High Small-Scale Variation of Leaf Traits And Their Plasticity Within And Among Genotypes of A Clonal Species

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Research Article

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Abstract

Genetic variation of plant traits and their phenotypic plasticity are two supplementary ways of plant adaptation to temporarily fluctuating and spatially heterogeneous environmental conditions. Genetic variability and plasticity of leaf traits have been studied extensively as important indicators of the plant survival. In the case of clonal species with a patchy local distribution of clonal individuals, it would be important to investigate leaf traits at a small spatial scale. Here, small-scale variability of leaf traits and their plasticity within and among clonally spread genotypes in small 2 x 2 m plots was examined on the example of the clonal legume *Trifolium alpestre*. Seven leaf traits, leaflet length, area, width, fresh and dry weights, dry matter content (LDMC), and specific leaf area (SLA), were measured for ramets of various clonal genotypes sampled from five natural populations of *T. alpestre*. High variation of leaf traits and their plasticity was detected among the individual ramets of genotypes in 2 x 2 m plots of within the same population, as well as differential variation among the genotypes from different populations. The extent of variation in leaf traits and plasticity was found to be specific for the particular trait, genotype and site. The observed high variation of leaf traits and their plasticity within and among the clonally spread genotypes in local sites of populations is attributed to their differential combined response on the small-scale heterogeneity in the habitat conditions and genetic factors. High variation of leaf traits and their plasticity allows plants effectively respond to spatiotemporally fluctuating environmental conditions.

Introduction

Persistence and stability of plant species in natural communities has been shown regulated by plant functional traits (Polley et al., 2020). Among the plant functional traits, leaf traits are of special importance because leaves have a key role in carbon assimilation by photosynthesis and transpiration, thus ensuring the use of light and nutrients for the plant growth (Wilson et al., 1999; Evans and Poorter, 2001; Hodgson et al., 2011). Numerous studies have analysed variability and plasticity of leaf traits among various plant species under diverge habitat conditions (review: Gratani, 2014). These studies have shown that variability and plasticity of leaf traits are important indicators of the persistence of plant species and their populations. Among the leaf traits, SLA and LDMC are the two most important leaf functional traits that are related to plant performance in natural communities but respond differently to particular environmental factors related to light conditions and soil fertility (Wilson et al., 1999; Hodgson et al., 2011). SLA has been shown positively associated with relative growth rate, carbon acquisition through photosynthesis and competitive ability of plant species across biomes at the global level (Reich et al., 1999). Poorter and Bongers, (2006) found that tree species with higher photosynthesis and respiration rates also higher SLA values. Majeková et al., (2014) showed that LDMC is the best predictor of population stability: herbaceous species with high LDMC were be more stable over 13 years in a grassland community studied. The above studies showed that LDMC and SLA vary largely among and within the species of different functional groups, indicating the need to study the extent of variability within individual species.

Among the plant functional groups, clonal plants are of special interest because the ability for the clonal growth favours the plant ability to survive in adverse habitat conditions. Clonal plants are widely represented in environments with increased stress conditions, dominating frequently in cold and wet habitats, in light- and nutrient-limited habitats, along forest edges and in species-rich semi-natural grasslands (reviews: Oborny and Bartha, 1995; Klimeš et al., 1997). They are characterized by the continuous horizontal spatial spread of individual plants (ramets of the same clone) during the growth period. Clonal plants form local patches of individuals that are interconnected by underground rhizomes or aboveground spacers and thus form spatial structure of genotypes (clones) at a local scale. Therefore, in the case of clonal species with a patchy local distribution of clonal individuals, it would be important to investigate leaf traits at a small spatial scale. Variation of leaf traits at the level of local populations has been studied for several clonal grasses of different growth form (Bittebiere et al., 2013; Bittebiere and Mony, 2015). It has been reported that higher plasticity and variation of functionally important leaf traits in clonal plants support the performance and survival of species, particularly in spatially and timely fluctuating conditions of light intensity, soil fertility and water availability (Navas and Garnier, 2002).

To my knowledge, the extent small-scale variation in leaf traits and their plasticity has not been studied in clonal species. Here, *Trifolium alpestre* L., growing in rare fragmented populations at its northern distribution range in Estonia, was used as a model clonal species for simultaneous assessing of variability in morphological and functional leaf traits and their plasticity among and within genets in small 2 x 2 m plots from different sites of natural populations. Given that both biotic and abiotic conditions are found to be variable at a local small spatial scales in various natural communities and that plants respond to micro-environmental variations by changes in plant phenotypes and physiology (review: Denney et al., 2020), I expect to find variation in leaf traits of plants at local sites. The major aim of the study is to measure the extent of small-scale variability in the leaf trait values and their plasticity within and between clones from different fragmented populations.

