Sulfate and Nitrate Assimilation in Leaves of *Quercus ilex* and *Quercus pubescens* Grown Near Natural CO_2 Springs in Central Italy

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Abstract The effect of long-term exposure to elevated pCO_2 concentrations on sulfate and nitrate assimilation was studied under field conditions using leaves from *Quercus ilex* and *Quercus pubescens* trees growing with ambient or elevated CO_2 concentrations in the vicinity of three natural CO_2 springs, Bossoleto, Laiatico and Sulfatara, in Tuscany, Italy. The activity of the key enzymes of sulfate assimilation, adenosine 5′-phosphosulfate reductase (APR) and nitrate assimilation, nitrate reductase (NR), were measured together with the levels of acid soluble thiols, and soluble non-proteinogenic nitrogen compounds. Whereas NR activity remained unaffected in *Q. ilex* or increased *Q. pubescence*, APR activity decreased in the area of CO_2 springs. The latter changes were often accompanied by increased GSH concentrations, apparently synthesized from H_2S and SO_2 present in the gas mixture emitted from the CO_2 springs. Thus, the diminished APR activity in leaves of *Q. ilex* and *Q. pubescence* from spring areas can best be explained by the exposure to gaseous sulfur compounds. Although the concentrations of H_2S and SO_2 in the gas mixture emitted from the vents at the CO_2 springs were low at the Bossoleto and Laiatico spring, these sulfur gases pose physiological effects, which may override consequences of elevated pCO_2.

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L.J. De Kok et al. (eds.), *Sulfur Metabolism in Plants*, Proceedings of the International Plant Sulfur Workshop 1, DOI 10.1007/978-94-007-4450-9_29,

The current increase in atmospheric pCO_2 may affect the physiology, the development and the growth of plants. Wealth of information concerning these effects has been published from experiments, in which plants were exposed to elevated pCO_2 under controlled conditions in different experimental set ups starting from controlled growth chambers, open top chambers and Free-Air CO_2 Enrichment (FACE) experiments. Within these experiments the pCO_2 concentration was enhanced from ambient to elevated pCO_2 levels within one step. This approach does not allow long-term acclimation to rising pCO_2 concentrations over several generations, especially relevant for plant communities and trees. Vegetation, that grows for generations in a naturally pCO_2-enriched atmosphere around pCO_2 springs, provide a unique opportunity to address questions of long-term adaptation. At these springs, pCO_2 of geological origin is continuously released to the atmosphere through natural vents, thus producing local increases in the atmospheric pCO_2 concentrations as expected in a future pCO_2-enriched world (Miglietta and Raschi 1993; Schulte et al. 1999). These conditions are ideal for assessing physiological responses (Körner and Miglietta 1994) and micro-evolutionary adaptations (Schulte et al. 2002) to long-term elevated pCO_2 that cannot be simulated experimentally.

At three selected spring sites, *i.e.* near Laiatico, Bossoleto and Solfatara, the pCO_2 enrichment was comparable to the pCO_2 concentrations expected at the end of this century (Miglietta et al. 1993; Körner and Miglietta 1994). However, the gas mixture emitted from these vents contains SO_2 and H_2S in addition to CO_2 (Table 1, Schulte et al. 1999), pollutants which have been shown to affect the physiology of plants (De Kok et al. 1998) and may even be phytotoxic (De Kok 1990). The average H_2S and SO_2 concentrations did not reach levels thought to mediate visible symptoms of injury (De Kok 1990; Schulte et al. 1999) and, indeed, symptoms of H_2S and SO_2 damage were not observed at the field sites. Nevertheless, it can be assumed that atmospheric sulfur is taken up by the leaves and is used as an additional sulfur source (Brunold and Erismann 1975; De Kok 1990). Exposure of the shoot to atmospheric H_2S or SO_2 normally enhances the GSH content of leaves and roots (De Kok 1990), but high levels of cysteine were also detected (Buwalda et al. 1988; De Kok et al. 1988). This increase in thiols in the presence of SO_2 and H_2S may be explained by a direct incorporation of the sulfur into cysteine and the usage of cysteine for the formation of GSH which have been shown to decrease the activity of ATP sulfurylase (Lappartient and Touraine 1996; Lappartient et al. 1999) and APS reductase (APR, Westerman et al. 2001; Vauclare et al. 2002).

