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STIMULATION OF PHENOLIC ACID AND LIGNIN BIOSYNTHESIS IN SWEDE ROOT TISSUE BY ETHYLENE

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Key Word Index—*Brassica napo-brassica*; Cruciferae; swede root; ethylene effect; lignin; phenolic acid; biosynthesis.

Abstract—Experiments using ^{14}C-phenylalanine have shown that ethylene treatment of swede root tissue promotes the utilization of phenylalanine as a precursor for the synthesis of phenolic compounds. Ethylene treatment increases the labelling of soluble cinnamic acid derivatives and of a fraction with the properties of lignin. A 6-day treatment of whole swede roots leads to a 3–8-fold increase in the concentration of soluble cinnamic acid derivatives and to 3-fold greater yields of lignin oxidation products. The ageing of disks of tissue in the presence of ethylene has very little effect on the concentration of soluble cinnamic acid derivatives but leads to a 3-fold greater yield of lignin oxidation products compared with freshly prepared disks. It was concluded that ethylene stimulates the pathway of phenolic acid biosynthesis leading to the lignification of the tissue. Cytological studies have shown that the secondary xylem parenchyma lignifies in response to ethylene. In the case of the disks, the response is restricted to a layer 2–3 cells thick on the surface of the disks.

INTRODUCTION

IN A previous paper[1] it was shown that treatment with low concentrations of ethylene of whole swede roots or of tissue disks derived from the whole root led to a large increase in the activity of the enzyme, phenylalanine ammonia lyase[2] (PAL) which controls the step in phenolic biosynthesis involving the deamination of phenylalanine to form cinnamic acid. The present paper describes tracer experiments designed to show changes in the patterns of phenylalanine metabolism of the tissue upon ethylene treatment. Two types of experiment are described. In one [U^{14}C]phenylalanine was fed to tissue prepared from whole swede roots which had previously either been stored in air or in air containing a low concentration of ethylene. In the other type, radioactive phenylalanine was supplied to disks of root tissue which had previously been aged for 20 hr either in air or in air containing a low concentration of ethylene. In both types of experiment, the tissue after incubation was extracted and the composition and radioactivity associated with various fractions was analysed. The results of these biochemical investigations and of parallel cytological studies clearly show that in swede tissue ethylene promotes phenolic biosynthesis and the main product is lignin.

RESULTS

Figures 1–3 and Tables 1–3 show the results of the experiment of incubating tissue from control and ethylene treated swede roots with ^{14}C-phenylalanine. Separate samples of tissue were taken, extracted and their PAL activity measured. The activity of PAL in the air sample was less than 0·1 e.u./10 g fr. wt and in the ethylene treated sample was 15·3 e.u./10 g fr. wt. Figure 1 shows the changes in uptake of labelled phenylalanine and the radioactivity associated with the ethanol soluble and ethanol insoluble fractions in the two sets of

[1] M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **10**, 1989 (1971).
[2] J. KOUKOL and E. C. CONN, *J. Biol. Chem.* **236**, 2692 (1971).

tissue incubated for periods up to 4 hr. The uptake of labelled precursor was almost identical for each set of disks. The increase in labelling of the ethanol soluble fraction was very similar for each type of tissue up to 1 hr incubation and then diverged markedly. In the control tissue the rate of labelling of the fraction continued to increase while in the ethylene treated tissue it slowed down and between 2 and 4 hr there was virtually no increase in the labelling, so that after 4 hr incubation the ethanol soluble fraction of the control tissue contained nearly 2·5 times as much radioactivity as the similar fraction from ethylene treated tissue. In the ethanol insoluble fraction after 4 hr incubation there was nearly 3·5 times as much radioactivity in the fraction from ethylene treated compared with control tissue. In the ethylene treated tissue between 2 and 4 hr there was a 2-fold increase in the labelling of the ethanol insoluble fraction while there was no change in labelling of the ethanol soluble fraction.

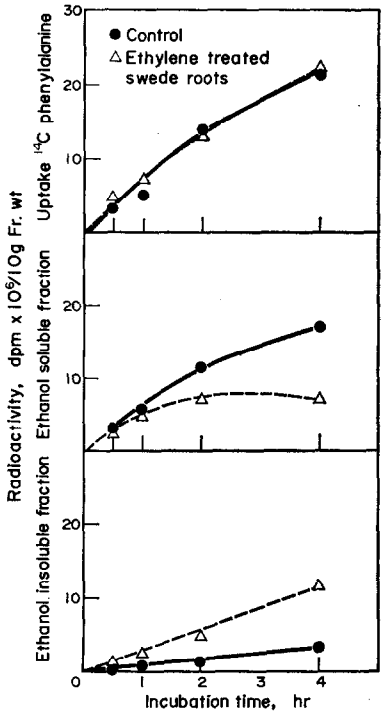

FIG. 1. THE UPTAKE OF LABELLED PRECURSOR AND THE RADIOACTIVITY IN THE ETHANOL SOLUBLE AND ETHANOL INSOLUBLE FRACTIONS OF TISSUE PREPARED FROM CONTROL AND ETHYLENE TREATED SWEDE ROOTS.

