UNIVERSITY OF ALBERTA

Physiological Bases for Age-Induced Loss of Apical Dominance, Sprout-Vigor and Rooting Potential in Potato (*Solanum tuberosum* L. cv. Russet Burbank) Seed-Tubers.

By

G. N. Mohan Kumar

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Physiological bases for age-induced loss of apical dominance, sprout-vigor and rooting potential in potato (*Solanum tuberosum* L. cv Russet Burbank) seed-tubers** submitted by G. N. Mohan Kumar in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **HORTICULTURE**.

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Dedication

To all my family members whose constant help and encouragement has guided me so far.

ABSTRACT

The focus of my research was to identify physiological processes that are associated with, and possibly contribute to, a loss of apical dominance, sprout-vigor and rooting potential during long-term aging (2 to 32 months) of potato (*Solanum tuberosum* L. cv. Russet Burbank) seed-tubers. Efforts to mitigate the effects of advanced age by treating seed-cores with indole-3-acetic acid (IAA) failed; while naphathaleneacetic acid restored apical dominance, sprout-vigor and rooting potential of older tubers. These effects may be related to age-induced differences in the ability of sprouts to transport and catabolize IAA. The potential for oxidation of IAA was higher in tuber and sprout tissues from older tubers, which also had higher specific activities of IAAoxidase (IAAox)/peroxidase (POX) relative to tissues from younger tubers. Age-reduced growth potential was also associated with increased lipid peroxidation in older seed-tubers, as evidenced by the accumulation of malondialdehyde (MDA), ethane and lipofuscin-like fluorescent compounds (FCs) with advancing seed-tuber age. The specific activities of phospholipase and lipoxygenase decreased, while those of free-radical (FR) scavenging enzymes increased with advancing age. The increased specific activity of superoxide dismutase (SOD) may be in response to a higher FR-titre in tissues from older seed-tubers, and higher activities of POX and catalase may be induced to scavenge the peroxide formed by SOD activity. Aging of tubers was also accompanied by a decrease in protein content and an increase in free-amino-acid content. Moreover, older tubers had a reduced ability to synthesize protein during sprouting and this may limit the ability of older tubers to synthesize the enzymes required for mobilization and translocation of reserves to developing sprouts.

A loss in membrane integrity occurs with advancing tuber age, and the possibility that 'leaky' membranes are a greater 'sink' for ATP was investigated. Plasma membranes (PMs) were isolated from young and old tubers at different stages of sprouting and H^+-ATPase activity was compared. Electron microscopy showed that the PM fractions (recovered from a liquid-polymer two-phase system) contained a high proportion of intact vesicles. Phosphotungstic acid-chromic acid staining, glucan synthase II activity, pH optimum of 6.5, and appreciably lower contamination from endoplasmic reticulum and Golgi membranes (as indicated by lower cytochrome c reductase and latent inosine diphosphatase activities) indicated that the upper phase (UP) fraction was composed of PMs. However, a relatively low sensitivity to vanadate inhibition indicated that the ATPase in vesicles from the UP may not be entirely of PM origin. Comparison of kinetic parameters of the H^+-ATPase isolated from young and old seed-tubers during sprouting, revealed a significant age-enhanced ATPase activity. Further work is required to understand the physiological significance and mechanism of activation of ATPase during aging of tubers.

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DATE: January 8, 1993. G. N. Mohan Kumar.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER IV

CHAPTER V

CHAPTER VI

LIST OF ABBREVIATIONS

alt	alternate (CN^- insensitive) respiration
APase	acid phosphatase (EC 3.1.3.2)
ATP	adenosine-5'-triphosphate
ATPase	adenosine triphosphatase (EC 3.6.1.35)
ANOVA	analysis of variance
BSA	bovine serum albumin
BHT	butylated hydroxytoluene
CAT	catalase (EC 1.11.1.6)
CCR	cytochrome c reductase (EC 1.6.99.3)
cyt c	cytochrome c
2,4-D	2,4-dichlorophenoxyacetic acid
DAP	days after planting
DBI	double-bond-index
DCP	dichlorophenol
DEX	dextran
DNA	deoxyribonucleic acid
DTT	DL-dithiothreitol
DMSO	dimethyl sulfoxide
dw	dry weight
EB	erythrosin B
EM	electron microscopy
ER	endoplasmic reticulum
FC	fluorescent compound
FID	flame-ionization detector
FR	free-radical
GC	gas chromatograph
GSII	glucan synthetase II (EC 2.4.1.34)
IAA	indole-3-acetic acid
IAAox	IAA oxidase
IBA	indole-3-butyric acid
IDP	inosine-5'-diphosphate
IDPase	inosine-5'-diphosphatase
K_m	Michaelis constant

LAI	leaf area index
LDH	lactate dehydrogenase (EC 1.1.1.28)
LOX	lipoxygenase (EC 1.13.1.13)
LP	lower phase
LPC	lysophosphotidylcholine
MDA	malondialdehyde
NAA	naphthaleneacetic acid
NBT	nitrobluetetrazolium
PEG	polyethyleneglycol
PK	pyruvate kinase (EC 2.7.1.40)
PL	phospholipid
PM	plasma membrane
PMSF	phenyl methyl sulfonyl fluoride
POX	peroxidase (EC 1.11.1.7)
PTAC	phosphotungstic acid-chromic acid
PUFA	polyunsaturated fatty acid
PVP	polyvinyl pyrolidone
RNA	ribonucleic acid
SHAM	salicylhydroxamic acid
SOD	superoxide dismutase (EC 1.15.1.1)
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEM	transmission electron microscopy
TLC	thin-layer chromatography
UDPG	uridine-5'-diphosphoglucose
UP	upper phase

Chapter I

INTRODUCTION

Potatoes (*Solanum tuberosum* L. cv. Russet Burbank) are propagated vegetatively by planting virus-free seed-tubers that were harvested the previous fall and stored for 7 to 8 months at 4°C and 95% RH. Though seed-tubers can be stored for up to 3 years under these conditions, storage beyond 7 to 8 months results in a loss in apical dominance, sprout vigor, rooting potential and subsequent plant-vigor (Mlkitzel and Knowles, 1990). Following harvest, seed-tubers enter a period of dormancy which lasts for about 3 months in the Russet Burbank cultivar. As tubers emerge from dormancy, a single vigorous sprout develops from the apical end of the tuber and exerts a strong correlative inhibition over lateral meristems. Sprout vigor increases with length of storage up to about 7 to 8 months. With advancing storage period, tubers initially lose apical dominance between nodes and, with further aging, apical dominance within each node is eventually lost. Consequently, multiple sprouts appear per node, signifying a loss of apical dominance between the meristems within each node. During the period over which apical dominance is lost, sprout vigor and root-growth potential also decline. Aging for about 28 to 36 months causes a complete loss of sprouting ability, and the axillary meristems directly form small tubers. The effects of seed-tuber age on sprouting characteristics of whole tubers and single-eye seedcores are illustrated in Fig. 1 (Chapter II). Single-eye-containing cores from the appropriate age of seed-tuber behave similarly to whole tubers. Krijthe (1962) found similar age-induced effects on sprout development for the 'Bintje' cultivar.

There is a paucity of information on the physiological and biochemical changes associated with aging and loss of vigor in vegetative propagules, although many such studies have been done with true seeds. Most studies on true seeds, however, involve an elevated temperature treatment to accelerate the aging process (Stewart and Bewley, 1980; Gidrol et al., 1989). Since at a constant temperature (4°C), changes in growth potential and associated physiological processes can be detected within months of harvest of potato, seed-tubers are well-suited for studies on the physiology of aging of vegetative propagules.

Auxin plays an important role in apical dominance and adventitious root formation. Synthesis, translocation, conjugation into biologically inactive forms, and potential for catabolism are some of the known mechanisms through which endogenous auxins are regulated. Of particular relevance, is the finding that the rate of auxin translocation is inversely related to physiological age of tissue in many plants (Smith and Jacobs 1969; Veen and Jacobs 1969; Davenport *et al.* 1980). Moreover, auxin content has been shown to be regulated enzymatically by IAAox and POX (Moore 1989; Beffa *et al.* 1990). In plants with higher activities of these enzymes, IAA generally fails to elicit growth responses, while synthetic auxins (eg., NAA) remain effective. IAAox and POX are unable to degrade synthetic auxins (Gianfangna 1987).

Though aging is distinctly different from senescence, the two processes have similarities at the biochemical level. For example, increased lipid peroxidation (Heath and Packer 1968), FR titre (Leshem *et al.* 1981; McRae and Thompson 1983; Lynch and Thompson 1984) and a gradual disruption of membrane integrity are common to both aging (Knowles and Knowles 1989) and senescing plant tissues (Paliyath *et al.*, 1987). As seeds advance in age, increased lipid peroxidation causes a gradual loss of membrane integrity (Wilson and McDonald 1986). Though the mechanism of membrane deterioration in aging potato seed-tubers is not fully understood, in senescing tissues and in aging seeds the action of lipolytic enzymes (Brown *et al.* 1991) and/or FRs (Fobel *et al.* 1987) inflict peroxidative damage on membranes. Increased peroxidation of membrane lipids results in enhancement of microviscocity. With the onset of senescence, activities of lipolytic enzymes (Lynch and Thompson 1984) often increase, and the activities of FR-scavenging enzymes often decline (Dhindsa *et al.* 1981). Membranes thus become a target for FR-mediated peroxidative damage. The net result is a loss in membrane integrity and increased permeability (Barber and Thompson 1980). Peroxidative damage to membranes alter the physical chemistry of the membrane bilayer and thus can influence the kinetic properties of membrane-bound enzymes (Carruthers and Melchior 1986). For example, ATPase is an intrinsic protein (Harper *et al.* 1989) whose activity can be modified by its lipid environment (Cooke and Burden 1990).

In addition to cellular membranes, FRs can damage a number of other macromolecules which are crucial to cellular homeostasis. The products of lipid peroxidation, FRs and MDA, are potential inhibitors of protein synthesis. FRs are highly reactive and capable of self-propagation. Besides inactivating enzymes directly, they are known to damage DNA, ribosomes and RNA synthesis (Osborne 1980). Exogenous addition of MDA was shown to inhibit *in vivo* and *in vitro* protein synthesis (Dhindsa 1982). During the processes of aging and senescence in plants, the equilibrium between the rates of synthesis and degradation of protein is known to shift in favour of degradation (Brady 1988; Thimman 1980; Dhindsa *et al.* 1981; Richardson 1981; Miller and Huffaker 1985; Lalonde and Dhindsa 1990). As in germinating seeds, protein synthesis is required for the mobilization of reserves during sprouting of potato tubers. An age-induced loss in the ability to synthesize proteins may thus adversely affect sprout-vigor.

Aging potato seed-tubers feature a loss of apical dominance and reduced sprout-vigor and rooting potential, in spite of an increased rate of respiration and ATP generation during sprouting (Mikitzel and Knowles, 1990). The goal of my research was to characterize the biochemical and physiological bases for age-induced changes in growth potential of 'Russet Burbank' seed-potatoes. The specific objectives were to:

1. determine if the age-induced loss of apical dominance and rooting potential is associated with reduced capacity for basipetal translocation and/or increased catabolism of auxin in developing sprouts.
2. characterize a potential mechanism (enzymatic, FR-driven or both) by which membranes deteriorate during aging of potato seed-tubers, and compare and contrast the deteriorative processes with that of studies reported on classical senescence.
3. compare protein-synthetic ability of young and old seed-tubers during sprouting, and to determine if age-reduced sprout-vigor is associated with reduced capacity for protein synthesis.

4. standardize a method for isolation of plasma membrane vesicles from potato tubers and compare kinetic parameters of the H^+-ATPase in vesicles isolated from different ages of tubers at various stages of sprout development.

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Chapter II

Involvement of Auxin in the Loss of Apical Dominance and Plant Growth Potential Accompanying Aging of Potato Seed-Tubers[1]

INTRODUCTION

Potato seed tubers are harvested and stored for 7-9 months at 4°C (95% RH) prior to serving as vegetative propagules for commercial production. Despite the relatively short commercial storage period, viability of some cultivars can be maintained as long as 3 years in cold storage; however, sprouting potential and subsequent plant vigor are greatly affected by age of the seed tuber. The potato is thus a good subject, incorporating generative and postharvest attributes, for basic studies of the metabolic processes associated with aging of plant tissues.

Figure 1 illustrates the effects of long-term aging (up to 3 years) on sprouting characteristics of 'Russet Burbank' seed-tubers, as compiled from a number of recent publications (15,18,19,20, 21). After harvest, 'Russet Burbank' tubers undergo a period of dormancy, which lasts approximately 3 months at 4°C. As tubers emerge from dormancy, a high degree of apical dominance is imposed on lateral meristems by the single sprout developing from the apical end of the tuber. Sprout vigor increases over the initial 7- to 9-month storage period. There is a gradual loss of apical dominance with advancing age, as evidenced by an increase in the number of sprouts developing per tuber. Apical dominance is initially lost between nodes (eyes) on the stem (tuber); however, storage beyond 14 months results in the production of multiple sprouts per node, indicating a further loss of apical dominance between the meristems within each node. Moreover, storage from about 9 to 21 months induces changes within the tubers and meristems that result in significant loss of

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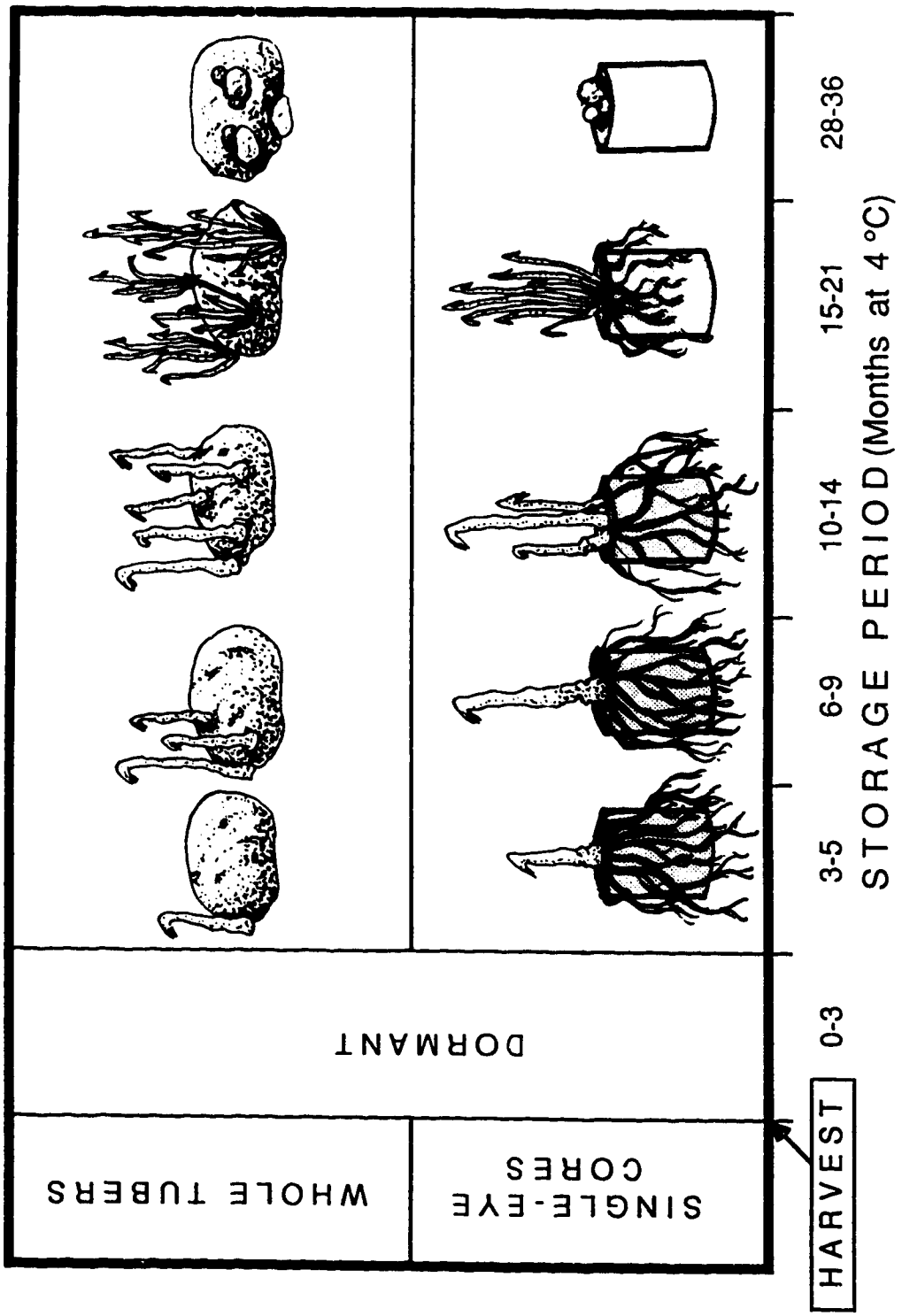
Fig. 1. Schematic presentation of the effects of long-term storage (4°C, 95 % RH) of Russet Burbank seed tubers on sprouting behavior of single-eye seed-cores and whole seed tubers. The illustrated effects were compiled from previous publications (15,18,19,20, 21).


WHOLE TUBERS
SINGLE-EYE CORES
DORMANT
HARVEST
0-3
3-5
6-9
10-14
15-21
28-36
STORAGE PERIOD (Months at 4 °C)

sprout vigor and root-growth potential. The tubers completely lose the ability to sprout at the 28 to 36-month-old stage, as evidenced by tuberization of the meristems within each node. Figure 1 also shows that single-eye-containing cores from various ages of seed tubers display the characteristic effects of age on sprouting capacity that are evident in whole tubers. Although the time scale is different, these age-induced effects on sprout development are consistent with those observed by Krijthe (16) for the Bintje cultivar.

The loss of apical dominance, sprout vigor and root-growth potential accompanying aging of seed tubers may be auxin-related. Indeed, IAA has been shown to regulate apical dominance and adventitious root formation in a number of plants (5). It was suggested that the loss of apical dominance and root-growth potential observed during sprouting of aged seed tubers may be due to: (i) reduced ability of older meristems to synthesize IAA during sprouting, (ii) reduced ability of older meristems to translocate IAA to lateral buds, (iii) increased ability of sprouts from older seed tubers to conjugate IAA into biologically inactive forms, and/or (iv) an increased ability of sprouts from older tubers to catabolize IAA enzymatically (20). Treatment of cut seed-pieces or single-eye cores from older seed tubers with NAA partly restores apical dominance and increases shoot and root growth (15,20). This suggests that auxin is involved in the age-induced changes in growth potential, as characterized in Figure 1; however, the efficacy of IAA in mitigating the deleterious effects of advanced age on sprout growth and development is not known.

In this study, pre-plant treatment of 18-month-old seed-cores with NAA restored apical dominance and enhanced sprout vigor and root-growth potential, while IAA was ineffective. However, both IAA and NAA had a negative effect on growth from seed-cores from 6-month-old seed tubers, suggesting that aging increases the ability of tuber and sprout tissues to break down IAA. The inability of exogenously applied IAA to affect plant growth from older tubers may be due to a greater ability of the older tuber tissue to catabolize exogenously applied IAA via IAAox/POX. Furthermore, age-induced loss of apical dominance, sprout vigor and root-growth potential may be manifested through increased catabolism of endogenous

IAA, or reduced ability of sprouts to translocate IAA basipetally. We examined these possibilities by profiling IAA translocation patterns, quantifying the rates of decarboxylation of IAA, and comparing the activities of IAAox and POX in tuber and sprout tissues during sprouting from single-eye seed-cores from different ages of seed tubers.

MATERIALS AND METHODS

Plant material

Certified potato (*Solanum tuberosum* L. cv Russet Burbank) seed tubers, obtained from a commercial seed grower at harvest, were stored at 4°C and 95% RH for up to 20 months. These conditions inhibited sprouting over the entire storage period. For discussion purposes, 6 to 8-month-old tubers are referred to as physiologically 'young' while 18 to 20-month-old tubers are referred to as physiologically 'old' or 'aged'.

Comparison of the effect of IAA and NAA on growth

Single-eye seed-cores were treated with equimolar concentrations of IAA (Sigma) or NAA (United States Biochemical Corporation, Ohio) to compare the relative effectiveness of these auxins on various growth parameters during plant establishment. Seed tubers of uniform size were removed from cold storage, washed and acclimated to room temperature for 24 h. Seed-cores (1.8 cm diameter X 2.4 cm length) were cut with a cork borer from the middle portion of 6- and 18-month-old seed tubers. The cores were rinsed in water and air dried for 30 min prior to immersing in solutions of 0.1% (w/w) Tween-20 containing 0, 270 and 540 µM IAA or NAA for 10 min. The IAA and NAA were initially solubilized in DMSO. The treated cores were air dried as above and planted (3 cores per pot) in 15 cm diameter pots containing peat-vermiculite (1:1 v/v). The plants were grown in a greenhouse from March 21 to April 10, 1991 where high-intensity sodium vapor lamps supplemented natural light. The greenhouse was maintained at 25°C day/18°C night with a 16 h photoperiod. The pots were watered as needed and the number of days to 100% sprout emergence was recorded. Sprout emergence was considered to be 100% when at least one sprout from each core had broken the soil surface.

The plants were harvested at 20 DAP and were divided into stems, leaves and roots. Stem number, leaf number and leaf area were determined. The stems, leaves and roots were then dried at 80°C for 72 h and dw recorded. The treatments were arranged factorially (2 ages X 2 auxins X 3 concentrations of each auxin) in a randomized complete block design with 4 replications (48 pots).

IAAoxidase and peroxidase activities during sprouting

In this study, 50-60 g seed pieces, cut from the apical portion of 8- and 20-month-old seed tubers, were planted in pots (one seed piece per pot) containing a peat-vermiculite mix and placed in the greenhouse. To prepare seed pieces, uniformly sized tubers were cut radially to produce apical halves weighing approximately 100 g, which were in turn cut longitudinally to produce two 50 g seed pieces. One seed piece of each pair was immediately frozen at -20°C to serve as the zero DAP sample; the other was planted as described above. IAAox activity was determined in the seed tuber tissue at 0, 15, 20, 25 and 30 DAP. After rinsing the harvested seed pieces with distilled water, IAAox was extracted by grinding 25 g of tissue in a mortar and pestle with 25 mL Hepes buffer (50 mM, pH 7) containing 2 mM $Na_2S_2O_5$. The crude homogenate was strained through a layer of mira-cloth and centrifuged for 30 min at 10,000 g. All manipulations were carried out at 4°C . IAAox activity was determined for the supernatants which were stored at -20°C prior to analysis (described below). Treatments in this study were arranged factorially (2 ages X 5 DAP) in a randomized complete block design with 4 replicates (40 pots).

In another study, IAAox and POX activities were measured in sections of etiolated sprouts and associated seed cores from 6- and 18-month-old seed tubers. Single-eye cores, prepared as previously described, were planted in peat-vermiculite and sprouted in the dark at 23°C . The one-week-old etiolated sprouts (2.5 to 4.0 cm long) were then divided into apical and basal halves for analysis. In addition, the uppermost portion of the seed-cores (1.8 cm diameter X 0.5 cm length) was analyzed for IAAox and POX activities. For the 18-month-old sprouted cores, only 2.5 to 4.0 cm long sprouts were selected for enzyme analysis.

Treatments were arranged factorially (2 ages X 3 portions) in a randomized complete block design with 3 replications. Ten sprouted cores constituted one replication.

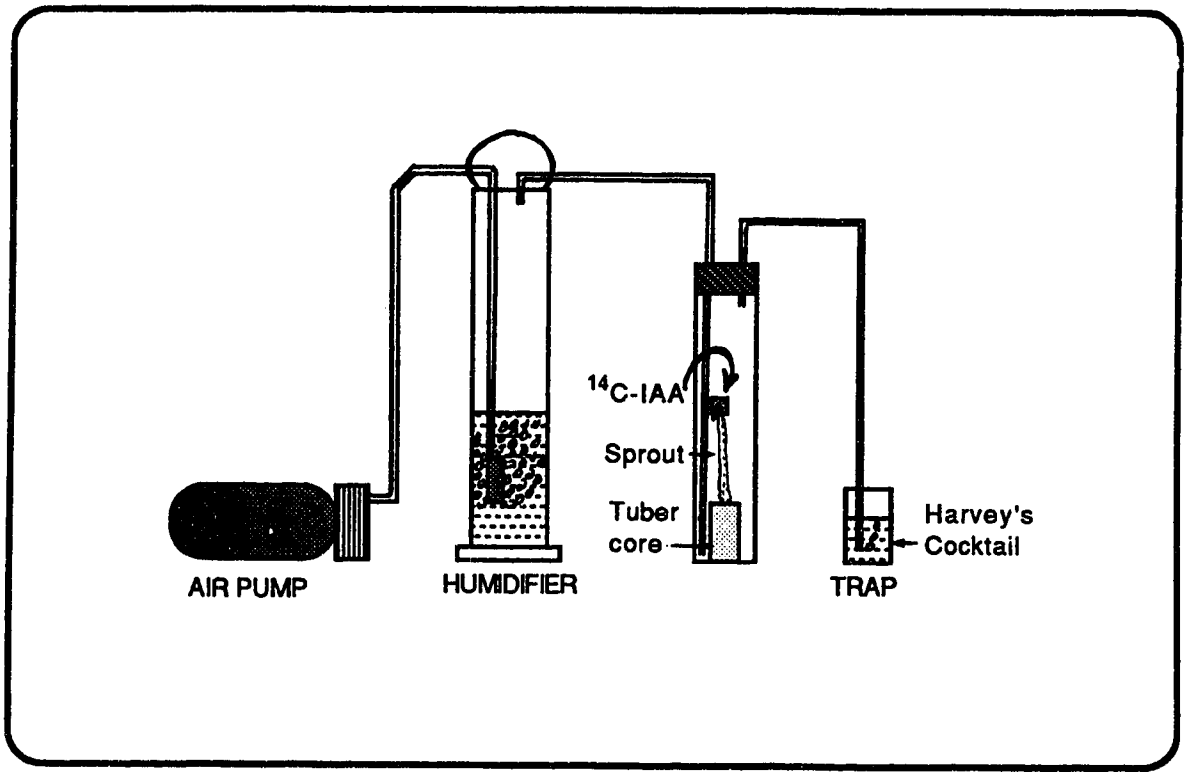
IAAox activity was determined polarographically with a YSI biological O_2 monitor by the method of Palmieri et al. (23). The reaction medium consisted of 1.1 mM $MnCl_2$, 1.1 mM DCP, 0.5 mM H_2O_2 and 750 µM IAA in 100 mM sodium acetate buffer (pH 3.7). The reaction at 30°C was initiated by addition of 100 µL enzyme extract (see above) to 2.4 mL of the reaction medium. Enzyme activity was expressed as nmol O_2 min^{-1} mg $protein^{-1}$.

POX activity was determined colorimetrically by the method of Soressi *et al.* (25). The reaction medium consisted of 0.03% (v/v) H_2O_2 and 15 mM guaiacol (Sigma) in sodium acetate buffer (50 mM, pH 6.0). The reaction (at 23°C) was initiated by adding 10 to 40 µL of the enzyme extract to 3 mL of the reaction medium. The change in absorbance at 470 nm due to oxidation of guaiacol was monitored on a recording sectrophotometer (Varian, Cary 219). The specific activity was expressed as the change in absorbance min^{-1} mg $protein^{-1}$. In all of these studies, protein was determined by a modified Lowry method using BSA as a standard (2).

Transport and decarboxylation of [1-^{14}C]-IAA

Tuber cores (1.6 cm diameter X 2.0 cm length), cut from the middle portion of 7- and 19-month-old tubers, were planted in peat-vermiculite and sprouted in the dark for 9 days. Cores with 7 to 8 cm long etiolated sprouts were selected for use in the translocation study. The 19-month-old cores were pruned to three uniform sprouts to approximate the fresh weight of the single, etiolated sprout from 7-month-old cores. The sprouted cores were placed inside 40 mL test-tubes (one core per tube). Approximately 162 nmoles of [1-^{14}C]-IAA (6 mCi/mmol) in Mes buffer (50 mM, pH 5.5) was incorporated into 1% (w/v) agar cylinders (0.5 cm diameter X 1.0 cm length) which were then placed on the intact apical meristems of the sprouts, so that the apical hook was imbedded in the agar to a depth of approximately 0.5 cm (Fig. 2). In addition, agar-cylinders containing [^{14}C]-IAA were imbedded on the tips of plungers from 1 mL syringes (8

Fig. 2. A flow-through system for studying [1-^{14}C]-IAA translocation and decarboxylation by etiolated sprouts from seed-cores from different ages of seed tubers. The [1-^{14}C]-IAA was incorporated into agar cylinders and applied to the intact apices of 9-day-old etiolated sprouts from 7- and 19-month-old tuber cores. Humidified air was passed through the plant chamber, and $^{14}CO_2$ released via decarboxylation was trapped in scintillation cocktail. Activity in the CO_2 traps was determined at 2 h intervals over a 10 h incubation period in the dark.


^{14}C-IAA
Sprout
Tuber
core
Harvey's
Cocktail
AIR PUMP
HUMIDIFIER
TRAP

cm long) which were placed inside test-tubes to account for spontaneous decarboxylation (if any) over the incubation interval. The tubes were closed with serum stoppers that had inlet and outlet ports. Air-flow to each tube was maintained at 85 mL min^{-1} via a manifold. Prior to entering the manifold, the air was humidified by passing it through a diffusion-stone submersed in distilled water (Fig. 2). The outlet air from each tube was bubbled through 10 mL of scintillation cocktail (R J Harvey Instruments Corporation, New Jersey) to trap $^{14}CO_2$. The CO_2 traps were changed every 2 h and activity in the traps was determined. After 10 h of incubation in the dark at 23°C, the agar-blocks were removed from the sprout apices and the sprout tips were rinsed with distilled water and blotted dry. Sprouts were excised from the cores, roots were excised from the sprouts, and the sprouts were further sectioned into apical and basal halves. Sprouts, roots and cores were lyophilized, weighed and combusted in a biological oxidizer (model OX 300, R J Harvey Instruments Corporation, New Jersey). Samples were then counted with a Minaxiß Tri-carb 4000 series liquid scintillation counter. Agar blocks were also combusted and counted. The experiment was constructed as a randomized complete block design (2 ages X 3 portions) with 3 replications. The main effects of tuber age and portion on translocation of the [^{14}C]-IAA (along with relevant interactions) were partitioned from treatment variation in the ANOVA.

RESULTS

Growth response to IAA and NAA treatment

On average, auxin treatment of single-eye seed-cores from 6- and 18-month-old seed tubers delayed emergence, and the effect was linear with increasing concentration. However, the magnitude of the effect depended upon tuber age, auxin type (IAA or NAA) and concentration, as characterized by a highly significant 3-way interaction among these treatments (Table 1). The auxin-induced delay in emergence was greater for NAA than IAA. Moreover, when compared with the response from older cores, NAA (540 µM) was approximately 1.7-fold more effective at delaying sprout emergence from younger cores.

