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REGULAR PAPER

Does *Bienertia cycloptera* with the single-cell system of C_4 photosynthesis exhibit a seasonal pattern of $\delta^{13}C$ values in nature similar to co-existing C_4 Chenopodiaceae having the dual-cell (Kranz) system?

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Abstract Family Chenopodiaceae is an intriguing lineage, having the largest number of C_4 species among dicots, including a number of anatomical variants of Kranz anatomy and three single-cell C_4 functioning species. In some previous studies, during the culture of *Bienertia cycloptera* Bunge ex Boiss., carbon isotope values ($\delta^{13}C$ values) of leaves deviated from C_4 to C_3–C_4 intermediate type, raising questions as to its mode of photosynthesis during growth in natural environments. This species usually co-occurs with several Kranz type C_4 annuals. The development of *B. cycloptera* morphologically and $\delta^{13}C$ values derived from plant samples (cotyledons, leaves, bracts, shoots) were analyzed over a complete growing season in a salt flat in north central Iran, along with eight Kranz type C_4 species and one C_3 species. For a number of species, plants were greenhouse-grown from seeds collected from the site, in order to examine leaf anatomy and C_4 biochemical subtype. Among the nine C_4 species, the cotyledons of *B. cycloptera*, and of the *Suaeda* spp. have the same respective forms of C_4 anatomy occurring in leaves, while cotyledons of members of tribe Caroxyloneae lack Kranz anatomy, which is reflected in the $\delta^{13}C$ values found in plants grown in the natural habitat. The nine C_4 species had average seasonal $\delta^{13}C$ values of −13.9‰ (with a range between species from −11.3 to −15.9‰). The measurements of $\delta^{13}C$ values over a complete growing season show that *B. cycloptera* performs C_4 photosynthesis during its life cycle in nature, similar to Kranz type species, with a seasonal average $\delta^{13}C$ value of −15.2‰.

Keywords *Bienertia cycloptera* · C_4 Plants · Carbon isotope discrimination · Chenopodiaceae · Kranz anatomy · NAD-ME Subtype

Abbreviations

$\delta^{13}C$ values	Carbon isotope values
NAD-ME	NAD-Malic enzyme
NADP-ME	NADP-Malic enzyme
PEP-CK	Phosphoenolpyruvate carboxykinase
PEPC	Phosphoenolpyruvate carboxylase
PPFD	Photosynthetic photon flux density
Rubisco	Ribulose bisphosphate carboxylase-oxygenase
Ci/Ca ratio	The relationship between the concentration of CO_2 in the intercellular air space of the leaf and that in the atmosphere
ϕ	Percentage leakage of CO_2 following decarboxylation of C_4 acids in the C_4 cycle

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Introduction

Family Chenopodiaceae contains the largest number of C_4 species among dicots (cf. Sage 2004). C_4 evolved in subfamilies Chenopodioideae (*Atriplex*), Suaedoideae (*Bienertia* and *Suaeda*), Salicornioideae (*Tecticornia*), and Salsoloideae (various genera), with at least 12 independent origins (Kadereit et al. 2003; Kapralov et al. 2006; Akhani et al. 2007; Voznesenskaya et al. 2008). The diversity currently found in the family includes a number of Kranz anatomical types (six major classifications, some of which have further minor variations in anatomy), two anatomical forms of single-cell C_4 functioning species, as well as the occurrence of two biochemical subtype C_4 species, NAD-malic enzyme (NAD-ME) and NADP-malic enzyme (NADP-ME), and C_3–C_4 intermediate species (Carolin et al. 1975; Akhani et al. 1997; Pyankov et al. 1992; Pyankov et al. 2001; Voznesenskaya et al. 2001a; Kadereit et al. 2003; Edwards et al. 2004; Akhani et al. 2005). Most of this diversification in C_4 traits occurred in Central Asian and Middle Eastern deserts, in a wide range of habitats from hyper-saline to extremely arid conditions (Butnik et al. 1991, 2001; Sage 2004; Akhani 2006).

Demonstration of the occurrence of C_4 photosynthesis in individual chlorenchyma cells in *Suaeda aralocaspica* (Bunge) Freitag & Schütze and *B. cycloptera* Bunge ex Boiss., *B. sinuspersici* Akhani broke the paradigm that Kranz anatomy is required for its function in terrestrial plants (Voznesenskaya et al., 2001b; Edwards et al., 2004; Akhani et al., 2005). Function of C_4 photosynthesis in leaves and cotyledons of these species (see Edwards et al. 2004), and surprisingly, in flowers of *B. cycloptera* and *S. aralocaspica* (Boyd et al. 2007), has been shown using a variety of methods. Differentiation to form C_4 type chlorenchyma cells occurs as young leaves mature (Voznesenskaya et al. 2003, 2005). The spatial separation to form two cytoplasmic compartments, including dimorphic chloroplasts and other organelles in single mature chlorenchyma cells, is supported by a well-developed cytoskeleton (Chuong et al. 2006). In *Bienertia*, the peripheral cytoplasm (having one type of chloroplast) and a central cytoplasmic compartment (a ball-like structure having another type of chloroplast) are proposed to function analogous to mesophyll and bundle sheath cells in Kranz type C_4 species. In *S. aralocaspica*, spatial separation of functions occurs because of dimorphic chloroplasts being oriented toward the distal and proximal ends of elongated mesophyll cells.

In C_4 plants, atmospheric CO_2 is converted to bicarbonate (by carbonic anhydrase) which is utilized by phosphoenolpyruvate carboxylase (PEPC), leading to the synthesis of C_4 acids. Effective donation of CO_2 from C_4 acids to Rubisco in bundle sheath cells restricts discrimination against ^{13}C by Rubisco resulting in more positive $\delta^{13}C$ values than C_3 plants (Farquhar 1983; Peisker and Henderson 1992). The $\delta^{13}C$ values for C_4 plants are approximately between −10‰ and −15‰; whereas, values for C_3 species are typically between −24 and −30‰ (Akhani et al. 1997; Cerling 1999). $\delta^{13}C$ values for herbarium specimens of *B. cycloptera*, collected from various sites, have values indicative of C_4 photosynthesis (Voznesenskaya et al. 2002; Akhani et al. 2005). However, Freitag and Stichler (2002) reported values of leaves of *B. cycloptera* grown in greenhouse conditions ranging from −15.5 to −21.1‰ (with the latest developing leaves being most negative), whereas, the single-cell C_4 functioning species *S. aralocaspica* and related Kranz type species had C_4 type values. Voznesenskaya et al. (2002) also reported lower values in young leaves than in mature leaves of *B. cycloptera* from plants grown in growth chamber conditions. This raised the question as to whether by nature this species might function as a facultative C_4 plant, i.e., in a C_3, intermediate or C_4 mode depending on environment (e.g., light level) or stage of development.

To test this hypothesis, during a complete growing season, we followed morphological development, and sampled and analyzed $\delta^{13}C$ values of photosynthetic organs of *B. cycloptera,* eight other C_4 species, and one C_3 species belonging to Chenopodiaceae which co-occur in a saline biotope in north central Iran. Seeds were collected from the site for a number of the C_4 species, and plants grown in a greenhouse; subsequently, the anatomy of cotyledons versus leaves, and the biochemical subtype of photosynthetic CO_2 fixation, were determined.

Materials and methods

Field studies and plant material

Field studies were conducted in salt flats near Mardabad, in saline soils near the Rude-Shur River, which is located 40 km west of Tehran. The studies lasted from early spring (beginning of March) to the middle of autumn (end of October), 2003. The cotyledons, young leaves on seedlings, and leaves and assimilating organs later in the life cycle (floral leaves, bracts and bracteoles, and photosynthetic shoots) were sampled. The species included *B. cycloptera* Bunge ex Boiss., and eight Kranz type C_4 species, *Atriplex leucoclada* Boiss., *Suaeda arcuata* Bunge, *S. cochlearifolia* Woloszczak ex Stapf, *Climacoptera turcomanica* (Litw.) Botsch., *Halimocnemis rarifolia* (C. Koch) Akhani, *Halimocnemis mollissima* Bunge, *Halimocnemis pilifera* Moq., and *Petrosimonia glauca* (Pall.) Bunge, and a C_3 species, *Atriplex verrucifera* M. Bieb. (as a control), all belonging to Chenopodiaceae.

They were growing either in association with *B. cycloptera* or in close proximity on salt flats and adjacent wastelands. Collections were made at regular intervals (~biweekly). At the time of collection, the developmental stage was noted and documented by herbarium vouchers and digital photography. All vouchers are present in the Botanical Biodiversity Research Laboratory of the School of Biology, University of Tehran.

