Plant Physiol. (1982) 69, 1339–1343
0032-0889/82/69/1339/05/$00.50/0

# Some Physiological Changes Occurring during the Senescence of Auxin-Deprived Pear Cells in Culture[1]

Received for publication October 9, 1981 and in revised form December 9, 1981

CLAUDINE BALAGUÉ, ALAIN LATCHÉ, JEAN FALLOT, AND JEAN-CLAUDE PECH[2]
*Ecole Nationale Superieure Agronomique de Toulouse, 145, Avenue de Muret 31076 Toulouse Cédex, France*

## ABSTRACT

**Part of the changes in the hormonal balance involved in plant senescence is due to an auxin limitation. Some of its physiological consequences are studied using pear (*Pyrus communis* L.) cells cultured in a continuously renewed medium in which 2,4-dichlorophenoxyacetic acid (2,4-D) was absent. In these conditions, an assessment was made of the absence of nutrient deficiency.**

**In the period preceding cell death, the rate of respiration and ethylene production remain low, and no major changes were observed in the total protein and RNA content of the cells. Beginning around day 9, an important efflux of three amino acids (serine, threonine, and aspartic acid) occurs among which serine represents more than 52%. However, exogenous serine supplied to the medium fails to show any senescence promoting effect. At the same time, leucine uptake and incorporation sharply and simultaneously increased. The presence of 2,4-D inhibits both these phenomena and prevents cell death. It is proposed that auxin deprivation is responsible for unmasking a program of synthesis of new proteins involved in cell death.**

It is now accepted that regulation of senescence in plants is associated with changes in the balance of senescence promoting hormones, ethylene and ABA, and senescence retarding hormones, auxins and cytokinins (11, 19, 30). According to this concept, auxin and cytokinin deficiencies are at least partly involved in triggering senescence. However, from an experimental point of view, the induction of hormone deficiency to unmask the program of senescence has proved to be possible only in a few cases through the use of hormone antagonists (8). Most of the studies have so far been conducted by applying exogenous auxins and cytokinins in order to elucidate their role in the maintenance of cell integrity.

A model system of senescent pear fruit cells was described previously (22) in which auxin-deprivation was performed by omitting 2,4-D from the continuously renewed medium. In these conditions, a sharp increase in protein and RNA synthesis was observed that appeared to be directly involved in cell death.

Here, several other changes are reported including amino acid efflux, [$^{14}$C]leucine uptake and ability to divide upon transfer in a medium with 2,4-D. Evidence is also provided for the inhibition by 2,4-D of the increase in protein synthesis and amino acid uptake and for the prevention of cell death.

## MATERIALS AND METHODS

**Maintenance of Cell Cultures.** Cell suspensions established in 1972 from young *Passe Crassane* pear fruits (*Pyrus communis* L.) were routinely maintained by successive transfers every 7 days in 1,000-ml Erlenmeyer flasks containing 500 ml standard medium supplemented with $4.5 \times 10^{-6}$ M 2,4-D (21). In all the experiments reported here the initial cell density of each subculture was adjusted to about $8 \times 10^5$ cells/ml by pouring an adequate inoculum.

**Preliminary Subculture in the Absence of 2,4-D.** Before transferring into the continuous system, the cells were grown in 5-L Erlenmeyer flasks for 9 days in the standard medium in the absence of 2,4-D (4).

**Culture in Closed Continuous System.** The continuous culture was carried as previously reported (4, 22) in a so-called "aging medium" (one-fourth concentration of the mineral and organic nutrients present in the standard medium + 0.37 M mannitol + 0.03 M sucrose). The culture system used is basically the same except that 4-L spherical vessels, each containing 3-L of suspension, have been substituted for Fernbach flasks; orbital shaking has been replaced by a magnetic stirrer supported by a glass rod rotating from the lid of the vessel. All bioreactors were incubated at 24 ± 1°C and exposed to 12 h of light at 5.0 to 5.5 w $m^{-2}$ followed by 12 h of darkness.

**Estimation of Cell Number and Dead Cells.** The total cell number was determined after enzymic dissociation as indicated in a previous paper (21). Dead cells were stained either by adding 1 volume of Evans's blue dye in fresh medium (0.5% w/v) to 1 volume of cell suspension (22) or by incubating 4 volumes of cell suspension in 5 volumes of Tris-HCl buffer 0.5 M (pH 7.5) and adding, 10 min later, 1 ml of the erythrosine dye solution of Phillips (23). For each point, eight samples of approximately 100 cells each were counted under a microscope.

