

# The capacity of antioxidant protection during modulated ageing of bean (*Phaseolus vulgaris* L.) cotyledons. 1. The antioxidant enzyme activities

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Reactive oxygen species are known to increase in plant senescence. We investigated the participation of antioxidative enzymes in initiation of cotyledon senescence. Senescence of bean (*Phaseolus vulgaris* L.) cotyledons was modulated by UV C irradiation and by the decapitation of plant apices. Senescence was accompanied by a decrease of protein content and by a decrease of photochemical efficiency. A drop in activity of antioxidative enzymes preceded the onset of senescence in control plants. In cotyledons with prolonged life span, the decrease of antioxidant activities and the markers of senescence onset appeared at a similar age as in controls. Thus we presumed that the period from senescence initiation to cotyledon abscission was extended. On the other hand, in UV C irradiated plants we did not observe actual senescence initiation, and antioxidant enzymes although elevated, did not effectively play their role. The decrease of antioxidant enzymes activity and the markers of senescence appeared at a similar age both in control and in decapitated (D) plants, so we can presume that we prolonged mainly the period from senescence onset to cotyledon abscission in D plants. In UV C irradiated plants the antioxidative enzymes were probably destroyed before the process of senescence could begin. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS — antioxidants; bean cotyledons; decapitation; senescence; UV C irradiation

ABBREVIATIONS — APOD, ascorbate peroxidase; C plants, control plants; CAT, catalase; Chl, chlorophyll; d, days; D plants, decapitated plants; DTT, dithiothreitol; DW, dry weight; EDTA, ethylenediamine tetraacetic acid;  $F_v/F_m$ , ratio of variable to maximum fluorescence—the quantum efficiency of open photosystem II centres; FW, fresh weight; GR, glutathione reductase; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]; PS II, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; UV plants, UV C irradiated plants.

## INTRODUCTION

Ageing has been classically defined as the accumulation of changes in plant development responsible for slow, progressive and sequential alterations that accompany the organism with time.<sup>1,2</sup> Senescence is considered to be the final developmental phase of a plant or a plant organ.<sup>3</sup> It is a genetically regulated

degradative process that involves a general decomposition of cellular structures and the mobilization of degradation products to younger or reproductive organs of the plant. Senescence is mainly characterized by a cessation of photosynthesis, disintegration of organelle structures, intensive losses of chlorophyll and proteins, and dramatic increases in lipid peroxidation and membrane leakiness.<sup>4</sup>

Senescence is induced at a certain age, which is specific for every plant species. However, different kinds of stress can induce senescence at any stage of the plant life cycle. The basic metabolic aspects of stress-induced senescence and of natural senescence are in many cases identical or at least very similar.

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This is probably caused by the fact that in both cases, the same adaptive mechanisms are triggered.<sup>5</sup> But stress-induced senescence and natural senescence are not always identical. Kanazawa *et al.*<sup>6</sup> found that changes in the activity of the antioxidative enzymes during dark-induced senescence are generally different from changes during natural senescence. Becker and Apel<sup>7</sup> found that there could be differences in gene expression between natural and stress-induced senescence.<sup>8</sup>

The nature of the inducer(s), initiating a natural senescence programme, is not yet understood. Amongst the various possibilities that are discussed, such as source-sink competitions during redirection of nutrients, changing hormone levels, or changes of the energy status of the cell,<sup>9–11</sup> the free-radical theory has attracted particular attention. The hypothesis was developed that ageing results either particular or predominantly from an accumulation of deleterious free radical reactions<sup>1</sup> and that the onset of senescence is mainly due to the uncontrolled strong enhancement in the generation of reactive oxygen species.<sup>12</sup> Reactions involving reactive oxygen species (ROS) are an intrinsic feature of plant senescence. They promote the process of oxidative deterioration that contributes to cell death.<sup>12</sup> Amongst these ROS are superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\cdot$ ), singlet oxygen ( $^1O_2$ ), alkoxy radicals ( $RO^\cdot$ ) or peroxy radicals ( $ROO^\cdot$ ), which are produced in various metabolic pathways.

Plant cells possess both enzymic and non-enzymic mechanisms, which can protect them against oxidative damage to their components.<sup>13</sup> Superoxide dismutases (SODs; EC 1.15.1.1.) are metalloenzymes, which catalyse the dismutation of  $O_2^-$  to  $H_2O_2$  and  $O_2$ . Removal of  $H_2O_2$  is catalyzed by ascorbate peroxidases (APOD; EC 1.11.1.11) and catalases (CAT; EC 1.11.1.6). Foyer and Halliwell<sup>14</sup> have shown that glutathione reductase (GR; EC 1.6.4.2) and reduced glutathione, one of the non-enzymic antioxidants, in association with ascorbate, another non-enzymic low-molecular weight antioxidant, form a protective system in the so-called Halliwell—Foyer—Asada cycle.

