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The capacity of antioxidant protection during modulated ageing of bean (*Phaseolus vulgaris* L.) cotyledons. 2. The low-molecular weight antioxidants

D. Procházková* and N. Wilhelmová

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Czech Republic

The aim of the present work was to evaluate non-enzymic antioxidants during natural and artificially modulated senescence. Senescence of bean (*Phaseolus vulgaris* L. cv. Jantar) cotyledons was modulated by UV C irradiation or by the decapitation of plants apices. The content of β-carotene and zeaxanthin decreased in control and decapitated plants but in UV C irradiated plants these contents increased. The degree of de-epoxidation increased in all cultivations with age. The content of total glutathione (sum of reduced and oxidized) sharply decreased in bean cotyledons grown in all conditions. Interestingly, the content of total ascorbate increased at the end of cotyledon life span of control and decapitated plants but decreased in UV plants. Decrease of reduced/oxidized ratio of ascorbate and glutathione during cotyledon ageing confirmed increasing oxidative stress during senescence in all cultivations. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS — ascorbate; β-carotene; decapitation; glutathione; senescence; UV C irradiation

ABBREVIATIONS — A, antheraxanthin; AsA, reduced ascorbate; C plants control plants; Chl, chlorophyll; D plants, decapitated plants; DHA, oxidized ascorbate; DTNB, 5,5′-dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; DW, dry weight; FW, fresh weight; GSH, reduced glutathione; GSSG, oxidized glutathione; PS II, photosystem II; ROS, reactive oxygen species; UV plants, UV C irradiated plants; V, violaxanthin; Z, zeaxanthin.

INTRODUCTION

Plant senescence is a natural process characterized by an intensive loss of proteins and chlorophyll, as well as severe increases in lipid peroxidation and membrane permeability due to a notable enhancement in the metabolism of activated oxygen.[1–3] Plant cells possess both enzymic and non-enzymic mechanisms, which can protect them against oxidative damage to their components.[4]

Carotenoids can directly deactivate singlet oxygen and quench the excited triplet state of chlorophyll, thus preventing the formation of singlet oxygen.[5,6] The decrease of β-carotene content with increasing age was observed in sage leaves[7] and in *Cistus clusii* leaves.[8] Another carotenoid, xanthophyll zeaxanthin, is involved in the de-excitation of excess energy via radiationless dissipation in the pigment bed via the xanthophyll cycle. The xanthophyll cycle involves the conversion of violaxanthin, a xanthophyll that contains two epoxide groups, through antheraxanthin with one epoxide group to zeaxanthin that has no epoxide group.[9] The xanthophyll pool size increased during leaf senescence of wheat.[10]

Reduced Ascorbate (AsA) is a primary antioxidant,[11,12] reacting directly with hydroxyl radicals, superoxide and singlet oxygen.[12,13] AsA plays an important role in photoprotection and regulation of photosynthesis,[6] in preserving the activities of enzymes that contain prosthetic transition metal ions. Ascorbate is also a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol.[12,14]

* Correspondence to: D. Procházková, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ-160 00 Praha 6, Czech Republic. Tel: +420 2 24 310 109. Fax: +420 2 24 310 113. E-mail: prochazkova@ueb.cas.cz

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Moreover, ascorbate acts as a co-factor for de-epoxidase reactions in course of xanthophylls de-epoxidation. The content of ascorbate decreased during ageing of chrysanthemum petals[15] and in senescing soybean nodules.[16]

Glutathione (GSH) may react directly with ROS or with oxidized substrates thereby reducing them. By doing so GSH is eventually transferred to its oxidized form (GSSG). GSH is also required for the regeneration of ascorbate, it may reduce disulphide bonds of proteins, is responsible for the removal of xenobiotics from the cytoplasm and regulates sulphur uptake at root level.[17]

An increase of GSH and GSSG in peroxisomes, mitochondria and the cytosol during senescence of pea leaves has been observed.[18] GSSG/GSH ratio increased with a concomitant GSH content decrease during senescence of soybean nodule[16] and during ageing of tomato seeds.[19]

The aim of the present investigation was to evaluate changes in low-molecular antioxidants in bean cotyledons with natural and with artificially shortened and prolonged life spans.