**Determinations of Organic Compounds.** The amount of total reducing sugars (or nonreducing sugars submitted to acid hydrolysis, HCl N, 10 min, 100°C) was estimated by the 3.5 dinitrosalicylic acid method (2).

For amino acid isolation, 50 ml output medium was adjusted to pH 3 to 4 with HCL N, passed through a column (1 × 10 cm) of cation exchange resin (Dowex 50, $H^+$ form, 80–100 mesh), eluted by 80 ml N $NH_4OH$ and 20 ml 2 N $NH_4OH$, evaporated twice to dryness after dissolving in 20 ml water. The analysis was performed using a Beckman automated amino acid analyzer.

Total cell proteins were isolated and estimated by the method described by Street (29).

Total RNA was extracted according to the method of Schneider (27) and determined by the orcinol method (24).

**Respiration and Ethylene Production.** $CO_2$ present in the effluent air was measured colorimetrically as previously described (22). Ethylene production was estimated by injecting 3 ml effluent air into a gas chromatograph.

**Determination of Mineral Compounds.** $K^+$, $Ca^{2+}$, and $Mg^{2+}$ were analyzed using an atomic absorption spectrometer (Perkin Elmer 290 model). Pi and $NH_4^+$ were measured colorimetrically respectively by the method of Allen (1) and by Technicon autoan-

[1] Supported by the Centre National de la Recherche Scientifique (Grant 031 874).

[2] To whom reprint requests should be addressed.

alyzer. Residual $NO_3^-$ in the medium was estimated by means of an Orion Model 601 ion electrode.

**Leucine Uptake and Incorporation into Proteins.** For leucine uptake determinations, 16 ml cell suspension containing a predetermined volume of cells (around $8 \times 10^5$ cells/ml) were incubated in 25-ml flasks at 25°C in a water bath shaker (100 rpm) in the presence of 0.8 $\mu$Ci of [U-$^{14}$C]leucine (318 mCi/mmol). Series of 1-ml samples were collected at time intervals ranging from 2 to 120 min and immediately filtered under vacuum on 2.5 cm Whatman GF/A glass fiber discs. The remaining free isotope was removed by two washings of the cells trapped on the filter with 7 ml fresh medium containing 1.7 $\mu$M of [$^{12}$C]leucine. Then the filters were dried, added to a dioxane based scintillator, and counted in a liquid scintillation spectrometer (LKB 1215 Rackbeta). All counts were corrected for quenching (external standard) and background.

[$^{14}$C]Leucine incorporation into proteins was estimated as previously described (22).

## RESULTS

In the experiments reported here, auxin deprivation was achieved by transferring cells to the "aging medium" in continuous culture after a previous culture of 9 days in the standard medium minus 2,4-D. In these conditions, almost no cell division occurs and the percentage of dead cells begins to increase around day 16 (Fig. 1). With some stock cells, however, cell death occurs later (Fig. 6). These fluctuations remain unpredictable despite the careful attention taken to achieve reproducible conditions (date of transfer, cell density, volume of flasks, rate of liquid and air renewal); they may be due to uncontrolled differential rates of 2,4-D depletion within the cells. For these reasons, all the comparative studies described in this paper have been carried out on the same individual stock cells.