Carbon isotope composition

For each species, on a given date samples were collected as follows with replicates dependent on availability: for cotyledons ($n = 2$–5); for other photosynthetic organs (leaves, floral leaves etc., $n = 1$–5). For a given date, replicates for each species were combined to form a single preparation for isotope analysis. When leaves were sampled during the growing season, generally the most newly developed and exposed leaves or bracts were taken. For determination of δ^{13}C values, cotyledons, leaves, or photosynthesizing shoots (depending on their age and availability) were analyzed according to the standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard as described by Bender et al. (1973) and Osmond et al. (1975). One to two measurements were made of the isotope value. The samples were ground to a fine powder, and then 1–2 mg placed in a tin capsule and combusted in a Heraeus CHN Eurovector elemental analyzer (GSF National Research Center for Environment and Health, Munich, Germany). The resulting N_2 and CO_2 were separated by gas chromatography and admitted into the inlet of a Finnigan MAT Delta S (GSF) or Micromass Isoprime isotope ratio mass spectrometer (IRMS) (Washington State University) for determination of $^{13}C/^{12}C$ ratios (R). Each sample was analyzed once or twice. δ^{13}C values were calculated where $\delta = 1000 \times (R_{sample}/R_{standard} - 1)$.

Material for anatomical studies

Seeds of *Bienertia cycloptera*, *Suaeda arcuata*, *S. cochlearifolia*, *Halimocnemis rarifolia*, *H. pilifera* and *Petrosimonia glauca* were collected from the study area and were germinated in Petri dishes at room temperature. Depending on the species, after 2–10 days, the young seedlings were transplanted into 10-cm diameter pots containing 10 parts commercial potting soil, one part clay, one part sand and 100 g gypsum, and grown for 3 weeks at room temperature under ~50 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After 3 weeks, the plants were transferred to a greenhouse and grown under natural sunlight plus supplemental light (sodium vapor lamps providing a PPFD of 400 μmol m^{-2} s^{-1}) with a maximum mid-day PPFD on clear days of 1750 μmol m^{-2} s^{-1}, and 25°C day and 18°C night temperatures. Plants were watered once weekly with 20:20:20 Peters Professional fertilizer (1 g l^{-1}) plus 150 mM NaCl per liter of water, or otherwise with water as needed.

Light microscopy

The cotyledons, and young and mature leaves, were fixed at 4°C in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 0.05 M PIPES buffer, pH 7.2. The samples were dehydrated with a graded ethanol series and embedded in London Resin White (LR White, Electron Microscopy Sciences, Fort Washington, PA, USA) acrylic resin. Cross sections were made on a Reichert Ultracut R ultramicrotome (Reichert-Jung GmbH, Heidelberg, Germany). Semi-thin sections were stained with 1% (w/v) Toluidine blue O in 1% (w/v) $Na_2B_4O_7$. The sections were photographed with a Nikon Optiphot-2 microscope coupled with a digital camera Muticam 2300 (School of Biology, University of Tehran). The anatomical features of *Halimocnemis mollissima*, *Climacoptera turcomanica*, and the two *Atriplex* species were studied using hand sections (not shown) to determine the type of anatomy.

Climate

The synoptical climatic data for 19 years (1985–2003) at station Karaj (35°55′N, 50°54′E, 1312.5 m), located at 25 km NW of the area, were obtained from the Iranian Meteorological Organization. Averages for the most important climatic data were obtained according to Walter's method (Walter and Lieth 1967). More detailed data for 2003, the year of the study, are presented for comparison with the long-term, 19-year average.

Protein extraction

Total soluble protein from different samples was extracted from greenhouse-grown species using a buffer containing 100 mM Tris–HCl, pH 7.3; 1 mM EDTA; 10 mM $MgCl_2$; 15 mM β-mercaptoethanol; 20% (v/v) glycerol; 1 mM NaF; 50 mM KH_2PO_4; 1 mM phenylmethylsulfonylfluoride; 1 mM dipyridyldisulfide; 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} chymostatin. The samples were ground completely in a cold mortar and centrifuged at 10,000*g* for 10 min at 4°C. Samples were diluted in 0.25 M Tris–HCl, pH 7.5; 2% (w/v) SDS; 0.5% (v/v) β-mercaptoethanol and 0.1% (v/v) bromophenol blue and boiled for 2 min for SDS-PAGE. Protein concentration was determined using the Bradford reagent (Biorad) using bovine serum albumin as standard.

Gel electrophoresis and Western blots

SDS-PAGE was performed in 8% (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane for immunoblotting according to Burnette (1981). Anti-*Amaranthus viridis* PEPC (1:200 dilution, Colombo et al. 1998); anti-maize 62 kDa NADP-ME (1:200, Saigo et al. 2004); anti-cucumber phosphoenolpyruvate carboxykinase (PEP-CK, 1:200, Walker et al. 1995); anti-*Amaranthus hypochondriacus* αNAD-ME (1:1,000, Long et al. 1994); and anti-spinach Rubisco large subunit (1:10,000, courtesy B. McFadden), were used. Bound antibodies were located by linking to AP-conjugated goat anti-rabbit IgG according to the manufacturer's instructions (Sigma).

Results

Study area, vegetation, and climate

The area is a saline flat biotope near the Rude-Shur (salty river), located ca. 40 km West of Tehran, 25 km SE of Karaj (35°43′26″N, 50°44′27″E, 1166 m) (Fig. 1). The saline flats around the Rude-Shur area are partly wasteland from former cultivated ground, which is subjected to temporary inundation and runoff water during the rainy season. The soil is alkaline and composed of clay with salinity levels ranging from 4.9 to 78 ds m^{-1}. The vegetation is dominated by annual C_4 halophytic communities with scattered *Tamarix* shrubs, indicative of a high water table in the area, and patches of perennial hemi-cryptophytic communities dominated by *Atriplex verrucifera* (see Akhani et al. 2003, and Fig. S1 for details on the vegetation and soil characterization). The long roots of most of the annual species (personal observations, H. Akhani) may reach the wetter parts of the soil horizon during the dry season. The vegetation period begins toward the end of March and ends in November for most halophytes growing in the area. However, this area is also rich in pseudohalophytes and ephemerals which benefit from lower soil salinity caused by leaching due to winter and early spring rainfalls. Most of the pseudohalophytes complete their life cycles, or die, due to increasing temperatures from May onward.

The diagram in Fig. 1 shows the averages of important climatic factors over 19 years. The area has received an average of 239.5 mm annual precipitation with an annual mean daily temperature of 14.9°C. Much of the precipitation occurs during late autumn, winter, and early spring, with the two maximums during December and March.

