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Photosynthetic pigments, betalains, proteins, sugars, and minerals during *Salicornia brachiata* senescence

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Abstract

Senescence is the last developmental stage in plants during which recycling of nutrients takes place from senescing organs to newly formed organs such as young leaves and developing seeds. In the present work, senescence induced alterations in mineral ions, chlorophylls, carotenoids, betacyanin, betaxanthin, proteins, amino acids, sugars, starch, and polyphenols were monitored in shoots of an extreme halophyte *Salicornia brachiata*. A sharp decline in the content of chlorophylls, carotenoids, and proteins in the shoot was noticed at middle and late stages of senescence in comparison with early stage. However, the content of betacyanin, betaxanthin, total soluble sugars, reducing sugars, and starch increased significantly in senescing shoots. The total free amino acid content decreased gradually with the progress of senescence. The content of major minerals did not change significantly with the progress of senescence, whereas marked changes in content of minor minerals were observed. From this study, it was concluded that the sugars and starch accumulating in senescing shoots might be transported into developing seeds to serve as storage nutrients. The accumulation of betacyanin and betaxanthin in senescing shoots suggests that these pigments may act as scavengers of reactive oxygen species during senescence. This study provides comprehensive information on the variations in the utilization of mineral nutrients and organic metabolites with progressing senescence in the halophyte *S. brachiata*.

Additional key words: amino acid, betacyanin, betaxanthin, carotenoids, chlorophylls, halophyte, polyphenols, starch.

Introduction

Senescence is a coordinated physiological process in plants occurring in the final stage of the development of the whole plant, organ, tissue, or the cell that ultimately leads to the death. It is a vital process for recycling of nutrients from mature and senescing source leaves to newly-formed sink organs such as young leaves and developing seeds (Kim *et al*. 2007, Watanabe *et al*. 2013). Senescence that occurs as a part of normal development of the plants when growth conditions are near optimal is commonly referred to as developmental or age-dependent senescence, and it is induced and controlled by endogenous factors (Lers 2007). However, senescence may be induced prematurely *via* exposure to harsh environment. Key environmental stresses inducing premature senescence in plants are extreme temperatures, excess of radiation, drought, nutrient deficiency, presence of toxic materials, and pathogen infection (Lers 2007, Obata and Fernie 2012).

Several phytohormones affect the processes during senescence. The cytokinin and ethylene have an extensive role in delaying or inducing leaf senescence, respectively (Sperotto *et al*. 2009, Davies and Gan 2012). Besides, other hormones, such as abscisic acid, auxins, gibberellic acid, jasmonic acid, and salicylic acid, also have a significant role in regulating the senescence processes (Schippers *et al*. 2007). The coordinated degradation of macromolecules and the remobilization of regained nutrients such as nitrogen, carbon, and minerals from senescing tissues into other parts of the plant are of vital importance (Zimmermann and Zentgraf 2005). The transport of metabolites from source leaves to sinks, such as developing seeds takes place through the vascular system and has a substantial role on crop yield and crop quality (Gregersen *et al*. 2013). Therefore, the vascular

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Abbreveations: ABTS - 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; Car - carotenoid; Chl - chlorophyll; DPPH - 2,2-diphenyl-1-picrylhydrazyl; NBT - nitroblue tetrazolium; PMS - phenazine methoslphate; ROS reactive oxygen species; TCA - trichloroacetic acid.
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system is retained until the very late stages of senescence (Gan and Amasino 1997). It has been reported that the content of various minerals such as potassium, phosphorrous, iron, zinc, chromium, sulphur, molybdenum, carbon, copper, and zinc is reduced by more than 25 % in senescent leaves of *Arabidopsis thaliana* as compared with the previous stages (Himelblau and Amasino 2001). This report suggests that mobilization of minerals takes place at the onset and during leaf senescence.

Leaf senescence is a highly regulated degenerative process involving a series of biochemical reactions (Buchanan-Wollaston *et al*. 2003, Lim and Nam 2005, Kim *et al*. 2007). The degradation of chlorophylls and breakdown of saccharides, lipids, proteins, and nucleic acids increase due to activation of hydrolytic enzymes during senescence, whereas the photosynthesis as well as protein synthesis decrease (Kim *et al*. 2007, Watanabe *et al*. 2013). Conversely, the fragmentation of DNA takes place at very late stage of senescence (Orzáez and Granell 1997, Zimmermann and Zentgraf 2005). The final stage of leaf senescence is a type of genetically controlled programmed cell death. During senescence, a massive change in gene expression occurs (Kim *et al*. 2007). The transcriptome analysis of senescing leaves showed that many genes are down-regulated but some genes are up-regulated (Kim *et al*. 2007, Watanabe *et al*. 2013). In addition, various transcription factors are associated with leaf senescence (Lin and Wu 2004).