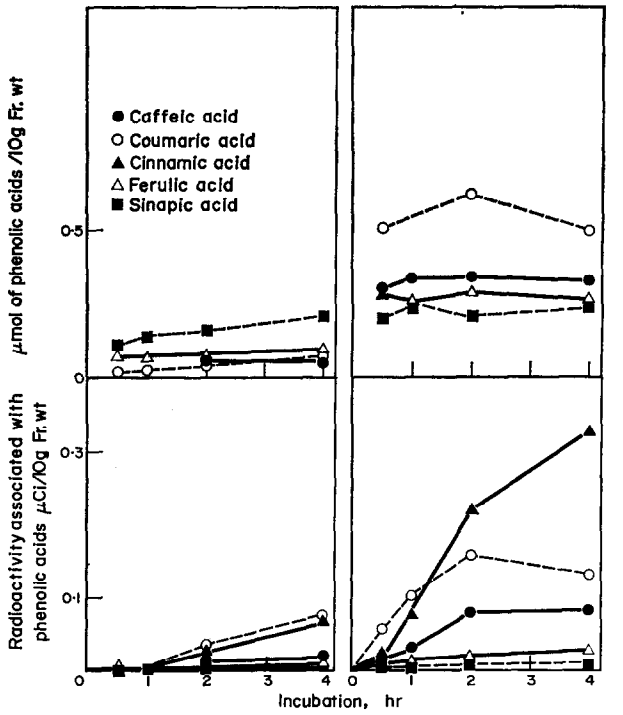

FIG. 2. THE CHANGES IN CONCENTRATION AND RADIOACTIVITY OF SOLUBLE PHENOLIC ACIDS SEPARATED FROM DISKS PREPARED FROM CONTROL (LEFT-HAND GRAPH) AND ETHYLENE TREATED (RIGHT-HAND GRAPH) SWEDE ROOTS.

The ethanol soluble fraction was extracted with light petrol. which removed some pigmented material but very little radioactivity. The ethanol soluble extract was further divided into ether soluble and insoluble fractions and the radioactivity associated with these fractions is shown in Table 1. The ether insoluble fraction showed essentially the same pattern

of labelling as the total ethanol soluble fraction from which it was derived. This fraction was examined by PC and the radioactivity present was shown to be associated almost exclusively with free phenylalanine.

The ether soluble portion of the ethanol soluble fraction after hydrolysis with NaOH was shown to contain phenolic acids, the major components being caffeic, cinnamic, coumaric, ferulic and sinapic acids. The radioactivity in this fraction of 4 hr incubated ethylene treated tissue represents about 32% of the total radioactivity of the ethanol soluble fraction while at the same stage in control tissue it represents less than 5% of the total radioactivity. Thus after 4 hr incubation there was 3 times as much radioactivity associated with this fraction in ethylene compared with control tissue.

TABLE 1. THE RADIOACTIVITY ASSOCIATED WITH EXTRACTS OF TISSUE PREPARED FROM EITHER ETHYLENE TREATED OR CONTROL SWEDE ROOTS AND INCUBATED IN THE PRESENCE OF ^{14}C-PHENYLALANINE

	DPM × 10^6/10 g fr. wt							
	Control tissue				Ethylene treated tissue			
	hr incubation							
Sample	0·5	1	2	4	0·5	1	2	4
Ethanol soluble fraction	3·2	4·7	11·5	17·2	2·6	4·1	7·2	7·2
Light petrol. soluble	0·002	0·002	0·01	0·003	0·007	0·002	0·003	0·001
Ether soluble	0·11	0·12	0·28	0·71	0·37	0·91	1·67	2·3
Ether insoluble	3·1	4·8	11·8	16·2	2·2	3·1	5·1	4·6

The quantitative analysis of soluble phenolic acids is shown in Fig. 2. The ethylene treated tissue had a much larger content of caffeic, coumaric and ferulic acids compared with the control tissue but the levels of sinapic acid were similar in both. In fact, in control tissue sinapic acid was the major phenolic acid while coumaric and ferulic were the major components of the ethylene treated tissue. The pattern of labelling showed a much faster rate of labelling of all the phenolic acids in the ethylene treated compared with the control tissue. Coumaric and cinnamic acids were most heavily labelled followed by caffeic, ferulic and sinapic acids.

The ethanol insoluble residue was subjected to acid hydrolysis and radioactive phenylalanine originally present as protein bound amino acid was isolated and counted. Figure 3a shows that the incorporation of labelled phenylalanine into protein was similar in the two sets of tissue throughout the incubation period. Subtracting the radioactivity associated with protein from the total radioactivity in the ethanol insoluble residue leaves radioactivity associated with a non-protein residue fraction (see Fig. 3b). In the case of the control tissue, protein bound radioactivity accounted for most of the radioactivity associated with the ethanol insoluble fraction up to 2 hr when the non-protein fraction started to become significant. In the ethylene treated tissue the protein bound phenylalanine accounted for only about 20% of the total radioactivity in the ethanol insoluble residue and thus the non-protein fraction in the ethylene treated tissue contained a major fraction of radioactivity. The possibility that this non-protein residual radioactivity was associated with phenolic acids[3] bound to the ethanol insoluble fraction was investigated by use of mild alkaline

[3] S. Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* 3, 627 (1964).

hydrolysis. With swede tissue such alkaline hydrolysis released less than 3% of the total radioactivity in the residue and no measurable quantities of phenolic acids.

