CERTAIN ASPECTS OF LIGHT AND PLANT HORMONES IN CONTROL OF SENESCENCE[1]

YIH-MING CHEN[2]

Abstract: The senescence of excised primary leaves of *Phaseolus vulgaris* L. was followed by measuring the net breakdown of chlorophyll, protein, RNA, proteinase and ribonuclease activity. A decline of chlorophyll, protein, and RNA mark the progress of senescence in excised leaves.

The chemical growth regulators, indoleacetic acid and gibberellic acid, were relatively ineffective in retarding senescence. N_6-Benzyladenine at the concentration of 1×10^{-5}M was effective in retarding the decline in the amount of chlorophyll, protein and RNA.

In the dark, L-serine at the concentration of 3×10^{-2}M promoted yellowing and RNA degradation of excised leaves. But in the light, it was ineffective in promoting senescence.

Fluorescent light of 1,000 Lux, was very effective in senescence retardation. In the dark, adding sucrose (5.8×10^{-2}M) to the culture medium replaced the light function in retarding the chlorophyll, protein and RNA loss. The senescence of excised leaves was retarded by cycloheximide (5 μg/ml.). It is assumed that, upon excision of leaves, the synthesis of some m-RNA responsible for the synthesis of destructive enzymes is activated, which ultimately leads to the breakdown of RNA and the protein synthesizing machinery. The protein inhibitors could then be expected to temporarily retard chlorophyll breakdown primarily by inhibiting the synthesis of destructive enzymes. The role of light and N_6-Benzyladenine in the retardation of senescence may act synchronously.

I. INTRODUCTION

The chemical changes that occur in leaves as they grow old have been well characterized for many species. The normal features of the aging leaf blade include loss of chlorophyll, irreversible yellowing, the declining level of protein and RNA, and the eventual death of the organ. These characteristic symptoms of senescence occur at an accelerated rate in excised mature leaves and in leaf discs floated on water. Over the years many proposals have been made to account for the accelerated deterioration of detached leaves. A new insight into the hastened senescence was proposed by Chibnall (1956) who suggested that the leaf integrity might be controlled by hormonal materials normally supplied by the roots. In a detached leaf the hormonal supply would be depleted and senescence would ensue. Support for this hypothesis was provided by the work of Richmond and Lang (1957) who demonstrated that kinetin could retard senescence of detached leaves of *Xanthium*. Moreover, the demonstration of the occurrence of kinetin-like materials in root exudates (1964) makes these compounds likely candidates for the hormonal material proposed by Chibnall (1956). However, the retardation of senescence by kinetin is not ubiquitous. It has been found that in some species the senescence which is unaffected by kinetin can be delayed by auxins. Auxins can delay senescence in

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(2) (陳益明), Lecturer in Botany Department, National Taiwan University.

Prunus leaves but does not effect *Xanthium* leaves. In contrast, kinetin retards senescence in *Xanthium* but is ineffective in *Prunus* (1965). Brain *et al.* (1959) reported that a third group of plant hormones, gibberellins, delayed the development of autumn coloration in leaves. The senescence delaying characteristics of this group of compounds have been demonstrated recently by Fletcher and Osborne (1965) and Beevers (1968). Fletcher and Osborne reported that gibberellic acid (GA3) or kinetin could delay the senescence of leaf discs of dandelion, *Taraxacum officinale*. They indicated that out of 17 species studied, only *Taraxacum* showed a GA regulation of senescence. Beevers reported that the nasturtium (*Tropaeolum majus*) leaf is similar to that of dandelion in its response. These findings of GA retarded senescence gain further significance in relation to the findings of Phillips and Jones (1964) that gibberellic acid may be synthesized in plant roots. Thus a continued supply of GA from roots may prevent senescence of leaves.

These various observations support the proposition that senescence of detached leaves or leaf discs may be a function of hormonal status, and thus any manipulation which varies the hormone level of the tissue may alter the rate of senescence. Inhibition of the effect of kinetin by actinomycin D has led to the proposal that the hormone influences the rate of senescence by regulating DNA-dependent RNA synthesis. Changes in permeability and free space are associated with senescence and have been shown to be susceptible to hormonal regulation in the fleshy leaves of *Rhoeo*. A substantial loss of protein occurred prior to detectable increase in free space. The endogenous factors which control and regulate these matters in plant cells, to a great extent, obscures, the problem of why the cell eventually dies. By using plant hormones, protein synthesis inhibitors, a cytokinin-antagonism L-serine and radiation of light, the author has attempted to examine the mode of action of plant hormones and light in the controlling of senescence.

