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Leaf physiological and structural plasticity of two *Asplenium* (Aspleniaceae) species coexisting in sun and shade conditions

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Background and aims – Relatively few studies have addressed the sun-shade response of fern species. Moreover, there is no information on species-specific plasticity patterns of such response, their relationship with species ecological requirements and the costs of such plasticity. The present study aims at filling these gaps by analysing the sun-shade plastic response of two *Asplenium* species that differ in their ecological requirements.

Methods – We measured 27 leaf morphological, anatomical and physiological parameters using standard methods for *A. ceterach* and *A. trichomanes* in the field. The parameters were combined through Principal Component Analysis in order to highlight an integrated sun-shade response across species. Linear regression analysis was carried out to highlight the relationship between the calculated species plasticity patterns and the structural control on photosynthetic process.

Key results – A significant degree of phenotypic plasticity was found for both species. Moreover, sun and shade leaves shared a common slope for the morpho-functional relationships reflecting no additional costs in terms of carbon assimilation. Even if the plastic responses of the two species scaled positively ($R^2 = 0.68$, $P = 4.667e–07$), *A. trichomanes* was characterized by a slightly higher anatomical plasticity (plasticity index = 0.19), while *A. ceterach* showed a higher physiological plasticity (0.60).

Conclusion – A remarkable acclimation capacity for the two *Asplenium* species in response to different light conditions was highlighted. Nevertheless, *A. ceterach* seems to be more suited to cope with full sunlight conditions as compared to *A. trichomanes*, according to species ecological requirements.

Keywords – Acclimation capability; ferns; leaf anatomy; leaf mass per area; photosynthesis; plasticity.

INTRODUCTION

Leptosporangiate ferns (Polypodiidae) are the most numerous group of extant spore-bearing vascular plants (Pteridophyte Phylogeny Group 2016). Despite their ancient origin, the vast majority of modern ferns has emerged in the period of the last 100 million years (Pryer & Schuettpelz 2009) together with the evolution and spreading of flowering plants (Schneider et al. 2004). However, evolutionary competition with angiosperms resulted in habitat diversification, specialization, and extension of biological diversity as well as increasing adaptability of many fern species. According to Page (2002), the "low-light photosynthetic ability" is among the twelve important advantages of pteridophyte biology, providing a broad framework for exploitation of ecological habitats.

Comparison of the main functional traits of ferns and their adaptive significance with that of seed plants has largely been made (Choy-Sin & Suan 1974; Ludlow & Wolf 1975; Bauer et al. 1991; Brach et al. 1993; Karst & Lechowicz 2007). In general, it was established that some important leaf traits and physiological characteristics of ferns have values that correspond to the lowest ones reported for seed plants (Karst & Lechowicz 2007; Tosens et al. 2016). This applies for instance to leaf mass per unit of leaf area (Karst & Lechowicz 2007), hydraulic conductivity of xylem (Brodrib & Holbrook 2004; Watkins et al. 2010), stomatal and mesophyll conductance (Nishida et al. 2015; Tosens et al. 2016), photosynthetic capacity (Gago et al. 2013; Tosens et al. 2016) and photosynthetic nitrogen use efficiency (Durand & Goldstein 2001; Stuntz & Zolt 2001; Tosen et al. 2016).

Apart from the differences between ferns and seed plants, relatively few studies have specifically addressed fern's plastic responses to different environment factors as different light conditions. Phenotypic plasticity is defined as the capacity of a given genotype to express different phenotypes under different environmental conditions (Valladeres et al. 2007) and it is considered to play an adaptive role in survival of the species in a changing environment. The standard plasticity experiment involves two or more taxa (clones, genotypes, families, populations, species) that grow in a series of different environments in the field, or under controlled condition (Schlichting 1986).

Some works dedicated to the sun/shade response between different fern species (Choy-Sin & Suan 1974; Ludlow & Wolf 1975; Winter et al. 1986; Brach et al. 1993; Arens 1997; Nurul Hafiza et al. 2014) highlighted that fern response to sun/shade agrees with that of seed plants. In fact, ferns growing in high light condition tend to be characterized by lower total chlorophyll content and higher chlorophyll a/b ratio, stomatal density, light compensation and saturation points, net photosynthetic rates and nitrogen contents per unit leaf area. However, there is still lack of information on the morpho-functional response of different fern species to different light environments. In particular, there is no information on the range of these differences. That is, how much morphological, anatomical and physiological plasticity can be expressed by different fern species in the sun/shade transition.

