Plant Physiol. (1981) 68, 494–498
0032-0889/81/68/0494/05/$00.50/0

Polyamine Oxidase in Oat Leaves: A Cell Wall-Localized Enzyme[1]

Received for publication November 10, 1980 and in revised form February 16, 1981

RAVINDAR KAUR-SAWHNEY, HECTOR E. FLORES, AND ARTHUR W. GALSTON
Department of Biology, Yale University, New Haven, Connecticut 06511

ABSTRACT

The localization and activity of polyamine oxidase (PAO; EC 1.5.3.3), was investigated in leaves and protoplasts of oat seedlings. Activity of the enzyme is highest with spermine as substrate; spermidine is also oxidized, but putrescine and cadaverine are unaffected by the enzyme. Protoplasts isolated following digestion of leaves with cellulase in hypertonic osmoticum showed no PAO activity, and about 80% of the total leaf PAO activity could be accounted for in the cell wall debris. Histochemical localization experiments showed intense PAO activity in guard cells and in vascular elements whose walls are not digested by cellulase. When protoplasts were cultured in a medium suitable for regeneration of cell wall, PAO activity could be detected as the cellulose wall developed. Thus, PAO appears to be localized in cell walls.

Since applied spermine and spermidine prevent senescence of detached leaves, PAO activity was investigated during leaf senescence. The specific activity of PAO declines with increasing age of attached leaves and with increasing senescence of excised leaves incubated in darkness. This decline in enzyme activity, which parallels the decreases in chlorophyll and protein content used as measures of leaf senescence, suggests that the enzyme is not involved in the control of senescence of oat leaves.

The polyamines spermidine and spermine and the related diamines putrescine and cadaverine are constituents of most if not all organisms. They appear to play an important role in cell growth, division, and differentiation (2, 4, 9), possibly because of their polycationic association with nucleic acids and other anionic macromolecules.

We have reported previously that the polyamines and their precursors L-arginine and L-lysine are potent inhibitors of senescence in oat mesophyll protoplasts and in leaf tissue of various monocots and dicots (1, 14). In addition to stabilizing protoplasts against lysis, they promote the synthesis of macromolecules including DNA, increase the frequency of mitotic divisions (15) and inhibit the senescence-linked rise in RNase, protease, and Chl breakdown activities in protoplasts and leaves (8, 13, 14). If the polyamines act *in vivo* to promote mitosis and prevent senescence, then it might be expected that polyamine titer would fall as cells cease division and enter senescence. At that point, enzymes oxidizing polyamines should increase in activity.

PAO,[2] also known as spermine oxidase (EC 1.5.3.3), is known to be especially active in leaves of cereals (26). Although the enzyme from oat leaves has been well characterized (25), its possible role in regulation of cell growth and senescence has not been explored. The present investigation was undertaken to determine the localization and changes in titer of PAO in attached and excised leaves of oats in connection with its possible role in regulation of senescence.

MATERIALS AND METHODS

Plant Material. Oat (*Avena sativa* L. var. Victory) seedlings were grown in vermiculite in controlled growth rooms with a 16-h photoperiod of 12,000 lux intensity as detailed in an earlier report (7). The first leaf of 1-week-old seedlings and protoplasts isolated from these leaves were used to study the localization of PAO. Activity of PAO was measured in the intact first leaf of 1- to 3-week-old seedlings and in excised first leaf of 2-week-old seedlings.

Preparation and Culture of Protoplasts. Protoplasts were prepared by a procedure (15) involving surface sterilization of the leaves, stripping of the lower epidermis and exposure of the peeled side of leaf tissue to cell wall-degrading enzyme (Cellulysin, Calbiochem) in 0.6 M mannitol. The isolated protoplasts were washed by centrifugation and resuspension in the osmoticum and cultured in B5 medium (10) containing additives (16) to promote cell wall regeneration. In addition, putrescine or spermidine (1 mM) was added to the cultures to stabilize the protoplasts against lysis and to increase their macromolecular biosynthetic activity (1). The protoplast suspensions were cultured as hanging drops (15) or as thin layers of 2 to 3 ml in 15 × 60 mm Petri dishes and incubated in moist chambers in the dark at room temperature. All manipulations were performed aseptically in a laminar flow hood. The partially digested leaf pieces remaining after removal of protoplasts were used to study the localization of PAO.

