

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

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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, functional 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 cutinization, cell wall thickening, slightly convolute and adaxially ribbed leaf blades, tissutal reinforcements by sclereids, pubescence, protected stomata and densely packed mesophyll. Abundant pectins, important in both 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, in terms of lipid peroxidation, indicative of membrane 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: antioxidant response; catalase; functional traits; pectines; xeromorphic traits.

Introduction

Geothermal alteration fields can be considered as the most spectacular and extreme manifestation on the earth crust surface of an active geothermal system. They occur where the hot geothermal fluids, arising from the depth of the earth crust, reach the soil surface and give rise to fumaroles, hot springs, mud pots, hot and thermally altered grounds. These sites are exceptionally restrictive environments, where both chemical and physical stress factors coexist and frequently form complex environmental gradients of stress intensity. High soil temperature and acidity, local intensity of gas emissions and ground micromorphology are the main stressors determining vegetation composition and zonation (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., CO₂, CH₄, NH₃, H₂S, Rn, As, Hg, B) frequently experienced in an active geothermal area (Loppi 2001), contributes to the selection of the specialized 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 able to live in geothermal environments activate a wide range of responses at different levels of organization (Stout et al. 1997; Feder & Hofmann 1999; Stout & Al-Niemi 2002; Rachmilevitch et al. 2006; Xu et al. 2007; Tian et al. 2009; 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 in plants the overproduction of reactive oxygen species (ROS) resulting in oxidative damage (Bartoli et al. 2013; Bartoli et al. 2014), counteracted by multiple antioxidant defense mechanisms, both enzymatic and non-enzymatic. Plant defense and tolerance ability are strictly dependent on species and on distance from geothermal manifestations (Bartoli et al. 2013; Bartoli et al. 2014) with distinct antioxidants having different importance.

Geothermal alteration field of Sasso Pisano (Tuscany, Italy), is considered as one of the most extreme geothermal alteration fields in terms of both soil acidity and hot temperatures and the vascular plants *Calluna vulgaris* and *Agrostis castellana* were found to be

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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).

the most widespread species tolerating the harshest environmental conditions of this site (Selvi & Bettarini 1999; Chiarucci et al. 2008). *A. castellana* is supposed to play a key role in the colonization of the highly selective substrates of the geothermal biotopes and its establishment on the hydrothermally altered soils seems to be allowed by the co-occurrence of the moss *Campylopus* and the shrub *Calluna vulgaris* (Selvi & Bettarini 1999). The stands of *C. vulgaris* can provide a thin layer of organic matter, on which the seeds of *A. castellana* can germinate, assisted by the thermic isolation and water retaining provided by the *Campylopus* tufts. Once *A. castellana* plants have rooted, the mosses tend to disappear and the vegetation tends to become a paucispecific *Agrostis-Calluna* community type, only rarely containing other acidophilic grasses (Selvi & Bettarini 1999).

Little is known about the life strategies of the pioneer plants living in the prohibitive environment of the active geothermal alteration field (Stout & Al-Niemi 2002; Rachmilevitch et al. 2006) and few investigations have been performed on the characterization of the morpho-anatomical and physiological traits having adaptive significance for these plants. The shrubs *C. vulgaris* and *Cistus salvifolius* are able to colonize this hostile environment, 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). Leaf features can be considered a product of the environment in which a certain plant lives (Dickison 2000) and then specific adaptations at leaf level are expected to enable the plants to survive in geothermal environments. In this study, we aimed to investigate the morpho-anatomical, functional and

physiological traits of *A. castellana* leaves, focusing on their putative ecological significance.

Material 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 characterised by emissions of steam vents containing H₂S, CO₂, boric acid and water vapor 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/>; accessed 10.03.2014) and the monthly averaged daily mean global insolation upon a normal surface was 7780.1 Wh/m² (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>; accessed 10.03.2014). 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. 1b), similar in size and growing at a distance of some meters from fumaroles, were screened and fully expanded mature leaves (at the same developmental phenological state) were sampled and then considered for morpho-anatomical, functional 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 and morpho-functional traits

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 100 mM 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% in aqueous solution) 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 were analysed. Images of each slide were taken using a Leica DFC 420 digital camera.

