Plant & Cell Physiol. 17: 323-331 (1976)

Aging induced changes in photosynthetic electron transport of detached barley leaves

U. C. Biswal and Prasanna Mohanty

School of Life Sciences, Jawaharlal Nehru University, New Delhi-110057, India

(Received November 15, 1975)

Primary leaf segments of 11-day-old seedlings of barley (*Hordeum vulgare* L. cv IB 65) were floated on distilled water in darkness at 25°C to induce senescence. This stress induced aging[1] brings significant loss in the total content of pigments, proteins and nucleic acids (DNA, RNA) of the leaves and of chloroplasts isolated from the senescing leaves. Of the three macromolecular components, RNA content of the isolated chloroplasts was found most susceptible to stress-induced aging.

Loss of DCPIP Hill activity of the isolated chloroplasts could be correlated, in a general way, with the loss of pigments, proteins and nucleic acids of the leaves and chloroplasts isolated from them. However, during the stress period, the ability of different exogenous electron donors like $MnCl_2$ and diphenylcarbazide (DPC) to feed electrons to Photo System II (PS II) was found to be different. $MnCl_2$ supported photoreduction of DCPIP only up to the fourth day, whereas DPC sustained its ability to donate electrons up to the seventh day of incubation of the leaves in darkness. These results suggest a sequential alteration of the sites in the electron-transport chain between H_2O and PS II reaction centers of chloroplasts during dark-induced senescence. Kinetin not only prevented the loss of pigments and proteins during senescence, but also preserved the integrity of the electron-transport chain.

Photosynthetic capacity of mature green leaves is known to decline with advance in age (*3*). Loss in the total chlorophyll and protein content (*19*) as well as the disorganization of the structure of the chloroplast membranes (*6*) together with the reduction in ability of photosynthetic CO_2 fixation have been reported (*10*). The structural changes in the chloroplast during senescence begin with a gradual disintegration of the stroma lamellae, followed by the disruption of the grana stackings (*6, 11*). The effects of aging on the functional characteristics of isolated chloroplasts have also been reported (*7*). Recently, the loss of Hill activity during aging of cucumber leaves has been observed (*8*). Although the general phenomenology of senescence induced alterations of some of the photosynthetic activities is known, very little is known about the specific sites of these alterations or the sequence of events which occur during these phenomena. Furthermore, the effect of growth-regulators, which in general retard the process of senescence (*19*), on the chloroplast structure and function has not been well characterized.

Abbreviations: TCA, trichloroacetic acid; BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenol indophenol; DPC, diphenylcarbazide; PS I, photosystem I; PS II, photosystem II.

[1] In this communication 'aging' and 'senescence' are used interchangably.

In this report, we have attempted to characterize the changes in photosynthetic ability of barley chloroplasts caused by stress-induced senescence. The effect of kinetin, a well known plant-growth regulator, on photosynthetic activities of chloroplasts has also been studied. Here, we report some specific sequential alterations in the site(s) for the entry of electrons between H_2O and PS II reaction centers of the photosynthetic apparatus during the dark stress period. The effect of kinetin, which seems to protect these site(s) from dark-induced aging, is also described.

Materials and methods

Barley (*Hordeum vulgare* L. cv IB 65) seeds were germinated in petri plates fitted with soaked filter sheets at 25°C, under continuous illumination (2,500 lux). Fully expanded primary leaves of 11-day-old seedlings were selected for experiments. Ten pieces of 7 cm long leaf segments were cut off from the apex and were floated on 15 ml of double distilled water with or without 50 μM kinetin (Serva, Germany). The petri plates were covered and kept in darkness at 25°C for 7 days. Several sets of replicates were always maintained unless mentioned otherwise. Samples were removed at 24 hr intervals and used for assays.

Estimation of leaf pigments

Pigments were extracted in cold 80% acetone in darkness. The amount of chlorophyll in the extracts were estimated according to Arnon (*1*). The carotenoid content was estimated spectrophotometrically from the same extract (*14*).