Senescence of Excised Leaf Segments. Four median 45-mm long leaf sections from which the lower epidermis had been stripped were floated, stripped side down, either on 5 ml of 1 mM phosphate buffer (pH 5.8), or on buffered solutions containing polyamines or other senescence inhibitors. The dishes were incubated at room temperature in the dark or light (12,000 lux) for 48 h. At intervals, the leaves were washed thoroughly with distilled H_2O and the crude enzyme extracted and assayed. Most of the experiments were done under aseptic conditions (14).

Enzyme Extraction. Two median 45-mm long leaf pieces (fresh weight about 65 mg) were homogenized in 1 ml of 100 mM phosphate buffer (pH 6.5) in chilled mortars. To obtain protoplast homogenates, pellets from 10^6 protoplast/ml suspensions were "vortexed" with 1 ml of the buffer. The homogenates were then placed in the cold for 30 min and centrifuged at 12,000*g* for 10 min at 4 C. The resulting clear supernatant fractions were assayed for PAO activity.

Enzyme Assay. PAO activity in leaves and protoplasts was determined by: (a) *Peroxidase/guaiacol method.* The procedure used was modified from Smith (24) and involved measurement of the rate of peroxidative oxidation of guaiacol by H_2O_2 released in enzyme extracts from leaf homogenates with spermidine or sperm-

[1] Supported by National Science Foundation Grant DAR 7813294 to A. W. G.

[2] Abbreviation: PAO, polyamine oxidase.

ine as substrates. The reaction mixture consisted of 1 ml of 100 mM phosphate buffer (pH 6.5), 0.05 ml each of 25 mM guaiacol and 1 mg/ml of peroxidase (Horseradish Type II, Sigma) and 0.1 ml of crude enzyme. After preincubation of mixture at room temperature for 2 min, 0.02 ml of 10 mM of spermidine or spermine was added and the *A* at 470 nm was measured. (b) *Starch Reagent Method.* To localize PAO activity in the leaf tissue, the histochemical method of Smith (22) was used. Four 45-mm long peeled leaf segments or partially digested leaves remaining after cellulytic digestion or 5×10^5 protoplasts contained in 100 λ suspension were floated on a reagent containing 1.3% soluble starch, 20 mM potassium iodide and 10 mM spermine in 1 mM phosphate buffer (pH 5.8, for protoplasts 0.6 M mannitol was included) for a period varying from 30 min to several hours. The development of the blue starch-iodine color was the result of I^- oxidation to I° by enzymically generated H_2O_2 formed following addition of selected polyamines. This color was presumed indicative of PAO activity. Histochemical localization of PAO activity on leaf pieces was established microscopically, and enzymic activity was determined from measurements of the absorbance of the blue iodine-starch complex at 550 nm.

Cell Wall Staining. The fluorescent brightener Calcofluor White (American Cyanamid Co.) was used to stain the cellulose of regenerating cell walls of the cultured protoplasts. The cells were incubated with 0.1% Calcofluor White for 5 min and then examined under a fluorescence microscope (20).

Chlorophyll Content. Chl from excised leaves was extracted in hot 80% ethanol and measured by determining *A* at 665 nm by means of a Beckman-Gilford spectrophotometer.

Protein Estimation. The soluble protein content of leaves was measured by the method of Lowry *et al.* (18), using BSA as a standard. The data presented are means ± SE from 2 to 6 experiments.

