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TITLE: Stress-induced changes to the flora in a geothermal field in central Italy

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Abstract

The vegetation profile and the photosynthetic efficiency, oxidative damage and stomatal conductance in the evergreen dwarf shrub *Calluna vulgaris* (L.) Hull were analyzed in a Mediterranean ecosystem characterized by intense geothermal activity. Among the higher plants present in the area, this species appears to be the sole to possess the ability to grow near the geothermal sources. The hot fluid springs strongly alter the environment in their proximity: the emitted water vapor, CO_2, H_2S, CH_4, H_3BO_3, SO_4^{2-} and NH_4^+ partly condensate and precipitate to the soil, thus leading to its extreme acidification and nutrient depletion. Furthermore, the temperature starts to rise sharply just a few centimeters under the soil surface. Under this multiple stress, the individuals of *C. vulgaris* growing within a few meters from the springs showed lower photosystem II efficiency, higher oxidative damage to the biomembranes and lower stomatal conductance than the individuals growing farther away. Drought and high air temperatures occurring in summer exacerbate these harsh conditions, but only the plants closer to the springs did undergo an acute, yet transient crisis, as shown by the analyzed parameters. These results suggest that the main factors of stress are related to the physical and chemical features of the soil, while the adverse climate conditions apparently are of secondary importance. The possible role of reduced stomatal conductivity in enhancing the resistance of *C. vulgaris* to this hostile environment is discussed.

Keywords

Drought; heat; membrane damage; nutrient deficiency; PSII efficiency

Author contribution

Alice Pippucci carried out the research work and contributed to the analysis of data, Roberto Lorenzi is the group leader and supervised all the activities, Carmelina Spanò contributed to manuscript writing and editing, Carlo Sorce planned the research work, contributed to the field activities, data analysis, manuscript writing and editing. All authors read and approved the final version of this manuscript.

Introduction

The survival of higher plants relies on their adaptation ability, which involves changes in physiology, ontogenesis, geographic distribution and ecological interactions with different species (Hughes 2002; Walther et al. 2002). Several authors (Albert et al. 2011 and references therein) have drawn attention to the importance of studying the response to multiple climatic factors, in order to take into account long-term acclimation. These issues may be effectively addressed by investigating plant communities of natural geothermal springs under field conditions (Peñuelas et al. 2001). The ecosystems with geothermal activity that are present in central Italy are characterized by high soil temperature, low nutrient availability and elevated atmospheric CO_2 concentration. Owing to their latitude and proximity to the sea, these environments often show a typical Mediterranean climate, where plant communities undergo long-term exposure to multiple stress factors. The geothermal field investigated in this work is located in southern Tuscany and its intense activity strongly affects plant life in the area, owing to the high temperatures of the root-growing zone and to the low soil pH (Chiarucci et al. 2008; Bartoli et al. 2013). The response to the multiple stress imposed by this environment was studied by carrying out a survey of the plant community along a gradient of stress conditions, which led us to focus our interest on a species that was selected for a physiological investigation. The area under study is characterized mainly by two types of wood communities: one of deciduous species and one of evergreen sclerophylls (Tomei et al. 2008). In this forest landscape, the geothermal fields represent a striking discontinuity. Less than 30 species (out of 155 identified in the whole area) are present within the geothermal fields and the number decreases further in proximity of the hot springs. Prairies of *Calluna vulgaris* (L.) Hull and *Agrostis castellana* Boiss. & Reut. prevail, that are the two species typical of the site (Fiori 1920), owing to their ability to colonize hot and hyper acid soils.

Around these prairies, further species grow: these are mainly shrubs tolerant to low soil pH, like *Cistus salviifolius* L., *Erica arborea* L. and *E. scoparia* L. The sole tree species that is present, although it is confined to the border of the fields, is *Quercus suber* L. The results of our vegetation survey allowed us to select *Calluna vulgaris* as the species of choice for the physiological investigation. It is an evergreen small shrub with a highly branched woody stem, scale-like leaves, apical racemes with small pink-violet, bell-shaped flowers that bloom in late summer. It is found in cold and temperate areas, from the mountains of Morocco to northern Russia, throughout Europe, as well as in western Asia, North America, southern Australia and New Zealand and may thrive from sea level up to 2500 m. It is an oligotrophic, calcifuge and acidophilus species, which grows in full sun and open spaces, where it can form wide prairies. Its average lifespan is 25-30 years (Gimingham 1960).

Stressful conditions lower photosynthetic efficiency, increasing the dissipation of pigment excitation energy, thus helping to prevent photooxidation (Figueroa et al. 1997). The measurement of chlorophyll fluorescence is a sensitive method for assessing the efficiency of photosystem II (PSII). In the present work, field measurements of the difference of maximum fluorescence (F_m) minus minimum fluorescence (F_0), divided by F_m, or F_v/F_m (Lichtenthaler 1988; Baker 1991; Bolhàr-Nordenkampf and Öquist 1993; Baker 2008), were performed on dark adapted leaves of the selected species. Furthermore, the degree of stress was investigated by measuring stomatal conductance (g_s) and by analyzing the concentration of malondialdehyde (MDA) in leaf tissues, which is an index of the degree of peroxidation of membrane lipids (Hodges et al. 1999; Blockhina et al. 2003).

Besides the floristic characterization of the site, the aim of our work was to evaluate which environmental components (i.e., the seasonal course of weather or the main features of the soil) might have the strongest impact on the physiology of the selected species, in order to gain more insight into the response to long-term exposure to the extreme conditions of this environment and, consequently, into the mechanisms of adaptation.

