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METABOLISM OF CHLOROPHENYLALANINES IN CROP AND WEED PLANTS IN RELATION TO THE FORMATION OF POTENTIAL HERBICIDAL END PRODUCTS*

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Key Word Index—*Phaseolus vulgaris; Glycine max.;* Leguminosae; *Zea mays; Amaranthus retroflexus; Chenopodium album; Setaria faberii;* metabolism; D,L-chlorophenylalanines; chlorophenylacetic acids; chlorobenzoic acids; chlorocinnamic acids; growth regulators.

Abstract—Metabolism of 12 synthetic D,L-chlorophenylalanines has been examined in several crop and weed plants. Twenty-five gram samples of excised shoots or leaves of bushbean, soybean, corn, pigweed, lambsquarters and giant foxtail were allowed to metabolize 10^{-4} M solutions of the D,L-chlorophenylalanines for 24 hr in continuous light. The plant samples were then extracted in 80% methanol and the soluble acidic metabolites fractionated into ether. Each ether concentrate was partially purified by fractional elution from a PrepSep C18 column and then analysed by HPLC. Collected fractions were esterified with pentafluorobenzylbromide and examined by GC-MS to demonstrate the presence of PFB-esters of chlorophenylacetic, chlorobenzoic and/or chlorocinnamic acids. Since certain of these metabolites are known to be potent plant growth-regulators and herbicides, the results are discussed in relation to the potential herbicidal action of certain chlorophenylalanines by the mechanism of 'lethal synthesis'.

INTRODUCTION

The first recognition of the selective herbicidal properties of synthetic auxins was made independently in the 1940's by scientists in the U.S.A. and Britain. Zimmerman and Hitchcock [1] first demonstrated that several chlorophenoxyacetic acids are more powerful auxins than naphthylacetic acid and this finding soon led to the discovery by Hamner and Tukey [2, 3] that certain of these compounds are very potent herbicides. During the same period and without knowledge of the American investigations, Slade, Templeman and Sexton in war-time Britain examined the effect of synthetic auxins on crop growth. They discovered and later reported [4] that naphthylacetic acid could selectively kill yellow charlock weeds in a stand of wheat. Certain of the most active chlorophenoxyacetic acids (eg. 2,4-D and MCPA) continue to be important herbicides used world-wide for weed control in cereal crops.

The growth-regulating activity and herbicidal properties of ring-substituted chlorophenylacetic acids [5–7], chlorobenzoic acids [8–10] and chloro-*cis*-cinnamic acids [11] were subsequently demonstrated. With the phenylacetic and cinnamic acids, the positional effect of monochloro- ring-substitution on enhancing the growth-regulating activity of the parent compound was in the order 2- > 3- > 4-. With dichloro-substitution, the 2,3- and 2,6-derivatives were very active auxins in both series of compounds, but the 2,3,6-trichloro-acids were the most potent growth regulators and were found to exhibit herbicidal activity equal to that of 2,4-D. In the chlorobenzoic acid series, none of the mono-substituted derivatives showed activity, but the 2,3-, 2,6- and especially the 2,5-dichloro-acids were active compounds. Again, the most active compound in the series was 2,3,6-trichlorobenzoic acid [8–10, 12], which was also found to be a very potent herbicide [6, 13].

Investigations at Wye in the early 1950's were the first to demonstrate that certain higher homologues of 2,4-D and 2,4,5-T can be converted in some plants, but not in others, to the highly active acetic derivative which then exhibits herbicidal activity [14]. Destruction of the susceptible species occurs due to 'lethal synthesis' of the herbicide molecule *in vivo* [15]. This approach to herbicide formation in susceptible species arose from the demonstration [16] that 2,4,5-trichlorophenoxybutyric, -caproic and -octanoic acids can be metabolized in some dicotyledonous plants, but not in others, to the highly active acetic derivative (2,4,5-T) by the process of β-oxidation. This finding led to the development of γ-2,4-dichlorophenoxybutyric acid (2,4-DB) and γ-2-methyl-4-chlorophenoxybutyric acid (MCPB) as selective herbicides for use in cereal, clover and celery crops [14, 15, 17].

In more recent studies on auxin metabolism carried out in this laboratory, the biosynthesis of the natural auxins,

*Part 4 in the series: Metabolism of chlorophenylalanines by multispecific aspartate-aromatic aminotransferases in crop and weed plants. This series is dedicated to the memory of our former colleague, Budhi Singh Rauthan, who first demonstrated in 1972 the *in vivo* conversion of L-phenylalanine to phenylacetic acid in higher plants. Mr. Rauthan was killed in the crash of the Air India Boeing 747 into the Atlantic Ocean, 23 June 1985.

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3-indolylacetic acid (IAA) and phenylacetic acid (PAA) has been extensively investigated [18, 19–22]. Tracer experiments with a range of plants have shown that both auxins are derived from their corresponding amino acids, L-tryptophan and L-phenylalanine, via two reaction pathways known as the arylpyruvate and arylethylamine pathways, depending on the nature of the first intermediate formed in each route. Thus, in PAA biosynthesis, L-phenylalanine is initially converted either to phenylpyruvate via an aminotransferase reaction, or to phenylethylamine via the action of a decarboxylase. Both intermediates are then metabolized by different enzymes to phenylacetaldehyde, which is then oxidized to PAA [23, 21, 22]. Phenylalanine is also metabolized in many plants and fungi to cinnamic acid by the enzyme, phenylalanine ammonia lyase [24, 25]. This C_6-C_3 acid is the main intermediate leading to the synthesis of a wide range of secondary compounds [26] and is also known to give rise to benzoic acid in both plants and fungi [25, 26]. Zenk [27] has proposed that this C_6-C_3 to C_6-C_1 chain-shortening reaction requires the involvement of a cinnamoyl-CoA derivative which could enter the β-oxidation pathway and so undergo β-oxidative cleavage to give benzoyl-CoA, which could then be split by a thiolesterase to yield benzoic acid. Evidence for a thylakoid bound enzyme system capable of converting L-phenylalanine to benzoic acid has been reported in thylakoid membranes of green and blue-green algae [28, 29] and higher plants [30]. However, there is also the possibility that benzoic acid could arise from the further metabolism of PAA, in a manner analogous to the known formation of 3-indolecarboxylic acid from IAA [31–33].

In view of the known routes of synthesis of PAA, cinnamic and benzoic acids in plants and our demonstration of the presence of a multispecific aminotransferase in bushbean shoots capable of transaminating chlorophenylalanines [34–36], it was logical to investigate whether bushbean and other crop or weed plants can selectively metabolize certain chlorophenylalanines to chloro-PAA, chlorobenzoic and/or chlorocinnamic acids, as outlined in Fig. 1. Many of these chlorophenyl acids have already been shown to possess strong growth-regulating and herbicidal properties [6, 7, 10, 11] and the formation of such phytotoxic end products from chlorophenylalanine metabolism would provide a further example of herbicidal action via lethal synthesis. This paper reports the results of preliminary studies examining the metabolism of a series of D,L-chlorophenylalanines in six crop and weed plants.

RESULTS AND DISCUSSION

Chemical syntheses

A series of 12 ring-substituted mono-, di- and trichloro-D,L-phenylalanines were synthesized as previously described [37] to provide the principal substrates for the *in vivo* plant metabolism experiments reported here. The corresponding chlorobenzoic acids (Cl-BzA's) and chlorophenylacetic acids (Cl-PAA's) were required as authentic standards for identification of the expected end products of chlorophenylalanine metabolism (Fig. 1). Thus, 9 di- and trichlorophenylacetic acids which were not commercially available were synthesized by a condensation of the appropriate benzyl halide with NaCN in DMSO to give the corresponding chlorophenylacetonitrile, the structure of which was confirmed by both IR and NMR spectroscopy. The nitrile was then base-hydrolysed to yield the required chlorophenylacetic acid. Overall yields by this synthetic route were *ca* 60–85%, and each acid was characterized by melting point, IR and GC-MS (Tables 1 and 2). The melting point data agreed closely with those reported by Pybus *et al.* [7]. HPLC of the chlorophenylacetic acids gave only one major peak in each case (relative retention times are reported in Table 1), indicating that the compounds were $\geq 95\%$ pure and therefore suitable for use as GC-MS standards. To our knowledge, the 2,3,4- and 2,4,5-trichlorophenylacetic acids have not previously been reported.

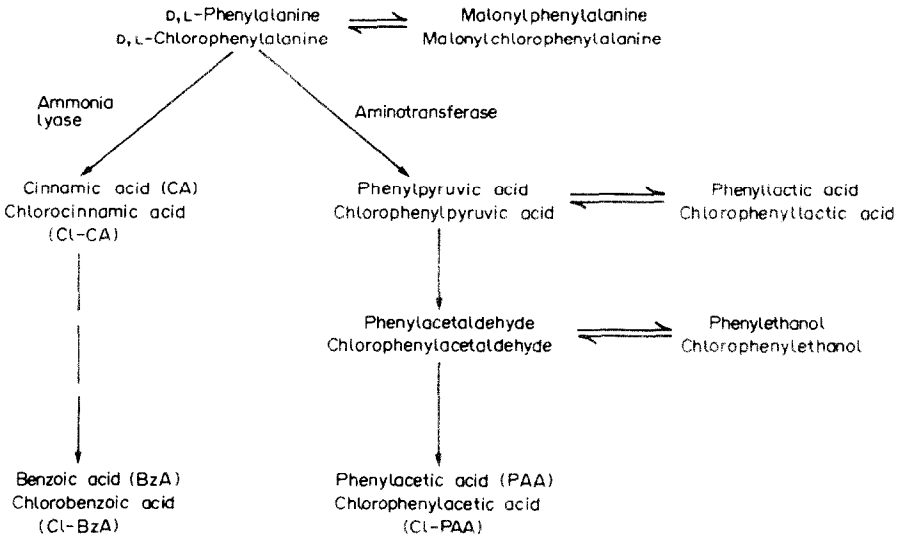


Fig. 1. Biochemical pathways for the conversion of D,L-phenylalanine and D,L-chlorophenylalanines to their corresponding phenylacetic (PAA), cinnamic (CA) and benzoic (BzA) acids in shoot tissues.

The four chlorobenzoic acids not commercially available were synthesized by classical methods and characterized as described in the Experimental. Melting point and HPLC retention times as well as the GC-MS fragmentation patterns for all synthetic chlorobenzoic and chlorocinnamic acids used as GC-MS standards are reported in Tables 1 and 2.

Metabolism studies

Development of methodology. Metabolism experiments designed to examine the potential for *in vivo* conversion of chlorophenylalanines to the corresponding chloro-PAA and/or chlorocinnamic and chlorobenzoic acids, were conducted by feeding the individual chlorophenylalanines to excised shoots or leaves and leaf sheaths of six species. Bushbean, soybean and corn were the crop plants used while pigweed, lambsquarters and giant foxtail were the weeds examined.

Samples of shoots or leaves (25 g fr. wt) were fed with 10^{-4} M solutions of the D,L-chlorophenylalanines under constant light and temperature conditions and when the feeding solutions had been absorbed (within 8 hr), the shoots were then supplied with distilled water for a further 16 hr to allow metabolism to occur. Three control experiments were also run: one sample of shoots was fed with 10^{-4} M D,L-phenylalanine at pH 7.0, a second sample was fed with distilled water at pH 7.0 and a third sample was frozen without feeding at $-22°$ until analysed.

After the 24 hr metabolism period, each sample of shoots or leaves was subjected to the extraction and fractionation procedure outlined in the Experimental for the isolation and identification of acidic metabolites with potential growth-regulating activity. The metabolites of interest were the chlorophenylacetic acids (Cl-PAA's) and chlorobenzoic acids (Cl-BzA's) and when standards were available, the chlorocinnamic acids (Cl-CA's) were also monitored. When an untreated bushbean shoot sample was extracted in 80% methanol immediately after harvesting and the extract spiked with 10 μg amounts of authentic 3-chloro-PAA, 3-chloro-BzA and 3-chloro-CA and then processed as outlined in the Experimental, overall recoveries of 75, 50 and 66% of the three standards were obtained. It was found that if a neutral ether fraction was removed prior to acid ether fractionation, recovery of added standards was reduced by more than 50% over the levels recovered when only an acid ether extraction was made. This indicated that considerable partitioning of the chloro-acids into the ether phase was occurring at neutral pH, a finding not predicatable from the dissociation constants of these chloro-acids [38]. Neutral ether fractionation was therefore not included as a partial purification step prior to acid ether extraction of the chlorophenyl metabolites.