II. MATERIAL AND METHODS

Seeds of *Phaseolus vulgaris* L. were germinated and were grown in sawdust and watered with Hoagland solution. The plants were grown in a green house with a temperature of 25±2°C under natural illumination. After 18 days, the primary leaves attained their maximum growth. Leaves were then selected for uniformity of green color. The excised leaves were weighed and then sterilized with 0.1% sodium hypochlorite for 10 minutes and then washed with sterile water four times. The sterilized leaves were incubated in petri dishes (containing one layer of filter paper) moistened by nutrient solution plus various plant hormones or other chemicals (see results). The nutrient solution was prepared with 8×10^{-2}M potassium nitrate, 0.01% Tween and 30 μg/ml streptomycin sulfate. All manipulations were carried out under sterile conditions. The cultures were maintained in a darkened room or under white fluorescent light (1,000 Lux) at 25±1°C. Data were normally expressed as the percentage of the initial amount remaining after experimental treatment. Duplicate tissue samples were used, and the experiment was repeated. The criteria of senescence here used are the net changes in chlorophyll, protein and RNA over time. Differences between experimental treatments are considered significant if the chlorophyll, protein or RNA contents differ by about 10% or more.

1. Extraction and estimation of chlorophyll:

Total chlorophyll was determined spectrophotometrically in 80% acetone according to Arnon (1949).

2. Extraction and estimation of protein and RNA contents:

The leaf discs were extracted three times with boiling 80% ethanol and then ground in 80% ethanol in a glass homogenizer. The homogenate was centrifuged at 3,000 xg for 15 min.. The RNA and protein were extracted from the alcohol insoluble residue by the methods described by Osborne (1962). RNA contents were estimated by referring OD reading of the extracts at 260 mμ using yeast RNA as a standard solution. Protein content of the alkaline extract of the alcohol insoluble residue was determined by the method of Lowry *et al* (1951).

3. Proteolytic enzyme assay:

Proteolytic enzyme activity of excised leaves was measured on the homogenates prepared by grinding two excised leaves (about 1 g) in a glass homogenizer with 10 ml. of a grinding solution consisting of 0.05 M tris-maleic acid buffer (pH 7.0). The resulting homogenate was centrifuged at 12,000 xg for 15 min. and the clear supernatant collected. Five milliliter of this supernatant was incubated for two hours at 37°C in reaction tubes containing 2 ml. of 0.6% casein (pH 7.0). The reaction was terminated by addition of 4 ml. of protein precipitant (containing 0.11 M Trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid) to stop enzyme reaction and was kept at 37°C for 30 min.. The resulting precipitates were removed by filtration. One milliliter of this filtrate was diluted with 4 ml. distilled water. The optical density of the diluted filtrate was measured at 275 mμ in a Beckman DU spectrophotometer and the readings were corrected by substracting the value of a blank solution in which the enzyme solution was mixed with protein precipitant before the casein solution was added. The results are expressed as △OD 275 mμ.

4. Ribonuclease enzyme assay:

The ribonuclease activity was measured according to the method of Shibaoka and Thimann (1970). Two excised leaves (about 1 g) were homogenized in a glass homogenizer with 10 ml. of ice-cold 0.1 M sodium acetate buffer (pH 5.5). Two milliliter of homogenate was mixed with 2 ml. of 10 mg/1 of yeast RNA in 0.1 M acetate buffer (pH 5.5) and was incubated for 60 min. at 37°C. The reaction was stopped, and the undigested RNA was precipitated by adding 4 ml. of McFadyens reagent (0.25% uranyl acetate in 2.5% trichloroacetic acid). After being centrifuged, one milliliter of supernatant was diluted with 7 milliliter distilled water and the absorbance of the supernatant was read at 260 mμ in a Beckman DU spectrophotometer.