Isolation of chloroplasts

The chloroplasts were isolated from 40 to 50 leaf segments by homogenizing them in a prechilled Waring type blendor with ice cold buffer, at maximum speed for 20 sec. The homogenizing medium contained 0.4 M sucrose, 0.01 M EDTA-Na_2 and 0.1 M phosphate buffer (pH 7.8). The homogenate was squeezed through 16 layers of cheese cloth and the filtrate was centrifuged at $500 \times g$ for 1 min in a clinical centrifuge. The supernatant was again centrifuged at $1{,}000 \times g$ for ten min and the pellet was collected by suspending it in a minimal volume of homogenizing medium. Chlorophyll was assayed according to Arnon (*1*). All operations were carried out at 4°C and in dim green light.

Estimation of nucleic acids

Both DNA and RNA contents of the leaf segments and isolated chloroplasts were determined according to Cherry (*5*). The sample was homogenized in cold methanol. The methanol insoluble residue was washed first with cold 0.2 M $HClO_4$ (−20°C); then, with cold ethanol and finally with ethanol-ether (2 : 1, v/v). The resulting residue was suspended in 0.5 N KOH and was incubated at 37°C in water bath for 16 hr. The alkali-hydrolyzed RNA was centrifuged and the supernatant was kept for estimation of total RNA. The total RNA was estimated spectrophotometrically after neutralizing the excess of alkali. The residue was extracted with 5% $HClO_4$ at 70°C for 40 min. The hydrolyzed DNA in the supernatant was removed and the pH was adjusted to 7.0 and again estimated spectrophotometrically (*5*).

Estimation of protein

All protein in the leaf homogenate was precipitated with 5% TCA, dissolved in 0.5 N NaOH and the total amount of protein was estimated according to Lowry et al. (*15*). BSA (Sigma, fraction V) was used as standard.

Measurement of DCPIP-Hill reaction

The photoreduction of DCPIP (dichlorophenol indophenol) was measured spectrophotometrically as described below. The chloroplast sample was illuminated for 30 sec with saturating ($\sim 7 \times 10^4$ ergs cm^{-1} sec^{-1}) white light. The light source was a 300-watt projection lamp. The incident beam was passed through a water and a C.S.3-69 cut-off filter to eliminate infra-red light. The reaction mixture, with a final volume of 3 ml, contained chloroplasts equivalent to 10–15 μg Chl; DCPIP, 15 μM; KCl, 100 mM; $MgSO_4$, 0.1 mM, and 10 mM phosphate buffer, adjusted to pH 6.8. The bleaching of DCPIP due to photoreduction was measured at 605 nm as described by Mohanty et al. (*17*). The concentration of exogenous electron donors such as manganous chloride ($MnCl_2$) and diphenylcarbazide (DPC) when used, were 3 mM and 0.5 mM respectively. The stock solution of DPC was made daily in methanol. The final concentration of methanol in the reaction mixture did not exceed 2%, and the methanol at this concentration had no effect on the DCPIP-Hill reaction. Chloroplasts were isolated in a medium without EDTA, when $MnCl_2$ was used as an electron donor for DCPIP reduction. When DPC was used as the electron donor, the reaction mixture contained 6 mM instead of 10 mM phosphate buffer.

Results

The visible symptom of aging induced by dark stress in detached barley leaves was the gradual yellowing of the leaf. Kinetin prevented the appearance of the yellow colour, as the leaf segments treated with it remained green even 7 days after incubation (data not shown here). The control leaf segments became almost yellow by the seventh day of incubation. Thus, for all our experiments, the leaf-segments were treated for 7 days.

1. *Pigment contents*

The loss of chlorophyll and carotenoid began after just 24 hr of incubation of the leaf segments floated on distilled water in darkness. Both pigments showed a steady decline throughout the experimental period of 7 days (Fig. 1). However, the percentage of loss of chlorophyll with time was higher than that of carotenoid (Fig. 1). This is also evident from the estimation of the ratio of total carotenoid to chlorophyll. These results suggest that the degradation of carotenoid is less compared with that of chlorophyll. Fig. 1 also shows that leaves treated with 50 μM kinetin did not show any significant decline in the levels of chlorophyll. The kinetin treated sample, however, showed a slight (9%) increase in the level of pigment up to the fifth day of dark incubation.

