Effects of Ethylene, Kinetin, and Calcium on Growth and Wall Composition of Pea Epicotyls

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ABSTRACT

Ethylene supplied with indoleacetic acid at 0.1 and 1 μM inhibited elongation and enhanced swelling in epicotyls of decapitated and derooted pea seedlings (*Pisum sativum* L., var. Alaska). These growth responses were correlated with the development of cell walls rich in weak acid-extractable materials and pectic uronic acids. Ethylene had no effect on the formation of hemicellulose, or hemicellulosic uronic acid. Ethylene stimulated the formation of residual materials at 0.1 μM indoleacetic acid but had little effect at 1 μM. With indoleacetic acid at 10 μM, ethylene modified neither the growth or wall composition appreciably. Growth and wall composition in intact seedlings were modified in similar fashion by ethylene. In intact seedlings ethylene promoted the development of walls high in weak acid-extractable materials and pectic uronic acid. These effects were less impressive in the first 24 hours than in the second 24 hours when the control plants suffered a net loss of these constituents. Ethylene considerably inhibited the formation of hemicellulose and residual wall materials in the apical sections but promoted it in the basal sections of the intact seedlings.

Measurements of ethylene production by decapitated and derooted pea seedlings suggest that Ca^{2+} and kinetin do not promote swelling through an effect on the formation of ethylene.

We propose that cells of ethylene-treated pea epicotyls lack polarity because their walls are abnormally rich in pectic substances.

It is known that pea epicotyls exhibit excessive swelling when exposed to ethylene (1, 5, 6, 13, 17), Ca^{2+} (16), kinetin (9, 16, 22), supraoptimal levels of IAA (5, 24) and compounds related to ethylene (11). Zimmerman and Hitchcock (25) and Chadwick and Burg (8) linked the regulation of root hair formation by ethylene to the modification of cell wall structure. Burg and Burg (6) noticed some distinct longitudinal "ribs" in the walls of ethylene-induced swollen cells of pea epicotyls and suggested that these ribs might bear some relationship to lateral expansion of cells. Apelbaum and Burg (1) later reported that such swelling is correlated with a decrease in the proportion of microfibrils oriented transversely to the long axis of the cell. According to Veen (24) swelling of pea stem sections promoted by supraoptimal levels of IAA is related to

the predominantly longitudinal orientation of microfibrils in the inner part of the walls of swollen cells. Recently Nance (16) demonstrated that swelling of pea epicotyls in response to Ca^{2+} and kinetin is correlated with the development of walls rich in pectic substances.

Although attempts have been made to relate cell shape to cell wall architecture, little attention has been paid to the possible relationship between cell shape and wall composition. The present study was undertaken to determine whether swelling induced by ethylene is associated with a change in wall composition and whether swelling enhanced by Ca^{2+} and kinetin is associated with increased production of ethylene.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* L., var. Alaska) were grown and used in these experiments generally as described earlier (16). An important change in procedure was that in the present studies the seedlings were not exposed to light before the termination of the experiment. The experiments were done with seedlings 4.5 days old from which the roots were removed just below the insertion of cotyledons and shoots at the base of epicotyl hooks. Because the epicotyl was cut below the bract, only the stem tissue of the first internode was left. The seedlings were marked 5 mm below the apex of the epicotyl stump with a permanent color pen of brand Esterbrook, USA 49. The pen was found to cause no apparent injury to the seedlings in any case. These operations and the subsequent ones involved in the setting up the experiment were carried out in a dark room under safe green light.

For the experiments concerning the effect of ethylene on the decapitated seedlings, the seedlings were incubated in 300 ml of test solution contained in Pyrex pie pans (21 × 4.5 cm). The seedlings were supported upside down on a rack of glass rods by their cotyledons with the apical 10-mm portion of each epicotyl immersed in the experimental solution. Laboratory tissues soaked with deionized H$_2$O were placed on the cotyledons and the Pyrex pan was set on a desiccator plate in a 9-liter desiccator. Thirty to 40 seedlings were used for each experimental treatment. A finger bowl, having 100 ml of a 15% (w/v) KOH solution, was placed beneath the desiccator plate. The desiccator top was then sealed in place. Four and one-half ml of pure ethylene were introduced into the desiccator with a syringe. The final concentration of ethylene inside each desiccator was approximately 500 μl/l. They were then left in the dark at 21 ± C.

