

Anthemis maritima L. in different coastal habitats: a tool to explore plant plasticity.
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ABSTRACT

Anthemis maritima, a plant which has the ability to colonise different stressful coastal environments, sand dunes and rocky cliff ecosystems, exhibits a high degree of leaf trait plasticity. The key parameters are the regulation of stomatal density and size, the succulence index and the specific antioxidant response. With the aim to explore plant plasticity, we analysed various morphological and physiological traits of the leaves of *A. maritima* populations dwelling in three different coastal areas of Italy. The highest values of stomatal density, leaf thickness, and succulence index were found in plants living in a sub-arid climate, on rocky cliffs, with the highest soil pH and salinity. Although this population exhibited the highest concentration of oxygen reactive species (hydrogen peroxide), it also had the lowest value of lipid peroxidation, an indicator of oxidative stress. Ascorbate was the main protective molecule in this population, while phenols appeared to carry out this role in plants living on soils with the lowest salinity and highest annual rainfall.

Keywords: *Anthemis maritima*; antioxidants; coastal dunes; plant morphology; oxidative stress; rocky cliffs

1. Introduction

Plants colonising sand dunes of coastal ecosystems, physically dynamic habitats (Defeo et al., 2009), are subjected to severe environmental stresses and disturbances with the main constraints being salt spray, and burial and drifting, drought, wind abrasion, and nutrient limitations (Rozema et al., 1985). To survive in such habitats, coastal plants have developed several morphological and anatomical adaptations, such as succulent tissues to store water, leaf roll, pubescent epidermis and a thick cuticle to reduce transpiration and water loss, and a network of rhizomes to withstand sand burial (Ciccarelli et al., 2009, 2010). In addition, both drought and salt stress induce physiological changes such as a decrease in cell water potential and stomata closing. This can lead to a decrease in CO₂ availability to green tissues, which promotes the Mehler reaction, with consequent overproduction of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals. Although ROS are important signalling molecules in triggering stress defence responses (Dat et al., 2000), when their concentration reaches critical levels they may cause damage to cellular components such as membrane lipids, nucleic acids, and proteins, resulting in oxidative stress (Parida and Das, 2005). Protection against ROS may be mediated by hydrogen peroxide scavenging enzymes such as catalase (CAT), glutathione peroxidase (GPX), and enzymes of the ascorbate glutathione cycle such as ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) (Lenher et al., 2006). Low molecular weight antioxidants, such as ascorbate and glutathione, can cooperate with enzymes to increase the resistance to oxidative injury. Leaf phenols are also important protective components of plant cells. The ability of phenolics to act as antioxidants derives mainly from their capacity to serve as hydrogen donors, reducing agents, and singlet O₂ quenchers (Rice-Evans et al., 1997). A close positive correlation has been found between antioxidant capacity and stress tolerance in a wide variety of species such as *Cakile maritima* (Amor et al., 2007), and *Acacia longifolia* (Morais et al., 2012). The capacity to scavenge ROS and reduce damage to macromolecules and structures represents an important stress tolerance trait (Cruz de Carvalho, 2008). The subject of the present study is the psammophyte *Anthemis maritima* L. (Asteraceae), a perennial species living on white dunes. *A. maritima*

populations are distributed throughout the Mediterranean area, where they contribute to coastal sand dune edification and restoration (De Lillis et al., 2004). Recently, a good deal of research attention has been focused on the chemical composition of *A. maritima* essential oil and its therapeutic applications (Collu et al., 2008; Darriet et al., 2009), although, in general, there is not a detailed knowledge of the biology of this plant (Gratani et al., 2009). The comparison among plants of *A. maritima*, living in both sand dunes and on rocky substrata of different coasts of Italy, could be valuable for studying the plasticity occurring in the plants of the same species growing under different environmental conditions. Our aim was to determine whether the ability of this species to survive in heterogeneous coastal environments result from plastic activation of different adaptive strategies. We hypothesise that plants of *A. maritima* evolve different morphological and anatomical leaf traits and antioxidant responses depending on the soil and climatic conditions. To test this hypothesis, morphological, anatomical and physiological traits were analysed in the leaves of *A. maritima* populations living in three different geographical coastal areas of Italy.

