

ECOLOGICAL ANATOMY IN HALOPHYTES WITH C₄ PHOTOSYNTHESIS: DISCUSSING ADAPTATIVE FEATURES IN ENDANGERED ECOSYSTEMS

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Abstract. The *Chenopodiaceae* halophyte species provide perhaps the ideal model to study the ecological adaptations in relations with extreme environmental conditions. Closely linked with saline habitats, the chenopods with Kranz anatomy represents a striking and intriguing example of coevolution. In this study, we investigate the Kranz anatomy in a holistic manner in halophytes vegetating in two nature reserves, here regarded as rare and endangered ecosystems. This issue, apart from its scientific interest – as an adaptive, ecological and evolutive feature – also suggests the compulsory necessity to protect these areas, in order to preserve the floristic diversity in such menaced ecosystems.

Keywords: halophytes, Kranz Anatomy, C₄ photosynthesis, strategy, evolution, adaptation.

1. INTRODUCTION

The Earth's surface area occupies about 13.2 billion ha, but no more than 7 billion ha are arable and 1.5 billion are cultivated (Massoud, 1981). Of the cultivated lands, about 340 million ha (23%) are saline (salt-affected) and another 560 million ha (37%) are sodic (sodium-affected) (Tanji, 2002). Here are many different projections, suggesting that human population will increase over 8 billion by the year 2020 that will worsen the current scenario about food insecurity (Athar & Ashraf, 2009). There are often not sufficient reservoirs of freshwater available and most of the agronomically used irrigation systems are leading to a permanent increase in the soil-salinity and slowly to growth conditions unacceptable for most of the common crops (Koyro et al., 2009).

Salt stress, together with water stress, became, in above described circumstances, one of the most interesting studied issue over the last years (Cheeseman, 1988; Shannon, 1992; Bohnert et al., 1995; Neumann, 1997; O'Leary, 2002; Sen et al. 2002; Yokoy et al., 2002).

Halophytes are plants able to vegetate and

reproduce in saline environments; despite the progresses recorded in the last decades, it is still very difficult to use a single-conventional definition of halophytes (Grigore, 2008a; 2008b; Grigore & Toma, 2010a; Grigore & Toma, 2010b).

C₄ photosynthesis is a series of biochemical and anatomical modifications that concentrate CO₂ around the carboxylating enzyme Rubisco (Sage, 2004). This photosynthetic type is not a single metabolic pathway; it is a series of biochemical and structural adjustments that have exploited phosphoenolpyruvate carboxylase (PEPCase) and other existing enzymes to concentrate CO₂ around Rubisco.

In the great majority of C₄ plants, functioning of the C₄ pathway requires metabolic cooperation of two closed and distinct chlorenchymatous tissues: an external one (or photosynthetic carbon assimilative - PCA) and an inner bundle sheath (or photosynthetic carbon reductive - PCR) tissues. These tissues are arranged concentrically with respect to vascular tissues, forming a structural pattern known as Kranz anatomy (Muhaidat et al., 2007). This structural type provides one of the best examples of the intimate

connection between plant form and function and represents a suite of structural characters that have evolved repeatedly from C₃ ancestors (Dengler & Nelson, 1999; Kellog, 1999; Sage, 2001; Sage, 2004). This internal architecture physically partitions the biochemical events of the C₄ pathway into two main phases. In the first step, atmospheric CO₂ is initially assimilated into C₄ acids by PCA-tissue-specific phosphoenolpyruvate carboxylase. In the second phase, these acids diffuse into the PCR compartment, where they are decarboxylated, and the released CO₂ is re-fixed by PCR-tissue-specific Rubisco. This biphasic C₄ system enhances CO₂ levels around Rubisco, suppressing photorespiration and improving plant carbon balance (Kanai & Edwards, 1999).

The aim of our work is to integrate the Kranz anatomy structure in the whole set of adaptive features of halophytes, especially addressing to ecological factors. Our investigations refer to five halophyte species; four of these have a limited distribution in Europe, as described above. In addition, several of investigated species were collected from two nature reserves. Therefore, discussing this evolutive pattern also referring to such fragile ecosystems could reveal several new aspects related to interrelations plant-soil.

