

Activities of antioxidant enzymes during senescence of *Prunus armeniaca* leaves

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Abstract

During the period of senescence of apricot leaves changes in photosynthetic pigment contents and in the activities of some antioxidant enzymes (superoxide dismutase, catalase, peroxidase and ascorbate peroxidase) were analysed. Significant changes in pigment contents were, in most cases, correlated with changes in activities of the antioxidant enzymes. Modifications in superoxide dismutase and catalase isoform patterns were also observed during the progression of senescence. Both enzyme activities and isoenzyme patterns proved to be genotype-dependent.

Additional key words: activated oxygen species, apricot, ascorbate peroxidase, catalase, peroxidase, superoxide dismutase.

Introduction

All multicellular organisms undergo a decline in physiological functions in the final stage of their development. This process, called senescence, occurs as a genetically programmed developmental step which consists of endogenously controlled deteriorative changes leading to death of cells, tissues, organs and whole organisms (Leopold 1975, Noodén and Leopold 1978). Senescence is a very complex process consisting of morphological, metabolic and physiological modifications which spread from changes at the ultrastructural level, to changes in respiration and photosynthesis due to modifications in mitochondria and chloroplasts. Proteolysis, changes in nucleic acids and new mRNAs and proteins synthesis have also been commonly observed (Thimann 1986, Buchanan-Wollaston 1997). One of the main features of senescence is the change in cellular membrane organisation, which results in progressive loss of function and structural integrity (Goldstein and Weissmann 1977, Thompson 1988).

There is evidence that a great contribution to these changes derives from activated oxygen species; they are in fact constitutively produced by the normal metabolism, but a strong enhancement in the production of these radicals has been observed to take place in plant tissues during senescence process (Thompson *et al.* 1987, Leshem 1988). Activated oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and

highly reactive hydrogen radical (HO^\bullet) have all types of biomolecules as targets, causing their damage and thus contributing to oxidative damage-mediated cell death (Thompson *et al.* 1987, Halliwell and Gutteridge 1989).

To provide protection against activated oxygen species, plants have evolved enzymatic and non-enzymatic antioxidant defence systems which scavenge these highly reactive oxygen forms. During senescence, changes in scavenging ability can induce increases in free radicals concentration, so that oxidative stress is produced. Even if senescence and changes in antioxidant enzyme activity are closely associated phenomena, contradictory reports about the trend of these changes have appeared for some enzymes (McRae and Thompson 1983, del Rio *et al.* 1998).

Senescence is a well known and documented phenomena, but most of the studies have been performed on the genetic mechanisms and molecular changes of this process (John *et al.* 1997, Kleber-Janke and Krupinska 1997, Panavas *et al.* 1999) and less attempts have been done to understand the modifications in the antioxidant enzymes activity, especially in fruit trees. Therefore, the aim of this work has been the characterisation of some of the free radical scavenging enzymes (superoxide dismutase, catalase, peroxidase and ascorbate peroxidase) in apricot leaves collected at the beginning and during their senescence.

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Abbreviations: APRX - ascorbate peroxidase; Car - carotenoids; CAT - catalase; Chl - chlorophyll; PRX - peroxidase; SOD - superoxide dismutase.

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Materials and methods

Plants: Apricot (*Prunus armeniaca* L. cultivars San Castrese and Monaco Bello) trees were grown in 20 dm³ pots and regularly fertilised and irrigated during the growing season. In the autumn, three samples of leaves were randomly collected from six to eight 4-year-old trees of each cultivar at three different sampling dates. Leaves were weighed, immediately frozen in liquid nitrogen and stored at -80 °C until use.

Pigments analysis: Three leaf discs (diameter 1 cm) were collected from three plants of each cultivar. Extractions were performed by shaking discs in 5 cm³ of methanol, in the dark at 4 °C for 72 h. Extracts were analysed spectrophotometrically and chlorophyll *a*, chlorophyll *b* and carotenoid contents were calculated using equations according to Lichtenthaler (1987).

