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Induced Senescence of Intact Wheat Seedlings and Its Reversibility[1]

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VERNON A. WITTENBACH
Central Research and Development Department, Experimental Station, E. I. du Pont de Nemours & Company, Wilmington, Delaware 19898

ABSTRACT

Intact wheat seedlings (*Triticum aestivum* L.) were induced to senesce by placing them in the dark and at various stages of senescence were placed back in the light and their recoverability observed. Seedlings demonstrated complete recovery of chlorophyll, protein, and rate of photosynthesis after 2 days in the dark, but were unable to recover fully after 4 days. This suggests the onset of an irreversible stage in senescence by day 4. Foliar applied cytokinins delayed senescence, and zeatin at 0.1 mM delayed the onset of the irreversible stage for 6 days. In addition to delaying the loss of total soluble protein, zeatin maintained the net protein recovery capacity of the tissue. Control seedlings, however, lost their potential for net protein recovery at a rate similar to their loss of total soluble protein. Treatment with zeatin had no apparent effect on dark respiration during senescence, and although treatment did delay the loss of membrane permeability to substrate, the change in permeability occurred too late to have a causal role in senescence.

Plant senescence has been studied in a wide variety of plant systems (3, 8, 23). The mechanism leading to the initiation of senescence is still unknown. One of the major problems associated with these studies is the lack of ability to separate possible causal changes from those which are only effects of senescence. Numerous catabolic processes, such as the breakdown of Chl, protein, nucleic acid, and membranes (12, 16, 23), occur nearly simultaneously. Most studies have used detached systems, which may include artifactual changes resulting from excision (10). The use of cytokinins (15) and other growth regulators (4, 17), as well as protein synthesis inhibitors (11, 21) to delay senescence has helped to separate some of these secondary changes. However, our lack of understanding of the mode of action of these growth substances and the inability to inhibit selectively the synthesis of certain proteins have limited further progress.

In the following experiments an intact wheat seedling system, similar to that developed by Peterson *et al.* (13), was used to study plant senescence. This system was chosen because intact seedlings, in contrast to excised plant parts, can be induced to senesce to various stages in the dark and then removed and their recoverability observed. The results demonstrate the initiation of an irreversible stage in senescence, which follows the initial loss of protein and Chl but precedes the loss of membrane permeability to substrate. Applications of cytokinin delayed the onset of the irreversible stage and markedly delayed leaf senescence.

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MATERIALS AND METHODS

Plant Material and Treatments. *Triticum aestivum* L. var. Chris was grown in vermiculite in 6-cm plastic pots for 7 days at 22 C under continuous light (1200 ft-c). At this time the second leaf had just emerged and the first leaf was about 15 cm long. Seedlings in each pot were sprayed to runoff with 0.02% Tween 80 (v/v) or Tween 80 plus growth regulator. After treatment the seedlings were placed back in the light an additional day for uptake of the compound. They were then transferred to a dark room (22 C) to induce senescence. At different times seedlings were removed, given nutrient solution, and placed back under continuous light for 4 days to study their recoverability. All experiments were terminated prior to a greater than 20% loss in fresh weight of the tissue analyzed.

Chlorophyll and Protein Determinations. For the determinations on the influence of growth regulators on senescence, Chl was extracted from primary leaves (8-cm sections cut 2 cm from the tip) using 80% ethanol. In all other studies, 4-cm sections (cut 4 cm from the tip) from primary leaves were weighed and extracted in 0.05 M HEPES (pH 7.5). Chlorophyll content was determined on an aliquot of the extract following the method of Arnon (1). The remaining extract was centrifuged at 30,000*g* for 15 min and an aliquot of the supernatant was taken for determination of total soluble protein based on the procedure of Lowry *et al.* (9) using a trichloroacetic acid precipitate.

Photosynthesis Measurements. Plants removed from the dark were placed back in the light for 30 min prior to taking sections for photosynthesis determinations. Four 1-cm sections were cut from the first leaf (4–8 cm from the tip) and placed adaxial side up in 25-ml flasks on 0.5 ml of 0.02 M MOPS,[2] (pH 7). The flasks were then placed on a photosynthetic Warburg bath and preincubated at 25 C and 3700 ft-c (74 nanoeinsteins/cm^2·sec, 400–700 nm) for 30 min to insure equilibration and opening of the stomata. Rates of photosynthesis were determined from $^{14}CO_2$ incorporation. The reaction was initiated by adding 0.1 μCi $^{14}CO_2$ (generated from [^{14}C]barium carbonate by lactic acid) to the stoppered flasks. After 5 min, the reaction was terminated by placing the flasks in the dark on dry ice. The sections were then digested and the ^{14}C-incorporation determined by liquid scintillation spectroscopy. All results were corrected for zero time controls.

