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Oxidative Stress, Growth Factor Production and Budding in Potato Tubers During Cold Storage

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In order to verify the role played by oxidation in the budding of potato tubers (*Solanum tuberosum* L. cv. Kennebec), the physiological events occurring below bud at 4°C have been studied for a period of 6 months. The low temperature storage induced an increase in the degree of unsaturation and a decrease in the ratio of saturated/unsaturated fatty acids of membrane polar lipids with a subsequent increase of lipid hydroperoxides (LOOH). Cold stress increased both enzymatic antioxidative activities (superoxide dismutase, SOD, E.C.1.15.1.1; catalase, CAT, E.C. 1.11.1.6), and α-tocopherol levels thus protecting membrane's polyunsaturated lipids. Between 0 and 15 days of storage SOD/CAT ratio, α-tocopherol, LOOH levels and the degree of lipid unsaturation showed strong variations. After 30 to 120/150 days the antioxidative system seemed to reach a homeostasis different from that of time 0, accompanied by a constant increase of indole-3-acetic acid (IAA) after 60 days. The antioxidative system, after 150 days, lost its efficiency while LOOH levels were maintained higher than time 0 and IAA concentration was sufficient to allow sprouting.

Keywords: Catalase; Indole-3-acetic acid; Lipid hydroperoxides; Potato tuber; Superoxide dismutase; α-tocopherol

Abbreviations: BHT, butylatedhydroxytoluene; CAT, catalase; 2,4 D, 2,4, dichlorophenoxyacetic acid; FA, fatty acids; IAA, indole-3-acetic acid; LAH, lipolytic acyl hydrolase; LOOH, lipid hydroperoxides; LOX, lipoxygenase; PL, polar lipids; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase

INTRODUCTION

Low temperature storage is used to store potato tubers following harvest. Storage temperature below optimum (i.e. 10°C)[1,2] results in starch breakdown (low temperature sweetening) that is responsible for the loss of processing value of potatoes. It is reported that the sugar accumu-

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lation over storage time could be related to membrane damage (i.e. biophysical modification of lipids and peroxidation) induced by low temperature stress.[1,3] Potato tubers respond to cold by enhancing lipid unsaturation to maintain cell membrane fluidity.[4] In addition the concentration of dissolved oxygen in the cell sap increases with the decrease of temperature[5] and this leads to the formation of reactive oxygen radicals, which could be among the causes of lipid hydroperoxides (LOOH) production. Furthermore, the high levels of lipolytic acyl hydrolase (LAH, E.C. 3.1.1.5) and lipoxygenase (LOX, E.C. 1.13.12.12) found in potato tubers can induce LOOH formation and an increase of these enzymes during cold-storage of tubers has been reported.[6,7] A positive correlation between the tuber sweetening and malondialdeyde formation (a marker of lipid peroxidation) has also been reported even though it is uncertain whether the two phenomena are strictly and immediately related.[1] In a previous work,[8] on potato tubers (cv Kennebec), we demonstrated that LOOH led, after wounding stress, to a rapid, short term increase (0–120 min) in the hormone auxin (indole-3-acetic acid, IAA) concentration associated with bud elongation, mitosis promotion and starch hydrolysis. Cold and wounding are both abiotic stresses, which could share a modulation of some physiological responses in plants. The role of IAA during potato cold-storage is underestimated although this compound is strictly associated with sprouting[9] a parameter implicated in the commercial value of potatoes.

In this paper the pattern of fatty acids (FA) in membrane polar lipids (PL), LOOH formation, the activity of enzymatic antioxidants (superoxide dismutase, SOD, E.C 1.15.1.1 and catalase, CAT, E.C. 1.11.1.6), the concentration of α-tocopherol and IAA have been monitored in below buds tissues.

The potential metabolic correlation between these parameters and IAA was evaluated to investigate the role played by IAA during potato cold-storage.

MATERIALS AND METHODS

Plant Material

Certified potato tubers (*Solanum tuberosum* L. cv Kennebec, kindly supplied by PAT-FRUT, Castel D'Aiano, Bologna, Italy) were stored at 4°C and were brought to room temperature 24 h prior to use and always kept in the dark. The experiments were carried out on 5 g potato slices (3 cm diameter and ~ 0.5 cm thick) excised below the bud (eye region). In each experiment five slices were randomly chosen among 30 slices from 10 potato tubers.

