Features of Photosynthesis in *Haloxylon* species of Chenopodiaceae that are Dominant Plants in Central Asian Deserts

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Haloxylon aphyllum and *H. persicum* of Chenopodiaceae are dominant plants in the continental deserts of the Asian Irano-Turanian region. The photosynthetic organs, assimilating shoots and leaf-like cotyledons of these two species were studied to characterize their photosynthetic types. $^{13}C/^{12}C$ isotope ratios, the cellular anatomy of assimilating organs, primary photosynthetic products, and activities of carbon metabolism enzymes, RUBP carboxylase, PEP carboxylase, malic enzymes, and aspartate aminotransferase, indicate different pathways of CO_2 fixation in the photosynthetic organs. Assimilating shoots had attributes of the C_4 photosynthesis entirely, while cotyledons lack Kranz-anatomy and incorporated CO_2 via C_3 photosynthesis. Cotyledons and seeds had lower $\delta^{13}C$ values compared to shoots, consistent with the contribution of C_3-like CO_2 assimilation. Two pathways of carbon donation to the C_3 cycle via decarboxylation of C_4 acids in bundle sheath cells are suggested to occur in shoots of *Haloxylon*. The primary photosynthetic product malate can be utilized through $NADP^+$-malic enzyme which occurs in high activity. NAD^+-malic enzyme may contribute to C_4 photosynthesis (some aspartate is formed as an initial product, the bundle sheath chloroplasts have some grana, and NAD^+-malic enzyme is found in bundle sheath cells of shoots, all criteria for NAD^+-malic enzyme type photosynthesis). We propose that organ diversity of CO_2 fixation pathway in *Haloxylon* species is an important factor for their growth, survival and reproduction in continental climate deserts.

Key words: Carbon isotopes — C_3 photosynthesis — C_4 photosynthesis — Cotyledons — Deserts — *Haloxylon*.

Haloxylon aphyllum and *H. persicum* (having the

common names of black and white saxauls) are dominant vegetation components of the sandy and clay deserts across Central Asia. They are very broadly distributed in Middle Asia, the Middle East (Karakum and Kyzylkum deserts), Iran, and Afganistan. *H. persicum* also inhabits North-West China (Kashgar and Dzhungar regions) and Near East deserts (Iljin 1936, Botschantzev 1953). Both species grow up to latitude 50° north, and in south Russia in the Aktjubinsk region (Sokolov et al. 1977). Both species are strong vegetation dominants of hot sandy deserts; *H. aphyllum* also dominates in clay and saline arid regions (Netchaeva et al. 1973, Rotov 1969). The area covered by *H. aphyllum* and *H. persicum* amounts to a minimum of 1 million square km across the Turanian deserts (Iljin 1936). In the desert areas of Central Asia the wood from both species have an enormous importance as fuel. *H. aphyllum* is more salt- and cold-resistant than *H. persicum* (Netchaeva et al. 1973); it exhibits an earlier seed germination and vegetation growth in natural environments and is distributed more to the north (Sokolov et al. 1977). Both species have tree life forms or grow as large shrubs depending on their age and environment (Iljin 1936, Netchaeva et al. 1973).

Haloxylon species have an unusual photosynthetic apparatus. The true leaves are quite reduced and the cortex of young annual cylindrical shoots is the main photosynthetic tissue. Shoots of both *Haloxylon* species have a similar cross sectional anatomical structure, suggesting a C_4 type of photosynthesis. The shoots have a Salsoloid type of Kranz-anatomy (Voznesenskaya and Gamaley 1986), a C_4-like $^{13}C/^{12}C$ ratio (Winter 1981, Zalensky and Glagoleva 1981, Akhani et al. 1997), primary $^{14}CO_2$ fixation products being C_4 dicarboxylic acids with a preponderance of malate (Gedemov 1974, Glagoleva et al. 1978, Zalensky and Glagoleva 1981, Pyankov 1984, Pyankov and Vakhrusheva 1989), and a high activity of C_4 photosynthesis enzymes (Pyankov et al. 1992). The occurrence of C_4 photosynthesis in trees was reported previously only with some Euphorbiaceae species in a Hawaiian rainforest (Pearcy and Troughton 1975).

The main focus in previous studies of the unique natural roles of *Haloxylon* species in Central Asian deserts was based on plant morphology and photosynthetic fea-

tures of the shoots only. However, the initial stages of plant development after germination are very important for the survival, growth and reproduction of these species in extreme desert conditions. Seed germination occurs usually near the end of March-April when temperatures are low and soil frost is very frequent. Drought is also common. Hence we focused our current studies on the photosynthetic features of cotyledons. Both saxauls have comparatively long-lived cotyledons, photosynthesizing for about one month after germination to support early plant growth. *Haloxylon* seeds lack endosperm; therefore, cotyledon photosynthesis is a major source of assimilates for young plant development. Previous studies have shown that *Haloxylon* cotyledons have an isolateral mesophyll structure without Kranz anatomy (Butnik et al.1991). The possible occurrence of some features of C_3 photosynthesis in cotyledons and a C_4 mechanism in assimilating shoots in *Haloxylon* species is surprising. Usually within a plant, all photosynthetic tissues have the same photosynthetic pathway, although some deviations are known to exist in epidermal leaf cells and reproductive tissues (Willmer et al. 1973, Edwards and Walker 1983). In other C_4 dicot species, including *Amaranthus retroflexus* (Usuda et al. 1971), *Amaranthus hypochondriacus* (Wang et al. 1993, Long and Berry 1996, Berry et al. 1997) and some species of Chenopodiaceae in the genera *Atriplex* (Osmond et al. 1980), *Bassia, Kochia* and *Salsola* (Pyankov et al. 1997), cotyledons as well as leaves perform C_4 photosynthesis. The objectives of this study were to characterize the photosynthetic biochemistry in assimilating shoots and cotyledons of two *Haloxylon* species. The combination of anatomical studies with biochemical analyses using materials from the same plant provided unequivocal evidence for the presence of C_3 photosynthesis in cotyledons versus C_4 photosynthesis in shoots which is suggested to involve two C_4 acid decarboxylase pathways. We also addressed whether biochemical diversity of CO_2 fixation pathways in assimilating shoots and reproductive organs may be an important factor for plant survival in the harsh environment of Central Asian deserts.

