Intraspecific variability of leaf traits and functional strategy of *Himantoglossum adriaticum* H. Baumann

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Abstract

Trait-based studies have become extremely common in plant ecology. In this work we analysed intraspecific trait variation of *Himantoglossum adriaticum*, a European endemic orchid species of Community interest, to investigate whether different populations growing on managed and abandoned semi-natural dry grasslands show differences in the CSR strategy. In seven populations occurring in Veneto Region (NE Italy), we measured *H. adriaticum* maximum vegetative height, leaf traits (LA, LDMC, SLA) and calculated the CSR strategy. Through CCA we investigated the relationship between plant traits and both plant community attributes (cover and height of herbs and shrubs), and geomorphologic features (aspects and slope). PERMANOVA test was used to investigate if the CSR strategy of *H. adriaticum* varied according to the management regime. Results showed that individuals of *H. adriaticum* develop different strategies when growing in different habitats. Specifically, individuals growing in managed fully sunny dry grasslands reached higher vegetative height (MH), had lower values of SLA and a higher relative contribution of the C parameter than individuals growing in abandoned dry grasslands, which, on the contrary, were shorter, had higher values of SLA (and correspondingly lower values of LDMC) and a higher relative contribution of the R parameter. Further data on reproductive traits (e.g. fruit and seed-set) may corroborate our results. Although the number of individuals addressed in this study is rather low, and our conclusions may not be considered of general validity for the species, our study demonstrated the applicability of the CSR strategy scheme in detecting functional strategies at intraspecific level.

Keywords

Introduction

Trait-based studies have become extremely common in plant ecology (Chelli et al. 2019). This conceptual framework implies describing species through their biological characteristics by measuring suites of traits, i.e. features measurable at the individual level, that are assumed to impact plant fitness and survival (Pierce et al. 2017), either directly or indirectly (Violle et al. 2007).

Although species identity provides major information for ecological studies, the traditional taxonomic description becomes less effective when we are to understand plant reactions to environmental changes, e.g. climate or natural/anthropogenic disturbance, and the processes behind them (Westoby et al. 2002; Garnier et al. 2004). As measurable properties of individuals, functional traits allow understanding the interactions between organisms and their environment (both abiotic and biotic) and bring a functional perspective to the study on biodiversity and how it affects processes at higher levels of organization. Indeed, plant traits have been proved to vary with e.g. environmental conditions (Tautenhahn et al. 2008; Garnier and Navas 2012; Ivanova et al. 2019), land-use (Purschke et al. 2013; Duflot et al. 2014), and species distributions (Violle et al. 2014) suggesting that traits reflect the strategies plants use to cope with the environment and directly influence species interactions and the properties of com-

munities and ecosystems (Grime 2006; Albert et al. 2011; Fantinato and Buffa 2019; Fantinato et al. 2019a, 2019b).

Several studies suggest the existence of sets of plant traits that co-vary, thereby allowing the identification of syndromes that affect survival (e.g. Díaz et al. 2004; Cerabolini et al. 2010; Funk et al. 2016); however, there is no consensus yet on any particular ecological scheme (Pierce et al. 2017). One of the first suggested frameworks is the so-called "leaf economic spectrum" (LES), proposed by Wright et al. (2004); it distinguishes species according to their resource uptake dynamics from quick (acquisitive) to slow return on investment of resources (conservative). Another notable and popular strategy scheme is the CSR (competitor, stress tolerator, ruderal) theory by Grime (1974, 1977), recently reviewed by Grime and Pierce (2012). According to this theory, plant species are assigned to three main strategies which represent trait combinations arising under conditions of competition, abiotic limitation to growth or periodic biomass destruction, respectively (Pierce et al. 2017). These primary strategies represent the extremes, and CSR classification reflects the full spectrum of possible intermediate types. The scheme has been so far adopted at different levels of organization, worldwide (e.g. Yildirim et al. 2012; Astuti et al. 2019; Giupponi and Giorgi 2019; Nyakunga et al. 2018), and values on CSR strategy are currently available for several species (Cerabolini et al. 2010; Pierce et al. 2017). In most trait databases, species are described by mean trait values, regardless of environmental or genetic context (Albert et al. 2010), mostly due to the search for general patterns (Albert et al. 2011). However, plants change their traits not only from species to species (interspecific trait variability) but also among populations of the same species (intraspecific trait variability) when growing in different environment. Recent examples (Lecerf and Chauvet 2008; Messier et al. 2010) showed that intraspecific trait variability plays a crucial role, especially at local scale (Albert et al. 2011). Volis et al. (2004) found that plants of wild barley (*Hordeum spontaneum* Koch) from favourable environments were better competitors than those from stressful environments. Similarly, Pierce et al. (2013) classified individual plants of the genus *Poa*, showing within populations trait variation. Intraspecific trait variability thus might help reveal a species' ability to respond to environmental variations and has been proven to have direct effects on e.g. community assembly (Jung et al. 2010) and nutrient cycles (Lecerf and Chauvet 2008).