RESULTS AND DISCUSSION

PAO in Oat Leaves. Using the coupled peroxidase/guaiacol reaction, we detected a stable enzyme in the first leaf of oat seedlings which oxidizes only spermidine and spermine. The pH for optimal stability and activity of the enzyme was 6.5 in phosphate buffer with either polyamine as substrate. The enzyme was more active with spermine than with spermidine. In this respect, the PAO from oats resembles that from barley but differs from PAO in maize, in which spermidine is the preferred substrate (26). The enzyme is inhibited by β-hydroxyethylhydrazine, resembling in this respect the polyamine oxidases from leaves of other cereals, but differing from legume amine oxidase (the only other intensively studied plant enzyme known to oxidize polyamines) in its inability to oxidize putrescine and cadaverine (24, 27).

Localization of PAO. Determinations of PAO activity in the leaf tissue, using the peroxidase/guaiacol assay, indicated that the enzyme is loosely bound to the cell wall. The results in Table I demonstrate that about 80% of the PAO activity is associated with cell wall debris in the Cellulysin digest while virtually no activity is found in protoplasts devoid of cell wall. These protoplasts were more than 90% intact, as judged by exclusion of Evans Blue (12). The residue of the enzymically digested leaves, consisting of vascular strands, guard cells, and a few intact cells, showed only about 22% of the total activity. These results are in contrast with the observation that the enzyme from homogenized barley leaves is associated with a dense particulate fraction that can be sedimented in low centrifugal fields (23). We believe that the evidence involving nondisruptive protoplast preparation is more conclusive than that from experiments in which leaves are homogenized. In the latter instance, a "sticky" PAO might become associated with charged particles in the cell. The report that PAO activity sediments in fields as low as 100 g (26) supports the view that cell wall fragments, rather than an organelle, may be involved.

Table I. *Polyamine Oxidase Activity in Oat Leaves Digested with Cellulysin*

Peeled leaves were floated on Cellulysin solution and PAO activity was measured in homogenates of the intact leaf, after cellulase digestion, in cellulase digest of leaf which contained cell wall debris, and in homogenates of protoplasts.

	PAO Activity	
	OD units/min·g fresh wt	*% undigested leaves*
Undigested leaves	63.7 ± 8.3	100
Digested leaves	14.0 ± 2.1	22
Cellulase digest of leaves (cell wall debris)	52.3 ± 9.8	80
Protoplasts	0	0

To ascertain further the localization of PAO, its activity in the cell wall was measured by the histochemical starch method. The intensity of the blue color, which develops with spermidine or spermine as substrates, shows that the activity is highest in the Cellulysin digest of leaves following cell wall dissolution. However, some activity was again specifically bound to vascular strands, guard cells, and cell walls of unreleased protoplasts in the partially digested leaves, while no activity was found in the protoplasts (Fig. 1). Using the same technique, Smith (22) also observed that most of the enzyme activity in maize leaves was associated with vascular strands.

Since the peroxidase/guaiacol and starch reagent assays are based on enzymically generated H_2O_2, it may be argued that the high endogenous levels of catalase in oat protoplasts may decompose H_2O_2 generated from polyamine oxidation, thus resulting in a spuriously negative test for PAO in protoplasts. Homogenates of protoplast were therefore treated with 2,4-dichlorophenol (10^{-4} M) specifically to inhibit catalase activity (11) prior to the determination of PAO activity. No activity was observed in these protoplast homogenates, suggesting that the enzyme activity is not associated with protoplasts.

Another possible reason for the absence of PAO activity in protoplasts would be the presence of a low molecular weight PAO inhibitor. However, activity determinations following extensive dialysis of the homogenates revealed no PAO activity, thus arguing against such an inhibitor. In another set of experiments, protoplast homogenates were mixed 1:1 with total leaf homogenates prior to the peroxidase/guaiacol assay. The observed activity was half of the leaf homogenate activity, again suggesting the absence of a PAO inhibitor in protoplasts.