Materials and methods

Site description

The geothermal area of the study is the Site of Regional Interest B12 ‘Campi di alterazione geotermica di Monterotondo Marittimo e Sasso Pisano’ (code n° IT5170102), located at 43°09’ N, 10°51’ E, 614 m asl. According to Duchi et al. (1991), the fluid and gas released by the ground contain mainly water vapor, $(NH_4)_2SO_4$, $CaSO_4$, $MgSO_4$, CO_2, HCO_3^-, CO_3^{2-}, CH_4, H_2S, HS^-, SO_4^{2-}, SO_2, Cl^-. The atmosphere near the soil surface is enriched in CO_2, H_2S, NH_3 and CH_4, with variable amounts of boric acid derivatives (Bussotti et al. 2003). The physiological investigation was carried out on *C. vulgaris* plants growing in two areas (stress area, A_s and control area, A_c), that differed for the intensity of the geothermal alteration. The two areas were chosen on the basis of the following criteria: distance from the springs, soil average pH and temperature, species distribution (data acquired from the vegetation transects), macroscopic effects of the stress based on visual estimation. The areas shared the following traits: microclimate, elevation (610 m asl), slope (flat ground), exposure (full sun), species abundance (*C. vulgaris* was the dominant species). The distance between A_s and A_c was 30 m.

Vegetation transect

Between April and June 2012, several surveys of the flora were carried out along a gradient of stress conditions, by the means of linear transects. A graduated ribbon was fixed to the ground by plastic stakes and stretched from the edge of a geothermal spring (site of most intense stress) to the closest individual of *Q. suber*, whose occurrence is a reliable index of the attenuation of the stress. Point intercept data (species present at each sampling point) were recorded at 1 m intervals starting from the spring and ending at an average distance of 15 m away from it, where the first individual of *Q. suber* was found. The severity of the stress conditions was evaluated by measuring, at each point, soil temperature and pH, as described in the following sections.

Soil characterization: temperature, pH and elemental analysis

Field measurements were performed by a Checktemp 1C HI 98509 pocket thermometer with ± 0.3 °C accuracy (Hanna instruments, Padova, Italy) in August 2012. The instrument probe was inserted at 0.1 m depth in the ground and left to stabilize for 2 min before recording the temperature. One series of measurements was made at 1 m interval along the path of the vegetation transect; at the same points, soil samples were taken at 0.1 m depth for pH determination. The values of temperature and pH were compared with the result of the vegetational survey, to highlight any possible correlation between these soil parameters and the pattern of distribution of the species.

Along the edge of each of the two areas that had been selected for the physiological investigation on *C. vulgaris*, i.e. A_s and A_c, six plants of this species were chosen. Three of them grew at the margin of the respective area facing the closest geothermal spring (‘spring side’) and the others at the opposite side, i.e. along the edge farther from the spring (‘wood side’). In this way, we tried to highlight if, within both A_s and A_c, the plants growing at the minimum distance from the nearest spring underwent different soil conditions in comparison to those growing at the maximum distance. In the rhizosphere of these 12 plants, soil temperature was measured and samples were taken at 0.1 m depth for pH determination. Samples of 20 g of soil were weighed, sieved and dried at 70 °C for 24 h. Ten g of dried soil were dissolved in 50 ml distilled water to obtain a homogeneous suspension, that was mixed for 30 s every 3 min for 5 times and then left to settle for 5 min. The pH of the resulting suspension was measured by a 3305 pH meter (Jenway, Staffordshire, UK).

For elemental analysis, soil samples (20 g) were dug from the rhizosphere of all the selected *C. vulgaris* plants, from both A_s and A_c, at a depth of 0.2-0.3 m by a small plastic shovel. They were put in sterile polythene bags and carried to the laboratory for the analysis. First, they were sieved (mesh < 2 mm), dried at 100 °C for 12 h and ground to a fine powder. Tablets were prepared by adding 0.6 ml of 20% polyvinyl alcohol glue to 3 g of soil powder and blending to obtain a dense mixture. This was heated at 100 °C for 3 h, then powdered again, mixed with an equal amount of boric acid and squeezed under a pressure of 3 t m^{-2}. The resulted tablets were analyzed by a Philips PW 1480 X-ray photoelectron spectroscope (Philips, Eindhoven, The Netherlands) to determine the elemental content of the main mineral nutrients. A fraction of each soil sample was sieved with mesh $\leq$ 0.25 mm and was used for determining the content of total nitrogen by the micro Kjeldahl technique.

Climate data

These data were gathered from the weather observatory of Castelnuovo Val di Cecina, located at about 2 km from our experimental site (43°12’ N, 10°54’ E), at a similar elevation (576 m asl), through a dedicated website (www.castelnuovometeo.it). To collect information on the recent course of the climate in the area, we compared the monthly rainfall and temperature values recorded in 2012 with the corresponding data averaged over the previous 20 years (1992-2011). To probe the correlation between the measured physiological parameters and climate course, we calculated the accumulated rainfall and average temperature for the period studied. Accumulated rainfall was expressed as the sum of precipitation for every interval between two sampling dates; moreover, the average temperatures for the above said intervals were also calculated. The accumulated rainfall and average temperature for the first sampling date were calculated from the respective values recorded throughout 10 days prior to that date. To estimate the degree of dryness of 2012, De Martonne Aridity Index (De Martonne 1926) was calculated with the following equation and compared with the values obtained for the previous 20 years (1992-2011):

$A_i = 12\ P / (10 + T)$

where P = monthly average precipitation and T = monthly average temperature

Selection of the plants for the physiological investigation

Twenty individuals of *C. vulgaris* from both A_c and A_s were chosen for our study: they were marked with a thin thread and measured to determine their overall size. The selected plants were of comparable age (5-6 years old), that was estimated by cutting one of the oldest branches, close to soil surface, and counting the annual rings of the stem under a stereomicroscope (SMZ-1, Nikon, Japan). The selected individuals of A_s grew within 6-9 m from a geothermal spring and their leaves did not show any evident symptom of stress (e.g. reddening, browning or desiccation). The marked plants of A_c were located near the border of a wood of *Q. suber* and *Castanea sativa* Mill., where they coexisted with sparsed individuals of the shrub species typical of this geothermal field.

