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The Positional Differentiation of Abscission Zones during the Development of Leaves of *Sambucus nigra* and the Response of the Cells to Auxin and Ethylene

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Summary. Abscission in the leaf rachis of *Sambucus nigra* L. is preceded by a positional differentiation of zone cells that enlarge and separate in response to ethylene but not to auxin. These cells are absent from youngest leaves, and such leaves do not abscind even in ethylene; other cells of the immature rachii will enlarge in response to auxin. These two classes of target cells are always recognisable by their opposing responses to auxin and ethylene. Prior to separation zone cells exposed to ethylene show considerable activation of the cytoplasm, many polysomes, elongate endoplasmic reticulum and highly dilated dictyosomes with many associated vesicles. Treatment with auxin precludes these changes, and abscission is always retarded: high levels of ethylene must be added to overcome the auxin inhibition. The differentiation of zone cells and their ethylene-stimulated growth and activation are prerequisites for rachis abscission in *Sambucus*. Such cell development may be of general occurrence prior to organ abscission in plants.

Introduction

In previous papers we have shown that special target cells are differentiated at leaf abscission zones, and that these cells differ from their neighbours in that they are stimulated to renewed expansion growth in response to ethylene, but not to auxin. In the mature primary leaf of *Phaseolus vulgaris* var. Canadian Wonder, for example, (Wright and Osborne, 1974) one row of such cells is positionally differentiated at the proximal side of the junction of pulvinus and petiole, and the enlargement of these cells in response to a critical level of ethylene is instrumental in evoking the shearing force that separates the petiole from the pulvinus above. A second row of cells, adjacent to the first, on the pertiole side shows a limited growth response to ethylene but only after abscission has occurred. In intact plants, ethylene produced by senescing distal tissue (Jackson and Osborne, 1970) – or by other nearby tissue in response to environmental stress (McMichael, Jordan and Powell, 1972; El-Beltagy and Hall, 1974) – is presumably responsible for the stimulation of abscission.

In senescing Atumn leaves of *Sambucus nigra* (Osborne and Sargent, 1976) a band of some 30–40 rows of flattened cells can be distinguished in the abscission zones of the rachis at the point of insertion of the leaflets. Ethylene produced by the yellowing leaflets (or applied ethylene), induces these special cells to expand and renewed cytoplasmic activity is seen in what previously appeared to be quiescent cells of the rachis abscission zone. In particular, dictyosomes produce numerous vesicles and the secretion of the intact vesicles or their contents through the plasmalemma or the branched plasmodesmata precedes dissolution of the middle lamellae and cell separation. As with *Phaseolus,* these special cells do not enlarge, nor does abscission occur, when the tissue is supplied with auxin. We have proposed that the positional differentiation of these special ethylene-sensitive cells could be the essential prerequisite for the formation of an abscission zone, and that without them abscission can not occur (Wright and Osborne, 1974; Osborne, 1973, 1974, 1976; Osborne and Sargent, 1976).

From a study of the growth and ultrastructure of the abscission zone region of the rachis of *Sambucus nigra* during the development of leaves from bud break until senescence, we now present evidence for the timing of the positional differentiation of the special ethylene-sensitive target cells, and confirm that until these target cells are seen to be present, abscission cannot be induced even by prolonged exposure to high concentrations of applied ethylene.

Abbreviation: IAA = indole-3yl-acetic acid

Materials and Methods

Buds and leaves were harvested from a wild stand of *Sambucus nigra* from the time of earliest bud break (April) until leaf fall (November). Two kinds of explants were excised from intact leaves, comprising either a) 5 mm segments of the rachis taken midway between the insertion of leaflets (rachis explants) or b) 5 mm segments cut from the rachis at the position of insertion of the leaflets (zone explants). For zone explants, leaflets were first removed with a sharp blade at their junction with the rachis (Osborne and Sargent, 1976).