In light of the above, this study aims at investigating whether populations of the Adriatic Lizard Orchid (*Himantoglossum adriaticum* H. Baumann), growing on managed and abandoned semi-natural dry grasslands show differences in the CSR strategy. The evaluation of intraspecific trait variability might be the key to understanding the interactions between *H. adriaticum* and the extant community and reveal its ability to respond to environmental variations.

Materials and method

Study Area

The study took place on two hilly massifs of the Veneto Region (NE Italy): the Berici Hills (N 45.443.909; E 11.516.229) and the Euganei Hills (N 45.315.955; E 11.702.056), originated from volcanic (basaltic and rhyolitic) bedrock (Macera et al. 2003), and located southward of the Pre-Alps. Average annual rainfall is 800-1000 mm, distributed according to an equinoctial pattern with two maxima in April and September and two minima in December and July. Annual mean temperature is about 13.0-14.0 °C; the highest average temperature values can be found in July, while the lowest in December (Fantinato et al. 2019c).

Sampling sites were represented by small- to medium-sized (0.2–2 ha) dry grasslands, dispersed in an agricultural landscape among forests and arable fields. Created by low-intensity agricultural land use, semi-natural dry grasslands represent unique and species-rich plant assemblages. Temperate dry grasslands are maintained by traditional management practices as mowing, pasturing and haymaking (Habel et al. 2013), which regulate species composition and richness (Valkó et al. 2012). When low-intensity agricultural regimes are maintained, dry grasslands host important orchid populations (Fantinato et al. 2016a, 2016b, 2017; Slaviero et al. 2016). Abandonment results in structural changes such as the development of thermophilous fringe vegetation and scrubland facies (Biondi et al. 2006; Slaviero et al. 2016), with possible impacts on orchid populations (Slaviero et al. 2016).

Target species

Himantoglossum adriaticum is a European endemic species of priority interest (Directive 92/43/ EEC, Annex II), suffering population decline in many European countries (Dostalova et al. 2013). Its range comprises Austria, Bosnia-Herzegovina, Croatia, Czech Republic, Hungary, Italy, Slovakia, and Slovenia (Dostalova et al. 2013). It is described in the global IUCN Red List (Dostalova et al. 2013) and in the Italian Red List (Rossi et al. 2013) as LC (Least Concern). At regional level (Buffa et al. 2016) the species is classified as NT (Near Threatened), but its status worsens in lowland areas. In Northern Italy, it is mostly found in dry grasslands and nearby ecotonal meso-xeric scrubland/woodland patches (Slaviero et al. 2016; Del Vecchio et al. 2019).

H. adriaticum is a robust, perennial, tuberous, orchid with an over-wintering rosette composed of lanceolate, pale green basal leaves. The generative shoots are on average 40–80 cm tall, but they can reach 120 cm. The inflorescence is elongate and lax, composed of several (15–40) flowers and typically 14–24 cm in length (Bódis et al. 2019).

The labellum is the main characteristic that differentiates *Himantoglossum* species (Perazza and Lorenz 2013). In *H. adriaticum*, the violet trilobal labellum can reach 7 cm in length, the central lob has some tufts of purple hairs while the laterals are very thin and shorter. The flowering season usually starts in May or June. Its pollination system is rather generalised (Fantinato et al. 2017). *H. adriaticum* is normally found in light to semi-shaded habitats (Delforge 2006), where it takes advantage of shadows produced by shrubs or slopes to avoid direct sunlight and drought conditions. However, if shrubs become dominant thereby increasing shadow, *H. adriaticum* can suffer limited growth or even local population extinction (Tornadore et al. 2006).

