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Leaf traits variation in *Sesleria nitida* growing at different altitudes in the Central Apennines

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Abstract

Global climate change may act as a potent agent of natural selection within species with Mediterranean mountain ecosystems being particularly vulnerable. The aim of this research was to analyze whether the phenotypic plasticity of *Sesleria nitida* Ten. could be indicative of its future adaptive capability to global warming. Morphological, anatomical, and physiological leaf traits of two populations of *S. nitida* growing at different altitudes on Mount Terminillo (Italy) were analyzed. The results showed that leaf mass per unit leaf area, leaf tissue density, and total leaf thickness were 19, 3, and 31% higher in leaves from the population growing at 1,895 m a.s.l. (B site) than in leaves from the population growing at 1,100 m a.s.l. (A site), respectively. Net photosynthetic rate (P_N) and respiration rate (R_D) peaked in June in both A and B leaves [9.4 ± 1.3 μmol(CO$_2$) m^{-2} s^{-1} and 2.9 ± 0.9 μmol(CO$_2$) m^{-2} s^{-1}, respectively] when mean air temperature was 16 ± 2°C. R_D/P_N was higher in B than in A leaves (0.35 ± 0.07 and 0.21 ± 0.03, respectively, mean of the study period). The mean plasticity index (PI = 0.24, mean of morphological, anatomical, and physiological leaf traits) reflected *S. nitida* adaptability to the environmental stress conditions at different altitudes on Mount Terminillo. Moreover, the leaf key traits of the two populations can be used to monitor wild populations over a long term in response to global change.

Additional key words: adaptation; leaf anatomy; photosynthesis; respiration.

Introduction

Environmental conditions for growth and reproduction of plants are generally considered to become more severe with increasing altitude in mountain areas (Taguchi and Wada 2001). Mountains are among the most fragile environments in the world and disproportionately exposed to climate change (Theurillat and Guisan 2001, Diaz *et al.* 2003). In high mountain environments, small changes in altitude can correspond to large shifts in temperature, humidity, exposure, and other abiotic factors (Körner 1999, Hovenden and Vander Schoor 2004, Neuner and Pramsohler 2006, Aryal and Neuner 2010, Gratani *et al.* 2012). The forecasted increase of air temperature at a global level could determine the loss of many European high-mountain plant species (Neuner *et al.* 1999, Thuiller *et al.* 2005, Neuner and Pramsohler 2006, Engler *et al.* 2011, Pauli *et al.* 2012). The Mediterranean region lies in a transition zone between the arid climate of North Africa and the temperate rainy climate of Central Europe (Ruiz-Labourdette *et al.* 2013). In this ecotone, even minor alterations can lead to substantial changes in climate

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Abbreviations: A site – 1,100 m a.s.l.; B site – 1,895 m a.s.l.; C – soil organic carbon content; CD – mesophyll cell density; DM – dry mass; DXV – diameter of the xylematic vessels; E – transpiration rate; ET$_{ab}$ – abaxial epidermis thickness; ET$_{ad}$ – adaxial epidermis thickness; g_s – stomatal conductance; HCB – height of the central vascular bundle; HLB – height of the major lateral vascular bundle; LA – leaf area; LMA – leaf mass per unit leaf area; LT – total leaf thickness; LTD – leaf tissue density; LW – leaf width; N – total soil nitrogen content; PI – mean plasticity index; PI$_a$ – anatomical plasticity index; PI$_m$ – morphological plasticity index; PI$_p$ – physiological plasticity index; P_N – net photosynthetic rate; R_D – respiration rate; RH – relative air humidity; SAI$_{ab}$ – abaxial stomatal area index; SAI$_{ad}$ – adaxial stomatal area index; SBC – total surface area of bulliform cells; SD$_{ab}$ – abaxial stomatal cell density; SD$_{ad}$ – adaxial stomatal cell density; SL$_{ab}$ – abaxial stomatal length; SL$_{ad}$ – adaxial stomatal length; SOM – soil organic matter content; SWC – soil water content; T – air temperature; T_l – leaf temperature; T_m– mean air temperature; T_{max} – mean maximum air temperature; T_{min} – mean minimum air temperature; UST – thickness of the upper sclerenchyma layers; WCB – width of central vascular bundle; WLB – width of the major lateral vascular bundle.

making Mediterranean mountains a potentially vulnerable region (Lionello *et al.* 2006). It is therefore of the utmost importance to assess the impact of future climate change upon the biodiversity of Mediterranean mountains (Ruiz-Labourdette *et al.* 2013).

One of the predicted consequences of global climate change is the movement of plants to higher elevations as the climate, to which they are adapted, is displaced (Jump and Peñuelas 2005). An increasing number of studies attests to a widespread upward movement along altitudinal gradients (Frei *et al.* 2010). Similar altitudinal shifts in species distribution, linked to climate warming, have been shown in Mediterranean mountains also at lower altitudes (Penuelas and Boada 2003). Moreover, summer drought in Mediterranean high mountains might impose important reproduction limitations on late-flowering plants, especially at lower altitudes (Cavieres *et al.* 2006). At the present time, the adaptive capability of plant species growing in mountain areas to the hypothesized increase of air temperature has been poorly understood (Byars *et al.* 2007, Gratani *et al*. 2012). Nevertheless, rates and patterns of these dynamics may be highly dependent on the habitat preference of a particular species and on the ecological tolerance to stress factors (Pauli *et al.* 2003, Lenoir *et al.* 2008, Gratani *et al.* 2012). Moreover, the current rate of global warming might be too rapid for natural migration to successfully deliver species to suitable habitats (Rice and Emery 2003, Aitken *et al.* 2008). Changes in species distribution in accordance with expectations for climate warming have been recognized by many studies (Parmesan 2006, Walther 2010). Nevertheless, it is not clear whether the majority of plant populations is able to maintain current distribution under global climate change (Jump and Penuelas 2005). Byars *et al.* (2007) underline the importance of local plant trait variations, which can indicate the potential of plants to adapt to different altitudes, with environmental factors changing over a relatively small scale.