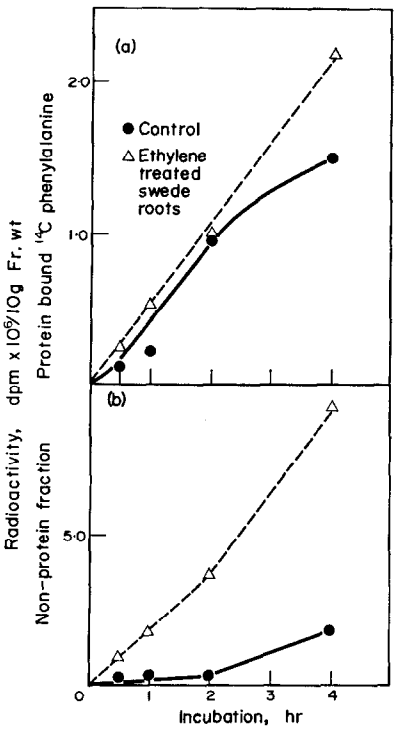

FIG. 3. CHANGES IN THE LABELLING OF PROTEIN BOUND PHENYLALANINE (UPPER GRAPHS) AND OF THE NON-PROTEIN RESIDUAL FRACTION (LOWER GRAPHS) ASSOCIATED WITH THE ETHANOL INSOLUBLE RESIDUE OF DISKS PREPARED FROM CONTROL AND ETHYLENE TREATED SWEDE ROOTS.

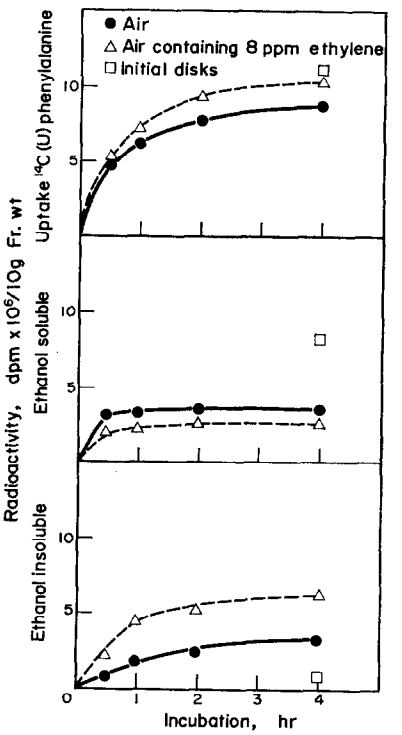

FIG. 4. THE UPTAKE OF LABELLED PRECURSOR AND THE RADIOACTIVITY ASSOCIATED WITH THE ETHANOL SOLUBLE AND ETHANOL INSOLUBLE FRACTIONS OF DISKS OF SWEDE ROOTS WHICH HAD BEEN AGED IN AIR OR IN AIR CONTAINING ETHYLENE FOR 20 hr. THE RADIOACTIVITY ASSOCIATED WITH THE FRACTIONS SEPARATED FROM FRESHLY PREPARED INITIAL DISKS IS ALSO SHOWN.

Aliquots of the ethanol insoluble residue were subjected to alkaline copper oxide oxidation[4] and two phenolic containing fractions were separated from the oxidation mixture. The major neutral phenolic products were *p*-hydroxybenzaldehyde and vanillin, the characteristic lignin oxidation products. Table 2 shows the radioactivity, mass and specific activities of *p*-hydroxybenzaldehyde and vanillin isolated from the oxidation mixtures of both types of tissue over the 4-hr incubation period. The results show that a three times greater yield of aldehydes was obtained on oxidation of ethylene treated compared with control tissue. The rates of labelling increased rapidly over the 4-hr period with a large difference in labelling of up to 10-fold appearing between the ethylene and control tissue. The specific activities show smaller differences than the total radioactivity of the products, mainly due to the large difference in yield of oxidation products between the two types of

[4] R. D. HARTLEY, *J. Chromatog.* **54**, 335 (1971).

TABLE 2. THE CONCENTRATION, RADIOACTIVITY AND SPECIFIC RADIOACTIVITY OF LIGNIN OXIDATION PRODUCTS PREPARED FROM CONTROL AND ETHYLENE TREATED WHOLE SWEDE ROOTS

	Control tissue				Ethylene treated tissue			
	hr incubation							
	0·5	1	2	4	0·5	1	2	4
p-Hydroxybenzaldehyde								
μmol/10 g	0·85	1·01	1·04	1·08	2·49	3·40	3·46	3·08
μCi/10 g	0·0033	0·013	0·019	0·067	0·027	0·054	0·136	0·28
μCi/mmol	3·8	13·0	18·6	61·8	10·9	16·0	39·4	90·9
Vanillin								
μmol/10 g	0·63	0·64	0·64	0·76	2·33	2·42	1·96	1·74
μCi/10 g	0·0014	0·007	0·0074	0·018	0·037	0·074	0·084	0·12
μCi/mmol	2·5	10·9	11·6	21·8	16·2	30·6	43·3	71·3

tissue. This difference in yield reflects an increase in endogenous unlabelled lignin during the course of the ethylene treatment of the whole swede roots. In common with many similar studies[5] the yields of aldehydes obtained on oxidation were low; the radioactivity in lignin aldehydes accounted for only 10–15% of the radioactivity in the non-protein residual fraction. There were no consistent differences in the percentage of the radioactivity associated with the non-protein residual fraction in recovered aldehyde oxidation products in the air and ethylene treated samples. Much of the remaining radioactivity was found in the acidic phenolic fraction and this probably represented condensed polyphenolic material. At least 70% of the radioactivity of the non-protein residual fraction could be accounted for as ether soluble products of the alkaline copper oxide oxidation. The oxidation of lignin is clearly a complicated process and the isolated aldehydes are merely the main small molecular weight components of a complex mixture of reaction products.[5]