III. RESULTS

A. The changes in the amount of chlorophyll, RNA and protein in excised leaves floated on water or other chemical growth regulators:

Excised leaves were tested in several chemical growth regulators in darkness for 8 days. The results indicate that the treatment with IAA or gibberellic acid did not differ significantly from the control. On the other hand, the N6-Benzyladenine (BA) reduced the loss of chlorophyll, RNA and protein from the excised leaves (Figs. 1, 2, 3). These results show that the continuous fall in the level of protein and RNA which are associated with the yellowing and loss of chlorophyll are symptomatic of senescence. In the presence of N6-Benzyladenine the fall in these levels is initially slower, and senescence is retarded though not prevented. Cycloheximide (5 μg/ml.) may retard the loss of chlorophyll, protein and the rate of RNA degradation.

B. Light-induced retardation of chlorophyll, RNA and protein loss in excised leaves:

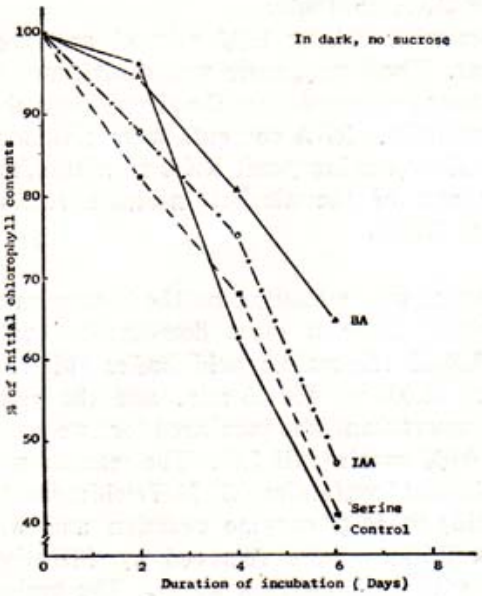

Fig. 1. The changes in the amount of chlorophyll in excised primary leaves of bean seedling in the dark on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, BA or IAA or Serine (3×10^{-2}M), and 30μg/ml streptomycin sulfate. The excised leaves floated on the culture media from day zero.
BA=N_6-Benzyladenine (1×10^{-5}M).
IAA=Indole-3-acetic acid (1×10^{-6}M).

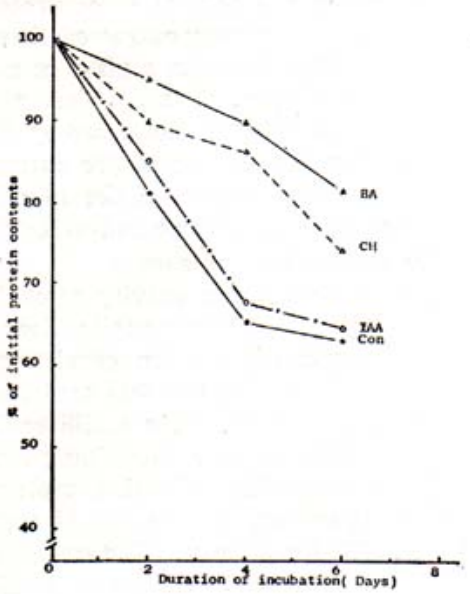

Fig. 2. The changes in the amount of protein in excised primary leaves of bean seedling in the dark on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml streptomycin sulfate and N_6-benzyladenine, 1×10^{-5}M, or IAA, 1×10^{-6}M, or Cycloheximide, 5μg/ml. The results are presented as the percentage of initial protein contents.

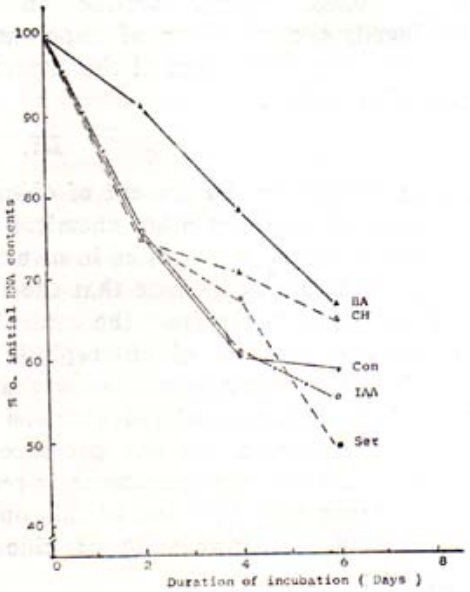

Fig. 3. The changes in the amount of RNA in excised primary leaves of bean seedling floated in the dark on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml streptomycin sulfate and 1×10^{-5}M BA, or 5μg/ml cycloheximide, or 1×10^{-6}M IAA or 3×10^{-2}M L-serine. The excised leaves floated on the culture media from day zero. The results are presented as the percentage of initial RNA contents.

