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The role of cytokinins and ethylene in bean cotyledon senescence. The effect of free radicals

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Abstract

During ageing of bean (*Phaseolus vulgaris* L.) cotyledons in plants with modified life span the time-course of four cytokinins, ethylene, and the end products of free radical attack, lipofuscin-like pigments (LFP), were studied. UV irradiation shortened cotyledon life span, while epicotyl decapitation prolonged it. In controls, LFP increased at the senescence onset but at the end of life span it returned to the initial level. Ethylene increased more than 3-fold at the time of abscission. The content of individual cytokinins (zeatin, zeatin riboside, isopentenyl adenine, isopentenyl adenine riboside) varied differently during ageing but they did not decreased in any case under level observed in young cotyledons at the time of abscission. UV irradiation resulted in 14-fold increase in LFP concentration at the end. Ethylene increased 8-fold 2 h after irradiation. Individual cytokinins increased after UV irradiation to a different extent and time-course, nevertheless cotyledon life span was shortened. Decapitation induced LFP decrease. On day 13, LFP abruptly increased and than decreased and stayed lowered until abscission. Ethylene was maximum on day 24, at the time of abscission, it was above 200 % of control. Decapitation produced transient decrease in some cytokinins namely zeatin and isopentenyl adenine riboside.

Additional key words: ageing epicotyl decapitation, lipid peroxidation, lipofuscin-like pigments, *Phaseolus vulgaris*, UV irradiation.

Introduction

Senescence is the final stage of plant development, which is functionally separated from the previous period. It is generally held that senescence is controlled by specific genes. The switching on of these genes in a given organ is caused by factors which are extrinsic, such as environmental factors (*e.g.* change in the length of the daylight) or hormonal signals originating from other parts of the plant.

In the earlier studies it has been found that abscisic acid (ABA) and ethylene accelerate the symptoms of senescence (Smart 1994) while cytokinins delayed the onset of senescence (Badenoch-Jones *et al.* 1996). Ethylene is not considered to be an initiator of senescence, however, an increase in its production accompanied senescence (Orzáez *et al.* 1999). Increased cytokinins, when applied exogenously (Van Staden *et al.* 1988) or increased endogenously in transgenic plants, effectively postpone senescence (Gan and Amasino 1995, Jordi *et al.* 2000, McCabe *et al*. 2001). On the other hand, content of some cytokinins decrease in the course of senescence (Van Staden 1996). Buchanan-Wollaston (1997) has postulated that cytokinins, either directly or indirectly via a signalling pathway, inhibit the transcription of senescence associated genes. With

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Abbreviations: ABA - abscisic acid; Chl - chlorophyll; d.m. - dry mass; f.m. - fresh mass; iP - N^6-(Δ^2isopentenyl)adenine; iPR - N^6-(Δ^2isopentenyl)adenine riboside; LFP - lipofuscin-like pigments; Z - zeatin; ZR - zeatin riboside.
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regard to the action of cytokinins it has been suggested that they act in synergy or in antagonism with other signals. This concept could explain why they are able to elicit many different biological responses (Brault and Maldinay 1999). For instance, cytokinins exert control on gravitropism that could be mediated by ethylene (Golan *et al.* 1996).

The free radical theory of ageing presumes a central role of reactive oxygen species (Harman and Piette 1966, Harman 1972). In plants, reactive oxygen species originate from photochemical reactions and together with other free radicals formed by various non-enzymic and enzymic reactions constitute standard reactants of plant cell metabolism. Early studies confirmed increased free radical-mediated lipid peroxidation in senescent leaves and a failure of the protective mechanisms against free radicals during senescence (Dhindsa *et al.* 1981).