Chlorophyll *a* fluorescence measurements

Components of chlorophyll fluorescence were quantified with a portable modulated fluorometer Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany). The subapical segment (located below the terminal inflorescence) of three sun-exposed shoots from the current year per plant were measured *in situ*. Leaves were dark adapted for 30 min by Dark Leaf Clips (Walz) before the measurment of F_0, F_m, and F_v/F_m. During June 2012 a series of measurements was performed to set up the operating parameters, then the analytical readings were taken weekly, around midday of sunny days, between July and November 2012.

Leaf MDA concentration

The degree of lipid peroxidation was evaluated by the TBARS assay, according to Dhindsa et al. (1981). Leaf samples (10 g) were taken at variable intervals, mainly along with fluorescence measurements. They were kept at 10 °C for about 3 h, then delivered to the laboratory, where they were stored at -80 °C until analysis. The concentration of MDA, expressed as nmol g^{-1} fresh weight, was calculated by subtracting the aspecific absorbance of the samples at 600 nm from the absorbance of the complex MDA-thiobarbituric acid (at 532 nm) and dividing the value obtained by the molar extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$.

Stomatal conductance

Stomatal conductance (mmol $H_2O\ m^{-2}\ s^{-1}$) was measured three times by a SC1 steady-state leaf porometer (Decagon Devices, Inc., Pullman, WA, USA) on the subapical segment of three sun-exposed shoots per selected plant, in both studied areas. The three times were:

1) July 29: prior to the expected maximum summer stress, when photosynthetic efficiency was still high and shortly after a rainfall.

2) August 26: at maximum summer stress, when we detected a marked decrease of F_v/F_m, at least in A_s and no rain had been recorded in the previous 26 days.

3) September 23: following the maximum summer stress, when photosynthetic efficiency showed a considerable increase in A_s and shortly after a rainfall.

Statistical analysis

Statistical analyses were performed using one value per area, obtained by averaging the values of the 6 plants measured per area in the case of chlorophyll *a* fluorescence and stomatal conductance and by pooling the material from different plants in the case of the TBARS test. All differences were considered significant at a probability level of $P < 0.05$. Data from control and stress plots were first compared by *t* test. Multiple linear regression analysis was performed between fluorescence values and climatic data (accumulated rainfall and temperature sum), separately for the control and the stress plot. The same was done for the TBARS test data. Pearson’s correlations were calculated between mean values of fluorescence and TBARS data, for A_c and A_s.

Results

Floristic pattern

The landscape in the studied area is characterized by hills that are covered mainly by *Quercus cerris* (L.). *Pinus pinaster* (Aiton) and *Castanea sativa* are also widespread, owing to past reforestation for wood and fruit production. The geothermal fields of Monterotondo Marittimo and Sasso Pisano represent a marked peculiarity, in that they are almost devoid of forest vegetation. The flora of ‘Biancane’ park exhibits distinctive traits in comparison to the surroundings, being characteristic of a typical mediterranean environment, where open areas are interspersed with stands of evergreen sclerophylls and the sole tree species is *Q. suber* (Tomei et al. 2008). The species diversity quickly declines while approaching to the springs. To acquire a detailed picture of the floral pattern of the studied site, a survey of the plant community along a gradient of stress conditions was carried out by the means of linear vegetation transects, departing radially from the edge of a spring to the closest individual of *Q. suber*. A representative result is reported in Table 1, where the linear distribution of the species may be compared with the measured gradients of soil temperature and pH: when moving towards the spring, it can be observed that the evergreen shrubs (*C. salviifolius, E. arborea, E. scoparia*) are progressively replaced by *A. castellana* in association with *C. vulgaris*, but the latter is the dominant species close to the geothermal sources. Soil temperature and pH progressively change along the transect. On average, within the belt around the sources, the temperature at 0.1 m depth drops from 98.9 ± 7.4 °C (at 1 m radial distance from the source) to 52.3 ± 5.7 °C (at 4 m from the source) and pH rises from 1.8 ± 0.32 to 2.35 ± 0.87. The ground looks coarse and grey, borate and sulphate concretions and sulphur crystals are frequently observable on the surface, whereas vascular plants are missing. These were found at a distance from the springs not shorter than 5 m and were all small and stunted shrubs of *C. vulgaris*, which could tolerate soil temperatures up to 52 °C and pH as low as 2.35. Milder conditions are found at increasing distances from the sources, allowing the population of *C. vulgaris* to become denser and, apparently, healthier. Significant differences arose when comparing the growth of individuals of the same age from A_s and from A_c (10 plants area^{-1}). The formers showed a flat shape, bore short branches and attained an average height of 0.25 ± 0.046 m; their roots were thin, with a prevailing plagiotropic orientation and did not grow deeper than 0.15 m. *Calluna vulgaris* plants of A_c were taller (0.55 ± 0.087 m) than those of A_s, with upright branches; roots were thicker and reached depths exceeding 1 m in many individuals. *Agrostis castellana* was not found within 8 m from the vents, *i.e.* in soils with temperature above 47 °C and pH below 3. The tolerance to the harsh conditions of *C. salvifolius, E. scoparia* and *E. arborea* is even lower, because they were found beyond a radius of 11 m from the springs, where the temperature of the ground did not exceed 36 °C and pH was higher than 3.7. Such crowded communities are unsuited for *C. vulgaris,* which gradually disappears. The sources and the edge of the stand of *Q. suber* are at least 14 m apart; this species grows on soils cooler than 34 °C and with pH above 3.9.

