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Seasonal dimorphism and winter chilling stress in *Thymus sibthorpii*

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Abstract

Seasonal dimorphism (summer/winter) has been so far studied only in a few plants and has been focused on summer drought stress. However, *Thymus sibthorpii* in the study area appears to be affected by winter chilling stress and not by summer drought stress. Thus, the winter leaves were thicker and more compact compared to the summer leaves and they had more stomata and peltate hairs, more sclerenchymatous fibers, vacuoles with phenolics, and chloroplasts than the summer leaves. In addition, their chloroplasts possessed large grana and starch grains. In the summer leaves, cell vacuoles in mesophyll did not contain phenolics, and chloroplasts were devoid of starch grains and had large plastoglobuli. Physiological measurements revealed higher net photosynthetic rate and chlorophyll content in the winter leaves than in the summer leaves. Proline and soluble sugar content along with antioxidative enzyme (superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase) activities were increased in the winter leaves.

Additional key words: antioxidative enzymes, chlorophyll, chloroplast, net photosynthetic rate, proline, sugars.

Introduction

The genus *Thymus* comprises approximately 350 species of perennial herbs and sub-shrubs. The species *T. sibthorpii* belongs to section *Serpyllum* and it is a seasonally dimorphic plant. Seasonal dimorphism is considered as an advanced strategy reflecting pronounced metamorphosis of plants in order to withstand successfully the stressful climatic conditions of winter or summer. Seasonally dimorphic plants, studied so far, have been reported to be adapted to heat/drought stress during the summer period and not to cold/chilling stress during the winter period. In these plants, the summer leaves (in comparison with the winter leaves) exhibit a smaller blade surface, as in *Euphorbia* (Margaris and Papadogianni 1975), *Phlomis* (Kyparissis and Manetas 1993), and *Cistus* (Gratani and Bombelli 2000), a thicker blade, as in *Ballota (*Christodoulakis and Bazos 1990), more epidermal glycoproteins, as in *Sarcopoterium* (Christodulakis *et al.* 1990), more mesophyll phenolics, as in *Phlomis* (Christodoulakis 1989), more sclerenchymatous fibers, as in *Ballota* (Christodoulakis and Bazos 1990) and *Euphorbia* (Diamantopoulos and Margaris 1981), and more non-glandular hairs and stomata, as in *Cistus* (Aronne and De Micco 2001) and *Phlomis* (Christodoulakis and Fasseas 1991). Physiologically, dimorphic plants have been found to be more efficient in photosynthesis during summer (Letchamo *et al.* 1995).

The aim of the present study on seasonal dimorphism of *Thymus sibthorpii* was to elucidate whether this plant exhibits the above-mentioned different structural and functional features in summer and winter leaves according to a geographic region where it grows. Further, results can reflect an adaptation to winter chilling stress, which has been inadequately studied so far.

Materials and methods

Plants: Native plants of *Thymus sibthorpii* Bentham (*Labiatae*) were studied in the region of Ormylia, Chalkidiki, Greece (40° 16' 53" N, 23° 31' 43" E altitude 51 m a.s.l.). In this region, the meteorological

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Abbreviations: APX - ascorbate peroxidase; Chl - chlorophyll; E - transpiration rate; GR - glutathione reductase; g_s - stomatal conductance; LM - light microscopy; PN - net photosynthetic rate; POD - peroxidase; SEM - scanning electron microscopy; SOD - superoxide dismutase; TEM - transmission electron microscopy.
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data in the years of study (2009 - 2011) show that during three winter months, average air temperature was 7.3 °C, relative humidity 78.0 %, and rainfall 1.9 mm. During three summer months, the climatic conditions were mild with average air temperature of 24.3 °C, average relative humidity of 63.3 %, and rainfall of 1.0 mm. Meteorological data were provided by the Regional Center for Plant Protection and Quality Control, Thermi, Thessaloniki, Greece. Winter sampling was performed in January and summer sampling in July. Fully-expanded leaves of annual shoots were used (3rd node from the shoot basis).

Twelve developed leaves obtained from different plants were used for measurements of the blade area separately for the winter and summer plants in each of the 3 years of the study (a total of 36 measurements in winter and 36 in summer). The leaf blade area was measured with an MK_2 area meter (*Delta-T Devices*, Cambridge, UK) connected to a *TC 7000* camera (*Burle Industries*, Lancaster, PA, USA).

