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Role of the ascorbate-glutathione cycle during senescence and programmed cell death in *Phaseolus* cotyledons.

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ABSTRACT
Programmed cell death is an integral part of normal plant development including leaf senescence. This study investigated the response of some component of ascorbate-glutathione cycle, chlorophylls.a & b, protein content, and membrane leakage during the developmental stages of *Phaseolus* cotyledons from imbibition till cotyledon death. It was found that during cotyledon senescence, lipid peroxidation increased while the enzymic and nonenzymic antioxidants were decreased, particularly at the final stage (cotyledon death). Moreover, chlorophylls a & b and protein contents were decreased. It could be concluded that natural senescence of *Phaseolus* cotyledons exhibited a pattern of programmed cell death underlined by increase in membrane leakage levels.
KEYWORDS: chlorophylls, protein, membrane leakage, development, *Phaseolus vulgaris*

INTRODUCTION

Programmed cell death (PCD) is a genetically defined process associated with common morphological and biochemical changes (Steller 1995). It is well established that PCD is an intrinsic part of the life cycle of all multicellular organisms studied so far, including both animals and plants (Pennell & Lamb 1997; Green 1998). This default cell-suicide process can be initiated by a variety of stimuli, including developmental signals and environmental cues (Vaux & Strasser 1996; Wertz & Hanley 1996). PCD is critical for normal development, maintenance of tissue homeostasis, and defense response (Mittler 1998). One important function in plant PCD is to remobilize nutritients to benefit the plant as a whole (Smart 1994). Cell death has been observed to occur during many stages of plant development (Greenberg 1996). It includes embryogenesis, floral organ abortion, root cap sloughing, senescence, and the development of gametophytes and vascular tissue (Jones & Dangl 1996; Pennell & Lamb 1997; Mittler 1998). During these processes, cell death may occur at the level of individual cells and can involve a single tissue layer or an entire organ. DNA fragmentation is the most widely evaluated criterion for PCD.

The final stage of leaf development is often characterized by the mobilization of nutrients stored within the leaf to other parts of the plant, and this mobilization and the changes that accompany it have been referred to as the " senescence syndrome" (Bleecker & Patterson 1997). Senescence is an active process under nuclear control with interference of a large number of genes regulating mRNAs, proteins, or coordinating activities (Bleecker & Patterson 1997; Buchanan-Wollaston 1997). An early target of the senescence process is the chloroplast, where approximately one-half of the protein content of leaves is found. It is not known which proteases are involved in mobilizing the nitrogen contained within chloroplast proteins, although ATP-dependent proteolysis has been observed within chloroplasts (Lindahl *et al*. 1995; Halperin & Adam 1996).

During leaf senescence, numerous metabolic changes take place, such as protein degradation, nucleic acid and chlorophyll breakdown, and lipid and nitrogen remobilization (Buchanan-Wollaston 1997). It was also reported that mRNA accumulates during natural senescence (Lohman *et al*. 1994; Nakashima *et al*. 1997; Weaver *et al*. 1998). These changes also affect the metabolism of leaf peroxisomes, as in the case of the levels of the enzymes of the glyoxylate cycle, malate synthase and isocitrate lyase, which increased in the peroxisomes of senescent leaves as a result of increased gene expression (Pastori & del Río 1997). Increased proliferation of potentially toxic active oxygen species (AOS) within plant systems is apparently a common denominator of many types of stress. AOS have also been implicated

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in induced or natural senescent processes and its dynamics (Philosoph-Hadas *et al*. 1994). Moreover, the implication of the activated oxygen metabolism of leaf peroxisomes in the oxidative mechanism of leaf senescence has been proposed (Pastori & del Río 1997; del Río *et al*. 1998).