In a comparison of derivatization methods suitable for GC-MS indentification of the chloro-acids, it was found

Table 1. Melting points and HPLC retention times of chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids

	Phenylacetic acids		Benzoic acids		Cinnamic acids†	
Chloro-substitution pattern	Melting point* °C	HPLC‡ retention time (min)	Melting point* °C	HPLC‡ retention time (min)	Melting point °C	HPLC‡ retention time (min)
Unsubstituted	77–78.5	20.5	122–123	22.9	128–130	31.0
2-chloro-	94–95 (94.5–95.5)	27.5	139–140 (138–140)	24.1	208–210	37.0
3-chloro-	95–96 (77–78)	31.0	150–151 (154–155)	34.4	159–161	38.7
4-chloro-	104–105 (105.5–106.5)	30.7	240–241 (243.5–245)	34.2	248–250	38.0
2,3-dichloro-	129–130.5 (131.5–134)	35.0	163–165 (167–169)	20.8	—	—
2,4-dichloro-	132–133 (131–132.5)	36.8	171–172 (162–163)	36.2	—	—
2,5-dichloro-	109–110 (104–106)	35.0	150–151 (151–153.5)	34.0	—	—
2,6-dichloro-	157–157.5 (156.5–162)	36.5	143–143.5 (142–145)	19.7	194–196	42.5
3,4-dichloro-	91–92 (87–89)	37.2	204–205 (207–208)	43.4	—	—
3,5-dichloro-	110–111 (112–115)	39.1	185–187.5 (188–190)	47.5	—	—
2,3,4-trichloro-	147–149.5	42.0	138–140	42.5	—	—
2,3,6-trichloro-	161–161.5 (159–161)	39.6	124–125 (125–127)	44.0	—	—
2,4,5-trichloro-	150–152	41.0	158–159	44.3	—	—

*Lit mps in brackets. References for phenylacetic acids [7], benzoic acids [10] and cinnamic acids [11].

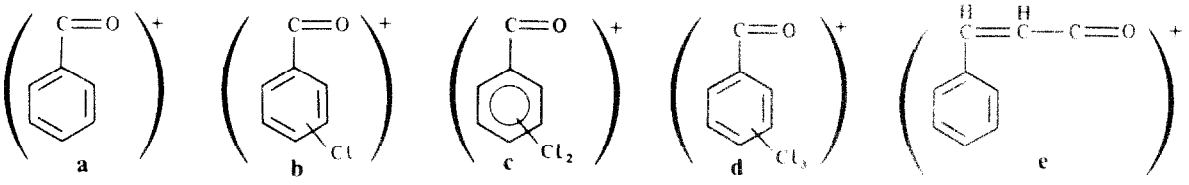
†Primarily *trans* form in these commercially-prepared compounds.

‡Retention times on Whatman P-10 reversed phase column on gradient specified in Experimental.

Table 2. Characteristic ion fragmentation patterns obtained by GC-MS

Degree of ring substitution	Phenylacetic acids			
	m/z Rel %	Character of fragment ion	*m/z* Rel %	Character of fragment ion
Unsubstituted	316 (7.6)	M^+ PFB-PAA	91 (100)	(Benzyl Grp)$^+$
Monochloro-substituted	350 (6.5)	M^+ PFB-ClPAA	125 (100)	(Cl-Benzyl Grp)$^+$
	352 (2.2)	M^+2 "	127 (49)	" containing ^{37}Cl
Dichloro-substituted	384 (12.3)	M^+ PFB-Cl_2PAA	159 (100)	(Cl_2-Benzyl)$^+$
	386 (7.7)	M^+2 "	161 (63.8)	2 heavy isotope ^{37}Cl peaks
	388 (1.7)	M^+4 "	163 (10.0)	
Trichloro-substituted	418 (6.0)	M^+ PFB-Cl_3PAA	193 (54)	(Cl_3-Benzyl)$^+$
	420 (5.9)	M^+2 "	195 (50.5)	
	422 (2.3)	M^+4 "	197 (18.1)	3 heavy isotope ^{37}Cl peaks
	424 (ND)	M^+6 "	199 (1.8)	

*Actual relative % values given in brackets beneath each *m/z*. Theoretical ND. Not detected; theoretical value below limit of detection (rel % 0.5).

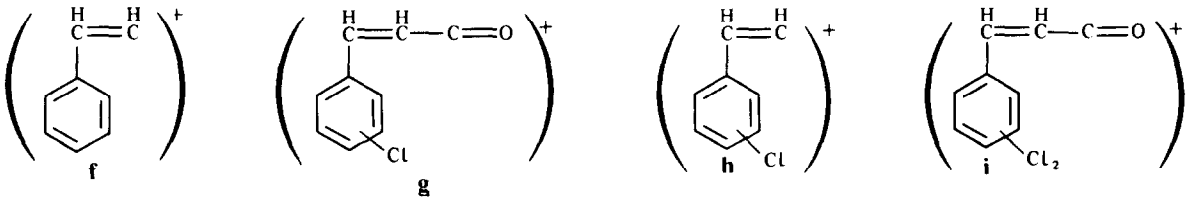


that methylation using the procedure of ref. [39] resulted in small amounts of alkylating agent and base remaining in the reaction mixture which caused destruction of the pre-column of the GC-MS. Using the Me-8 alkylating reagent circumvented this problem since it allowed direct injection of the derivatization mixture into the GC-MS without damage to the GC column. However, it was found that methyl esters of the chloro-acids gave weaker total ion current (TIC) signals from the mass spectrometer in comparison to equivalent amounts of the corresponding pentafluorobenzyl (PFB) esters (see Fig. 2). This indicated that statistically, the PFB ester ionized more strongly than the corresponding methyl ester which resulted in a greater electron cross section and therefore a stronger TIC signal. Since the amount of each chloro-acid metabolite was to be estimated by measuring the area of its molecular ion peak, relative to the area given by a known amount of standard, it was decided to use the PFB

of pentafluorobenzyl esters of chlorophenylacetic, chlorobenzoic and chlorocinnamic acids*

Benzoic acids				Cinnamic acids			
m/z Rel %	Character of fragment ion	*m/z* Rel %	Character of fragment ion	*m/z* Rel %	Character of fragment ion	*m/z* Rel %	Character of fragment ion
302 (27.9)	M^+ PFB-BzA	105 (100)	**a**	328 (55.5)	M^+ PFB-CA	131 (100)	**e**
						103 (77.9)	**f**
336 (51)	M^+ PFB-ClBzA	139 (100)	**b**	362 (6.7)	M^+ PFB-ClCA	165 (12.3)	**g**
338 (18.3)	M^+2 "	141 (30.6)	" containing ^{37}Cl	364 (2.8)	M^+2 "	167 (3.8)	1 heavy isotope peak
						137 (12.1)	**h**
						139 (4.2)	1 heavy isotope peak
370 (23.6)	M^+ PFB-Cl_2BzA	173 (75.7)	**c**	396 (3.2)	M^+ PFB-Cl_2CA	199 (7.6)	**i**
372 (16.4)	M^+2 "	175 (49.5)		398 (23.4)	M^+2 "		
			2 heavy isotope	ND	M^+4 "		
374 (2.5)	M^+4 "	177 (8.3)	^{37}Cl peaks	361 (23.4)	$M^+ - Cl$	201 (4.4)	2 heavy isotope ^{37}Cl peaks
				363 (7.7)	$M^+2 - Cl$	203 (0.9)	
404 (15.8)	M^+ PFB-Cl_3BzA	207 (38.8)	**d**				
					Not available		Not available
406 (16.4)	M^+2 "	209 (36.6)					
408 (5.2)	M^+4 "	211 (12.4)	3 heavy isotope ^{37}Cl peaks				
410 (ND)	M^+6 "	213 (1.5)					

rel % ratios: Monochloro- 3:1; Dichloro- 1:0.6:0.1; Trichloro- 1:0.9:0.3:0.03; see ref. [42].



derivatization procedure since these esters not only gave the strongest TIC signals, but also gave very characteristic fragmentation patterns in the mass spectrometer (Table 2).

Metabolism of phenylalanine by crop and weed plants. The amounts of PAA, BzA and CA found in excised shoots or leaves of each of the six species examined after 24 hr feeding with D,L-phenylalanine or distilled water, compared to initial endogenous levels of these acids in untreated shoots is shown in Table 3. Initial levels of PAA were found to be in the 40–80 ng range per g fresh weight of shoot or leaf tissue for all six plants examined. These values are similar to those reported previously from this laboratory for untreated shoots; for example, Wightman and Lichty [40] reported a range of 70–220 ng PAA/g fresh weight in shoots of several crop plants using gas chromatography of methylated extracts containing an internal standard as the method of analysis. Recently,

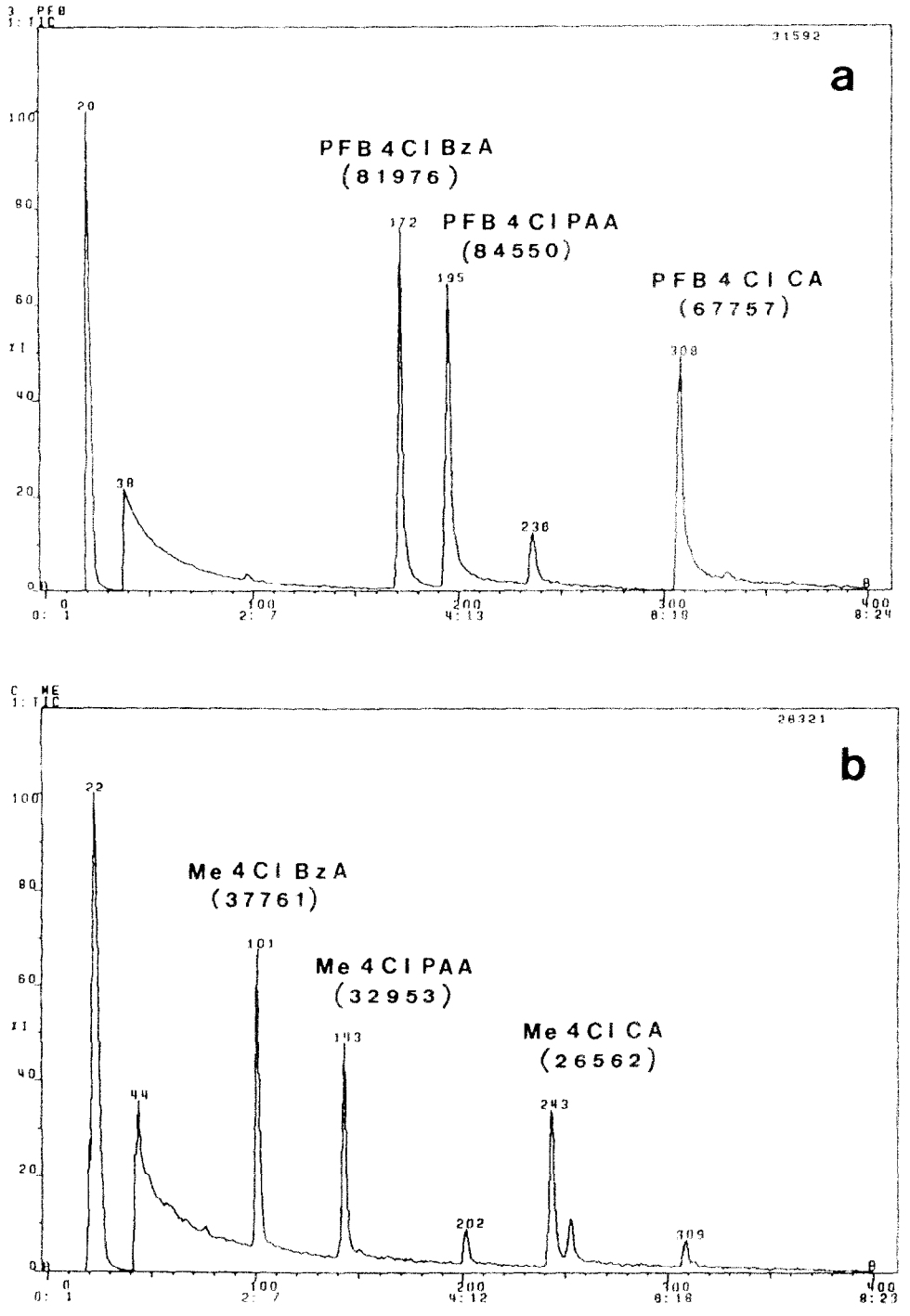


Fig. 2. Comparison of TIC scans and peak areas given by equivalent amounts of pentafluorobenzyl- (a) and methyl- (b) esters of 4-chlorobenzoic acid (4-ClBzA), 4-chlorophenylacetic acid (4-ClPAA) and 4-chlorocinnamic acid (4-ClCA).

