Morpho-anatomical and physiological traits of *Agrostis castellana* living in an active geothermal alteration field

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Running title: *Agrostis castellana* of an active geothermal alteration field

Abstract

Agrostis castellana is one of the few plants colonizing one of the most extreme geothermal alteration fields characterized by low pH and high temperature of soil.

The study of species surviving in these multi-stress habitats can help to know more in deep the adaptive ability of plants. In this work morpho-anatomical and physiological traits of leaves of *A. castellana* living few meters from fumaroles were studied, focusing on their putative ecological significance.

Some typical xeromorphic traits occurred in the leaves of these plants: abundant cutinisation, cell wall thickening, slightly convolute and adaxially ribbed leaf blades, tissutal reinforcements by sclereids, pubescence, protected stomata and densely packed mesophyll. Additionally abundant pectins, important in water balance adjusting and as monosaccharide source, were observed in epidermal cell walls. Despite the low value of relative water content, indicative of a disturbed hydric state, oxidative damage was significantly lower than in other plants of the same environment, probably tanks to an adequate antioxidant response based mainly on enzymatic machinery. Interestingly catalase activity was not inhibited by extreme conditions of the geothermal alteration field as in other plants of the same habitat. In conclusion a cooperation of xeromorphic traits and antioxidant response seems to make *A. castellana* able to survive in such a prohibitive environment.

Key words: *Agrostis castellana*, antioxidant response, catalase, geothermal alteration field, pectins, xeromorphic traits

Introduction

Geothermal alteration fields are extremely restrictive environments where both chemical and physical stress factors coexist. High soil temperature and acidity, local intensity of gas emissions and ground micromorphology are the main stressors determining vegetation composition (Selvi & Bettarini 1999; Bonini et al. 2005; Chiarucci et al. 2008). Moreover 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) frequently experienced in an active geothermal area (Loppi 2001), contributes to the selection of the specialised flora typical of the geothermal areas. The study of plants of these sites is of particular interest to have a whole picture of adaptive mechanisms in natural habitats, that could not be adequately described by investigations made applying a single stress factor to plants under laboratory controlled conditions (Miller et al. 2010). In fact, in natural habitat, different stressors often coexist inducing a specific response, different from the sum of responses to individual stress factors (Rizhsky et al. 2002). The few plants living in geothermal environment activate a wide range of responses at different levels of organization (Feder & Hofmann 1999) developing specific morpho-anatomical and physiological traits (Bartoli et al. 2013; Bartoli et al. 2014) that help species to cope the geothermal constraints.

The prohibitive conditions of geothermal alteration field can induce the overproduction of reactive oxygen species (ROS) resulting in oxidative damage (Bartoli et al. 2013; Bartoli et al. 2014), counteracted by multiple antioxidant defence mechanisms, both enzymatic and non-enzymatic. Plant defence is strictly dependent on species and distance from fumaroles (Bartoli et al. 2013; Bartoli et al. 2014) with distinct antioxidants having different importance.

Agrostis castellana is a perennial caespitose grass that, as few other pioneer plants (eg. *Calluna vulgaris* and *Cistus salviifolius*) colonises the geothermal alteration field of Sasso Pisano (Tuscany, Italy), one of the most extreme geothermal alteration fields in terms of both soil acidity and hot temperatures (Chiarucci et al. 2008). The aim of this work is to analyze the morpho-anatomical and physiological traits of *A. castellana* leaves, focusing on their putative ecological significance.

Materials and Methods

Site description

The collection site was the geothermal alteration field (Fig. 1a) near to Sasso Pisano town (Castelnuovo Val di Cecina, Pisa province, Italy), in the site of Regional Interest (RIS 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 characterized by emissions of steam vents containing H_2S, CO_2, boric acid and water vapour from cracks in the rocks that contribute to soil overheating and acidification (Chiarucci et al. 2008).

The considered geothermal alteration field is part of the geothermoelectric basin of Larderello, in southern Tuscany. The geological structure of the Larderello geothermal area is described in Bertini et al. (2006).

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 annual temperature of 13.3 °C (Barazzuoli et al. 1993) and a mean annual rainfall of 1107 mm (Chiarucci et al. 2008).

