Morpho-physiological plasticity contributes to tolerance of *Calluna vulgaris* in an active geothermal field

Running head: *C. vulgaris* adaptations in a geothermal field

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Abstract

Geothermal alteration fields are very prohibitive environments, limiting vegetation establishment and growth. In this study, the ecological specialisation of the pioneer plant *Calluna vulgaris* was investigated, assuming that its ability to survive in geothermal habitats derives from a fine regulation of morpho-physiological traits. Mature leaves of *C. vulgaris* were sampled from plants close to a fumarole (near), and from plants living some meters (intermediate) or about 1 km (distant) distant. Along the sampling sites, a gradient of soil pH and temperature values occurred: near plants facing the highest soil temperature and the lowest soil pH. A regulation of constitutive morpho-anatomical and physiological traits in response to different stress levels occurred. A progressive reduction of leaf exposed surface and hair density and mucilages, combined with a gradual increase of oxidative stress levels, of phenols and ascorbate was observed from distant to near plants. Near plants showed an increase in stomatal density, in lignin and cuticle thickness and the highest activity of ascorbate peroxidase. Except for high glutathione levels, in distant plants antioxidant machinery was consistently less active. The apparent morphological and physiological plasticity demonstrated in this research contributed to the capability of these plants to tolerate the prohibitive, highly changing, environmental conditions of the geothermal field.

Introduction

Geothermal alteration fields, present in different areas of the world, are interesting environments with utility for electricity generation (Bertani 2012), direct heat applications (Lund *et al* 2011), as well as for tourism and conservation of unique habitats characterized by distinctive vegetation.

They are restrictive environments with a combination of both chemical and physical stressors. Steep soil temperature gradients, low pH values, low content of carbon and nitrogen, high heavy metal concentration in soil, limit the establishment, growth and development of surrounding vegetation (Convey *et al.* 2000; Boothroyd 2009). The substratum is a siliceous gravel with a coarse texture, superficially altered by chemical processes and heated up to 60°C at about 30 cm of soil depth (Selvi and Bettarini 1999). In addition, the emission of water steam mixed with different gases, toxic elements and compounds (e.g. CO_2, CH_4, NH_3, H_2S, Rn, As, Hg, B, etc.) are common in an active geothermal area (Loppi 2001) and strongly contribute to the evolution of specialised flora that live in this environment. The pioneer plant communities are exclusively grass-shrub type, and colonise areas of geothermal alteration in a mosaic pattern which is a function mainly of the intensity of fumarolic emissions, soil temperature and acidity (Bonini *et al*. 2005; Chiarucci *et al*. 2008); all these factors frequently lead to complex gradients in the environment.

High temperature and low pH of soil are restrictive for plant development, inhibiting root growth, water and nutrient uptake (Huang and Xu 2000; Wu and Zhang 2002). High soil temperature inhibits hormone synthesis and transport (Udomprasert *et al*. 1995). In addition, this abiotic stressor induces more damage to leaves and accelerates more severe leaf senescence than high air temperature (Huang *et al*. 1998; Liu and Huang 2000). Furthermore the bio-availability of some heavy metals in soil is strongly affected by low pH (Wu and

Zhang 2002) and an increased toxicity to pollutants has been reported in conditions of soil acidity (Richards *et al*. 1998).

As in other stress conditions (Bowler *et al*. 1992; Zhang and Kirkham 1994; Bartoli *et al*. 2010), the constraints of a geothermal alteration field can cause oxidative damage by reactive oxygen species (ROS). Leaf injury has been associated with pronounced lipid peroxidation and decrease in antioxidative activity, particularly in plants with roots exposed to high temperatures (Huang *et al*. 2001).

In order to survive in stressful conditions, pioneer plants activate a wide range of responses at different levels of organisation (Feder and Hofmann 1999), consisting in altered gene expression, cell metabolism modifications, specific morpho-anatomical trait evolution and changes in growth rate and yield (Kacperska 2004). The study of these plants, able to live and reproduce in extreme environments, is of great interest and might be very useful also in attempts to improve stress tolerance in crops. However, though the knowledge of their adaptive strategies is of great interest, little information exists about plants living in a geothermal environment.

Calluna vulgaris (L.) Hull is the most frequent vascular plant inhabiting in the geothermal alteration field of Sasso Pisano (Italy), one of the most extreme geothermal alteration fields in terms of both soil acidity and hot temperatures (Chiarucci *et al*. 2008). It is an ericaceous acidophilic shrub, with a distribution through temperate oceanic and sub-oceanic climatic regions in western Europe, and in many natural and semi-natural heathland communities in the oceanic north west of Europe, growing on a wide range of superficially different soil types (Marrs and Bannister 1978). It is an interesting plant for soil decontamination, due to its ability to accumulate heavy metals, such as tungsten and antimony (Pratas *et al*. 2005), and radionuclides such as cesium (Bystrzejewska-Piotrowska *et al*. 2005).

On the basis of the above-mentioned considerations, we focused our study on *Calluna*

vulgaris plants growing in the area of Sasso Pisano. Taking into account that some leaf morphological traits are under both genetic and environmental control (Xu *et al*. 2008) and that restrictive conditions of geothermal fields are known as inducing oxidative stress, we wanted to address the following issues:

1) Do the leaves of *C. vulgaris* exhibit stress-induced morphogenic and physiological responses, aimed at avoiding or tolerating sudden and severe environmental condition changes? and

2) Do these responses change depending on the distance from geothermal hot spots?

We hypothesise that the ability of *C. vulgaris* plants to tolerate the extreme conditions of this unusual stressful environment is derived from a high acclimation capacity, mediated by a finely tuned regulation of morpho-physiological traits in response to the specific environmental conditions it grows. To test this hypothesis, plants living close to a fumarole, some meters or about 1 km distant, along a gradient of soil temperature and pH, were studied.