Protein extraction: Frozen leaves were ground in liquid nitrogen with mortar and pestle and the powder was suspended in 0.1 M Tris buffer, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 0.1 % (m/v) polyvinylpyrrolidone, 0.1 % (m/v) ascorbic acid and 1 % (v/v) β-mercaptoethanol. Extracts were centrifuged twice at 26 000 *g* for 20 min (4 °C) to remove cellular debris and the clarified supernatant was used for enzyme activity determinations. Protein content was determined according to Bradford (1976) using a *Bio-Rad* protein assay kit and bovine serum albumin as a standard.

Enzyme analyses: Enzyme activities were measured at 25 °C using a thermostated spectrophotometer. Superoxide dismutase (E.C.1.15.1.1) activity was measured and expressed according to Madamanchi *et al.* (1994). Different amount (5, 10, 20 and 40 mm³) of crude extract were added to a reaction mixture (3 cm³) containing 50 mM sodium phosphate buffer, pH 7.8, 0.1 mM ethylene diamine tetraacetic acid, 13 mM methionine, 2 μM riboflavin and 75 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The reaction was started exposing the mixture to cool white fluorescent light for 15 min. After this period the light was switched off, the tubes were mixed and the absorbance was measured at 560 nm.

Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture (3 cm³) composed of 50 mM potassium phosphate buffer pH 7.0 to which 30 % (m/v) H₂O₂ was added to reach, at 240 nm, an absorbance value in the range 0.520 - 0.550. The reaction was started by adding the reaction solution to 10 mm³ of crude extract and the activity was followed by monitoring the decrease in absorbance at 240 nm as a consequence of H₂O₂ consumption (Aebi 1984).

Peroxidase (E.C.1.11.1.7) activity was assayed in a reaction mixture (3 cm³) containing 10 mM potassium phosphate buffer, pH 7.0, 10 mM H₂O₂ solution, 20 mM

guaiacol and 10 mm³ of crude extract (Maehly and Chance 1954). The reaction was started by adding H₂O₂ and guaiacol solutions and the activity was determined by monitoring the increase in absorbance at 470 nm, as a result of guaiacol oxidation.

Ascorbate peroxidase (EC 1.11.1.11) activity was determined, according to Chen and Asada (1989), in a reaction volume of 1 cm³ containing 100 mM potassium phosphate buffer, pH 7.5, 0.5 mM ascorbate, 0.2 mM H₂O₂ and 10 mm³ of the crude extract, following the decrease in absorbance at 290 nm.

Native PAGE and isoform staining: Discontinuous electrophoresis was performed with a *MiniProtean* electrophoresis unit using 5 % stacking gel and a 7.5 % running gel. Equal amounts of protein from each sample were subjected to electrophoretic separation (Tris-glycine buffer, pH 8.3, 150 V for 45 min, 4 °C).

To determine superoxide dismutase activity gels were incubated for 30 min at 30 °C in the dark, in a solution composed of 4.8 μM MTT dissolved in 50 mM phosphate buffer, pH 7.5. Gels were then transferred to a solution composed of 25 cm³ of 50 mM phosphate buffer, pH 7.5, with 2.65 μM N,N,N',N'-tetramethylethylenediamine (TEMED) and 2.4 μM riboflavin. The reaction was started exposing the gel to white cool fluorescent light (Beauchamp and Fridovich 1971).

Catalase isoforms were revealed by incubating the gel in a solution made of 5 mm³ of H₂O₂ (30 % m/v) in 50 cm³ of distilled water for 10 min in the dark, then for 10 min in 50 cm³ of a solution with 3.7 mM FeCl₃ and 3 mM potassium ferricyanide (Woodbury *et al.* 1971).