Among the southern European grasses, the genus *Sesleria* constitutes a complex group of similar and closely related taxa mostly distributed throughout Europe, where they colonize high mountain pastures and calcareous slopes (Pignatti 2005, Di Pietro 2007, Kuzmanović *et al.* 2009). The taxonomy of the genus is not clearly defined due to the intricate phenotypic variation patterns and delimitation of distribution areas for most of the species (Di Pietro 2007). Thus, it is important to increase the data set of the genus *Sesleria* (Deyl 1946, Stebbins 1956, Di Pietro 2007). The distribution area of the genus extends as far as Caucasus, Lebanon, and Persia to the East; Iceland, southern Scandinavia, and Saint Petersburg to the North; Spain to the West, and Marocco and Libya to the South (Deyl 1946). Among the species of the genus, *Sesleria nitida* Ten. grows in the Balkan Peninsula and in the north of Albania (Tutin *et al.* 1980, Kuzmanović *et al.* 2009). *S. nitida* is a mesophyllous species, which colonizes mainly arid soils, mildly basic or neutral-basophil and oligotrophic (Pignatti 2005). In Italy, *S. nitida* colonizes the Central and Southern Apennines from 600 to 2,000 m a.s.l. (Tutin *et al.* 1980, Pignatti 1982).

The aim of this research was to analyze morphological, anatomical, and physiological leaf traits of two wild populations of *S. nitida* growing at different altitudes on Mount Terminillo (Central Appenines). Recent studies indicate that under rapid climate change phenotypic plasticity rather than genetic diversity is likely to play a crucial role in allowing plants to persist in their environments (Vitasse *et al.* 2010). When environments within the distribution area of a species differ, it is unlikely that any single phenotype confers high fitness in all situations. In particular, phenotypic plasticity determines the short-term ecological response of a species to climate change and, in some cases, may directly buffer the effects of such change (Theurillat and Guisan 2001). We addressed the question of whether the plasticity of *S. nitida* leaf traits could be indicative of its future adaptive capability to global warming. Moreover, key traits that mainly contribute to the adaptive strategy of this species can be used to monitor these wild populations over a long period of time**.** The examined traits can also be used for systematic issues.

Materials and methods

Study area, climate, and soil characterization: The study was carried out from May to August 2012 in two wild populations of *Sesleria nitida* Ten. growing at 1,100 m a.s.l. (site A; 42°27’47” N, 12°56’12” E) and at 1,895 m a.s.l. (site B; 42°28’28’’ N, 13°00’24’’ E), respectively, both on calcareous rocky slopes (Pelorosso *et al.* 2011) on Mount Terminillo (Latium, Italy). The maximum vegetative activity of *S. nitida* was observed in June in A and B sites, followed by flowering and seed formation. Morphological, anatomical, and physiological measurements were carried out on representative plants (13 per population) randomly selected in their natural environments (A and B sites).

The climate of the site A was characterized by a mean minimum air temperature (T_{min}) of the coldest month (February) of –3.2°C, a mean maximum air temperature (T_{max}) of the hottest month (August) of 24.3°C, and a mean annual air temperature (T_{m}) of 8.7°C. Total annual rainfall was 1,180 mm and snow occurred from November to April (data from the Meteorological Station of Pian de Rosce, Centro Appenninico Carlo Jucci, for the years 1997–2012). At site B, T_{min} of the coldest month (February) was –4.3°C, T_{max} of the hottest month (August) was 20.9°C, and T_{m} was 6.6°C. Total annual rainfall was 1,249 mm and snow occurred from November to May (data from the Meteorological Station of Colle Scampetti, Centro Appenninico Carlo Jucci, for the years 1997–2012). In the period January–August 2012, at site A, T_{m} was 9.4°C and total rainfall 707 mm. At site B, T_m was 7.4°C and total rainfall 874 mm.

Soil samples (500 g each, 3 samples per sites A and B) were collected at the beginning of June, at least 5 d after the last rainfall, by a hand auger at 40 cm depth. Analysis was performed according to Violante (2000): pH, soil water content (SWC), total soil nitrogen content (N), soil organic matter content (SOM), and soil organic carbon content (C) were determined. Soil samples were air dried and then passed through a 2 mm sieve. The pH (in H_2O) was determined by a glass electrode pH meter on a 1:2.5 soil-water suspension. N was determined by the Kjeldahl method, and C by the oxidation method using potassium dichromate-sulfuric acid (Sims and Haby 1971). The ratio between carbon and nitrogen content (C/N) was calculated. SWC was calculated by the ratio between soil fresh mass and soil dry mass after oven-dried at 100–110°C to a constant mass.

Microclimatic measurements: Air temperature (T) and relative air humidity (RH) were measured at A and B sites by portable thermo-hygrometers (*HD8901*, *Delta Ohm*, Italy) when gas-exchange measurements were taken (from May to August).

Anatomical leaf traits: Measurements of anatomical leaf traits were conducted on fully expanded sun leaves ($n = 13$ per population) collected at the end of June from randomly selected plants per each of the two populations in their natural environments (A and B sites) and analyzed by light microscopy using an image analysis system (*Axiovision AC software*) at the midpoint of the leaf, according to Allard *et al.* (1991).

