Plant Physiol. (1985) 78, 29–33
0032-0889/85/78/0029/05/$01.00/0

Interaction between Senescence and Wounding in Oat Leaves[1]

Received for publication September 18, 1984 and in revised form December 24, 1984

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ABSTRACT

A study was made of the influence of wounding on the senescence of standard oat leaf segments in the dark. Wounding was by either subdividing the 3 centimeter long segments into 5 millimeter subsegments, gently scraping the adaxial surface of the segments with a sharp blade, making transverse linear cuts, or by making many small holes with a needle. Wounding considerably delayed the loss of both chlorophyll and protein in the dark and the amount of inhibition was roughly proportional to the intensity of wounding. With surface wounding, the inhibition of senescence was detectable from the first day of dark incubation; other methods caused moderate promotion of senescence for the first 2 days but decreased the loss of chlorophyll and protein thereafter. A number of senescence-modifying substances acted similarly on both unwounded and wounded segments, but the amount of chlorophyll and protein in the wounded segments was always more than in the respective controls. Cytokinins, however, provided an exception, since their effect was actually decreased by wounding. The proteases operating at pH 4.1 and 6.6 were both clearly less active in the wounded leaves than in controls. The possible mechanism of this inhibitory effect of wounding on senescence is discussed.

Foliar senescence has been studied in many laboratories by following the loss of Chl, proteins, and nucleic acids and the corresponding accumulation of free amino acids or amino nitrogen. The choice of plant has varied, including monocots and dicots, and some studies have been made with leaves attached to the plant, but more with the leaves detached. In the latter case, discs or segments cut from larger leaves have often been used. However, when such areas are cut out of larger leaves, they are then surrounded by wounded cells and the resulting wound effects could well interfere with the changes due to senescence proper. Where the effects of wounding have been studied, as in plant storage tissues, they have been found to include changes in ultrastructure and biogenesis of cell organelles, stimulation of cell division, and changes in metabolic pathways (1). The concept of a 'wound hormone,' introduced by Haberlandt as long ago as 1913, and given experimental support by Reiche (14), led later to Wehnelt's work with bean pods and the use of his bioassay by Bonner and English (2) for the isolation of traumatic acid. But these later studies have not been concerned with leaves, and the role of traumatic acid remains unclear. The only clear relationship between wounding and the senescence of leaves so far elucidated has been that due to 'wound ethylene' (23). Apparently, however, the production of ethylene by leaves due to wounding is usually small in amount and relatively short-lived (see "Discussion").

For these reasons, it seemed important to explore the role, if any, that wounding plays in the syndrome of leaf senescence. From the beginning of our work the long narrow monocotyledonous leaf was selected, partly because its use minimized the amount of wounding as compared to that in discs cut from the leaves of dicots. Thus, in a 1-cm diameter disc the length of the wounded edge is about 3.15 cm and the area of leaf is 0.8 cm^2—a ratio of about 4. In a 3-cm oat leaf segment 5 mm wide, the corresponding figures are 1 cm and 1.5 cm^2 or a ratio of 0.66. Not only has the disc some 6 times as much wounded tissue per leaf area, but since the effect of wounding is known to extend three or four cells in from the cut surface the actual ratio is larger still.

In our experiments with leaf segments floating on water or test solutions in the dark, with loss of about 75% of the Chl in 4 d, it was noticed that any minor injury of the segment due to handling caused a slight retention of Chl on that part, while the rest of the segment senesced normally. There was also a narrow region 1 to 2 mm wide along the cut edge where some Chl tended to be retained. A series of studies on the interaction of wounding with leaf senescence has therefore been initiated.

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* cv. Victory) supplied by Svalöf AB, International Division, Svalöv, Sweden, were presoaked in water and sown in vermiculite. The seeds were grown under continuous light from daylight fluorescent lamps giving 30 $\mu E\ m^{-2}\ s^{-1}$ at plant level and at 25°C. Three-cm long subapical segments from 3 mm below the tip were cut from the first leaves of the 7- to 8-d-old seedlings and floated on distilled H_2O or test solutions in the dark for up to 4 d.

