Plant Physiol. (1980) 65, 94–97
0032-0889/80/65/0094/04/$00.50/0

Comparative Metabolism of 2,4-Dichlorophenoxyacetic Acid in Cotyledon and Leaf Callus from Two Varieties of Soybean[1]

Received for publication April 17, 1979 and in revised form August 7, 1979

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ABSTRACT

Only quantitative differences were observed in the metabolism of [1-^{14}C]2,4-dichlorophenoxyacetic acid (2,4-D) by soybean cotyledon callus as influenced by variety (Acme and Amsoy), source of tissue (cotyledon, leaf and root) and age. All tissues metabolized 2,4-D to water-soluble (glycosides) and ether-soluble (primarily amino acid conjugates) metabolites.

In young (3- to 4-week-old) Amsoy cotyledon or leaf callus tissue the free 2,4-D increased proportionately with the external 2,4-D concentration while in older (6- to 8-week-old) tissue the amino acid conjugates increased in this manner. Thus, in the older Amsoy tissue apparent regulation of the internal 2,4-D level (1.5–3.5 nmoles per gram fresh weight) was observed. In 3- or 6-week-old Acme cotyledon callus 2,4-D accumulated with an increase in the external 2,4-D concentration with no evidence for regulation of internal 2,4-D levels.

We have previously studied the metabolism of the herbicide 2,4-dichlorophenoxyacetic acid in callus tissue cultures from soybean and several other plant species (2–5). The variations were mostly quantitative; however, major differences were observed between monocots and dicots. An important observation was that the metabolism of 2,4-D was shown to change with the age of soybean callus tissue (2). Nine-week-old soybean root callus tissue formed much greater amounts of amino acid conjugates than did 3-week-old tissue. The conjugation appeared to regulate the level of 2,4-D in the older tissue, because the conjugates increased in proportion to the external 2,4-D concentration while the internal 2,4-D level was fairly constant.

Plant tissue cultures have been proposed to be an ideal tool for at least initial metabolism studies with pesticides or other xenobiotics (8), but one must determine how various factors affect metabolism and what sort of variations may be encountered. Arnison and Boll (1) showed that plant callus cultures derived from different organs of the same plant differ in their metabolic capabilities. Root, hypocotyl, and cotyledon bean callus tissues differed in their complement and activity of peroxidase isozymes and other enzymes. We now report the results of the comparative metabolism of 2,4-D in two varieties of soybean cotyledon callus tissue (Amsoy and Acme) and in Amsoy cotyledon, leaf and root callus (2) as well as excised roots (2) of Amsoy.

MATERIALS AND METHODS

Soybean leaf callus cultures were derived from mature soybean leaves (*Glycine max.* [L.] Merrill, var. Amsoy). Leaves were surface-sterilized in 2.5% sodium hypochlorite, rinsed in sterile water, and cut into 2- to 3-mm pieces. Amsoy soybean cotyledon callus cultures were derived from 3-day-old excised cotyledons. Soybean callus cotyledon var. Acme has been maintained for several years in our laboratory (3). Cultures were grown at 25 C under continuous, low intensity fluorescent light (0.5 $\mu E/m^2 \cdot s$) on agar solidified Miller's medium (7).

Callus tissue (2–8 g) was transferred aseptically to 125-ml flasks containing 50 ml of liquid medium (without added auxin) to which [1-^{14}C]2,4-D was added (0.9–4.4 μM, 52 mCi/mmol or 55 mCi/mmol, Amersham/Searle). The flasks were incubated for 48 h on a shaker at room temperature (21–22 C) under ambient fluorescent light (17 $\mu E/m^2 \cdot s$).