In *A. thaliana* shoots, not only the content of water-soluble non protein-SH compounds, but also the organic N in the shoot increased significantly by SO_2 exposure (Van der Kooij et al. 1997), indicating an interaction between sulfur and nitrogen metabolism. Such an interaction has been established for a long time (reviewed in Brunold 1993; Brunold et al. 2003; Kopriva and Rennenberg 2004) and has also been described at the whole plant level (Kruse et al. 2007). An interaction between sulfur and carbon metabolism is evident from experiments with *Arabidopsis* and *Lemna*. Here APR activity depends on light, is increased by sucrose and glucose treatment and is strongly reduced when CO_2 is omitted (Kopriva et al. 1999, 2002; Hesse et al. 2003). Also nitrate reductase (NR), the key enzyme of nitrate assimilation,

Table 1 Summary of the mean CO_2, SO_2 and H_2S concentration measured at the three natural CO_2-springs (Schulte et al. 1999)

	Geographical coordinates	CO_2 (µl l^{-1})	H_2S (nl l^{-1})	SO_2 (nl l^{-1})	Time of emission
Bossoleto (Rapolano)	43°17′N; 11°35′E	1,074	22	12	Night
Laiatico	43°24′N; 10°50′E	795	22	4	Day
Solfatara	42°30′N; 12°08′E	797	245	18	Day and Night

interacts with carbon metabolism. Elevated pCO_2 reduced the decline of NR activity during the second part of the photoperiod and partially reversed the dark inactivation in herbaceous plants (Scheible et al. 1997; Geiger et al. 1998). The increased stability of NR at elevated CO_2 might be a result of the higher level of sugars at elevated pCO_2 (Kaiser and Huber 1994). In tobacco elevated pCO_2 increased the turn-over of amino N, most remarkable Glu and Ala, in mature leaves indicating enhanced nitrate reduction (Kruse et al. 2003).

Effects of elevated pCO_2 in oak depend on the origin of *Q. ilex* acorns (Schulte et al. 2002). Elevated pCO_2 decreased APR activity and thiol levels in *Q. ilex* leaves when acorns were collected from control area. However, although thiols were reduced at elevated pCO_2, when acorns were collected from the Laiatico spring area, APR activity was not (Schulte et al. 2002). NR increased at elevated pCO_2 when *Q. ilex* trees were grown from acorns originating from the spring area. As these results indicate long-term acclimation to elevated pCO_2, micro evolutionary adaptation of sulfur and nitrogen nutrition as a consequence to elevated pCO_2 in *Q. ilex* and *Q. pubescens* are expected. To test this assumption, three natural springs with elevated pCO_2 and corresponding control areas with ambient atmospheric pCO_2 were selected in Tuscany, Italy. The key enzymes of both pathways, *i.e.* APR and NR, were investigated in combination with analyses of reduced S and N compounds.

Mature leaves were randomly harvested between 10 am and 14 pm in June 1995 and 1996 from 2 years old twigs of six *Q. ilex* and *Q. pubescens* trees grown at three different natural CO_2-springs or at the corresponding control areas each. The Bossoleto (Rapolano) spring is situated at about 40 km SE of Siena at 250 m (Körner and Miglietta 1994) and released pCO_2 mainly in the night (Table 1, Schulte et al. 1999). Thereby the atmospheric pCO_2 ranged from 325 µl l^{-1} up to peak values of 8,097 µl l^{-1} in the night. The emitted gas mixture also contained trace amounts of H_2S (2–83 nl l^{-1} H_2S) and SO_2 (5–23 nl l^{-1} SO_2, Schulte et al. 1999). The spring is surrounded by a typical Mediterranean forest composed mainly of *Q. ilex* and *Q. pubescens*. The control site was at a distance of 5 km from the source at Poggia San Cecilia, where the vegetation, the climate and the soil were comparable to the Bossoleto spring (A. Raschi, personal communication). The Laiatico spring is situated on the slope of a hill near the village of Laiatico, which is at 10 km W from the city of Volterra. CO_2 is emitted from one major and a number of smaller vents at the foot of the slope mainly during the day. The atmospheric pCO_2 ranged from 209 up to 2,815 µl l^{-1} (Schulte et al. 1999). The gas mixture at the Laiatico spring was