Schneider *et al.* [41] found 58.1 ng PAA per g fresh weight in pea epicotyls using single ion monitoring with an internal deuterated standard as the method of quantitation. In the present study, corn leaves were found to contain quite low levels of endogenous PAA (*ca* 4 ng/g fr. wt), which was less than one tenth of the amount found in other plants. Since the shoot apical meristem in a corn seedling is very close to ground level, it was not excised with the leaves and sheath tissues used in the present experiments. It is possible that the separation of the stem and apical meristem from the excised leaves removed a large proportion of the endogenous PAA normally present in the entire corn shoot system.

The shoots and leaves of all plants fed with D,L-

Table 3. Amounts of phenyl metabolites produced in excised shoots or leaves of crop and weed plants fed with a 10^{-4} M solution of D, L-phenylalanine (PHE) or with distilled water for 24 hr

Plant examined	Feeding solution*	Metabolites produced: Phenylacetic acid Total ng per sample†	Phenylacetic acid ng per g fresh wt	Benzoic acid Total ng per sample†	Benzoic acid ng per g fresh wt	Cinnamic acid Total ng per sample†	Cinnamic acid ng per g fresh wt
Bushbean	PHE	5020	200	600	24	230	9.2
	Water	2500	100	550	22	350	14
	Unfed	810	32.5	280	11	280	11.2
Soybean	PHE	6800	270	52500	2100	1500	60
	Water	3700	150	20200	808	540	21.6
	Unfed	1020	41	16560	662	410	16.4
Corn	PHE	135	5.5	280	11.2	—	—
	Water	120	4.8	265	10.6	—	—
	Unfed	100	4.0	240	9.6	—	—
Pigweed	PHE	4930	197	2540	102	65	2.6
	Water	1830	73	1300	52	—	—
	Unfed	1330	53	1035	41	—	—
Lambs-quarters	PHE	5150	206	1400	56	165	6.6
	Water	2820	113	5220	209	400	16
	Unfed	1500	60	1050	42	80	3.2
Giant foxtail	PHE	6060	242	3300	132	—	—
	Water	4230	169	1790	72	—	—
	Unfed	2120	85	510	20	—	—

*Twenty five gram samples of shoot or leaf tissue were supplied with either 10^{-4} M solutions of D, L-PHE (25 ml) or with distilled water for 24 hr. Unfed tissue was frozen immediately after harvesting at $-22°$.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard. —: metabolite not detected.

phenylalanine generally showed a 3- to 4-fold increase in amounts of PAA over initial levels (Table 3), indicating that a PAA biosynthetic pathway was operating in all plants. This finding agrees with previous results which showed that ^{14}C-PAA was produced in excised shoots of several crop plants during the metabolism of exogenously supplied [3-^{14}C]-D,L-phenylalanine [19, 21]. In the present study, water-fed control shoots also consistently showed an increase in PAA over initial endogenous levels. This increase may reflect an enhanced production of aromatic and phenolic compounds (secondary metabolites) by excised shoots and leaves in response to the stress of excision.

The amounts of benzoic acid in the shoots and leaves fed with D,L-phenylalanine, or water, generally increased above the initial endogenous levels in proportions similar to the observed increases in PAA. In contrast, the detectable levels of endogenous cinnamic acid were quite low and with the exception of soybean, no obvious pattern of change in this metabolite was apparent in any of the shoots after feeding with D,L-phenylalanine or with water. Since benzoic acid was produced in appreciable amounts in all shoots supplied with D,L-phenylalanine, except corn, these results suggest that in addition to its known formation from cinnamic acid [24, 25], benzoic acid may also arise from the catabolism of PAA in a manner analogous to the known formation of 3-indolecarboxylic acid (ICA) from 3-indoleacetic acid (IAA) in higher plants [31–33]. However, this possibility requires further study utilizing radiolabelled PAA.

Metabolism of chloro-phenylalanines in bushbean—a typical crop plant. Bushbean was chosen as the typical crop plant for initial examination of chlorophenylalanine metabolism since previous studies in our laboratory had shown that some of these synthetic amino acids were metabolized *in vitro* by a multispecific aspartate-aromatic aminotransferase purified from bushbean shoots [34, 35]. This transamination reaction would be the initial step in the proposed arylpyruvate pathway leading from a chlorophenylalanine to the corresponding chlorophenylacetic acid, as indicated in Fig. 1.

The data obtained from experiments in which bushbean shoots were fed with each of the 12 D,L-chlorophenylalanines show that with the monochloro-analogues, only 3-chlorophenylalanine was selectively metabolized, presumably via the arylpyruvate pathway, to yield high levels of the potent 3-chloro-PAA (Table 4). The calculated molar rate of conversion of 3-chlorophenylalanine to 3-chloro-PAA was between 1.4 and 2.8%, depending on whether the plant was able to use both D- and L-forms of 3-chlorophenylalanine, or whether only the L-isomer was metabolized. While we have shown that the multispecific asparatate-aromatic aminotransferase present in bushbean shoots is L-phenylalanine-specific [35], there is always the possibility for *in vivo* conversion of the D-isomer to its L-form by a racemase. Alternatively, the D-chlorophenylalanine isomer may be metabolized by a D-amino acid oxidase to the optically inactive chlorophenylpyruvate which could then be metabolized to the corresponding chloro-PAA via the pathway outlined in Fig. 1.

Of the dichlorophenylalanines tested, only the 3,4-

Table 4. Amounts of chloro-phenyl metabolites produced in excised shoots of bushbean fed with 10^{-4} M solutions of different D,L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	ng per g fresh wt
2-Cl-	—	—	175	7	—	—
3-Cl-	6000	240	175	7	45	2
4-Cl-	—	—	2500	100	460	18
2,3-Cl_2-	—	—	—	—	ND	ND
2,4-Cl_2-	—	—	45	2	ND	ND
2,5-Cl_2-	—	—	585	24	ND	ND
2,6-Cl_2-	—	—	—	—	—	—
3,4-Cl_2-	295	12	4850	194	ND	ND
3,5-Cl_2-	—	—	3200	128	ND	ND
2,3,4-Cl_3-	—	—	—	—	ND	ND
2,3,6-Cl_3-	—	—	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*Twenty-five gram samples of bushbean shoots were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

—Metabolite not detected. ND: Not determined.

dichloro-compound was metabolized to produce the corresponding 3,4-dichloro-PAA. Here the molar conversion rate was 0.06–0.12%, about 25-fold lower than that observed for 3-chlorophenylalanine, discussed above. None of the trichloro-phenylalanines were converted to their corresponding trichloro-PAA's. Of the possible other acidic metabolites, all three monochlorobenzoic acids and four of the six possible dichlorobenzoic acids were produced in bushbean shoots, among them the potent growth regulator, 2,5-dichlorobenzoic acid [10]. The 3- and 4-chlorocinnamic acids were also produced in this plant. Without information from metabolism experiments using radiolabelled chloro-intermediates, it is premature to say whether 3-chlorobenzoic acid, for example, arose from the corresponding chloro-cinnamic acid, or from catabolism of the corresponding chloro-PAA.

Typical GC–MS data used to establish the identification of the chloro-phenyl metabolites produced in bushbean shoots fed with chloro-phenylalanines are presented in Figs 3–6, for 3-chlorobenzoic acid (3-ClBzA), 3-chlorophenylacetic acid (3-ClPAA) and 3-chlorocinnamic acid (3-ClCA) isolated from shoots supplied with 3-chlorophenylalanine. All other chloro-phenyl metabolites isolated from the other crop and weed plants used in this study were identified in a similar manner.

Figure 3a shows the total ion current (TIC) scan obtained for 1 μg amounts of the standard PFB esters of 3ClBzA, 3-ClPAA and 3-ClCA. Figure 3b shows the TIC of the pentafluorobenzylated acid ether extract, equivalent to 2.5 g fresh, weight, of bushbean shoots fed with 10^{-4} M 3-chlorophenylalanine. Small peaks of suspected PFB-esters of 3-ClBzA and 3-ClCA are present and there is a very prominent ester peak for the major metabolite, 3-ClPAA. Based on the ratio of the area for the molecular ion peak in the TIC scan of the plant sample relative to that given by the corresponding standard, the amount of metabolite present was estimated (in ng) as described in the Experimental. The values obtained for bushbean are reported in Table 4.

The corresponding mass spectra of the PFB esters of authentic 3-ClBzA and of suspected 3-ClBzA isolated from bushbean shoots fed with 3-chlorophenylalanine are essentially identical, with a molecular ion (M^+) peak at 336 and characteristic fragmentation ion peaks at 181, 139 and 111 (Fig. 4). The ratio of the relative abundance of the molecular ion to base ion is 0.40 for the authentic 3-ClBzA and 0.36 for the suspected 3-ClBzA, further confirming the identification of the latter. The mass spectra of the PFB esters of authentic 3-ClPAA and suspected 3-ClPAA isolated from bushbean shoots fed with 3-chlorophenylalanine are also identical with a molecular ion at 350 and the expected fragment ions at 181, 125 and 91 (Fig. 5). The ratio of the relative abundance of the molecular ion to base ion in one is 0.20 while that in the other is 0.23. The mass spectra of the PFB esters of authentic and suspected 3-ClCA each showed a molecular ion at 362 and characteristic fragment ions at 181, 165 and 137 (Fig. 6). The relative abundance ratio for the molecular ion to base ion is 0.83 for the authentic compound and 0.70 for the suspected 3-ClCA.