The carotenoid content of the kinetin-treated samples showed a slight increase both absolutely and compared to the chlorophyll content after about the fourth day of incubation. However, the extent of increase in this ratio of carotenoids to

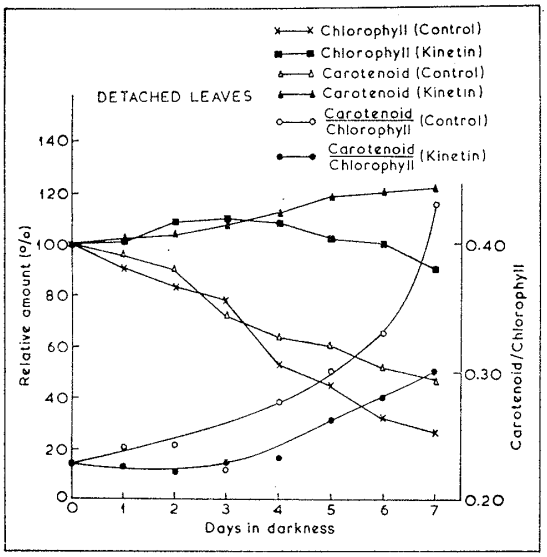

Fig. 1.

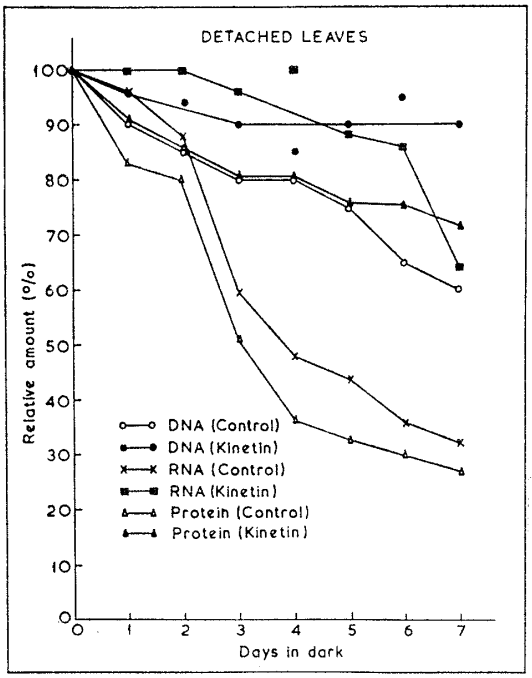

Fig. 2.

Fig. 1. *Changes in the pigments of detached barley leaves incubated in darkness for 7 days in water or in 50 μM kinetin.* The initial (zero time) values for chlorophyll (1.1 mg/g fresh weight) and for carotenoid (0.25 mg/g fresh weight) were set equal to 100 per cent. Each point represents an average of values for five experiments.

Fig. 2. *Changes in the level of protein and nucleic acids of the detached barley leaves incubated in darkness for 7 days in water or in 50 μM kinetin.* The values of initial (zero time) measurements of controls (protein 35 mg/g fresh weight; RNA 1.25 mg/g fresh weight; DNA 95 μg/g fresh weight) were set equal to 100 per cent. Each point represents the average of five experiments.

chlorophylls is much less than that of the control. The cause of the slight increase of chlorophyll content in the kinetin-treated samples incubated in darkness is not clearly known. The results shown in Fig. 1, confirm earlier observations by other workers (*22*) that the loss of chlorophyll and carotenoid is the characteristic of leaf aging.

2. *Changes in the level of nucleic acids and proteins*

The changes in the total protein, DNA and RNA content in both untreated controls (incubated with distilled water) and kinetin treated leaves are shown in Fig. 2. In the controls, the level of protein, RNA and DNA showed a continuous decline throughout the period of incubation. However, the rate of decline in the content of DNA is appreciably slower than the rest. As shown in Fig. 2, the decline in the content of DNA is insignificant in the kinetin-treated leaves. The protein content of kinetin treated leaves suffered a loss of about 30% by the end of the dark-incubation period. The total RNA content began to decline after the fourth day in the kinetin-treated leaves and showed a sharp decline after the sixth day. The loss of DNA in the control sample by the end of the seventh day is