Measurement of Respiration Rates. Oxygen uptake was determined at 25 C using a YSI O_2 electrode. Two 5-cm sections (cut 4 cm from the tip) from primary leaves of intact plants at different stages of senescence were placed around the outside of the chamber with the abaxial side toward the center, and rates of dark respiration were determined polarographically.

Determination of Membrane Permeability. Changes in membrane permeability to substrate were determined using D-glucose

[2] Abbreviation: MOPS: morpholinopropane sulfonic acid.

($U^{14}C$). Eight 4-mm sections (cut 4–8 cm from the tip) were placed adaxial side up on 0.4 ml of 5 mM glucose (1 μCi/ml in 20 mM MOPS, pH 7) in 10-ml vials. The sections were incubated for 1.5 hr at 25 C on a water bath with slow continual shaking. They were then rinsed three times with distilled H_2O over a 30-min period, transferred to vials containing 0.5 ml 20 mM MOPS (pH 7) and incubated for 30 min on the shaker bath for elution of the radioactivity. Determination of dpm in the elution buffer and in the tissue was determined by liquid scintillation spectroscopy.

RESULTS AND DISCUSSION

When wheat seedlings were induced to senesce by placing them in the dark, there occurred a rapid loss of Chl. After 5 days in the dark, the first leaf had lost nearly 80% of its Chl. Exogenous applications of ABA, GA_3, and Ethephon (2-[chloroethyl]-phosphonic acid [Amchem Products, Inc.]) had little or no effect on the rate of senescence, and only the highest concentration of naphthaleneacetic acid was able to delay senescence significantly (Fig. 1). However, BA (also zeatin and kinetin although not presented) at 1 μl/l markedly delayed senescence and at higher concentrations had an even greater effect. Thimann *et al.* (20) have demonstrated a similar effect for BA and kinetin on senescence of intact oat seedlings.

The loss of Chl and total soluble protein during senescence was nearly linear for the first 4 days (Fig. 2). Application of zeatin at 0.1 mM delayed the loss of both, although the effect on Chl was more pronounced. Control seedlings after 2 days in the dark were able to recover to their initial levels of Chl and protein after 4 days in the light (Fig. 2 arrows). However, after 4 days in the dark control plants were no longer capable of complete recovery and after 6 days no recovery was observed. The lack of complete recovery after 4 days indicates the onset of an irreversible stage in senescence. Changes occurring prior to this time may be causal agents of senescence, whereas later changes are only effects of senescence. The fact that some recovery still occurs after 4 days probably reflects the pattern of senescence in wheat, in that senescence proceeds from the tip of the leaf to the base. After 4 days even though an irreversible stage has been initiated in the section, it has not progressed the entire length of the section, and therefore, some recovery is still evident. Treatment of seedlings with zeatin extended this period of complete recoverability to 8 days, and even after 12 days in the dark, seedlings continued to exhibit recoverability (Fig. 2). Vonshak and Richmond (24) following protein synthesis in chloroplasts isolated from detached tobacco leaves induced to senesce in the dark were also able to demonstrate the onset of an irreversible phase. In their system induction occurred between 1 and 2 days, perhaps reflecting the faster rate of senescence in detached systems.

Photosynthesis was closely correlated with the level of protein in the leaf (Fig. 2). The initial decline in rate appeared to follow the loss of protein, but thereafter the rate of decline was similar to the rate of loss of protein. Moreover, recoverability of photosynthesis closely followed the recovery of protein. These data

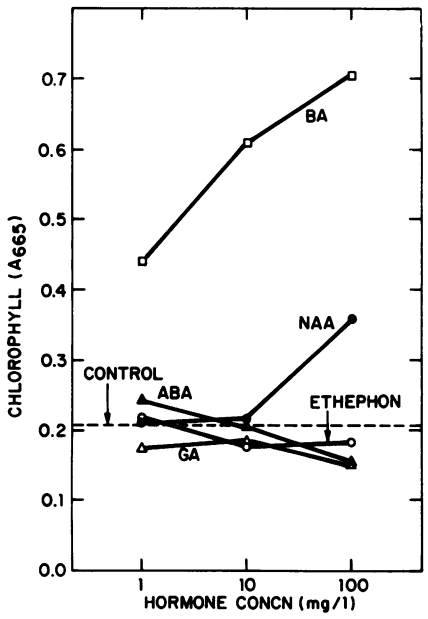

FIG. 1. Effect of growth regulators on the dark-induced senescence of intact wheat seedlings. Chl content of primary leaves was determined after 5 days in the dark. BA, □; NAA, ●; ABA, ▲; Ethephon, ○; GA, △. BA at 100 mg/l was autoclaved into solution.