Salicornia brachiata is a succulent and leafless annual. It is an obligate halophyte growing in the intertidal zones and salt marshes of Indian coast. It belongs to the family *Chenopodiaceae*. Whereas *Mesembranthemum crystallinum* shifts photosynthesis from the C_3 mode to CAM to overcome high salinity, *Salicornia* species are C_3 plants. However, *Salicornia* species have the capability of efficient compartmentalization of toxic ions (Iyengar and Reddy 1997). In *Salicornia*, the spongy mesophyll cells are large and able to store a quantity of salts, whereas palisade tissue, where 80 % of the photosynthetic pigments are located, remains relatively free of Na^+ and Cl^- ions (Iyengar and Reddy 1997), and it is able to photosynthesize normally under high salinity (Iyengar and Reddy 1997). High ATPase activity is required to efflux toxic ions from palisade tissue to other non-photosynthetic tissues. Compartmentation of ions and high ATPase activity may be the adaptive features in *Salicornia* species to overcome high salinity (Iyengar and Reddy 1997). The developmental senescence of *S. brachiata* is visibly indicated by changes in shoot colour from green at an early stage to greenish yellow at middle stage and finally red at the terminal stage.

Senescence induced metabolic changes have been reported mostly in annual crops such as barley, maize, rice, wheat and some legumes (Crafts-Brandner *et al*. 1998; Yang *et al*. 2003; Robson *et al*. 2004; Parrott *et al*. 2005; Weng *et al*. 2005; Pick *et al*. 2011) and in model plant *Arabidopsis* (Diaz *et al*. 2005, Otegui *et al*. 2005, Watnabe *et al*. 2013). However, to the best of our knowledge, the changes in metabolites between pre-senescent and senescent tissues of the halophytes have not been investigated in detail so far. Therefore, in this work, a comprehensive study of senescence induced changes in the metabolites, including pigments, proteins, sugars, starch, amino acids, polyphenols, and mineral ions, have been carried out in the halophyte *S. brachiata* using shoots of three consecutive developmental stages.

Materials and methods

Plants: In January, the *Salicornia brachiata* Roxb. plants at different developmental stages categorized into mature plants (green), early senescence (greenish-yellow), and late senescence (red) were collected from a single community of salt marshes in Diu, Gujarat, India (latitude 20°44.5´N and longitude 70°56.0´E). The shoot tissue was used for the measurement of various parameters and most of the methods mentioned below were described in detail previously (Parida and Jha 2004, 2013).

Estimation of ion content: The shoot samples (0.5 g) were dried in an oven at 70 ºC for 48 h, homogenized, and placed in a 100-cm^3 flask. The samples were digested by adding 10 cm^3 of a mixture of HNO_3 and $HClO_4$ (9:4) until the production of red NO_2 fumes ceased. The content was further evaporated until the volume was reduced to 3 - 5 cm^3. After cooling, the deionized water (20 cm^3) was added and the solution was filtered through *Whatman No. 1* filter paper. Aliquots of this solution were used for the determination of Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} content by inductively coupled plasma atomic absorption spectrometry (*Optima 2000DV*, *Perkin Elmer*, Waltham, MA, USA).

For estimation of nitrogen and phosphorous, the dried samples were digested with concentrated H_2SO_4 at 200 °C and decolorized using H_2O_2 (30 %, v/v). The total nitrogen content in shoot samples was determined following the colorimetric procedure and the absorbance was read at 650 nm. The nitrogen content in the sample was determined from a standard curve prepared using $(NH_4)_2SO_4$ solution. The phosphorous content was estimated following the formation of a phosphomolybdate complex by adding 2.3 cm^3 of a reagent made of 1 part of 10 % (m/v) ascorbic acid and 6 parts of 0.42 % (m/v) $(NH_4)_6MO_7O_{24}$. 4 H_2O in 0.5 M H_2SO_4. In order to complete formation of the complex, the solution was processed at 45 °C for 10 min. The absorbance was measured at 820 nm, and phosphorus content was read from a calibration curve prepared using analytical-grade KH_2PO_4.

Estimation of chlorophylls and carotenoids: About

500-mg fresh shoot samples were homogenized with 80 % acetone (v/v) in a pre-chilled pestle and mortar in the dark. The homogenates were centrifuged at 15 000 *g* and 4º C for 10 min in a refrigerated centrifuge (*Thermofisher Scientific*, Waltham, USA). The absorption of the supernatant was read at 663.2, 646.8, and 470.0 nm in a microplate spectrophotometer using a quartz microtiter plate. Chlorophyll (Chl) *a*, Chl *b*, total Chl, and carotenoids (Cars) were estimated following the method of Litchenthaler (1987).

