ACCUMULATION OF 2,4-D, AN AUXIN HERBICIDE, IN TISSUE CULTURES FROM 2,4-D SENSITIVE AND RESISTANT VARIETIES OF SOLANUM TUBEROSUM

by

DR. B. FALUDI – DR. ÁGNES FALUDI-DÁNIEL – DR. I. GYURJÁN and MÁRIA PACSÉRY

Department of Phylogenetics and Genetics of the Eötvös Loránd University, Budapest

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Phenoxyacetic acid derivatives are mostly used in agriculture. In Hungary Dikonirt (2,4-D) was used first in 1956 in a quantity of 240 tons. In 1960 the amount of this herbicide used up in a year was 600 tons and in 1965 as much as 3300 tons (data supplied by the Section of Plant Protection of the Department of Agriculture), which, however, still lags behind the yearly consumption by other countries.

The advantage of phenoxyacetic acid derivatives over other types of herbicides consists in their hormone action, on the one hand, and in the fact that they disappear from the soil within some months, on the other (A u d u s 1951). In the plants, however, they are firmly absorbed and do not diffuse out even from isolated organs (S e r g e n t and B l a c k m a n 1962).

There are a number of papers dealing with the transport of 2,4-D (C r a f t s 1951, 1956 a,b, 1960; B r i a n and R i d e a l 1952, F a n g and B u t t s 1954a, C r a f t s and Y a m a g u c h i 1960). The experiments described in the above publications were carried out with intact plants or sometimes with isolated organs and the data reported do not differentiate between the three main factors of transport, i.e. penetration through the cuticle, translocation in the conducting vessels and absorption by the tissues proper. Therefore, such experiments yield only some indirect evidence as to the extent and character of the accumulation at the site of action.

It has been shown that the ontogenic phase i.e. the degree of differentiation has an influence on accumulation (L i n s c o t t and M c C a r t y 1962). With young *Vernonia baldwinii* plants the highest concentration of 2,4-D was found in newly formed young shoots, whereas with fully developed plants it was in the roots.

In *Phaseolus vulgaris* a very active apicocaudal transport was demonstrated with a minimum amount of 2,4-D getting back into the soil (H o l l e y et al. 1950). When the 2,4-D content of etiolated and normal leaves was compared no difference concerning accumulation was found (J a v o r s k y et al. 1955)

Rates of accumulation are influenced by the degree of metabolization and by the factors acting on it. It has been established that in *Phaseolus vulgaris* kept in the light 2,4-D is converted very rapidly into an alcohol insoluble product (Weintraub et al. 1962). In the same plant species the 2,4-D concentration attained remains at a constant level in both stems and roots (Fang and Butts 1954b).

For a thourough investigation of accumulation as a factor of transport non-differentiating tissue cultures prepared from potato tubers seemed to be exceptionally suitable. With them neither cuticular penetration nor translocation has to be taken into consideration. In addition, the optimum growth rate of such tissue cultures was observed to be in the dark (Faludi 1957) and exceedingly quick transformation processes do not interfer with the evaluation of the results.

Material and Methods

Tissue cultures were prepared from tuber tissues of two varieties of *Solanum tuberosum*. One variety, "Gül Baba" was previously found to be sensitive and the other variety "Margit" to be relatively resistant to 2,4-D (Faludi et al. 1961). The cultures were prepared and maintained using a method which had been particularly designed for potatoes (Faludi 1957, 1966–67, Faludi et al. 1962, 1965a). The results have been based in the average values obtained for 50 to 100 explants and their mean errors.

"Auxin-type growth" was established partly by morphological features and partly by changes in water content, dry weight and total nitrogen content. These latter were determined by weighings and by the Nessler-method modified by Bálint and Hegedüs (Bálint and Hegedüs 1955).

For the determination of the concentration of 2,4-D inside the tissues 2,4-D solutions of known specific activities and of different concentrations were used. The active ingredient of these solutions was labeled either in the C-1 atom of the ring or in the – COOH group of the side chain.

In experiments in which the determination of the accumulation of the total activity originating from 2,4-D was aimed at, the cultures were washed prior to the measurements three times for 5 minutes with a solution containing non-labeled 2,4-D in order to remove surface activity. The tissues were then extracted in 80 per cent alcohol, five times their weight. The residue was washed with 80 per cent, 90 per cent and absolute alcohol, dried with ether, combined with the washing medium, evaporated at room temperature and taken up in a known volume of 50 per cent alcohol. Aliquots of both the alcoholic extract and the residue were plated on planchettes and the residue was fixed with polyvynyl acetate dissolved in 0.5 per cent acetone. Radioactivity was measured in a gas-flow counter with a geometry of 2π and a 46 per cent efficiency. The measurements had an accuracy of about 1 to 5 per cent. The self-absorption corresponding to the thickness of the samples was expressed in terms of an infinitely thin layer of 2,4-D dissolved in sucrose. The value thus obtained was but slightly higher than the self-absorption of the alcoholic extract and somewhat lower than that of the residue.