In the mass spectra obtained for all the chlorophenylalanine metabolites, there was the added diagnostic feature of additional molecular ion peaks due to the presence of the heavy isotope of chlorine, ^{37}Cl. The number and percentage intensity of these heavy isotope peaks ($M+2$, $M+4$, $M+6$) relative to the parent molecular ion peak (M^+) is indicative of the number of chlorine atoms present in the molecule, as calculated by Beynon [42]. For example, in the mass spectrum of the PFB ester of 3-ClPAA, a compound containing 1 chlorine atom, the theoretical ratio of the intensity of the parent molecular ion peak (M^+) to that of the heavy isotope peak due to

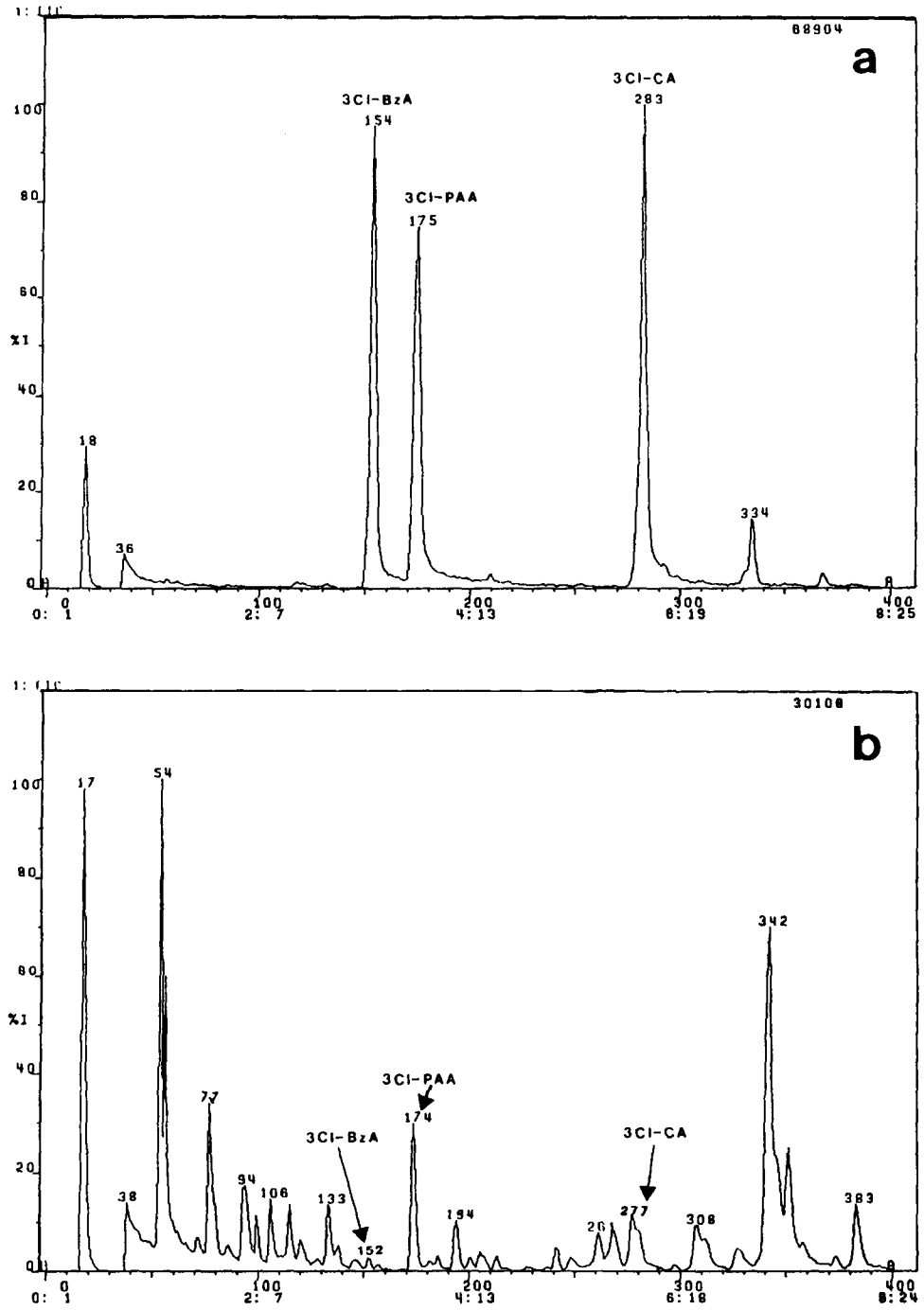


Fig. 3. (a) TIC scan of pentafluorobenzyl esters of authentic 3-chlorobenzoic, -phenylacetic and -cinnamic acids and (b) TIC scan of pentafluorobenzylated HPLC fraction of acid ether extract of bushbean shoots fed with 3-chlorophenylalanine.

^{37}Cl (M + 2) is, 100:32.6. This theoretical 3:1 relative intensity ratio was indeed observed; the molecular ion peak (M^+) at 350 is about three times the height or intensity of the M + 2 peak at 352. The same ratio can be observed for the normal and heavy isotope-containing fragments of the base ion (3-chlorobenzyl$^+$) at 125 and 127, respectively. The presence of the heavy isotope of chlorine as a 'tag' for the products of chlorophenylalanine metabolism adds further proof that these chloro-metabolites had indeed arisen by *in vivo* metabolism. None of the chlorophenylacetic, -benzoic or -cinnamic acids was indigenous to bushbean or any of the other plants examined in this study, a finding supported in Engvild's recent review of chlorine-containing natural

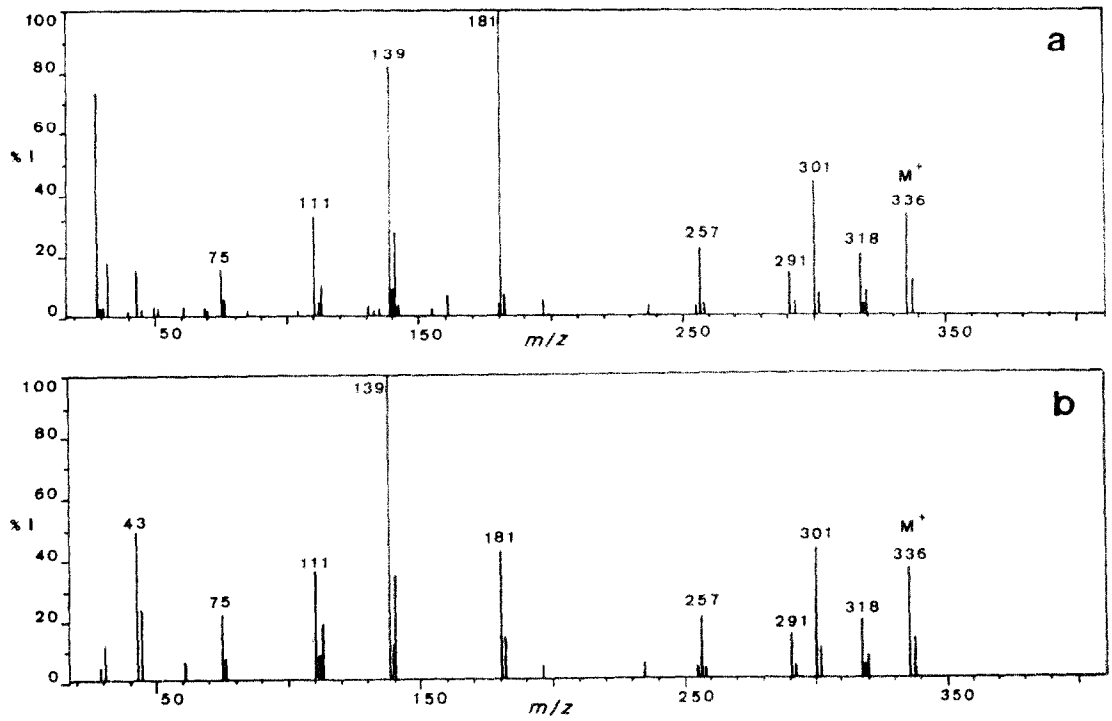


Fig. 4. Mass spectra of PFB esters of authentic 3-chlorobenzoic acid (a) and of suspected 3-ClBzA (b) isolated from bushbean shoots fed with 3-chlorophenylalanine.

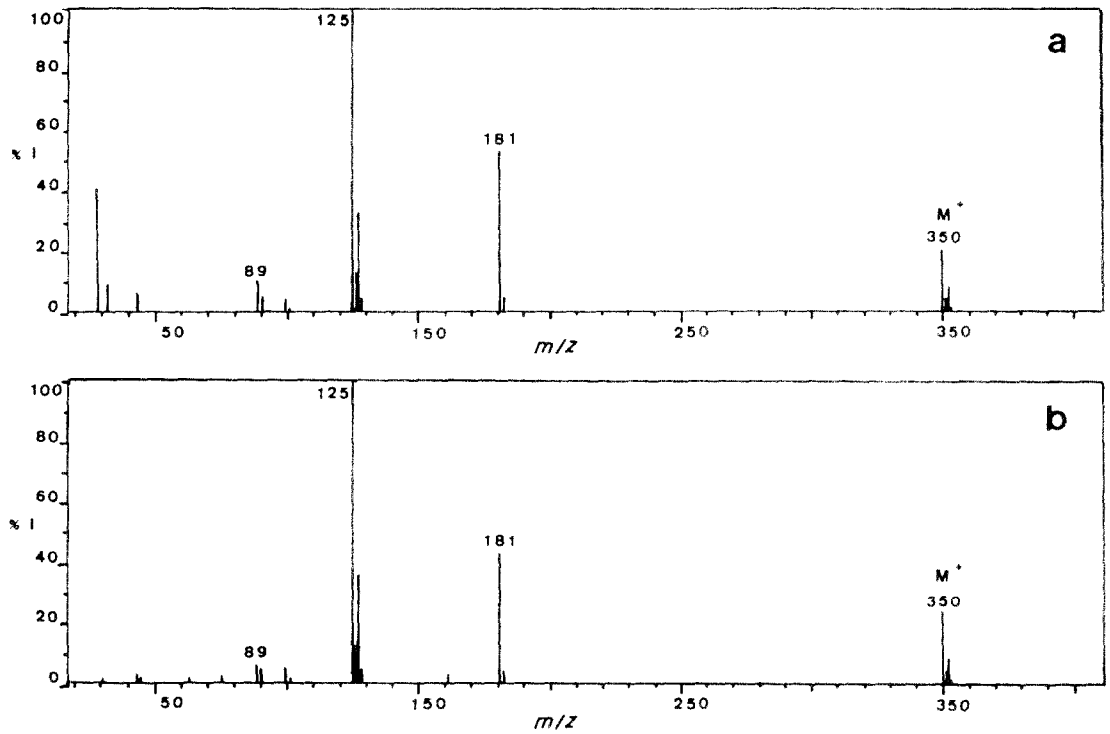


Fig. 5. Mass spectra of PFB esters of authentic 3-chlorophenylacetic acid (a) and of suspected 3-ClPAA (b) isolated from bushbean shoots fed with 3-chlorophenylalanine.

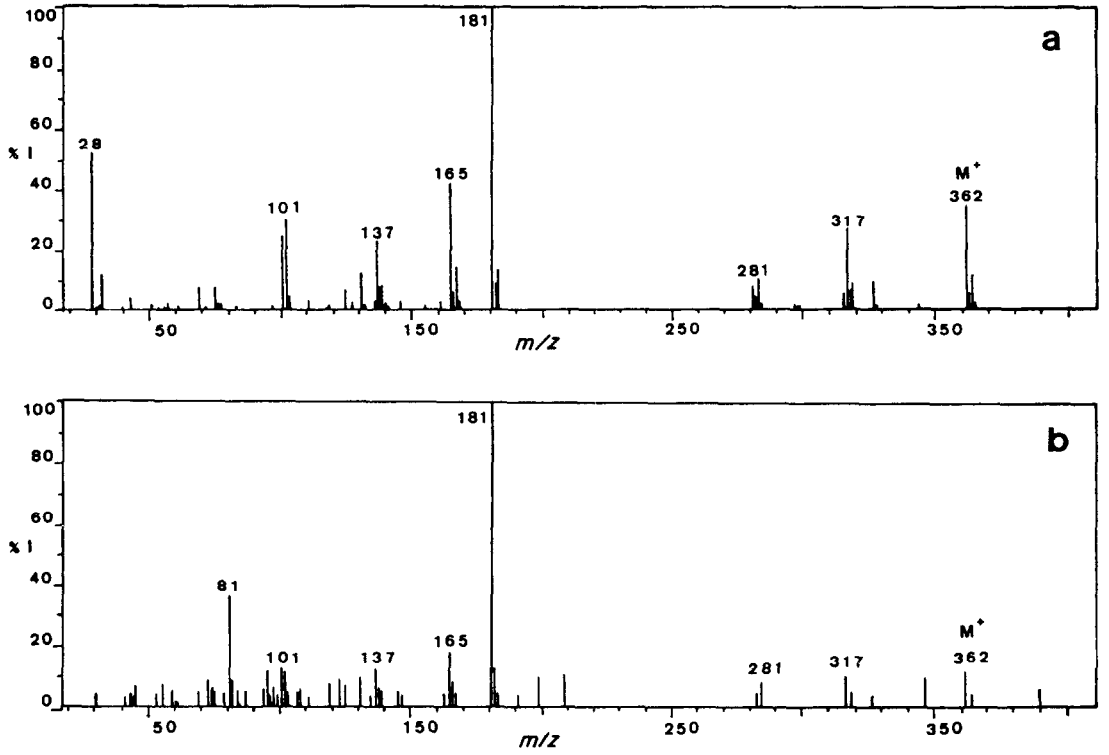


Fig. 6. Mass spectra of PFB esters of authentic 3-chlorocinnamic acid (a) and of suspected 3-ClCA (b) isolated from bushbean shoots fed with 3-chlorophenylalanine.

compounds in higher plants [43]. In contrast, the potent auxin, 4-chloro-3-indoleacetic acid (4-ClIAA), has been isolated from immature seeds of pea [44,45] and *Vicia* [46], and the methyl ester of this compound is known to be present in seeds of *Lathyrus*, *Vicia* and *Pisum* [47]. Recently Schneider *et al.* [41] working in this laboratory demonstrated the presence of 4-ClIAA in the root, cotyledon and epicotyl tissues of 3-day-old etiolated pea seedlings by HPLC and GC-MS procedures.