Materials And Methods

Study species and sites

Trifolium alpestre L. is a perennial diploid (2n = 16) clover species which is distributed throughout central, southern and east Europe, but has sparse distribution mainly in dry meadows, forest fringes, steppes and light woodlands (Coombe, 1968). The species is able to reproduce both sexually from seeds and further by the local clonal spread of sexually derived individuals (Kaljund et al., 1998). In Estonia, its distribution of this species is limited only to the Island Saaremaa in the westernmost part of Estonia where the species is at its northern range edge and grows in small and isolated forest-edge populations on thin calcareous soils. My common garden observations showed that the species has epigeogenous, phalanx type of rhizomes with short spacer length that favours clumped clonal spread. In our common garden experiment, we observed that each mother plant formed new shoots from the two outer buds that grew up to 12–15 cm in a sandy soil during the summer growth period. Field excavations showed that genets formed nets of interconnected rhizomes with short spacers of about 5–10 cm length, indicating phalanx

type clonal growth form for this species. Therefore, 2 x 2 m plots with sixteen 25 x 25 cm subplots were chosen as suitable for the study.

Ramets from five 2 x 2 m local plots from five different sites of high plant density in the Viidumäe Nature Reserve on the island Saaremaa (58º17'54" N 22º 2'55" E) were collected for analyses. Sites 1 and 2 were growing near a road verge, about 200 m apart from each other. Sites 3 and 4 were from roadside forest gaps, separated by a 40 m coppice, while site 5 was under a sparse pine forest about 50 km kilometres apart from other populations. In total, 80 individuals were sampled among the five plots studied. All plots are derived from similar thin calcareous soils that are characteristic for the whole study area. However, the plots differed in the density and species identity of neighbour plants.

Leaf sampling and trait measurements

Fully expanded healthy trifoliate leaves were sampled at the peak of flowering time from the upper part of individual plants (ramets) within a day. The collected leaves were immediately placed in sealed plastic bags, kept in a cold box until transportation to a lab and then hold in a cooler. Leaf traits were measured from 16 equal 50 x 50 cm subplots of the 2 x 2 m plots (one of the 4–9 ramets present in the subplot was sampled). In total, 90 leaves were sampled. The following leaf traits were measured using the central leaflet as a proxy: leaflet length, width and area, leaflet fresh and dry weights, dry matter content (LDMC) and specific leaf area (SLA). The individual fresh leaflets were weighed on a digital balance and scanned on a flatbed scanner CanoScann 8800F. Leaflet areas (cm^2) were measured with the Image J software (https://imagej.net/). The leaflets were then oven-dried at 80°C for 3–4 hours to a constant weight and weighed with an analytical balance. Specific leaf area (SLA) is expressed as the ratio of the leaf area to the leaf dry weight in mm^2/mg. The leaflet length was measured along the midrib and widths were measured at the broadest site in mm. Leaflet dry matter content (LDMC, %) was calculated dividing the leaflet dry weight by the fresh weight to the fresh and multiplied by 100. A lateral leaflet of individual leaves was used for performing genotyping with the use of allozymes of seven polymorphic isozymes of five enzymes (aspartate aminotransferase (EC 2.6.1.1), esterase (EC 3.1.1.-), leucine aminopeptidase (EC 3.4.11.1), peroxidase (EC 1.11.1.7) and superoxide dismutase (EC 1.15.1.1), detected with the use of polyacrylamide gel electrophoresis as described in Kaljund et al., (2018). Allozyme genotypes (AGs) for each individual plant are defined by differences in allozymes at the seven isozyme loci.

Data analysis

Freeware PAST (Hammer et al., 2001) was used to calculate the character means, standard deviations and coefficients of variation (CV), as well as correlations and significance of differences among traits and plasticity. Trait means and standard deviations were calculated for each MLG. Correlations and differences among leaf traits and plasticity were analysed with a Pearson's *t* test. Associations among the seven leaf traits and differentiation among clones and traits was analysed with a principal component analysis (PCA). The plasticity among the ramets of the same MLG and among different

MLGs was described by the coefficient of variation (CV, %).The CV was calculated by dividing standard deviation by trait mean and multiplied by 100.