Materials and Methods

Site description

Plants of *Calluna vulgaris* (L.) Hull were collected in the geothermal field of Larderello (Pisa, Italy), near to Sasso Pisano town (Municipality of Castelnuovo Val di Cecina), in the site of Regional Interest (SIR B12, IT 5160103) named “Monterotondo Marittimo and Sasso Pisano geothermal fields”, hosting two types of natural habitats listed in the Natura 2000 EU directive: the “Forests of *Castanea sativa* Miller (cod 9260)” and the “Lava fields and natural cavities (cod 8320)” (Bonini *et al*. 2005). The sampling site is characterised by

emissions of steam vents containing H_2S, CO_2, boric acid and water vapour from cracks in the rocks with surface soil temperature above 50-60°C and extremely low pH values (pH 1.13) (Chiarucci *et al*. 2008).

The considered geothermal alteration field is part of the geothermoelectric basin of Larderello, in southern Tuscany. The sampling site is located in the upper valley of Cornia river, at an elevation of about 550 m a.s.l. The climate is Mediterranean, with a mean yearly temperature of 13.3°C (Barazzuoli *et al*. 1993) and a mean yearly rainfall of 1107.2 mm (Chiarucci *et al*. 2008). At the time of the sampling (May 2010), the mean monthly temperature recorded was 13.4°C (min: 8.9°C; max: 17.7°C) and the mean monthly rainfall was 218.00 mm (data from the "Osservatorio Meteo a norma O.M.M. di Castelnuovo Val di Cecina (PI)" available at http://www.castelnuovometeo.it/). The geological structure of the Larderello geothermal area is described in Bertini *et al*. (2006).

Plant material

Ten plants (similar in size, sun exposition, etc.) from the three different locations from the fumarole were considered:

- plants growing at a distance of 0.1-0.3 m from fumaroles (near plants);
- plants growing at a distance of 4-6 m from fumaroles (intermediate plants);
- plants growing out of the active geothermal alteration field, about 1000 m distant from fumaroles (distant plants).

From each experimental group, mature leaves (at the same developmental phenological state) were sampled in May 2010 and then considered for morphoanatomical and histochemical analyses (fresh material or chemically fixed). Material from the same plants was collected for physiological determinations (fresh materials or fixed in liquid nitrogen

and stored at -20°C until use). For physiological parameters at least three measurements of three independent extractions on mature leaves randomly collected from 10 individuals were made for each site.

Field conditions: soil temperature and pH determinations

Soil temperature was measured in correspondence of near, intermediate and distant plants at 10 cm of depth by a portable thermometer (Hanna Instruments 9060) daily in three different time period (7 a.m.; 1 p.m.; 8 p.m.). For pH determination soil samples (5–10 cm of depth) were collected in each site and sieved at 2 mm. A 1:2.5 soil-water suspension was used for a potentiometric measure of pH.

Light, fluorescence and scanning electron microscopy

Twenty leaves collected from ten individuals for each experimental group (two leaves per plant), were fixed for 24 h in FAA fixative (Formalin - Acetic acid - Alcohol), dehydrated in a graded ethanol series, and embedded in LR-White medium Grade (London Resin Company). Semi-thin sections (5 μm) were cut with an ultramicrotome Ultratome Nova LKB and stained with different dyes: Toluidine blue O (TBO) (0.05% in 100 mM benzoate buffer at pH 4.4) for general cytological investigations (Feder and O'Brien 1968), Periodic acid-Schiff (PAS)–TBO for non cellulosic polysaccharides detection (Jensen 1962; O' Brien and Mc Cully 1981), Sudan black B (0.07% in saturated solution of ethanol 70%) for lipid compounds detection (Bayliss and Adams 1972), Coomassie Brilliant Blue R-250 (0.05% in Carnoy solution) for protein staining (Fisher 1968), Ruthenium red (0.1%) for pectic substances characterisation (Jensen 1962) and Iodine-Potassium Iodide

(IKI) for starch detection (Ruzin 1999). The sections were cleared in xylene, air dried, mounted in DPX Mountant (Sigma) and then observed with a LEITZ DIAPLAN light microscope. Images of each slide were taken using a Leica DFC 420. In order to evidence essential oils and oleoresins, fresh leaf portions were treated with Nadi Reagent (David and Carde 1964) and subsequently observed and acquired.

For lignin detection, leaf sections were examined for autofluorescence with the epifluorescence microscope (Zeiss Axio Observer.Z1) in green excitation (excitation: 540 nm; emission: 615 nm) and the corresponding images were then captured with Axiocam MRc5. The exposure was strictly kept identical to insure the comparability among the different samples.

For semi-quantitative histochemical studies, twenty images of slices randomly selected from each sample were captured, and at least 200 cells for each sample were analyzed and processed by computer-aided image analysis system (ImageJ software) in order to evaluate the arbitrary units of integrate density, corresponding to the staining intensity.

For groove width determination, two cross histological sections of the middle region of 20 leaves randomly collected from 10 individuals (two leaves for each plant) were analyzed by using the open source UTHSCSA Imagetool software, available at http://ddsdx.uthscsa.edu/dig/itdesc.html.

Stomatal and hair densities into the groove were calculated according to Aronne and De Micco (2001), with some modifications. In detail, stomatal and hair densities were calculated on 20 serial cross sections by counting respectively the number of stomata and hairs occurring along the sections and measuring the length of the groove. Considering the section thickness (5 µm), the number of serial sections analysed (20) for each determination, the observed stomata or hairs and the length of the groove, stomatal and hair densities were calculated and respectively expressed as stomata or hairs *per* mm^2.

Hairs on the adaxial surface were counted three-dimensionally with the adjustment of the microscope plane of focus. The analysed slices belonged, for each group, to the same middle region of the leaf, that is the most representative part, including the highest width of the groove and most of the photosynthetic tissues and vascular bundles. For calculating mean and standard error, the average stomatal and hair densities, and the average groove width for each individual plant were one statistical observation.

For scanning electron microscopy (SEM) observations, the samples were fixed in 3% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 24 h and then dehydrated in a graded ethanol series. After critical point drying, whole leaves and portions were coated with gold, examined and captured with a JEOL/JSM-5410 scanning electron microscope.