comparable with that observed at the Bossoleto spring (Table 1). During the day the atmospheric H_2S concentration ranged from 2 to 65 nl l^{-1}, the atmospheric SO_2 concentration from 0.25 to 4 nl l^{-1}. The slope is covered with a dense, 20 year old forest consisting mainly of *Q. ilex* and *Q. pubescens* and a few *Fraxinus excelsior* trees. Leaves from trees at distances up to 200 m from the main hole of the vent were harvested for analyses. The Solfatara spring is situated close to the village Grotte San Stefano, which is located near the city of Viterbo. At the Solfatara site there are a large number of holes along a brook emitting gas during the day and the night with peak values of atmospheric pCO_2 of 2,044 µl l^{-1} (Miglietta et al. 1993; Schulte et al. 1999). The gas mixture emitted at the Solfatara site contained sulfur gases in much higher concentrations compared to the two other sites (Table 1). pH_2S showed peak values up to 850 nl l^{-1} (Schulte et al. 1999), the atmospheric pSO_2 concentration increased up to 140 nl l^{-1}. The control site was at 400 m E from these holes. The vegetation, the climate and the soil were similar in the whole transect.

Thiols were extracted from approx. 50 mg powdered leaf material. Extraction, reduction and derivatization were performed as described (Schulte et al. 2002). Thiol derivatives were separated and quantified after HPLC analysis by fluorescence detection (Schupp and Rennenberg 1988). Powdered leaf samples were taken for the extraction of soluble amino compounds based on Winter et al. (1992) and were separated by ion exchange chromatography as previously described (Schneider et al. 1996). After post-column derivatization with ninhydrin absorption of the amino-ninhydrin derivatives was measured at 440 and 570 nm. For measurement of nitrate reductase and APR activity, leaves stored in liquid nitrogen were homogenised in the frozen state using a micro-dismembrator (B. Braun, Melsungen AG, Melsungen, Germany). The resulting frozen powder was transferred into phosphate buffer (0.1 M, pH 7.7) containing 1% (v/v) Tween 80, 4% (w/v) PVPP K30, 5 mM EDTA, 10 mM DTE, 10 mM L-cysteine and 20 µM FAD, using 10 parts (w/v) of buffer per one part of frozen leaf material. The resulting suspension was homogenized using a Polytron (Kinematica, Littau, Switzerland). The homogenates were made cell-free by passing through two layers of viscose fleece (Millette, Migros, Switzerland). An aliquot of 190 µl of this crude extract was added to 810 µl of the assay mixture for NR composed of 25 mM phosphate buffer, pH 7.5, 3.5 mM KNO_3 and 0.15 mM of each NADPH and NADP (Neyra and Hagemann 1975). These reductants were omitted from the blanks. The NR activity was linear at 30°C for at least 20 min of incubation. After 20 min the reaction was stopped by adding 400 µl of 125 mM zinc acetate. The nitrite produced in an aliquot was detected spectrophotometrically at 540 nm after deazotization in a 1:1 mixture of 1% (w/v) sulfanylamide in 1.5 M HCl and 0.02% (w/v) N-(1-naphtyl) ethylene diamine dihydrochloride. APR activity was determined according to Brunold and Suter (1990) by measuring the acid volatile radioactivity produced from $AP^{35}S$ using DTE as the reductant (Suter et al. 2000). Radioactivity was determined by liquid scintillation spectrometry using a Betamatic instrument (Kontron Instruments, Zürich, Switzerland). Proteins in the crude extracts were determined according to Bradford (1976) after precipitation with 10% trichloric acid and subsequent solubilization in 0.1 M KOH using BSA as a standard.