At the time of the sample collection (May 2013), the mean monthly temperature recorded was 12.9 °C (min: 8.8 °C; max: 17.6 °C) and the mean monthly rainfall was 120.0 mm (data from the weather station of Castelnuovo Val di Cecina, Pisa available at http://www.castelnuovometeo.it/) and the monthly averaged daily mean global insulation upon a normal surface was 7780.1 Wh/m^2 (data from ‘‘Atlante Italiano della radiazione solare’’, edited by ‘‘ENEA, Agenzia nazionale per le nuove tecnologie, l’energia e lo sviluppo economico sostenibile’’, available at http://www.solaritaly.enea.it/index.php). In the sampling site, the daily temperature of the soil was recorded 30.4 °C (min: 25 °C; max: 35 °C) and soil pH was 3.8 (Bartoli et al. 2013)

Experimental plant material

Ten healthy plants (8-10 tillers) of *A. castellana* (Fig. 1.b), similar in size and growing at a distance of some meters from fumaroles, were screened and fully expanded mature leaves (at the same developmental phonological state) were sampled and then considered for morphoanatomical and histochemical analyses (fresh material or chemically fixed) and for physiological determinations (fresh materials or fixed in liquid nitrogen and stored at -20°C until use).

Light microscopy

Leaf portions from the leaf sheath and the leaf blade were excised from *A. castellana* leaves and were fixed for 24 h in FAA fixative (10 % formaldehyde–5 % acetic acid–45 % ethanol), dehydrated in a graded ethanol series, and embedded in LR-White medium Grade (London Resin Company). Semi-thin sections (3 μm) were cut with an ultramicrotome (Ultratome Nova LKB Producter, Bomma, Sweden) and stained with different dyes, as follows: toluidine blue O (TBO) (0.05% in 100mM benzoate buffer at pH 4.4) for general cytological investigations (Feder & O'Brien 1968), Sudan black B (0.07% in saturated solution of ethanol 70%) for lipid compounds detection (Bayliss & Adams 1972), Coomassie brilliant blue R-250 (0.05% in Carnoy solution) for protein staining (Fisher 1968), Ruthenium red (0.1%) for pectic-substance 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. At least 100 histological sections for each experimental group were analysed. Images of each slide were taken using a Leica DFC 420.

Epidermal stripping of both adaxial and abaxial surfaces of fresh leaves were made to determine stomatal density (SD), expressed as the number of stomata per square millimetre leaf area (Radoglou et al. 1990) and hair density (HD), expressed as the number of hairs per square millimetre leaf area. For each experimental group, 20 leaves collected from the selected plants (10 individuals) were analysed. Counts were made on both surfaces of the leaves for a total of 40 counts per experimental group.

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 of leaf samples at 100°C for 24 h. 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 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 & Choudhuri (1982) using titanium chloride in H_2SO_4 for peroxide detection. The intensity of the yellow colour 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 □mol 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 (reduced form, GSH+ oxidized form, 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 base 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.

APX (EC 1.11.1.11) activity was measured according to Nakano & 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).

DHAR (EC 1.8.5.1) activity was determined as described by Nakano & 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).

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).

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 H_2O_2. 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}.

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.

Data analysis

All the experiments were performed at least in triplicate. Value in the tables indicates mean value ± SE based on three independent experiments and was significantly different as assessed 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

Morphoanatomical and histochemical leaf characterization

Mature leaves of *A. castellana* were simple and alternate (Fig. 1b). They consisted in a tubular sheath with free overlapping margins and in a lanceolate blade, slightly curved from both sides of the abaxially prominent mid vein and acuminate at the apex (Fig. 1b; Fig. 2 a, b).

The outer and the inner epidermises of the leaf sheath delimited a thin mesophyll, consisting in a wide aerenchyma interspersed with collateral bundles, accompanied by sclerenchyma (Fig. 2a, g). With respect to the cells of the inner epidermis (Fig. 2d), those of the outer one were lobed in shape (Fig. 2c) and evidenced thicker cuticle and walls (mainly the outer periclinal ones) (Fig. 2g). Additionally, outer epidermis showed elongate-sinuous phytolites arranged in linear rows (Fig. 2c) and a greater stomatal and hair densities than the inner epidermis (Table 1).