In experiments on the influence of 2,4-D analogues and of β-indolylacetic acid on absorption the same procedure was followed as that used in other studies on their growth regulatory action (F a l u d i et al. 1964, 1965a).

To test varietal differences experiments were carried out with 2,4-D labeled either in the ring or in the carboxyl group. For the tentative identification of the degradation products the alcoholic extracts were subjected to paper chromatography in a mixture of propanol: ammonia: water = 10 : 1 : 1 (v/v/v) using Schleicher-Schüll 204 3b filter paper. The radioactivity spotted for each chromatographic run was 10000 cpm. For autoradiography an exposition time of 4 weeks was taken.

Results

Before studying the accumulation of radioactivity deriving from 2,4-D we had to get some information about the type of growth which has a significant influence on the evaluation of the results. When the tissues contained 2,4-D in an optimum concentration of 10^{-4} M they were of a transparent, creamy white colour. At an early stage of culturing (4^{th} to 5^{th} day) a great number of tubercles appeared which gave the explants a cauliflower like appearance characteristic of an "auxin-type" growth (Fig. 1).

In the picture some large and several small tubercles are to be seen. This is a typical picture of a so called hyperhydric growth and gives some hints as to an "auxin-type" growth. This is unequivocally supported by the data shown in Table 1.

It is clear from Table 1 that the major part of tissue growth is due to induced water uptake. For several decades this phenomenon has been considered as the most important feature of "auxin-type" growth (R e i n d e r s 1938). The induced water uptake of the variety which is sensitive to 2,4-D is 100 per cent higher than that of the resistant variety. In addition to a more intensive water uptake there is also an increase in dry matter content. It is important to note, however, that at the same time the change in total nitrogen content on a percentage basis is exactly the same with the two varieties which exhibit a significant difference in their growth rate.

During our studies on the accumulation of ^{14}C originating from labeled 2,4-D, changes in the 2,4-D* concentration related to 1 ml of the culture medium and to 1 mg tissue, respectively, were, first recorded up to the end of non-differentiated growth, i.e. until the 14^{th} day (F a l u d i 1966).

As seen in Table 2, most of the activity was present in the alcoholic fraction. Concentrations of $5 \cdot 10^{-5}$ M, 10^{-4} M and $2 \cdot 10^{-4}$ caused a 2 to 4 fold weight increase, respectively (F a l u d i 1966). The 2,4-D* concentration in this zone was uniformly 2. 2 times higher than that in the culture medium. This value may be regarded as the saturation level regulated by growth. This is shown also by the fact that in the case of a culture medium containing 10^{-3} M 2,4-D* (10^{-6} M/ml) where growth stops, the rate of the degree of concentration falls below 1 as compared to a 5 times higher concentration in the culture medium.

Table 1.

Effect of 2,4-D used in the optimum concentration of 10^{-4} M on changes in water content, dry matter content and total N content of the potato varieties Gül Baba and Margit*

Age of tissue (days)	Gül Baba						Margit					
	Water content mg/explant	Change per cent	Dry matter content mg/explant	Change per cent	Total N mg/explant	Change per cent	Water content mg/explant	Change per cent	Dry matter content mg/explant	Change per cent	Total N mg/explant	Change per cent
0	23,5±0,35	–	4,8±0,40	–	0,099±0,0012	–	21,8±0,45	–	6,4±0,35	–	0,078±0,0012	–
14	116,4±1,30	+395	6,1±0,17	+27	0,129±0,0038	+61	68,1±0,45	+214	5,6±0,10	−12	0,129±0,0039	+60

* Thanks are due to E. Kovács for the determination of total-N contents

Fig. 1. Characteristic picture of a growing tissue induced by 2,4-D

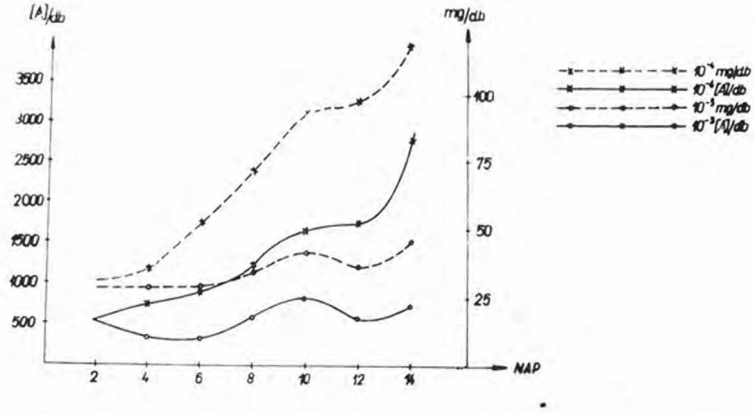

Fig. 2. Growth of potato tissue cultures and accumulation of 2,4-D labeled with ^{14}C in the ring during culturing. (The ^{14}C label was the same in both the 10^{-4} M and 10^{-3}M culture media)

Table 2.