Extraction and Estimation of Chl and Protein. The leaf segments were extracted with boiling 80% ethanol for 20 min and Chl was estimated by absorption at 660 nm. Protein was extracted from the segments by 1 N NaOH in a boiling water bath for 30 min and estimated by the method of Lowry *et al.* (11).

Methods of Wounding. Mechanical wounding of the segments was done by four different methods: (a) the 3-cm segments were subdivided into 5-mm subsegments; (b) the adaxial surface of the segments was gently scraped with a sharp blade; (c) 50 linear wounds were made lightly across the segments without severing them; (d) 50 roughly equidistant punctures were made with a sharp needle.

Extraction and Determination of Proteases. The procedure was based on work of Drivdahl and Thimann (6). The segments were chilled, minced with scissors, and homogenized with a precooled pestle and mortar in a 0.05 M phosphate-citrate buffer (pH 6.0) containing 5 mM sodium metabisulfite. The crude homogenate was filtered through four layers of cheesecloth and centrifuged at 25,000*g* for 60 min. All operations were carried out at 0 to 4°C.

For the protease assay, the clear supernatant was allowed to

[1] Supported in part by a grant from the National Science Foundation to K.V.T. (No. PCM 80-21584).

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act on acid-denatured hemoglobin (Sigma Chemical Co.). The reaction medium for the acid protease contained 1.25 ml of 0.05 M sodium acetate buffer (pH 4.1), 0.25 ml of 4% hemoglobin suspension, and 0.25 ml of the supernatant. For neutral protease, the reaction mixture contained 1.5 ml of 0.05 M phosphate-citrate buffer (pH 6.6), 10 mM β-mercaptoethanol, 0.25 ml 4% hemoglobin suspension, and 0.25 ml of the supernatant. After 90 min in a shaking water bath at 50°C, the reaction was terminated by adding 0.25 ml of 40% TCA. For blanks, the TCA was added prior to addition of the enzyme solution. Insoluble protein was removed by centrifugation and free amino nitrogen determined in the supernatant by the method of Moore and Stein (13).

RESULTS

It has often been observed that, after 4 d in darkness when most of the visible Chl has disappeared, a thin greenish line 1 to 2 mm wide remains along both of the cut edges. To investigate this effect further, the four types of wounding described above were inflicted. When the 3-cm segments were subdivided into 5-mm segments, the cut edges of each subsegment showed distinct retention of Chl after 4 d in the dark. When the surface of the segments was scraped, so that the wounding was uniform throughout, the whole segment remained green. When the wounding was done by methods (c) and (d), retention of Chl close to the cuts or punctures was clearly evident. Data on the Chl content of the ethanol extracts in such an experiment are presented in Table I. While the controls retained only some 25% of the initial Chl content, all the wounded segments retained much larger fractions. The extent of inhibition of Chl loss was evidently roughly proportional to the area wounded, the punctures having the smallest effect.

Since the loss of Chl during foliar senescence in the dark is accompanied by (and perhaps due to) the hydrolysis of proteins, determinations of protein content in parallel with that of Chl, after 4 d in the dark, are included in Table I. While 75% of the initial Chl content was lost in the controls, the protein loss was only 54%. All four types of wounding acted in about the same way, scraping the surface of the segments being the most effective and puncturing the least. Although the accumulation of free amino nitrogen has been most frequently reported on in the earlier work (17), the wounded segments underwent such marked exudation of nitrogen into the medium that values for the free α-amino nitrogen were unreliable.

Table II shows that scraping the basal 1.5 cm of the segments is more effective for retention of Chl than similar scraping of the apical 1.5 cm. The difference in protein content, though small, suggests the apex-to-base transport of the free amino nitrogen described earlier (22).

The time course of the loss of Chl from intact and wounded (method b) segments is shown in Figure 1A. The loss of Chl is slight or zero during the 1st d (*cf.* Fig. 1 of Ref. 12), but thereafter the Chl content decreases rapidly to reach about 30% of the initial value by day 4. In the subdivided segments (method 3), the rate of Chl loss was somewhat different, as shown in Figure 1B. Wounding caused an initial modest promotion of the loss of Chl during the first 2 d, but by day 3 when the Chl continues to decrease in the controls, further decrease of Chl is arrested in the wounded segments. Thus, on day 3 the Chl contents of the control and wounded segments were comparable.