Abscission of Entire Leaves. Developing Spring buds were placed with their basal ends in 0.9% agar and entire excised leaves were maintained with their petioles dipping into water. The plant material was enclosed under 10 l belljars with suba seal ports and maintained in the light. A solution of mercuric perchlorate 0.25 M in 0.25 M perchloric acid was included in the air controls to maintain an ethylene-depleted atmosphere. Ethylene was introduced through the ports by hypodermic syringe to achieve concentrations of 5, or 10 µl/l. Belljars were aerated and recharged with ethylene at least once daily, and the time of abscission recorded.

Ethylene Production. Samples of approximately 3.5 g of buds or leaves were enclosed in a 50 ml container fitted with a suba seal port, and at hourly or two-hourly intervals 1 ml samples of the gas phase were withdrawn for ethylene determinations by gas-solid chromatography on a Pye Series 104 gas chromatograph fitted with a silica gel column and a flame ionization detector (Jackson and Osborne, 1970). The rate of ethylene produced was monitored over periods of several hours after the wound ethylene production had subsided. Containers were aerated after each set of assays.

Separation in Excised Abscission Zones and Determinations of Fresh Weight Change in Zone and Rachis Explants. Explants were placed with the basal cut surface in contact with 0.9% agar in petri dishes enclosed within the belljars as described above. The agar was made up either with distilled water or with an aqueous solution of the sodium salt of indole-3yl-acetic acid (IAA) which was introduced just prior to setting of the gel. With the exception of the ethylene treatments a beaker containing a solution of mercuric perchlorate 0.25 M in 0.25 M perchloric acid was included in the belljars to absorb ethylene. A range of concentrations of IAA was tested, but only those at 10^{-4} M and higher were effective in retarding abscission; zone explants in the lower concentrations separated at the same time as the air controls. Concentrations of 10^{-4} M or 2×10^{-4} M were therefore routinely used for the auxin-agar experiments. At intervals during the course of the experiments, each explant was removed from the agar, blotted and weighed, and abscission recorded.

Preparation of Tissue for Light and Electron Microscopy. Small segments of rachis tissue were excised from immature leaves at the insertion of the second pair of leaflets from the distal end (a position in which a rachis abscission zone could be expected to develop) or from differentiated zones in explants from older leaves. Excision was conducted beneath the surface of a solution of fixative consisting of 6% glutaraldehyde in 0.1 M sodium cacodylate buffer adjusted to pH 7.1. After fixation for 2 h at room temperature and subsequent rinsing for 2 h in buffer the tissues were post-fixed in a 2% solution of osmium tetroxide in buffer. They were then dehydrated through an ethanol series, embedded in Spurr's resin and polymerised at 100° C for 90 min. 3 µm median longitudinal sections of each entire segment were cut on an LKB Pyramitome and stained with crystal violet (1% in 50% acetone) for light microscopy. For transmission electron microscopy thin longitudinal sections were cut from various regions of the segments, collected on uncoated grids and stained with uranium and lead.

Results

Abscission and Growth of Immature Leaves

Until the second pair of leaves in the Spring buds was more than 20 mm long, leaflet or rachis abscission could not be induced by a continuous exposure to ethylene at 10 µl for 12–17 days. Also, during this stage of rapid leaf expansion, the buds maintained on agar and enclosed with ethylene showed a reduced growth increment (as measured by leaf elongation and increase in total bud fresh weight) when compared with buds maintained in ethylene-depleted air (Table 1). However, when buds contained a second pair of leaves with a tip-to-base length of 30–35 mm or more, abscission could be induced in all but the smallest leaves present within 48–96 h in ethylene. If leaves longer than 30–35 mm were excised from the bud and maintained instead with their petioles in water, abscission also occurred within 2–3 days in ethylene.