The adaxial side of the leaf blade was organized in more ribs alternating with furrows (Fig. 2b). The ribs, with single, centrally arranged vascular bundles, were symmetrically arranged on each side of the midrib. Both adaxial and abaxial epidermises evidenced a costal and intercostal zonation with fusiform cells, stomata, unicellular prickle-hairs and elongate-sinuous silica bodies arranged in rows (Fig. 2.e, f). However, with respect to the adaxial epidermis, the abaxial one was characterized by cells with thicker outer periclinal walls and cuticle (Fig. 2.h) and by lower stomatal and hair densities (Table 1). Between two consecutive adaxial ribs, linear rows of bulliform cells and slightly sunken stomata occurred along the vascular system (Fig. 2b, h). Bulliform cells, contained abundant pectins in their outer periclinal walls and little lipidic bodies in their extensive vacuoles (Fig. 2k,

m). Girders consisting in few sclerenchymatic fibres occurred underlying the epidermis at the adaxial tip of each rib and abaxially, at level of both vascular bundles and bulliform cell clusters (Fig. 2b). In the mesophyll, the photosynthetic parenchyma was not differentiated into palisade and spongy parenchyma (Fig. 2b, 2h). Its cells, containing abundant chloroplasts, well defined nuclei, little lipid deposits and no significant starch deposits (Fig. 2h, j, l, m), were tightly arranged, so that the mesophyll resulted densely packed, except at the level of the stomata, where well developed sub-stomatal chambers occurred (Fig. 2.h). The outermost mesophyll cells contained crystals (Fig. 2.i). The collateral vascular bundles showed a poorly differentiated xylem and few tracheal elements; they were surrounded by a mestome accompanied by a layer of round parenchymatic cells with chloroplasts (Fig. 2.h).

Leaf water content, RWC and pigments

Leaf water content was 62.67% and its relative water content (RWC) was 68.67% (Table 2). The content of chlorophyll *a*, *b*, and carotenoids were 2.54, 0.99, and 0.77 respectively (Table 2).

Oxidative stress evaluation

ROS production was monitored by detecting the concentration of hydrogen peroxide. The content of this molecule was about 200 μmol g^{-1}DW. The measurement of lipid peroxidation (Tab. 2), indirectly indicating membrane damage, was detected as TBARS and was 367.33 nmol g^{-1}DW.

Enzymatic and non-enzymatic antioxidants

The content of phenols and ascorbate/glutathione cycle metabolites were detected (Tab. 2). Phenol content, measured as gallic acid equivalents, was about 6 mg GAE g^{-1}DW, ascorbate pool was 2.13 mg g^{-1}DW, and glutathione pool was about 226 nmol g^{-1}DW. Both ascorbate and glutathione were predominantly in the oxidised form, with AsA/DHA and GSH/GSSG ratios of 0.85 each.

Among the hydrogen peroxide scavenging enzymes, APX and GPX had activities higher than 500 U g^{-1}protein while catalase had an activity that amounted on about 100 U g^{-1}protein (Table 2). DHAR activity was about 811 U g^{-1}protein and GR activity was 64.53 U g^{-1}protein.

Discussion

Geothermal alteration field of Sasso Pisano, as many other geothermal ecosystems in the world, is a multi-stress habitat that poses significant constraints to plant colonization and surviving. However, few pioneer species, including *Calluna vulgaris* and *Cistus salviifolius*, are able to live in this site, especially through the expression of specific morphoanatomical and physiological traits that can be conveniently modulated in response to specific and sudden environmental situations, sometimes having to mediate between opposite requirements (eg. toxicants defence, water balance) (Bartoli et al. 2013; Bartoli et al. 2014)). Selvi & Bettarini (1999) observed that most plant species of solfataras and fumaroles with strongly acid soil, developed xeromorphic traits that can make these plants more adapted to support various kinds of environmental stresses (Bomfleur et al. 2011; Bartoli et al. 2013; Bartoli et al. 2014). Accordingly, also *A. castellana* plants living some meters distant from geothermal manifestations exhibited some xeromorphic characters in their leaves: abundant cutinisation and cell wall thickening, leaf blades slightly convolute and adaxially ribbed with well developed bulliform cells and sclerenchymatic reinforcements, pubescence, protected stomata, densely packed mesophyll. Epidermis constitutes the first barrier against geothermal stressors. Thus, abundant cutinisation and wall thickening occurred mainly in the epidermal cells of the surfaces particularly subjected to geothermal constraints. Additionally, epidermal cell walls of *A. castellana* leaf blades evidenced abundant pectins, as observed also in the leaves of *C. vulgaris* and *C. salviifolius* plants living in the same geothermal site (Bartoli et al. 2013; Bartoli et al. 2014). Pectins can be involved both in fixation of metal cations deriving from the environment or in water storage (Pellerin & O'Neill 1998; Bartoli et al. 2013). In particular, abundant pectins occurred in bulliform cell walls and can represent a way to adjust in the short term the water balance of these cells that modulate the leaf rolling in response to the adverse and sometime unpredictable environmental conditions of geothermal habitats.