TABLE 3. THE RADIOACTIVITY ASSOCIATED WITH KLASON LIGNIN AND WITH PHENOLIC ALDEHYDE OXIDATION PRODUCTS

Sample	Incubation time (hr)	Radioactivity associated with isolated Klason lignin DPM × 10^6/10 g fr. wt	Specific activities of phenolic aldehydes produced on oxidation of Klason lignin μCi/mmol		Specific activities of phenolic aldehydes produced on oxidation of the ethanol insoluble fraction μCi/mmol	
			p-Hydroxy-benzaldehyde	Vanillin	*p*-Hydroxy-benzaldehyde	Vanillin
Control	4	0·5	—	—	—	—
Ethylene treated	4	5·6	93·0	66·0	90·9	71·3
Ethylene treated	2	1·3	35·0	40·2	39·4	43·3

Klason lignin was isolated from the ethanol insoluble residue[6] and the product was light brown in colour and stained red with phloroglucinol–HCl. On alkaline copper oxide

[5] K. V. SARKENEN and C. H. LUDWIG, *The Lignins, Occurrence, Formation, Structure and Reactions* (edited by K. V. SARKENEN and C. H. LUDWIG), Wiley, New York (1971).
[6] D. G. SMITH and A. C. NEISH, *Phytochem.* 3, 609 (1964).

oxidation (see Table 3) the Klason lignin yielded *p*-hydroxybenzaldehyde and vanillin of the same specific activity as the oxidation products derived from the ethanol insoluble residue. When tissue from control and ethylene treated samples are compared, more radioactivity is associated with the Klason lignin isolated from ethylene treated samples.

TABLE 4. THE RADIOACTIVITY PRESENT IN VARIOUS FRACTIONS FOLLOWING THE INCUBATION IN THE PRESENCE OF ^{14}C-PHENYLALANINE OF FRESHLY PREPARED AND AGED DISKS OF SWEDE ROOTS

	DPM × 10^6/10 g fr. wt								
	Initial disks	Air aged disks				Ethylene aged disks			
	Incubation time (hr)								
	4	0·5	1	2	4	0·5	1	2	4
Ethanol soluble fraction	8·4	3·3	3·6	3·6	3·6	2·1	2·2	2·7	2·7
Light petrol. soluble	0·004	0·003	0·005	0·006	0·004	0·004	0·004	0·006	0·006
Ether soluble	0·37	0·45	0·56	0·85	0·92	0·67	0·75	0·68	0·59
Ether insoluble	7·4	2·6	3·1	2·7	2·5	1·78	1·67	1·67	1·98

Figures 4–6 and Tables 4 and 5 show the results of the experiment in which [U^{14}C]-phenylalanine was fed for periods of up to 4 hr to freshly prepared disks of swede root (initial disks) or disks which had previously been aged for 20 hr either in air (control) or in air containing 8 ppm ethylene (ethylene treated). The levels of PAL activity in the initial, 20 hr air and 20 hr ethylene treated disks were $<0{\cdot}1$, 1·8 and 7·5 e.u./10 g fr. wt. For the two sets of aged disks the time course of incubation was followed over a 4-hr period whereas for the initial tissue only a single point after 4 hr incubation was taken. Figure 4 shows the uptake of labelled precursor and the radioactivity associated with the ethanol soluble and ethanol insoluble fractions during incubation. The uptake of precursor shows some differences between the three samples of tissue, being greatest in the initial disks and least in the air-aged tissue. The maximum difference between the air- and ethylene-aged tissue was about 15%. The distribution of radioactivity between the ethanol soluble and insoluble fractions shows the same pattern as in the whole root experiment. The air-aged disks have more radioactivity associated with the ethanol soluble and less associated with the ethanol insoluble compared with ethylene treated samples. The initial sample shows an even more extreme situation with only 11·5% of the radioactivity taken up associated with the ethanol insoluble fraction compared with 39 and 65% respectively in the case of the air- and ethylene-aged disks.

Table 4 shows the radioactivity associated with the ether soluble and ether insoluble sub-fractions of the ethanol soluble fractions of both control and ethylene treated disks. During the first hour of the incubation period there was a greater accumulation of radioactivity in the ether soluble fraction of ethylene treated compared with control disks but subsequently the labelling of this fraction decreased in the ethylene treated sample while it continued to increase in the air control disks so that after 4 hr there was about 30% more radioactivity in the air control tissue. There was a greater accumulation of radioactivity in the ether insoluble fraction of air compared with ethylene treated tissue throughout the incubation period. The radioactivity in this fraction was shown by PC to be associated almost exclusively with unchanged phenylalanine.

The ether soluble fraction was separated by paper chromatography and nearly all the radioactivity of this fraction was shown to be associated with phenolic acids which were subsequently analysed by UV spectrophotometry and liquid scintillation counting. The levels of cinnamic, coumaric, ferulic and sinapic acids were very similar in both ethylene- and air-aged tissue whereas ethylene treated tissue had nearly double the level of caffeic acid. Aged disks had similar levels of cinnamic, ferulic and sinapic acids compared with initial disks but had roughly twice the concentration of caffeic and coumaric acids. The labelling pattern of these phenolic acids during the 4-hr incubation period is shown in Fig. 5.