Microscopy: From a sample of 18 leaves (3 leaves × 6 plants), 5 leaves were randomly selected for light microscopy (LM) and transmission electron microscopy (TEM), and another 5 leaves for scanning electron microscopy (SEM). Leaves for LM and TEM were cut into small pieces which were subsequently fixed with 5 % (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 3 h. After washing in the buffer, the specimens were post-fixed with 2 % (m/v) osmium tetroxide for 4 h. The temperature in all solutions was kept at 0 °C to avoid leaching phenols during fixation. Samples were then dehydrated in an ethyl alcohol series (50 - 100 %) and finally embedded in Spurr's resin. Semithin sections (1 μm thick) for LM were obtained with a *Reichert OM U_2* microtome (*Reichert Optische Werke AG*, Vienna, Austria), stained with toluidine blue O and photographed on a *Nikon Eclipse i80* microscope (*Nikon Instruments*, Amstelvee, The Netherlands). Ultrathin sections (100 nm thick) for TEM were cut using a *Reichert-Jung Ultracut E* ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a *Jeol JEM 1011* transmission electron microscope (*Jeol*, Tokyo, Japan). For SEM, the specimens, after fixation and dehydration, were critical-point dried in a *Balzers CPD 030* device (*Balzers Union AG*, Liechtenstein) and then carbon-coated in a *Jeol JEE-4X* vacuum evaporator. Observations were made with a *Jeol JSM 840-A* scanning electron microscope.

For the morphometric assessment of the relative volume of the leaf histological components, a square lattice of point arrays, 10 mm apart, was projected over 36 LM micrographs of leaf cross-sections (Steer 1981). The same technique was applied to 36 TEM micrographs to assess the volume fractions of grana, starch grains, and plastoglobuli per chloroplast. The densities of stomata, and glandular and non-glandular hairs on both leaf surfaces were determined using 36 SEM micrographs.

Gas exchange and chlorophyll content: Measurements of photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO_2 concentration were performed using a portable gas exchange system (*LC pro+*, *ADC Bioscientific*, Herts, UK). Pieces of shoots bearing 3 nodes with fully developed leaves which were randomly selected from different plants were used. Three measurements in winter (4 repetitions each time) and another three in summer (also 4 repetitions) were conducted every year (2009 - 2011). For determination of chlorophyll, proline, and soluble sugar content, a total of 60 developed leaves (5 leaves per plant × 12 plants) were used each year separately for winter and summer. The same number of leaves was used for assessment of antioxidant enzymes.

Leaf chlorophyll content was determined after extraction of leaves (0.1 g) with 96 % (v/v) ethanol in a water bath (78 °C) for 30 min and then absorptions at 649 and 665 nm were measured using a *LKB Ultraspec II* spectrophotometer (*LKB Vertriebs*, Vienna, Austria). Total chlorophyll content (Chl *a*+*b*) was assessed using the equations of Wintermans and Mots (1965).

Proline and soluble sugars: Fully developed leaves (0.1 g) were cut into small pieces, placed in a glass vial containing 10 cm³ of 80 % (v/v) ethanol, and heated at 60 °C for 30 min. The extract was then filtered and diluted with 80 % (v/v) ethanol up to a volume of 20 cm³ (Khan *et al.* 2000). The leaf content of free proline and soluble sugars was determined in this extract following the acid ninhydrin method and the anthrone method, respectively (Plummer 1987, Khan *et al*. 2000). For proline, 2 cm³ of the alcoholic extract was transferred into a test tube and 2 cm³ of acid ninhydrin was added. With glass marbles on top to minimize evaporation, the test tube was maintained at 95 °C for 60 min in a water bath and then allowed to cool at room temperature. Toluene (4 cm^3) was added and thoroughly mixed. After separation of the solution layers, the toluene layer was carefully removed, placed in a glass cuvette, and absorption was measured at 518 nm. For soluble sugars, the ethanolic extract, as used for the proline assay, was diluted 10 times with 80 % (v/v) ethanol. The diluted extract was added drop-by-drop in 2 cm³ of anthrone reagent in a test tube in an ice bath and left to mix the content. The fully mixed sample was incubated in a water bath at 90 °C for 15 min and cooled. Absorbance was read at 625 nm. For proline and sugar measurements, the *LKB Ultraspec II* spectrophotometer was used.