The response of excised leaves to light are shown in Figs. 4–8. The retardation of the loss of chlorophyll and protein induced by light is observed in excised leaves under fluorescent light at 1,000 Lux. treatment. But under light treatment there was no significant retardation in the RNA loss.

C. Effect of sucrose on retardation of senescence in excised leaves:

The effect of sucrose on retardation of senescence in excised leaves is shown in Figs. 9, 10. The results indicate that the treatment of excised leaves in the dark with 5.8×10^{-2}M sucrose will retard the loss of protein and chlorophyll. These results are similar to the report of Goldthwait and Laetsch (1967).

D. Effect of N6-Benzyladenine, light and sucrose on retardation of senescence in excised leaves:

The response of excised leaves to the combination of N6-Benzyladenine, light and sucrose are shown in Figs. 11–13. Incubation of N6-Benzyladenine-treated excised leaves under 1,000 Lux fluorescent light, showed the greatly increased effectiveness of N6-Benzyladenine. Incubation of non-treated excised leaves in light also greatly retarded chlorophyll loss. In the dark, when excised leaves were incubated in the culture medium containing 5.8×10^{-2}M sucrose and 1×10^{-5}M N6-Benzyladenine, there was more effective retardation of chlorophyll loss than when incubated in the

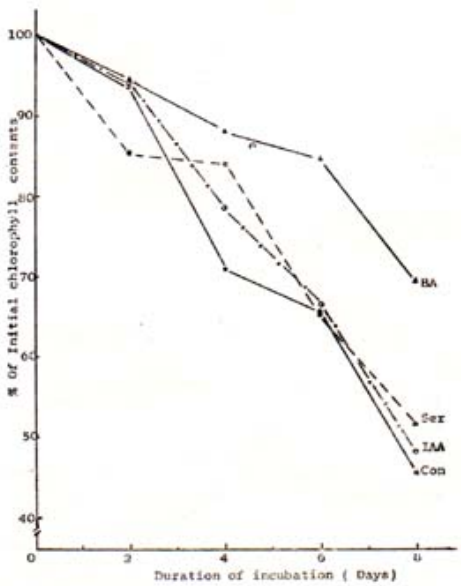

Fig. 4. The changes in the amout of chlorophyll in excised leaves of bean seedling floated in the light on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml streptomycin sulfate and BA or IAA or Ser. The excised leaves floated on the culture media from day zero. Light intensity=1,000 Lux.

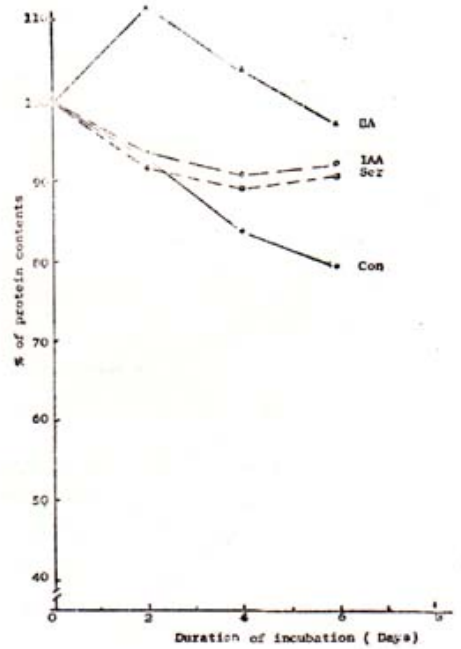

Fig. 5. The changes in the amount of protein in excised primary leaves of bean seedling in the light on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml sterptomycin sulfate and 1×10^{-5}M BA, or 1×10^{-6}M IAA, or 3×10^{-2}M serine. Light intensity=1,000 Lux.