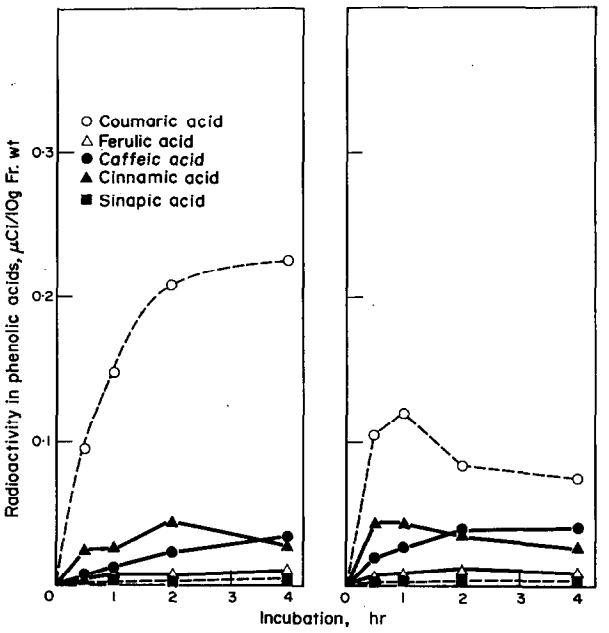

FIG. 5. CHANGES IN THE RADIOACTIVITY ASSOCIATED WITH SOLUBLE PHENOLIC ACIDS SEPARATED FROM DISKS AGED FOR 20 hr EITHER IN AIR (LEFT-HAND CURVES) OR IN THE PRESENCE OF 8 ppm ETHYLENE (RIGHT-HAND CURVES).

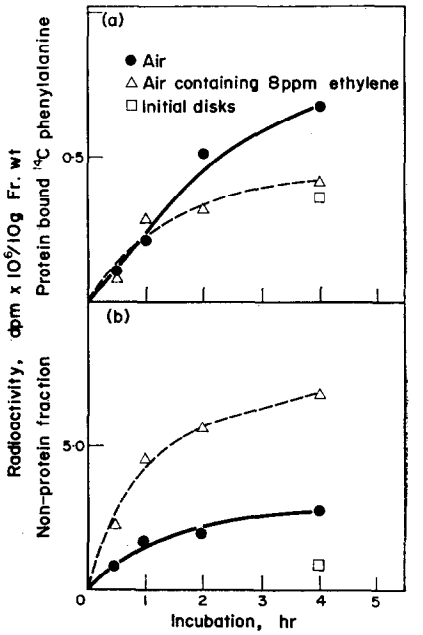

FIG. 6. THE RADIOACTIVITY IN PROTEIN BOUND PHENYLALANINE (UPPER CURVES) AND IN THE NON-PROTEIN RESIDUAL FRACTION (LOWER CURVES) OF THE ETHANOL INSOLUBLE FRACTION OF DISKS AGED FOR 20 hr EITHER IN AIR OR IN AIR CONTAINING ETHYLENE. THE RADIOACTIVITY ASSOCIATED WITH THESE FRACTIONS SEPARATED FROM FRESHLY PREPARED INITIAL DISKS IS ALSO SHOWN.

The ethylene treated tissue showed a higher rate of labelling of caffeic acid throughout the 4-hr period. In the case of coumaric acid, the major labelled component, the rate of labelling in the two types of aged tissue was very similar over one hour but thereafter while the air treated disks continued to accumulate radioactivity in coumarate, the ethylene treated tissue showed a fall. The rates of labelling of sinapic and ferulic acids were similar in both samples.

The ethanol insoluble fraction was subjected to acid hydrolysis and the radioactivity associated with protein bound phenylalanine determined as previously described. Figure 6a shows that the labelling of protein bound phenylalanine was higher in the air-aged disks than in either the ethylene treated or initial disks. The radioactivity associated with protein

accounted for only about 10% of the radioactivity of the ethanol insoluble fraction of the ethylene treated tissue but in the air treated and initial disks it reached as high as 40%. When a correction for the radioactivity associated with protein is applied to the ethanol insoluble residue the remaining non-protein residual fraction (shown in Fig. 6b) shows a 3-fold greater labelling in the ethylene treated compared with the air-aged tissue and over 6-fold greater than in the initial disks.

TABLE 5. THE CONCENTRATION, RADIOACTIVITY AND SPECIFIC RADIOACTIVITY OF LIGNIN OXIDATION PRODUCTS PRODUCED BY THE OXIDATION OF THE ETHANOL INSOLUBLE RESIDUES OF INITIAL AND AGED SWEDE DISKS

	DPM × 10^6/10 g fr. wt								
	Initial disks	Air aged disks				Ethylene aged disks			
	Incubation time (hr)								
	4	0·5	1	2	4	0·5	1	2	4
p-Hydroxybenzaldehyde									
μmol/10 g	0·3	0·56	0·63	0·69	0·58	0·93	1·04	0·96	0·98
μCi/10 g	0·020	0·031	0·051	0·055	0·069	0·067	0·122	0·145	0·16
μCi/mmol	64·4	55·6	94·0	80·0	118·0	71·0	117·0	152·0	163·0
Vanillin									
μmol/10 g	0·18	0·23	0·29	0·27	0·25	0·42	0·36	0·42	0·43
μCi/10 g	0·0041	0·0037	0·0069	0·0076	0·0089	0·0196	0·024	0·039	0·046
μCi/mmol	8·0	15·8	23·0	28·0	34·0	47·2	68·8	91·3	106·3

The ethanol insoluble fractions were again subjected to alkaline copper oxide oxidation and the yield and labelling of the phenolic aldehydes produced was determined. Table 5 shows that the average yield for *p*-hydroxybenzaldehyde was 0·30 μmol/10 g for the initial disks, 0·61 μmol/10 g for the air-aged disks and 0·98 μmol/10 g for the ethylene-aged disks. The corresponding figures for vanillin were 0·18, 0·26 and 0·41. This indicates increases in lignin during ageing of roughly 2-fold in air and three fold in ethylene. There was a 3- to 4-fold greater degree of labelling of *p*-hydroxybenzaldehyde and vanillin in the ethylene treated disks compared with the air disks in the incubation of 4 hr. This indicates a sustained difference in the rates of lignin synthesis in the two types of tissue. In initial disks, the rate of labelling of lignin oxidation products was very low. The specific activities of the two aldehydes is also shown in Table 5. Here again marked difference in specific activity between the ethylene and air treated tissue are displayed but these are smaller than the difference in radioactivity, mainly due to the higher yields of oxidation products from the ethylene treated tissue.

