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Role of Ethylene in Chlorophyll Degradation in Radish Cotyledons

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Abstract. The effect of age of radish seedlings on changes in chlorophyll concentration caused by ethylene was examined. Ethylene was produced at 2–4 nl g^{-1} h^{-1} following excision of cotyledons from 5- to 20-day-old seedlings. The youngest cotyledons maintained this rate, whereas ethylene synthesis declined by as much as 80% during a 24-h period in older cotyledons. The youngest cotyledons continued to accumulate chlorophyll in the dark, but after 7 days cotyledons lost chlorophyll and the proportion of chlorophyll lost increased with age. Ethylene promoted, and norbornadiene inhibited, this loss of chlorophyll; in combined treatments the effects of ethylene and norbornadiene were competitive. The maximal rate of chlorophyll loss occurred in 1 μl L^{-1} ethylene; extrapolation of the response to concentration indicated that half-maximum loss would occur at 0.005–0.01 μl L^{-1} ethylene. In cotyledons from 20-day-old seedlings, chlorophyll degradation occurred mainly after 24 h from excision and transfer to the dark. Chlorophyll degradation during 48 h in the dark was affected by norbornadiene or ethylene applied from 0–24 h or from 24–48 h.

Plant responses to phytohormones may result from changes in concentration or changes in "sensitivity" (Firn 1986, Trewavas 1982). Although there has been extensive debate about this subject (Hoad et al. 1987) there are too few examples in the literature to support a general conclusion that hormone concentration is more or less important than sensitivity in the regulation of plant development. In addition, the more fundamental question whether an aspect of development is strictly dependent on a phytohormone is often unresolved. Jackson (1987) has proposed criteria to establish dependence and, particularly, that changes in hormone concentration are involved. These questions about involvement of plant hormones need to be resolved in order to develop strategies for practical regulation of crop plant growth and development. Earlier work demonstrated that low concentrations of ethylene become effective in inducing ethylene synthesis during development of apple fruit (Knee et al. 1987). This paper is concerned with the development of a response to ethylene in radish cotyledons. In addition to testing the effects of exogenous ethylene, it was important to measure endogenous concentrations in order to assess their potential role.

Chlorophyll degradation is a visible and convenient indicator of leaf senescence. The promotion of chlorophyll degradation by ethylene treatment of leaf tissue is well known, but the role of endogenous ethylene in leaf senescence is still in doubt (Roberts et al. 1985). Ethylene synthesis rises late in the senescence of some leaves, but "the possibility that endogenous ethylene plays a considerable role in the regulation of leaf senescence, even in its early stage" is not excluded (Aharoni et al. 1979). Recently, Zacharias and Reid (1990) showed that leaf discs from an ethylene-resistant line of *Arabidopsis thaliana* retained their chlorophyll longer than wild-type discs, irrespective of the presence or absence of illumination or ethylene during incubation. Thus, lowering the capacity to respond to ethylene did retard senescence in this species.

Radish cotyledons are convenient for the study of senescence because reproducible material of known age is readily available and senescence occurs rapidly in the dark (Kawakami and Watanabe 1988). Preliminary work showed that a commonly used inhibitor of ethylene synthesis, amino ethoxyvinylglycine, promoted chlorophyll loss in radish cotyledons (Knee 1989). A commonly used inhibitor of ethylene action, silver thiosulfate, inhibited chlorophyll loss, but this effect was not reversible by ethylene treatment; in terms of Jackson's (1987) criteria for hormonal involvement, "deletion and reinstatement" of ethylene action could not be

Table 1. Chlorophyll concentration and fresh weight of cotyledons from greenhouse-grown radish seedlings and their ethylene production during incubation in the dark.