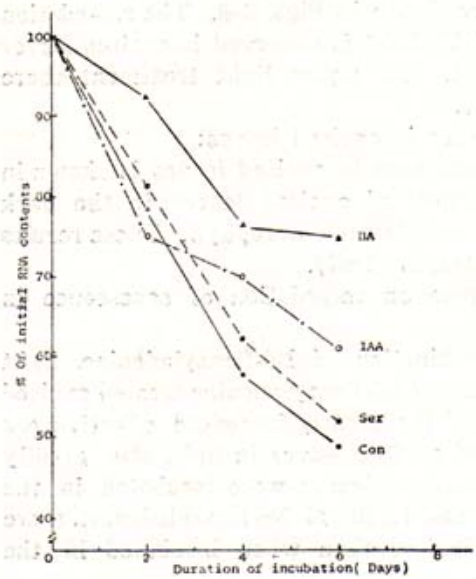

Fig. 6. The changes in the amount of RNA in excised primary leaves of bean seedling floated in the light on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 1×10^{-5}M BA or 1×10^{-6} M IAA, or 3×10^{-2}M Serine. Light intensity =1,000 Lux. The results are presented as the percentage of initial RNA contents.

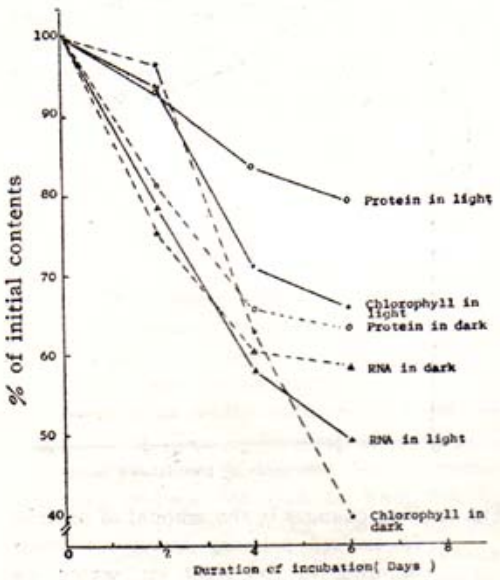

Fig. 7. Effect of light on excised primary leaves senescence.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml streptomycin sulfate and incubated under fluorescent light (intensity=1,000 Lux).

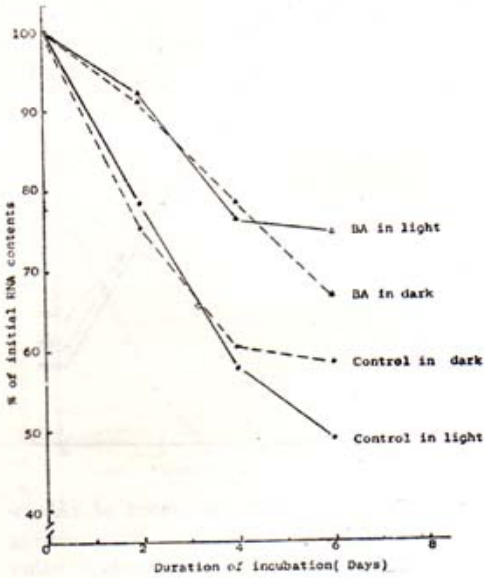

Fig. 8. Effects of N^6-benzyladenine and light on retardation of RNA loss in excised primary leaves

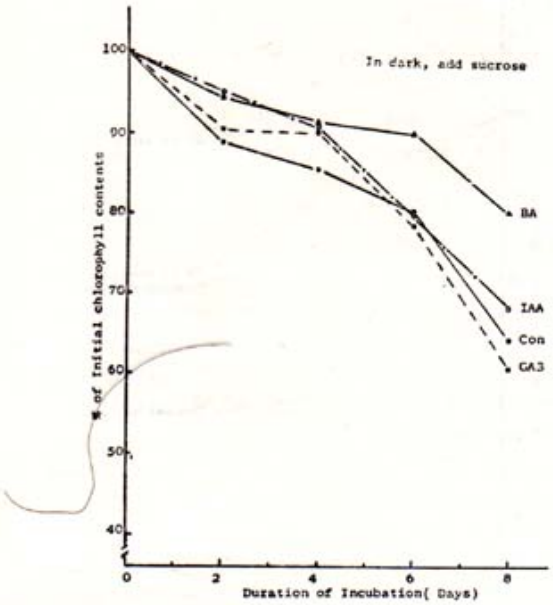

Fig. 9. The changes in the amount of chlorophyll in excised primary leaves of bean seedling floated in the dark on water or other test solutions.

