Leaf traits of C3- and C4-plants indicating climatic adaptation along a latitudinal gradient in Southern Siberia and Mongolia☆


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ABSTRACT
Increasing aridity is one of the most important trends of current climate change. Leaf functional traits suggest a substantial basis for assessing the aridity effects on vegetation. However, since plants possess diverse leaf morphology and anatomy due to different evolutionary history of taxa, the effect of aridity can hardly be revealed in a multi-species analysis. We studied leaf functional traits for 317 samples of 193 plant species in steppe and desert communities along a 1600-km latitudinal gradient in Southern Siberia (Transbaikalia, Russia) and Mongolia. We determined morphological leaf traits, quantitative anatomical parameters, physiological parameters, and photosynthetic pigments content. Different relevance of leaf traits for indication of plant response to climate has been demonstrated. The clearest changes in site-mean values along the aridity gradient were shown for leaf thickness, total chloroplast number per leaf area (Nchl/A) and total surface area of chloroplasts (Achl/A) and cells (Ames/A) per leaf area. Unlike leaf size and leaf mass per area, these quantitative mesophyll parameters related to plant photosynthetic capacity were strongly correlated with climate. We found no evidence for a decrease in sizes of mesophyll cells with aridity, but cell volume as well as chloroplast number per cell were linked with plant functional type (PFT). We revealed an increase in Nchl/A and Achl/A in desert-steppe species in comparison to steppe and forest-steppe vegetation types within each PFT of C3-plants (C3-dicot herbs, C3-dicot shrubs, C3-monocots and C3-succulents). C4-plants generally characterized by low Achl/A and Ames/A, but had higher rate of CO2-transfer through mesophyll and chloroplast surfaces. C3- and C4-plants differed in response to aridity and showed opposite trends in changes of leaf traits along the aridity gradient. We conclude that leaf mesophyll traits contribute to important mechanism of climatic adaptation in different PFTs along a large latitudinal gradient.

Nomenclature
Catalogue of Life (www.catalogueoflife.org; accessed on 1 February 2018), and The Plant List (http://www.thep plantlist.org; accessed on 1 February 2018).

1. Introduction
Increasing aridity is one of the most important climatic factors of current climate change in step with a general global trend towards climate warming and drying (Burke et al., 2006; Hansen et al., 2010;...
Acidification is a complex climatic process combining long-term changes in temperature, the amount and seasonality of precipitation and insolation. These environmental variables are among the key determinants of physiology, biomass allocation and fitness of organisms, and increasing aridity is thus expected to have profound consequences on biodiversity and the distribution of vegetation types (Woodward, 1987; Chapin et al., 2000; Parmesan, 2006; Rustad, 2008). Studying plant responses to climate across latitudinal gradients is a useful approach to examining the effects of climate warming and aridity on vegetation (Reich and Oleksyn, 2004; De Frenne et al., 2013). Vegetation of current sites has evolved with the warming and aridity on vegetation (Voronin et al., 2003; Poorter et al., 2013). We hypothesized that mesophyll functional traits clearly indicate the response of plants with various leaf structure and photosynthetic performance along the aridity gradient in Central Asia. The aims of this study were 1) to find out which leaf traits among C3- and C4-plants, from whole-leaf to cell-level, respond to aridity, 2) to analyze the distribution of plant functional types (PFTs) along the aridity gradient and to define their influence on the site-mean values of leaf traits, 3) to reveal the role of mesophyll structure changes in photosynthetic performance of different PFTs along an aridity gradient.

2. Methods

2.1. Study sites

Studies were conducted in Russia and Mongolia along a 1600-km transect from Severobaikalsk (Transbaikalia in Southern Siberia, Russia) in the north to Ekhin-Gol (Southern Mongolia) in the south (Fig. 1, Table 1). A transect was located in an area with predominantly sharply continental cryoarid climate. Transbaikalia and Mongolia are characterized by sharp contrasts in relief where latitudinal climate zones are mixed with elevation gradients. In our case, the elevation of studied regions varied from 400 to 2000 m however these elevations corresponded to the common elevation level of current landscape and represented plain sites which are flat areas with zonal soils and vegetation. We choose sites with characteristic zonal communities which would be appropriate for analysis along a latitudinal gradient. To allow the direct comparison of the studied sites we used universal and simple parameters for the assessment of climate. The climate of the study sites was characterized by the De Martonne aridity index (Ia) calculated using the equation: \[ Ia = \frac{MAP}{MAT + 10} \], where MAP is mean annual precipitation (mm) and MAT is mean annual temperature (°C). Temperature and precipitation data for the period 1980–2010 were taken using the equation: \[ Ia = \frac{MAP}{MAT + 10} \].

2.2. Plant material

Vascular plant species which are quite common in the steppe zone of Southern Siberia and in the steppe and desert zones of Mongolia were used for this study. In total, 317 samples of 193 plant species were investigated in 13 sites (Table A1 in Supplementary material). Plants
selected were the dominant and most abundant species in each of the thirteen communities (Table 1), defined as comprising the main part of the projective foliage cover. Almost all species belonged to perennial plants – herbs, semishrubs, dwarf shrubs and shrubs. Species from 31 families were included in this study, the most dominant families being Asteraceae (28 species), Fabaceae (26 species), Chenopodiaceae (23 species) and Poaceae (19 species).