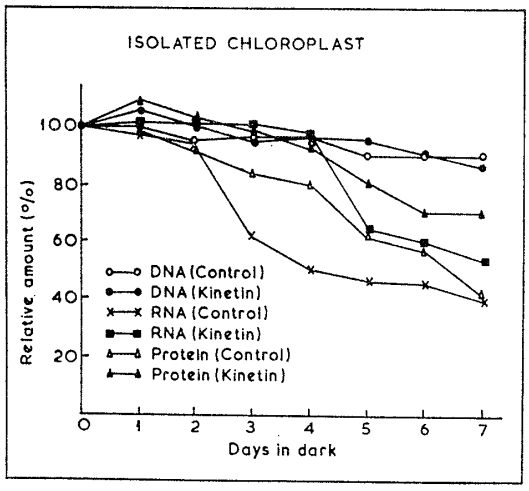

Fig. 3.

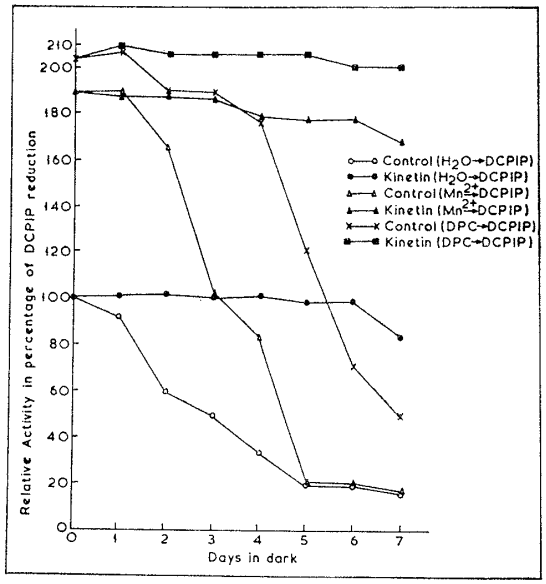

Fig. 4.

Fig. 3. *Changes in the protein and nucleic acids of the isolated chloroplasts.* The leaves were incubated in darkness for 7 days in water or 50 μM kinetin. The initial (zero) values for 100% = 2.6 mg/mg Chl for protein; 200 μg/mg Chl for RNA and 10 μg/mg Chl for DNA. Each point represents the average of five determinations.

Fig. 4. *Changes in the rates of DCPIP photoreduction with or without exogenous donors by chloroplasts isolated from detached senescing barley leaves incubated in dark for 7 days in water or treated with 50 μM kinetin; initial (zero time) rate representing 100% = 54 μmoles DCPIP reduced/mg Chl/hr with H_2O as electron donor.* Each point represents the average of determinations of five experiments.

approximately 35–40% of the initial level; however, roughly 65–70% of the protein and RNA were lost by the same time. Also, the extent of the loss of chlorophyll is roughly the same (65–70%) by the end of the incubation period.

Fig. 3 shows the variations in the content of nucleic acids and protein of chloroplasts isolated from leaf segments incubated with and without kinetin, as a function of the dark-incubation period. The amount of DNA remained unaltered both in kinetin treated and untreated samples. However, the RNA content of the chloroplasts isolated from the water-floated leaf segments began to decrease after the second day of incubation and continued to decline till the fourth day. The loss of RNA during this period is about 50% of the original. After the fourth day, the further loss of RNA is insignificant. Kinetin treatment prevented the loss of RNA up to the fourth day after which a drop of about 40% was noticed.

The loss of TCA precipitable protein of the chloroplasts in the control leaf segments was slow and gradual. Approximately 60% of the protein was degraded by the end of the seventh day, while the kinetin-treated samples suffered a loss of protein roughly to the extent of 30% during the same period.