Materials and Methods

Plant material—Seeds of *H. aphyllum* and *H. persicum* were collected during the Fall of 1996 from the Samarkand desert regions of Uzbekistan and stored at 4°C. The species names were according to Czerepanov (1995). Plants were grown in a greenhouse from February to July at Washington State University, Pullman, U.S.A. and at the field station of the Urals State University, near Ekaterinburg, Russia. Seeds were either incubated for 2 weeks on moist filter paper in Petri dishes at 4°C, which promotes germination in some cases, or germinated in Petri dishes in the greenhouse. After germination, seedlings were planted in soil. The temperatures in the greenhouse were maintained at 25/20°C day/night, with a maximum photosynthetic radiation of 1,500 μmol quanta $m^{-2} s^{-1}$ on sunny days (supplemented with

600 W sodium vapor lamps) and a photoperiod of 14/10 h. Cotyledons were fully expanded after 10 to 12 d of growth in soil; these were used for enzyme activity determination and fixed for anatomical studies. Photosynthetic shoots were taken from plants 2 to 4 months of age.

Light microscopy—For light microscopy, samples of shoots and cotyledons were fixed in a 2% paraformaldehyde~2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) overnight in a refrigerator, postfixed in 2% OsO_4 and then after a usual procedure of dehydration embedded in a mixture of Epon and Araldite. Cross sections were obtained using an ultramicrotome (Ultracut, Austria). The sections were stained with 1% Toluidine blue O on 1% $Na_2B_4O_7$.

Enzyme extraction and assay—Enzymes were extracted from illuminated cotyledons during the daytime. Usually about 0.1 g of tissue was ground in a prechilled mortar with 1 ml of grinding medium and sand. The grinding medium contained 50 mM Tris-HCl (pH 7.0), 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EDTA-Na_2, 5 mM DTT, 10% glycerol, 5% insoluble PVP and 1% soluble PVP (MW 40,000). The crude extract was immediately centrifuged at 15,000 × g for 10 min at 4°C. Usually the supernatant was desalted by centrifugation through a Sephadex G-25 column which was prequilibrated with the grinding medium without insoluble PVP. Extracts were immediately used for assaying enzymes. Enzymes were assayed at room temperature.

RUBP carboxylase (RUBPC) activity was assayed by the rate of ^{14}C-bicarbonate incorporation into acid-stable products. The reaction mixture (150 μl) contained 50 mM Tris-HCl at pH 8.0, 20 mM $MgCl_2$, 5 mM DTT, 20 mM $NaH^{14}CO_3$, 0.83 mM RUBP, and usually 50 μl of enzyme extract. After preincubation the reaction was initiated by the addition of RUBP. The reaction was carried out for 1 min and then stopped with 80 μl 20% trichloroacetic acid, and the acid-stable ^{14}C counts were determined.

PEP carboxylase (PEPC), NAD-ME (malic enzyme), NADP-ME, and AAT activities were measured spectrophotometrically. The assay medium for PEPC (1 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM DTT, 1 mM $NaHCO_3$, 0.5 mM glucose-6-phosphate, 0.2 mM NADH, 10 mM PEP, 3 units malate dehydrogenase (MDH) and 10 μl extract. The reaction was started with PEP.

The reaction mixture for assay of NAD-ME (1 ml) contained 25 mM HEPES-KOH (pH 7.2), 2.5 mM NAD, 25 μM NADH, 5 mM DTT, 0.2 mM EDTA-Na_2, 5 mM malate, 3 units MDH, 75 μM CoA or 25 μM acetyl CoA, 10 mM $MnCl_2$, and 20 μl extract (see Hatch et al. 1982). The reaction was started by addition of $MnCl_2$. The reaction mixture for assay of NADP-ME (1 ml) contained 25 mM Tricine-KOH (pH 8.3), 5 mM Na-malate, 0.5 mM NADP, 0.1 mM Na_2-EDTA, 20 mM $MgCl_2$ and 10 μl extract. The reaction was started with malate.

The assay medium for AAT (1 ml) contained 25 mM Tris-HCl (pH 8.0), 2.5 mM 2-oxoglutarate, 2.5 mM Na-aspartate, 5 μM pyridoxal-5-phosphate, 0.2 mM NADH, 2 mM Na_2-EDTA, 3 units MDH, and 10 μl extract. The reaction was started by adding aspartate. All rates were obtained within a range where enzyme activities were linear with time and extract concentration.

