Physiological characterization of photosynthesis, chloroplast ultrastructure, and nutrient content in bracts and rosette leaves from *Glaucium flavum*

S. REDONDO-GÓMEZ[*,+], E. MATEOS-NARANJO[*], and F. J. MORENO[**]

Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 - Sevilla, Spain[*]
Departamento de Biología Celular, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain[**]

Abstract

Glaucium flavum is a biennial plant that bears a rosette of leaves, producing a flower stalk, bracteate monochasium, in its second year. The aims of this work were both to investigate the contribution of bracts to gas-exchange activities in this species and to compare this contribution to that of rosette leaves. In addition, we investigated the extent to which its responses can be explained by chloroplast ultrastructure, as well as the possible role of nutrient concentrations in the physiological responses of both leaf types. Gas exchange and plant characteristics regarding chlorophyll fluorescence were examined in a field experiment; we also determined leaf relative water content, tissue concentrations of photosynthetic pigments, chloroplast ultrastructure and nutrient contents. Although bracts indeed contributed to gas-exchange activities of *G. flavum*, rosette leaves showed higher values of net photosynthetic rate and stomatal conductance to CO_2 for photosynthetic photon flux density above 200 μmol m^{-2} s^{-1}. The incongruities in photosynthetic rates between bracts and leaves may be explained by the bigger chloroplasts of rosette leaves, which results in a larger membrane surface area. This agrees with the higher pigment concentrations and quantum efficiency of photosystem II values recorded as well for rosette leaves. On the other hand, bracts showed higher sodium concentrations, which could be a mechanism for salt tolerance of *G. flavum*.

Additional key words: carotenoids; chlorophyll content; chlorophyll fluorescence; photosystem II; stomatal conductance.

Introduction

Glaucium flavum Crantz. (Papaveraceae), known as yellow horned-poppy, is a biennial plant that bears a rosette of leaves, producing a single central flower stalk in its second year. Its inflorescences are a spreading bracteate monochasium; the bracts are semi-amplexicaul and auriculate, those below being more or less leaf-like in appearance, while those above become instead much smaller, wider, less divided, and less hairy (Scott 1963). This species is a widespread plant along the coasts of the Mediterranean basin and Western Europe (Peled *et al.* 1988). However, it is has been cited as an endangered species in some locations (Bercu *et al.* 2006).

Since *G. flavum* is useful for the restoration of coastal shingle (Walmsley and Davy 1997a), germination biology (Thanos *et al.* 1989, Walmsley and Davy 1997b, Davy *et al.* 2001) and anatomical studies (Bercu *et al.* 2006) of this species have been approached. In contrast to this, experimental data are lacking on photosynthetic gas exchange. However, evidence that in other plants like cotton certain organs (Bondada and Oosterhuis 2000), in particular the bracts of the floral bud, may participate in gas-exchange activities, renders this aspect worthy of study in the case of *G. flavum*. Thus, the aims of the present study were: (*1*) compare the physiological responses of bracts and rosette leaves; (*2*) examine chloroplast ultrastructure of both leaf types. In this

Received 2 April 2010, *accepted* 1 August 2010.
[+]Corresponding author; tel.: +34-95-4557165, fax: +34-95-4615780, e-mail: susana@us.es
Abbreviations: C_i – intercellular CO_2 concentration; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; C*x*+*c* – carotenoids; F_0 – minimal fluorescence level in the dark-adapted state; F_m – maximal fluorescence level in the dark-adapted state; F_s – steady state fluorescence yield; F_v – variable fluorescence level in the dark-adapted state; F_v/F_m – maximum quantum efficiency of PSII photochemistry; g_s – stomatal conductance; NPQ – non-photochemical quenching; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; RWC – relative water content; WUE – water-use efficiency; Φ_{PSII} – quantum efficiency of PSII.
Acknowledgements: We are grateful to Mr. Jesús Cambrollé for technical assistance. We also thank the Directorate of the Odiel Marshes Natural Park for collaboration.

respect, despite the fact that detailed anatomical characteristics of *G. flavum* and ultrastructure of the laticifers of the stem and seeds have been studied (Bercu *et al.* 2006), chloroplast ultrastructure of bracts and rosette leaves have not been previously documented. A study of this nature would therefore aid in establishing a functional relationship with photosynthesis in the two leaf types (Bondada and Oosterhuis 2003). And (*3*) examine the possible role of nutrient concentrations in both leaf types, which can be of help for understanding physiological responses.