The activity of antioxidative enzymes during senescence usually decreases but the results vary depending on the plant and/or the organ studied. Total SOD activity declined with increasing age in bean cotyledons.<sup>15</sup> In tobacco leaves the activity started to decline after a full leaf expansion was reached.<sup>16</sup> APOD activity decreased in senescent pea leaves,<sup>17</sup> in maize leaves<sup>18</sup> and in senescing detached rice leaves grown both in light and dark conditions.<sup>19</sup> However, APOD activity

increased during senescence in both natural and dark grown senescing cucumber cotyledons.<sup>6</sup> CAT activity decreased during senescence in bean cotyledons,<sup>15</sup> tobacco leaves,<sup>16</sup> cucumber cotyledons<sup>6</sup> and rice leaves.<sup>20</sup> GR activity decreased in ageing of chrysanthemum petals.<sup>21</sup> Its activity decreased during natural and dark induced senescence of cucumber cotyledons.<sup>6</sup> On the contrary, in rice leaves, GR activity increased in light grown senescing leaves but slightly decreased in dark grown senescing leaves.<sup>19</sup>

We studied bean cotyledons as they have a quite definite life span. This was further modulated in order to find the role of antioxidant protection at the onset of senescence in the same organ but with different lengths of life span. The life span length was either prolonged by apical decapitation or shortened by UV irradiation.

## MATERIALS AND METHODS

### *Plant material*

Bean (*Phaseolus vulgaris* L., cv. Jantar) cotyledons were studied. Bean plants were grown in sand in plastic trays.

Control plants (C plants) were grown in a growing chamber (Klimax 1300, Czechoslovakia) with 16 h photoperiod under the following conditions: temperature 24°/18°C, air humidity 60/80% and irradiation of 220/0  $\mu\text{mol (PAR) m}^{-2} \text{s}^{-1}$  day/night, respectively.<sup>22</sup> Supplemental irradiation with UV C 253.7 nm was used to shorten the life span in the case of UV plants (UV lamp Philips TUV 30 W, 6.1 W/cm<sup>2</sup> at leaf surface from 8th day for 8 h per day). Cotyledon life span was extended by repeated decapitation of plant shoots (D plants). The whole shoot above the cotyledons was excised on the 7th day (d) and then whenever needed.

All plants were watered daily with distilled water and twice a week with Hewitt solution. The cotyledons of C plants finished their life span at the age of 16 d and were harvested at plant age 8, 10, 13 and 16 d. The cotyledons of D plants finished their life span at the age of 28 d and were harvested at plant age 8, 10, 13, 16, 21 and 28 d. The first sampling of UV plants was carried out on the 8th d before start of UV C irradiation and the second sampling was carried out 2 h after start of irradiation. Cotyledons of UV plants finished their life span at the age of 13 d and were harvested at plant age 8, 10 and 13 d. Samples were deep frozen in liquid nitrogen immediately after harvesting and stored at  $-70^\circ\text{C}$  until analysis for content of proteins, pigments and antioxidants.

### *Antioxidant enzymes activities*

For APOD, CAT and GR assays, bean cotyledons were homogenized in the extraction medium (0.1 M Tris, 1 mM dithiothreitol, 1 mM EDTA Na<sub>2</sub>, 1% Triton X-100, 5 mM ascorbic acid, pH 7.8), 5 ml/1 g fresh weight (FW) using an Ultra Turrax (Ika, Germany) at half speed. The samples were processed for 2 min by ultrasound in an ice bath and incubated in ice for 30 min in the dark. Finally they were centrifuged at 20 000 g and 2°C for 10 min.

For SOD assay, bean cotyledons were homogenized in the extraction medium (0.01 M phosphate buffer, 0.03 M KCl, pH 7.4), 5 ml/1 g FW. The samples were processed by ultrasound in an ice bath as for other enzymes. After centrifugation for 10 min, 20 000 g at 2°C the supernatant was desalted through Sephadex G-25 column, using the extraction medium for the preparation of the column. The supernatants were frozen in liquid nitrogen and stored at -70°C for further use.

The activities of SOD, APOD and GR were measured spectrophotometrically (Hitachi U 3300, Japan) at 25°C. SOD activity was measured at 550 nm with the cytochrome c method.<sup>23</sup> One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the cytochrome c reduction rate. APOD activity was determined by the decrease in absorbance at 290 nm due to ascorbate oxidation.<sup>24</sup> GR activity was determined by the decrease in absorbance at 340 nm due to the oxidation of NADPH.<sup>25</sup> CAT activity was estimated polarographically<sup>26</sup> using a liquid-phase oxygen electrode (Hansatech Instruments, Great Britain).