We classified the species under study according to the photosynthetic pathway, phylogeny, growth form and mesophyll anatomy into 9 plant functional types (PFTs): C_3-DH-DV – C_3-dicotyledonous herbs with dorsiventral mesophyll, C_3-DH-IP – C_3-dicotyledonous herbs with isopalisade mesophyll, C_3-DS-IP – C_3-dicotyledonous shrubs (semishrubs, dwarf-shrubs) with isopalisade mesophyll, C_3-M-Gr – C_3-monocotyledonous herbs with a graminoid leaf (Poaceae, Cyperaceae), Gymn – Gymnosperms (species of genus Ephedra), C_3-Succ – C_3-herbs and shrubs with succulent leaves (Chenopodiaceae, Crassulaceae, Zygophyllaceae, Tamaricaceae, Alliaceae), C_4-D-AK – C_4-dicotyledonous herbs and shrubs with atriplecoid and kochioid types of kranz-anatomy,

![Fig. 1. Locations of the study sites on the map of GlobCover-2009 (according to Bontemps et al., 2010). Sites numbers are given according to Table 1.](image-url)
C₄-M-Pan – C₄-monocotyledonous herbs with panicoid type of kranz-anatomy (Poaceae). C₄-D-Sals – C₄-dicotyledonous herbs and shrubs with salsoloid type of kranz-anatomy. Only two species among studied plants exhibit Crassulacean Acid Metabolism (CAM) – with salsoloid type of kranz-anatomy. Only two species among studied species were taken for measuring chlorophyll and carotenoid content, and leaf thickness, leaf mass per area and leaf dry matter content, 9–12 leaves were used for measuring leaf area, photosynthetic organs projection. 

20–30 leaves were fixed in tubes in a solution of glutaraldehyde (3.5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4) for anatomical measurements. Physiological and biochemical parameters were measured in a lesser number of samples with n = 110 than the parameters of leaf structure with n = 315.

### Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Site (coordinates)</th>
<th>Type of community</th>
<th>Vegetation</th>
<th>Elev</th>
<th>R</th>
<th>MAT</th>
<th>MAP</th>
<th>LA</th>
<th>n</th>
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<tr>
<td>1</td>
<td>Severobaikalsk</td>
<td>Relict sites of meadow steppe in the forest zone</td>
<td>South taiga</td>
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<tr>
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<td>Grass-low herb petrophytic steppe</td>
<td>Mountain forest-steppe</td>
<td>669</td>
<td>3.42</td>
<td>−1.4</td>
<td>315</td>
<td>36.6</td>
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<td>Mountain forest-steppe</td>
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<td>28.0</td>
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<td>Forb-sedge steppe</td>
<td>Meadow degraded steppe</td>
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<td>True steppe</td>
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<td>−0.2</td>
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<td>Dry steppe</td>
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<td>1.5</td>
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<td>Dry steppe</td>
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<td>Mountain desert steppe</td>
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<td>4.36</td>
<td>3.5</td>
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<td>11.9</td>
<td>14</td>
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<td>9</td>
<td>Bulgan (43°52′54″E 103°34′42″)</td>
<td>Shrub-forb- bunchgrass steppe</td>
<td>Mountain steppe</td>
<td>1720</td>
<td>4.43</td>
<td>3.2</td>
<td>118</td>
<td>8.9</td>
<td>11</td>
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<tr>
<td>10</td>
<td>Bulgan (40°00′45″E 103°33′31″)</td>
<td>Semishrub-onion-small bunchgrass steppe</td>
<td>Desert steppe</td>
<td>1442</td>
<td>4.41</td>
<td>4.2</td>
<td>117</td>
<td>8.2</td>
<td>24</td>
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<td>Desiccated coarse-shrub desert</td>
<td>True desert</td>
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<td>Mountain desert steppe</td>
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<td>Sparse shrub desert</td>
<td>Hyper-arid desert</td>
<td>985</td>
<td>4.69</td>
<td>8.5</td>
<td>58</td>
<td>3.1</td>
<td>9</td>
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</table>

Geographical location, climatic and vegetation characteristics of the study sites. No. – site number, Elev, elevation (m); R, solar radiation incident on a horizontal surface (kWh m⁻² d⁻¹); MAT, mean annual temperature (°C); MAP, mean annual precipitation (mm); Ia, De Martonne aridity index; n – number of studied species.
per species, one leaf per individual). Cell sizes were determined in cell suspension after maceration of leaf pieces in 1 N HCl under heating up to 40–50 °C in 30 replicates per sample (5 leaf pieces from the middle parts of leaf blades, one piece per leaf, and one leaf per individual). Cell projection area and perimeter were measured separately for each type of photosynthetic tissue (palisade and spongy mesophyll for C3-plants, mesophyll and bundle sheath cells for C4-plants, simple and segmented mesophyll cells for grasses) with a light microscope (Axiostar plus, Zeiss, Germany) and the image analysis system SIAMS Mesoplant (SIAMS, Yekaterinburg, Russia). The mesophyll cell volume (Vcel, 103 μm3) and the chloroplast volume (Vchl, μm3) as well as their surface areas (Scel, 103 μm2, and Schl, μm2) were calculated by the projection method described in detail by Ivanova and Pyankov (2002) and Ivanova et al. (2018). Chloroplast number per cell (Chl per cell) was determined in 30 replicates per sample in the same cells that were measured for cell sizes.