Materials and methods

Plant material: The work was carried out on a nontidal area at Odiel Marshes (37º15´N, 6º58´W; SW Spain), where *G. flavum* grows on sandy soil, 5% fine sand and 95% coarse sand. The climate of the area is Mediterranean, affected by oceanic influences. Winter is wet and with mild temperatures (mean temperature *ca*. 11ºC in January) and summer is long and dry (mean temperature is *ca*. 25ºC). Mean annual rainfall reaches 510 mm, with an interannual variation coefficient of 31%.

To evaluate ecophysiological differences between bract and rosette leaves of *G. flavum*, ten adult plants of similar size were randomly selected in October 2006.

Gas-exchange measurements were taken in randomly-selected, fully expanded bract and rosette leaves (n = 10) using an infrared gas analyzer in an open system (*LI-6400*, *Li-COR Inc*., Lincoln, NE, USA). Net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i) and stomatal conductance to CO_2 (g_s) were determined at an ambient CO_2 concentration of 380 µmol mol^{-1}, temperature of 20/24ºC, 50 ± 5% relative humidity and fourteen photon flux densities (between 0 and 2,500 µmol m^{-2} s^{-1}), using a LED light source (*6400-02B, LI-COR Biosciences Inc*., Lincoln, Nebraska, USA). P_N, C_i and g_s were calculated automatically by the infrared gas analyzer using the standard formulae of von Caemmerer and Farquhar (1981). As bracts did not occupy the leaf chamber surface, their photosynthetic areas were calculated by superimposing the surface of each leaf over a mm-square paper. The water-use efficiency (WUE) was calculated as the ratio between P_N and transpiration rate [mmol(CO_2 assimilated) mol^{-1}(H_2O transpired)].

Chlorophyll fluorescence was measured in randomly-selected, fully expanded bract and rosette leaves (n = 10) using a portable modulated fluorimeter (*FMS-2*, *Hansatech Instrument Ltd*., King´s Lynn, UK). Light- and dark-adapted fluorescence parameters were measured at dawn (stable 50 µmol m^{-2} s^{-1} ambient light) and at midday (1600 µmol m^{-2} s^{-1}, Redondo-Gómez *et al.* 2008).

Plants were dark-adapted for 30 min, using leaf–clips designed for this purpose. The minimal fluorescence level in the dark-adapted state (F_0) was measured using a modulated pulse (<0.05 µmol m^{-2} s^{-1} for 1.8 µs) too small to induce significant physiological changes in the plant. The data stored were an average taken over a 1.6-second period. Maximal fluorescence in this state (F_m) was measured after applying a saturating actinic light pulse of 15,000 µmol m^{-2} s^{-1} for 0.7 s. Values of the variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry (F_v/F_m) were calculated from F_0 and F_m. This ratio correlates with the number of functional PSII reaction centres (Maxwell and Johnson 2000).

The same leaf section of each plant was used to measure light-adapted parameters, always making sure that the leaf section was fully sunlit. Steady-state fluorescence yield (F_s) was recorded after adapting plants to ambient light conditions for 30 min. A saturating actinic light pulse of 15,000 µmol m^{-2} s^{-1} for 0.7 s was then used to produce the maximum fluorescence yield (F_m') by temporarily saturating PSII photochemistry.

Using fluorescence parameters determined in both light- and dark-adapted states, the following were calculated: quantum efficiency of PSII [$\Phi_{PSII} = (F_m' - F_s)/F_m'$], photochemical quenching [$q_P = (F_m' - F_s)/(F_m' - F_0')$, where F_0', which was measured, corresponds with open reaction center traps in the light-acclimated state], and non-photochemical quenching [$NPQ = (F_m - F_m')/F_m'$; Redondo-Gómez *et al.* 2006].