Characteristic end-products of lipid peroxidation are represented by the so called lipofuscin-like pigments (LFP). These autofluorescent compounds originate from the reactions of aldehydic decomposition products. After their discovery in the animal material (Chio and Tappel 1969), they were also observed by several authors in plant ageing (Maguire and Haard 1975, Wilhelm and Wilhelmová 1981). Accumulation of LFP was observed in microsomal membranes from senescing bean cotyledons (Pauls and Thompson 1984). The formation of LFP can be modulated by treatment with exogenous substances. An increase in LFP concentration was observed in chloroplasts isolated from corn and rice leaves treated with ABA (Sun and Lin 1988). On the other hand, treatment of the leaves of rice, amaranth, and corn with α-tocopherol, a free radical scavenger, inhibited the formation of LFP (Wang *et al.* 1988). In senescent cotyledons, the percentage of LFP decreased in cytoplasm and increased in microsomal membranes (Hudák *et al.* 1995). We have characterized LFP in ageing of bean cotyledons and their time-course after decapitation of the plant epicotyl (Wilhelmová *et al.* 1997).

Free radicals interact with the processes of hormonal regulation. Correlations have been found between superoxide formation and the increase in ethylene production (Kacperska and Kubacka-Zebalska 1985). It appears that free radical-mediated membrane damage and ethylene biosynthesis are closely related (Paulin *et al.* 1986, Matoo *et al.* 1992, Lidon *et al.* 1995). Membrane lipid peroxidation may be triggered by ferrous ions and ascorbate that are concomitantly used in ethylene biosynthesis (Moya-Leon and John 1995).

Because of their central role in the regulation of senescence, the question of the relationship of cytokinins to free radicals is extremely important. It has been shown that cytokinins inhibit the activity of xanthine oxidase, one of the cellular free radical sources, and also act as a direct free radical scavenger (Leshem *et al.* 1981).

In the present study we have manipulated the life span of bean cotyledons by two different treatments which either prolonged (epicotyl decapitation) or shortened (UV irradiation as a source of free radicals) their life span in relation to controls. We have studied how these changes affected the concentrations of cytokinis, ethylene, and the end-products of free radical reactions in the course of senescence. The results imply involvement of free radicals as a signal in senescence.

Materials and methods

Plants: Bean (*Phaseolus vulgaris* L. cv. Jantar) cotyledons were used for our experiments. Plants were grown in plastic trays filled with sterilized sand. Plants were watered by distilled water daily and twice a week by Hewitt nutrient solution [4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 1.5 mM $MgSO_4$, 1.33 mM $NaHPO_4$, 0.5 mM $FeNa_2EDTA$, 10 μM $MnSO_4$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 30 μM H_3BO_3, 0.1 mM NaCl, 0.5 μM Na_2MoO_4].

We manipulated cotyledon life span in order to found effect of different ageing on the contents of hormones. Control plants were grown in a growth chamber (*Klimabox 1300*, *Kovodružstvo*, Slaný, Czech Republic) at day/night temperature 18/16 °C, air humidity 60/80 % and irradiation (400 - 700 nm) of 220 μmol(photon) m^{-2} s^{-1} during 12-h photoperiod. In order to obtain cotyledons with different life span we conditioned the plants by various means. The life span was lengthened up to 28 d by plant decapitation. The whole shoot above cotyledons was excised when the plant raised up (8th d) and always when needed. The life span was shortened to 13 d when bean plants grews under supplementary UV irradiation started on day 8. The UV lamp *Philips TUV* (Eindhoven, The Netherlands; 30 W, 253.7 nm) was employed 8 h daily with irradiance 6.1 W m^{-2} at plant surface.

The cotyledons were harvested at plant age 8, 10, 13, 16, 21, 28 d in the case of decapitated plants, control plants finished their life span at the age of 16 d, UV irradiated plants at the age of 13 d. Samples were deep frozen in liquid nitrogen immediately after harvesting and stored at -70 °C until use. We used bean cotyledons of various ages as a source for cytokinins, LFP and chlorophyll determinations. Ethylene was determined in a freshly harvested material.