Figure 2 shows the time course of the loss of protein from intact segments and those wounded by methods a and b. The controls show the usual daily decrease while the scraped segments showed inhibition of the decrease from the start, as with the Chl in Figure 1A. The subdivided segments (method a) showed smaller effects, but the pattern of protein decrease was comparable with that of Chl, with a cross-over point on day 3. We conclude that wounding, irrespective of the method used, causes clear inhibition of the senescence process.

Wounding in the Presence of Agents That Modify Senescence. The ways in which foliar senescence is modified by metabolic inhibitors, chelators, metals, hormones, and atmospheric gases have been extensively studied in several laboratories (see review in 20). When our experiments made it clear that mechanical wounding could considerably delay foliar senescence, an exploration of the effects of those substances on the senescence of wounded leaves was undertaken. The results are shown in Tables III and IV. Table III shows that three compounds that have been reported to promote Chl loss, namely ACC,[3] ABA, and methyl jasmonate, had the same effect when the segments were wounded, but in each case the Chl content was clearly increased (*i.e.* the loss due to senescence was reduced) by the wounding. Similarly, five compounds, namely CHI, Sm, Spd, EDTA, and DiPyr, all of which delay Chl loss in the dark (8, 20), delayed Chl loss also in wounded segments. Again, however, the Chl values in wounded segments were much higher than in their respective unwounded controls. The combination of wounding with CHI or α,α-DiPyr, indeed, results in the almost complete stability of Chl for 4 d in the dark. Thus, the Chl retaining capacity due to wounding is still present no matter which reagent is used. On the other hand, the two cytokinins tested provide an exception. Both strongly inhibited the loss of Chl in control segments, but failed to do so appreciably in the presence of wounding. Traumatic acid (Δ-9,decene-1,10-dicarboxylic acid) was completely without effect.

The modifications in protein loss caused by wounding in the presence of a similar group of reagents are shown in Table IV. In general, the differences in protein loss due to wounding were comparable to those in the loss of Chl. With the exception of kinetin and, in this case, methyl jasmonate, the protein contents

[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CHI, cycloheximide; Sm, spermine; Spd, spermidine; DiPyr, α,α-dipyridyl; PPIF, Proteinase Inhibitor-Inducing Factor.

Table I. *Effects of Four Types of Wounding on the Loss of Chl and Protein in Detached 3-cm Oat Leaf Segments*

Data were taken after 4 d in darkness.

Type of Wounding	Chl		Protein	
	μg/segment	*% of initial*	*μg/segment*	*% of initial*
Initial values	41.1	100	538	100
Control, unwounded	10.6	25	250	46
Wounded				
Method a	19.8	48	338	63
Method b	25.6	62	375	70
Method c	23.2	56	338	63
Method d	16.0	39	263	49

Table II. *Effect of Scraping the Apical or Basal Half (1.5 cm) of the Segments*

Data were taken after 4 d in darkness.

Procedure	Chl	Protein
	μg/segment	
Initial values	41.0	531
Control, unwounded	10.0	204
Whole segment scraped	21.0	325
Apical half scraped	13.2	251
Basal half scraped	19.5	285

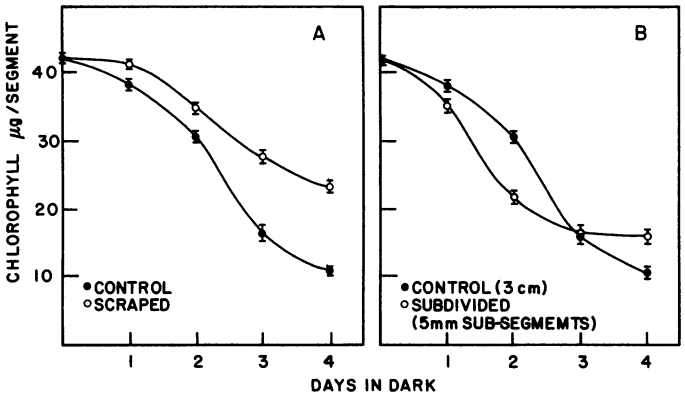

FIG. 1. A, Time course of Chl loss from 3-cm oat leaf segments on water in the dark at 25°C. (●), Controls; (○), adaxial surfaces gently scraped. B, As A, but segments subdivided into six equal pieces (○). Vertical bars, probable errors of the means (four experiments).