The DNA content of the chloroplasts expressed on a unit basis of chlorophyll from kinetin-treated and untreated samples remained about the same for the entire period of incubation (Fig. 3). Thus, it seems that in the untreated sample, the loss of DNA from the plastids is not significant. Here it is important to note

that the chlorophyll content of the untreated leaves does not remain constant, but decreases with time in dark incubation as shown in Fig. 1. The kinetics of the loss of chloroplast DNA in untreated samples (Fig. 3) if expressed in terms of fresh weight of the tissue from which they were isolated, would appear quite similar to that of the loss of chlorophyll. Similarly, the time-dependent loss of chloroplast RNA and protein, if expressed in terms of the fresh tissue weight of chlorophyll, would appear much more severe than what is shown in Fig. 3. However, the general trend of the time-dependent alterations in the contents of chloroplast protein and RNA remain almost the same as shown in Fig. 3. Thus, the results shown in Fig. 3 give fair representation of the relative extent of the losses in the contents of chloroplast RNA and protein during stress-induced aging. It is interesting to note that the loss of protein from leaves is about 70% of the initial amount at the end of third day (Fig. 2), while the chloroplasts lost 25–20% of the initial level by the same time (Fig. 3). The time course of degradation of RNA in the leaves and the isolated chloroplasts in the untreated sample seem to be parallel.

3. *Hill reaction*

The time course of the decline in the rate of DCPIP-Hill reaction by the chloroplasts isolated both from control and kinetin treated leaves is shown in Fig. 4. Photoreduction of DCPIP in the chloroplasts isolated from control leaves (floated on water) showed a gradual decline till the seventh day of incubation. The initial rate of DCPIP reduction in chloroplasts isolated from kinetin-treated leaves remained unchanged till the sixth day and it decreased slightly (about 10%) on the last day. As shown in Fig. 4, an almost two-fold increase in the rate of reduction of DCPIP was observed with the application of exogenous donors like $MnCl_2$ and diphenylcarbazide (DPC) to the reaction mixture. However, the ability of these donors to donate electrons for the photoreduction of DCPIP was found to be dependent on the stage of dark-stress-induced senescence of the leaves floated on water. A fast decline in the ability of $MnCl_2$ to support DCPIP Hill-reaction was noted after just 24 hr of incubation. $MnCl_2$ could support Hill-reaction and bring about an enhancement in the rates of photoreduction of DCPIP till the fourth day, after which it became completely ineffective. On the other hand, DPC could effectively support Hill reaction till the fourth day after which a steady decline was observed. However, unlike $MnCl_2$, DPC was found to support DCPIP reduction and bring about an enhancement in the rates of photoreduction of DCPIP over that of the samples where H_2O served as electron donors, till the last day of incubation.

It is to be noted that the progressive decline in the rate of photoreduction of DCPIP by chloroplasts during dark incubation of leaves would appear much more severe if the rates are expressed in terms of fresh weight of the tissue, rather than unit weight of the chlorophyll as shown in Fig. 4. However, the kinetic patterns of the losses in the Hill activity would remain very similar to the one shown in Fig. 4. In contrast to the case of the control chloroplasts, the ability of DPC to support the enhanced rate of DCPIP Hill reaction remained almost unchanged throughout the entire experimental period for the chloroplasts isolated from the leaves treated with kinetin. But the rate of $MnCl_2$ supported DCPIP photo-reduction by the chloroplasts isolated from kinetin treated leaves, showed a slow

and gradual decline. The rate decreased by about 10% of the initial value by the third day and then a subsequent decrease of another 10% was noted by the last day of incubation.

It is, however, interesting to note that the rate of loss of ability of both the exogenous donors to promote DCPIP reduction is much faster compared with that of water during dark-stress-induced senescence (Fig. 4), and that there is a temporal difference between the two exogenous donors in their ability to support high rates of photoreduction of DCPIP by the chloroplasts.

Discussion

Decline in the total content of chlorophyll and nucleic acids (RNA and DNA) is the usual symptom of aging of plants. Similar trends were also observed in our experiments with detached barley leaves kept in dark, suggesting a close similarity between stress-induced senescence and the normal aging process of leaves attached to plants. Chloroplasts isolated from the leaves at different times of dark incubation showed the same major symptoms of senescence. We observed that the degradation of macromolecules was suppressed by the application of kinetin, a well known inhibitor of senescence in plants as reported by others (*18*, *20*).