One of the major characteristics of senescence in plant tissues is also increased lipid oxidation (Lacan & Baccou 1998) with concomitant production of malondialdehyde (MDA), a secondary end product of polyunsaturated fatty acid oxidation. It acts as an estimator of the degree of oxidative stress experienced by the tissue (Hodges *et al*. 1999). Plants contain complements of enzymic and non-enzymic antioxidants, which play an important role in regulating the levels of AOS. These antioxidants include the enzymes ascorbate peroxidase (ASPX), catalase (CAT), and superoxide dismutase (SOD). They also include the water-soluble compounds such as ascorbate and glutathione. SOD catalyses the dismutaion superoxide radical (O_2^-) to H_2O_2, which is further reduced to H_2O by CAT and by ASPX throught the concomitant oxidation of ascorbate. Glutathione serves to reduce oxidized ascorbate; both ascorbate and glutathione can also react directly with and scavenge certain AOS (Foyer *et al*. 1994).

The relation between leaf oxidative and antioxidative potentials has been implicated in the dynamics of senescence (Kunert & Ederer 1985). It has been demonstrated that general leaf reduction levels were negatively correlated with the rate of senescence in a number of herbaceous species (Philosoph-Hadas *et al*. 1994; Meir *et al*. 1995). During leaf senescence some antioxidants increase while others decrease (Kingston-Smith *et al*. 1997), with specific profiles probably dependent upon the species examined and whether senescence is natural or stress-induced. Increased levels of activated oxygen measured in senescing tissues could either occur through an enhanced production of activated species or a decline of the various defense systems that normally afford protection against oxidative injury. Both mechanisms seem to be realized in senescing tissues, so that investigating the regulation of these scavenging systems probably offers an opportunity to characterize the factors and parameters regulating senescence.

The present study was performed to investigate the response of some ascorbate-glutathione cycle components to natural senescence and programmed cell death parameters during the developmental stages of *Phaseolus* cotyledons from imbibition to cotyledon death.

MATERIALS AND METHODS

Phaseolus vulgaris seeds were sterilized with 3% sod. hypochlorite for 15 seconds and rinsed thoroughly with distilled water three times. They were allowed to imbibe, then planted in 15-cm diameter pots, half-filled with sandy soil and allowed to germinate and grow. The developmental stages, through which *Phaseolus* cotyledons pass from imbibition (stage 1) through early expansion growth (stages 2, 3) to maturity (stage 4) then senescence (stage 5) and finally cell death (stage 6), were defined on the basis of chlorophyll content. These changes took 11 days. Samples were taken at six stages (1, 3, 5, 7, 9 and 11 days post imbibition).

Chlorophyll content: was measured in fresh cotyledones, according to the method of Witham *et al*. (1971).

Ascorbate content: was measured using the method of Sadasivam & Theymoli (1987).

Antioxidant enzymes: Enzyme extracts were prepared by homogenizing *Phaseolus* cotyledones in a prechilled mortar in 20 ml chilled extraction buffer (pH 7.5). Extracts were then centrifuged at 6000 rpm for 20 min at 5 °C. Enzyme assays were conducted immediately following extraction.

ASPX activity: was determined using the method of Nakano & Asada (1987). The assay moisture contained 90 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.65 mM ascorbate, and 1.0 mM H_2O_2. Activity was determined by following the H_2O_2- dependent decomposition of ascorbate at 290 nm.