Lipid Analysis

The slices were washed three times in sterilized distilled water, and extracted three times with 15 ml chloroform/methanol (2/1 v/v) in the presence of 100 μg of butylated hydroxytoluene (BHT) as antioxidant. The extracted lipids, after filtration and vacuum evaporation, were fractionated by thin layer chromatography (TLC) and the PL fraction was recovered as previously reported[10] with minor modification. Before extraction, tricosanoic acid (C23:0) was added as internal standard. The extracted lipids were trans-esterified by borontrifluoride (BF3 10% in CH_3OH) and the resulting FA methyl-esters were analyzed by gas chromatography (GC) on a capillary column FFA-P 50 m × 0.32 mm × 0.52 μm.[11]

IAA Detection

Analyses of IAA were performed at different storage times. Samples were extracted three times for 1 h with 20 ml ethylacetate in presence of 100 μg BHT as antioxidant and 5 μg of 2,4 dichlorophenoxyacetic acid (2,4 D) used as internal standard. The extracts were filtered, vacuum evaporated and then silylated. The trimethyl-silyl-ether (TMS)-derivatives were analyzed by GC-MS on a 5890 Hewlett-Packard (Palo Alto, CA, USA) gas chromatographer

coupled with a 5970 Hewlett-Packard Mass Spectrometer using a Supelco (Bellefonte, PA, USA) SPBTM-1 fused silica capillary column (30 m × 0.20 mm × 0.30 μm) and splitless injection of 2 μl of sample. Helium was used as carrier gas. For the analyses, oven temperature was brought from 110 to 240°C, at rate of 5°C min^{-1}, and from 240 to 280°C, held for 20 min, at rate of 30°C min^{-1} with an injector temperature of 250°C. Quantitative analyses were performed in SIM (single ion monitoring) mode, selecting the ions having $m{\cdot}z^{-1}$ 319, 304, 202 for the trimethylsilyl derivative of IAA and ions of $m{\cdot}z^{-1}$ 292, 257, 219 for the derivative of 2,4 D. Calibration curves were constructed by plotting the ratios of the integrated peak areas of IAA and 2,4 D against their amount and performing a linear regression using equal weighting. The method was linear ($R > 0.99$) in the analyzed concentration range of 1–50 ng IAA μl^{-1}.

Detection and Quantification of α-tocopherol

Analyses of α-tocopherol were performed at different storage times, as previously reported, on 5 g fresh weight of tuber sliced below bud. Samples were extracted three times in chloroform/methanol (2/1 v/v) for 1 h in presence of 100 μg BHT as antioxidant and 5 μg heptadecanoic acid (C17:0) used as internal standard. The chloroform/methanol mixture recovered was extracted three times in hexane, filtered, vacuum evaporated and then silylated with TMS. The TMS-derivatives were analyzed by GC-MS as previously described for IAA assay. The oven temperature was brought from 110 to 280°C, at rate of 15°C min^{-1}, with an injector and detector temperature respectively of 250 and 280°C. Quantitative analyses were performed in SIM mode, selecting the ions having $m{\cdot}z^{-1}$ 502, 277, 237 for the trimethylsilyl derivative of α-tocopherol and ions of $m{\cdot}z^{-1}$ 342, 327 for the derivative of C17:0. Calibration curves were performed as reported for the IAA analysis, the method was linear ($R > 0.99$) in the range of 1–50 ng α-tocopherol μl^{-1}.