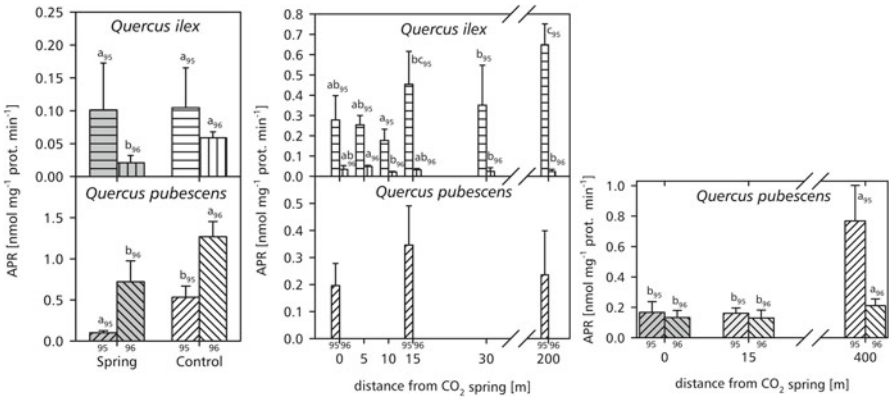

Fig. 1 Activity of APR in leaves of *Q. ilex* and *Q. pubescens* trees growing at the CO_2 springs at Bossoleto (left), Laiatico (middle), and Sulfatara (right) and at corresponding control sites. Mean values from six leaves collected from six different trees (±SD) are presented for 1995 (*striped diagonal up* for *Q. pubescence*; *striped horizontal* for *Q. ilex*) and 1996 (*striped diagonal down* for *Q. pubescence*; *striped vertical* for *Q. ilex*). Values carrying different letters are different at $P \leq 0.05$ within one sampling date. APR was not measured in *Q. pubescens* from the Laiatico spring in 1996

Data shown represent means (±SD) of measurements from individual mature leaves from different trees (n = 6) of comparable size. Statistical analysis of the results was done using the Student's t-test after testing for normality and equal variance and the multifactorial Duncan test (SPSS for Windows, 7.0). If normality was not present, a Mann–Whitney rank sum test was used.

Comparison of the activity of APR from leaves of oak trees growing near the springs with those from various control sites results in two patterns: Either the levels were the same (*Q. ilex,* Bossoleto 1995 and Laiatico 1996; *Q. pubescens,* Laiatico 1995) or APR levels at the spring area were significantly lower than at the control areas (*Q. ilex,* Bossoleto 1996, Laiatico 1995; *Q. pubescens,* Bossoleto 1995 and 1996, Solfatara 1995 and 1996) (Fig. 1). In the leaves of *Q. pubescens* from the Bossoleto and the Solfatara site APR activities increased with increasing distance from the spring area in parallel with decreasing NR activities (Fig. 2). At the Laiatico site, NR activity of *Q. ilex* leaves was not significantly different between the spring area, the control area at 200 m distance, and areas of intermediate distances from the spring (Fig. 2). In *Q. pubescens* leaves a significantly lower NR activity was measured at the spring than at 15 m distance and at the control area in 1995 (Fig. 2). NR activities of *Q. pubescens* leaves were much lower in 1995 than in 1996. The same pattern was found in leaves of *Q. pubescens* from the Bossoleto site, indicating that an additional ecological factor was regulating NR activity in 1995. At the Bossoleto site, the level of NR activity was typically higher at the spring than at the control area. However, significant differences were only found in *Q. pubescens* leaves in 1996 (Fig. 2). At Solfatara site, NR activity in leaves of *Q. pubescens* was