Estimation of betalains: The betalains (betacyanin and betaxanthin) were extracted and purified following the procedure of Wang *et al.* (2006) with slight modifications. The shoot samples (approximately 5.0 g) were homogenized in liquid nitrogen in a pre-chilled pestle and mortar. The homogenate was mixed with 10 volumes of methanol and centrifuged at 15 000 *g* and 4 °C for 15 min. The supernatant was discarded, and the pellet was re-extracted in 10 volumes of ethanol and was again centrifuged for 15 min. This procedure was repeated once more to remove Chl, Cars, ascorbic acid, and tocopherol. To extract betalains, the pellet was then re-extracted with 50 % ethanol for 30 min and centrifuged at the same conditions for 15 min. The supernatant was mixed with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1) and again centrifuged to remove proteins. The supernatant was re-extracted with a mixture of chloroform and isoamyl alcohol (24:1) to remove phenols. Then phenol phase was discarded, and the chloroform and isoamyl alcohol fraction was suspended with 50 mM potassium phosphate buffer (pH 6.5), vortexed thoroughly and then centrifuged at 15 000 g for 10 min. The aqueous phase was collected, and the concentration of purified betacyanin and betaxanthin was estimated using the formula of Stintzing *et al.* (2003) after recording the absorbance of supernatant at 538 and 480 nm.

Estimation of total protein content: Approximately 0.5 g of fresh shoot sample was homogenized with 5 cm^3 of pre-chilled 10 % (m/v) trichloroacetic acid (TCA) in 100 % acetone solution containing 0.07 % β-mercapto-ethanol in a chilled pestle and mortar. The homogenate was kept at -20 °C for 2 h to precipitate the protein and then centrifuged at 10 000 *g* and 4 °C for 15 min. The supernatant was discarded, and the pellet was washed with 100 % pre-chilled acetone and centrifuged under the same conditions to collect the protein pellet. The washing step was repeated three times. The supernatant was discarded, and the pellet was dissolved in 1 M NaOH. The protein concentration was estimated by taking the absorption at 660 nm following the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard.

Estimation of content of total soluble sugars, reducing sugars, and starch: Approximately, 5.0 g of fresh shoots was extracted with 10 cm^3 of 80 % ethanol (v/v) in a pestle and mortar. The homogenate was incubated in water bath at 70 ºC for 10 min and then centrifuged at 10 000 *g* for 10 min. The pellet was re-extracted twice with 80 % ethanol, and the supernatants were pooled. An aliquot of this supernatant was evaporated to dryness in a rotary evaporator (*R205*, Buchi, Germany); the residue was re-dissolved in distilled water and utilized for the estimation of total soluble sugars, reducing sugars, and polyphenols. The pellet left after ethanolic extraction was solubilised with 52 % perchloric acid and utilized for the estimation of starch.

Total soluble sugars and starch were estimated by anthrone-sulphuric acid using 0.2 % (m/v) anthrone in concentrated H_2SO_4 as a reagent. Spectrophotometric readings were taken at 630 nm. The standard curve was plotted with 0 - 100 µg of glucose. Reducing sugars were estimated following alkaline copper method using arsenomolybdate reagent. Absorbance was recorded at 510 nm and reducing sugar content was determined from a standard curve prepared from pure glucose (0 - 50 µg).

Estimation of total free amino acids: An aliquot of the ethanolic extract obtained above was evaporated to dryness in a rotary evaporator. The residue was re-dissolved in 0.2 M citrate buffer (pH 5.0). Total free amino acids were estimated using ninhydrin reagent. The absorbance was recorded at 570 nm. The concentration of amino acid was calculated from a standard curve prepared using glycine (0 -100 µg).

Estimation of polyphenols: The polyphenol content was estimated from the ethanolic extract using Folin-Ciocalteau reagent. The absorbance was recorded at 650 nm. A standard curve was prepared using several different concentrations of catechol (0 - 100 µg).

Measurement of ROS scavenging activities: Fresh shoots (5 g) were grounded to a fine powder in liquid nitrogen and extracted in 50 cm^3 of methanol (80 %, v/v) by shaking at 100 rpm for 24 h at room temperature. The extract was then freeze-dried in a lyophilizer and stored at -20 °C for further analysis.

DPPH radical scavenging activity of crude methanolic extract and purified betalain solution was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Prior *et al.* 2005). Freshly prepared DPPH methanol solution (0.1 mM) was mixed with different aliquots (20 - 100 mm^3) of crude methanolic extract or betalin solution and incubated for 30 min at room temperature in the dark. After incubation, the absorbance (A) was measured at 517 nm. The scavenging activity was estimated using the following equation: scavenging [%] = $[(A_{control} - A_{sample})/A_{control}] \times 100$.