The number of cells per leaf area unit (Ncel/A, 106 cm−2) was evaluated in 20 replicates in cell suspension using a hemocytometer (“Goryaev chamber”, Minimed, Bryansk, Russia) after maceration of leaf pieces of known area in 2–3 ml of 20% KOH after heating them to 90 °C (5 leaf pieces from the middle parts of leaf blades, one piece per leaf, and one leaf per individual). The chloroplast number per leaf area (Nchl/A, 106 cm−2) was calculated by multiplying the chloroplast number per cell and the cell number per leaf area for palisade and spongy mesophyll, or mesophyll and bundle sheath cells, or for the whole mesophyll in the case of uniform or irregular cells.

The total cell / chloroplast surface area per leaf area unit (Acel/A and Achl/A) was determined by multiplying the average cell / chloroplast surface area and the cell / chloroplast number per leaf area. We determined also the area of cell surface covered by chloroplasts (k) using the equation: k = (Achl/A)/(2Acel/A). Acel/A and Achl/A describe the surface areas through which CO2 penetrates from the intercellular air space to the sites of Rubisco carboxylation in the chloroplast. In C3-plants this process occurs similarly in cells of both palisade and spongy mesophyll. Therefore, in the case of C3-species Acel/A and Achl/A were separately calculated for palisade and spongy cells and then summarized. In the case of C4-plants, mesophyll and bundle sheath cells and chloroplasts in them are functionally different, because diffusion from the intercellular spaces in the cell cytosol and the initial carboxylation by phosphoenolpyruvate carboxylase (PEPC) occur only...
in the mesophyll cells, while carbon dioxide fixation is performed only in the chloroplasts of the bundle sheath cells. For this reason, we calculated $A_{\text{mes}}/A$ for C₄-plants as the surface area of mesophyll cells only and $A_{\text{chl}}/A$ as the surface area of the chloroplasts of bundle sheath cells only.

To determine the content of chlorophyll $a + b$ per leaf area ($C_{ab}/A$) pigments were immediately extracted with 80% acetone from fresh leaf pieces and measured using the Odyssey DR/2500 portable spectrophotometer (“HACH”, USA). $C_{ab}/A$ was calculated according to Lichtenthaler and Wellburn (1983). The chlorophyll $a + b$ content per chloroplast ($C_{ab}/\text{chl}$, $10^{-9}$ mg) was derived by dividing the pigment content per unit leaf area ($C_{ab}/A$) by the chloroplasts number per unit area ($N_{\text{chl}}/A$).

Light-saturated photosynthetic rate per leaf area unit ($A_{\text{max}}$) and transpiration rate (E) were determined in the first half of the day – from 9:00 till 14:00 – depending on the daily photosynthetic activity peak of the species. The measurements were carried out on intact mature leaves of three to five individuals per species using an infrared gas analyzer Li-6400xt (Li-COR, USA) under ambient CO₂ concentration, controlled leaf temperature (24 °C) and photosynthetic photon flux density (PPFD) of 1500 μmol photons m⁻² s⁻¹ for C₃-species and 2000 μmol photons m⁻² s⁻¹ for C₄-species. The photosynthetic activity of the chloroplast ($A_{\text{max}}/\text{chl}$, $10^{-10}$ μmol CO₂ s⁻¹) was calculated by dividing the photosynthetic rate per unit leaf area ($A_{\text{max}}$) by the number of chloroplasts per leaf area ($N_{\text{chl}}/A$). The rate of CO₂-transfer through mesophyll surface area ($TR_{\text{mes}}$) and chloroplast surface area ($TR_{\text{chl}}$) were calculated by dividing the maximum rate of CO₂-uptake per leaf area ($A_{\text{max}}$) by $A_{\text{mes}}/A$ or $A_{\text{chl}}/A$, respectively.

2.4. Statistical analysis

For some traits, such as leaf area or cell volume, the data were log transformed to meet the assumption of normality. The coefficient of determination was used to reveal the relationship between aridity index
and leaf parameters. To test for differences between categories \(C_2\) versus \(C_4\)-plants, PFTs and vegetation types following one-way ANOVAs we used Tukey-post hoc tests, and in appropriate cases a t-test was used. Differences were considered to be significant at \(p \leq 0.05\). All statistical analyses were carried out in Statistica 6.0 (StatSoft Inc.).