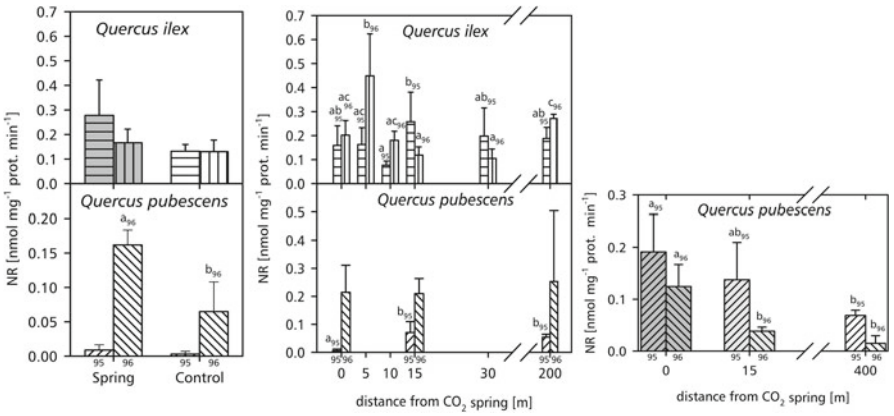

Fig. 2 Activity of NR in leaves of *Q. ilex* and *Q. pubescens* trees growing at the CO_2 springs near Bossoleto (left), Laiatico (middle) and Sulfatara (right) and at corresponding control sites. Mean values from six leaves collected from six different trees (±SD) are presented for 1995 (*striped diagonal up* for *Q. pubescence*; *striped horizontal* for *Q. ilex*) and 1996 (*striped diagonal down* for *Q. pubescence*; *striped vertical* for *Q. ilex*). Values carrying different letters are different at $P \leq 0.05$ within one sampling date. At the Solfatara spring only *Q. pubescens* was present

always higher at the spring than at the control area at 400 m distance from the spring in both years, 1995 and 1996 (Fig. 2). Even at intermediate distance from the spring, NR activity was lower than at the spring area.

At the Bossoleto and the Solfatara site, the level of cysteine and GSH measured in the leaves of both species was typically higher at the spring than at the control area (Fig. 3). Only in 1995 *Q. ilex* leaves contained comparable levels of cysteine and GSH at the Bossoleto spring and the control area (Fig. 3). A higher level of γ-EC was detected at the Solfatara spring in *Q. pubescens* leaves harvested in 1995 (Fig. 3). At the Laiatico site, significant differences in cysteine, γ-EC or GSH contents between leaves from the control and the spring area for both oak species were not observed (Fig. 3). The lower levels of cysteine and GSH in the leaves of *Q. ilex* in 1996 (Fig. 3) corresponded to lower levels of APR activity in that year (Fig. 1). In the leaves of *Q. pubescens*, TSNN, main amino acids and ammonium contents were typically not significantly different in leaves from the three springs and their corresponding control areas (Fig. 4). Only at the Bossoleto site the ammonium content was significantly lower at the spring than at the control area (Fig. 4). *Q. ilex* leaves from the Laiatico spring area contained more ammonium than at the control area (Fig. 4). At the Bossoleto site, *Q. ilex* leaves contained significantly more alanine and arginine at the control than at the spring area (Fig. 4) despite similar NR activities (Fig. 2). All other main amino acids and TSNN were comparable at the Laiatico and Bossoleto springs and their corresponding control area (Fig. 4).

When the results of the present field study were compared with a cross exchange experiment under controlled conditions with acorns of *Q. ilex* collected from the Laiatico spring (Schulte et al. 2002), some important differences could be identified.

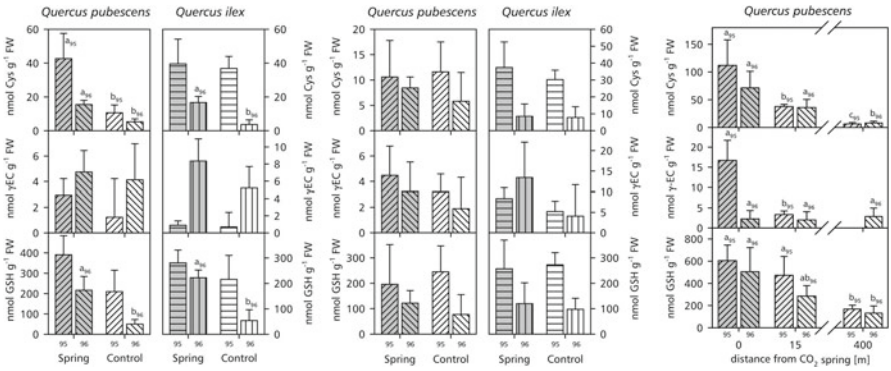

Fig. 3 Cysteine (Cys), γ-EC and glutathione (GSH) in leaves of *Q. ilex* and *Q. pubescens* trees growing at the CO_2 springs at Bossoleto (left two columns), Laiatico (middle two columns), and Sulfatara (right column) and at corresponding control sites. Mean values from six leaves collected from six different trees (±SD) are presented for 1995 (*striped diagonal up* for *Q. pubescence*; *striped horizontal* for *Q. ilex*) and 1996 (*striped diagonal down* for *Q. pubescence*; *striped vertical* for *Q. ilex*). Values carrying different letters are different at $P \leq 0.05$ within one sampling date