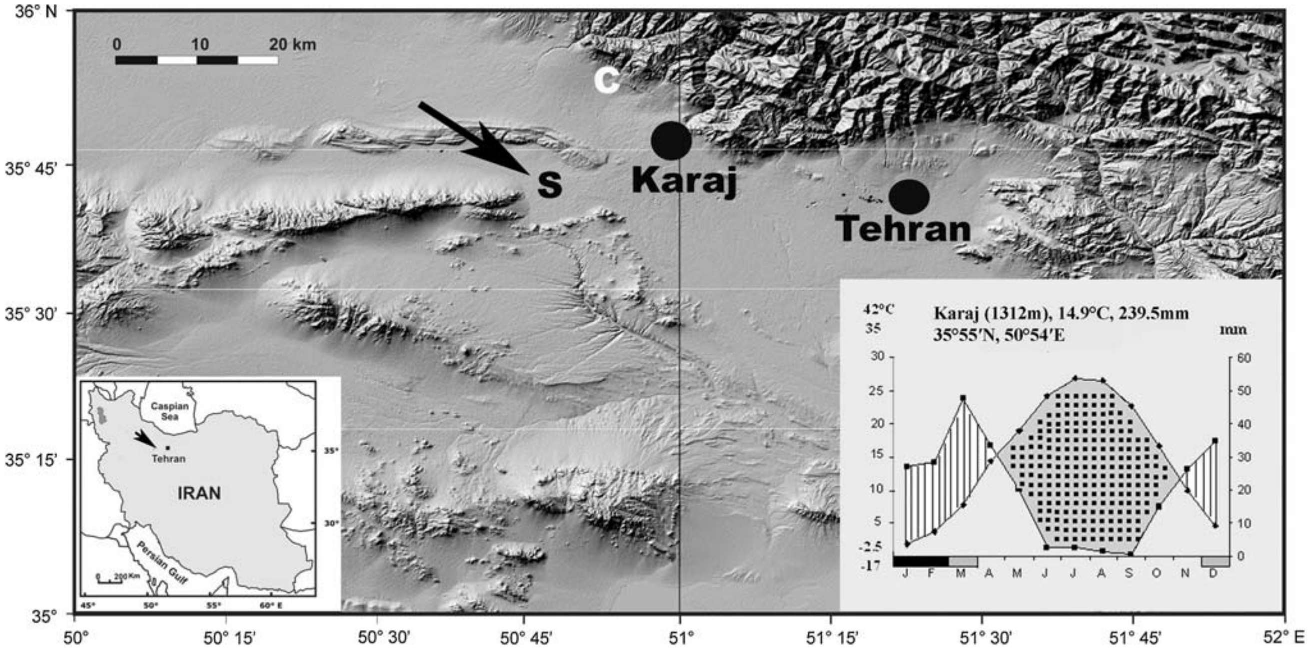

Fig. 1 Relief map of the study area showing its geographical location in North-Central Iran (lower inset left). Right side inset shows the climatic diagram of Karaj station based on an average over 19 years. ■, average mean daily temperature; ▲, average monthly precipitation. The data above the diagram shows elevation of the area from sea level, mean annual temperature (°C), average annual precipitation (mm), and geographical coordinates. Dotted area in the diagram represents the dry period and vertically hatched areas indicate the wet period. The black area on the x-axis represents months with mean daily minimum temperature below 0°C and shaded areas show months with absolute minimum temperature below 0°C, i.e., with early or late frosts. S: location of sampling; C: location of climatic station

Table 1 Climatic data from station Karaj during 2003

Factor	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Ave. minimum temperature (°C)	0.0	0.6	3.0	*8.4*	*10.9*	*15.3*	*18.7*	*19.3*	*14.5*	*13.4*	4.0	0.2
Ave. maximum temperature (°C)	8.9	9.3	12.9	*18.7*	*24.7*	*31.5*	*36.1*	*34.3*	*30.0*	*26.1*	14.0	8.0
Ave. mean daily temperature (°C)	4.4	4.9	8.0	*13.5*	*17.8*	*23.4*	*27.4*	*26.8*	*22.3*	*19.8*	9.0	4.1
Temperature records lowest (°C)	−4.8	−5.4	−6.6	*−1.4*	*1.8*	*7.2*	*14.2*	*15.4*	*9.4*	*8.0*	−4.0	−5.0
Temperature records highest (°C)	15.4	14.8	18.4	*27.8*	*29.2*	*37.0*	*40.6*	*38.0*	*35.0*	*30.0*	20.0	15.2
Average relative humidity (%)	64	60	55	*58*	*40*	*34*	*36*	*32*	*36*	*34*	52	67
Monthly precipitation (mm)	11.9	34.4	48.2	*80.0*	*18.3*	*0.0*	*0.0*	*0.4*	*0.0*	*20.0*	18.0	37.2
# days with precipitation (#)	6	10	10	*14*	*8*	*1*	*0*	*1*	*0*	*6*	5	12
Monthly total sunshine (h)	163.9	147.8	197.7	*123.3*	*287.6*	*338.1*	*344.6*	*352.7*	*310.6*	*249.4*	192.8	139.5

Courtesy of the Iranian Meteorological Organization. The months featuring most active growth are highlighted in italics

From April to November, the area is arid with almost no rainfall from June to September. These conditions, combined with low humidity, ranging on average from 32 to 67%, indicate an extreme continental climate. Based on observations during this study, the area was found to be very windy due to interaction between cool mountain and warm desert conditions. Table 1 shows data for nine major factors for 2003. The April precipitation of 80 mm during 2003 was exceptionally high, compared to the 19-year average, dropping quickly to 18.3 mm in May. In 2003 the seasonal change in mean daily temperatures, and the essential lack of precipitation in June through September, was similar to conditions averaged over 19 years. The large difference (29.2°C) between the lowest and the highest temperatures recorded during this period is indicative of the great variation between night and day temperatures, and also the rapid increase of temperature in the summer.

Taxonomy, comparative morphology

A list of the 10 chenopod species studied, their photosynthetic anatomical types, and biochemical subtypes among the C_4 species, their life forms, distributions, and previous reports on $\delta^{13}C$ values are given in Table 2. Pictures and descriptions of live specimens of all species at different developmental stages are shown in Fig. S1 (Supplementary material available at *Photosynthesis Res* online). The two *Atriplex* species are subshrubs belonging to subfamily Chenopodioideae, tribe Atripliceae. The remaining species are annual plants with succulent leaves belonging to subfamily Suaedoideae, tribes Bienertieae (*Bienertia*) and Suaedeae (*Suaeda*), and subfamily Salsoloideae, tribe Caroxyloneae (*Climacoptera*, *Halimocnemis* s.l. and *Petrosimonia*). The C_4 species *A. leucoclada* is widespread, occurring in warmer parts of SW Asia in many disturbed saline habitats. The C_3 species *A. verrucifera* is widely distributed in temperate deserts and semi-arid areas of SW Asia and has a more northerly range. The range of other species is centered in the Iranian temperate deserts and salines, and radiates into Central Asia as the Irano-Turanian elements. The morphological development and phenology of annual species are more or less similar. All species germinate during March; during April, May, and June, they are densely leafy, and thereafter begin flowering. As flowers develop during late June and early July, the larger vegetative leaves begin to senesce, and are replaced by shorter floral leaves and bracts.

Anatomy of leaves and cotyledons

The types of leaves of the C_4 chenopod species studied fall into five groups, which are summarized in Table 2. (i) *Bienertia cycloptera*, with Bienertioid type anatomy, has semi-terete linear-cylindrical leaves with three layers of chlorenchyma tissue internal to the epidermal cells and 2–3 layers of water storage cells in the central part of the leaf (Fig. 2a). (ii) *Suaeda arcuata* has cylindrical leaves with Salsinoid type anatomy consisting of a palisade mesophyll layer adjacent to the epidermal tissue and a bundle sheath (Kranz) layer surrounding the aqueous tissue, with vascular bundles imbedded in the center (Fig. 2b). (iii) *Suaeda cochlearifolia*, with Schoberiod type anatomy, has flat, succulent, ovate leaves and a unique Kranz anatomy with centrifugal chloroplasts in the bundle sheath cells surrounding the vascular bundles (Fig. 2c). (iv) The remaining five C_4 species in tribe Caroxyloneae have Salsoloid type anatomy with semi-terete, cylindrical to filiform leaves. The leaves are composed of an epidermal layer, followed by a single layer of palisade chlorenchyma cells and a layer of Kranz cells, illustrated with *Halimocnemis pilifera*, *H. rarifolia*, *and Petrosimonia glauca* in Fig. 2d–f. (v) *Atriplex leucoclada* has laminate leaves with Atriplicoid type anatomy in which an interrupted Kranz layer encircles the central and lateral vascular bundles. On both the adaxial and abaxial sides, there is one layer of palisade

Table 2 List of species studied, their anatomical type, C_4 subtype, life form, distribution, and previous records of $\delta^{13}C$ values from herbarium field collected specimens. Abbreviations: AT: anatomical type

Species	Leaf AT	Cotyledon leaf AT	C_4 Sub-type	Life form	Distribution	$\delta^{13}C$ Values, previous reports
C_4 Species						
Bienertia cycloptera Bunge ex Boiss.	Bienertioid (non-Kranz)	Bienertioid	NAD[a]	Annual	Temperate SW Asia, penetrates to Central Asia, mainly in Iran	−14.34[j], (−15.6, −14.2, −14.1, −13.1, −14.8, −13.1)[k]
Atriplex leucoclada Boiss.	Atriplicoid	Atriplicoid	NAD[b]	Dwarf shrub	SW and Central Asian, mainly in Southern and warmer parts	−13.56[j]
Climacoptera turcomanica (Litw.) Botsch.	Salsoloid	±Bifacial, non Kranz	NAD[c]	Annual	Central Asia, penetrates to SW Asia	−13.34[j]
Halimocnemis mollissima Bunge	Salsoloid	±Bifacial, non Kranz	NAD[d]	Annual	Iran, Afghanistan, Central Asia	−12.46[j]
H. pilifera Moq.	Salsoloid	±Bifacial, non Kranz	NAD[e]	Annual	Central Asia, Iran	−12.52[j]
H. rarifolia (C. Koch) Akhani	Salsoloid	±Bifacial, non Kranz	NAD[f]	Annual	Mainly Iran, occurs also in E Turkey and Armenia	−13.65[j]
Petrosimonia glauca (Pall.) Bunge	Salsoloid	±Bifacial, non Kranz	NAD[g]	Annual	Central Asia, Caucasus, Iran	−13.69[j]
Suaeda arcuata Bune	Salsinoid	Salsinoid	NAD[h]	Annual	Middle Asia, Iran, Afghanistan, Pakistan	−12.75[j]
S. cochlearifolia Woloszczak ex Stapf	Schoberiod	Schoberiod	NAD[i]	Annual	Iran, Iraq	−13.38[j]
C_3 species						
Atriplex verrucifera M. Bieb.	Non Kranz	Non Kranz	–	Dwarf shrub	SE Europe, SW Asia, Central Asia	−25.66[j]