2. MATERIAL AND METHODS

2.1. Material

The material subjected to our analysis is represented by leaves of halophytes, collected from saline habitats, in plants anthesis phenophase. The taxa subjected to our investigations are: *Atriplex tatarica* L., *Camphorosma annua* Pall., *Camphorosma monspeliaca* L., *Petrosimonia oppositifolia* (Pall.) Litv. and *Petrosimonia triandra* (Pall.) Simonk, from Chenopodiaceae family. *P. oppositifolia* has been collected from a salty habitat, on Cotnari (Iași) from a salinized slope on Belcești (Iași), *C. annua* and *A. tatarica* from 'Valea Ilenei' (Iași) natural reserve, *P. triandra* and *C. monspeliaca* from 'Valea lui David' (Iași), during years of 2005-2007.

The ecological characterization, our short notes in the field as well as other histo-anatomical features suggest that all investigated taxa are xero-halophytes (Grigore and Toma, 2010a), appart from *A. tatarica*, which is a species with a wider ecological spectrum, a non-obligatory halophyte. This species has been classified by Romanian plant ecologists as preferential halophyte (Țopa, 1954), neohalophyte (Bucur, 1961) and supporting

halophyte (Șerbănescu, 1965). Remaining species have been considered as obligatory halophytes (Țopa, 1954; Șerbănescu, 1965), euhalophytes (Bucur, 1960). Anyway, attention should be paid on the fact that in Aronson's database (1989) referring on halophytes, only *P. crassifolia* and *A. tatarica* have been included in. This could be explained by limited inputs in collating this database. In addition, appart from *A. tatarica*, considered as weedy plant and having a wide distribution in Europe, other taxa have a quite restricted distribution in Europe. *Camphorosma monspeliaca* occurs in saline soils and dry waste places, and it is confined to South of Europe, extending northwards to 53° North In East Russia while *C. annua* vegetates in saline habitats, restricted to East and Center of Europe, extending to Bulgaria and Central Ukraine (Edmonson, 1993). *Petrosimonia* species, all growing in saline habitats, have a more local distribution, occurring only in South East of Europe (Albania, Romania and Russia) (Edmonson, 1993); in Romania, *P. oppositifolia* is a very rare species (Grigore, 2008a).

'Valea Ilenei' (Iași) is a quite small but very interesting natural reserve of saline soils from Romania. It occupies only 10 hectares and is located to approximately 4 km NV from Letcani rail station, at confluence of Valea Ilenei and Bahlui rivers (Nicoară & Bomher, 2010). Data regarding the flora and vegetation of this unique nature reserve are very scattered (Burduja, 1939; Răvărut, 1941; Mititelu, et al., 1987). Recently, a preliminary study regarding the ecology of halophytes in this area has been published (Grigore & Toma, 2011), but the "Valea Ilenei" nature reserve still requires a special attention and long-term monitoring studies. As we revealed (Grigore & Toma, 2011) this limited-surface reserve provides perhaps the most striking habitat where interrelationships halophytes-ecological factors are to be deeply studied. The large biodiversity, referring on microhabitats, as well on specific flora in this reserve argue again for extending the researches in this reserve.

'Fânețele seculare de la Valea lui David' (Iași) is a floristic reserve, located to approximately 5 km V from Iași, at 1000 m from Iași-Targu Frumos road (Nicoară & Bomher, 2010). As in the case of 'Valea Ilenei', here also vegetate many rare, vulnerable and endemic species, also included in 'Cartea Roșie a județului Iași' (Nicoară & Bomher, 2010).

2.2. Methods

For subsequent histo-anatomical investigations, the material was fixed and preserved in ethanol (70°).