CAT activity: was assayed in a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 1.0 mM EDTA, 60.0 mM H_2O_2, and approximately 30 µg extract protein in a method following Aebi (1983). Activity was determined by following the decomposition of H_2O_2 at 240 nm.
Peroxidase activity: was determined by following the dehydrogenation of guaicol at 436nm (Malik & Singh 1980).
Total protein content: was extracted from the seeds with 1M NaOH. The extracts were centrifuged and an aliquot of the supernatant was analyzed for protein by the method of Lowery *et al*. (1951) using bovine serum albumin as standard.
Glutathione: Total glutathione was determined spectrophotometrically following the method described by Griffith (1980).
Lipid peroxidation: Estimation of lipid peroxidation was assessed spectrophotometrically using TBA-MDA assay. Lipid peroxides were extracted from 0.5 g of cotyledons with 5 mL of 5% (w/v) metaphosphoric acid and 100 µL of 2% (w/v in ethanol) butyl hydroxytoluene (Minotti & Aust 1987). The extract was filtered centrifuged at 6,000*g* for 20 min. An aliquot of the supernatant was reacted with thiobarbituric acid at low pH and 95°C and cooled to room temperature. The resulting thiobarbituric acid-malondialdehyde adduct was extracted with 1-butanol.
Membrane leakage: Measurement of ion leakage from *Phaseolus* cotyledons was performed as described by Panavas *et al*. (1998). One g cotyledons were incubated in 50 ml double distilled water for 1 h under gentle shaking. This solution was tested for sample conductivity. Then, the cotyledons were boiled in 50 ml of double distilled water for 5 min, and this solution was measured to obtain the subtotal conductivity. Membrane leakage is represented by the relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductivity and subtotal conductivity).
Statistical analysis: Analysis of variance (ANOVA) and correlation tests were performed on all data using SPSS program (version 8.0).

RESULTS

The data in Table 1 show that Chlorophyll a&b content increased significantly from stage 1 to stage 4. However, Chl. a started to decrease at stage 5 with a sudden drop at stage 6. Chlorophyll b decreased at stage 6. Similarly, the changes of total proteins showed a progressive decrease from stage 5 to 6.

Table 1: Content of chlorophyll a&b and protein in *Phaseolus* cotyledons at different developmental stages. Data are means of three measurements

Stages	Chl. a (µg/g)	Chl. b (µg/g)	Total protein (mg/g)
1 (1-day old)	43.0	17.0	4.03
2 (3-day old)	47.0	31.0	4.28
3 (5-day old)	59.0	59.0	4.48
4 (7-day old)	156.0	61.0	4.73
5 (9-day old)	135.0	73.0	2.34
6 (11-day old)	44.0	41.0	1.90
F	125.70	30.35	80.07
P value	6.19 E-10	2.06 E-06	8.57 E-09

There is a significant positive correlation between Chl.a and Chl.b ($r= 0.75$, $p<0.01$), but, no correlation between the total protein and either chlorophyll a ($r= 0.08$, n.s.) or b ($r= -0.15$, n.s.).

The data in Table 2 show that the enzymic as well as non-enzymic antioxidants reached their minimum levels at stage 6. Glutathione content was increased from stage 1 to stage 3 then decreased significantly till stage 6. Conversely, the decrease in total ascorbate was more obvious during the developmental stages of the cotyledons. Catalase and peroxidase

activities were significantly increased to stages 3&5, respectively then decreased afterwards. ASPX activity decreased significantly with the increase in cotyledon age.

Table 2: Contents of enzymic and non enzymic antioxidants of *Phaseolus* cotyledons at different developmental stages. Data are means of three measurements

	Non enzymic antioxidants		Enzymic antioxidants		
Stages	Glutathione (mg/g)	Ascorbate (mg/ 100g)	Catalase (unit/ g)	Peroxidase (unit/ g)	ASPX (unit/ g)
1 (1-day old)	0.63	180	0.4 X 10^3	0.995	2.23
2 (3-day old)	5.26	270	4.0 X 10^3	0.597	1.57
3 (5-day old)	5.68	126	3.6 X 10^3	0.896	1.33
4 (7-day old)	4.74	135	1.2 X 10^3	2.190	1.23
5 (9-day old)	3.58	108	0.8 X 10^3	3.680	1.11
6 (11-day old)	2.00	99	0.4 X 10^3	1.090	1.01
F	115.89	349.30	309.71	434.04	71.23
P value	9.96 E-10	1.47 E-12	3 E-12	4.03 E-13	1.69 E-08

Table 3 illustrates that, as *Phaseolus* cotyledons aged, the membrane leakage (as measured by relative conductivity) and lipid peroxidation levels increased significantly (F=17.18 and 294.48, respectively).