The following parameters were measured: total leaf thickness (LT); abaxial and adaxial epidermis thickness (ET_{ab} and ET_{ad}), mesophyll cell density (CD), abaxial and adaxial stomatal length (SL_{ab} and SL_{ad}), abaxial and adaxial stomatal cell density (SD_{ab} and SD_{ad}), the number of bulliform cells, and total surface area of bulliform cells (SBC). The abaxial and adaxial stomata area index (SAI_{ab} and SAI_{ad}) was calculated by the product of the mean stomatal length and SD (Ashton and Berlyn 1994, Gratani and Varone 2004). The thickness of the upper sclerenchyma layers (UST), height, and width of the major lateral vascular bundle (HLB and WLB, respectively), and of the central vascular bundle (HCB and WCB, respectively) were measured according to Kuzmanović *et al.* (2009). The diameter of the xylematic vessels (DXV) was measured, according to Nardini *et al.* (2012).

Morphological leaf traits were measured on fully expanded, sun leaves ($n = 13$ per population), collected at the end of June from randomly selected plants in A and B sites. The following parameters were measured: leaf area (LA), excluding sheath, and leaf width (LW) measured at the midpoint of the leaf, by an *Image Analysis System* (*Delta-T Devices*, UK); dry mass (DM) after drying at 80°C to constant mass. Leaf mass per unit leaf area (LMA) was calculated by the ratio between DM and LA (Reich *et al.* 1992) and leaf tissue density (LTD) by the ratio between LMA and LT (Wright *et al.* 2004).

Gas-exchange measurements were carried out by an infrared gas analyser (*LCA-Pro, ADC*, UK) equipped with a leaf chamber (*PLC*, *Parkinson Leaf Chamber*, UK). Measurements were made in the field (A and B sites) (13 fully expanded sun leaves per population on each sampling occasion) at the end of May, June, July, and August. P_N, stomatal conductance (g_s), transpiration rate (E), leaf temperature (T_l), and PAR were measured under natural conditions on cloud-free days, in the morning (from 9:00 to 12:00 h) to ensure that maximum P_N were measured (Reich *et al.* 1995, Varone and Gratani 2007). PAR was ≥1,500 μmol(photon) m^{-2} s^{-1} during the measurements.

On each sampling occasion, R_D was measured on the same leaves after P_N measurements, as CO_2 efflux after darkening a leaf chamber by a black paper for 30 min prior to measurements to avoid transient post-illumination bursts of CO_2 releasing (Atkin *et al.* 1998). R_D and P_N were recorded as the mean measured rates for three following days per month characterized by the same weather conditions. The ratio between R_D and P_N (R_D/P_N) was calculated according to Galmés *et al.* (2007).

Leaf plasticity: The plasticity index was calculated as the difference between the minimum and the maximum mean value divided by the maximum mean value, for those variables that were significantly different between the two populations (LT, HLB, WLB, HCB, WCB, DXV, CD, UST, SBC, SL_{ad}, ET_{ab}, ET_{ad}, LMA, LW, P_N, g_s, E, R_D, R_D/P_N), according to Valladares *et al.* (2000). The mean plasticity index (PI) was calculated by averaging the plasticity index for all the selected anatomical (PI_a), morphological (PI_m), and physiological (PI_p) leaf traits (Valladares *et al.* 2000).

Statistics: Differences in morphological anatomical leaf traits between A and B leaves were analyzed by one-way analysis of variance (*ANOVA*).

A two-way *ANOVA* was performed on the physiological variables in order to evaluate the effect of the main factors (month and site) and their interaction (month *vs.* site) on the response variables. Multiple comparisons were analyzed by a *Tukey*'s test. A regression analysis was carried out to evaluate the correlations between P_N and g_s and between E and g_s.

Of the 27 variables sampled (*i.e.* 5 morphological variables, 17 anatomical variables, and 5 physiological variables measured in June), the most relevant variables discriminating between the two populations of *S. nitida* (A and B sites) were determined. To decide which variables should be retained and which ones could be dropped, a stepwise selection procedure was used. To avoid overfitting (when number of predictors > number of observations) and multicolinearity (high correlations between several variables) an F-test (*ANOVA*) was first conducted on each variable and only variables with a $p<0.01$ were retained. A correlation analysis was conducted on the variables. In all cases, when two or more variables were too highly correlated (*i.e.* $R^2 > 0.9$) only one of them was retained within the descriptor set. A logistic regression and Akaike information criteria (AIC; Burnham and Anderson 2002) was applied to search for combinations of n out of the 14 variables retained by the previous two steps that best discriminated between the two *S. nitida* populations. The function 'glmulti' (Calcagno and De Mazancourt 2010), which was a part of an *R package* (*R Development Core Team* 2012) for automated model selection, and multimodel inference with generalized linear models, was used. From a list of explanatory variables, the function 'glmulti' built all possible models involving these variables allowing the selection of a subset of variables that best explained the response variable of interest (Calcagno and De Mazancourt 2010). To separate responsive from nonresponsive combinations of variables, all models were ranked by their AIC values, and only models within two AIC units above the best model were retained (Calcagno and De Mazancourt 2010). For each of the 21 models retained, the relative evidence weight was computed as $\exp(-\Delta\text{AIC}/2)$, where ΔAIC was the difference in AIC between a model and the best model. The relative evidence weights of each variable in the 'best' models were computed as the sum of the relative evidence weights of all models, in which the variable appeared (Calcagno and De Mazancourt 2010).

Results

Soil characterization: The measured soil parameters are shown in Table 1. The pH was not significantly different between A and B sites, while SWC was 48% higher in A than in B site. N and SOM were 82% and 90% higher in B than in A, respectively. C/N was not significantly different between A and B sites.

Microclimatic measurements: The microclimatic measurements are shown in Table 2. The T and RH were significantly higher in A than in B site (29% and 39%, respectively, mean of study period).

Table 1. Soil characterization at A site (1,100 m a.s.l.) and B site (1,895 m a.s.l.). N – total soil nitrogen content; SOM – soil organic matter content; SWC – soil water content; C/N – ratio between carbon and nitrogen content. Mean values (± SE) are shown (n = 3). *Different letters* indicate significant differences between A and B sites (*ANOVA*, $p<0.05$).