Data collection

We selected seven populations of *H. adriaticum*; three at the Euganei Hills and four at the Berici Hills. Between the 1st and the 10th of June, we placed a plot of 2 m × 2 m at the core of each population. In each plot, the plant community attributes were described by recording the composition of vascular plant species and visually estimating the percentage cover and average height of the herb and shrub layer (C_h and C_s; H_h and H_s, respectively). To account for geomorphic variability, in each plot topographic data were collected: aspect (AS), expressed in degrees clockwise from the North and slope (SL), measured in percentage steepness with respect to the horizontal plane (steepness = 0%).

Given the threatened status of the species, we restricted the sampling to a low number of individuals per population (less than 15% of the overall individuals' abundance) in order to avoid negative impacts on the populations' persistence (Astuti et al., 2019). We collected individuals by cutting the stem at 1 cm from the ground and we rehydrated them for 24 hours to reach full turgidity. For each individual, we measured the maximum vegetative height, i.e. the height of the whole foliage (MH) and we chose at least two undamaged and well-developed leaves for the subsequent measurements (Pérez-Harguindeguy et al. 2013). Laboratory measurements followed the standardized methodologies detailed by Pérez-Harguindeguy et al. (2013). We determined leaf fresh weight (LFW) and leaf area (LA, i.e. a single-sided leaf area with petiole). LA was determined using the Leaf Area Measurement Software (Askew 2003). Leaf dry weight (LDW) was then determined following drying for 72 h at 60 °C, and the specific leaf area (SLA; the ratio of leaf area to leaf dry mass) and the leaf dry matter content (LDMC; the ratio of leaf dry mass to fresh mass) were calculated.

LA, LDMC and SLA provide strong indications about vascular plant adaptations under different environmental conditions and are at the basis of the calculation of the CSR strategies (Pierce et al. 2013). They can be defined as follows.

- ***Leaf area (LA)***: it is the size of the photosynthetic organ. It measures how much a plant invests in the photosynthesis. The leaf area is directly linked to the plant capacity to intercept light, and hence to the plant productivity. Leaf area can vary with changes in climate, altitude, and stress, such as soil aridity. LA decreases under increasing stressful conditions (Pérez-Harguindeguy et al. 2013).

- ***Leaf dry matter content (LDMC)***: it quantifies leaf tissue density and nutrient retention capacity. High values of LDMC indicate a preference in conserving nutrients. Species with high level of LDMC show tough leaves, highly resistant to hazards (Freschet et al. 2010; Lienin and Kleyer 2012).

- ***Specific leaf area (SLA)***: it is defined as the ratio of total leaf area to total leaf dry mass. In other words, it describes the amount of leaf area for light capture per unit of biomass invested. High SLA values are generally recorded in resource-rich environments, while low values in resource-poor environments (Pérez-Harguindeguy et al. 2013; Freschet et al. 2010).

Data analysis

We used the canonical correspondence analysis (CCA) to study the relationship between plant traits, plant community attributes and plot geomorphological features. In the CCA ordination, the sample units were the individuals of *H. adriaticum* collected in each population. We used values of LDMC, SLA and the maximum height of the whole foliage (MH) as dependent variables, while the percentage cover and average height of the herb and shrub layer (C_h and C_s; H_h and H_s), plot aspect (AS) and slope (SL) as predictor variables. We used 1,000 Monte Carlo permutations to assess statistical significance of the association between plant traits, plant community attributes and plot geomorphological features. To define the groups in the CCA diagram we performed a cluster analysis on the plots × plant community attributes (using average-linkage method and Bray–Curtis distance).