TABLE. 1. Number of days for 100 % emergence of sprouts from IAA- and NAA-treated single-eye-seed cores from 6-and 18-month-old seed tubers.

		Days to sprout emergence	
Auxin	[Auxin] µM	6-month-old	18-month-old
IAA	0	8.5	10.5
	270	9.5	11.0
	540	10.0	10.5
NAA	0	8.5	10.5
	270	14.5	13.2
	540	15.0	14.2

Age[a]	NS[b]
Auxin	0.01
$[Auxin]_L$	0.01
$[Auxin]_D$	0.05
Age X Auxin	NS
Age X$[Auxin]_L$	0.05
Age X $[Auxin]_D$	NS
Auxin X $[Auxin]_L$	0.01
Auxin X $[Auxin]_D$	NS
Age X Auxin X $[Auxin]_L$	0.01
Age X Auxin X $[Auxin]_D$	NS

[a]Sources of variation (L and D subscripts indicate linear and deviations, respectively).

[b]Significance levels for indicated sources of variation (NS, non-significant).

Treatment with IAA had no effect on the time to sprout emergence from 18-month-old cores, but significantly delayed sprout emergence from 6-month-old cores.

The effects of seed tuber age, auxin type and concentration on growth (per seed-core basis) and overall morphologies of plants from the 6- and 18-month-old single-eye seed-cores are illustrated in the polygonal diagrams of Figure 3. For clarity, polygonal diagrams depicting the 270 µM auxin treatments were not included in the figure. However, a summary of the ANOVA for the effects of all treatments (including 270 µM auxin) on each of the yield components displayed in Figure 3 is presented in Table 2. Plant growth on a per stem basis was also analyzed and is presented in Table 3. Untreated single-eye cores from 6-month-old seed tubers produced only one stem per core over the 20-day growth interval, while cores from 18-month-old tubers averaged 7.5 stems each (Fig. 3), illustrating a significant loss of apical dominance with advanced age (Table 2). As with the effects on emergence, it is evident from the analysis presented in Table 2 that tuber age, auxin type and auxin concentration interacted to affect the degree of apical dominance (stem number per seed-core). While IAA was ineffective, NAA (540 µM) reduced the total number of stems from older seed-cores to 2.6 and thus partially restored apical dominance. Auxin treatment of seed-cores from 6-month-old seed tubers did not affect stem number.

At 20 DAP, plants from untreated 18-month-old seed-cores had 3.5 times as many leaves as those from 6-month-old tuber cores (Fig. 3). However, on a per stem basis, plants from younger cores produced approximately twice as many leaves as did those from older cores (Table 3). Furthermore, age interacted with auxin type and auxin concentration in affecting the number of leaves produced per stem. Auxin type had no effect on the number of leaves per stem on plants from older cores. On younger cores, a linear decrease in leaf number per stem was evident with increasing concentration of NAA (Leaf no. per stem = $7.7 - 4.44e^{-3}$[NAA], $r = -0.99$, $P<0.01$); this effect was no doubt related to the previously described delayed emergence caused by this auxin.

Fig. 3. Polygonal diagrams showing the effects of preplant applications of IAA or NAA to single-eye seed-cores from 6- and 18-month-old seed tubers on growth and dry matter partitioning in 20-day-old plants. The seed-cores were immersed in the appropriate concentration of IAA or NAA for 5 min before planting. The axes are defined on the 540 μM NAA polygonal diagrams. A summary of the statistical analyses for each yield component is presented in Table 2.

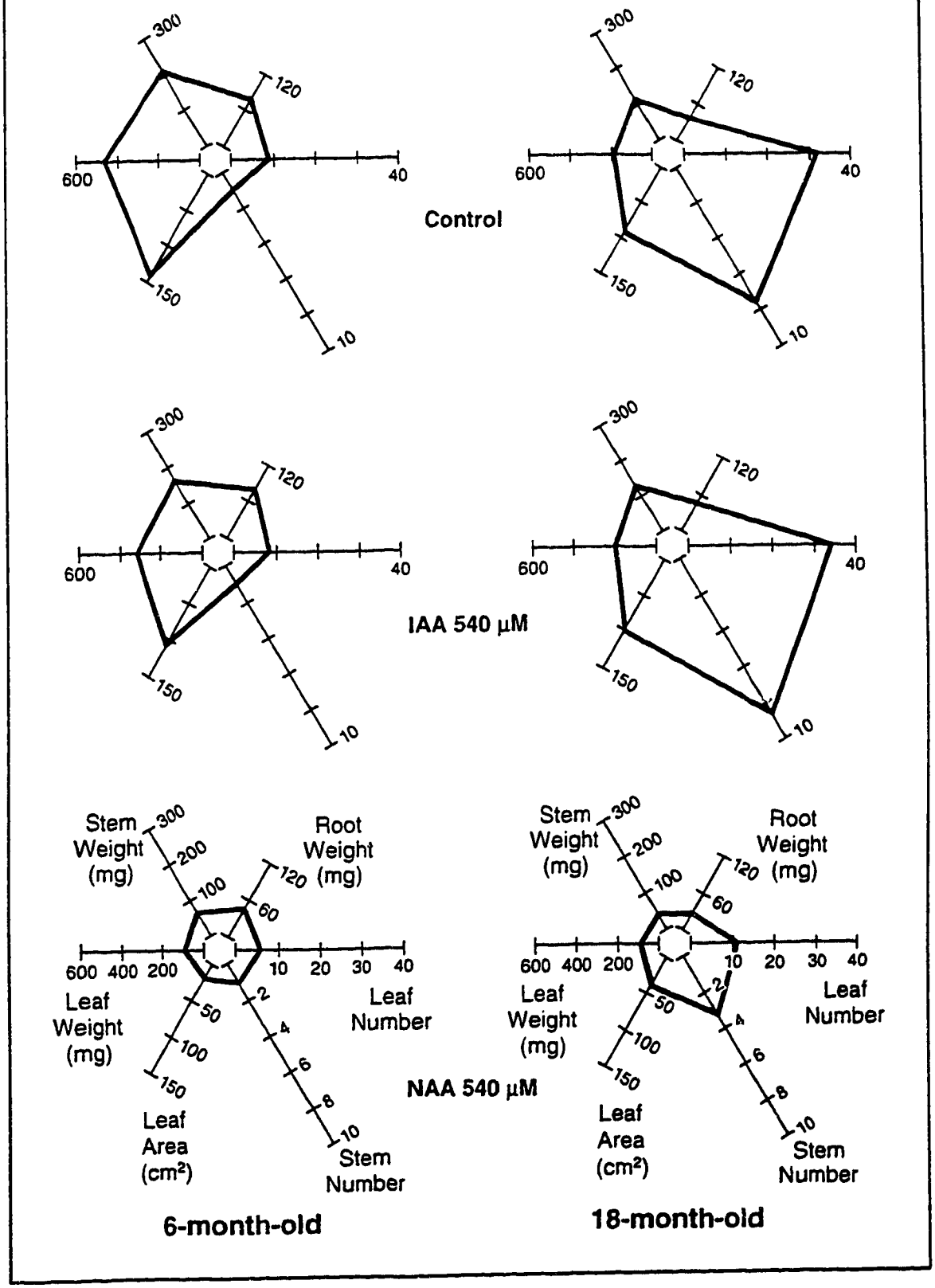

Control
IAA 540 µM
NAA 540 µM
Stem Weight (mg)
Root Weight (mg)
Leaf Weight (mg)
Leaf Number
Leaf Area (cm²)
Stem Number
6-month-old
18-month-old

TABLE. 2. Summary of analyses of variance for various yield components of 20-day-old plants grown from single-eye seed cores from 6- and 18-month-old potato seed tubers.

Source of variation	Stems (No./core)	Leaves (No./core)	Dry weight (mg/core)			Leaf area (cm^2/core)
			Stems	Leaves	Roots	
Age[a]	0.01[b]	0.01	0.01	0.01	0.01	0.05
Auxin	0.01	0.01	0.01	0.01	NS	0.01
$[Auxin]_L$	0.05	NS	0.01	0.01	NS	0.01
$[Auxin]_D$	NS	NS	NS	0.05	NS	0.05
AgeX Auxin	0.01	0.05	NS	0.01	NS	0.05
Age X $[Auxin]_L$	NS	NS	0.01	0.01	NS	0.01
Age X $[Auxin]_D$	NS	NS	NS	0.01	NS	NS
Auxin X $[Auxin]_L$	0.01	0.05	0.01	0.01	NS	0.01
Auxin X $[Auxin]_D$	NS	NS	NS	NS	NS	0.05
Age X Auxin X $[Auxin]_L$	0.01	NS	NS	NS	NS	NS
Age X Auxin X $[Auxin]_D$	NS	NS	NS	NS	NS	NS

NOTE: Seed cores were treated with 0, 270 or 540 µM IAA or NAA prior to planting. The values refer to the levels of significance of F values for the indicated sources of variation. Data are presented in Fig. 3.

[a]Sources of variation (auxin concentration is indicated by [auxin]; L and D subscripts indicate linear and deviations, respectively).

[b]Significance levels for indicated sources of variation (NS, non-significant).

TABLE. 3. Growth and dry matter partitioning in 20-day-old plants obtained from IAA- and NAA-treated single-eye seed cores from 6- and 18-month-old seed tubers.

		Dry weight (mg/stem)						Leaf area				Leaf number	
		Stem		Root		Leaf		cm^2/stem		cm^2/leaf		(No./stem)	
Age (months)		6	18	6	18	6	18	6	18	6	18	6	18
Auxin	[Auxin] (μM)												
IAA	0	186	15	85	5	391	25	128.3	12.2	17.1	2.9	7.9	4.0
	270	162	17	86	5	324	31	111.5	11.2	12.3	2.6	8.9	4.2
	540	161	15	77	5	326	24	110.0	11.6	13.2	2.9	8.3	4.0
NAA	0	189	15	85	5	391	25	128.3	12.2	17.1	2.9	7.7	4.0
	270	84	23	48	12	132	50	39.8	22.6	6.0	4.8	6.5	4.7
	540	67	19	45	11	86	38	22.3	16.1	4.0	3.9	5.3	4.1

Age[a]	0.01[b]	0.01	0.01	0.01	0.01	0.01
Auxin	0.01	NS	0.01	0.01	0.01	0.01
$[Auxin]_L$	0.01	NS	0.01	0.01	0.01	NS
$[Auxin]_D$	NS	NS	NS	NS	0.05	NS
Age X Auxin	0.01	0.05	0.01	0.01	0.01	0.01
Age X $[Auxin]_L$	0.01	NS	0.01	0.01	0.01	NS
Age X $[Auxin]_D$	0.05	NS	0.05	0.05	0.01	NS
Auxin X $[Auxin]_L$	0.01	NS	0.01	0.01	0.05	0.05
Auxin X $[Auxin]_D$	NS	NS	NS	NS	NS	NS
Age X Auxin X $[Auxin]_L$	0.01	NS	0.01	0.01	0.01	0.05
Age X Auxin X $[Auxin]_D$	NS	NS	NS	NS	NS	NS

[a]Sources of variation (L and D subscripts indicate linear and deviations, respectively).
[b]Significance levels for indicated sources of variation (NS, non-significant).

On a per core basis, significant age X auxin type and auxin type X concentration (linear) interactions characterized the effects of auxin on total leaf number (Table 2, Fig. 3). Average leaf number per core was unaffected by IAA, but declined by 4.5 leaves for every 200 μM increase in the concentration of NAA. Furthermore, when compared with the leaf number of plants from IAA-treated seed-cores, NAA more effectively reduced the number of leaves on plants from 18-month-old seed-cores than on those from 6-month-old seed-cores.

The leaf area of plants from untreated older cores was 36% less than that of plants from younger cores (Fig. 3, Table 2). When averaged over both ages, leaf area per core declined linearly by 4.4 cm^2 and 31.6 cm^2 for every 200 μM increase in the concentration of IAA and NAA, respectively (auxin X $[auxin]_{linear}$, $P<0.01$). Moreover, the average effect of the two auxins was to reduce the total leaf area by 58% in plants from 6-month-old cores, as compared with 23% in plants from 18-month-old cores (age X $[auxin]_{linear}$, $P<0.01$).

The effect of NAA on restoring apical dominance to 18-month-old seed-cores was greater than its effect on reducing total plant leaf area, as evident from an 85% increase in leaf area per stem with 270 μM NAA relative to the untreated seed-cores (Table 3). Treating 18-month-old seed-cores with 270μM and 540μM NAA also induced a 66% and 34% increase in average leaf area per leaf, respectively, compared with the untreated seed-cores. In contrast, leaf area decreased by 39.5 cm^2 $stem^{-1}$ and 4.9 cm^2 $leaf^{-1}$ for every 200 μM increase in NAA concentration applied to 6-month-old cores. The IAA treatment also significantly reduced the leaf area of plants from younger cores, but was much less effective than NAA in this regard (for every 200 μM increase in [IAA], leaf area of plants from younger cores decreased by 6.7 cm^2 $stem^{-1}$ and 1.5 cm^2 $leaf^{-1}$). The IAA treatment had no effect on the leaf area of plants developing from 18-month-old seed-cores.

Age-induced differences in stem, leaf and root dw were also apparent (Table 3). Despite the fact that older seed-cores produced an average of 7.5 stems per core, their combined dw accounted for only 46% of that produced by the single stem from younger seed-cores. Tuber age, auxin type and concentration interacted to affect stem and leaf dw. In general, IAA and

NAA induced highly significant reductions in stem and leaf dw of plants developing from younger seed-cores relative to untreated controls; however, both auxins significantly increased the dw of these yield components when applied to older seed-cores. For example, treatment of older seed-cores with 270 µM NAA (optimal concentration) caused a 53% increase in dw of individual stems, a 100% increase in leaf dw per stem (70% increase per leaf), and a 140% increase in root dw per stem, relative to untreated controls. In contrast, 540 µM NAA applied to younger seed-cores inhibited dry matter accumulation by 65%, 78% and 47% for stems, leaves and roots, respectively, relative to untreated controls.

Depending on the yield component, IAA was either ineffective or caused slight increases in growth from 18-month-old seed-cores (Table 3, Fig. 3). The IAA treatment inhibited growth of almost all plant yield components when applied to 6-month-old seed-cores. This interaction of age with IAA to affect overall plant morphology is clearly evident when comparing the areas encompassed by the polygonal diagrams of Figure 3. The decrease in area of the 6-month-old, 540 µM polygonal diagram indicates that seed-cores from younger tubers were much more responsive to IAA treatment than those from older seed tubers.

IAAoxidase and peroxidase activities

The relative inability of IAA to alter many of the growth parameters of plants from 18-month-old seed-cores (e.g. time to sprout emergence; leaf number per core and per stem; leaf area per core, per stem, and per leaf; root dw) may be due to an increased capacity of older tuber tissues to quickly catabolize this natural auxin. To test this possibility, IAAox activity was determined in tuber tissues from 8 and 20-month-old seed pieces at 0, 15, 20, 25 and 30 DAP. At 0 DAP (directly from a 4^{o}C storage), the specific activity of IAAox in older seed tuber tissue was 3-fold greater than that from younger seed tuber tissue (Fig. 4). IAAox activity increased in tissues from both seed tuber ages during plant establishment (DAP); however, the rate of increase was greater in the 20-month-old (relative to the 8-month-old) seed pieces (age X DAP, $P<0.05$).

Fig. 4. Change in IAAox activity in tuber tissue from seed-pieces from 8 and 20-month-old seed tubers at various intervals after planting. Enzyme activity was determined polarographically at 30°C by the methods of Palmieri *et al.* (23). *F*-values for the main effects of tuber age and time (DAP), and their interaction, were significant at the 0.01 level.

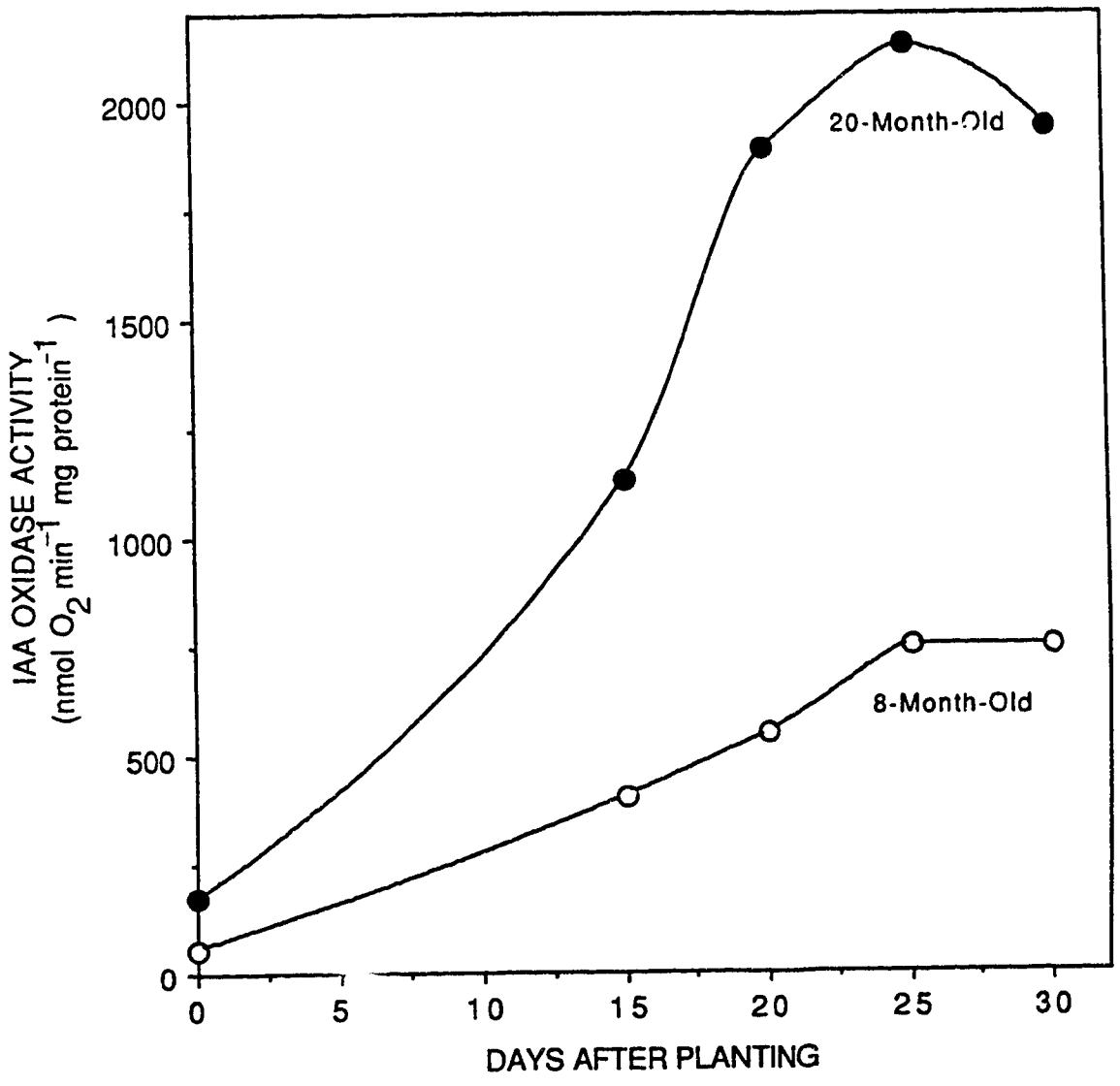

2000
1500
1000
500
0
0
5
10
15
20
25
30
20-Month-Old
8-Month-Old
IAA OXIDASE ACTIVITY
(nmol O_2 min^{-1} mg $protein^{-1}$)
DAYS AFTER PLANTING

TABLE. 4. IAAoxidase (IAAox) and peroxidase (POX) activities in sprout and associated tuber tissues from single-eye potato seed cores from 6- and 18-month-old seed tubers.

	IAAox (nmol O_2 min^{-1} mg $protein^{-1}$)		POX (Δ A 470 nm min^{-1} mg $protein^{1}$)	
Tissue	6-month-old	18-month-old	6-month-old	18-month-old
Sprout apex (A)	28	563	3.5	4.9
Sprout base (B)	838	2330	25.0	64.0
Tuber core (C)	1142	3444	20.0	56.6
Age[a]	0.01[b]		0.01	
A/B	0.01		0.01	
AB/C	0.01		0.01	
Age X AB	NS		0.01	
Age X AB/C	0.05		0.10	

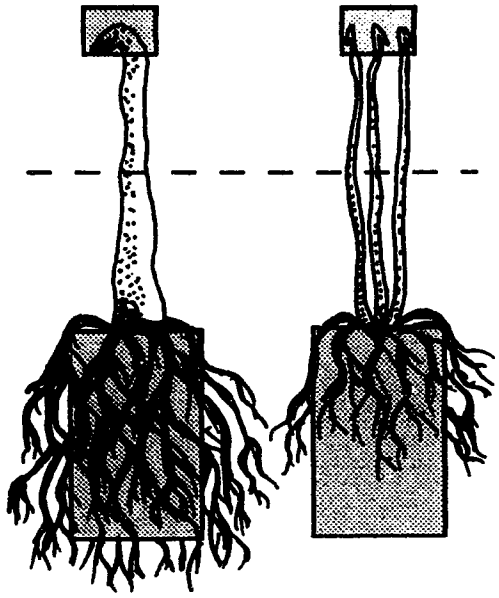
NOTE: Single-eye seed cores were sprouted for 7 days in the dark at 23 °C. The 2.5 to 4.0-cm-long etiolated sprouts were then divided into apical and basal halves, and enzyme activity was quantified in each half. The upper 0.5 cm length (including the eye with surrounding periderm) of the seed core was analysed. [a]Sources of variation; [b]Significance levels for indicated sources of variation (NS, non-significant).

To further characterize age-induced differences in the potential for IAA catabolism during sprouting, IAAox and POX specific activities were measured in tissues from the apical and basal halves of etiolated sprouts, and in the uppermost portion (0.5 cm including the eye with surrounding periderm) of single-eye seed-cores from 6- and 18-month-old seed tubers. On average, IAAox and POX activities were lower in the apical half than in the basal half of etiolated sprout tissues (Table 4). However, the highest IAAox activity overall was observed in the uppermost portion of seed-cores. POX activity was approximately 15% lower in tuber tissue relative to basal sprout tissue. Sprout and tuber tissues from 18-month-old seed-cores had substantially higher IAAox and POX activities than those from 6-month-old seed-cores. More importantly, seed tuber age interacted with source of plant tissue to affect IAAox and POX activities and their distribution. When compared with specific activities in tissues from younger seed-cores, IAAox activity was 20-fold higher in the apical half of sprouts from older seed-cores, and 2.8- and 3-fold higher in the sprout base and seed-core tissues, respectively.

Transport and decarboxylation of [1-^{14}C]-IAA

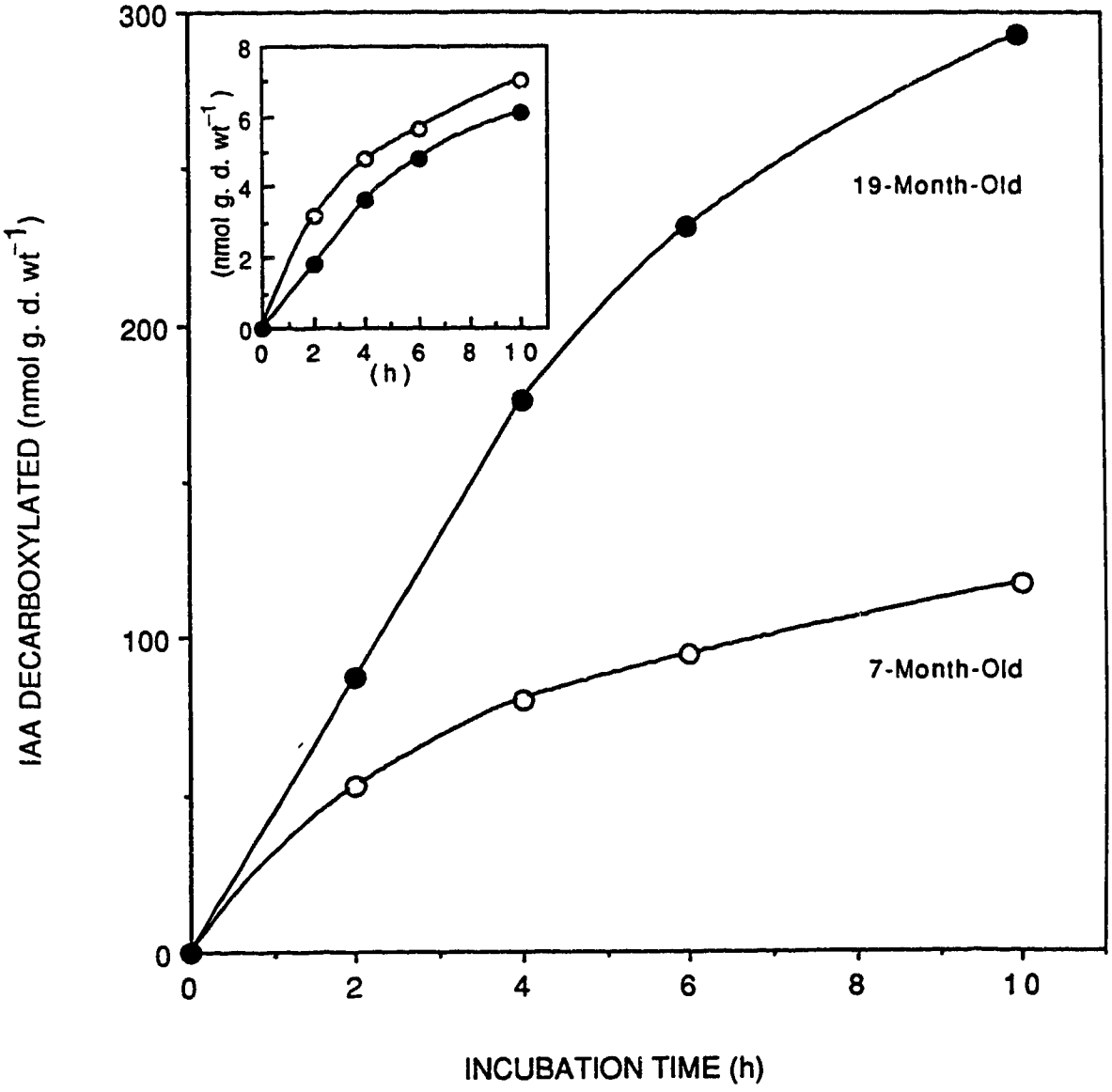
The effect of seed tuber age on the ability of intact etiolated sprouts to transport radiolabeled IAA basipetally was determined over a 10 h incubation interval. The open air-flow system utilized in these studies (Fig. 2) also allowed for quantification of *in vivo* IAA catabolism via decarboxylation. Of the 162 nmoles of [1-^{14}C]-IAA applied to the apices of sprouts from 7- and 19-month-old seed-cores at zero-time, 36.7 nmoles (23%) was accounted for collectively in tissues and CO_2 traps after 10 h incubation. Interestingly, the three sprouts from 19-month-old seed-cores processed (translocated and decarboxylated) about the same total amount of IAA equivalent (36.1 nmoles) as the single sprout from 7-month-old seed-cores (37.3 nmoles), despite the fact that sprout and associated root dw from older seed-cores was 3.4-fold less than that from younger seed-cores. The absolute amount of radiolabel (nmol IAA equivalent) in similar sections of plant tissues was thus compared between the two ages (Fig. 5). Age and tissue portion interacted to affect the amount of [^{14}C] recovered in each section. Of the total amount of [^{14}C] recovered in plant tissue after 10 h incubation, 65% (equivalent to 23.5 nmoles

Fig. 5. Translocation of [1-^{14}C]-IAA by etiolated sprouts from 7- and 19-month-old seed tubers. Agar cylinders, each containing approximately 162 nmoles of [1-^{14}C]-IAA (6 mCi/mmol) were placed on the intact apices of 9-day-old sprouts. After a 10 h incubation in the apparatus depicted in Fig. 2, the 7- to 8-cm-long sprouts were divided into apical and basal halves, and the total activity in each half was expressed as nmole IAA equivalent. The amount of [^{14}C] from seed core tissue and the total amount of [^{14}C]-IAA decarboxyla are also determined. [a]nmol equivalents of IAA decaboxylated by the tissue over the 10 h incubation interval; [b]sources of variation; [c]significance levels for nmol IAA equivalent; [d]significance levels for nmol IAA equivalent/g. dw.

Tissue	Tuber age (months)		[1-^{14}C]-IAA equivalent (nmoles)		(nmoles/g. dw)	
	7	19	7	19	7	19
Sprout apex (A)			16.1	23.5	500	2285
Sprout base (B)			1.3	0.7	46	99
Roots (R)			3.4	0.7	327	115
Seed core			7.9	5.0	7	5
Decarboxylated[a]			8.6	6.2	-	-

Age[b]	NS[c]	0.01[d]
Tissue	0.01	0.01
Age X A/B	0.05	0.01
Age X AB/R	0.10	0.01

Fig. 6. Time-course of the decarboxylation of [1-^{14}C]-IAA in 7- to 8-cm-long etiolated sprouts growing from 7- and 19-month-old single-eye seed-cores. At zero-time, agar cylinders containing approximately 162 nmoles of [1-^{14}C]-IAA (6 mCi/mmol) each, were placed on the intact apices of 9-day-old sprouts as depicted in Fig. 2. Decarboxylation over the 10 h incubation interval is expressed on the basis of sprout and associated root dw. *F*-values for the main effects of seed tuber age and time, and their interaction, were significant at the 0.01 level. Decarboxylation on a total dw basis (including the seed-core dw) is depicted in the inset.


300
200
100
0
0
2
4
6
8
10
IAA DECARBOXYLATED (nmol g. d. wt^{-1})
INCUBATION TIME (h)
19-Month-Old
7-Month-Old
(nmol g. d. wt^{-1})
(h)

of IAA) remained in the apical 3.5 cm of sprouts from 19-month-old seed cores, compared with only 43% (16.1 nmoles) in the apical section from 7-month-old seed cores (a significant difference, $P<0.01$). Only 3.5% (1.3 nmoles) and 1.9% (0.7 nmoles) of the total activity recovered remained in the basal 3.5 cm of sprouts from younger and older seed cores, respectively, and the interaction of age X sprout section (apical vs. basal) was significant ($P<0.05$). The [^{14}C] levels in the roots of apically dominant sprouts from 7-month-old seed cores accounted for 9.1% (3.4 nmoles) of the total recovered radioactivity, compared with 1.9% (0.7 nmole) in those of the multiple sprouts from 19-month-old seed cores. Moreover, the interaction of age X sprout vs. roots was significant ($P<0.1$), indicating that sprouts from younger seed-cores were better able to transport the radiolabeled IAA from sprout apex to roots than those from older seed-cores. Approximately 21% (7.9 nmoles) and 14% (5.0 nmoles) of the distributed label was recovered basipetally as reflected by a 23-fold higher level of [^{14}C] in apical, compared with basal, sections from the 7- and 19-month-old seed-cores, respectively. Finally, plant and seed-core tissues from 7-month-old seed tubers decarboxylated 8.6 nmoles of IAA (23.1% of the total amount processed), compared with 6.2 nmoles of IAA decarboxylated by tissues from 19-month-old seed tubers (17.2% of the total amount processed) over the 10 h period.