We used 0.6 M mannitol solution as the osmoticum for protoplast isolation. It has been reported that mannitol at concentrations of 0.6 to 0.8 M inhibits *in vitro* phenylalanine ammonia-lyase activity in protoplasts isolated from tobacco leaves (17). To ascertain that the 0.6 M mannitol used in our experiments is not responsible for lack of PAO activity in the protoplast homogenates, we added 0.6 M mannitol to leaf homogenates prior to the assay. No decrease in PAO activity occurred. These results show that protoplasts isolated from oat leaves neither have PAO activity nor contain an inhibitor of PAO.

PAO Activity in Protoplast Cultures. To confirm the localization of PAO activity in the cell wall, protoplasts were cultured in a medium optimal for regeneration of the cell wall. The results in Figure 2 show that there was little or no PAO activity in the freshly isolated protoplasts or in the 24-h-old cultures. The enzyme was first detected in 48-h cultures, after which its activity continued to increase with time, showing a peak in 7-day-old cultures. The development of the PAO activity appeared to coincide with regeneration of the cell wall, as observed by Calcofluor White staining (16). This confirms that PAO activity is associated with the cell wall and not with protoplasts.

FIG. 1. Localization of PAO activity in oat leaves following oxidation of I^- to I° by H_2O_2 generated in polyamine oxidation. Activity is shown by the dark blue color of the starch reagent in vascular strands and stomatal guard cells of leaves partially digested by Cellulysin while protoplasts remain green in color.

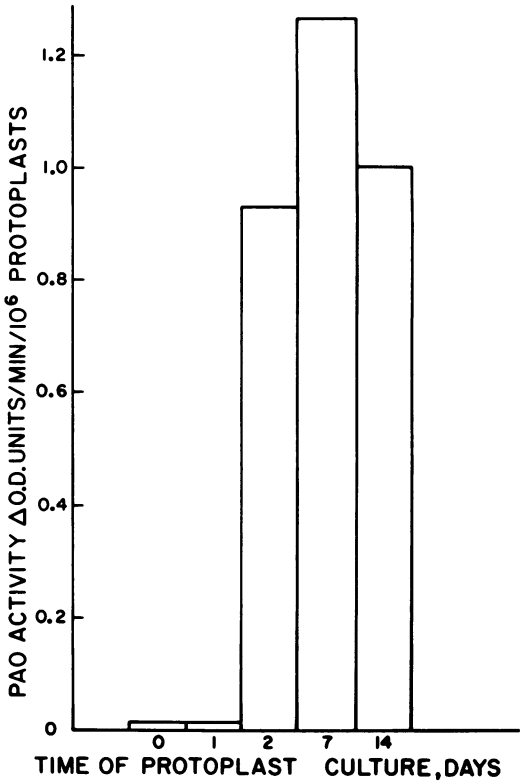

FIG. 2. Development of PAO activity in cultures of oat leaf protoplasts. The SE for protoplast cultures are ±0.04 for 2 days, ±0.09 for 7 days and ±0.06 for 14 days.

Table II. *Substrate Specificity of PAO Activity in Oat Leaves Using the Starch Reagent Assay*

Substrate	Absorbance, 550 nm
	units
None	0
Cadaverine, 10 mM	0
Putrescine, 10 mM	0
Spermidine, 10 mM	0.351
Spermine, 10 mM	2.570

Enzyme Specificity. The results in Table II show that the enzyme in leaf segments exhibits specificity for spermidine and spermine, as determined by the starch reagent assay. No activity was detected when the diamines putrescine or cadaverine were substituted for the polyamines. Similar substrate specificity was observed in cultured protoplasts. All these observations were confirmed by the peroxidase/guaiacol assay.

Thus, the absence of PAO activity in freshly isolated protoplasts and its presence in protoplasts with regenerated cell walls indicates that the enzyme is located in the cell wall. Furthermore, similarity of enzyme specificity in homogenates of the leaf and the protoplasts suggest that the same enzyme is formed in the cultured protoplasts.