Changes of photosynthetic rates across leaves with different structure and chemistry have been successfully expressed by the leaf economics spectrum (Wright et al. 2004). Relationships between leaf structure and function in ferns have received less attention, with two exceptions: Karst & Lechowicz (2007) analysed the so-called 'frond economics spectrum' by including 20 understory fern species and, by exploring only area-based correlations, found that the relationships between foliar traits were in agreement with those of the leaf economic spectrum. However, the mass-based photosynthetic capacity characterizes the biochemical capacity of single cells and is the key player in the worldwide trade-off between the physiological and structural characteristics of leaves (Westoby et al. 2013; Niinemets et al. 2004; Tosens et al. 2016). Accordingly, Tosens et al. (2016), analysing the bivariate relationships between leaf traits in 35 ferns grown under non-stressful conditions found mass-based relationships to be stronger than the area-based ones, in agreement

with Wright et al. (2004) for seed plants, but in contrast with Karst & Lechowicz (2007). Despite this evidence, at the best of our knowledge there is no previous study that has addressed how the sun-shade transition differentially affect the relationship between leaf structure and function in ferns. As a result, we do not know how the expected changes in leaf structure affect changes in mass-based net photosynthesis in ferns growing in different light environments (i.e. cost in terms of carbon assimilation). This is of particular concern since bivariate relationships between leaf structure and function are useful tool for predicting species-specific adaptations to particular environments (Wright et al. 2004).

Considering these gaps of knowledge, the present study aimed at analysing the leaf morpho-physiological response of two *Asplenium* L. species coexisting under both sun and shade conditions in the field but characterized by different ecological preferences. *Asplenium ceterach* L. is a thermophilic species that tends to colonize habitats characterized by higher radiation levels, while *Asplenium trichomanes* L. is more linked to shaded and humid environments (see below).

In particular, we wanted to address: (i) whether leaf morphological trait variations pose a physiological cost in the sun/shade transition independently of the species and (ii) whether growing under full sunlight can be achieved through a common pattern of response across coexisting (and congeneric) species.

We hypothesized that: (i) sun leaves may be characterized by a greater cost in terms of carbon assimilation, as highlighted by a steeper slope for the relationships involving mass based net photosynthesis and leaf structural traits, due to a greater structural investment in leaves; (ii) even if a common pattern of response (in terms of mean values) could be highlighted, we still expected that the selected species could differ in plasticity of key traits (i.e. high range of variability) that could reflect their different ecological requirements.

MATERIALS AND METHODS

Abbreviations

Aa – Net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}); AC – *Asplenium ceterach*; Am – Mass based net photosynthesis (nmol CO_2 g^{-1} s^{-1}); AT – *Asplenium trichomanes*; DM – Leaf dry mass (g); DSt – Stomatal density (number mm^{-2}); E – Transpiration rate (mmol H_2O m^{-2} s^{-1}); gs – Stomatal conductance (mol H_2O m^{-2} s^{-1}); iWUE – Intrinsic water-use efficiency (μmol CO_2 mol H_2O^{-1}); LA – Leaf area (cm^2); LMA – Leaf mass per unit of leaf area (g m^{-2}); LT – Total leaf thickness (μm); LTD – Leaf tissue density (mg cm^{-3}); PI – Phenotypic plasticity index; PPFD – Photosynthetic photon flux density (μmol photon m^{-2} s^{-1}); WUE –Water-use efficiency (μmol CO_2 mmol H_2O^{-1}).

Plant material

Two largely distributed *Asplenium* L. species were selected. Both species are perennials with overwintering leaves that persist 12–18 months in the study area. Previous studies have demonstrated that longer-term leaf traits versus light rela-

tionships are stable during most of the growing season (Niinemets et al. 2004; Hallik et al. 2012).

Asplenium trichomanes L. grows in a range of habitats from open to shaded rocky ones, but prefers calcareous rocks (such as limestone and dolomite) or mortared walls. It sometimes occurs in terrestrial habitats. It is widespread in temperate and subarctic areas of northern hemisphere and also occurs in mountainous regions in the tropics extending to Australia and western regions of South America. In Italy, it occurs up to 2910 m a.s.l. (Pignatti 1982; Marchetti 2001; Soster 2001; Preston et al. 2002; Tutin et al. 2010).

Asplenium ceterach L. (*Ceterach officinarum* Willd.) occurs mainly on exposed and sunny cliffs, cracks in stone, scree slopes of basic rocks, especially limestone. It is widespread in Mediterranean region, with general distribution in Europe, North Africa and Southwestern Asia. In Italy, it attains altitudes up to 2000 m a.s.l (Pignatti 1982; Marchetti 2001; Soster 2001; Preston et al. 2002; Tutin et al. 2010). *Asplenium ceterach* is a desiccation-tolerant fern (Proctor & Tuba 2002).