Total antioxidant activity of crude methanolic extract and purified betalain solution were measured by 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) decolorization assay according to the method of Re *et al.* (1999). In this assay, $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS in H_2O with 2.45 mM potassium

persulfate ($K_2S_2O_8$) and incubating in the dark at room temperature for 16 h. Prior to use, the $ABTS^{\bullet+}$ solution was diluted with 0.1 M sodium phosphate buffer (pH 7.4) to give an absorbance of 0.750 ± 0.025 at 734 nm. Then, 1 cm^3 of $ABTS^{\bullet+}$ solution was added to crude methanolic extract or betalain solution. The absorbance was recorded after 1 min. The antioxidant capacity was calculated using the same equation as mentioned above.

The superoxide anion radical scavenging assay was done according to the method described by Saeed *et al*. (2012). Samples of crude methanolic extract or purified betalain solution were mixed with reaction mixture containing 0.5 cm^3 of 50 mM phosphate buffer (pH 7.6), 0.3 cm^3 of 50 mM riboflavin, 0.1 cm^3 of 0.5 mM nitroblue tetrazolium (NBT), and 0.25 cm^3 of 20 mM phenazine methosulphate (PMS). After 20 min of incubation under fluorescent lamp, the absorbance was measured at 560 nm. The scavenging ability of the extract was determined by the same equation as mentioned above

Hydrogen peroxide scavenging activity was measured following the procedure of Saeed *et al*. (2012). Hydrogen peroxide solution (2 mM) was prepared in 50 mM potassium phosphate buffer (pH 7.4). Aliquots of methanolic extract or betalain solution was transferred into the test tubes, and their volumes were made up to 0.4 cm^3 with 50 mM phosphate buffer (pH 7.4) After addition of 0.6 cm^3 of hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide was determined after 10 min at 230 nm, against a blank. Hydrogen peroxide scavenging activity was calculated using the same equation as mentioned above.

All the spectrophotometric analyses were performed in a UV-visible microplate spectrophotometer (*Epoch 120821B*, *Biotek*, Winooski, USA) using *Gen5 v. 2.01.14* software (*Biotek*).

Statistical analyses: All the experiments were conducted with a minimum of three replicates, and the results were expressed as the mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (*ANOVA*) and Duncan's multiple-range test ($P \leq 0.05$) using the *Sigma Plot v. 12.0* (*Systat Software*, Chicago, IL, USA).

Results

Salicornia brachiata shows different phenotypic characteristics at various stages of development. The mature shoots are green, then the colour of shoots turns to greenish-yellow, and at the end of senescence the shoots are red (Fig. 1). In order to decipher the mechanisms involved in senescence of this species, various biochemical indicators were studied in shoots at different developmental stages. The Na^+ content decreased by 57 % in greenish-yellow plants and increased by 7 % in the red plants as compared to green plants. The Ca^{2+} content increased by 17 % in greenish-yellow plants and decreased by 6 % in red plants. The nitrogen content declined by 29 and 38 %, respectively, in greenish-yellow and red plants as compared to green plants. The Fe^{2+} content increased by 8 % in greenish-yellow plants and decreased by 25 % in red plants. In comparison to green plants, the Zn^{2+} content increased by 29 and 23 %, respectively, in greenish-yellow and red plants. The Mn^{2+} content increased by 18 % in greenish-yellow plants, and a marginal increase of 9 % was noticed in red plants. No significant change in the Cu^{2+} content was observed between green and greenish-yellow plants, however, about 29 % decrease in Cu^{2+} content was observed in red plants in comparison to green plants. However, there were no significant changes in K^+, Mg^{2+}, Ni^{2+}, and P content during plant development (Table 1).

The content of Chl *a*, Chl *b*, total Chl, and Cars decreased significantly with the progress of senescence (Table 2). It was observed that the Chl *a* content decreased by 61 and 85 % in greenish-yellow and red plants, respectively, as compared to green plants, whereas the Chl *b* content decreased by 59.8 % in greenish-yellow and by 80 % in red plants. Similarly, the Cars content decreased by 27.5 % in greenish-yellow plants and by 66 % in red plants in comparison to green plants. The Chl *a*/*b* ratio decreased by 18 % at greenish-yellow plants and by 33 % in red plants (Table 2). Conversely, both betacyanin and betaxanthin content increased in *S. brachiata* shoots with the progress of senescence (Table 2). In comparison to green plants, the

Fig. 1. *Salicornia brachiata* plants showing different phenotypic characteristics at different stages of development: *A* - mature green plants, *B* - greenish-yellow plants (early senescence), *C* - senescent red plants.

Table 1. Changes in the content of macro- and micro-nutrients in shoots of *S. brachiata* during different developmental stages. Means ± SDs, $n = 4$. Means followed by different letters are significantly different at $P \leq 0.05$.