Anatomical Studies and Light Microscopy

In buds with a second pair of leaves of 24 mm or less in length, sections of the rachis at the insertion of leaflets, viewed by light microscopy, indicate that the cells of the cortical parenchyma are all of a uni-

Table 1. Abscission of leaves and increase in fresh weight of expanding Spring buds of *Sambucus nigra* exposed continuously to ethylene 10 µl/l or ethylene-depleted air (+mercuric perchlorate, MP)

Leaf length (2nd pair of leaves) mm.	Duration of Treatment	% Abscission		% Increase in F.wt. of bud	
		Air+MP	C_2H_4 10 µl/l	Air+MP	C_2H_4 10 µl/l
12	17 days	0	0	60.3 ± 6.9	46.1 ± 3.6
20	12 days	0	0	49.2 ± 5.6	32.0 ± 7.1
30	4 days	0	100	17.7 ± 2.0	28.8 ± 3.9

Table 2. Ethylene production by buds and intact leaves at different stages of development

Stage of development	C_2H_4 nl/g Fwt/h
Buds – earliest expansion (1–2 cm)	1.8 ± 0.2
Expanding leaves (2–3 cm)	0.7 ± 0.1
Expanding leaves (6–8 cm)	0.1 ± 0.1
Senescing leaves	7.0 ± 2.5

Table 3. Effect of IAA 2×10^{-4} M and C_2H_4 5 µl/l on the increase in fresh weight and abscission of rachis and zone explants from expanding leaves (8–10 cm, May) ($p=0.05$)

	% Increase in Fresh Weight		
	IAA	Air + MP	C_2H_4
Rachis explants			
48 h	24.9 ± 1.8	14.1 ± 0.8	13.2 ± 1.0
144 h	25.3 ± 3.1	16.9 ± 1.8	13.8 ± 1.3
Zone explants			
45 h	38.4 ± 1.5	27.0 ± 1.6	60.4 ± 5.7
		% Abscission	
45 h	0%	0%	80%

form shape and size with no evidence for the differentiation of the band of laterally elongated cells that typify the abscission zone in the rachis of older leaves (Osborne and Sargent, 1976). Coupled with the data that show that abscission cannot be induced in such leaves (20 mm or 12 mm) when held continuously in ethylene (Table 1) the result is taken to indicate that the target cells of the abscission zone are not present at the 24 mm (or smaller) stage of leaf development in Spring buds. By the 30 mm stage, however, abscission is induced by ethylene and the laterally elongated cells are just distinguishable in sections of zone tissue from 35 mm leaves. Abscission zones are clearly differentiated in larger leaves.

Ethylene Production

Determinations of ethylene production from early bud break to well-expanded leaves show that during the earliest stage of leaf emergence, very high levels of the gas are synthesised (Table 2). The rates decline as the leaf enlarges. Similar results have been obtained