Soil temperature and pH

The temperatures recorded in the rhizosphere of *C. vulgaris* (Table 2) showed that at 0.1 m depth the roots experienced an average of 23.88 ± 1.35 °C in A_c and 38.13 ± 2.19 °C in A_s (these mean values were calculated from the data reported in Table 2). The means were significantly different. In both areas the highest value was recorded in the rhizosphere of the plant growing near the point located at the shortest distance from the geothermal vent (‘spring’ side, Table 2), while the lowest temperature was measured for the individual on the opposite end of the respective area (‘wood’ side), but these differences were not statistically significant. It is worth noting that the highest temperature recorded in A_c was considerably lower than the lowest one of A_s.

The mean values of soil pH (Table 2) were 4.33 ± 0.2 in A_c and 2.99 ± 0.7 in A_s; the means of the two areas differed significantly. In both areas, the lowest value was detected on the ‘spring’ side (4.2 in A_c and 2.35 in A_s) and the highest one on the ‘wood’ side (4.45 in A_c and

3.7 in A_s), but neither in this case the differences within the areas were statistically significant. According to USDA classification (http://soils.usda.gov/technical/handbook/contents/part618.html) the soil of A_c is extremely acid ($3.5 < pH < 4.4$) and that of A_s is ultra acid ($1.8 < pH < 3.4$).

Nutrient content

The concentrations of the most relevant mineral nutrients (Table 3) were higher in A_c than in A_s. The sole exception was Mn, which did not differ significantly between the studied areas. In comparison to the reference values that are reported by Greenwood and Earnshaw (1985), Batjes (1996) and Pais and Jones (1997), many nutrients may be considered deficient in this soil: Ca, Mg, K, Fe, Zn and Mn concentrations were low and A_s seemed to be poor also in P. Conversely, N did not appear to be potentially limiting for growth.

Climate data

According to Köppen-Geiger climate classification (Kottek et al. 2006), the climate of this area is comprised in the subcategory ‘Csa’ (Mediterranean climate), characterized by long, hot, dry summers and mild, rainy winters. Figure 1 shows the course of the mean temperatures, rainfall and De Martonne Aridity Index measured monthly (or calculated monthly, in the case of the Aridity Index) near the studied site in 2012. In this year the temperatures were slightly higher than in the previous 20 years (data not shown), over several months and precipitations were below the average throughout 9 months. Overall, 2012 has been warmer and drier than the average of the previous 20 years. Although June and July 2012 were relatively rainy, drought occurred in spring and in the period spanning from mid summer to late autumn. De Martonne Aridity Index showed that 2012 has been an arid year, especially in May, August and September (Fig. 2; the lower the A_i value, the drier and warmer is the climate), thus turning out to be particularly hard for plant growth and survival.

Photosynthetic efficiency

The maximum quantum efficiency of PSII was evaluated by measuring the components of chlorophyll fluorescence. The results of our investigation are reported in Figure 3. Photosynthetic efficiency was high, both in A_c and in A_s, at the start of the studied period, i.e. in early summer. *Calluna vulgaris* plants growing in A_c showed a greater maximum quantum yield of PSII than those in A_s, throughout the period studied. Only for the average values of July 8 the difference between the two studied areas was not significant. Overall, F_v/F_m changed little for A_c plants, while marked fluctuations were observed in the A_s group. The latter showed, from July 23, a progressive decrease of photosynthetic efficiency, which attained the lowest value (0.412) on August 26. The maximum quantum yield of PSII then recovered appreciably within one month, although it was still lower than in A_c individuals. A further, yet transient, decrease of F_v/F_m of A_s plants occurred in October, while the values recorded in November were comparable to those of early July.

Oxidative damage

Leaf MDA concentration is an index of lipid peroxidation, therefore it represents a reliable estimation of the severity of the oxidative damage to the leaves. Our analyses started as soon as we detected a distinct changing of the pattern of photosynthetic efficiency of A_s plants, which occurred at the end of July, when the F_v/F_m values of the A_s group began to decrease.

This was assumed to be a clue of incipient chronic photoinhibition and prompted us to investigate the relationship between the decline of photosynthetic performance and the degree of lipid peroxidation. Oxidative damage was more severe in A_s than in A_c plants: across the studied period, MDA concentration was higher in the former area, with the exception of the average values of July 29 and August 6, that did not differ significantly between the control and the stress group (Fig. 4). The leaves of A_c individuals showed the greatest MDA concentration on August 26, with an average value of 162.3 ± 0.325 nmol MDA g^{-1} f wt. Afterward, the degree of lipid peroxidation declined throughout September and early October, with a rise at the end of the studied period. Also in A_s the highest value of MDA concentration was attained on August 26, with an average value of 252.9 ± 0.325 nmol MDA g^{-1} f wt. During the subsequent weeks, the time course of MDA concentration of A_s plants was similar to that of A_c, with a marked decrease in September up to early October, followed by a rise at the last sampling date.