2. Material and methods

2.1 Study site and plant material

Plant material was collected at three coastal sites of Italy in May 2010: A: sand-dune ecosystems in Principina a Mare (Grosseto) within Maremma Regional Park (42° 41' 16.8" N, 10° 59' 53.1" E, 30 m from the water line, shifting dunes with *Ammophila arenaria*). This area is characterised by a C1 type of climate, Mediterranean sub-arid, with an annual average temperature of 15.6 °C (mean Tmin 10 °C, Tmax 21.2 °C) and an annual average rainfalls of 618 mm (total rainfall during summer drought: 54 mm) (Pinna, 1985). B: rocky cliffs on Pianosa island (Leghorn) in Tuscan Archipelago National Park (42° 34' 34.0" N, 10° 06' 09.8" E, 10 m from the water line, coastal *Helichrysum garrigues*). This isle is characterised by a D type of climate, Mediterranean semi-arid, with an annual average temperature of 16.9 °C (mean Tmin 11.2 °C, Tmax 22.6 °C) and annual average rainfalls of 418 mm (total rainfall during summer drought: 37 mm) (Foggi et al., 2008). C: sand-dune habitats in Casalbordino Lido (Chieti) (42° 11' 53.7" N, 14° 37' 21.0" E, 15 m from the water line, shifting dunes with *Ammophila arenaria*) close to one Site of Community Interest called "Punta Aderci-Punta della Penna". This area is characterised by a C2 type of climate, Mediterranean sub-humid, with an annual average temperature of 15.8 °C (mean Tmin 11.8 °C, Tmax 19.8 °C) and an annual average rainfalls of 658 mm (total rainfall during summer drought: 105 mm) (Pirone et al., 2001). Mature leaves were randomly collected from 10 individuals of *A. maritima* at each site for histological

analyses and biochemical determinations (fresh material, chemically fixed or frozen in liquid nitrogen and stored at -20 °C until use).

2.2 Soil and plant nutrient status

Soil physical and chemical characteristics of the three sites were determined using standard soil analysis techniques. Each sample represented approximately 3 kg of soil randomly collected from 10 soil cores (20 mm diameter, 0-10 cm depth) for each site. Specifically, soil pH was determined in water as detailed by McLean (1982). Electrical conductivity was determined by fixed-ratio extract (Rhodes, 1982). Total soil nitrogen (N) was obtained by micro-Kjeldahl digestion (Allen, 1989). Available phosphorus (P) was obtained by the Olsen et al. (1954) method. Na⁺ was determined by atomic absorption spectrophotometry (Thomas, 1982). Leaf N concentration, leaf P concentration, and leaf C concentration were determined following Kjeldahl methods (Allen, 1989) and expressed on a dry matter basis (%).

2.3 Leaf anatomy and morphology

Leaves of *A. maritima* were isolated and fixed in FAA (10% formaldehyde, 5% acetic acid and 45% ethanol) (Sass, 1958) for 24 h, dehydrated in an ethanol series (70%, 96%, 100%) and subsequently embedded in LR White acrylic resin (SIGMA). Leaf samples were placed in gelatine capsules together with embedding resin which was then hardened at 60 °C over night. Leaf serial cross sections (3 µm) were cut by means of an ultracut (Ultratome Nova, LKB, Stockholm, Sweden) using a glass knife. Leaf sections were adhered to gelatine coated slides and stained with toluidine blue solution (TBO) (0.05% in 0.1M phosphate buffer at pH 6.8) as a generic stain (tannins will appear green to blue-green; O'Brien and Mc Cully, 1981) and with periodic acid Schiff (PAS) for non-cellulosic polysaccharides (O'Brien and McCully, 1981). Histological analysis was performed by optical microscopy (OM; Leitz, Diaplan) to assess leaf anatomy. The epidermal cell thickness (ET) was also determined. Epidermal strips (approx 0.3 x 0.4 cm) were taken from between the major lateral veins of both surfaces of fresh leaves, as described by Fischer (1968), to determine stomatal density (SD), expressed as the number of stomata per square mm leaf area and stomatal size (SS), defined as the length (µm) between the junctions of the guard cells at each end of the stoma, and may indicate the maximum potential opening of the stomatal pore (Zhenzhu and Guangsheng, 2008). For each site, 20 mature leaves were randomly collected and analysed. Leaf area (LA) of the same leaves was determined as one-sided projected surface area of a fresh leaf, expressed in mm². Cross sections of the fresh leaves were analysed by software (Leica Application Suite V3) in order to measure leaf thickness (LT, mm). Succulence index (SI) was calculated as the ratio of the difference between the

oven-dry mass of a leaf and its water-saturated fresh mass to the leaf surface area, expressed in mg cm^{-2} (Read et al., 2005). Leaves were stored in moist paper in a cool box and rehydrated in laboratory for at least 6 h at 4 °C before measurements (Cornelissen et al., 2003). A Leitz diaphan light microscopy equipped with a Leica DC 300F camera was used for image acquisition. Leaf projected area was acquired with a CanoScan LiDE 90 (Canon) and determined by CompuEye, Leaf & Symptom Area software (<http://www.ehabsoft.com/CompuEye/LeafSArea/>).