Leaf cross sections were obtained using a razor blade and a microtome. The cross sections obtained were subsequently subjected to the “classical” stages of a common histo-anatomical procedure: immersion in sodium hypochlorite for 20-30 min, washing with acetic water and tap water, then staining: first with iodine green (for 1 minute) and washing in ethanol (90°) bath then second with red carmine (for 20 min.), washing with water and finally fixation in glycerol-gelatine.

Permanent slides were examined with a light microscope and micrographs have been taken using a NOVEX (Holland) microscope, with a Canon photo digital camera.

For obtaining an accurate picture of environmental factors, the soil' pH and electrical conductivity were determined, using a Crison pH Meter and an electrical conductivimeter, respectively.

3. RESULTS

The results of our investigations relieved Kranz anatomy architecture on lamina level structure, in all investigated halophytes belonging to Chenopodiaceae. Our findings are correlated with additional data stating that these species have C₄ pathway (Mateu-Andrés, 1993a, 1993b; Sage et al., 1999a).

Thus, on *A. tatarica*, some bundle sheaths have been observed, well expressed around the small, lateral vascular bundle; these form an incomplete layer around the lateral veins and a more less developed arch around the big, main vascular bundle (Fig. 1) (the black arrows indicate the chlorenchymatic tissues).

These vascular sheaths may be easily observed on superficial leaf sections. Here, between the veins network, some groups of epidermal cells, surrounded by polygonal cells perpendicularly by veins can be well evidenced (Fig. 2). These perpendicular cells represent in fact the “crown” of cells surrounding the veins, a typical arrangement called Kranz anatomy (Waisel, 1972). There are many classifications and variations of this structural pattern; the sub-type evidenced by us correspond with the *atriplicoid* one (Jacobs, 2001; Muhaidat et al., 2007). This sub-type has been also observed on other *Atriplex* species: *A. lampa* (Pyykkö, 1966), *A. buchananii* (Troughton & Card, 1974), *A. sibirica* (Gamaley, 1985; Frey & Kürschner, 1983). Anyway, it seems like about 111 *Atriplex* species have C₄

photosynthesis and implicitly Kranz anatomy (Sage et al., 1999a).

In *Petrosimonia oppositifolia*, we evidenced in the structure of lamina, the *kochioid* Kranz anatomy sub-type (Fig. 3), also related with C₄ photosynthesis. The cross sections must be analyzed carefully, because the continuous/discontinuous character of internal chlorenchyma imposes the true “diagnosis” regarding different sub-types. The *kochioid* configuration is very similar to the *salsoloid* one, the single difference being related to the fact that in the last situation, the concentric layers of chlorenchyma are continuous (Gamaley, 1985; Voznesenskaya, 1999; Jacobs, 2001; Muhaidat et al., 2007; Pyankov et al., 1997; Pyankov et al., 2001), as in *P. brachiata*, investigated by Frey & Kürschner (1983). Moreover, as a novel and interesting observation, this type of structure was also evidenced by us in the top of the stem, where the structure is discontinuous in various areas of circular contour of the cross section (Fig. 4). The presence of Kranz anatomy pattern at this level of the stem is very interesting, because we don't have evidenced this configuration on other Romanian *Petrosimonia* species, *P. triandra*.

P. triandra has also the *kochioid* sub-type in the lamina's structure: external chlorenchyma, internal chlorenchyma - at whose periphery scattered vascular elements are located (Fig. 5) - and central water storage tissue, in which a big, central vascular bundle is embedded, as well in the case of *P. oppositifolia*.

In the two studied species of *Camphorosma*, we have evidenced the *kochioid* sub-type (Gamaley, 1985; Voznesenskaya, 1999; Jacobs, 2001; Muhaidat et al., 2007; Pyankov et al., 1997), being, therefore a C₄ species, as *C. monspeliaca* (Frey & Kürschner, 1983) or *C. lessingii* (Pyankov et al., 2001).

In contrast with *Petrosimonia* species, on *Camphorosma annua*, a hypodermis, located between epidermis and external chlorenchyma (palisade tissue) occurs (Fig. 6); the remaining structure is similar with those of *Petrosimonia*, with a central water storage tissue in which a big vascular bundle is prominent.