Kinetics of PAO Activity in Attached and Detached Leaves. The enzyme activity as measured by the peroxidase/guaiacol assay in attached leaves was highest in young leaves and decreased with age. Levels of protein and Chl, used as measure of senescence (14) also decreased with age (Fig. 3).

A similar decrease in PAO activity and Chl content of detached leaves occurred with increasing time of incubation in the dark or light (Fig. 4). However, the rate of the decrease in PAO activity was greater than the decline in Chl content. In light, PAO activity declined more rapidly than in the dark during the first 24 h, while the decrease in PAO activity was the same in both light and dark at the end of the 48-h incubation period. In contrast, Chl degradation occurred at the same rate during the first 24 h but decreased more rapidly in the dark than in the light during the next 24 h of incubation (Fig. 4). While both PAO activity and Chl content decrease with senescence of oat leaves, hydrolytic enzymes such as RNase and proteases are known to increase dramatically (14, 28, 29). Since high levels of hydrolytic enzymes are associated with senescence and polyamines prevent their increase, it might have been expected that PAO activity also should have increased with senescence. However, the decrease in its activity suggests that PAO is not involved in oat leaf senescence; rather, our unpublished results indicate that senescence is correlated with a decline in polyamine biosynthesis.

Effect of Senescence Inhibitors on PAO Activity. The effects of the senescence inhibitors cytokinins and cycloheximide (13, 19) and polyamines (14) show (Table III) that while spermidine, spermine, cycloheximide, and kinetin all prevent Chl breakdown as compared with controls, their effect on PAO activity is varied. Spermidine and cycloheximide have no effect on the decline of PAO activity, while treatments with spermine drastically promote and kinetin partially prevents the decline. The decline in PAO activity during senescence does not seem to require protein synthesis, as indicated by the cycloheximide treatments. While all

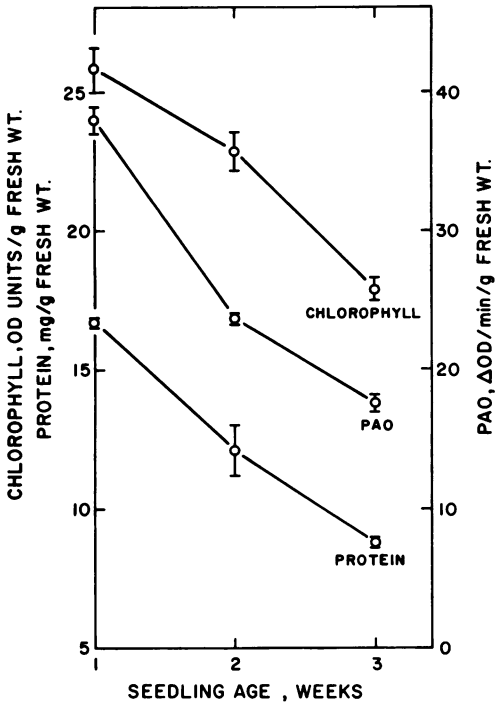

FIG. 3. Changes in PAO activity, Chl and protein content in attached leaves of oat seedlings of various ages.