Determination of water content and of relative water content

Calculations of leaf fresh weight, dry weight and moisture content were based on weights determined before and after oven drying (100°C) of leaf samples to constant weight. Water content percentage was estimated on the fresh weight basis. Leaf relative water content (RWC; Turner 1981 with minor modifications) was calculated with the formula:

RWC = [(FW-DW)/(TW-DW)] x 100

FW = Fresh weight

DW = Dry weight

TW = Turgid weight

Fresh weight was obtained by weighing the fresh leaves. The leaves were then immersed in water over night, blotted dry and then weighed to get the turgid weight. The leaves were

then dried overnight in an oven at 100°C to constant weight and reweighed to obtain the dry weight.

Chlorophyll and carotenoid determination

Chlorophylls (*a*, *b* and total) and carotenoids were extracted in 80 % acetone and determined according to Hassanzadeh *et al*. (2009) and to Lichtenthaler (1987) respectively. 100 mg of fresh leaves were homogenised and the extracts were centrifuged for 10 min at 6000 x g at 4°C. The supernatants were collected and the pellets were resuspended and extracted with 80% acetone until they resulted colourless. The collected supernatants were read using spectrophotometer at 645, 663 and 470 nm. Pigment contents were expressed as mg g^{-1}DW.

Hydrogen peroxide and lipid peroxidation

H_2O_2 content of leaves was determined according to Jana and Choudhuri (1982) using titanium chloride in H_2SO_4 for peroxide detection. The intensity of the yellow color of the supernatant was measured at 410 nm. The amount of H_2O_2 in the extracts, calculated from a standard curve, was expressed as μmol g^{-1}DW.

The amount of lipid peroxidation products in leaves was estimated by determining the malonyldialdehyde (MDA) content in the leaves according to Hartley-Whitaker *et al*. (2001) with minor modifications as in Spanò *et al*. (2007). Powder from freeze-dried leaves material was mixed with TBA reagent, heated (95°C), cooled and centrifuged. The level of MDA (155 $mM^{-1}cm^{-1}$ extinction coefficient) was measured as specific absorbance at 532 nm and by subtracting the non-specific absorbance at 600 nm (De Vos *et al*. 1989).

Non enzymatic antioxidants

Level of phenolic compounds was calculated as equivalent of gallic acid (GAE mg g^{-1}DW) according to Arezki *et al*. (2001) using the Folin-Ciocalteu reagent.

Ascorbate (ASA) and dehydroascorbate (DHA) extraction and determination were performed according to Kampfenkel *et al*. (1995) with minor modifications. Total ascorbate (ASA+DHA) was determined at 525 nm after reduction of DHA to ASA by dithiothreitol and DHA level was estimated on the basis of the difference between total ascorbate and ASA value. Calculations were made on the base of a standard curve and ascorbate content was expressed as mg g^{-1}DW.

Glutathione was extracted and determined according to Gossett *et al*. (1994). Total glutathione (GSH+GSSG) was determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure (Anderson *et al*. 1992) and the reaction was monitored as the rate of change in absorbance at 412 nm. GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. Calculations were made on the basis of a standard curve and content was expressed as nmol g^{-1}DW.

Enzymatic antioxidants

For enzyme extraction acetonic powders were made according to Saari *et al*. (1996) with modifications. Briefly, leaves were homogenised in ice-cold acetone. Homogenates were filtered through a Buckner filter with Macherey-Nagel MN 618 filter paper under vacuum and washings were repeated until the powder resulted colourless. After complete removal of acetone under vacuum, acetonic powders were extracted in 100 mM potassium

phosphate buffer pH 7.5 containing 1 mM EDTA, and 1% (w/v) polyvinylpyrrolidone (PVP-40) as in Spanò *et al*. (2011). All the extractions were performed at 4°C. The homogenate was then centrifuged at 15000 x g for 20 min. For ascorbate peroxidase, 2 mM ascorbate was added to the extraction medium. For glutathione reductase the supernatant was desalted on a Sephadex G-25 column. Supernatants were collected and stored in liquid nitrogen until their use for enzymatic assays.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) with modifications. Enzyme activity was assayed from the decrease in absorbance at 290 nm (extinction coefficient 2.8 $mM^{-1}cm^{-1}$) as ascorbate was oxidised. The reaction mixture contained 100 mM potassium phosphate pH 7.5, 0.5 mM ascorbate and enzyme extract (25 μg protein ml^{-1}). The reaction was started by adding 0.2 mM H_2O_2. Correction was made for the low, non enzymatic oxidation of ascorbate by hydrogen peroxide (blank).

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was determined as described by Nakano and Asada (1981). The activity of DHAR was determined by monitoring the glutathione-dependent reduction of dehydroascorbate. Enzymatic extract contained 12.5 μg protein ml^{-1}. The activity was determined by measuring the increase in absorbance at 265 nm for 3 min. Specific activity was calculated from the 14 mM^{-1} cm^{-1} extinction coefficient. A correction for the non-enzymatic reduction of DHA by GSH was carried out in the absence of the enzyme sample (blank).

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) was assayed as in Zhang and Kirkham (1996) by monitoring the decrease in absorbance at 340 nm because of the oxidation of NADH (extinction coefficient 6.2 mM^{-1} cm^{-1}). The reaction mixture contained: 0.1 mM NADH, 2.5 mM ASA, 50 mM Na-phosphate buffer pH 7.6 and 50 μg protein ml^{-1}. The reaction was started by the addition of ascorbate oxidase (4 U).

Correction was made by subtracting value obtained in the absence of ascorbate oxidase (blank).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined as described by Rao *et al*. (1995) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). Enzymatic extract contained 25 µg protein ml^{-1}. A correction for the non-enzymatic reduction of GSSG was carried out in the absence of the enzyme sample (blank).

Ascorbate oxidase (AO, EC 1.10.3.3) activity was measured as described by Mosery and Kanellis (1994). The activity was determined by monitoring ASA oxidation following the decrease in absorbance at 265 nm for 3 min. Enzymatic extract contained 50 µg protein ml^{-1}. Specific activity was calculated from the 14 mM^{-1} cm^{-1} extinction coefficient.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its reaction with that of GR, using as substrate 0.45 mM H2O2. The activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM-1 cm-1). Enzymatic extract contained 25 µg protein ml-1.

Catalase (CAT, EC 1.11.1.6) activity was determined as described by Aebi (1984). Enzymatic extract contained 25 µg protein ml^{-1}. A blank containing only the enzymatic solution was made. Specific activity was calculated from the 39.4 mM^{-1} cm^{-1} extinction coefficient.