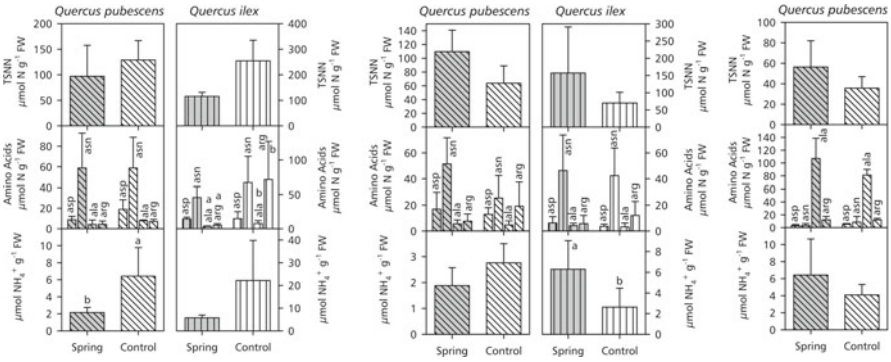

Fig. 4 Total soluble non-proteinogenic nitrogen compounds (TSNN), main amino acids and ammonium (NH_4^+) content in leaves of *Q. ilex* and *Q. pubescens* trees growing at the CO_2 springs at Bossoleto (left two columns), Laiatico (middle two columns), and Sulfatara (right column) and at corresponding control sites. Mean values from six leaves collected from six different trees in 1996 ± S.D. (*striped diagonal down* for *Q. pubescence*; *striped vertical* for *Q. ilex*) are presented. Values carrying different letters are different at $P \leq 0.05$

When the acorns collected from the Laiatico spring area were cultivated at elevated pCO_2 under controlled conditions, APR activity was comparable to that determined in *Q. ilex* leaves at ambient pCO_2 (Schulte et al. 2002). In contrast, APR activity was down regulated in *Q. ilex* leaves of the spring area in the field in 1995, whereas thiol levels were not affected. Under controlled conditions Cys and GSH contents were reduced in leaves of *Q. ilex* plants cultivated at elevated pCO_2 (Schulte et al. 2002). This clearly indicates that the sulfur metabolism reacts differently under controlled and field condition, which was not observed for nitrogen metabolism. TSNN in

Q. ilex leaves from the Laiatico spring was slightly enhanced at the spring area in the present field study and also under control conditions at elevated pCO_2 (Schulte et al. 2002). This goes parallel with unchanged levels of major amino compounds in *Q. ilex* leaves under field (present study) and controlled conditions (Schulte et al. 2002). Also total leaf nitrogen concentration of *Q. ilex* and *Q. pubescens* was not significantly different between plants growing at elevated pCO_2 close to the spring or at atmospheric pCO_2 (Körner and Miglietta 1994). Thus, effects from elevated pCO_2 on nitrogen metabolism were not observed for *Q. ilex* neither in the field nor under controlled conditions. In contrast, sulfur assimilation from atmospheric sulfur gases in the field seems to counteract the effects observed under controlled conditions.

From several studies with herbaceous (for review see De Kok 1990, further literature: Herschbach et al. 1995a, b; Durenkamp and De Kok 2004; Westerman et al. 2000, 2001; Yang et al. 2006; Koralewska et al. 2007) and deciduous (Herschbach et al. 2000) plants it is well known that sulfur gases effects thiol contents and, as a consequence, APR activity (Westerman et al. 2001; Lang et al. 2007; Durenkamp et al. 2007; Koralewska et al. 2008). APR activity tends to decrease in leaves collected at the spring area of the Bossoleto and Sulfatara site, although not at any time and in any oak species, and also in *Q ilex* leaves from the Laiatico spring in 1996. Cysteine and GSH levels were higher in leaves from trees at the spring area of the Bossoleto and Sulfatara site, but unaffected at the Laiatico site. These results indicate a strong correlation between thiol contents and APR activity also under field conditions and are consistent with current knowledge of feedback inhibition of APR (for review see Kopriva and Rennenberg 2004; Kopriva 2006; Davidian and Kopriva 2010).