2.4 Chlorophyll determination

Chlorophylls (*a*, *b*, and total) were extracted by soaking leaves in N,N-dimethylformamide for 24h at 2°C, in the dark. Pigments were determined spectrophotometrically according to Moran (1982) and expressed as mmol g^{-1} DW.

2.5 Oxidative stress and antioxidant response

H₂O₂ concentration of leaves was determined according to Jana and Choudhuri (1982) using titanium chloride in H₂SO₄ for peroxide detection. The intensity of the yellow colour of the supernatant was measured at 410 nm by spectrophotometer. The amount of H₂O₂ in the extracts, calculated from a standard curve, was expressed as mmol g^{-1} DW. Lipid peroxidation was estimated by determining the malonyldialdehyde (MDA) concentration in the leaves according to Hartley-Whitaker et al. (2001), with minor modifications. Powder from freeze-dried leaves material 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. The level of phenolic compounds was calculated as the 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 was determined at 525 nm by spectrophotometer 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 concentration was expressed as mg g^{-1} DW. Glutathione was extracted and determined according to Gossett et al. (1994). Total glutathione (reduced, GSH +oxidised, GSSG forms) was determined by the 5,5'- dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure 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. For enzyme assays, leaves were ground in liquid nitrogen with a mortar and pestle with extraction at 4°C, according to Spanò et

al. (2011), with minor modifications. In particular, extraction buffer contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), and 30% (w/v) polyvinylpyrrolidone (PVP-40). Ascorbate peroxidase (APX, EC 1.11.1.11) was extracted using the same buffer containing also 2 mM ascorbate; the supernatant of glutathione reductase (GR, EC 1.6.4.2) was desalted on a Sephadex G-25 column. The activity of APX was determined following the decrease in absorbance at 290 nm as ascorbate was oxidised (Nakano and Asada, 1981). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) was determined by monitoring the glutathione-dependent reduction of dehydroascorbate at 265 nm (Nakano and Asada, 1981). The activity of GR was determined as described by Rao et al. (1995) following the oxidation of NADPH at 340 nm. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) was assayed as in Zhang and Kirkham (1996) by monitoring the decrease in absorbance at 340 nm because of the oxidation of NADH. Ascorbate oxidase (AO, EC 1.10.3.3) activity was measured as described by Moser and Kanellis (1994). The activity was determined by monitoring ASA oxidation following the decrease in absorbance at 265 nm. Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its reaction with that of GR. The activity was determined by following the oxidation of NADPH at 340 nm. Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi (1984) by monitoring the decomposition of H₂O₂ at 240 nm. All enzymatic activities were determined at 25 °C and expressed as U g⁻¹protein. Protein measurement was performed according to Bradford (1976), using BSA as standard.

2.6 Statistical analysis

Differences in morphological and physiological leaf traits were determined by analysis of variance (ANOVA). Bartlett's test was applied to the data to verify the homogeneity of variances. When the variances were not homogeneous, we compared the data using the nonparametric test of Kruskal-Wallis. Constrained correspondence analysis (CCA) was used to find correlations between the environmental parameters and leaf traits. All statistical tests were performed using R 2.14.0 software (vegan package for CCA, Oksanen et al., 2011, <http://www.r-project.org/>).

3. Results

3.1 Soil and plant nutrient status

The soil underlying all the three populations was alkaline (Table 1). The substrata of areas A and C (Table 1) were relatively similar, while the soil from B, characterised by the highest conductivity and Na⁺ concentration, was relatively nutrient-rich (high N and P concentration). The A and C

populations lived on typical sand dune ecosystems, characterised by an overwhelming preponderance of sand and low percentages of clay and silt (A: sand: 96.2%, A: clay: 3%, A: silt: 0.8%; C: sand: 93.5%, C: clay: 5.3%, C: silt: 1.2%). By contrast, the plants at B colonised rocky cliffs, characterised by a soil texture with a considerably lower percentage of sand and greater amounts of clay and silt (B: sand: 80.1%, B: clay: 6%, B: silt: 13.9%). Plants at B exhibited the highest value of leaf Na⁺ concentration ($P < 0.001$) and the lowest concentration of leaf nitrogen ($P < 0.001$) and phosphorus ($P < 0.001$). The highest value of mean leaf carbon concentration ($P < 0.001$) was detected in the B population (Table 1).