To determine peroxidase isoforms pattern gels were incubated for about 10 min in a solution composed of 5.8 mM *o*-dianisidine dissolved in 95 % (v/v) ethanol, 7 cm³ of Na-acetate/acetic acid buffer, pH 6.0, and 500 mm³ of 3 % (m/v) H₂O₂ solution. The staining was then fixed in Na-acetate/acetic acid pH 5.0 and the gels washed with distilled water (Brewbaker *et al.* 1968).

For ascorbate peroxidases isoforms determination, gel were pre-run using Tris-glycine buffer to which 2 mM ascorbate was added. After electrophoretic separation, gels were incubated for 30 min in 25 cm³ of potassium phosphate buffer, pH 7.0, with 2 mM ascorbate, then for 20 min in 25 cm³ of buffer with 4 mM ascorbate and 2 mM H₂O₂ and finally in 25 cm³ of buffer with 28 mM TEMED and 2.45 mM MTT until bands developed (Rao *et al.* 1996).

Statistical analysis: Determinations of pigment content and enzyme activities were performed on three independent experiments with three replications each. Analysis of variance (*ANOVA*) was performed using the *SYSTAT* (Evanston, USA) software and means were evaluated by Fischer's LSD test at *P* ≤ 0.05.

Results

In both cultivars analysed, significant differences in pigment contents were observed; in particular chlorophyll (Chl) and carotenoid (Car) content decreased between the first and the second samplings and further decreased, even if less, at the third sampling (Fig. 1). This trend was

consistent with the observation of a gradual leaf yellowing and progression to a state of senescence. No significant differences in pigment content were observed between the two cultivars.

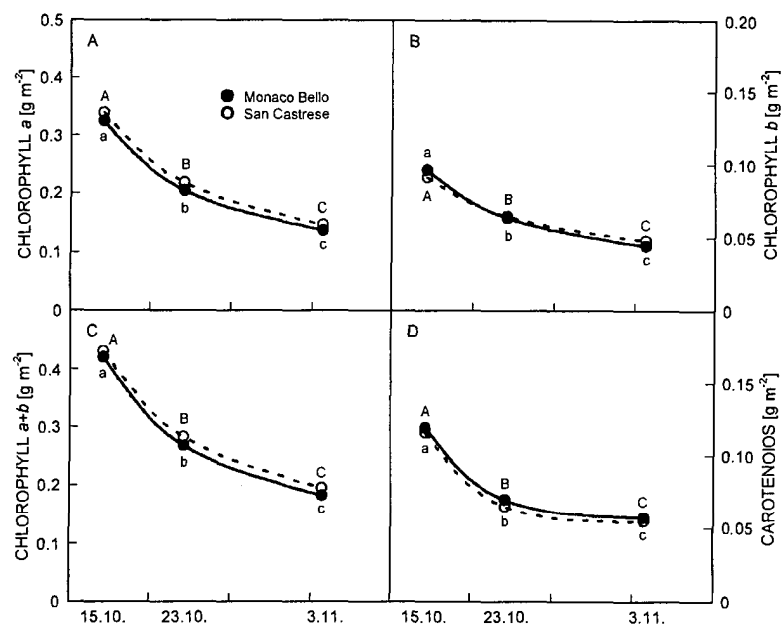


Fig. 1. Effect of natural senescence on chlorophyll *a* (A), chlorophyll *b* (B), chlorophyll *a+b* (C) and carotenoid (D) content in leaves of apricot (*Prunus armeniaca* L) cvs. Monaco Bello and San Castrese, collected at three different sampling dates. For each cultivar the same letter indicates mean values that do not significantly differ according to Fischer's LSD test ($P \leq 0.05$).

