

LEAF MORPHOLOGY AND GAS EXCHANGE IN HOLM OAK (*Quercus ilex* L.) TREES IN DIFFERENT ENVIRONMENTS

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Abstract

Laakso K., Paoletti E., Huttunen S.: Leaf morphology and gas exchange in holm oak (*Quercus ilex* L.) trees in different environments. *Ekológia (Bratislava)*, Vol. 25, No. 3, p. 223–233, 2006.

Two holm oak (*Quercus ilex* L.) sites in Tuscany, Central Italy, were studied in July 2000. One of the sites (Cala Violina) was xeric with low ozone concentration (daily hourly mean for July–August 2000 13.3 nmol mol⁻¹). The other site (Colognole) was mesic with a higher ozone concentration (daily hourly mean for July–August 2000 54.2 nmol mol⁻¹). Leaf morphological characteristics (area, dry weight, specific dry weight, water content, epicuticular wax amount, stomatal density) and leaf fluorescence were measured. Ozone flux into the leaves was calculated on a stomatal conductance basis. Among the measured parameters, only stomatal density and wax amount significantly differed between the sites. Despite they are usually reported to increase with water stress, both were higher at the mesic and more polluted site than at the xeric one. This response might indicate an adaptation to air pollutants, of which ozone might be of the greatest importance – as also stressed by the higher ozone flux into the leaves at the mesic site – even if other not measured parameters, such as nitrogen deposition, may also have contributed to the result.

Key words: air pollution, drought, Mediterranean forests, O₃, tropospheric ozone

Introduction

Oak decline is considered a complex effect of diseases and environmental factors, with abiotic factors, such as water deficiency and pollution, serving as predisposing or inciting agents, and biotic factors causing the final decline (Manion, 1991). The forest area affected by oak decline in Italy accounts for an average 30% of the total oak forest cover (Ragazzi, 2000).

Holm oak (*Quercus ilex* L.) is a climax species in the forests of the Mediterranean basin (Barbero et al., 1992). It prevents severe summer water stress by reducing stomatal activity during the warmest hours, i.e. is a drought-avoidant and water-saving species (Manes et al., 1997). However, under moderate water constraint, holm oak maintains elevated stomatal conductance for a longer period than deciduous mesophile oaks (Nardini et al., 1999) and can become a water spending species where there is no shortage of water (Sala, Tenhunen, 1994). Water conditions are also known to influence the morphological traits of holm oak leaves (Sabaté et al., 1999; Bussotti et al., 2002) together with their position on the crown (Terradas, Savé, 1992) and microclimate (Gratani, 1996).

Drought is believed to be a predisposing factor for oak decline in the Mediterranean basin (Paoletti, 2000). Oak species can show morphological and anatomical features to improve their tolerance to drought, such as small leaf size, increased leaf blade and cuticle thickness, small stomata, high stomatal density, covering structures (hair, wax), deep rooting system, the presence of a tap-root and high root proliferation (Cellerino, Gennaro, 2000).

Tropospheric ozone (O_3) is considered the main pollutant causing decline in forest trees in S Italy (de Vries et al., 2000). Only a few experimental studies on the impact of O_3 on holm oak have been published in international journals. Manes et al. (1998), who studied the O_3 impact on holm oak saplings, suggested the xeromorphic adaptations of holm oak effectively protect it from O_3 injuries. Ozone concentration of $300 \text{ nmol mol}^{-1}$ or a cumulative exposure of $3.6 \text{ } \mu\text{mol mol}^{-1} \text{ h}$ may form a threshold above which distinct injuries occur (Manes et al., 1998). Saplings of *Quercus ilex* ssp. *ballota* seemed to be more sensitive to O_3 than ones of ssp. *ilex* (Inclan et al., 1999).

The aim of this paper was to compare leaf structural and functional characteristics of holm oak trees at two climatically different sites in C Italy (dry Cala Violina and mesic Colognole) and to determine the possible relation to ozone.