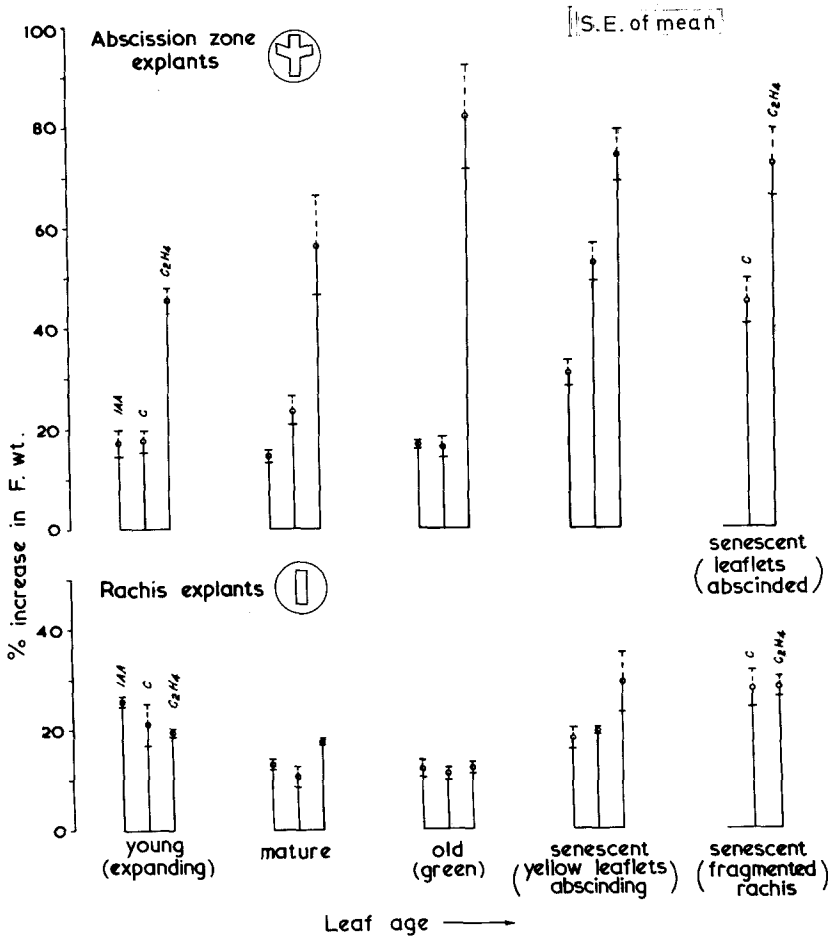

Fig. 1. Increase in fresh weight of zone and rachis explants collected in late August from leaves at different stages of developmental. Explants treated with ethylene 5 µl/l or IAA 10^{-4} M. Mercuric perchlorate (MP) was enclosed with control and IAA treated explants. SE. $p=0.01$

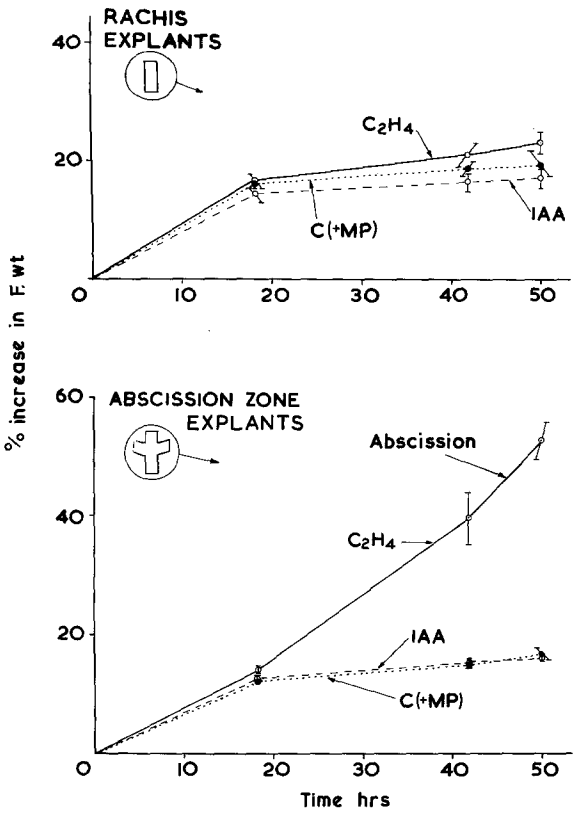

Fig. 2. Time course of increase in fresh weight by zone and rachis explants exposed to ethylene 5 µl/l or treated with IAA 10^{-4} M (May). Some of these explants were subsequently used for electron microscopy (Fig. 3). Mercuric perchlorate (MP) was enclosed with control and IAA treated explants. SE. $p=0.01$

for both green (Burg and Burg, 1968) and etiolated (Goeschl, Pratt and Bonner, 1967) pea shoots where the highest levels are also associated with the rapidly developing apical bud, the rates decreasing as the tissue matures. The production by yellowing *Sambucus* leaves is, however, even higher than that of the expanding buds, reaching nearly 10 nl/g/h.