[a] Current study and Voznesenskaya et al. (2002); [b] Not determined for this species, but *Atriplex* species are reported to be NAD-ME subtype (Sage et al. 1999); [c] Unpublished results from MV Lara and H Akhani; and NAD-ME subtype reported in this genus by Sage et al. (1999) and Pyankov et al. (2000a, b); [d] Not determined, but some other species in genus are reported to be NAD-ME from the current study, and by Sage et al. (1999); [e] Current study; [f] Current study; [g] Current study, and in this genus by Sage et al. (1999); [h] Voznesenskaya et al. (2007); [i] Current study; [j] Akhani et al. (1997); [k] see Akhani et al. (2005). ±, bifacial with slight differences on adaxial/abaxial sides

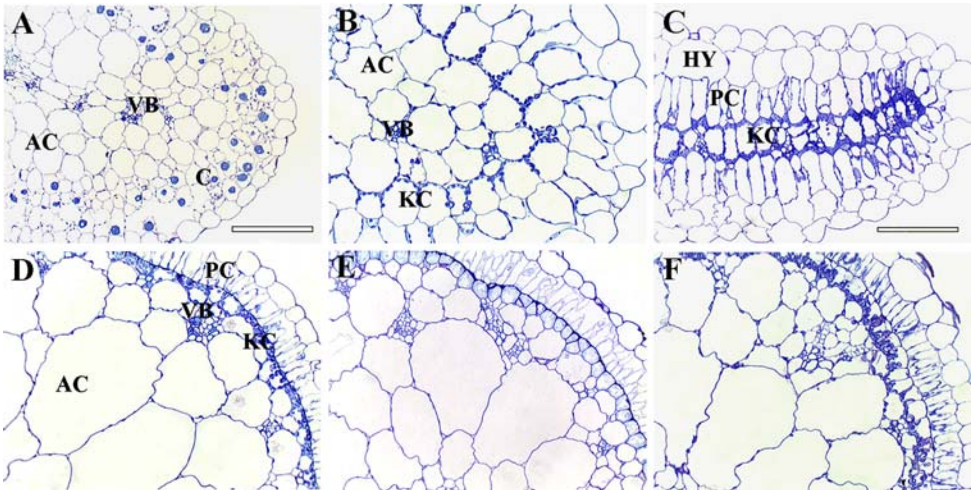

Fig. 2 Light microscopy of anatomical cross sections of leaves of *Bienertia cycloptera* and some other chenopods at the study site. **(a)** *Bienertia cycloptera*, **(b)** *Suaeda arcuata*, **(c)** *Suaeda cochearifolia*, **(d)** *Halimocnemis pilifera*, **(e)** *Halimocnemis rarifolia*, and **(f)** *Petrosimonia glauca*. Abbreviations: C: chlorenchyma cells of single-cell C_4 functioning species; AC: aqueous cells; VB: vascular bundle, KC: Kranz cells, HY: hypodermis; PC: palisade chlorenchyma. Scale bar for **a** is 200 µm; all other panels have the same scale bar as **c** which is 100 µm

chlorenchyma with large cells, followed by a second layer of smaller palisade chlorenchyma, which encircles the Kranz layer. *A. verrucifera* is a C_3 species having laminate leaves and non-Kranz anatomy (not shown).

The C_4 species in the study can be classified into two main groups with respect to type of anatomy of cotyledons. In one group cotyledons have Kranz type anatomy, with each species having the same form of C_4 anatomy in cotyledons as that appears in leaves, which includes *B. cycloptera*, *A. leucoclada*, and the two *Suaeda* species. This is illustrated with *S. cochlearifolia* with cotyledons having Schoberoid type anatomy (Fig. 3a), similar to that of leaves (Fig. 2c). In the other group, which consists of species in tribe Caroxyloneae, the cotyledons have C_3 type anatomy (illustrated for *H. pilifera*, *H. rarifolia*, and *P. glauca*, Fig. 3b, c), while leaves of these species have Salsoloid type Kranz anatomy (Fig. 2d–f).

Western blots of some key enzymes involved in photosynthesis

Western blots (Fig. 4) clearly show that the five representative species/genera analyzed in this study have high levels of PEPC along with Rubisco, characteristic of C_4 species. All species show immunolabeling for the C_4 acid decarboxylase NAD-ME. There was no immunolabeling with antibody for NADP-ME (lanes 2–6), while lane 1 shows immunolabeling with the control *Zea mays*, a known NADP-ME type C_4 species. There were no immunoreactive bands with antibody for PEP-CK (lanes 2–6), while lane 1 shows reactivity with the control *Spartina alternifolia*, a known PEP-CK type C_4 species (for other controls, see Voznesenskaya et al. 2006). All C_4 species in the study have NAD-ME subtype biochemistry.

Carbon isotope variation

Cotyledons

δ^{13}C values for cotyledons (Table 3) can be grouped into three categories: (i) *A. verrucifera*, with a value of –26.7‰, clearly lies in the C_3 range. (ii) The three species of Caroxyloneae, with values between −20.4‰ in *H. mollissima* and −22.7‰ in *P. glauca*, have C_3-like values. (iii) Species belonging to *Suaeda* and *Bienertia*, have C_4 type values which range from −11.8‰ in *S. arcuata* to −15.0‰ in *B. cycloptera*.

Leaves

The seasonal pattern of change in carbon isotope composition, and the seasonal average δ^{13}C value for each species, based on samples (leaves, and later in the life cycle floral leaves and bracts) taken between April and October are shown in Fig. 5. Leaves of *A. verrucifera* have a C_3 type isotope value (seasonal average −27.0‰), similar to that of its cotyledons. Leaves of all other species have C_4 type isotope values, with seasonal averages ranging from δ^{13}C of −11.3‰ in *H. mollissima* to −15.9‰ in *A. leucoclada*. Among the C_4 species examined, the lowest values were observed in genus *Halimocnemis* (with an average δ^{13}C for three species of −12.6‰). The highest seasonal variation based on the standard deviation (Fig. 5) was in *Suaeda arcuata* (±1.61) and the lowest in *H. mollissima* (±0.43).

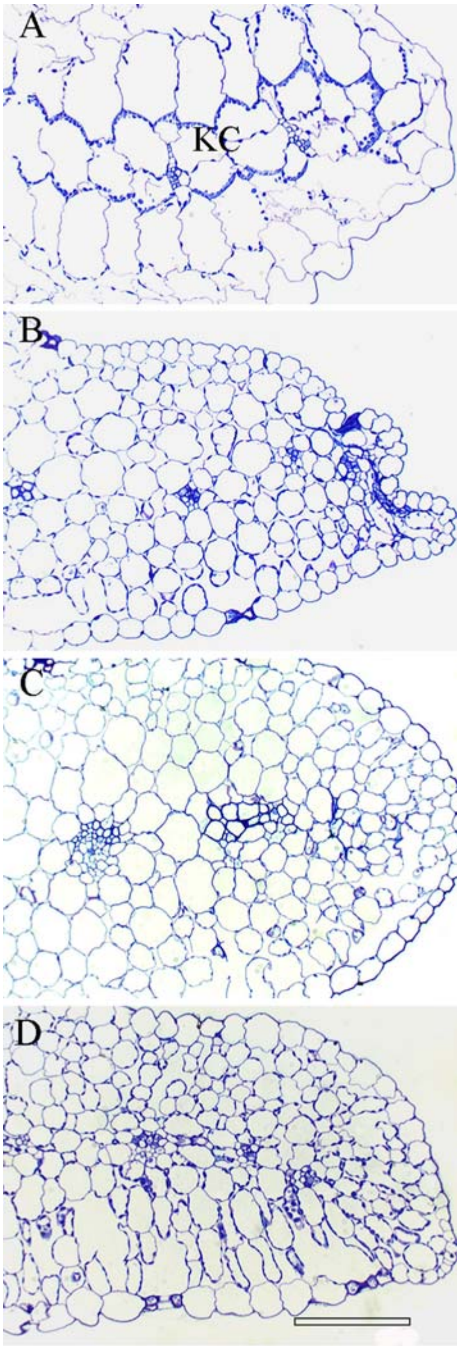

Fig. 3 Light microscopy of cross sections of cotyledon leaves of four selected species illustrating the occurrence of Kranz or C_3 type anatomy. **(a)** *Suaeda cochlearifolia* (C_4), **(b)** *Halimocnemis pilifera* (C_3), **(c)** *Halimocnemis rarifolia* (C_3), and **(d)** *Petrosimonia glauca* (C_3). KC: Kranz cells. Scale bars for all panels 100 µm