Immediately after the experiment was set up, a sample of 30 or 40 plants previously set aside as the material representative of the initial state of the plants was excised at positions marked previously. The sections were washed several times, and their fresh weight and lengths were measured. No attempt was made to measure the stem diameters. As a measure of

swelling, tables include values designated "index of swelling." This is defined as the percentage increase in fresh weight divided by the percentage increase in length. The sections were covered with water and frozen for cell wall analysis. This set of sections was referred to as "initial" sample. After 24 hr the seedlings were removed from solutions and subjected to operations identical to those for the initial sample.

For the experiment concerning the effect of ethylene on intact seedlings, the seedlings were grown on expanded vermiculite in culture dishes (16 × 8.5 cm) in the dark at 21 ± C. The dishes were placed in plastic pans and covered as usual with glass plates. When they were 4.5 days old, the seedlings were marked first at the base of the epicotyl hook, then 5 mm and 10 mm below the first mark. The dishes containing the seedlings were placed in desiccators which were then sealed. Into each of two desiccators 4.5 ml of ethylene were introduced. All desiccators were finally left in the dark at 21 ± C. Two of the desiccators, one control and the other containing ethylene, were incubated for 24 hr and the rest for 48 hr. At the end of the incubation period, the seedlings were removed, washed, and sections were excised at the marks. The sections between first and second markings were designated apical sections and the rest basal sections. Subsequent operations were identical to those described for the initial sample of the experiment involving decapitated plants.

For the experiments involving the effects of $CaCl_2$ and kinetin on the growth and production of ethylene by decapitated seedlings, the seedlings were grown and prepared as stated for the first set of experiments. An especially designed Pyrex glass unit was used for the experiments. This unit accommodated 8 seedlings suspended from a small rack by their cotyledons, the epicotyl tips dipped into 10 ml of experimental solution. A side-arm sealed with a rubber serum cap facilitated the removal of gas samples. The unit was then left in the dark at 21 ± C. At the end of 24 hr, a small gas volume of 1 or 2 ml was withdrawn for the measurement of ethylene by gas chromatograph. The seedlings were taken out of the unit and 5-mm sections were excised as usual. Their fresh weight and lengths were then measured.

Cell wall material was isolated following a modification (16) of a method described by Bean and Ordin (3). The wall material was dried in an oven set at 65 C for 1.5 hr. After further drying in a desiccator over P_2O_5 overnight, the sample was quickly weighed. The dried wall material was extracted with weak acid (0.05 N H_2SO_4), then with N HCl by procedures described earlier (16). Uronic acids of the weak acid extract ("pectic substances") and the N HCl extract ("hemicellulose") were determined by the method of Bitter and Muir (4).

RESULTS

Ethylene, in the presence of IAA at 0.1 μM and 1 μM, inhibited elongation of decapitated epicotyls (Table I). In IAA at 0.1 μM, the epicotyls displayed no swelling. Indoleacetic acid at 1 μM induced slight swelling. Ethylene supplied along with each of these IAA levels enhanced swelling. The swelling was observed in tissues exposed to 10 μM IAA alone. Ethylene accompanying IAA at 10 μM did not promote any additional swelling. In the absence of exogenous IAA, ethylene caused swelling of epicotyls suggesting that the response involved endogenous IAA of the stem. At the two lower levels of IAA, ethylene promoted the development of wall appreciably higher in weak acid-extractable materials and pectic uronic acid. There was no effect on the formation of hemicellulose, hemicellulosic uronic acid. Ethylene promoted the formation of residual materials at 0.1 μM IAA but had little effect at the next higher concentration. With IAA at 10 μM, ethylene had no appreciable effect on the composition of the wall.

Both $CaCl_2$ and kinetin in the presence of IAA promoted lateral enlargement of pea stems and the change was associated with the formation of wall high in weak acid-extractable materials and pectic uronic acid (Table II). Whereas tissues exposed to $CaCl_2$ were lower in the amounts of hemicelluloses and residual materials, those treated with kinetin were higher in these wall constituents. A similar observation was made by Nance (16). It is also evident from the results that even in the presence of saturating levels of ethylene, both $CaCl_2$ and kinetin, added separately, enhanced swelling of epicotyls to an appreciable extent. The response was particularly impressive in the case of kinetin. One might not expect any additional swelling if $CaCl_2$ or kinetin were to promote enlargement of the epicotyls through their effects on ethylene production.