Parameter	Site A	Site B
pH	7.9 ± 0.01^{a}	7.9 ± 0.15^{a}
N [%]	0.39 ± 0.04^{a}	0.71 ± 0.08^{b}
SOM [%]	6.8 ± 0.18^{a}	12.9 ± 0.56^{b}
SWC [%]	3.1 ± 0.02^{a}	2.1 ± 0.01^{b}
C/N	17.7 ± 1.6^{a}	18.4 ± 2.6^{a}

Anatomical and morphological leaf traits: The measured anatomical parameters are shown in Table 3. LT was 31% higher in B than in A leaves. ET_{ab} and ET_{ad} were 6% and 8% thicker in B than in A leaves, respectively. SD was significantly higher in the adaxial than in the abaxial epidermis for both A and B leaves. SD_{ab} and SAI_{ab} were 22% and 31% higher in B than in A leaves, respectively, while SAI_{ad} was not significantly different between A and B leaves. Bulliform cells ($n = 9 \pm 1$ both in A and B leaves) occurred on the sides of central vascular bundle in the adaxial surface of the leaf. UST was 46% thicker in B than in A leaves. SBC, CD, HCB, HLB, WLB, and WCB were 8, 23, 64, 43, 32, and 48% higher in B than in A leaves, respectively. DXV was 22% higher in B than in A leaves. The leaf width was 25% larger in A than in B leaves, and LMA and LTD were 19% and 3% lower in A than in B leaves, respectively (Table 4).

Gas-exchange measurements: The highest P_{N} were monitored in June both in A and B leaves [10.3 ± 0.7 and 8.5 ± 0.6 μmol(CO$_2$) m^{-2} s^{-1}, respectively] decreasing by

Table 2. Trend of microclimate at sites A and B during the study period. T – air temperature; RH – relative air humidity. *Lowercase letters* show significant T differences, *capital letters* show significant RH differences at p=0.05.

Month	Site A T [°C]	RH [%]	Site B T [°C]	RH [%]
May	12.6 ± 2.8^{a}	74.8 ± 11.1^{A}	7.7 ± 3.3^{b}	63.4 ± 22.6^{B}
June	18.9 ± 3.0^{a}	70.1 ± 10.8^{A}	14.7 ± 3.8^{b}	50.1 ± 24.6^{B}
July	21.4 ± 1.9^{a}	60.6 ± 13.4^{A}	17.2 ± 2.7^{b}	42.3 ± 22.9^{B}
August	21.6 ± 1.7^{a}	61.2 ± 8.6^{A}	17.9 ± 2.0^{b}	36.4 ± 16.6^{B}

Table 3. Anatomical leaf traits of *Sesleria nitida* from populations growing at A site (1,100 m a.s.l.) and B site (1,895 m a.s.l.). LT – total leaf thickness; ET$_{ab}$ – abaxial epidermis thickness; ET$_{ad}$ – adaxial epidermis thickness; CD – mesophyll cell density; UST – thickness of the upper sclerenchyma layers; HLB – height of the major lateral vascular bundle; WLB – width of the major lateral vascular bundle; HCB – height of the central vascular bundle; WCB – width of the central vascular bundle; DXV – diameter of xylematic vessels; SBC – total surface area of bulliform cells; SL$_{ab}$ – abaxial stomatal length; SL$_{ad}$ – adaxial stomatal length; SD$_{ab}$ – abaxial stomatal cell density; SD$_{ad}$ – adaxial stomatal cell density; SAI$_{ab}$ – abaxial stomatal area index; SAI$_{ad}$ – adaxial stomatal area index. Mean values (± SE) are shown (n = 13 leaves). *Different letters* indicate significant differences between A and B leaves (*ANOVA*, $p<0.05$).

Parameter	Site A	Site B
LT [μm]	256.0 ± 7.4^{a}	336.3 ± 21.7^{b}
ET$_{ab}$ [μm]	25.7 ± 0.8^{a}	27.3 ± 2.3^{b}
ET$_{ad}$ [μm]	14.1 ± 0.6^{a}	15.2 ± 1.1^{b}
CD [cells μm^{-2}]	0.0017 ± 0.0003^{a}	0.0021 ± 0.0002^{b}
UST [μm]	64.1 ± 4.3^{a}	93.6 ± 26.2^{b}
HLB [μm]	103.8 ± 6.9^{a}	148.9 ± 2.7^{b}
WLB [μm]	69.4 ± 3.3^{a}	91.9 ± 5.1^{b}
HCB [μm]	75.9 ± 2.2^{a}	124.7 ± 3.4^{b}
WCB [μm]	64.3 ± 2.4^{a}	95.1 ± 4.6^{b}
DXV [μm]	11.6 ± 0.9^{a}	14.2 ± 1.2^{b}
SBC [μm^{2}]	8241.8 ± 217.9^{a}	8862.8 ± 524.0^{b}
SL$_{ab}$ [mm]	0.031 ± 0.007^{a}	0.034 ± 0.004^{a}
SL$_{ad}$ [mm]	0.031 ± 0.003^{a}	0.029 ± 0.002^{b}
SD$_{ab}$ [n mm^{-2}]	4.09 ± 5.48^{a}	4.99 ± 1.75^{a}
SD$_{ad}$ [n mm^{-2}]	293.1 ± 144.7^{a}	295.5 ± 89.8^{a}
SAI$_{ab}$	0.00013 ± 0.0017^{a}	0.00017 ± 0.0006^{a}
SAI$_{ad}$	0.0091 ± 0.0045^{a}	0.0087 ± 0.0026^{a}

Table 4. Morphological leaf traits of *Sesleria nitida* from populations growing at A site (1,100 m a.s.l.) and B site (1,895 m a.s.l.). LA – leaf area; DM – dry mass; LW – leaf width; LMA – leaf mass per unit leaf area; LTD – leaf tissue density. Mean values (± SE) are shown (n = 13 leaves). *Different letters* indicate significant differences between A and B leaves (*ANOVA*, $p<0.05$).