The levels of radiolabel in the various tissue sections were compared on a dw basis, and the effects of age were similar to those described above for the absolute amounts of radiolabel (IAA equivalent) in each section. Radiolabel from applied IAA in the apices of sprouts from 19-month-old seed-cores was 4.6-fold more concentrated after 10 h incubation than that from 7-month-old seed-cores ($P<0.01$) (Fig. 5). Moreover, age-reduced ability to transport auxin of sprouts from 19-month-old seed-cores. By comparison, apical sections of sprouts from younger seed-cores were only 11-fold higher in [^{14}C] label than basal sprout sections after 10 h incubation (age X sprout apex vs. base, $P<0.01$). The root system of sprouts from older seed-cores was 3-fold lower in [^{14}C] than that of sprouts from younger seed-cores ($P<0.05$), and the age X sprout vs. root interaction was highly significant ($P<0.01$). Collectively, these

results characterize a reduced ability of sprouts developing from older seed-cores to transport IAA, or IAA metabolites, basipetally.

The effect of age on the ability of tissues to decarboxylate [^{14}C]-IAA was assessed by quantifying radioactivity in CO_2 traps at various times over the 10 h incubation interval (see Fig. 2). The absolute amount of IAA that was decarboxylated was about the same for tissues from the two ages of seed-cores (avg. 7.4 nmoles) over the course of the study. Hence, on an entire-tissue dw basis (sprouts plus roots plus seed-cores), age had little effect on the rate and total amount of IAA decarboxylated (Fig. 6, inset). However, from the per g sprout plus root dw, it was evident that plant tissues developing from the 19-month-old seed-cores were much more efficient at decarboxylating IAA than those from 7-month-old seed-cores (Fig. 6). These results are thus consistent with the higher specific activities of peroxidase and IAA oxidase (responsible for decarboxylating IAA) profiled in sprout tissues from older seed-cores (Table 4).

DISCUSSION

Consistent with a previous study (20), we have found that the deleterious effects of advanced seed tuber age on sprouting and subsequent plant growth can be ameliorated by treating seed pieces with NAA prior to planting. Hence, a significant number of the effects of advanced seed tuber age on plant development are most likely mediated through alterations in auxin transport, metabolism or action. This study was undertaken to further characterize the involvement of auxin in the age-induced loss in sprout and plant growth potential. Specifically, the efficacies of IAA and NAA in mitigating the effects of seed tuber age on plant growth were compared. In addition, auxin transport efficiency and potential for auxin catabolism were determined during sprouting for potato seed-tubers of various ages.

NAA was more effective than IAA in altering growth from the single-eye seed-cores. More importantly, NAA treatment of older seed-cores partially restored apical dominance and stimulated growth on a per-stem basis. The NAA treatment of younger cores was not only inhibitory to overall plant growth, but created a hormonal imbalance which favored production

of adventitious roots directly from the seed-core tissue. These results are consistent with the previously proposed concept that NAA treatment of older seed-cores compensates for either lower endogenous auxin levels in meristematic tissues, or loss in tissue sensitivity to auxin during plant establishment (15,20).

Although IAA affected growth of plants from younger tuber cores, it failed to elicit a response from older tuber cores, suggesting that age may increase the ability of tissues to inactivate or catabolize this natural auxin. Endogenous auxin levels have been shown to be enzymatically regulated through oxidation by IAAox/POX in many plant species (1,3,22). Activities of these enzymes were also reported to increase with age of plant tissue (12,14) and thus lack of response to exogenous IAA may be partly due to enhanced catabolic ability of tissues. On the other hand, treatment with synthetic auxins (e.g. NAA, 2,4-D) remains effective in altering growth, as they are not substrates for the enzymes which catabolize IAA (11,13). In preliminanry studies, IAAoxidase was unable to decarboxylate NAA. Consistent with these reports, my results indicate that the specific activity of IAAox increased in seed tuber tissues over a 30 d interval of plant establishment (i.e. during aging of the seed piece). Moreover, during sprouting and early plant establishment, IAAox activity was initially higher and increased much faster in tissue from 20-month-old seed tubers as compared with that from 8-month-old seed tubers (Fig. 4). The trend in POX activity in tissues from young and old seed tubers during sprouting (see chapter IV) was similar to that for IAAox (17). Hence, the inability of exogenously applied IAA to alter growth from older seed-cores (Fig. 3, Table 3) may be due in part to rapid auxin catabolism via IAAox/POX. The fact that younger seed-core tissue also showed significant IAAox/POX activity (albeit much less than older seed-cores) may account for the reduced effectiveness of IAA in altering growth relative to that characterized for NAA.

In many plants, the rate of basipetal translocation of auxin is significantly reduced in physiologically older tissues (4,24,26,27,28,29). The reduced basipetal transport of radiolabel from IAA characterized for sprouts growing from seed-cores from older seed tubers in this study cannot be attributed completely to greater catabolism via decarboxylation by IAAox/POX,

since a greater proportion of the applied radiolabel remained in the sprout apices (Fig. 5). Several reports have demonstrated that basipetal transport of IAA is dependent on the concomitant acropetal transport of calcium (6,7,8), and chelation of calcium by EDTA markedly retards IAA transport (8). It was even possible to restore IAA transport ability to EDTA-treated stem segments from sunflower by supplying calcium (8). Interestingly, sprouts developing from older seed tubers eventually develop apical tip necrosis which results from a calcium deficiency (9,10). The apparent reduced ability to mobilize and/or translocate calcium to apical meristems during sprouting of older tubers may be related to the age-reduced ability for basipetal transport of IAA, as characterized in this study. Alternatively, Suttle (26) has demonstrated that the age-induced loss in polar transport of IAA in *Helianthus* hypocotyls is associated with loss of function of the phytotropin-sensitive IAA efflux system. Regardless of the mechanism, the reduced ability of sprouts from older seed tubers to transport auxin to developing roots and lateral buds (along with increased potential for IAA catabolism in sprout and tuber tissues from older seed tubers) may result in loss of apical dominance and plant growth potential during aging of potato seed tubers.

The results of this study demonstrate that the efficacy of IAA and NAA in restoring apical dominance and growth potential of potato seed tubers depends upon their age. Tuber tissues from older seed tubers have a higher capacity to catabolize IAA enzymatically, which correlates well with the inability of this auxin (relative to NAA) to compensate for the negative effects of advanced age on growth and development. Moreover, the ability of sprouts developing from older seed tubers to translocate IAA basipetally is reduced compared with those from younger seed tubers. When coupled with much higher specific activities of IAAox/POX in apical and basal sprout tissues, this may result in lower levels of auxin in lateral meristems, effectively releasing them from correlative inhibition and thereby reducing apical dominance.

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Chapter III

Evidence for Lipid Peroxidation and Associated Changes in Lipolytic and Free-Radical Scavenging Enzyme Activities During Aging and Sprouting of Potato Seed-Tubers.

INTRODUCTION

Aging and senescence are distinctly different but overlapping developmental processes. Aging encompasses the entire lifespan of an organism whereas senescence can be thought of as the final developmental phase that culminates in death. Evidence suggests that whole-plant (or plant-organ) senescence is a well-defined, genetically programmed phase of development, that is mediated by a highly-regulated set of anabolic and catabolic reactions which catalyze the ultimate deterioration and death of tissues (Nooden, 1988). In contrast, the degenerative processes involved in aging are thought to be more stochastic in nature. Abscission of petals following pollination and gradual loss of seed viability during storage represent examples of senescence and aging, respectively (Nooden, 1988).

The knowledge pertaining to the physiological mechanisms mediating the deteriorative processes of aging is much less than that for senescence. Part of the problem in elucidating the mechanisms of aging in plants is selection of an ideal intact plant system. The potato, being vegetatively propagated by seed-tuber cuttings, provides such a system. Russet Burbank potato seed-tubers remain viable for approximately three years from harvest (at 4^{0}C, 95% RH); however, the process of aging from about 7 through 24 months results in a gradual loss of sprout vigor (Mikitzel and Knowles, 1990; Kumar and Knowles, 1993a). Further aging (through 36 months) leads to complete loss of sprouting ability, and the axillary meristems within each node of the tuber precociously form small tubers (Kumar and Knowles, 1993a; Krijthe, 1962). Aging and senescence are the result of complex changes in basic plant metabolism and, while the two are distinguishable, they also share similarities at the biochemical level. For example, a gradual disruption of membrane integrity, resulting in loss of compartmentation of cytoplasmic organelles

and increased permeability of the plasma membrane, are widely-reported phenomena common to both progressive aging and senescence of plant tissues (Thompson, 1988). The age-induced loss in membrane integrity is often caused by an increase in saturation of membrane phospholipids, which results in decreased fluidity and increased permeability (Pauls and Thompson, 1980; 1981). Although a high double-bond-index of membrane lipids is often important in conferring fluidity to plant membranes, the degree of fatty-acid saturation and a loss in membrane fluidity do not always go hand-in-hand (Uemura and Yoshida, 1984). Increased microviscocity can also be induced by peroxidation of membrane lipids (Fobel et al., 1987) which renders the membranes more permeable (Paliyath et al., 1987). In potato tubers, a progressive loss of membrane integrity during aging was highly correlated with a decrease in double-bond-index, and thus an increase in the saturation of membrane lipids (Knowles and Knowles, 1989).

In view of the reported loss of membrane integrity during aging of seed-tubers (Knowles and Knowles, 1989), and the increased lipid peroxidation that occurs during senescence of plant tissues (Thompson et al., 1987) and during accelerated aging of seeds (Wilson and McDonald, 1986), lipid peroxidation was examined as a possible mechanism for membrane deterioration during seed-tuber aging. Ethane formation, MDA and FC content were quantified as indices of lipid peroxidation (Konze and Elstner, 1978; Heath and Packer, 1968; Dhindsa et al., 1981). In addition, the effects of seed-tuber age on the activities of phospholipase, LOX, SOD, POX and CAT were characterized. Since activities of the lipolytic and FR-scavenging enzymes were lower and higher, respectively, in tissues from older seed-tubers, it is proposed that the loss in membrane integrity that occurs with progressive aging (Knowles and Knowles, 1989) is most likely due to a shift in the equilibrium between FR production and consumption towards production. A resulting gradual buildup of FRs with age would not only peroxidize membrane lipids, but would also damage other macromolecules in the cell. It appears that the process of aging in potato seed-tubers is similar to that in true seeds, and therefore more congruent with Nooden's (1988) definition of aging as 'wear and tear accumulated over time'.

MATERIALS AND METHODS

Plant material

Certified potato (*Solanum tuberosum* L. cv Russet Burbank) seed-tubers, obtained from a commercial seed grower at harvest, were stored at 4°C and 95% RH for up to 32 months. These conditions inhibited sprouting over the entire storage period. Seed-tuber ages reported in these studies were all calculated from harvest.

Indices of lipid peroxidation

Ethane, MDA and lipofuscin-like FCs accumulating in tissues from different ages of seed-tubers were determined as indices of lipid peroxidation (Konze and Elstner, 1978; Heath and Packer, 1968; Dhindsa et al., 1981). Disks of tissue (1 cm diameter, 1 mm thick), cut from 1-, 13- and 25-month-old seed-tubers, were used in initial studies to compare the effects of seed-tuber age on ability to generate ethane *in vivo*. Two uniformly sized seed-tubers of each age were selected from a 4°C cold storage (95% RH) and washed. The apical and basal ends of each tuber were excised, leaving a central portion approximately 6 cm long. Cores of tissue were then cut with a cork borer from this portion longitudinally through both cortical and pith tissues. The cores of tissue were reinserted into the tuber from which they arose, and the entire central portion was then sliced into 1 mm thick sections with a food processor. The resulting disks were collected from the incubation buffer in the reservoir of the food processor. The incubation buffer consisted of 140 mM Mes (pH 5.5) containing 1 mM DTT, 0.5% (w/v) PVP (mol wt 40,000), and 0.25 mM PMSF. After an additional rinse in incubation buffer, disks were placed in 25 mL erlenmeyer flasks (10 disks per flask) containing 0, 1, 2, 4 or 8 mM $CaCl_2$ in 2 mL of incubation medium. The flasks were then closed with serum stoppers. Ethane was analyzed in 1 mL gas samples taken from the head-space of each flask after 16 h incubation at 23°C by FID-GC (see below). Treatments in this study were arranged factorially (3 tissue ages and 5 calcium concentrations) in a randomized complete block design with three replications (45 experimental units). The effects of tuber age and calcium on the ability of tissues to produce ethane were thus determined.

The effect of seed-tuber age on the ability of tissue disks to produce ethane from exogenously supplied linoleic acid (Sigma) was also characterized. Tuber disks (10 disks/flask containing 2 mL buffer as described above) from 5- and 17-month-old seed-tubers were incubated at 23°C for 16 h. Ethane formation in response to linoleic acid concentration (0, 0.5, 1.0 and 2.0 mM) was determined in 1 mL gas samples withdrawn from the head-space of each flask. The treatments were arranged factorially (2 ages and 4 linoleate concentrations) in a randomized block design with three replications.

In a separate study, internal ethane concentration of seedpieces from 8- and 20-month-old seed-tubers was profiled and compared at 5-day intervals from 15 to 30 days after planting. Seedpieces (50-60 g fresh wt) were planted (1 seedpiece/pot) in peat/vermiculite (1:1, v/v) in 15 cm diameter pots and placed in a greenhouse (23°C/18°C day/night, 16 h photoperiod). The seedpieces were harvested at 15, 20, 25 and 30 DAP for determination of ethane content by the methods of Beyer and Morgan (1970) with minor modifications. Following harvest, the seedpieces were rinsed, cut in half longitudinally, and one half (approximately 25 g) was cut into several small pieces. The pieces of tuber tissue were then submerged in degassed (by vacuum) buffer (50 mM Hepes, pH 7.0) and subjected to a vacuum of 330 mm Hg for 5 min to extract ethane from the tissue. One milliliter of the displaced gas sample from each seedpiece was analyzed for ethane. The 1 mL gas samples were injected into a Hewlett-Packard model 5890A gas chromatograph equipped with a FID and a 3.05-m stainless steel column (3.2 mm o.d.) packed with 80/100 mesh Porapak N (Supelco, Canada). The N_2 flow rate was 30 mL min^{-1}, and the oven temperature was isothermal at 95°C. Injection port and detector temperatures were 250°C. Treatments in this study were arranged factorially (2 ages X 5 DAP) in a randomized block design with four replications (40 pots). Internal ethane concentration was expressed as microliters ethane per liter of gas extracted. Following extraction of ethane, the tissue was frozen at -20°C and subsequently used for determining the activities of phospholipase, LOX, SOD, POX and CAT.

Malondialdehyde, also a product of lipid peroxidation, was extracted from lyophilized tissue from seed-tubers which had previously been stored for 2 to 32 months (4°C, 95% RH) from

harvest. To prepare tissue for analysis, five tubers (170-200 g/tuber) of each age (11 ages total) were cored longitudinally (1.2 cm diameter) through the pith, and the cores were sliced and lyophilized. The lyophilized tuber tissue was ground into a fine powder with a mortar and pestle. For each of the tuber ages, 2 g of tissue were extracted with 10 mL of 0.1% (w/v) TCA containing 2 mM $Na_2S_2O_5$. The crude extracts were strained through a layer of mira cloth and centrifuged at 10,000 g for 10 min. A 1 mL aliquot of each supernatant was then vortexed with 4 mL 20% (w/v) TCA containing 0.5% (w/v) TBA, and the resulting solution was heated for 30 min at 95°C. The samples were cooled on ice for 5 min, recentrifuged for 20 min at 1640 g, and non-specific absorbance of the supernatants at 600 nm was subtracted from the absorbance at 532 nm. MDA content was calculated on the resulting difference using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968; Dhindsa et al., 1981). Reducing sugar content of the lyophilized tissue was also determined, using a glucose standard and the methods of Nelson (1944) and Somogyi (1952).

Extraction and determination of lipofuscin-like FCs in tuber tissue from the apical, middle and basal portions of 5-, 17- and 29-month-old seed-tubers was accomplished according to the methods of Meir et al. (1991). Tuber tissue, cut from the appropriate tuber portion, (Fig. 1), was frozen in liquid N_2 and lyophilized. Samples (500 mg each) of lyophilized tissue were extracted with 5 mL of chloroform/methanol (2:1) containing 20 mg mL^{-1} BHT using a polytron. The resulting homogenates were heated for 5 min at 45°C, mixed with 5 mL of 15 µM $CaCl_2$ and centrifuged at 1640 g for 10 min to hasten phase separation. A portion of the chloroform-phase (500 µL) from each sample was passed through a Sep-Pak silica cartridge (Waters Associates, Mass., USA) which had been prewashed with 3 mL of chloroform containing 0.7% (v/v) ethanol. The column was then rinsed with 5 mL of chloroform which was discarded. FCs were eluted from the column with 3 mL of methanol into a tube containing 0.5 mg BHT in 25 µL methanol, and fluorescence intensity was determined on a Varian spectrofluorometer (model SF 330). Maximum excitation ranged between 300 and 340 nm, and maximum emission ranged between 400 and

Fig. 1. Schematic of the procedure for sampling potato tuber tissue for analysis of lipofuscin-like fluorescent compounds. A longitudinal slice (1 cm thick) was cut from the tuber. Blocks of tissue (4 cm by 1 cm) were then cut (periderm included) from apical and basal ends of the slice. In addition, two blocks of tissue (2 cm by 1 cm each) were cut from the slice and combined to represent the middle portion of the tuber. Tissue was frozen in liquid nitrogen and lyophilized. The results are presented in Fig. 5.

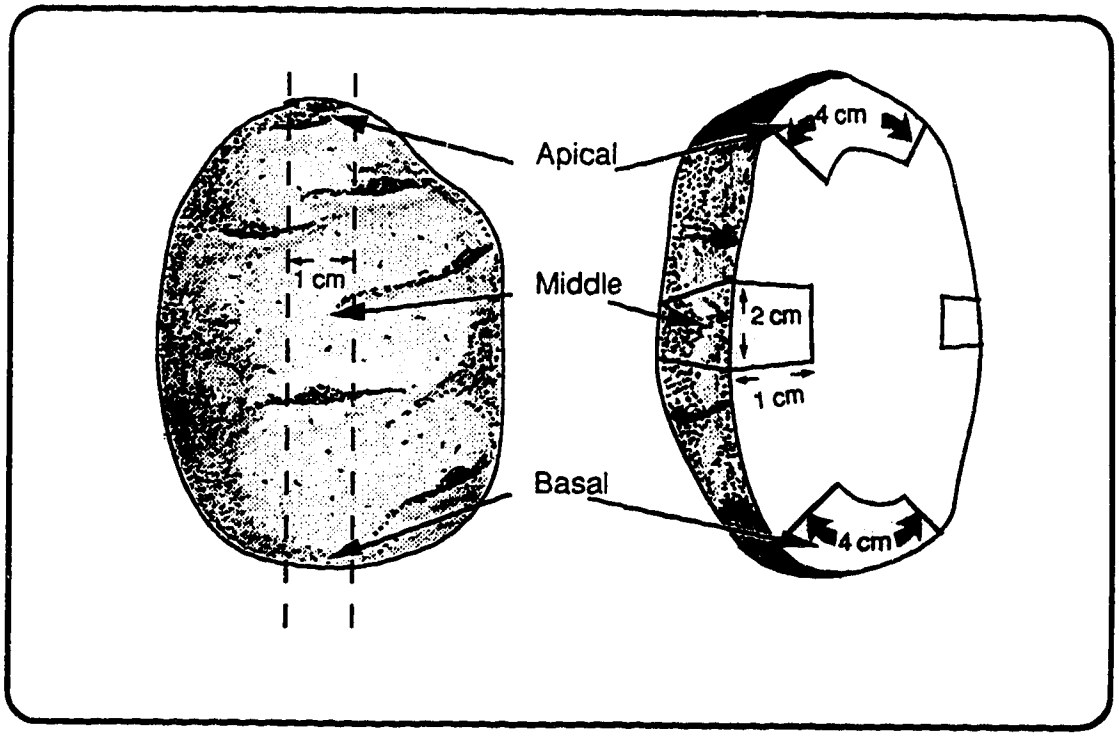
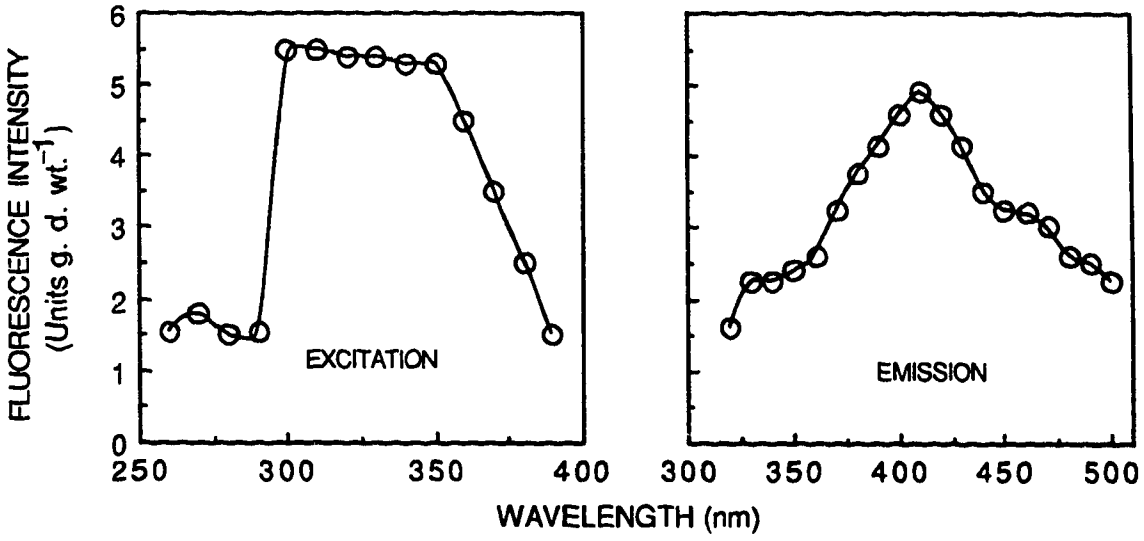

Apical
Middle
Basal
1 cm
4 cm
2 cm
1 cm
4 cm

Fig. 2. Representative excitation and emission spectra for fluoresce... of lipofuscin-like compounds in lipid extracts from lyophilized potato tuber tissue.


FLUORESCENCE INTENSITY
(Units g. d. wt.$^{-1}$)
0
1
2
3
4
5
6
250
300
350
400
EXCITATION
300
350
400
450
500
EMISSION
WAVELENGTH (nm)

425 nm (Fig. 2). Therefore, 300 nm and 410 nm were used as excitation and emission wavelengths, respectively. Quinine sulfate (1 μg mL^{-1}) was used to standardize the spectrofluorometer. The treatments were arranged factorially (3 ages X 3 tuber portions) in a randomized complete block design with 3 replications.

Lipolytic and free-radical scavenging enzymes

To determine the effects of age and plant development on the activities of various lipolytic and FR scavenging enzymes, frozen seedpiece samples from the internal ethane study (described previously), including a 0 DAP sample (directly from a 4°C storage), were examined for phospholipase (0 DAP sample only), LOX, SOD, POX and CAT activities. Tuber tissue (25 g fresh wt.) from each seedpiece was extracted (mortar and pestle) with 25 mL Hepes buffer (50 mM, pH 7.0) containing 2 mM $Na_2S_2O_5$. The extract was strained through a layer of mira cloth and centrifuged for 30 min at 10,000 g. All manipulations were carried out at 4°C. The supernatants were stored at -20°C and were used as a source of phospholipase, LOX, SOD, POX and CAT. Treatments were arranged in a randomized complete block factorial design (2 ages and 5 DAP) with 4 replications.

Phospholipase activity in tissue from seed-tubers stored for 8 and 20 months at 4°C was assayed by the methods of Moreau and Rawa (1984). Ten microlitres of L-3-phosphotidyl choline-1-palmitoyl-2-[1-^{14}C] palmitoyl (25 μCi/mL, 55 mCi/mmol; Amersham, Canada) were transferred to a test tube and the solvent (1:1 v/v ethanol/toluene) was evaporated under nitrogen. This was followed by the addition of 15 μL of phosphotidylcholine (from soybean 100mg/mL, 99% pure, Sigma), 60 mg Triton X-100 and 15 mL Tricine buffer (100 mM, pH 7) containing 2 mM $CaCl_2$ to the radiolabeled substrate. The tube was then flushed with nitrogen and sonicated for 2 h in a Branson bath sonicator. The assay mixture, containing 500 μL of the substrate so prepared, 500 μL of Tricine buffer (as above) and 20 μL of the enzyme extract, was incubated at 32°C for up to 32 min. The reaction was terminated by addition of 50 μL glacial acetic acid. Fifty microliters of 10 mM linoleic acid was then added, and the lipid fraction was partitioned by adding 7 mL hexane/2-propanol (3:2, v/v) and 5 mL 6.7% (w/v) Na_2SO_4. The hexane/isopropanol fraction was

transferred to a test tube and dried under nitrogen at 40°C. The residue was dissolved in 100 µL of chloroform and 20 µL of this was spotted on a 250 µm thick Silica Gel-G TLC plate. TLC plates were developed in 70:30:1.5 (v/v/v) hexane/diethyl ether/acetic acid and were visualized in an iodine tank. The FFA-zone was identified as that which cochromatographed with a linoleic acid standard. The origin and FFA-zone on the TLC plate were scraped into scintillation vials and the samples were counted in 5 mL of Scintevarse E (Fischer Scientific) with a Minaxiß Tri-Carb 4000 liquid scintillation counter. Phospholipase activity was expressed as nmol [1-^{14}C] palmitoyl cleaved mg protein^{-1} and was plotted against incubation time.

LOX activity was determined polarographically using a Clark oxygen electrode (Gaillard, 1970). The reaction mixture consisted of 50 µL of enzyme extract in 2.5 mL of potassium phosphate buffer (50 mM, pH 6.5) containing 1 mM Na linoleate (saturating concentration). The assay was carried out at 25°C and the enzyme activity was expressed as nmoles O_2 [illegible] mg protein^{-1}. The substrate for LOX was prepared following the method of Axelrod et al. (1981). Specifically, 75 mg each of Na-linoleate and Tween-20 were weighed into a test tube and dissolved in 4 mL of degassed distilled water. After adding 550 µL of 0.5 N NaOH, the volume was adjusted to 25 mL with degassed water and the resulting solution was distributed among 4 mL vials. Each vial was flushed with nitrogen, stoppered and stored at -20°C until further use.

The ability of seedpiece enzyme extracts to inhibit light-mediated reduction of NBT was the basis for the SOD assay (Beauchamp and Fridovich, 1971). Enzyme extracts were added to the reaction medium which consisted of 13 mM methionine, 75 µM NBT, 2 mM riboflavin and 0.1 mM EDTA in a total of 3 mL potassium phosphate buffer (50 mM, pH 7.8). The reaction was started by adding riboflavin, which initiated the light mediated reaction. Tubes containing the reactants were incubated under fluorescent light (400 µEm^{-2} sec^{-1}) at 25°C. After 10 min, the reaction was stopped by transferring the test-tubes to darkness, and the absorbance at 560 nm was recorded. In the absence of light, the reaction mixture failed to develop color and served as a blank to zero the spectrophotometer. The reaction mixture had a maximum absorbance when no enzyme was present. A standard curve of percent inhibition of color development was plotted as a function of

volume of enzyme extract (0-100 µL). The volume of enzyme extract causing 50% inhibition was considered as one unit of SOD activity (Dhindsa et al., 1981).

POX activity was determined colorimetrically by the methods of Soressi et al. (1974). The reaction medium consisted of 0.03% (v/v) H_2O_2 and 15 mM guaiacol in sodium acetate buffer (50 mM, pH 6.0). The reaction was initiated by adding 10 to 40 µL of the enzyme extract to 3 mL of reaction medium. Enzyme activity was determined as the change in absorbance at 470 nm on a recording spectrophotometer (Varian, model Cary 219). Specific activity was expressed as change in the absorbance $min^{-1}mg\ protein^{-1}$.

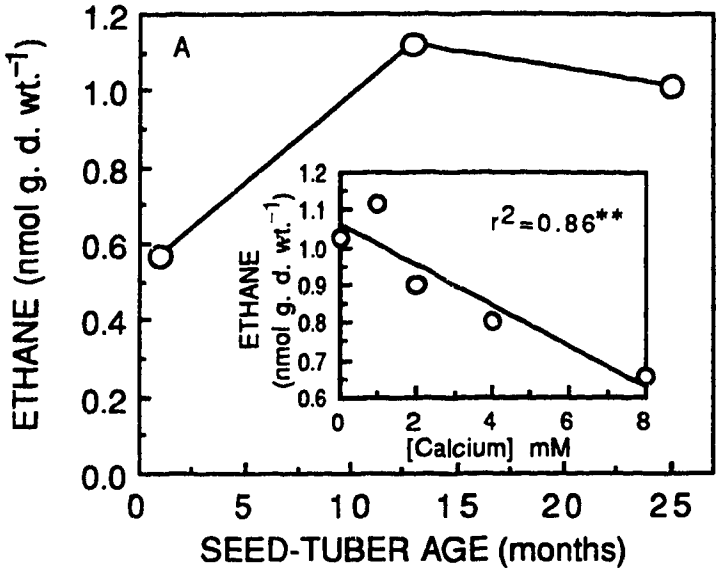
CAT activity was determined by following the enzymatic degradation of H_2O_2 (Chance and Maehly, 1955). The reaction media of 3 mL consisted of 12 mM H_2O_2 in 100 mM sodium phosphate buffer (pH 6.0) and 30 µL of enzyme extract. Enzyme activity was expressed as the change in absorbance at 240 nm min^{-1} mg $protein^{-1}$. In all studies, protein was determined by a modified Lowry method using BSA as a standard (Bensadoun and Weinstein, 1976).