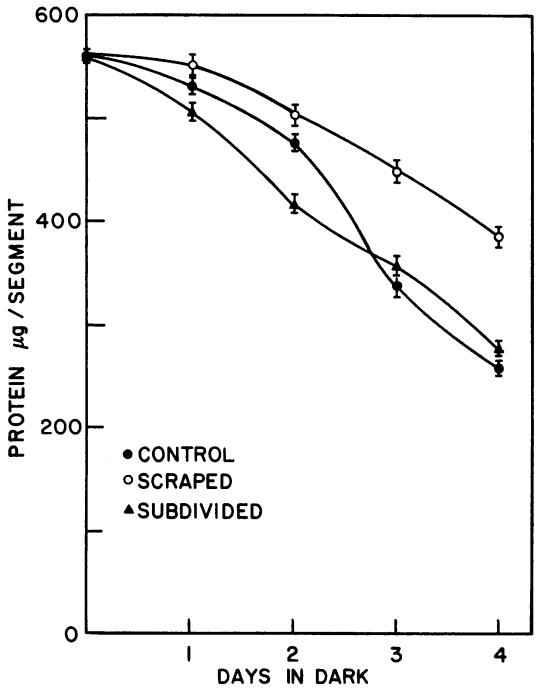

FIG. 2. Time course of protein loss from 3-cm segments similar to Figure 1. (●), Controls; (○), adaxial surfaces gently scraped; (▲), segments subdivided into six equal pieces.

of the wounded segments were higher than in their respective unwounded controls in all cases, whether the reagents were promoters or inhibitors.

The Effects of Wounding on the Development of Proteases during Foliar Senescence. The decrease in the loss of protein shown above indicates that the wounding may influence the activities of proteases. The protease activity at pH 4.1 and 6.6 was accordingly determined as described in "Materials and Methods." Table V shows that in controls the activity of the acid protease increased almost 3 times and that of the neutral protease 7 times by the 4th d in darkness. When the segments were wounded (method b), the activities were only one-half as great. Thus, wounding decreases the activity of both types of protease.

Table III. *Effect of Wounding (Method a) on Chl Loss by 3-cm Oat Leaf Segments in Presence of Senescence-Modifying Substances*

Treatment	Concn.	Chl Content	
		Intact	Subdivided (6 × 5 mm)
	mM	*μg/segment*	
Initial value		39.6	
Water—dark control		10.2	17.4
ACC	0.2	8.2	16.2
ABA	0.05	8.6	15.4
Methyl jasmonate	0.05	6.8	10.2
CHI	0.10	26.6	38.2
Sm	1.0	18.4	30.4
Spd	1.0	18.0	28.0
EDTA	5.0	16.0	24.0
DiPyr	0.10	33.6	35.0
Kinetin	0.10	34.0	14.8
Benzylaminopurine	0.10	38.0	18.0

DISCUSSION

That wounding should delay or inhibit the senescence process is somewhat surprising, since a number of general indications could lead one to expect the reverse. Many seedlings if wounded on one side undergo curvature toward that side, showing that wounding reduces the growth rate locally. This was one of the findings of the early work on growth rates and their control (see *e.g.* 5, 8) and it received its explanation from the discovery that auxin is destroyed in wounded tissue (19). Comparable evidence is not available for other hormones. But in any case senescence does not involve growth, although it is indeed an active process, since it depends on protein synthesis and stomatal movement, consumes energy, and is strongly modified by visible light.