Table 3: Membrane leakage (relative conductivity) and lipid peroxidation levels of *Phaseolus* cotyledons at different developmental stages. Data are means of three measurements

Stages	Membrane leakage	Lipid peroxidation
1 (1-day old)	0.77	0.045
2 (3-day old)	0.75	0.050
3 (5-day old)	0.77	0.089
4 (7-day old)	0.83	0.091
5 (9-day old)	0.86	0.116
6 (11-day old)	0.95	0.136
F	17.18	294.48
p-value(p>0.01)	4.2E-05	4.05E-12

DISCUSSION

Senescence has been defined as a genetically regulated process, which leads to the death of cells, organs, or whole organism (Kawakami & Watanabe 1988; Nooden & Guiamet 1989). In plants, morphological changes and/or alterations in biochemical and biophysical properties of metabolism accompany senescence. It was found from table 1 that Chlorophylls a & b and protein content increased significantly with increasing cotyledons age, then declined gradually when the cotyledons became senescent. These results confirm those of Humbeck *et al*. (1996) who reported that during the process of leaf senescence, chlorophyll and photosynthetic proteins are degraded.

Leaf senescence in particular may involve degradation of proteins (Lutts *et al*. 1996), chlorophyll (Fang *et al*. 1998) and nucleic acids (Buchanan-Wollaston 1997). Senescence in plants can refer to at least two distinct processes (Greenberg 1996): the aging of various tissues and organs as the whole plant matures (best studied in leaves, petals and fruits) and the process of whole plant death that sometimes occurs after fertilization (monocarpic senescence).

Leaf senescence is believed to initiate from signals from the chloroplast (Smart 1994). During the hypersensitive response of tobacco to tobacco mosaic virus, an increase in monomeric chloroplast DNA occurred before gross changes in nuclear morphology and significant chromatin cleavage (Mittler 1998).

L-Ascorbic acid (AsA) is an important component of the plant antioxidant system (Smirnoff 1996; Noctor & Foyer 1998; Smirnoff & Wheeler 1999). In addition to its well-established antioxidant role, AsA has been proposed to have roles in regulation of photosynthesis (Noctor & Foyer 1998), cell expansion (Smirnoff 1996) and trans-membrane electron transport (Horemans *et al*. 1994). AsA is readily oxidized to monodehydroascorbate (MDA) as part of its antioxidant function. Oxidation is catalysed by two enzymes: ascorbate peroxidase (APX)

and ascorbate oxidase (AO). MDA disproportionates to dehydroascorbate (DHA) and AsA if it is not immediately reduced.

It was found from the present study that as *Phaseolus* cotyledons aged, the ascorbate content decreased. It was also found that glutathione level increased significantly to stage 4 (7-day old) and then declined gradually, as *Phaseolus* cotyledons became senescent. These findings are in agreement with those obtained by Hodges & Forney (2000). They reported that concentrations of total ascorbate dropped significantly in spinach leaves following detachment, while total glutathione levels increased significantly in spinach leaves held in ambient air for longer than 14 day then declined after 35 day. The decrease in ascorbate could be attributed to the decrease in ASA-GSH cycle enzymes and the disturbance in glutathione levels, which stabilize AsA content by preventing dehydroascorbate accumulation (Foyer *et al*. 1995). Increases in the levels of glutathione might represent an attempt by cotyledons to accommodate oxidative stress generated by declining activities of ASPX and CAT and overall concentrations of ascorbate. NADPH is known to be essential for defense against oxidative stress, as it is the cofactor required for the reduction of oxidized glutathione by glutathione reductase, a component of the ascorbate-glutathione cycle that is present in leaf peroxisomes (Jiménez *et al*. 1998).

The data in table 2 highlights that the enzymic antioxidants CAT, peroxidase and ADPX) reached their minimum levels at stage 6. Hodges & Forney (2000) found that activities of ASPX and CAT significantly declined in detached spinach leaves stored in ambient air over 35 day. Declining activities of CAT, peroxidase and ASPX during the developmental period of *Phaseolus* cotyledons suggest that the potential of enzyme-catalyzed H_2O_2 scavenging became progressively decreased. The requirement for ASPX capacity might have declined due to decreasing concentrations of total available ascorbate.