Lipid Hydroperoxides Detection

Lipid hydroperoxides detection (in absorbance at 556 nm) was carried out at different times after storage on lipids extracted from 5 g fresh weight of tuber slices and spectrophotometrically assayed using *N,N*-diethyl-1,4-phenylene diammoniumsulphate (DEPD) according to Nazzaro-Porro *et al.* (1986).[12]

Superoxide Dismutase and Catalase Assay

Potato tubers (5 g fresh weight) were homogenized in 0,1 M phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged at 6000*g* for 15 min at 4°C. Protein concentration of plant tissues was determined in the supernatant by Bradford test and expressed as mg/ml. Superoxide dismutase (SOD) activities were evaluated by spectrophotometer.[13] In this competitive inhibition assay, superoxide generated by xanthine–xanthine oxidase is detected by monitoring the reduction of nitroblue tetrazolium at 505 nm. Total SOD activity was measured at pH 7.8 in Tris-HCl 0.2 M. The standard curve was performed using bovine SOD (Sigma) at different concentrations (0.1, 0.25, 0.5, 1, 2 U/ml). One unit of activity was defined as the amount of protein that yields 50% of maximal inhibition of nitroblue tetrazolium reduction by superoxide. The results were reported as units of SOD/mg of proteins.

Catalase (CAT) activity was determined by the rate of disappearance of hydrogen peroxide (10 mM) in a phosphate buffer at pH 7.4 and was measured at 240 nm by spectrophotometer.[14] One unit of CAT is defined as the amount of enzyme that degrades 1 μM of H_2O_2. The results were reported as units of CAT/mg of proteins.

Statistical Analysis

In all experiments, mean values were compared using Student's *t*-test.

RESULTS

Pattern of PL Fatty Acids in Below Bud Potato Slices During Storage

Fatty acids composition (%) of PL during storage is shown in Table I. An increase in the unsaturated FA (C18:1 + C18:2 + C18:3) was detected during the time. Among the polyunsaturated fatty acids (PUFA) present, C18:3 increased during the storage and the degree of unsaturation, reported as C18:3/C18:2 ratio, significantly ($P < 0.001$) increased from time 0 (0.25) up to 180 days (0.49).

During early periods of storage (0–30 days) the saturated/unsaturated ratio significantly ($P < 0.01$) decreased from 0.82 ± 0.02 to 0.65 ± 0.01, followed by a significant ($P < 0.01$) increase of the ratio (0.72 ± 0.01) up to 60 days, after this time it decreased to 0.57 ± 0.02. The saturated/unsaturated ratio, however, remained at lower levels than time 0 during all the time course (Fig. 1).

Detection of Lipid Hydroperoxides and α-tocopherol in Below Bud Potato Slices During Storage

Some fluctuations were observed during the time course of below bud LOOH levels (Fig. 2). The LOOH levels increased rapidly after time 0 and peaked at 21 days (from 0.10 ± 0.01 to 0.22 ± 0.01 in absorbance; $P < 0.001$) followed by a decrease. Between 60 and 90 days a significant further increase (from 0.15 ± 0.01 to 0.21 ± 0.02 in absorbance; $P < 0.01$) related to the enhancement of FA unsaturation of PL ($R = -0.99$; $P < 0.001$) was observed, however, during all the time points the level of LOOH was maintained higher than time 0.

The concentration of α-tocopherol has a steep increase from time 0 to 21 days (94.4%; $P < 0.001$) (Fig. 2), related both to membrane unsaturation ($R = 0.75$; $P < 0.01$) and LOOH formation ($R = 0.92$; $P < 0.001$). After this time point the α-tocopherol concentration still increased up to 90 days but at slower rate (65%), the trend from 90 to 150 days was almost linear and was followed by a decrease after 150 days.

Enzymatic Antioxidative SOD and CAT Activities During the Time Course

From 0 to 15 days a decrease of SOD activity was evident (Fig. 3) while CAT activity increased. After this period SOD started to raise (15–75 days) from 1.62 ± 0.28 to 3.27 ± 0.09 U/mg protein ($P < 0.01$), reaching the time 0 value after 30 days. From 75 to 150 days SOD activity set at a higher level than time 0. Catalase activity returned to the level of time 0 at 30 days, and then presented a slower but significant increase

TABLE I Composition (%) of FA of PL fraction and degree of unsaturation (C18:3/C18:2) in potato tuber slices (5 g fresh weight) cv. Kennebec at different times (days) after storage at 4°C. The results are reported as percentage of each fatty acid/total fatty acids analyzed and represent the mean value (±S.E.) of five determinations