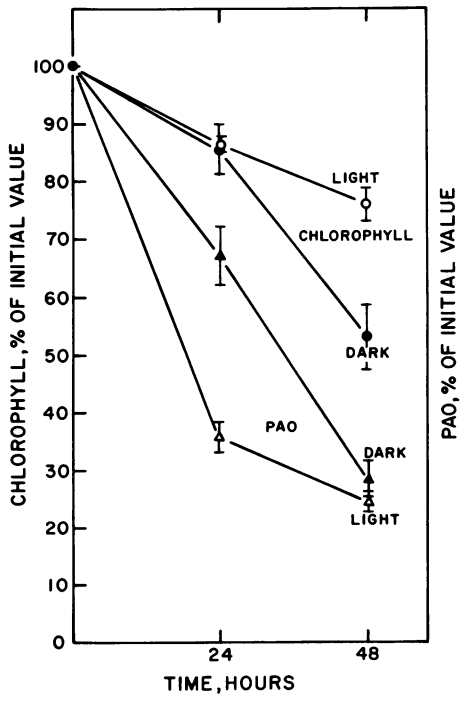

FIG. 4. Changes in PAO activity and Chl content in excised leaf segments with increasing time of incubation in light or dark. Initial value for Chl is 26.9 ± 0.5 OD units/g fresh wt, for PAO is 42.9 ± 3.7 ΔOD units/min·g fresh wt.

senescence inhibitors have been shown to prevent the rise in RNase, protease, and Chl breakdown activities (13, 14, 19, 30), these inhibitors, with the exception of spermine, do not induce a parallel decrease in PAO activity. Also, while 0.1 mM β-hydroxyethylhydrazine completely inhibits PAO activity, it has practically no effect on prevention of Chl breakdown when compared with

Table III. *Effect of Inhibitors of Senescence and Polyamine Oxidase on Chlorophyll Content and PAO Activity of Excised Oat Leaves*

Analysis made after 48 h in dark at 24 C; referred to time zero values. Initial value for Chl is 24.8 ± 0.4 OD units/g fresh weight, for PAO is 37.2 ± 1.8 ΔOD units/min·g fresh weight.

	Chlorophyll	PAO activity
	% initial value	
Control	44 ± 0.5	36 ± 0.5
Spermidine, 1 mM	97 ± 2.0	44 ± 3.5
Spermine, 1 mM	90 ± 3.5	1 ± 0
Cycloheximide, 1 μg/ml	93 ± 7.5	39 ± 0.5
Kinetin, 1 μg/ml	91 ± 4.5	73 ± 8.5
β-Hydroxyethylhydrazine, 0.1 mM	50 ± 3.5	0

control treatments (Table III). Thus PAO, which is restricted to the cell wall, is apparently not involved in processes controlling senescence in oat leaves.

If PAO is in the wall, one presumes that its substrates, spermidine and spermine, would also be located there, as well as in other cellular locales. What possible role could polycationic polyamines have in the apoplast? It is significant that cellulosic walls with high negative charge densities can bind large quantities of cations (6), and that such an apoplastic continuum probably serves as a pathway for ion transport between flexor and extensor motor cells of leguminous pulvini (3). Thus, metabolism of polycationic polyamines might furnish a regulable and variable parameter for influencing and changing the flux of such important ions as Ca^{2+} and K^+ in cell walls. The fact that the activity of one of the major polyamine biosynthetic enzymes, arginine decarboxylase, is regulated by the state of phytochrome (5), which also regulates apoplastic salt flux (21) gives further support to this possible connection.