SOD and GR isoenzymes were visualised on 7.5% homogenous gels after discontinuous PAGE under non-denaturing and non-reducing conditions. The methods used for SOD detection were standard methods for gel staining with nitroterazolium blue chloride.<sup>27</sup> The different isoenzymes were distinguished by their sensitivity to inhibition by 2 mM KCN and 5 mM H<sub>2</sub>O<sub>2</sub>. Mn SOD is resistant to both KCN and H<sub>2</sub>O<sub>2</sub>, Fe SOD is resistant to KCN but inhibited by H<sub>2</sub>O<sub>2</sub>, Cu/Zn SOD is inhibited by both inhibitors.<sup>28</sup> For detection of GR isoenzymes detection a staining method with 3-(4,5-dimethylthiazol-2-4)-2,5diphenyl tetrazolium bromide (MTT) and 2,6-dichlorophenolindophenol was used.<sup>29</sup> The duplicate gels without GSSG were used as a control.

### *Content of pigments*

The pigment contents 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 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) 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.

### *Fluorescence parameters*

Photochemical efficiency of electron transport through photosystem II (PS II) was specified from chlorophyll fluorescence induction kinetics. It was measured after a 15 min dark period with the PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) on detached fresh cotyledons at room temperature. Three to five cotyledons (depending on size) were stuck on a tape to cover the whole area of a chamber. Measuring irradiation was 0.35 µmol m<sup>-2</sup> s<sup>-1</sup>, actinic irradiation 200 µmol m<sup>-2</sup> s<sup>-1</sup>, 700 ms saturated flash 2500 µmol m<sup>-2</sup> s<sup>-1</sup>. The DA 100 Data Acquisition System (Walz, Effeltrich, Germany) was used for sampling and calculation. The chlorophyll fluorescence parameter  $F_v/F_m$  ( $F_v = F_m - F_0$ ) was calculated using the nomenclature according to van Kooten and Snel.<sup>30</sup>

### *Protein content*

Protein content was determined by the method of Bradford,<sup>31</sup> with standard curves prepared using bovine serum albumin.

### *Statistical evaluation*

The data of all measurements are means from four experiments with three replications, except the data concerning pigment analysis that are 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 Jr (NCSS, USA) in the case of enzyme activities and photochemical efficiency and with 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 sowing. On the other hand, in plants with a shortened life span (UV plants), cotyledons fell off on the 13th d after sowing. Their cotyledons turned brown probably due to protective pigments.

The content of soluble proteins in cotyledons decreased at the end of the life span of bean cotyledons, that is on the 13th, 16th and 28th d in the case of UV, C and D plants, respectively (Figure 1A). This content in senescent cotyledons was not significantly different from the content in 8 d old plants in the case of C plants. In 28 d old D plants the content was significantly higher compared to 8 d old D plants. At the end of the life span of UV plants the level was almost two times higher than at the end of life span of C plants.

The content of total chlorophyll in C plants reached its maximum on the 13th d (Figure 1B). The highest content was in D plants. Its level increased until the 13th d, for next three days it remained practically constant and then decreased during the whole remaining life span. In the case of UV plants the chlorophyll content increased during the whole life period.

The  $F_v/F_m$  ratio, which reflects the maximum photochemical efficiency of PS II, decreased during ageing in all studied cultivations (Figure 1C). The optimum value of the  $F_v/F_m$  ratio of a mature healthy plant is in the range 0.75–0.85. C plants fell to a value lower than 0.75 after the 13th d and D plants on the 16th d. In the case of UV plants this ratio was already lower after 2 h of irradiation and decreased during the whole remaining life span.

During ageing the activity of antioxidant enzymes usually decreased. Total SOD activity attained a maximum value on the 10th d in C plants, in D plants it was almost constant until the decrease on the 28th d (Figure 2A). Total SOD activity in UV plants attained the highest values of all the studied cultivations with the maximum on the 10th d. By using particular inhibitors we probed individual isoenzyme activities. The presence of Fe SOD in bean cotyledons was not detected. The main activity of SOD was represented by Cu/Zn SOD (Figure 2B). Its course was very similar to the course of total SOD activity. Activity of Mn SOD was highest in C and D plants on the 13th d (Figure 2C). In UV plants its course was the same as in the case of total SOD and Cu/Zn SOD.

Five bands of SOD activity were distinguishable in zymograms after gel staining of overall activity; one band for Mn SOD, four bands for Cu/Zn SOD (Figure 3). Individual Cu/Zn SOD isoenzymes were