All enzymatic activities were determined at 25°C and expressed as U g^{-1} protein. Protein measurement was performed according to Bradford (1976), using BSA as standard.

Statistical analysis

Statistical analyses were performed using statistical package Primer of Biostatistics (Glantz, Statistical Software Program Version 6.0, McGraw Hill 2005). Data were analysed

by the analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test, with values of $p<0.01$ sufficient to reject the null hypothesis

Results

Soil temperature and pH determination

The mean soil temperature ranged between 21.5°C and 38.2°C and the pH values ranged between 1.84 and 4.65 (Table 1). Near plants experienced the lowest soil pH (1.8) and the highest soil temperature (43°C). A gradual decrease in soil temperature and a gradual increase in soil pH was recorded from near to distant plants (Table 1).

Morpho-anatomical and histochemical leaf characterisation

C. vulgaris leaves from distant, intermediate and near plants appeared stiff, small, with inrolled margins, sessile and sagittate in shape, characterised by a deep and extended abaxial groove with long unicellular hairs, whose density was the lowest in near plants, and paracytic stomata, with a significantly higher density in near plants than in the other samples (Table 2). Stomata protruded into the groove by club-shaped and thin-walled subsidiary cells (Figs. 1c, f, i, j and 2a). The groove opening was wider in distant plants (Fig. 1b, c) than in intermediate and near ones (Fig.1e, f and h, i, respectively), these latter plants having however the greatest groove surface (Table 2). In all the samples, rare stomata occurred in the leaf adaxial surface.

Stiff and protective trichomes, localised along the leaf margins (Fig. 1) and at the middle of the adaxial surface (Fig. 2b), showed the highest density in distant plants. This was

reduced to half in intermediate plants and decreased more drastically in near plants (Fig. 1, Table 2). Glandular hairs, displaying a slight Nadi positivity to essential oils, were localised on the adaxial surface and along leaf margins (Fig. 2c) in all the samples.

Epidermis, constituted by thick-walled cells, had a roughened cuticle, particularly thick in leaves from near plants (Fig. 2d, e, f), and mucilages, localised between the plasmalemma and the inner periclinal cell walls (Figs. 1c, f, i and 3a-f). Total polysaccharides, mainly occurring in the thick outer periclinal walls and in the mucilages of these cells (Figs. 3a-c and 4), were significantly less abundant in near plants, that showed also the lowest content (less than half) of wall pectic polysaccarides compared with the other samples (Figs. 3d-f and 4).

Mesophyll was peripherally constituted by a palisade parenchyma, multilayered and tightly packed mostly beneath the abaxial leaf surface, and by lateral vascular bundles. A major central vascular bundle, strengthened by mechanical fibers at the side of the groove, was surrounded by a well developed aerenchyma (Fig. 1c, f, i, k). Palisade parenchyma chloroplasts showed a comparable protein content in all the samples (Figs. 3g-i and 4), lipid deposits only in intermediate plants (Fig. 3k) and starch only in distant plants (Fig. 3m). Epifluorescence analyses showed a significant increase of red fluorescence, due to lignin content, in the mesophyll cell walls and vascular bundles of near plant leaves with respect to distant and intermediate ones (Figs. 3 p-r and 4). Below living leaves, dead leaves remained attached closely to the stem in all the samples.

Leaf water content, RWC and pigments

Water content (Table 2) showed slight differences among near, intermediate and distant plants (61, 66 and 61% respectively). RWC (Table 2), on the other hand, was higher in

intermediate and distant plants (82%) than in near plants (70%). The content of chlorophyll *a*, *b*, of total chlorophyll and carotenoids was similar in all plants (Table 2).

Oxidative stress and antioxidant response

Plants near to the fumarole had higher levels of hydrogen peroxide (1.9 times higher than distant plants) and suffered a greater degree of lipid peroxidation than plants from further away (Table 3). Similarly plants near to the fumarole contained higher levels of the antioxidants phenols and ascorbate (Table 3). The content of ascorbate was particularly low in distant plants that, in contrast, had the highest content of total glutathione (Table 3), while intermediate plants showed the lowest value of this thiolic compound. Ascorbate was always present predominantly in the oxidised form with similar reduced/oxidised ascorbate ratio values (0.60, 0.52 and 0.62 for distant, intermediate and near plants respectively). Reduced/oxidised glutathione ratio reached the highest value in distant plants (6.73).

With the exception of CAT, all antioxidant enzymes had the lowest activity in distant plants (Table 3). APX had an activity approximately twofold higher in near than in intermediate and distant plants. On the contrary the activity of GR (Table 3) was about twofold higher in intermediate than in near plants. MDHAR, GPX and AO (Table 3) activities were slightly higher in intermediate than in near plants while DHAR activity (Table 3) was lower in intermediate than in near plants. The CAT activity (Table 3), very low in near plants, gradually increased with the distance from fumaroles.

Discussion

A peculiar feature shared by most plants from solfataras and fumaroles with strongly acid soil, is the development of xeromorphic adaptive traits (Selvi and Bettarini 1999). Thus *C. vulgaris* leaves from plants growing in the Sasso Pisano geothermal alteration field, characterised by particularly extreme values of soil temperatures and pH, displayed some xeromorphic traits such as reduced size, hairiness, thick cuticle, stomata mainly placed into a deep and protected groove. Leaf epidermal cells showed, moreover, thick periclinal walls mainly composed by pectins, which could be involved both in fixation of metal cations deriving from the environment (Pellerin and O'Neill 1998) and in water storage. Additionally, dead leaves attached to the lower portion of the stem may provide a protective shield to the upper living leaves, especially for plants close to fumarolic vents.

In accordance with the assumption that plasticity is essential for adaptation to heterogeneous environments (Xu et al. 2008), the expression of some of the previously mentioned traits was conveniently fine-tuned by plants living at different distances from geothermal hot spots, along a gradient of soil temperature and pH, in response to the more extreme environmental conditions. A progressive increase of cuticle thickness, a decrease of the groove opening and trichome density in the adaxial leaf surface occurred with increasing stress levels. Moreover, only in near plants, were significant reduction of hair density and an increase of stomatal density into the groove observed.