FA	*Time* (days)			
	0	30	90	180
C14:0	1.33 ± 0.01	0.82 ± 0.02	0.83 ± 0.03	0.82 ± 0.02
C16:0	32.25 ± 0.39	27.08 ± 0.18	27.22 ± 1.76	25.67 ± 0.39
C18:0	10.24 ± 0.27	8.85 ± 0.43	10.37 ± 0.39	9.93 ± 0.27
C18:1	0.96 ± 0.05	0.62 ± 0.02	0.72 ± 0.05	0.71 ± 0.05
C18:2	41.79 ± 0.61	45.36 ± 0.67	39.47 ± 1.51	40.26 ± 0.61
C18:3	10.44 ± 1.29	14.27 ± 0.07	18.39 ± 0.59	19.60 ± 0.6
Others	3 ± 0.5	3 ± 1.1	3 ± 1.2	3 ± 0.8
C18:3/C18:2	0.25	0.31	0.47	0.49

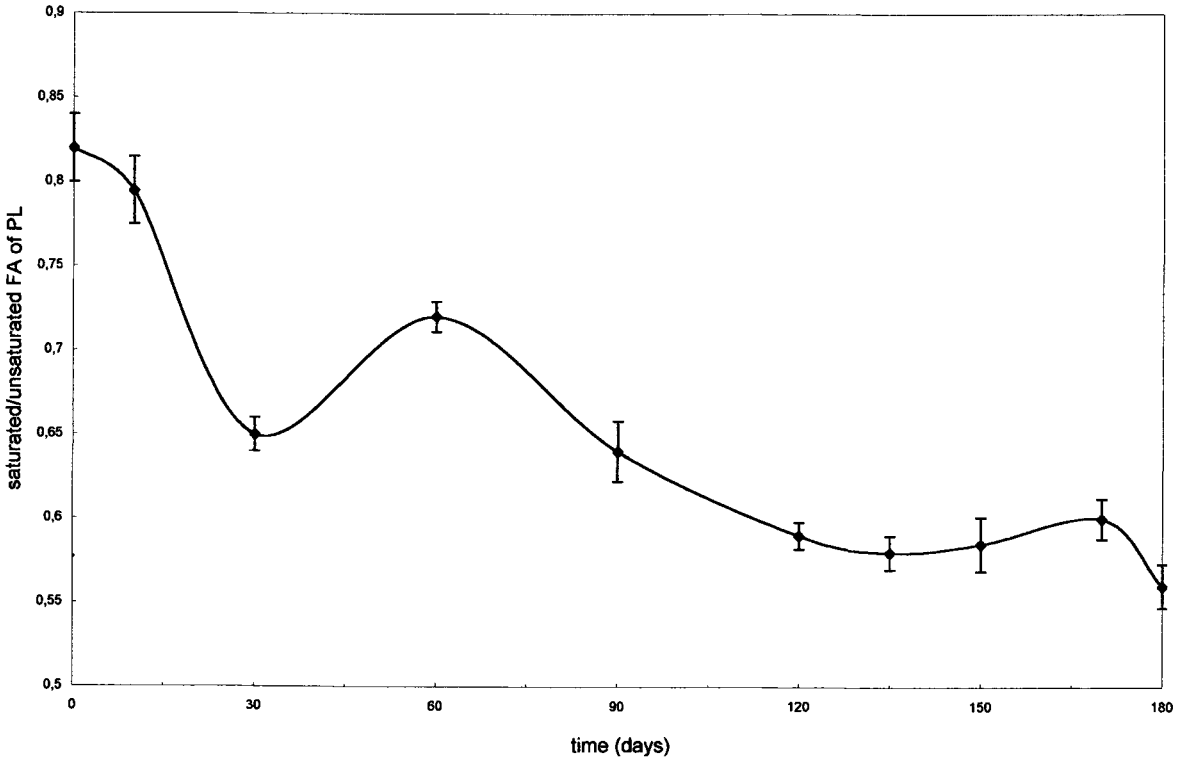

FIGURE 1 Ratio of saturated/unsaturated FA of PL in potato tuber slice from 0 to 180 days of storage at 4°C. Results are reported as mean value (±S.E.) of five determinations.

from 30 days (0.14 ± 0.03 U/mg protein) to 150 days (0.33 ± 0.05 U/mg protein; $P < 0.01$).

