

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

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

10

11 **Abstract**

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

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

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

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

## 1 **Introduction**

2 Geothermal alteration fields are extremely restrictive environments where both chemical and  
3 physical stress factors coexist. High soil temperature and acidity, local intensity of gas emissions  
4 and ground micromorphology are the main stressors determining vegetation composition (Selvi &  
5 Bettarini 1999; Bonini et al. 2005; Chiarucci et al. 2008). Moreover the emission of water steam  
6 mixed with different gases, toxic elements and compounds (e.g. CO<sub>2</sub>, CH<sub>4</sub>, NH<sub>3</sub>, H<sub>2</sub>S, Rn, As, Hg,  
7 B) frequently experienced in an active geothermal area (Loppi 2001), contributes to the selection of  
8 the specialised flora typical of the geothermal areas. The study of plants of these sites is of  
9 particular interest to have a whole picture of adaptive mechanisms in natural habitats, that could not  
10 be adequately described by investigations made applying a single stress factor to plants under  
11 laboratory controlled conditions (Miller et al. 2010). In fact, in natural habitat, different stressors  
12 often coexist inducing a specific response, different from the sum of responses to individual stress  
13 factors (Rizhsky et al. 2002). The few plants living in geothermal environment activate a wide  
14 range of responses at different levels of organization (Feder & Hofmann 1999) developing specific  
15 morpho-anatomical and physiological traits (Bartoli et al. 2013; Bartoli et al. 2014) that help species  
16 to cope the geothermal constraints.

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

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

27

## 1 **Materials and Methods**

2

### 3 *Site description*

4 The collection site was the geothermal alteration field (Fig. 1a) near to Sasso Pisano town  
5 (Castelnuovo Val di Cecina, Pisa province, Italy), in the site of Regional Interest (RIS B12, IT  
6 5160103) named “Monterotondo Marittimo and Sasso Pisano geothermal fields”, hosting two types  
7 of natural habitats listed in the Natura 2000 EU directive: the “Forests of *Castanea sativa* Miller  
8 (cod 9260)” and the “Lava fields and natural cavities (cod 8320)” (Bonini et al. 2005). The  
9 sampling site is characterized by emissions of steam vents containing H<sub>2</sub>S, CO<sub>2</sub>, boric acid and  
10 water vapour from cracks in the rocks that contribute to soil overheating and acidification  
11 (Chiarucci et al. 2008).

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

15 The sampling site is located in the upper valley of Cornia river, at an elevation of about 550 m a.s.l.  
16 The climate is Mediterranean, with a mean annual temperature of 13.3 °C (Barazzuoli et al. 1993)  
17 and a mean annual rainfall of 1107 mm (Chiarucci et al. 2008).

18 At the time of the sample collection (May 2013), the mean monthly temperature recorded was 12.9  
19 °C (min: 8.8 °C; max: 17.6 °C) and the mean monthly rainfall was 120.0 mm (data from the  
20 weather station of Castelnuovo Val di Cecina, Pisa available at <http://www.castelnuovometeo.it/>)  
21 and the monthly averaged daily mean global insolation upon a normal surface was 7780.1 Wh/m<sup>2</sup>  
22 (data from “Atlante Italiano della radiazione solare”, edited by “ENEA, Agenzia nazionale per le  
23 nuove tecnologie, l’energia e lo sviluppo economico sostenibile”, available at  
24 <http://www.solaritaly.enea.it/index.php>). In the sampling site, the daily temperature of the soil was  
25 recorded 30.4 °C (min: 25 °C; max: 35 °C) and soil pH was 3.8 (Bartoli et al. 2013)