Following incubation the tissue was collected by filtration through Whatman No. 1 paper (Büchner funnel), rinsed with cold distilled H_2O and weighed. The tissue was homogenized with five times its weight of hot 95% ethanol. The homogenate was filtered and the residue washed extensively with 80% ethanol. The filtrate was concentrated to about 50 ml at 40 C on a rotating evaporator (aspirator), the pH adjusted to 3 (3 N H_3PO_4), and partitioned three times with equal volumes of diethyl ether. The aqueous portion was again partitioned three times with water-saturated 1-butanol. The aqueous concentrate from the 1-butanol fraction was treated with Emulsin (U.S. Biochemical Corp.) as previously described (2). The diethyl ether fraction was further partitióned three times with 5% $NaHCO_3$. The combined bicarbonate fraction was acidified (pH 3, 3 N H_3PO_4), concentrated and reextracted with diethyl ether. The radioactivity of each fraction was measured by liquid scintillation counting in Aquasol. The ethanol-insoluble residue was combusted by the O_2 flask method prior to counting (6).

The fractions were examined by TLC on silica gel plates (Supelcosil 12A plus fluorescent indicator). Metabolites were cochromatographed with nonlabeled standards and located by autoradiography. The following solvent systems were used: I, diethyl ether-petroleum ether (38–46 C)-formic acid (70:30:2, v/v/v); II, 1-butanol-acetic acid-water (90:20:10, v/v/v); and III, benzene-dioxane-acetic acid (90:25:4, v/v/v).

RESULTS AND DISCUSSION

The uptake and metabolism of 2,4-D by the callus tissue varied with concentration of 2,4-D, with tissue variety, source, and age. All tissue metabolized 2,4-D to water-soluble (glycosides) and ether-soluble (primarily amino acid conjugates) metabolites, but quantitative differences did exist. Almost all of the applied radioactivity could be accounted for by the tissue fractions (residue plus ethanol-soluble) and the incubation medium. The latter contained mainly 2,4-D (80–90%) and some metabolites similar to those in the tissue.

The uptake of 2,4-D by either young (3-week) or old (6-week) soybean cotyledon callus was similar but Amsoy took up about

[1] Authorized for publication as Paper No. 5699 in the Journal Series of the Pennsylvania Experiment Station. Supported in part by the Northeastern Regional Project NE-115 and Regional Research Funds.

Table I. *Relative Percentage of Metabolic Products of 2,4-D after Incubation of Acme and Amsoy Cotyledon Callus Tissue with [1-^{14}C]2,4-D*

Fraction	Acme[a]		Amsoy[b]		
	3-Week	6-Week	3-Week	4-Week	6-Week
Tissue uptake[c]	70.5 ± 6.3	77.0 ± 9.0	36.2 ± 6.2	56.0 ± 6.8	34.0 ± 8.5
Residue[d]	12.2 ± 4.5	9.7 ± 0.8	11.2 ± 5.7	19.0 ± 5.7	17.5 ± 2.1
Ethanol-soluble[d]	87.8 ± 4.5	90.3 ± 0.8	88.7 ± 5.7	81.0 ± 5.7	82.5 ± 2.1
Free 2,4-D	59.1 ± 3.8	49.4 ± 1.0	39.8 ± 10.8	27.3 ± 1.8	16.8 ± 2.3
Aqueous-soluble	18.0 ± 3.3	25.3 ± 7.3	13.9 ± 1.5	20.0 ± 3.3	19.0 ± 2.5
Ether-soluble	10.7 ± 2.8	15.5 ± 5.4	35.0 ± 15.2	33.6 ± 3.5	46.7 ± 4.3

[a] Represents eight and two replicates, respectively.
[b] Represents four, five, and three replicates, respectively.
[c] As a percentage of the [1-^{14}C]2,4-D supplied.
[d] As a percentage of the tissue uptake.

Table II. *Relative Percentage of 2,4-D Metabolites after Incubation of Amsoy Leaf Callus Tissue with [1-^{14}C]2,4-D for 48 h*

Fraction	Amsoy	
	4-week[a]	8-week[b]
Tissue uptake[c]	71.0 ± 7.3	60.7 ± 8.9
Residue[d]	15.5 ± 4.8	17.2 ± 7.7
Ethanol-soluble[d]	84.5 ± 4.8	82.8 ± 7.7
Free 2,4-D	41.4 ± 9.3	17.6 ± 2.8
Aqueous-soluble	30.7 ± 3.7	35.0 ± 7.2
Ether-soluble	12.6 ± 5.0	30.2 ± 8.2

[a] Represents four replicates.
[b] Represents three replicates.
[c] As a percentage of the [1-^{14}C]2,4-D supplied.
[d] As a percentage of the tissue uptake.