Immunoblotting—For western immunoblot analysis of PEPC and malic enzymes, control species were used which represent different photosynthetic subtypes: *Flaveria robusta* (C_3), *Zea mays* (C_4, NADP-ME subtype), *Amaranthus cruentus* (C_4, NAD-ME subtype) and *Urochloa panicoides* (C_4, PEP-CK subtype). Protein was extracted from cotyledons and shoots of *Haloxylon* plants using a phenol extraction procedure of van Etten et al.

(1979). Proteins were resolved by SDS-PAGE (Laemmli 1972) using a linear 7.5–15% acrylamide resolving gel and 5% acrylamide stacking gel. Each gel carried a prestained SDS-PAGE low molecular weight marker (Bio-Rad) and 17.5 μg protein/sample. After electrophoresis, proteins on the gel were electro-transferred to nitrocellulose membrane (Towbin et al. 1979) and probed with an appropriate antibody overnight. The three purified antibodies used were anti-maize 62 kDa NADP-ME IgG (1 : 200 dilution) (courtesy of C. Andreo, Maurino et al. 1996), anti-maize PEPC IgG (1 : 6,000 dilution) (courtesy of R. Chollet) and anti-*Amaranthus hypochondriacus* mitochondrial NAD-ME IgG (1 : 1,000 dilution) which was prepared against the 65 kDa α-subunit (courtesy of J. Berry, Long et al. 1994). Goat anti-rabbit IgG-AP conjugate (Bio-Rad) was used as the secondary antibody for detection of the enzymes. All blots were air dried and used for image analysis.

Primary ^{14}C-labeled products—Plants were grown under field conditions in the summer (mid June–mid July) at the Urals State University field station, about 50 km from Ekaterinburg, Russia. Young, 30 to 40 d old shoots and 10 to 15 d old cotyledons (about 0.5–1 g FW) were placed into a 1.5 dm^2 photosynthetic chamber (0.25 liter). The gas, containing ^{14}CO$_2$ (about 0.04%), was injected into the leaf chamber; the specific activity was about 5 mCi liter^{-1}. Plant samples were fixed after 10 s of ^{14}CO$_2$ assimilation by plunging into boiling alcohol. The temperature during experiments was near 25°C and the light intensity was 2,000 μmol quanta m^{-2} s^{-1}. Labeled products were extracted and separated using two dimensional paper chromatography where the first solvent contained butanol–formic acid–water (13 : 12 : 75), and the second solvent was 80% phenol. The positions of labeled compounds were identified using X-ray film. After elution from the chromatography paper the radioactivity for individual compounds was determined with a Geiger-Müller counter (Veb Robotron-Messelektronik 20046 model, Germany).

δ^{13}C carbon isotope determination—Carbon isotope fractionation values were determined on dried shoots and cotyledons from plants grown in the greenhouse and under field conditions, using standard procedure relative to PDB (Pee Dee Belemnite) limestone carbon isotope standard (Bender et al. 1973).

Results

Photosynthetic structure of assimilating organs—*Haloxylon* species have cylindrical assimilating shoots. Both species have a layer of subepidermal cells and two layers of chlorenchyma on the stem periphery, an outer layer of palisade cells and an inner layer of bundle sheath cells as shown for *H. persicum* in Fig. 1. The central portion of the shoot is occupied by water-storage tissue with the main vascular bundles located in the center. Central bundles are thus separated from Kranz-type cells by layers of water storage cells. There are some small peripheral bundles that have contact with bundle sheath cells. All three tissues, mesophyll, bundle sheath and water storage, contain chloroplasts, but the concentration in water-storage tissue is very low in comparison with either mesophyll or bundle sheath cells. This specialized anatomy, termed Salsoloid (Carolin et al. 1975), is typical for succulent C$_4$ species of *Salsola* and closely related species. Shoots of *H. aphyllum* have the same Kranz anatomy-

type structure as *H. persicum*, except that its epidermal cell structure and its water-storage tissue cells are slightly larger (not shown).

The cotyledons of both species have an oval cross section and an isolateral mesophyll structure. There are 1 or 2 layers of palisade-like cells on abaxial and adaxial sides and 2 to 3 layers of spongy parenchyma around vascular bundles; but there is no Kranz-type cell arrangement. Cells of both palisade and spongy tissues contain chloroplasts (Fig. 1).

δ^{13}C/^{12}C values—Measurements of ^{13}C/^{12}C carbon isotope fractionation show differences in isotopic composition between shoots and cotyledons for the two saxaul species (Table 1). The discrimination values (δ^{13}C) in assimilating shoots ranged from −13.1 to −14.8‰, while in cotyledons the values are more negative ranging from
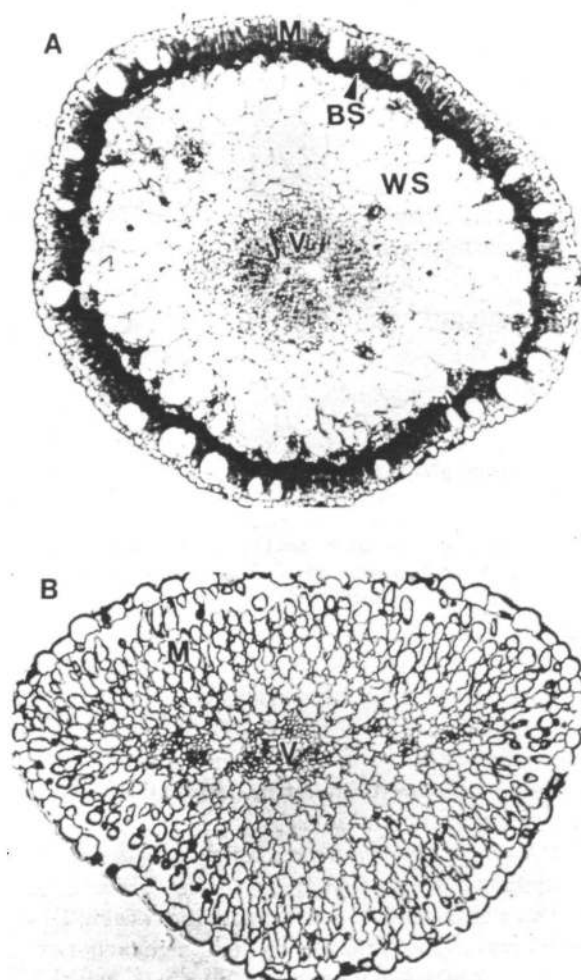