Perhaps the most interesting result in the bushbean experiments was the inability of 4-chlorophenylalanine to be metabolized to the corresponding 4-ClPAA. We have previously demonstrated that a cytoplasmic multispecific aspartate-aromatic aminotransferase purified from bushbean shoots was able to transminate all three monochloro-phenylalanines to the corresponding chlorophenyl-pyruvic acids [35]. D,L-4-Chlorophenylalanine showed a very high rate of transamination by the multispecific aminotransferase in *in vitro* experiments; *ca* 250% of the rate observed with unsubstituted D,L-phenylalanine. However, it is clear from the present study, that despite its high transamination potential *in vitro*, 4-chlorophenylanine was not metabolized to the corresponding 4-ClPAA in bushbean shoots, which suggests that some form of enzymic regulation was occurring *in vivo* at a metabolic step beyond the aminotransferase reaction in the PAA-biosynthetic pathway (see Fig. 1).

Metabolism of chlorophenylalanines by other crop and weed plants. Using identical methods to those already described for the bushbean experiments, metabolism of the series of D,L-chlorophenylalanines was next examined in two other crop plants, soybean and corn, and in three weeds, pigweed, lambsquarters and giant foxtail. The results of these experiments are presented in this section.

Data from the metabolism of different chlorophenylalanines in soybean shoots are summarized in Table 5. As in bushbean, with the monochlorophenylalanines, only the 3-chloro-compound was metabolized to any extent and produced a relatively large amount of the very active growth regulator, 3-ClPAA (molar rate of conversion *ca* 1.9 to 3.8%). 4-Chloro- and 3,4-dichlorophenylalanines gave rise to low levels of the corresponding chloro-PAA's. Also as in bushbean, all three monochlorobenzoic acids were produced, with 4-chlorobenzoic acid again being formed in the largest amount. There was concomitant formation of 4-chlorocinnamic acid, also at a relatively high rate. The potent growth regulator, 2,5-dichlorobenzoic acid, was not however produced in soybean.

In corn, the pattern of metabolism was similar to that observed in soybean. 3-Chloro-, 4-chloro- and 3,4-dichloro-PAA's were all produced (Table 6) but the highest molar rates of conversion were for the 3-chloro- and 3,4-dichloro-PAA's at *ca* 0.25–0.50%. 3,5-Dichloro-PAA was also produced in appreciable amounts, but this compound has no activity as a growth regulator. Of the potential benzoic acid metabolites, the 4-chloro- and 3,4-dichloro- acids were formed in the highest amount and small quantities of the 3-chloro- and 3,5-dichloro-benzoic acids were produced. Relatively large amounts of 4-chlorocinnamic acid were synthesized in corn, similar to the activity found in bushbean and soybean resulting from 4-chlorophenylalanine metabolism (Tables 4 and 5).

The data obtained from the metabolism of chlorophenylalanines in three weed plants are presented

Table 5. Amounts of chlorophenyl metabolites produced in excised shoots of soybean fed with 10^{-4} M solutions of different D,L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	Chlorophenylacetic acid ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	Chlorobenzoic acid ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	Chlorocinnamic acid ng per g fresh wt
2-Cl-	—	—	100	4	—	—
3-Cl-	8200	328	1250	50	570	23
4-Cl-	210	8.4	14760	590	3560	142
2,3-Cl_2-	—	—	—	—	ND	ND
2,4-Cl_2-	—	—	80	3.2	ND	ND
2,5-Cl_2-	—	—	—	—	ND	ND
2,6-Cl_2-	—	—	—	—	—	—
3,4-Cl_2-	200	8	9600	384	ND	ND
3,5-Cl_2-	—	—	750	30	ND	ND
2,3,4-Cl_3-	—	—	—	—	ND	ND
2,3,6-Cl_3-	—	—	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*Twenty-five gram samples of soybean shoots were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

—Metabolite not detected. ND: Not determined.

Table 6. Amounts of chlorophenyl metabolites produced in excised corn leaves fed with 10^{-4} M solutions of different D,L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	Chlorophenylacetic acid ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	Chlorobenzoic acid ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	Chlorocinnamic acid ng per g fresh wt
2-Cl-	—	—	—	—	—	—
3-Cl-	950	38	380	15.2	1620	65
4-Cl-	595	24	7320	293	3025	121
2,3-Cl_2-	—	—	—	—	ND	ND
2,4-Cl_2-	—	—	—	—	ND	ND
2,5-Cl_2-	—	—	—	—	ND	ND
2,6-Cl_2-	—	—	—	—	—	—
3,4-Cl_2-	1280	51	5325	213	ND	ND
3,5-Cl_2-	675	27	130	5.2	ND	ND
2,3,4-Cl_3-	—	—	—	—	ND	ND
2,3,6-Cl_3-	—	—	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*25 g samples of corn leaves were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

—Metabolite not detected. ND: Not determined.

in Tables 7–9. In redroot pigweed (*Amaranthus retroflexus*) there was no conversion of the 3-chloro- or 3,4-dichlorophenylalanines to their corresponding chloro-PAA's (Table 7), a result quite different from that found with the three crop plants. The 4-chloro-, 2,4-, 2,5- and 3,5-dichlorophenylalanines, however, were metabolized to their corresponding chloro-PAA's but only in relatively small amounts. None of the trichlorophenylalanines was converted to the corresponding chloro-PAA; the monochlorobenzoic acids were produced in amounts comparable to those observed in the three crop plants studied. Of the possible chlorocinnamic acid metabolites in pigweed, only 2,6-dichlorocinnamic acid was produced in appreciable amounts. The *cis*-form of this compound is known to be a potent growth regulator [11]. This metabolite was absent in all three crop plants examined.

Table 7. Amounts of chlorophenyl metabolites produced in excised shoots of redroot pigweed fed with 10^{-4} M solutions of different D, L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	Chlorophenylacetic acid ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	Chlorobenzoic acid ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	Chlorocinnamic acid ng per g fresh wt
2-Cl-	—	—	1100	44	—	—
3-Cl-	—	—	1050	42	230	9.2
4-Cl-	175	7.0	5950	238	150	6.0
2,3-Cl_2-	—	—	—	—	—	—
2,4-Cl_2-	285	11.4	290	11.6	ND	ND
2,5-Cl_2-	350	14.0	125	5.0	ND	ND
2,6-Cl_2-	—	—	170	6.8	1950	78
3,4-Cl_2-	—	—	700	28	ND	ND
3,5-Cl_2-	80	3.2	380	15.2	ND	ND
2,3,4-Cl_3-	—	—	—	—	—	ND
2,3,6-Cl_3-	—	—	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*25 g samples of pigweed shoots were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

—Metabolite not detected. ND: Not determined.

Table 8. Amounts of chloro-phenyl metabolites produced in excised leaves of giant foxtail fed with 10^{-4} M solutions of different D,L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	Chlorophenylacetic acid ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	Chlorobenzoic acid ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	Chlorocinnamic acid ng per g fresh wt
2-Cl-	—	—	45	1.8	—	—
3-Cl-	435	17.4	1060	42.4	93	—
4-Cl-	240	9.6	21730	869	3325	133
2,3-Cl_2-	—	—	—	—	ND	ND
2,4-Cl_2-	—	—	—	—	ND	ND
2,5-Cl_2-	—	—	—	—	ND	ND
2,6-Cl_2-	—	—	—	—	—	—
3,4-Cl_2-	—	—	—	—	ND	ND
3,5-Cl_2-	—	—	—	—	ND	ND
2,3,4-Cl_3-	—	—	—	—	ND	ND
2,3,6-Cl_3-	—	—	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*25 g samples of giant foxtail leaves were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

—Metabolite not detected. ND: Not determined.

In the giant foxtail (*Setaria feberii*), very little chlorophenylalanine metabolism occurred (Table 8). The 3- and 4-chlorophenylalanines gave rise to relatively low levels of the corresponding chloro-PAA's, but as in the other plants examined, all three monochlorobenzoic acids were produced with 4-chlorobenzoic acid being the most prominent metabolite. Comparatively high levels of the 3- and 4-chlorocinnamic acids were also detected in foxtail. However, this plant was unable to convert any of the di- or trichlorophenylalanines to the corresponding chloro-PAA's or chlorobenzoic acids.

The most interesting results on chlorophenylalanine metabolism in terms of their conversion to potentially herbicidal end products, occurred in lambsquarters (*Chenopodium album*). The metabolism data are summarized in Table 9. In contrast to the five other plants

Table 9. Amounts of chlorophenyl metabolites produced in excised shoots of lambsquarters fed with 10^{-4} M solutions of different D,L-chlorophenylalanines for 24 hr

Chloro-phenylalanine supplied*	Metabolites produced: Chlorophenylacetic acid Total ng per sample†	Chlorophenylacetic acid ng per g fresh wt	Chlorobenzoic acid Total ng per sample†	Chlorobenzoic acid ng per g fresh wt	Chlorocinnamic acid Total ng per sample†	Chlorocinnamic acid ng per g fresh wt
2-Cl-	—	—	120	4.8	—	—
3-Cl-	—	—	—	—	70	2.8
4-Cl-	—	—	4425	177	190	7.6
2,3-Cl_2-	—	—	—	—	ND	ND
2,4-Cl_2-	—	—	290	11.6	ND	ND
2,5-Cl_2-	—	—	—	—	ND	ND
2,6-Cl_2-	14000	560	1025	41	4180	167
3,4-Cl_2-	—	—	—	—	ND	ND
3,5-Cl_2-	—	—	—	—	ND	ND
2,3,4-Cl_3-	—	—	—	—	ND	ND
2,3,6-Cl_3-	3000	120	—	—	ND	ND
2,4,5-Cl_3-	—	—	—	—	ND	ND

*25 g samples of lambsquarters shoots were supplied with 10^{-4} M solutions of chlorophenylalanines (25 ml) followed by distilled water for 24 hr.

†Amount estimated from area of molecular ion peak in the TIC scan of PFB-derivatized HPLC fractions, relative to that of a known amount of standard.