Antioxidant enzymes: Fully developed leaves were homogenized by a pestle and mortar and extracted with a cold buffer (1:4 m/v). For superoxide dismutase (SOD), peroxidase (POD), and glutathione reductase (GR), the buffer consisted of 50 mM K_2PO_4 (pH 7.5), 1 mM EDTA, 2 % (m/v) polyvinylpyrolidone (PVPP), 0.05 % (v/v) *Triton X-100*, and 1 mM phenylmethylsulphonyl fluoride (PMSF). For ascorbate peroxidase (APX), the

buffer additionally contained 5 mM ascorbic acid. The homogenates were centrifuged at 20 000 *g* to remove cell remnants and then used for antioxidant enzyme activity determinations using the above mentioned spectrophotometer. SOD was determined at 560 nm according to Beauchamp and Fridovich (1971), POD at 590 nm according to Ngo and Lenhoff (1980), APX at 290 nm according to Nakano and Asada (1987), and GR at 340 nm according to Halliwell and Foyer (1978).

Statistics: Statistical analysis was performed with the *SPSS* package (*SPSS Inc*, Chicago, USA) using *ANOVA* for comparison of means between treatments. Significance of differences was determined at $\alpha = 0.05$.

Results

During winter, *T. sibthorpii* consisted of short shoots (about 9 cm in length) which were densely arranged to create a cluster (Fig. 1*A*,*C*). The shoots bore small leaves (average length 6.9 mm, average width 1.6 mm, average area per leaf side 12.0 mm^2) with dark green appearance. By the summer period, *T. sibthorpii* had already shed its spring inflorescences and consisted only of leaf-bearing shoots with length of about 15 cm (Fig. 1*B*,*D*). These shoots arose from the short winter shoots through growth and elongation. At each shoot node, two large opposite summer leaves existed (decussate phyllotaxis) along with several small winter leaves. The summer leaves had average length of 16.6 mm, average width of 3.1 mm, and average area per leaf side of 33.5 mm^2. Their coloration was not dark green as in the winter leaves, but bright-green to yellowish. In November, leaves became dry and the same happened later to shoot axes. At the beginning of winter, the buds at the bottom of the plant broke dormancy and started elongating generating many short shoots arranged in a cluster (the winter form of the plant).

Scanning electron microscopy of the winter and summer leaves of *T. sibthorpii* show that in the winter leaves, stomata were more numerous and they were of the diacytic type (Fig. 2*C*, Table 1). Among stomata, many essential oil-secreting peltate hairs existed, the density of

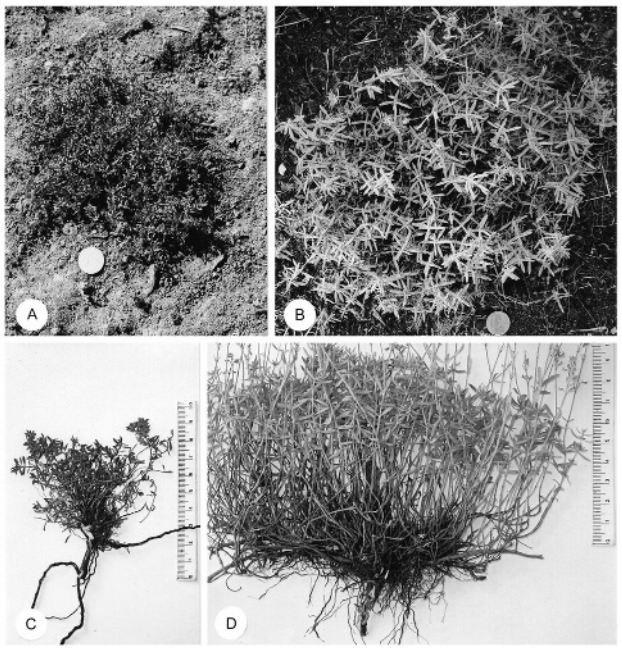

Fig. 1. *Thymus sibthorpii*. Surface view of a winter plant (*A*) and a summer plant (*B*) in the field. A coin has 2.4 cm diameter. Lateral view of herbarium winter (*C*) and summer (*D*) plants. Compare the size and density of shoots and leaves.