Fig. 1 Transverse sections of assimilating shoots and cotyledons of *Haloxylon persicum*. (A) Aphyllous shoot (×59.5). (B) Cotyledon with abaxial side of the organ on the top (×93.5). M, mesophyll cells; BS, bundle sheath cells; WS, water storage tissue; V, vascular tissue.

Table 1 Structural and biochemical characteristics of the photosynthetic apparatus in assimilating shoots and cotyledons of two *Haloxylon* species

Characteristics	Species	Cotyledons	Shoots
$^{13}C/^{12}C$ isotope discrimination (‰) [a, b]	*H. aphyllum*	-17.5, -19.3^*	-14.0, -14.8, -14.3
	H. persicum	-17.5, -17.8^*	-14.3, -13.1^*
Mesophyll structure [c]	*H. aphyllum*	non-Kranz, IP	Kranz-type, SALS
	H. persicum	non-Kranz, IP	Kranz-type, SALS
Enzyme activity μmol (mg Chl)$^{-1}$ h^{-1}			
RUBP carboxylase	*H. aphyllum*	177.0	165.0
	H. persicum	206.0	122.0
PEP carboxylase	*H. aphyllum*	47.5	1396.5
	H. persicum	41.9	1867.8
NADP-ME	*H. aphyllum*	14.0	253.8
	H. persicum	30.0	219.3
NAD-ME	*H. aphyllum*	5.9	16.5
	H. persicum	9.1	15.9
AAT	*H. aphyllum*	126.8	337.1
	H. persicum	138.0	509.0
Immunodetection			
PEP carboxylase	*H. aphyllum*	not detected	present
	H. persicum	not detected	present
NADP-ME	*H. aphyllum*	not detected	present
	H. persicum	not detected	present
NAD-ME	*H. aphyllum*	present	present
	H. persicum	present	present
Biochemical subtype	*H. aphyllum*	C_3	NADP-ME/NAD-ME
	H. persicum	C_3	NADP-ME/NAD-ME

[a] Plants grown in greenhouse or field conditions near Ekaterinburg (marked by asterisks).
[b] $\delta^{13}C$ values for seeds plus husks of *H. aphyllum*, -16.1; and *H. persicum*, -15.7.
[c] SALS, Salsoloid; IP, isopalisade.

-17.5 to -19.28‰. The values for the seeds harvested from natural environmental conditions in the Kyzylkum desert were about -16.1‰ for *H. aphyllum* and -15.7‰ for *H. persicum*. Carbon isotope discrimination values of assimilating organs were similar in both plants, and independent of growing conditions.

Activity of photosynthetic enzymes—The photosynthetic organs of these *Haloxylon* species differ in their patterns of photosynthetic enzyme activities (Table 1). The young shoots from both species had high activities of PEPC, ca. 1,400–1,900 μmol (mg Chl)$^{-1}$ h^{-1}, which was 10–15 times RUBPC activity. High activities of NADP-ME, ca. 220–250 μmol (mg Chl)$^{-1}$ h^{-1}, and of AAT, ca. 340–510 μmol (mg Chl)$^{-1}$ h^{-1}, occurred in shoots also. We detected high NADP-ME activity in shoots, and, in addition, low activity of NAD-ME (ca. 16 μmol (mg Chl)$^{-1}$

h^{-1}). In contrast, cotyledons from both species had low activity of PEPC (about 40–50 μmol (mg Chl)$^{-1}$ h^{-1}), which was 4 to 5 times lower than RUBPC activity. The level of RUBPC in cotyledons was slightly higher than in assimilating shoots. The activities of NAD-ME and AAT were about 2 to 3 times lower in cotyledons than in the shoots. NADP-ME was also detected in cotyledons, but its level was about 10 times less than those in shoots.