Seedling age (days)	Chlorophyll (μg/concentration cotyledon)	Weight (mg/cotyledon)	Ethylene production (nl g^{-1} h^{-1})			
			1 h	4 h	27 h	51 h
5	5.9	12	8.38	1.44	1.89	2.26
10	29.9	54	3.28	2.54	1.80	1.02
15	44.6	67	3.58	1.27	0.94	0.40
20	63.6	98	2.29	0.71	0.28	0.15

The probabilities of null effects on ethylene production were 0.0001 for seedling age, 0.0001 for time of incubation, and 0.0208 for their interaction.

demonstrated with this inhibitor. Norbornadiene has not commonly been used in experiments on leaf senescence, since its introduction by Sisler and Pian (1973). However, inhibition of chlorophyll breakdown in radish cotyledons by this compound was fully reversible by ethylene (Knee 1989). Norbornadiene has also been shown to be a specific inhibitor of ethylene action in promotion of growth of deep water rice (Bleecker et al. 1987), inhibition of growth of etiolated pea (Sisler and Yang 1984), and senescence of carnation flowers (Wang and Woodson 1989).

Materials and Methods

Radish (*Raphanus sativus* L. cv "Sparkler White Tip") seedlings were grown in a "peat-lite" mix (Metromix 250, W.R. Grace Co.) in 20 row seedling flats in a greenhouse at 20–25°C with ambient illumination. Alternatively, seedlings were grown in the bases of 15 × 60 mm petri dishes in a growth room at 20°C with cool white fluorescent tubes providing 12 h illumination/day at 2000 lux. For up to 10 days' growth 20 seeds were placed in the dish which was then filled with horticultural vermiculite and 15 ml water was added.

For ethylene and norbornadiene treatment, excised cotyledons (20) were held in 15 × 60 mm petri dishes on Whatman 1 paper with 0.5 ml water. Excised cotyledons or intact seedlings were put in 2-L capacity Mason jars, also containing a petri dish with 2 ml M NaOH to absorb CO_2, and kept in the dark in a room at 20°C usually for 48 h. When no ethylene was to be applied, 10 g of an ethylene absorbent (Purafil) was enclosed in the jars; however, this was not done in experiments involving norbornadiene. Ethylene was injected into the jars to give the desired concentration, and norbornadiene was applied as a liquid to a filter-paper circle in the jar, immediately before sealing. The chlorophyll content of cotyledons was measured by spectrophotometry (Arnon 1949) after homogenization in 80% aqueous acetone and centrifugation. Ethylene production by 20 excised cotyledons was measured after enclosure in a 50-ml syringe also containing a circle of filter paper wetted with 0.5 ml water. To measure ethylene production by intact seedlings, a petri dish with seedlings was enclosed in a 220-ml capacity Mason jar. Internal ethylene concentrations were measured after extraction of tissue under vacuum (Beyer and Morgan 1970). Ethylene was analyzed by gas chromatography on a 500 × 0.4 mm column of alumina (80–100 mesh) at 100°C with nitrogen carrier gas at 60 ml min^{-1} and a flame ionization detector. This was calibrated by reference to a commercial standard mixture of ethylene in air.

Most data are means of duplicate analyses but means of triplicates are shown in Table 4 and pooled observations for seedlings and detached cotyledons in Fig. 2. The significance of effects of treatments was estimated by analysis of variance. Interactions were analyzed only if main effects were significant ($p < 0.05$). Ethylene production data were transformed to logs before analysis and the means shown in Tables 1–3 were backtransformed.

Results

Ethylene concentrations were measured in all jars containing cotyledons or seedlings. When no exogenous ethylene was added, the concentration represents that produced by the tissue. Even when this was absorbed by permanganate, a concentration gradient would have existed so that tissue contained a minimal level of ethylene. Knowing the rate of production (*EP*, μl g^{-1} h^{-1}) and the internal ethylene concentration (*EC*, μl L^{-1}), a resistance factor ($R' = EC/EP$, g h L^{-1}) could be calculated. This is equivalent to the internal concentration (μl L^{-1}) at a production rate of 1 μl g^{-1} h^{-1}. For intact 3-day-old seedlings R' was 35–36 g h L^{-1} and for cotyledons from 20-day-old seedlings 54–71 g h L^{-1}.