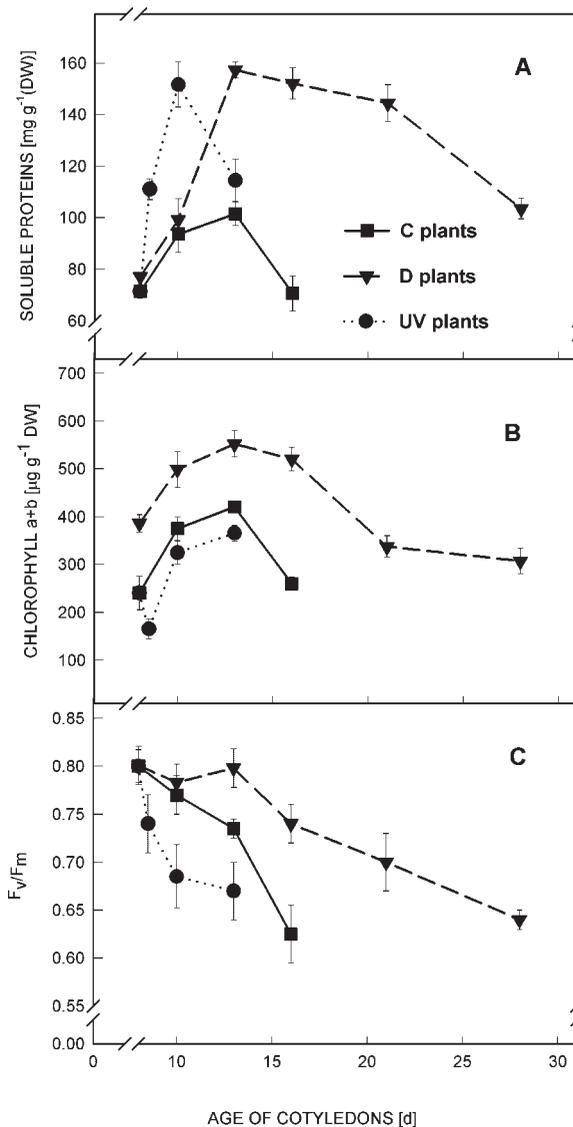


Figure 1. Content of soluble proteins (A), total chlorophyll (B) and maximal efficiency of photosystem II ( $F_v/F_m$ ) (C) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Measurements were repeated three times. Means and standard deviations are given. Where bars are not shown, they are smaller than the symbols in the plot

marked by numbers 2–5 according to increasing mobility on the gel. Bands 3 and 4 showed decreasing intensity with increasing age in all cultivations.

The activity of APOD in C plants attained a maximum value on the 10th d (Figure 4A). In 8 d old D plants the activity was lower than in the case of other

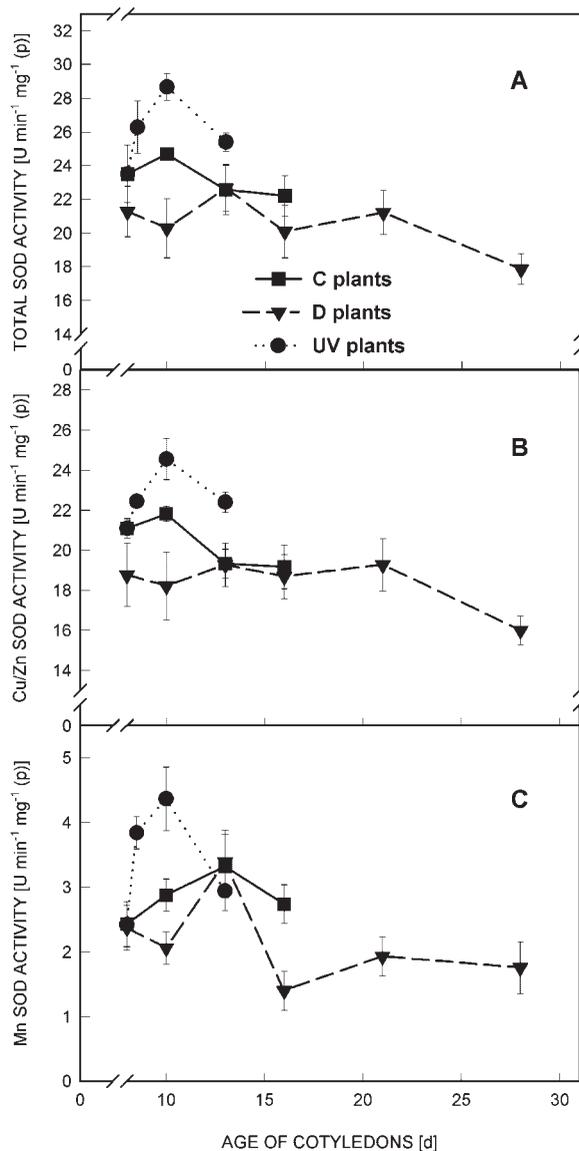


Figure 2. Activity of total superoxide dismutase (A), Cu/Zn superoxide dismutase (B) and Mn superoxide dismutase (C) per mg protein (p) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. One unit of superoxide dismutase was defined as the amount of enzyme necessary to produce a 50% inhibition of the cytochrome c reduction rate. 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

cultivations and increased on the 10th d. On the 13th d it decreased, but by the 16th d it doubled and then started to decrease again. In UV plants the activity of APOD was higher during the whole life span than