Reduction in H_2O_2 -scavenging potential might have significantly contributed to enhanced oxidative stress as measured by lipid peroxidation. The results in table 3 indicate the significant increase in membrane leakage and lipid peroxidation during the aging stages of *Phaseolus* cotyledons. Similarly, Hodges & Forney (2000) found a significant increase in MDA content of detached spinach leaves during storage.

Free radicals are thought to play an essential role in senescence, especially those derived from oxygen. The activated oxygen metabolism of peroxisomes was found to be involved in the mechanism of senescence (Pastori & del Río 1997; del Río *et al*. 1998). Levels of MDA as a secondary by-product of lipid peroxidation, indicate the degree of plant oxidative stress (Hodges *et al*. 1999), while decreases in Chlorophyll and soluble protein concentrations often serve to estimate the extent of senescence (Meir *et al*. 1995). Lipid peroxidation and active oxygen speciec (AOS) have been implicated in the direct or indirect degradation and/or bleaching of Chl (Hodges *et al*. 1996; Toivonen & Sweeney 1998), thus the significant increases in MDA and decreases in Chl contents in *Phaseolus* cotyledons appeared to be concomitants.

Decreased antioxidant potential concomitant with an increased AOS production may be an important component in the induction and sequence of senescence and programmed cell death. Lacan & Baccou (1998) investigated the senescence process in two muskmelon (*Cucumis melo* L.) varieties, Clipper and Jerac, which differ in their storage life. Their results indicated that senescence in Jerac (the short-storage-life variety, less than 7-d) is the result of lipid peroxidation by free radicals, membrane phospholipid breakdown, and a drop in the level of antioxidants, resulting in increased membrane leakiness. The high levels of two enzymes implicated in antioxidative defence, superoxide dismutase (SOD) and catalase, are involved in delaying the senescence process in Clipper and this could explain, at least, to some extent, the long storage life of Clipper (longer than 14 d).

Zentgraf *et al.* (2000) revealed that the breakdown of macromolecules and the mobilization of nutrients from the senescing tissues characterize leaf senescence. The rapid loss of chlorophyll, the lowering of protein and RNA levels or the leakiness of the cell membranes

might be attributed to selective activation of gene expression where a shutoff of certain RNA and/or protein synthesis is likely to initiate and regulate this process.

In the present work, it could be concluded that during natural senescence of *Phaseolus* cotyledons, some antioxidants increase while others decrease. Increased levels of lipid peroxidation could be attributed to enhanced production of activated oxygen species or to a decline of the various defense systems that normally afford protection against oxidative injury. It is suggested that senescence in *Phaseolus* cotyledons can be considered as programmed cell death, incorporating chlorophylls decline and increased membrane leakage.

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الملخص العربى

دور دورة الجلوتاثيون-أسكوربات عند الشيخوخة والموت المبرمج للخلايا فى فلقات الفاصوليا

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يعتبر الموت المبرمج للخلايا جزء من التطور الطبيعى للنبات والذى يشمل شيخوخة الورقة. تحقق هذه الدراسة فى تأثير مكونات دورة الجلوتاثيون-أسكوربات ، محتوى كلوروفيل أ ، ب ، محتوى البروتين، و ارتشاح الأغشية الخلوية أثناء مراحل تطور فلقات الفاصوليا من فترة التشرب وحتى فترة موت الفلقات. وقد وجد أنه أثناء شيخوخة الفلقات زاد مستوى فوق أكسدة الدهون بينما قلت مضادات الأكسدة الإنزيمية وغير الإنزيمية وذلك فى فترة موت الفلقات. بالإضافة إلى ذلك فإنه قد حدث تحلل لكلوروفيل أ ، ب والبروتين. ومن ذلك تم استنتاج أن الشيخوخة الطبيعية لفلقات الفاصوليا ممكن أن تعتبر نوع من الموت المبرمج للخلايا حيث زاد مستوى ارتشاح الأغشية الخلوية.