Parameter	Site A	Site B
LA [cm^{2}]	5.0 ± 1.7^{a}	4.8 ± 1.4^{a}
DM [g]	0.034 ± 0.01^{a}	0.041 ± 0.02^{a}
LW [mm]	4.5 ± 0.6^{a}	3.6 ± 0.5^{b}
LMA [mg cm^{-2}]	6.8 ± 0.9^{a}	8.1 ± 1.1^{b}
LTD [mg cm^{-3}]	250.1 ± 22.0^{a}	257.2 ± 16.4^{a}

46 and 49%, in July, respectively, and by 83 and 82% in August, respectively (Fig. 1*A*). T_l was 4.3°C higher in A than in B leaves (Fig. 1*F*). The g_s had the same P_N trend showing the highest values [0.036 ± 0.004 and 0.022 ± 0.002 mol(H_2O) m^{-2} s^{-1} in A and B leaves, respectively] in June decreasing by 66% and 27%, respectively, in August (Fig. 1*B*). The regression analysis was attested by the significant ($p<0.001$) correlations between g_s and P_N (Fig. 2*A*) and g_s and E (Fig. 2*B*).

The highest R_D were measured in June [2.3 ± 0.3 and 3.6 ± 0.2 μmol(CO_2) m^{-2} s^{-1} in A and B leaves, respectively] and the lowest values [0.30 ± 0.07 and 0.40 ± 0.15 μmol(CO_2) m^{-2} s^{-1} in A and B leaves, respectively] in August (Fig. 1*C*). The highest R_D/P_N (0.23 ± 0.03 and 0.43 ± 0.03 in A and B leaves, respectively) was monitored in June and the lowest one in August (0.18 ± 0.04 and 0.29 ± 0.12 in A and B leaves, respectively) (Fig. 1*E*). On an average, R_D/P_N was 64% higher in B than in A leaves.

Statistics and plasticity index: Two-way *ANOVA* showed a significant effect ($p<0.01$) of month, site, and their interaction on the physiological leaf variables (Table 5). In particular, most of the variability for P_N, g_s, E, and R_D was explained by the factor of month, while the factor site affected mainly R_D/P_N. On the whole, the month factor affected the variability more than the site factor or their interaction (F = 254.42, 85.87, 6.88, respectively; mean values).

The 14 variables retained by the stepwise selection procedure and their relative evidence weights are shown in Fig. 3. The set of 21 best variable combinations is shown in Table 6. The Akaike information criteria was computed as AIC = 2 k – 2 ln(L) where k was the number of parameters in the statistical model and L the maximized value of the likelihood function for the estimated model. Therefore, all models in Table 6 had maximum discrimination power for the two populations of *S. nitida* (*i.e.* L = 1 for all the models). The difference was only in the number of variables in the models, where a more parsimonious model was usually considered a better model than a less parsimonious one. Among the variables retained by the stepwise selection procedure, HCB, LT, and the R_D/P_N had the highest discriminant ability between the two populations.

PI$_p$ was higher (0.35) than PI$_m$ (0.18) and PI$_a$ (0.20) (Table 7). The HCB had the highest plasticity index (0.39) among the considered leaf morphological traits, LW (0.21) among the leaf anatomical traits, and R_D/P_N (0.47) among the leaf physiological traits. PI was 0.24.

Discussion

The assessment of plant response to the environmental factors contributes to the understanding of ecological differences among species (Bazzaz 1996). A fundamental question in a global change context is how plant species will respond to new environmental scenarios and what mechanisms will be involved in the process (Parmesan 2006). Many studies show the relevance of using plant traits to identify plant response to environmental changes