— Metabolite not detected. ND: Not determined.

examined, none of the monochlorophenylalanines was metabolized to the corresponding chloro-PAA. Similarly, most of the dichloro- and trichlorophenylalanines were also not metabolized along this route. However, both 2,6-dichloro- and 2,3,6-trichlorophenylalanines were selectively metabolized in lambsquarters to yield comparatively high amounts of the corresponding chloro-PAA's, with molar conversion rates of 2.7–5.4% and 0.5–1.0%, respectively, being found. Both 2,6- and 2,3,6-chloro-PAA's are known to be very potent growth regulators and at certain concentrations will exhibit herbicidal properties comparable to 2,4-D [6, 7]. 2,6-Dichlorophenylalanine also produced considerable amounts of 2,6-dichloro-benzoic and cinnamic acids and these compounds have also been shown to possess strong growth-regulating properties [10, 11]. Figure 7(a) shows the TIC scan of the authentic PFB-esters of 2,6-dichloro-benzoic, -phenylacetic and -cinnamic acids, while Fig. 7(b) reproduces the TIC scan of a pentafluorobenzylated HPLC fraction from the ether extract of lambsquarter shoots fed with 2,6-dichlorophenylalanine. The PFB-esters of the three suspected chloro-metabolites are indicated by arrows. When the mass spectra from these metabolites were compared with those of the corresponding authentic PFB-esters, the spectrum for each metabolite contained all of the major ion fragments characteristic of the authentic compound (see Table 2 for MS fragmentation patterns). Similarly, Fig. 8(a) shows the TIC scan of the authentic PFB esters of 2,3,6-trichlorophenylacetic and benzoic acids, and Fig. 8(b) shows the TIC scan of a pentafluorobenzylated HPLC fraction from the ether extract of lambsquarter shoots fed with 2,3,6-trichlorophenylalanine. A peak for the suspected 2,3,6-trichloro-PAA-PFB ester can be clearly seen. When the mass spectrum of this peak was compared with that of the authentic PFB ester, identical fragmentation patterns indicated that the metabolite was indeed, 2,3,6-trichlorophenylacetic acid. It should be especially noted that the mass spectra of all four suspected metabolites contained the correct number of diagnostic heavy isotope peaks for ^{37}Cl, with relative intensities very closely approaching the theoretical ratios predicted by Beynon [42] (see Table 2).

A summary of the metabolites produced during chlorophenylalanine metabolism in the crop and weed plants studied in this investigation is shown in Table 10, while Table 11 shows the growth-regulating activity in the wheat coleoptile cylinder test obtained in earlier studies [7, 10, 11] with all the chlorophenylacetic, chlorobenzoic and chlorocinnamic acids considered as potential metabolites in the present study. The three crop plants, bushbean, soybean and corn, showed similar patterns of metabolism, most notably with respect to the formation of chlorophenylacetic acids, both 3-chloro- and 3,4-dichloro-PAA's were produced in all three plants. In contrast, among the weed plants, only the giant foxtail produced detectable levels of the 3-chloro-PAA; neither of the dicototyledonous weeds (pigweed and lambsquarters) produced the 3-chloro- or 3,4-dichloro-PAA's. However, the weed plants did produce four potent, growth-regulating chloro-phenylacetic acids, namely, the 2,4, 2,5-, 2,6- and 2,3,6-chloro-PAA's which were not synthesized in the three crop plants. In addition, the two dicot weeds were able to metabolize 2,6-dichlorophenylalanine to the potent 2,6-dichlorocinnamic acid, presumably through PAL activity, whereas none of the crop plants produced this metabolite. These differences in metabolism between the crop and weed plants examined in this study are examples of the type of selective metabolism which can be exploited when herbicidal action by lethal synthesis is desired.

It should be noted that in this investigation, we were primarily interested in determining whether the chloro-

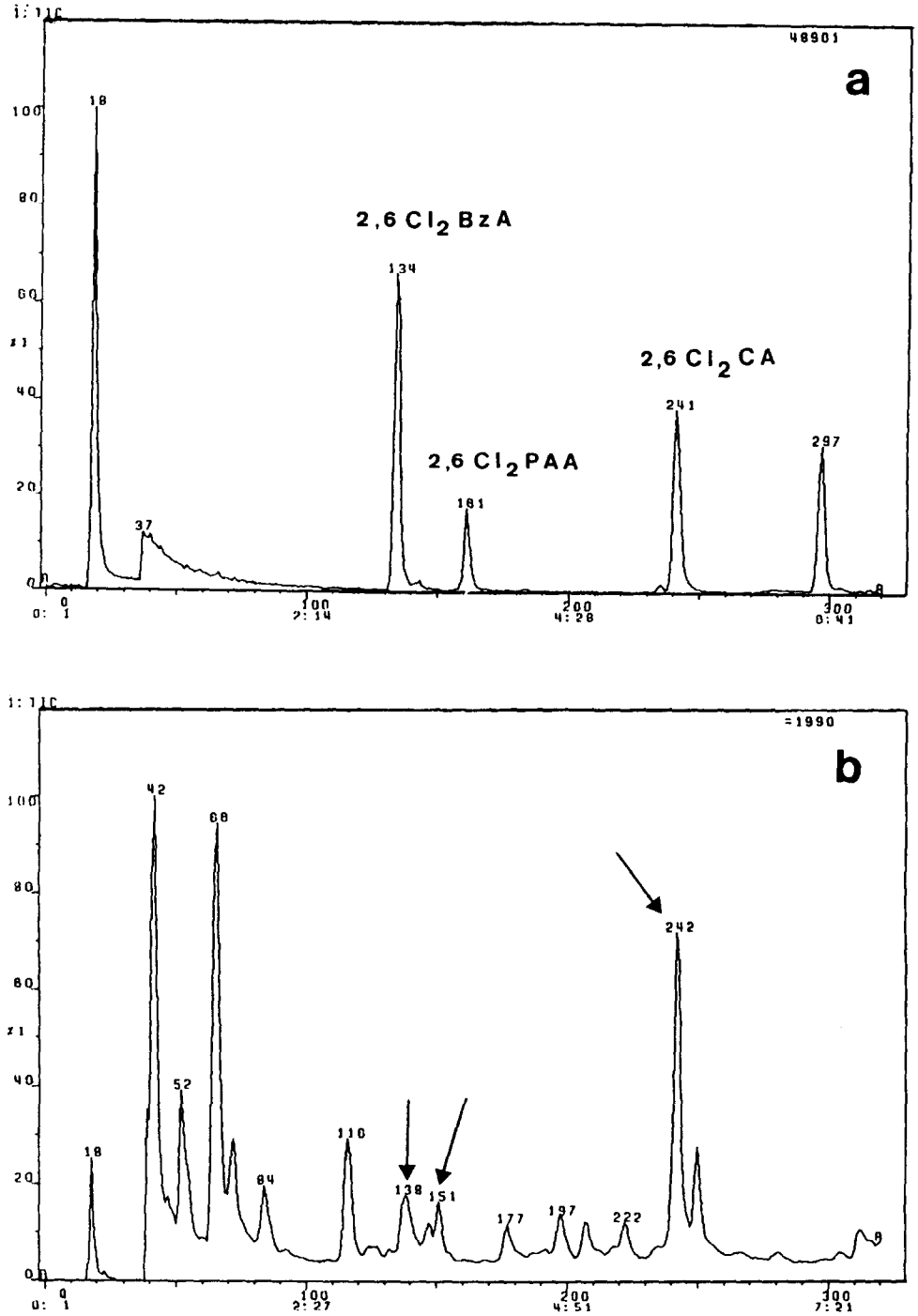


Fig. 7. (a) TIC scan of PFB esters of authentic 2,6-dichlorobenzoic, -phenylacetic and -cinnamic acids and (b) TIC scan of pentafluorobenzylated HPLC fraction of acid ether extract of lambsquarters shoots fed with 2,6-dichlorophenylalanine.

phenylalanines could be selectively metabolized in the crop and weed plants examined. Thus, shoot feedings were performed at only one concentration of a chlorophenylalanine and metabolism was allowed to occur over a relatively short period of time (24 hr), in order to maintain plant turgor and minimize stress effects on normal metabolism due to shoot excision. While some potentially phytotoxic chlorophenyl metabolites were produced (e.g. 2,6- and 2,3,6-trichloro-PAA's in lambsquarters), none of the early morphogenic effects normally associated with direct application of such auxin herbicides (e.g. leaf epinasty and upper stem curling) was observed in the plants. Normally, such growth abnormalities take 2–3 days to appear [48].

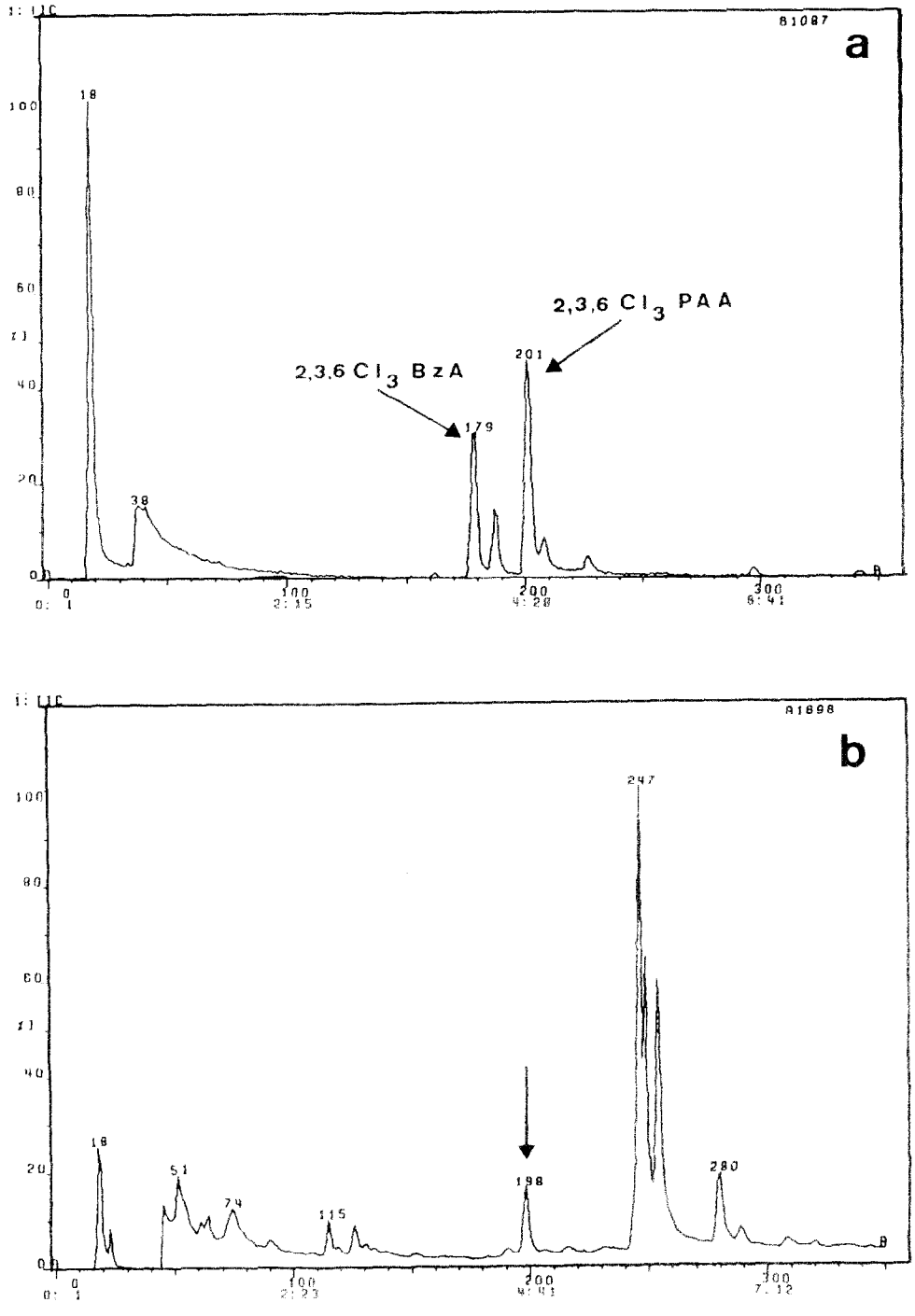


Fig. 8. (a) TIC scan of PFB esters of authentic 2,3,6-trichloro-benzoic and -phenylacetic acids and (b) TIC scan of pentafluorobenzylated HPLC fraction of acid ether extract of lambsquarters shoots fed with 2,3,6-trichlorophenylalanine.