MATERIALS AND METHODS

Plant material

Bean (*Phaseolus vulgaris* L., cv. Jantar) cotyledons were studied. The growth conditions and time of harvesting are described in the accompanying paper 'The capacity of antioxidant protection during modulated ageing of bean (*Phaseolus vulgaris* L.). 1. The antioxidant enzyme activities' (this issue).

Non-enzymic antioxidant contents

The contents of β-carotene, zeaxanthin, violaxanthin and antheraxanthin were determined in acetone extracts by high performance liquid chromatography (Spectra-Physics, USA) using a reverse phase column (Sepharon SGX C 18, Tessek, Czech Republic). Four to ten cotyledons were homogenized in 1 ml acetone with a mortar and pestle and centrifuged. The supernatant was dried by gaseous nitrogen and the sediment was dissolved in 50 µl acetone. The solvent system was acetonitrile/methanol/water (80/12/6 v/v/v) applied for 8 min followed by 100% methanol for 13 min. The gradient run was 25 min, the flow rate 1 cm/min, and the detection wavelength was 445 nm.

The contents of AsA and GSH were measured spectrophotometrically (Hitachi U 3300, Japan) at 25°C.

The ascorbate assay was based on the reduction of Fe^{3+} by AsA in acidic solution. Fe^{2+} forms complexes with bipirydyl, giving a pink colour with a maximum absorbance at 525 nm.[21] Cotyledons—one g fresh weight (FW)—were homogenized with a pestle in ice-cold 5% metaphosphoric acid (1/10, w/v) in a cold mortar. Total ascorbate (AsA + dehydroascorbate (DHA)) content was measured after a prior reduction of dehydroascorbate to ascorbate with dithiothreitol. The concentration of dehydroascorbate was estimated from the difference of total ascorbate and reduced ascorbate. For the glutathione assay, cotyledons (1 g FW) were homogenized with a pestle in ice-cold 5% sulfosalicylic acid (1/10, w/v) in a cold mortar. Total glutathione (GSH + GSSG) content was determined on the supernatant by the 5,5′-dithio-bis-nitrobenzoic acid (DTNB)—glutathione reductase recycling procedure.[22] The reaction was monitored as the rate in absorbance change at 412 nm. GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. GSH was calculated by subtracting the amount of GSSG from total glutathione.

Statistical evaluation

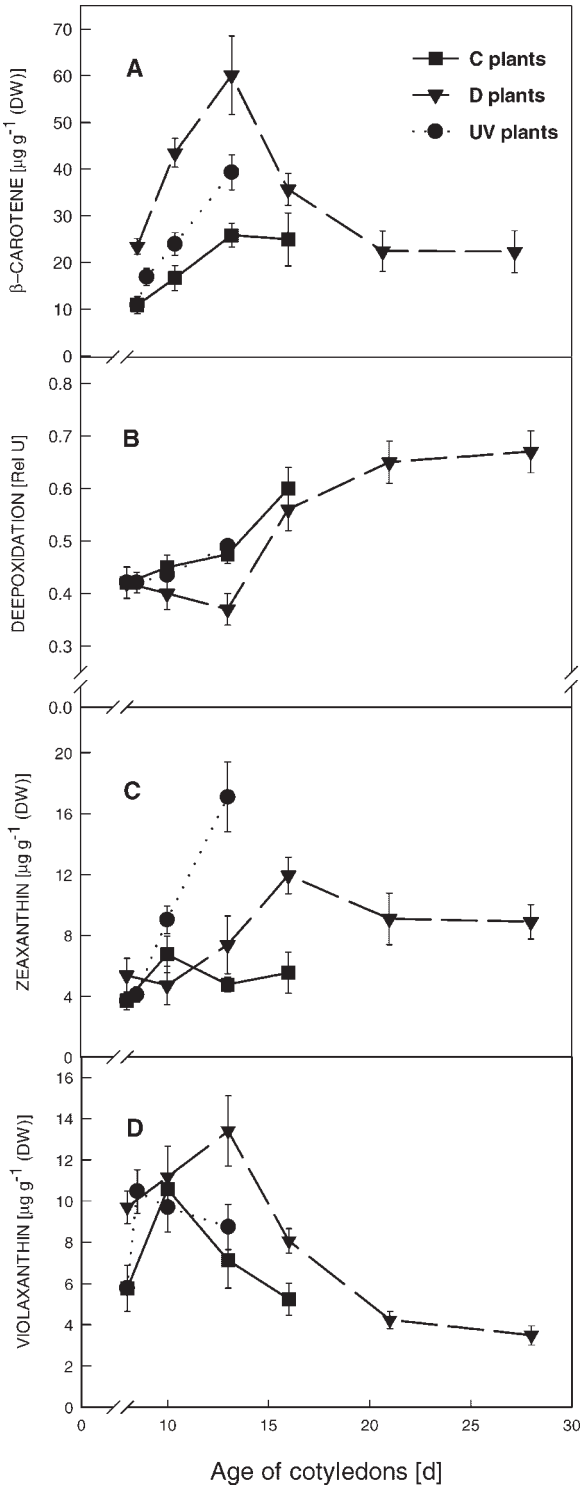
The data reported here are from four experiments with three replications, the data concerning pigment analysis are means from two experiments with three replications. Data and statistical significance of difference were evaluated with analysis of variance (ANOVA) using program NCSS 6.0.21 (NCSS, USA) in the case of ascorbate and glutathione contents and with a t-test in the case of pigment contents.