Thanks to the occurrence of sclerenchymatic fibres and silica bodies, both increasing tissutal stiffness and toughness (Turner 1994; Ma & Yamaji 2006), the leaves of *A. castellana* resulted less liable to be damaged. Silica, other than in tissutal strengthening, is involved in mitigation of many chemical and physical abiotic stresses (eg. salt, metal toxicity, nutrient imbalance, drought, high temperature, UV) (Banowetz et al. 2008; Ma & Yamaji 2006).

Both leaf blade and sheath of *A. castellana* leaves were amphystomatic and presented morphological and anatomical strategies aimed at stomata defence, such as stomata localization in furrows or in the inner surface of the leaf sheath, stomata sinking below the plane of the epidermis

and protection by hairs. These strategies are aimed to enhance boundary layers around leaves of *A. castellana* thereby buffering the evaporative demand of the atmosphere and retarding transpirational water loss (Hill 1998; Benz et al. 2006; Roth-Nebelsick 2007). Accordingly, an increased morpho-anatomical protection might explain the higher stomatal density occurring on the adaxial side than in the abaxial one of the leaf blade. Regarding pubescence, the thrichomes can also play a role in protection from excess radiation, including avoidance of overheating (Jordan et al. 2005).

A densely packed mesophyll was observed in *A. castellana* leaves. This trait, reducing the intercellular spaces, is useful in minimizing water transpiration and can improve photosynthetic process increasing the amount of chloroplasts per leaf surface unit. The photosynthetic process can be also improved by the abundant crystals occurring in the outer layer of the mesophyll. In fact, crystals in palisade cells, other than a reserve of Ca^{2+} for cell metabolism, can represent a tool to improve photosynthesis: druses have multiple, radial oriented facets with potential reflective properties that would help disperse light to the surrounding chloroplasts (Kuo-Huang et al. 2007).

While leaf water content was not significantly different from other plants living in the same environment (*Calluna vulgaris* and *Cistus salviifolius*, Bartoli et al. 2013; Bartoli et al. 2014 respectively), relative water content (RWC), useful indicator of the state of the water balance in plants (González & González-Vilar 2001), was significantly lower, suggesting a worse hydric state in *A. castellana*. In particular, the RWC value, well below 80%, could produce changes in the metabolism (González & González-Vilar 2001) affecting photosynthetic physiology, carbon assimilation and energy use (Lawlor & Cornic 2002). A signal of a partially altered carbon metabolism could be the starch absence in chloroplasts, probably counterbalanced by pectin deposits observed in cell walls. In fact these storage molecules may be remobilised as monosaccharides, important in short-term osmotic and ionic adjustments (Clifford et al. 2002; Ghanem et al. 2010) and could be a source of energy in prohibitive environmental conditions (Bartoli et al. 2013). Despite the disturbance in water balance, chloroplasts were abundant and did not show any histologically evident alteration in their morphology and carotenoid/total chlorophyll ratio did not differ significantly with respect to the other plants of the geothermal alteration field (Bartoli et al. 2013; Bartoli et al. 2014). In addition, hydrogen peroxide content and lipid peroxidation, indicative of membrane damage and measured as TBARS, were significantly lower in *A. castellana* than in *Calluna vulgaris* and *Cistus salviifolius* of the same habitat. To this respect, the little lipid deposits observed in leaf tissues could be used in membrane repair events, thus explaining their relative low oxidative damage. As a matter of fact, there was a good ability of our species to counteract oxidative damage commonly associated with stressors typical of this

environment such as soil low pH and high temperature (Huang et al. 2001; Bartoli et al. 2013; Bartoli et al. 2014). In fact, plants have evolved complex enzymatic and non enzymatic protective system to keep under control stress-related ROS evolution. *A. castellana* had a lower phenol and low molecular weight antioxidant content in comparison with the two other plants of the same habitat. Of particular interest is the low reducing power of GSH/GSSG couple (0.85), significantly lower ($p < 0.01$) than *C. vulgaris* and *C. salviifolius* (2.53 and 9.25 respectively). The low content of non-enzymatic antioxidant is counteracted by a high activity of hydrogen peroxide scavenging enzymes such as APX and CAT. The high CAT activity is a peculiar trait of *Agrostis* as previous studies had detected a low catalase activity, probably due to the inhibitory effect the extreme conditions of the geothermal environment could have an on this enzyme (Bartoli et al. 2013). APX and CAT were assisted in their role by GPX, characterized by values of activity similar to *C. vulgaris* and *C. salviifolius.*

In conclusion *Agrostis castellana*, as expected, showed typical xeromorphic traits (eg. cutinisation and wall thickenings, pubescence, protected stomata, etc.) that together with an active antioxidant response, based mainly on enzymatic systems, could help this plant to survive under the prohibitive conditions of the geothermal alteration field.