Table 1. Morphometric assessments of *Thymus sibthorpii* winter and summer leaves. Means ± SD, $n = 36$). Means with different letters within the row are significantly different at $P \leq 0.05$. There are no non-glandular hairs on the lower leaf side of the winter leaves and no starch grains in chloroplasts of the summer leaves.

Characteristics	Winter leaves	Summer leaves
Leaf lamina surface (per leaf side) [mm^2]	12.0 ± 3.4 a	33.5 ± 4.1 b
Leaf thickness [μm]	322.7 ± 39.3 a	218.2 ± 25.1 b
Density of stomata on upper side [mm^{-2}]	226.3 ± 24.9 a	168.5 ± 20.8 b
Density of stomata on lower side [mm^{-2}]	366.8 ± 46.5 a	170.2 ± 27.6 b
Density of peltate glandular hairs on upper side [mm^{-2}]	20.7 ± 2.9 a	12.8 ± 2.1 b
Density of peltate glandular hairs on lower side [mm^{-2}]	8.8 ± 0.3 a	7.7 ± 0.4 b
Density of conical non-glandular hairs on upper side [mm^{-2}]	149.6 ± 17.6 a	164.5 ± 19.3 b
Density of conical non-glandular hairs on lower side [mm^{-2}]	-	62.6 ± 5.3
Number of chloroplasts per palisade cell section	12.5 ± 2.4 a	6.2 ± 1.7 b
Number of chloroplasts per spongy cell section	7.3 ± 1.6 a	3.8 ± 0.8 b
Relative volume of grana per chloroplast [%]	44.7 ± 5.9 a	16.7 ± 3.0 b
Relative volume of starch grains per chloroplast [%]	27.3 ± 4.8	-
Relative volume of plastoglobuli per chloroplast [%]	12.8 ± 2.2 a	33.7 ± 6.1 b

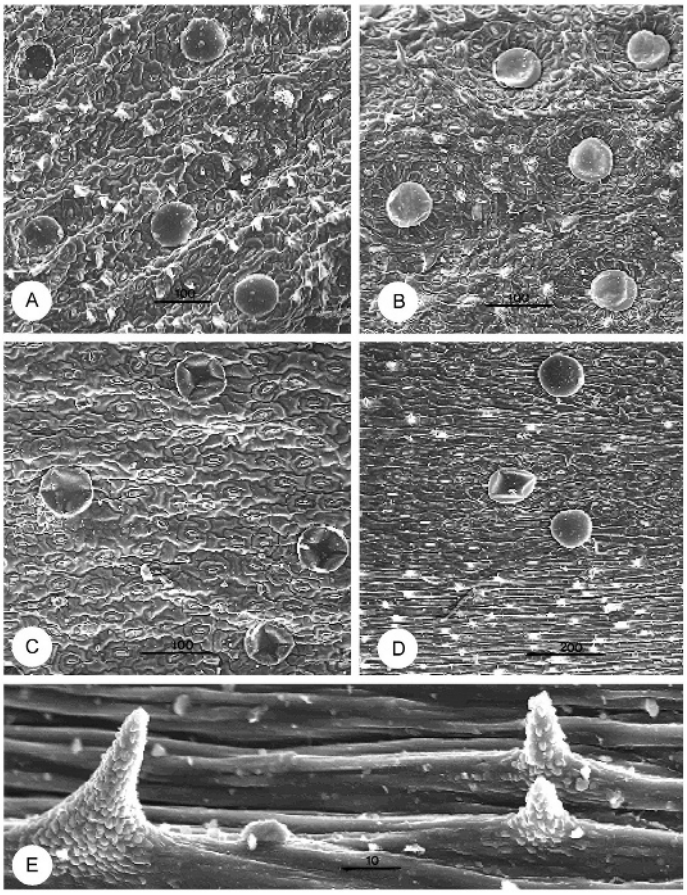

Fig. 2. SEM view of the upper side of a winter leaf (*A*) and a summer leaf (*B*) and the lower side of a winter leaf (*C*) and a summer leaf (*D*). Conical non-glandular hairs covered with granula found on winter leaves (*E*). *Bars* in μm.

which was higher in the winter leaves than in the summer leaves (Fig. 2*A*-*D*, Table 1). Glandular capitate hairs of any type were not observed in both the winter and summer leaves on both the leaf surfaces. Non-glandular hairs were short (approximately 40 μm in length), nearly conical in shape, and covered with granula (Fig. 2*E*).