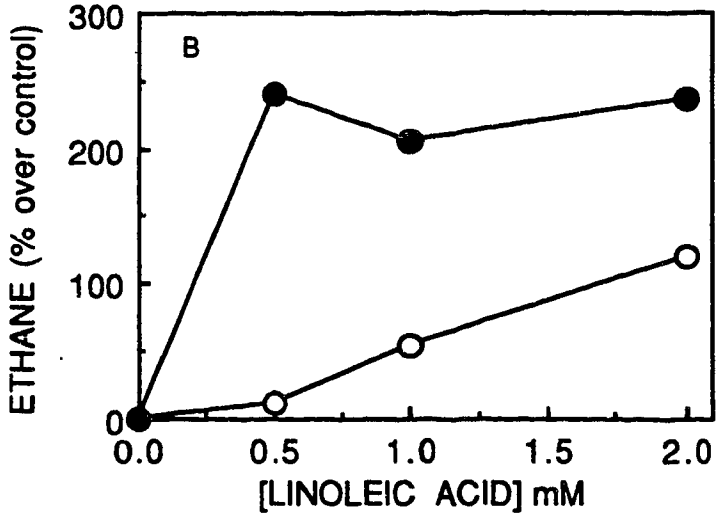
RESULTS

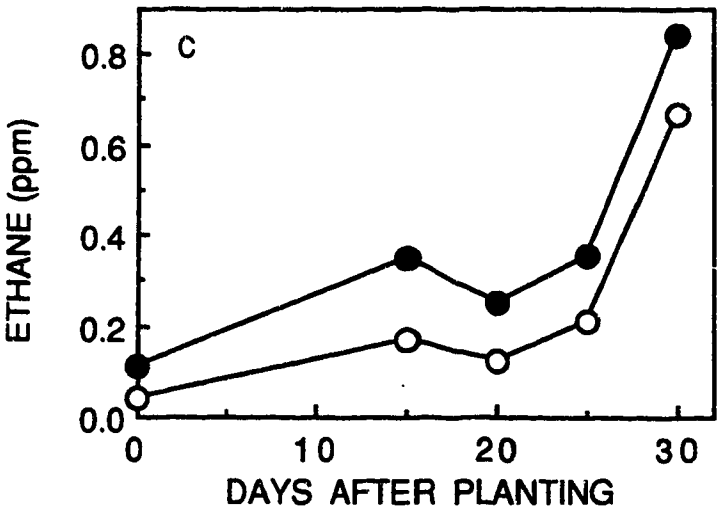
Lipid peroxidation

Free-radical-mediated peroxidation of fatty acids in tissues results in the formation of ethane (Konze and Elstner, 1978). Tissue disks from 13- and 25-month-old tubers produced 96 and 78% more ethane, respectively, than those from 1-month-old tubers (Fig. 3A), indicating a higher level of lipid peroxidation in the older tissues. Moreover, addition of calcium to the incubation medium significantly suppressed ethane production, and the effect was directly related to concentration. Since ethane production was not effected by an interaction between tuber age and calcium concentration, only the main effects are illustrated (Fig. 3A and inset). Addition of linoleic acid substrate to the incubation medium greatly stimulated ethane production *n vivo* by tissue disks from 5- and 17-month-old seed-tubers over their respective controls lacking linoleic acid (Fig. 3B). On average, linoleic acid enhanced ethane production from older tissue by 228%,

Fig. 3. (A) Effects of seed-tuber age and calcium (inset) on ethane evolution from disks of tissue cut from 1-, 13- and 25-month-old potato seed-tubers. Tuber disks were incubated for 16 h at 23°C in 140 mM Mes buffer (pH 5.5) containing 1 mM DTT, 0.25 mM PMSF, 0.5% (w/v) PVP, and 0 to 8 mM $CaCl_2$. Ethane in the headspace of the incubation flasks was quantified after 16 h by FID-GC. *F*-values for the main effects of age and calcium concentration were significant at the 0.01 level. Inset: **Correlation coefficient for the linear model was significant at the 0.01 level; [ethane] = $1.06 - 5.39e^{-2}$ [calcium]. (B) Percent increase (over respective controls lacking linoleic acid) in ethane evolution from disks of tissue cut from 5- (O) and 17-month-old (●) seed-tubers, as affected by linoleic acid concentration of the incubation medium. F-values for the effects of age, linoleic acid concentration and their interaction were significant at the 0.01 level. (C) Change in internal ethane content of seedpieces (50 to 60 g) from 8- (O) and 20-month-old (●) seed-tubers during plant establishment. Seedpieces were planted and ethane was vacuum extracted from the tuber tissue on the specified DAP. F-values for the main effects of age and DAP were significant at the 0.01 level.


A
ETHANE (nmol g. d. wt.$^{-1}$)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
SEED-TUBER AGE (months)
ETHANE (nmol g. d. wt.$^{-1}$)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
r^2=0.86**
0
2
4
6
8
[Calcium] mM


B
ETHANE (% over control)
300
200
100
0
0.0
0.5
1.0
1.5
2.0
[LINOLEIC ACID] mM


C
ETHANE (ppm)
0.8
0.6
0.4
0.2
0.0
0
10
20
30
DAYS AFTER PLANTING

compared with only 61% from younger tissue, reflecting a highly significant interaction between tuber age and substrate concentration. These results indicate that tissues from older tubers have a much greater ability to undergo peroxidative degradation of polyunsaturated fatty acids than tissues from younger tubers.

The internal ethane content of seedpieces from 8- and 20-month-old seed-tubers remained fairly constant between 15 and 25 days after planting, and then increased at similar rates between 25 and 30 days after planting (Fig. 3C). More importantly, 20-month-old seedpieces maintained a higher concentration of internal ethane than 8-month-old seedpieces throughout plant establishment. Since the trend and rate of increase in seedpiece ethane during plant establishment were similar for both ages of seed-tubers, the results indicate similar degrees of peroxidative damage (as indicated by ethane evolution) during this period. It is evident that older tubers accumulated more ethane as a result of peroxidative reactions occuring over a much longer storage period than younger tubers, hence the significant main effect of age on ethane content.

From 2 to 7 months of storage ($4^{o}C$, 95% RH), MDA concentration of tuber tissues decreased from 123 to 61 nmol g dry wt^{-1} (Fig. 4A). As age advanced from 7 months, MDA content increased linearly in tubers, reaching a maximum of 298 nmol g dry wt^{-1} at 28 months. The change in MDA concentration over the entire 32-month storage period was best described by a cubic polynomial ([MDA] = 154.4 - 24.9(Age) + 2.14$(Age)^2$ - 3.88$e^{-2}(Age)^3$. Similarly, the trend in concentration of tuber reducing sugars with advancing age (Fig. 4B) was cubic ([reducing sugars] = 74.4 - 12.8(Age) + 1.03$(Age)^2$ - 1.88$e^{-2}(Age)^3$. A positive correlation between the concentrations of reducing sugars and MDA was thus established over the 32-month storage interval (Fig. 4C). Since MDA is an indirect product of lipid peroxidation, the results show that sweetening of potatoes during prolonged storage is highly correlated to increased peroxidative damage.

Lipofuscin-like FCs were 1.8-fold (on average) more concentrated in tissues from 17- and 29-month-old seed-tubers than in tissue from 5-month-old seed-tubers (Fig. 5). Furthermore, tissue from the apical portion of seed-tubers had a significantly higher content of FCs (36% on

Fig. 4. Changes in MDA (A) and reducing sugar (B) concentrations of potato seed-tubers over a 32-month storage (4^{0}C, 95% RH) interval, as measured from harvest. **F-values for the cubic trends with advancing tuber age were significant at the 0.01 level (see Results for line equations). (C) **Correlation between MDA and reducing sugar concentrations was significant at the 0.01 level; [MDA] = 2.66[reducing sugars] - 5.62, ($P<0.01$).

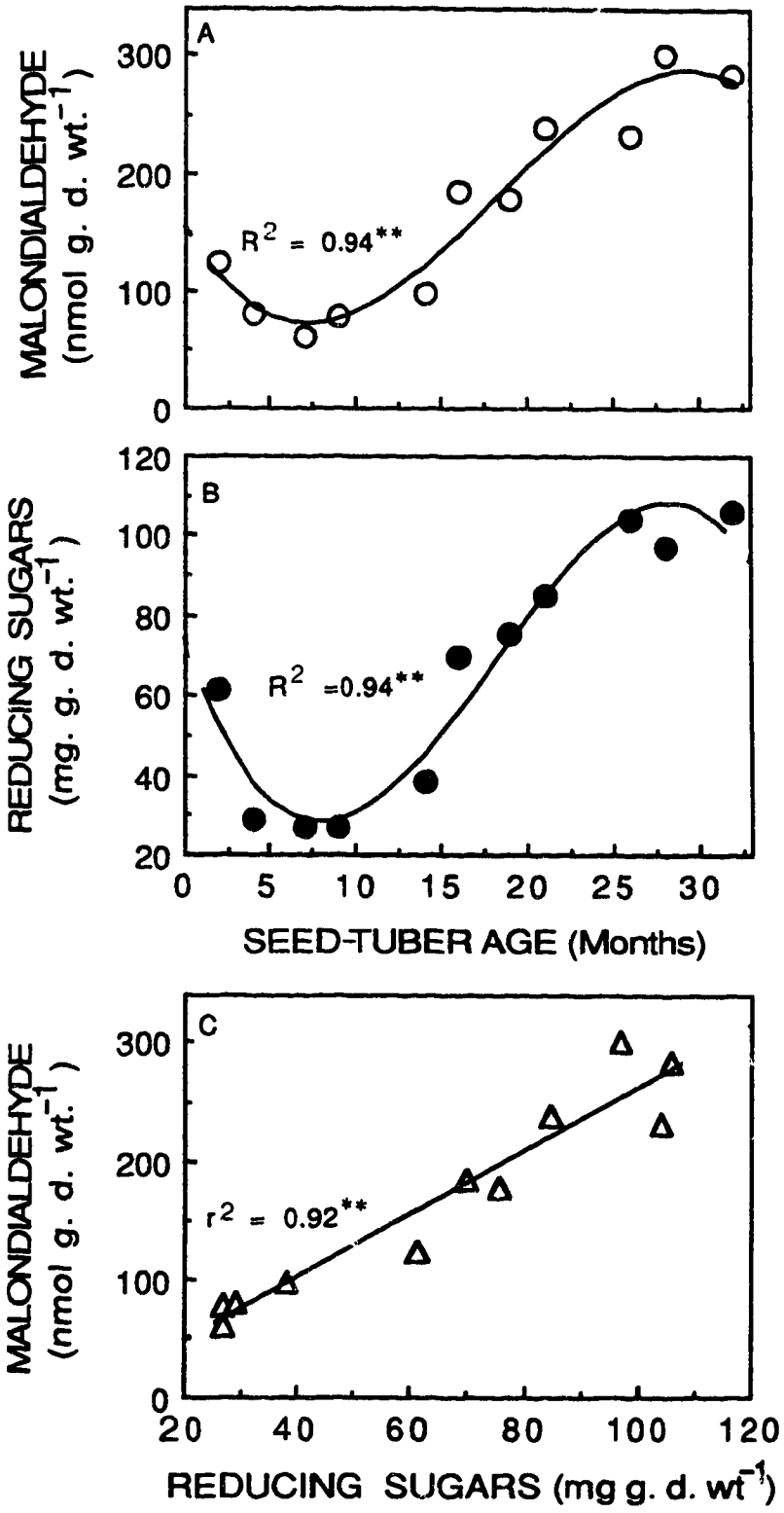
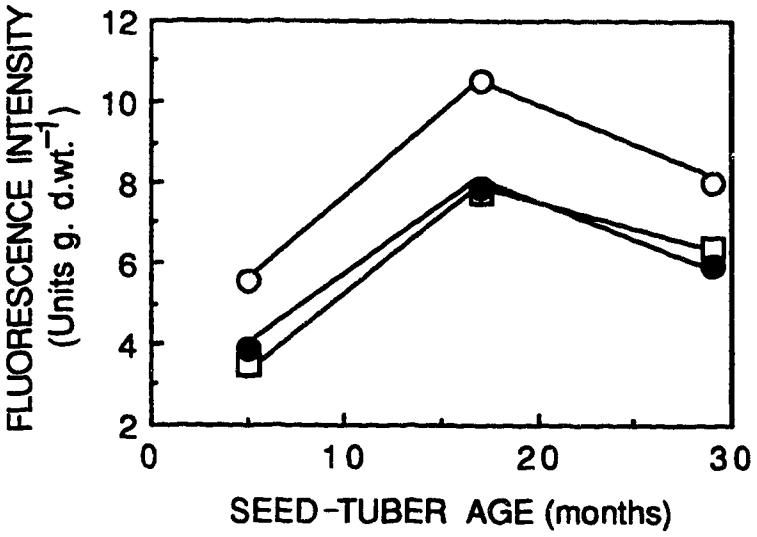

A
MALONDIALDEHYDE (nmol g. d. wt.$^{-1}$)
300
200
100
0
R^2 = 0.94**
B
REDUCING SUGARS (mg. g. d. wt.$^{-1}$)
120
100
80
60
40
20
R^2 =0.94**
0 5 10 15 20 25 30
SEED-TUBER AGE (Months)
C
MALONDIALDEHYDE (nmol g. d. wt.$^{-1}$)
300
200
100
0
r^2 = 0.92**
20 40 60 80 100 120
REDUCING SUGARS (mg g. d. wt^{-1})

Fig. 5. Relative fluorescence of lipofuscin-like compounds in lipid extracts of lyophilized tissue from 5-, 17- and 29-month-old potato seed-tubers. The concentration of FCs was determined for tissue from the apical (○), middle (●) and basal (□) portions of the seed-tubers (see Fig. 1). F-values for the main effects of seed-tuber age and portion were significant at the 0.01 and 0.05 levels, respectively.


FLUORESCENCE INTENSITY
(Units g. d.wt.$^{-1}$)
12
10
8
6
4
2
0
10
20
30
SEED-TUBER AGE (months)

average) compared with tissue from the middle and basal portions, and the main effects of seed-tuber age and portion were significant at the 0.01 and 0.05 levels, respectively.

Lipolytic and free-radical scavenging enzymes

In many plant tissues, phospholipase activity increases during senescence, resulting in deesterification of PUFA from membrane phospholipids (Leshem, 1987) . The free PUFA thus released (especially linoleate and linolenate) can then become substrates for oxidation by LOX, which in turn generates hydroperoxides and oxy-FRs. In contrast to senescing tissue, the specific activity of phospholipase in tissue from 8-month-old tubers was significantly higher than that from 20-month-old tubers (Fig. 6A and inset). In both ages, activity increased quadratically through 32 min of incubation, and the specific activity of phospholipase from 8-month-old seed-tubers increased 2.5-fold faster than that from 20-month-old seed-tubers over the incubation interval (Fig. 6 inset). Similarly, the specific activity of LOX (Fig. 6B) was significantly higher (33%) than that from 20-month-old tubers at zero-time (directly from a 4^{o}C storage). Over 30 days of plant establishment, LOX activity in 8-month-old seedpieces increased linearly ($P<0.01$). In contrast, LOX activity remained fairly constant in 20-month-old seedpieces through 25 days of sprouting, and then declined. Tuber age and time after planting thus interacted to effect the specific activity of LOX in seed-tuber tissues.

The specific activities of SOD, POX and CAT (Fig. 7), which have a protective role in scavenging FRs, were significantly higher in seedpieces from older seed-tubers than in those from younger seed-tubers at zero-time (directly from a 4^{o}C storage). A linear increase in SOD activity (SOD activity = $8.686\ e^{-4} + 0.2654X$, $r = 0.96$) was evident in seed-tubers stored from 4 to 32 months (at 4^{o}C) (data not shown). Over 30 days of plant establishment, SOD and POX activity increased linearly ($P<0.01$) in seedpieces from both seed-tuber ages; however, the rate of increase was 2-fold and 3-fold greater, respectively, in the older seed-tubers. In contrast, CAT activity in 8-month-old seedpieces remained constant with time, while the activity in 20-month-old seedpieces increased by 270% from 0 to 25 DAP (Fig. 7C). Seed-tuber soluble pro[illegible]

Fig. 6. (A) Phospholipase activity in tissue from seed-tubers stored for 8- (O)and 20-months (●) at 4°C (95% RH). Enzyme extracts were incubated at 32°C with L-3-phosphotidyl choline-1-palmitoyl-2-[1-^{14}C] palmitoyl substrate. The reaction was terminated at various intervals through 32 min, and dpm in the free fatty acid fraction was determined. **For 8-month-old tubers, phospholipase activity = $0.11085 - 1.0505e^{-2}$ (min) + $2.5068e^{-3}$ $(min)^2$ ($P<0.01$); for 20-month-old tubers, phospholipase activity = $4.6452e^{-3} - 6.0171e^{-3}$ (min) + $9.9953e^{-4}$ $(min)^2$ ($P<0.01$). Inset: Change in the specific activity of phospholipase from 8- and 20-month-old seed-tubers during incubation. (B) Change in LOX activity during plant establishment from seedpieces (50 to 60 g) prepared from 8- (O) and 20-month-old (●) seed-tubers. Seedpieces were planted and LOX activity was determined polarographically in enzyme extracts from the tuber tissue on the specified DAP. F-values for the effects of age, DAP (linear), and the interaction of age with DAP (linear) were significant at the 0.01 level. **For 8-month-old tubers, LOX activity = 637.2 + 12.78(DAP), ($P<0.01$). *For 20-month-old tubers, LOX activity = 456.7 + $20.35(DAP) - 74.93e^{-2}(DAP)^2$, ($P<0.05$).

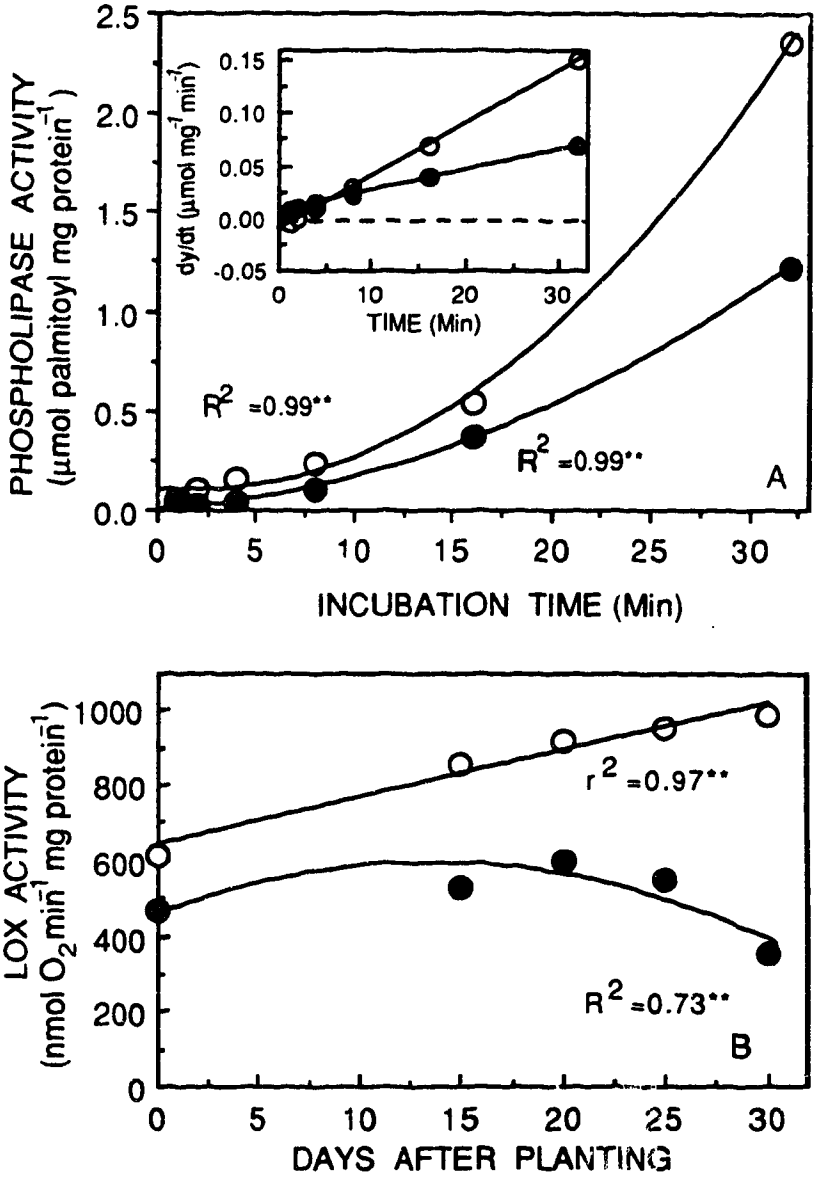

PHOSPHOLIPASE ACTIVITY
(µmol palmitoyl mg protein^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
INCUBATION TIME (Min)
R^2 =0.99**
R^2 =0.99**
A
dy/dt (µmol mg^{-1} min^{-1})
0.15
0.10
0.05
0.00
-0.05
0
10
20
30
TIME (Min)
LOX ACTIVITY
(nmol O_2 min^{-1} mg protein^{-1})
1000
800
600
400
200
0
0
5
10
15
20
25
30
DAYS AFTER PLANTING
r^2 =0.97**
R^2 =0.73**
B

Fig. 7. Changes in SOD, POX and CAT activities during plant establishment from seedpieces prepared from 8- (○) and 20-month-old (●) seed-tubers. Seedpieces were planted, and enzyme activities were determined in extracts of the tuber tissue on the specified DAP. For each enzyme, F-values for the effects of seed-tuber age, DAP and the interaction of age with DAP were significant at the 0.01 level. **For 8-month-old seed-tubers, SOD activity = $4.17 + 2.18e^{-1}$(DAP), ($P<0.01$); POX activity = $1.87 + 4.05e^{-1}$(DAP), ($P<0.01$); CAT activity = $2.66e^{-1} + 6.52e^{-3}$(DAP), ($P<0.01$) **For 20-month-old seed-tubers, SOD activity = $6.84 + 4.34e^{-1}$(DAP), ($P<0.01$); POX activity = $4.39 + 13.58e^{-1}$(DAP), ($P<0.01$); from 15 to 30 DAP, CAT activity = $6.52e^{-1} + 1.11e^{-1}$(DAP), ($P<0.01$).

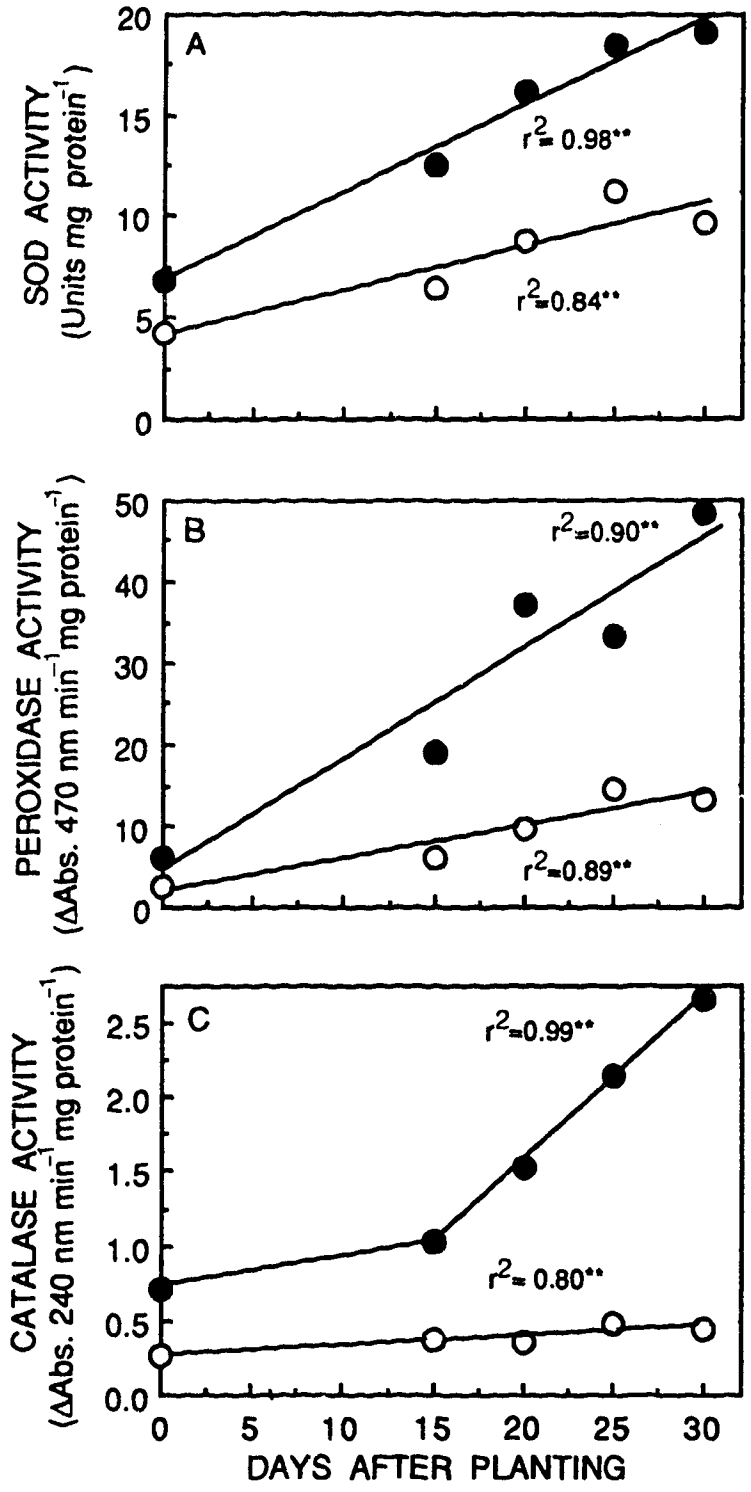
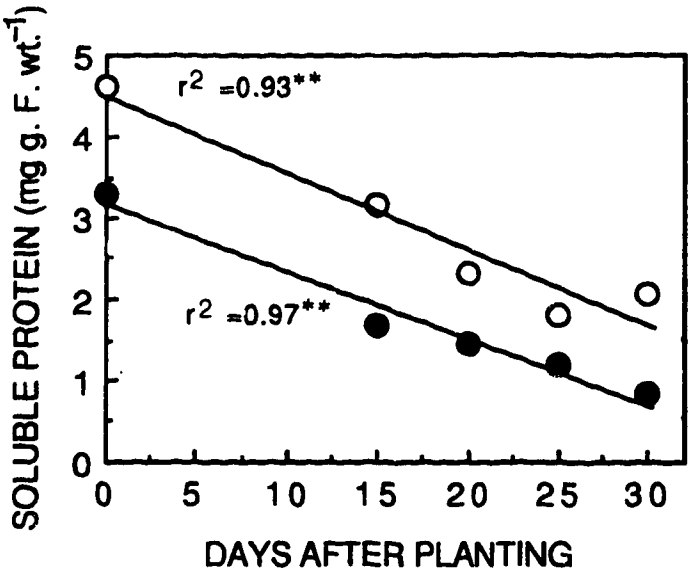

A
SOD ACTIVITY
(Units mg protein^{-1})
r^2= 0.98**
r^2=0.84**
B
PEROXIDASE ACTIVITY
(ΔAbs. 470 nm min^{-1}mg protein^{-1})
r^2=0.90**
r^2=0.89**
C
CATALASE ACTIVITY
(ΔAbs. 240 nm min^{-1}mg protein^{-1})
r^2=0.99**
r^2= 0.80**
DAYS AFTER PLANTING

Fig. 8. Change in concentration of soluble protein of seed-tuber tissue during plant establishment from seedpieces from 8- (○) and 20-month-old (●) seed-tubers. Seedpieces were harvested on the specified DAP for protein analysis. F-values for the main effects of tuber age and DAP were significant at the 0.01 level. **For 8-month-old seed-tubers, [soluble protein] = $4.50 - 9.51e^{-2}$(DAP), ($P<0.01$). For 20-month-old seed-tubers [soluble protein] = $3.16 - 8.19e^{-2}$(DAP), ($P<0.01$).


SOLUBLE PROTEIN (mg g. F. wt.$^{-1}$)
r^2 =0.93**
r^2 =0.97**
0
1
2
3
4
5
0
5
10
15
20
25
30
DAYS AFTER PLANTING

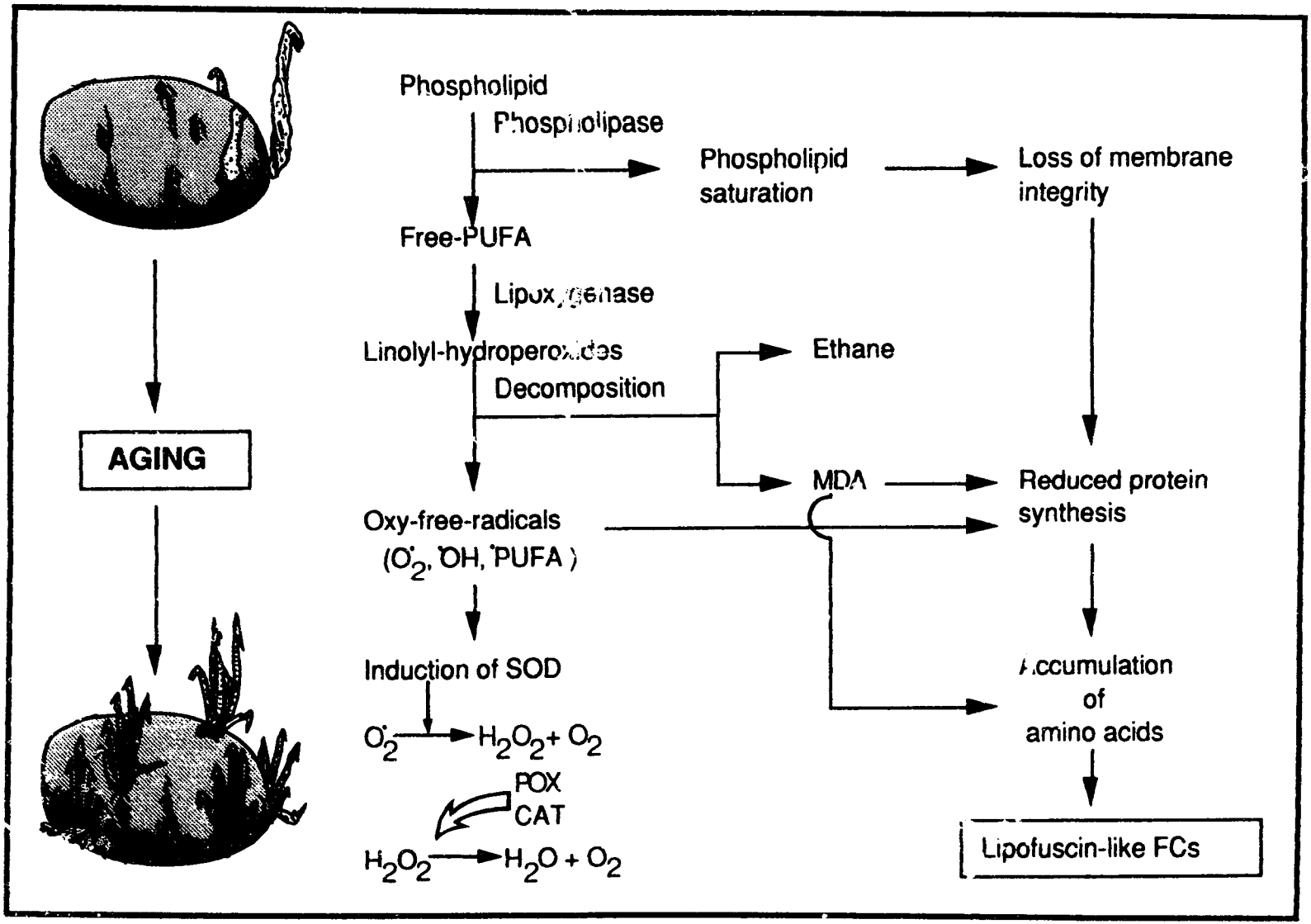
concentration declined linearly ($P<0.01$) over 30 days of plant establishment, and 8-month-old seed-tubers maintained approximately 1 mg g fresh wt^{-1} more protein than 20-month-old seed-tubers throughout the study (Fig. 8).