3. Results

Initial analysis of site-mean values across all species in the study failed to reveal changes in leaf traits except an increase in leaf thickness (LT) and leaf mass per area (LMA) with a decrease in aridity index (IA). Based on the separate analysis of \(C_2\) and \(C_4\)-plants, we found significant trends in five out of more than 20 leaf parameters for \(C_2\) plants along the aridity gradient (Fig. 2). All parameters that showed strong relationships between site-means and aridity belonged to the characteristics of leaf structure. The strongest correlations with climate were found for integrated mesophyll parameters per leaf area – chloroplast number \((N_{chl}/A)\), total surface area of mesophyll \((A_{mes}/A)\) and chloroplasts \((A_{chl}/A)\). These mesophyll traits substantially increased with a decrease in IA, indicating the increasing aridity along the latitudinal gradient. Among whole-leaf traits, LT and LMA also increased with aridity in \(C_4\)-species. Variation in the leaf area (LA) and leaf dry matter content (LDMC) could not be reliably explained by climate. Within a site, different \(C_2\)-species showed widely ranging values of cell volume and chloroplast number per cell (Fig. 2), that showed no patterning along the gradient. Within \(C_2\)-plants, the cell number per leaf area varied to a lesser degree than cell size, while in \(C_4\)-plants it was reversed. Physiological and biochemical parameters such as photosynthesis and transpiration rates, water use efficiency and pigment content did not show tendencies to change with aridity. Contrary to \(C_2\)-plants, \(C_4\)-species were abundant only at the southern end of the gradient, where there was an increase in IA, chlorophyll a + b content per leaf area and chloroplast number per cell and a decrease in LDMC and \(A_{mes}/A\) in \(C_4\)-plants.

We found common features as well as changes in the distribution of PFTs among all species in the study along the aridity gradient (Fig. 3A). \(C_2\)-dicot herbs and shrubs with isopalisade mesophyll dominated across the entire gradient. The proportion of \(C_2\)-dicots with dorsiventral mesophyll was higher at the northern part of the gradient and this PFT disappeared in southern sites. The proportion of \(C_4\)-plants, especially \(C_4\)-salsoloid type, increased with aridity. A clear tendency was also found in the distribution of zonal vegetation types of plants (Fig. 3B). Forest-steppe species were presented in the northern half of the gradient whereas desert-steppe plants appeared at the values of IA than 13. Desert species dominated at the southern end of the gradient with values of IA from 3 to 7.

The main differences between PFTs consisted of LT, LMA and mesophyll proportion in the leaf (Fig. 4). \(C_2\)-succulents, \(C_4\)-salsoloids and gymnosperms (species of genus Ephedra) had two- to three-fold higher LT than other PFTs. \(C_2\)-dicots are characterized by the largest values of the relative volume of mesophyll \((RV_{mes})\) which reached 70–80% of the leaf. In the case of \(C_2\)-grasses, Ephedra type and \(C_4\)-dicots, \(RV_{mes}\) was lesser – 50–60%. Minimal values of \(RV_{mes}\) were found for \(C_4\)-Salsoloid type – 35–40%. Very high values of LMA were intrinsic for Ephedra and \(C_4\)-Salsoloid type. We found significant differences in cell size or chloroplast number per cell for \(C_4\)-succulents which had 4–5-fold larger cells with a huge amount of chloroplasts in them than other PFTs. Leaf traits of two CAM-species Orostachys spinosa and O. malacophylla were similar to \(C_2\)-succulents with high water content – 95% in both species, thick leaves – 1930 μm in O. spinosa and 960 μm in O. malacophylla but had much larger mesophyll cells – 3.8106 μm3 and 1.1106 μm3 accordingly, and lower \(A_{chl}/A\) – 6.7 and 7.5. Integrated mesophyll parameters mostly differed between \(C_2\) and \(C_4\)-plants with lower values in the latter. Among \(C_2\)-plants, \(N_{chl}/A\) and \(A_{mes}/A\) were higher in isopalisade species, graninoidics, succulents and gymnosperms than those in plants with dorsiventral mesophyll.

The differences in leaf structure between zonal vegetation types can be seen in Fig. 5. Plants with \(C_2\)-photosynthesis did not show any clear difference in leaf parameters between steppe, desert-steppe and desert species. In the case of \(C_4\)-plants, desert and desert-steppe species differed from steppe and forest-steppe plants by higher \(N_{chl}/A\) and \(A_{chl}/A\). In addition, montane-desert plants possessed the thickest leaves with the largest values of cell volume and chloroplast number per cell.

Physiological and biochemical data show comparable rates of photosynthesis and transpiration per leaf area unit between different PFTs as well as between different zonal vegetation types (Table 2). However, PFTs were distinguished by maximum photosynthetic activity per chloroplast and CO2-transfer rate per mesophyll \((TR_{chl})\) and chloroplast \((TR_{chl})\) surface with maximal values in \(C_2\)-plants and minimal values in gymnosperms. Zonal vegetation types were characterized by different water use efficiency (WUE) and pigment forms ratio. Compared to forest-steppe and steppe species, desert-steppe \(C_4\)-plants had a 1.5-fold increase in WUE. At the same time, desert-steppe \(C_2\)-plants did not differ from steppe either in LT or LMA, but exhibited higher \(N_{chl}/A\) and \(A_{chl}/A\) as well as a larger share of cell surface occupied by chloroplasts (k) (Fig. 5). Desert plants possessed lower values of chlorophyll a/b and a higher chlorophyll/carotenoid ratio than other zonal vegetation types (Table 2).