The reduction of hair densities and the increase of stomatal number into the groove with the increasing of the stress, may appear contradictory with the general response displayed by plants in regulating their water balance. However, considering that near plant leaves are subjected to high temperatures uneven both for intensity and for frequency, we can assume that these plants have the necessity not only to control water balance, but even more to find a way for leaf cooling, increasing the transpiration levels by the high stomatal density and the lower number of hairs. The reduced trichome number can also facilitate the prompt

dissipation of dangerous geothermal steam. In addition, the peculiar groove stomata accessory cells, varying their turgor, could increase the protrusion of stomata into the groove itself, further allowing a fast leaf cooling. The relatively high activity of AO and of the high level of DHA observed in near and intermediate plant leaves, support the hypotheses that stomata opening and closure has to be finely adjusted in these stressed plants, given that DHA is involved in the early activation of stomatal closure (Fotopoulos et al. 2008).

In the light of these considerations, C. vulgaris seems to give priority to defence strategies against high temperatures and toxic steams at the expense of controlling the leaf water content. Accordingly, near plants showed the lowest value of RWC, a useful indicator of the state of the water balance of plants (González and González-Vilar 2001), affecting photosynthetic physiology, in the same way as carbon assimilation and energy use (Lawlor and Cornic 2002) mainly when RWC level is below 80% (González and González-Vilar 2001) as in near plants. These alterations could produce changes in the metabolism involving also the use of different and uncommon storage substances such as pectins and mucilages. They may be remobilised as monosaccharide sugars, possibly important in short term osmotic and ionic adjustments (Clifford et al. 2002; Ghanem et al. 2010) and might provide a form of energy, when environmental conditions become prohibitive. This is consistent with the reduction of total polysaccharides, and mainly of pectins, and with the absence of chloroplast starch deposits in these more stressed plants. In spite of the less efficient water balance, near plants showed only a slight decrease in chlorophyll content and no histologically evident alteration of chloroplast morphology, indicating a good adaptability of C. vulgaris to fumaroles. Excluding significant variations in chlorophyll content, the increase of red fluorescence, observed mainly in the walls of palisade cells and vascular bundles of near plants, could be due to an increased lignin content, that could be

beneficial for leaves, resulting in greater structural rigidity and longer life of tissues (Polle et al. 1994). Indeed lignin production can be considered a putatively compensatory response to cell wall damage caused by oxidative stress (Denness et al. 2011).

As expected, considering the extreme environmental conditions typical of geothermal fields, oxidative stress was recorded in plants living in the proximity of the geothermal hot spots. Stress parameters gradually increased from distant to near plants, that showed the highest lipid peroxidation level, indicative of membrane damage, in accordance with the observed severe lipid oxidation in conditions of high soil temperature (Huang et al. 2001). Membranes are surely a target of oxidative stress and their repair is an important adaptive trait (Hall 2002), and so the absence in near plants of intracellular deposits of lipids could be explained by their use in repair events. Accordingly, intermediate plants, less stressed than near ones can partially store lipid deposits, absent in distant plants that do not need to accumulate them.

As H_2O_2 level was well correlated with the level of MDA, environmental conditions of an active geothermal field might lead to oxidative stress via lipid peroxidation mediated by ROS. On the other hand, ROS accumulation during abiotic stresses has also been proposed as part of the signalling cascade leading to common stress responses in plants (Prasad et al. 1994; Mittler 2002) that have developed a complex protective system, including antioxidants and enzymes.

A good ability to counteract oxidative stress is detected in near plants which had a high antioxidant molecule content and antioxidant enzyme activity. Antioxidative response, though not able to avoid oxidative stress, was however sufficient to allow survival and growth of near plants. Antioxidant machinery was consistently less observed in distant plants with the one interesting exception of the high level of glutathione. Distant plants had not only the highest level of this antioxidant but also the highest reduced/oxidised

glutathione ratio, keeping a pool of easily available reducing power. Unexpectedly, CAT activity was lower in plants in strict proximity of fumaroles than in plants from further away. However, it is known from literature that peroxisomal CAT protein is very sensitive to salt and high temperatures stress (Hertwig et al. 1992; Foyer and Noctor 2000) and that leaf catalase is also sensitive to high radiation levels (Polidoros and Scandalios 1997). The idea that toxic metals can deplete antioxidative defences with inhibition of the activity of CAT and other antioxidant enzymes, has already been expressed (Schutzendubel and Polle 2002). So the extreme conditions of the geothermal environment could have an inhibitory effect on this enzyme. Consistently, a higher activity was detected in plants, far away from fumaroles. APX could have a more important role than CAT in the control of ROS during stress (Gill and Tuteja 2010); in fact it was the main H_2O_2-scavenging enzyme in the most stressed near plants and was assisted by GPX that could play an important role in the control of hydrogen peroxide in intermediate plants. These plants were characterised also by a higher GR activity and so glutathione metabolism could be more active in intermediate than in near plants.

In conclusion, the results of this study underline the presence in *C. vulgaris* leaves of a complex adaptive response to the peculiar environment of the geothermal alteration field, where plants have often to mediate between opposite requirements. This response results from the integration of leaf morpho-anatomical characters and physiological adjustments.

The fine-tuned regulation of morphological traits including stomatal and hair density, along with the content/activity of antioxidant molecules/enzymes contribute to the capability of these plants to tolerate the restrictive, highly changing, environmental conditions that characterise this habitat.

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Legends of figures

Fig. 1. SEM micrographs of adaxial and abaxial leaf surfaces in distant (a, b), intermediate (d, e), and near (g, h) plants of *C. vulgaris*. Cross medial section of distant (c), intermediate (f), and near (i) plant leaves. Stomata with large subsidiary cells and hairs localized into the groove (j) and mechanical fibers associated to the major central vascular bundle (k) in a leaf from an intermediate plant (TBO staining); sc: subsidiary cells, fb: fibers, vb: vascular bundle, gr: groove, ae: aerenchyma, h: hair.