DISCUSSION

The association of declining membrane integrity with advancing age of potato seed-tubers is well established (Knowles and Knowles, 1989). In senescing plant tissues, lipid peroxidation plays a key role in the loss of membrane integrity (Leshem, 1987; Gidrol et al., 1989). The present study gathered evidence for the involvement of lipid peroxidation in the loss of membrane integrity in aging potato seed-tubers.

Selected features of the deteriorative processes that generally accompany aging and senescence in plant tissues are presented in Fig. 9. During senescence, the activities of lipolytic enzymes (e.g. phospholipases) increase, resulting in the release of membrane-bound PUFA. The free-PUFA are then oxidized by LOX to generate oxy-FRs and hydroperoxides. The peroxidized-PUFA, when reinserted into membranes, increase membrane permeability (Hildebrand, 1989) and the hydroperoxides serve as potential endogenous calcium-ionophores. Thus, increased calcium entry into the cytoplasm (not shown in the schematic) triggers a cascade of events in which PUFA (released by calcium-calmodulin-activated-phospholipases) are degraded into hydroperoxides and FRs. The products of PUFA-peroxidation accumulate in lipid bilayers, further destabilizing membranes. The action of LOX on free-PUFA produces jasmonic acid (Vick and Zimmerman, 1983), the derivatives of which are regarded as potential 'death hormones' (Engvild, 1989). Jasmonic acid also interferes with membrane integrity, as Vilhar et al. (1991) has demonstrated decreased fluidity of membranes in potato protoplasts isolated from plants grown on jasmonic acid. In addition, hydroperoxides can potentially inactivate the cellular mechanism of protein synthesis (Hildebrand, 1989); an effect which is characterized for potato in the second paper of this series (Kumar and Knowles, 1993b).

Fig. 9. An overall view of phospholipid catabolism in senescing plant tissues, as applicable to aging potato seed-tubers. PUFA released by various lipolytic enzymes (e.g. phospholipase) are oxidized by LOX to generate oxy-FRs and hydroperoxides. Removal of PUFA from PLs increases the saturation and PL:sterol ratio of membranes, thus reducing the fluidity, a characterstic of deteriorating membranes. The lipid hydroperoxides, resulting from LOX-mediated degradation of free-PUFA, decompose into MDA, ethane and FRs. MDA can then react with free-amino acids to form lipofuscin-like FCs. The FRs attack various macromolecules within cells, including membranes, protein, RNA and DNA. Among other factors, loss of membrane integrity (Knowles and Knowles, 1989) and reduced ability for protein synthesis during sprouting (Kumar and Knowles, 1993b), are coincident with, and may contribute to, reduced growth potential of aged potato seed-tubers. The age-induced increase in SOD activity is most likely due to higher FR titre of older tubers. Moreover, the higher POX and CAT activities of older tubers is probably a response to the increased H_2O_2 produced from the SOD reaction.


Phospholipid
Phospholipase
Phospholipid saturation
Loss of membrane integrity
Free-PUFA
Lipoxygenase
Linolyl-hydroperoxides
Ethane
Decomposition
AGING
MDA
Reduced protein synthesis
Oxy-free-radicals
(O2·, ·OH, ·PUFA)
Induction of SOD
O2· → H2O2 + O2
POX
CAT
H2O2 → H2O + O2
Accumulation of amino acids
Lipofuscin-like FCs

In view of the above, an increase in the activities of lipolytic enzymes during aging/senescence would be expected (Lynch and Thompson, 1984). However, lower activities of phospholipase and LOX do not preclude aging of potato tissue, as demonstrated in this study. The specific activities of phospholipase and LOX were substantially lower in tissue from older seed-tubers (Fig. 6). It is worth noting that the various phospholipases responsible for PL degradation exhibit preferences for particular molecular species of PL (Brown et al., 1987, 1991). Notwithstanding the age-induced decline in phospholipase activity, gradual changes in membrane architecture (which favor saturation during aging of tubers) may render membrane PLs more susceptible to phospholipases. If this were the case, lower activity of a phospholipase, as demonstrated by utilizing one molecular species of phospholipid as substrate, may not represent the true potential for phospholipid catabolism *in vivo*. It would thus be of interest to characterize changes in molecular species of phospholipids as a function of advancing tuber age and sprouting, and relate these changes to specific effects on phospholipase activity.

With regard to LOX activity, the decrease which evidently occurs during aging of potato seed-tubers is not unique, and has been observed in other plant organs that truly senesce. For example, a decline in LOX activity occurs during senescence of wheat and rye leaves (Kar and Feirabend, 1984) and soybean cotyledons (Peterman and Siedow, 1985). The activity of membrane-associated LOX, responsible for initiating peroxidative damage following release of membrane PUFA by phospholipases, represents a small portion of the total LOX activity (Lynch and Thompson, 1984). Accordingly, it would be interesting to determine what percentage of the total LOX activity (as measured in this study) was due to membrane associated-LOX, as this may more accurately reflect the tissue's susceptibility to peroxidation.

Since FRs are capable of self-propagation once formed (Fobel et al., 1987), and can peroxidize membrane lipids directly, increased LOX activity need not be indicative of higher peroxidative potential of older tissue. Further, it is doubtful if LOX of plant origin is capable of oxidizing membrane-associated PUFA directly (Hildebrand, 1989). Increased LOX activity during germination of seeds enhances mobilization of reserves to the developing embryo by

accelerating disruption of cellular membranes (Hildebrand, 1989). Thus, reduced LOX activity of older seed-tubers may actually contribute to age-reduced growth potential, by imparing reserve mobilization and translocation to developing sprouts. Age-induced dysfunctions in the ability to mobilize tuber carbohydrate and nitrogen reserves to developing sprouts have been characterized (Knowles, 1987; Mikitzel and Knowles, 1989). LOX also has a role in wound-healing, through the production of traumatin which stimulates periderm formation (Zimmerman and Coudron, 1979). In general, younger seed-tubers have a greater capacity for periderm formation during wound-healing than older seed-tubers (Lange and Rosenstock, 1964). Enhanced LOX activity therefore need not always correlate with aging, particularly in potato.

Lipid peroxides formed during senescence (by the activities of phospholipase and LOX) decompose to ethane and MDA, and the latter reacts with free amino acids and their esters to form lipofuscin-like FCs (Fletcher et al., 1973). Ethane, MDA and lipofuscin-like FCs are thus regarded as sensitive markers of lipid peroxidation (Konze and Elstner, 1978; Heath and Packer, 1968; Dhindsa et al., 1981). Cell-membranes are particularly susceptible to peroxidation because of a relatively higher content of PUFA (Meir et al., 1991). Potato tissue disks from older tubers produced higher amounts of ethane than those from younger tubers (Fig. 3A). Moreover, calcium suppressed ethane production from the tuber disks in a concentration-dependent manner (Fig. 3A, inset). This effect may be due to increased stabilization of the membranes by calcium (Evensen, 1984), although the exact mechanism requires further investigation. In addition, tissue from older tubers exhibited a higher capacity for peroxidation, as evidenced by increased ethane formation when provided with linoleic acid substrate (Fig. 3B). In older tubers, ethane formation is most likely limited by the availability of free-PUFA substrate. In fact, tissues from 19-month-old tubers contained 2- to 4-fold less free-linoleate and linolenate than did those from 7-month-old tubers (Knowles and Knowles, 1990). Moreover, the double-bond-index of phospholipids decreased as seed-tuber age advanced, indicating a trend toward increased saturation of membranes with advancing age (Knowles and Knowles, 1989). During sprouting, though the internal ethane content of both young and older tubers increased, old tubers consistently had a

higher ethane content than younger tubers (Fig. 3C). The results show that the extent of peroxidation in older tubers is similar to that in younger tubers during plant establishment; however, older tubers accumulated more ethane due to the comparatively longer interval over which peroxidation occured during storage.

It is interesting to note that MDA and reducing sugars followed similar trends and were positively correlated (Fig. 4). The accumulation of reducing sugars during prolonged storage of potato tubers is referred to as 'senescent sweetening' (Burton, 1969; Isherwood, 1976), and is most likely due to the progressive degeneration of amyloplast membranes which facilitates the enzymatic hydrolysis of starch. Sowokinos et al., (1987) reported degeneration of amyloplast membranes after prolonged storage (9 to 10 months) of Norchip potatoes. Moreover, Spychalla and Desborough (1990a) characterized an inverse relation between membrane lipid unsaturation and sugar content in potato tubers. Increased saturation of PLs during aging of potato seed-tubers correlated well with increased leakage of electrolytes and thus age-induced loss of membrane integrity (Knowles and Knowles, 1989). Although the data is correlative, these results suggest that the age-induced loss in amyloplast membrane integrity which leads to senescent sweetening may be due to a gradual peroxidation of amyloplast membrane lipids.

Senescence is often accompanied by an accumulation of lipofuscin-like FCs which are formed by the reaction of MDA with free amino acids or their esters (Fletcher et al., 1973). MDA (via lipid peroxidation) and free amino acids (via reduced incorporation into protein, increased proteolysis or both) accumulate in plant tissues during senescence (Brady, 1988; Frost, 1986). Aging of potato tubers, is accompanied by a linear increase in free amino acid concentration and a linear decrease in soluble protein concentration (Kumar and Knowles, 1993b), thus favoring the formation of FCs in older tubers (Fig. 5). Furthermore, the apical portion of seed-tubers had a higher content of FCs than the basal and middle portions, suggesting a higher degree of peroxidative damage at the stem apex. Over a 29-month storage interval, growth potential of meristems located at the apical end of potato tubers declined faster than that for basal meristems

(Knowles et al., 1985). The relation between lipid peroxidation and age-induced changes in topophysis in potato seed-tubers warrants further investigation.

Though lipid peroxidation is usually associated with loss of membrane integrity, Gidrol et al., (1989) could not detect membrane damage in aged sunflower seeds, in spite of a high degree of lipid peroxidation. This was attributed to higher lipid reserves in sunflower seeds serving as a detoxifying trap. In contrast to oilseeds, potato tubers contain relatively small amounts of lipid, associated mainly with membranes. In the absence of a detoxifying lipid trap, it appears that the membrane lipids in potato tubers become the primary target for damage.

A loss in the ability to scavenge FRs effectively is another generally-acknowledged feature of senescence. Since FRs are highly reactive, potentially dangerous and formed even during normal metabolism, their effective removal is very important for the well-being of living organisms (Fridovich, 1976). One mechanism by which plants defend against the deleterious effects of FRs is the induction of SOD, which catalyzes the dismutation of superoxide-radicals to molecular oxygen and H_2O_2 (Fig. 9). POX and CAT then catalyze the breakdown of H_2O_2 to H_2O and O_2. By eliminating H_2O_2 accumulation, POX and CAT prevent the further formation of potent FRs. The activities of FR-scavenging enzymes (SOD, POX, CAT) usually decrease during senescence of plant tissues (Dhindsa et al., 1981). In contrast, Spychalla and Desborough (1990b) showed that the activities of SOD and CAT increased in potato tubers over 40-weeks of storage at 3°C. While my studies focused on differences over a much longer storage period, the results demonstrate significant increases in activities of these protective enzymes due to advanced tuber age (Fig. 7).

SOD is an inducible enzyme (Hassan and Scandalios, 1990). Bowler et al., (1989) found that stress, C_2H_4, or pathogenesis increased the specific activity of SOD in *Nicotiana plumbaginifolia*. Ethylene concentration of tuber tissue increases with age (Mikitzel and Knowles, 1989), and thus may stimulate a higher SOD activity in older tubers. It is also likely that POX and CAT activities are higher in older tubers, in response to the highly reactive peroxide produced from the SOD reaction (Finazzi-Agro et al., 1986; Scott et al., 1987; Spychalla and Desborough,

1990b). In spite of the higher FR scavenging activity of these enzymes, aging potato seed-tubers show higher peroxidative damage. If FRs are directly involved in the peroxidative reactions within aging potato seed-tubers, then FR production exceeds catabolism by the SOD-POX-CAT system. A decrease in non-enzymatic FR scavengers (such as ascorbate, vitamin E, polyamines, etc.) may also contribute to a shift in the balance of FR metabolism (towards accumulation) during aging of tubers. Polyamines are efficient FR scavengers (Drolet et al., 1986), and older seed-tubers contain less spermidine and spermine than younger tubers (Mikitzel and Knowles, 1989).

Potato tubers are vegetative propagules, and are therefore different from senescence-targeted organs such as flowers. Since tubers are poised for sprouting at any time, protection from FRs is essential for growth to occur. Thus, in aged seed-tubers, induction of FR scavenging enzymes is most likely induced by increased FR content. These studies implicate lipid peroxidation as a possible mechanism for the previously reported loss of membrane integrity during aging of potato seed-tubers (Knowles and Knowles, 1989). The accumulation of MDA, ethane and lipofuscin-like FCs in tissues of older seed-tubers supports this view. The increase in activities of lipolytic enzymes (phospholipase and LOX) and decrease in activities of FR-scavenging enzymes (SOD, POX and CAT) that are frequently reported during senescence of plant tissues did not occur in aging potato seed-tubers; in contrast, opposite trends were characterized. It thus appears that the progressive loss of membrane integrity associated with aging of potato seed-tubers is a function of increasing peroxidative damage over time. Higher lipolytic enzyme activity may not be necessary to mediate this damage, since self-propagating FRs are able to directly damage various cellular components, including membranes. Age-induced changes in membrane lipid molecular species may also increase the susceptibilty of membrane phospholipids to lipolytic catabolism (Brown et al., 1987; 1991). The higher activities of SOD, POX and CAT in aged potato seed-tubers are probably a futile response on the part of the cell to quench an age-induced buildup of FRs.

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Chapter IV

Age of Potato Seed-Tubers Influences Protein Synthesis During Sprouting.

INTRODUCTION

During senescence of plant tissues, the equilibrium between the rates of synthesis and degradation of protein shifts in favor of degradation (Brady, 1988; Thimman, 1980; Dhindsa, et al., 1981; Richardson, 1981; Miller and Huffaker, 1985; Lalonde and Dhindsa, 1990). Moreover, loss of protein-synthetic ability is coincident with age-induced loss of vigor in seeds (Bewley and Black, 1985). The reduced ability for protein synthesis may be due to failure to translate, process or protect RNA (Skadsen and Cherry, 1983). For example, in oat leaves, a rapid decline in total protein, total RNA and poly $(A)^+$-RNA (Lalonde and Dhindsa, 1990), and a reduction in the population of translatable mRNAs (Malik, 1987) are observed during senescence. Transcription factors, tRNA turn-over rates and aminoacyl tRNA synthetase activities are all altered during aging (Strehler et. al., 1971; Cherry and Anderson, 1972; Skadsen and Cherry, 1983).

Increased lipid peroxidation (Packer et. al., 1967) and FR titre of tissues are also evident during plant aging and senescence (Leshem et. al., 1981; Mayak et. al., 1983; McRae and Thompson, 1983; Lynch and Thompson, 1984). The products of lipid peroxidation, FRs and MDA, are potential inhibitors of protein synthesis. For example, exogenous addition of MDA inhibits protein synthesis *in vivo* in *Pisum sativum* leaf disks and *in vitro* in the polyribosomes of *Tortula ruralis and Cratoneuron* (Dhindsa, 1982). MDA also adversely affects enzyme activity directly by binding to protein (Manwaring and Csallany, 1988). FRs damage DNA, reduce ribosomal integrity and RNA synthesis, inactivate enzymes and destabilize membranes through direct lipid peroxidation (Osborne, 1980).

Gradual loss of apical dominance, sprout vigor and root growth potential are characteristic features of long-term aging (up to 36 months of storage at 4^oC, 95% RH) of potato seed-tubers (Mikitzel and Knowles 1990; Kumar and Knowles, 1993a). Aging for 7 to 8 months from harvest results in an increase in the vigor of individual sprouts; however, a progressive loss in growth

potential occurs with further aging. While the products of lipid peroxidation (MDA, ethane and lipofuscin-like FCs) increased with advancing seed-tuber age, 20-month-old seed-tubers had 39% less protein (mg g fresh wt^{-1}) than 8-month-old seed-tubers (Kumar and Knowles, 1993b). In this study, aging of potato seed-tubers resulted in a linear decrease in tuber protein concentration. The decline in protein was coincident with a linear increase in the concentration of free amino acids, suggesting that increased protein degradation, decreased protein synthesis, or both are responsible for the age-induced loss of protein. As in seeds, an age-induced loss in the ability to synthesize enzymes involved in the mobilization and translocation of seed-tuber reserves to developing sprouts may limit plant growth potential. Age-induced dysfunctions in carbohydrate and nitrogen mobilization have been characterized for potato (Knowles, 1987; Mikitzel and Knowles, 1989). Hence, the objective of this study was to compare protein synthesizing ability of young and old seed-tubers during various stages of sprout development.

MATERIALS AND METHODS

Analysis of tuber protein and free amino acids

Virus-free (certified) potato (*Solanum tuberosum* L. cv. Russet Burbank) seed-tubers, obtained directly from a local grower at harvest, were stored for up to 32 months under conditions that inhibited sprouting (4°C, 95% RH). Tubers were taken from storage at 3 to 4 month intervals to profile the effects of age on protein and free amino acid concentrations. To prepare tissue for analysis, 5 seed-tubers were selected (170 to 200 g/tuber) at each harvest and cored (1.2 cm diameter) longitudinally through the central pith region (1 core/tuber). The cores were sliced into thin sections, lyophilized and collectively ground with mortar and pestle. Soluble protein was extracted (mortar and pestle) from 1 g of the lyophilized tissue with 10 mL of cold (4°C) Hepes buffer (50 mM, pH 7) containing 0.1% (w/v) $Na_2S_2O_5$. The crude nomogenate was centrifuged at 10,000 g for 30 min. Protein was determined on a 50 µL aliquot of supernatant by a modified Lowry method (Bensadoun and Weinstein, 1976), using BSA (15.6% nitrogen, Sigma) as a standard. For free amino acid determination, 25 mg of lyophilized tuber tissue was extracted

(mortar and pestle) with 5 mL of cold (4°C) potassium phosphate buffer (20 mM, pH 7.2) containing 0.1% (w/v) $Na_2S_2O_5$, and the homogenate was centrifuged as above. A 50 µL aliquot of the supernatant was analyzed for free amino acid content by the methods of Rosen (1956), using leucine (10.7% nitrogen) as a standard.

***In vivo* protein synthesis**

The capacity of tissue from 5- and 17-month-old tubers to synthesize protein at similar stages of sprouting (as determined by sprout dry weight) was also compared. To induce sprouting, uniform size (approximately 200 g fresh weight) 5- and 17-month-old seed-tubers were selected from a 4°C storage, rinsed, weighed and placed at 23°C in the dark in 5-L glass chambers (3 tubers/chamber). The sprouting chambers had inlet and outlet ports through which air-flow was maintained at 200 mL min^{-1} with a flow-board/manifold system (see appendix I). The air was humidified by passing it through a diffusion stone submersed in distilled water prior to flowing into the manifold.

Comparison of protein-synthetic capacity of tuber tissue at similar stages of sprout development necessitated synchronization of sprouting by the 5- and 17-month-old seed-tubers. Since 17-month-old tubers sprout faster than 5-month-old tubers, the younger tubers were placed into the chambers 14 to 18 days (depending on the degree of sprout development desired) before the older tubers. Both ages of seed-tubers were also placed into the sprouting chambers in reverse chronological order, so that tubers showing different degrees of sprout development could be harvested simultaneously for determination of protein synthesizing ability. Thirty days after initiation of the study, 5-month-old tubers that had been sprouting for 0 (directly from 4°C storage), 18, 24 and 30 days were harvested from the chambers, and the various degrees of sprout development were designated stages I, II, III and IV, respectively. Comparable sprouting stages were obtained for 17-month-old tubers, by harvesting (at 30 days) tubers that had been sprouting in the chambers for 0, 4, 8 and 12 days, respectively.

Whole-tuber respiration was measured for a more precise characterization of the physiological status of the 5- and 17-month-old tubers during sprouting in the chambers.

Respiration rates were quantified by calculating the difference in CO_2 concentration between inlet and outlet ports of each chamber at 2 to 4 h intervals over the first 48 h, and at 24 h intervals over the remaining sprouting period. Carbon dioxide was analyzed by injecting 1 mL gas samples from the ports of each chamber into a Hewlett-Packard 5890A gas chromatograph. The GC had a 2.4 m stainless steel column (3.2 mm o.d.) packed with HayeSep T (Hewlett-Packard) and a thermal conductivity detector. The carrier gas (He) flow rate was 30 mL min^{-1}, and the column was isothermal at 100°C. Injection and detector port temperatures were 140°C. Tuber respiration rates were expressed as mL CO_2 h^{-1} kg^{-1} fresh weight.

The ability of tissue disks from the 5- and 17-month-old sprouted tubers to synthesize protein was compared by quantifying the rate of incorporation of radiolabeled amino acids (^{14}C-protein hydrolysate, 50 µCi mL^{-1}, 50 mCi milliatom $carbon^{-1}$; Amersham, Canada) into TCA-precipitable protein. Sprouted 5- and 17-month-old tubers were removed from the chambers, desprouted, and sprouts were dried at 80°C (72 h) for dry weight determination. Disks (1 mm thick) of cortical tissue were prepared from cores (1 cm diameter) cut longitudinally (2 mm interior to the periderm) from the apical halves of the young and old tubers for each of the four sprouting stages. The disks were incubated for 24 h at room temperature in 5 mM Mes buffer (pH 6.5) containing 1 mM DTT, 0.25 mM PMSF, 5 mM $CaCl_2$, 0.5% (w/v) PVP (M. wt. 40,000) and 0.15 mM chloramphenicol (180 disks per 50 mL incubation medium). The flasks containing the disks were placed on a rotary shaker, and the incubation medium was changed five times over the 24 h period. This incubation period was necessary to allow the tissue slices to revert back to the conventional respiratory pathway of whole-tubers, where glycolysis is no longer inhibited by slice-induced liberation of free-fatty-acids (Radamoss et. al., 1976) and cabohydrate (rather than lipid) serves as the respirable substrate (Jacobson et. al., 1970). Consistent with previous studies on potato (Kahl, 1971) and sweet potato (Sakano and Asahi, 1969), rates of protein synthesis were much higher (8.7-fold relative to freshly cut disks) if tissue was incubated for 24 h prior to the incorporation studies (data not shown).

Following the 24 h incubation, rates of incorporation of ^{14}C-amino acids into protein were compared by incubating disks for 30, 60, 120 and 240 min at 30^{o}C in 50 mM Mes buffer (pH 6.5) containing 0.5 µCi mL^{-1} of the protein hydrolysate (15 disks/treatment/10mL). Dithiothreitol, PMSF, $CaCl_2$, chloramphenicol and PVP were also included in the incorporation buffer, as previously described. The treatments (replicated 3 times) consisted of two seed-tuber ages, four stages of sprout development and four tissue incubation times (96 experimental units). At each harvest, disks were rinsed three times with 10 mL of incubation medium lacking ^{14}C-protein hydrolysate. This effectively removed unincorporated radiolabel adhering to the tissue, as evidenced by a level of dpm in the final rinse equivalent to background. The harvested tissue disks were lyophilized and weighed.

Soluble protein was extracted (mortar and pestle) from the lyophilized tissue with cold (4^{o}C) potassium phosphate buffer (20 mM, pH 7.2) containing 0.1% (w/v) $Na_2S_2O_5$ (approximately 350 mg tissue/10 mL buffer), and the crude homogenate was centrifuged at 30,000 g for 20 min. Total uptake of labeled amino acids by the tissue disks was determined by counting 100 µL of the supernatant. Soluble protein content of the supernatant was quantified as previously described. Seven hundred and fifty microliters of 1% (w/v) sodium desoxycholate was then added to 5 mL of supernatant and the mixture was vortexed. After 15 min, soluble protein was precipitated by adding 750 µL of 60% (w/v) TCA to the supernatant. The mixture was again vortexed and stored over night at 4^{o}C. The TCA-precipitated protein was centrifuged at 1640 g for 20 min, and the pellet was resuspended in 10 mL of 10% (w/v) TCA. The protein suspension was then filtered through a 25-mm diameter MSI micron separation filter (0.45 µm, Micron Separations Inc., U.S.A.) under vacuum. Protein captured by the filter was washed three times with 10 mL of 10% (w/v) TCA. The separation filter was transferred to a scintillation vial containing 10 mL of Scintiverse E (which dissolved the filter), and dpm in the protein precipitate was determined with a Minaxi Tri-Carb 4000 liquid scintillation counter.

For each treatment, incorporation of radiolabeled amino acids into TCA-precipitable protein was plotted over the 240 min incubation interval, and the regression coefficients (dpm mg protein

$^{-1}$ min^{-1}) were compared as a measure of protein-synthetic capacity. The treatments (2 seed-tuber ages, 4 stages of sprout development, 4 tissue incubation times) were arranged factorially in a randomized block design with 3 replications. Data were subjected to analysis of variance and, where appropriate, sums of squares were partitioned into individual degree-of-freedom components of main effects and interactions.

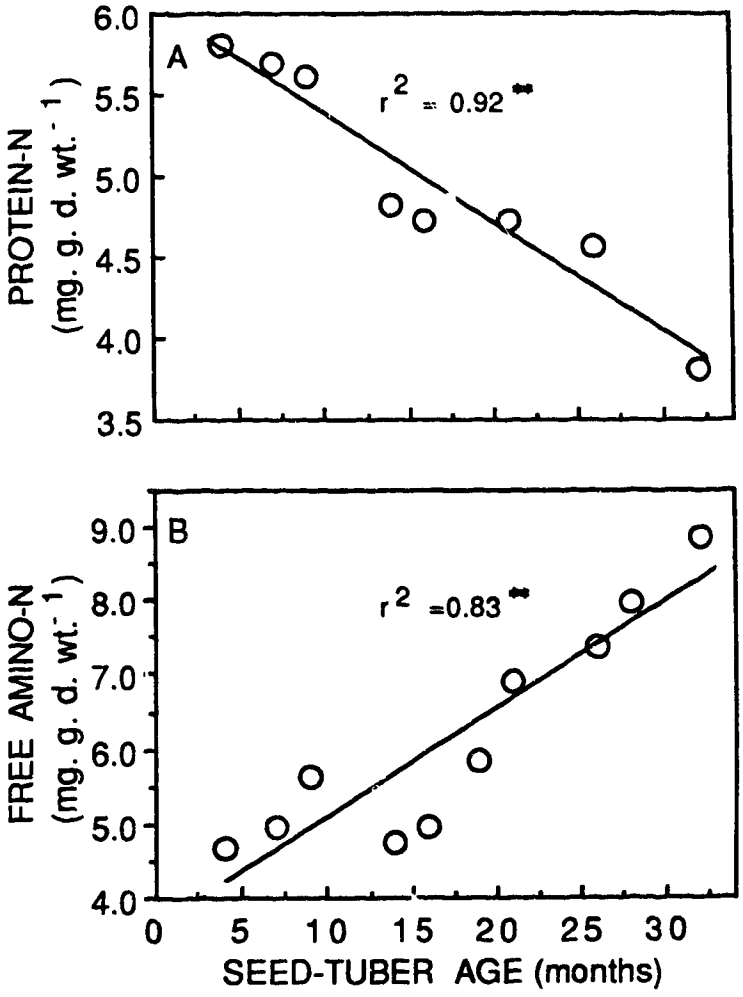
RESULTS

The concentration (mg g dry wt^{-1}) of tuber soluble protein-N decreased linearly as seed-tuber age increased from 4 to 32 months (Fig. 1A). In contrast, over the same storage period, the concentration of tuber free amino acid-N increased (Fig. 1 B). The rate of increase in free amino-N (143 µg N g dry wt.$^{-1}$ month^{-1}) was greater than twice the rate at which protein-N was lost (67.4 µg N g dry wt.$^{-1}$ month^{-1}) during aging of the seed-tubers. Age-induced buildup in free amino-N may thus be due to increased proteolysis, decreased protein synthesis, or both.

Since reduced capacity for protein synthesis accompanies, and possibly contributes to, a loss of vigor in seeds (Bewley and Black, 1985), and age-reduced growth potential has been well characterized for potato (Mikitzel and Knowles, 1990; Kumar and Knowles, 1993a), the effect of tuber age on protein-synthetic capacity during various stages of sprout development was compared. In seeds, rates of protein synthesis in the endosperm change from imbibition through seedling establishment (Mayer and Poljakoff-Mayber, 1982). Hence, sprouting of 5- and 17-month-old seed-tubers was synchronized so that protein-synthetic capacity of tuber tissues could be compared at similar stages of sprout development (based on sprout dry weight).

Sprouting of 5-month old tubers was slower than that from 17-month-old tubers, as evident by a lower rate of total sprout dry-matter accumulation (Fig. 2). Based on total sprout dry matter, younger tubers sprouted for 18 days (stage II) were developmentally equivalent to older tubers sprouted for 8 days (stage III). Similarly, sprouting 5-month-old tubers for 30 days (stage IV) was approximately equal to sprouting 17-month-old tubers for 12 days (stage IV). The fact that older

Fig. 1. Trends in potato tuber soluble protein and amino acid nitrogen concentrations with advancing age. Tubers were taken for analysis from a 4°C (95% RH) storage at various intervals from harvest through 32 months. **Coefficients of determination for linear trends were significant at the 0.001 level; protein-N = 6.06 - $6.74e^{-2}$ (Age), Free amino-N = 3.63 + $1.46e^{-1}$(Age).


A
r^2 = 0.92**
PROTEIN-N (mg. g. d. wt.$^{-1}$)
6.0
5.5
5.0
4.5
4.0
3.5
B
r^2 =0.83**
FREE AMINO-N (mg. g. d. wt.$^{-1}$)
9.0
8.0
7.0
6.0
5.0
4.0
0 5 10 15 20 25 30
SEED-TUBER AGE (months)

tubers sprouted faster than younger tubers should not be construed as contradictory to the concept of reduced sprout vigor with advanced seed-tuber age. Relative to 17-month-old tubers, delayed sprouting of younger tubers is attributed to incomplete release from dormancy prior to initiation of the study. Furthermore, the study focused only on the very early stages of sprouting (up to 300 mg sprout dry weight production). Previous results have shown that the relative delay in sprouting of younger tubers, as they emerge from dormancy, is more than compensated for by much higher plant growth rates (compared with plants from older tubers) in establishment (Knowles, 1987; Mikitzel and Knowles, 1989). Of further relevance, was the fact that apical dominance between sprouts developing from 17-month-old seed-tubers was nonexistent, resulting in many sprouts per node (Fig. 3). In contrast, 5-month-old seed-tubers showed a high degree of apical dominance within, as well as between, nodes on the seed-tuber. The average number of sprouts produced per single-eye core from a 17-month-old seed-tuber is five (Mikitzel, 1989). Hence, on an individual basis, sprout vigor (dry-matter accumulation per unit time) was substantially greater from the younger tubers in this study, as illustrated in Figure 3.