Despite the fact that both species often prefer rocky outcrops and can grow together, *A. trichomanes* has much wider ecological amplitude and geographical range. According to Didukh's (2011) approach for the estimation of ecological scales, both species have similar requirements to damping ($\omega = 0.05–0.25$), soil aeration (Ae = 50–95), and salt regime (30–200 mg/l). *Asplenium trichomanes* grows in a wide range of soil humidity (Wnp = 55–250 mm), light condition (from scyophytes to heliophytes), and thermal climate (25–85 kcal cm^{-2} year^{-1}). On the other hand, ecological requirements of *A. ceterach* are shifted to warmer (Wnp = 25–75 mm) and higher insolation condition (it belongs to the group of sub-heliophytes or heliophytes).

For both species nine representative plants were randomly chosen along two transects in open and shade areas along the calcareous slopes. The length of the transects reached 200 m for the open area and about 150 m for the shade area. All selected plants were fertile with leaves of adult form approaching maximum size and complexity inherent in mature individuals. The number of leaves per rhizome was 7–13 for open area and up to 20 for shade area.

Study area and climate

The research was carried out in Central Apennine Mountains at Lucretili Mountains (Parco Naturale Regionale dei Monti Lucretili, Rome, Italy) in May 2017. The open area ("sun plants", 42°4′44.57″N, 12°53′28.13″E; 475 m a.s.l.) was characterized by a mean photosynthetic photon flux density (PPFD, µmol photon m^{-2} s^{-1}) of 1895±175, mean temperature (Tm, °C) of 24.9±1.3, red far red ratio (R/FR) of 2.02. The shade area was situated in the understory of *Fraxinus ornus* L., *Acer monspessulanum* L. and *Carpinus betulus* L. ("shade plants", 42°4′45.02″N, 12°53′29.82″E; 475 m a.s.l.; PPFD = 15±1; Tm = 17.5±0.9, R/FR = 0.60). The area is characterized by a Mediterranean climate. The mean minimum air temperature (Tmin) of the coldest months (January and February) is 2.0±1.6°C, the mean maximum air temperature (Tmax) of the hottest months (July and August) is 33.0 ± 1.7°C and the mean yearly air temperature (Tm)

is 14.1±1.5°C. The dry period begins in June and ends in August (35.5±23.5 mm total rainfall). Total annual rainfall is 1177.99 mm, most of which occurs in autumn and winter (data from the Meteorological Station of Licenza – loc. Colle Franco, Rome, 460 m a.s.l., ARSIAL – Lazio Regional Agency for Development and Agricultural Innovation, for the years 2007–2017).

Morphological leaf traits

Morphological measurements were carried out on fully expanded fertile leaves from each selected plant (n = 9). Measurements included leaf area (LA, cm^2), leaf length (cm) and leaf width (cm) measured by an Image Analysis System (Delta–T Devices, UK) and leaf dry mass (DM, g), measured after desiccation at 80°C to constant mass. LA and DM of the whole lamina without rachis (for *A. trichomanes*) and massive midrib (for *A. ceterach*) were considered. Leaf width was measured at the midpoint of the leaf.

Leaf mass per unit of leaf area (LMA, g m^{-2}) was calculated by the ratio between DM and LA and leaf tissue density (LTD, mg cm^{-3}) by the ratio between LMA and total leaf thickness (LT, µm) (Wright et al. 2004).

Anatomical leaf traits

Anatomical measurements were carried out on pinnae (AT) or lateral lobes (AC) from the central part of the fully expanded fresh leaves (n = 9) and analysed by light microscopy (Zeiss Axiocam MRc 5 digital camera (Carl Zeiss) using an Image Analysis System (Axiovision AC software). The following parameters were determined in transverse sections: total leaf thickness (µm), abaxial and adaxial cuticle with cell wall thickness (µm), abaxial and adaxial epidermis thickness (µm), mesophyll tissue thickness (µm).

Epidermal features were determined from nail varnish impressions (n = 9) of the adaxial and abaxial surfaces of the lamina, according to Sack et al. (2003). The following parameters were measured: adaxial epidermal cell length (µm), adaxial epidermal cell width (µm), abaxial epidermal cell length (µm), abaxial epidermal cell width (µm), stomatal cell length and width (µm), stomatal density (number mm^{-2}).

Gas exchange measurements

Measurements of gas exchange were carried out using the infrared gas analysers ADC LCPro+ (UK) equipped with a leaf chamber (PLC, Parkinson Leaf Chamber). Measurements were made on three leaves per each sun and shade plants (n = 9). Net photosynthetic rate (Aa, µmol CO_2 m^{-2} s^{-1}), photosynthetic photon flux density (PPFD, µmol photon m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}), were measured. Measurements were carried out under natural conditions, on cloud-free days at saturating PPFD (> 1,500 µmol photon m^{-2} s^{-1} in sun conditions), in the morning from 8:00 am to 12:00 am. CO_2 concentration in the leaf chamber was set at 400 µmol CO_2 mol^{-1}air, and relative air humidity of the incoming air ranged between 40% and 60%. The intrinsic water-use efficiency (iWUE, µmol CO_2 mol H_2O^{-1}) was calculated as the ratio between Aa and gs. Water-use efficiency (WUE, µmol

CO_2 mmol H_2O^{-1}) was calculated as the ratio between Aa and E. Mass based net photosynthesis (Am, nmol CO_2 $g^{-1}s^{-1}$) was calculated as the ratio between Aa and LMA (Wright et al. 2004).