The SOD/CAT ratio, which is a parameter to evaluate the sensitivity of cells to pro-oxidants,[15] decreased from time 0 to 15 days and thus the superoxide anions produced during cold storage were not scavenged by the low level of SOD. The SOD/CAT ratio progressively increased from 30 to 75 days maintaining a level higher than time 0 until 150 days. This could lead to an enhancement of SOD-generated H_2O_2 and of hydroxyl radical formation by the Fenton reaction, which could be related to a slight but continuous generation of LOOH from 60 to 150 days. After this period SOD/CAT ratio rapidly decreased.

IAA Content in Below Bud Potato Tuber Slices During Storage

In the period from time 0 to 60 days IAA did not seem to be affected by the rapid changes in the other parameters tested (SOD, CAT, α-tocopherol and LOOH). Subsequently the IAA concentration showed a significant ($P < 0.001$) increase starting from 60 days (432.7 ± 32.3 ng/g fresh weight) up to 180 days (1123.5 ± 38.3 ng/g fresh weight) (Fig. 4), which was probably related to the increase of the bulk of LOOH, connected to the antioxidants unbalance, compared to time 0.

Correlation Among Increase (%) of Antioxidant System, PUFA and IAA

The increase in percentage, in comparison with time 0, of the bulk of the antioxidants (α − tocopherol + CAT + SOD) is reported in Table II. This accumulation is related both to IAA content and to PUFA, reported as sum of C18 : 2 + C18 : 3, in each time point (0–180 days). The trend of the modification of the antioxidant system fits well both with PUFA ($R = 0.99$; $P < 0.001$) and