Immunoblot analysis—The western blots showed that PEPC, NAD-ME and NADP-ME are present at different levels in the protein extracts of shoots and cotyledons from both species (Fig. 2, summarized in Table 1). Using the maize PEPC antibody, high levels of an immunoreactive form of 100 kDa was detected in extracts of shoots, but not in cotyledons, which have low PEPC activities. The C_4 control plants, maize, *U. panicoides*, and *A. cruentus*, all
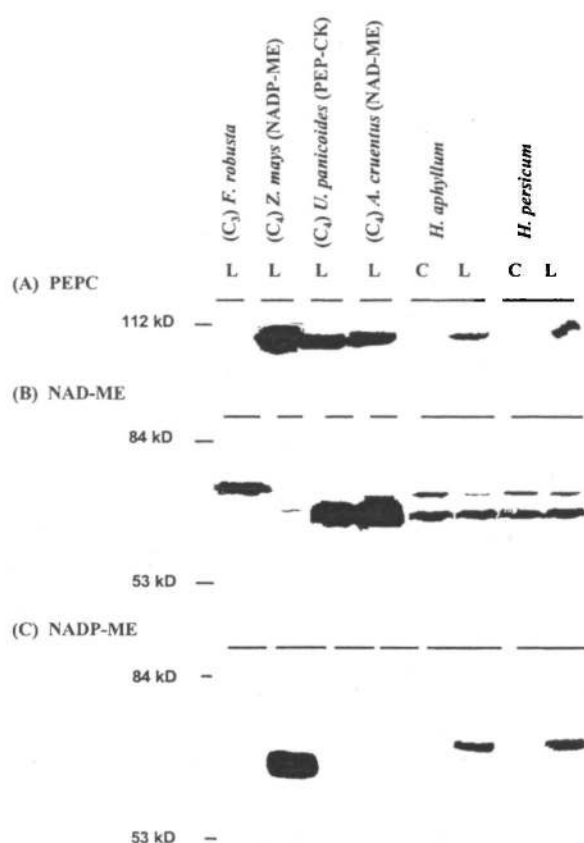
Fig. 2 Immunoblots of PEP carboxylase (A), NAD-ME (B), and NADP-ME (C) for total protein extracted from assimilating shoots and cotyledons of *H. aphyllum* and *H. persicum*. Control species were *Flaveria robusta* (C₃), *Zea mays* (C₄, NADP-ME subtype), *Urochloa panicoides* (C₄, PEP-CK subtype), and *Amaranthus cruentus* (C₄, NAD-ME subtype). C, cotyledon; L, assimilating shoots.

extremely low immuno-reaction.

Using an antibody raised against the a-subunit of NAD-ME from *Amaranthus hypochondriacus*, two immunoreactive forms of NAD-ME (70 and 65 kDa) were found in shoots and cotyledons of the two *Haloxylon* species. The smaller molecular mass form gave the largest immuno-reaction which has the same mass as the form occurring in the control C_4 species which utilize NAD-ME in C_4 photosynthesis (the NAD-ME C_4 species *A. cruentus* and the PEP-CK type C_4 plant *U. panicoides*). On the other hand, maize, a NADP-ME type species, only has traces of a reactive band with an apparent molecular mass of 67 kDa. With the C_3 species *F. robusta*, a "larger" form of NAD-ME (73 kDa) was detected.

NADP-ME was detected only in shoots of *Haloxylon* by immunoblotting (Fig. 2c). As expected, the NADP-ME subtype C_4 plant maize contains a large amount of NADP-ME in leaves while the C_3 *F. robusta* and the other two C_4 subtypes do not. The molecular mass of this enzyme in *Haloxylon* species was larger (72 kDa) in comparison to NADP-ME from *Zea mays* (62 kDa) (Fig. 2c).

Primary photosynthetic products—After 10-s of $^{14}CO_2$ fixation, assimilating shoots incorporated about 80% of the ^{14}C into dicarboxylic acids (Table 2). Malate was the main ^{14}C-labeled product in shoots with both *Haloxylon* species which accounted for a little over 50% of the total assimilated $^{14}CO_2$ while about 28% of the CO_2 was incorporated into aspartate. About 3–5% of the radioactivity was detected in alanine, and 12–14% was incorporated into C_3 products. On the other hand, cotyledons from both species incorporated about 70% of their assimilated $^{14}CO_2$ into C_3 products, PGA and sugar-phosphates; and only about 10% was found in C_4 products, malate and aspartate, after a 10-s $^{14}CO_2$ exposure (Table 2).

Discussion

The nature of Haloxylon—*H. persicum* and *H. aphyl-*

exhibited substantial immuno-reaction for the PEPC polypeptide at a similar molecular mass as the *Haloxylon* shoots while in the C_3 control plant, *F. robusta*, there was

Table 2 Percentage of incorporation of ^{14}C into photosynthetic products after 10-s assimilation of $^{14}CO_2$ by young shoots and cotyledons of *H. aphyllum* and *H. persicum*

Compounds	*H. aphyllum*		*H. persicum*	
	Cotyledons	Shoots	Cotyledons	Shoots
Sugar-P esters	13.6	5.6	22.7	9.7
PGA	61.6	6.7	46.3	3.8
Malate	6.0	50.5	4.0	54.0
Aspartate	4.9	29.0	5.9	27.2
Alanine	1.6	3.6	4.8	0.9
Sucrose	3.0	0.5	2.4	0.9
Serine + glycine	4.4	0.3	4.9	0.8
Not identified	4.9	3.8	9.0	2.7

Labeling conditions were temperature of 25°C and light intensity of 2,000 μmol quanta m^{-2} s^{-1}.