To characterize the physiological status of the 5- and 17-month-old seed-tubers, profiles of respiration were established during each stage of sprouting (Fig. 3). Respiration of 5-month-old tubers increased to a maximum at 48 to 60 h of acclimation to room temperature in the sprouting chambers. Respiration of the younger tubers then declined, reaching a basal rate (4.25 mL CO_2 kg^{-1} h^{-1}) at approximately 8 days of sprouting, and this was maintained for the duration of the study (30 days). A similar climacteric in respiration was evident from 17-month-old tubers; however, the maximum rate was achieved about 24 h earlier and was 1.2-fold higher (averaged over sprouting stages) than that from younger tubers. Respiration of older tubers declined from the maximum, reaching a minimum at 4 to 5 days, and then increased with sprout dry weight (Fig. 2) through 12 days. Thus, at similar stages of sprout development (dry-weight production), respiration of older tubers was 2- to 3-fold greater than that of younger tubers (e.g. compare final rates between young and old seed-tubers at stages III and IV of sprouting in Fig. 3).

Fig. 2. Total sprout dry matter production from 5- (O) and 17-month-old (Δ) seed-tubers. At zero-time, tubers were taken from a 4°C (95% RH) storage and placed in the dark at 23°C to sprout. Roman numerals indicate the various stages of sprout development, as illustrated in figure 3. **Coefficients of determination for linear trends were significant at the 0.01 level. For 17-month-old seed-tubers, the linear regression was calculated on data from 4 to 12 days.

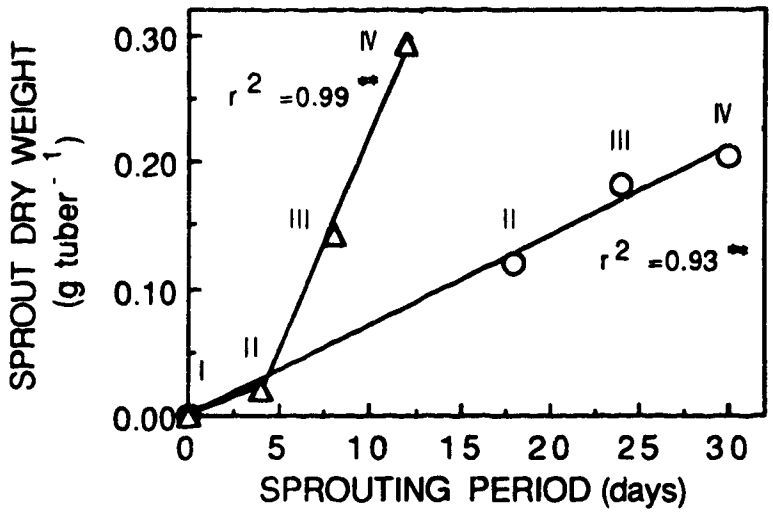

SPROUT DRY WEIGHT
(g tuber⁻¹)
0.30
0.20
0.10
0.00
0
5
10
15
20
25
30
SPROUTING PERIOD (days)
r 2 =0.99 **
r 2 =0.93 **
I
II
III
IV
II
III
IV

Fig. 3. Respiratory profiles of 5- and 17-month-old seed-tubers through different stages of sprouting. Insets depict the sprout growth characteristics of the young and old seed-tubers as observed at the end of each sprouting period. The seed-tubers were sprouted in the dark at 23°C for different intervals, so that similar stages of sprout development (based on sprout dry weight) could be harvested simultaneously (at 30 days) for quantification of protein-synthetic capacity. Sprout dry weights and protein-synthetic capacity of tuber tissue at each stage of sprout development are depicted in figures 2 and 4, respectively.

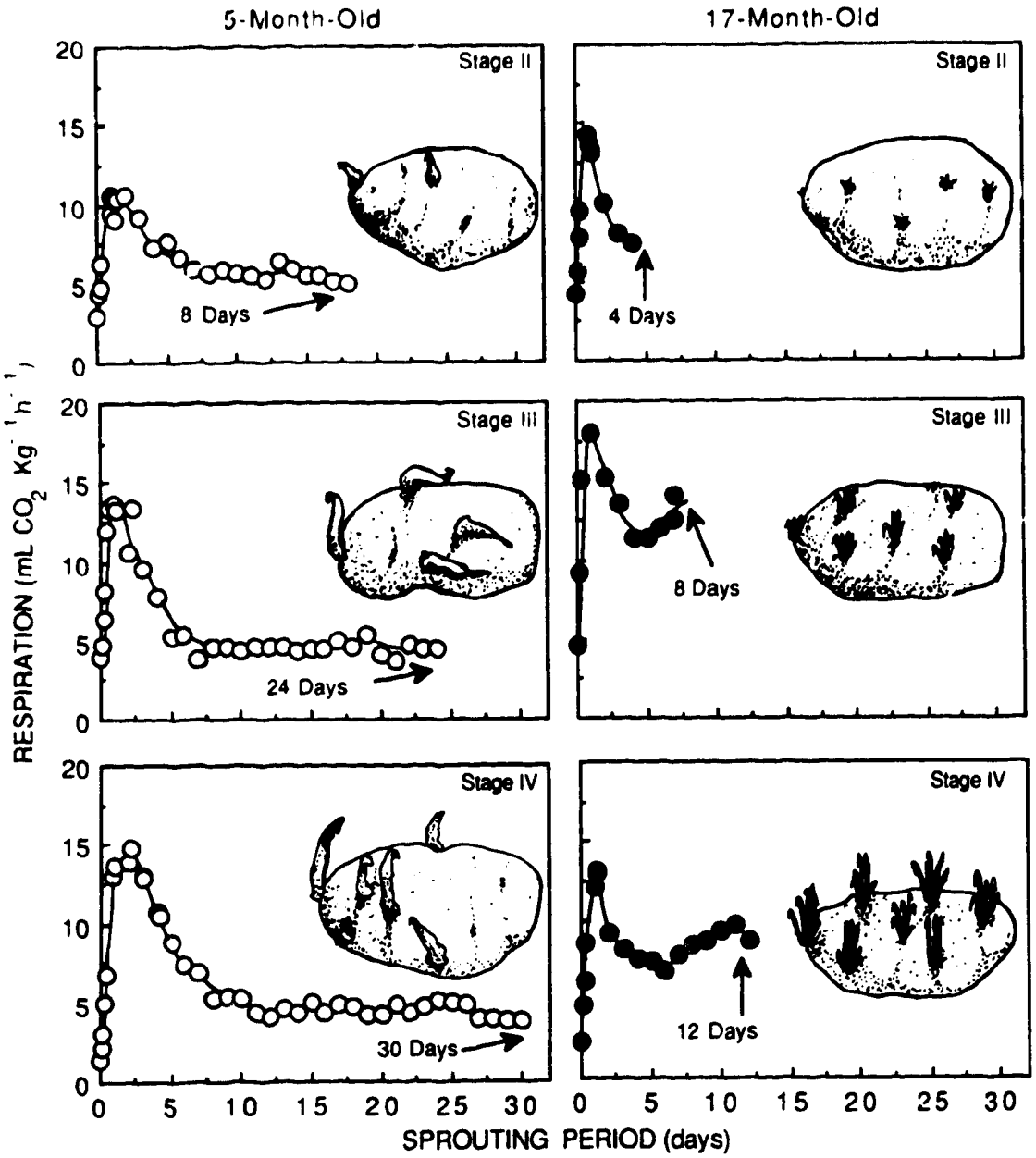
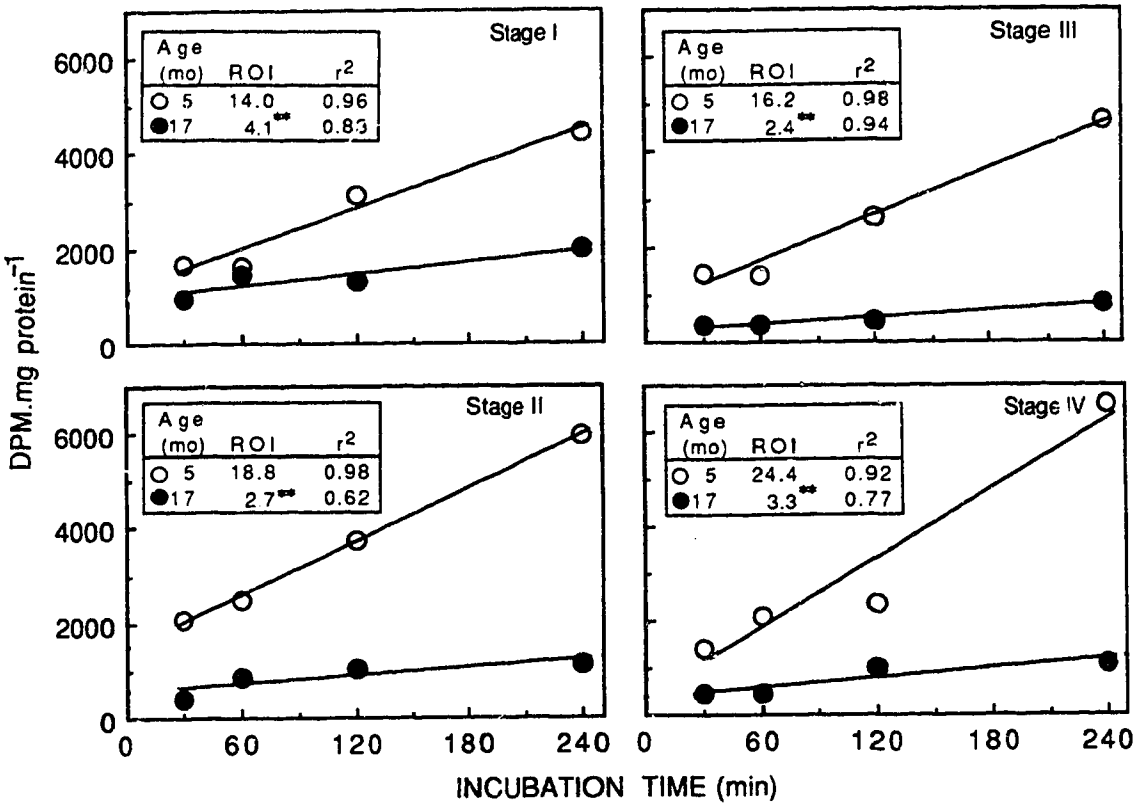

5-Month-Old
17-Month-Old
Stage II
Stage II
8 Days
4 Days
Stage III
Stage III
24 Days
8 Days
Stage IV
Stage IV
30 Days
12 Days
RESPIRATION (mL CO_2 Kg^{-1} h^{-1})
SPROUTING PERIOD (days)
0
5
10
15
20
25
30

Fig. 4. Incorporation of radiolabeled amino acids into TCA-precipitable protein of tuber disks from 5- and 17-month-old seed-tubers at different stages of sprouting. Freshly cut tuber disks were aged for 24 h and then incubated at 30°C in a mixture of radiolabeled amino acids (^{14}C-protein hydrolysate) for various intervals through 240 min. The rate of incorporation (ROI) was determined by calculating the linear regression coefficients (dpm mg protein^{-1} min^{-1}) over the incubation interval (insets). Stages of sprout development are illustrated in figure 3. F-values for the main effects of seed-tuber age and incubation time (linear) were significant at the 0.01 level. The interaction of seed-tuber age with stage of sprout development was significant at the 0.05 level.


Stage I
Age (mo) ROI r²
5 14.0 0.96
17 4.1** 0.83
Stage III
Age (mo) ROI r²
5 16.2 0.98
17 2.4** 0.94
Stage II
Age (mo) ROI r²
5 18.8 0.98
17 2.7** 0.62
Stage IV
Age (mo) ROI r²
5 24.4 0.92
17 3.3** 0.77
DPM.mg protein⁻¹
6000
4000
2000
0
0
60
120
180
240
INCUBATION TIME (min)

Protein-synthetic capacity of tissue from the young and old seed-tubers was compared at the various stages of sprout development depicted in Figure 3. Incorporation of radiolabeled amino acids into soluble protein of tuber tissue disks was linear ($P<0.01$) over a 240 min incubation interval (Fig. 4). At all stages of sprout development, the rate of protein synthesis in tissue from 5-month-old seed-tubers was significantly ($P<0.01$) higher (5.9-fold on average) than that from 17-month-old seed-tubers (Fig. 4 insets). More importantly, seed-tuber age interacted ($P<0.05$) with stage of sprout development to affect the rate of incorporation (ROI) of amino acids into protein by tuber tissue. Protein-synthetic rate increased linearly ($P<0.05$, $r=0.82$) by 8.5 dpm mg protein^{-1} min^{-1} (based on linear model) in tissue from younger tubers as sprouting advanced from stage I to IV (a 61% increase). In contrast, the rate of protein synthesis in tissue from 17-month-old seed-tubers remained relatively constant at 3.1 dpm mg protein^{-1} min^{-1} (on average) during sprouting. In this study, the difference in free amino acid content between 5- and 17-month-old tubers was about 50% (Fig. 1). This agrees well with the studies of Knowles (1987). THe diffrerence in rate of incorporation of labeled amino acids into protein between 5- and 17-month-old was 612% (averaged over stage of sprouting). Hence, the differences in the rate of incorporation cannot be attributed to the dilution of the specific activity of exogenous radiolabeled amino acids by the initial differences in endogenous amino acids. Furtherrmore, the rate of incorporation of amino acids in older tuber tissue did not change during sprouting. Previous research (Knowles, 1987) has shown that differences in amino acid content between 5- and 17-month-old tubers decline linearly over a 24 day sprouting interval. Since rate of incorporation did not change with stage of sprouting in tissues in tissues from 17-month-old tubers, the age-induced differences in rate of incorporation were not manifested by differences in endogenous free amino acid content over the study interval. Thus a substantial impairment of protein-synthetic capacity during sprouting is directly attributable to advanced seed-tuber age.

DISCUSSION

Potato seed-tubers stored beyond 7 to 8 months (4^{o}C, 95% RH) begin to develop symptoms of aging, such as reduced apical dominance, sprout vigor, and plant and root growth potential (Kumar and Knowles, 1993a; Mikitzel and Knowles, 1990a). In general, the processes of aging and senescence in plant foliar tissues are often accompanied by reduced protein synthesis and increased protein degradation, resulting in the accumulation of free amino acids (Brady, 1988; Thimman, 1980; Dhindsa et. al., 1981; Richardson 1981; Miller and Huffaker, 1985; Lalonde and Dhindsa, 1990). Hence, the decrease in protein and increase in amino acid concentration with advancing age of potato seed-tubers (Fig. 1) are somewhat consistent with the process of senescence in vegetative plant tissues.

Increased protein synthesis is a salient feature of germinating seeds (Brooker et. al., 1977), and many of the newly synthesized enzymes mediate the mobilization and translocation of seed reserves to the developing seedling (Eldan and Mayer, 1974). Sprouting of potato seed-tubers is somewhat analogous to seed germination, in that food reserves must be mobilized enzymatically to support growth of developing sprouts. A dysfunction in the ability of potato seed-tubers to synthesize protein during sprouting could thus restrict sprout growth and development. In light of a substantial reduction in tuber protein content with advancing age (Fig. 1), we compared age-induced differences in protein synthesizing capacity, and examined whether reduced vigor of sprouts from older tubers is associated with a general deficiency in protein synthesis during sprouting.

Although the effect of seed-tuber age on respiration is well documented, respiratory rates of 5- and 17-month-old seed-tubers were determined during sprouting to enable a more definitive characterization, and thus selection, of the most appropriate stages of sprout development for comparisons of protein synthesizing ability. Consistent with previous studies (Knowles, 1983; Mikitzel and Knowles, 1990b), tuber respiration rate increased during the initial 24 to 40 h period of acclimation (from a 4^{o}C storage) to room temperature (Fig. 3). This transitory increase was induced by a combination of the direct effect of temperature on respiration (Q_{10}), and

temperature-dependent carbohydrate conversions within the tubers serving as an increased sink for ATP (Isherwood, 1976).

Increased respiration during imbibition and germination of seeds is necessary to meet the higher energy requirements of these metabolically active phases of development (Rodaway et. al., 1979; Mayer and Poljakoff-Mayber, 1982). In contrast to seeds, respiration rate of relatively young (e.g. stored for 7 months at 4°C) potato seed-tubers remains constant during the initial stages of sprouting (Mikitzel and Knowles, 1990b), and does not increase until sprout fresh weight reaches 1% of tuber fresh weight (Burton et. al., 1955). This increased respiration of tubers later in sprouting no doubt fuels a greater demand by the sprouts for tuber reserves. The higher respiratory rate of 17-month-old seed-tubers during early sprouting (stages III and IV, Fig. 3) has been attributed to a higher rate of alternative (cyanide-insensitive) respiration; rates of cyt-mediated respiration are equal for young and old seed-tubers (Mikitzel and Knowles, 1990b). Paradoxically, older seed-tubers (e.g. stored for 19 months at 4°C), while exhibiting a significant loss of sprout vigor, had substantially higher rates of respiration than younger tubers during sprouting (Mikitzel and Knowles, 1990b). Since the degree of coupling of oxidative phosphorylation to electron transport is unaffected by seed-tuber age (Knowles, 1983), the increased energy (ATP) derived from respiration of older tubers is evidently directed toward metabolic processes (sinks) that do not directly benefit sprout growth.

Prior to sprouting, protein-synthetic capacity of tissue from 5-month-old tubers was greater than that from 17-month-old tubers (Fig. 4 inset, stage I). Of greater significance with regard to sprout development, was the fact that the rate of protein synthesis increased in younger tubers with sprouting, while the rate in tissue from older tubers remained constant (Fig. 4, stages II to IV). The basal rate of respiration established during sprouting of younger tubers was evidently sufficient to support the increasing rate of protein synthesis; however, despite a substantial increase in respiration of older tubers during sprouting, protein synthesis remained at a relatively low and constant rate. Protein synthesis is thus a realtively small sink for metabolic energy during sprouting of aged seed-tubers. Since protein synthesis is an energy-dependent process (Brady,

1988; Cocucci and Marre, 1973) that is positively correlated with seed vigor (Heydecker, 1977), the apparent reduction in allocation of energy (ATP) to support protein synthesis during sprouting may be closely associated with the age-induced loss in sprout vigor of potato seed-tubers.

Protein synthesis requires a functional membrane system 'because ribosomes directly associate with internal membranes and because many of the products of protein synthesis need to be transferred through or correctly deposited within membranes' (Brady, 1988). Hence, alterations of membrane architecture and loss of membrane integrity can have profound effects on the efficiency of protein synthesis. Loss of membrane integrity with advancing age of potato seed-tubers has been characterized, and is accompanied by a decrease in the double-bond-index of membrane phosphclipids (Knowles and Knowles, 1989). Moreover, as tubers age, products of lipid peroxidation (ethane, MDA and lipofuscin-like FCs) accumulate in tuber tissues (Kumar and Knowles, 1993b). Although the evidence is largely circumstantial, the peroxidative damage of membranes during tuber aging appears to be mediated by a buildup of FRs. MDA cross-links membrane-bound proteins, rendering them immobile, and thereby further destabilizes the membrane lipid bilayer (Manwaring and Csallany, 1988). MDA and FRs can also directly inhibit protein synthesis (Osborne, 1980; Dhindsa, 1982). A buildup in the associated products of lipid peroxidation may therefore reduce protein-synthesizing ability of aged seed-tubers. Moreover, loss in the ability to synthesize enzymes involved in the mobilization and translocation of tuber reserves to developing sprouts may be an important contributor to age-induced loss of growth potential. The mechanism by which protein synthesis is impaired, and the consequence of reduced protein synthesis for the production of enzymes that have specific importance to sprouting, warrants further investigation. The results of this study are consistent with those of aging in seeds (Priestley, 1986), and support the concept that age-induced loss of protein-synthetic capacity contributes to loss in growth potential of potato seed-tubers.

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Chapter V

Effects of Seed-Tuber Age and Sprouting on Plasma Membrane H^+-ATPase Activity from Potato Tuber Cortex Tissue.

INTRODUCTION

Although a wealth of information exists on the physiological and biochemical changes associated with aging and loss of vigor in seeds, the physiological processes involved in degeneration and loss of growth potential during aging of vegetative propagules have received much less attention. Many studies on seed aging involve exposing seeds to high temperature to accelerate the aging process. In contrast, potato seed-tubers stored at 4°C begin to show age-induced changes in growth potential within months of harvest.

Vigor and growth potential of vegetative meristems of potato tubers depends in large part on the physiological age of the seed-tuber. In temperate production zones, commercial plantings are made in the spring from seed-tubers harvested the previous fall and stored for 7 to 8 months (4°C, 95% RH). Upon sprouting, these relatively young tubers typically display a high degree of apical dominance, producing a few shoots with vigorous leaf area and root development (Mikitzel and Knowles, 1990a; Kumar and Knowles, 1993a), which leads to a greater yield potential compared with that from older tubers (Knowles et al., 1985). When seed-tuber age advances beyond 7 to 8 months, growth potential and apical dominance gradually decline, resulting in the production of many shoots with low specific leaf area and sparse root systems. Such plants produce small tubers and yield poorly relative to those from younger seed-tubers (Knowles et al., 1985). Seed-tubers stored at 4°C (95% RH) can remain viable for about 3 years; however, they eventually lose their ability to sprout and the meristems develop directly into small tubers toward the end of this period (Krijthe, 1962). The physiological basis for loss in growth potential accompanying aging of potato seed-tubers is under investigation in this laboratory.

During the process of aging in true seeds, lipid peroxidation (mediated either by free-radicals or lipolytic enzymes) causes a gradual loss of membrane integrity (Wilson and McDonald,

1986). In potato seed-tubers, as age advances from 6 to 24 months, membrane permeability electrolytes increases and this age-induced decline in membrane integrity is highly correlated with a decrease in double-bond-index of fatty acids within the membrane lipid fraction (Knowles and Knowles, 1989). Although the mechanism of declining membrane integrity is not completely known for aging potato seed-tubers, free-radical-mediated lipid peroxidation appears to play a central role in the loss of membrane integrity (Kumar and Knowles, 1993b).

It is well documented that increased saturation of membrane lipids results in organizational changes that favor disruption of membrane integrity and increased permeability (Barber and Thompson, 1980; Pauls and Thompson, 1981). Alterations in the lipid microenvironment of a membrane would also affect membrane-protein mobility (Shinitzky and Inbar, 1976), resulting in potential alterations in the activities and kinetic properties of membrane-bound enzymes notably transport proteins (Carruthers and Melchior, 1986). The activity of membrane-bound ATPase would thus be subject to changes in its lipid environment (Cooke and Burden, 1990). For example, altered lipid composition in senescing plants leads to a loss in membrane fluidity (Pauls and Thompson, 1980, 1981; Itzhaki et al., 1990) and a decline in ATPase activity (Adam et al., 1983; Paliyath and Thompson, 1988; Venken et al., 1991). As potato seed-tubers age, the saturation of membrane lipids increases with a concomitant loss of membrane integrity (Knowles and Knowles, 1989). Since age-induced saturation of membrane lipids could affect fluidity (Pauls and Thompson, 1980, 1981), it is reasonable to expect that the activity of membrane-bound ATPases would be altered in potato.

In view of the decline in membrane integrity that occurs during aging of potato, the impact of seed-tuber age on membrane-bound ATPase activity was examined. Isolation and characterization of PMs, and comparative evaluation of kinetic parameters of PM ATPase from young and old seed-tubers (as reported in this study), represent the initial steps toward fulfilment of a long-term objective of identifying alternate 'sinks' for the apparent increase in energy metabolizing ability that occurs during sprouting of older seed-tubers. In spite of higher amounts of ATP generated via a combination of alt and cyt-pathways (Mikitzel and Knowles, 1990b), the

of ATP generated via a combination of alt and cyt-pathways (Mikitzel and Knowles, 1990b), the vigor of individual sprouts from aged tubers is less than that in sprouts from younger tubers; this suggests that in older seed-tubers ATP is diverted away from processes directly involved with sprout growth. A potential 'sink' for ATP could thus be ATPase in a PM which has become 'leaky' with advanced age. Indeed, the results show an age-induced increase in the V_{max} of the PM H^+-ATPase. The physiological implications of increased ATPase activity are discussed with reference to age-related differences in respiratory metabolism and sprout-vigor, and the probable mechanisms of activation of ATPase are considered in relation to changes in membrane architecture.

MATERIALS AND METHODS

Plant material

Certified potato *(Solanum tuberosum* L. cv. Russet Burbank) seed-tubers, obtained directly from a local grower at harvest, were stored for 7 to 19 months under conditions inhibitory to sprouting (4°C, 95% RH). For discussion purposes, 7-month-old tubers are referred to as physiologically 'young' and 19-month-old tubers as physiologically 'old' or 'aged'. Seed-tuber age was calculated from harvest. Characterization of the physiological status of the young and old tubers during sprouting was accomplished by determining respiration.

Whole-tuber respiration

Tuber respiration was followed over a 10 day sprouting interval in the dark at 23°C. Treatments consisted of two seed-tuber ages and three harvest dates (0, 5 and 10 days of sprouting), each replicated three times. Each replication was represented by three tubers. Uniform size (180-200 g) 7- and 19-month-old tubers were selected from a 4°C storage, rinsed, weighed and sealed in 1.5-L high-density polyethylene chambers equipped with inlet and outlet ports (3 tubers per chamber). Air-flow to each chamber was maintained at 125 mL min^{-1} via a flow-board manifold system (see Appendix I). The air was humidified through a diffusion stone submersed in distilled water prior to flowing into the manifold. Respiration rates were quantified by

calculating the difference in CO_2 concentration between inlet and outlet ports of each chamber at 2 to 4 h intervals over the first 48 h, and at 24 h intervals over the remaining sprouting period. At each sampling, 1 mL gas samples from each chamber were injected into a Hewlett-Packard 5890A gas chromatograph equipped with a thermal conductivity detector and a 2.4 m stainless steel column (3.2 mm o.d.) packed with HayeSep T (Hewlett-Packard). The carrier gas (He) flow rate was 30 mL min^{-1}, and the column temperature was isothermal at 100°C. Injection and detector port temperatures were 140°C. Tuber respiration rates were expressed as mL CO_2 kg^{-1} h^{-1}. At 5 and 10 days of sprouting, tubers were harvested from the chambers, PM vesicles were isolated for determination of H^+-ATPase activity. Similarly, H^+-ATPase activity in PMs isolated from the two tuber ages at zero-time (directly from 4°C storage) was compared.

Isolation of plasma membranes

Plasma membrane vesicles were isolated from potato tuber tissue by the methods of Iswari and Palta (1989) with minor modifications. After the appropriate sprouting interval, tubers were removed from the chambers, desprouted, and sprout number was recorded. The sprouts were dried at 80°C (72 h) for dry weight determination. The tubers were halved, and the outer cortical tissue from the apical halves was excised (extending from the periderm inward 1 cm) and grated in a food processor. The resulting tissue was collected in 50 mL of cold (4°C) grinding medium in the reservoir of the food processor. The grinding medium consisted of 75 mM Mes buffer (pH 7.8) containing 250 mM sucrose, 5 mM EGTA, 2 mM PMSF, 2 mM SHAM, 2.5 mM $Na_2S_2O_5$, 1.5% (w/v) PVP, 0.5% (w/v) protease free-BSA, 10 µg mL^{-1} BHT and 1 mM DTT. All chemicals were obtained from Sigma. The tissue was then ground with mortar and pestle in an additional 50 mL of grinding medium, filtered through two layers of mira-cloth, and the filtrate was centrifuged at 20,000g for 25 min. The resulting supernatant was centrifuged at 105,000g for 45 min to pellet the microsomal membranes. The microsomal pellet was suspended in 9 mL of 10 mM potassium-phosphate buffer (pH 7.8) containing 250 mM sucrose. Three milliliters of the microsomal suspension were washed in 20 mL of phase-dilution buffer (5 mM Mops, pH 7.8; containing 250 mM sucrose, 1 mM EGTA, 0.5 mM PMSF, 10 µg mL^{-1} BHT, 1 mM DTT and 10 mM KCl) and the

microsomes were pelleted at 105,000g for 45 min. The microsomal vesicles, suspended in 5 mL of phase dilution buffer, were stored at -80°C in 1.5 mL microfuge vials. Plasma membranes were isolated from the remaining 6 mL of the microsomal suspension by a two-step PEG-DEX (5.8% w/w) two-phase system containing 15 mM NaCl. The remaining microsomal suspension (6 mL) was added to 24 g of the two phase system at 4°C. The mixture was inverted 40 times and phase separation was facilitated by centrifugation (2,500 g, 20 min) in a swinging-bucket centrifuge (Himac centrifuge, Hitachi, Japan). The UP and LP were diluted 4 and 10 times (v/v), respectively, with phase dilution buffer and centrifuged at 105,000 g to pellet the membrane vesicles. All manipulations were carried out at 4°C. The final pellets from the UP and LP were suspended in 5 mL of phase dilution buffer, and stored at -80°C in 1.5 mL microfuge vials.

Characterization of plasma membranes

Membrane pellets from the microsomal, UP and LP fractions, along with tissue sections from tuber cortex, were fixed in 2.5% (v/v) glutaraldehyde in Millonig's (1961) phosphate buffer (pH 7.2, 200 mM) for 16 h, followed by 1% (v/v) OsO_4 for 2 h. The fixed membranes were then treated with 1% (w/v) tannic acid for 30 min prior to embedding in CY 212 Araldite. The embedded specimens were sectioned, stained with 4% (w/v) uranyl acetate and 0.2% (w/v) lead citrate, and viewed in a Hitachi H 7000 TEM.

The epoxy-embedded sections were also stained with PTAC, a specific morphological stain for the PM (Roland, 1972). The sections were transferred to nickel grids, destained with periodic acid (1%, v/v) for 30 min, and washed 3 times (10 min each) by successive transfers to drops of distilled water (Hall, 1988). The sections were then stained for 5 min in a solution of 1% (w/v) PTAC in 10% aqueous chromic acid, rinsed in distilled water and viewed by TEM as described above.