RESULTS

Cotyledons of C plants were abscised on the 16th d after sowing. At this stage they were yellow and had shrunk extensively. The cotyledons of D plants remained in place up to the 28th d after planting. UV plants cotyledons fell off on the 13th d after sowing.

In C and D plants the maximum of β-carotene content was attained on the 13th d (Figure 1A) but the content in 13 d old D plants was more than twice that in C plants of the same age. UV plants were the only case where the content of β-carotene increased during the whole life span.

With one exception the degree of de-epoxidation in all the cultivations studied increased during the whole life span (Figure 1B). The only exception was in the case of D plants, where a decrease occurred during the first 13 d.



In C and D plants the maximum zeaxanthin content was attained on the 10th and 16th d, respectively (Figure 1C). In UV plants zeaxanthin content increased during the whole life span. In C and D plants the maximum of violaxanthin content was attained on the 10th and 13th d, respectively (Figure 1D). In UV plants the content sharply increased after 2 h of irradiation, then it decreased.

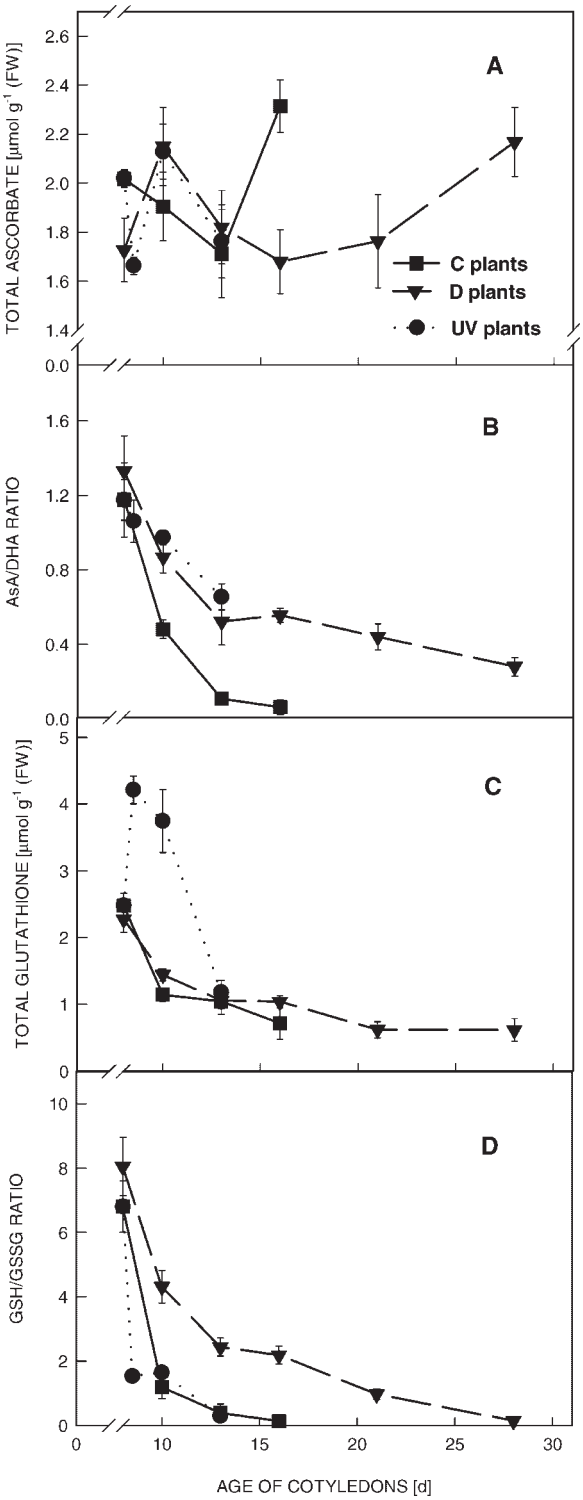
The content of total ascorbate (Figure 2A) in C plants decreased until the 13th d, and on the 16th d it abruptly increased. In D plants, similar to UV plants, a maximum was observed on the 10th d. In D plants the content of total ascorbate between the 10th and 16th d significantly decreased. The content began to rise again on the 21st d and on the 28th d it attained approximately the same value it had on the 10th d. The maximum content in UV plants was observed on the 10th d and UV plants were the only case where the content did not increase at the end of the cotyledon life span.