Data analysis

Standardized major axis regression (SMA, Warton et al. 2006) was used to analyse the relationships LMA–LT, LMA–LTD, LTD–LT and between Am and LMA, LT and LTD with *Light* as the main factor. The differences in terms of slopes and intercepts were tested by the Likelihood Ratio and Wald statistic, respectively. All analyses were run with the R library SMATR (Warton et al. 2012) on log-transformed data.

To test if species shared a common pattern of response in the sun-shade transition, a Principal Component Analysis (PCA) by including all the considered traits grouped by *Species×Light* environment was carried out. Then, a Two-way ANOVA was performed in order to test the effect of factors *Species*, *Light* and their interaction (*Species×Light*) on the extracted principal components (i.e. PC1 and PC2).

Multiple comparisons were analysed by a Tukey test. Such approach was used in order to reduce multiple testing, considering that the use of emerging collective properties (expressed by PCs) as primary variables allows for an equally robust approach (Giuliani 2017).

The phenotypic plasticity index (PI, Valladares et al. 2000) was calculated for each species (hereafter PI_{AC} and PI_{AT}) in order to quantify the degree of phenotypic plasticity for each of the considered traits in response to different light environments. PI was calculated as the difference between the minimum and the maximum mean value divided by the maximum mean value per each trait. Then, PI_{AT} was regressed against PI_{AC} via linear regression analysis (n = 25).

RESULTS

Traits variability and bivariate relationships

The results highlighted an overall difference in the considered leaf traits of sun and shade leaves for the two considered species (fig. 1, table 1).
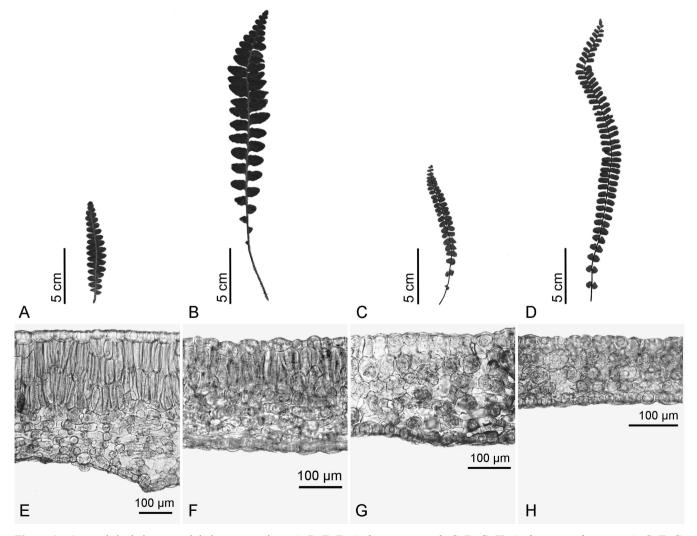

Figure 1 – Sun and shade leaves and their cross sections. **A**, **B**, **E**, **F**. *Asplenium ceterach*. **C**, **D**, **G**, **H**. *Asplenium trichomanes*. **A**, **C**, **E**, **G**. Sun leaves. **B**, **D**, **F**, **H**. Shade leaves.
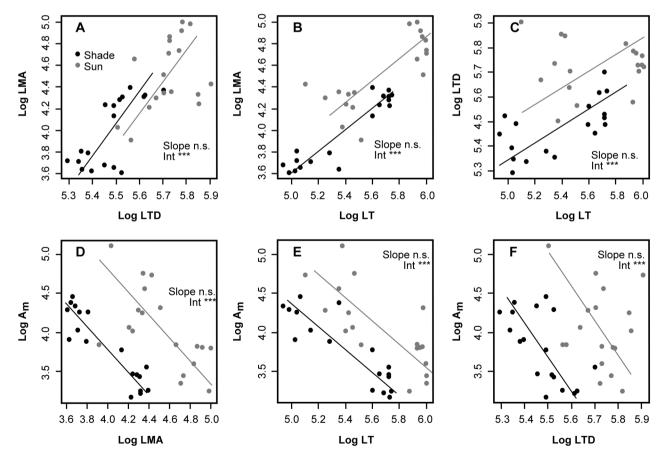
Table 1 – **Morphological, anatomical and physiological parameters of *Asplenium ceterach* and *Asplenium trichomanes* (mean value ± standard deviation).**
* Mesophyll is not differentiated.