Leaf relative water content (RWC): Ten bract and rosette leaves were collected at Odiel Marshes (n = 10) to determine RWC, which was calculated as:

$$RWC = (FM - DM)/(TM - DM) \times 100 \quad (1)$$

where FM is the fresh mass of the leaf, TM is the turgid mass after rehydrating the leaf in distilled water for 24 h, and DM is the dry mass after oven-drying at 80ºC for 48 h.

Photosynthetic pigments in ten randomly-selected, fully expanded bract and rosette leaves were extracted using 0.05 g of fresh material in 10 ml of 80% aqueous acetone. After filtering, 1 ml of the suspension was diluted with a further 2 ml of 80% aqueous acetone and chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (C*x*+*c*) contents were determined with a *Hitachi U-2001* spectrophotometer (*Hitachi Ltd*., Japan), using three wavelengths (663.2, 646.8, and 470.0 nm). Concentrations of pigments were obtained by calculation, using the method of Lichtenthaler (1987).

Analysis of chloroplasts: Small fragments of rosette leaves and bracts were fixed in 4% (v/v) glutaraldehyde prepared in 0.1 M cacodylate buffer, pH 7.2, for 3 h at

4°C and postfixed in 1% OsO_4 for 2 h at 4°C. Samples were dehydrated in an acetone series and embedded in Epon (epoxy embedding medium). Thin sections (60- to 80-nm thick) were stained with uranyl acetate and lead citrate and examined in an electron microscope (*Philips CM-10*, Eindhoven, Netherlands). The images were captured by means of a digital camera (*Megaview 3*, *Leica*, Solms, Germany). The analysis of chloroplasts was carried out on 80 images of cells per a study group. Mesophyll cells were chosen at random. Chloroplast areas of mesophyll cell were analysed using an image analysis software (*Leica Q-win*, *Leica*, Solms, Germany).

Determination of nutrient content: Bract and rosette leaves (n = 5) were carefully washed with distilled water before any further analysis, and dried at 80ºC for 48 h and ground. 0.5 g samples were subsequently digested with 6 ml HNO_3, 0.5 ml HF, and 1 ml H_2O_2. Al, As, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Si, and Zn were measured by inductively coupled plasma spectroscopy (*ICP ARL-Fison 3410*, *Fisons Instruments Inc.*, Carmel Valley, CA, USA). Total N and C concentrations were determined for undigested dry samples with an elemental analyzer (*Leco CHNS-932*, Spain).

Statistical analysis was carried out using a statistic software (*Statistica v. 6.0*, *Statsoft Inc.*, Tulsa, OK, USA). Pearson coefficients were calculated to assess correlation between different variables. Data were analyzed using one- and two-way analyses of variance (F-tests) and first tested for normality with the *Kolmogorov-Smirnov* test and for homogeneity of variance with the *Brown-Forsythe* test. Significant test results were followed by *Tukey* tests for identification of important contrasts, while differences between bract and rosette leaves were compared by the Student test (t-test).

Results

Gas exchange: Net respiration rate was –2.5 µmol m^{-2} s^{-1} and the compensation point to light was recorded at 25 µmol(photon) m^{-2} s^{-1} for both leaves´ types. Nevertheless, bract and rosette leaves showed different response curves to increasing photosynthetic photon flux density (PPFD) between 200 and 2,500 µmol m^{-2} s^{-1}, the latter recording higher net photosynthetic rates (P_N; two-way *ANOVA*, p<0.0001). The maximum values of P_N, recorded at 2,500 µmol m^{-2} s^{-1}, were 14.8 and 22.5 µmol m^{-2} s^{-1} for bract and rosette leaves, respectively (Fig. 1*A*).

g_s was similar for the two leaf types in darkness (0.27 mmol m^{-2} s^{-1}), but g_s values of rosette leaves increased with increasing external PPFD, while stomatal conductance of bracts declined between 0 and 200 µmol m^{-2} s^{-1}, before stabilizing at around 0.21 mmol m^{-2} s^{-1} (Fig. 1*B*). As to C_i, it responded similarly in bract and rosette leaves, decreasing with increasing PPFD (Fig. 1*C*). Water-use efficiency (WUE) ranged from –1.1 to 5.0 mmol mol^{-1}, increasing with PPFD (Fig. 1*D*).