Determination of cytokinins: Cytokinins were determinated by ELISA test after HPLC resolution of individual cytokinins in methanolic extracts purified by DEAE-cellulose chromatography and *Sep-pak* (*Waters*

Corp., Milford, USA) cartridge according to method of Macháčková *et al.* (1993). Each sample was assayed in two concentrations in triplicates.

Ethylene assay: Cotyledons of experimental plants were excised and placed to vials (2 cotyledons per 20 cm^3) tightly closed with rubber septum and exposed there for 4 h at 24 °C in the darkness. Ethylene was assayed by gas chromatography (*GC Hewlett Packard 5890*, Houston, USA) equipped with FID (5). Measurements were repeated four times.

LFP assay: LFP were estimated in lipophilic extracts of whole cotyledons in chloroform after HPLC (*Shimadzu LC9*, Kyoto, Japan) resolution. They were characterized by blue fluorescence emission after excitation in UV using fluorimeter *Perkin Elmer LS5* (Boston, USA; Wilhelmová *et al.* 1997). The fluorometer was calibrated with the standard *No. 5* of the instrument manufacturer. For the determination of LFP concentration wavelengths 355/400 nm (excitation/emission) were used and fluorescence was expressed in relative fluorescence units (RFU).

Chlorophyll content was estimated after fresh cotyledon homogenisation in 85 % acetone. Concentrations were determined on *Hitachi U-3300* (Tokyo, Japan) spectrophotometer after double centrifugation at 5 000 *g* for 10 min according to Lichtenthaler (1987).

Statistical analysis: Data reported are means of three measurements (for ethylene analysis four measurements) and standard errors. Analysis of variance was done by a statistical analysis program *ANOVA* (*StatView*, *Abacus Concepts*, Inc., Berkeley, USA). Statistical significance of the observed differences was evaluated by the *post-hoc* test of Scheffé´s procedure.

Results

Changes in chlorophyll content: Chl content of the control group decreased significantly between day 13 and 16, indicating the onset of senescence. The cotyledons in UV irradiated plants abscised on the 13th day, and the content of Chl decreased only to about 80 %, whilst in controls it decreased to about 40 %. Decapitation of epicotyl resulted in Chl decrease on day 21 and from this day on Chl content decreased linearly until day 28 when it reached about 20 % of initial value (Fig. 1).

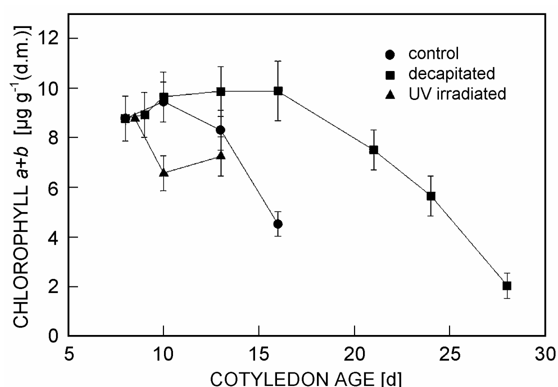

Fig. 1. Chlorophyll content in bean cotyledons with manipulated life span. *Circles* - controls; *triangles* - UV irradiated cotyledons; *squares* - cotyledons of decapitated plants.

Changes in LFP content: LFP content (Fig. 2*A*) significantly increased in controls only on day 13 (to 162 %), at the end of life span it returned to the initial level. UV irradiation (Fig. 2*B*) induced a sharp elevation in LFP content, *i.e.* it increased 8-fold 2 d after irradiation and was maximum (14-fold of the initial level) at the end of life span on day 13. One day after decapitation a LFP significantly decreased. Then LFP stayed below 50 % of initial level until day 10, on day 13 it abruptly increased to about 240 % of initial value and then decreased to 60 % on day 16 and continued to decrease to 33 % on day 21. On day 24 there was an increase in LFP content and then it was slightly reduced after abscission on day 28.