The anatomical study revealed that the winter leaves were much thicker than the summer leaves with more palisade cell layers, and they had higher density of palisade cells. The winter leaves also contained more sclerenchymatous fibers, more chloroplasts, and more vacuolar phenolics in their epidermal and mesophyll cells (Fig. 3, Table 1). TEM of the winter leaves revealed that vacuoles in mesophyll cells were filled with dark, fine-granular phenolics (Fig. 4*A*). Chloroplasts consisted of many grana with up to 20 thylakoids and in their stroma large starch grains occured (Fig. 4*A*, *B*). In the summer leaves, vacuoles of mesophyll cells did not contain phenolics, whereas chloroplasts were almost fully occupied by plastoglobuli with no apparent presence of starch grains (Fig. 4*C*). Grana were scanty and usually consisted of 2 - 6 thylakoids.

Comparison of the physiological measurements show that net photosynthetic rate (P_N) in the winter leaves was about twice as in the summer leaves (Fig. 5). Transpiration rate (E) and stomatal conductance (g_s) were higher in the summer leaves, specifically from morning to noon. Maximal values of all parameters (P_N, E, g_s, and intercellular CO_2 concentration) were at 12:00 in the winter leaves and at 10:00 in the summer leaves.

Biochemical analyses show higher content of proline and soluble sugars in the winter leaves than in the summer leaves (Table 2). Antioxidative enzymes included SOD, POD, APX, and GR, and their activities were higher in the winter leaves (Table 2).

Discussion

Contrary to the previous reports on seasonal dimorphism in which all studied plants undergo summer drought stress, *T. sibthorpii* in the study area was found to be well-adapted to cope with winter chilling stress. Under chilling stress, curling and reduction of the leaf blade surface may be associated with avoidance of exposure of

an expanded blade to low temperatures and not with protection from water loss. Furthermore, leaf non-

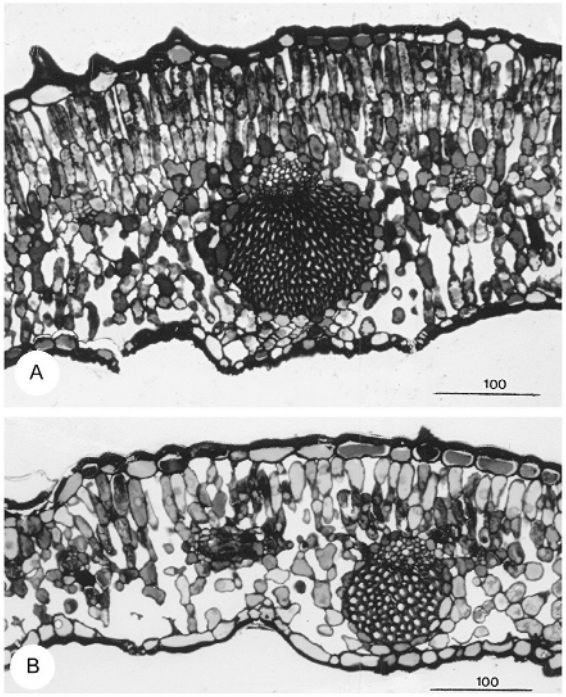

Fig. 3. Cross-sections of a winter leaf (*A*) and a summer leaf (*B*). The winter leaf is thicker (3 palisade cell layers) and contains more sclerenchymatous fibers, more chloroplasts and more phenolics in the epidermal and mesophyll cells. *Bars* in μm.