Results

The studied five plots were found to have each a different dominating AG that were clustered in neighbouring subplots, indicating their patchy small-scale clonal spread as expected for a species with short epigeogenous, phalanx type rhizomes of limited annual spread (Table 1). The same dominant AG was recorded in 9–16 subplots of each plot and from one to three different AGs in boarder subplots (data not shown, but see Kaljund et al. 2018). The spatial aggregation of dominating AGs indicates that they are derived through the clonal spread and represent clones. The data show remarkable differences in the variability by trait means and in their plasticity (CV) of leaflet traits among individual plants of AGs spread in 2 x 2 m plots (Table 1). The data also show that intra-clonal variability and plasticity of the seven leaf traits in local microsites are specific for particular traits and clones. The two functional leaf traits, LDMC and SLA, showed moderate, but differential variation among the five clones in their mean values. Thus, AGs 1–3 had similar lower LDMC values (31.0-32.3) in comparison with clones 4 and 5 (39.8 and 37.4, $p < 0.5$). In contrast, the mean SLA values of plants in AGs 2 and 5 (11.5 and 11.2) are much lower than in AG1 and AG3 (18.3.2–22.9, $p < 0.01$).). LDMC had a lower and less variable plasticity with CV ranging between 7.2–13.4 % (mean 10.2) in comparison with SLA (CV range 11.2–22.9 %, mean 15.3). The data suggest that LDMC and SLA values have opposite trends of variation among genotypes.

Table 1. Variation in the traits of central leaflets (mean ± SD and CV – coefficient of variation, %) among five dominant allozyme genotypes (AGs) of *Trifolium alpestre* dominating in 2 x 2 m quadrats from five natural populations (P). The number of ramets comprising the clonally spread AG dominating in the plot is given after the AG number. Short designations of leaflet traits: FW, fresh weight; DW, dry weight; LDWC, leaf dry weight content (% = DW/FW x 100); SLA, area per dry weight.

Traits/units	AG1-12		AG2-16		AG3-15		AG4-15		AG5-12	
Length, mm	52.1±6.4	12.4	42.0±5.5	13.1	55.3±4.9	8.9	50.6±5.8	11.4	42.4±6.3	14.9
Width, mm	9.5±1.0	10.9	10.1±0.9	8.7	9.8±1.0	10.1	10.1±1.2	11.8	8.9±1.0	11.2
Area, cm2	4.0±0.9	22.5	3.1±0.7	22.6	4.1±1.0	17.3	3.7±0.8	20.8	2.8±0.7	24.9
FW, mg	52.1±14	27.5	41.8±10.9	26.2	74.1±18.9	25.6	47.2±9.9	21.0	44.4±15.0	33.7
DW, mg	16.8±5.0	29.8	13.1±3.0	23.3	23.0±6.5	28.3	18.8±3.9	20.6	16.6±4.8	29.1
LDMC, %	32.2±3.2	9.8	31.3±3.7	11.7	31.0±4.2	13.4	39.8±2.9	7.2	37.4±3.4	8.9
SLA, mm2/mg	23.8±4.5	18.3	23.7±2.7	11.5	17.8±4.3	22.9	19.7±2.5	12.5	16.9±1.9	11.2

Leaf fresh and dry weights are generally the most variable and plastic traits within clones, showing the CV values ranging from 20.6 to 33.7 % among the clonemates of the five clones, followed by the leaflet area (CV range 17.3–24.9 %). Statistical analysis of the data in Table 1 for Pearson's linear *r* coefficients shows that all differences between trait means within clones are statistically significant at $P \leq 0.01$, except AG3 at $P < 0.02$. Principal component analysis shows that the five AGs (C1-C5) are well distinguished from each other by their PC 1 and PC 2 values, illustrating statistically significant genetic differentiation between all five clones (Fig. 1). Remarkably, the clone pair AG1 and AG2 as well as AG3 and AG4 originating from closely spaced populations are clearly differentiated by PC1 and PC2. In contrast, spatially far populations AG4 and AG5 from spatially far populations appear in the same PC1 and PC2 sector, presumably indicating that rather differences in habits than geographic distances cause larger differentiation between clones.