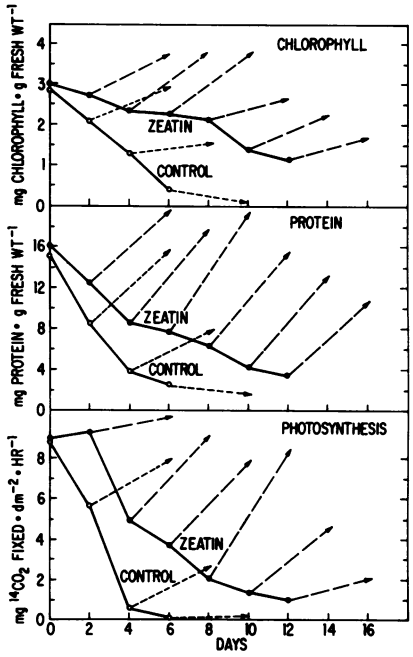

FIG. 2. Changes in Chl, total soluble protein, and rate of photosynthesis in the primary leaves of intact wheat seedlings during dark-induced senescence (○●) and subsequent recovery after 4 days in the light (→). Seedlings were given a foliar application of 0.02% Tween 80 (control) or Tween 80 plus 0.1 mM zeatin 24 hr prior to being placed in the dark.

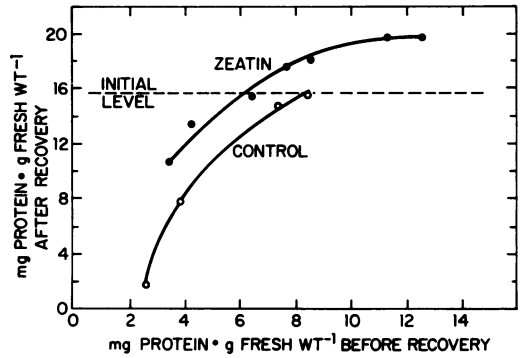

FIG. 3. Net soluble protein content of the primary leaf of seedlings after a 4-day recovery in the light as a function of protein content prior to recovery in control and 0.1 mM zeatin treated seedlings. These results represent the combination of two experiments like that reported in Figure 2.

Table I. Net Increase in Total Soluble Protein Content of Leaves after Returning Plants to the Light for a Four Day Recovery Period

Sections from primary leaves of seedlings were assayed for total soluble protein after various times in the dark and after a 4 day recovery period (see text for details). The following data represent the net change in protein content during recovery and consist of combined results from two experiments

Days in Dark	Δ mg Protein · g Fresh Wt^{-1}	
	Control	10^{-4} M Zeatin
2	7.0	7.0
4	4.0	9.2
6	-0.8	9.7
8		8.9
10		9.0

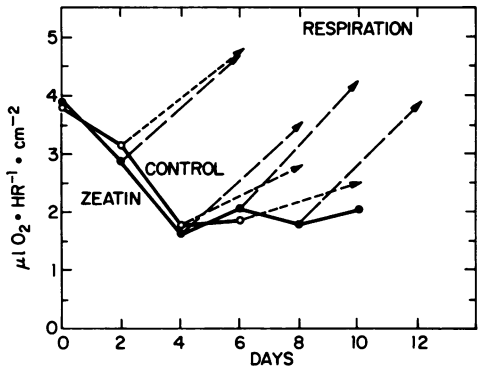

FIG. 4. Dark respiration rates in primary leaves of seedlings during senescence (O●) and after a 4-day recovery period (→). Rates were determined polarographically using an O_2 electrode. Seedlings were treated with 0 or 0.1 mM zeatin.

most probably reflect the fact that approximately 50% of the total soluble protein consists of ribulose bisphosphate carboxylase (13).