Table III shows the results of an experiment in which the effects of ethylene on intact pea seedlings were investigated. It is evident that both apical and basal sections, which did not swell normally, exhibited this response on exposure to ethylene. The swelling was more intense with apical sections than with basal sections. In ethylene, the apical and basal sections made

Table I. *Effects of IAA Alone and Ethylene in Presence of IAA on Growth and Cell Wall Composition of Epicotyls of Etiolated Pea Seedlings*

Roots and epicotyl hooks of the seedlings were excised. IAA solutions were in Pyrex dishes, each in a desiccator. Ethylene was added to the desiccators as indicated to give a final concentration of 500 μl/l. Index of swelling is the percentage increase in fresh weight divided by the percentage increase in length.

Experimental Period	IAA	Ethylene	Fresh Wt	Length	Index of Swelling	Cell Wall Constituents				
						Weak acid extract		Hemicelluloses		Residue
						Total	Uronate	Total	Uronate	
	μM	μl/l	mg/section	mm				mg/100 sections		
Initial			15.0	5.3		11.2	3.4	5.0	0.5	3.8
Final	0.1	0	29.5	10.2	1.1	24.5	6.0	13.7	1.9	13.8
		500	27.0	6.6	3.3	29.0	7.7	13.7	1.9	17.1
	1	0	47.8	10.7	2.2	23.5	6.9	15.8	2.8	21.2
		500	39.3	7.2	4.5	29.4	8.7	15.0	2.4	20.9
	10	0	48.5	8.0	4.4	29.8	8.3	15.0	2.8	22.9
		500	43.5	7.4	4.8	30.1	8.3	16.0	2.9	22.4
	0	500	25.0	6.4	3.2	29.0	7.7	14.0	1.7	15.3

Table II. *Effects of CaCl₂ and Kinetin on Growth and Cell Wall Composition of Epicotyls of Etiolated Pea Seedlings Treated with IAA and or Ethylene*

Roots and epicotyl hooks were excised. All experimental solutions contained IAA at 10 μM. Calcium chloride was added at 10 mM and kinetin at 10 μM. The solutions were in Pyrex dishes, each in a desiccator. Ethylene was added to the desiccator as indicated to give a final concentration of 500 μl/l.

Experimental Solution	Fresh Wt	Length	Index of Swelling	Cell Wall Constituents				
				Weak acid extract		Hemicelluloses		Residue
				Total	Uronate	Total	Uronate	
	mg/section	*mm*		*mg/100 sections*				
Initial	17.3	5.5		12.7	3.5	5.0	0.5	3.3
IAA	45.0	7.8	3.8	25.0	7.7	15.0	2.5	16.0
IAA, CaCl₂	35.0	6.6	5.2	28.7	9.3	11.3	2.0	12.0
IAA, kinetin	50.3	7.1	6.6	34.7	10.3	16.0	2.8	22.0
IAA, ethylene	43.3	7.4	4.4	27.7	7.7	15.7	2.5	16.7
IAA, ethylene, CaCl₂	35.0	6.5	5.4	29.3	9.7	11.7	1.8	12.3
IAA, ethylene, kinetin	45.0	6.6	8.0	36.3	11.7	17.7	3.3	22.3

Table III. *Effect of Ethylene on Growth and Cell Wall Composition of Intact Etiolated Pea Seedlings*

Pea seedlings were first marked at the base of the epicotyl hook. Additional markings were made 5 mm and 10 mm from the first mark. They were set in a desiccator which then received either 500 μl/l of ethylene or none. The sections between the first and the second markings are designated apical sections and those between the second and the third markings basal sections.