Fig. 2. Leaf epidermis characteristics in *C. vulgaris* evidenced by SEM (a, b) and light microscopy (c, d, e, f). Hairs and stomata into the groove (a), hairs localised on the adaxial leaf surface (b) from an intermediate plant leaf. Glandular hairs (c) on the adaxial surface of a distant plant leaf (Nadi staining). Cross sections of *C. vulgaris* leaves showing cuticle and mucilages in epidermal cells of distant (d), intermediate (e) and near (f) plant leaves (TBO staining); ct= cuticle, mc= mucilages.

Fig. 3. Variation in *C. vulgaris* leaf anatomy of distant (a, d, g, j, m, p), intermediate (b, e, h, k, n, q) and near (c, f, i, l, o, r) plants to fumarole vents as evidenced by: total polysaccharides (wall polysaccharides and mucilages; a, b, c; PAS-TBO double staining); pectic substances (d, e, f; Ruthenium red staining); proteins (g, h, i; Coomassie Brilliant Blue R-250 staining); lipids (j, k, l; Sudan black staining); starch (m, n, o; IKI staining); and lignin epifluorescence (p, q, r;). sc: subsidiary cells, fb: fibers, vb: vascular bundle, gr: groove, ae: aerenchyma, h: hair.

Fig. 4. Semiquantitative analyses of carbohydrates, pectins, total proteins and lignine contents (arbitrary units) in *Calluna vulgaris* leaves, from distant, intermediate and near plants after staining with PAS/TBO, Ruthenium red, Coomassie or in autofluorescence respectively. Different letters show means that are significantly different at $p < 0.01$.

Figure 1

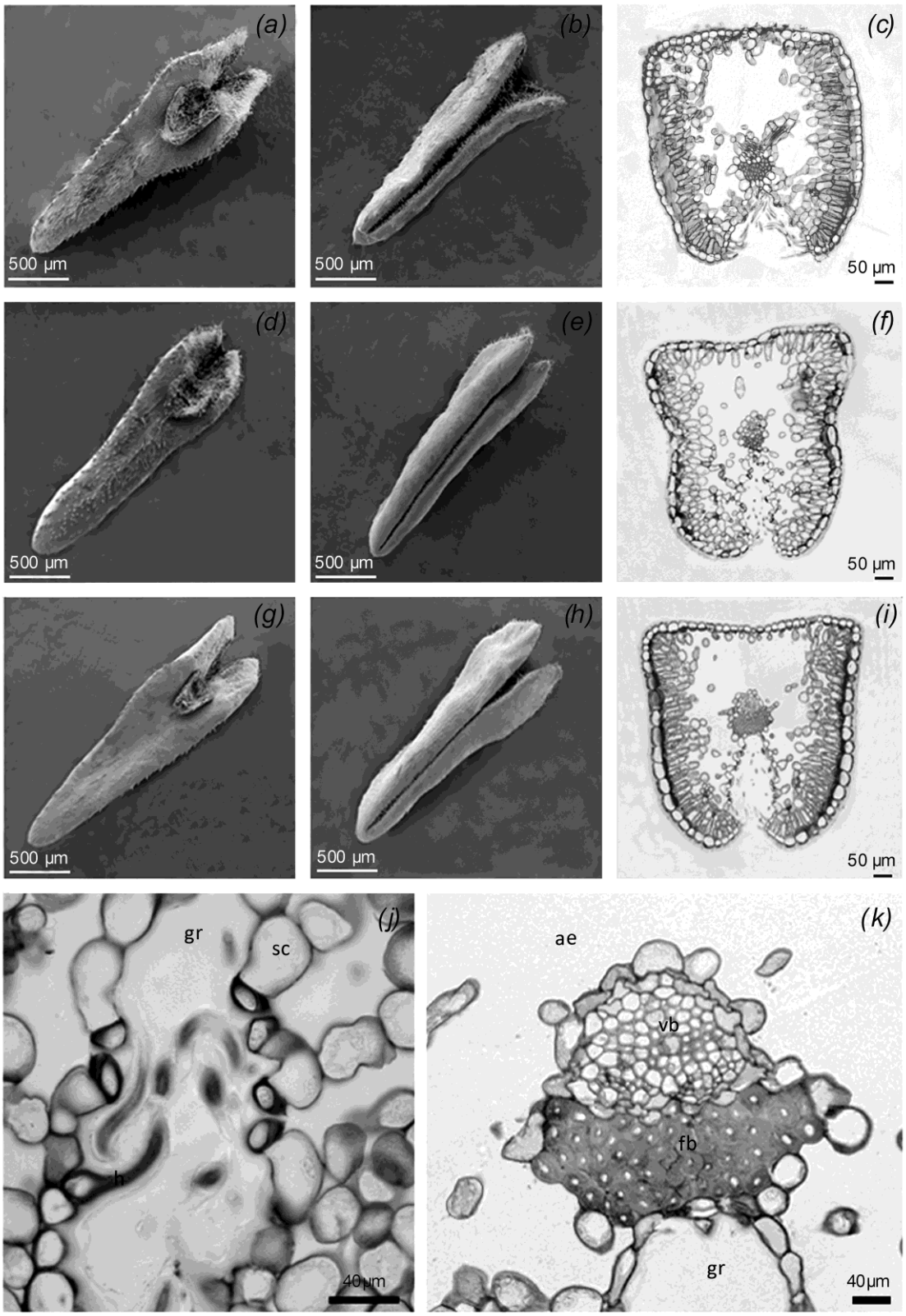
(a)
500 µm
(b)
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Figure 2