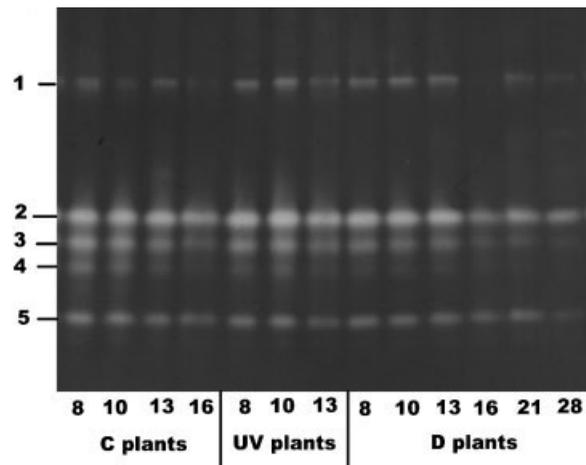


Figure 3. Zymograms of superoxide dismutase isoenzymes in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Non denaturing electrophoresis was carried out on 7.5% gels and stained with nitrobluetetrazolium blue chloride. Band marked No. 1 is Mn superoxide dismutase, bands No. 2–5 are Cu/Zn superoxide dismutase

in the case of cotyledons of the same age in other cultivations. They attained a maximum value on the 10th d.

The maximum GR activity in C plants was observed on the 10th d (Figure 4B). In D plants, its activity decreased from the 13th d up to the end of their life span. The highest GR activity was observed again in UV plants. It had already increased two-fold 2 h after start of irradiation and attained its highest value on the 10th d.

Five isoenzymes of GR were distinguishable in zymograms after gel staining with MTT (Figure 5). Individual isoenzymes were marked by numbers 1–5 according to increasing mobility on the gel. The maximum changes in intensity were observed in band number 4 whose intensity increased on the 13th d in C plants, on the 16th d in D plants and was very intense in 10 and 13 d old UV plants. The changes in intensity of other bands during ageing were not so conspicuous.

The CAT activity continuously decreased in C plants during the whole life span (Figure 4C). The same situation occurred in D plants with only one exception—an insignificant increase between 13th and 16th d. On the other hand, CAT activity increased in UV plants during the whole life span.

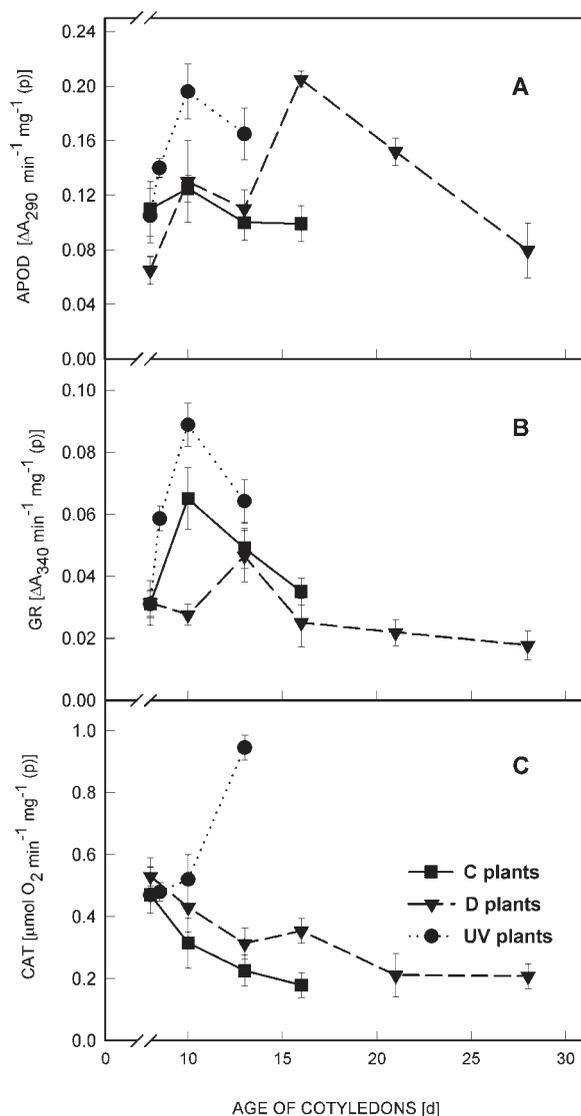


Figure 4. Activity of ascorbate peroxidase (A), glutathione reductase (B) and catalase (C) per mg protein (p) in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Measurements were repeated three times. Means and standard deviations are given. Where bars are not shown, they are smaller than symbols in the plot

## DISCUSSION

The aim of this study was to examine any relationship between changes in antioxidative capacity and the onset of senescence dependent on the length of cotyledon life span.