Minerals	Green	Greenish-yellow	Red
Na^+ [mg g^{-1} (d.m.)]	73.36 ± 0.83a	31.53 ± 7.62b	78.24 ± 12.34ac
K^+ [mg g^{-1} (d.m.)]	12.31 ± 1.61a	14.73 ± 3.76a	13.65 ± 2.34a
Ca^{2+} [mg g^{-1} (d.m.)]	2.71 ± 0.26a	3.17 ± 0.69b	2.54 ± 0.14ac
Mg^{2+}[mg g^{-1} (d.m.)]	3.68 ± 0.13a	3.72 ± 0.35a	3.82 ± 0.15a
N [mg g^{-1} (d.m.)]	4.24 ± 0.74b	3.01 ± 0.47a	2.61 ± 0.30a
P [mg g^{-1} (d.m.)]	0.21 ± 0.07a	0.27 ± 0.05a	0.23 ± 0.05a
Fe^{2+} [µg g^{-1} (d.m.)]	458.08 ± 34.8a	495.08 ± 56.3ab	345.96 ± 40.7c
Zn^{2+} [µg g^{-1} (d.m.)]	37.93 ± 5.71a	48.97 ± 9.78b	46.53 ± 6.89bc
Mn^{2+} [µg g^{-1} (d.m.)]	27.09 ± 2.12a	31.91 ± 8.05b	29.63 ± 1.90ac
Cu^{2+} [µg g^{-1} (d.m.)]	5.02 ± 0.72a	4.81 ± 0.79a	3.56 ± 0.13b
Ni^{2+} [µg g^{-1} (d.m.)]	3.16 ± 0 .21a	3.19 ± 0.44a	3.42 ± 0.54a

Table 2. Changes in the content of various pigments in shoots of *S. brachiata* during different developmental stages. Means ± SDs, $n = 4$. Means followed by different letters are significantly different at $P \leq 0.05$.

Pigments	Green	Greenish-yellow	Red
Chl *a* [µg g^{-1}(d.m.)]	574.6 ± 68.8a	221.9 ± 57.3b	84.3 ± 21.9c
Chl *b* [µg g^{-1}(d.m.)]	170.6 ± 21.4a	68.5 ± 15.8b	35.0 ± 10.8c
Chl *a*/*b*	3.3 ± 0.1a	2.7 ± 0.6b	2.2 ± 0.4b
Total Chl [µg g^{-1}(d.m.)]	746.5 ± 88.9a	307.0 ± 80.6b	119.5 ± 32.6c
Cars [µg g^{-1}(d.m.)]	433.8 ± 46.0a	314.2 ± 72.0b	147.5 ± 53.8c
Betacyanin [µg g^{-1}(d.m.)]	8.9 ± 1.1a	30.0 ± 5.5b	32.5 ± 2.9b
Betaxanthin [µg g^{-1}(d.m.)]	28.7 ± 5.3a	49.0 ± 6.9b	62.3 ± 8.7c

betacyanin content increased by 237 and 265 %, respectively, in greenish-yellow and red plants and the betaxanthin content increased by 70 and 117 %, respectively, in greenish-yellow and red plants (Table 2).

It was observed that content of total free amino acids and proteins in shoot decreased gradually with the progress of senescence. As compared to green plants, the content of free amino acids decreased by 56 % in greenish-yellow plants and by 72 % in red plants (Table 3). The total protein content decreased by 26 % in greenish-yellow plants and by 31 % in red plants as compared to green plants (Table 3). On the contrary, the total soluble sugarr, reducing sugars, and starch content increased significantly during development. As compared to green plants, the total soluble sugars content increased by 170 and 55 % in greenish-yellow and red plants, respectively. The reducing sugars content increased by 95 and 140 %, respectively, in greenish-yellow and red plants as compared to green plants. The starch content increased by 64 - 68 % in greenish-yellow and red plants as compared to green plants (Table 3). The total polyphenol content decreased by 31 % in greenish-yellow plants and by 8 % in red plants as compared to green plants (Table 3).

The ROS scavenging activities of *S. brachiata* shoot were measured as DPPH radical scavenging, ABTS radical cation decolorization, and $O_2^{\cdot-}$ and H_2O_2 scavenging assays. The crude extracts and purified

Table 3. The content of various organic compounds [mg g^{-1}(d.m.)] in shoots of *S. brachiata* during different developmental stages. Means ± SDs, $n = 4$. Different letters indicate statistically different means at $P \leq 0.05$.