It is well-known that wounding induces the formation of ethylene in most tissues, and this is equally true of leaves. Indirect evidence of the production of ethylene by chopped, shredded, and infected leaves was plentiful in the earlier literature and this has been summarized by Burg (3). However, most of the dramatic increases often recorded as due to cutting or wounding are given by fruit tissue, and this is especially true of those cases where the emanation was identified as ethylene. Leaves of *Nymphoides*, on detachment, show a rise in ethylene from an initial value of 4 to a peak at 8 h of 30 nl g^{-1} fresh weight h^{-1}, but it falls rapidly thereafter (7). Simple rubbing of bean hypocotyls gives rise to an 'elicitor' which can be eluted from the tissue without crushing, and causes production of ethylene for about 12 h (16). But such effects of relatively short duration would not fit well with the slow onset of the wound effect shown in Figure 1B. A few preliminary determinations of ethylene evolution by scraped oat leaves have shown increases averaging no more than 50% above the normal small output, and these are limited to the first 24 h. However, what more convincingly eliminates ethylene as a cause is the fact that ethylene acts to promote or accelerate senescence (especially in the light) rather than to delay it as here. Peeling has indeed been shown to release ethylene, as well as to have important effects on senescence (10), but those effects probably rested on an entirely different basis, namely the removal of

Table IV. *Effect of Wounding on Senescence of Oat Leaf Segments in the Dark in Presence of Senescence-Modifying Reagents*

Wounding was by scraping the surface (method b).

Treatment	Concn.	Chl		Protein	
		Intact	Scraped	Intact	Scraped
	mM	*μg/segment*			
A. Data after 3 d in darkness					
Initial Value	—	40.0		583	
Water	—	15.0	30.1	283	356
CHI	0.10	25.0	40.0	317	350
ABA	0.05	12.0	25.0	225	275
Kinetin	0.10	32.0	20.0	475	433
B. Data after 4 d in darkness					
Water	—	12.0	22.0	256	305
DiPyr	0.10	42.0	43.0	506	512
Methyl jasmonate	0.10	7.5	17.0	293	250
ACC	3.0	8.0	20.0	218	275
Sm	1.0	16.0	27.5	281	368

Table V. *Development of Protease Activities in the Control and Wounded Leaf Segments Data after 4 Days in Darkness*

	Acid Protease	Neutral Protease
	nmol/h·mg protein	
Initial value	23	10
Control	63	70
Wounded (method b)	35	35

stomatal resistance (21).

A wound-induced agent nearer to the senescence process than the known plant hormones is the PIIF brought to light by Ryan and his co-workers (18). In that work, it was shown that a proteinase inhibitor is produced as a result of wounding and that it is formed in a leaf adjacent to the leaf that was actually wounded (9). The timing is nearer to that observed in our experiments, since the inhibitor continues to accumulate for up to 100 h. The transport is ascribed to an inducing factor, which appears to be a polysaccharide of mol wt more than 5000, and causes inhibition of the proteinase within a few hours (15). The direct evidence that leaf proteinases are in fact inhibited in our experiments (Table V) strengthens the possibility that the wound response described does result from formation of a proteinase inhibitor. However, the inhibitor in oat leaves cannot be exactly the same as Ryan's PIIF because the latter is accumulated only in light (9). Furthermore, the similarity is somewhat weakened by another fact, namely that a study of the leaf proteins by gel electrophoresis (data not presented here) shows that wounding does not simply retain existing proteins but also involves the appearance of some new ones. Formation of new proteins could explain the inability of the cytokinins to operate in the presence of wounding, since by maintaining some of the original protein they would decrease the level of amino acids needed for synthesis.

The conclusion that changes in proteins are in any event the dominant factor in the wound effects here described has an important corollary, namely that Chl destruction in senescence is dependent on proteolysis, *i.e.* that Chl becomes susceptible to enzymic destruction when it is liberated from its protein-bound state in the thylakoid. That deduction was first made when proteolysis was found to precede Chl loss in oat leaves by some 20 h (12), and was supported when isolated chloroplasts, in which Chl is much more stable than in the intact leaf, were found to undergo only very slight proteolysis (4). The unfortunate fact that the effective proteolysis inhibitors, like leupeptin or the PIIF, are molecules too large to be taken up into living cells makes it difficult to get unequivocal evidence for that dependence at present.

A number, though not all, of the reagents reported to delay senescence in the dark have been found to exert the opposite effect, *i.e.* to promote senescence, in white light. Wounding effects show several comparable reversals of action in light, and these will be the subject of a separate communication.

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