The culture media consisted of 8×10^{-2}M KNO_3, 5.8×10^{-2}M sucrose, 0.01% Tween, 30 μg/ml streptomycin sulfate and various plant hormones. The results are presented as the percentage of initial chlorophyll contents.
BA = N_6-benzyladenine (1×10^{-5}M).
IAA = Indole-3-acetic acid (1×10^{-6}M).
GA = Gibberellic acid (1×10^{-6}M).
Con = Control.

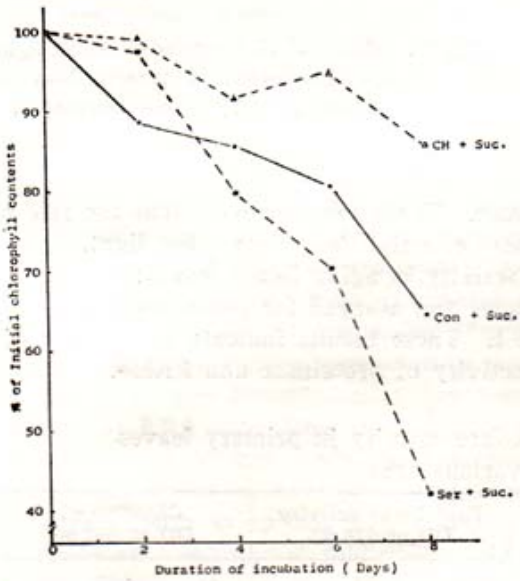

Fig. 10. Effect of L-serine and cycloheximide on senescence of excised primary leaves of bean seedling floated in the dark on test solution.

The culture media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30μg/ml streptomycin sulfate and 3×10^{-2}M L-serine or 5μg/ml cycloheximide and 5.8×10^{-2}M sucrose. The excised leaves floated on the culture media from day zero. Suc. = Sucrose; Ser = Serine; CH = cycloheximide. Con = Control.

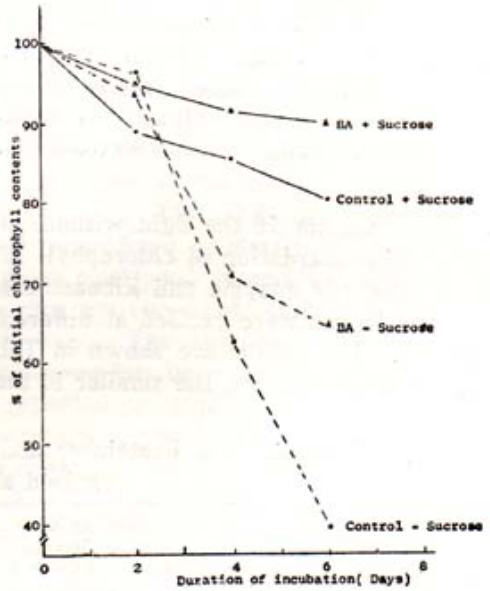

Fig. 11. Effect of sucrose and N_6-benzyladenine on excised primary leaves senescence.

The media consisted of 8×10^{-2}M KNO_3, 0.01% Tween, 30 μg/ml streptomycin sulfate and 5.8×10^{-2}M sucrose or no sucrose. The cultures were incubated in the dark.

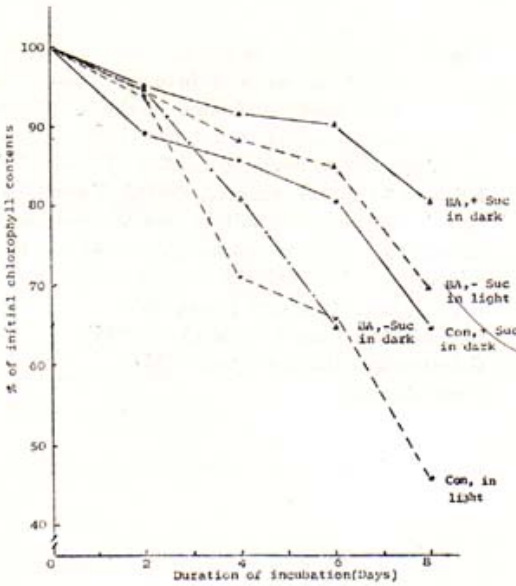

Fig. 12. The retardation of loss of chlorophyll from senescent excised primary leaves of bean seedling by N_6-benzyladenine, light and sucrose treatment.