Parameters	*Asplenium ceterach*		*Asplenium trichomanes*	
	Shade	Sun	Shade	Sun
Morphological parameters				
Petiole length, cm	4.64 ± 2.35	1.70 ± 0.61	4.57 ± 1.56	1.38 ± 1.15
Lamina length, cm	16.79 ± 3.37	10.00 ± 2.52	22.76 ± 4.68	13.12 ± 3.13
Lamina width, cm	3.08 ± 0.48	1.74 ± 0.15	1.96 ± 0.24	1.17 ± 0.17
Leaf dry mass, g	0.17 ± 0.05	0.10 ± 0.03	0.10 ± 0.01	0.05 ± 0.02
Leaf area, cm^2	23.87 ± 6.29	8.42 ± 2.13	24.04 ± 2.96	7.52 ± 2.30
Leaf mass per unit of area, $g\ m^{-2}$	73.04 ± 0.57	123.04 ± 1.91	40.37 ± 0.28	70.24 ± 1.08
Leaf tissue density, $mg\ cm^{-3}$	258.85 ± 21.14	310.09 ± 20.55	221.47 ± 17.18	305.62 ± 41.55
Water content, %	64.02 ± 1.88	58.34 ± 2.91	62.78 ± 2.06	58.08 ± 2.29
Anatomical parameters				
Tissue thickness				
Total leaf, μm	294.62 ± 18.85	388.21 ± 27.70	164.53 ± 23.78	216.31 ± 27.85
Adaxial cuticle, μm	3.89 ± 0.58	4.45 ± 0.53	3.50 ± 0.49	4.81 ± 0.95
Adaxial epidermis, μm	26.35 ± 2.73	29.39 ± 3.14	30.95 ± 6.68	43.12 ± 9.91
Palisade, μm Spongy, μm	104.07 ± 11.91 131.08 ± 16.24	182.36 ± 22.38 152.31 ± 17.25	103.99 ± 25.22*	141.41 ± 24.63*
Abaxial epidermis, μm	23.33 ± 4.90	23.33 ± 6.09	24.22 ± 3.22	25.91 ± 4.52
Abaxial cuticle, μm	2.78 ± 0.46	2.62 ± 0.31	2.02 ± 0.23	2.76 ± 0.64
Epidermal features				
Adaxial cell length (μm)	88.20 ± 9.25	70.90 ± 10.62	79.53 ± 9.63	71.06 ± 10.74
Adaxial cell width (μm)	54.10 ± 5.16	47.75 ± 8.37	39.93 ± 5.73	40.43 ± 6.03
Abaxial cell length (μm)	89.47 ± 10.65	75.70 ± 9.71	108.63 ± 17.23	81.32 ± 11.28
Abaxial cell width (μm)	47.92 ± 6.50	42.82 ± 6.29	48.94 ± 6.83	40.45 ± 4.12
Stomatal parameters				
Length (μm)	38.03 ± 3.01	37.17 ± 2.21	47.38 ± 2.50	46.94 ± 3.69
Width (μm)	28.46 ± 2.64	29.19 ± 2.07	31.30 ± 2.45	29.87 ± 1.69
Density ($n\ mm^{-2}$)	108.60 ± 16.88	167.49 ± 18.17	35.10 ± 12.76	115.51 ± 47.99
Physiological parameters				
Area based net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	2.21 ± 0.38	5.04 ± 1.38	2.73 ± 0.51	6.22 ± 2.67
Mass based net photosynthesis ($nmol\ CO_2\ g^{-1}\ s^{-1}$)	30.43 ± 6.30	41.95 ± 14.64	67.85 ± 13.32	88.14 ± 38.32
Stomatal conductance ($mol\ H_2O\ m^{-2}\ s^{-1}$)	0.03 ± 0.01	0.11 ± 0.02	0.02 ± 0.00	0.05 ± 0.02
Transpiration rate ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	0.60 ± 0.23	4.34 ± 0.58	0.48 ± 0.03	2.06 ± 0.76
Intrinsic water-use efficiency ($\mu mol\ CO_2\ mol\ H_2O^{-1}$)	85.56 ± 46.83	44.80 ± 11.39	126.61 ± 48.61	139.44 ± 49.57
Water-use efficiency ($\mu mol\ CO_2\ mmol\ H_2O^{-1}$)	4.36 ± 2.14	1.16 ± 0.26	5.70 ± 0.93	2.99 ± 0.61

Table 2 – Common slope, intercept, *R²* and *P* of the Standardized major Axis Regression for the log--log relationships.
The log–log relationships between leaf dry mass per unit leaf area (LMA) and net photosynthetic rate per unit of leaf dry mass (Am), leaf thickness (LT) and Am, leaf tissue density (LTD) and Am, LTD and LMA, LT and LMA and LTD and LT for sun and shade leaves. *P* in bold indicates significance at < 0.05.