On *C. monspeliaca*, the Kranz structure maintains the same as in the case of previous species, with the little difference referring on less individualized hypodermis, between epidermis and external chlorenchyma (Fig. 7).

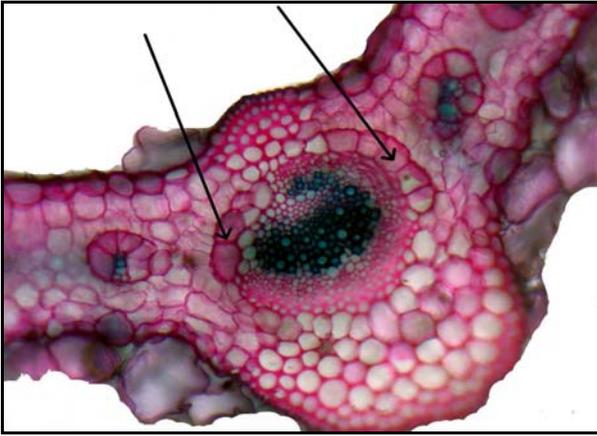


Figure 1. Micrograph of cross section through the lamina of *Atriplex tatarica* (X200)

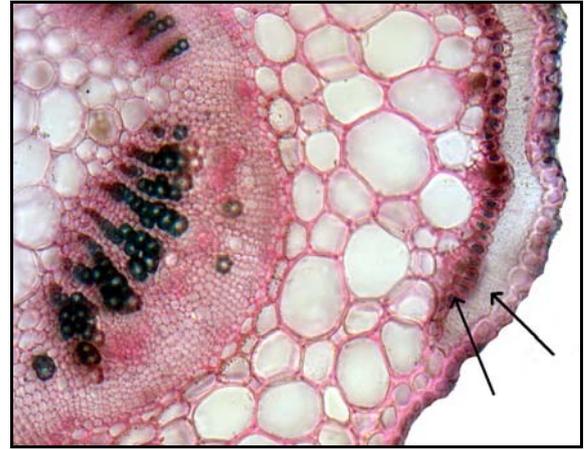


Figure 4. Micrograph of cross section through the stem of *Petrosimonia oppositifolia* (X200)

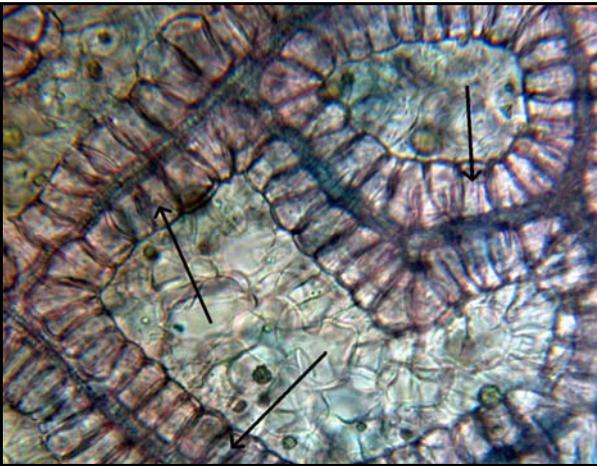


Figure 2. Micrograph of lower epidermis of lamina of *Atriplex tatarica* (surface view) (X400)

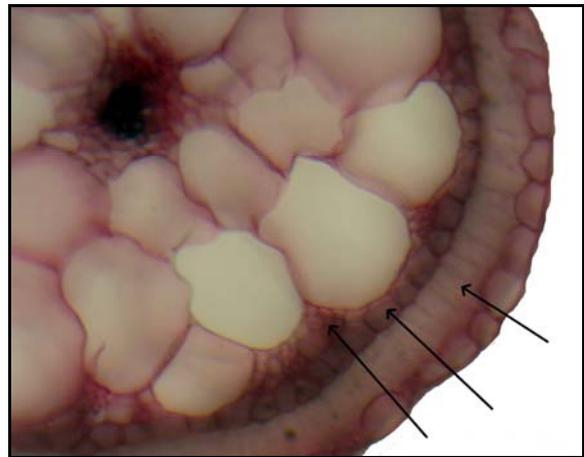


Figure 5. Micrograph of cross section through the lamina of *Petrosimonia triandra* (X200)