lum are very important desert plant species that have some specific ecological, morphological, biochemical and physiological features. *H. aphyllum* is a tree that can grow 8–9 m high and can live up to 100 years (Netchaeva et al. 1973) often forming forest-type woodlands in some desert environments. *H. persicum* has been described as a tree (Iljin 1936, Butnik et al. 1991), or large shrub (Netchaeva et al. 1973), or intermediate form that is up to 5 m high and lives 25 to 30 years (Nikitin 1966). *H. aphyllum* is more cold- and salt-resistant than *H. persicum* (Netchaeva et al. 1973). In addition, *H. aphyllum* is a very polymorphic species (Botschantzev 1944). A striking environmental trait of *Haloxylon* species is their presence in areas with large daily and annual temperature fluctuations. In the Karakum desert near the Ashkhabad region (37°N latitude, 58°E longitude), frosts with temperatures as low as ca. −4°C in April have been reported, whereas air temperatures are commonly about 50°C in July (Müller 1982). The minimum air temperatures near the north distribution limit of saxauls (Turgai, 49°NL, 63°EL) are −6°C in April and September (Müller 1982), i.e. during periods of active vegetative growth low freezing temperatures are common. *Haloxylon* species from the Karakum desert are reported to show net photosynthesis at air temperatures between −5 and 50°C and to exhibit a broad temperature optimum between 20 to 40°C (Zalensky 1975, Voznesenskii 1977). These physiological and ecological features of *Haloxylon* suggest that they have characteristics which allow them to function photosynthetically under extreme conditions.

Organ specific differences in photosynthetic carbon assimilation in Haloxylon—Our findings here show that different organs of *Haloxylon* species vary in their main pathway of photosynthetic CO_2 fixation. The carbon isotope fractionation values in shoots of *H. aphyllum* and *H. persicum* obtained in this study (−12 to −14‰), do not differ from other reports with *Haloxylon* (Shomer-Ilan et al. 1981, Winter 1981, Akhani et al. 1997), and are within the range of typical values for C_4 plants of −7 to −15‰ (Bender 1971, Smith and Epstein 1971, Ehleringer 1988). However, cotyledons of *Haloxylon* have $\delta^{13}C$ values ranging from −17.5 to −19.3‰, which are intermediate between the values of C_3 (−20 to −35‰) and C_4 plants (Ehleringer 1988). Analyses of mature true leaves and assimilating stems of more than 400 species of Chenopodiaceae from Middle Asia, Middle East, North Africa and Europe (Shomer-Ilan et al. 1981, Winter 1981, Zalensky and Glagoleva 1981, Akhani et al. 1997, Pyankov et al. 1997) showed a range of −9 to −15‰ for C_4 species and −20 to −32‰ for C_3 plants. No published data on carbon isotope discrimination in cotyledons are available. The absence of Kranz-anatomy, a low activity of enzymes of the C_4 dicarboxylic pathway, and initial product analysis in cotyledons of *Haloxylon* give strong evidence for CO_2 fixation in saxaul cotyledons via the C_3 cycle. The non

typical $\delta^{13}C$ values in cotyledons can be accounted for by a mixing of "old" C_4 products from the parent plant which were stored in the cotyledons during seed formation and "fresh" metabolites formed during C_3 photosynthesis by cotyledons following germination. Embryo formation in *Haloxylon* species begins about 45 d after flowering (near June in the field). Cotyledons and the embryonic radical differentiate in mid September, and seeds develop fully by the end of October (Konycheva 1983). This temporal sequence means that embryos and cotyledons are formed mostly from C_4 assimilates produced by shoots with a smaller contribution from C_3 photosynthesis. Seeds collected in natural Kyzylkum desert environments have a $\delta^{13}C$ value of −16.1‰ for *H. aphyllum* and −15.7‰ for *H. persicum* (Table 1). Those seeds were used in our laboratory experiments. It is clear that embryonic seeds (including cotyledons) were fed mostly assimilates from C_4 photosynthesis, but following germination they begin to assimilate atmospheric CO_2 directly by RUBPC. Thus, as cotyledons mature they contain a mixture of assimilates which originated from C_4 and C_3 photosynthesis. After seed germination, in 10–12 d the cotyledons are fully developed and about 2 cm long. These cotyledons exhibit all structural and biochemical features of C_3 photosynthesis. Thus, the $\delta^{13}C$ values in cotyledons, being more negative and closer to that of C_3 result from a mixture of metabolites from different assimilatory sources.

Malic enzymes in Haloxylon—The much higher activity of NADP-ME in shoots than cotyledons, and its prominent immuno-reaction with the antibody to maize C_4 isoform of NADP-ME indicate its association with C_4 photosynthesis in *Haloxylon*. The enzyme is a homotetramer and the C_4 isoform is located in bundle sheath chloroplasts of maize (Maurino et al. 1997); the single band of immuno-reactivity in *Haloxylon* indicates there is a major C_4 isoform.

NAD-ME activity was about 2 to 3 fold higher in shoots than cotyledons (Table 1). With respect to western blots, we detected two polypeptides using the antibodies against the *a*-subunit of the C_4 NAD-ME of *Amaranthus hypochondriacus* (Fig. 2). Studies with this species show that NAD-ME, which is composed of two 65 kDa *a*-subunits and two 60 kDa *β*-subunits, is located specifically in the mitochondria of bundle sheath cells (Long et al. 1994). The *a*-subunit is responsible for catalytic acitivity, and the antibody against this subunit does not react with the *β*-subunit which appears to be a completely different protein (see Long et al. 1994). Two immuno-reactive forms of NAD-ME were found in shoots and cotyledons with apparent molecular masses of 65 and 70 kDa (Fig. 2). The 65 kDa form, which has a similar molecular mass to that in *A. cruentus*, is more dominant than the 70 kDa form. If the antibody only cross reacts with the *a*-subunit of NAD-ME (Long et al. 1994), the results suggest that two differ-

ent *a*-subunits, and thus two different native isoforms of NAD-ME occur in *Haloxylon* species. Immunolocalization studies in shoots of *Haloxylon* indicate that NAD-ME is specifically located in bundle sheath cells (E. Voznesenskaya, V. Franceschi and G. Edwards, unpublished), suggesting it is involved in C_4 photosynthesis. The presence of both forms in cotyledons which have C_3 photosynthesis indicates these may have been pre-existing constitutive forms in tissue having C_3 photosynthesis.