LITERATURE CITED

1. ALTMAN A, R KAUR-SAWHNEY, AW GALSTON 1977 Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant Physiol 60: 570–574
2. BACHRACH U 1973 Function of Naturally Occurring Polyamines. Academic Press, New York
3. CAMPBELL NA, RL SATTER, RC GARBER 1981 Apoplastic transport of ions in the motor organ of *Samanea*. Proc Natl Acad Sci USA In press
4. COHEN SS 1971 Introduction to the Polyamines. Prentice-Hall, Englewood Cliffs, NJ
5. DAI Y-R, AW GALSTON 1981 Simultaneous phytochrome-controlled promotion and inhibition of arginine decarboxylase activity in buds and epicotyls of etiolated peas. Plant Physiol 67: 266–269
6. DE MARTY M, A AYADI, A MONNIER, C MORVAN, M THELLIER 1977 Electrochemical properties of isolated cell walls of *Lemna minor* L. In M Theiller, A Monnier, M De Marty, J Dainty, eds, Transmembrane Ionic Exchanges in Plants. CNRS et Université de Rouen Rouen, pp 61–73
7. FUCHS Y, AW GALSTON 1976 Macromolecular synthesis in oat leaf protoplasts. Plant Cell Physiol 17: 475–482
8. GALSTON AW, A ALTMAN, R KAUR-SAWHNEY 1978 Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci Lett 11: 69–79
9. GALSTON AW, R KAUR-SAWHNEY 1980 Polyamines and plant cells. What's New in Plant Physiol 11: 5–8
10. GAMBORG OL, RA MILLER, K OJIMA 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158
11. GOLDACRE PL, AW GALSTON 1953 The specific inhibition of catalase by substituted phenols. Arch Biochem Biophys 43: 358–373
12. KANAI R, GE EDWARDS 1973 Purification of enzymically isolated mesophyll protoplasts from C_3, C_4, and Crassulacean acid metabolism plants using an aqueous dextran-polyethylene glycol two-phase system. Plant Physiol 52: 484–490
13. KAUR-SAWHNEY R, WR ADAMS JR, J TSANG, AW GALSTON 1977 Leaf pretreatment with senescence retardants as a basis for oat protoplast improvement. Plant Cell Physiol 18: 1309–1317
14. KAUR-SAWHNEY R, AW GALSTON 1979 Interaction of polyamines and light on biochemical processes involved in leaf senescence. Plant Cell Environ 2: 189–196
15. KAUR-SAWHNEY R, HE FLORE, AW GALSTON 1980 Polyamine-induced DNA synthesis and mitosis in oat leaf protoplasts. Plant Physiol 65: 368–371
16. KINNERSLEY AM, RH RACUSEN, AW GALSTON 1978 A comparison of regener-

ated cell walls in tobacco and cereal protoplasts. Planta 139: 155–158
17. KOPP M, B FRITIG, L HIRTH 1977 Phenylalanine ammonia-lyase activity of protoplasts: *In vitro* inhibition by mannitol and sorbitol. Phytochemistry 16: 895–898
18. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
19. MARTIN C, KV THIMANN 1972 The role of protein synthesis in the senescence of leaves. 1. The formation of protease. Plant Physiol 49: 64–71
20. NAGATA T, I TAKEBE 1970 Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92: 301–308
21. SATTER RL, AW GALSTON 1971 Potassium flux: a common feature of *Albizzia* leaflet movement controlled by phytochrome or endogenous rhythm. Science 174: 518–520
22. SMITH TA 1970 Polyamine oxidase in higher plants. Biochem Biophys Research Commun 41: 1452–1456
23. SMITH TA 1972 Purification and properties of the polyamine oxidase of barley plants. Phytochemistry 11: 899–910
24. SMITH TA 1974 Polyamine oxidation by enzymes from *Hordeum vulgare* and *Pisum sativum* seedlings. Phytochemistry 13: 1075–1081
25. SMITH TA 1977 Further properties of the polyamine oxidase from oat seedlings. Phytochemistry 16: 1647–1649
26. SMITH TA 1977 Recent advances in the biochemistry of plant amines. Prog in Phytochem 4: 27–81
27. SURESH MR, S RAMAKRISHNA, PR ADIGA 1976 Diamine oxidase of *Lathyrus sativus* seedlings. Phytochemistry 15: 483–485
28. THIMANN KV 1978 Senescence. *In* Controlling Factors in Plant Development. Botanical Magazine, Tokyo, Special Issue 1: 19–43
29. UDVARDY J, GL FARKAS, E MARRÈ 1969 On RNase and other hydrolytic enzymes in excised *Avena* leaf tissues. Plant Cell Physiol 10: 375–386
30. WYEN NV, S ERDEI, J UDVARDY, G BAGI, GL FARKAS 1972 Hormonal control of nuclease level in excised *Avena* leaf tissues. J Exp Bot 23: 37–44