In addition to PTAC staining, differential sensitivity of the ATPase to azide, molybdate, nitrate, vanadate and EB was used to characterize further the UP membrane vesicles. Vanadate (Na_3VO_4) was prepared by the method of Surowy and Sussman (1986). Since ATPase activity in the tuber microsomal, UP and LP membrane fractions was relatively insensitive to VO_4 (maximum

27% inhibition), VO_4-sensitivity of microsomal membranes isolated from potato (cv. Norland) leaf tissue (50 g F.wt.) by the methods of Iswari and Palta (1989) was also tested to substantiate the effectiveness of this isolation procedure, and to confirm the validity of the relatively insensitive PM ATPase from tuber tissue. VO_4-sensitive ATPase activity was determined simultaneously in both leaf and tuber membranes (described under 'ATPase assays').

Since VO_4 and EB inhibition of ATPase activity in membrane fractions from potato tubers was not substantial, GS II activity was quantified as a marker for PMs. GS II activity was determined in microsomal as well as in UP and LP membrane fractions by the methods of Widell and Larsson (1990) with minor modifications. The reaction medium (100 µL) consisted of 330 mM sucrose, 0.8 mM spermine, 16 mM cellobiose (Serva, Heidelberg, Germany), 4 mM EGTA, 4 mM $CaCl_2$, 0.5 mM DTT, 0.015 % (w/v) digitonin, 0.5 mM UDPG and 0.476 nmol ^{14}C-UDPG (25 µCi/mL, 315 mCi/mmol; Amersham, Canada) in Hepes-KOH buffer (50 mM, pH 7.25), along with 25 µL of membrane suspension. All chemicals except ^{14}C-UDPG and cellobiose were obtained from Sigma. Following incubation at 25^{o}C for 30 min, the reaction was terminated by adding 1 mL of 70% (v/v) ethanol, 50 µL of 50 mM $MgCl_2$, and 150 µL of boiled crude microsomal preparation, and the mixture was allowed to stand overnight at 4^{o}C. This was followed by centrifugation at 1640 g for 15 min. The supernatant was discarded and the pellet was washed with 2.5 mL ethanol four times which effectively removed unreacted ^{14}C-UDPG. The pellet was transfered to a scintillation vial and counted in 10 mL of scintiVerse E with a Minaxiß Tri-Carb 4000 liquid scintillation counter. GS II activity was expressed as nmol glucan formed mg protein^{-1} h^{-1}.

The activity of latent IDPase (Golgi marker) was assayed by the methods of Green (1988). The reaction was carried out in a total volume of 1.0 mL containing 17 µg membrane protein, 3 mM IDP and 1 mM $MgCl_2$ in Tris buffer (50 mM, pH 7.5). The reaction medium was incubated at 25^{o}C for 60 min and the reaction was stopped by adding 400 µL of 24% (w/v) TCA. The Pi released from IDP hydrolysis was determined colorimetrically (described below). The IDPase activity was assayed both on the day of membrane isolation and after a 3 day incubation period at 4^{o}C, to account for enzyme latency.

Cytochrome c reductase (cyanide and antimycin A insensitive) activity (EF_i marker) was assayed by the methods of Briskin et al., (1987). The reaction medium (1.0 mL) consisted of 2.5 mM NaCN, 0.18 mM cyt c, 0.15 mM NADH (Sigma) and 2 µM antimycin (Sigma) in sodium phosphate buffer (50 mM, pH 7.5), along with 300 µL of membrane suspension. The change in absorbance due to reduction of cyt c was followed at 550 nm on a chart recorder. The reduction of cyt c was quantified using the extinction coefficient of 28 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as µmol cyt c reduced min^{-1} mg $protein^{-1}$.

ATPase assays

For the inhibitor studies, ATPase activity was determined by colorimetric measurement of Pi released from ATP hydrolysis (Serrano et al., 1976). The ATPase assay was carried out by the methods of Surowy and Sussman (1986). The reaction medium (0.5 mL) consisted of 5 mM each of ATP (vanadium-free, Sigma), PEP (to regenerate ATP), $MgCl_2$ and, where indicated, NaN_3 (inhibitor of mitochondrial ATPase), 100 mM KNO_3 (inhibitor of tonoplast ATPase), 1 mM $NaMoO_4$ (inhibitor of APases), 100 µM Na_3VO_4, 0.1 mg mL^{-1} LPC and 24 units of PK (Sigma) in 10 mM Pipes buffer (pH 6.5). The membrane suspension (10 µL microsomes or 100 µL UP) was added to the reaction medium and the mixture was incubated at 32°C. The reaction was terminated after 60 min by addition of 400 µL of 24% (w/v) TCA. One milliliter of ammonium molybdate (0.7% w/v in 0.72 N H_2SO_4) and 50 µL of 0.1% (w/v) ascorbic acid were then added, and the absorbance at 750 nm was determined after 10 min at room temperature. The Pi standards contained the ingredients as above (except ATP) and were incubated similarly to the ATPase assay mixture. Orthovanadate at 100 µM was included in the Pi-standards to account for VO_4 interference in absorption at 750 nm.

The pH optimum and kinetic parameters of H^+-ATPase activity were determined by coupling ATP hydrolysis to NADH oxidation via PK and LDH (Auffret and Hanke, 1981). The assay was performed at 23°C in a total volume of 1.0 mL. The reaction was initiated by the addition of NADH (125 µM final concentration) to a reaction medium containing 10 to 2000 µM ATP (vanadium-free, Sigma), 5 mM each of PEP, $MgCl_2$, NaN_3, 100 mM KNO_3, 1 mM Na_2MoO_4 in Pipes buffer (10

mM, pH 6.5), 30 units each of PK and LDH enzymes (Sigma), and 100 µL of membrane suspension. The spectrophotometer was zeroed on the reaction medium containing all ingredients except NADH. The oxidation of NADH was monitored at 340 nm on a recording spectrophotometer (Varian, Cary 219). The NADH oxidation was quantified by using the extinction coefficient of 6.22 μmol^{-1} cm^{-1}, and was expressed on a protein basis. The kinetic parameters were computed using the *Fig. P* program *(Biosoft* Corporation, New Jersey, USA).

The optimal reaction pH (6.0 to 6.5) was determined by profiling ATPase activity of microsomal and UP membrane fractions from pH 4.5 to 9.0 in the presence of inhibitors of tonoplast and mitochondrial ATPases and APase. The buffers (sodium acetate, Mes, Pipes and BTP, 10 mM each), ATP, PEP, $MgCl_2$, NaN_3, KNO_3 and Na_2MoO_4 (as above) were dissolved in distilled H_2O, distributed into 10 test-tubes, and the pH was set from 4.5 to 9.0. ATPase activity was quantified by following NADH oxidation as described above. Protein content of the membrane suspensions was determined by a modified Lowry procedure (Bensadoun and Weinstein, 1976), using BSA as standard.

Statistical procedures

Randomized complete block designs were used in the growth, inhibitor and kinetic studies reported herein. Treatments were factorially arranged. Sprout growth, respiration and ATPase activities were subjected to ANOVA, and sums of squares were partitioned into main effects and interactions, where appropriate. Marker enzyme analysis involved triplicate samples, and means with associated standard deviations are reported.

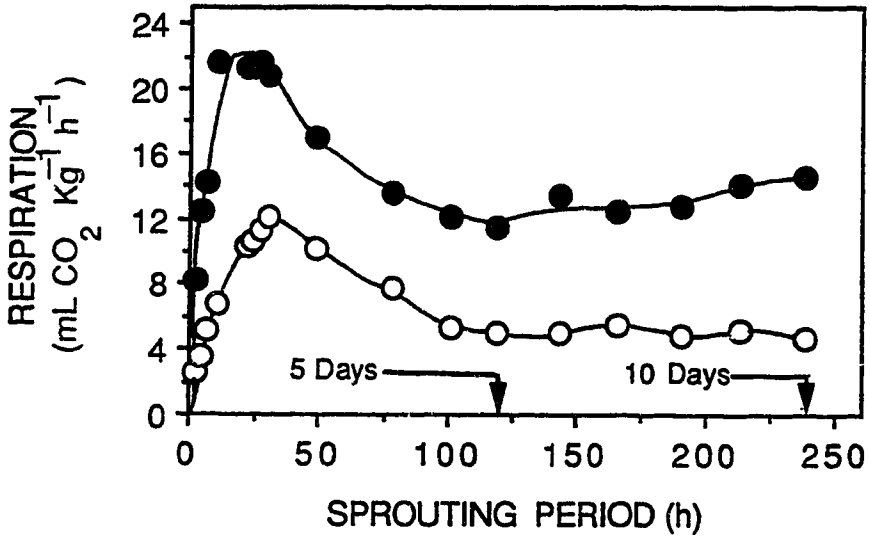
RESULTS

Whole-tuber respiration

To characterize more precisely the metabolic status of tubers prior to isolation of PM vesicles, respiration of 7- and 19-month-old tubers was determined over a 10-day sprouting interval. On average, 19-month-old tubers had a higher rate of respiration than 7-month-old

Fig. 1. Respiratory profiles of 7- and 19-month-old whole tubers in dark at 23^{o}C. Arrows indicate the time of harvest for isolation of plasma membranes for determination of H+-ATPase activity. Number of sprouts and their dry weight per tuber basis and per sprout basis at 10 days of sprouting, are presented in the box above. **F-values for the effects of seed-tuber age on the number of sprouts per tuber and the dry weight per sprout were significant at the 0.01 level.

Tuber age (months)	Sprouts (No./tuber)	Sprout dry weight (mg/tuber)	Sprout dry weight (mg/sprout)
○ 7	3.0	105.6	35.2
● 19	28.0**	137.6	4.9**



tubers throughout the sprouting period (Fig. 1). A transitory rise in respiration was evident during the initial 24 h of acclimation to room temperature. At 24 h, 19-month-old tubers had a 2-fold greater rate of respiration than 7-month-old tubers. Respiration rates gradually declined from the maximum, stabilizing after 100 h at 5.1 mL CO_2 kg^{-1} h^{-1} for 7-month-old tubers and 12.7 mL CO_2 kg^{-1} h^{-1} for 19-month-old tubers. Thus, during sprouting, the respiration rate of 19-month-old tubers was approximately 2.5-fold greater than that of 7-month-old tubers.

Although 19-month-old tubers produced over 9 times as many sprouts as 7-month-old tubers after sprouting for 10 days, dry weight of individual sprouts from younger tubers was 7 times greater than that of sprouts from older seed-tubers (Fig. 1). The total sprout dry weight/tuber, however, did not differ significantly between the two tuber ages. Tubers were harvested at 0 (directly from a 4°C storage), 5 and 10 days of sprouting (arrows in Fig. 1) to characterize the effects of seed-tuber age and sprouting period on PM H^+- ATPase activity.

Characterization of plasma membranes

Potato tuber microsomal membranes (isolated by differential centrifugation) and PMs (isolated by liquid-polymer two-phase system) were intact vesicles as evidenced by TEM studies (Fig. 2). Membrane vesicle sections were stained by PTAC (a PM morphological marker, Fig. 2DEF), indicating that all fractions contained PMs. Reliance on PTAC staining as a PM marker required that the specificity of the stain toward PMs be demonstrated on intact sections of tuber tissue. Cortical tissue sections from tubers were thus stained with uranyl acetate/lead citrate or PTAC and compared. Uranyl acetate/lead citrate stained the tonoplast, mitochondria, ER and PM, and was therefore nonspecific. When uranyl acetate/lead citrate stained sections were destained and then restained with PTAC, the PMs selectively stained relative to the other membrane-bound organelles (Fig. 3BCD). Hence, PTAC staining qualitatively indicated the presence of PM-derived vesicles in the UP fraction (Fig. 2 E). Moreover, PTAC stain appears to be a good morphological marker for PMs in sections from potato tuber tissue.

ATPase activity in microsomal and UP membrane vesicles was determined in the presence

Fig. 2. Transmission electron micrographs of microsomal (AD), UP (BE) and LP (CF) membrane fractions isolated from cortical tissue of potato tubers and stained with uranyl acetate-lead citrate (ABC) and and PM specific PTAC stain (DEF) (X 9000). The proportion of vesicles stained with PTAC did not differ between microsomes, UP or LP membrane fractions.

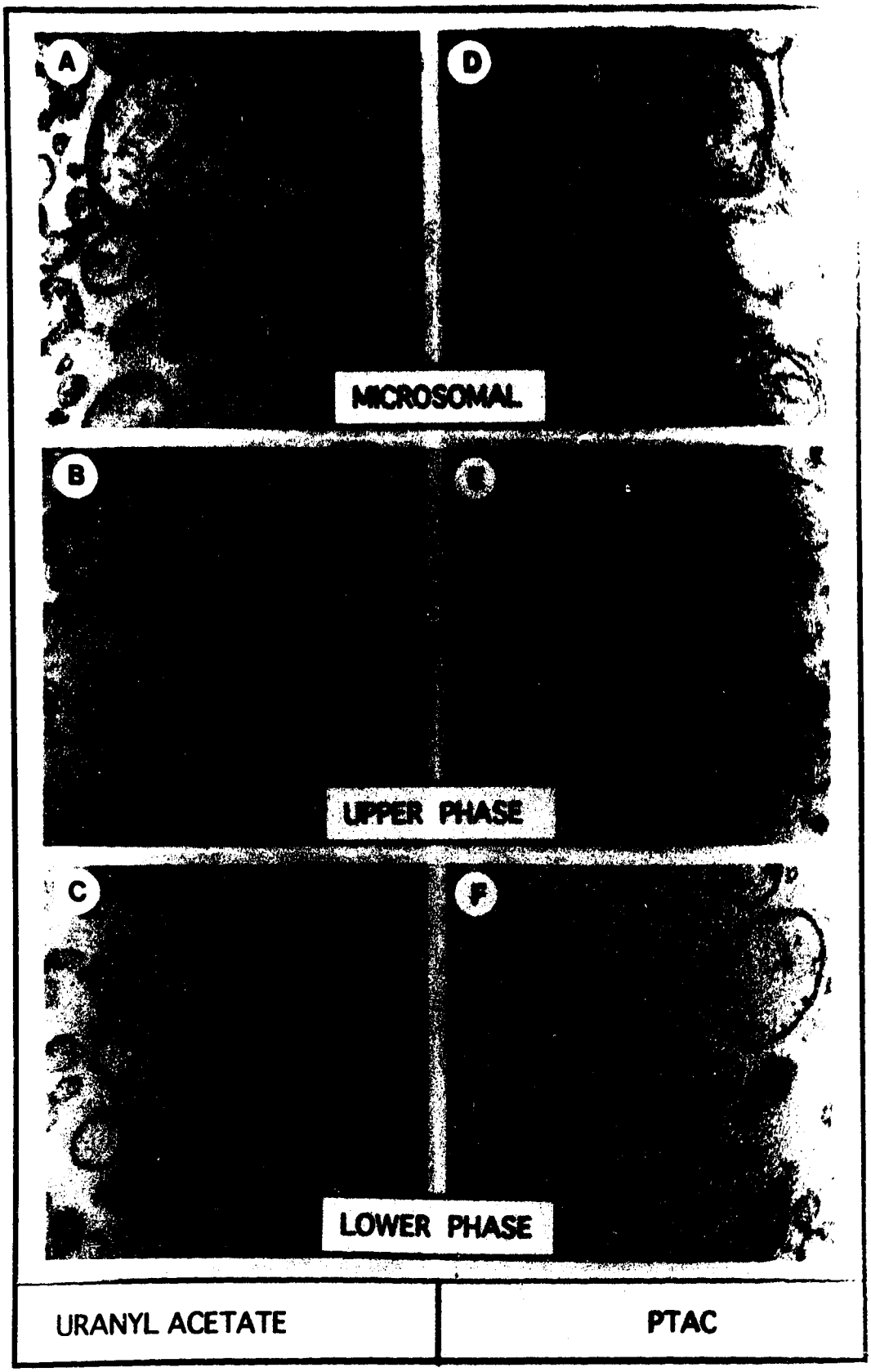
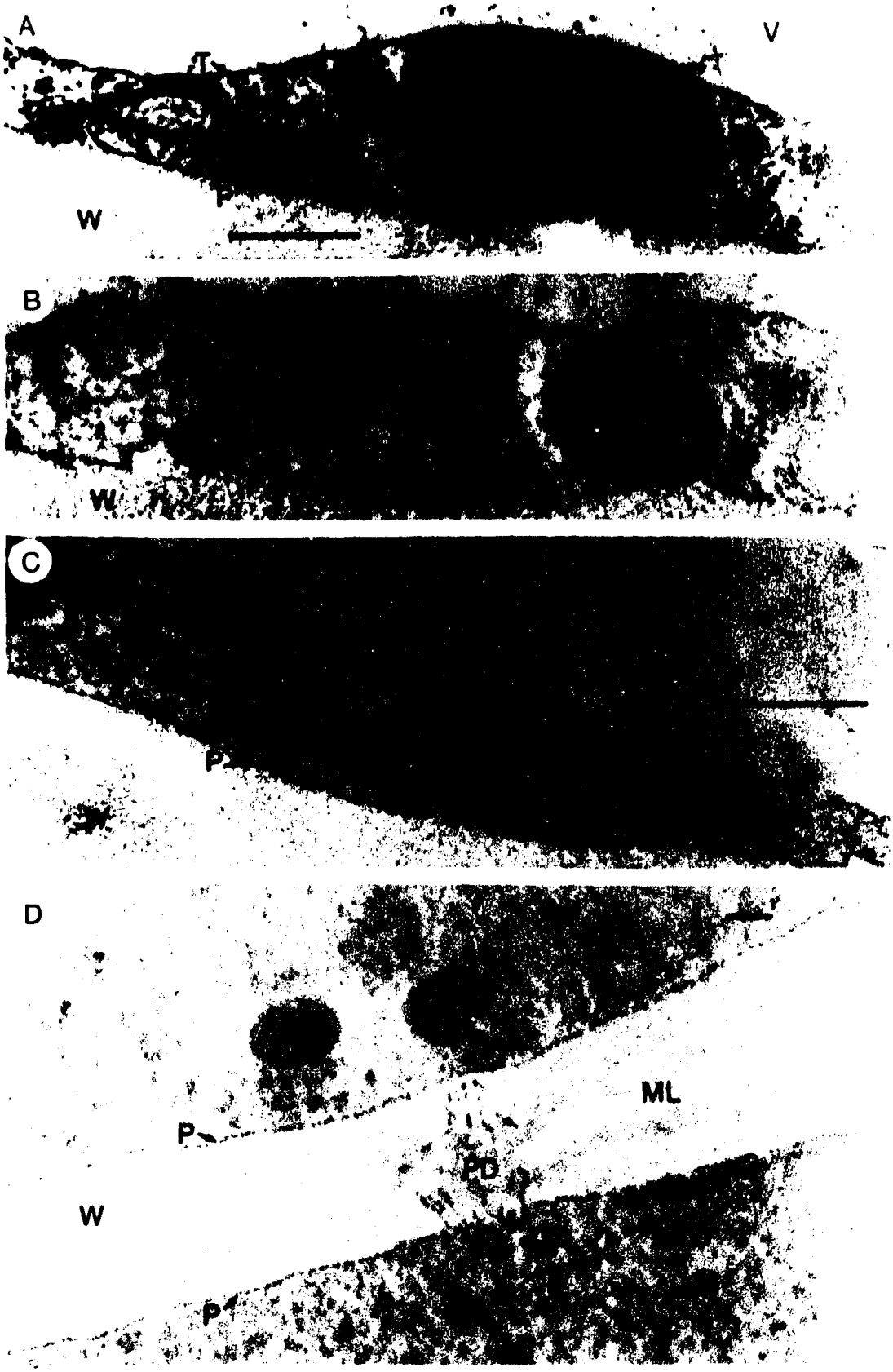

A
D
MICROSOMAL
B
UPPER PHASE
C
F
LOWER PHASE
URANYL ACETATE
PTAC

Fig. 3. Tranmission electron micrographs of cortical tissue stained with uranyl acetate-lead citrate (A), and PM specific PTAC stain (B, C and D). ER, endoplasmic reticulum; P, plasma membrane; T, tonoplast; W, cell wall; V, vacuole; M, mitochondria and PD, plasmodesmata. Scale bars equal 0.5 μm.


A
V
W
P
B
W
C
P
D
ML
P
PD
W
P

and absence of inhibitors of mitochondrial, tonoplast and PM ATPases, and APase. In the microsomal fraction, the contribution to total ATPase activity by mitochondrial and tonoplastic ATPases and APases accounted for 16, 28 and 8%, respectively (Table I). Inhibition of ATPase activity by vanadate in the microsomal fraction was 20%. Incubation of the membrane vesicles with all of the inhibitors resulted in 36% inhibition, relative to controls with no inhibitors. Fractionation of microsomal membranes into UP on a liquid-polymer two-phase system significantly reduced the levels of contamination by mitochondrial and tonoplastic ATPases (to 5 and 14% of control, respectively). Molybdate-sensitive APase activity in vesicles from the UP was 4.8 μmol P mg^{-1} h^{-1}, and was very close to the activity found in the microsomal fraction (4.7 μmol P mg^{-1} h^{-1}). Vanadate inhibition of ATPase activity in UP vesicles was significantly greater (7%) than that for microsomal vesicles. The stimulation of ATPase activity in UP vesicles by LPC was only 9%, indicating a possibility of 'inside out' vesicles in the UP.

In contrast to microsomes isolated from tuber tissue, VO_4 inhibited the ATPase activity in the microsomal membrane fraction from potato leaves by 69%. Thus, VO_4 was relatively less effective on PM ATPase of potato tubers. The inhibitory effect of EB, another inhibitor of PM ATPase activity (Cocucci, 1986; Cocucci and Marre, 1986; Poder and Penot, 1992), exceeded that of VO_4 only marginally (by 5%, at 100 μM). Furthermore, PM ATPase activity was not stimulated by KCl, which is consistent with the results of Demandre (1978) who failed to detect K-stimulated ATPase activity in PMs from potato tuber tissue. The pH optimum (6.5) of potato tuber PM found in this study (Fig. 4) is well within the range of pH values (6 to 7) reported for plant PM ATPases.

The specific activities of GS II in the microsomal, UP and LP membrane fractions from leaves and tubers are presented in Table. II. GS II activity in the tuber microsomes was comparable to the activity in the UP of leaf, suggesting that tuber microsomes are inherently enriched in PMs. GS II activity in the tuber UP was 2.5-fold greater than that of the microsomal fraction, indicating that the UP was more enriched in PM than the crude microsomal fraction. IDPase activity did not increase appreciably over the three day incubation period at 4°C, reflecting an absence of latency and thus

Table. I. Contribution of Mitochondrial, Tonoplastic and PM ATPases, and APase to Total ATPase Activity in Microsomal and UP Membrane Vesicle Fractions Isolated from Cortical Tissue from 7-Month-Old Potato Seed-Tubers.

ATPase activity was assayed by the colorimetric determination of Pi released from ATP hydrolysis. The reaction mixture (5 mM each of ATP, PEP, $MgCl_2$, 0.1 mg LPC mL^{-1} and 24 units of PK in 10 mM Pipes buffer, pH 6.5) along with membrane suspension was incubated at 32°C for 1h. NaN_3 (5mM), KNO_3 (100mM), Na_3VO_4 (0.1mM) and $NaMoO_4$ (1.0 mM) were included in the reaction medium to inhibit mitochondrial, tonoplast and PM ATPases, and APases, respectively.

Treatment	ATPase Activity			
	Microsomal		Upper Phase	
	SA	%[c]	SA	%
No Inhibitors (NI)	57.14	100	55.38	100
+NaN_3	47.81	84	52.46	95
+KNO_3	41.28	72	47.63	86
+$NaMoO_4$	52.43	92	50.60	92
+Na_3VO_4	45.59	80	40.61	73
+All inhibitors	36.61	64	35.83	65

Membrane Fraction (MF)[a]	n. s.[b]
Inhibitors	0.01
NaN_3 vs. NI X MF	0.01
KNO_3 vs. NI X MF	0.01
$NaMoO_4$ vs. NI X MF	n. s.
Na_3VO_4 vs. NI X MF	0.01
All Inhibitors vs. NI X MF	n. s.

SA, specific activity (µmol Pi mg^{-1} h^{-1}); [a]source of variation; [b]significance levels for indicated sources of variation (n. s, not significant); [c]percent reduction in ATPase activity over control (no inhibitors)

Fig. 4. Effect of pH on ATPase activity of microsomal (O) and plasma membranes (Δ) isolated from 7-month-old tubers at 0 day (directly from 4°C storage) in the presence of inhibitors of tonoplast (KNO_3) and mitochondrial ATPases (NaN_3) and inhibitor of acid phosphatase ($Na\ MoO_4$).

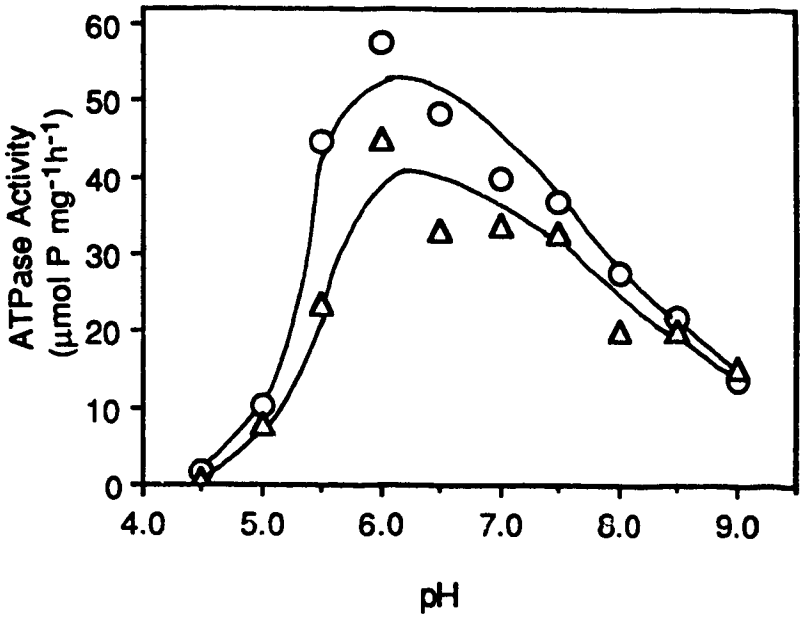

ATPase Activity
(µmol P mg-1h-1)
60
50
40
30
20
10
0
4.0
5.0
6.0
7.0
8.0
9.0
pH

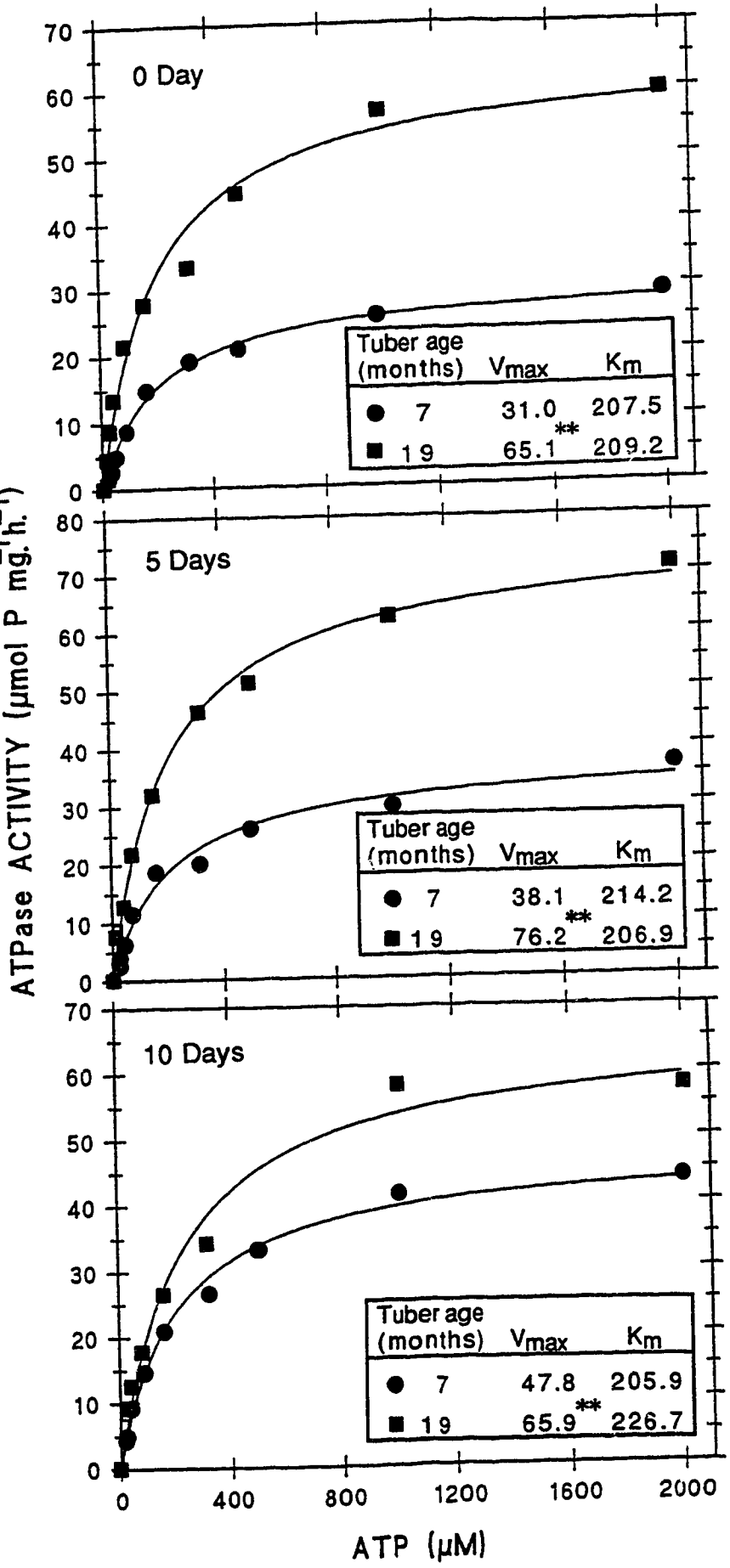
Table. II. Marker Enzyme Analysis of Membrane Vesicle Fractions Isolated From Tuber and Leaf Tissues of Potato.

GS II, CCR and IDPase activities were determined as markers for PM, ER and golgi, respectively. GS II activity was assayed by determining the synthesis of glucan from ^{14}C-UDPG. Latent IDPase was assayed by quantifying Pi released from IDP, and CCR activity was determined by following the reduction of cyt c at 550 nm on a recording spectrophotometer. CCR activity could not be detected in the UP membrane fractions. Enzyme activities are a mean of three determinations ± SD.

Tissue	Membrane Fraction	GS II Activity[a]	CCR Activity[b]	IDPase Activity[c] 0 day	IDPase Activity[c] 3 days
Tuber	Microsomal	264.2 ±29.7	16.75 ±4.38	14.73 ±0.89	15.44 ±0.47
	Upper Phase	655.1 ±61.0	n. d.	3.59 ±0.14	4.75 ±0.18
	Lower Phase	352.4 ±10.1	11.95 ±0.18	20.45 ±0.80	21.26 ±0.38
Leaf	Microsomal	82.5 ± 6.8	14.23 ±3.97	12.70 ±1.31	13.22 ±0.04
	Upper Phase	260.1 ±16.5	n. d.	0.30 ±0.37	0.54 ±0.09
	Lower Phase	104.7 ± 8.9	13.26 ±0.25	15.15 ±0.64	14.74 ±0.32

[a]nmol glucan mg protein^{-1}h^{-1}; [b]µmol cyt c reduced mg protein^{-1} min^{-1}; [c]µmol P mg protein^{-1} h^{-1}; n. d., not detected.