Material and methods

Site description

The study was carried out between the 3rd and 13th July 2000 in two ICP forests (International Co-operative Programme on Monitoring and Assessment of Air Pollution Effects on Forests) in W Tuscany (Italy), where holm oak is the main tree species. The forests are permanent monitoring plots in the MON.I.TO-program (MONitoraggio Intensivo foreste TOscane), which monitors the condition of forests in Tuscany. The two sites are called Cala Violina (CV) and Colognole (CL). CV is close to the sea and near the city of Grosseto. CL is more inland from the coastline and near the industrial city of Livorno. CV is very xeric, as it yearly experiences five months of soil water deficit (Bigi, Rustici, 1984). At CL a very superficial water-table guarantees water supply even in the driest period (Bussotti et al., 2002). The distance between the sites is about 80 km. The main site characteristics are in Table 1 (Bartolozzi et al., 1996; Bussotti et al., 2002). At the time of sampling, sporadic insect attacks on holm oaks were recorded at both sites, caused by the moth *Lymantria dispar* at CV and the aphid *Phylloxera quercus* at CL.

Table 1. Main characteristics of the two study sites, Colognole and Cala Violina, in Tuscany, Italy.

	Colognole (mesic site)	Cala Violina (xeric site)
Location	10 km inland from the coast line	A few hundred meters inland from the sea
Ozone measurement station	Gabbro (3.25 km south from the site)	Puntone (3.75 km north of the site)
Altitude (m a.s.l.)	250	5
Bedrock	clayey schists	sandstone
Precipitation (mm/year)	978	637
Temperature (°C)	15.1	14.7
% of holm oak out of all trees	39	69
Dominant age (years)	40–60	40–60
Crown transparency (%)	28.8	36.1

Pollution climate

At CL, the concentrations of SO₂ and NO_x have earlier been found to be fairly low with some occasional peak values, and O₃ has been considered the most detrimental air pollutant (Ferretti et al., 1993). The O₃ data for July–August 1999 and 2000 were recorded at the Puntone station 3.75 km north of CL and at the Gabbro station, which is 3.25 km south of CL. Ozone concentrations were higher at Gabbro than at Puntone in both the summers (Table 2). Despite air pollution may vary with meteorological conditions year by year, and measurements were recorded outside the forests, O₃ data from Gabbro and Puntone are the only ones available at present and in this study are considered representative for CL and CV, respectively.

Table 2. Hourly ozone concentrations (nmol mol⁻¹) at the measurement stations (Gabbro, close to Colognole; Puntone, close to Cala Violina) in the summers 1999 and 2000 (July and August): M24 – daily 24-h mean; M1 – mean of daily maximum values; M7 – mean value between 10:00 and 17:00; Max – absolute maximum value.

Year	Ozone	Gabbro	Puntone
1999	M24	50.5	15.1
	M1	65.3	34.4
	M7	55.8	24.9
	Max	98.7	50.0
2000	M24	54.2	13.3
	M1	70.2	25.3
	M7	62.1	19.6
	Max	92.1	35.6

Study procedure

Five holm oak trees were sampled at both sites. The trees were growing in open sunny places, were about 8 m tall and approximately 17 cm in diameter at chest height, and had sun branches on the south-eastern to south-western

side of the trunk which were possible to reach from ground level. All measurements were performed on mature sun leaves from spring sprouts, avoiding branchlets with June sprouts.

Crown transparency of each sampled tree was assessed by two people working together with the help of photographs provided by the European Union transparency guidebook (Ferretti, 1994).

Amount of epicuticular waxes, and leaf area and dry weight

Approximately 3 g of leaves (7 to 26 in number) were collected into plastic bags from each tree, to measure the amount of epicuticular waxes, and the leaf area and dry weight, and to calculate specific dry weight (ratio dry weight to area) and water content $((FW-DW) / FW * 100)$. Fresh weight was measured between 12:00 and 14:00 by using a portable balance (Sartorius Basic plus Bp110, Goettingen, Germany).

The leaves were kept in cold (+4 °C) until washing in 40 ml of chloroform in preweighed aluminium dishes for 15 s. The extract was filtered through a glass fibre pre-filter (Millipore, type AP20, diameter ~ 8 µm) in the Millipore filtering system, poured back into the dish, left to evaporate in a fume chamber for 24 h under a paper sheet that kept the dust out, and weighed. The leaves were dried in an oven at 70 °C until dry weight was achieved. The leaf area was measured by using a photo analyser (MicroScale TC/TM, Version 2.10, Digithurst Ltd, Herts, UK). The quantity of wax was calculated as per leaf area and dry weight based on the differences in the dish weights before and after the extraction.