Cytological Localization of Newly Formed Lignin

The tissue used for all the present work was the secondary xylem of swedes in their first season of growth. Such tissue is only very weakly lignified and consists of many unlignified paerenchymatous cells together with a few lignified tracheidal elements. Using phloroglucinol–HCl stain for light microscopy and UV microscopy with light of wavelength 254 nm it was shown that during the ethylene treatment of the whole swede roots the newly formed lignin is laid down in the parenchyma of the secondary xylem.

When initial disks were sectioned, only tracheidal elements stained with phloroglucinol. On ageing, either in air or in air containing low concentrations of ethylene, the parenchyma cells on the surface of the disk stain with phloroglucinol–HCl and absorb UV light (see Fig. 7). The region of the disks responding is only 2–3 cells thick and this represents lignification of the surface of the disk. The air and ethylene treated disks show the same location of response but the response is greater in the ethylene treated tissue.

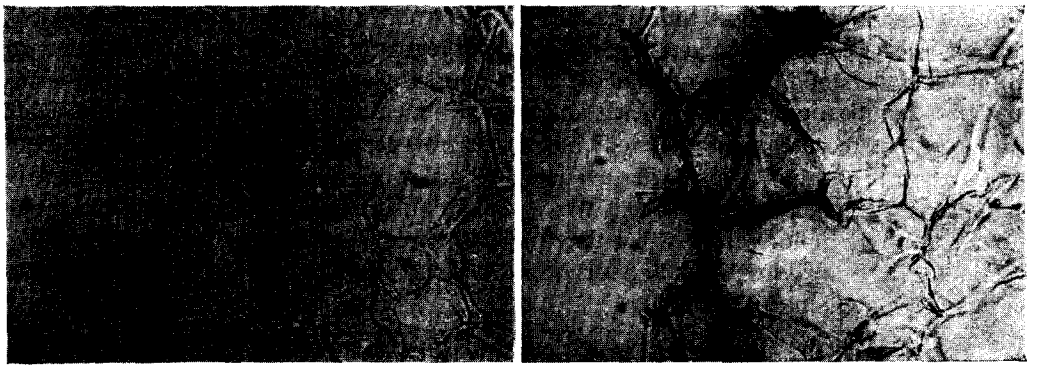

FIG. 7. UV MICROGRAPHS OF SECTIONS OF INITIAL DISKS (LEFT) AND OF DISKS AGED IN THE PRESENCE OF 9 ppm OF ETHYLENE (RIGHT). IN EACH CASE THE SECTION SHOWS PART OF THE EDGE OF THE DISK. MAGNIFICATION × 109. WAVELENGTH UV LIGHT USED 254 nm.

DISCUSSION

The enhanced rate of utilization of phenylalanine as a precursor of cinnamic acid derivatives in tissue treated with ethylene is in agreement with the increased levels of extractable PAL in ethylene treated tissue described in an earlier paper.[1] The main product of this increased phenolic biosynthesis has many of the properties of lignin. With the precise structural formula of lignin still a matter of doubt[7] and the criteria for defining a material as lignin open to various interpretations,[8] the precise characterisation of any product as lignin is clearly difficult. However, our product is phenolic in nature, is associated with plant cell walls, absorbs UV light, stains heavily with phloroglucinol–HCl, is stable to acid and to mild alkaline hydrolysis and on mild alkaline oxidation yields the characteristic lignin phenolic aldehydes. In addition to this lignin-like product, ethylene enhances the biosynthesis of phenolic acids, which exist in the cell in the combined state, probably as either esters or glycosides, and are known precursors of lignin. In untreated whole roots, sinapic acid is the major component but on ethylene treatment the levels of caffeic, coumaric and ferulic acids rise above the level of sinapic acid. In aged disks sinapic acid is always the major phenolic acid but again on ethylene treatment the level of caffeic acid rises.

Both whole swede roots and tissue disks derived from them respond to ethylene with enhanced lignin biosynthesis. In both tissues the secondary xylem parenchyma lignifies but in the disks the response is localised in a layer 2–3 cells thick on the surface of the disks whereas in the whole root the response is more uniform. The significance of this and the factors determining the changed locus of response are not understood. However, Mina-

[7] K. FREUDENBERG, *Constitution and Biosynthesis of Lignin* (edited by K. FREUDENBERG and A. C. NEISH), Springer, Berlin (1968).
[8] I. A. PEARL, *The Chemistry of Lignin*, p. 1, Arnold, London (1967).

mikawa and Uritani[9] showed that the increase in PAL activity on incubation of slices of sweet potato root was greatest at the surface of the disk and it is probably true for the respiratory increase of tissue disks during ageing that the surface cells of the disk are particularly active.[10]

Ethylene has been shown to stimulate the biosynthesis of specific phenolic compounds including isocoumarin in carrot roots[11] and chlorogenic acid in sweet potato tissue.[12] It also has been shown to stimulate PAL activity in sweet potato root slices[13] and in grapefruit flavedo tissue.[14] It also stimulates the activity of both PAL[15] and cinnamate-4-hydroxylase[16] in excised pea epicotyl tissue but its role in regulating the activity of these enzymes is not yet understood. Hormonal factors other than ethylene have been implicated in the control of lignification[5] in plant tissues and these include auxin, kinetin and giberellic acid. This work has recently been reviewed[5] but, in general, very little information on the hormonal control of lignification is yet available. The secondary xylem of the swede root is composed largely of parenchyma cells which remain unlignified until the time of the rapid expansion of the inflorescence when they undergo rapid lignification. This tissue readily responds to hormonal treatment and it is hoped to study the interaction of hormonal effects on lignin synthesis in this material.