3.2 Leaf anatomy and morphology

Leaves were more or less orthotropic, very close to the stem, with a dorsi-ventral rolled lamina and biseriate glandular hairs, typical for the *Asteraceae*. In mature leaves, the glandular hairs were distributed very thickly over the abaxial surface, within depressions of the epidermis. Epidermal stripping of the leaves revealed large cells with a lobed shape on the adaxial side (Fig. 1a) and a smooth contour on the abaxial side (Fig. 1b). The epidermis was uniseriate, especially well-developed in height, with a thick, roughened cuticle that sometimes rolled up to form several ridges with longitudinal microchannels (Fig. 1c, d). The cell walls were thick, especially the outer one, and stained intensely with TBO and PAS (Fig. 1d). The thickness of the epidermal cells, including the cuticle, was significantly greater in plants from B than in plants from A ($P < 0.001$) and C ($P < 0.001$) (Table 2). Stomata were present on both leaf surfaces (Fig. 1a-b, e-g) and overlaid a wide substomatal chamber. Some stomata on the adaxial side of the leaf protruded above the other epidermal cells (Fig. 1h). The stomatal density (SD) of the adaxial surface was significantly higher than that of the abaxial one for all the three sites ($P < 0.001$). The B plants had the highest total value of SD, as compared to plants from A ($P < 0.001$) and C ($P = 0.028$), and smaller-sized stomata than the plants from A ($P = 0.015$) and C ($P < 0.001$) (Table 2). Hydathodes, characterised by few, thin-walled epithem cells, were located along the leaf margins in all the populations studied (Fig. 1i). The mesophyll showed a palisade parenchyma, characterised by the presence of numerous and large substomatal chambers on both leaf sides and several vascular bundles (Fig. 1e-g). Only the mesophyll of leaves collected at B was characterised by the presence of sub-epidermal cells containing tannins (Fig. 1l). In all populations, a water-storage parenchyma made of large, thin-walled cells was present in the middle layers of the mesophyll (Fig. 1e-g). The B plants exhibited the highest values of leaf thickness and succulence index, differing significantly in these parameters from both A ($P < 0.001$ for LT, $P = 0.025$ for SI) and C plants ($P < 0.001$ for LT, $P = 0.043$ for SI) (Table 2). Leaf area did not vary significantly among the populations (Table 2).

3.3 Chlorophyll

There was no significant difference in total chlorophyll concentration between the plants from A and C, while a significantly lower chlorophyll concentration was detected in B plants ($P < 0.01$, Table 2).

3.4 Oxidative stress and antioxidant response

The H₂O₂ concentration (Table 3) was significantly higher in B plants than in plants from the other two sites ($P < 0.001$). Membrane damage, evaluated as lipid peroxidation (Table 3), was approximately 1.6 and 1.4 times higher in A and C plants, respectively, than in B plants ($P < 0.001$). Antioxidant level was assessed by monitoring variations in phenols and ascorbate/glutathione cycle metabolites. Phenol concentration (Table 3) was significantly higher in C plants than in the other populations ($P < 0.001$). The ascorbate pool (Table 3) was instead significantly higher in A plants and, especially, in B plants than in C plants ($P < 0.001$). In addition, the B population showed the highest ASA/DHA ratio (0.78), versus C (0.34) and A (0.40). Glutathione was undetectable. No DHAR or GR activity was detectable. APX (Table 3) exhibited different activity levels in plants from the different populations, with a minimum in A and a maximum in B plants ($P < 0.001$). MDHAR activity (Table 3) and the barely detectable CAT activity (data not shown) did not differ significantly among the three populations. The activity of GPX was 4-5 times higher in B plants than in A and C plants ($P < 0.001$, Table 3). Low AO activity was detected only in B plants (Table 3). Lastly, constrained correspondence analysis (CCA) revealed that the three coastal sites were well separated in the two-dimensional space (Fig. 2). Annual rainfall and average temperature were the most important environmental factors influencing plant responses.