Log–Log Relationship	Light condition	Common Slope	Intercept	R^2	P
Am–LMA	sun	−1.48	10.73	0.41	**0.004**
	shade		9.70	0.84	**8.36E–08**
Am–LT	sun	−1.48	12.45	0.55	**0.0004**
	shade		11.79	0.79	**8.57E–07**
Am–LTD	sun	−4.41	29.27	0.04	0.42
	shade		27.93	0.39	**0.005**
LMA–LTD	sun	2.94	−12.10	0.18	0.08
	shade		−12.31	0.53	**0.0006**
LMA–LT	sun	0.99	−1.35	0.87	**1.18E–08**
	shade		−1.10	0.59	**0.0002**
LTD–LT	sun	0.34	3.80	0.0005	0.93
	shade		3.64	0.42	**0.003**

Figure 2 – Log–log relationships between: **A**, leaf tissue density (LTD) and leaf dry mass per unit area (LMA); **B**, total leaf thickness (LT) and LMA; **C**, LTD and LT; **D**, LMA and net photosynthetic rate per unit of leaf dry mass (Am); **E**, LT and Am; **F**, LTD and Am for sun (grey dots) and shade (black dots) leaves. Data were pooled across species per light condition. The estimated common slopes, intercepts, *R²* and *P* are shown in table 2. (Standardized major Axis Regression, n = 27). n.s. = not significant; *** = *P* < 0.01.

Table 3 – Results of the two-way ANOVA (*F* and *P* values) for the first and second principal component (PC1 and PC2, respectively) extracted.

The main effects of *Species*, *Light* and the first order interaction (*Species×Light*) are shown. The factor effect was considered significant at $P < 0.05$.

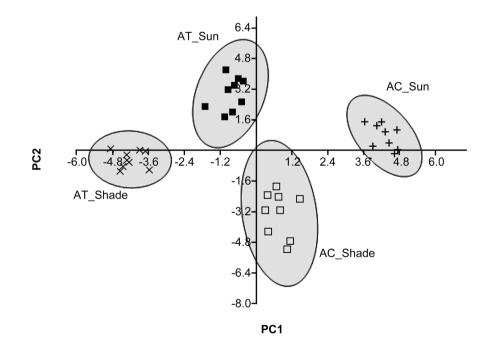| Response variable	Factors	Degrees of freedom	*F*-value	*P*-value
PC1	*Species*	1.32	775.426	**< 2E–16**
	Light	1.32	1368.73	**< 2E–16**
	Species×Light	1.32	0.214	0.647
PC2	*Species*	1.32	150.854	**1.19E–13**
	Light	1.32	85.568	**1.47E–10**
	Species×Light	1.32	3.807	0.0598

Changes in LMA were due to changes in both LTD (R^2 = 0.53; P = 0.0006) and LT (R^2 = 0.59; P = 0.0002) for shade leaves while in sun leaves only the relationship LMA–LT (R^2 = 0.87; P = 1.18E–08) was significant (fig. 2A–C, table 2). LT and LTD were in turn significantly and positively related only in shade leaves (R^2 = 0.42; P = 0.003). Moreover, LMA and LT scaled negatively ($P < 0.05$) with Am in both sun and shade leaves while the relationship Am–LTD was only significant in shade leaves (R^2 = 0.39; P = 0.005) (fig. 2D–F, table 2). Significant differences between slopes for the considered bivariate relationships were not found. However, intercepts were always found to significantly differ between sun and shade leaves (fig. 2A–F) except for the relationship LMA–LTD.

Patterns of phenotypic plasticity: convergences and divergences between species

The PCA returned two axes of variation with percentage of explained variance of 38 % and 21 % for PC1 and PC2, respectively (fig. 3; supplementary file 1). PC1 was significantly and positively related to gs, E, LMA, LTD, LT, mesophyll thickness and stomatal density while negatively with WUE, leaf length and abaxial epidermal cell length. PC2 was significantly and positively related to Aa, Am and adaxial cuticle thickness while negatively with adaxial epidermal cell width, iWUE, LP, water content and stomatal cell length showed the same magnitude of correlation with both of the PCs.

The Two-way ANOVA showed that the factors *Species* and *Light* were significant for both the PCs while the interaction terms were not significant in both of the analyses (table 3). Overall, as expected, *Light* accounted for most of the variance for PC1 while the factor *Species* for PC2.