References

Aebi H. 1984. Catalase *in vitro*. Method. Enzymol. **105**: 121–126.

Anderson J.V., Chevone B.I. & Hess J.L. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. Plant Physiol. **98**: 501–508.

Arezki O., Boxus P., Kevrs C. & Gaspar T. 2001 Changes in peroxidase activity and level compounds during light-induced plantlet regeneration from *Eucalyptus camaldulensis* Dehn. nodes in vitro. Plant Growth Regul. **33**: 215–219.

Banowetz G.M., Azevedo M.D. & Stout R. 2008. Morphological adaptations of hot springs panic grass (*Dichanthelium lanigunosum* var *sericeum* (Schmoll) to thermal stress. J. Therm. Biol. **33**: 106–116.

Barazzuoli P., Guasparri G. & Salleolini M. 1993. Il clima, pp. 114–171. In: Giusti F. (ed), La storia Naturale della Toscana Meridionale. Pizzi: Cinisello Balsamo, Milano, Italia.

Bartoli G., Bottega S., Forino L.M.C., Ciccarelli D. & Spanò C. 2014. Plant adaptation to extreme environments: the example of *Cistus salviifolius* of an active geothermal alteration field. C. R .Biologies **337**: 101–110.

Bartoli G., Bottega S., Forino L.M.C., Ruffini Castiglione M., Tagliasacchi A.M., Grilli I. & Spanò C. 2013. Morpho-physiological plasticity contributes to tolerance of *Calluna vulgaris* in an active geothermal field. Austr. J. Bot. **61**: 107–118.

Bayliss O.B. & Adams C.W.M. 1972. Bromine–Sudan black (BSB): a general stain for tissue lipids including free cholesterol. Histochem. J. **4**: 505–515.

Benz B.W., Martin C.E. 2006. Foliar trichomes, boundary layers, and gas exchange in 12 species of epiphytic *Tillandsia* (Bromeliaceae). J. Plant Physiol. **163**: 648–656.

Bertini G., Casini M., Granelli G. & Pandeli E. 2006. Geological structure of a long-living geothermal system, Larderello, Italy. Terra Nova **18**: 163–169.

Bomfleur B., Pott C. & Kerp H. 2011. Plant assemblages from the Shafer Peak formation (Lower Jurassic), north Victoria Land, Transantarctic Mountains. Antartic Sci. **23**: 188–208.

Bonini I., Casini F., Chiarucci A. & De Dominicis V. 2005. The bryophyte flora of the geothermal field of Sasso Pisano (Pisa, Italy). Cryptogamie Bryol. **26**: 291–299.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Chiarucci A., Calderisi M., Casini F. & Bonini I. 2008. Vegetation at the limits for vegetation: vascular plants, bryophytes and lichens in a geothermal field. Folia Geobot. **43**: 19–33.

Clifford S.C., Arndt S.K., Popp M. & Jones H.G. 2002. Mucilages and polysaccharides in *Ziziphus* species (Rhamnaceae): localization, composition and physiological roles during drought-stress. J. Exp. Bot. **53**: 131–138.

De Vos C.H.R., Vooijs R., Schat H. & Ernst W.H.O. 1989. Copper-induced damage to the permeability barrier in roots of *Silene cucubalus*. J. Plant Physiol. **135**: 164–169.

Feder M.E. & Hofmann G.E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. **61**: 243–282.

Feder N. & O'Brien T.P. 1968. Plant microtechnique: some principles and new methods. Am. J. Bot. **55**: 123–142.

Fisher D.B. 1968. Protein staining of ribboned epon sections for light microscopy. Histochemie. Histochemistry. Histochimie **16**: 92–96.

Ghanem M.E., Han R.M., Classen B., Quetin-Leclerq J., Mahy G., Ruan C.J., Qin P., Pérez-Alfocea F. & Lutts S. 2010. Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. J. Plant Physiol. **167**: 382–392.

González L. & González-Vilar M. 2001. Determination of relative water content, pp. 207–212. In Reigosa Roger M.J. (eds), Handbook of plant ecophysiology techniques, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Gossett D.R., Millhollon E.P. & Lucas M.C. 1994. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. **34**: 706–714.

Hartley-Whitaker J., Ainsworth G., Vooijs R., Bookum W.T., Schat H. & Merag A.A. 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. Plant Physiol. **126**: 299–306.