Radish cotyledons continued to expand and gain chlorophyll on a per organ basis up to 20 days after sowing (Table 1). Ethylene production by freshly cut cotyledons was high; production declined during 48-h incubation and, in general, the rates were inversely proportional to the age of seedlings (Table 1). Ethylene production by intact seedlings was high at the beginning but declined during incubation in the dark (Table 2). The extent of chlorophyll loss during dark incubation increased with the age of seedling from which cotyledons were excised (Fig.

Table 2. Chlorophyll concentration and fresh weight of cotyledons, fresh weight of intact seedlings of radish grown in the growth room, and ethylene production by seedlings during a 2-day incubation in the dark.

Seedling age (days)	Chlorophyll (concentration μg/cotyledon)	Weight (mg/cotyledon)	Weight (mg/seedling)	Ethylene production (nl g^{-1} h^{-1}) on day		
				0	1	2
5	4.6	9	96	1.53	1.51	0.95
7	8.6	11	112	2.16	0.94	1.01
10	10.4	13	137	0.77	0.39	0.38

The probabilities of null effects on ethylene production were 0.092 for seedling age and 0.170 for time of incubation.

1). Exogenous ethylene, up to 10 μl L^{-1}, accelerated the loss of chlorophyll to about the same extent for cotyledons from 10-, 15-, and 20-day-old seedlings (Fig. 1).

Cotyledons from 5-day-old seedlings showed a slight gain in chlorophyll during incubation in the dark (Fig. 1). Further experiments with detached cotyledons and intact seedlings from 3–5 days old confirmed that this increase occurred but there was not a consistent effect of ethylene (data not shown). A factorial experiment involving seedlings of three ages, incubation of detached cotyledons or intact seedlings, and presence or absence of ethylene and norbornadiene was conducted. Analysis of variance showed that there was no significant effect of excision on changes in chlorophyll in cotyledons. The experiment showed a transition in chlorophyll changes from an increase in cotyledons from 5-day-old seedlings to a decline in 10-day-old cotyledons (Fig. 2). The presence of 1000 μl L^{-1} norbornadiene enhanced the increase or diminished the loss of chlorophyll. Ethylene at 10 μl L^{-1} had the opposite effect and reversed the effects of norbornadiene.

Data from five experiments involving cotyledons from 20-day-old seedlings and a range of ethylene concentrations were combined (Fig. 3). Data were normalized with respect to the maximum loss of chlorophyll, observed in each experiment, which occurred at 1–10 μl L^{-1} ethylene. A gradient of ethylene concentration was assumed to exist so that cotyledons contained 30 nl L^{-1} more ethylene than their surrounding atmosphere. This was derived from R' and the average rate of ethylene production observed during a 48-h incubation of similar cotyledons. Over 50% of the maximum loss of chlorophyll was observed even at the lowest ethylene levels; extrapolation from a graph of relative chlorophyll loss and ethylene concentration suggested that 50% maximum loss would have occurred at 0.005–0.01 μl L^{-1} ethylene.