Abscission and Fresh Weight Increment of Rachis and Zone Explants in Response to Ethylene or IAA

Explants of both the rachis or the abscission zones of *expanding* leaves (8–10 cm, May), when placed on auxin-agar, increase in fresh weight considerably more than the controls (Table 3). This accords with the usual auxin-enhanced growth in cells of immature shoot tissue (Went, 1928). Also in accord with most other shoot tissue ethylene does not induce a fresh weight enhancement in the rachis above that of controls. In zone explants, however, ethylene *does* enhance the fresh weight increase and this is attributed to the presence in the explant of ethylene-responsive cells of the differentiated abscission zone *in addition to* the immature auxin-responsive cells, comparable with those of the rachis. Despite the increased fresh weight increment in response to both auxin and ethylene in these zone explants, only ethylene induces the enhanced growth of the specific cells that comprise the abscission zone, and only treatments lead to cell separation and abscission.

A similar interpretation can be extended to the data for the ethylene-enhanced increase in fresh weight of *buds* which contain a second pair of leaves of 30 mm length (Table 1); only those of a leaf length of 30 mm or more appear to possess the cells which enlarge in response to ethylene.

In the experiments set out in Figure 1 the values for the percentage increases in fresh weight are shown for the period up to 41 h in explants prepared from leaves collected in late August at different stages of expansion up to, and including, senescent stages of development. In these experiments, no abscission occurred before 41 h, but separation was subsequently completed by 72 h in all zone explants exposed to ethylene 5 µl/l and in most of the control zone explants from senescing yellowing leaves. There was no abscission of auxin-treated zone explants, all rachis explants remained intact, and none showed a significantly enhanced fresh weight increase in response to ethylene.

Auxin and Ethylene Competition

The experiment set out in Table 4 was designed to demonstrate that abscission in *Sambucus* is always retarded by auxin, but that the cells of the zone always remain sensitive to ethylene. Freshly-gathered leaves 8–10 cm long were dipped into water, or an aqueous solution of IAA 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5} M and then maintained on damp tissue for 3 h before removal of the leaflets. The isolated rachii containing abscission zones were maintained vertically with their basal ends dipping into flasks of distilled water. Batches of 10 rachii from each treatment were then maintained at high humidity in 6.5 l desiccators containing either a dish of mercuric perchlorate solution (to deplete the gas phase of ethylene), or one of the following concentrations of ethylene (0.1, 1.0 or 10 µl/l). The results show that the control explants, or those from leaves treated with the lowest concentrations of IAA were already abscinding by 24 h in the 1 or 10 µl/l ethylene. The rachii that took longest to fragment were those that had been maintained in ethylene-depleted air and had been treated with auxin: even after 10 days, none had abscinded. Further, such