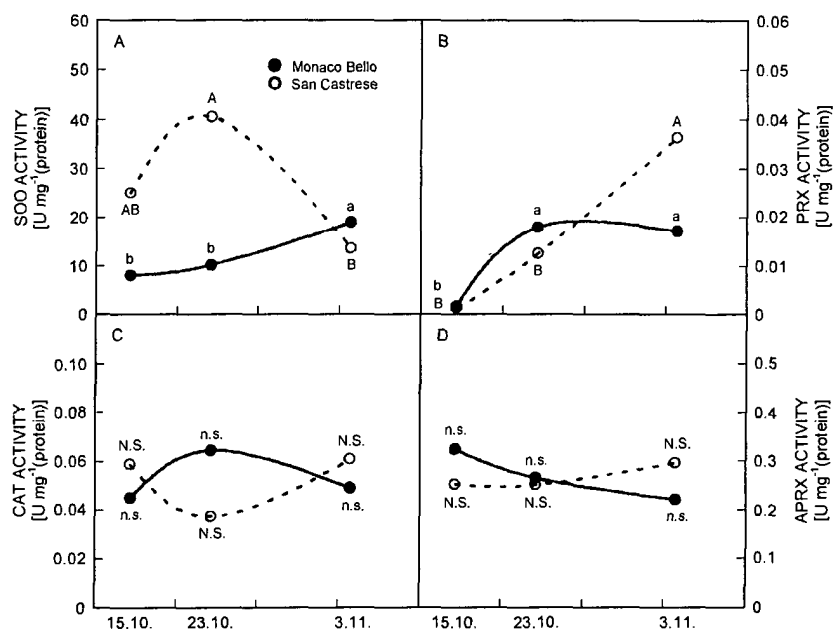


Fig. 2. Changes in activity of superoxide dismutase (A), peroxidase (B), catalase (C) and ascorbate peroxidase (D) in leaves of apricot cvs. Monaco Bello and San Castrese, collected at the beginning and in following stages of senescence. For each cultivar the same letter indicates mean values that do not significantly differ according to Fischer's LSD test ($P \leq 0.05$).

The main antioxidant enzymes were spectrophotometrically analysed to investigate the senescence-induced modifications in their activities (Fig. 2). Results showed no significant differences in catalase (CAT, Fig. 2C) and ascorbate peroxidase (APRX, Fig. 2D) activities from the beginning to the following stages of senescence neither in San Castrese, nor in Monaco Bello. Significant differences both between stages and between cultivars were observed for superoxide dismutase (SOD) and peroxidase (PRX) activities. In particular a slight increase in PRX activity was observed in both cultivars, even if in San Castrese this increase was delayed while in Monaco Bello it was between the first and the second sampling (Fig. 2B). SOD activity showed a different trend for the two cultivars; a gradual and significant increase was observed in Monaco Bello, while in San Castrese, a significant decrease was found between the second and

the third sampling (Fig. 2A).

Activities of antioxidant enzymes were also plotted against the Chl $a+b$ content and the correlations between these variables were analysed (Fig. 3). The correlations between SOD activity and Chl concentration (Fig. 3A) highlighted a differential behaviour between the two cultivars; in Monaco Bello changes in SOD activity was negatively correlated ($r^2 = 0.62$) with changes in Chl content, while in San Castrese this correlation was not observed ($r^2 = 0.03$). Negative correlation between PRX activity and Chl (Fig. 3B) was observed in both cultivars ($r^2 = 0.64$ and $r^2 = 0.67$ in Monaco Bello and San Castrese, respectively). CAT activity was unchanged while Chl content decreased (Fig 3C). The correlation of APRX activity with Chl content (Fig. 3D) was weak in Monaco Bello ($r^2 = 0.23$) and absent in San Castrese ($r^2 = 0.06$).