4. Discussion

Many of the morphological features observed in the leaves of *Anthemis maritima* can be considered as the adaptations to the constraints imposed by coastal habitats. The dorsi-ventral rolling of the leaf, also observed in other psammophytes (Ciccarelli et al., 2009, 2010), could be viewed as an adaptation to reduce the adaxial surface and to limit transpiration and water loss through the formation of a sort of crypt with a significantly higher stomatal density than the abaxial surface. Moreover, the presence of stomata on both leaf surfaces could enable more efficient gas exchange in partially buried leaves, thereby providing some protection against the effects of sand burial. Hydathodes were found along the leaf margins of all the *A. maritima* populations. It has been suggested that these structures are involved in eliminating mineral salts in guttation solution (Chen and Chen, 2006) and would thus serve to prevent excessive accumulation of minerals within the plant.

Although the populations studied did not reveal any significant differences in terms of leaf area, the B plants exhibited the highest leaf thickness value. This is due to the larger size of the epidermal cells and the water-storage parenchyma, which is also consistent with the high succulence index detected in this population. This is a common trait in plants subjected to drought-stress (Beck, 2005) and consistent with the climatic conditions at B, which was the driest of the three areas. In addition, the thicker epidermal cells in the B plants may represent a reserve of water, at the level of vacuole and cell wall, and could play an important role in reducing the risk of overheating of the underlying parenchyma. Moreover, the presence of sub-epidermal cells containing tannins in the B population alone may be linked to the relatively high soil salinity levels, as NaCl is known to stimulate production of condensed tannins, which are important protective molecules (Reinoso et al., 2004). Increased levels of NaCl induce progressive absorption of Na⁺ (Turan et al., 2007), so it is not surprising that the B plants, which grew on the most saline soil, exhibited the highest sodium concentration. Although the soil at B was relatively nutrient-rich (high concentration of N and P), according to Cha-Um and Kirdmanee (2009), its higher salinity would negatively influence nutrient uptake. According to Cordovilla et al. (1995), sodium decreased N concentration in shoot tissues, and the negative effects of NaCl on plant nitrogen concentration could be explained by the antagonism between Cl⁻ and NO₃⁻, as also reported by Wehrmann and Hahndel (1984). Despite the lower concentration of chlorophyll and the negative effect of salinity on carbon assimilation, the highest total carbon concentration, indicative of a higher photosynthetic capacity (Hgaza et al. 2009), was detected in the B plants. This contradiction, however, may be explained by smaller stomata and higher stomatal density, that characterise B plants. Smaller stomata have, in fact, faster response times and have been correlated with a higher photosynthetic capacity (Drake et al. 2013). In addition, Spence et al. (1986) reported that small guard cells may allow stomata to remain open in drought conditions, ensuring a balance between photosynthetic carbon assimilation and excessive water loss, a particularly important factor for the B plants, which live in the driest conditions. This balance could be further ensured by the higher stomatal density of B plants, another trait often detected in plants subjected to water deficit. It has frequently been reported that salt and drought stresses cause oxidative damage to plant tissues (Cruz de Carvalho, 2008; Gomez et al., 1999). Under stress conditions, ROS production overwhelms the antioxidant system, and oxidative stress ensues. However, plants have evolved a number of protective mechanisms, both enzymatic and non-enzymatic, to protect cellular structures from damage. According to Gill and Tuteja (2010), the environmental constraints of coastal habitats produced oxidative stress in *A. maritima* leaves with significantly higher amounts of H₂O₂ in the B plants, which lived under the most stressful conditions and exhibited the highest Na⁺ leaf concentration. However, lipid peroxidation, often used as an indicator of oxidative stress (Gomez et