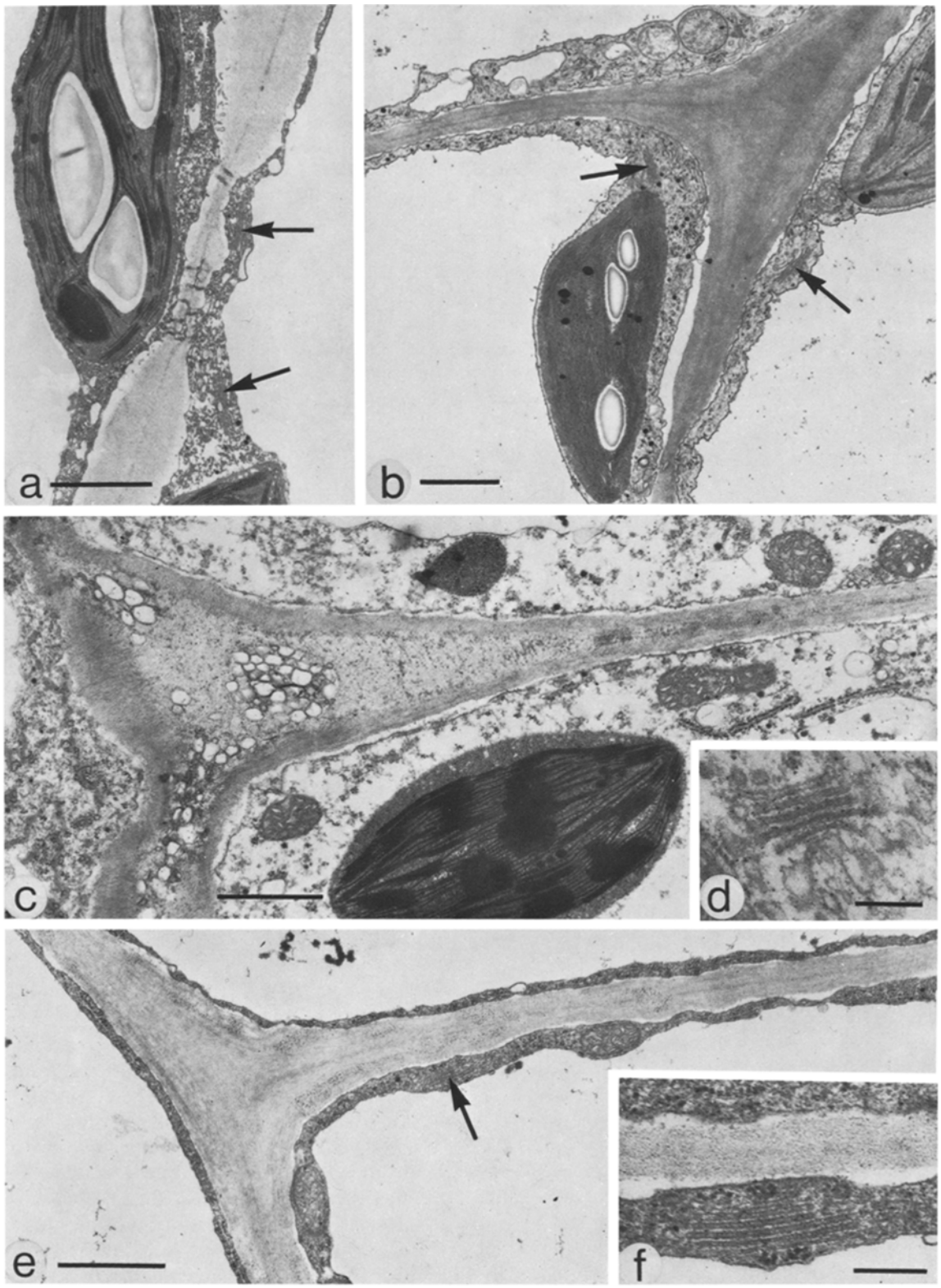

Fig. 3a–f. Electron micrographs of longitudinal sections of cortical cells from the rachis abscission zone region of explants of *Sambucus nigra* leaves (May). **a** 0 h control; **b** 48 h in air (+mercuric perchlorate); **c**, **d** 48 h in ethylene (5 µl/l); **e**, **f** 48 h treated with IAA 10^{-4} M (+mercuric perchlorate). Dictyosomes (arrows) are quiescent in a, e and f, somewhat activated in b and highly activated in d. Scale bar: a, b, c, e = 1 µm; d, f = 0.2 µm

rachii showed a considerable auxin-induced elongation. The abscission zones were in no way impaired by these prolonged treatments for subsequent exposure of the rachii to 1 µl/l ethylene resulted in 100% abscission of zones in all but the IAA 10^{-2} M material within 48 h.