Stomatal density

Five leaves were collected from each tree, avoiding leaves with insect damage, and air-dried. Two pieces (~0.5 cm²) were taken from the middle portion of each leaf, one from each side of the main vein. The trichomes were removed by using a scalpel, and the samples were sputtered with gold and palladium (80:20) and examined under a Field Emission Scanning Electron Microscope (FESEM, JEOL JSM-6300 F) (500x, 5 kV). Five micrographs were taken randomly from each leaf piece by avoiding veins, the total number of micrographs being 10 per leaf. The total area covered on one leaf was 0.34 mm². Number of stomata was counted in each micrograph (partly shown stomata were included).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured once at both sites at 9:00, 11:00, 13:00, 15:00, 17:00 and 19:00 to obtain information on daily variation. Measurements were also carried out on two further days at 13:00. Eight leaves were measured in each tree. The leaves were adapted to dark for 40 min, after which the initial fluorescence (F_0), the maximum fluorescence (F_m) and the ratio of variable to maximum fluorescence (F_v/F_m) were measured by using a fluorometer Fim 1500 (ADC Ltd, Hoddesdon, England) with a light intensity of 1500 µmol m⁻² s⁻¹.

Gas exchange

Measurements of net photosynthesis (A) were carried out every 15 min from 11:30 to 16:00 on three different days per site (one fully-sunny leaf per day) with an infra-red gas-analyzer (CIRAS-1 PP-Systems, Herts, UK) equipped with a Parkinson leaf chamber, set at environmental CO₂, PAR, and air temperature inside the cuvette. Stomatal conductance (g_s) and apparent internal CO₂ concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981) and related to one-sided leaf areas.

The potential O₃ flux (FO₃) into the leaves was calculated from the hourly mean values of g_s and O₃ (Wieser et al., 1998), according to the first Fick's law. Ozone concentration inside the leaf was assumed to be zero (Laisk et al., 1989). The conductance for O₃ was completely ascribed to the stomatal component, which was calculated multiplying g_s by 0.613, the ratio of the binary diffusivities of water vapour and O₃.

Statistical analysis

The data were tested by using the Mann-Whitney U-test (2-tailed) of SPSS for Windows 10.0. The results were given as mean values with standard deviation. The statistical unit was the individual tree – mean of three days – for morphology and fluorescence data, and the single record for A and g_s . Correlations were tested by using the Spearman's rank procedure (SPSS for Windows 10.0) on all independent data for the parameters that did not significantly differ by site, and on single-site data when significant site-related differences were recorded.

Results

The crown transparency of the samples trees did not differ significantly between the sites (21 vs 36% at CL and CV, respectively) and were not correlated with any of the other measured parameters. There were no significant differences in leaf dry weight, leaf area, specific dry weight, and leaf water content between the sites (Fig. 1).

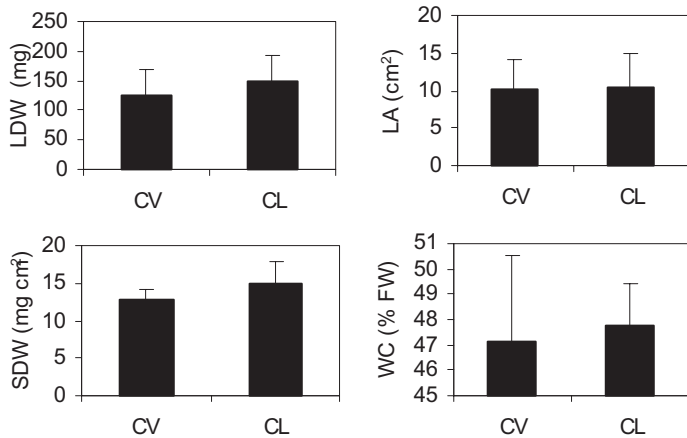


Fig. 1. Leaf dry weight (LDW, mg), area (LA, cm²), specific dry weight (SDW, ratio of leaf dry weight to area, mg cm⁻²), and water content (WC, % of fresh weight) in Cala Violina (CV) and Colognole (CL). Vertical bars show standard deviations. n = 5.