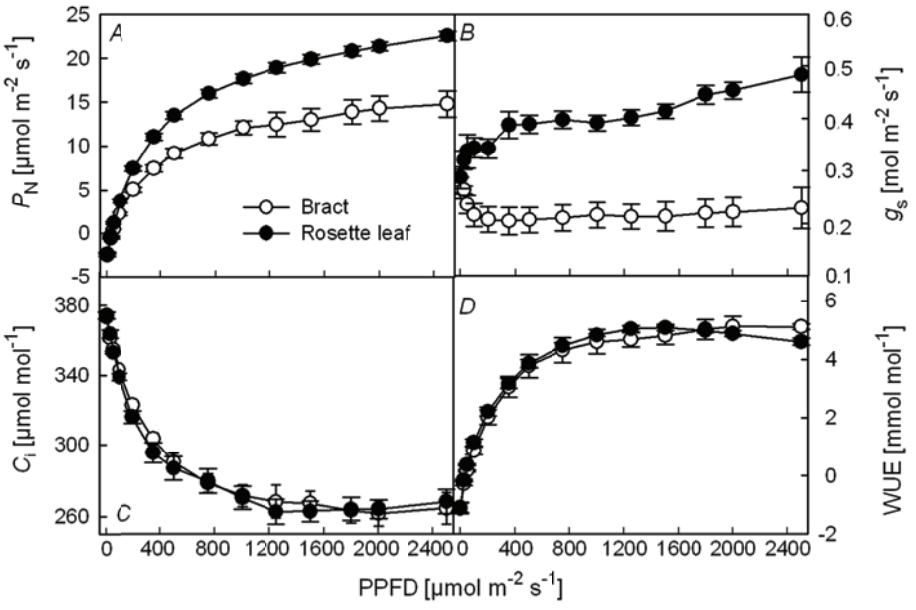

Fig. 1. Net photosynthetic rate, P_N (*A*), stomatal conductance to CO_2, g_s (*B*), intercellular CO_2 concentration, C_i (*C*) and water-use efficiency, WUE (*D*) in random fully expanded bract (o) and rosette leaves (•) of *Glaucium flavum* in response to fourteen photosynthetic photon flux densities (PPFD) between 0 and 2,500 µmol m^{-2} s^{-1}. Values represent mean ± standard error, n = 10.

Table 1. Maximum quantum efficiency of PSII photochemistry (F_v/F_m), quantum efficiency of PSII (Φ_{PSII}), photochemical quenching (q_P) and non-photochemical quenching (NPQ), at midday and dawn in bract and rosette leaves of *Glaucium flavum*. Values are mean ± standard error (n = 10); *t*-test compared fluorescence parameters in bract versus rosette leaves. *p<0.05, ** p<0.01, ***p<0.001, n.s. = not significant.

Leaf type	F_v/F_m	Φ_{PSII}	q_P	NPQ
Midday measurements [relative]				
Bracts	0.82 ± 0.01	0.27 ± 0.03	0.43 ± 0.05	1.17 ± 0.12
Rosette leaves	0.82 ± 0.01	0.42 ± 0.03	0.67 ± 0.05	0.90 ± 0.07
t-test	–0.07 (n.s.)	4.31***	3.19**	–3.07**
Dawn measurements [relative]				
Bracts	0.86 ± 0.01	0.79 ± 0.01	0.93 ± 0.01	0.20 ± 0.06
Rosette leaves	0.88 ± 0.00	0.83 ± 0.00	0.96 ± 0.00	0.13 ± 0.02
t-test	3.10**	3.41**	2.70*	–2.22*

Table 2. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (C*x*+*c*) concentrations and Chl *a*/*b* ratio for bract and rosette leaves of *Glaucium flavum*. Values represent mean ± standard error, n = 10; *t*-test compared concentrations in bract versus rosette leaves. *p<0.05, n.s. = not significant.

Leaf type	Chl *a* [μg g^{-1}(FM)]	Chl *b* [μg g^{-1}(FM)]	C*x*+*c* [μg g^{-1}(FM)]	Chl *a*/*b*
Bracts	4.7 ± 0.27	1.6 ± 0.08	1.4 ± 0.07	3.0 ± 0.04
Rosette leaves	5.5 ± 0.21	1.8 ± 0.08	1.6 ± 0.04	3.1 ± 0.07
t-test	2.38*	2.11*	2.15*	0.84 (n.s.)