Figure 3. Micrograph of cross section through lamina of *Petrosimonia oppositifolia* (X 400)

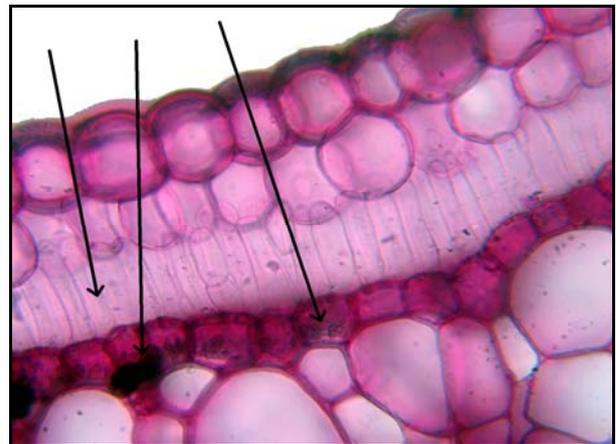


Figure 6. Micrograph of cross section through the lamina of *Camphorosma annua* (X400)

4. DISCUSSION

As regards the nomenclature and the division of Kranz anatomy structures in different sub-types, here some difficulties might occur.

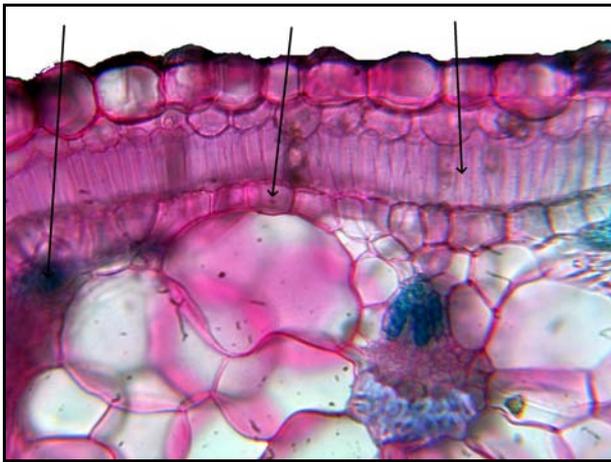


Figure 7. Micrograph of cross section through the lamina of *Camphorosma monspeliaca* (X400)

Mostly, the differences between the two subtypes (kochioid and salsoid) are referring on continuous or discontinuous character of internal chlorenchyma layer. In addition, there are also many differences in using a language nominating these tissues; for instance, for external chlorenchyma, it is use the term “outer mesophyll” or “palisade parenchyma” and for internal chlorenchyma, “chlorenchyma-sheath”. If we are going deeper, we can find that old botanists, such as Monteil (1906) used the term “endodermic sheath” in his drawings, with reference with internal chlorenchyma.

A major point of discussion is related to the fact that over years it was considered that vascular sheaths must be located very close to the vascular bundles. With the exception of *A. tatarica*, these chlorenchymatous layers are present at some distance by the main vascular bundle. In many studies - relatively old for the issue discussed by us – it has been shown this topographic reality, despite the fact that the C_4 pathway has been proved by physiological and biochemical methodology. The same is true for *Suaeda monoica*, a C_4 plant, with a structure analogous with those of species investigated by us. The authors investigating this species (Shomer-Ilan et al., 1975) concluded that C_4 metabolism can exist even in plants with such chlorenchyma located at some distance from the vascular bundle.

This is a precocious observation, in the whole context of further divisions that will be made by the first authors describing and developing anatomical “syndromes” of Kranz anatomy pattern (Carolín et al., 1975; 1978; 1982).

There are many questions and debates concerning the evolution of this photosynthetic pathway: why such a new metabolic pathway was necessary to occur and especially what

environmental imperatives would have induced the development of associated mechanisms with C_4 photosynthesis? There are some real adaptive advantages of this photosynthetic type on halophytes?