The interaction of norbornadiene and ethylene

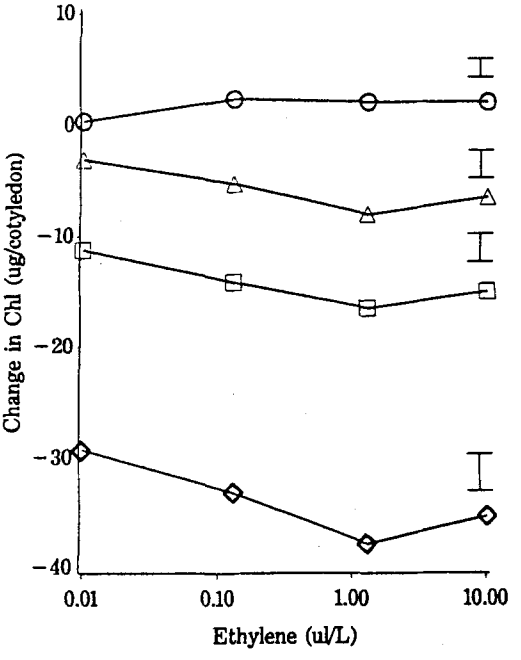

Fig. 1. Effect of exogenous ethylene concentration on changes in chlorophyll concentration during 48 h in the dark in cotyledons excised from greenhouse-grown radish seedlings of different ages: ○—○, 5 days; △—△, 10 days; □—□, 15 days; ◇—◇, 20 days. Vertical bars show the standard error of the difference between means (SEDM) for each seedling age. Control (nonethylene treated) cotyledons were assumed to contain 0.01 μl L^{-1} ethylene. The change in chlorophyll was calculated from the difference in concentrations at the beginning and end of incubation. The probability of null effects was 0.0001 for age of seedling and 0.009 for ethylene (when 5-day samples were excluded).

was investigated further with cotyledons from 20-day-old seedlings. Norbornadiene at 1000 μl L^{-1} stimulated ethylene production by at least 100% (Table 3) and inhibited chlorophyll loss by about 30% (Fig. 4). Approximately 1 μl L^{-1} ethylene restored the chlorophyll loss to that of the untreated control (Fig. 4). Most of the chlorophyll loss occurred during the second day of incubation in the

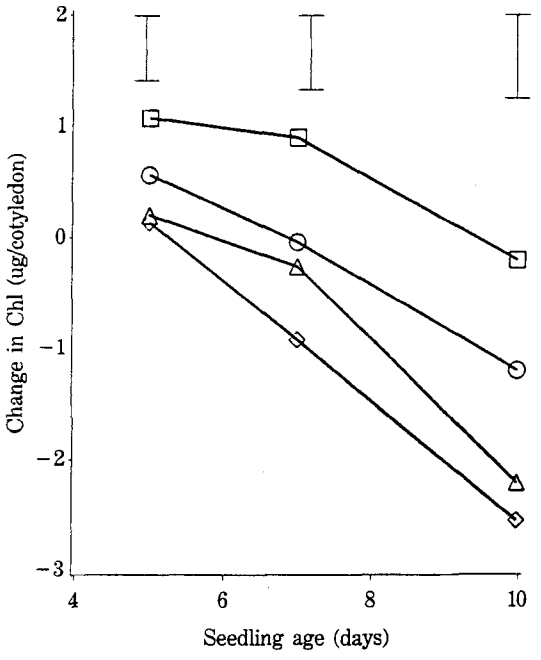

Fig. 2. Effect of seedling age, norbornadiene, and ethylene on changes in chlorophyll concentration in radish cotyledons (grown in the growth room) during a 48-h incubation in the dark (means from incubation of intact seedlings and detached cotyledons): ○—○, no additions; □—□, 1000 μl L^{-1} norbornadiene; △—△, 10 μl L^{-1} ethylene; ◇—◇, ethylene and norbornadiene. Vertical bars show SEDM for each seedling age. The probabilities of null effects were 0.0002 for age of seedling, 0.005 for ethylene, 0.54 for norbornadiene, and 0.53 for excision of cotyledons.