The reduced/oxidized ascorbate ratio decreased in all the cultivations with one exception—a statistically non-significant increase in 16 d old D plants (Figure 2B). The lowest value was observed at the end of the C plant life span, when the AsA/DHA ratio, as compared with D plants of the same age, was almost 17-fold lower.

The content of total glutathione (Figure 2C) decreased during ageing of cotyledons in all cultivations. Only in UV plants was a marked increase apparent immediately after 2 h of irradiation. However, from then on, the content decreased and at the end of the life span it was not significantly different from C plants.

The GSH/GSSG ratio (Figure 2D) markedly decreased in all cultivations with age. The most significant decrease occurred between the 8th and the 10th d in C and D plants. A sharp decrease also occurred 2 h after UV C irradiation in UV plants. Between the 8th and 10th d, the ratio in UV plants non-significantly increased, then decreased again. There were no statistical differences between cultivations at the end of plant's life span.

Figure 1. Content of β-carotene (A), de-epoxidation state (B) of the xanthophyll cycle pigments (calculated as $(Z + 1/2A)/(V + A + Z)$, where Z is zeaxanthin, A is anteraxanthin and V is violaxanthin), content of zeaxanthin (C) and violaxanthin (D) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Measurements were repeated three times. Means and standard deviations are given. Where bars are not shown, they are smaller than the symbols in the plot



DISCUSSION

Ageing of plants and/or plant organs is associated with increased accumulation of ROS. Their metabolism is under control of enzymic and low molecular weight antioxidative systems. We studied age-dependent changes in low molecular antioxidants, that is carotenoids, ascorbate and GSH during natural and modulated ageing of bean cotyledons. We have previously observed a decline in antioxidative enzyme systems in bean cotyledons[23] and in maize leaves,[24] with increasing age either aged naturally or under mild stress.

Carotenoids are disappear at a much lower rate than chlorophylls.[25] This difference in the degradation rate perhaps reflects the persistence of the photoprotective role of carotenoids, which dissipate excessive radiation energy and protect against damage caused by free radicals.[26] In bean cotyledons, the content of β-carotene increased up to the 13th d in both C and D plants. Results obtained from C and D plants are in agreement with the experiments of Trebst and Depka[27] and Depka *et al.*,[28] who have discussed the role of β-carotene in the rapid turnover and assembly of the D1 protein into the photosystem II centre of the green alga.

In UV plants β-carotene content increased during their whole life span. The number of experiments carried out on carotene radioprotection against UV C irradiation is not large and the published results are somewhat contradictory. In published reports there was practically no difference in UV C survival between carotenoid-containing wild type and colourless mutants of the bacteria *Micrococcus* sp. and *Sarcina lutea*.[29] A high intake of carotenoids protected guinea pigs against lipid peroxidation.[30] Cerdá-Olmedo *et al.*[31] suggested that β-carotene may not quench ROS effectively *in vivo*. However, considering the high induction of β-carotene in UV bean plants, we suggest that β-carotene represents a significant factor in the protection of plants against oxidative damage caused by UV C radiation.