EXPERIMENTAL

Whole root experiment. Roots were harvested from 6-month-old swede plants and stored at +1° until required for analysis. Samples of roots were then equilibrated at +15° for 3 days and divided into two batches one was treated for 6 days in an air stream and the other for 6 days in an air stream containing 30 ppm o ethylene. After this treatment, roots were taken from each batch and disks (10 × 2 mm) prepared from them as described previously.[1] Samples of 79 disks (10 g) from each treatment were incubated at 30° in 7 ml of a medium containing 0·25 M mannitol, 0·05 M potassium phosphate (pH 6·0), 50 μg/ml D-threo-chloramphenicol and 10·4 μCi [U^{14}C]phenylalanine (Radiochemical Centre, Amersham—specific activity 10 mC/mmol). Four such incubations were set up for each treatment and allowed to run for 0·5 1, 2 and 4 hr respectively.

Aged disk experiment. Disks (10 × 2 mm) were prepared from swede roots and aged for 20 hr either in air or in air containing 8 ppm ethylene as previously described.[1] After 20 hr, 10 g (79 disks) samples of control and ethylene treated disks were incubated for periods of 0·5, 1, 2 and 4 hr in 7 ml of a medium containing 0·25 M mannitol, 0·05 M potassium phosphate (pH 6·0), 50 μg/ml *D*-threo-chloramphenicol and 5·2 μCi [U^{14}C]phenylalanine (specific activity 10 mCi/mmol—Radiochemical Centre, Amersham).

Extraction procedure. The subsequent extraction of the incubated tissue was identical in both types of experiment. After incubation, the disks were separated from the incubation medium, washed with 0·01 M ^{12}C-phenylalanine and the washings and media made up to 100 ml and samples taken for counting as an estimate of the uptake of the labelled precursor by the disks during the incubation. The disks were frozen in liquid N_2 and subsequently fixed in boiling EtOH and homogenised with an Ultra-turrax homogenizer. The residue after centrifugation was re-extracted with absolute EtOH and then subjected to 5 further extractions in the cold with 80% alcohol. In the third of these extractions 0·01 M ^{12}C-phenylalanine was included to exchange any loosely bound radioactive material associated with the residue. The residue was finally re-extracted with absolute EtOH, EtOH–Et_2O (1:1) and finally with Et_2O. All the EtOH and Et_2O extracts were combined as the EtOH soluble fraction while the residue was dried *in vacuo* over P_2O_5 and designated the EtOH insoluble fraction.

Fractionation of the EtOH *soluble fraction.* The EtOH soluble fraction after evaporation under reduced pressure was extracted with light petrol. (40–60°), and the aqueous phase hydrolysed with 2 N NaOH at

[9] T. MINAMIKAWA and I. URITANI, *Agric. Biol. Chem.* **29**, 1021 (1965).
[10] G. G. LATIES, in *Control Mechanisms in Respiration and Fermentation* (edited by B. WRIGHT), p. 129, Ronald Press, New York (1961).
[11] E. CHALUTZ, J. E. DEVAY and E. C. MAXIE, *Plant Physiol. Lancaster* **44**, 235 (1969).
[12] H. IMASEKI, T. ASAHI and I. URITANI, in *Biochemical Regulation in Diseased Plants or Injury* (edited by T. HIRAI), pp. 189–201, Kyoritan Printing, Tokyo (1968).
[13] H. IMASEKI, M. UCHIYAMA and I. URITANI, *Agric. Biol. Chem.* **32**, 387 (1968).
[14] J. RIOV, S. P. MONSELISE and R. S. KAHAM, *Plant Physiol. Lancaster* **44**, 631 (1969).
[15] H. HYODO and S. F. YANG, *Plant Physiol.* **47**, 765 (1971).
[16] H. HYODO and S. F. YANG, *Arch. Biochem. Biophys.* **143**, 338 (1971).

0° for 16 hr in N_2.[3] The hydrolysate was acidified, extracted with Et_2O and the aqueous and Et_2O soluble fractions separated. The aqueous phase was analysed by ion exchange chromatography on Dowex 50 and by PC using *n*-BuOH–HoAc–H_2O (4:1:5); the only labelled compound detected was shown to be phenylalanine. The ether phase was re-extracted with 1% freshly prepared $NaHCO_3$, the aqueous phase was re-acidified and extracted 5× with Et_2O. The combined Et_2O fractions were evaporated to dryness in N_2 and then taken up in 0·5 ml absolute EtOH. It was shown that more than 80% of the radioactivity of the fraction was associated with cinnamic, coumaric, caffeic, ferulic and sinapic acids. The identity of these phenolic acids was confirmed by their R_f in 2 solvents on TLC and 3 solvents on PCs, their characteristic alkaline UV absorption spectra after elution from the chromatograms, their appearance when viewed under UV light and their specific colour reactions with *o*-dianisidine diazonium salt and with diazotised *p*-nitroaniline. For the quantitative determination of the phenolic acids and the radioactivity associated with them, aliquots of the final ether fraction were applied to Whatman No. 20 filter paper and run in; (a) one dimension using toluene–HOAc–H_2O (10:7:3), or (b) in two dimensions using toluene–HCO_2Et–HCO_2H (5:4:1) and toluene–HOAc–H_2O (10:7:3). The spots were located under UV light, cut out and eluted with 5 ml 0·1 N NaOH, except for caffeic acid which was eluted with 5 ml absolute EtOH. UV spectra from 400–250 μm were read against a blank area of the chromatogram similarly extracted. Aliquots of the elutates were analysed by liquid scintillation counting using Triton X100-toluene phosphor.[17] The above method was shown in separate experiments to give better than 95% recoveries of known amounts of standards taken through the chromatographic procedures.