A gradual decline in Hill activity with the dark-incubation time has also been observed (*16*). However, the decline in Hill activity seems to be correlated with the loss of chlorophyll, protein and RNA contents of the incubated leaves (Fig. 1, 2, 3). Of all the macromolecular components of the chloroplasts, RNA seemed to be most susceptible in stress-induced aging (Fig. 3). Even kinetin could not preserve the RNA content of the chloroplasts after the fourth day of dark treatment. It is interesting to note that decline in the amount of chloroplast protein is very small.

The most interesting finding of this investigation is the differential effectiveness of the two exogenous electron donors in sustaining electron transport during stress-induced senescence. Manganous ion could sustain high rates of photoreduction of DCPIP by isolated chloroplasts only up to 24 hr, after which the rate of photoreduction declined very fast, while DPC could support a high rate of DCPIP Hill reaction up to 4 days, after which a fast decay in the rate was observed. It is known that both Mn^{2+} ions (*13*) and DPC (*21*) feed electrons to PS II. But Mn^{2+} ions stop supporting DCPIP reduction after the fourth day, while DPC would sustain Hill reaction activity till the seventh day of incubation. These observations suggest that these two exogenous donors probably feed in at different sites in the electron transport chain between H_2O and PS II reaction centers and that dark-stress-induced senescence causes a sequential disruption in the function of these sites. Alternatively, it is possible that this differential behavior may be indicative of the degree of alteration in the integrity of the O_2 evolving system. It has been shown that addition of Mn^{2+} ions can restore the oxygen evolving system damaged by mild treatment or due to partial loss of endogenous manganese (Mn^{2+}) from chloroplasts (*4*, *12*). Stress-induced senescence may cause the loss of endogenous Mn^{2+} and thus the addition of $MnCl_2$ to the chloroplasts may effect the restoration of the oxygen

evolving capacity. The inability of exogenous Mn^{2+} to support DCPIP photoreduction after the fourth day of incubation may, thus, imply complete loss of endogenous Mn^{2+}, which is involved in photo-oxidation of water (*4*). It has also been shown that a severe loss of endogenous manganese (Mn^{2+}) may cause loss of this ability ot restore (*4*, *9*). However, this alternative appears unlikely in our case from the observation that both DPC and $MnCl_2$ stimulate photoreduction of DCPIP by the chloroplasts isolated from fresh leaves (initial day of incubation) to about the same extent (Fig. 4), even though, loss of endogenous manganese (Mn^{2+}) in these preparations is expected to be minimal. This indicates that enhancement of photoreduction of DCPIP by addition of manganese (Mn^{2+}) is mostly due to the feeding of electrons by Mn^{2+} ions as in the case of DPC; the latter is known to be a specific electron donor to PS II (*21*).

Furthermore, even though both $MnCl_2$ and DPC stimulate DCPIP Hill reaction by roughly about the same extent on the first day of incubation, their abilities to support this photoreduction begin to decline at different times, which indicates that these two exogenous donors may not be acting at the same site. Thus, our results confirm and amplify the earlier suggestions (*2*, *16*) that Mn^{2+} ions and DPC feed electrons at two different sites and that stress-induced aging brings about a sequential loss of these sites for the entry of Mn^{2+} ions and DPC to feed electrons to PS II. As DPC supports DCPIP reduction till the last day of incubation, it is possible that DPC may feed at a site close to PS II reaction center.

The ability of both the exogenous electron donors to reduce DCPIP with almost identical efficiency throughout the period of incubation in the leaves treated with kinetin, suggests that the possible role of kinetin is to protect the integrity of the sites between H_2O and PS II reaction centers during the dark-stress period, even though the kinetin treatment did not insure complete preservation of the content of protein and RNA of the chloroplasts.

We thank Professor P. N. Srivastava, Dean of the School of Life Sciences, and Dr. G. S. Singhal for support and Mr. P. Vijayan for kindly reading the manuscript.

References

(*1*) Arnon, D. I.: Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* 24: 1–15 (1949).

(*2*) Braun, B. Z. and Govindjee: Antibodies against an intermediate on the waterside of photosystem II of photosynthesis. *FEBS Lett.* 25: 143–146 (1972).