The changes in xanthophyll cycle pigments during senescence have not been fully characterized, but it seems that these changes are dependent on the type of organ. For example an increase in zeaxanthin

Figure 2. Content of total ascorbate (A), reduced/oxidised ascorbate ratio (B), content of total glutathione (C) and reduced/oxidised glutathione ratio (D) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Measurements were repeated three times. Means and standard deviations are given. Where bars are not shown, they are smaller than the symbols in the plot

content was described in wheat leaves during senescence.[10] Zeaxanthin is well known for its role in the quenching of the excited triplet state of chlorophyll, singlet oxygen, free radicals and particularly for the thermal dissipation of excess absorbed energy. In our experiments the decrease of zeaxanthin occurred in C on the 13th d and in D plants on the 21st d. The late decrease of zeaxanthin content in D plants is probably caused by the need to prevent cotyledons' photosynthetic function from damage as long as possible because cotyledons took over the function of the missing leaves. Cotyledons of D plants were also under direct radiation without the protection of leaf cover. The continuous increase of zeaxanthin content in UV plants is in accordance with the evidence for a role of zeaxanthin as a stabilizer of thylakoid membrane function and structure under stress conditions.[32,33]

The source of protective xanthophyll pigments antheraxanthin and zeaxanthin, that is violaxanthin, started to decrease quite early in C and D plants, and in UV plants even from the very beginning of their life span. This was in consequence of the high accumulation of zeaxanthin in these cotyledons with age. The xanthophyll cycle is effective in protecting a plant cell against excessive light that cannot be utilized. The de-epoxidation state of the xanthophyll cycle pigments is correlated with the so-called non-photochemical quenching, that is with the dissipation of excess excitation energy, interpreted as a photoprotective mechanism.[34] The de-epoxidation state increased with cotyledon age in all cultivations studied.

Two non-enzymic antioxidants ascorbate and GSH play an important role in antioxidative defence acting as reductants. We observed an increase in total ascorbate content in C and D plants at the final stage of development. This has been attributed mainly to the oxidized form as the ratio of reduced to oxidized ascorbate dramatically declined during the whole life span. This is in contrast to the widely accepted opinion that total ascorbate content is lowered in senescence.[35] However, in cotyledons, where proliferation of peroxisomes was described in senescence,[36] an increase in content of total ascorbate has been already described.[18,37] Further, Kar and Feierabend[38] also found a slight increase in total ascorbate in detached senescent rye leaves.

The decreasing ratio of reduced to oxidized ascorbate in all cultivations suggests the decreasing antioxidative capacity of the ascorbate pool. This decline is assumed to be due to various factors such as faster utilization of reduced ascorbate and slower re-reduction. Foyer *et al*.[4] suggested that the AsA pool could be lowered by oxidative stress when the capacity of regenerative systems is exceeded. In consequence, if reduced ascorbate is lost, the ascorbate-glutathione cycle malfunctions.[39] Actually, we observed a sharp decrease in the activity of ascorbate peroxidase with the beginning of senescence in cotyledons of all cultivations (Procházková and Wilhelmová, submitted).

In all cultivations the decline of the GSH/GSSG ratio also occurred. This was caused by the increasing GSSG content as the result of an increased GSH oxidation and a decreased GSSG reduction.[19] GSSG content exceeded GSH content in C plants on the 10th d, in UV plants on the 13th d and in D plants on the 16th d. The decrease in reduction of GSSG could be caused by the lack of NADPH, whose production decreases with decreased photosynthesis (the decrease of photochemical efficiency was observed in all cultivations with increasing age, Procházková and Wilhelmová, submitted). We also found that activities of glutathione reductase in cotyledons of all cultivations sharply decreased at the final stage of life span (Procházková and Wilhelmová, submitted).

The shift towards an oxidized status of ascorbate and GSH, which appeared at the end of the life span in all cultivations studied, is in full agreement with the theory that either natural or stress induced ageing involves oxidative stress.[19,40–42]

The decrease in content of reduced low molecular antioxidants content, observed with progressing age in bean cotyledons could further contribute to enhanced oxidative stress. The exceptions are steadily increasing contents of β-carotene and zeaxanthin in cotyledons under harsh UV stress but they had no power to overcome the oxidative stress.

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