Changes in ethylene production: Ethylene evolution (Fig. 3) in control cotyledons increased more than 3-fold on the day 16 at the time of their abscission. In UV irradiated cotyledons ethylene abruptly increased about 8-fold already after 2 h, within 2 d relatively decreased, but it was still more than 2-fold higher than before irradiation and on this level stayed until abscission on day 13. After decapitation ethylene was steadily increasing, reaching a maximum on day 24. At the time of abscission it was above 200 % of initial level.

Changes in zeatin and zeatin riboside contents: Z content increased in control cotyledons until day 13 to about 200 % and at the time of abscission on day 16 it returned to the initial value. ZR increased in controls on day 10 to 250 %, on day 13 decreased to 200 % and on this level stayed until abscission. In UV irradiated group Z increased two-fold on day 10 and was still increased to about 150 % on day 13 when cotyledons abscised. On the other hand, ZR increased immediately after irradiation more than 7-fold, further increased on day 10 (totally more than 12-fold), and on day 13 it was still 4-fold higher than the initial value. In cotyledons of decapitated plants Z was decreased immediately after decapitation and stayed below the initial value until day 21. On day 24 it increased to 140 % and at the time of cotyledon

abscission it returned to the initial level. Conversely, ZR increased after decapitation more than twice and stayed at this level until day 10. On day 13 ZR sharply increased (totally 6-fold), and on day 16 it returned to the level 4-fold higher compared to initial value and with slight variations it stayed at this level until day 28 (Fig. 4).

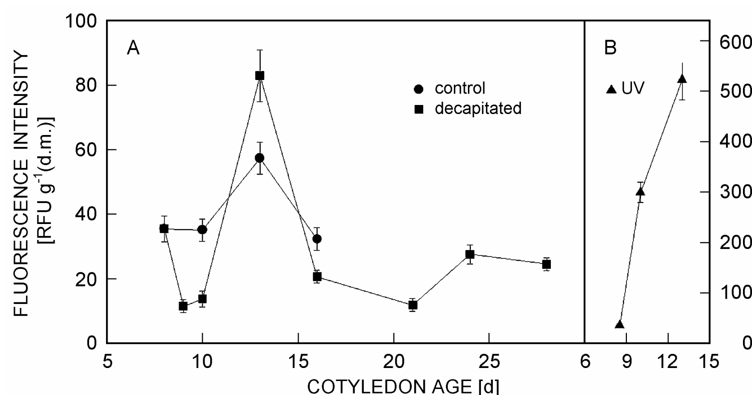

Fig. 2. Time-course of the content of lipofuscin-like pigments (LFP) during ontogeny of bean cotyledons with manipulated life span. *A* - controls and decapitated, *B* - UV irradiated plants.

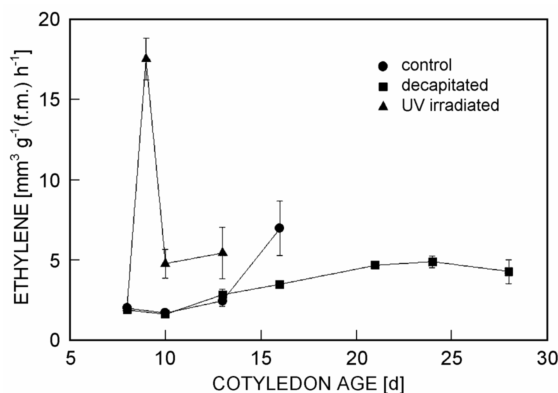

Fig. 3. Time-course of the production of ethylene during ontogeny of bean cotyledons with manipulated life span.