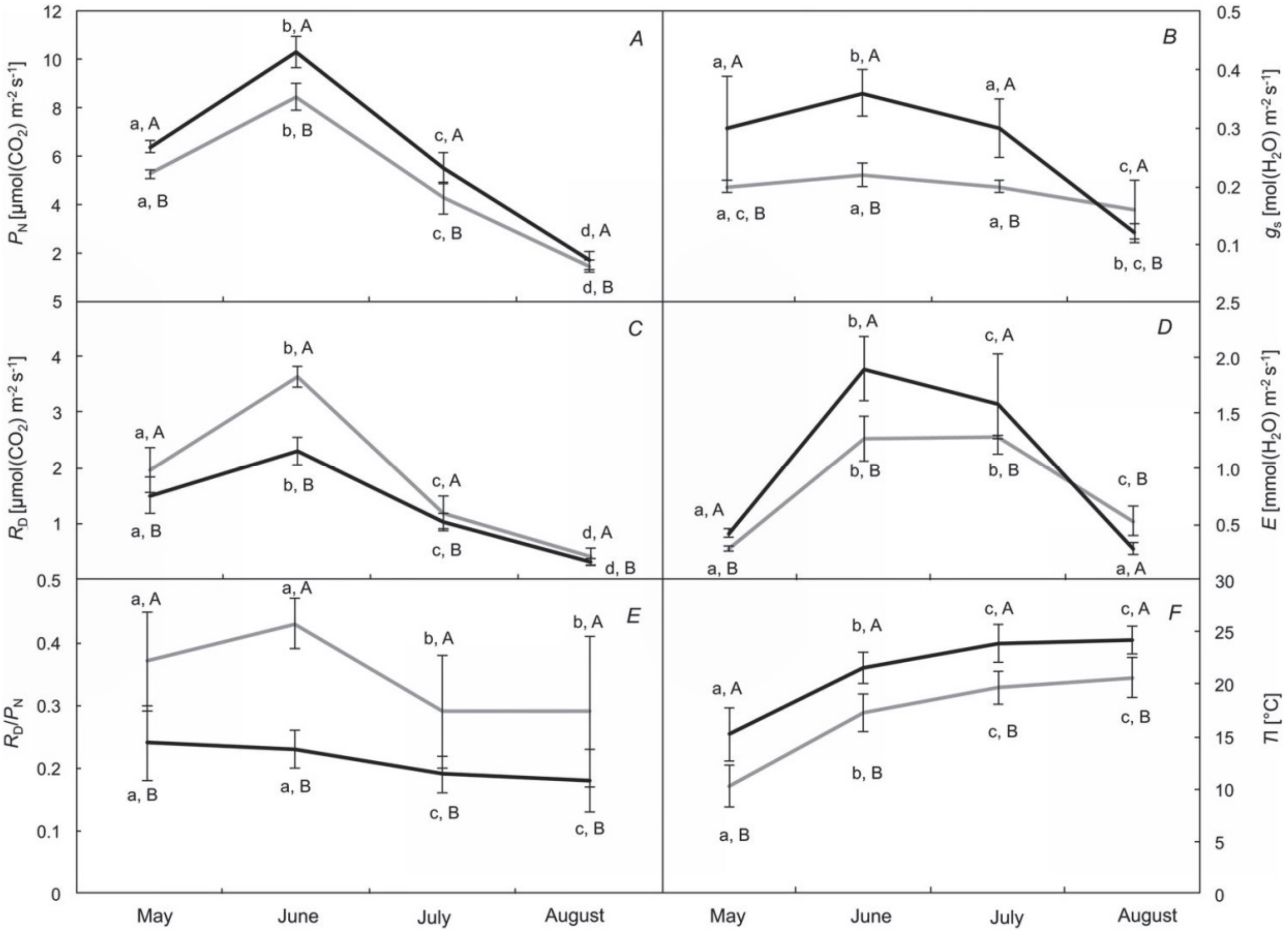

Fig. 1. Trends of net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), respiration rate (R_D) (*C*), transpiration rate (E) (*D*), ratio between R_D and P_N (R_D/P_N) (*E*), and leaf temperature (T_l) (*F*) of *Sesleria nitida* growing at 1,100 m a.s.l. (site A, *black line*) and at 1,895 m a.s.l. (site B, *gray line*) during the study period. The mean value (± SE) is shown (n = 13 leaves). *Lowercase letters* show significant intrapopulation differences, *capital letters* show significant interpopulation differences at p=0.05.

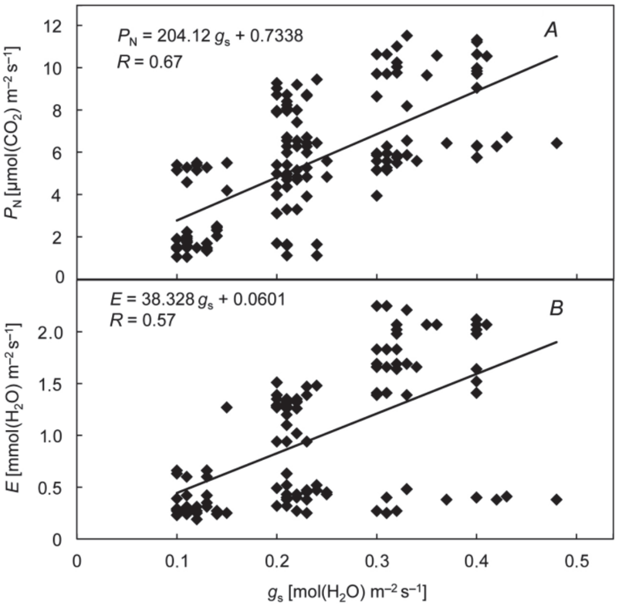

Fig. 2. Regression analysis between (*A*) net photosynthetic rate (P_N) and stomatal conductance (g_s), and (*B*) between transpiration rate (E) and stomatal conductance (g_s) of *Sesleria nitida*. Individual daily measurements, collected from May to August 2012 at sites A and B, were used as experimental units (n = 152, p<0.001).

(Doyle *et al.* 2008, Kahmen and Poschlod 2008). One way in which plants respond to environmental factor variations is through phenotypic plasticity (Pigliucci 2001). Plastic responses can affect the performance and reproductive success of individual plants and their ecological breadth (Sultan 2001, González and Gianoli 2004, Matesanz *et al.* 2010).

Our results on the whole show the capability of *S. nitida* to change anatomical, morphological, and physiological leaf traits in response to environmental factor variations at different altitudes on Mount Terminillo in the Central Apennines. With regards to leaf morphology and anatomy, a 19% higher LMA in B than in A leaves is

Table 5. Results of the two-way *ANOVA* (*F* values) for the five leaf physiological study variables for *Sesleria nitida*. P_N – net photosynthetic rate; g_s – stomatal conductance; E – transpiration rate; R_D – respiration rate; R_D/P_N – ratio between R_D and P_N. The main effects (site and month with 1 and 3 d.f., respectively) and the first order interaction (site × month with 2 d.f.) are shown. * – significant at $p<0.01$; ** – significant at $p<0.001$; ns – not significant.