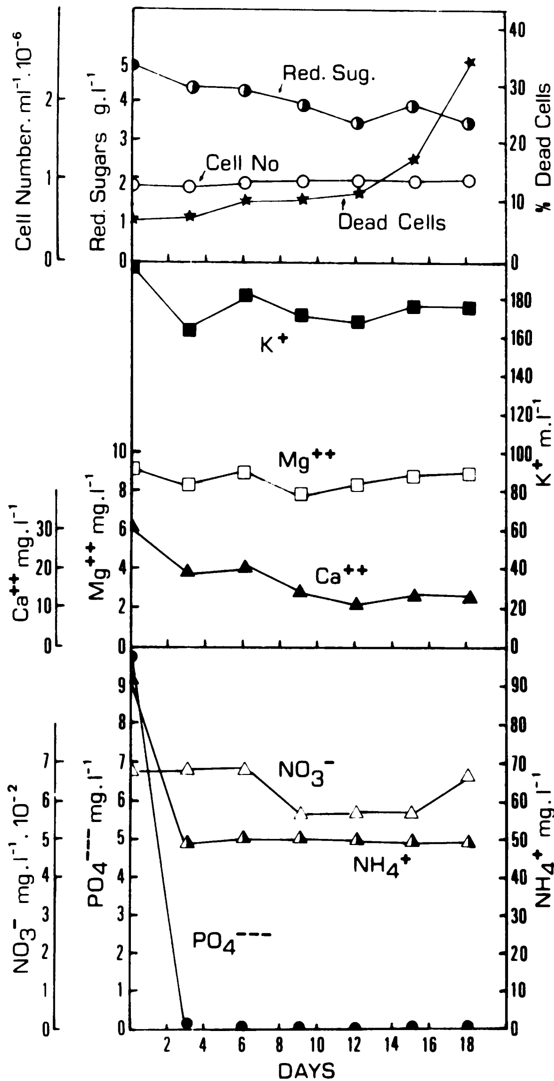


FIG. 1. Cell number, percentage of dead cells, and nutrients in the output medium in 2, 4-D-deprived pear fruit cells cultured with continuous renewal of medium. Medium flow rate: 430 ml day$^{-1}$. Air flow rate 66 ml min$^{-1}$.

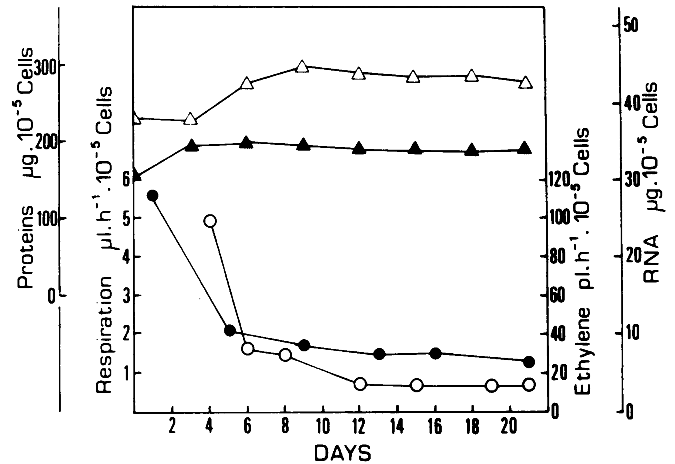


FIG. 2. Respiration rate (●——●), ethylene production rate (○——○), protein (▲——▲) and RNA content (△——△) of 2,4-D-deprived pear fruit cells cultured with continuous renewal of medium. Each point represents mean of four samples, two each from duplicate cultures.

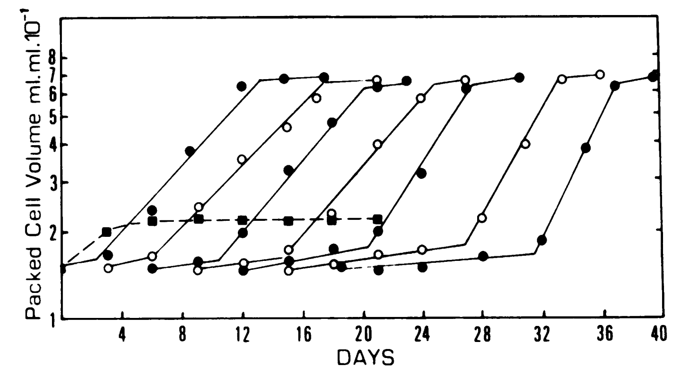


FIG. 3. Growth of pear fruit cells upon transfer into the standard medium plus 2, 4-D (●, ○) at different time intervals during the continuous culture (■——■).

**Nutrient Consumption during the Continuous Culture in the Absence of 2,4-D.** In order to assess that no nutrient deficiency occurs during the continuous culture, the level of sucrose and some minerals was measured at intervals in the output medium (Fig. 1). At any time, no depletion in any of the components under analysis were observed except for $PO_4^{3-}$ which is rapidly absorbed. It corresponds to an important accumulation of phosphate within the cell and is not a cause of deficiency.