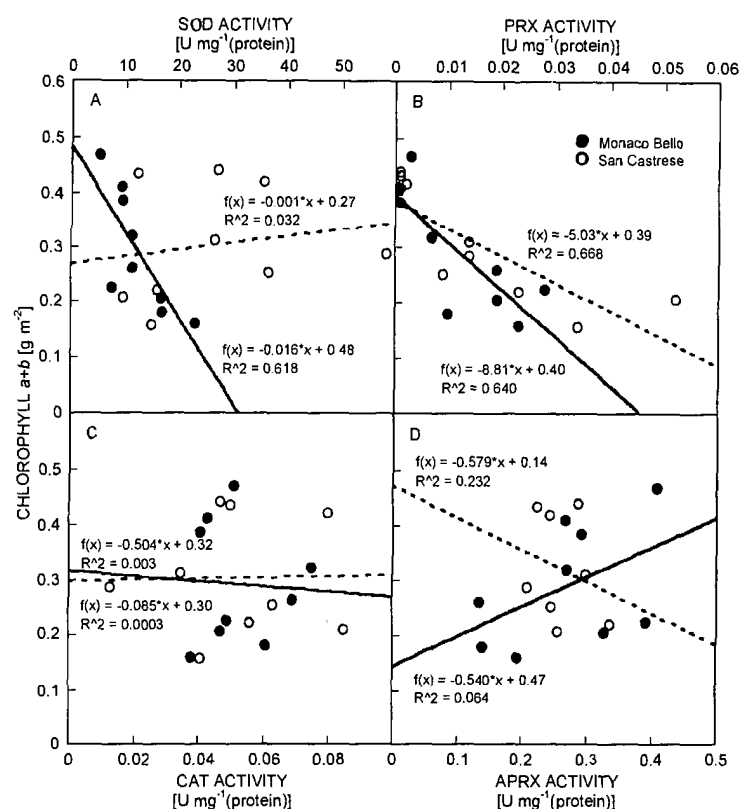


Fig. 3. Correlation between superoxide dismutase (A), guaiacol peroxidase (B), catalase (C), ascorbate peroxidase (D) activity and chlorophyll $a+b$ content in senescent leaves of apricot cvs. Monaco Bello and San Castrese.

As changes in enzyme activities can be not only quantitative but also qualitative, changes in antioxidant isoenzyme pattern during senescence process were also investigated. From the analysis of PRX and APRX isoforms patterns no qualitative differences were found; bands revealed by the enzyme staining were exactly the same in the different stages of senescence and in the two cultivars studied, even if their intensities differed (Figs. 5

and 7). Differences in the number of isoforms revealed for SOD were observed. In San Castrese for all the samplings, one more band at low molecular mass was detected (Fig. 4, arrow 2). Moreover, in Monaco Bello the highest molecular mass band was not visible at the first sampling date (Fig. 4, arrow 1). As regards gel for CAT, differences in band width were observed in both cultivars from the first to third sampling (Fig. 6).

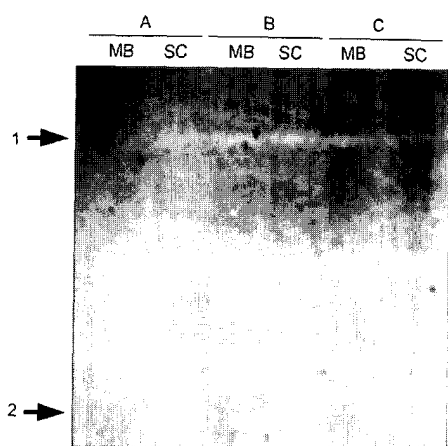


Fig. 4. Superoxide dismutase isoforms in apricot leaves collected at three different sampling dates. *Arrow 1* shows a band which is not expressed at the first sampling neither in Monaco Bello, nor in San Castrese. *Arrow 2* shows a band which, at the three sampling dates, is expressed only in San Castrese.