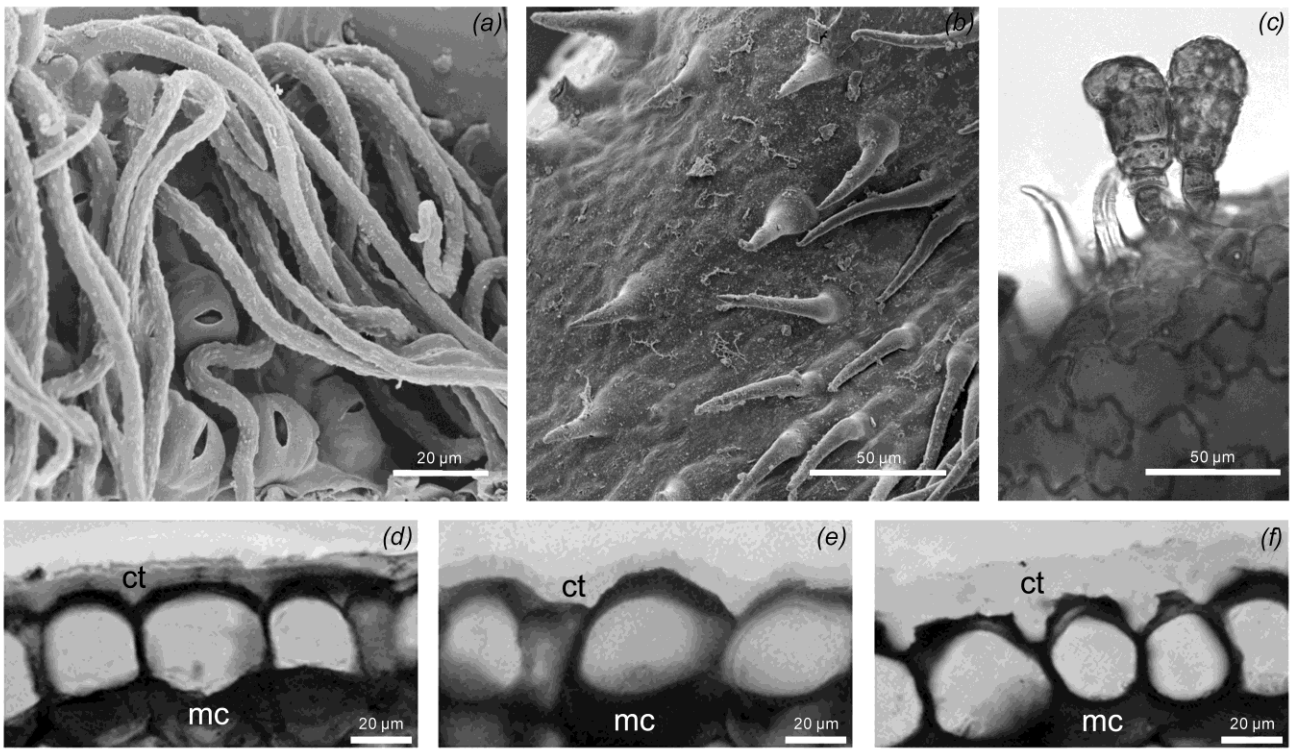
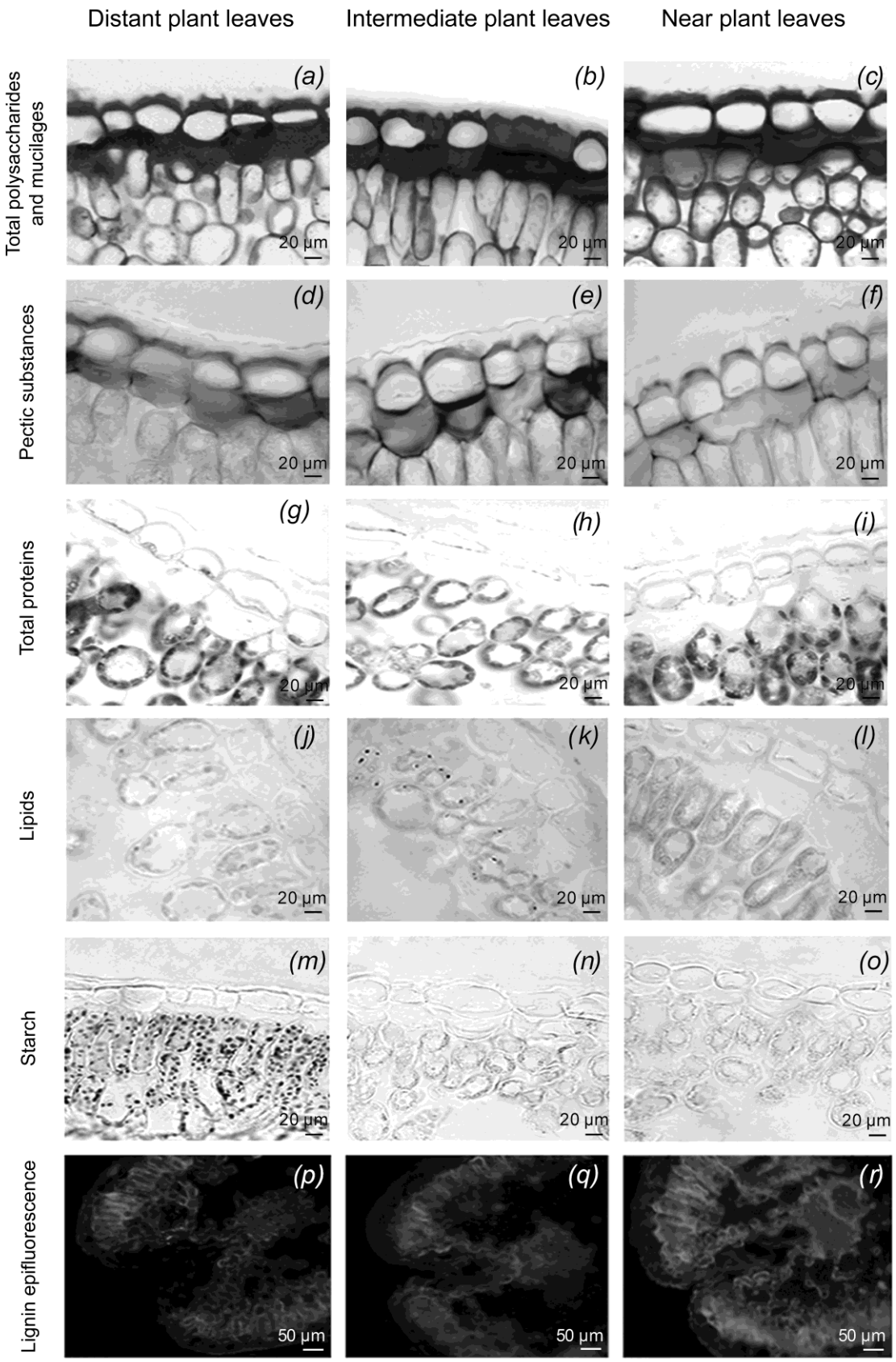