The relative contribution (%) of C, S, and R parameters to the tertiary CSR strategy was automatically calculated and represented by a ternary plot using the spreadsheet provided by Pierce et al. (2017). The CSR classification investigates the trade-off between the traits LA, LDMC and SLA (high values of these traits are mutually-exclusive and represent extremes of leaf economic and size) to calculate the relative proportion of C-, S- and R-selection exhibited by the species. We quantified the relative contribution (%) of C, S, and R parameters to the tertiary CSR strategy for each sampled leaf and results were averaged per individual. In order to evaluate intraspecific trait variability, in terms of percentage of C, S and R parameters, among individuals of *H. adriaticum* growing in the different groups revealed by the cluster analysis, we performed a one-way PERMANOVA applying the Bray–Curtis similarity index with 9,999 (Anderson and Ter Braak 2003; Past 3.0 Software). Then, the Tukey HSD test was used to determine which parameters differed between the two groups.

Results

We sampled 29 individuals of *H. adriaticum*. The number of sampled individuals varied from 1 to 15, with an average number of 4.5 ± 5.2 (mean ± SD) individuals per population (Table 1).

The two CCA axes explained 88.81% (p = 0.018) and 11.19% (p = 0.002) of the variance, respectively (Trace = 0.021; P < 0.001). The first CCA axis was strongly positively related to the percentage cover of the herb (C_h; 0.71) and shrub layer (C_s; 0.63), to the average high of the shrub layer (H_s; 0.62) and to the plot slope (SL; 0.53). The largest loadings on the second axis were from plot aspect (AS; -0.27) and slope (SL; 0.25). The CCA revealed a strong positive relationship between SLA, community attributes (i.e., C_h, C_s, H_h and H_s) and plot geomorphological features (i.e., AS and SL; Fig. 1), and a slightly negative relationship between LDMC, community attributes and plot geomorphological features (Fig. 1). The cluster analysis highlighted that sampled plots belonged to two distinct groups; the first corresponding to flat, managed grasslands without shrubs, while the second to steep, abandoned grasslands, highly encroached by shrubs (Table 1).

The overall strategy of *H. adriaticum* obtained by averaging single individual strategy irrespectively of the habitat type was CR (C:S:R; 60:0:40 %). Significant differences in the CSR strategy of *H. adriaticum* growing in managed vs. abandoned dry grasslands were revealed by PERMANOVA (F= 28.4; d.f. = 40.71; P < 0.0001). Tukey test revealed that individuals of *H. adriaticum* growing in managed dry grasslands showed a significantly higher percentage of the C parameter (P < 0.0001; Fig. 2), and a significantly lower percentage of the R parameter (P < 0.0001; Fig. 2) than those growing in abandoned dry grasslands (Table 2).

In other words, our results suggest that individuals of *H. adriaticum* show different strategies when growing in different habitats (Fig. 3). In our study, individuals growing in managed dry grasslands were more competitive than individuals growing in abandoned dry grasslands, which, on the contrary, were more ruderal.

Discussion

Plant traits are increasingly used to detect plant responses to changes in environmental conditions, since they allow to determine species ecological roles in the environment (Violle et al. 2007) and to detect their ecological strategies (Grime and Pierce 2012; Pierce et al. 2017). Based on the results of this study, leaf traits and Grime's (1974; 1977) theoretical triangular scheme of competitor, stress tolerator and ruderal plant strategies (CSR theory) proved to be applicable for assessing the functional strategies at intraspecific level.

According to Delforge (2006), *H. adriaticum* is a species of light or semi-shaded habitats on shallow rocky soils with neutral or basic reaction. It does not show any strong phytocoenological preference. Its primary habitat includes dry grasslands (*Festuco-Brometea* Br.-Bl. & Tüxen ex Br.-Bl. 1949 class) and open shrubby communities (*Rhamno-Prunetea* Rivas Goday & Borja ex Tüxen 1962 class) with a mosaic of fully sunny and shaded patches (Bódis et al. 2019), however it can persist in a wide range of habitats including mesic grasslands (*Molinio-Arrhenatheretea* Tüxen 1937 class), and can also colonise secondary habitats such as roadsides or abandoned vineyards (Fekete et al. 2017).