35% of the 2,4-D supplied while Acme took up twice as much (Table I). The same trend is shown when uptake is expressed as nmol/g fresh weight (Table III). Amsoy leaf and root callus however took up almost as much 2,4-D as Acme cotyledon callus (Tables II and III). Of course 2,4-D uptake (nmol/g fresh weight) was related to the concentration supplied and it appeared to be accumulated by the tissue.

The ethanol fraction accounted for 80 to 90% of the total radioactivity taken up and the remaining radioactivity in the residue was not further characterized. The radioactivity in the ethanol, diethyl ether, and aqueous fractions is expressed as nmol of ^{14}C label (calculated as [1-^{14}C]2,4-D) per g fresh weight.

The ethanol fraction contained all soluble constituents including amino acid conjugates, glycosides, other metabolites and free 2,4-D. Since the 2,4-D in the diethyl ether fraction was expressed separately, the remaining ether-soluble fraction consisted primarily of the 2,4-D conjugates of glutamic and aspartic acids (3, 4). Although the aqueous extract contained a number of glycoside metabolites no effort was made to identify them. The 1-butanol extract of the aqueous fraction after removal of the 1-butanol yielded several aglycons following treatment with Emulsin. As we reported previously (2, 3) these consisted primarily of 4-hydroxy-2,5-dichlorophenoxyacetic acid (major), 4-hydroxy-2,3-dichlorophenoxyacetic acid, 5-hydroxy-2,4-dichlorophenoxyacetic acid, and 2,4-D. Presumably the 2,4-D released by Emulsin treatment is derived from the glucose ester of 2,4-D (5).

In general as the concentration of 2,4-D in the medium was increased the radioactivity in the ethanol fraction expressed as nmol/g fresh weight increased. Since the weight of tissue per flask was not determined until after incubation with [1-^{14}C]2,4-D and some variation in uptake was observed in individual experiments it was found more reliable to use the ethanol-soluble radioactivity as an index of uptake (2). The three ethanol-soluble subfractions are plotted (nmol/g fresh weight) as a function of this increase in radioactivity of the ethanol fraction (Fig. 1). The 3-week-old Acme cotyledon callus showed no evidence for regulation of 2,4-D level (Fig. 1). All three ethanol-soluble subfractions increased in a linear manner with an increase in the total ethanol-soluble radioactivity; however, the 2,4-D increased more rapidly and was the major component present. These results are similar to our previous results (2) with 3-week-old Amsoy root callus.

The relative percentage of 2,4-D and its metabolites found in Acme and Amsoy cotyledon callus of various ages is presented in Table I. These data are averages of between two to eight experiments. Although the concentration of 2,4-D applied per gram fresh weight varied (18.8–77.6 nmol/g fresh weight) and influenced the results, the data are expressed as averages for comparative purposes. The standard deviations provide an indication of observed variations and the influence of concentration differences.

Six-week-old Acme cotyledon callus took up somewhat more of the 2,4-D and metabolized it to aqueous and ether-soluble products to a slightly greater extent than 3-week-old cotyledon callus tissue (Table I). Six-week-old Acme cotyledon callus did not exhibit any marked increase in amino acid conjugate formation or regulation of the level of 2,4-D in the tissue. Acme cotyledon callus does not approach senescence (browning) until approximately 9 weeks of age and perhaps still older tissue might exhibit 2,4-D regulation.

The Amsoy cotyledon callus took up only about 35% (3 and 6 weeks) to 56% (4 weeks) of the applied 2,4-D and the 2,4-D was metabolized more by Amsoy than by Acme cotyledon callus (Table I). In 3-week-old Amsoy cotyledon callus the amino acid conjugates (ether-soluble) represent about one-third of the radioactivity in the tissue, while free 2,4-D makes up about 40% of the radioactivity in the tissue (Table I). After 4 and 6 weeks the per cent 2,4-D in the tissue decreased while the aqueous and especially the ether-soluble fraction (amino acid conjugates) increased. In the 6-week-old Amsoy cotyledon tissue the level of amino acid conjugates comprised just less than 50% of the total label accumulated. By this time, the tissue regulated the level of free 2,4-D in the tissue at 1.5 nmol/g fresh weight (Table III) which represented only 16.8% of the radioactivity in the tissue.