The phenotypic plasticity patterns obtained for the two considered species in response to light were significantly related (R^2 = 0.68; P = 4.667e–07; fig. 4). However, on average, *A. trichomanes* was characterized by a slightly higher anatomical plasticity as compared to *A. ceterach* (0.19 and 0.14, respectively), while the latter showed a greater physiological plasticity (0.60 and 0.46 in *A. ceterach* and *A. trichomanes*, respectively). The two species roughly converged in terms of the magnitude of morphological plasticity (0.35 and 0.38 in *A. ceterach* and *A. trichomanes*, respectively).
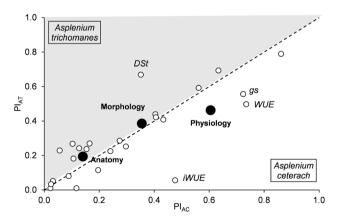

Figure 3 – Principal Component Analysis (PCA) on a correlation matrix including all the 25 morphological, anatomical and physiological leaf traits (see supplementary file 1) for sun and shade leaves of *Asplenium trichomanes* (AT) and *Asplenium ceterach* (AC). The % of explained variance for each PC and the correlation of each variables with both PC1 and PC2 are shown in supplementary file 1.

DISCUSSION

Plant responses to reduced light environment have been investigated in several ecological and eco-physiological studies (see Valladares & Niinemets 2008 for a review) but ferns have often been disregarded. In the present study, the response of two congeneric fern species coexisting under sun and shade conditions in the field was analysed.

Traits variability and bivariate relationships

Overall, the results highlight significant divergence between leaves developed under full sunlight and shade conditions (mean PPFD = 1895±175 and 15±1, respectively) in terms of mean values of the considered traits. In particular, as expected, we found an increase in Aa and gs for sun leaves, and the values are in the range of that reported for fern species (Aa generally < 10 and gs between 0.017 and 0.177, Tosens et al. 2016). In our study, LMA and LT ranged between 40.67–123.04 g m^{-2} and 164–388 μm, respectively. Interestingly, such values were slightly outside the ranges reported for these traits in the literature (Tosens et al. 2016; Vasheka et al. 2016) suggesting that sun-shade transition represents a strong driver of LMA and LT variations in ferns. Accordingly, we found that both LMA and LT scaled negatively with photosynthetic rate on a mass basis (Am) and that LT was the main determinant of LMA changes in both sun and shade leaves. Moreover, the scaling relationships differ in terms of intercepts, reflecting species acclimation to different light conditions. It is in fact well known that different environmental conditions may determine different proportional changes of the parameters on the x- and y-axis, with the result that intercepts significantly differ (Wright et al. 2001; Atkinson et al. 2010), as observed here. However, an eventual cost in terms of Am due to changes in LMA and LT would be out-

lined by differences in slopes between sun and shade leaves, but we did not observe any difference in slopes. Moreover, we want to stress that the slope we found for the relationship Am–LMA (−1.48) was identical to that found by Tosens et al. (2016) by including data for 35 fern species (−1.47).

We argue that the common slope shared by sun and shade leaves for the relationship Am–LMA and Am–LT, together with differences in intercepts, reflects the ability of the considered species to adapt morphological and anatomical leaf traits in response to light with no further costs in terms of carbon assimilation. Additionally, there was a general lack of relationship between LTD and the rest of the considered parameters only for sun leaves, possibly highlighting a tendency of the considered species to better modulate LT under full sunlight conditions, supported by the lack of relationship LTD–LT. A similar result was obtained by Puglielli et al. (2017) for a shrub species growing in different light environments. Moreover, LTD is a costly trait (de la Riva et al. 2016; Puglielli & Varone 2018), as also attested by the greatest slope of the relationship LTD–Am (−4.41) as compared to Am–LMA and Am–LT (mean slope = −1.48). Therefore, such response can allow sun leaves to avoid excessive costs in terms of Am by preferably modulating LT in order to avoid a longer pay-back time for leaf construction costs (i.e. longer leaf life-span). On the contrary, this may happen in shade conditions even if this aspect needs further investigation.

Concerning the anatomical traits, few comparisons with other studies could be made even if the measured values are in agreement with Vasheka et al. (2016).

Patterns of phenotypic plasticity: convergences and divergences between species

The ANOVA carried out on the two PCs extracted was decisive to determine if species shared a common pattern of response in the shade-sun transition. The analysis highlighted a greater effect of *Light* than *Species* on PC1 as compared to PC2. Such result can be easily interpreted. In fact, for mathematical construction the PC1 captures the highest variance within the data, so it is reasonable to expect greater differences in terms of *Light* than *Species* along this axis. On the contrary, PC2 should also include species-specific differences for the considered traits, thus resulting in a greater effect of *Species* than *Light*, as observed. Moreover, the lack of significance for the interaction term (*Species×Light*) for both PC1 and PC2 demonstrates that such response patterns are independent of species-specific differences in trait values. If so, a convergence in terms of phenotypic plasticity patterns for the considered species could be expected. Accordingly, the plastic responses of the two species scaled positively (R^2 = 0.68, P = 4.667 e −07) with most of the points falling tightly around the 1:1 line in fig. 4. Mean plasticity values plotted in the same graph showed that *A. trichomanes* tends to be characterized by a slightly higher anatomical plasticity (mean value falling above the 1:1 line) while *A. ceterach* is characterized by a higher physiological plasticity (mean value falling below the 1:1 line). Such a discrepancy mainly comes from different plastic behaviour in few parameters. In fact, *A. ceterach* is characterized by a greater