‘Valea Ilenei’ nature reserve is a unique salinized ecosystem, because in a very small area here is a large heterogeneity in environmental factors and a big diversity in halophytes distribution and adaptations, consequently (Grigore & Toma, 2011). The soil pH is basic, with values varying from 7.92 to 9.78, while the salinity shows surprisingly huge variations, from 0.57 up to 11.82 dS/m (values obtained from soil samples in the summer of 2011). Tree different microhabitats have been described in this area, where salt and water stresses play an important role in halophytes adaptations (Grigore & Toma, 2011). Within Kranz anatomy, osmolytes biosynthesis is also involved allowing chenopods to cope with stressful factors. Thus, proline is synthesized in small amount, while glycine-betaine accumulates in huge quantity (Grigore *et al*, unpublished data).

‘Fânețele seculare Valea lui David’ comprises salinized marshes, meadows, and Ponto-Sarmatic steppes; here are scattered small dry and salinized surfaces, where salinity ranges from 1.05 to 4.62 dS/m.

It has been shown that aridity and salinity are important factors promoting stomatal closure and thus reduce intercellular CO_2 levels, stimulating photorespiration and aggravating a CO_2 substrate deficiency (Guy et al., 1980; Adam, 1990). Together, the combination of drought, increased salinity, low humidity and high temperature produces the greatest potential for photorespiration and CO_2 deficiency (Ehleringer & Monson, 1993). In addition, drought or salinity stresses further increase CO_2 compensation points, because lower stomatal conductance and photosynthetic capacity reduce carbon income, allowing respiration to consume proportionally more of carbon acquired by the plant (Sage, 2004).

Evolutionarily speaking, it seems like anatomical modifications (Kranz type) represented a preconditioning step in occurrence of this photosynthetic type (Sage, 2004); to evolve an effective CO_2 concentration mechanism, the distance between mesophyll and bundle sheath cells has to decline to allow for rapid diffusion of metabolites (Raghavendra, 1980; Ehleringer et al., 1997).

Even with all exposed data at our disposal, it is still difficult to find a direct correlation between salinity factor and Kranz anatomy structures. All investigated species by us are xero-halophytes and

obligatory halophytes, excepting *A. tatarica*. In its native distribution area of Middle and western part of Central Asia, this species occupies solonetz sandy and clayey banks of rivers and lakes, coastal solonchaks, solonetz alluvial trails, and is frequently found as a weed in roadside ditches and in villages (Kochánková & Mandák, 2008).

C₄ species form a particularly high proportion of the herbaceous flora of saline environments, even in cool temperate regions (Long & Mason, 1983). Apparently, the inherently higher water use efficiency of C₄ species would have two theoretical advantages in saline environments (Long, 1999). First, saline soils have a soil water potential of around - 2.5 MPa; to extract water, the halophytes must generate a lower water potential, even though this exceeds limits that can apparently be tolerated by many mesophytic vascular plants. Transpiration must be minimal, and the higher water use efficiency of C₄ species would confer the advantage of maximizing carbon gain per unit of water lost. Second, plant mineral content is inversely correlated to water use efficiency as an assumed result of increased passive uptake with increased transpiration. For a halophyte, increased transpiration increases the energy needed to exclude Na⁺ and Cl⁻ (Long & Mason, 1983).

It has been suggested that halophytes are, in fact, a special case among xerophytes (Wiesner, 1889; Henslow, 1895; Schimper, 1903; Kearney, 1904; Warming 1909; Clements, 1920; McDougall, 1941; Grigore & Toma, 2010a). This implies the occurrence of some mechanisms serving to protect the water reserves of the plant in periods of drought or high potential evapotranspiration when soil water potential falls. A cost of xeromorphy is increased resistance to diffusion of CO₂ to the mesophyll; because of the low leaf intercellular pressure, necessary to saturate C₄ photosynthesis, this cost is minimized in C₄ species.