Amsoy leaf callus tissue metabolized 2,4-D in a similar manner to that of cotyledon callus tissue (Table II). In 4-week-old tissue free 2,4-D predominated and the amino acid conjugates (ether fraction) were minor metabolites (12.6%). In 8-week-old tissue free 2,4-D only represented about 17% while the amino acid conjugates increased to 30% of the tissue radioactivity. Thus, the older Amsoy leaf callus tissue appears to show 2,4-D regulation (3.5 nmol/g fresh weight, Table III) through amino acid conjugate formation similar to Amsoy cotyledon and root callus tissues (2). An interesting observation was the larger amount of aqueous-soluble metabolites (hydroxylated 2,4-D glycosides) in this leaf callus tissue (Table II) compared to the cotyledon callus tissue (Table I).

A summary of the uptake of radioactivity and the 2,4-D levels (in nmol/g fresh weight) by the various callus tissues examined is presented in Table III. Included for comparison are our (2) previous results with Amsoy root callus. Older Amsoy leaf and

Table III. *Summary of Total Uptake and Internal 2,4-D Concentration Found in Various Ages of Acme and Amsoy Soybean Callus Tissues Incubated with [1-^{14}C]2,4-D for 48 h*

Age of Tissue	Acme		Amsoy					
	Cotyledon		Cotyledon		Leaf		Root[a]	
	Total uptake	Free 2,4-D	Total uptake	Free 2,4-D	Total uptake	Free 2,4-D	Total uptake	Free 2,4-D
weeks	*nmol per gram of fresh weight*							
3	26.2 ± 8.5[b]	15.8 ± 5.6[b]	17.6 ± 7.9	7.6 ± 4.9			24.5 ± 9.5	13.0 ± 7.9
4			19.6 ± 3.2	5.3 ± 0.5	26.3 ± 12.0[c]	10.0 ± 5.4[c]		
6	26.2 ± 1.5	13.0 ± 1.0	8.8 ± 0.2	1.5 ± 0.1[d]				
8					20.2 ± 10.5	3.5 ± 1.7[d]		
9							19.8 ± 8.8	3.2 ± 1.2[d]

[a] Data taken from reference 2.
[b] Data selected from a range of 13.4 to 37.0 nmol/g fresh weight of 2,4-D taken up by the tissue.
[c] Data selected from a range of 9.3 to 37.6 nmol/g fresh weight of 2,4-D taken up by the tissue.
[d] Tissues found to regulate 2,4-D are underlined and represent a range of 8.3 to 32.2 nmol/g fresh weight of 2,4-D taken up by the tissue.