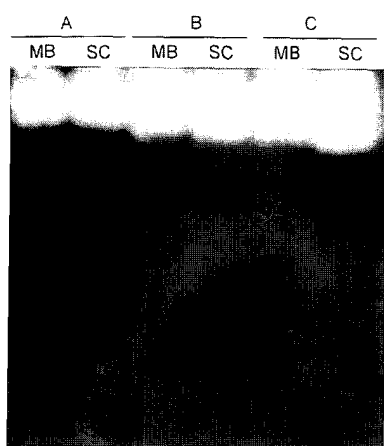


Fig. 6. Catalase isoforms in apricot leaves collected at three different sampling dates. Both in Monaco Bello and San Castrese wider bands at the third sampling can be observed.

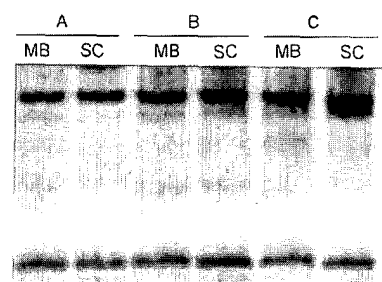


Fig. 5. Peroxidase isoforms in apricot leaves collected at three different sampling dates.

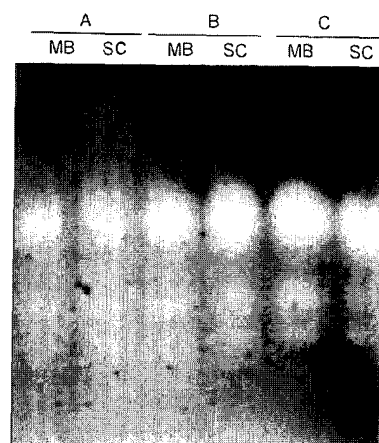


Fig. 7. Ascorbate peroxidase isoforms in apricot leaves collected at three different sampling dates.

Discussion

The clearest evidence of senescence in plants is represented by a leaf yellowing due to decrease in Chl content which together with protein loss, induces a progressive decrease in photosynthetic ability (Thompson *et al.* 1987). Differences in pigment content have been found in our samples during ageing. In particular a decrease, both in chlorophyll and carotenoid content, was observed from the beginning, when pigment content in leaves was still unchanged in comparison to that of mature leaves (data not shown), to the more advanced stages of senescence. This proved that pigment content is a good marker for senescence.

Chl and protein degradation is mainly due to the

strong enhancement in the concentration of activated oxygen species in plant tissues during senescence (Leshem 1988) depending on the activity of antioxidant systems. The results of our experiments proved, at least for some of the enzymes analysed, a good correlation between changes in enzyme activity and progression of the senescence. In the cultivar Monaco Bello, with the exception of CAT, the antioxidant enzymes showed to be positively (PRX and SOD) or negatively (APRX) correlated with decrease in Chl content during senescence. On the contrary in San Castrese only PRX activity was correlated with Chl content. Therefore, as the kinetics of pigment degradation do not differ in the two

cultivars, the antioxidant enzyme activities seem to be differentially regulated in Monaco Bello and San Castrese during senescence.

Differences observed in leaf antioxidant enzymes were overall changes in activities, but antioxidant enzymes can exist in multiple molecular forms with different localisation in individual cell organelles (Scandalios 1997). Consequently a separate role for each isoform in response to senescence can be postulated. The PRX isoform patterns did not differ during senescence in both genotypes, so the increase in activity observed was probably induced by higher enzyme synthesis. On the contrary CAT activity, which has not showed any significant correlation with Chl content, displayed senescence-induced changes in band width from the first

to the third sampling, both in Monaco Bello and in San Castrese. Therefore, the response of the two enzymes to senescence was different.

Moreover, also genotype seemed to play an important role in inducing different kind of response. In APRX and SOD the correlation between enzyme activity and senescence was genotype dependent. This behaviour, at least for SOD, could be explained by the differences observed in isoforms pattern.

In conclusion, during autumn the progression to senescence in apricot leaves is linked to complex changes observed in antioxidant enzymes. The multiform response pattern observed for antioxidant enzymes could be explained considering that for different genotypes, each enzyme can be specifically regulated and localised.

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