Stomatal conductance

An attempt was made to highlight differences, at stomatal level, in the response of plants to the environmental conditions. Few data were collected, but they could allow to detect if leaf gas exchanges had been significantly affected by environmental factors other than soil water content, since the latter could be expected to be similar for the two experimental areas at the three selected dates. We tried to characterize stomatal regulation in response to the environment and to the seasonal course by comparing the values recorded at the time of putative maximum stress, as well as shortly before and after such time. The data are reported in Table 4 and show significant differences between A_c and A_s and between sampling dates.

Plants of A_s always displayed a lower stomatal conductance than those of A_c. The values changed significantly also with time, reaching the lowest level on August 26.

Relationship between photosynthetic efficiency and oxidative stress in leaves

The comparison of the values of F_v/F_m with those of leaf MDA concentration was carried out within each area (A_c and A_s), by calculating Pearson's correlation coefficients. These highlighted that there was almost no correlation between photosynthetic efficiency and oxidative stress in leaves of A_c plants, because the Pearson's coefficient was -0.03989. Instead, for stressed plants we obtained a lower coefficient value (-0.74171), which indicates a negative correlation between lipid peroxidation and photosynthetic performance.

Relationship between physiological parameters and climate

We sought for correlations between the main physiological parameters measured on *C. vulgaris* plants and the seasonal pattern, by comparing the time course of photosynthetic efficiency and lipid peroxidation with cumulated rainfalls and average temperatures, within each interval of the period studied. The impact of the two main climatic factors, i.e. rainfall and temperature, on the physiology of the studied *C. vulgaris* individuals was estimated through multiple linear regression analysis. The independent variables were the cumulated rainfall and average temperature, while the dependent ones were the values of the measured physiological parameters. We chose a multiple regression analysis, because we aimed at evaluating the effects of the joined action of precipitation regime and temperature. Prior to running each analysis, we checked the multicollinearity between the chosen predictors. The values of the Variance Inflationary Factor that we obtained were 1.6750 for photosynthetic efficiency and 1.1086 for MDA (two different values were calculated, because the two sets of data were of different size). Based on these values, we could exclude the existence of a significant correlation between the two independent variables, because the calculated parameters were smaller than 5. The latter is the upper limit that, when exceeded, is an index of significant multicollinearity, according to Snee (1973). The analysis revealed a statistically significant relationship only between photosynthetic efficiency and climate for *C. vulgaris* plants growing in A_c (Table 5). The r^2 coefficients suggest that an appreciable correlation exists between photosynthetic efficiency and the two climatic factors in A_c. Both predictors influenced PSII energy conversion of *C. vulgaris* plants growing in A_c: the significance of the t values of the two independent variables were 0.0012 for rainfall and 0.0153 for average temperature. Overall, the two predictors explain a limited fraction of the observed variability of both photosynthetic efficiency and lipid peroxidation data, as shown by the low values of the r^2 coefficients.

Discussion

The geothermal activity in ‘Biancane’ park deeply alters the chemical and physical features of the soil, thus establishing hard ecological conditions. Several soil traits (high temperatures near the surface, extremely low pH, low water retention, nutrient deficiency, occurrence of chemical toxicants) combine to bring about a stressful environment, which determines the distribution of plant species in this site. Plant growth on acidic soils is hampered by multiple causes, that are related to the chemical and physical features of the substrate (Marschner 1991). One primary consequence of the low pH is the little cation exchange capacity (Haynes and Swift 1986; Violante 2002) and, accordingly, our analyses revealed that these soils are deficient in Ca, Mg, K, Fe, Zn and Mn. The depletion of mineral nutrients was more pronounced in A_s, where also P concentration seemed to be potentially limiting for plant growth. Our data confirm the existence of gradients of soil temperature and pH: the former declines and the latter rises progressively at increasing distances from the emission sources, similarly to what has been observed by Bartoli et al. (2013) in a nearby site. The floristic pattern is the result of the degree of tolerance to soil conditions which characterizes each species. The role of soil temperature in determining the distribution of the species in those areas that are affected by volcanism has been discussed by several authors (e.g., Glime and Iwatsuki 1994; Burns 1997). Floristic patterns may be substantially influenced also by the pH and nutrient availability of the substrate, that in turn are affected by the condensation of the geothermal vapors on the ground surface. The poor biodiversity of the site is attributable to its strong selective pressure, which is successfully withstood by *C. vulgaris,* whose ecophysiological traits are suitable for surviving even at highly unfavorable spots. Indeed, *C. vulgaris* is the vascular plant species that is able to survive closest to the geothermal sources, as previously observed by several authors (Bargagli-Petrucci 1916; Fiori 1920; Gimingham 1960; Selvi and Bettarini 1999; Bartoli et al. 2013). Nevertheless, the individuals of A_s underwent a depression of growth involving both the aerial part and the root apparatus. Nutrient deficiency (particularly of Ca^{2+}) and high concentrations of toxic metals, that are a consequence of the extreme acidity of the soil, were likely responsible for shoot and root growth depression in A_s (Singh and Agrawal 2008). However, the observed developmental pattern of the roots in A_s may be attributed mostly to the high temperature of the soil, which prevented roots from growing deeper and restricted their presence to the upper soil layer. In depth investigation on *Agrostis stolonifera* has demonstrated that both root and shoot growth are restrained under high soil temperatures, owing to the impairment of many physiological processes (Xu and Huang 2000; Huang et al. 2001; Wang et al. 2003; Liu and Huang 2005; Lyons et al. 2007).