Overall, *H. adriaticum* exhibited a Competitive-Ruderal (CR) secondary strategy, incorporating substantial competitive and, to a lesser extent, ruderal features. Interestingly, neither the species nor the two groups of individuals showed the stress-tolerant component. This low
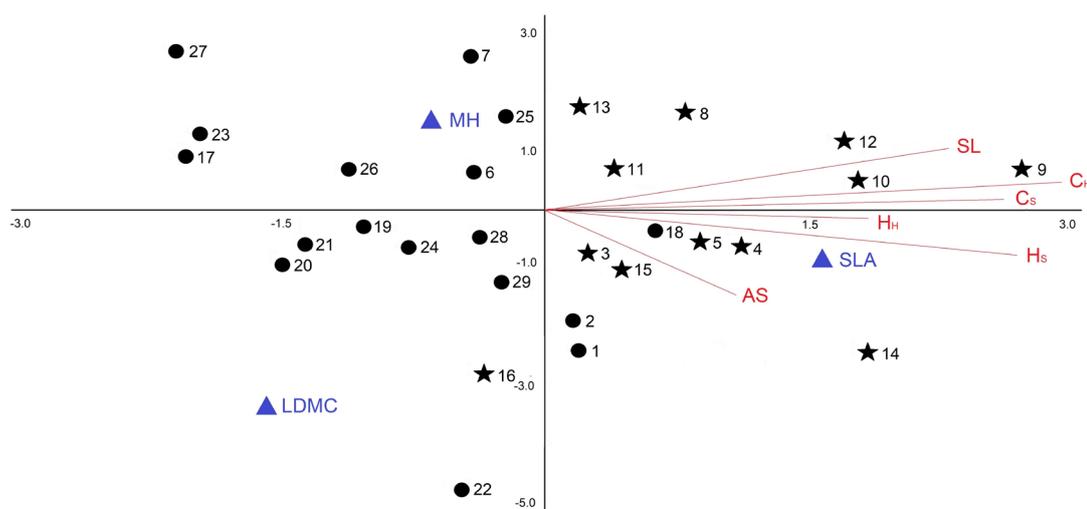

Figure 1. Scatterplot of the canonical correspondence analysis (CCA) utilized to investigate the relationship between plant traits (blue triangles), plant community attributes and plot geomorphological features (red lines). Different symbols indicate individuals of *H. adriaticum* growing in managed (black dots) or in abandoned grasslands (black stars), as revealed by the cluster analysis.

Table 1. Plant community attributes and geomorphological features of sampled populations.

| Population ID	Sampling site	Total number of individuals	Number of sampled individuals	Aspect (degrees)	Slope (%)	Herb layer cover (%)	Shrub layer cover (%)	Herb height (cm)	Shrub height (cm)	Management regime
1	Euganei	15	2	315	5	70	-	20	0	Managed
2	Euganei	115	13	45	5	40	-	25	0	Managed
3	Euganei	96	3	0	0	90	30	20	300	Abandoned
4	Berici	15	2	90	35	100	28	70	200	Abandoned
5	Berici	23	1	135	20	65	50	40	250	Abandoned
6	Berici	62	3	135	15	80	65	40	180	Abandoned
7	Berici	101	3	135	10	75	50	30	400	Abandoned
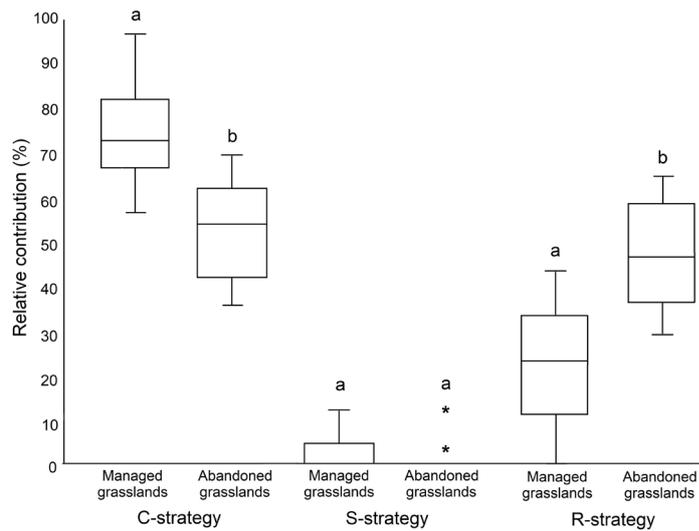
Figure 2. Differences in the relative contribution (%) of C, S and R parameters, among individuals of *H. adriaticum* growing in managed vs. abandoned grasslands. Different letters indicate significant differences according to Tukey's test.