The analysis of mesophyll structure for PFTs and zonal vegetation types allowed us to generalize the structural and functional diversity of studied plants in a common scheme (Fig. 6). A combination of PFTs and vegetation types is described by points on a plane with two main traits as axes. Since cell volume and \(A_{mes}/A\) belong to the key parameters of mesophyll structure, contributing to the main differences in photosynthetic tissues anatomy between PFTs and vegetation types, one axis on a plane is represented by mesophyll cell volume, another axis is expressed by \(A_{chl}/A\). When plant groups were more finely separated by PFT and zonal vegetation type, we found local differences in cell volume and integrated parameters of mesophyll. For example, the cells of steppe plants in \(C_2\)-dicot shrubs with IP type were twice as small as those of desert-steppe (t-test, \(p < 0.001\)). Among steppe plants the cell volume of \(C_2\)-dicot herbs was on average three-times larger than that in \(C_2\)-shrubs (t-test, \(p < 0.05\)).

4. Discussion

4.1. The changes of leaf functional traits along an aridity gradient

We investigated a big data set on leaf functional traits along an extensive latitudinal gradient that allowed us to test our hypothesis about the indicative significance of leaf functional traits for plant response to climate in semi-arid and arid regions of Central Asia. Our results demonstrated the differing relevance of leaf traits as response indicators among PFTs. Some leaf traits known to be sensitive to climate, as in our case, were not related to aridity. Globally, site-mean leaf size typically scales with water availability and temperature (Givnish, 1984; Royer et al., 2005; Peppe et al., 2011). Leaf size, shape, and teeth are widely claimed in paleoclimatic reconstructions as good predictors of environmental variables (Royer et al., 2005; Peppe et al., 2011). However, such an approach mostly concerns biomes dominated by woody dicots. The percentage of herbs and monocots (especially grasses, sedges, and onions among them) is very high in semi-arid and arid areas of Central Asia, where strong aridity stress leads to convergent leaf traits in particular simple shape and small size with a reduction of the leaf lamina in some species. Therefore trait–environment studies require other traits instead of leaf size and shape.

An increase in LMA and LT among both \(C_2\) and \(C_4\)-plants with dryness as well as a weak tendency for LDMC of \(C_2\)-plants was found in many cases (Fonseca et al., 2000; Niinemets, 2001; Lavorel et al., 2008; Prentice et al., 2011). In our previous study in European Russian steppes, no differences in LMA and LT were found in \(C_2\)-plants between forest steppe and desert steppe, which were twice as diverse in the
We found a general increase in LT and LMA among C₃-plants along the extensive aridity gradient investigated in this study (Fig. 2); however, these trends reflected the general differences between vegetation zones. Two groups of the sites studied within the gradient could be distinguished. The first group unites site-mean values from forest steppe to true steppe with LT 300–350 μm and LMA 70–90 g m⁻², and the second group at the other part of the gradient brings together sites from dry steppe to desert where LT reaches more than 500 μm and LMA is more than 100 g m⁻². Notably, most C₄-species manifested the highest values of these parameters in sites at the southern end of the transect. Differences occurring in LT and LMA between PFTs were higher than those between sites along the aridity gradient (Fig. 4). This finding confirms the predominant effect of PFT on LMA and LT variation (see also Castro-Diez et al., 2000; Poorter et al., 2009; Ivanova et al., 2018).

The strongest relationship with the aridity index across the entire gradient was found for mesophyll traits (Fig. 2). The fact that site-mean values of Nchl/A, Ames/A and Achl/A among C₃-plants vary in response to aridity indicates that species having low values of integrated mesophyll traits are replaced by species with high values in a more arid climate. At the same time the cell-level mechanism of these traits regulation represents a trade-off between cell size and cell number depending on functional peculiarities and evolutionary history of the species. Remarkably, the maximal rate of photosynthesis has comparable site-mean values in all sites of the gradient. The occurrence of C₄-plants being restricted to a much shorter latitudinal gradient could influence the character of relationships between leaf traits and aridity. However we suppose that biochemical peculiarities which contribute to mesophyll conductance

Fig. 4. Distribution of leaf traits for plants of different plant functional types (PFTs). C₃-DH-DV – C₃-dicotyledonous herbs with dorsiventral mesophyll, C₃-DH-IP – C₃-dicotyledonous herbs with isopalisade mesophyll, C₃-DS-IP – C₃-dicotyledonous shrubs with isopalisade mesophyll, C₃-M-Gr – C₃-monocotyledonous herbs with graminoid type of mesophyll, C₃-Succ – C₃-herbs and shrubs with succulent leaves, Gymn – Gymnosperms (Ephedra), C₄-D-AK – C₄-dicotyledonous herbs and shrubs with ariplecoid and kochioid types of kranz-anatomy, C₄-M-Pan – C₄-monocotyledonous herbs with panicoid type of kranz-anatomy, C₄-D-Sals – C₄-dicotyledonous herbs and shrubs with salisoloid type of kranz-anatomy. The results of one-way ANOVA are presented: F-criteria and p-level. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001, ns – not significant.
In C₄-plants may also lead to changing the trait–climate relationships for these plants in comparison to C₃-species (see below).