Changes in N^6-(Δ^2isopentenyl)adenine and N^6-(Δ^2isopentenyl)adenine riboside contents: The time course of iP (Fig. 5*A*) in control cotyledons was characterized by an abrupt increase to 250 % on day 13 and a return to the initial value at the abscission on day 16. The time course of iPR content (Fig. 5*B*) was similar. In UV irradiated cotyledons the maximum increase of iP was observed immediately after irradiation (more than 8-fold) and during following 5 d the concentration of iP steadily decreased to the level 4-fold higher than the initial one on day 13. Relatively lowest response was observed with iPR after UV irradiation. It increased to 160 % immediately after irradiation and stayed at this level until day 10, on day 13 it returned to the initial level. Decapitation resulted in a significantly decreased content of iP, which steadily increased. On day 21 it increased more than 4-fold, on day 24 it was decreased (only twice of initial level) and on this level it stayed until day 28. Contrary to iP, iPR stayed below the initial level during the whole life span of the cotyledons in decapitated plants.

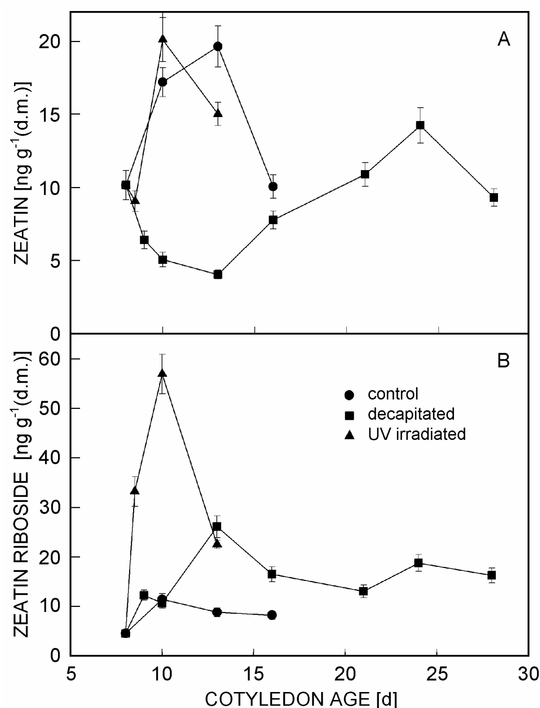

Fig. 4. Time-course of the contents of cytokinins zeatin, Z (*A*) and zeatin riboside, ZR (*B*) during ontogeny of bean cotyledons with manipulated life span.

Discussion

Free radicals are produced in various pathways throughout the whole life span of plants and their high reactivity might be responsible for the stochastic character of loss of physiological functions observed with increasing age. One of the targets of free radical species is represented by Chl. Induction of senescence is accompanied by increased Chl degradation (Humbeck *et al.* 1996, Matile *et al.* 1997) and this could be an indication of free radical involvement in senescence programme.

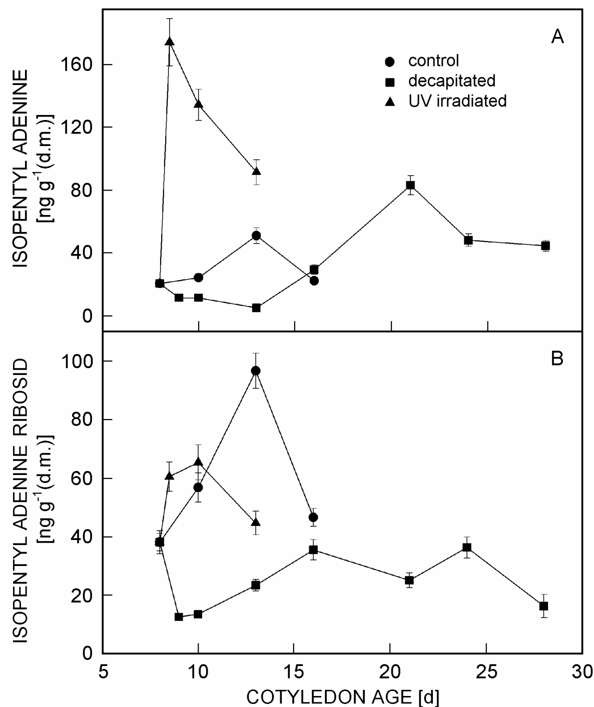

Fig. 5. Time-course of the contents of cytokinins N^6-(Δ^2isopentenyl)adenine, iP (*A*) and N^6-(Δ^2isopentenyl)-adenine riboside, iPR (*B*) during ontogeny of bean cotyledons with manipulated life span.