inclination for the S- strategy may be explained by the particular phenology of *H. adriaticum*. Contrary to other dry grassland orchid species, its growing period starts in late spring, and the time of flowering is usually around late May or June. The capsules mature in 4–6 weeks, and seeds are shed rather quickly, in July or August (Bódis et al. 2019). This period corresponds with the rainfall season in the study area, with the average peak of rain in April; at the same time, temperatures, although increasing, do not have reached their peak, which is in July (Fantinato et al. 2017). Thus, *H. adriaticum* can take advantage of climatic conditions that allow it to complete its life cycle without incurring in stress conditions. Conversely, its characteristic perennial features and persistent rootstock account for the species' ability to compete and allow a rapid growth and development of a large standing biomass.

Despite these general characteristics, the CCA ordination showed that specific leaf area (SLA) and leaf dry matter content (LDMC) of *H. adriaticum* varied along with community attributes and plot geomorphological features, reaching, respectively, the highest and the lowest values in partially shaded, sloping grasslands encroached by shrubs. These traits are known to reflect crucial life history
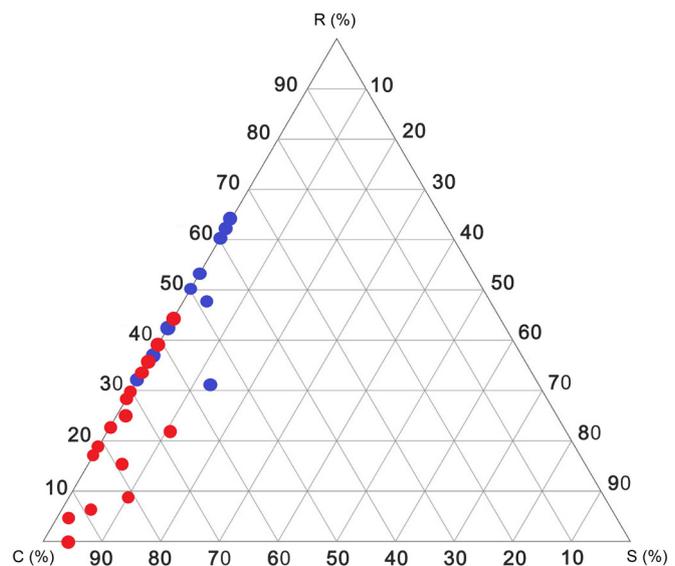

Figure 3. CSR triangular scheme for the individuals of *H. adriaticum* growing in managed (red dots) and abandoned grasslands (blue dots).

Table 2. Trait values and CSR strategy of sampled individuals of *H. adriaticum*.

Managed grasslands

ID	Maximum vegetative height (cm)	LA (mm²)	LDMC (%)	SLA (mm² mg⁻¹)	%C	%S	%R	Tertiary strategy
1	22.7	2.347.033	9.937	17.685	77.43	0	22.57	C/CR
2	27.4	1.935.357	11.290	20.531	67.94	0	32.06	C/CR
6	38.7	1.869.641	11.597	21.933	64.49	0	35.51	C/CR
7	45.2	1.766.543	10.141	23.411	61.14	0	38.86	C/CR
17	40.6	2.936.839	12.720	12.847	95.71	4.29	0	C
18	31.1	1.346.199	10.101	24.798	56.52	0	43.48	CR
19	33.9	1.804.074	11.796	16.346	81.46	0	18.54	C
20	33.7	2.301.610	12.784	14.276	89.15	4.69	6.159	C
21	35.2	1.091.375	12.789	15.440	79.25	5.38	15.37	C
22	24.2	1.315.645	13.901	17.281	67.44	10.9	21.7	C/CR
23	45.6	1.672.339	13.610	14.696	80.97	10.1	8.909	C
24	34.0	1.755.570	12.237	18.075	73.72	1	25.28	C/CR
25	35.8	1.650.154	9.290	20.705	66.4	0	33.6	C/CR
26	36.0	2.073.885	11.139	16.157	82.95	0	17.05	C
27	49.1	2.709.226	12.363	14.069	93.29	1.94	4.776	C
28	32.7	1.809.762	11.325	19.574	70	0	30	C/CR
29	28.5	1.531.902	10.782	18.836	71.08	0	28.92	C/CR