It is well known that dryness adversely affects the photosynthetic process in plants (Flexas et al., 2004; Chaves et al., 2009). However, A<sub>max</sub> has even been shown to increase with site aridity in a global multispecies analysis (Maire et al., 2015). Photosynthetic traits can be influenced not only by climate but also by soil properties such as soil pH, available phosphorus, nitrogen and others. Considering this circumstance we excluded all saline as well as disturbed sites from our analysis since the leaf traits of halophyte or ruderal species as well their response to climate should be subjects of special studies. In our case, we found the constancy of site-mean A<sub>max</sub> along the aridity gradient that can be achieved by replacement of PFTs related to leaf structure and functioning. A large within-site variation in most physiological parameters was observed in all parts of the gradient. This variation increased to the most arid end of the gradient (Fig. 2) and can be explained by diversity of PFTs in every site (see below).

4.2. Functional diversity of plants and their change along an aridity gradient

Patterns of C₃- and C₄-plant leaf types across semi-arid and arid

Fig. 5. Differences in leaf traits between plant species belonging to different zonal vegetation types inside C₃-plants (red box plots) and C₄-plants (green box plots). X-axis represents zonal vegetation types: FS – forest-steppe species, Sm – montane-steppe, S – steppe, DS – desert-steppe, Dm – montane-desert, D – desert species. The results of one-way ANOVA separately for C₃ and C₄-plants are presented: F- criteria and p-level. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001, ns – not significant (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
regions in Central Asia have been generalized on a plane of mesophyll parameters as axes (Fig. 6). One axis represented by mesophyll cell area and $A_{chl}/A$ rather than mesophyll cell number reflected plant response to arid climate. Clear differences between C3 and C4-plants with very low values of $A_{chl}/A$ for the latter are evident from Fig. 6. As mentioned earlier by Longstreth et al. (1980), most C4-species are generally characterized by low $A_{chl}/A$ in comparison to C3-species. Low values of integrated mesophyll parameters were also demonstrated for C3-species in European steppes (Ivanova et al., 2018).

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**Table 2**

Leaf functional traits of plant species belonging to different plant functions types (PFTs) and zonal vegetation types. C3-DH-DV – C3-dicotyledonous herbs with dorsiventral mesophyll, C3-DH-IP – C3-dicotyledonous herbs with isopalisade mesophyll, C3-DS-IP – C3-dicotyledonous shrubs with isopalisade mesophyll, C3-M-Gr – C3-monocotyledonous herbs with graminoid type, Gymn – Gymnosperms, C3-Succ – C3-herbs and shrubs with leaf succulence including species from Alliaceae. Gymnosperms (Ephedra), Succ – herbs and shrubs with leaf succulence including species from Alliaceae. The significance of differences between leaf structural types using $t$-test at $*$, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. fs – forest-steppe, s – steppe, ds – desert-steppe. Cell volume: the total chloroplast surface area per leaf area unit, cm$^2$ cm$^{-2}$; $A_{max}$ – maximal rate of photosynthesis per leaf area, μmol m$^{-2}$ s$^{-1}$; $E$ – transpiration rate per leaf area, mmol m$^{-2}$ s$^{-1}$; WUE – water use efficiency, μmol mmol$^{-1}$; $TR_{mes}$ – rate of CO$_2$ transfer through the mesophyll surface, μmol m$^{-2}$ s$^{-1}$; $TR_{chl}$ – rate of CO$_2$ transfer through the chloroplast surface, μmol m$^{-2}$ s$^{-1}$; $Cab/chl$ – chlorophyll a/b content per chloroplast, 10$^9$ mg; $A_{max}/chl$ – maximal rate of photosynthesis per chloroplast, 10$^{-10}$ μmol CO$_2$ s$^{-1}$; $a/b$ – chlorophyll a/b ratio, g g$^{-1}$; chl/car – chlorophylls/carotenoids ratio, g g$^{-1}$.