The chlorophyll fluorescence parameters did neither differ significantly between the sites as a mean of the midday F_v/F_m values nor as daily trend, except F_0 at 9:00 ($U = 1$, $p = 0.032$) and F_m at 17:00 ($U = 1$, $p = 0.038$) (Fig. 2). Midday F_v/F_m ratio was positively correlated to leaf water content ($p = 0.022$).

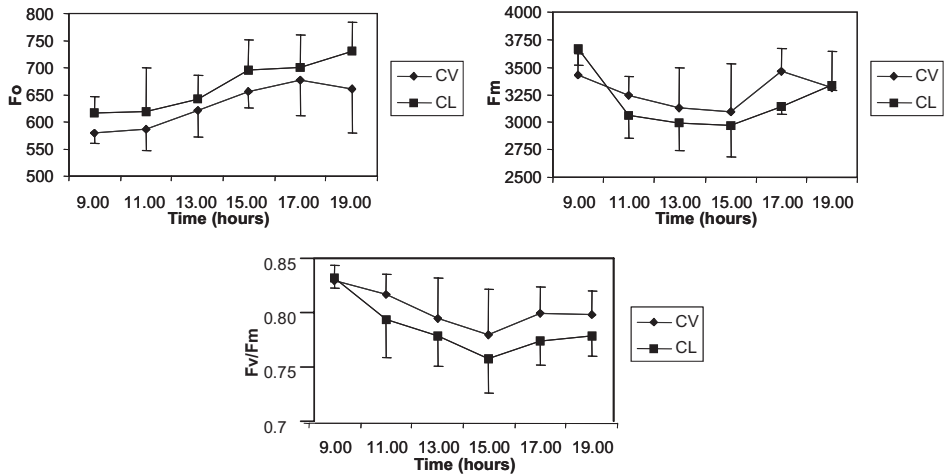


Fig. 2. Daily variation of initial chlorophyll fluorescence (F_0), maximum fluorescence (F_m) and the ratio of variable to maximum fluorescence (F_v/F_m) in Cala Violina (CV) and Colognole (CL). $n = 5$ in CV, except at 19:00 $n = 3$. $n = 4$ in CL, except at 12:00 $n = 5$. Vertical bars show standard deviations.

Stomatal conductance and net photosynthesis were about two and three times higher at CL, respectively ($U = 223$ and 97 , $p = 0.000$), so that ozone flux into leaves was also about three times higher ($U = 4$, $p = 0.0076$), despite mean hourly O_3 concentration during gas exchange measurements was only 31% higher at CL than at CV ($U = 0$, $p = 0.009$) (Table 3).

Table 3. Mean hourly values of stomatal conductance (g_s), net photosynthesis (A), ozone flux into leaves (FO_3) and related environmental parameters at the sites during three days of measurements between 11:30 and 16:00. Standard deviations are in parenthesis. Differences between the sites were always significant ($U = 0 \div 223$, $p < 0.01$).

Parameter	Colognole	Cala Violina
g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	125 (38)	56 (38)
A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	11.07 (3.59)	3.54 (1.27)
FO_3 ($\text{nmol m}^{-2} \text{s}^{-1}$)	4.14 (1.57)	1.50 (0.18)
O_3 (nmol mol^{-1})	55 (4.2)	42 (2.1)
PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	1285 (383)	820 (443)
Cuvette air temperature ($^{\circ}\text{C}$)	29.2 (2.1)	33.4 (1.5)

A linear regression on all A and g_s data (records every 15 min) gave highly significant results: $A = 0.0392 g_s + 5.8135$, $R^2 = 0.2236$, $p < 0.001$ at CL; $A = 0.0239 g_s + 2.311$, $R^2 = 0.3192$, $p < 0.001$ at CV.

The stomatal density was 12% higher at CL than at CV ($U = 0.000$, $p = 0.014$) (Fig. 3) and did not correlate with any other of the leaf morphology parameters. The holm oak trees at CL had 14% more epicuticular waxes on their leaves than the trees at CV when calculated per area ($U = 1$, $p = 0.016$), while the wax amount per dry weight did not differ between the sites (Fig. 3).

Discussion

The crown transparencies were similar to the means already assessed for both sites (30 holm oak trees per site, Table 1) and subjected to Quality Assurance procedures (Bartolozzi et al., 1996), thus confirming that the chosen trees were representative of the site.