Abandoned grasslands

ID	Maximum vegetative height (cm)	LA (mm²)	LDMC (%)	SLA (mm² mg⁻¹)	%C	%S	%R	Tertiary strategy
3	29.4	2.381.676	9.724	21.063	68.02	0	31.98	C/CR
4	29.4	2.067.571	9.769	26.650	57.07	0	42.93	CR
5	27.0	1.934.965	9.010	22.896	62.78	0	37.22	C/CR
8	38.7	1.547.654	9.624	30.906	49.52	0	50.48	CR
9	45.2	854.140	7.471	40.698	35.96	0	64.04	R/CR
10	41.2	1.018.072	10.568	40.301	37.84	0	62.16	R/CR
11	32.3	2.131.742	9.468	23.533	62.28	0	37.72	C/CR
12	39.5	868.181	8.752	35.873	39.62	0	60.38	R/CR
13	33.4	1.184.001	9.111	23.704	57.33	0	42.67	CR
14	37.4	1.404.920	11.609	32.653	46.9	0	53.1	CR
15	37.2	1.273.419	13.051	28.582	48.44	4.23	47.33	CR
16	27.2	2.379.992	15.152	22.667	56.45	12.7	30.87	C/CR

trade-offs between the investment of resources in further resource acquisition vs. conservation (Pierce et al. 2017). High values of SLA ensure for high rates of resource acquisition in productive habitats, while high values of LDMC indicate a conservative leaf economics, implying the acquisition of traits responsible for retention of resources in unproductive conditions. Thus, strategies arise as an adaptive response of plants to site ecological condition (Cerabolini et al. 2010), and our results suggest that individuals of *H. adriaticum* develop different strategies when growing in different habitats. Specifically, individuals growing in managed fully sunny dry grasslands had lower values of SLA and a higher relative contribution of the C parameter than individuals growing in abandoned dry grasslands, which, on the contrary, had higher values of SLA (and correspondingly lower values of LDMC) and a higher relative contribution of the R parameter. The relatively higher ruderal component of individuals of *H. adriaticum* growing in abandoned dry grasslands, may be explained considering the local geomorphological characteristics. Dry grassland abandonment started from steep slopes, where management practices were more difficult. Steep slopes are generally characterized by high levels of natural disturbance, such as accelerated erosion processes from stormwater runoff and soil loss, with vegetation destruction and fragmentation. Under these unstable environmental conditions, individuals of *H. adriaticum* may have been selected according to their strategy, with the ruderal component being favoured on the competitive one.

We are aware that the number of individuals addressed in this study is rather low, and that our conclusions may not be considered of general validity for the species. However, as reported by Astuti et al. (2019), in the case of rare and threatened species, the use of small samples can be justified for conservation purposes. Although further research is needed to disentangle to what extent the morphological and ecological variability of the species is due to phenotypic plasticity or genetic variability of the populations, these results are stimulating as they show that plant species present adaptations to changing environmental conditions and extant community attributes that can be detected through the CSR scheme, at a very small spatial scale. Moreover, and consistently to other previous studies (e.g. May et al. 2017; Giupponi and Giorgi 2019), this research confirms that all components (Competitive, Stress tolerant and Ruderal component) of the CSR functional strategy can vary significantly among individuals of the same species. In this regard, a further step towards the comprehension of

processes underlying patterns of trait variation should account for regeneration traits of *H. adriaticum*. Trait-based ecology has so far mainly focused on adult plants despite early stages of a plant life history being very sensitive to environmental factors (Fantinato et al. 2018; Del Vecchio et al. 2020a, 2020b). Specifically, beside differing in leaf traits, the two components (competitive and ruderal) also vary in trade-offs of resource allocation to seed production, with competitors primarily composed of species with relatively low seed production and ruderals allocating resources mainly to seeds. Thus, reproductive traits and precisely fruit and seed-set may corroborate our results.

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