Accumulation of 2,4-D labeled with ^{14}C in the ring in potato tissue cultures as a function of 2,4-D concentration in the culture medium

2,4-D* M/ml culture medium	2,4-D* 10^{-7} M/g tissue			activity related to that of the culture medium
	in the acohol soluble fraction	in the residue after alcoholic extraction	total activity	
$5 \cdot 10^{-8}$	1.08	0.03	1.11	2.18
10^{-7}	2.14	0.03	2.17	2.18
$2 \cdot 10^{-7}$	4.25	0.07	4.32	2.16
10^{-5}	8.37	0.30	8.67	0.90

A similar correlation is indicated by the data in Fig. 2, which shown the accumulation of 2,4-D in the course of culturing. It may be seen that in a culture medium containing 10^{-4} M 2,4-D there is a very rapid accumalation during the lag-period of growth. Later on accumulation is proportionate to growth. On a culture medium containing 10^{-3} M 2,4-D no such phenomenon was observed.

When in addition to 2,4-D* the basic molecule (phenoxyacetic acid) and analogues were added together to the culture medium it was found that some analogues as well as the non-labeled 2,4-D which acted just as a factor to increase concentration, had the same inhibitory effect on accumulation. Some other analogues, however, inhibited accumulation to a much higher extent than did 2,4-D (Table 3).

Table 3

Effect of 2,4-D analogues on the growth of potato tissue cultures and on the accumulation of 2,4-D in them
(Date of culturing December)

Active ingredients	$\frac{10^{-7}\text{ M} + 10^{-7}\text{ M}}{\text{ml culture medium}}$	Weight of tissues mg	Per cent	Accumulation 10^{-7} M/g fr. w.	Per cent
2,4-D*		119	100	3.3	100
2,4-D* + 2,4-D		101	84	2.6	79
2,4-D* + 2,3-D		99	82	1.9	58
2,4-D* + 2,6-D		101	84	2.5	76
2,4-D* + 3,5-D		76	70	2.4	73
2,4-D* + 2,5-D		97	81	1.8	55
2,4-D* + 3,4-D		82	69	2.1	64
2,4-D* + phenoxyacetic acid		100	84	1.9	58

Such analogues were 2,3-D, 2,5-D, 3,4-D and phenoxyacetic acid. It is also evident that induction of growth and inhibition of accumulation did not always run in step (2,3-D and phenoxyacetic acid).

We get the same overall picture when 2,4-D* and β-indolylacetic acid in different concentrations are used together (Table 4).

Table 4.

Accumulation of 2,4-D* in potato tissue cultures in the presence of ß-indolylacetic acid
(Date of culturing: October)

Concentration M/ml culture medium	Weight of tissues mg	2,4-D* 10^{-7} M/g tissue		Total activity 10^{-7} M/g	Per cent
		in the alcohol soluble fraction	in the residue after alcoholic extraction		
10^{-7} 2,4-D*	76	2.14	0.07	2.21	100
10^{-7} 2,4-D* + 10^{-7} IAA	72	2 72	0.03	2.75	124
10^{-7} 2,4-D* + 10^{-6} IAA	92	2.48	0.03	2.51	114
10^{-7} 2,4-D* + 2.10^{-6} IAA	59	3.54	0.03	3.57	161

The data in Table 4 clearly show that whenever IAA was used in a high enough concentration or even in concentrations which antagonize the stimulatory effect of 2,4-D upon growth it enhanced the accumulation of radioactivity deriving from 2,4-D.

It has been known for a long time that within the same species there exist very delicate differences in the sensitivity to 2,4-D (D e r s c h e i d et al. 1952) and that these varietal differences appear at the tissue level as well (F a l u d i et al. 1961, 1965b). The question arose in how much this phenomenon can be attributed to differences in accumulation. Since some authors claim that the main reason for these differences is to be sought for in various degrees of decarboxylation (E d g e r t o n 1961, E d g e r t o n and H o f f m a n 1961) the problem has been reinvestigated by using 2,4-D which was labeled either in the ring or in the carboxyl group (Table 5).

Table 5.