Principal component analysis (PCA) also shows a clear differentiation of leaf traits from each other by their PC 1 and PC 2 values (Fig. 2). SLA is negatively correlated with LDMC and leaflet length by PC2 that also revealed negative association of leaflet fresh with leaflet dry weight, area and width which covariate at PC2. Statistical analysis of traits by Pearson's correlation coefficients showed that leaf length, area, fresh and dry weights are significantly and positively correlated (Table 2). These three traits are negatively correlated with LDMC (P□0.05) and SLA (non-significantly). LDMC and SLA are significantly negatively correlated (P□0.05). Leaf width is variably correlated with other traits. SLA was a more plastic than LDMC, with SLA CV values ranging from 11.2 to 23.8 (mean 15.2) and LDMC CV values ranging from 7.2 to 13.4 (mean 10.2).

Table 2. Pearson's linear r correlations between leaf traits. Short designations of leaflet traits: FW, fresh weight; DW, dry weight; LDWC, leaf dry weight content (% = DW/FW x 100); SLA, area per dry weight. Significance values: *P□0.05.

Traits	L	W	A	FW	DW	LDMC	SLA
L	-						
W	0,256*	-					
A	0,969*	0,39*	-				
FW	0,816*	0,088	0,738*	-			
DW	0,828*	0,026	0,676*	0,889*	-		
LDMC	-0,186*	-0,17*	-0,319*	-0,445*	0,0146	-	
SLA	-0,091	0,45*	0,154*	-0,362*	-0,617*	-0,446*	-

Given the importance of variability in traits and their plasticity of individual plants to environmental conditions, it is important to evaluate associations between them. Comparison of the data in Table 1 shows that character means and CVs of clones are not correlated: higher trait means in clones often have

lower CV values and reversely. For example, leaflet length of AG5 had the lowest mean 42.4 among AGs, but the highest CV. Reversely, LDMC of AG4 had the highest value among AGs, but the lowest CV. Similarly, leaflet fresh weight of AG3 hat the highest value among AGs, but a low value of CV. Statistical correlation analysis of the data showed that the mean linear Pearson's coefficient *r* values between the trait mean and plasticity values varied largely between clones from negative (AG1 -0.049; AG2 -0.029; and AG4 -0.27) to positive (AG3 0.11 and AG5 0.062). This result implies that the trait values and their plasticity vary differentially and independently among the clones.

Discussion

This study describes for the first time small-scale variation of leaf traits concomitantly with their plasticity on the example of the clonal legume *Trifolium alpestre*. The most impressive result of the study is finding remarkably high fine-scale variation of leaf traits and their plasticity among individual ramets within the same clones (genets) in small 2 x 2 m plots. Thus, the CV values for LDMC and SLA individual genotypes (AGs) of *T. alpestre* (table 1) are largely overlapping those estimated for various clonal specie (grasses and forbs) at the population level by Bittebiere et al. (2013). Such high small-scale intraclonal variation of leaf traits most likely reflects plastic responses of ramets to fine-scale heterogeneous habitat conditions existing even within such small plots. Apparently, leaf traits of the same genet respond differentially to the fine-scale variation in surrounding environmental conditions. This raises a question about the kind of habitat variables that might cause so notable fine-scale differences in the leaf traits. *Trifolium alpestre* populations in Saaremaa are adapted to similar thin calcareous soils that are characteristic for all study sites. Resource sharing among the plants of the same clone through the clonal integration is shown to diminish the effect of soil heterogeneity on the leaf traits in clonal species (review: Liu et al., 2016a). Therefore, the high variability of leaf traits within the allozyme-based clones (AGs) is somewhat surprising. Several studies have shown large effects of biotic neighbourhood, the local vegetation density and the species identity on various plant functional traits at small spatial scales (Bittebiere and Mony, 2015; Abakumova et al., 2016). The density and height of neighbour plants will cause differential shading of individual plants, and numerous studies have shown that light intensity and quality greatly affect the plant performance and morphology (Liu et al., 2016b). In addition, variable density and species composition of neighbour plants will affect the traits measures between clonal ramets through the competition for various abiotic resources, e.g. light, water and nutrients (Bittebiere and Mony, 2015; Wang et al., 2016). Based on the above literature data, we assume that variation in the density and species identity may be the major factor for the observed high variability of leaf traits within clones.