Electron Microscopy

Electron microscopy of cells of explants from expanding leaves collected in May has confirmed the differential effects of auxin and ethylene on cell growth and abscission. From the time course of increase in fresh weight of rachis and zone explants (Fig. 2) it is clear that the growth changes precede abscission. The ultrastructural responses by abscission zone cells from the zone explants at 0 and 48 h are shown in Figure 3. Cells from freshly-collected tissue possess dense cytoplasm containing compact, relatively quiescent dictyosomes (Fig. 3a) and adjacent cell walls are closely adpressed. When zone explants are maintained in an ethylene-depleted environment for 48 h, no difference can be seen in the cell wall but slight changes occur in the cytoplasm (Fig. 3b); the cytoplasmic volume appears to increase and there is an aggregation of ribosomes and some dilation of the dictyosomes. These changes are indicative of an early response to ethylene by abscission zone cells, as reported for mature and senescing leaves (Osborne and Sargent, 1976) and probably result here from the elevated level of ethylene production following wounding when the explants are excised. The zone cells from these immature leaves are therefore as fully capable of responding to ethylene as those from mature or senescing leaves. After 48 h in 10 µl/l ethylene the cytoplasm appears highly activated; the number of polysomal units is increased, the endoplasmic reticulum profiles are longer (Fig. 3c) and dictyosomes (Fig. 3d) are highly dilated and indicate the secretion of numerous vesicles. The middle lamellae show considerable degradation, so that the walls of adjacent cells become separated and many vesicles appear in the enlarging lacunae between them. None of these changes takes place in the presence of IAA (Fig. 3e and 3f) and the cells retain at 48 h the appearance of 0 h controls. Auxin therefore suppresses even the degree of cytoplasmic change observed in the control explants over 48 h.

Discussion

These results illustrate two important features in abscission of *Sambucus nigra*:

(a) That abscission does not occur and cannot be induced by ethylene until after the visible differentiation of special cells whose growth is stimulated by ethylene. The position at which these special cells are formed constitutes a potential separation zone. The cells enlarge and undergo cytoplasmic activation in response to concentrations of ethylene above a certain threshold level. The cytoplasmic activation includes the formation of highly vesiculate dictyosomes and the apparent discharge of dictyosome vesicles or their contents to the wall, partly via the plasmalemma and also through the branched desmotubules of the plasmodesmata (Osborne and Sargent, 1976). Associated with the appearance of the vesicles within the wall, the middle lamellae become degraded, leaving cell wall microfibrils free within the solubilized wall matrix. Since greatest degradation is associated with accumulation of the vesicles it is presumed that special wall hydrolyzing enzymes are contained within the vesicles and that they are synthesized and secreted in response to an ethylene stimulus.

(b) The cells of the abscission zone are clearly distinct from those of the rest of the rachis. The latter are like those of other immature shoot tissues and exhibit an enhanced growth (and increase in fresh weight) when supplied with additional auxin. Such growth does not occur in zone cells of rachis explants supplied with auxin, and abscission is delayed or entirely prevented depending upon the concentration supplied. The ultrastructural changes that occur in the zone during ethylene treatments, or to a limited extent in the controls after 48 h, are entirely repressed by auxin—despite the fact that the auxin treatment causes a large auxin-induced ethylene production.

It could be argued that the ethylene response of abscission zones is saturated by the high levels of auxin-induced ethylene and is overridden by the presence of auxin. However, it has been demonstrated before for explants of *Phaseolus* (Osborne, unpublished) and leaves of *Euonymus* (Hallaway and Osborne, 1967) that increasing levels of ethylene are required to prevent the increasing delay in abscission that result from applications of progressively higher concentrations of auxin. As now shown for *Sambucus* (Table 4), if the level of IAA in leaves is increased before excising the rachis, the level of ethylene in which they are subsequently maintained must also be increased to ensure early abscission of the rachis zones. Such results confirm that the zone cells of *Sambucus* are indeed of a class that enlarges and separates in response to ethylene, but are always inhibited from doing so by auxin.