Among the other minerals, some are consumed at a high rate without major changes during the culture. They include $K^+$ (with a calculated consumption of about 0.55 $\mu$g/day/$10^5$ cells), $NH_4^+$ (about 1.2) and $Ca^{2+}$ (about 0.4). Surprisingly $NO_3^-$ and $Mg^{2+}$ are not assimilated in measurable proportions.

As for sucrose, only trace amounts can be found in the efflux medium indicating that it is completely hydrolyzed within the cell suspension. The level of the residual reducing sugars decreases

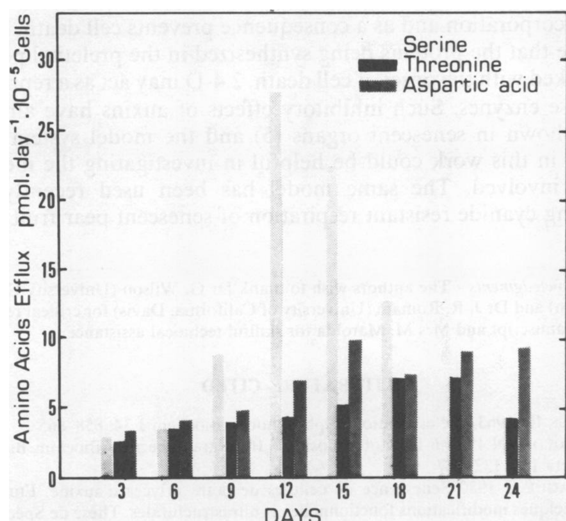


FIG. 4. Efflux of serine, threonine, and aspartic acid in the output medium measured at time intervals during the continuous culture of 2,4-D-deprived pear fruit cells. Each value represents mean of two samples.

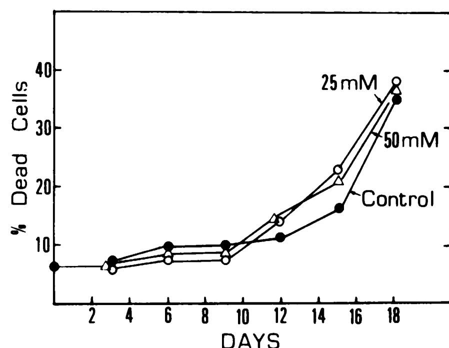


FIG. 5. Effects of added serine (25 and 50 mM) to the fresh medium on cell death of 2,4-D-deprived pear fruit cells cultured with continuous renewal of medium.

slightly up to day 9 and then remains almost unchanged. The consumption rate varies from 20 $\mu$g/day $\cdot 10^5$ cells at day 3 to 34.5 $\mu$g/day $\cdot 10^5$ cells at day 12.

**Changes in Respiration Rate, Ethylene Production, Protein, and RNA Content.** The respiration rate and the ethylene production rate both decreased markedly during the first 6 days of culture (Fig. 2). During the same period, protein and RNA content increased slightly to reach a constant level by days 4 and 8, respectively.

**Changes in the Ability to Divide upon Transfer into a 2,4-D Provided Medium.** The capacity of the senescent cells to resume division upon transfer into the standard culture medium was examined using 250-ml flasks inoculated with $5 \times 10^5$ cells/ml taken from the bioreactor at time intervals up to day 18. Cell growth was measured by determining cell number every 3 days (Fig. 3). No important change in the slope of the exponential phase (doubling times approximately 96 h) or in the final yield of cells (around 700%) was observed, however the length of the lag phase progressively increased from 72 h at day 3 to 336 h at day 18.

**Amino Acids Leakage into the Medium.** Three major amino acids, serine, threonine, and aspartic acid (representing 80% of the amino acid fraction) could be detected in the output medium. Among them, aspartic acid, and threonine undergo a slow rise in the efflux rate until the end of the culture (Fig. 4). Serine efflux followed a different pattern with a sharp increase starting at day 9 and reaching values as high as 27 $\mu$mol/day $\cdot 10^5$ cells (corresponding to about 1 mmol/L of culture medium and 52% of the amino acid fraction).