Fig. 5. ATPase activity in PMs isolated from 7- and 19-month-old potato seed-tubers at 0 (directly from $4^{o}C$ storage), 5 and 10 days during sprouting. ATPase activity was determined by coupling ATP hydrolysis to NADH oxidation via PK and LDH enzymes. The assay was carried out in presence of 5 mM NaN_3, 100 mM KNO_3, 1 mM Na MoO_4 and 1 mg mL^{-1}LPC. The reaction was carried out at $23^{o}C$ and NADH oxidation was monitored at 340 nm on a recording spectrophotometer. V_{max} (µmol P mg $protein^{-1}$ h^{-1}) and K_m (µM ATP) of PM ATPase at 0, 5 and 10 days were computed using *Fig. P* program (*BIOSOFT* corporation, New Jersey, USA) and are presented in the insets. **F-value for the effect of seed-tuber age on V_{max} was significant at 0.01 level. Seed-tuber age, sprouting period and their interaction did not affect the K_m of the ATPase.


0 Day
Tuber age (months) Vmax Km
7 31.0 207.5
19 65.1** 209.2
5 Days
Tuber age (months) Vmax Km
7 38.1 214.2
19 76.2** 206.9
10 Days
Tuber age (months) Vmax Km
7 47.8 205.9
19 65.9** 226.7
ATPase ACTIVITY (µmol P mg-1h-1)
ATP (µM)
0
10
20
30
40
50
60
70
80
400
800
1200
1600
2000

minimal contamination by Golgi. Antimycin-A-resistant CCR activity (ER marker) was undetectable in the UP of both leaf and tubers. However, the specific activities of CCR in microsomal and LP fractions from both leaves and tubers was substantial. Thus, the two phase system employed for partitioning PM from potato tubers into UP was effective in reducing ER and Golgi contamination.

Kinetics of plasma membrane ATPase

ATPase activities in PMs isolated from 7- and 19-month-old seed-tubers at 0 (directly from a 4^0C storage), 5 and 10 days of sprouting are presented in Fig. 5, and kinetic parameters (V_{max} and K_m) at the respective stages of sprouting are presented in the insets. The younger tubers had an average (over stage of sprouting) V_{max} of 39 µmol Pi mg^{-1} h^{-1}, while the older tubers had an average V_{max} of 69 µmol Pi mg^{-1} h^{-1} (a 77% increase with age). Stage of sprouting, and the interaction of seed-tuber age with stage of sprouting, did not effect the V_{max} or K_m. Moreover, the K_m averaged 212 µmol ATP and was not altered by tuber age.

DISCUSSION

As in previous studies (Knowles, 1983; Mikitzel and Kr[illegible]s, 1990b), seed-tubers displayed a transitory rise in respiration during the first 24 h of acclimation to room temperature (Fig. 1); this is attributed to a combination of the direct effects of increased temperature on respiration and temperature-dependent starch-to-sugar conversions acting as a 'sink' for ATP within the tubers (Isherwood, 1976). The magnitude of the respiratory increase depended upon seed-tuber age. At the maximum, respiration of 19-month-old tubers was substantially greater than that of 7-month-old tubers. Age-induced respiratory differences during acclimation, though not always as distinct as in this study, are most likely related to differences in initial substrate levels. Senescent sweetening, which arises from a gradual disintegration of amyloplast membranes during long-term storage (Sowokinos et al., 1987), results in extremely high levels of soluble carbohydrates (esp. reducing sugars) in older tubers (Isherwood, 1976; Kumar and Knowles, 1993b). The concentration of reducing sugars in single-eye seedcores from 19-month-old seed-tubers (taken directly from a 4^0C storage) was 2-fold greater than that from 7-month-old

seedcores (Mikitzel and Knowles, 1989). Moreover, reducing-sugar concentration increased 2.3-fold faster in seedcores from 19- versus 7-month-old seed-tubers during sprouting, despite significantly lower sprout dry weight (vigor) from the older seedcores (Mikitzel and Knowles, 1989). Positive relationships between respiration rate and glucose/sucrose content have been established for potato (Solomos and Laties, 1975). Hence, the high rates of respiration observed in older tubers during acclimation may be effected by increased availability of substrate for respiration.

During sprouting, respiration stabilized at a higher rate in 19-month-old tubers. Mikitzel and Knowles (1990b), however, did not find an increase in respiration rate of older whole tubers during sprouting. The discrepancy in results between the two studies is probably related to the number of tubers utilized in the respiration chambers. Whole-tuber respiration data presented in the latter study was an average for 9 tubers, while in this study each chamber had only 3 tubers. Variation in developmental state among individual tubers could have masked differences in whole-tuber respiration between ages in the previous study. This is supported by the fact that, when respiration was measured in tissue taken from randomly selected 7- and 19-month-old tubers at various intervals during sprouting, older tubers consistently showed a 30 to 50% higher respiration than younger tubers (Mik... .nd Knowles, 1990b). Moreover, six additional replicated experiments using only a few tubers per respiration chamber consistently showed higher respiratory rates from the sprouting older tubers (data not shown).

A constant level of respiration is maintained during the early stages of sprout development from potato seed-tubers. In fact, respiratory rates do not respond to sprouting until sprout fresh weight reaches at least 1% of tuber fresh weight (Burton et al., 1955). At 10 days, sprout fresh weight of 19-month-old tubers accounted for only 0.37% of tuber weight. Though sprouts of 7-month-old tubers accounted for a relatively higher percentage (0.49%) of tuber weight, the rate of respiration of 7-month-old tubers remained lower than that of 19-month-old sprouting tubers. It is therefore unlikely that age-induced differences in respiratory rates during the early stages of sprout development are directly attributable to differences in the metabolic activities of sprouts.

The greater respiration rate of sprouting older tubers has been attributed to an increase in alternate respiration, as the rate of cyt-mediated respiration is not affected by seed-tuber age (Mikitzel and Knowles, 1990b). Although alt-respiration is only one-third as efficient as *cyt*-mediated respiration (Laties, 1982), older tubers generate more ATP during sprouting through a combination of the two respiratory pathways (Mikitzel and Knowles, 1990b). Evidently, the greater ATP production of older tubers is not fueling processes which directly contribute to sprout growth, as evidenced by the age-reduced sprout vigor. The increased energy metabolizing ability of sprouting older tubers may be a response to compensate for the loss of membrane integrity during tuber aging (Knowles and Knowles, 1989). The 'leaky' membranes of older tubers may comprise a greater 'sink' for ATP than the relatively intact membranes of younger tubers. Since H^+-ATPases consume 25 to 50% of total cellular ATP content (Felle, 1982), and these enzymes have a central role in maintaining electrochemical gradient across the membrane and ionic balance for the cell, differences in PM ATPase activity may partly account for age-induced differences in respiratory metabolism and ATP generation during sprouting. As an initial step in testing this hypothesis, PMs were isolated from young and old seed-tubers during sprouting for characterization of H^+-ATPase activity.

Membrane vesicles, isolated from potato tuber cortex tissue, were characterized as PMs by PTAC staining, GS II activity and pH optimum of the H^+-ATPase. According to Widell and Larsson (1990), PTAC staining is a highly reliable method for characterizing PMs, and they suggest that this technique be used to assess the purity of PM in an isolated membrane fraction. However, Hall (1988) maintains that reliance on PTAC staining as a PM marker should be attempted only after substantiating the specificity of this stain on intact tissue sections. Accordingly, cortical tissue sections from tubers were stained with uranyl acetate/lead citrate and PTAC and compared. Uranyl acetate/lead citrate stained a number of cellular organelles including PMs (Fig. 3A), while PTAC selectively stained PM (Fig. 3BCD). Vesicles from the microsomal, UP and LP membrane fractions were well stained with PTAC, and there was no apparant difference in the effectiveness

of the stain among these three fractions (Fig. 2 DEF). This suggests that vesicles in all fractions were derived from PM.

In general, the optimum pH for PM ATPase from plant tissues ranges between 6.0 and 7.0 (Sze, 1985; Palmgren and Sommarin, 1989), while that of potato tuber PM ATPase falls between 6.0 and 6.75 (Jolliot et al., 1976; Bagdasaryan et al., 1983). A pH of 6.5 was optimal for ATPase from microsomal and UP membrane fractions in this study, suggesting minimal contamination by vesicles derived from mitochondria or tonoplast and/or effective inhibition of mitochondrial and tonoplast ATPases and APases by NaN_3, KNO_3 and $NaMoO_4$, respectively.

The specific activity of GS II (PM marker; Ray, 1979) in the UP membrane fraction was 2.5-fold greater than that of microsomal membranes (Table II) suggesting a 2.5-fold enrichment in PMs. Surprisingly, the LP also showed an enrichment in GS II activity which is consistant with the results of Kjellbom and Larsson (1984). Though a complete elimination of mitochondrial and tonoplast ATPase activities could not be achieved, their contamination was reduced substantially by phase partitioning. Moreover, residual activities of mitochondrial and tonoplast ATPases and APases in UP vesicles were effectively suppressed by routine incorporation of suitable inhibitors in the assay medium. The two known inhibitors of PM ATPase, VO_4 (Sze, 1985) and EB (Cocucci, 1986; Cocucci and Marre, 1986; Poder and Penot, 1992), were only effective in inhibiting activity of potato tuber PM ATPase to a maximum of 27% in this study. It may be possible to obtain greater sensitivity to these inhibitors by incorporating more than two phase partitioning steps in isolation procedure. However, in microsomal vesicles isolated from potato roots, VO_4 inhibition of ATPase activity did not exceed 24% (McArthur and Knowles, 1993). Preliminary data showed that LPC, KCL, or both did not improve VO_4-sensitivity of potato tuber microsomes (data not shown). It is important to note that VO_4-sensitive ATPase activity is also present on other membranes (Berczi et al., 1989), and EB inhibits both PM and tonoplast ATPases (Cocucci, 1986). Therefore, VO_4- and EB-sensitive ATPase activity as PM markers can be misleading. In potato-tuber disks, EB (100 µM) caused only a 9% inhibition of PM ATPase activity as measured by Pi uptake (Poder and Penot, 1992). In this study, the inhibitory effect of

EB on microsomal or PM ATPase did not exceed that caused by VO_4. Contaminants such as Golgi and ER (in UP) were minimal, as evidenced by IDPase and CCR assays (Table II). Phosphotungstic acid-chromic acid staining, glucan synthase II activity, pH optimum of 6.5, and appreciably lower contamination from endoplasmic reticulum and Golgi membranes (as indicated by lower cytochrome c reductase and latent inosine diphosphatase activities) suggest that the UP fraction was composed of PMs. However, a relatively low sensitivity to vanadate inhibition indicated that the ATPase in vesicles from the UP may not be entirely of PM origin.

PM ATPase is an important regulatory enzyme in cellular events such as adaptation to cold stress and developmental changes (Sussman and Surowy, 1987). In this study, aging caused an increase in the V_{max} of the ATPase, though K_m remained unaffected. Fernandes et al., (1988) compared Ca^{+2} Mg^{+2}-ATPase activities in young and old human erythrocytes and found similar results. Since ATPase is a membrane-bound enzyme, its activity can be potentially modified by membrane architecture (Cooke and Burden, 1990). Changes in bilayer organization can effect configurational changes in membrane-bound enzymes, which may then alter kinetic properties (Carruthers and Melchior, 1986). ATPase activity is regulated by variation in lipid polar head group, fatty acid chain length (Sinsnsky et al., 1979; Kasamo and Yamanishi, 1991) and degree of unsaturation (Palmgren et al., 1988; Palmgren and Sommarin, 1989). Kasamo (1982, 1990) also suggested direct interactions between active sites of the enzyme and the hydrophobic environment of the PM (especially the fatty acid acyl chains of the phospholipid) in regulating ATPase activity. The age-induced increase in PM H^+-ATPase activity may be influenced, in part by changes in membrane architecture favoring gel phase. Though phase transition per se has not been established for aging potato seed-tubers, a reduction in double-bond-index of phospholipids signifying increased saturation is well estabilshed (Knowles and Knowles, 1989).

The mechanism of activation and the physiological significance of higher-activity ATPase in aging tubers remains speculative. A higher ATPase activity in aged seed-tubers could partly be related to the breakdown of electrochemical gradient as a consequence of reduced membrane

integrity. Indeed, ionophore-mediated breakdown of electrochemical gradient across membranes activates ion pumping and ATP hydrolysis (Sze, 1985).

The age-induced increase in respiration and ATP production (Mikitzel and Knowles, 1990b), concomitant with reduced sprout vigor, with a loss of membrane integrity, and increased PM ATPase activity, suggest that 'leaky' membranes may be a greater 'sink' for metabolic energy in older tubers during sprouting. The increased oxidative phosphorylation during sprouting of older tubers (Mikitzel and Knowles, 1990b) may be in response to the higher ATP utilization by PM ATPase. Characterizing the effects of seed-tuber age and sprouting on energy charge, phosphate potential, PM ATPase concentration, and degree of coupling of ATP hydrolysis to proton translocation by the ATPase will clarify the physiological significance of the higher PM ATPase activity of older potato seed-tubers.

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Chapter VI

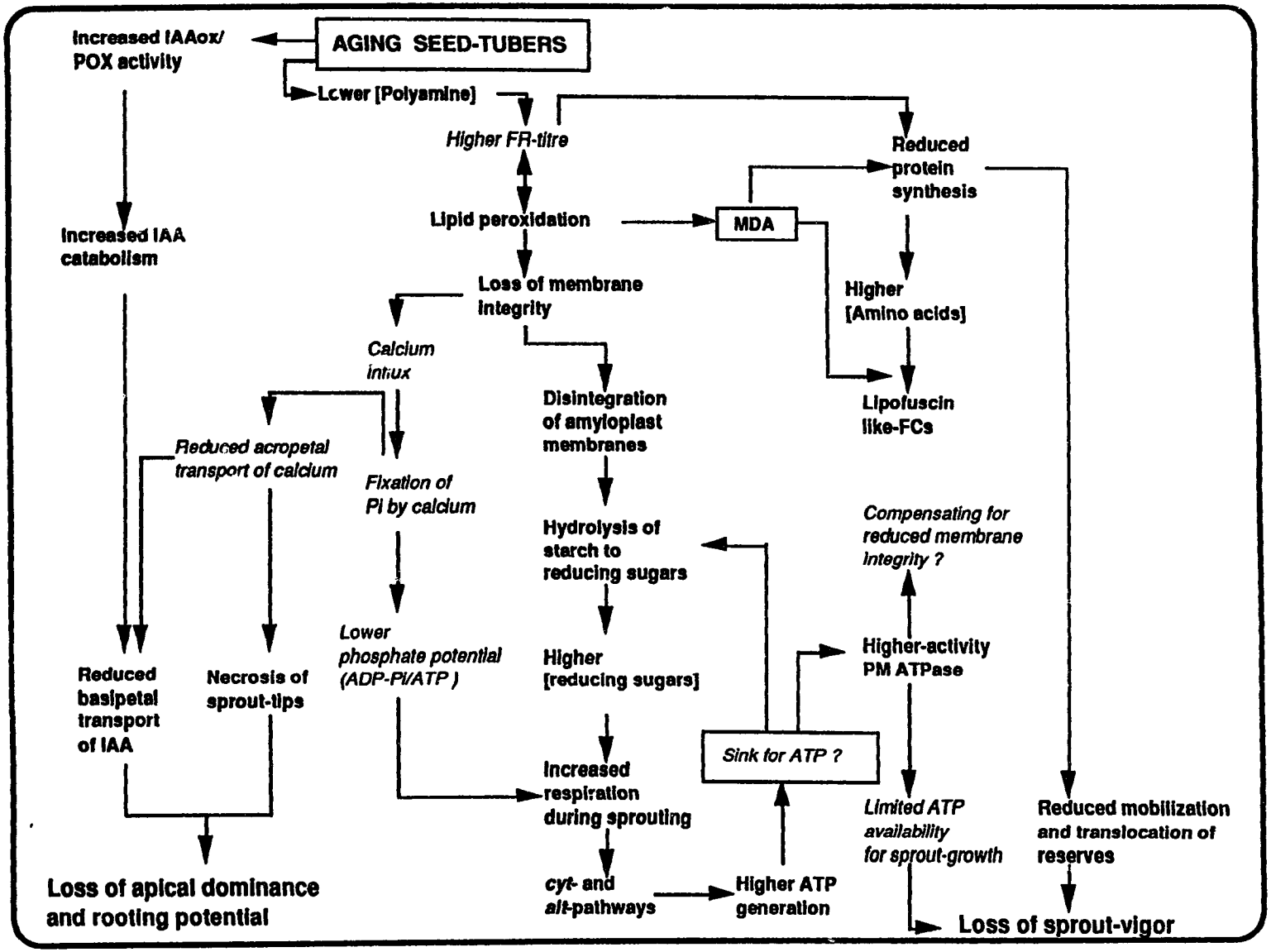
SUMMARY AND CONCLUSIONS

The studies reported herein focused on identifying the physiological processes that are effected during aging of potato seed-tubers. Age-induced factors that were characterized as being associated with, and possibly contributing to, loss of apical dominance and root- and shoot-growth potential included: reduced basipetal transport of auxin in developing sprouts, coupled with increased potential for auxin catabolism; lipid-peroxidation-mediated deterioration of cellular membranes; reduced protein-synthesizing ability of seed-tubers during sprouting; and a potential increase in ATP consumption by PM-bound ATPase during sprouting (Fig. 1). The latter effect may be a response to compensate for an age-induced loss of membrane integrity, and thus may divert metabolic energy away from processes that directly contribute to sprout-vigor.

The probable involvement of auxin in age-related growth characteristics was supported by partial amelioration of age-linked effects with NAA (Knowles *et al.*, 1985; Mikitzel and Knowles 1990a). In the present study, NAA partly restored apical dominance and stimulated plant growth (on a per-stem basis) in seedcores from older tubers. When applied to younger seedcores, NAA inhibited overall plant growth. On the other hand, attempts to restore apical dominance and sprout vigor in older seedcores with IAA failed, while IAA remained effective at inhibiting growth from younger seedcores. This suggested that aging may have affected the ability of tissues to catabolize this natural auxin. IAAox and POX are involved in the regulation of endogenous auxin levels (Chaturvedi and Laloraya 1983; Moore 1989; Beffa *et al.*, 1990), and an increase in their activities during aging of other plant tissues has been reported (Galston and Dalberg 1954; Kerstetter and Keit 1966). Indeed, sprout and tuber tissues from older tubers contained substantially higher specific activities of IAAox/POX, and this may explain the lack of response of older seed-tubers to IAA treatment. On the other hand, a synthetic auxin such as NAA was very effective at altering growth from older seed-tubers, as it is not a substrate for the auxin-catabolyzing enzymes (Gianfangna 1987; Goldschmidt *et al.*, 1967).

In addition to increased potential for auxin catabolism, sprouts developing from older seed-

Fig. 1. Schematic presentation of the physiological processes associated with aging of potato seed-tubers. Bold text indicate processes for which experimental evidence is available. Italicized text indicates factors that could be associated with aging of seed-tubers, and thus represent areas for future work.


AGING SEED-TUBERS
Increased IAAox/ POX activity
Lower [Polyamine]
Higher FR-titre
Reduced protein synthesis
Increased IAA catabolism
Lipid peroxidation
MDA
Loss of membrane integrity
Higher [Amino acids]
Calcium influx
Disintegration of amyloplast membranes
Lipofuscin like-FCs
Reduced acropetal transport of calcium
Fixation of Pi by calcium
Hydrolysis of starch to reducing sugars
Compensating for reduced membrane integrity ?
Lower phosphate potential (ADP-Pi/ATP)
Higher [reducing sugars]
Higher-activity PM ATPase
Reduced basipetal transport of IAA
Necrosis of sprout-tips
Sink for ATP ?
Increased respiration during sprouting
Limited ATP availability for sprout-growth
Reduced mobilization and translocation of reserves
cyt- and alt-pathways
Higher ATP generation
Loss of apical dominance and rooting potential
Loss of sprout-vigor

tubers had a reduced ability to translocate auxin basipetally. Similar effects of age on auxin translocation have been reported for other plant species (Smith and Jacobs 1969; Veen 1969; Veen and Jacobs 1969; Werblin and Jacobs 1967; Davenport *et al.*, 1980; Suttle 1991). Although the mechanism by which plants lose the ability to translocate auxin with age is not understood, loss in ability to translocate calcium acropetally may be linked to loss in the ability for basipetal auxin translocation (De Guzman and Dela Fuente 1984; Dela Fuente 1984). In this regard, apices of sprouts developing from older tubers often become necrotic from calcium deficiency (Dyson and Digby 1975ab), which suggests that age may also affect the ability for acropetal translocation of calcium. It would be interesting to determine the extent to which age-reduced basipetal transport of IAA is related to reduced acropetal transport of calcium in potato. Thus, a loss in the ability to translocate auxin, coupled with a greater potential for auxin catabolism may contribute to the loss of apical dominance and root-growth potential in aging potato seed-tubers.

As seed-tuber age advances, membrane integrity declines, resulting in a measurable increase in leakage of electrolytes (Knowles and Knowles 1989). Moreover, the age-induced increase in membrane permeability is coincident with an increase in saturation of membrane lipids. My results suggest that FR-mediated lipid peroxidation is the mechanism by which membranes deteriorate during aging of seed-tubers. The products of lipid peroxidation (MDA, ethane and lipofuscin-like FCs) increased with advancing seed-tuber age. It is possible that the age-induced increase in membrane permeability results in an elevated calcium concentration in the cytosol, and increased phospholipid catabolism via calcium-calmodulin-activated phospholipases (Leshem, 1987). In addition, increased cytosolic free-calcium has the potential to fix inorganic phosphate (Farber, 1981; Blaustein, 1985). Thus the basipetal transport of IAA may also be adversely affected by the loss of calcium to calcium phosphate as shown in Fig. 1. Indeed, older tubers appear to be less efficient at translocating calcium and phosphorus from the seed-tuber to the developing sprouts (Kumar and Knowles, unpublished).

Activities of phospholipase and LOX were lower in older tubers compared with younger tubers, while the activities of FR-scavenging enzymes (SOD, POX and CAT) were higher in older

tubers. It is possible that the FR-scavenging enzymes were induced by higher FR-titre of older tubers. Increased phospholipase and LOX activity is not necessarily a prerequisite to greater membrane damage. FRs are self-propagating, and can damage the membrane lipid bilayer directly (Fobel *et al.*, 1987). In addition, age-induced changes in acyl-chain molecular species can render phospholipids more susceptible to enzymatic catabolism (Brown *et al.*, 1987, 1991), and this would not be reflected as higher phospholipase activity in assays employing one molecular species of PL as substrate. SOD is inducible by such factors as stress, pathogenesis and ethylene (Bowler *et al.*, 1989), and tuber ethylene content increases with aging (Mikitzel and Knowles, 1989). Since a product of the increased SOD activity is the cytotoxic peroxide, activities of POX and CAT would increase to catabolize this highly reactive compound (Finazzi-Agaro *et al.*, 1986; Scott *et al.*, 1987; Spychalla and Desborough 1990). Mikitzel and Knowles (1989) also found a lower polyamine titre in older seed-tubers during plant establishment. Since one role for polyamines is to scavenge FRs (Drolet *et al.*, 1986), an age-induced deficiency in polyamine titre of seed-tubers may increase FR-mediated lipid peroxidation and thus membrane deterioration.

Loss of protein-synthesizing ability of older seed-tubers during sprouting was also characterized, and this response is thus consistent with that reported for many senescing plant tissues (Dhindsa *et al.*, 1981; Miller and Huffaker 1985; Lalonde and Dhindsa,1990). The age-induced loss of protein-synthesizing ability may be related to the accumulation of lipid peroxidation products (FRs and MDA) in tuber tissues, which are potential inhibitors of protein synthesis (Osborne 1980; Dhindsa 1982). Thus, increased activities of FR-scavenging enzymes, accumulation of lipid peroxidation products, and a deficiency in protein synthesis during sprouting, collectively suggest the involvement of FR-driven lipid peroxidation as a mechanism for the loss of membrane integrity during aging of seed-tubers.

Although aged seed-tubers respire at a higher rate (Kumar and Knowles, 1993) and generate higher amounts of ATP via a combination of alt- and cyt-mediated respiration (Mikitzel and Knowles 1990b) during sprouting, a large portion of this metabolic energy apparently supports processes which do not directly benefit sprout growth. Among other factors, higher rate of respiration in older

seed-tubers may be related to the influx of calcium into cytoplasm. Free cytosolic calcium would fix the inorganic phosphate (Farber, 1981; Blaustein, 1985) affecting ADP-Pi/ATP ratio which is a powerful regulator of glycolysis via phosphofructokinase activity (Krebs, 1978). In view of declining membrane integrity, higher potential for ATP generation and reduced sprout vigor, Mikitzel and Knowles (1990b) suggested maintenance of membrane integrity as a possible metabolic sink for ATP generated in older seed-tubers. As an initial step in this direction, PM H^+-ATPase activity of young and old seed-tubers during sprouting was determined. PMs were isolated on a liquid-polymer two-phase system and the presence of PMs in the different fractions was confirmed by PTAC staining, pH optimum and GS II activity. It was interesting to note that the specific activity of PM H^+-ATPase was higher in older tubers, though seed-tuber age and sprouting did not affect K_m. Similar effects of age on $Ca^{+2}Mg^{+2}$-ATPase were reported by Fernandes *et al.*, (1988) in aging human erythrocytes. Elucidation of the physiological significance and the *modus operandi* of activation of proton pump in older seed-tubers requires further work. The probable exploratory areas would include the effect of altered membrane architecture (phospholipid molecular make-up) on ATPase activity, quantification of ATPase molecules in the PM and the possibility of activation of ATPase by the intermediates of the signal transduction pathway (not shown in Fig. 1). It is likely that aging as a stress-signal is processed via signal transduction pathway culminating in enhancement of ATPase activity by phosphotidylinositols (intermediates of the signal transduction pathway). Activation of ATPase by phosphotidylinositols is well supported in literature (Memon *et al.*, 1989; Memon and Boss, 1990; Chen and Boss,1991; Patton and Lester, 1992). The physiological significance of higher-activity ATPase is not clearly established in the present study. However, the task of maintaining degenerating membranes in older seed-tubers might require a greater ATP input, and an increase in oxidative phosphorylation may be to serve the needs of higher-activity ATPase. To clarify these possibilities, it is necessary to measure ATP concentration, the energy charge of adenine nucleotides and to determine coupling of ATP hydrolysis to proton translocation.

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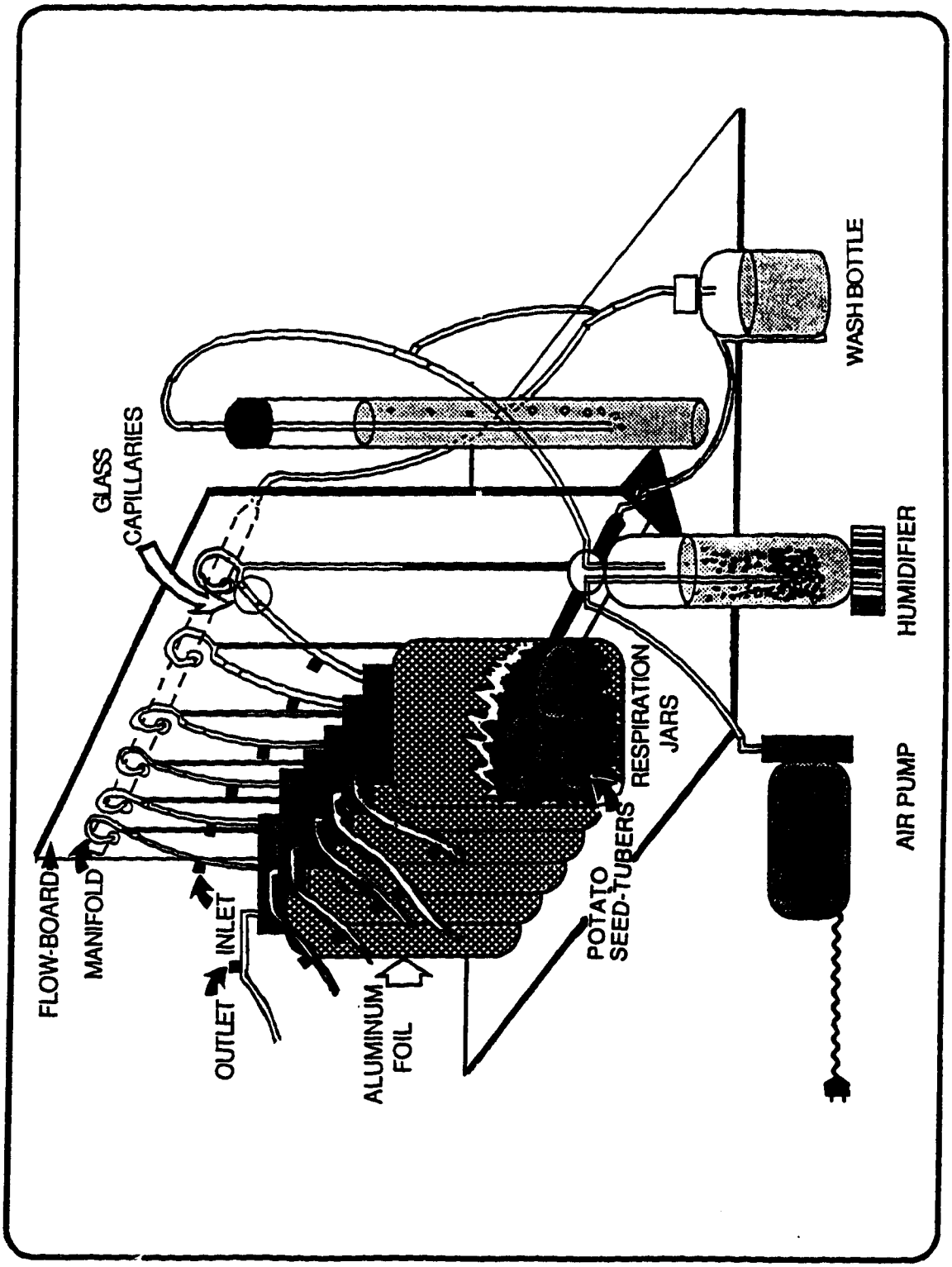
Appendix I. Flow-through system used for monitoring respiration of whole tubers during sprouting.


FLOW-BOARD
MANIFOLD
OUTLET
INLET
ALUMINUM FOIL
GLASS CAPILLARIES
POTATO SEED-TUBERS
RESPIRATION JARS
AIR PUMP
HUMIDIFIER
WASH BOTTLE