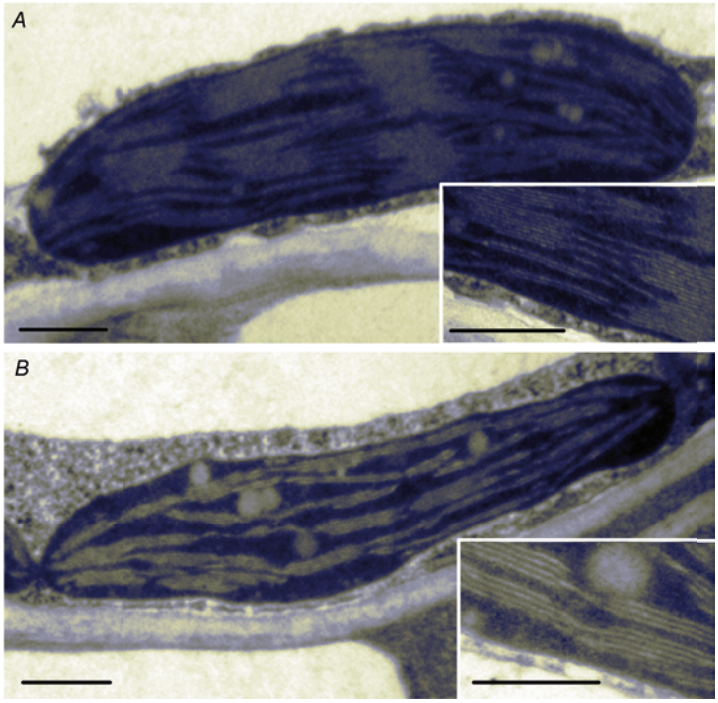

Fig. 2. Transverse section of chloroplast of rosette leaves (*A*) and bracts (*B*) observed using electron microscopy. *Bar* = 0.5 μm.

Chlorophyll fluorescence: Values of F_v/F_m were different for bract and rosette leaves at dawn (0.86 ± 0.01 and 0.88 ± 0.00 respectively; *t*-test, p<0.05), but not at midday, with values varying around 0.82 (Table 1). The lower F_v/F_m values of bracts at dawn resulted mainly from lower values of F_m (data not presented).

The quantum efficiency of PSII (Φ_{PSII}) was lower for bracts at both dawn and midday; and dawn values were significantly higher than midday values for the two leaf types (*t*-test, p<0.05; Table 1). The lower Φ_{PSII} of bracts was accompanied by lower photochemical quenching (q_P) and higher non-photochemical quenching values (*t*-test, p<0.05; Table 1).

Table 3. Total carbon, total nitrogen, total sodium and total calcium concentrations for bract and rosette leaves of *Glaucium flavum*. Values represent mean ± standard error, $n = 5$; *t*-test compared concentrations in bract versus rosette leaves. *$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Leaf type	C [%]	N [%]	C/N ratio	Na [mg kg^{-1}]	Ca [g kg^{-1}]
Bracts	36.5 ± 0.27	4.2 ± 0.12	8.7 ± 0.32	411 ± 16	50.1 ± 2.4
Rosette leaves	39.5 ± 0.28	5.0 ± 0.15	7.9 ± 0.21	304 ± 35	31.5 ± 3.34
t-test	–7.69****	–3.96***	–2.34*	2.45*	4.46**

RWC was similar for bract (97 ± 1.2%) and rosette leaves (96 ± 2.1%; $p>0.05$).

Photosynthetic pigment concentration: Concentrations of Chl *a*, Chl *b* and Cx+c were higher for rosette leaves (*t*-test, $p<0.05$). There were no significant differences for Chl *a*/*b* ratio between bract and rosette leaves, this ratio being only slightly higher for rosette leaves (Table 2).