Organic metabolites	Green	Greenish-yellow	Red
Total soluble sugars	39.9 ± 4.1a	107.9 ± 14.0b	100.3 ± 15.1b
Reducing sugars	10.6 ± 2.1a	20.7 ± 1.6b	25.6 ± 2.8c
Starch	28.2 ± 4.6a	47.3 ± 12.1b	46.5 ± 11.7b
Proteins	38.0 ± 5.6a	28.1 ± 1.7b	26.2 ± 4.2b
Amino acids	2.4 ± 0.3a	1.4 ± 0.2b	1.0 ± 0.05c
Polyphenols	3.9 ± 0.1a	3.0 ± 0.1b	3.7 ± 0.2a

betalain solutions from green, greenish-yellow, and red shoots were used (Fig. 2). The overall antioxidant and ROS scavenging activities of crude methanolic extracts were the highest in the greenish-yellow shoots, moderate in the red shoots, and the lowest in the green shoots (Fig. 2*A*). However, when betalain solutions were used, all the ROS scavenging activities increased in red shoots as compared to green and greenish-yellow shoots (Fig. 2*B*).

Discussion

Senescence is a synchronized process during which a plant or a part of the plant retrieve nutrients and remobilize them to younger tissues or to developing seeds (Ricachenevsky *et al*. 2013). In the present work, development associated changes in mineral nutrients and metabolites such as pigments, protein, amino acids, sugars, and polyphenols were studied using samples collected from an extreme halophyte *S. brachiata* at different developmental stages with the aim to ascertain the mechanism of nutrient recycling and metabolic shift during senescence. Some of mineral elements are

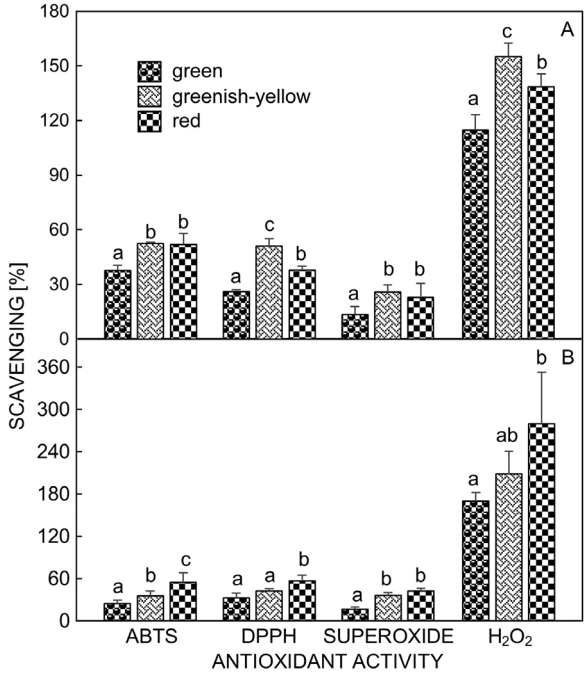

Fig. 2. Antioxidant properties of *S. brachiata* measured in terms of DPPH radical, ABTS radical, $O_2^{\bullet-}$ radical, and H_2O_2 scavenging activities in shoots of different developmental stages. *A* - Crude methanolic extracts (200 µg mm^{-3}); *B* - betalain solutions (100 µg cm^{-3}). Means ± SDs, $n = 4$. Different letters indicate statistically different means at $P \leq 0.05$.

required in large quantities (macronutrients N, P, S, K, Ca, and Mg) and some in small quantities (micronutrients Fe, Mn, Zn, Cu, B, Mo, Cl, and Ni) (Fischer 2007). Rather high accumulation of Na^+ is a typical feature of the halophytic plants (Zhu 2003). Most of the halophytes compartmentalize Na^+ ions into vacuoles to mitigate the toxic effects of Na^+. In addition, Na^+ accumulation in the succulent stems is one of the strategies of succulent halophytes (Rabhi *et al*. 2010). It has been reported that plants grown under high salinity usually absorb more Na^+ and less K^+ than control plants (Shi and Wang 2005, Yang *et al*. 2007). *S. brachiata* is an extreme halophytic species and grows in salt marshes. The high absorption of Na^+ in *S. brachiata* growing under extreme salinity inhibits the K^+ absorption due to competitive inhibition between Na^+ and K^+. Moreover, high saline conditions can break the ion balance and disturb the K^+ and Ca^{2+} distribution in the cell (Yang *et al*. 2007). The plants need to establish the ion balance in the cell under salinity for the tolerance and survival. In *S. brachiata* Na^+ is considered as the main inorganic osmolyte under saline conditions, but in the absence of NaCl, K^+ mainly acts as the inorganic osmolyte (Yang *et al*. 2007). Higher Na^+ content as compared to K^+ content has also been reported in the *Aneurolepidium chinense* (Shi and Wang 2005), *Kochia sieversiana* (Yang et al. 2007), and many other halophytes. The mineral nutrients from senescing plant parts are re-translocated to the seeds or other surviving structures such as bulbs and roots (Fischer 2007). In *S. brachiata*, the marginal changes in Na^+ and Ca^{2+} content of the shoot were observed at a late stage of senescence, and there were no significant changes in K^+ and Mg^{2+} content. In contrast to our results, it has been reported that K^+ easily leaches from senescing tissues of wheat or *Fagus* (Debrunner and Feller 1995, Tyler 2005). The N content of shoot declined gradually with the progress of senescence together with amino acid and protein content. The decline of protein content may be attributed to the increased proteolysis (Hortensteiner and Feller 2002). On the contrary, there was no significant difference in the phosphorous content of green, greenish-yellow and red shoots. The Zn^{2+} and Mn^{2+} content of shoot increased significantly at the late stage of senescence, whereas a marked decrease in Fe^{2+} and Cu^{2+} content was observed in *S. brachiata* with the progress of senescence. Our results suggest that important macronutrients are maintained whereas content of micronutrients changes during the senescence.