Epidermal stripping of both leaf blade (adaxial and abaxial surfaces) and sheath (outer and inner sides) of fresh leaves were made in order to determine stomatal density (SD), expressed as the number of stomata per square millimeter leaf area (Radoglou et al. 1990) and hair density (HD), expressed as the number of hairs per square millimeter leaf area. 20 leaves collected from the selected plants (10 individuals) were analysed and the counts for SD and HD determinations were made on both surfaces of the leaves, for a total of 40 counts.

Because of the large differences in thickness occurring along leaf width, in both leaf blade and sheath, the mean leaf blade thickness (LBT) and the mean leaf sheath thickness (LST) were calculated as the ratio between the area of the transverse section of blade or sheath respectively and its width (Garnier & Laurent 1994). Then, 20 histological median cross-sections randomly collected from 10 leaf samples were used for the determination of leaf blade thickness (LBT) and the mean leaf sheath thickness (LST), all expressed in mm. Observations and image acquisition for SD, HD, LBT and LST determinations were made by using a LEITZ DIAPLAN light microscope and a Leica DFC 420 digital camera respectively. Analyses were performed by using the open source UTHSCSA Imagetool software (available at <http://ddsdx.uthscsa.edu/dig/itdesc.html>; accessed 15.04.2014).

Leaf area (LA) was the one-sided projected surface area of a fresh leaf, expressed in mm^2 . Specific leaf area (SLA) was the one-sided area of a fresh leaf divided by its oven dry mass, expressed in mm^2/mg . Leaf dry matter content (LDMC) was the oven dry mass of a leaf divided by its water-saturated fresh mass, expressed in mg/g . (Read et al. 2005; Omae et al. 2007). 20 leaves from 10 individuals were considered. Sample storing and processing

followed the standardized methodologies detailed by Cornelissen et al. (2003). Leaf projected area was acquired with a CanoScan LiDE 90 (Canon) and determined by using the open source UTHSCSA Imagetool software (available at <http://ddsdx.uthscsa.edu/dig/itdesc.html>; accessed 15.04.2014).

Leaf biomass and water status

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:

$$\text{RWC} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100$$

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 *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 $\text{mg g}^{-1}\text{DW}$.

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 $\mu\text{mol g}^{-1}\text{DW}$.

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 was mixed with TBA reagent, heated (95°C), cooled and centrifuged. The level of MDA ($155 \text{ mM}^{-1} \text{ cm}^{-1}$ 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 $\text{mg g}^{-1}\text{DW}$) 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⁻¹DW.

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 derivatization. Calculations were made on the base of a standard curve and content was expressed as nmol g⁻¹DW.

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 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 H₂O₂. 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 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.

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

For calculating mean and standard error, the average SD, HD, LBT, LST, LA, SLA, and LDMC for each individual plant was one statistical observation. For physiological parameters, at least three measurements of three independent extractions on mature leaves randomly collected from 10 individuals were made. Value in the tables indicates mean value ± SE. Regarding the parameters stomatal density (SD) and hair density (HD), the values were 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 and discussion

Species of the genus *Agrostis* are often found in geothermally altered or polluted soils (Selvi & Bettarini 1999; Stout & Al-Niemi 2002; Rachmilevitch et al. 2006; Austruy et al. 2013; Elmarsdóttir et al. 2015) suggesting a good adaptability of these plants to the different environmental constraints.

A. castellana plants living near to manifestations of the geothermal alteration field of Sasso Pisano exhibited some interesting morpho-anatomical features in their leaves that can play significant roles in the adaptive strategy of this plant to geothermal environment.