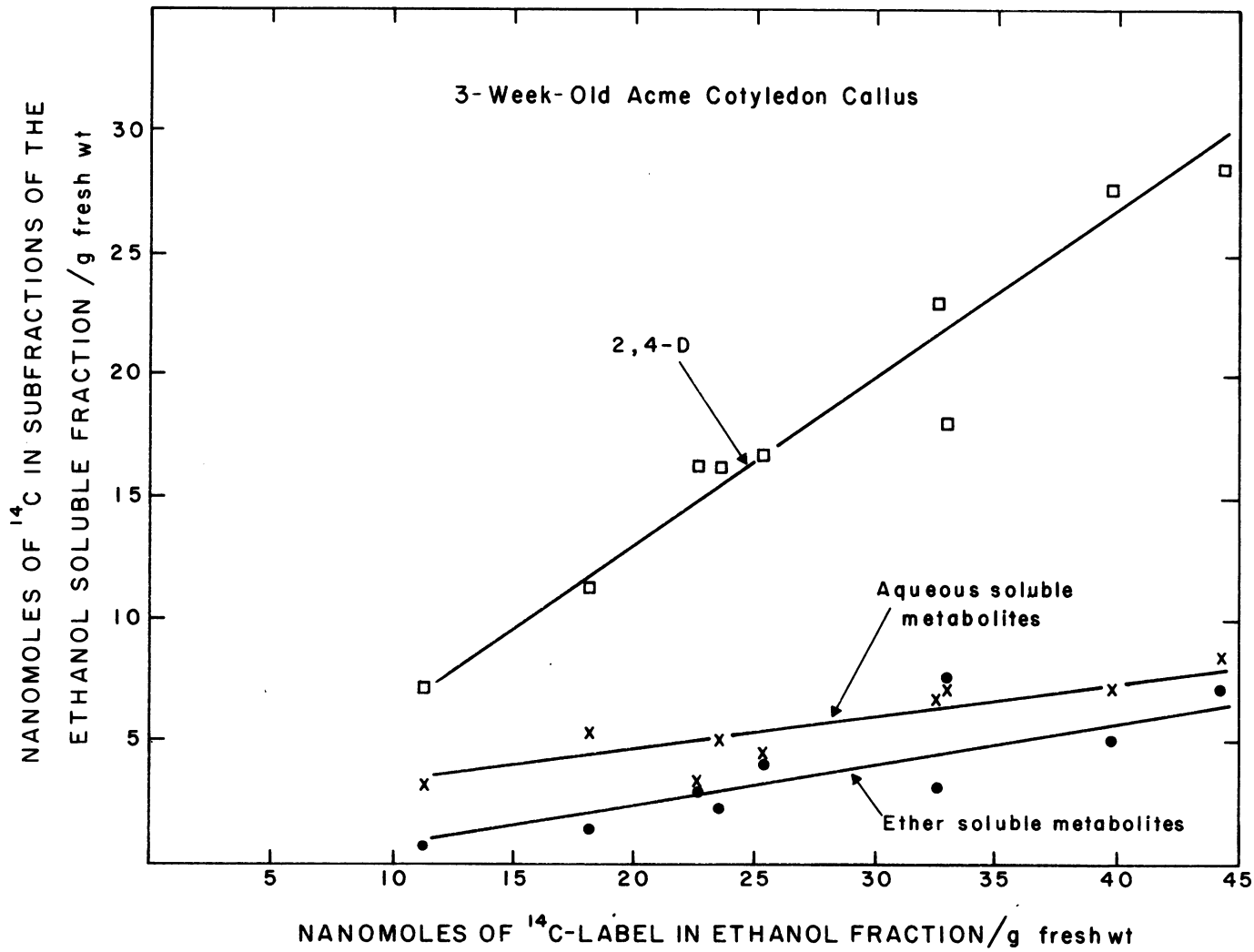

FIG. 1. Concentration of 2,4-D or its metabolites found in three subfractions of the total ethanol extract of soybean cotyledon callus following 48-h incubation with various levels of [1-^{14}C]2,4-D (1.1–4.4 μM). The free 2,4-D content was subtracted from the total ether-soluble fraction to yield ether-soluble metabolites (mostly amino acid conjugates).

root callus were similar and regulated 2,4-D at 3–3.5 nmol/g fresh weight while Amsoy cotyledon callus regulated 2,4-D at about 1.5 nmol/g fresh weight. Previously we observed 2,4-D regulation (2.0 nmol/g fresh weight) with differentiated Amsoy roots in culture (2). Acme cotyledon callus (3- and 6-week-old) and young Amsoy callus (3- and 4-week-old) tissues did not regulate but accumulated 2,4-D as the external concentration increased. There is a trend toward lower 2,4-D uptake by the older tissues which regulated 2,4-D levels. This change in which older tissues regulate 2,4-D levels may be due to an increase in the level of conjugated

enzyme(s) with increasing age of the tissues as well as an increase in the pools of free amino acids. This difference may be related to physiological changes associated with the onset of senescence. Amsoy cotyledon callus tissue was usually approaching senescence (browning) 6 weeks after subculture while the slower growing Acme cotyledon callus tissue was only becoming senescent 9 weeks after subculture. The apparent varietal differences observed in the two soybean cotyledon callus cultures may be due to the differences in their physiological ages. The utilization of these 2,4-D conjugates when older Amsoy callus tissues are placed on fresh medium has now been observed and will be the subject of a forthcoming report.

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