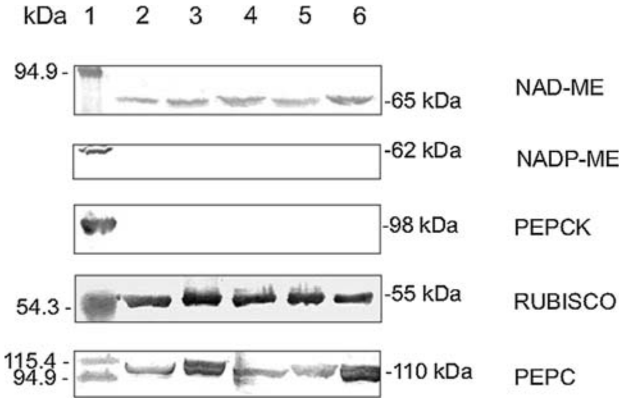

Fig. 4 Western blot analysis of total protein extracted from leaves revealed with antibodies raised against *Amaranthus hypochondriacus* αNAD-ME, recombinant 62 kDa NADP-ME from *Zea mays* leaves, cucumber PEP-CK, spinach Rubisco large subunit, and *A. viridis* PEPC. For *Bienertia cycloptera* (**2**), *Suaeda cochlearifolia* (**3**), *Halimocnemis rarifolia* (**4**), *Halimocnemis pilifera* (**5**), and *Petrosimonia glauca* (**6**) 5 µg of protein was loaded for Western blots for Rubisco and 20 µg for Western blots for other peptides. For the Western blots for NAD-ME, Rubisco and PEPC, selected bands from use of molecular weight markers are shown in lane (**1**). For the Western blots with antibodies against NADP-ME, there was no reactivity (lanes **2–6**); lane (**1**) shows reactivity with antibody to NADP-ME using extracts from leaves of *Zea mays*, a control NADP-ME type C_4 species. Western blots with antibodies against PEP-CK showed no reactivity (lanes **2–6**); lane (**1**) shows reactivity with antibody to PEP-CK using extracts from leaves of *Spartina alterniflora*, a control PEP-CK type C_4 species. Molecular masses of the immunoreactive bands are shown on the right

Discussion

The genus *Bienertia* is composed of two species which perform C_4 photosynthesis without Kranz anatomy. *B. cycloptera* is distributed in temperate salty deserts in Iran and the surrounding areas, whereas its vicariant, *B. sinuspersici*, inhabits warm saline and coastal areas around the Persian Gulf and the Gulf of Oman (Akhani et al. 2005). The ecology of *B. cycloptera* shows that it associates with C_4-dominated annual chenopod communities (Akhani et al. 2003). In the study area, it is common and associated with several other C_4 species, *Climacoptera turcomanica*, *Halimocnemis rarifolia*, *Petrosimonia glauca*, and *Suaeda cochlearifolia,* which are growing on clay soils. In close proximity there are several other Chenopodiaceae species, including the C_4 species *Halimocnemis mollissima* and *H. pilifera*, which occur in drier and less saline soils, the C_4 species *Atriplex leucoclada*, growing in disturbed areas, and the C_3 species *Atriplex verrucifera*, growing on highly saline and heavy clay and wet soils. The domination by NAD-ME C_4 subtypes in this community (Table 2) might be related to availability of nitrogen from edaphic conditions with nitrified soils due to previous agricultural activities. Studies on C_4 grasses indicate NAD-ME type species have a lower nitrogen use efficiency (photosynthesis and biomass produced per unit leaf N) than NADP-ME type species suggesting the latter might be more competitive on N-poor soils (Ghannoum et al. 2005; Akhani and Ghasemkhani 2007).

Table 3 Carbon isotope composition of cotyledons of *Bienertia cycloptera* and associated C_4 and C_3 species

Species	Anatomy	$\delta^{13}C$ values (‰)			
Date		02.04.03	15.04.03	26.04.03	08.05.03
Atriplex verrucifera	C_3	–	–	−26.67	–
Bienertia cycloptera	C_4	–	−15.03	−14.50	−14.56
Suaeda arcuata	C_4	−11.78	−11.78	–	–
Suaeda cochlearifolia	C_4	−14.77	−13.44	–	−13.28
Tribe Caroxyloneae					
Climacoptera turcomanica	C_3	−21.95	–	–	−22.07
Halimocnemis mollissima	C_3	–	–	–	−20.43
Petrosimonia glauca	C_3	−22.65	–	–	–

Carbon isotope values of *B. cycloptera* relative to other species in this study

The discovery of single-cell type C_4 photosynthesis in family Chenopodiaceae raised the question of whether these plants are facultative (C_3 or C_4 depending on environmental conditions) or obligate C_4 plants (See Introduction). From studies of plants grown in controlled environments, during leaf development the chlorenchyma cells have a developmental transition from a "C_3 default" mode with one type of chloroplast, to differentiation to form two types of chloroplasts and two cytoplasmic compartments for C_4 photosynthesis (Voznesenskaya et al. 2003, 2005). Also, the question has been raised as to whether the single-cell C_4 system in *Bienertia* may have greater leakage of CO_2 from site of donation of CO_2 from C_4 acids to Rubisco than Kranz type plants. In either case, development of C_3 type chlorenchyma, or less efficiency in function of C_4, should lead to more negative $\delta^{13}C$ values than typical for C_4 plants. With *B. cycloptera* cultivated in growth chambers, younger leaves had more negative $\delta^{13}C$ values (by 2–4‰) than mature leaves, indicating there is a stage of development where there is incomplete function of C_4 photosynthesis (Voznesenskaya et al. 2002, 2005). Freitag and Stichler (2002) found with *B. cycloptera* cultivated under greenhouse conditions that the $\delta^{13}C$ values ranged from approximately −15.5‰ to values as low as −21‰ in the uppermost leaves in the fall, which they suggested may be due development under low light conditions. Studies have shown that discrimination against fixing $^{13}CO_2$ does occur in C_4 plants under lower light, and the point at which this occurs may be species-dependent (Henderson et al. 1992, 1998; Kubasek et al. 2007). These results raised the question of whether by nature, the leaves may function in a C_3 or intermediate mode under some conditions, or whether they are programmed to develop C_4 chlorenchyma independent of environmental conditions.

Among the nine C_4 species in this study, the seasonal average $\delta^{13}C$ values ranged from −11.3 to −15.9‰ (Fig. 5). The results show that *B. cycloptera* is performing C_4 photosynthesis during the life cycle in this natural habitat, having an annual seasonal average $\delta^{13}C$ of −15.2‰. This is consistent with results from herbarium specimens of *Bienertia* collected from various habitats and several countries, which indicate function of C_4 photosynthesis, where $\delta^{13}C$ values of samples (leaves, stems, flowers, roots) for *B. cycloptera* were on average −13.9‰ ($n = 16$), and −13.8‰ ($n = 7$) for *B. sinsupersici* (Akhani et al. 2005).