The metabolites screened in this study were limited to the free acid form of the chloro-phenylacetic, -benzoic and -cinnamic acids. There is, however, a strong possibility that many of these chlorophenyl carboxylic acids were converted *in vivo* to their corresponding β-D-glucosyl- or L-aspartyl-esters in a manner similar to the known formation of these conjugates with 3-indolylacetic acid in shoots of higher plants [20, 49, 50]. *N*-benzoyl- and *N*-phenylacetyl-aspartate conjugates have been found in pea seeds [51]. Since PAA has been shown to be a naturally-occurring plant auxin [19, 20, 22, 23, 40, 52], a detailed study of the vegetative tissue conjugates of this growth regulator, along with those of its related synthetic analogues, appears to be warranted.

Table 10. Summary of metabolites produced in excised shoots or leaves of crop and weed plants fed with 10^{-4} M solutions of D,L-chlorophenylalanines

Plant examined	Metabolites produced†		
	Chlorophenylacetic acid	Chlorobenzoic acid	Chlorocinnamic acid
Bushbean	3-**	2- 2,4-	3-
	3,4-*	3- 2,5-**	4-
		4- 3,4-	
		3,5-	
Soybean	3-**	2- 2,4-	3-
	4-*	3- 3,4-	4-
	3,4-**	4- 3,5-	
Corn	3-**	3- 3,4-	3-
	4-*	4- 3,5-	4-
	3,4-*		
	3,5-		
Redroot pigweed	4-*	2- 2,5-*	3-
	2,4-*	3- 2,6-	4-
	2,5-*	4- 3,4-	2,6-**
	3,5-	2,4- 3,5-	
Giant foxtail	3-**	2-	3-
	4-*	3-	4-
		4-	
Lambs-quarters	2,6-**	2- 2,4-	3-
	2,3,6-**	4- 2,6-	4-
			2,6-**

†Compound known to possess high (*) or very high (**) growth-regulating and herbicidal activity at 10^{-5} M, relative to phenylacetic, benzoic and cinnamic acids at the same concentration.

Probable chlorophenylalanine metabolites not determined in the present study were the *N*-malonyl-chlorophenylalanines. Rosa and Neish [53] have shown that excised shoots of several species supplied with ^{14}C-labelled D,L-phenylalanine formed [^{14}C]-*N*-malonyl-phenylalanine as the main metabolite. This was subsequently confirmed in our laboratory [19, 21, 23]. The formation of this simple peptide probably represents a detoxification mechanism in shoots operating in response to abnormally high concentrations of phenylalanine [53]. There is no evidence, however, that such *N*-malonyl conjugates of phenylalanine or chlorophenylalanines exhibit any plant growth-regulating or herbicidal activity.

Another possibility for consideration is that some of the chlorophenylalanines may act as selective competitive inhibitors of certain L-phenylalanine-metabolizing enzymes. For example, competitive inhibition of the cytosolic aromatic aminotransferase in plants, such as that purified from bushbean shoots [34–36], could cause a reduction in normal auxin biosynthesis, since this enzyme catalyses the first step in the biosynthetic pathway leading to PAA and IAA from phenylalanine and tryptophan, respectively [20, 23] (Fig. 1). Similarly, competitive inhibition of PAL activity by a chlorophenylalanine comparable to that reported for D,L-3-fluorophenylalanine [54], would greatly inhibit lignin and flavonoid biosynthesis. These possibilities require further study.

In conclusion, this initial investigation of the metabolism of synthetic D,L-chlorophenylalanines in six crop and weed plants has shown that some significant species-specific conversions to potentially herbicidal end-products can occur. In particular, several potent chlorophenylacetic acids were produced, indicating that all the enzymes comprising the PAA biosynthesis system in these plants are sufficiently non-specific to allow them to metabolize chlorophenyl intermediates. Chlorobenzoic acids were the most prevalent metabolites formed in all plants examined, but it is not clear whether these chlorobenzoic acids arose from corresponding chlorocinnamic acid intermediates, or whether they were formed as a result of further metabolism of the corresponding chlorophenylacetic acids. Future experiments with radiolabelled chlorocinnamic acids and corresponding chlorophenylacetic acids should resolve this question.

EXPERIMENTAL

Plant material. The crop plants examined in the present study were bushbean (*Phaseolus vulgaris* L., var. Pencil Pod Black Wax), soybean (*Glycine max.* L. Merrill, var. Maple Arrow) and corn (*Zea mays* L., var. Seneca Chief). The weed plants studied were pigweed (*Amaranthus retroflexus* L.), lambsquarters (*Chenopodium album* L.) and giant foxtail (*Setaria faberii* W. Hermann). Seeds of weed plants were supplied by Dr Suzanne Warwick of the Biosystematics Division, Central Experimental Farm, Ottawa, and were collected at different locations in Ontario. All seeds were soaked in H_2O at room temperature overnight and then either sown in moist vermiculite for plants

Table 11. Growth-regulating activity of chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids in the wheat coleoptile cylinder test [7, 10, 11]

Compound tested	Wheat coleoptile cylinder test† concentration tested (M)			
	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Phenylacetic acid (PAA)	0	0	20*	42*
2-Chloro-PAA	25*	48*	56*	50*
3-Chloro-PAA	20*	51*	47*	46*
4-Chloro-PAA	0	18*	50*	36*
2,3-Dichloro-PAA	57*	60*	58*	40*
2,4-Dichloro-PAA	3	20*	45*	44*
2,5-Dichloro-PAA	2	37*	46*	36*
2,6-Dichloro-PAA	13*	47*	54*	41*
3,4-Dichloro-PAA	4	40*	36*	8
3,5-Dichloro-PAA	0	0	6	0
2,3,6-Trichloro-PAA	58*	60*	57*	46*
Benzoic acid (BzA)	0	0	0	0
2-Chloro-BzA	0	0	0	10*
3-Chloro-BzA	0	0	0	0
4-Chloro-BzA	0	0	0	0
2,3-Dichloro-BzA	0	0	29*	27*
2,4-Dichloro-BzA	0	0	0	0
2,5-Dichloro-BzA	0	24*	43*	36*
2,6-Dichloro-BzA	0	0	0	54*
3,4-Dichloro-BzA	0	0	0	0
3,5-Dichloro-BzA	0	0	0	0
2,3,6-Trichloro-BzA	0	21*	50*	45*
2,4,6-Trichloro-BzA	0	0	0	0
	1 ppm	10 ppm	100 ppm	
Cinnamic acid (CA)‡	1	38*	45*	
2-Chloro-CA	24*	49*	52*	
3-Chloro-CA	1	25*	34*	
4-Chloro-CA	19*	50*	49*	
2,3-Dichloro-CA	6	14*	2	
2,4-Dichloro-CA	2	38*	22*	
2,5-Dichloro-CA	1	5	8*	
2,6-Dichloro-CA	37*	56*	5T	
3,4-Dichloro-CA	2	27*	T	
3,5-Dichloro-CA	0	8*	0	

*Activity statistically significant from water controls at the 0.1% level.

†Activity is expressed as per cent increase in length over water controls.

‡Only the *cis*-acids were active, all *trans*-acids were inactive in this test.

T: Toxic effects were observed.

grown only for 2 weeks, or in soil for plants grown for 4–8 weeks. Seeds were germinated in a growth cabinet at 25/22° day/night temperatures and seedlings were exposed to 16 hr daily photoperiods of 4600–4800 $\mu W/cm^2$. The time of planting was taken as day 0, and metabolism experiments were conducted with 14-day-old bushbean, soybean and corn plants, 4-week-old foxtail and 8-week-old lambsquarters and pigweed plants.

Chemicals. The ring-substituted mono-, di- and trichloro-DL-phenylalanines used in this investigation were synthesized by an acetamidomalonate condensation with the appropriate chlorobenzyl halide, followed by acid hydrolysis, as described previously [37]. The chlorophenylalanines were characterized by m.p. and IR, ^1H NMR, GC-MS and CIMS spectroscopy.

The corresponding ring-substituted mono-, di- and trichlorophenylacetic acids and benzoic acids were required as authentic standards for the identification of possible end-products of chlorophenylalanine metabolism. When commercially available, the corresponding chlorocinnamic acids were also obtained, again to use as standards for the identification of expected end-products resulting from the activity of phenylalanine ammonia-lyase (PAL). Phenylacetic acid (PAA) and the 2-, 3- and 4-monochlorophenylacetic acids were purchased from Aldrich. All of the di- and trichlorophenylacetic acids were synthesized from the corresponding ring-chlorinated benzyl halides as described in the following section. Benzoic acid and all other required chlorobenzoic acids were obtained from Aldrich Chemical Co., Pfaltz and Bauer Research Chemicals or ICN Pharmaceuticals, except the 2-, 2,6- and 2,4,5-chlorobenzoic acids which were synthesized as reported below. Cinnamic acid, the 2-, 3- and 4- monochlorocinnamic acids, 2,6-dichlorocinnamic acid, 2-chloro- and 2,6-dichlorobenzaldehydes, 2,4,5-trichlorotoluene, 3,5-dichlorobenzyl alcohol and 4-chlorobenzyl chloride were purchased from Aldrich. 2,3-dichlorotoluene, and the 2,4-, 2,6- and 3,4-dichlorobenzyl chlorides were acquired from Pfaltz and Bauer Research Chemicals. Pentafluoro-benzyl bromide (PFB-Br), DMF-dimethyl acetal (Me-8) and dimethylsulfate, sequanal-grade diisoprophylethylamine and acetonitrile were obtained from Pierce (Rockford, Ill., U.S.A.). All other chemicals utilized in this study were of analytical grade.

Synthesis of di- and trichloro-phenylacetic acids. All 6 dichloro- and 3 trichlorophenylacetic acids were synthesized from the corresponding di- or trichlorobenzyl halides by a nucleophilic displacement reaction with NaCN in DMSO [55]. The reaction product, a di- or trichlorophenylacetonitrile, was hydrolysed in base to give the corresponding chlorophenylacetic acid [56]. Details of the syntheses are as follows: The 2,5-, 3,5- and 2,3,4-chlorobenzyl chlorides were prepared from the corresponding chlorobenzyl alcohols by chlorination of the alcohol (0.075 mol) with $SOCl_2$ (0.10 mol) in benzene (150 ml) containing one drop of pyridine, as previously described [37]. The 2,3-, 2,3,6- and 2,4,5-chlorobenzyl bromides were synthesized from the corresponding chlorotoluenes by bromination of the toluene (0.030 mol) with *N*-bromosuccinimide (0.033 mol) in CCl_4 (80 ml) with benzoyl peroxide (0.1 g) as the catalyst, as reported previously [37]. The other chlorobenzyl halides (2,4-, 2,6- and 3,4-) were obtained commercially as indicated above.

To prepare the chlorophenylacetonitrile, the chlorobenzyl halide (0.025 mol) in 5 ml DMSO was added to a rapidly stirred slurry of NaCN (0.0275 mol, 1.34 g) in DMSO (25 ml) over 10–15 min. During addition of the halide, the reaction became exothermic and ice bath cooling was necessary to keep the reaction temperature at 40–50°, to minimize a reaction between the solvent and the halide. Stirring was continued for 5 hr after the addition of the halide was complete. After cooling, the reaction mixture was poured into H_2O, extracted × 3 with ether, the combined ether extracts washed with 6 N HCl to hydrolyse any noxious isocyanide, followed by H_2O, and dried over dry Na_2SO_4. The ether was removed on a rotary evaporator to give crude chlorophenylacetonitrile either as a light brown solid or a viscous brown liquid, depending on the chloro-substitution pattern. Success of the displacement reaction was confirmed by IR and NMR spectroscopy. An upfield shift in the benzylic protons from *ca* 4.7 ppm to *ca* 3.8 ppm indicated that the halide had been displaced by the nitrile in the NMR analysis, and by appearance of a strong C≡N absorption band at *ca* 2240 cm^{-1} in the IR analysis. The yield of chlorophenylacetonitrile was 80–90% in all cases.