The maximum quantum efficiency of PSII was comparable to that reported by Llorens et al. (2004) and by Albert et al. (2011), who suggested that the optimal maximum quantum yield of PSII in *C. vulgaris* is between 0.74 and 0.797. The values recorded were generally higher in A_c than in A_s. Nevertheless, the individuals growing around the emission sources exhibited an excellent degree of stress tolerance, at least for what concerns the photochemical process: photosynthetic efficiency was somewhat high for A_s plants, with the exception of the sampling dates around the midpoint of the studied period, when the hot and dry climate seemed to induce an acute suffering, yet the relationship between this physiological trait and climate (mean air temperatures and precipitations) was not statistically significant in A_s. Similarly, there was not any significant correlation between lipid peroxidation and climate course for *C. vulgaris* individuals of A_s, although the highest values of MDA concentration were recorded during the hottest and driest period. Hence, climate had a weak effect on the changes of F_v/F_m and MDA concentration in A_s. The opposite was true for A_c plants, but only for photosynthetic efficiency, which displayed a higher sensitivity to climate fluctuations, likely because these plants grew on a more favorable soil, which did not affect markedly their physiology. However, the value of F_v/F_m changed little with time in A_c plants, that showed a high tolerance to summer drought, as demonstrated also by the relatively low values of MDA concentration.

A relationship may exist between the maximum quantum efficiency of PSII and the concentration of MDA in leaves, since peroxidation of lipidic molecules of chloroplast thylakoids is a primary cause of the decrease of photosynthetic efficiency that occurs under stress conditions (Mishra and Singhal 1992). Furthermore, the accumulation of ROS prevents the repair of damaged PSII (Takahashi et al. 2009). We found a significant, negative correlation between photosynthetic efficiency and lipid peroxidation only in the leaves of A_s plants, likely because the stressful conditions heavily and concurrently affected both measured physiological parameters.

In conclusion, *C. vulgaris* is able to effectively tolerate the multiple stress induced by this peculiar environment: the physiology of this species approaches to a critical state only when severe drought conditions occur. The physical and chemical features of the soils of ‘Biancane’ park, that are a consequence of the geothermal activity, may be considered the primary cause of the observed physiological differences between A_c and A_s plants throughout the studied period, while climate would have played only a subordinate role. *Calluna vulgaris* might be affected also by the direct exposure to the emissions from the springs. We did not investigate the effect of spring vapor condensation on the leaves, but it could exacerbate the stress induced by the adverse soil environment. Indeed, the toxic effect of the effluents has been demonstrated by Bussotti et al. (2003), whose work in a nearby site showed a decrease of photosynthetic efficiency in *Quercus pubescens* plants growing near the springs. Further analyses that were made in a geothermal area very similar to the one investigated in the present study demonstrated that the plants growing around the springs are exposed to high CO_2 concentrations (Tognetti et al. 2000). This CO_2-enriched air, as well as the possible toxic effects from other gaseous compounds, might indirectly strengthen the resistance of A_s plants to drought stress by lowering their stomatal conductance. Plant water use efficiency would thereby be enhanced, although the elevated CO_2 might also down-regulate photosynthesis, as suggested by Albert et al. (2011). Our data show that stomatal conductance was lower in A_s than in A_c plants, even after a rainfall, when soil water content would not have been limiting. Such restriction of leaf gas exchange might be crucial for increasing the ability of A_s plants to overcome the driest period in summer. Further physiological and morpho-anatomical traits have been shown to contribute to the achievement of the high degree of tolerance to the geothermal-induced stress, like, for instance, leaf cuticle thickening and enhanced oxidant-scavenging capacity, according to Bartoli et al. 2013. Notably, these authors found that the leaves of *C. vulgaris* plants growing near the springs in a geothermal field adjacent to our investigation site displayed a higher stomatal density and a lower hairiness: they suggest that these leaf traits may allow for a more precise and faster regulation of transpiration, which would help these plants to cope with the unpredictable temperature peaks to which they are continuously exposed.

Calluna vulgaris may represent a valid model to foster our understanding of the response of plants to the geothermal-induced stress and to establish a base of ecological and ecophysiological data which may help to scale up our current knowledge to a higher level.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 Satellite image of the study site, indicating the stress area (A_s) and the control area (A_c), where the physiological investigation on *C. vulgaris* was performed and the geothermal spring (S) located at the shortest distance from both A_s and A_c. The distance between the two areas was 30 m

Fig. 2 Monthly average temperatures (line), rainfall (empty bars) and De Martonne Aridity Index (Ai; filled bars) of 2012

Fig. 3 Maximum quantum efficiency of PSII (F_v/F_m) of *C. vulgaris*. White symbols = control (A_c plants); black symbols = stress (A_s plants). Values of A_c and A_s plants were significantly different ($p < 0.05$), except at July 8

Fig. 4 Concentration of MDA in leaves of *C. vulgaris*. White symbols = control (A_c plants); black symbols = stress (A_s plants). Values of A_c and A_s plants were significantly different ($p < 0.05$), except at July 29 and August 6