Hassanzadeh M., Ebadi A., Panahyan-e-Kivi M., Eshghi A.G., Jamaati-e-Somarin Sh., Saeidi M. & Zabihi-e-Mahmoodabad R. 2009. Evaluation of drought stress on relative water content and chlorophyll content of sesame (*Sesamum indicum* L.) genotypes at early flowering stage. Res. J. Environ. Sci. **3**: 345–350.

Hill R.S. 1998. Fossil evidence for the onset of xeromorphy and scleromorphy in Australian Proteaceae. Aust. Syst. Bot. **11**: 391–400 .

Huang B., Liu X. & Xu Q. 2001. Supraoptimal soil temperatures induced oxidative stress in leaves of creeping bentgrass cultivars differing in heat tolerance. Crop Sci. **41**: 430–435.

Jana S. & Choudhuri M.A. 1982 Glycolate metabolism of three submerged aquatic angiosperm during aging. Aquatic Bot. **12**: 345–354.

Jensen W.A. 1962. Botanical histochemistry: principles and practice. W. Freeman & Co., San Francisco, 408 pp.

Jordan G.J., Dillon R.A. & Weston P.H. 2005. Solar radiation as a factor in the evolution of scleromorphic leaf anatomy in Proteaceae. Am. J. Bot. **92**: 789–796.

Kampfenkel K., Montagu M.V. & Inzé D. 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal. Biochem. **225**: 165–167.

Kuo-Huang L.L., Ku M.S.B. & Franceschi V.R. 2007. Correlations between calcium oxalate crystals and photosynthetic activities in palisade cells of shade-adapted *Peperomia glabella*. Bot. Stud. **48**: 155–164.

Lawlor D.W. & Cornic G. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ. **25**: 275–294.

Lichtenthaler H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method. Enzymol. **148**: 350–382.

Loppi S. 2001. Environmental distribution of mercury and other trace elements in the geothermal area of Bagnore (Mt Amiata, Italy). Chemosphere **45**:991–995.

Ma J.F. & Yamaji N. 2006. Silicon uptake and accumulation in higher plants. Trends Plant Sci **11**: 392–397.

Miller G., Nobuhiro S., Ciftci-Yimalaz S. & Mittler R. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses, Plant Cell Environ. **33**: 453–467.

Nakano Y. & Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. **22**: 867–880.

Navari-Izzo F., Meneguzzo S., Loggini B., Vazzana C. & Sgherri C.L.M. 1997. The role of the glutathione system during dehydration of *Boea hygroscopica*. Physiol. Plant. **99**: 23–30.

Pellerin P. & O'Neill M.A. 1998. The interaction of the pectic polysaccharide rhamnogalacturonan II with heavy metals and lanthanides in wines and fruit juices. Analysis **26**: 32–36.

Rao M.V., Beverley A.H. & Ormrod D.P. 1995 Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Role of antioxidant enzymes. Plant Physiol. **109**: 421–432.

Radoglou K.M. & Jarvis P.G. 1990. Effects of CO_2 enrichment on four poplar clones II. Leaf surface properties. Ann. Bot. **65**: 627–632.

Rizhsky L., Liang H. & Mittler R. 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. **130**:1143–1151.

Roth-Nebelsick A. 2007. Computer-based studies of diffusion through stomata of different architecture. Ann. Bot. **100** : 23–32.

Ruzin S.E. 1999. Plant microtechnique and microscopy. Oxford University Press, Oxford, UK, 322 pp.

Saari N., Fujita S., Yamaguchi S. & Tono T. 1996. Distribution of ascorbate oxidase in *Citrus* fruits. Food Sci. Techn. Int. **2**: 154–156.

Selvi F. & Bettarini I. 1999. Geothermal biotopes in central-western Italy from a botanical view point, pp. 1-12. In: Raschi A., Vaccari F.P. Miglietta F. (eds), Ecosystem response to CO_2: the MAPLE project results. Office for Official Publications of the European Communities, Luxembourg.

Spanò C., Bottega S., Grilli I. & Lorenzi R. 2011. Responses to desiccation injury in developing wheat embryos from naturally- and artificially-dried grains. Plant Physiol. Biochem. **49**: 363–367.

Spanò C., Buselli R., Ruffini Castiglione M., Bottega S. & Grilli I. 2007. RNases and nucleases in embryos and endosperms from naturally aged wheat seeds stored in different conditions. J. Plant Physiol. **164**: 487–495.

Turner I.M. 1994. Sclerophylly – primarily protective? Funct. Ecol. **8**: 669–675.

Turner N.C. 1981. Techniques and experimental approaches for the measurement of plant water status. Plant Soil **58**: 339–366.