Mature leaves (Fig. 1b) consisted in a tubular sheath, with free overlapping margins, and in a lanceolate and slightly curled blade, adaxially arranged in ribs and furrows (Fig. 2a, b). Significant differences occurred between the blade and the sheath. In the sheath (Fig. 2c, d), only the outer epidermis was constituted by lobed cells (Fig. 2c) and showed a greater density of both stomata and hairs (Table 1) than the inner one. In the blade, the epidermis evidenced a zonation in costal and intercostal regions, with rows of fusiform cells, stomata and unicellular prickle-hairs, especially marked on the adaxial side, with well-defined rows of slightly sunken stomata and bulliform cells (Fig. 2b, e, f, h). These ones, showed abundant pectins in their outer periclinal walls (Fig. 2k) and little lipid bodies in their extensive vacuoles (Fig. 2m). The abaxial epidermis of the blade evidenced a lower stomatal and hair densities (Table 1) than the adaxial one. Elongate-sinuous phytolites (Fig. 2c, e, f) and thickenings in cuticle and wall (Fig. 2g, h) occurred in both blade and sheath epidermal cells. Unlike the sheath mesophyll, consisting in a very wide aerenchyma (Fig. 2g), the blade mesophyll consisted in a densely packed photosynthetic parenchyma arranged around collateral vascular bundles (Fig. 2h). Photosynthetic cells, provided by abundant chloroplasts and crystals (Fig. 2h, i, l), evidenced no significant starch deposits and little lipid deposits (Fig. 2j, m). Sclerenchymatic strengthening occurred in both leaf blade and sheath (Fig. 2a, b, g, h).