In addition to the effects of environmental factors, the genetic differences among the genets should also be considered. The allozyme genotypes analysed for the leaf traits (putative genets) may consist of several sub-clones differing by changes in the DNA structure of genes affecting the expression of leaf traits by inclusion of mutations that were detected in plant species with the use of various hypervariable DNA markers (review: Nybom, 2004). In addition, the accumulation of epigenetic changes has been shown to be a characteristic feature of clonal plants that has contributed to their ability for rapid plastic

response to environmental variables (review: Douhovnikoff and Dodd, 2015). Epigenetic mutations caused by the methylation of histones and gene promoters induce heritable changes in the expression of respective genes and in the plant characters that they control. It is appropriate to assume that the observed fine-scale variability of leaf traits among MGs is partially caused by the differential accumulation of various DNA-based mutations in the same AG.

The results of the study show differential variability of plasticity of traits (CV) among clonal genotypes from different local populations. Principal component analysis showed that the five AGs derived from different populations are well distinguished from each other by their PC 1 and PC 2 values, illustrating significant differentiation between all clones. Inter-clonal competition among the initial sexual recruits and selection of plastic genotypes adapted to the particular local site has presumably contributed to the formation of differently adapted local AGs 1–5. The formation clonal clumps in natural populations in result of clonal competition and selection of superior genotypes that are adapted for a set of local environmental conditions has been shown for several clonal species (Arens et al., 2005; Vandepitte et al., 2009). It is thus reasonable to assume that clonal competition has contributed the spread of genotypes with more plastic leaf traits that are better fit to the local microenvironments in natural populations of *T. alpestre* with different sets of biotic conditions. The genotypes with differently variable leaf traits likely have superior competitive ability that favours their subsequent spread due to a better fit to local microsite habitats. Inter-clonal competition among the initial sexual recruits and selection of plastic genotypes adapted to the particular local site has presumably also contributed to the formation of differently adapted local clones.

The Principal Component Analysis data (Fig. 1) also show that leaf traits are largely differentiated among the AGs (C 1–5) that have spread and occupied local sites, exemplified by 2 x 2 m plots. *Trifolium alpestre* natural populations in Saaremaa are adapted to similar thin calcareous soils that are characteristic for all study sites. Resource sharing among the plants of the same clonal AG through the clonal integration should further minimize the effect of soil heterogeneity on the plant traits (Liu et al., 2016). Therefore, the fine-scale biotic heterogeneity in the density and species composition of neighbour plants is presumably the main cause of the high intra-clonal variation of leaf traits among plants in small 2 x 2 m plots, as described in several studies (Bittebiere and Mony, 2015; Abakumova et al., 2016). The density and height of neighbour plants will cause differential shading of individual plants, and numerous studies have shown that light intensity and quality greatly affect the plant performance and morphology (Liu et al., 2016). In addition, variable density and species composition of neighbour plants will affect the traits measures between clonal ramets through the competition for various abiotic resources, e.g. light, water and nutrients (Bittebiere and Mony, 2015; Wang et al., 2016). High flexibility and plasticity of leaf traits observed in a single genotype will provide greater opportunity for its persistence in spatially and temporarily variable conditions.

The SLA values among the Estonian clones of *T. alpestre* varied between 16.9 and 23.8 mm^2/mg (Table 1), being within 15.7 and 39.2 mm^2/mg reported for this species in the TRY database for the genotypes of the Central European populations (Kattge et al., 2020). This comparison shows that clones

from an Estonian northern population that has persisted since the post-glacial colonisation up to our days are characterised by the same range of SLA values as the Central European populations. However, the mean SLA plasticity CV value 32.7 computed from the TRY data for the eleven Central European plants of *T. alpestre*, is much higher than the mean CV 15.3 among the five Estonian clones. The higher SLA plasticity range among the Central European plants may be explained because they were sampled from more variable habitat conditions than the Estonian plants that grow in similar thin calcareous soils. The main difference among the plots studied was in the density and species identity of plants that affect the neighbouring light conditions for each ramet and may be the main reason for the observed variation in the SLA values and plasticity. This assumption is supported by the study of Poorter and Evans (1998) who showed that the SLA values among six herb species were 1.8–2.1 times higher under five times lower irradiance than at high irradiance. Meziane and Shipley (1999) found that SLA of herbaceous species varies depending on the interacting light intensity and nutrient availability combinations. Similarly, Navas and Garnier (2002) showed that SLA of a small clonal shrub *Rubia pergrina* varies significantly depending on the light, nutrient and water availability, indicating plastic responses to these abiotic factors. The five *T. alpestre* clones studied are collected from forest roadside sites with similar calcareous soils, further indicating that rather variable light conditions caused by different neighbour plants and competition among the neighbouring plants may be the prime factors that caused the observed high intra-clonal variation in the leaf traits, but not differences in soil conditions.