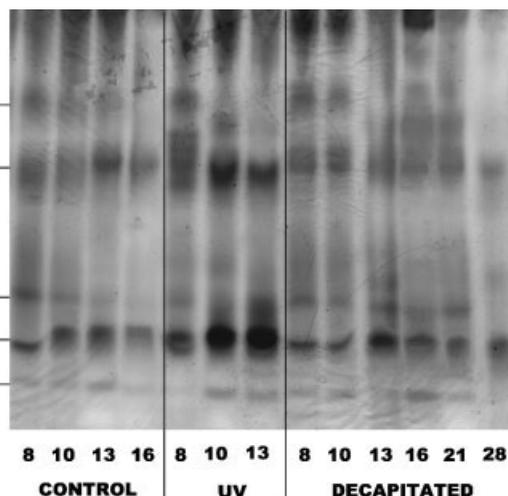


Figure 5. Zymograms of GR isoenzymes in bean (*Phaseolus vulgaris* L.) cotyledons with natural life span (C plants) and with artificially prolonged (D plants) and shortened (UV plants) life span. Non denaturing electrophoresis was carried out on 7.5% gels and stained with 3-(4,5-dimethylazol-2-4)-2,5diphenyl tetrazolium bromide

Chlorophyll content has been widely used as a benchmark of leaf senescence.<sup>9,32</sup> In senescent leaves chlorophyll is generally degraded and the rate of photosynthesis declines.<sup>33</sup> We used photochemical efficiency ( $F_v/F_m$  ratio) and protein content as further markers for the determination of senescence. The information available about changes of photochemical efficiency during cotyledon ageing is very limited, because the majority of the work has been done on leaves. Our results indicate that the  $F_v/F_m$  changes in bean cotyledons are very similar to the changes seen in leaves. We can conclude from decreases in both chlorophyll and  $F_v/F_m$  values that onset of senescence occurred after the 13th d in C plants. Finally, it was in agreement with another marker of senescence: the sharp decrease of protein content. Leaf senescence involves the transport of nutrients resulting from protein degradation to other regions of plant.<sup>34</sup> According to Thimann's theory and from measurements of markers we concluded that senescence of bean cotyledons was initiated in the 13th d in control plants.

The maximum activity of SOD, APOD and GR in C plants occurred on the 10th d. The ratio of individual Cu/Zn SOD isoforms was influenced by the age of the plants: The third and fourth isoforms practically disappeared in old plants. It could be explained by observations that oxidative stress induced different changes in SOD isoforms.<sup>35,36</sup>

A drop in activities of these enzymes preceded the onset of senescence in C plants. Thus the hypothesis is that this drop allows ROS to increase in concentration and act as a signal and/or cause of oxidative stress.

On the contrary, CAT activity declined during the whole life span of C plants. As a consequence of the decrease in CAT activity an accumulation of H<sub>2</sub>O<sub>2</sub> in peroxisomes would normally take place. A certain amount of H<sub>2</sub>O<sub>2</sub> may leak into the cytosol. This could be in agreement with the conclusion of Kanazawa *et al.*<sup>6</sup> that peroxisomes are responsible for the increased oxidative damage of senescent cells by generating H<sub>2</sub>O<sub>2</sub>. The senescence-inducing H<sub>2</sub>O<sub>2</sub> leaked from peroxisomes might also act in the cytosol as a second messenger in cellular signal transduction pathways that lead to specific gene expression.<sup>37,38</sup>

Surprisingly, the chlorophyll content increased during the whole life span in UV plants. Exceptions to the rule of chlorophyll content decrease as a marker of onset of senescence have been described previously<sup>33,39</sup> and this could be a case of such exceptions as follows from other parameters. Moreover, in UV plants the chlorophyll breakdown was probably effectively prevented by a brown layer on the surface of their cotyledons. We presume that this layer consists mainly of phenolic compounds, which have an antioxidant protective function. Isolated anthocyanins were found to have greater antioxidative ability than that of  $\alpha$ -tocopherol.<sup>40</sup>

The  $F_v/F_m$  ratio, reflecting photochemical efficiency, was already lower than the optimum value (0.85–0.75) after 2 h of UV C irradiation. This suggests that from the very beginning of UV C irradiation, these plants were under a severe stress, which remained during their whole life span. Casati and Andreo<sup>41</sup> found that UV C irradiation enhanced the levels of ROS and caused inhibition and damage to the photosynthetic apparatus, such as PS II degradation. Simultaneous enhancement of the protein content in whole life span of UV cotyledons reflected stress response expression of stress proteins. On the other hand, the decrease of chlorophyll content as a principal marker of senescence did not occur. So we can hypothesize that cotyledons in this cultivation suffered from strong oxidative stress that prevented the senescence program from being initiated. UV C irradiation is very effectively absorbed by nucleic acids<sup>42</sup> causing their direct damage. As is well known, the initiation of senescence requires the coordinated activation of a suite of specific genes<sup>34</sup> and therefore in the case of UV plants, senescence was probably prevented due to impairment of genetic apparatus.

In UV plants we observed increased activities of all antioxidant enzymes compared to controls. They were probably induced by the imposed oxidative stress. It is known that plants activate protective enzymes when production of oxygen radicals is stimulated by stress.<sup>43</sup> The maximum activities of antioxidant activities except CAT were observed on the 10th d. The observed decrease of the activity of SOD, APOD and GR on the 13th d could be connected with destruction of proteins and inhibition of protein synthesis. On the other hand, CAT activity increased during their whole life span. Boldt and Scandalios<sup>44</sup> observed that UV light in the range of 240–400 nm can cause a strong transient induction of CAT. Maybe the 8 h long period of UV C irradiation used in our experiments was long enough to induce a strong induction of CAT. During the following 16 h period without irradiation, the CAT activity remained at the same level and the next 8 h of irradiation caused another strong induction of CAT.