Figure 1

Figure 2

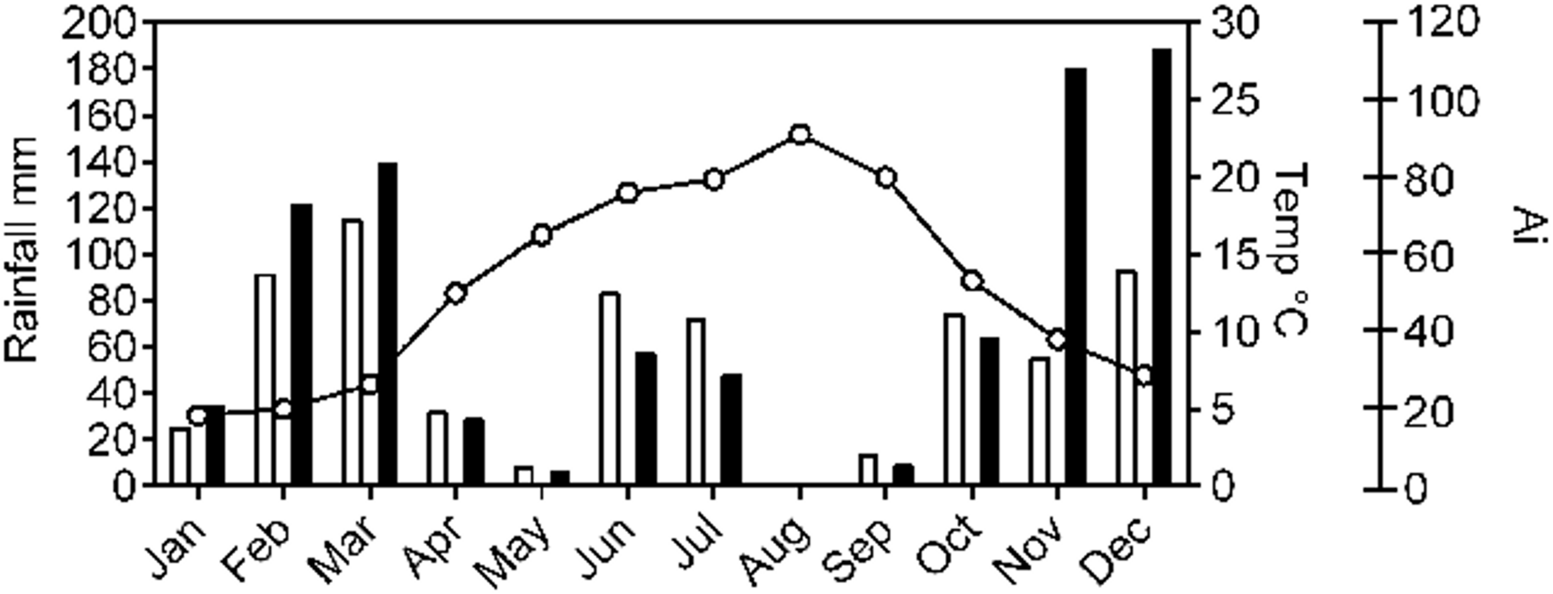

Rainfall mm
200
180
160
140
120
100
80
60
40
20
0
Temp °C
30
25
20
15
10
5
0
Ai
120
100
80
60
40
20
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 3

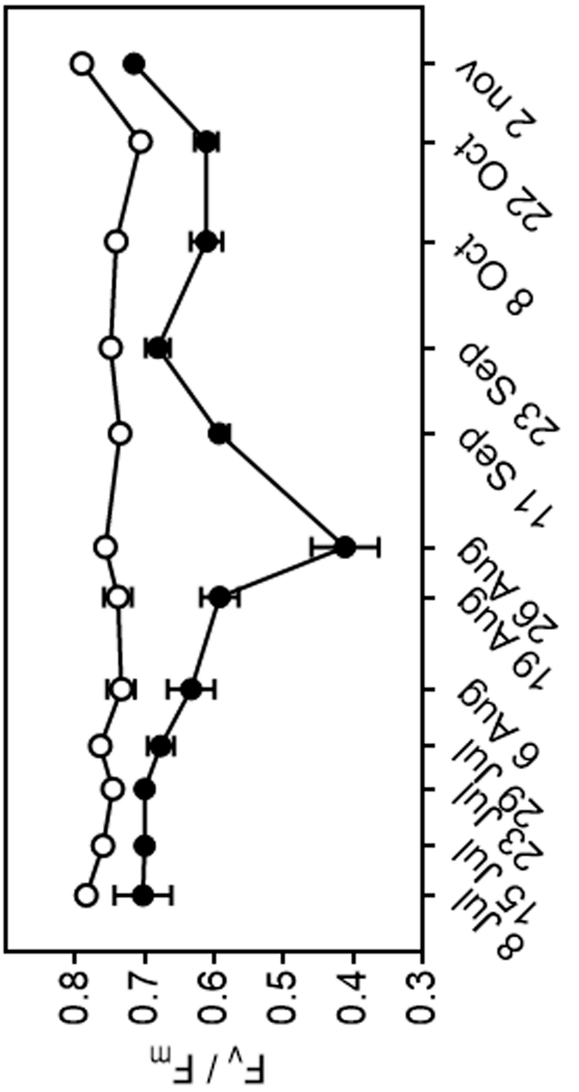
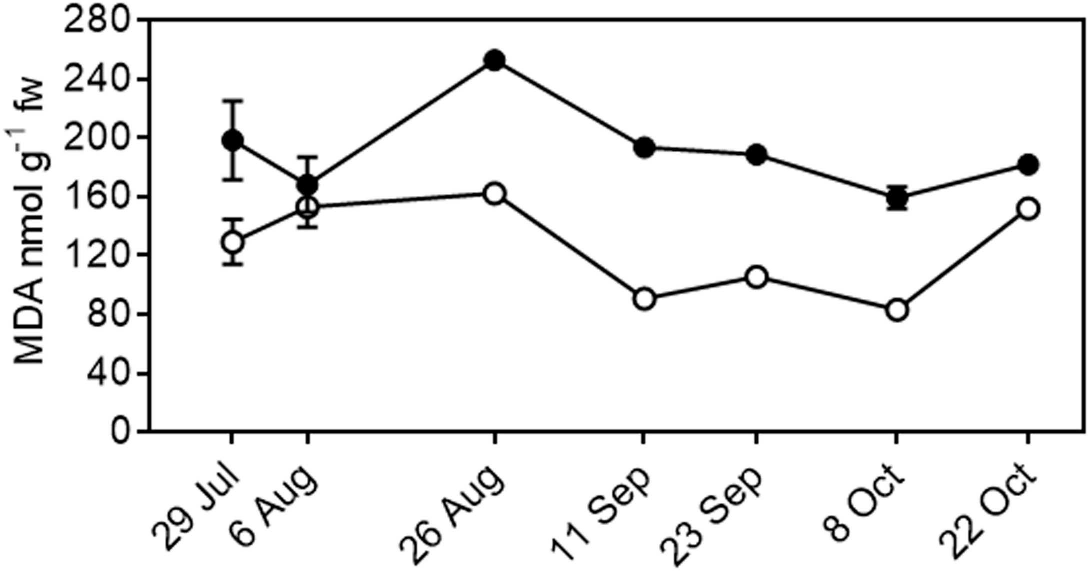