When recovery in total soluble protein is plotted against the level of protein prior to recovery (Fig. 3), it is evident that zeatin not only delays the loss of protein, but also increases the potential for recovery. Leaf sections from zeatin-treated seedlings demonstrated a greater net increase in total soluble protein/mg protein during recovery than control leaf sections at all stages of senescence. There is an apparent stabilization of the recovery capacity in treated tissues despite the general breakdown of protein. This is further illustrated in Table I where it can be seen that although both control and cytokinin treated seedlings demonstrate the same degree of recovery after 2 days in the dark, thereafter their response is much different. Control seedlings lose their capacity to undergo a net increase in protein recovery at a linear rate similar to the rate of loss of total soluble protein (Fig. 2). Cytokinin-treated seedlings, although also exhibiting a nearly linear loss of soluble protein, do not demonstrate a loss in net recovery of protein for up to 10 days in the dark.

Most recent work suggests that cytokinins retard senescence by inhibiting protein degradation (6, 7, 18). Our results do not refute this hypothesis, although they do indicate that cytokinins maintain the capacity for protein recovery. Whether this recovery is maintained by inhibiting breakdown of proteins (7, 18), stabilizing the components of protein synthesis (2, 5), or increasing protein synthesis *per se* (22) is not evident from the present data.

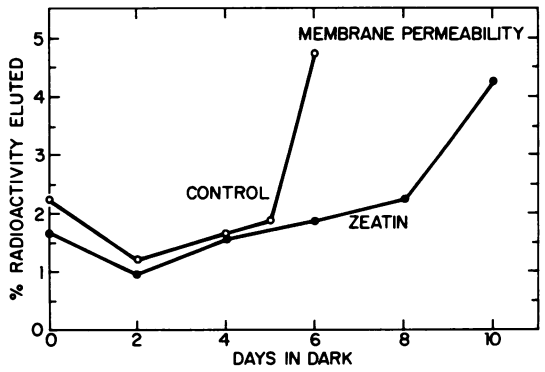

FIG. 5. Per cent efflux of radioactivity from sections of primary leaves taken from control and treated (0.1 mM zeatin) seedlings after various periods in the dark. Sections were incubated on 5 mM glucose (1 μCi/ml) for 1.5 hr, rinsed three times, and eluted for 30 min.

Recently emphasis has been placed on the role of cytokinins in maintaining tight coupling of respiration (19) and in maintaining membrane permeability (14) during senescence. Hence the influence of zeatin on both processes in the intact wheat seedling system was studied. Zeatin treatment had no significant effect on the rate of dark respiration during senescence (O●), though it did have a pronounced effect on recoverability (→) (Fig. 4) similar to that observed for photosynthesis (Fig. 2). Control tissue did not fully recover after 4 days, whereas treated tissue showed complete recovery even after 8 days in the dark. The reason for the lack of an effect during dark-induced senescence followed by an effect on recovery may be the result of substrate availability for respiration. When plants are placed in the dark their substrate supply is slowly used up, thereby resulting in a decline in respiration rate. Inasmuch as cytokinin-treated seedlings are capable of complete recovery of photosynthetic rates after 8 days in the dark they are also capable of renewing initial substrate levels, whereas control seedlings lose this ability after 2 days in the dark.

A loss of membrane permeability has been shown to occur during senescence (16). Hence, the change in permeability to substrate was observed to see how closely it corresponded with the loss of ability of seedlings to recover. The data (Fig. 5) indicate a marked change in permeability during senescence; however, the change did not occur until after 6 days in the dark. Calcium (5 mM $CaCl_2$), which is known to stabilize membranes, only influenced the loss of radioactivity after the initial loss in permeability had occurred. From the aforementioned results on recovery, this change would appear to occur too late to have a causal role in senescence. It would seem to be correlated with senescence, since zeatin treatment delayed the loss in permeability (Fig. 5).

In summary, these data indicate that senescence in the intact wheat seedling system is completely reversible for up to 2 days. Between 2 and 4 days, an irreversible stage in senescence is initiated. Cytokinins delay senescence and delay the onset of the irreversible phase. They apparently act by delaying the loss of total soluble protein and by maintaining the protein recovery capacity of the tissue. Zeatin seemed to have no direct effect on respiration, and although treatment did influence membrane permeability to substrate, the change in permeability occurred too late to have a causal role in senescence. Since the soluble protein fraction, which consists of many essential enzymes, responds rapidly to induced senescence and recovery, the loss of one or more of these enzymes may well determine the onset of irreversibility.

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