In *S. brachiata*, a significant loss of Chl and Car content was observed with the progress of senescence. Our results are in agreement with Lee *et al*. (2003), who have reported that both Chl and Car are broken down during senescence. However, usually more of the Car are retained than Chl resulting in yellow coloration of leaves during senescence (Hörtensteiner and Lee 2007). It has been reported that Chl breakdown is an important

catabolic process during fruit ripening (Hörtensteiner and Kräutler 2011). Lim *et al*. (2007) reported that loss of green colour visually marks the initiation of metabolic changes that occur during senescence. In addition, senescence is accompanied by decline in photosynthesis and the massive degradation of cellular proteins (Sakuraba *et al*. 2012). It has been reported that the Chl degradation produces various colourless catabolites known as non-fluorescent chlorophyll catabolites (NCCs) (Kraütler 2008, Hörtensteiner 2009, Hörtensteiner and Kraütler 2011, Sakuraba *et al.* 2012). NCCs are the low molecular mass tetrapyrrolic compounds having effective antioxidant properties (Kraütler 2008). These NCCs are photodynamically safe, because they do not absorb visible radiation thereby protecting the plants from photo-oxidative damage during senescence (Hörtensteiner and Kraütler 2011). In *S. brachiata*, it was observed that the chlorophyll degradation in senescing shoots was positively correlated with the ROS scavenging activities. The above evidences suggest that the chlorophyll degradation in *S. brachiata* might have some role in ROS detoxification during senescence. The declining Chl *a*/*b* ratio at a late stage of senescence suggests that the light-harvesting complexes of thylakoid membranes are affected by senescence in *S. brachiata* (Parida and Jha 2013).

It has been reported that betacyanins together with betaxanthins belong to a class of nitrogenous chromo-alkaloids known as betalains. Although anthocyanins are widely distributed in higher plants, betacyanins accumulate only in ten families of the order *Caryophyllales* (*e.g.*, *Amaranthaceae*, *Cactaceae*, and *Chenopodiaceae*). In these species, betacyanins replace anthocyanins, and these pigments are not found simultaneously in the same plant (Stafford 1994). *Salicornia brachiata* belongs to the family *Chenopodiaceae*, and red pigmentation of its shoots is due to the presence of betacyanin (Davy *et al*. 2001). A considerable increase in betacyanin and betaxanthin content was observed in the shoot of *S. brachiata* with the progress of senescence. Wang *et al*. (2006) have reported that betacyanin synthesis is induced by low temperature and high salinity in the halophyte *Suaeda salsa*. In *Suaeda japonica*, the betacyanin accumulation increases under significant drop in temperature (Hayakawa and Agarie 2010). Vogt *et al*. (1999) reported that betacyanin synthesis is induced by UV-A in *Mesembryanthemum crystallinum*. It has been reported that betalains act as ROS scavengers (Hayakawa and Agarie 2010). In *S. brachiata*, ROS scavenging activities were positively correlated with betalain accumulation in shoots. Our results showed that betalains (betacyanin and betaxanthin) production increased with the progress of senescence and it was important for scavenging potentially cytotoxic ROS. Both water-soluble pigments anthocyanins and betalains accumulate in the vacuoles (Tanaka *et al*. 2008). H_2O_2 rapidly accumulates in the chloroplast stroma during photoinhibition and further diffuses to other cell compartments (Nakano and Asada 1981). The juvenile and senescing leaves seem to have increased need of photoprotection. Therefore, an anthocyanin or betalain-rich vacuole acts as a potential sink for excess H_2O_2 produced in the chloroplast, alleviating the photo-oxidative damage to the plants (Kytridis and Manetas 2006). In addition to protection from photo-oxidative damage, the anthocyanins or betalains may act as the osmolytes (Hughes 2011). In *S. brachiata*, enhanced biosynthesis of betacyanin and betaxanthin with the progress of senescence may take part in both ROS scavenging and osmotic adjustment.