In considering species-to-species variation in carbon isotope composition of biomass of C_4 plants, there are several factors which can cause differences in $\delta^{13}C$ values. C_4 plants have more positive $\delta^{13}C$ values than C_3 plants because the C_4 cycle concentrates CO_2 around Rubisco in a semi-closed compartment. Not all the CO_2 delivered is fixed by Rubisco; leakage of a percentage of the CO_2 generated from decarboxylation of C_4 acids (ϕ) results in more negative $\delta^{13}C$ values (with a given enzymatic capacity of the C_4 and C_3 cycles), as predicted according to theory by Farquhar (1983) and as shown experimentally by Henderson et al. (1992). Variations between species may be due to intrinsic differences (biochemistry and diffusive resistance to CO_2 leakage and environmental factors). From estimates of leakage from online measurements of discrimination with a number of C_4 species, an average of ~20% for ϕ values was obtained by Henderson et al. (1992). A study by Kubasek et al. (2007), using both online measurements and dry matter analyses of $\delta^{13}C$ values to estimate leakage in a number of C_4 species, showed a range in ϕ approximately from 20 to 40% (Kubasek et al. 2007). In addition to the effect of CO_2 leakage on $\delta^{13}C$, the relationship between the CO_2 concentration in the intercellular air space of the leaf and that in the atmosphere (Ci/Ca ratio), which is influenced by stomatal conductance, can also affect isotope discrimination in C_4 plants. With a ϕ value of 20%, decreasing the Ci/Ca ratio is predicted to lead to more negative $\delta^{13}C$ values (maximum of ~4‰ by decreasing Ci/Ca from 1 to 0). With leakiness values of ~40%, a change in Ci/Ca has no effect on discrimination. With higher ϕ values, where the C_4 system is very ineffective in concentrating CO_2 around Rubisco,

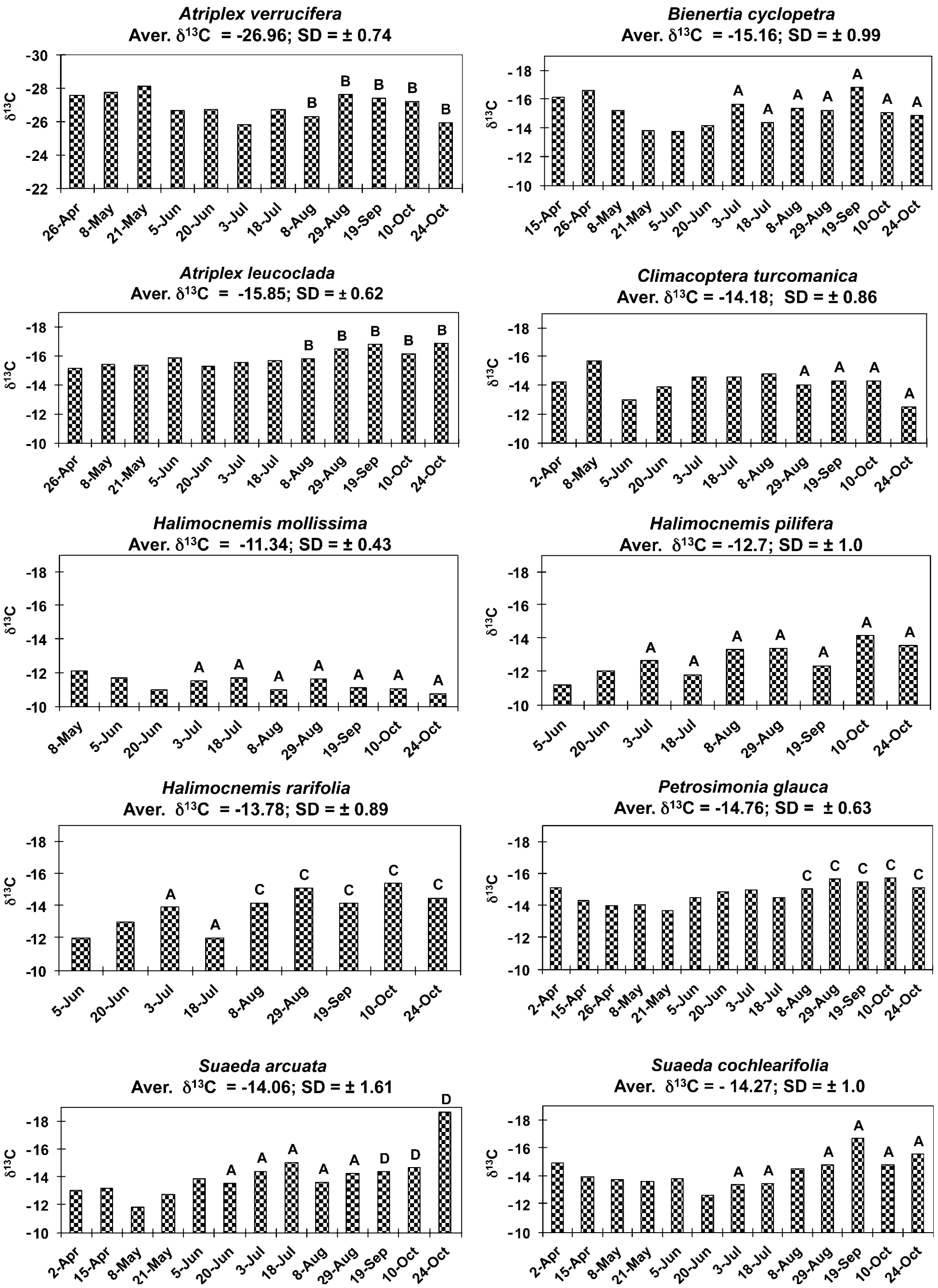

Fig. 5 Histogram showing seasonal variation of $\delta^{13}C$ values in *Bienertia cycloptera* and nine other Chenopodiaceae species studied. Samples were taken from leaves, floral leaves, bracts, and shoots. The seasonal average $\delta^{13}C$ values are given at the upper left side of each diagram. Values not indicated above each histogram represent normal leaves; other cases include: A—Floral leaves or bracts and bracteoles; B—upper leaves; C—Flowering or fruiting shoots; and D—Uppermost inflorescence branch (green flowers, very small floral leaves, and stem)

decreasing Ci/Ca values (e.g., due to increased diffusive resistance to CO_2 by drought) would have a reverse effect with less discrimination against $^{13}CO_2$ (more positive $\delta^{13}C$ values), as observed in C_3 plants (Farquhar 1983; Henderson et al. 1992). A study on *Sorghum* showed discrimination varies between different lines because of differences in the Ci/Ca ratio; lines having lower Ci/Ca ratios had increased discrimination (Henderson et al. 1998). In this case, differences in discrimination are not because of differences in the efficiency of C_4 photosynthesis (e.g., due to variation in ϕ).

$\delta^{13}C$ values in dry matter tend to be more negative (e.g., $\delta^{13}C$ values 1–2‰) than values obtained from online measurements of discrimination during photosynthesis. This occurs for non-photosynthetic reasons, which are not well defined, but they include fractionation occurring during respiration, and during biosynthesis (e.g., synthesis of lignin and lipid) (Henderson et al. 1992; Kubasek et al. 2007). Thus, differences in the discrimination following fixation of CO_2 could contribute to variation in the $\delta^{13}C$ values between species, and to seasonal changes in $\delta^{13}C$ within a species.

$\delta^{13}C$ values relative to features of C_4 species in the study

Cotyledons

In family Chenopodiaceae, there are two groups of C_4 plants, some having C_4 anatomy/photosynthesis in both leaves and cotyledons, others having C_4 photosynthesis/anatomy in leaves and C_3 photosynthesis/anatomy in cotyledons (Butnik 1974; Voznesenskaya et al. 1999; Pyankov et al. 2000b; Pyankov et al. 2001; Akhani and Ghasemkhani 2007). In this study, there is a relatively high frequency of species in tribe Caroxyloneae at the study site, in which the cotyledons have C_3 type and the leaves C_4 type anatomy (e.g., *Climacoptera turcomanica*, *Halimocnemis* spp. and *Petrosimonia glauca*). The temperate conditions which occur early in the life cycle in this habitat, would minimize photorespiratory losses of CO_2 in cotyledons performing C_3 photosynthesis. The first measurements of $\delta^{13}C$ on the various species were on cotyledons in the spring (see Table 3). In the three species analyzed in tribe Caroxyloneae, the $\delta^{13}C$ values of the cotyledons were more positive than typical values for C_3 plants. The values range from −20.4‰ in *H. mollissima*, to −22.7‰ in *P. glauca*. In plants which have C_4 photosynthesis in leaves, the carbon exported to sink tissue, including developing seeds, will have C_4 type isotope composition. Following germination, as seed reserves (carrying the C_4 type isotope signature) are utilized (normally photosynthate is transferred from cotyledons to leaves, not vice versa) and as C_3 type cotyledons of the above species are performing C_3 photosynthesis, the isotope values should shift toward C_3 type values. This could explain the "intermediate" isotope values of cotyledons in species in tribe Caroxyloneae. Also, in a study of several C_4 species in a saline biotope in Golestan National Park, NE Iran, C_3 type cotyledons had C_3-like carbon isotope values: *Haloxylon ammodendron* (−22.2‰), *Noaea mucronata* (−23.8‰), *Suaeda microphylla* (−23.7‰), and *Pyankovia brachiata* (−23.3‰) (Akhani and Ghasemkhani 2007).

Leaves, bracts, shoots

As indicated, the nine C_4 species in the study have average seasonal differences in $\delta^{13}C$ values ranging from −11.3 to −15.9‰ (a range of ~4.5‰, Fig. 5). All nine species are classified as NAD-ME type C_4 plants (Table 2, Fig. 4); so, variation in $\delta^{13}C$ is not considered to be because of differences in the type of C_4 biochemistry. All of the species grow in open flat habitats, and except for possible differences in soil salinity and water availability in the microhabitats, there are no other apparent abiotic differences which may affect $\delta^{13}C$ values.