Experimental Period	Experimental Treatment	Type of Section	Fresh Weight	Length	Index of Swelling	Cell Wall Constituents				
						Weak acid extract		Hemicelluloses		Residue
						Total	Uronate	Total	Uronate	
			mg/section	*mm*		*mg/100 sections*				
Initial		Apical	14.0	4.9		11.7	2.8	3.7	0.3	2.3
		Basal	15.3	4.9		8.4	2.3	3.7	0.3	2.3
24 hr	Control	Apical	59.0	15.6	1.5	25.3	7.8	19.7	2.5	22.6
		Basal	27.3	6.8	2.0	14.3	3.8	10.0	1.1	11.0
	Ethylene	Apical	29.0	5.8	6.0	29.4	8.2	12.6	1.6	16.3
		Basal	29.0	6.3	3.2	17.3	4.1	11.3	1.6	14.7
48 hr	Control	Apical	59.3	16.2	1.5	20.7	6.0	21.0	3.9	27.3
		Basal	27.0	6.8	2.0	11.3	3.3	8.7	2.0	16.0
	Ethylene	Apical	34.4	6.5	5.0	31.7	10.5	20.6	3.9	23.0
		Basal	29.3	6.2	3.7	19.3	5.6	15.0	2.7	20.0

only small increases in length during the entire experiment. Control apical sections elongated appreciably in the first 24-hr period only, whereas their basal sections elongated only slightly more than those of the ethylene-treated seedlings. Apical and basal sections of both control and experimental plants increased in fresh weight during the first 24 hr, with little change in the second 24 hr. Weight gains by apical sections of the control plants were 2 or 3 times greater than those for the ethylene-treated ones. Weight gains by basal sections of the two treatments were not appreciably different. Ethylene favored the development of cell wall rich in weak acid-extractable materials and pectic uronic acid. This effect is more impressive in the second 24 hr than in the first 24 hr. In confirmation of earlier work (16), it is seen that in the second 24 hr there was a net loss of weak acid-extractable materials and pectic uronic acid from the control seedlings. The plants in ethylene gained in both values in the same period. N HCl-extractable materials and residual material increase throughout the experiment with no consistent modification in the presence of ethylene. A small decrease in the N HCl extract in the controls from 24 to 48 hr may be an error because the uronic acid content of the control nearly doubled in the same interval. The value for the extract

obtained by a weight difference is less reliable than for the uronic acid value which is a direct determination. Ethylene appreciably inhibited the formation of hemicellulose and residual materials in the apical sections but promoted it in the basal sections.

Nance (16) reported recently that Ca^{2+} enhanced swelling of pea epicotyls when IAA was present at 10 μM. In view of this observation, it seemed desirable to determine whether Ca^{2+} enhances the formation of ethylene. Because IAA alone at 10 μM promotes swelling and ethylene production, lower concentrations of the auxin were also used. It is seen from Table IV that although Ca^{2+} in the presence of IAA at 0.1 μM and 1 μM promoted swelling to a considerable extent, it did not significantly modify the production of ethylene. Sections treated with IAA at concentrations lower than 10 μM did not show any swelling or produce any significant quantity of ethylene.

In a similar experiment (Table V), kinetin caused enhancement of swelling and ethylene production at all levels of IAA used. However, there seems to be little correspondence between the magnitude of swelling and the production of ethylene induced. For example, the amount of ethylene produced by IAA

TABLE IV. *Effect of CaCl₂ in Presence of Several Levels of IAA on Growth and Production of Ethylene by Epicotyls of Etiolated Pea Seedlings*

Roots and epicotyl hooks were excised. Pea epicotyls were supported by a rack inside a glass unit. Test solutions contained IAA alone or with $CaCl_2$ at concentrations as indicated.

Experimental Period	IAA	CaCl₂	Fresh Wt	Length	Index of Swelling	Ethylene Production
	μM	mM	mg/section	mm		nl/g·hr
Initial			14.0	5.2		
Final	0.1	0	46.0	14.1	1.4	0.93
		10	34.7	8.0	3.0	1.04
	1	0	48.3	11.3	2.1	1.86
		10	34.3	7.2	4.0	1.75
	10	0	41.3	8.1	3.6	12.70
		10	30.3	6.3	5.8	13.20

Table V. *Effect of Kinetin in the Presence of Several Levels of IAA on Growth and Production of Ethylene by Epicotyls of Etiolated Pea Seedlings*

Roots and epicotyl hooks were excised. Pea epicotyls were supported by a rack inside a glass unit. Test solutions contained IAA alone or with kinetin at concentrations as indicated.

Experimental Period	IAA	Kinetin	Fresh Wt	Length	Index of Swelling	Ethylene Production
	μM	μM	mg/section	mm		nl/g·hr
Initial			15.0	5.4		
Final	0.1	0	47.0	14.4	1.3	0.95
		10	48.3	7.6	5.5	3.00
	1	0	50.0	11.8	1.9	1.92
		10	47.3	6.8	8.3	11.50
	10	0	43.3	8.3	3.5	12.50
		10	45.7	6.7	8.5	36.50

at 10 μM is 12.5 nl whereas that formed by kinetin and 0.1 μM IAA is only 3 ml, yet the swelling by the former is less than that by the latter. This suggests that kinetin-induced ethylene production cannot account entirely for the response.