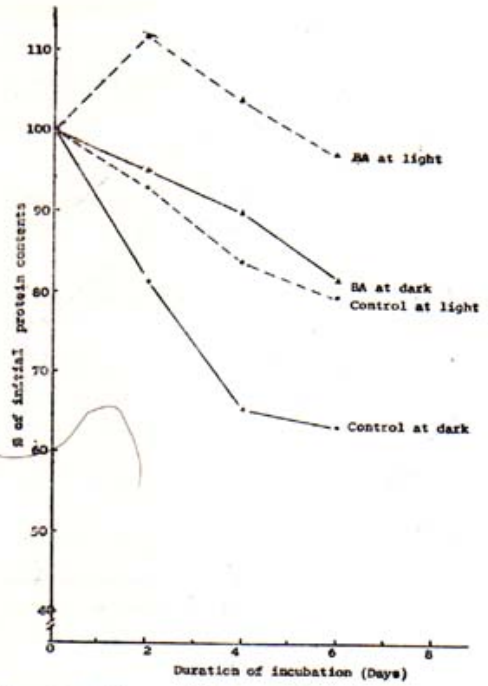

Fig. 13. Effects of N_6-benzyladenine and light on retardation of protein loss in excised primary leaves senescence.

culture medium in the light without sucrose. These results reveal that the role of sucrose in retardation of chlorophyll is more effective than fluorescent light.

E. Proteolytic enzyme and Ribonuclease activity in aging intact leaves:

The leaves were excised at different ages and assayed for proteinase and RNase activity. The results are shown in Table 1. These results indicate that the older the age of the leaves, the smaller is the activity of proteinase and RNase.

Table 1. The Proteinase and RNase activity in primary leaves excised at various ages

Kind of sample	RNase activity OD at 260 mμ	Proteinase activity OD at 275 mμ	Chlorophyll OD at 652 mμ
young leaves	0.657	0.094	1.52
mature leaves	0.186	—	1.12
half yellowing	0.131	—	0.58
complete yellowing	0.064	0.036	0.05

The primary leaves were excised at different ages and assayed for proteinase and RNase activity (see methods). One milliliter of homogenate was mixed with 4 ml. acetone and kept in the refrigerator overnight and then centrifuged. The supernatant from this was used for the determination of chlorophyll.

IV. DISCUSSION

As found by other workers (Osborne, 1962; Srivastava, 1967), there was a decrease in the amount of chlorophyll, protein and RNA in excised bean leaves floated on water. All these changes were retarded in leaves floated on N6-Benzyladenine solution (See results). Srivastava (1967) studied the effect of kinetin on biochemical changes in excised barley leaves and he found that kinetin could suppress the activities of ribonuclease and deoxyribonuclease in leaves floated on that solution. Furthermore he confirmed that kinetin principally preserves the ribosomal RNA in the excised leaves. From histological observations he showed that in excised barley leaves floated on water, a transient increase in nuclear size was later followed by a gradual decrease in the size of both nuclei and chloroplasts. The number of chloroplasts per cell were also greatly reduced in leaves floated on water. On the other hand, in leaves floated on kinetin solution, after a transient decrease in nuclear size, the normal size of both nuclei and chloroplasts were maintained during the duration of the experiment. The decrease in the size of nuclei and chloroplasts in leaves floated on water was accompanied by a loss of RNA and protein from them and these losses were retarded by kinetin. The results of this experiment show that the N6–Benzyladenine is an effective chemical in the retardation of senescence in excised leaves.