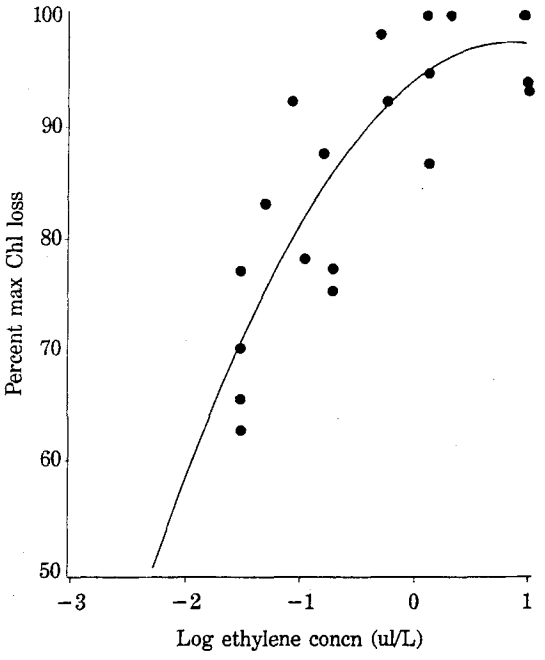

Fig. 3. Effect of concentration of ethylene on chlorophyll loss in cotyledons from seedlings grown 20 days in the greenhouse and incubated 48 h in the dark. Data from five experiments were pooled; chlorophyll loss is expressed as a percentage of the maximum recorded in each experiment. Untreated cotyledons were assumed to contain 0.03 μl L^{-1} ethylene in their intercellular spaces. Treated cotyledons were assumed to contain the exogenous concentration plus 0.03 μl L^{-1}. The line represents a fitted quadratic which had a slightly higher correlation (r = 0.86) than a linear regression (r = 0.82).

dark. Norbornadiene and ethylene were applied during the first or second day in all possible sequences. Norbornadiene or ethylene treatment during the second day had more effect on total chlorophyll loss than treatments during the first (Table 4). However, ethylene caused some chlorophyll loss in the first day and the presence of norbornadiene during the first day had a carry over effect on chlorophyll loss in the second day.

Table 3. Effect of 1000 μl L^{-1} norbornadiene on ethylene production, during a 48-h incubation in the dark, by cotyledons from radish cotyledons grown 20 days in the greenhouse.

Norbornadiene present		Ethylene production (nl g^{-1} h^{-1}) at				
0–24 h	24–48 h	1 h	4 h	24 h	28 h	47 h
−	−	3.33	3.22	0.72	0.58	0.20
−	+				0.37	2.32
+	+	5.53	7.63	1.84	1.01	0.87
+	−				1.22	0.66

The probabilities of null effects were 0.0039 for norbornadiene, 0.0002 for time, and 0.561 for their interaction.

Discussion

The tendency of radish cotyledons to lose chlorophyll on incubation in the dark increases with age, whereas ethylene production declines. The effect of decline in production on tissue ethylene levels could be offset by an increase in resistance to diffusion, but it is unlikely that higher ethylene concentrations occurred in older than in younger seedlings. If a change of ethylene concentration is excluded as an explanation of the change in extent of chlorophyll degradation, a change in sensitivity to ethylene is an alternate possibility. The response to exogenous ethylene may have increased between 5 and 10 days, but it remained constant from 10 to 20 days when the major increase of chlorophyll loss occurred in non-ethylene-treated tissue. This acceleration could have resulted from an increased response to endogenous ethylene. This raises the