DISCUSSION

It is recognized that the wall fraction procedures used in these studies suffer from certain inherent limitations (23). The fractions obtained do not correspond to defined chemical entities, and it is likely that degradation of some wall units occurs. As studies of wall composition, they have the sort of significance that applies to other work in which the "classical" methods of wall fraction have been used. It seems that acid extraction is a relatively innocuous procedure for the solubilization of uronic acid-containing components (21). The results seem to be appreciably more valid, therefore, so far as they follow changes in the uronic acid levels of the wall.

Swelling of decapitated pea epicotyls in response to ethylene, like that in response to Ca^{2+} and kinetin (16), is correlated with the development of walls rich in pectic substances. Intact seedlings respond similarly to ethylene. It is evident that Ca^{2+} does not promote swelling through an effect on the synthesis of ethylene. A more complex situation is seen with kinetin. Epicotyls in IAA at 0.1 μM and kinetin were more swollen but produced only one fourth as much ethylene as those in IAA at 10 μM (Table V). Swelling by epicotyls in IAA and kinetin

each at 10 μM was about the same as those in kinetin with IAA at 1 μM even though in the latter case one third as much ethylene was produced (Table V). Of similar significance, the effects of kinetin and ethylene are additive even with the latter at 500 μl/l, well beyond a saturating level (Table II). These observations suggest that kinetin promotes swelling of pea epicotyls by processes independent of its effect on ethylene production. Apelbaum and Burg (1) came to a similar conclusion. On the basis of elegant, newly developed procedures for the determination of the composition of the cell wall, Keegstra et al. have proposed a structure for the primary cell wall of sycamore cells grown in suspension culture (10). Although our results and earlier one (16) do not seem to be inconsistent with the proposed structure, they suggest that a model of walls of cells growing in the pea epicotyl must allow for changes during the course of cell enlargement. Keegstra et al. (10) do not consider this possibility for suspension-cultured sycamore cells. It may be significant that, unlike cells of pea epicotyl, such sycamore cells do not appear to possess polarity.

It has been proposed that pea epicotyls in solutions containing Ca^{2+} or kinetin swell because wall stress (resistance to wall deformation) in the direction transverse to the long axis of the shoot is inadequate relative to that in the longitudinal direction (7, 16). Pectic substances contribute to wall stress (2) but since they are randomly oriented their effect is equal in the transverse and longitudinal directions. Any treatment that increases the proportion of pectic substances in the wall results in the formation of a less polar wall and therefore a more nearly isodiametric cell. This analysis seems applicable to the effects of ethylene.

Absolute losses in pectic substances occurred in the untreated, intact shoots in contrast to gains in the same materials in seedlings exposed to ethylene. These results, similar reports of net losses of pectic substances in elongating pea epicotyles (12, 16, 18), as well as results reported earlier (16) and here of the development of walls abnormally high in pectic substances suggest that the normal enlargement of tubular cells involves, among other things, maintenance of an appropriate balance between synthesis and degradation of these substances in the wall. Breakdown of pectic substances would make the wall more extensible and so promote cell enlargement. It would also increase the ratio of transverse stress to longitudinal stress, and thereby favor the development of a tubular cell. Pectic substances may be involved in another mechanism by which wall stress is diminished, namely the displacement of Ca^{2+} by monovalent ions such as K^+ or H^+ (2). Other work (14, 16) suggests that displacement of Ca^{2+} from the wall leads to the loss of pectic substances.

It follows from this model that if cell enlargement is to occur, there must be a prior synthesis of pectic substances. Work by Bonner and his co-workers (2), Ray and Baker (19), Nance (16), Mondal (15) and the present study suggest that an important role of IAA in cell enlargement is its promotion of the synthesis of pectic substances. Ridge and Osborne report that a response to ethylene occurs only in the presence of auxin (20). The swelling effect of ethylene shown in the absence of an external supply of IAA in this study (Table I) probably reflects the use of plant material having a relatively high level of endogenous IAA.

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