Figure 4 – Relationship between the phenotypic plasticity index measured in response to light for *Asplenium trichomanes* (PI$_{AT}$) and *Asplenium ceterach* (PI$_{AT}$) for the 25 morphological, anatomical and physiological leaf traits considered (see table 3). Values of PI calculated per each parameter and species were used as experimental units (n = 25). R^2 = 0.68; P = 4.667e−07. The 1:1 line, mean values for each trait pool (black dots) and the parameters that mainly drive mean differences are also shown: stomatal density (DSt), stomatal conductance (gs), water use efficiency (WUE), intrinsic water use efficiency (iWUE).

plasticity in gs, iWUE and WUE which drives the mean below the 1:1 line, while the higher anatomical plasticity in *A. trichomanes* is driven by a greater plasticity of stomatal density. Concerning *A. ceterach*, its response is due to less stomatal closure under full sunlight conditions resulting in a lower iWUE and WUE as compared to shade leaves. On the other hand, *A. trichomanes* tends to have a more conservative water use strategy. For this species increasing stomatal density under full sunlight condition expands opportunity to better control stomatal conductance in relation to photosynthesis (i.e. iWUE) (Heterington & Woodward 2003; Puglielli et al. 2017). However, this is not enough to increase iWUE to a great extent in sun as compared to shade leaves, possibly reflecting hydraulic limitations to photosynthesis. Strong hydraulic limitation of photosynthesis in ferns is generally reported (Zhang et al. 2014; Tosens et al. 2015), and it seems to be due to the fact that water and CO_2 share a significant fraction of the outside-xylem pathways in photosynthetic organs (Flexas et al. 2013). While this hypothesis is valid for *A. trichomanes*, *A. ceterach* seems to display no hydraulic limitation of photosynthesis or, at least, to a lesser extent. These differences can be also reflected by the different structure of mesophyll tissues between the species (fig. 1). The mesophyll of *A. ceterach* is well differentiated on palisade and spongy tissues whereas *A. trichomanes* has undifferentiated mesophyll which can further justify different water use and carbon fixation strategy in *A. ceterach* as compared to *A. trichomanes*. Additionally, *A. ceterach* belongs to the group of desiccation-tolerant ferns (Proctor & Tuba 2002; Hietz 2010) and has some additional adaptations for control of water loss such as leaf curling and densely covered abaxial leaf surface by peltate scales, the effect of which on water uptake and loss of the desiccation-tolerant epiphytic fern was recently established (John & Hasenstein 2017).

Most studies have investigated phenotypic plasticity of leaf traits in angiosperms and values between 0.20 and 0.60 are generally reported (as reviewed by Gratani 2014 and Chelli et al. 2019). On the other hand, gymnosperms display much lower phenotypic plasticity, with PI ranging between 0.09 and 0.12 for anatomical and 0.14 and 0.32 for physiological traits (Wyka et al. 2007). Our results show that the investigated *Asplenium* species display PI values comparable to those of seed plants and that further comparisons are needed in order to place phenotypic plasticity of ferns in an evolutionary context.

CONCLUSIONS

On the whole, the present work highlights a significant degree of phenotypic plasticity for the considered species in response to different light conditions reflecting a remarkable acclimation capacity. This is supported by the lack of costs in terms of carbon assimilation possibly due to the species ability to modulate leaf thickness under full sunlight conditions. This allows reducing the leaf construction costs thus resulting in the same slopes for the considered bivariate relationships between sun and shade leaves.

Moreover, the study provides the first evidence of a common pattern of response to different light conditions in two fern species. Despite the similarities in the response to sun-shade transition we found that *A. ceterach* and *A. trichomanes* diverge in terms of water use strategies which reflect a different tolerance to full sunlight conditions. In particular, *A ceterach* seems to be more suited to cope with full sunlight conditions than *A. trichomanes*, according to the ecological requirements of the species. In fact, while both species prefer substrates with little water storage capacity such as rocks, cliffs, cracks in stone and scree slopes, *A. trichomanes* occurs, however, in more shady places or locations with continuous water supply.

This work contributes to the so far overlooked response of ferns to different light conditions which can further elucidate the ecological success of this group of vascular plants. Moreover, we identified a set of traits and provide data analysis procedure that can be used to test integrated sun-shade patterns of response in other species of ferns.

SUPPLEMENTARY FILE

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