Leaf dry matter content (LDMC) is another important leaf functional trait related to the plant performance in variable environments, being associated with multiple environmental conditions (Wilson et al., 1999). LDMC is considered a better indicator of soil fertility than SLA (Hodgson et al., 2011), biomass production and population stability (Majeková et al., 2014; Smart et al., 2017) than SLA. The LDMC among the five Estonian clones varied from 31.0 to 39.8. This range is similar to 30.6 to 34.8 retrieved for the Central European individuals from the TRY database. However, the mean LDMC plasticity value 10.2 among the five Estonian clones is much higher than the mean 5.8 among nine Central European individuals. SLA is a more plastic than LDMC among the clones from the Estonian populations, 15.3 and 10.2 respectively. This result is consistent with the study of Harze et al. (2016) who reported higher SLA plasticity range in comparison with the LDMC plasticity for four calcareous grassland herbs from three populations.

Our study revealed a significantly negative correlation between LDMC and SLA. This is consistent with the literature data showing overall negative association between LDMC and SLA in perennial herbs (Li et al., 2005). The negative association between LDMC and SLA has evidently contributed to their role as alternative indicators of plant responses to the use of habitat resources (Wilson et al., 1999; Hodgson et al., 2011). Significant positive association between leaf length, area, fresh and dry weights evidently indicates their similar inherent response to the existing ecological conditions.

Overall, our data show that the two leaf functional traits generally are more variable within clones than between them. Several studies have shown that SLA and LDMC vary notably depending on the specific environmental factors and multiple interactions between them (Price et al,. 2017; Stark et al., 2017). The results obtained illustrate astonishingly large fine-scale variation in leaf traits and their plasticity both

among and within clones of *T. alpestre* that has presumably contributed to the persistence of this locally rare clonal species at its northern range limit.

Declarations

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Author contribution statement. V.J. conceived and designed the experiments, also analysed the data and wrote the manuscript.