Despite not significantly, leaf area and water content showed the same trend already reported by Bussotti et al. (2002) for sun leaves in the same sites, i.e. lower values at CV than at CL, that is consistent with the expected response to water availability (Sabaté et al., 1999). The significant results by Bussotti et al. (2002) may be due to the fact that the gathering was performed from the upper third portions of the crown, where stress symptoms are shown as first in holm oak.

No significant difference was also observed in the chlorophyll fluorescence parameters between the xeric CV and the mesic CL, despite a lower F_v/F_m ratio in holm oak has been related to lack of water (Méthy et al., 1996), as confirmed by the positive correlation between F_v/F_m and water content in our leaves. The lack of difference between the sites with such a different water supply is surprising, as F_v/F_m ratio is considered a sensitive stress parameter (Lichtenthaler et al., 1986). As the measurements were carried out at the hottest time of the year with the highest irradiation intensity, it is possible that the PS II was affected similarly in the sun-exposed leaves at both the sites independently of the water conditions. It is also possible that the similar response in F_v/F_m at the sites was caused by different site-depending factors. Ferretti et al. (1993) considered tropospheric O_3 the most detrimental air pollutant at CL, which is close to the industrialised city of Livorno. The mean hourly concentrations at CL, measured over July and August, were more than two fold of the ones at CV in 1999 and 2000. Chlorophyll fluorescence could have been limited by the lack of water at CV and by O_3 at CL, even if F_v/F_m in holm oak leaves are affected only by high peak doses of O_3 (Manes et al., 1998). Ozone has been found to cause a decrease in F_v/F_m for example in

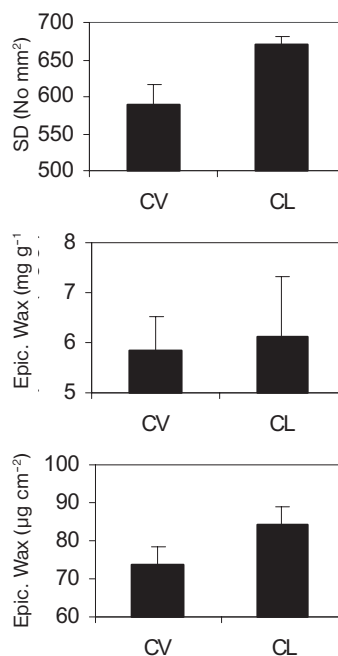


Fig. 3. Stomatal density (SD, number of stomata per leaf mm²), amount of epicuticular waxes per leaf dry weight (mg g⁻¹) and per leaf area (µg cm⁻²) in Cala Violina (CV) and Colognole (CL). $n = 5$ at both sites. Vertical bars show standard deviations.

poplar (Bortier et al., 2000) and *Fagus* species (Günthardt-Goerg et al., 1999). Kellomäki and Wang (1997) concluded that O_3 decreased photosynthesis in Scots pine by reducing Fv/Fm ratio rather than by decreasing stomatal conductance. Non stomatal limitations to carbon assimilation under O_3 have been already reported in deciduous broadleaved tree species (Paoletti et al., 2002). In our conditions, anyway, A and g_s were significantly coupled, indicating that stomatal control of CO₂ diffusion played an important role in controlling photosynthesis at both sites.

The differences in instantaneous gas exchange values between sites were likely to be caused by different local meteorological conditions, among which the higher radiation and mainly the lower air temperature at CL may account for the higher gas exchanging ability at this site. Leaf temperature allowing the highest photosynthetic and stomatal conductance rates of holm oak are in the range 17.5–29 °C (Gratani, 2000). Moreover, O_3 concentrations during measurements were 40–60 nmol mol⁻¹ at both sites and not likely to induce a significant photosynthetic decrease, while an increase is possible at these concentrations, as shown in holm oaks exposed to short-term O_3 fumigations (Manes et al., 1998). Many studies report a stimulus to stomata opening at O_3 concentrations below damage threshold (Darral, 1989). Whatever the cause, the elevated g_s entails an elevated flux of O_3 entering the leaf and amplifies the potential for injury due to the higher summer O_3 concentration at CL in 1999–2000, even if reassurance is needed that conductance values, measured on individual leaves, are representative for a canopy, and that O_3 measurements 3–4 km far from the sites are representative for values in forest.