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**Fig. 6.** General schema describing the functional diversity of semi-arid and arid vegetation. PFTs and zonal vegetation types are presented on a plane with two main traits of mesophyll structure as axes: C3 – plants: AK – atriplericeid and kochioid types of kranz-anatomy, Pan – panicoid type. C3 – plants: DH-DV – dicotyledonous herbs with dorsiventral mesophyll, DH-IP – dicotyledonous herbs with isopalisade mesophyll, Gr-M – dicotyledonous herbs with dicotyledonous herbs with graminoid type of mesophyll, Gymn – Gymnosperms (Ephedra), Succ – herbs and shrubs with leaf succulence including species from Alliaceae.
The results presented here provide an evident explanation of how different PFTs within C3-plants reach similar values of integrated parameters in the current climate. The cell-level mechanism is probably connected with biological peculiarities of species. For example, shrubs in comparison to herbs usually have deeper root systems that reach underground water supplies. Therefore, because of longer roots reaching the groundwater table, shrubs are usually more resistant to drought and, in our opinion, can use available water for maintaining cell turgor pressure. Only shrubs increase cell size and chloroplast number per cell with increasing aridity (Fig. 6). This mechanism is beneficial and is structurally less expensive than cell proliferation, involving increasing Ncell/A and Nchl/A. Herbs with a rather short root system are adapted to aridity by an increase in cell concentration per leaf area unit. Steppe grasses as well as steppe dicotyledonous herbs possess smaller cells than those in forest-steppe. In the latter case, the decrease in cell size is a probable mechanism contributing to WUE in the herbal growth form. We identified a considerable increase in cell number per leaf area for desert-steppe grasses as well as for dicotyledonous herbs in comparison with steppe and forest-steppe – a trait that definitely leads to enhanced Amax/A and Achl/A. Another type of leaf shown in figures). Here an exceptional structural type is Ephemera-type, representing the group of congenic species of gymnosperms, in which aphyllous assimilating branches are characterized by high thickness (1.2–1.4 mm) and density (400–800 g m⁻²), low proportion of photosynthetic tissue (45–60%) and low water content (45–55%). Nevertheless, the definite combination of cell size and number forms a large internal assimilation surface in Ephemera branches represented by the values of Amax/A and Achl/A, that are appropriate for adaptation to steppe vegetation environment. Indeed, the majority of Ephemera-species are characterized by botanists as montane-steppe (Grubov, 1982; Malyshev and Peshkova, 1984) (Table A.1 in Supplementary material).

Currently, there is a lack of data on the proportion of plants with different mesophyll types along geographical gradients (Gillison, 2013). In European steppes, a decrease in the proportion of species with DV mesophyll and an increase in isopalisade- and kranz-anatomy with aridity were shown (Ivanova et al., 2018). The same tendency to change in the occurrence of the mesophyll structures of C₄-plants along a humidity and light gradient was demonstrated for boreal plants in the Ural region, where in wet and shady habitats species with homogeneous mesophyll were predominant, in middle humid and moderately insulated places species with dorsiventral mesophyll, and finally in dry and well insulated sites species with isopalisade mesophyll (Ivanova, 2014). These works also demonstrate that the integrated mesophyll traits change along the moisture gradient. In the current study, which is more extensive, we also observed the change in the distribution of PFTs and zonal vegetation types along the aridity gradient that underpinned the shifts in site-mean values of mesophyll parameters inside C₃-plants.

The increase in proportion of C₄-plants with aridity has been shown for Mongolia (Pyankov et al., 2000). Among C₄-plants, the biochemical subtype is shown to be the most indicative trait response to environment. According to our findings, C₃-Salsoloid species have much thicker and denser leaves or aphyllous branches with higher Amax/A and Achl/A, compared to C₄-Atriplexoid and C₄-Kochioid types, and especially than C₄-Panicoid type (C₄-grasses). Indeed, arborescent species having a salsoloid type of Kranz anatomy and NADP-malic enzyme type were the most resistant to drought stress and severe environments in extreme arid Gobi deserts with less than 100 mm of annual precipitation, whereas C₄-grasses with NAD-ME and PEP-carboxykinase photosynthetic types were mainly annual and perennial herbs that occurred mostly in the steppe zone where they are often predominant in pasture ecosystems (Pyankov et al., 2000).

4.3. Why mesophyll traits are relevant indicators of response to climate aridity

Our results imply an important role of mesophyll structure and primarily the value of summarized cell surface area (Amax/A) and chloroplast surface area (Achl/A) per leaf area unit in plant adaptation to aridity. These parameters are not only anatomical features but also characterize exchange surfaces for CO₂ when it passes from the intercellular spaces to the chloroplast stroma. Therefore these parameters contribute substantially to mesophyll conductance (gch). The change of gch can influence Amax independently of stomatal conductance (gs) (Flexas et al., 2013; Bahar et al., 2018). It was shown earlier that dry-site species tend to have higher Amax at a given stomatal conductance (Wright et al., 2001, 2003; Reich et al., 2003), which can cause a decrease of intercellular CO₂ concentration (Ci) (Wright et al., 2001; Prentice et al., 2014). High atmospheric vapour pressure deficit implies low Ci for plants in dry climates (Stewart et al., 1995; Prentice et al., 2014). Therefore, changes in mesophyll traits as the basic determinants of gch should be important for plants for maintaining the vitally important level of Amax in arid climates.