Analysis of chloroplasts: Notwithstanding the fact that, morphologically and structurally, bract chloroplasts were analogous to leaf chloroplasts, the chloroplasts of rosette leaves had higher area in section (4.51 ± 0.18 μm^2) than those of bracts (3.78 ± 0.15 μm^2; *t*-test, $p<0.01$; Fig. 2).

Nutrient content: Tissue carbon and nitrogen concentrations were greater in rosette leaves than in bracts. In contrast, leaf tissue sodium and calcium concentrations and C/N ratio were higher in bracts (Table 3). There were no significant differences for Al, As, Co, Cr, Cu, Fe, K, Mg, Mn, Ni, P, S, Si, and Zn concentrations between bract and rosette leaves (*t*-test, $p>0.05$; data not presented).

Discussion

Bracts proved to be important organs contributing to gas exchange activities of *G. flavum* (bracts make up 30–35% of the photosynthetic area of the plant), although rosette leaves were more physiologically active, with greater values of P_N and g_s for PPFD above 200 μmol m^{-2} s^{-1}. Wullschleger and Oosterhuis (1991) also found that leaves of cotton showed greater values for P_N and g_s than the bracts of the floral bud. In our study, the lower g_s values of bracts were not accompanied either by a loss of leaf water content or by a decrease in WUE, and therefore they are most likely to be the result of a chemical signalling rather than of a general loss of turgor. The differences in g_s, which did not entail differences in C_i, may be related to differences in the K/Ca ratio in the guard cells and/or to differences in the abscisic acid concentration, which controls the stomatal movement (Marschner 1999). Consequently, we recorded a higher Ca content in bracts.

Bracts also showed higher sodium concentrations, which could be a mechanism for salt tolerance. Leaves of *G. flavum* accumulate organic solutes as glaucine for osmotic adjustment under salt stress (Peled *et al.* 1988). Likewise, compartmentation in bracts may contribute to minimize the concentration of salt in rosette leaves, and so prevent it from building up in the cytoplasm or cell wall (Munns 2002).

In addition to the higher g_s values, the greater P_N of rosette leaves could be explained by a higher content of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This enzyme can contain up to 75% of leaf N, and this contribution underlies the well-known association between photosynthetic capacity and biomass N content (Vrede *et al.* 2004). Therefore, our results showed lower values of foliar C/N ratio for rosette leaves.

On the other hand, Bondada and Oosterhuis (2003) stated that the incongruities in photosynthetic rates between bracts and leaves of cotton could be elucidated by probing the ultrastructure of their chloroplasts. In this way, we found that chloroplasts of rosette leaves were bigger than those of bracts, and had the greatest grana number, which perhaps conferred them with the largest membrane surface area. This is in accordance with the higher Chl *a*/*b* ratio recorded for rosette leaves. According to Bondada and Oosterhuis (2003), a decline in grana abundance could contribute to a lower accumulation of photosynthetic pigments in the bracts. This explains the low pigment concentrations of the bracts compared with the rosette leaves.

Photosystem II (PSII) is localized to the membrane system of grana; hence, the larger the surface area, the greater the capture of light energy (Anderson and Melis 1983). This agrees with Φ_{PSII} data recorded for *G. flavum*, which was higher for rosette leaves at both dawn and midday. Furthermore, Φ_{PSII} of both leaf types did show a significant reduction at midday compared to dawn values. At midday, Φ_{PSII} decreased as a consequence of the decrease in q_P and the increase in NPQ, which indicates that the plants dissipated excess energy in the antenna, thereby protecting the leaf from light-induced damage (Maxwell and Johnson 2000). Also F_v/F_m demonstrated the difference between sampling times. At midday, the reduction in F_v/F_m values indicated that *G. flavum* experienced slight reduction in PSII activity at the higher light flux. This reduction might have been caused by a

lower proportion of open reaction centers (lower values of F_m) resulting from a saturation of photosynthesis by light (Redondo-Gómez *et al.* 2009). However, bracts and rosette leaves showed optimal values for unstressed plants at dawn and midday (Björkman and Demmig 1987).

References

Anderson, J.M., Melis, A.: Localization of different photosystems in separate regions of chloroplast membranes. – Proc. Natl. Acad. Sci. USA **80**: 745-749, 1983.