Variable	Site	Month	Site × Month
P_N	139.59**	797.529**	4.159*
g_s	1.563^{ns}	46.609**	16.904**
E	45.54**	223.04**	11.05**
R_D	178.025**	199.525**	1.841^{ns}
R_D/P_N	64.621**	5.318*	0.434^{ns}

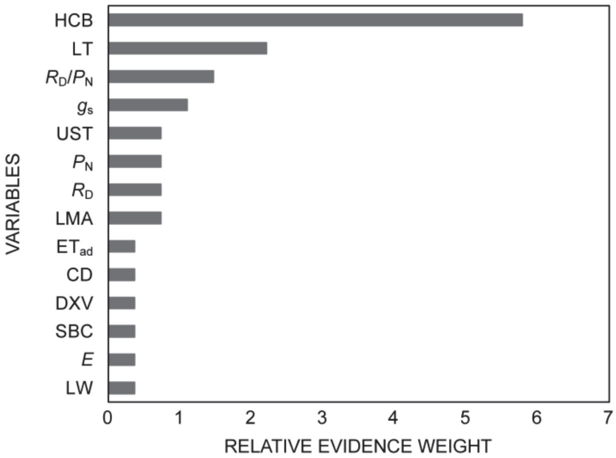

Fig. 3. The 14 variables for *Sesleria nitida* retained by the stepwise selection procedure together with their relative evidence weights are shown. HCB – height of the central vascular bundle; LT – total leaf thickness; R_D/P_N – ratio between respiration rate (R_D) in June and net photosynthetic rate (P_N) in June; g_s – stomatal conductance in June; UST – thickness of the upper sclerenchyma layers; P_N – net photosynthetic rate in June; R_D – respiration rate in June; LMA – leaf mass per unit leaf area; ET_{ad} – adaxial epidermis thickness; CD – mesophyll cell density; DXV – diameter of the xylematic vessels; SBC – total surface area of bulliform cells; E – transpiration rate in June; LW – leaf width.

associated to a higher leaf thickness (31%) and upper sclerenchyma thickness (46%). Moreover, the larger cell density (23%) in B than in A leaves determines a higher leaf compactness, which was expressed by a 3% higher LTD. The higher LMA and LTD, associated to a lower LA and LW (4% and 20%, respectively) in B leaves reduce leaf transpiration (20% lower in B, mean of study period), according to the results of Wright *et al.* (2004) and Gratani *et al.* (2012) for other mountain species. Moreover, *S. nitida* showed a 99% lower SAI in the abaxial leaf epidermis than in the adaxial one, which contributes to limit leaf transpiration. In fact, a lower SAI_{ab} than SAI_{ad} is a morphological feature accepted as the drought avoidance mechanism for other species of the same genus (Lloyd and Woolhouse 1978, Dixon 1986).

Table 6. List of the combinations of variables for *Sesleria nitida* contained in the best models selected by the "glmulti" procedure together with their AIC values. Int – Intercept; HCB – height of the central vascular bundle; LT – total leaf thickness; g_s – stomatal conductance in June; R_D – respiration rate in June; R_D/P_N – ratio between R_D and P_N in June; P_N – net photosynthetic rate in June; UST – thickness of the upper sclerenchyma layers; ET_{ad} – adaxial epidermis thickness; SBC – total surface area of bulliform cells; CD – mesophyll cell density; LW – leaf width; DXV – diameter of the xylematic vessels; LMA – leaf mass per unit leaf area; E – transpiration rate in June.

Variables combination	AIC
Int + HCB	4
Int + LT + g_s	6
Int + LT + R_D	6
Int + LT + R_D/P_N	6
Int + g_s + R_D/P_N	6
Int + LT + P_N	6
Int + LT + UST	6
Int + LT + HCB	6
Int + ET_{ad} + HCB	6
Int + HCB + UST	6
Int + HCB + SBC	6
Int + HCB + P_N	6
Int + HCB + R_D/P_N	6
Int + HCB + g_s	6
Int + HCB + CD	6
Int + HCB + LW	6
Int + HCB + R_D	6
Int + HCB + DXV	6
Int + HCB + LMA	6
Int + HCB + E	6
Int + R_D/P_N + LMA	6

In leaf physiology, the highest P_N [9.4 ± 1.3 μmol(CO_2) m^{-2} s^{-1}, mean of A and B leaves] was measured in June when mean air temperature was around 16 ± 2°C (mean value of A and B sites). In this period (*i.e.* plant vegetative activity), the highest R_D [2.9 ± 0.9 μmol(CO_2) m^{-2} s^{-1}, mean of A and B leaves] is necessary for new leaf formation which requires carbon skeletons necessary for biosynthetic reactions (Atkin *et al.* 2000). P_N and R_D decreased by 83 and 88% (mean of A and B leaves), respectively, in August. g_s and P_N were significantly correlated (R = 0.67). The 21% lower P_N in B than in A leaves (mean of the study period) was justified by the relatively lower air temperature (22%) and SWC (32%) in B than in A site. Moreover, the 22% larger DXV in B leaves allows a better hydraulic conductivity, as suggested by Nardini *et al.* (2012), and the higher R_D (40%, mean of the study period) may be justified by the larger energy requirement of plants in the more stressed environment. This result was also attested by the higher R_D/P_N in B than in A leaves (0.35 ± 0.07 and 0.21 ± 0.03, respectively, mean of the study period), which underlines a greater proportion of the fixed carbon consumed by respiration (Liang *et al.* 2013). The ratio R_D/P_N (*i.e.* moles of CO_2

Table 7. Plasticity index for the considered anatomical, morphological, and physiological leaf traits of *Sesleria nitida.* The anatomical plasticity index (PI_a), morphological plasticity index (PI_m), and physiological plasticity index (PI_p) are shown. The mean plasticity index (PI, mean of PI_a, PI_m, and PI_p) is shown. LT – total leaf thickness; HLB – height of the major lateral vascular bundle; WLB – width of the major lateral vascular bundle; HCB – height of the central vascular bundle; WCB – width of central vascular bundle; DXV – diameter of the xylematic vessels; CD – mesophyll cell density; UST – thickness of the upper sclerenchyma layers; SBC – total surface area of bulliform cells; SL_{ad} – adaxial stomatal length; ET_{ab} – abaxial epidermis thickness; ET_{ad} – adaxial epidermis thickness; LMA – leaf mass per unit leaf area; LW – leaf width; P_N – net photosynthetic rate in June; g_s – stomatal conductance in June; E – transpiration rate in June; R_D – respiration rate in June; R_D/P_N – ratio between R_D and P_N in June.