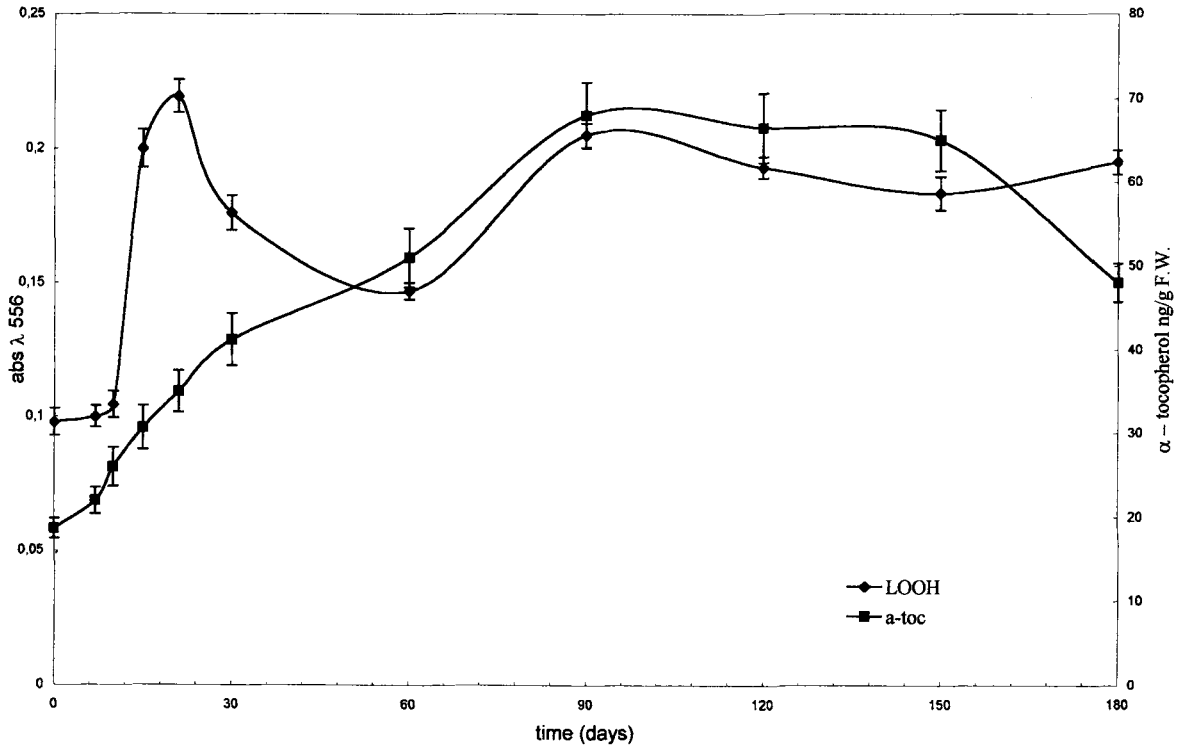

FIGURE 2 Detection of LOOH and α-tocopherol (ng/g fresh weight) in potato tuber slice from 0 to 180 days of storage at 4°C. Results are reported as mean value (±S.E.) of five determinations.

IAA ($R = 0.97$; $P < 0.001$) increase with respect to time 0, and demonstrates that IAA production is associated with a significant alteration of both parameters.

DISCUSSION

The study carried out on below bud tissue for a period of 6 months shows a relationship among PL modifications, antioxidative system alterations, LOOH formation and IAA increase during cold stress. Potato tubers react to cold stress by increasing the amount of lipid unsaturation in order to keep a membrane fluidity, which allows the carrying on of metabolic processes.[4] Desaturases may be involved in this phenomenon during cold acclimation[16], and its stimulation could be mediated by a signal mechanism that leads to the enhancement of the expression of desaturase genes.[17,18] Moreover, low temperature storage leads to an increase of both LAH, responsible for the FA cleavage from the membrane PL, and LOX activity with the formation of LOOH and superoxide anion.[6,7,19] In the early phases of storage (0–30 days), the increase of α-tocopherol seems to be associated to the PUFA increase ($R = 0.75$; $P < 0.01$) and probably the tuber reacts to the membrane oxidative damage, induced by the formation of lipid hydroperoxides, by stimulating the production of α-tocopherol, a well known membrane antioxidant able to inhibit LOOH formation. However α-tocopherol could be also responsible for non-oxidant molecular mechanisms such as activation of the expression of genes related to some growth factor as in animal model.[20] Furthermore, from 0 to 15 days the oxidative burst, not scavenged by SOD (decrease of SOD/CAT ratio) together with LOX activity[7]