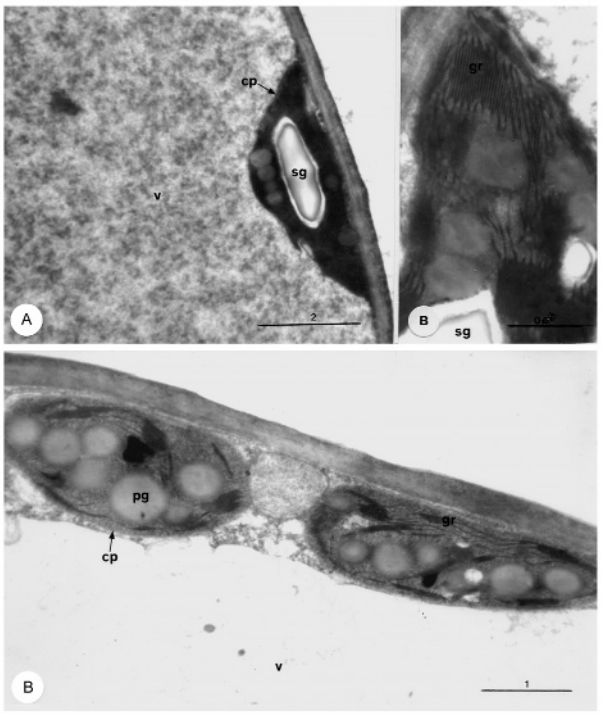

Fig. 4. Ultrastructure of mesophyll cells of winter leaves (*A*) and summer leaves (*B*). In the winter leaves, chloroplasts (cp) contain large starch grains (sg) and multi-thylakoid grana (gr). Vacuoles (v) are filled with dark fine-granular phenolics. In the summer leaves, chloroplasts are devoid of starch grains, contain many large plastoglobuli (pg), and also grana (gr) with a highly reduced number of thylakoids. Vacuoles (v) are free of phenolics. *Bars* in μm.

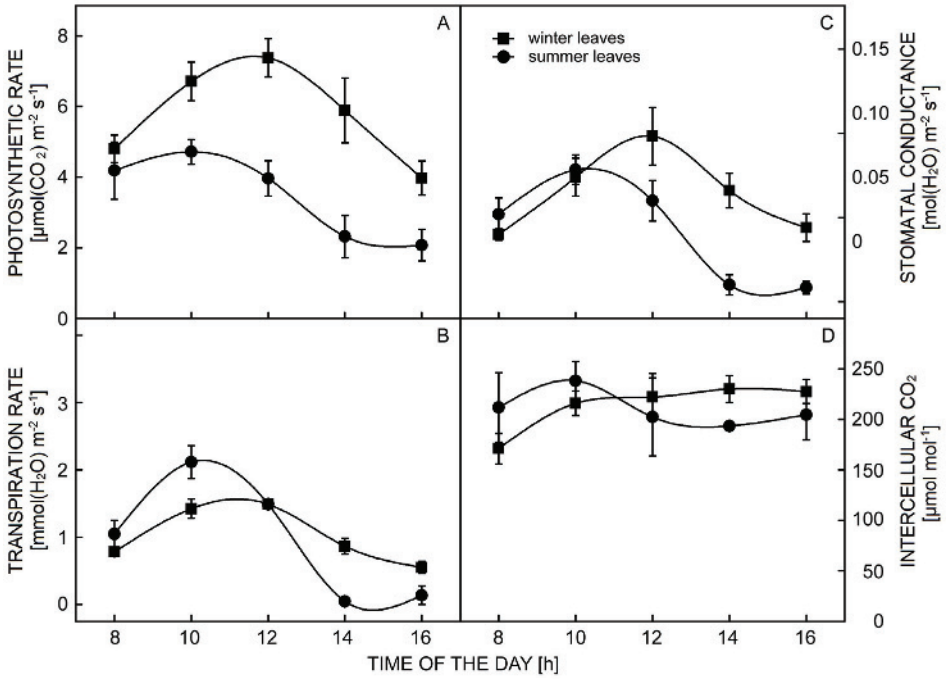

Fig. 5. Gas exchange parameters measured in expanded winter and summer leaves: *A* - photosynthetic rate, *B* - transpiration rate, *C* - stomatal conductance, and *D* - intercellular CO_2.concentration. Means ± SD, $n = 36$.

Table 2. Content of proline, soluble sugars, chlorophyll, and activities of antioxidant enzymes (SOD, POD, APX, GR) in winter and summer leaves. Means ± SD, $n = 9$. Means with different letters in the row are significantly different at $P \leq 0.05$.

Parameters	Winter leaves	Summer leaves
Proline [μmol g^{-1}(f.m.)]	1.4 ± 0.2 a	0.6 ± 0.1 b
Soluble sugars [μmol g^{-1}(f.m.)]	17.8 ± 2.4 a	12.4 ± 2.2 b
Chlorophyll *a*+*b* [mg g^{-1}(f.m.)]	1.2 ± 0.3 a	0.7 ± 0.1 b
Chlorophyll *a*+*b* [mg g^{-1}(d.m.)]	4.2 ± 0.7 a	1.9 ± 0.3 b
SOD [U mg^{-1}(protein]	2.9 ± 0.5 a	1.8 ± 0.3 b
POD [U mg^{-1}(protein]	26.8 ± 1.4 a	20.7 ± 1.2 b
APX [U mg^{-1}(protein]	0.73 ± 0.06 a	0.20 ± 0.02 b
GR [U mg^{-1}(protein]	0.25 ± 0.04 a	0.16 ± 0.03 b