Carbon isotope fractionation values of seeds—The phenomenon of a more negative $\delta^{13}C$ value in seeds in comparison to shoots of *H. aphyllum* ($-16.1‰$ versus -14.0 to $-14.8‰$) and *H. persicum* ($-15.7‰$ versus -13.1 to $-14.3‰$) (see Results section) is interesting. This could occur either by a slightly more negative isotope value of photosynthetic products during seed formation (i.e. by some direct fixation of atmospheric CO_2 by Rubisco), or by some further discrimination against ^{13}C during conversion of carbohydrates to biomass in seeds. *H. aphyllum* shoots demonstrate a positive Warburg effect with photosynthesis being inhibited 16% by normal O_2 versus 2% O_2 during cool temperature and low light intensity, for example during sandy storms (Ledyaikina and Voznesenskii 1982). Furthermore, decreasing temperature from 35 to 5°C induces some change in the primary pathway of carbon assimilation, with an increased incorporation of $^{14}CO_2$ into PGA and sugar-esters (Pyankov 1984). Low temperature may impair function of the C_4 cycle through cold lability of enzymes and limitations on chloroplast metabolite transport (Hatch 1979, Sugiyama et al. 1979, Edwards and Huber 1981, Potvin and Simon 1990, Kingston-Smith et al. 1997). The saxaul species have a long vegetation period from the end of March-April to October when temperatures below 0°C with frosts can be encountered (Voznesenskii 1977, Müller 1982).

C_3 photosynthesis in cotyledons of Haloxylon—The occurrence of C_3 photosynthesis in cotyledons of a C_4 species is an unusual phenomenon considering other reports in the literature. C_3 photosynthesis in *Haloxylon* cotyledons may have important ecological and evolutionary implications. After germination the cotyledons of *H. aphyllum* and *H. persicum* are fully developed in the field desert conditions in 10–12 d, and live for an additional month (Vernik 1983, Butnik et al. 1991). Since seeds of saxaul species have no endosperm (Vernik 1983), cotyledon photosynthesis is the sole source of new assimilates for plant development. Cotyledons in natural Kyzylkum desert conditions appear from the end of March (*H. aphyllum*) to the end of April (*H. persicum*) (Butnik et al.1991) when temperatures are low and soil moisture remains high after winter and spring precipitation, conditions favoring C_3 over C_4 photosynthesis (Black 1973, Ehleringer 1978). Thus, the C_3 pathway of CO_2 fixation in *Haloxylon* cotyledons can be of ecological importance for

plant survival during early development in cool climatic conditions. This mechanism of photosynthesis may be particularly important for *Haloxylon* to survive near the northern border of its distribution, at about 50°N (Sokolov et al. 1977). At the same time cotyledons also have some features (e.g. isopalisade mesophyll structure and rapid formation of a primary vascular system), which are characteristic of adaptations to arid conditions (Butnik 1983).

From an evolutionary view point, *Haloxylon* is a comparatively young genus that originated from *Salsola*. According to Iljin (1937) and Botschantzev (1969) the lifting of a large Asian mountain system, Tien-Shan and Pamirs, occurred at the end of the Tertiary. Large clay and sandy landscapes formed during the Pliocene. Some shrub species originated during this time which do well in sandy conditions and all shrub genera of the Salsoleae tribe, including *Haloxylon*, are derivatives of the section Arbuscula (Botschantzev 1969), which were subsequently included in section Coccosalsola (Botschantzev 1976), and their origin is dated to Pliocene. Thus, this C_3 type of cotyledon may also occur amongst *Salsola* ancestors of *Haloxylon* species.

C_4 photosynthesis in shoots of Haloxylon—The high resistance of *Haloxylon* species to hot summer, drought conditions may be attributed in part to C_4 photosynthesis in assimilating shoots as the main photosynthetic organs. Assimilating shoots of *H. aphyllum* and *H. persicum* have a typical Salsoloid type of Kranz anatomy and ultrastructural dimorphism of chloroplasts in mesophyll (granal) and bundle sheath (slightly agranal) cells (Voznesenskaya 1976, Voznesenskaya and Gamaley 1986). The photosynthesizing cortex of cylindrical shoots has the same origin as reduced leaves, being formed from the peripheral meristem of the shoot apex (Vasilevskaya 1955, Fahn and Arzee 1959).