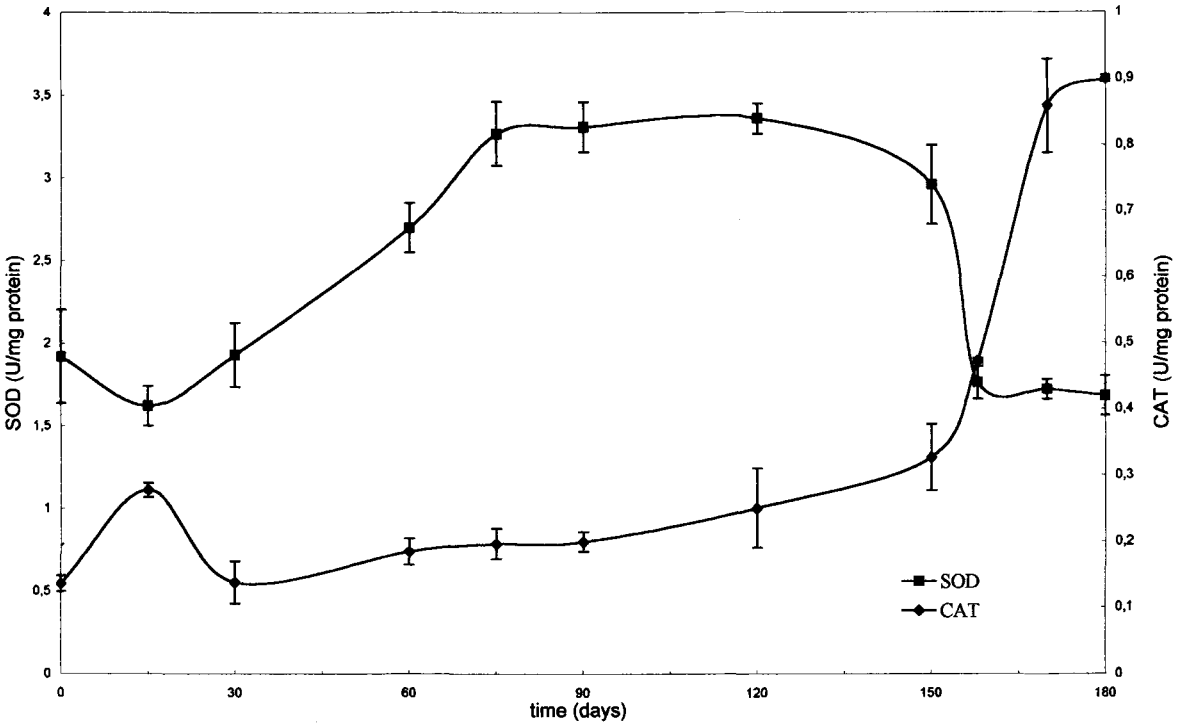

FIGURE 3 SOD and CAT activity (U/mg of protein) in potato tuber slice from 0 to 180 days of storage at 4°C. Results are reported as mean value (±S.E.) of five determinations.

could be responsible of LOOH formation, which is correlated with the steep increase of unsaturated bulk of membrane PL since it could lead to an enhancement of the potential substrate for the formation of LOOH. The peak of LOOH at 21 days might in fact originate from superoxide anion formation, NADPH oxidase system and LOX dependent[19,21] and from hydroperoxyl radical ($HO_2^{\cdot}$) which in plant tissue exists in equilibrium with O_{2^-}.[22] The decrease of SOD activity is then probably due to the trigger of radical species during the early stage of storage which could inhibit enzyme activity.[23] Upon this oxidative stress, after 30 days, the potato tuber seems to react with a steep increase of α-tocopherol, a progressive increase of SOD/CAT ratio, starting from a level similar to time 0 and from 60 days with IAA enhancement

TABLE II Percentage increase of different parameters analyzed over time 0

Days of storage	*α-tocopherol* (ng/g)	*CAT* (U/mg)	*SOD* (U/mg)	*Σ antioxidants*	*IAA* (ng/g)	*PUFA*
15	64.27±3.25	103.74±9.23	−15.62±4.07	152.39±19.11	0.59±0.07	9.11±1.32
30	120.67±11.23	1.54±0.32	0.52±0.04	122.73±18.66	11.92±2.98	15.0±2.42
45	141.02±12.35	10.13±2.01	17.19±4.89	168.35±20.44	−4.19±1.01	6.0±1.04
60	173.16±13.36	35.83±2.66	40.62±5.66	249.62±25.12	22.82±3.77	6.41±1.25
90	264.22±17.28	46.84±4.02	72.39±5.99	383.46±29.79	68.90±4.87	14.31±3.62
120	256.18±20.11	83.92± 5.42	54.16±4.75	394.27±32.14	141.28±13.42	22.62±4.21
150	248.15±22.31	139.79±12.76	−8.33±2.33	379.61±37.65	190.96±27.21	26.78±2.52
180	157.09±12.33	560.79±34.22	−11.46±3.43	706.43±53.21	218.92±43.66	24.70±3.55