Fig. 2 shows the protein synthesis inhibitor, cycloheximide, was effective in retarding the breakdown of chlorophyll of excised leaves in the dark. If it is assumed that, upon excision of a leaf, the synthesis of some m-RNA, responsible for the synthesis of destructive enzymes is activated, which ultimately leads to the breakdown of RNA and protein-synthesizing mechinery, then kinetin could be assumed to retard senescence by inhibiting the synthesis of such m-RNA's and by promoting the synthesis of other m-RNA's (synthesizing useful enzymes) thereby preserving the integrity of the RNA and protein-synthesizing systems. The inhibitors would then be expected to temporarily retard chlorophyll breakdown primarily by inhibiting the synthesis of destructive enzymes. The above-mentioned assumptions may also explain the suppressive effect of kinetin on the activities of certain enzymes (Udvardy *et al.*, 1964) and respiration (Sugiura, 1963). The increased synthesis of m-RNA in excised tissues compared to intact tissues has been reported (Cherry & Huystee, 1965) and benzyladenine has been reported to stimulate or inhibit the synthesis of RNA depending upon its concentration (Ockerse *et al.*, 1966). In this experiment, IAA or gibberellic acid seems to show very little effect on the retardation of the aging of excised bean leaves. (Fig. 3). But Beevers (1968) reported the characteristic symptoms of senescence can be delayed by floating leaf discs of nasturtium on a solution of gibberellic acid or kinetin. This may be due to different kind of experimental materials. Fletcher and Osborne (1965) indicated that of 17 species studied, only nasturtium showed a GA regulation of senescence.

Treatment of excised leaves with light alone greatly retarded their senescence as compared with dark control (Fig. 7). Incubation of BA-treated excised leaves in 1,000 Lux fluorescence light did greatly increase the effectiveness of retarding senescence. (Fig. 14). But Goldthwaite and Laetsch (1967) reported that incubation of kinetin-treated discs in 180ft-c light did not greatly increase the effectiveness of kinetin. Vickery *et al.* (1937) observed light-induced senescence retardation and showed that active photosynthesis was occurring in the light treated leaves. He proposed that photosynthetic production of substrates for respiration spared the

protein in the leaves but he had no direct evidence on this point. Mothes and coworkers (1964) observed that dark treatment of tobacco leaves or leaf parts hastened their yellowing and loss of protein and that light treatment would stimulate uptake and incorporation of amino acids into the protein of excised leaf discs. These workers felt that this light effect was caused by ATP production from photosynthetic phosphorylation. Goldthwaite & Laetsch (1967) reported that a light induced regulation of pigment and protein loss in bean leaves is dependent on photosynthesis. On the other hand, Sugiura (1963) presented evidence for a phytochrome mediated control of protein and chlorophyll loss in tobacco leaf discs. Although no strong evidence on the question of which product of photosynthesis (ATP, NADPH or carbon compounds) is functional in delaying senescence in the leaf tissue, yet the possible substitution of sucrose in the dark for the light effect would implicate nothing more specific than sucrose or some product of sucrose metabolism.

Light can retard the chlorophyll and protein loss, but cannot retard the RNA loss (Fig. 7). The role of light in retarding senescence of excised leaves may be quite different from the N6-benzyladenine action which enhances the RNA synthesis in excised leaves.

The significance of light as a factor regulating the natural senescence of older leaves on the intact plant is difficult to estimate at present. The acceleration of chlorophyll loss and proteolysis induced by darkening of mature leaves on the plant may be the basis for senescence of lower leaves in species which have a compact arrangement of leaves and stem. But many species of plants do not have a compact arrangement of leaves at the base of the stem, so light may be only one of the factors regulating senescence.

N6-benzyladenine is effective in retarding senescence of excised leaves (see results). This may be of considerable importance as a factor limiting the senescence process in older leaves on the plants. In fact, endogenous cytokinin, which is found in high amounts in young actively growing regions of the shoot (developing fruits, young leaves and buds) may well accelerate the senescence of older leaves by the mobilization of low molecular weight compounds and inorganic ions in the younger parts of the plant.

L-serine is evidently a kind of "senescence substance" in that it promotes degradation of chlorophyll, RNA and yellowing. But in this study the use of L-serine, was found to only promote the degradation of protein and RNA in the dark. Studying the RNase and proteinase activity in intact leaves at various ages, the results indicate that the older the age of the intact leaf, the less enzyme activity is evident in the tissue. From their studies on excised leaves, many authors have concluded that cytokinin may inhibit RNase activity. If the inhibition of RNase activity is the prime basis for the action of kinetin in retarding senescence, it is difficult to explain why young leaves have higher RNase activity. So the actual mode of the action of cytokinin in the control of senescence needs further investigation. From experiments we are led to the conclusion that the retardation of senescence of excised leaves is a synchronous effect of cytokinin and light. The retardation of senescence is a complex physiological phenomenom.

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