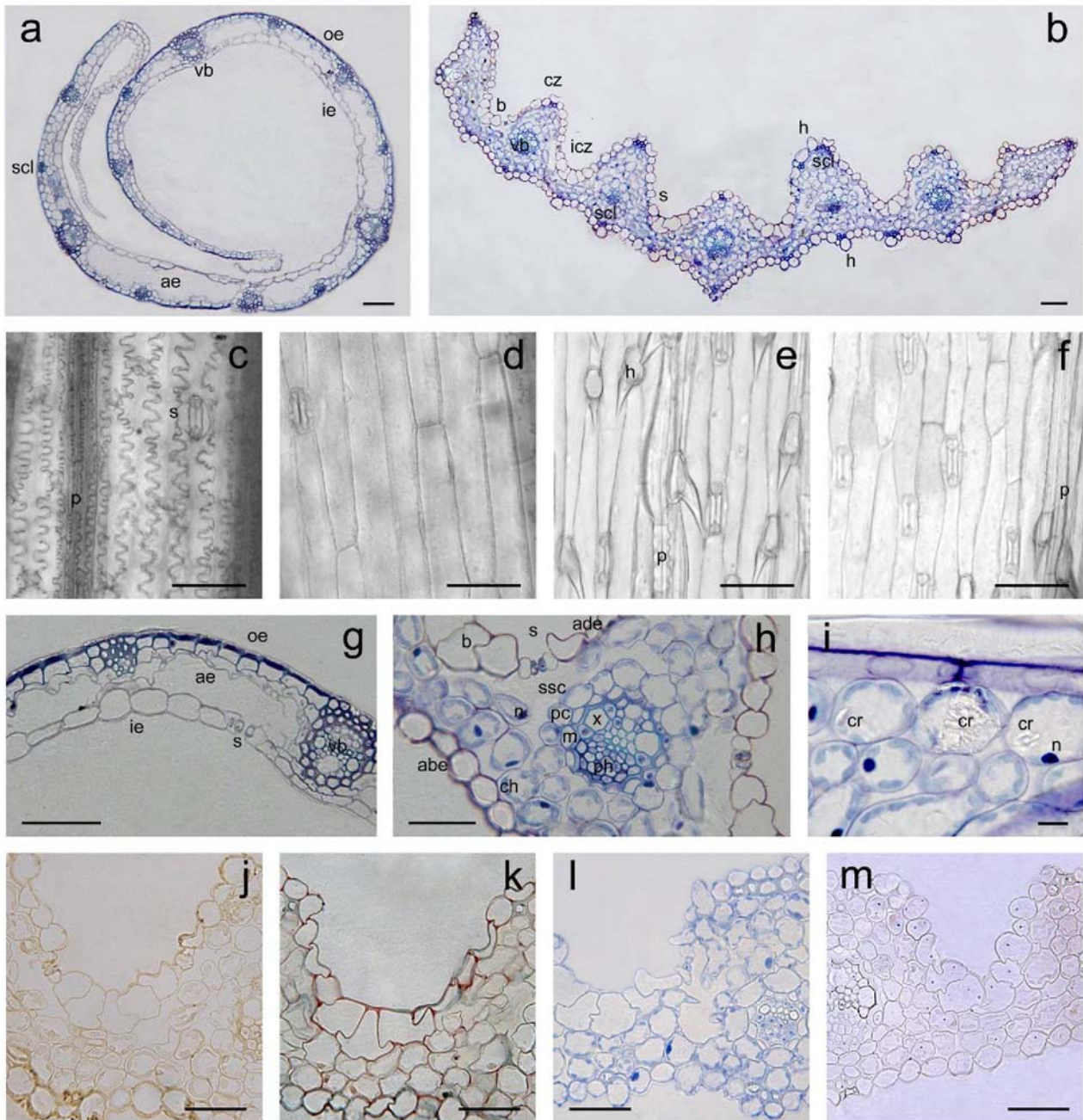


Fig. 2. Leaf anatomy of *A. castellana*. Transverse section of the leaf sheath (a) and leaf blade (b) (TBO, bar = 50 μ m). Stripping of outer and inner epidermis of the leaf sheath (c and d respectively, bar = 50 μ m) and of adaxial and abaxial epidermis of the leaf blade (e and f respectively, bar = 50 μ m). 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 μ m). Transverse section of leaf blade, showing the adaxial and abaxial epidermis and mesophyll (h; TBO, bar = 30 μ m). Particular of leaf blade, showing the adaxial epidermis and chlorenchyma cells with crystals (i; TBO, bar = 50 μ m). Istochemical determination of starch (j; iodine-potassium iodide staining, bar = 30 μ m), pectic substances (k; Ruthenium-red staining, bar = 30 μ m), proteins (l; Coomassie brilliant blue R-250 staining, bar = 30 μ m) and lipid deposits (m; Sudan black staining, bar = 30 μ m) 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, intercostal 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.

Many of the leaf morpho-anatomical features exhibited by *A. castellana* can be considered as xeromorphic traits that make these plants more adapted to support the various kinds of environmental stresses of an active geothermal alteration field. Xeromorphic traits with an adaptive significance were also evidenced in other plants living in solfataras or fumarolic fields,

with strongly acid soil (Selvi & Bettarini 1999; Bomfleur et al. 2011; Bartoli et al. 2013; Bartoli et al. 2014). Epidermis is the first barrier against geothermal stressors. Thus, abundant cutinization 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

Table 1. Morpho-functional traits in leaves of *A. castellana*.

Morpho-functional parameters	
SD (stomata/mm ²)	
Outer sheath	17.00 ± 0.63 ^c
Inner sheath	4.10 ± 0.76 ^d
Adaxial blade	108.25 ± 3.58 ^a
Abaxial blade	28.95 ± 1.79 ^b
HD (hairs/mm ²)	
Outer sheath	5.60 ± 1.75 ^c
Inner sheath	0.00 ± 0.00 ^c
Adaxial blade	130.35 ± 3.89 ^a
Abaxial blade	116.85 ± 4.33 ^b
LBT (mm)	0.1027 ± 0.009
LST (mm)	0.0438 ± 0.0028
LA (mm ²)	232.03 ± 22.68
SLA (mm ² /mg)	32.35 ± 1.16
LDMC (%)	25.67 ± 0.92

Data are mean ± standard error. Regarding the parameters stomatal density (SD) and hair density (HD), the values within a column followed by the same superscript letter do not differ significantly (at *P* = 0.01). SD, stomatal density; HD, hair density; LBT, leaf blade thickness; LST, leaf sheath thickness; LA, leaf area; SLA, specific leaf area; LDMC, leaf dry matter content.

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

Physiological parameters	
Water content (%)	62.67 ± 0.33
RWC (%)	68.67 ± 2.67
Chl <i>a</i> (mg g ⁻¹ DW)	2.54 ± 0.12
Chl <i>b</i> (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 (GAE mg 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.

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 (Fig. 2k) and can be interpreted as 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 fibers

and silica bodies (Fig. 2c, e, f, i), both increasing tissutal stiffness and toughness (Turner 1994; Ma & Yamaji 2006), the highly thin leaves of *A. castellana* (Table 1) 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) (Ma & Yamaji 2006; Banowetz et al. 2008). Interestingly, *A. castellana* leaves presented morphological and anatomical strategies aimed at stomata defence, such as stomata localization in furrows (Fig. 2b, h) or in the inner surface of the leaf sheath (Fig. 2d), stomata sinking below the plane of the epidermis and protection by hairs (Fig. 2e, h). 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 improved morpho-anatomical protection might explain the higher stomatal density occurring on the adaxial side than in the abaxial one of the leaf blade (Table 1). Regarding pubescence, significantly higher on the adaxial blade side than elsewhere (Table 1), the trichomes (Fig. 2e) can also play a role in protection from excess radiation, including avoidance of overheating (Jordan et al. 2005).