There are five anatomical forms of C_4 represented among the nine C_4 species, which could influence diffusive resistance to CO_2 and the fraction of leakage (ϕ). Factors which could influence leakage include thickness and composition of bundle sheath cell walls in Kranz type species, distances for diffusion of CO_2 in the liquid phase for leakage relative to fixation by Rubisco, and, in NAD-ME type C_4 species, the position of mitochondria (the site of C_4 acid decarboxylation) relative to Rubisco-containing chloroplasts (von Caemmerer and Furbank 2003). However, the average seasonal range of $\delta^{13}C$ values of leaves among the C_4 species in the study does not correlate with differences in anatomical types. The leaves of *B. cycloptera*, with single-cell C_4 photosynthesis, had a seasonal average of −15.2‰, which is more positive than *A. leucoclada* with Atriplicoid type Kranz anatomy (−15.9‰) and very close to *P. glauca* with Salsoloid type Kranz anatomy (−14.8‰). *Climacoptera turcomanica* (Salsoloid type) had a seasonal average value of −14.2‰. *S. arcuata* (*Salsina* type) and *S. cochlearifolia* (*Schoberia* type) had values of −14.1 and −14.3‰, respectively.

The most positive average seasonal $\delta^{13}C$ values among the species investigated were observed in Salsoloid Kranz type species belonging to *Halimocnemis* s.l., with a seasonal average for three members of −12.6‰ (*H. mollissima* (−11.3‰), *H. pilifera* (−12.6‰), and *H. rarifolia* (−13.8‰)). This result is in accordance with previous studies of a larger number of species belonging to *Halanthium*, *Physandera*, and *Halarchon* showing an average $\delta^{13}C$ value of −12.1‰ for 11 species sampled (Akhani et al, 1997). All these species, which belong to *Halimocnemis* s.l., grow in xerohalophytic communities on very dry and mostly gypsum soils. Thus, the most important factor influencing $\delta^{13}C$ values in these species may be their degree of xerophytism compared with other species at the study site. Mechanistically, why these species have more positive $\delta^{13}C$ values is not clear; it could be due to lower ϕ or differences in capacity of the C_4 versus C_3 cycle.

Seasonal variation in carbon isotope composition

Besides differences between the nine C_4 species in seasonal average $\delta^{13}C$ values, within each species there is temporal variation, which resulted in individual values ranging approximately from −11 to −17‰. In the single-cell C_4 species *B. cycloptera*, there is relatively low seasonal variation in $\delta^{13}C$ values, which indicates performance of C_4 photosynthesis in leaves throughout the life cycle. The standard deviation over the season for *B. cycloptera* of ±0.99 is lower than for Kranz type *S. arcuata* and similar to those of *S. cochlearifolia*, *H. pilifera*, and *C. turcomanica*, (see Fig. 5). Our regular sampling from early spring to late autumn of *Bienertia*, eight other C_4, and one C_3 member of Chenopodiaceae, demonstrates that *Bienertia* shows temporal variation of $\delta^{13}C$ values similar to a number of C_4 species having Kranz anatomy (Table 3, Fig. 5).

The seasonal patterns showed that in most species $\delta^{13}C$ values of leaves tended to be most positive in late May and/or early June and more negative in early spring and late summer. During early growth (April–May) there is rainfall and temperatures are cooler, late May and early June temperatures are warmer, while in July and thereafter conditions are hot and dry. In young leaves of *B. cycloptera* sampled in April the average $\delta^{13}C$ was $-16.4‰$. These C_4-like values may suggest a more leaky C_4 system early in the growing season in young leaves (greater overcycling by the C_4 pathway), partial direct fixation of atmospheric CO_2 by Rubisco, and/or increased post-CO_2 fixation discrimination through biosynthesis. The more negative values which occur in a number of C_4 species late in the growing season (between August and October) (*B. cycloptera*, *A. leucoclada*, *H. pilifera*, *H. rarifolia*, *P. glauca*, *and S. cochlearifolia*), may be related to warm temperatures combined with low precipitation reported in 2003 in the area (Table 1). In September, the decrease in $\delta^{13}C$ in *B. cycloptera* ($-16.8‰$) is similar to that in *A. leucoclada* and *S. cochlearifolia* (Fig. 5). These climate conditions could result in decreased stomatal conductance and lower Ci/Ca ratios which, as noted earlier, can increase carbon isotope discrimination against $^{13}CO_2$. In *S. arcuata*, rather negative isotope values were obtained from bracts and fruiting branches in October ($\delta^{13}C$ of $-18.6‰$ which is $\sim 4‰$ more negative than values in samples collected earlier in the month). Since leaves were showing signs of senescence at this time, either increased ϕ values (CO_2 leakage) or increased respiratory losses could lead to more negative values.

In conclusion, *B. cycloptera* is a single-cell functioning C_4 species, which shows C_4 type carbon isotope values in nature with a seasonal pattern of values similar to those of other co-occurring C_4 species having Kranz type of anatomy. This, and values for both species of *Bienertia* from herbarium specimens from different sites, indicates that they perform C_4 photosynthesis in nature. There is no support for the hypothesis of Freitag and Stichler (2002) that this is a facultative C_4/C_3 species. During culture under certain conditions, the rate of development of mature C_4 type chlorenchyma cells may be slower, resulting in more negative carbon isotope values, particularly under limiting light. The study by Chuong et al. (2006) shows that the spatial separation of organelles in mature C_4 type chlorenchyma cells of *Bienertia* is supported by an extensive cytoskeleton network and vacuolar development, which may limit potential for reversions to C_3 type chlorenchyma in response to climatic conditions.

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References

Akhani H (2006) Biodiversity of halophytic and sabkha ecosystems in Iran. In: Khan A, Böer B, Kust GS, Barth H-J (eds) Sabkha ecosystems: vol II: West and Central Asia. Springer, New York, pp 71–88

Akhani H, Ghasemkhani M (2007) Diversity of photosynthetic organs in Chenopodiaceae from Golestan National Park (NE Iran) based on carbon isotope composition and anatomy of leaves and cotyledons. Nova Hedw Suppl 131:265–277

Akhani H, Trimborn P, Ziegler H (1997) Photosynthetic pathways in Chenopodiaceae from Africa, Asia and Europe with their ecological, phytogeographical and taxonomical importance. Plant Syst Evol 206:187–221. doi:10.1007/BF00987948

Akhani H, Ghobadnejhad M, Hashemi SMH (2003) Ecology, biogeography and pollen morphology of *Bienertia cycloptera* Bunge ex Boiss. (Chenopodiaceae), an enigmatic C_4 plant without Kranz anatomy. Plant Biol 5:167–178. doi:10.1055/s-2003-40724

Akhani H, Barroca J, Koteeva N, Voznesenskaya E, Franceschi V, Edwards GE, Ghaffari SM, Ziegler H (2005) *Bienertia sinuspersici* (Chenopodiaceae): a new species from Southwest Asia and discovery of a third terrestrial C_4 plant without Kranz anatomy. Syst Bot 30:290–301. doi:10.1600/0363644054223684

Akhani H, Edwards G, Roalson EH (2007) Diversification of the Old World Salsoleae s.l. (Chenopodiaceae): molecular phylogenetic analysis of nuclear and chloroplast data sets and a revised classification. Int J Plant Sci 168:931–956. doi:10.1086/518263

Bender MM, Rouhani I, Vines HM, Black CC Jr (1973) $^{13}C/^{12}C$ ratio changes in Crassulacean acid metabolism plants. Plant Physiol 52:427–430

Boyd CN, Franceschi VR, Chuong SDX, Akhani H, Kiirats O, Smith M, Edwards GE (2007) Flowers of *Bienertia cycloptera* and *Suaeda aralocaspica* (Chenopodiaceae) complete the life cycle performing single-cell C_4 photosynthesis. Funct Plant Biol 34:268–281. doi:10.1071/FP06283

Burnette WN (1981) “Western Blotting” Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195–203. doi:10.1016/0003-2697(81)90281-5

Butnik AA (1974) Structural types of cotyledons in chenopods (Chenopodiaceae). Morphobiological features of wild-growing plants of Uzbekistan. FAN, Tashkent, pp 43–49

Butnik AA, Nigmanova RN, Paisieva SA, Saidov DK (1991) Ecological anatomy of desert plants of Middle Asia. V.1. Trees, shrubs, semi-shrubs. FAN, Tashkent

Butnik AA, Ashurmetov OA, Nigmatova RN, Paizieva SA (2001) Subshrubs, subshrublets, vol 2. FAN, Tashkent (in Russian)