UV plants were exposed to strong oxidative stress and the senescence programme did not proceed. The senescence initiation was prevented by cellular oxidative damage. The severe oxidative stress was supported by our previous findings of an extremely high level of lipid peroxidation, which increased steadily with cotyledon age of UV plants.<sup>45</sup> Antioxidative protection finally failed.

Decapitation prolonged cotyledons life span up to 28 d. The removal of epicotyls leads to an elevation in the ratio of cytokinins to auxins in the rest of the plant.<sup>46</sup> Cotyledons of D plants were influenced by three factors: 1) after the removal of the leaves the cotyledons took over the function of the leaves, 2) cotyledons were without leaf cover and were exposed to higher irradiation than controls, 3) plants were damaged by repeated wounding.

Similar to C plants, the decrease of antioxidant enzymes activity preceded the decrease of chlorophyll content and photochemical efficiency. Although the decrease of antioxidative protection and the markers of senescence appeared after the 13th and 16th d respectively, the period between the onset of senescence and abscission was prolonged for up to 28th d. Considering the time when the decrease of antioxidative protection and the markers of senescence appeared, we can presume that we prolonged the period from senescence onset to cotyledon abscission.

We found that the time course of antioxidant enzymes activities in cotyledons with manipulated life span differed from those in control plants. The stress imposed through our interventions interfered with the senescence programme. The strength of stress was

decisive: In UV plants the oxidative stress was very strong and prevailed. In D plants, peaks of antioxidant enzyme activities did not closely precede cotyledon death. The age when their maximum activities was achieved corresponded, surprisingly, to that of controls and also initiation of senescence was at a similar age i.e. fairly long before cotyledon death. Thus, the onset of senescence corresponded with the decrease of antioxidant enzyme activities in these two cultivations.

The onset of senescence programme which is inherent in bean cotyledons and is induced in normal conditions could be overcome by imposing a mild stress. Prolonged life span was only due to a prolonged period of senescence, not due to its postponed onset.

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#### REFERENCES

- Harman D. The aging process. *Proc Nat Acad Sci USA* 1981; **78**: 7124–7128.
- Harman D. The aging process: major risk factor for disease and death. *Proc Nat Acad Sci USA* 1991; **88**: 5360–5363.
- Leopold AC. Aging and senescence in plant development. In *Senescence in Plants*, Thimann KV (ed.). CRS Press, Boca Raton: Florida 1980; 1–12.
- Buchanan-Wollaston V. The molecular biology of leaf senescence. *J Exp Bot* 1997; **307**: 181–199.
- Olsson M. Alterations in lipid composition, lipid peroxidation and anti-oxidative protection during senescence in drought stressed plants and non-drought stressed plants of *Pisum sativum*. *Plant Physiol Biochem* 1995; **33**: 547–553.
- Kanazawa S, Sano S, Koshiha T, Ushimaru T. Changes in antioxidative enzymes in cucumber cotyledons during natural senescence: comparison with those during dark-induced senescence. *Physiol Plant* 2000; **109**: 211–216.
- Becker W, Apel K. Differences in gene expression between natural and artificially induced leaf senescence. *Planta* 1993; **189**: 74–79.
- Weaver L, Himelblau E, Amasino R. 1997 Leaf senescence: gene expression and regulation. In *genetic Engineering. Principles and Methods*, Setlow JK (ed.). Plenum Press: New York, 1997; 215–234.
- Thimann KV. The senescence of leaves. In *Senescence in Plants*, Thimann KV (ed.). CRS Press, Boca Raton: Florida, 1980; 86–115.
- Thomas H, Stoddart JL. Leaf senescence. *Ann Rev Plant Physiol* 1980; **31**: 83–111.
- Kar M, Feierabend J. Metabolism of activated oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. *Planta* 1984; **160**: 385–391.
- Thompson JE, Barber RF. The role of free radicals in senescence and wounding. *New Phytol* 1987; **105**: 317–344.
- Foyer CH, Descourvieres P, Kunert KJ. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant Cell Environ* 1994; **17**: 507–523.
- Foyer CH, Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* 1976; **133**: 21–25.
- Pauls KP, Thompson JE. Evidence for the accumulation of peroxidized lipids in membranes of senescing cotyledons. *Plant Physiol* 1984; **75**: 1152–1157.
- Dhindsa RS, Plumb-Dhindsa P, Thorpe TA. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 1981; **126**: 93–101.
- Jiménez A, Hernandez JA, Pastori G, del Río LA, Sevilla F. Role of ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. *Plant Physiol* 1998; **118**: 1327–1335.
- Procházková D, Sairam RK, Srivastava GC, Singh DV. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Sci* 2001; **161**: 765–771.
- Chang CJ, Kao H. H<sub>2</sub>O<sub>2</sub> metabolism during senescence of rice leaves: changes in enzyme activities in light and darkness. *Plant Growth Regul* 1998; **25**: 11–15.
- Lin J-N, CH Kao. Catalase activity and senescence of detached rice leaves. *Chinese Agron J* 1998; **8**: 143–149.
- Bartoli CG, Simontacchi M, Montaldi ER, Puntarulo S. Oxidants and antioxidants during aging of chrysanthemum petals. *Plant Sci* 1997; **129**: 157–165.
- Wilhelmová N, Wilhelm J, Kutík J, Haisel D. Changes in French bean cotyledon composition associated with modulated lifespan. *Photosynthetica* 1997; **34**: 377–391.
- McCord JM, Fridovich I. Superoxide dismutase: an enzymatic function of erythrocyte. *J Biol Chem* 1969; **244**: 6049–6055.
- Nakano Y, Asada K. Hydrogen peroxide scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 1981; **22**: 867–880.
- Schaedle M, Bassham JA. Chloroplasts glutathione reductase. *Plant Physiol* 1977; **59**: 1011–1012.
- del Río LA, Ortega MG, Lopez AL, Gorge JL. A more sensitive modification of the catalase assay with Clark oxygen electrode. Application to the kinetic study of the pea-leaf enzyme. *Anal Biochem* 1977; **80**: 409–415.
- Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; **44**: 276–287.
- Fridovich I. Superoxide dismutases. *Advances in Enzymol* 1986; **58**: 61–97.
- Rao MV, Paliyath G, Ormrod DP. Ultraviolet B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol* 1996; **110**: 125–136.
- van Kooten O, Snel JFH. The use of chlorophyll fluorescence and nomenclature in plant stress physiology. *Photosynth Res* 1990; **25**: 147–150.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
- Matile P. Chloroplast senescence. In *Crop Photosynthesis: Spatial and Temporal Determinants*, Baker NR, Thomas H (eds). Elsevier: Amsterdam, 1992; 413–440.
- Thomas H. Ultrastructure, polypeptide composition and photochemical activity of chloroplasts during foliar senescence of a non-yellowing mutant genotype of *Festuca pratensis* Huds. *Planta* 1977; **137**: 53–60.
- Lohman KN, Gan S, John MC, Amasino RM. Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiol Plant* 1994; **92**: 322–328.