The densely packed mesophyll observed in *A. castellana* leaf blade (Fig. 2b), with reduced intercellular spaces, is useful in minimizing water transpiration and can improve photosynthetic process increasing the amount of chloroplasts per leaf surface unit. The photosynthesis can be also improved by the crystals occurrence, especially in the outer layer of the mesophyll (Fig. 2i). Crystals, other than a useful reserve of Ca²⁺ for cell metabolism, would help light dispersion to the surrounding chloroplasts by means of its multiple, radially oriented facets with potential reflective properties (Kuo-Huang et al. 2007).

Leaf water content (62.67%) 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). On the contrary, relative water content (RWC), useful indicator of the state of the water balance in plants (González & González-Vilar 2001), was significantly lower (68.67%, Table 2), suggesting a worse hydric state in *A. castellana*. This is in accordance with functional traits (Table 1), SLA and LDMC, respectively higher and lower than in *C. salviifolius* of the same environment. These values, not different from those observed in *Agrostis* sp. that lived in unstressed conditions (Fort et al. 2013), could indicate a higher growth rate of *Agrostis* plant but, at the same time, an increased vulnerability to desiccation (Ramírez-Valiente et al. 2014) in comparison with *C. salviifolius*. 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 (Fig. 2j), probably counterbalanced by pectin deposits observed in cell walls (Fig. 2k). In fact, these storage molecules may be remobilized 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 (Fig. 2l) and the content of chlorophyll (Table 2) was not lower than those of the other plants of the geothermal alteration field (Bartoli et al. 2013; Bartoli et al. 2014) and also carotenoid/total chlorophyll ratio did not differ significantly with respect to the other. ROS production was monitored by detecting the concentration of hydrogen peroxide. The content of this molecule was about 200 $\mu\text{mol g}^{-1}\text{DW}$. The measurement of lipid peroxidation (Tab. 2), indirectly indicating membrane damage and measured as TBARS, was 367.33 $\text{nmol g}^{-1}\text{DW}$. Both these stress parameters were significantly lower in *A. castellana* than in *Calluna vulgaris* and *Cistus salviifolius* of the same habitat. To this respect, the little lipid deposits (Fig. 2m) observed in leaf tissues could be used in membrane repair events, thus explaining their relative low oxidative damage. As a matter of fact, a good membrane stability has been already underlined as an important adaptive trait in heat stress conditions in another *Agrostis* species (Tian et al. 2009). 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. Phenol content, measured as gallic acid equivalents, was about 6 GAE $\text{mg g}^{-1}\text{DW}$, ascorbate pool was 2.13 $\text{mg g}^{-1}\text{DW}$, and glutathione pool was about 147 $\text{nmol g}^{-1}\text{DW}$ (Tab. 2). Therefore *A. castellana* had a lower phenol and low molecular weight antioxidant content in comparison with the two other plants of the same habitat. Both ascorbate and glutathione were predominantly in the oxidised form, with AsA/DHA and GSH/GSSG ratios of 0.85 each. Of particular interest is the low reducing power of GSH/GSSG couple, significantly lower ($p < 0.01$) than in *C. vulgaris* and *C. salviifolius* (2.53 and 9.25 respectively). The low content of non-enzymatic antioxidants is counteracted by a relatively high activity of hydrogen peroxide scavenging enzymes. APX had an activity higher than 500 $\text{U g}^{-1}\text{protein}$ while catalase had an activity that amounted on about 100 $\text{U g}^{-1}\text{protein}$ (Table 2). The relatively 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 on this enzyme (Bartoli et al. 2013). APX and CAT were assisted in their role by GPX, characterized by values of activity (about 600 $\text{U g}^{-1}\text{protein}$) similar to *C. vulgaris* and *C. salviifolius*. DHAR activity

was about 811 $\text{U g}^{-1}\text{protein}$ and GR activity was 64.53 $\text{U g}^{-1}\text{protein}$ (Table 2).

In conclusion, *Agrostis castellana*, as expected, showed typical xeromorphic traits (eg. cutinization 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.

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