Figure 3


Distant plant leaves
Intermediate plant leaves
Near plant leaves
Total polysaccharides and mucilages
Pectic substances
Total proteins
Lipids
Starch
Lignin epifluorescence
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
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(p)
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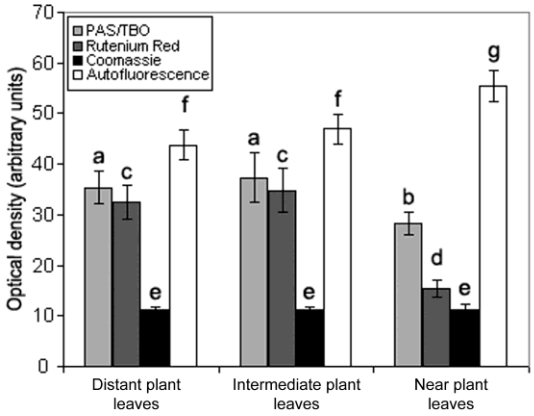
(r)
20 µm
50 µm

1

Figure 4


PAS/TBO
Ruthenium Red
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Autofluorescence
Optical density (arbitrary units)
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Distant plant leaves
Intermediate plant leaves
Near plant leaves

Table 1. Soil temperature and pH values in the three sampling sites

Data are means (± SE). Different letters indicate significant differences ($p<0.01$). Distant plant site: 1000 m from fumaroles. Intermediate plant site: 4-6 m from fumaroles. Near plant site: 0.1-0.3 m from fumaroles

	Distant plant site	Intermediate plant site	Near plant site
Soil temperature (°C)	21.50 ± 0.45^{a}	30.40 ± 1.08^{b}	38.20 ± 1.08^{c}
Temperature range (min-max)	20-24	25-35	31-43
Soil pH	4.65 ± 0.23^{a}	3.82 ± 0.13^{b}	1.84 ± 0.09^{c}

Table 2. Comparison of morphological and physiological parameters in leaves from plants of *C. vulgaris* from three distances to the geothermal sites

Data are means (± SE). Different letters indicate significant differences ($p<0.01$). Distant plants: plants growing 1000 m from fumaroles. Intermediate plants: plants growing 4-6 m from fumaroles. Near plants: 0.1-0.3 m from fumaroles

Leaf trait	Distant plants	Intermediate plants	Near plants
Groove stomatal density (No.mm^{-2})	2271.0±40.3^{a}	2234.9±75.5^{a}	2516.9±45.3^{b}
Adaxial hair density (No. mm^{-2})	820.0±37.4^{a}	420.0±23.6^{b}	65.0±16.6^{c}
Groove hair density (No. mm^{-2})	3105.0±129.6^{a}	2839.6±230.8^{a}	2085.8±99.8^{b}
Groove width (μm)	339.2±6.0^{a}	355.6±12.6^{a}	404.3±10.5^{b}
Water content (%)	61.0±0.5^{b}	66.0±2.0^{a}	61.0±1.0^{b}
Relative water content (RWC, %)	82.0±1.5^{a}	82.0±1.0^{a}	70.0±1.0^{b}
Chlorophyll *a* (mg g^{-1}DW)	1.67±0.06^{a}	1.74±0.06^{a}	1.54±0.05^{b}
Chlorophyll *b* (mg g^{-1}DW)	0.77±0.06^{a}	0.79±0.06^{a}	0.72±0.05^{a}
Total chlorophylls (mg g^{-1}DW)	2.44±0.10^{a}	2.53±0.10^{a}	2.26±0.10^{b}
Carotenoids (mg g^{-1}DW)	0.46±0.05^{a}	0.50±0.06^{a}	0.50±0.06^{a}

Table 3. Comparison of physiological parameters in leaves of *C. vulgaris* collected from three distances to the geothermal site

Malonyldialdehyde (MDA), equivalent of gallic acid (GAE), ascorbate (ASA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidised glutathione (GSSG), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalase (CAT), ascorbate oxidase (AO). Data are means (± SE). Different letters indicate significant differences ($p<0.01$). Distant plants: plants growing 1000 m from fumaroles. Intermediate plants: plants growing 4-6 m from fumaroles. Near plants: 0.1-0.3 m from fumaroles

Physiological trait	Distant plants	Intermediate plants	Near plants
Hydrogen peroxide (μmol g^{-1}DW)	178.67^{c}±1.54	384.56^{b}±3.61	509.89^{a}±3.89
Lipid peroxidation (MDA nmol g^{-1}DW)	714.00^{c}±17.00	1057.50^{b}±46.50	1351.00^{a}±55.01
Phenols (mg GAE g^{-1}DW)	20.13^{c}±0.23	21.50^{b}±0.25	31.80^{a}±0.46
AsA (mg g^{-1}DW)	3.96^{c}±0.07	6.10^{b}±0.17	8.80^{a}±0.40
DHA (mg g^{-1}DW)	6.59^{c}±0.12	11.71^{b}±0.18	14.19^{a}±0.60
GSH (nmol g^{-1}DW)	131.13^{a}±2.75	11.15^{c}±0.01	30.15^{b}±0.55
GSSG (nmol g^{-1}DW)	19.49^{a}±0.01	4.41^{c}±0.01	10.51^{b}±0.01
APX (U g^{-1} protein)	230.01^{b}±12.01	214.02^{b}±14.01	448.01^{a}±5.02
MDHAR (U g^{-1} protein)	29.50^{c}±2.50	68.34^{a}±0.44	53.80^{b}±3.00
DHAR (U g^{-1} protein)	400.01^{c}±6.01	632.03^{b}±1.80	691.01^{a}±0.80
GR (U g^{-1} protein)	32.00^{c}±0.10	135.50^{a}±0.01	64.50^{b}±0.01
GPX (U g^{-1} protein)	341.50^{c}±6.50	680.01^{a}±10.00	639.00^{b}±0.01
CAT (U g^{-1} protein)	40.79^{a}±0.03	27.23^{b}±0.02	13.62^{c}±0.01
AO (U g^{-1} protein)	29.00^{c}±1.00	60.01^{a}±0.10	51.40^{b}±0.01