It is clear that *Haloxylon* shoots perform C_4 and evidence exists for a NADP-ME type C_4 shuttle. Malate is a predominant product, there is substantial activity of NADP-ME, and the bundle sheath chloroplasts have less grana than mesophyll chloroplasts, all characteristics of NADP-ME subtype. However, there are also reasons to suggest that *Haloxylon* species operate in addition a NAD-ME type shuttle, the extent of which remains to be investigated. Aspartate is a significant initial photosynthetic product (about 28%, Table 2); the bundle sheath chloroplasts have significant grana development though less than mesophyll chloroplasts (Voznesenskaya and Gamaley 1986); and NAD-ME is found in shoots of *Haloxylon* of the same molecular mass as occurs in C_4 species utilizing NAD-ME in a C_4 shuttle. Consistently, previous studies of primary photosynthetic products of *Haloxylon* species in natural desert environments gave similar results; 18–28% of the initially fixed C was found in

aspartic acid (Pyankov 1984, Pyankov and Vakhrusheva 1989, Gamaley et al. 1992). Analysis of the ultrastructure of mesophyll and bundle sheath chloroplasts of the greenhouse grown plants in the present study showed grana in both chloroplast types. The granal index (the ratio of lengths of appressed thylakoid membranes to length of all thylakoid membranes) in *H. aphyllum* shoots was 0.62 in mesophyll chloroplasts versus 0.48 in bundle sheath chloroplasts; and in *H. persicum* 0.52 for mesophyll chloroplasts versus 0.31 for bundle sheath chloroplasts; similar result were obtained with plants grown in nature (E. Voznesenskaya, unpublished data). In comparison, bundle sheath chloroplasts of some NADP-ME monocots are agranal (grasses of Andropogoneae tribe, especially some *Sorghum* species). In NADP-ME type of C_4 photosynthesis malate contributes both CO_2 and reductive power to agranal bundle sheath chloroplasts which are deficient in production of NADPH. Granal bundle sheath chloroplasts, as occurs in NAD-ME species, allows for the production of NADPH and utilization of aspartate which donates CO_2 but not reductive power (Edwards and Walker 1983). Thus, variation of the degree of grana stacking may allow flexibility in relative formation of aspartate versus malate which may have some benefit in acclimation to rapid variations in climate or to different edaphic conditions.

The activity of NAD-ME in shoots of *Haloxylon* is 2 to 3 fold higher than in cotyledons. Although the activity in shoots was low compared to that of NADP-ME, immunoblots indicate the presence of significant amounts of NAD-ME protein in *Haloxylon*. This suggests there is some loss of activity during extraction or assay under suboptimum conditions. In addition, using immulocalization methods both NADP-ME and NAD-ME were found to be selectively located in bundle sheath cells of shoots of *Haloxylon* species suggesting both malic enzymes may be functioning in C_4 photosynthesis (Voznesenskaya et al., unpublished).

Further studies will be required to determine the relative capacity for C_4 acid cycles through the two malic enzymes in *Haloxylon*. Analyses of the kinetics of labeling of initial photosynthetic products do not distinguish between metabolism through the two decarboxylases. In NAD-ME type species aspartate formed in mesophyll cells is further metabolized in bundle sheath mitochondria through AAT, NAD-malate dehydrogenase and NAD-ME. In NADP-ME species like maize, malate is metabolized by bundle sheath chloroplasts, but not aspartate. However, in one case, in a study with a C_4 *Flaveria* species, in addition to malate, aspartate can serve as a carbon donor to bundle sheath cells via metabolism through AAT, NADP-malate dehydrogenase and NADP-ME (Meister et al. 1996). Further study of enzyme compartmentation and aspartate utilization using isolated bundle sheath prepara-

tions of *Haloxylon* will be required for a more quantitative assessment of the capacity for carbon flow through the respective decarboxylases.

NAD-ME subtype C_4 plants in Chenopodiaceae are known to be more resistant to stress than species of the NADP-ME subtypes (Pyankov and Vakhrusheva 1989, Gamaley et al. 1992). The NAD-ME subtype plants dominate the halophytic desert vegetation of Middle Asia and Middle East (Shomer-Ilan et al. 1981, Vogel et al. 1986, Pyankov and Vakhrusheva 1989, Gamaley et al. 1992, Pyankov et al. 1992, 1997). The ability to operate two pathways for decarboxylation of C_4 acids may allow these *Haloxylon* species to occupy a broader range of ecological and edaphic conditions which may be most important in *H. aphyllum*. In natural desert conditions we found a lower activity of NADP-ME in *Haloxylon* species during hot and arid summer conditions (Pyankov et al. 1992), relative to that of greenhouse grown plants.

Our results provide evidence for a unique diversity and combination of photosynthetic metabolic pathways in *Haloxylon* species. This is a rare example of combining C_3 and C_4 pathways in the same plant. We propose that developmental changes in CO_2 fixation from C_3 in cotyledons to C_4 in the main green assimilating shoots, and metabolic shifts between NAD-ME and NADP-ME carbon flow in C_4 photosynthesis may be important factors in the adaptation of these plants to extremes of temperature and water supply under desert environments of Central Asia. The phenomenon of true C_3 and C_4 pathways functioning in one plant makes it possible to suggest a new type of plant which combines C_3 and C_4 photosynthesis in which the two photosynthetic modes are separated temporally and developmentally in different organs, in comparison to C_3-C_4 intermediate plants like *Flaveria* in which the two photosynthetic modes operate simultaneously in the same leaf (Edwards and Ku 1987, Brown and Bouton 1993). It also seems likely that other derivative genera of Salsoleae, such as *Aellenia, Hammada, Iljinia, Seidlitzia,* or *Sevada* (Botschantzev 1969), may have the same photosynthetic dimorphism in cotyledons versus their main assimilating organs, shoots or leaves. The temporal and spatial separation of C_3 and C_4 photosynthesis in *Haloxylon* species also make them an ideal natural experimental system for studying organ-specific regulation of different photosynthetic CO_2 fixation pathways in plants and the regulatory expression of C_4 photosynthesis genes (Ku et al. 1996).

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