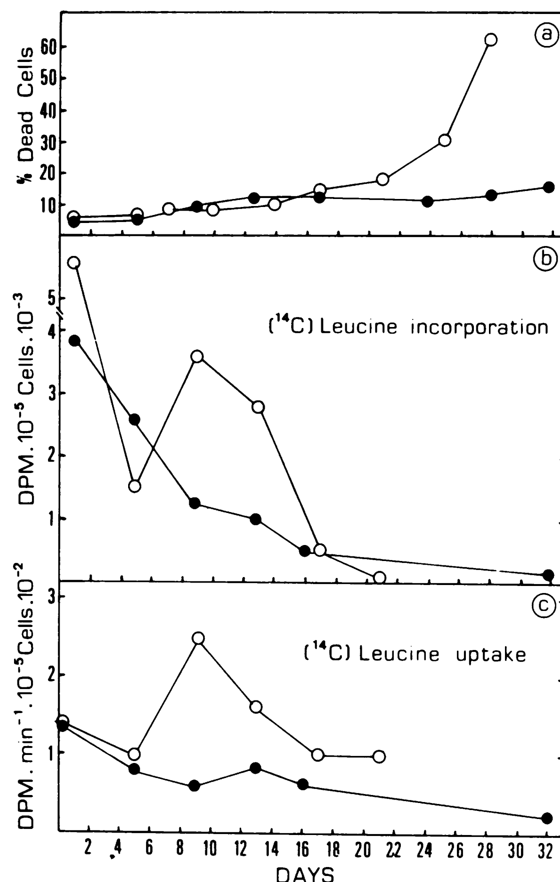


FIG. 6. Percentage of dead cells (a), [$^{14}$C]leucine incorporation (b) and [$^{14}$C]leucine uptake (c) in pear fruit cells cultured with continuous renewal of medium in the absence of 2, 4-D (○) or in the presence of $2.25 \times 10^{-6}$ M 2,4-D (●). Each point represents mean of two duplicates.

Since serine was found to have a senescence promoting effect in leaves (13), a series of three bioreactors was started with 0, 25, and 50 mg/L of L-serine added to the medium (corresponding approximatively to one-fourth and one-half the maximum rate of serine efflux). The results presented in Figure 5 demonstrate the failure of added serine to advance cell death in pear cell cultures.

**Leucine Uptake and Incorporation into Proteins in the Presence or Absence of 2,4-D.** Previous experiments had shown that cell death of 2,4-D deprived cells is preceded by a burst in protein synthesis (22). Figure 6 gives a new demonstration of this phenomenon. It further indicates that leucine uptake either measured by incubating the cells in their own medium (Fig. 6) or by transferring into a fresh medium (data not shown) follows the same trend with a maximum velocity around day 9.

The addition of $2.25 \times 10^{-6}$ M 2,4-D to the medium (Fig. 6) medium fails to induce cell division but greatly reduces the rate of leucine uptake and incorporation into proteins. As a consequence, cell death is greatly delayed. No major changes are observed in the percentage of cell death until the end of the experiment at day 32. In duplicate experiments the culture was conducted up to 45 days without occurence of cell death.

## DISCUSSION

A deficiency in senescence-retarding hormones is part of the triggering factors of senescence in whole plants or plant organs (19, 30). Nutrient withdrawal or deficiency may play a role but it is not considered as a primary factor (19). Our data show that no

organic or mineral deficiency occurred among the components analyzed in our experiments. The metabolic events observed can therefore be considered as a specific response to 2,4-D deprivation. These results give further information on the nutritional behavior of quiescent cells in culture. Surprisingly, some minerals are consumed at a high rate ($NH_4^+$, $K^+$ and $Ca^{2+}$) while some others are not consumed at all ($NO_3^-$ and $Mg^{2+}$). Sugars are taken up at an average rate of 30 $\mu$g/day·$10^5$ cells. By comparison, we have found that cells at the onset of the stationary phase in standard conditions consume sugars at around 120 $\mu$g/day·$10^5$ cells. Unlike growing cells, however, medium optimization proves to be difficult, inasmuch as no readily measureable parameters exist as base for the response to various levels of nutrients. But it can be assumed that nutrient deficiencies would tend to accelerate senescence while high levels of nutrients would delay it through an auxin-like effect (16). The disappearance of $PO_4^{3-}$ in the output medium is probably due to a very high capacity of the cells to accumulate phosphorus as demonstrated by Heller (9) and Kato *et al.* (10) rather than an actual deficiency.