glandular and glandular hairs along with thick cuticle and waxy coat may create an insulating epidermal layer which prevents penetration of the cold into the mesophyll (Satil *et al*. 2005, Marin *et al.* 2008). The extremely high accumulations of sclerenchymatous fibers within the mesophyll of *T. sibthorpii* winter leaf were highly unlikely to be related to the support of the leaf, since the winter leaf was very small (0.7 cm long). Thus, it could be argued that fibre aggregations might constitute "radiators" within the mesophyll which stored a significant amount of air having higher temperature compared to that surrounding the leaf. It might also be that sclerenchymatous fibers operate as optical fibers (Karabourniotis 1998, Gratani and Bombelli 1999) which transmit solar radiation into the mesophyll, thus increasing photosynthesis (the winter leaves had higher P_N compared to the summer leaves).

The presence of phenolics in the vacuoles of epidermal and mesophyll cells of the winter leaves could be related to protection from low temperatures and not from UV-B radiation, as reported for the summer leaves. Thus, low temperatures (0 - 10 °C) activate oxidative stress (release of reactive oxygen species, ROS) which may cause damage to the cells (Imahori *et al.* 2008, Mohammadian *et al*. 2011, Pongprasert *et al*. 2011). Cells respond by biosynthesizing phenolics which have antioxidant properties (Huang and Guo 2005, Crifo *et al*. 2011). Winter low temperatures do not seem to be the only stimulus for ROS production. Short winter photoperiod may also be a major factor (Queval *et al.* 2007, Lepistö and Rintamäki 2012, Michelet and Krieger-Liszkay 2012). Thus, temperature and photoperiod appear to be principal parameters that govern the mechanism of initiation of seasonal dimorphism.

Comparative measurements of gas exchange in the winter and the summer leaves of *T. sibthorpii* show that the former had higher P_N. This was closely correlated to the anatomical and ultrastructural observations in which the winter leaves appear to have more photosynthetic tissue (2 - 3 layers of palisade parenchyma cells) compared to the summer leaves (1 - 2 layers). Moreover, palisade cells in the winter leaves were more densely arranged and contained higher number of chloroplasts with bigger size and multi-thylakoid grana. The higher number of chlorenchymatous cells per mesophyll volume unit in the winter leaves reflected a larger total cell wall surface area facing the intercellular spaces (a larger surface area for CO_2 uptake) and, therefore, higher P_N. The increased P_N of the winter leaves resulted in the production of higher amounts of photosynthates which are largely invested in the biosynthesis of secondary metabolites needed for protection against the chilling stress. Such secondary metabolites were phenolics, essential oils, cutin, wax, cellulose, lignin, *etc*.

One of the major effects of low temperatures on plants is dehydration of cells by movement of intracellular water to intercellular spaces (Beck *et al.* 2007). The cells react by biosynthesis of proline, soluble sugars, and other osmolytes which are accumulated in the cell sap, thus adjusting osmotic potential (Patakas and Noitsakis 2001, Yadav 2010). Plants under stress, therefore, have higher content of proline and soluble sugars compared to unstressed plants (Hare and Cress 1997, Kumar *et al.* 2011). Measurements of proline and soluble sugar content in the winter and summer leaves of *T. sibthorpii* show significantly higher values in the winter leaves.

Stress conditions induce biosynthesis of ROS in chloroplasts, mitochondria, and peroxisomes which cause oxidative damage to the cells (Bafeel and Ibrahim 2008). Cells defend themselves by utilizing antioxidative enzymes, among which SOD, POD, APX, and GR are very important (Mittler *et al.* 2004, Zhang *et al.* 2010). Measurements of the activities of the above antioxidative enzymes in the winter and summer leaves of *T. sibthorpii* show that the values are remarkably higher in the winter leaves.

In conclusion, the presented results (morphological, anatomical, ultrastructural, morphometrical, physiological, and biochemical) provide evidence that *T. sibthorpii* in the study area underwent stress in winter (chilling stress) and not in summer (drought stress).

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