Leaf traits	PI
Anatomical traits	PI_a
LT	0.24
HLB	0.30
WLB	0.24
HCB	0.39
WCB	0.32
DXV	0.18
CD	0.19
UST	0.31
SBC	0.07
SL_{ad}	0.06
ET_{ab}	0.06
ET_{ad}	0.07
Mean value	0.20
Morphological traits	PI_m
LMA	0.15
LW	0.21
Mean value	0.18
Physiological traits	PI_p
P_N	0.18
g_s	0.39
E	0.33
R_D	0.37
R_D/P_N	0.47
Mean value	0.35
PI	0.24

respired per moles of CO_2 incorporated) can be used as a simple approach to characterize leaf carbon balance (Galmés *et al.* 2007). It is indicative of the plant's capability to produce new biomass for growth and development of reproductive structures (Poorter *et al.* 1992). The results concerning the two-way *ANOVA* showed a significant effect of the month, site, and their interaction on leaf variables. In particular, most of the variability for P_N, g_s, E, and R_D is explained by the factor of month, and it is justified by the response of leaf physiology to environmental factor variations in a short-term, according to the results of Gremer *et al.* (2012). Moreover, the two-way *ANOVA* showed that R_D/P_N was the only variable affected by the factor of site, despite photosynthetic and respiration rates being affected mainly by the factor of month. This means that P_N and R_D change in the same proportion throughout the months allowing *S. nitida* to maintain an unchanged carbon balance according to Liang *et al.* (2013). Thus, the physiological trait variations among the two populations assess to what extent carbon balance can cope with different environmental conditions at the two sites. It becomes indicative of the plant capacity to produce new biomass (Cavaleri *et al.* 2008) giving an indication of the efficiency of plant carbon use (Loveys *et al.* 2002). The results underline the importance of a positive carbon balance for *S. nitida* over the period of vegetative activity and reproduction.

Biological traits, which are advantageous under specific ecological conditions, should be present in a large proportion of the species within an ecosystem, where those specific conditions prevail. As climatic conditions change, the frequency of certain plant traits is expected to change (Pellissier *et al.* 2010). Thus, it is important to identify plant functional traits in which plasticity is likely to be important to species responses to climate change and to contribute in predicting species distribution changes and shifts (Nicotra *et al.* 2010). Functional trait variability at the intraspecific level is usually assumed to have an adaptive significance (Scheepens *et al.* 2010). In the context of a rapid climate change, phenotypic plasticity can be a crucial determinant of plant response, both in the short- and long term (Nicotra *et al.* 2010). On the whole, the measured PI (0.24) reflects *S. nitida* capability to maintain function under different environmental stress conditions at different altitudes on Mount Terminillo, and to sustain the air temperature increase through a potential shift toward higher elevations, according to the results of Gratani *et al.* (2012) for other mountain species. Moreover, our results underlined a higher plasticity for physiological leaf traits ($PI_p = 0.35$) than for morphological ($PI_m = 0.18$) and anatomical ones ($PI_a = 0.20$). This result agrees with the importance of plant acclimatization to adverse environments where morphological and anatomical plasticity play a secondary role (Zunzunegui *et al.* 2009). In fact, plants growing in stress conditions tend to have conservative leaf morphological patterns to avoid the production of structures too expensive to be sustained (Chapin *et al.* 1993, Valladares *et al.* 2000). This is due to the scarce soil nutrients because of low mineralization rates under low temperatures in high mountain areas (Morecroft *et al.* 1992), as attested by the high C/N (18.1 ± 0.5, mean value) in both A and B sites. On the contrary, the largest physiological leaf plasticity ensures an instantaneous adjustment of gas exchange to changes in stress factors intensity which characterizes the environments where *S. nitida* grows, according to the results of Zunzunegui *et al.* (2009).

Predictions of future climate scenarios indicate a likely increase in air temperature and a decrease in rainfall

(Solomon *et al.* 2007) which may negatively affect plant carbon assimilation. Climate change could make these mountain species more vulnerable because of the rocky soil where they develop (Gratani *et al.* 2003). The results concerning the plasticity index show the capability of *S. nitida* to acclimate to environmental conditions by moving up in response to the hypothesized increase in air temperature. The hypothesized shift of *S. nitida* to a higher altitude could reduce available space creating a competition with the native species already growing there, according to the results of Breshears *et al.* (2009), and creating pressure for upward migration of those species (Jump and Penuelas 2005, Byars *et al.* 2007). In this context, the different adaptability (*i.e.* phenotypic plasticity) of the species co-occurring in the same area could have an important role in selection amongst them. Nevertheless, we must also take into account that interspecific interactions and competitive responses of species under climate change can be complex and unexpected (Suttle *et al.* 2007). Moreover, if rising aridity is actually the driver of the observed species loss on many Mediterranean summits, this trend is likely to continue during the coming decades, because climatic models predict increasing temperatures, decreasing rainfall, and an extension of the dry period in southern Europe (Benito *et al.* 2011, Pauli *et al.* 2012).

The results of the logistic regression showed that HCB, LT, and R_D/P_N are the most discriminating traits between the two populations. Among them, the ratio R_D/P_N underlines the importance of carbon balance maintenance in response to environmental factor variations at different altitudes on Mount Terminillo. Thus, considering that climate change could be a potent selective factor leading to the adaptive evolution of key plant traits (Franks *et al.* 2007, Valladares *et al.* 2007), the selected leaf traits could be used to monitor *S. nitida* wild populations over the long-term period in response to global climate change. Moreover, considering that the taxonomy of the genus *Sesleria* is not completely clear because of the large phenotypic variations in the species (Di Pietro 2007) and their extremely variable ecology (Tammaro and Catonica 2000), the identified morphological and anatomical leaf traits of *S. nitida* can be used for systematic comparison among the species of this genus.

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