By the criterion of Chl degradation senescence proceeded between day 13 and 16 in the control cotyledons. The marker of free radical damage, LFP, increased on the 13th day. This could indicate the beginning of increased free radical production with commencing of senescence. However, this LFP increase was only temporary, at the time of abscission its concentration returned to the initial level.

Decapitation of the shoot prolonged the life span of cotyledon to 28 d. Chl content started to decrease from 21st day and on the day 28 it was lower than in control cotyledons at the time of abscission. LFP increased transiently on the 13th day similarly as in controls. It suggests that in decapitated plants the increase in free radical production is not associated with the onset of senescence. Nevertheless, the time-course of LFP has a character of a signal - it sharply increases during a short time period and then returns to the basic level. On the other hand, ethylene content does not show this signalling behaviour. In the control group it sharply increased in the abscised cotyledons, while in the decapitated plants it started to increase slowly from day 13, reaching a maximum on day 24. At the time of abscission of cotyledons on day 28 the ethylene content decreased relatively to day 24. These results also imply that there is not any association between free radicals production and generation of ethylene.

UV irradiation of plants induces generation of free radicals, manifested in the continuous increase in LFP content during the shortened life span of 13 d. Ethylene production sharply increased within 2 h after irradiation while LFP accumulation appeared several hours later. Thus, ethylene and LFP responded to the free radical generation by different kinetics and therefore, ethylene and LFP most probably do not interact. This view is supported by their reciprocal time-courses. Ethylene production is the result of stress response induced by UV irradiation (Abeles 1973).

Although UV irradiation shortened the life span of the cotyledons, Chl content at the time of abscission was reduced only slightly. This might indicate that UV irradiation does not induce senescence, but the abscission of cotyledons proceeds by a different mechanism differing from the senescence programme.

Individual cytokinins had different time courses in the control plants, suggesting specific metabolic roles. We have not found any correlation between the onset of senescence and the decreased cytokinin contents. Decapitation, which prolongs the life span of the cotyledons, results in a decrease contents of all measured cytokinins except ZR, which increased two-fold immediately after decapitation. Similar effect of decapitation on the concentration of ZR was observed in chickpea (Turnbull 1997). However, in our experiments ZR stayed elevated during the time of abscission and no correlation with the onset of senescence was observed.

UV irradiation increased cytokinins to various extent and with different time course. Again, no relationship between the shortened life span and cytokinins concentration was observed.

Our experiments did not prove involvement of any of the measured parameters in the regulation of the onset of senescence in bean cotyledons. Closest to the concept of the signal triggering senescence was the increased content of LFP in control plants. However, in the decapitated plants with prolonged cotyledon life span this signal occurred well before the degradation of chlorophyll has started and it is thus difficult to relate it to the onset of senescence. The time course of ethylene in control and decapitated plants indicate that the relationship between ethylene production and senescence is not a causal one.

Ethylene production increases at the end of cotyledon life span in controls, but in the decapitated plants this increase starts earlier, is slower, and is not maximum at the time of abscission. This increase in ethylene production could be induced by wounding caused by removal of leaves (O'Donnell 1996).

The investigated cytokinins did not decrease in the period of senescence. On the other hand, increased contents of cytokinins in decapitated plants did not prevent cotyledon senescence. So we did not observe a direct involvement of cytokinins in senescence regulation. This may present cotyledons as a special case, because in leaves increased cytokinin contents were associated with prolonged life span (Gan and Amasino 1995, McCabe *et al.* 2001). On the other hand, also in tobacco leaves with decreased cytokinin content their life span was surprisingly prolonged (Werner *et al*. 2001). The low contents of cytokinins are not thus the signal that triggers senescence.

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