35. Edwards EA, Enard C, Creissen GP, Mullineaux PM. Synthesis and properties of glutathione reductase in stressed peas. *Planta* 1994; **192**: 137–143.
36. Lacan D, Baccou J-C. High levels of antioxidant enzymes correlate with delayed senescence in non-netted muskmelon fruits. *Planta* 1998; **204**: 377–382.
37. Baeuerle PA, Rupec RA, Pahl HL. Reactive oxygen intermediates as second messengers of a general pathogen response. *Pathol Biol* 1996; **44**: 29–35.
38. del Río LA, Pastori GM, Palma JM, Sandalio LM, Sevilla F, Corpas FJ, Jiménez A, López-Huertas E, Hernández JA. The activated oxygen role of peroxisomes in senescence. *Plant Physiol* 1998; **116**: 1195–1200.
39. Grabau LJ. Physiological mechanisms of plant senescence. In *Handbook of Plant and Crop Physiology*, Pessarakli M (ed.). Marcel Dekker: New York, Basel, Hong Kong, 1995; 483–496.
40. Ishii G, Mori M, Umamura Y. Antioxidative activity and food chemical properties of anthocyanins from colored tuber flesh of potatoes. *J Jap Soc Food Sci Technol* 1996; **43**: 962–966.
41. Casati P, Andreo CS. UV-B and UV-C induction of NADP-malic enzyme in tissues of different cultivars of *Phaseolus vulgaris* (bean). *Plant, Cell Environ* 2001; **24**: 621–630.
42. Cerdá-Olmedo E, Martín-Rojas V, Cubero B. Causes of cell death following ultraviolet B and C exposures and the role of carotenes. *Photochem Photobiol* 1996; **64**: 547–551.
43. Elstner EF, Wagner GA, Schultz W. Activated oxygen in green plants in relation to stress situations. *Curr Topics Plant Biochem Physiol* 1988; **7**: 159–187.
44. Boldt R, Scandalios JG. Influence of UV-light on the expression of the CAT 2 and CAT 3 catalase genes in maize. *Free Radical Biol* 1997; **23**: 505–514.
45. Wilhelmová N, Procházková D, Macháčková I, Vágner M, Srbová M, Wilhelm J. The role of cytokinins and ethylene in bean cotyledon senescence. The effect of free radicals. *Biol Plant* 2004; **48**: 523–528.
46. Slab K, Šebánek JP. Content of endogenous cytokinins in intact and transversally cut leaf blades of *Bryophyllum crenatum*. *Plant Growth Regul* 1984; **2**: 111–116.