The time at which the leaves of the developing Spring buds first exhibit an abscission response (of either leaflets or rachis) to applied ethylene (Table 1)

Table 4. Effect of different concentrations of auxin and ethylene on the timing of abscission in debladed rachii. Leaves dipped in water of IAA 10^{-5}–10^{-2} M, debladed after 3 h, then maintained for 10 days in air + mercuric perchlorate (to deplete ethylene) or in ethylene 0.1, 1.0 or 10 µl/l. On day 10 all explants were transferred to ethylene 1 µl/l and abscission recorded for a further 2 days

		% Abscission							% Abscission in presence of 1 µl/l C_2H_4	
		24 h	41 h	49 h	64 h	74 h	98 h	10 days	24 h	48 h
Air+MP	H_2O	0	0	0	0	0	0	11	16	100
	IAA 10^{-5} M	0	0	0	0	0	0	0	17	100
	IAA 10^{-4} M	0	0	0	0	0	0	0	11	100
	IAA 10^{-3} M	0	0	0	0	0	0	0	0	100
	IAA 10^{-2} M	0	0	0	0	0	0	0	0	62
C_2H_4 0.1 µl/l	H_2O	0	38	48	71	76	81	100	–	–
	IAA 10^{-5} M	0	16	16	32	37	47	100	–	–
	IAA 10^{-4} M	0	0	0	11	28	33	100	–	–
	IAA 10^{-3} M	0	0	0	0	0	0	75	90	100
	IAA 10^{-2} M	0	0	0	0	0	0	50	60	75
C_2H_4 1.0 µl/l	H_2O	16	84	95	100	–	–	–	–	–
	IAA 10^{-5} M	25	90	95	100	–	–	–	–	–
	IAA 10^{-4} M	5	81	90	100	–	–	–	–	–
	IAA 10^{-3} M	0	21	37	89	95	95	100	–	–
	IAA 10^{-2} M	0	0	0	10	15	30	90	95	100
C_2H_4 10 µl/l	H_2O	25	100	–	–	–	–	–	–	–
	IAA 10^{-5} M	50	100	–	–	–	–	–	–	–
	IAA 10^{-4} M	40	90	100	–	–	–	–	–	–
	IAA 10^{-3} M	20	70	90	100	–	–	–	–	–
	IAA 10^{-2} M	0	19	29	57	62	71	100	–	–

coincides with the stage when a distinct zone of flattened cells, can be distinguished across the rachis at the position of leaflet attachment. Until such cells are recognisable by light microscopy, exposure to continuous ethylene at 10 µl/l does not induce separation. The leaves from the youngest Spring buds are too small to permit the excision of rachis explants, so the timing of abscission refers both to leaflet abscission and to rachis fragmentation. Since no buds have been observed in which leaflets abscinded but in which the rachis remained intact, it is presumed that the ethylene-responsive cells of both sites of abscission zones are differentiated at closely similar times.

It has not yet been established whether the differentiation of the abscission zones involves cell division for the production of the special ethylene-responsive cells, or whether existing cells undergo a biochemical differentiation that renders them subject to growth stimulation by ethylene. Such studies are currently under way. It is clear, however, that the presence of differentiated ethylene-sensitive abscission zone cells in the leaves of dormant buds could be a major ecological disadvantage at Spring bud break. Very high levels of ethylene are then produced (Table 2) by the rapidly expanding bud tissues and premature leaflet abscission or rachis fragmentation is doubtless precluded by the absence of ethylene-responsive cells until after the rate of ethylene production declines to a safer level.

We conclude that in leaves of *Sambucus nigra* abscission takes place only following the positional differentiation of special "target" cells at the precise position of a zone, and that their growth and loss of mutual adhesion occurs in response to ethylene but not to auxin. We believe that the differentiation of these special ethylene-responsive cells is the causative reason for the auxin retardation and ethylene acceleration of leaf abscission. We further suggest that the regulatory mechanisms we have described during the course of leaf development in *Sambucus nigra* may be general for the control of abscission processes in plants.

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