26

### 27 *Experimental plant material*

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

33

1 *Light microscopy*

2 Leaf portions from the leaf sheath and the leaf blade were excised from *A. castellana* leaves and  
3 were fixed for 24 h in FAA fixative (10 % formaldehyde–5 % acetic acid–45 % ethanol),  
4 dehydrated in a graded ethanol series, and embedded in LR-White medium Grade (London Resin  
5 Company). Semi-thin sections (3 µm) were cut with an ultramicrotome (Ultratome Nova LKB  
6 Producter, Bomma, Sweden) and stained with different dyes, as follows: toluidine blue O (TBO)  
7 (0.05% in 100mM benzoate buffer at pH 4.4) for general cytological investigations (Feder &  
8 O'Brien 1968), Sudan black B (0.07% in saturated solution of ethanol 70%) for lipid compounds  
9 detection (Bayliss & Adams 1972), Coomassie brilliant blue R-250 (0.05% in Carnoy solution) for  
10 protein staining (Fisher 1968), Ruthenium red (0.1%) for pectic-substance characterisation (Jensen  
11 1962) and iodine–potassium iodide (IKI) for starch detection (Ruzin 1999).The sections were  
12 cleared in xylene, air dried, mounted in DPX Mountant (Sigma) and then observed with a LEITZ  
13 DIAPLAN light microscope. At least 100 histological sections for each experimental group were  
14 analysed. Images of each slide were taken using a Leica DFC 420.

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

21

22 *Determination of water content and of relative water content*

23 Calculations of leaf fresh weight, dry weight and moisture content were based on weights  
24 determined before and after oven drying of leaf samples at 100°C for 24 h. Water content  
25 percentage was estimated on the fresh weight basis. Leaf relative water content (RWC; Turner 1981  
26 with minor modifications) was calculated with the formula:

27 
$$RWC = [(FW-DW)/(TW-DW)] \times 100$$

28 FW = Fresh weight

29 DW = Dry weight

30 TW = Turgid weight

31 Fresh weight was obtained by weighing the fresh leaves. The leaves were then immersed in water  
32 over night, blotted dry and then weighed to get the turgid weight. The leaves were then dried in an  
33 oven at 100°C to constant weight and reweighed to obtain the dry weight.

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### *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<sup>-1</sup>DW.

### *Hydrogen peroxide and lipid peroxidation*

H<sub>2</sub>O<sub>2</sub> content of leaves was determined according to Jana & Choudhuri (1982) using titanium chloride in H<sub>2</sub>SO<sub>4</sub> for peroxide detection. The intensity of the yellow colour of the supernatant was measured at 410 nm. The amount of H<sub>2</sub>O<sub>2</sub> in the extracts, calculated from a standard curve, was expressed as  $\mu$ mol g<sup>-1</sup>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<sup>-1</sup>cm<sup>-1</sup> 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<sup>-1</sup>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<sup>-1</sup>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

1 GSH from the sample extract by 2-vinylpyridine derivatisation. Calculations were made on the base  
2 of a standard curve and content was expressed as nmol g<sup>-1</sup>DW.

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#### 4 *Enzymatic antioxidants*

5 For enzyme extraction acetonic powders were made according to Saari et al. (1996) with  
6 modifications. Briefly, leaves were homogenised in ice-cold acetone. Homogenates were filtered  
7 through a Buckner filter with Macherey-Nagel MN 618 filter paper under vacuum and washings  
8 were repeated until the powder resulted colourless. After complete removal of acetone under  
9 vacuum, acetonic powders were extracted in 100 mM potassium phosphate buffer pH 7.5  
10 containing 1 mM EDTA, and 1% (w/v) polyvinylpyrrolidone (PVP-40) as in Spanò et al. (2011). All  
11 the extractions were performed at 4°C. The homogenate was then centrifuged at 15000 x g for 20  
12 min. For ascorbate peroxidase, 2 mM ascorbate was added to the extraction medium. For  
13 glutathione reductase the supernatant was desalted on a Sephadex G-25 column. Supernatants were  
14 collected and stored in liquid nitrogen until their use for enzymatic assays.

15 APX (EC 1.11.1.11) activity was measured according to Nakano & Asada (1981) with  
16 modifications. Enzyme activity was assayed from the decrease in absorbance at 290 nm (extinction  
17 coefficient 2.8 mM<sup>-1</sup>cm<sup>-1</sup>) as ascorbate was oxidised. The reaction mixture contained 100 mM  
18 potassium phosphate pH 7.5, 0.5 mM ascorbate and enzyme extract (25 µg protein ml<sup>-1</sup>). The  
19 reaction was started by adding 0.2 mM H<sub>2</sub>O<sub>2</sub>. Correction was made for the low, non enzymatic  
20 oxidation of ascorbate by hydrogen peroxide (blank).