The chlorophenylacetronitrile was hydrolysed without further

purification. A solution of the nitrile (0.020 mol) in EtOH (45 ml) was hydrolysed by refluxing overnight with 25% NaOH (45 ml) until NH_3 ceased to be evolved. The EtOH was distilled off and the aq. solution acidified with 20% H_2SO_4. The pptd chlorophenylacetic acid was separated by filtration, dissolved in Et_2O and the Et_2O fraction backwashed with H_2O followed by 10% bicarbonate and dried. After removal of the Et_2O, the chlorophenylacetic acid remained as a light yellow solid, in yields of 70–95%. Recrystallization from bp 65–110° petrol or 30% ethanol yielded pale white crystals of the pure chlorophenylacetic acid. Analysis by IR spectrometry showed a broad stretch due to C=O at *ca* 1710 cm^{-1} and a very broad OH stretch from *ca* 3400 to 2500 cm^{-1}, typical of carboxylic acids [57]. There was no evidence of nitrile absorption at *ca* 2240 cm^{-1} by unreacted chlorophenylacetonitrile. Each chlorophenylacetic acid was further characterized by mp, HPLC (Table 1) and by GC-MS analysis of its pentafluorobenzyl (PFB) ester derivative (Table 2).

Synthesis of 2-, 2,6- *and* 2,4,5-*chlorobenzoic acids.* The 2- and 2,6-chlorobenzoic acids were synthesized separately from the corresponding benzaldehydes by oxidation with Jones reagent [58]. The chlorobenzaldehyde (0.035 mol) was dissolved in 30 ml Me_2CO, the mixture cooled in an ice bath and treated dropwise with 25 ml Jones reagent. Following addition of the oxidizing reagent, the orange-brown mixture was left at 0° for a further 20 min and then diluted with 60 ml saturated aq. NaCl and extracted with 3 vol. of 10% Na_2CO_3. The aqueous phase was then acidified with 2 N H_2SO_4, saturated with NaCl, and extracted with three vol. of Et_2O. The combined Et_2O fraction was backwashed with a small vol. of H_2O and then dried. Removal of the Et_2O gave light yellow crystals of the chlorobenzoic acid, with yields of *ca* 70%. Upon recrystallization from 10% EtOH, 2-chlorobenzoic acid, mp 138–139° (lit. mp 142° [59]) and 2,6-dichlorobenzoic acid, mp 142° (lit. mp 144° [59]) were obtained. Structures were confirmed by GC-MS analysis of the pentafluorobenzyl derivate of each acid.

2,4,5-Trichlorobenzoic acid was synthesized from the corresponding toluene [60]. 2,4,5-Trichlorotoluene (0.005 mol) was added to a solution of $KMnO_4$ (0.025 mol) and Na_2CO_3 (1.2 g) in H_2O (75 ml). The mixture was heated under reflux with continual stirring for 6 hr by which time the permanganate colour had disappeared. The mixture was filtered, the cake of hydrated MnO_2 was washed with two 100 ml portions of hot H_2O, the combined filtrates were acidified by cautiously adding conc H_2SO_4 with stirring and the white pptd carboxylic acid was collected by filtration and washed with cold H_2O. Recrystallization of the crude acid from 20% EtOH gave crystals of the 2,4,5-trichlorobenzoic acid, mp 158–159°, in a 74% yield. The structure was confirmed by GC-MS analysis of the PFB-ester.

Metabolism experiments. Vegetative shoots or young leaves of crop and weed plants were excised 3–5 cm above soil level and the basal part of the stem or leaf sheath immediately placed in distilled H_2O. A second cut was then made under H_2O to remove another 2 cm of the basal tissue to ensure that air locks did not develop in the vascular tissue of the shoots or leaves. After the second excision, shoot or leaf samples equivalent to 25 g fr. wt were immediately placed in 25 ml of a 10^{-4} M solution of D,L-phenylalanine or D,L-chlorophenylalanine. Control samples were suppled with distilled H_2O or were promptly frozen and stored at −10° for subsequent analysis. The experimental tissue was placed in a growth chamber at 25° and maintained under constant illumination of 4600–4800 $\mu W/cm^2$ for 24 hr. The treatment solutions were taken up by the tissue within 5–8 hr, the rate of uptake being slowest with the monocot leaf samples. After most (80%) of the feeding solution had been adsorbed, the shoots or leaves were supplied with successive 25 ml aliquots of distilled H_2O to maintain the tissue in a fully turgid condition for the remaining 16–19 hr of the test period. Aliquots of the D,L-phenylalanine solution were taken before and after the test period in each monocot and dicot metabolism experiments and these were subjected to OPA-HPLC, as previously described [34], to determine the amount of phenylalanine adsorbed by the tissue during the 24 hr feeding period. It was found that in all cases, ≥ 95% of the D,L-phenylalanine was taken up by the shoots or leaf tissue. The level of residual phenylalaninine was below the limit of detection by OPA-HPLC.

Isolation of acidic growth-regulating metabolites. After the metabolism period, each sample of treated shoots or leaves was subjected to the following extraction and fractionation procedures for the isolation of acidic growth-regulating metabolites. 25 g of tissue were homogenized for 2 min in a Waring Blender containing 200 ml of grinding medium composed of 160 ml methanol, 20 ml 1 M ammonium acetate buffer, pH 6.5, 9.8 ml permanganate double-distilled H_2O and 0.2 ml 2-mercaptoethanol. The homogenate was stirred overnight at 4° in the dark to allow for complete extraction of the tissue; the extract was then suction filtered through Whatman No.1 paper, concd to the aqueous phase and frozen at −22° to ppt. the plant pigments and other cellular debris. These were later removed by centrifugation at 10000 rpm in a refrigerated Sorvall RC-2B centrifuge using a SS-34 fixed-angle rotor. The aqueous supernatant was adjusted to pH 2.5 and extracted × 3 with equal vols of re-distilled Et_2O. The combined acid ether extract, which should contain PAA or chloro-PAA, benzoic or chlorobenzoic acid and cinnamic or chlorocinnamic acid, was concentrated to a volume of *ca* 0.5 ml and then stored at −22° until analysed by HPLC. Prior to HPLC, each acid ether concentrate was purified 4–5 fold by passage through a C_{18} PrepSep extraction column (Allied-Fisher Scientific, Springfield, New Jersey). The PrepSep column was preconditioned with two, 2 ml vols of HPLC-grade MeOH containing 0.1 N HCO_2H, followed by two, 2 ml washings with double distilled H_2O containing 0.1 N HCO_2H. The acid Et_2O concentrate was dild to 4 ml in 20% MeOH containing 0.1 N HCO_2H, filtered through a 0.45 μm Millipore filter (Millipore Ltd, Mississauga, Ont.), applied to the PrepSep column and drawn through with a 10 ml syringe at 1 drop/sec. The eluant was discarded. The adsorbed sample was washed first with 2 ml double distilled H_2O containing 0.1 N HCO_2H followed by 500 μl of 35% MeOH containing 0.1 N HCO_2H. The washings removed many strong UV-absorbing contaminants from the sample but left the acidic metabolites of interest (e.g. PAA, benzoic acid, cinnamic acid and their corresponding chloro-acids) still adsorbed on the C_{18} column. These were then eluted with 500 μl of MeOH containing 0.1 N HCO_2H. Analyses performed on plant extracts spiked with ng amounts of authentic PAA, benzoic acid and cinnamic acid, or their corresponding chloro-acids, indicated that after selected adsorption and elution of the plant extract from the PrepSep column using the procedures described above, recoveries of the authentic compounds were 95% in all cases.

High performance liquid chromatography. HPLC was carried out using a Beckman-Altex system consisting of two Model 100A pumps controlled by a Model 420 microprocessor and connected to a Whatman P/10 ODS-3 Magnum 9-25 reversed phase prep. HPLC column (25 cc × 9.4 mm internal diameter; 10 μm particle size). The flow rate was 2.5 ml/min and the UV detector (Model 152) was set at 254 nm. Each acid Et_2O concentrate eluted after PrepSep purification was diluted to 1.1 ml with the starting solvent (acidic 20% MeOH) and filtered through a 0.45 μm Millipore filter. The 1 ml concentrate was then injected into the 1 ml injection loop of the HPLC. Reverse phase chromatography was carried out by gradient elution. Solvent A

was 0.1 N HCO_2H in H_2O and Solvent B was 0.1 N HCO_2H in MeOH. The gradient employed was: 20–50% B in A over 10 min, isocratic elution at 50% B for 8 min, then 50–65% B over 10 min, isocratic elution at 65% B for 22 min, then 65–100% B over 5 min. The HPLC retention times of PAA, benzoic acid, cinnamic acid and their corresponding ring-chlorinated analogues using this gradient system are listed in Table 1.

Derivatization of metabolites. Pentafluorobenzyl (PFB) esterification of purified metabolites was performed according to ref. [61]. 20 mg K_2CO_3 and 1.5 ml of a solution of pentafluorobenzylbromide (PFBBr) in acetone (5 μl PFBBr ml^{-1}) were added to each sample vial. The vials were closed with septum caps and heated at 60° for 2 hr in a Pierce Reactitherm heating module (Pierce Chemical Co., Rockford, Illinois). The residual solvent was evapd in N_2 at 35° and the residue dissolved in 1 ml distilled H_2O. The PFB esters were extracted from the aq. solution with EtOAc (3 × 1.5 ml). The combined EtOAc extract was placed in a 1.0 ml Reacti-vial (Pierce) and the solvent removed in N_2 at 35°. The residue was re-dissolved in 10 μl of EtOAc and an aliquot injected into the GC-MS.

Methylation of metabolites was carried out by two different methods. The first was that in which the HPLC-purified sample was dissolved in 450 μl acetonitrile in a 5 ml Reacti-vial fitted with a Mininert valve (Pierce). Diisopropylethylamine (98 μl, 100 μmol) and Me_2SO_4 (9 μl, 50 μmol) were added and the mixt. stirred at room temp. for 3 hr. The excess alkylating reagent and base were removed from the reaction mixture by rapid extraction with 1 ml distilled H_2O. An aliquot of the reaction mixture was then injected directly into the GC-MS [39]. In the second methylation method 1 ml of Methyl-8 (2 mEq/ml in pyridine) was added to the sample, the vial capped and heated to 100° for 20 min [62]. Aliquots of the reaction mixture were then injected directly into the GC-MS.

Gas chromatography-mass spectroscopy. Mass spectra were obtained using a VG 7070E instrument (VG Analytical, Manchester, N.Y.) employing a 30 m Megabore DB5 gas chromatography column (J & W Scientific Co.). For analysis of the unsubstituted and monochloro-substituted pentafluorobenzyl esters, the starting temp. was 200° and after a 2 min isothermal period, a temp. gradient of 10°/min was applied upto 320°. For analysis of the di- and trichloropentafluorobenzyl esters, the starting temp. was 220° and again after a 2 min isothermal period, a gradient of 10°/min was applied. The characteristic fragmentation ions for the PFB esters of unsubstituted phenylacetic, benzoic and cinnamic acids and of their corresponding chloro-substituted acids are reported in Table 2. The amount of metabolite produced in each feeding experiment was established from the area of the molecular ion peak of the PFB ester in the total in current (TIC) scan, relative to the area given by 1 μg of the PFB-ester of the appropriate standard using the following formula:

$$\text{Amount of metabolite in } \mu\text{g) in acid } Et_2O \text{ extract equivalent to 2.5 g fr. wt of shoots} = \frac{\text{Area of molecular ion peak of suspected metabolite}}{\text{Area of molecular ion peak/}\mu\text{g of standard}} = A\,(\mu\text{g}).$$

$$\text{Amount of metabolite (in ng) in acid } Et_2O \text{ extract equal to 25 g fr wt of shoots} = A\,(\mu\text{g}) \times \frac{1000\text{ ng} \times 10}{1\ \mu\text{g}}$$

For the methyl ester analyses, the starting temp. was 150° with a 2 min isothermal period followed by gradient of 10°/min up to 320°.

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