Despite the fact that C₄ species represent only about 8000 of the estimated 250,000 to 300,000 land plants species (Sage et al., 1999b), they are major components of biomes that cover more than 35 % of the earth's land surface area. These species are dominant in tropical and subtropical grassland and savanna, warm temperate grassland and savanna, arid steppe, beach dunes, saltmarshes, salt desert, hot deserts and semideserts.

C₄ also represents an important ecological strategy in certain desert shrubs, most notably species of *Atriplex*, particularly in saline soils (Keeley & Rundel, 2003). In these species, the key adaptation is the ability to maintain growth under high summer temperatures and drought conditions at

a time when C₃ species are dormant. The maximal rates of photosynthesis in these desert C₄ species are generally no higher than that of concurring C₃ species, but the water use efficiency is far greater. In addition, C₄ plants have higher nitrogen use efficiency.

Some studies certify the close relationship between C₄ photosynthesis and extreme habitats, such as deserts and salinized areas. Thus, Wang (2007), identified among species vegetating in the deserts of China that 36.5% of the *Chenopodiaceae* species were found with C₄ photosynthesis, which was about 48 % of the total C₄ species. These taxa were predominantly members of the genera *Anabasis*, *Atriplex*, *Kochia*, *Salsola*, and *Suaeda*.

Other studies sustain the facts above mentioned: there is a close relationship between some special morphotypes and respective photosynthetic type. In an ecological work, it was observed that halophytes and xerophytes with articulated stems and stem succulents of *Anabasis*-type are exclusively C₄. Leaf succulent halophytes and xerophytes are also predominantly C₄ (Akhani et al., 1997).

Additional results obtained by Pyankov et al. (2000) referring on C₄ plants from Mongolia, also suggest the relevance of this photosynthetic pathway on plants growing in extreme environmental conditions. The *Chenopodiaceae* comprises the greatest number of C₄ plants (about 41 species). Additionally, the C₄ *Chenopodiaceae* make up 45 % of the total chenopods and are very important ecologically in saline areas and cold arid deserts. NADP-ME tree-like species with a salsoloid type of Kranz anatomy, such as *Haloxylon ammodendron* and *Iljinia regelii*, plus shrubby *Salsola* and *Anabasis* species, were the plant most resistant to environmental stresses. Most of the annual C₄ chenopods species are halophytes, succulent and occurred in saline and arid habitats.

5. CONCLUSIONS

The occurrence of Kranz anatomy in halophytes collected from two nature reserve may be regarded as an adaptive feature. This is related to salt and water stress that impose different responses in plants exposed to such harsh conditions.

It is obvious that here is a logical causal and relational connection between halophytes, Kranz anatomy-C₄ photosynthesis and ecological conditions with stressful potential for plants life.

‘Sărăturile din Valea Ilenei’ (Iași) and ‘Fânețele seculare de la Valea lui David’ (Iași) represent two protected nature reserve where vegetate a great number of chenopods halophytes

having C₄ photosynthesis. These reserves are perhaps among the few and the last ecosystems from Moldova where the intimate relationships between environmental factors and halophytes' adaptations can be yet studied. This is because here some rare, vulnerable and sub endemic halophyte species still grow, despite the fragile character of these typical ecosystems. Their evolution, in terms of dynamic of vegetation and persistent state of several species is quite unpredictable, since there is a disputable management regarding the conservation of biodiversity. Uncontrolled grazing, mowing or setting fire to vegetation could negatively affect the distribution and ecology of these rare halophytes, especially in 'Valea Ilenei'.

In this context, such endangered ecosystems really needs a special and realistic management plan in order to protect halophytes species, as an valuable tools of study of some interesting and intriguing features, for instance, Kranz anatomy.

ACKNOWLEDGEMENTS

This paper was published with support provided by the POSDRU/89/1.5/S/49944 project "Developing the innovation capacity and improving the impact of research through post-doctoral programmes".

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Received at: 13. 01. 2011

Revised at: 04. 01. 2012

Accepted for publication at: 14. 01. 2012

Published online at: 20. 01. 2012