The cessation of growth resulting from auxin deprivation is immediate in the results reported here where the initial cell density is about $8 \times 10^5$ cells/ml. At higher cell densities, some divisions still proceed until day 3 to 6 (22). The quiescence is probably due to an arrest of the cell cycle at the $G_1$ phase as previously demonstrated in carrot cells (17). However, the progressive increase in the duration of the lag phase upon transfer into the standard medium plus 2,4-D in renewed conditions (results reported here) as well as the complete loss of mitotic activity in non renewed conditions as observed previously (4) remain unexplained. The reduced rate of the nuclear RNA processing of 2,4-D deprived *Acer pseudoplatanus* cells in culture (14) give arguments in favor of an increasing duration of some phases of the cell cycle as auxin removal becomes more complete. It is interesting to compare these results with the observation that plant and animal tissue put into culture at several stages of growth and development (especially during senescence) also show a decline in the ability to grow (12, 15, 18).

Another observation made in this study is that relatively high amounts of serine are excreted with a maximum efflux around day 9. Everett *et al.* (6) had previously found large amounts of serine in the intracellular pool of amino acids in *Acer* cultures under 2,4-D deprivation with a maximum accumulation just before the cessation of growth. It is probable that in our case the serine present in the output medium may be the result of a leakage of internal serine previously accumulated inside the cell.

Ultrastructural alterations of cytomembranes, similar to those observed in other senescent cells, can be observed at day 13 by electron microscopy (3). Serine leakage at day 9 may, therefore, be an early indicator of changes in membrane permeability. However, since added serine had no influence on the timing of cell death no role as a senescence promoting substance could be demonstrated. Nonetheless, the internal pool is probably sufficient to exert the metabolic effects described by Shibaoka and Thimann (28) in leaves.

It was demonstrated previously that cell death is preceded by a sharp increase in protein and RNA synthesis which could be inhibited (at least in short time studies) by cycloheximide and actinomycin D respectively (22). The results reported here show a close parallel between leucine uptake and incorporation into proteins. Similar observations have been made in cells or protoplasts in culture (7, 20, 26) suggesting an interdependence between the two phenomena. The synthesis of proteins by depleting the intracellular pools of amino acids could act to regulate the uptake of precursors from the medium. Serine acccumulation and leakage may also be involved by acting on the uptake of other amino acids.

Surprisingly, the addition of 2,4-D inhibits both leucine uptake and incorporation and as a consequence prevents cell death. If we assume that the proteins being synthesized in the prelethal period are linked with the onset of cell death, 2,4-D may act as a repressor of these enzynes. Such inhibitory effects of auxins have already been shown in senescent organs (5) and the model system presented in this work could be helpful in investigating the mechanisms involved. The same model has been used recently for studying cyanide resistant respiration of senescent pear fruit cells (25).

*Acknowledgments*—The authors wish to thank Dr G. Wilson (University College of Dublin) and Dr J. R. Romani (University of California, Davis) for critical reading of the manuscript and Mrs M. Marolda for skillful technical assistance.