21 DHAR (EC 1.8.5.1) activity was determined as described by Nakano & Asada (1981). The activity  
22 of DHAR was determined by monitoring the glutathione-dependent reduction of dehydroascorbate.  
23 Enzymatic extract contained 12.5 µg protein ml<sup>-1</sup>. The activity was determined by measuring the  
24 increase in absorbance at 265 nm for 3 min. Specific activity was calculated from the 14 mM<sup>-1</sup> cm<sup>-1</sup>  
25 extinction coefficient. A correction for the non-enzymatic reduction of DHA by GSH was carried  
26 out in the absence of the enzyme sample (blank).

27 GR (EC 1.6.4.2) activity was determined as described by Rao et al. (1995) following the oxidation  
28 of NADPH at 340 nm (extinction coefficient 6.2 mM<sup>-1</sup> cm<sup>-1</sup>). Enzymatic extract contained 25 µg  
29 protein ml<sup>-1</sup>. A correction for the non-enzymatic reduction of GSSG was carried out in the absence  
30 of the enzyme sample (blank).

31 GPX (EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its  
32 reaction with that of GR, using as substrate 0.45 mM H<sub>2</sub>O<sub>2</sub>. The activity was determined by  
33 following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM<sup>-1</sup> cm<sup>-1</sup>). Enzymatic

1 extract contained 25  $\mu\text{g}$  protein  $\text{ml}^{-1}$ .  
2 CAT (EC 1.11.1.6) activity was determined as described by Aebi (1984). Enzymatic extract  
3 contained 25  $\mu\text{g}$  protein  $\text{ml}^{-1}$ . A blank containing only the enzymatic solution was made. Specific  
4 activity was calculated from the 39,4  $\text{mM}^{-1} \text{cm}^{-1}$  extinction coefficient.  
5 All enzymatic activities were determined at 25°C and expressed as U  $\text{g}^{-1}$  protein. Protein  
6 measurement was performed according to Bradford (1976), using BSA as standard.

#### 7 8 *Data analysis*

9 All the experiments were performed at least in triplicate. Value in the tables indicates mean value  $\pm$   
10 SE based on three independent experiments and was significantly different as assessed by the  
11 analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* test, with values of  $p < 0.01$   
12 sufficient to reject the null hypothesis.

## 13 14 **Results**

### 15 *Morphoanatomical and histochemical leaf characterization*

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

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

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

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

11

#### 12 *Leaf water content, RWC and pigments*

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

15

#### 16 *Oxidative stress evaluation*

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

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#### 21 *Enzymatic and non-enzymatic antioxidants*