Figure legends

Fig. 1: The sampling site in the geothermal alteration field of Sasso Pisano (Pisa, Italy) with significant steam emissions from the geothermally altered soil (a) and plants of *Agrostis castellana* living close to a fumarole (b).

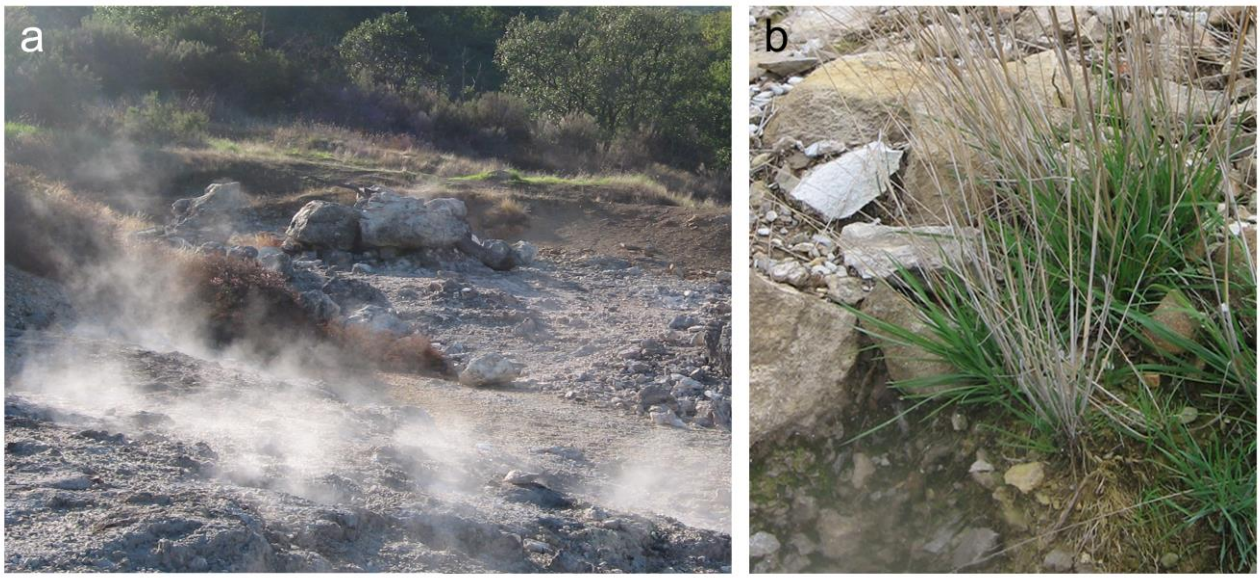

Fig. 2. Leaf anatomy of *A. castellana*. Transverse section of the leaf sheath (a) and leaf blade (b) (TBO, Bar = 50 µm). Stripping of outer and inner epidermis of the leaf sheath (c and d respectively, Bar = 50 µm) and of outer and inner epidermis of the leaf blade (e and f respectively, Bar = 50 µm). Particular of leaf sheath in transverse section, showing the outer epidermis (with cells having thickened walls and cuticle), the inner epidermis and the mesophyll, consisting in a wide aerenchyma intermingled with vascular bundles (g; TBO, Bar = 50 µm). Transverse section of leaf blade, showing the outer epidermis, and mesophyll organization around a vascular bundle (h; TBO, Bar = 30 µm). Particular of leaf blade, showing the outer epidermis, chlorenchyma cells with crystals (i; TBO, Bar = 50 µm). Istochemical determination of starch (j; iodine-potassium iodide staining, Bar = 30 µm), pectic substances (k; Ruthenium-red staining, Bar = 30 µm), proteins (l; Coomassie brilliant blue R-250 staining, Bar = 30 µm) and lipid deposits (m; Sudan black staining, Bar = 30 µm) in leaf tissues. **abe**: abaxial epidermis; **ade**: adaxial epidermis; **ae**: aerenchyma; **b**: bulliform cell; **ch**: chloroplast; **cr**: crystal; **cz**: costal zone; **h**: hair; **ie**: inner epidermis; **icz**: intercostals zone; **m**: mestome; **n**: nucleus; **oe**: outer epidermis; **p**: phytolite; **pc**: parenchymatic cell; **ph**: phloem; **s**: stoma; **scl**: sclerenchyma; **ssc**: sub-stomatal chamber; **vb**: vascular bundle; **x**: xylem.