(*3*) Butler, R. D. and E. W. Simon: Ultrastructural aspects of senescence in plants. In *Advances in Gerentological Research* 3. Edited by Bernad, L. Strehler, p. 73–123. Academic Press Inc., New York, N.Y., 1971.

(*4*) Cheniae, G. M. and I. F. Martin: Sites of function of manganese within Photosystem II. Roles in O_2 evolution and System II. *Biochim. Biophys. Acta* 197: 219–239 (1970).

(*5*) Cherry, J. H.: Nucleic acid determination in storage tissues of higher plants. *Plant Physiol.* 37: 670–678 (1962).

(*6*) Dodge, J. D.: Changes in chloroplast fine structure during the autumnal senescence of *Betula* leaves. *Ann. Bot.* 34: 817–824 (1970).

(*7*) Harnischfeger, G.: Chloroplast degradation in aging cotyledons of pumpkin. *J. Expt. Bot.* 24: 1236–1246 (1973).

(*8*) Harnischfeger, G.: Studies on chloroplast degradation in vivo II. Effects of aging on Hill activity of plastids from *Cucurbita* cotyledons. *Z. Pflanzenphysiol.* 71: 301–312 (1974).

(*9*) Heath, R. L. and G. Hind: On the functional site of manganese in photosynthetic oxygen evolution. *Biochim. Biophys. Acta* 189: 222–223 (1969).

(*10*) Hernandez-Cil, R. and M. Schaedle: Functional and structural changes in senescing *Populus deltoides* chloroplasts. *Plant Physiol.* 51: 245–249 (1973).

(*11*) Ikeda, T. and R. Ueda: Light and electron microscopical studies on the senescence of chloroplast in *Elodea* leaf cells. *Bot. Mag.* 77: 336–341 (1964).

(*12*) Itoh, M., K. Yamashita, T. Nishi, K. Konishi and K. Shibata: The site of manganese function in photosynthetic electron transport system. *Biochim. Biophys. Acta* 180: 509–519 (1969).

(*13*) Izawa, S.: Photoreduction of 2,6-dichlorophenol indophenol by chloroplasts with exogenous Mn^{2+} as electron donor. ibid. 197: 328–331 (1970).

(*14*) Liaaen-Jensen, S. and A. Jensen: Quantitative determination of carotenoids in photosynthetic tissue. In *Methods in Enzymology* 23. Edited by Anthony San Pietro. p. 586–602. Academic Press Inc., New York, N.Y., 1971.

(*15*) Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall: Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265–275 (1951).

(*16*) Margulies, M. M.: Electron transport properties of chloroplasts from aged bean leaves and their relationship to the manganese content of the chloroplasts. In *Proceed. IInd Internatl. Cong. On Photosynthesis Res., Stressa.* 1: Edited by Giorgio Forti, Mordhay Avron and Andrea Melandri. p. 539–545. Dr. W. Junk N. V. Publishers, The Hague, 1971.

(*17*) Mohanty, P., G. Papageorgiou and Govindjee: Fluorescence induction in the red alga *Porphyridium cruentum*. *Photochem. Photobiol.* 14: 667–682 (1971).

(*18*) Osborne, D. J.: Effect of kinetin on protein and nucleic acid metabolism in *Xanthium* leaves during senescence. *Plant Physiol.* 37: 595–602 (1962).

(*19*) Person, C., D. J. Samborski and F. R. Forsyth: Effect of benzimidazole on detached wheat leaves. *Nature* 180: 1294–1295 (1957).

(*20*) Richmond, A. E. and A. Lang: Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* 125: 650–651 (1957).

(*21*) Vernon, L. P. and E. R. Shaw: Oxidation of 1,5-diphenylcarbazide as a measure of photosystem 2 activity in sub-chloroplast fragments. *Biochem. Biophys. Res. Comm.* 36: 878–884 (1969).

(*22*) Whitfield, D. M. and K. S. Rowan: Changes in the chlorophyll and carotenoids of leaves of *Nicotiana tobacum* during senescence. *Phytochemistry* 13: 77–83 (1974).