Bercu, R., Fagaraş, M., Jianu, L.D.: Anatomy of the endangered plant Glaucium flavum Cr., occurring on the Romanian Black Sea littoral. – In: Gafta, D., Akeroyd, J. (ed.): Nature Conservation: Concepts and Practique. Pp 273-280. Springer, Heidelberg 2006.

Bondada, B.R., Oosterhuis, D.M.: Comparative epidermal ultrastructure of cotton (*Gossypium hirsutum* L.) leaf, bract and capsule wall. – Ann. Bot. **86**: 1143-1152, 2000.

Bondada, B.R., Oosterhuis, D.M.: Morphometric analysis of chloroplasts of cotton leaf and fruiting organs. – Biol. Plant. **47**: 281-284, 2003.

Björkman, O., Demmig, B.: Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins. – Planta **170**: 489-504, 1987.

Davy, A.J., Willis, A.J., Beerling D.J.: The plant environment: aspects of the ecophysiology of shingle species. – In: Packman, J.R., Randall, R.E., Barnes, R.S.K., Neal, A. (ed.): Ecology and Geomosphology of Coastal Shingle. Pp 191-201. Westbury Academic & Scientific Publishing, Yorkshire 2001.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Colowick, P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 148. Pp. 350-382. Academic Press, San Diego – New York – Berkeley – Boston – London – Sydney – Tokyo – Toronto 1987.

Maxwell, K., Johnson, G.N.: Chorophyll fluorescence - a practical guide. – J. Exp. Bot. **51**: 659-668, 2000.

Marschner, H.: Mineral Nutrition in Higher Plants. Pp. 285-307. – Academic Press, Harcourt B. Company Publishers, London 1999.

Munns, R.: Comparative physiology of salt and water stress. – Plant Cell Environ. **25**: 239-250, 2002.

Peled, B., Waisel, Y., Carmeli, S.: Alkaloid content in various chemoecotypes of *Glaucium flavum* from Israel. – Phytochemistry **27**: 1021-1024, 1988.

Redondo-Gómez, S., Wharmby, C., Castillo, J.M., Mateos-Naranjo, E., Luque, C.J., de Cires, A., Luque, T., Davy, A.J., Figueroa, M.E.: Growth and photosynthetic responses to salinity in an extreme halophyte, *Sarcocornia fruticosa*. – Physiol. Plant. **128**: 116-124, 2006.

Redondo-Gómez, S., Mateos-Naranjo, E., Cambrollé, J., Luque, T., Figueroa, M.E., Davy, A.J.: Carry-over of differential salt tolerance in plants grown from dimorphic seeds of *Suaeda splendens*. – Ann. Bot. **102**: 103-112, 2008.

Redondo-Gómez, S., Mateos-Naranjo, E., Figueroa, M.E.: Synergic effect of salinity and light-chilling on photosystem II photochemistry of the halophyte, *Sarcocornia fruticosa*. – J. Arid Environ. **73**: 586-589, 2009.

Scott, G.A.M.: *Glaucium flavum* Crantz. – J. Ecol. **51**: 743-754, 1963.

Thanos, C.A., Georghiou, K., Skarou, F.: *Glaucium flavum* seed germination – an ecophysiological approach. – Ann. Bot. **63**: 121-130, 1989.

Vrede, T., Dobberfuhl, D.R., Kooijman, S.A.L.M., Elser, J.J.: Fundamental connections among organism C:N:P stoichiometry, macromolecular composition, and growth. – Ecology **85**: 1217-1229, 2004.

von Caemmerer, S., Farquhar, G.D.: Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. – Planta **153**: 377-387, 1981.

Walmsley, C.A., Davy, J.A.: The restoration of coastal shingle vegetation: effects of substrate composition on the establishment of seedlings. – J. Appl. Ecol. **34**: 143-153, 1997a.

Walmsley, C.A., Davy, J.A.: Germination characteristics of shingle beach species, effects of seed ageing and their implications for vegetation restoration. – J. Appl. Ecol. **34**: 131-142, 1997b.

Wullschleger, S.D., Oosterhuis, D.M.: Photosynthesis, transpiration, and water use efficiency of cotton leaves and fruit. – Photosynthetica **25**: 505-515, 1991.