al., 1999), was the lowest in the B plants, which appeared well protected against oxidative damage. Thus, although protection against oxidative stress seems to depend mainly on non-enzymatic antioxidants, the relative importance of the different protective mechanisms appears to be different in the three populations. Phenols turned out to be the main protective molecule in the C plants, which grow on the lowest salinity soil, and under the most favourable climatic conditions. In contrast, ascorbate concentration, correlated with plant protection against salt and drought stresses (Cruz de Carvalho, 2008; Jaleel et al., 2008), was high in the A plants and reached its highest value in the B plants. Considering that B plants, despite their high peroxide concentration, exhibit the lowest degree of damage in terms of lipid peroxidation, it seems that a high ascorbate pool and ASA/DHA ratio are particularly protective, confirming that high levels of endogenous ascorbate are essential for maintaining the protective antioxidant system. The maximum ascorbate concentration, found in the B plants, is associated with the highest ascorbate peroxidase activity. This enzyme is a major constituent of the H₂O₂ scavenging system in chloroplasts (Logan et al., 2006). Moreover, the significantly higher carbon concentration found in the B plants (despite their lower N and chlorophyll concentration) with respect to the other two populations could in part be explained by high-capacity chloroplast protection by ascorbate peroxidase. The presence of ascorbate oxidase solely in the B plants is consistent with the high DHA value detected in this population. As dehydroascorbate has been hypothesised as an early activator of stomatal closure (Fotopoulos et al., 2008), the activity of ascorbate oxidase may be related to the need for fine regulation of stomatal opening under the particularly stressful conditions of area B, further emphasising the pivotal role of stomatal regulation in stress conditions. The low catalase activity detectable in the dune plants is rather surprising, but could be explained, at least in part, by the reported phenomenon of down-regulation of this enzyme by salt, high temperatures, and high radiation levels (Foyer and Noctor, 2000; Hertwig et al., 1992). Glutathione peroxidase activity, particularly high in the B plants, was present in all populations. As glutathione was not detectable, the enzyme probably had a substrate different from this thiolic compound. This is supported by studies showing that some plant and yeast GSH peroxidases can reduce peroxides much more efficiently, or sometimes even exclusively, by using the thioredoxin system rather than GSH as a reductant (Navrot et al., 2006). On the whole, antioxidant enzyme activities were particularly high in the B plants, in which the highest H₂O₂ concentration was revealed, thus confirming the function of this ROS as a signalling molecule for activation of antioxidant enzymes (Chaparzadeh et al., 2004). The CCA analyses enabled a clear separation of the three coastal sites in the multivariate space. Interestingly, the most important ecological factors influencing plant responses were rainfall and temperature, hence suggesting that drought conditions are more stressful than salinity for *A. maritima* populations.

In conclusion, there is no single strategy in the response of *A. maritima* populations to the constraints of different coastal environments. This species is able to trigger different morphological, anatomical and physiological traits to survive in these stressful habitats. *A. maritima* exhibits a high degree of leaf trait plasticity changing stomatal density and size, leaf thickness and succulence index, and activating different, mainly ascorbate- or phenol-based responses to oxidative stress. In particular, the high capacity for regulating the number and the size of stomata, as well as the response to oxidative stress seem to be of great eco-physiological significance. In addition, the different adaptive response of *A. maritima* populations appears to be linked to climatic conditions, especially severe water deficit and high temperatures, rather than to soil characteristics of the three study areas and this is a further evidence of the importance of plant plasticity also in view of climate changes (Nicotra et al., 2010).

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Figure captions

Fig. 1. Leaf anatomy of *Anthemis maritima*. a, b - Epidermal stripping of the adaxial and the abaxial side of a leaf from Casalbordino (C). c – Particular of the abaxial epidermis of a leaf: two kidney shaped guard cells of a stoma and roughened cuticle are evident. d – Uniseriate epidermis with a thick rolled cuticle that forms longitudinal microchannels (arrows). e, f, g - Cross sections of leaves, collected at Pianosa (B), Principina (A), and Casalbordino (C) respectively. Parallel vascular bundles (arrows) immersed in the water-storage parenchyma which consists of large cells and small intercellular spaces. h – Stomata in the adaxial side of the leaf protruding above the other epidermal cells. i – Hydathode with two stomata (arrows) and a large sub-stomatal chamber in contact with the bundle of tracheids. j – Subepidermal cells containing tannins (arrows) in a leaf from Pianosa (B) (c = substomatal chamber, s = stomata, t = tracheids, w = water-storage parenchyma). Scale bars = 45 μm (a, b, l); 10 μm (c, d); 80 μm (e, f, g); 60 μm (h, i). Section d was stained with toluidine blue (TBO) and periodic acid Schiff (PAS); e-j with TBO.

Fig. 2. Constrained correspondence analysis (CCA) plot showing the three coastal sites: Principina (A), Pianosa (B), Casalbordino (C), the most important environmental factors (rainfall and temperature) and morphological and physiological leaf traits (SD: stomatal density, SS: stomatal size, LT: leaf thickness, LA: leaf area, SI: succulence index, Chl: chlorophyll concentration, H_2O_2 : hydrogen peroxide, Perlip: lipid peroxidation, Phe: phenolic compounds, Asc: ascorbate, APX: ascorbate peroxidase, MDHAR: monodehydroascorbate reductase, GPX: glutathione peroxidase). Eigenvalues for constrained axes were 0.115 for CCA1 and 0.004 for CCA2.

Table 1

Chemical characteristics of samples of *Anthemis maritima* and soils from the three investigated coastal sites.