F_v / F_m
0.8
0.7
0.6
0.5
0.4
0.3
8 Jul
15 Jul
23 Jul
29 Jul
6 Aug
19 Aug
26 Aug
11 Sep
23 Sep
8 Oct
22 Oct
2 nov

Figure 4


280
240
200
160
120
80
40
0
MDA nmol g-1 fw
29 Jul
6 Aug
26 Aug
11 Sep
23 Sep
8 Oct
22 Oct

Tables

Table 1 Representative result of the vegetational survey and of soil temperature and pH recording (measured at 10 cm depth) along a linear transect from the edge of a geothermal spring (site of most intense stress) to the closest individual of *Quercus suber*

	Distance (m)	Temperature (°C)	pH
Aphytoic area	0 - 4.5	> 52	< 2.35
Calluna vulgaris	4.5 - 8	≤ 52	≥ 2.35
Agrostis castellana *Calluna vulgaris*	8 - 11	≤ 47	≥ 3.0
Cistus salvifolius *Erica arborea* *Erica scoparia*	11 - 14	≤ 36	≥ 3.7
Quercus suber	> 14	≤ 34	≥ 3.9

Table 2 Soil temperature and pH measured in the rhizosphere of selected plants of *C. vulgaris* from A_c and A_s. Within each area, three individuals grew near the margin facing the closest geothermal spring ('spring side') and the others at the opposite side, i.e. along the edge farther from the spring ('wood side'). Values of both mean soil temperature and pH differed significantly between A_c and A_s

Plant #	Area	Side	Soil T (°C)	Soil pH
1	A_c	Spring	25.3	4.20
1	A_s	Spring	38.6	2.35
2	A_c	Wood	22.8	4.45
2	A_s	Wood	36.8	3.70
3	A_c	Spring	24.7	4.29
3	A_s	Spring	42.0	2.41
4	A_c	Wood	23.3	4.41
4	A_s	Wood	36.0	3.48
5	A_c	Spring	24.2	4.22
5	A_s	Spring	38.0	2.37
6	A_c	Wood	23.0	4.43
6	A_s	Wood	37.4	3.65

Table 3 Concentration (ppm, except N = %) of the main mineral nutrients in the soil of A_s and A_c. Mean SD of the analytical values was ± 6.8%.

	N	P	K	Ca	Mg	S	Fe	Zn	Mn	Cu	Cl
A_s	0.67^{**}	500^{*}	5100^{***}	900^{*}	2200^{***}	790^{**}	9300^{*}	9.66^{*}	80	9.5^{*}	19^{*}
A_c	4.22	1600	10900	4800	3800	1374	27100	30	100	13.3	204

Asterisks indicate that the concentration of an element differs significantly between A_s and A_c ($^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$)

Table 4 Mean stomatal conductance (mmol H_2O m^{-2} s^{-1} ± SD) of the selected plants of *C. vulgaris* in A_c and A_s (*n* = 20 per area). Values differed significantly between A_c and A_s. Within each area, the value of August 26 differed significantly from those of July 29 and September 23, whereas the latter were not significantly different

Date	A_c	A_s
Jul 29	195.02 ± 8.047	68.05 ± 2.417
Aug 26	92.67 ± 1.825	27.95 ± 3.491
Sep 23	113.10 ± 6.085	51.92 ± 3.155

Table 5 Summary of the results of the multiple linear regression analyses. Predictor variables were the cumulated rainfall and average temperatures, calculated as described in the text, while the dependent variables were photosynthetic efficiency (F_v/F_m) and lipid peroxidation (MDA). The level of significance was 0.05

						t stat P value	
Parameter	Area	Mean	r^2	Standard Error	ANOVA Significance F	Cumulated Rain	avg. T°
F_v/F_m	A_c	0.737	0.7068	0.014	0.0040	0.0012	0.0153
F_v/F_m	A_s	0.601	0.2704	0.079	0.2421	0.5778	0.3564
MDA	A_c	126.68	0.0134	35.357	0.9668	0.9748	0.8081
MDA	A_s	190.22	0.3802	26.115	0.3024	0.4396	0.145