When the observed results from the three locations are related to the sulfur gas concentrations of the emitted gas mixture and to the diurnal course of emission controversial results obtained. The Sulfatara site exhibits a higher atmospheric pH_2S concentration compared to the Bossoleto and Laiatico spring that correlates with the highest Cys, γ-EC and GSH content in *Q. pubescence* leaves. Hence at the Sulfatara site a clear correlation between gas emission, thiol increment and APR activity was observed that agrees with the common knowledge (Kopriva and Rennenberg 2004; Kopriva 2006).

The mean value of the sulfur gas concentration of the Bossoleto and Laiatico spring was comparable. Thus, differences in sulfur metabolism observed between these sites could not be explained by differences in the gas composition, but probably by the time of sulfur gas emission. At the Bossoleto site the gas is mainly emitted during the early morning with peak values up to 8,097 µl l^{-1} pCO_2 (Schulte et al. 1999). During daytime, *i.e.* between 9 am and 6 pm, mean pCO_2 was 368 ± 54 µl l^{-1}. Net photosynthesis of *Q. ilex* and *Q pubescence* leaves at this site was higher at the spring area compared to the control area (Tognetti et al. 1998; Blaschke et al. 2001) and leaf conductance was reduced at the spring site resulting in higher water use efficiency (Tognetti et al. 1998, 1999). At the Laiatico spring, where the gas is emitted during the day (Schulte et al. 1999), *Q. pubescence* leaves showed higher net photosynthesis and slightly reduced leaf conductance (Stylinski et al. 2000). As neither H_2S nor SO_2 seem to penetrate the cuticula in appreciable amounts (Taylor and Tingey 1983; De Kok et al. 1991) and stomatal conductance is generally

around zero during the night, uptake of H_2S and SO_2 should be restricted to the daytime when concentrations of both sulfur gases were low at the Bossoleto site. However, at the Bossoleto spring cysteine and GSH contents were enhanced indicating uptake and usage of the emitted sulfur gases. From these considerations the observed results seem contradictory. However, also other environmental factors such as drought can influence GSH contents in leaves. At drought stress Marabottini et al. (2001) observed higher GSH contents in *Q. pubescence* leaves collected from the Bossoleto spring area than from the control area and unchanged GSH contents in *Q. ilex*. Although meteorological data are not available, it can be assumed from observations at the Bossoleto site that a drought period prior the harvest in 1996 had influenced the GSH content in *Q. pubescence* leaves.

On the other hand, elevated sulfur availability by sulfur gases emitted from the vents at the spring areas itself could function as an environmental factor influencing nitrogen assimilation. As nitrate reductase activity increased, decreased or remained constant at elevated pCO_2 (reviewed in Stitt and Krapp 1999, see also Natali et al. 2009) the findings of increased NR activity in *Q. pubescence* leaves from the Bossoleto and Sulfatara site as well as its decrease at the Laiatico spring in 1995 are not surprising. However, nitrogen assimilation and, thus, NR activity also depend on the sulfur supply (Brunold 1993). Sulfur deficiency diminishes NR activity (Brunold 1993), but information of a surplus of sulfur on NR activity is scarce. In *Q. pubescence* leaves from the Bossoleto and the Sulfatara spring area NR was higher compared to the control areas, whereas APR activity was lower. As the lower APR activity correlates with enhanced reduced sulfur levels, elevated NR activity may be an indication of an enhanced demand for reduced nitrogen for protein synthesis. This view is supported by the decreased leaf NH_4^+ content of *Q. pubescence* at the Bossoleto spring. In conclusion, the presented results support the diversity of C, N and S interactions and, additionally, the complex environmental influences on these interactions.

Acknowledgements This study was financially supported by the European Commission (MAPLE – Project EV5VCT940432), and the Swiss National Science Foundation. The authors thank M. Stalder for excellent technical assistance.

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