Carolin RC, Jacobs SWL, Vesk M (1975) Leaf structure in Chenopodiaceae. Bot Jahrb Syst Pflanzengesch Pflanzengeogr 95:226–255

Cerling TE (1999) Paleorecords of C_4 plants and ecosystems. In: Sage RF, Monson RK (eds) C_4 Plant Biology. Academic Press, New York, pp 445–469

Chuong SDX, Franceschi VR, Edwards GE (2006) The cytoskeleton maintains organelle partitioning required for single-cell C_4 photosynthesis in Chenopodiaceae species. Plant Cell 18:2207–2223. doi:10.1105/tpc.105.036186

Colombo SL, Andreo CS, Chollet R (1998) The interaction of shikimic acid and protein phosphorylation with PEP carboxylase from the C_4 dicot *Amaranthus viridis*. Phytochemistry 48:55–59. doi:10.1016/S0031-9422(97)01100-X

Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Biol 55:173–196. doi:10.1146/annurev.arplant.55.031903.141725

Farquhar GD (1983) On the nature of carbon isotope discrimination in C_4 species. Aust J Plant Physiol 10:205–226

Freitag H, Stichler W (2002) *Bienertia cycloptera* Bunge ex Boiss., Chenopodiaceae, another C_4 plant without Kranz tissues. Plant Biol 4:121–132. doi:10.1055/s-2002-20444

Ghannoum O, Evans JR, Chow WS, Andrews TJ, Conroy JP, von Caemmerer S (2005) Faster Rubisco is the key to superior nitrogen-use efficiency in NADP-malic enzyme relative to NAD-malic enzyme C_4 grasses. Plant Physiol 137:638–650. doi:10.1104/pp.104.054759

Henderson S, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. Aust J Plant Physiol 19:263–285

Henderson S, von Caemmerer S, Farquhar GD, Wade L, Hammer G (1998) Correlation between carbon isotope discrimination and transpiration efficiency in lines of the C_4 species *Sorghum bicolor* in the glasshouse and the field. Aust J Plant Physiol 25:111–123

Kadereit G, Borsch T, Weising K, Freitag H (2003) Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C_4 photosynthesis. Int J Plant Sci 164:959–986. doi:10.1086/378649

Kapralov MV, Akhani H, Voznesenskaya E, Edwards G, Franceschi VR, Roalson EH (2006) Phylogenetic relationships in the Salicornioideae/Suaedoideae/Salsoloideae sl (Chenopodiaceae) clade and a clarification of the phylogenetic position of *Bienertia* and *Alexandra* using multiple DNA sequence datasets. Syst Bot 31:571–585

Kubasek J, Setlik J, Dwyer S, Santrucek J (2007) Light and growth temperature alter carbon isotope discrimination and estimated bundle sheath leakiness in C-4 grasses and dicots. Photosynth Res 91:47–58. doi:10.1007/s11120-007-9136-6

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0

Long JJ, Wang J-L, Berry JO (1994) Cloning and analysis of the C_4 photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. J Biol Chem 269:2827–2833

Osmond C, Ziegler H, Stichler W, Trimborn P (1975) Carbon isotope discrimination in alpine succulent plants supposed to be capable of Crassulacean acid metabolism (CAM). Oecologia 28:323–328

Peisker M, Henderson SA (1992) Carbon: terrestrial C_4 plants. Plant Cell Environ 15:987–1004. doi:10.1111/j.1365-3040.1992.tb01651.x

Pyankov VI, Kuzmin AN, Demidov ED, Maslov AI (1992) Diversity of biochemical pathways of CO_2 fixation in plants of the families Poaceae and Chenopodiaceae from the arid zone of Central Asia. Sov Plant Physiol 39:411–420

Pyankov VI, Voznesenskaya EV, Kuz'min AN, Ku MSB, Ganko E, Franceschi VR, Black CC Jr, Edwards GE (2000a) Occurrence of C_3 and C_4 photosynthesis in cotyledons and leaves of *Salsola* species (Chenopodiaceae). Photosynth Res 63:69–84. doi:10.1023/A:1006377708156

Pyankov VI, Gunin PD, Tsoog S, Black CC (2000b) C_4 plants in the vegetation of Mongolia: their natural occurrence and geographical distribution in relation to climate. Oecologia 123:15–31. doi:10.1007/s004420050985

Pyankov VI, Ziegler H, Kuz'min A, Edwards GE (2001) Origin and evolution of C_4 photosynthesis in the tribe Salsoleae (Chenopodiaceae) based on anatomical and biochemical types in leaves and cotyledons. Plant Syst Evol 230:43–74. doi:10.1007/s006060170004

Sage RF (2004) The evolution of C_4 photosynthesis. New Phytol 161:341–370. doi:10.1111/j.1469-8137.2004.00974.x

Sage RF, Li M, Monson RK (1999) The taxonomic distribution of C_4 photosynthesis. In: Sage RF, Monson RK (eds) C_4 Plant Biology. Academic Press, New York, pp 551–584

Saigo M, Bologna F, Maurino VG, Detarsio E, Andreo CS, Drincovich MF (2004) Maize recombinant non-C_4 NADP-malic enzyme: a novel dimeric malic enzyme with high specific activity. Plant Mol Biol 55:97–107. doi:10.1007/s11103-004-0472-z

von Caemmerer S, Furbank RT (2003) The C_4 pathway: an efficient CO_2 pump. Photosynth Res 77:191–207. doi:10.1023/A:1025830019591

Voznesenskaya EV, Franceschi VR, Pyankov VI, Edwards GE (1999) Anatomy, chloroplast structure and compartmentation of enzymes relative to photosynthetic mechanisms in leaves and cotyledons of species in the tribe Salsoleae (Chenopodiaceae). J Exp Bot 50:1779–1795. doi:10.1093/jexbot/50.341.1779

Voznesenskaya EV, Artyusheva EG, Franceschi VR, Pyankov VI, Kiirats O, Ku MSB, Edwards GE (2001a) *Salsola arbusculiformis*, a C_3–C_4 intermediate in Salsoleae (Chenopodiaceae). Ann Bot (Lond) 88:337–348. doi:10.1006/anbo.2001.1457

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001b) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414:543–546. doi:10.1038/35107073

Voznesenskaya EV, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE (2002) Proof of C_4 photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). Plant J 31:649–662. doi:10.1046/j.1365-313X.2002.01385.x

Voznesenskaya EV, Edwards GE, Kiirats O, Artyusheva EG, Franceschi VR (2003) Development of biochemical specialization and organelle partitioning in the single celled C_4 system in leaves of *Borszczowia aralocaspica* (Chenopodiaceae). Am J Bot 90:1669–1680. doi:10.3732/ajb.90.12.1669

Voznesenskaya EV, Koteyeva NK, Chuong SDX, Edwards GE, Akhani H, Franceschi VR (2005) Differentiation of cellular and biochemical features of the single-cell C_4 syndrome during leaf development in *Bienertia cycloptera* (Chenopodiaceae). Am J Bot 92:1784–1795. doi:10.3732/ajb.92.11.1784

Voznesenskaya EV, Franceschi VR, Chuong SDX, Edwards GE (2006) Functional characterization of phosphoenolpyruvate carboxykinase-type C_4 leaf anatomy: Immuno-, cytochemical and ultrastructural analyses. Ann Bot (Lond) 98:77–91. doi:10.1093/aob/mcl096

Voznesenskaya EV, Chuong S, Koteyeva N, Franceschi VR, Freitag H, Edwards GE (2007) Structural, biochemical and physiological characterization of C_4 photosynthesis in species having two vastly different types of Kranz anatomy in genus *Suaeda*

(Chenopodiaceae). Plant Biol 9:745–757. doi:10.1055/s-2007-965579

Voznesenskaya EV, Akhani H, Koteyeva NK, Chuong SDX, Roalson EH, Kiirats O, Franceschi VR, Edwards GE (2008) Structural, biochemical and physiological characterization of photosynthesis in two C_4 subspecies of *Tecticornia indica* and the C_3 species *Tecticornia pergranulata* (Chenopodiaceae). J Exp Bot 59:1715–1734. doi:10.1093/jxb/ern028

Walker RP, Achenson RM, Tecsi LI, Leegood RC (1995) Phosphoenolpyruvate carboxykinase in C_4 plants: purification from cucumber and evidence of rapid protelolytic cleavage on extracts from a range of plant-tissues. Planta 196:58–63. doi:10.1007/BF00193217

Walter H, Lieth H (1967) Klimadiagramm-Weltatlas. Gustav Fischer, Jena