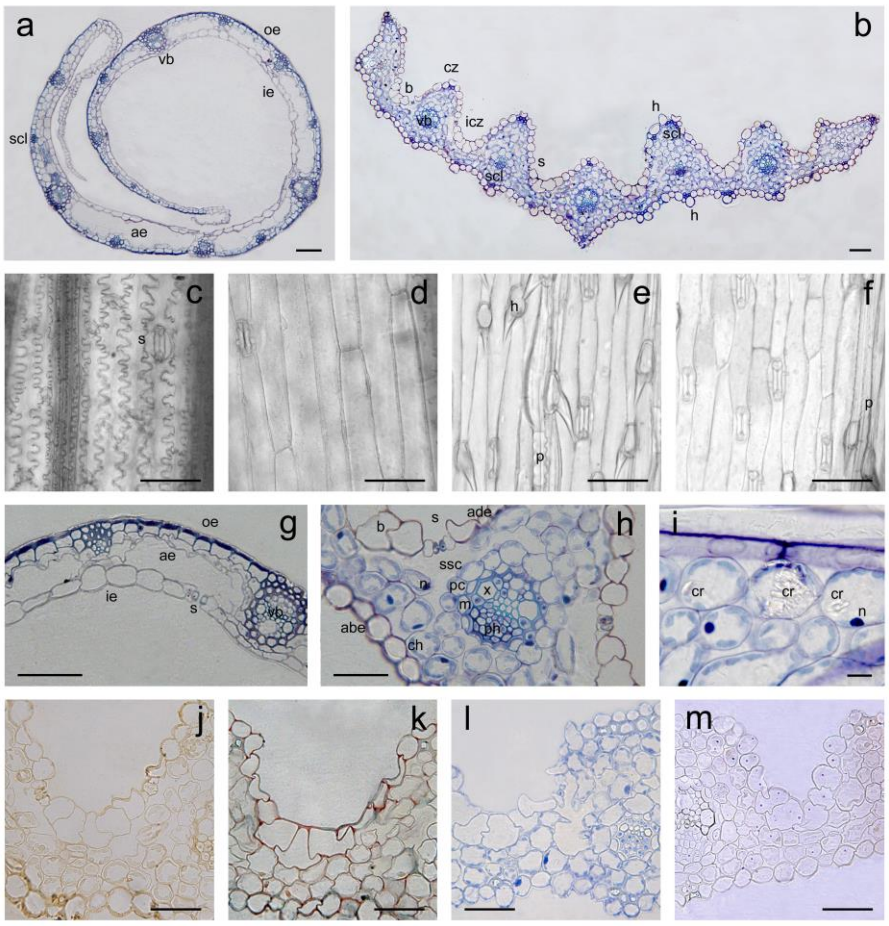

Table 1. Stomatal and hair densities in leaves of *Agrostis castellana*.

	Outer sheath	Inner sheath	Adaxial blade	Abaxial blade
Stomatal density (stomata/mm^2)	17.00±0.63 c	4.10±0.76 d	108.25±3.58 a	28.95±1.79 b
Hair density (hairs/mm^2)	5.60±1.75 c	0.00±0.00 c	130.35±3.89 a	116.85±4.33 b

Data are mean ±SE. Values within a row followed by the same superscript letter do not differ significantly (at P=0.01).

Table 2. Physiological parameters in *Agrostis castellana* of the geothermal alteration field of Sasso Pisano (Italy).

Water content (%)	62.67±0.33
RWC (%)	68. 67±2.67
Chl *a* (mg g^{-1}DW)	2.54±0.12
Chl *b* (mg g^{-1}DW)	0.99±0.05
Total Chl (mg g^{-1}DW)	3.53±0.18
Carotenoids (mg g^{-1}DW)	0.77±0.04
Car/Tot Chl	0.22±0.01
Hydrogen peroxide (μmol g^{-1}DW)	199.74±12.41
TBARS (nmol g^{-1}DW)	367.33±5.70
Phenols (mg GAE g^{-1}DW)	6.07±0.20
ASA (mg g^{-1}DW)	0.98±0.01
DHA (mg g^{-1}DW)	1.15±0.05
GSH (nmol g^{-1}DW)	67.43±2.04
GSSG (nmol g^{-1}DW)	79.49±0.01
APX (U g^{-1}protein)	525.15±28.65
DHAR (U g^{-1}protein)	811.40±25.6
GR (U g^{-1}protein)	64.53±7.17
GPX (U g^{-1}protein)	599.95±22.45
CAT (U g^{-1}protein)	100.42±1.70

Data are the mean of at least three replications of three independent experiments ± standard error.