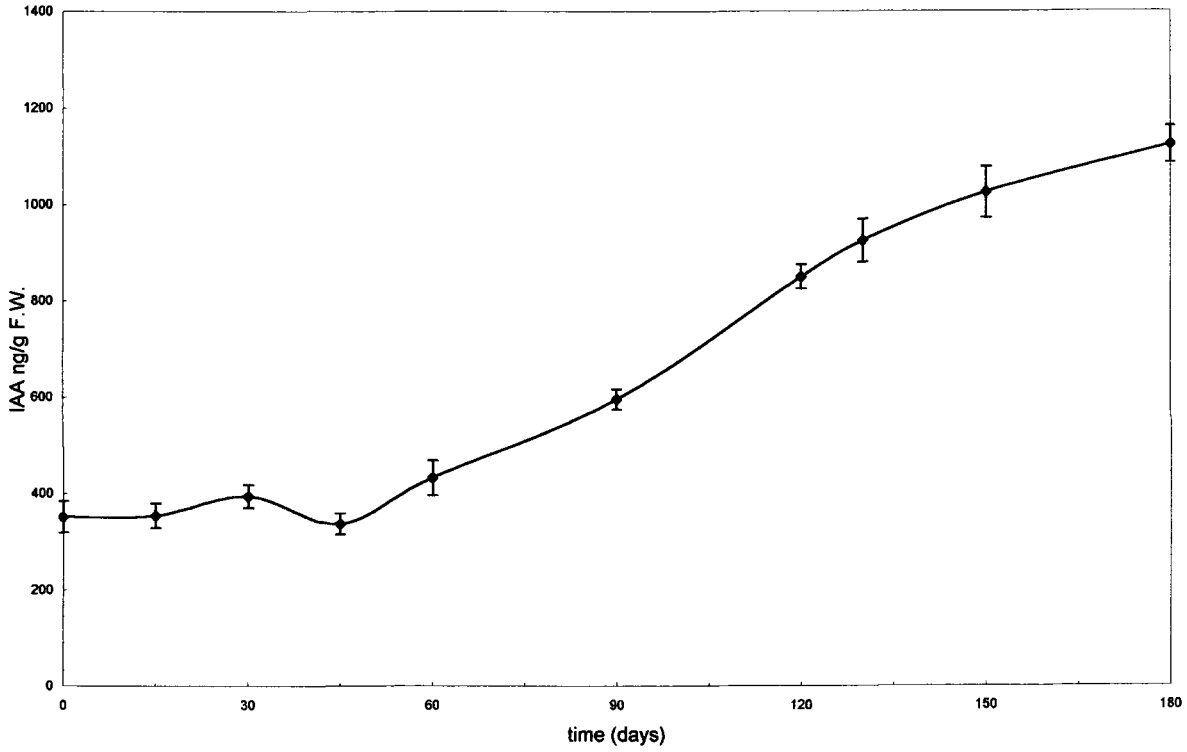

FIGURE 4 Indole-3-acetic acid (IAA, ng/g fresh weight) in potato tuber slice from 0 to 180 days of storage at 4°C. Results are reported as mean value (±S.E.) of five determinations.

in the presence of LOOH levels higher than time 0. Between 75 and 120/150 days the antioxidative system sets to an equilibrium in which SOD/CAT, LOOH, α-tocopherol have a different value in comparison with time 0 and there is a continuous production of reactive oxygen species, which is not completely scavenged. In addition, IAA levels are constantly increasing. After 120/150 days, when LOOH reaches the highest value, the antioxidative system loses its efficiency and the IAA concentration achieves a sufficient level to allow visible sprouting.

In this general picture the significant ($P < 0.01$) increase of LOOH (between 60 and 90 days) might have a relevance in signaling processes. The trend of IAA accumulation below the bud of potato tuber during cold storage is a scarcely studied process and in our experimental conditions, auxin significantly increases from 60 until 180 days. As previously demonstrated in potato tubers,[8] endogenously enhanced (through wounding) and exogenously added LOOH (8–10 μg) stimulated IAA production, which subsequently led to cytological events among which activation and increase of mitoses, starch hydrolysis, reactivation of cell cycle and bud sprouting. The presence of LOOH during the storage, always higher than time 0 by 2–3 μg, induced both oxidative stress and probably IAA enhancement. Furthermore the alteration of antioxidants is related both to the increase of PUFA and IAA formation as reported in Table II and LOOH is correlated with PUFA. We can thus speculate from these evidences that the unbalance of antioxidant system and the chronic production of pro-oxidant species such as lipid hydroperoxides, even if there is no statistical

correlation between these species and IAA, could set up an indirect relationship.

In conclusion, in wounding, potato cells respond to an "acute" stress with a rapid increase of LOOH (from 0 to 20 min) closely related to IAA increase and, in cold stress, respond to a "chronic" oxidative stress with a similar pattern, which suggests that different kinds of abiotic stresses can share a common pathway.

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