A gradual decrease in total free amino acid content was observed in *S. brachiata* with the progress of senescence. In a similar pattern, a decrease in free amino acid content has also been reported in tobacco and oat leaves (Masclaux *et al*. 2000, Soudry *et al*. 2005). The decrease in the content of amino acids may be due to membrane leakage during senescence (Soudry *et al*. 2005). However, in *Arabidopsis*, both the attached and detached leaves exhibit a gradual increase in amino acid content during senescence (Soudry *et al*. 2005). These results suggest that total free amino acid pools do not have a universal regulatory role in triggering senescence (Masclaux *et al*. 2000, Soudry *et al*. 2005). It has been reported that senescence-related proteases play significant roles in leaf senescence by regulating protein degradation and nutrient recycling (Wang *et al*. 2013, Wu *et al*. 2016). In *S. brachiata* the total protein content gradually decreased with the progression of senescence. The decline in the protein content may be due to the proteolysis associated with onset of senescence. In most plant tissues, the proteins contain the largest fraction of organic nitrogen, which is potentially available for remobilization during senescence (Fischer 2007, Wu *et al*. 2016). The decrease in the content of total protein may be the reason for the decline in the nitrogen content of the senescing shoots of *S. brachiata.*

Like other cellular constituents, sugars, starch, and polyphenols are also affected by senescence in *S. brachiata*. A significant increase in total sugars, reducing sugars, and starch content was observed in *S. brachiata* with the progress of senescence. Conversely, all individual sugars and starch content decrease with increasing age of *Lactuca sativa* (Witkowska and Woltering 2013). In agreement with our results, Masclaux *et al*. (2000) have reported an increase in sugar content with a concomitant decline in photosynthesis in tobacco leaves during senescence. It has been reported that cytokinin production increases during senescence in lettuce resulting an abnormally high accumulation of sugars in upper leaves that lead to premature senescence (McCabe *et al*. 2001). It has been proposed that the accumulation of sugars in mature leaves leads to a decline of photosynthetic activity and a certain threshold content of sugars may act as a senescence signal (Kim *et al*. 2007). The increase in sugar content with the progression of senescence has been reported in many plant species. It has been reported that increase in the sugar content with a concomitant decline in nitrogen content in leaves play a

role in the induction of leaf senescence (Hortensteiner and Feller 2002), which is in accordance with our results. The expression of senescence-associated gene *SAG12*, which is highly senescence specific, is induced several-fold by growth on glucose in combination with low nitrogen (Pourtau *et al*. 2004, Wingler *et al*. 2006). *SAG12* is expressed late during the senescence process, and it has been argued that late *SAGs* are sugar-repressible, whereas early SAGs are sugar-inducible (Paul and Pellny 2003, Wingler *et al*. 2006). Thus, some soluble sugars probably act as important regulatory molecules for triggering senescence (Dai *et al*. 1999, Xiao *et al*. 2000, Soudry *et al*. 2005). Generally, a decline in starch content of leaf with a concomitant increase in sugar content is common in many plant species subjected to abiotic stresses (Parida and Jha 2013). However, in *S. brachiata* concomitant increase in total soluble sugars and starch content was observed during senescence. The increase in starch content may be due to the synthesis of starch from sugars. Sugars may be extensively available for starch synthesis because they may not be consumed in plant growth during the senescence.

The polyphenols are considered as powerful non-enzymatic ROS scavengers in plants (Yildiz-Aktas *et al*. 2009, Parida and Jha 2013) and they help the plants to adapt to harsh environmental conditions. There are several reports of the role of polyphenols in energy dissipation and ROS scavenging (Edreva 2005, Yildiz-Aktas *et al*. 2009, Parida and Jha 2013), and the synthesis of these metabolites is stimulated in plants under salt stress (Reginato *et al*. 2014). Apart from defence against ROS, the polyphenols (predominantly flavonoids) also have other roles such as UV screening and developmental regulators (Ferdinando *et al*. 2014). In *S. brachiata*, a marginal increase in polyphenol content was observed at the late stage of senescence. It has been reported that the polyphenol content in leaves does not differ between maturity stages of *Lactuca sativa* (Witkowska and Woltering 2013). The contrasting results between *S. brachiata* and *L. sativa* suggest that the polyphenols do not have a universal regulatory role in triggering senescence.

In conclusion, the data presented in this work reveal that the content of important major mineral nutrients was preserved in *S. brachiata*, whereas the content of minor nutrients varied with the progress of senescence. The enhanced biosynthesis of both betacyanin and betaxanthin with progress of senescence took place in ROS scavenging and osmotic adjustment. In *S. brachiata* total free amino acids pools did not have a regulatory role in triggering senescence. The decline in the protein content in *S. brachiata* with progress of senescence may be due to the proteolysis associated with onset of senescence and may be the reason for the decline in the nitrogen content in the senescing shoots. The soluble sugars serve as important regulators for triggering senescence in this plant.

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