Analysis of the ethanol insoluble fraction. Mild alkaline hydrolysis. 50 mg aliquots of the EtOH insoluble residue were hydrolysed in 4 ml of 1 N NaOH at 30° for 4 hr.[18] The hydrolysate was examined for radioactivity and the presence of phenolic acids.

Hydrolysis of protein associated with the EtOH insoluble residue. Weighed aliquots of the EtOH insoluble residue were subjected to the standard protein hydrolysis procedure, namely 6 N HCl in a sealed vial at 105° for 15 hr. After hydrolysis, the hydrolysate was purified by ion exchange[19] and PC. Essentially all the radioactivity in the fraction was associated with the phenylalanine spot. Tyrosine, although present in the extract, was unlabelled.

Oxidation of lignin by alkaline copper oxide. 25 mg of EtOH insoluble residue was oxidised in a stainless steel bomb with 170 mg $CuSO_4$ and 1·5 ml 3 N NaOH at 180° for 2·5 hr.[4] After oxidation the residue was removed by centrifugation and the supernatant was acidified and then extracted with Et_2O. The combined Et_2O extracts were extracted once with 5 ml of freshly prepared 1% $NaHCO_3$ and the Et_2O and aqueous fractions separated. The Et_2O phase contains non-acidic phenolic compounds including phenolic aldehydes. The ether fraction was taken to dryness in N_2 and re-dissolved in 0·5 ml dry abs. EtOH and an aliquot taken for counting. Two further aliquots, one of 0·4 ml and the other 0·02 ml, were applied to Whatman No. 1 chromatography paper together with suitable standards. The chromatogram was developed in light petrol.–Bu_2O–HOAc–H_2O (6:1:1:2) and the absorbing aldehydes located by viewing under UV light. The strip of paper corresponding to the smaller aliquot of extract was used to locate the radioactive areas of the chromatogram. One radioactive region was close to the base line of the chromatogram and was associated with brown pigmented material which reacted strongly with $FeCl_3$–$K_3Fe(CN)_6$ spray and was probably polyphenolic. The other two radioactive areas were subsequently shown to be associated with *p*-hydroxybenzaldehyde and vanillin. The identity of these radioactive areas was confirmed by the correspondence of radioactivity with the positions of *p*-hydroxybenzaldehyde and vanillin in two dimensional TLC separations using toluene–HoAc–H_2O (10:7:3) and *n*-BuoH–NH_4OH–H_2O (4:1:5) and from their R_fs on paper in the latter solvent. In all cases the alkaline UV absorption spectra of the radioactive areas eluted with 2% KOH in alcohol[20] agreed closely with those given by pure *p*-hydroxybenzaldehyde and vanillin. In no case has evidence for the presence of syringaldehyde in our extracts been obtained. This agrees with the findings of Asada and Matsumoto[21] using Japanese radish, a member of the same plant family as the swede, in which there was no evidence for the formation of syringaldehyde on oxidation of lignin. For quantitative estimation of the phenolic aldehyde oxidation products, the absorbing areas corresponding to *p*-hydroxybenzaldehyde and vanillin from the chromatogram of the larger aliquot of the Et_2O extract were cut out and eluted with 2% KOH in absolute alcohol. The concentration of aldehyde was determined by UV spectrophotometry and the radioactivity by liquid scintillation counting by methods similar to those already described for soluble phenolic acids. The $NaHCO_3$ phase after separation of the neutral phenolic fraction was again acidified and re-extracted with Et_2O. This ether fraction was analysed by chromatograph on Whatman No. 20 paper in exactly the same way as the soluble phenolic acid fraction. In this case, nearly all the radioactivity was associated with material remaining on the base line and probably of high MW

[17] M. S. Patterson and R. C. Greene, *Analyt. Chem.* **37**, 554 (1965).
[18] S. El-Basyouni and G. H. N. Towers, *Can. J. Biochem.* **42**, 203 (1964).
[19] A. C. Hulme, *Food Sci. Abs.* **28**, 345 (1956).
[20] H. W. Lemon, *Analyt. Chem.* **19**, 846 (1947).
[21] Y. Asada and I. Matsumoto, *Ann. Phytopath. Soc. Japan* **35**, 160 (1969).

polyphenolic character. The remaining radioactivity was distributed between a number of very minor components.

Isolation and oxidation of Klason lignin. Klason lignin was isolated[6] from weighed aliquots of the EtOH insoluble residue, subjected to alkaline copper oxide oxidation and the phenolic aldehyde products analysed in the usual way.

Cytochemical examination of tissue. Thin sections of the tissue disks were cut on a freezing microtome and viewed either in a high resolution light microscope after staining with phloroglucinol–HCl or in a UV light microscope using light of wavelength 254 nm.

Phenylalanine ammonia lyase activity. Samples of swede root tissue were extracted and their content of PAL assayed by the methods described by Rhodes and Wooltorton.[1] The enzyme unit was defined as the amount of enzyme capable of producing 1 μmol of cinnamic acid per hr under the experimental conditions.

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