high mountain conditions, plant growth could be inhibited because of factors such as high light intensity, low temperature, severe wind, and nutrient-water deficiency and genetic limitations. Several biochemical and physiological processes are essential for strong light acclimation [19, 20, 21]. Cui *et al.* [22] suggested that the high radiation on the Tibet Plateau is likely to induce rapidly reversible photoinhibition which is related closely to plant architecture.

Chlorophyll concentrations when expressed on a leaf area basis decreased with increasing altitude in mediterranean species [23] however, in present study chlorophyll concentrations as proportion of dry mass significantly increased with increasing altitude. Investigations on several species show that increased chlorophyll content parallels an increase in carotenoid content [3]. However, in *J. excelsa,* the single gymnosperm species, low chlorophyll a and b values at high altitude were found. This finding was similar to that of Polle *et al.* [1, 24] who also found decreased chlorophyll content in spruce trees at high altitudes.

At high altitudes, increased α-tocopherol contents indicate that the protection of the cell membranes against oxidative damage can be necessery for growth at high altitude. Hydrophobic α-tocopherol reduces free radicals such as lipid hydroperoxyl radicals which arise in biological membranes. In the process, tocopherol is oxidised to the relatively stable tocopheroxyl radical. The tocopheroxyl radical is reduced back to tocopherol by ascorbic acid [25]. The role of α-tocopherol in protecting cell membranes is closely related to ascorbate concentration and its balance between oxidation-reduction reaction [1].

There are significant differences among the plants with regards to SOD activity [3]. SOD activity is higher in plants from high altitude due to defense against the free radical formation and photoinhibition triggered by the low temperature stress [20]. Polle *et al.* [24] proved that SOD activity does not depend upon the altitude in the needles of *Picea abies.* Under temperature-water stress combinations, SOD activity maintained by increased synthesis of the chloroplastic MnSOD while under high temperature-salt stress combinations increased synthesis cytosolic Cu/Zn SOD in wheat seedlings [26]. egulation of SOD activity also appears to be sensitive to environmental stresses presumably as a consequences of increased oxygen radical formation [7].

Glutathione reductase (GR) has a key role in protecting of chloroplasts against oxidative damage and could be accepted as complementary to SOD activity for removing the $H_2O_2$, product of SOD activity. Maintaining low concentrations of $H_2O_2$ is important in plant cells because $H_2O_2$ at low concentration inhibits chloroplasts SH-enzymes [27]. GR activity increases at some environmental stress condition such as drought. Keleş and Öncel [28]. showed that GR activity significantly increased only under severe stress conditions such as drought-high temperature or salinity-high temperature combinations. In *Picea abies* genotypes from high altitude, GR activity decreased, possibly because of stress sensitivity [1].

This study shows that there is a relationship between the altitude and the levels of antioxidant defense components in Mediterranean shrubs and trees. However, the adaptations to altitude in species of angiosperm and gymnosperm may relay on different physiological and morphological mechanisms [1, 3, 21, 24]. Moreover, biochemical mechanisms that include in antioxidant defense components have an effective function in the adaptation of plant to oxidative stress conditions of high altitudes. Further work is necessary to evaluate the significance of these mechanisms in many numerous angiosperm and gymnosperm species.

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