The stomata were fewer in number in the xeric CV than in the mesic CL. This result contradicts those reported in the literature about drought stress being related to higher stomatal density (Ticha, 1982). The nature of the relationship between stomatal density and air pollutants is still to be defined. The greater stomatal density of an O_3 -sensitive *Prunus serotina* genotype may lead to greater stomatal conductances and greater O_3 uptake into leaf (Ferdinand et al., 2000). Accordingly, NO₂ or O_3 exposure reduced stomatal density in poplar leaves (Matyssek et al., 1993; Günthardt-Goerg et al., 1996). On the other hand, O_3 exposure increased stomatal density in birch (Pääkkönen et al., 1997) and olive trees (Minnocci et al., 1999). Trees would benefit from increased stomatal density, as it may reduce the gas exchange and air pollutant load per single stoma, resulting in a more even distribution of pollutants inside the leaf (Pääkkönen et al., 1997). As a greater number of stomata in holm oak leaves at CL compared to that at CV cannot be explained by the difference in the water availability between the sites, it might work as a longer-term adaptation to the higher O_3 concentration at CL.

There is now evidence that waxes are sensitive to environmental change and atmospheric pollutants (Holroyd et al., 2002) and that alterations in wax composition can affect stomatal development (Gray et al., 2000). The amount of epicuticular waxes differed from the usual drought-related pattern as well. Epicuticular waxes tend to be more abundant in leaves exposed to water stress (Holloway, 1982). The amount of epicuticular waxes was lower in holm oak leaves at the dry CV than at the mesic CL. Bussotti et al. (2002) found at the same sites that the upper cuticular layer of holm oak leaves was thicker at CV than at CL. However, they did not specify whether the difference was found in a particular layer of the

cuticle. Ozone may cause degradation of epicuticular waxes (Karnosky et al., 1999). No response of increased epicuticular wax to O₃ has been reported, even if most of the studies have been carried out on saplings in short-term experiments. Some field studies have shown that trees tend to have more epicuticular waxes in polluted environments (Percy et al., 1993; Sase et al., 1998). To some extent, long-living plants might be able to protect themselves against moderate concentrations of certain pollutants by thickening the epicuticular wax layer. The greater amount of epicuticular waxes in holm oak leaves at CL compared to CV could serve as a protective mechanism against the impact of the relatively high O₃ concentration prevailing at the site.

The higher stomatal density and the greater amount of epicuticular waxes observed in holm oaks at the more polluted and mesic site might indicate an adaptation to air pollutants, of which tropospheric O₃ appeared of the greatest importance at the sites, as also stressed by the calculation of ozone flux into the leaves. The great natural phenotypic plasticity of holm oak (Barbero et al., 1992) and other not measured environmental factors, such as atmospheric nitrogen deposition and soil moisture deficit, may also have contributed to the results.

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Laakso K., Paoletti E., Huttunen S.: **Morfológia listu a výmena plynov pri *Quercus ilex* L. v rôznom prostredí.**

V júli 2000 sme skúmali dve stanovišťa *Quercus ilex* L. v Toskánsku (stredné Taliansko). Stanovište (Cala Violina) bolo xerické s nízkou koncentráciou ozónu (denný hodinový stred v mesiacoch júl–august 2000 bol 13.3 nmol mol⁻¹). Druhé stanovište (Colognole) bolo mezické s vyššou koncentráciou ozónu (denný hodinový stred v mesiacoch júl–august 2000 bol 54.2 nmol mol⁻¹). Zmerali sme morfológické znaky listu (plochu, hmotnosť sušiny, špecifickú hmotnosť sušiny, obsah vody, množstvo epikutikulárneho vosku) a listovú fluorescenciu. Prenikanie ozónu do listov sa vypočítalo na základe vodivosti prieduchov. Z meraných parametrov sme zaznamenali významné rozdiely iba v stomatálnej hustote a množstve vosku. Napriek tomu, že obyčajne sa stresom vyvolaným vodou hodnoty zvyšujú, boli vyššie na mezickom a znečistenom stanovišti ako na xerickom. Tento fakt môže indikovať adaptáciu na látky znečisťujúce ovzdušie, z ktorých najdôležitejšou by mohol byť ozón – svedčí o tom tiež vyššie prenikanie ozónu do listov na mezickom stanovišti – aj keď ostatné namerané parametre, ako je depozícia dusíka, by mohli skreslovať výsledok.