	Principina (A)	Pianosa (B)	Casalbordino (C)
<i>Soil*</i>			
pH	7.72 ^b ± 0.07	8.21 ^a ± 0.01	8.04 ^a ± 0.07
Conductivity (µS cm ⁻¹)	39.70 ^b ± 0.70	154.77 ^a ± 10.54	36.57 ^b ± 1.05
Total N (g kg ⁻¹)	0.72 ^b ± 0.02	2.23 ^a ± 0.16	0.18 ^c ± 0.003
Available P (ppm)	1.03 ^c ± 0.17	17.73 ^a ± 0.64	1.93 ^b ± 0.04
Na ⁺ (ppm)	25.79 ^b ± 1.37	129.50 ^a ± 4.94	12.15 ^c ± 0.03
<i>Plant material*</i>			
Leaf N content (%)	1.25 ^b ± 0.01	1.06 ^c ± 0.01	1.48 ^a ± 0.02
Leaf P content (%)	4.02 ^a ± 0.08	2.68 ^c ± 0.07	3.32 ^b ± 0.05
Leaf C content (%)	37.79 ^b ± 0.56	42.17 ^a ± 0.42	32.03 ^c ± 0.43
Na ⁺ (%)	0.58 ^c ± 0.01	2.23 ^a ± 0.06	0.82 ^b ± 0.01

Data are the mean of three replicates ± SE. Means followed by the same letters are not significantly different at 5% according to one-way ANOVA test.

* Data are expressed on a dry matter basis (%).

Table 2

Morphological traits and chlorophyll content of *A. maritima* from the three investigated coastal sites.

	Principina (A)	Pianosa (B)	Casalbordino (C)
Adaxial stomatal density (No. stomata mm ⁻²)	50.56 ^b ± 1.03	59.78 ^a ± 1.81	54.87 ^a ± 1.65
Abaxial stomatal density (No. stomata mm ⁻²)	29.02 ^b ± 0.97	34.33 ^a ± 2.15	28.95 ^{ab} ± 1.69
Total stomatal density (No. stomata mm ⁻²)	39.79 ^b ± 0.73	47.05 ^a ± 1.60	41.91 ^b ± 1.44
Stomatal size (µm)	35.41 ^a ± 0.68	32.69 ^b ± 0.74	37.02 ^a ± 0.49
Leaf thickness (mm)	0.79 ^b ± 0.02	1.20 ^a ± 0.02	0.77 ^b ± 0.02
Epidermal cells thickness (µm)	20.41 ^b ± 0.71	33.91 ^a ± 0.48	20.31 ^b ± 0.45
Leaf area (mm ²)	100.11 ^a ± 11.3	126.03 ^a ± 9.1	126.32 ^a ± 11.1
Succulence index (mg cm ⁻²)	59.44 ^b ± 2.76	68.76 ^a ± 4.47	61.35 ^b ± 1.28
Total chlorophyll (mmol g ⁻¹ DW)	8.74 ^a ± 0.15	6.85 ^b ± 0.21	9.05 ^a ± 0.32
Chlorophyll <i>a/b</i>	2.36 ^a ± 0.07	2.36 ^a ± 0.04	2.41 ^a ± 0.04

Data are the mean of ten replicates ± SE. Means followed by the same letters are not significantly different at 5% according to one-way ANOVA test.

Table 3

Selected physiological characteristics of *A. maritima* from the three investigated coastal sites.

	Principina (A)	Pianosa (B)	Casalbordino (C)
Hydrogen peroxide ($\mu\text{mol g}^{-1}\text{DW}$)	106.89 ^b \pm 0.22	153.22 ^a \pm 0.44	100.88 ^c \pm 0.22
Lipid peroxidation (MDA nmol g^{-1}DW)	393.72 ^a \pm 7.04	241.99 ^c \pm 4.46	336.00 ^b \pm 5.13
Phenols (mg GAE g^{-1}DW)	2.10 ^b \pm 0.02	1.90 ^c \pm 0.01	3.70 ^a \pm 0.09
Total ascorbate (mg g^{-1}DW)	8.00 ^b \pm 0.08	13.19 ^a \pm 0.75	3.96 ^c \pm 0.08
ASA/DHA	0.40 ^b \pm 0.01	0.78 ^a \pm 0.09	0.34 ^b \pm 0.01
APX (U $\text{g}^{-1}\text{protein}$)	199.98 ^c \pm 8.25	263.99 ^a \pm 4.12	240.02 ^b \pm 8.15
MDHAR (U $\text{g}^{-1}\text{protein}$)	35.03 ^b \pm 1.84	38.81 ^a \pm 1.87	31.99 ^c \pm 1.85
GPX (U $\text{g}^{-1}\text{protein}$)	164.17 ^b \pm 2.79	722.40 ^a \pm 29.79	151.77 ^c \pm 1.86
AO (U $\text{g}^{-1}\text{protein}$)	–	23.00 \pm 3.62	–

Data are the mean of ten replicates \pm SE. Means followed by the same letters are not significantly different at 5% according to one-way ANOVA test.