## LITERATURE CITED

1. Allen RJ 1963 The estimation of phosphorus. Biochem J 34: 858–865
2. Arnold WN 1965 $\beta$-Fructofuranosidase from grape berries. Biochim Biophys Acta 110: 137–147
3. Balagué C 1980 Senescence de cellules de poire privées d'auxine. Etude de quelques modifications fonctionnelles et ultrastructurales. Thèse de Spécialité, Toulouse, France
4. Codron H, A Latché, JC Pech, B Nebié, J Fallot 1979 Control of quiescence and viability in auxin-deprived pear cells in batch and continuous culture. Plant Sci Lett 17: 29–35
5. De Leo P, JA Sacher 1970 Control of RNase and acid phosphatase by auxin and ABA during senescence of *Rhoeo* leaf sections. Plant Physiol 46: 806–811
6. Everett NP, HE Street 1979 Studies on the growth in culture of plant cells XXIV. Effects of 2,4-Dichlorophenoxyacetic acid and light on the growth and metabolism of *Acer pseudoplatanus* L. suspension cultures. J Exp Bot 30: 409–417
7. Francki RI, M Zaitlin, RG Jensen 1971 Metabolism of separated leaf cells II. Uptake and incorporation of protein and ribonucleic acid precursors by tobacco cells. Plant Physiol 48: 14–18
8. Frenkel C, NF Haard 1973 Initiation of ripening in Bartlett pear with an antiauxin $\alpha$(*p*-chlorophenoxy) isobutyric acid. Plant Physiol 52: 380–384
9. Heller R 1953 Recherches sur la nutrition minérale des tissus végétaux cultivés *in vitro*. Doctorat d'Etat thesis, Paris
10. Kato A, A Fukasawa, Y Shimizu, Y Soh, S Nagai 1977 Requirements of $PO_4^{3-}$, $NO_3^-$, $SO_4^{2-}$, $K^+$, and $Ca^{2+}$ for the growth of tobacco cells in suspension culture. J Ferment Technol 55: 207–212
11. Lieberman M 1979 Biosynthesis and action of ethylene. Annu Rev Plant Physiol 30: 533–591
12. Macieira-Coelho A 1973 Aging and cell division. *In* L Robert, B Robert, eds, Frontiers of Matrix Biology. Vol 1. Karger, Basel, pp 46–77
13. Martin C, KV Thimann 1972 Role of protein synthesis in the senescence of leaves II. The infuence of amino acids on senescence. Plant Physiol 50: 432–437
14. Miassod R, JP Cecchini 1978 Hormone effect on the half-life of the 42 S pre-RNA of cultured sycomore cells. Febs Lett 96: 277–282
15. Mohan Ram HY, FC Steward 1964 The induction of growth in explanted tissue of the banana fruit. Can J Bot 42: 1559–1579
16. Morel G 1970 Le problème de la transformation tumorale chez les végétaux. Physiol Vég 8: 189–204
17. Nishi A, K Kato, M Takahashi, R Yoshida 1977 Partial synchronisation of carrot cell culture by auxin deprivation. Physiol Plant 39: 9–12
18. Nitsch JP, T Ashira, LM Rossini, C Nitsch 1970 Bases physiologiques de la production de chair de pomme et de poire *in vitro*. Bull Soc Bot Fr 117: 479–491
19. Noodén LD 1980 Senescence in the whole plant. *In* KV Thimann, ed, Senescence in Plants, Chap 10. CRC Press, Boca Raton, pp 219–258
20. Paszkowski J, H Lörz, I Potrykus, C. Dierks-Ventling 1980 Amino acid uptake and protein synthesis in cultured cells and protoplasts of *Zea mays* L. Z Pflanzenphysiol 99: 251–259
21. Pech, JC, C Ambid, A Latché, A Diarra, J Fallot 1974 Culture de tissus et de suspensions cellulaires de fruits. Moyen d'étude de certains aspects de la maturation. *In* R Ulrich, ed, Facteurs et Régulation de la Maturation des Fruits. Colloque CNRS, pp 211–220
22. Pech JC, RJ Romani 1979 Senescence of pear fruit cells cultured in a continuously renewed, auxin-deprived medium. Plant Physiol 63: 814–817
23. Phillips HJ 1973 Dye exclusion tests for cell viability. *In* PK Kruse, MK Patterson, eds, Tissue Culture. Methods and Applications, Chap 3. Academic Press, New York, pp 406–408
24. Pilet PE, R Braun 1967 The interrelation of RNA, auxin and auxin-oxidases in lentil roots. Physiol Plant 20: 870–878
25. Romani RJ, TJ Bos, JC Pech 1981 Cycloheximide stimulation of cyanide-resistant respiration in suspension cultures of senescent pear fruit cells. Plant Physiol 68: 823–826
26. Ruesink AW 1978 Leucine uptake and incorporation by *Convolvulus* tissue culture cells and protoplasts under severe osmotic stress. Physiol Plant 44: 48–56
27. Schneider WC 1945 Phosphorus compounds in animal tissues. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J Biol

Chem 161: 293-302

28. SHIBAOKA H, KV THIMANN 1970 Antagonisms between kinetin and amino acids. Experiments on the mode of action of cytokinins. Plant Physiol 46: 212-220
29. STREET HE 1973 Cell suspension cultures techniques. *In* HE Street, ed, Plant Tissue and Cell Culture, Chap 4. Blackwell Scientific Publications, Oxford, pp 59-99
30. THIMANN KV 1980 The senescence of leaves. *In* KV Thimann, ed, Senescence in Plants, Chap 5. CRC Press, Boca Raton, pp 85-115