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Figures

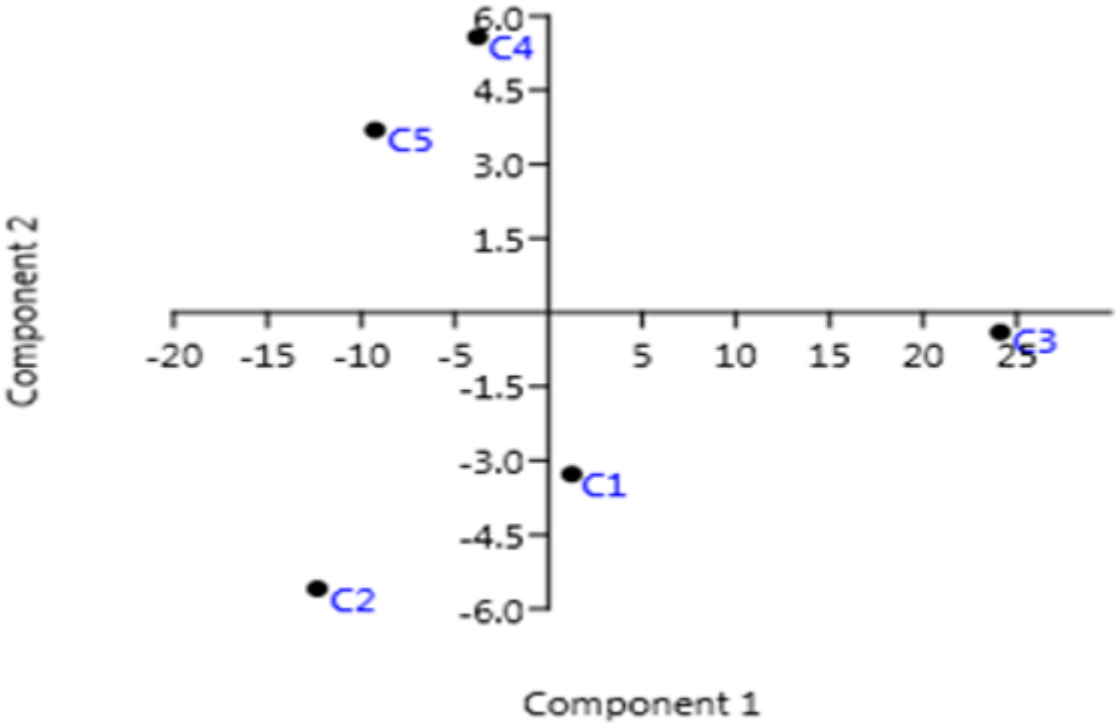

Figure 1

PCA analysis of C1-C5 of T. alpestre according to the mean values of leaf traits (A) and means of leaf traits (B). Designations: L – leaflet length, W – leaflet width, A – area, FW .– fresh weight, DW – dry weight, LDMC – leaf dry matter content, SLA – specific leaf area, Component 1 – PC1, Component 2 – PC2. PCA scores C1 C2 C3 C4 C5 PC 1 1.25 -12.3 24.1 -3.77 -9.25 PC2 -3.28 -5.59 -0.40 5.58 3.69 PC Eigenvalue % variance 1 208.5 85.3 2 21.7 5.63

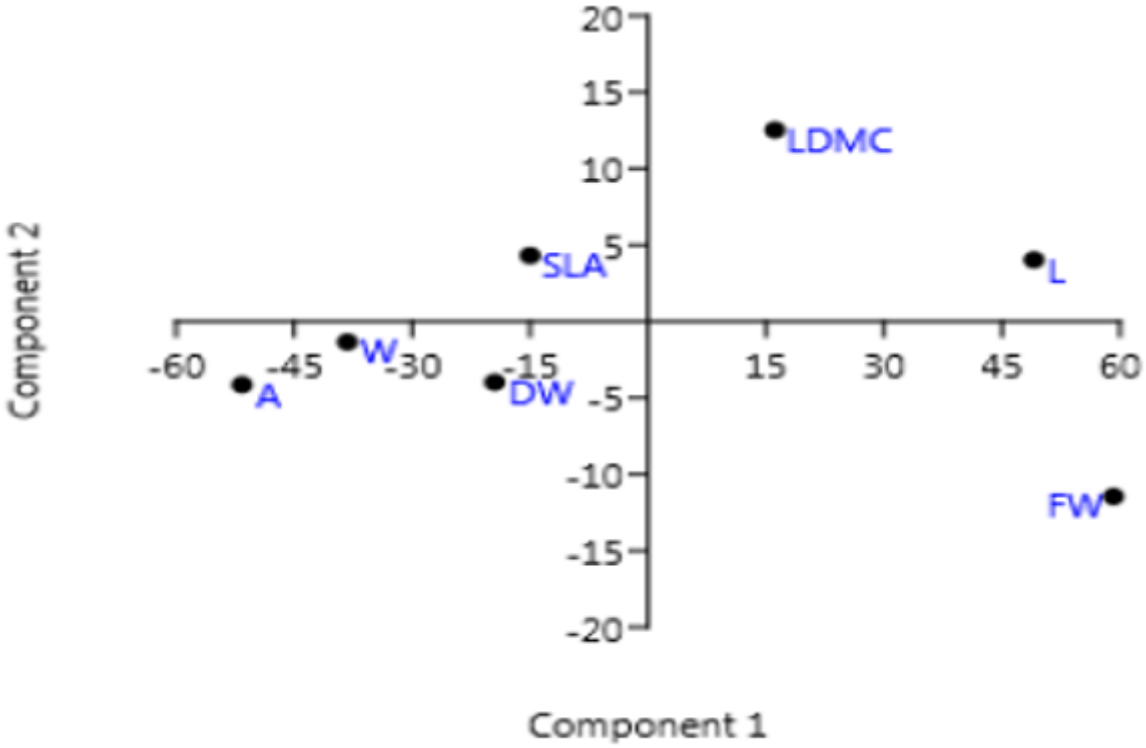

Figure 2

PCA analysis of C1-C5 of T. alpestre according to the mean values of leaf traits (A) and means of leaf traits (B). Designations: L – leaflet length, W – leaflet width, A – area, FW – fresh weight, DW – dry weight, LDMC – leaf dry matter content, SLA – specific leaf area, Component 1 – PC1, Component 2 – PC2. PCA scores L W A FW DW LDMC SLA PC 1 49.1 -38.2 -51.5 59.3 -19.4 15.7 -15.0 PC 2 4.21 -1.32 -4.14 -11.4 -4.01 12.2 4.43 PC Eigenvalue % variance 1 1814.67 96.088 2 58.3329 3.0888