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

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



## 1 **Discussion**

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

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

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

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

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

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

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

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

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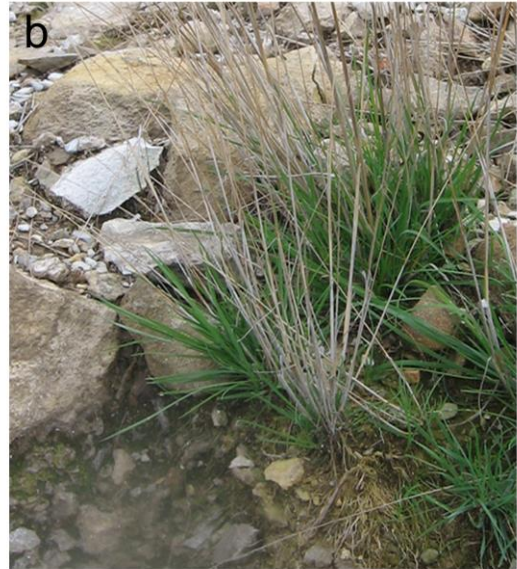
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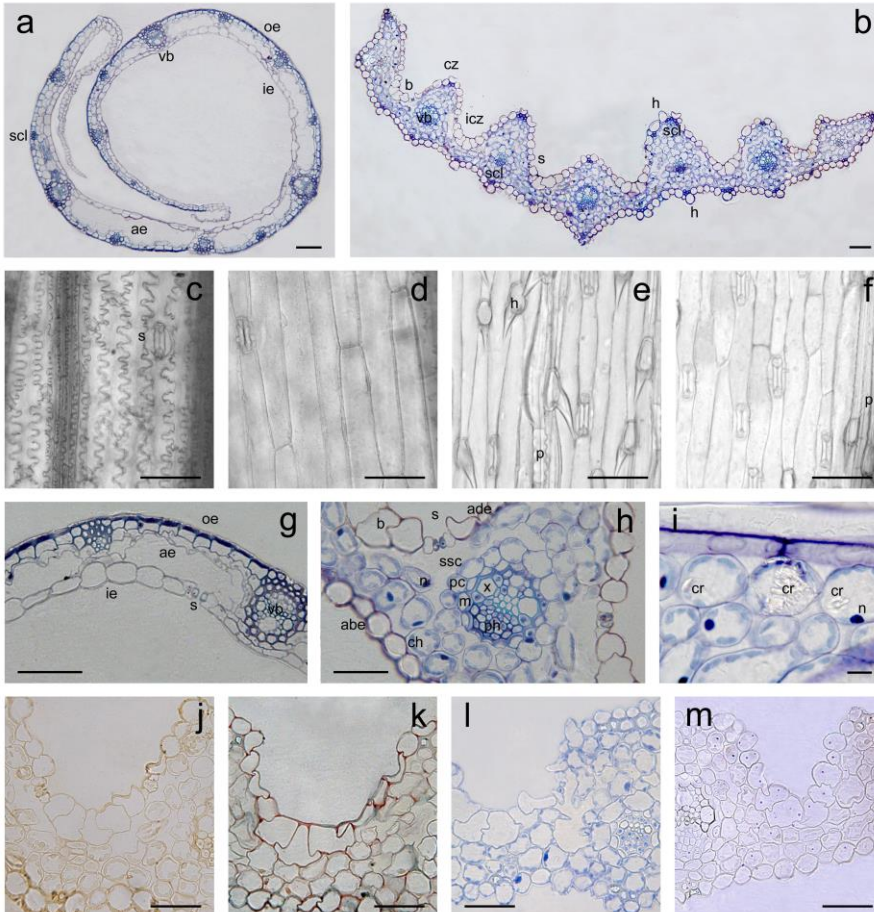
1 **Figure legends**

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





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



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

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

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

4

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

Water content (%)	62.67±0.33
RWC (%)	68.67±2.67
Chl <i>a</i> (mg g <sup>-1</sup> DW)	2.54±0.12
Chl <i>b</i> (mg g <sup>-1</sup> DW)	0.99±0.05
Total Chl (mg g <sup>-1</sup> DW)	3.53±0.18
Carotenoids (mg g <sup>-1</sup> DW)	0.77±0.04
Car/Tot Chl	0.22±0.01
Hydrogen peroxide (μmol g <sup>-1</sup> DW)	199.74±12.41
TBARS (nmol g <sup>-1</sup> DW)	367.33±5.70
Phenols (mg GAE g <sup>-1</sup> DW)	6.07±0.20
ASA (mg g <sup>-1</sup> DW)	0.98±0.01
DHA (mg g <sup>-1</sup> DW)	1.15±0.05
GSH (nmol g <sup>-1</sup> DW)	67.43±2.04
GSSG (nmol g <sup>-1</sup> DW)	79.49±0.01
APX (U g <sup>-1</sup> protein)	525.15±28.65
DHAR (U g <sup>-1</sup> protein)	811.40±25.6
GR (U g <sup>-1</sup> protein)	64.53±7.17
GPX (U g <sup>-1</sup> protein)	599.95±22.45
CAT (U g <sup>-1</sup> protein)	100.42±1.70

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

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