According to the first Fick’s law, there are two main mechanisms for regulation of CO₂ diffusion inside a leaf – a change in concentration gradient and a change in exchange surface area (Nobel, 1999; Tøsen et al., 2012), which can be expressed by indices Amax/A and Achl/A. C₃- and C₄-plants differ in these mechanisms regulating the CO₂ flow rate. C₃-plants possess a special CO₂-concentrating mechanism (Black, 1971; Caldwell et al., 1977), which provides steeper concentration gradients. Indeed, the CO₂ diffusion conductance in mesophyll is higher in C₄-plants than in C₃-plants (Table 2). Furthermore, according to other sources (Su, 2010) photosynthesis rate for desert C₄-plants could be much higher than observed in the majority of our data. In the case of Haloxylon ammodendron and Calligonum mongolicum, Amax was shown 36.1 μmol CO₂ m⁻² s⁻¹ and 47.1 μmol CO₂ m⁻² s⁻¹ accordingly. These data for Amax could mean that species of C₄-Salsoloid type could have even much higher values of TRmax – up to 2.9 μmol CO₂ m⁻² s⁻¹, and higher TRchl – up to 15–17 μmol CO₂ m⁻² s⁻¹, and higher photosynthetic activity of chloroplasts in bundle sheath cells (Achl/chl) up to 2.2×10⁻¹⁰ μmol CO₂ s⁻¹. The high rate of CO₂ transfer through the mesophyll and chloroplast surface in C₄-plants allows them to have lower area of the intra-leaf exchange surfaces and to invest less resources for the support the structural complexity of the mesophyll; as a result, C₃-plants even in the hyperarid climate of Gobi desert are able to keep the same low values Amax/A and Achl/A as indicated in C₃-plants in a humid climate (Pyankov et al., 1998; Ivanova, 2014; Ivanova et al., 2018).

C₃-plants having a C₃-carboxylation reaction are constrained in regulation of CO₂ flow rate mainly by changes in the size of exchange surfaces. In the leaves of C₃-plants CO₂ diffusion but not the biochemical capacity of CO₂-assimilation is predominantly affected by drought stress (Flexas et al., 2004). In C₃ plants, mesophyll conductance has been linked to WUE (Barbour et al., 2010) and has been proposed as a way to enhance WUE whilst avoiding the reductions in photosynthesis typically seen when reducing gs (Flexas et al., 2013). Indeed, we found a considerable increase of WUE in desert-steppe C₃-plants in comparison to steppe and forest-steppe (Table 2). The desert-steppe plants did not differ from the latter in LT or LMA, but had twice the Amax/A. An increase in Achl/A can furthermore provide the maximal degree of covering the cell surface by chloroplasts (k), as is shown in Fig. 5 by high values of parameter k for desert-steppe and desert C₃-plants. Only a few C₄-plants were found in hyper-arid deserts, where C₄-plants were
predominant. Desert C₃-plants were characterized by the same high values of integrated mesophyll traits as desert-steppe ones, and besides they had other values of pigment forms ratio – decreased chlorophyll a/b and increased chlorophylls/carotenoids. Similar trends in the pigments forms' ratio was found in steppes in the South Ural as a possible adaptation to aridity (Ivanov et al., 2013) – a factor that underpins changes in the functioning of photosynthetic units in chloroplasts under severe arid stress. The changes in carboxylation such as Rubisco activity (Wright et al., 2003; Prentice et al., 2014) or other biochemical mechanisms facilitating the diffusion of CO₂ (Evans et al., 2009) could be also expected in a few non-succulent C₃-species capable of surviving in a severe arid climate when they reach possible structural limits by an increase in mesophyll exchange surface. Our results clearly show that the increase of intra-leaf assimilation surface is one of the important mechanisms of adaptation of C₃-plants to arid climate and thereby such mesophyll parameters as Nₐ/d/A, A₉/M/A and A₉/A are confirmed as good predictors of plant response throughout global aridity gradients. In C₃-plants having other mechanisms of adaptation to aridity concerning their biochemical peculiarities probably the biochemical sub-type is predominant for adaptation to environment.

Thus, we found strong correlations of site-mean values of mesophyll traits with aridity along the large latitudinal gradient in Southern Siberia and Mongolia for C₃-plants. Leaf traits of C₃ and C₄-plants differed in response to aridity and showed opposite trends due to different mechanisms contributing to leaf mesophyll conductance. Patterns of C₃ and C₄-plant leaf types across semi-arid and arid regions in Central Asia have been generalized on a plane of mesophyll parameters. Cell size as well as chloroplast number per cell were clearly linked with plant functional type (PFT) whereas integrated mesophyll parameters were in greater degree related to vegetation types. Nₐ/d/A and A₉/d/A increased from forest-steppe and steppe to desert-steppe and desert C₃-plants. C₄-plants possess lower values of integrated mesophyll parameters compared to C₃-plants but higher rate of CO₂-transfer through the cell and chloroplast surfaces. We suppose that quantitative changes in leaf mesophyll structure allow different PFTs to maintain the photosynthetic performance under arid stress and to adapt to climate along a large latitudinal gradient.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.flora.2018.10.008.

References