Fig.1

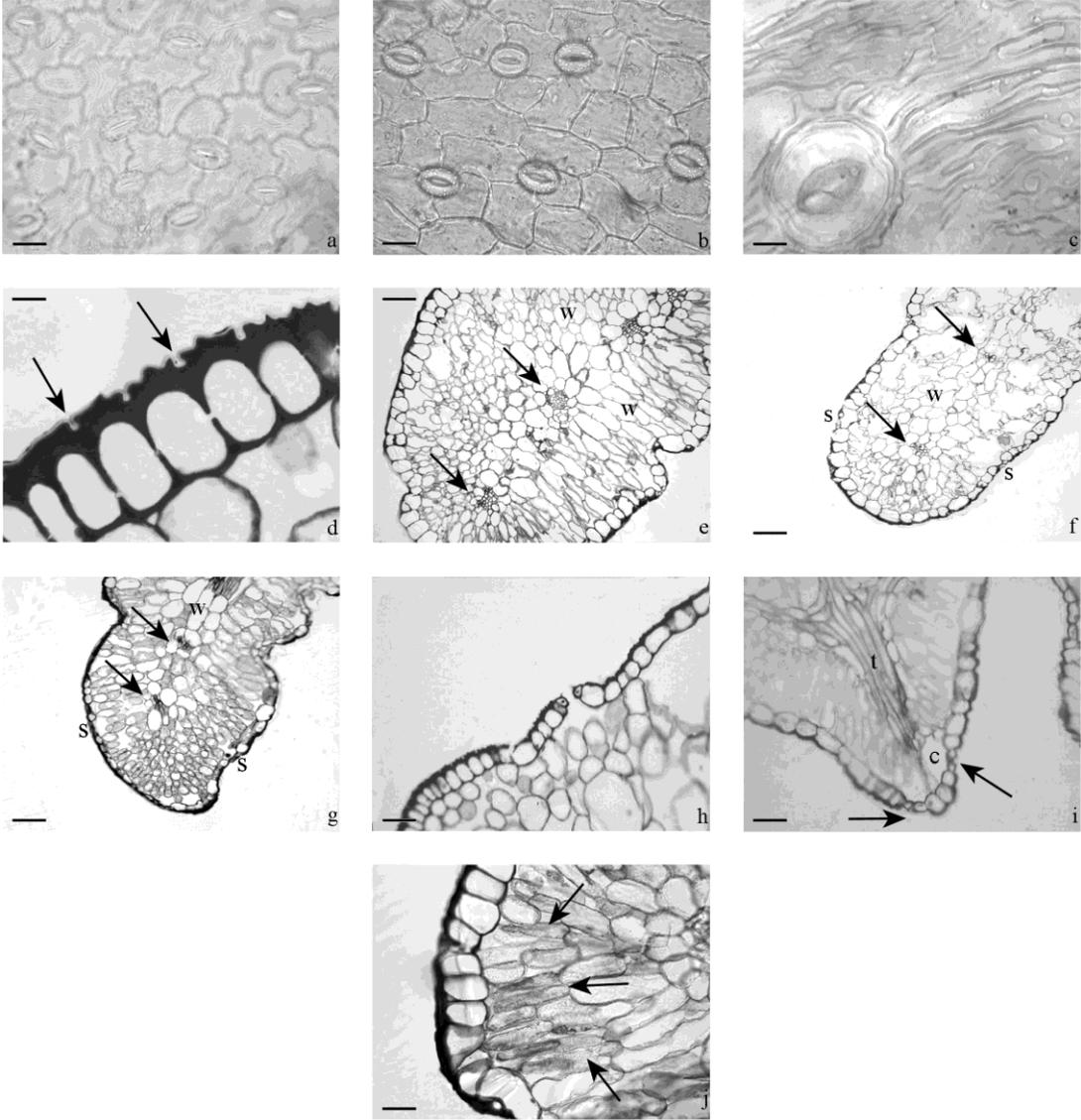


Fig.2