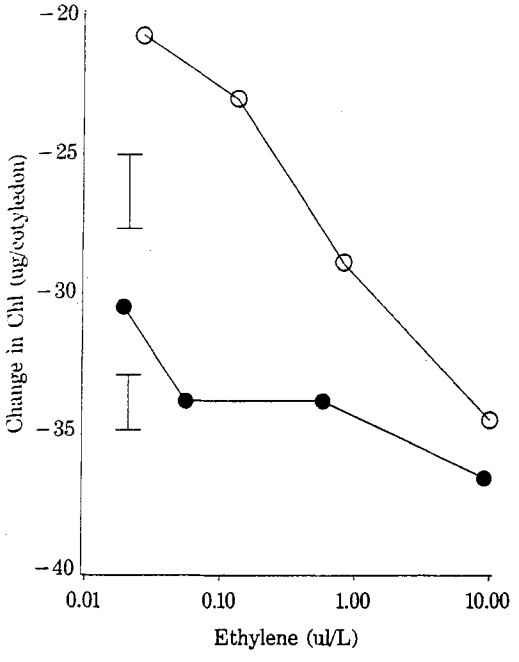

Fig. 4. Effect of ethylene concentration and 1000 μl L^{-1} norbornadiene on chlorophyll loss in cotyledons from radish seedlings grown 20 days in the greenhouse and incubated 48 h in the dark: ●—●, control; ○—○, norbornadiene. Vertical bars show SEDM for norbornadiene-treated and untreated cotyledons. The probabilities of null effects were 0.0059 for ethylene, 0.0010 for norbornadiene, and 0.231 for their interaction.

Table 4. Effect of 1000 μl L^{-1} norbornadiene and 2.5 μl L^{-1} ethylene, at different times during a 48-h incubation in the dark, on chlorophyll loss in cotyledons from 20-day-old greenhouse-grown radish seedlings.

	Change in chlorophyll (μg/cotyledon)			
		0–48 h after 24–48 h in		
Treatment 0–24 h	0–24 h	C	E	N
C	−0.9	−37.3	−42.7	−28.5
E	−5.0	−36.6	−39.8	−33.3
N	+0.8	−30.1	−33.9	−25.0

	Probability of null effect on change in chlorophyll		
		0–48 h after treatment for	
Treatment	0–24 h	0–24 h	24–48 h
E	0.174	0.222	0.013
N	0.448	0.032	0.005

C, control; E, ethylene; N, norbornadiene.

question whether endogenous ethylene regulates chlorophyll degradation. The estimated concentration of 0.005–0.01 μl L^{-1} ethylene for half-maximal chlorophyll destruction is lower than generally found for plant-ethylene responses (Goeschl and Kays 1975), but it is likely to be a high estimate. An internal ethylene concentration calculated from ethylene production rates in the absence of exogenous ethylene was added to the exogenous concentration recorded in the experiment. Since ethylene synthesis in vegetative tissue is inhibited by ethylene (Yang and Hoffman 1984) actual internal concentrations were probably lower than calculated. Relating chlorophyll loss to exogenous concentrations results in an even lower estimate of concentration for half maximum loss.

The role of endogenous ethylene was further tested by use of norbornadiene. The reversibility of norbornadiene effects by ethylene confirms that it is a specific probe for ethylene involvement. Its effect on chlorophyll levels at all stages of seedling development implies that endogenous ethylene affects the rate of chlorophyll degradation. The effectiveness of norbornadiene was offset by its promotion of ethylene synthesis. This is a consequence of the inhibition by norbornadiene of the well-known action of ethylene on its own synthesis (Yang and Hoffman 1984). The effects of norbornadiene on chlorophyll at all stages of seedling growth imply that the tissue was responding to endogenous ethylene at all times. Changes in the extent of chlorophyll degradation probably result from changes in capacity for chlorophyll metabolism rather than changes in sensitivity to ethylene.

One possible change in chlorophyll metabolism is a decreasing capacity for chlorophyll synthesis in the dark. This could have led to the increase in net chlorophyll degradation during seedling development. Ethylene seemed to affect chlorophyll degradation rather than chlorophyll synthesis; thus, a declining capacity for synthesis could result in increased response to ethylene even if the rate of degradation does not change. Kawakami and Watanabe (1988) report transcriptional changes in radish cotyledons after a 12-h incubation in the dark, but before a loss of chlorophyll was apparent. The effects of ethylene and norbornadiene present in the first 24 h on subsequent chlorophyll changes imply that the early events are ethylene sensitive and result in loss of chlorophyll.

These results reinforce the view of ethylene as an accessory factor or modulator rather than a dominant regulator in leaf senescence. Changes in the rate of senescence may not be associated with changes in concentrations of endogenous ethylene or sensitivity to ethylene. However, control of visible symptoms of senescence may yet be possible through the manipulation of ethylene physiology.

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