Accumulation of 2,4-D-^{14}C and 2,4-D-1^{14}C labeled in the ring in tissue cultures of the potato varieties Gül Baba and Margit
(Date of culturing February)

2,4-D label	10^{-7}M/g	
	Gül Baba	Margit
2,4-D*	3.3 ± 0.25	2.2 ± 0.58
2,4-D-1^{14}C	2.8 ± 0.46	2.3 ± 0.18
Decarboxylation 10^{-7} M/g 2,4-D	0.6	0.0

It may be seen from Table 5 that the sensitive variety accumulated more 2,4-D than did the resistant one and that about 20 per cent of this amount was decarboxylated. In the resistant variety there was no detectable decarboxylation. If the differences in growth between the two varieties are taken into

consideration one may conclude that no significant difference in accumulation exists between them. Therefore neither accumulation nor decarboxylation seem to be responsible for the varietal differences.

To get some more insight into the problems concerned paper chromatographic studies were also made (Fig. 3).

The chromatograms show that with both varieties about half of the original activity had an Rf value corresponding to that of 2,4-D. The alcoholic extracts of tissues treated with 2,4-D substituted either in the ring or in the carboxyl group gave two more spots in addition to that of 2,4-D: one with a higher and another one with a lower Rf value than that of 2,4-D. With both types of substituted derivatives the varietal difference appeared in the spot with the higher Rf value. This phenomenon may be explained by two alternative hypotheses: the product formed is either a compound with an increased mobility and a higher activity than the unchanged 2,4-D, or accumulation plays no role whatsoever in varietal differences and the difference merely depends on the manner the target tissue reacts. The fact that the spots with Rf values lower than that of 2,4-D behave just opposite to those with the higher Rf values stresses the importance of metabolization in the differences.

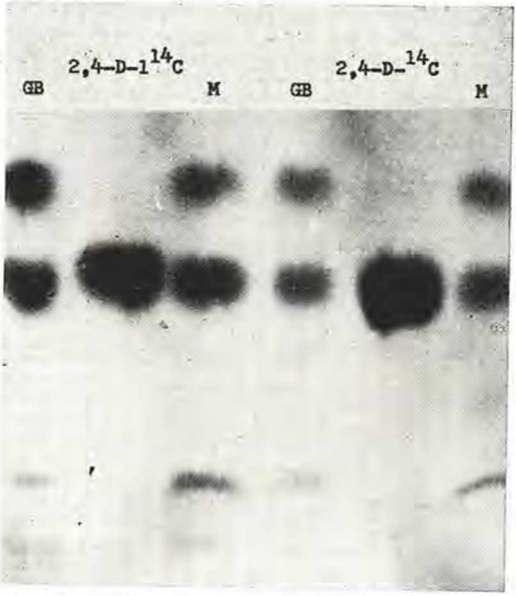

Fig. 3. Conversion of 2,4-D labeled either in the ring or in the carboxyl group in tissue cultures of potatoes of the varieties Gül Baba and Margit, respectively

Discussion and conclusions

It has for long been a debated question whether water uptake induced by auxin-like substances is of an osmotic (H a y n e s 1950, L e v i t t 1954) or of a nonosmotic nature (B o g e n 1953, D a i n t y 1963). During our studies on varietal differences it has been shown that between 2,4-D sensitive and resistant varieties there is originally no considerable difference in water content whereas dry matter content is somewhat higher in the resistant variety. When, however tissue cultures are treated with 2,4-D in a concentration which has a growth inducing effect the water uptake of the sensitive variety will become almost the double of that of the resistant one. This fact is in line with the assumption that in such cases water uptake is of a non-osmotic nature.

As far as changes in total nitrogen content are concerned some authors have found that in various plants it decreases with increasing water uptake (R a k i-

tin and Zemskaya 1958, Wort 1961) some others have established that it increases proportionally to (Kandler and Fink 1955) and or to a higher extent than water uptake (Kandler and Neumair 1954). In our experiments total nitrogen content increased almost at the same rate with both varieties which had different rates of water uptake and this increase was far from being proportionate to water uptake.

Penetration was formerly considered to be a more important factor than accumulation with respect to both growth induction by and selectivity of auxin herbicides (Hansen and Buchholtz 1952). Recently the delicate varietal differences in growth rates and herbicidal selectivity are thought to be due to morphophysiological effects with genetical implications (Williams 1953, 1954). Furthermore it is being emphasized that differences in penetration are practically of no importance (Williams et al. 1960). This latter assumption is supported by recent findings which also claim that the effect is exerted at the plasmatic level (Biebl 1963, Faludi and F. Dániel 1960). The connection with the induction of growth can be understood by assuming that growth will compensate for accumulation as long as it is possible. Later on both accumulation and growth may stop without being connected in any way. Similar conclusions were drawn from experiments with intact *Phaseolus vulgaris* plants. It has been namely shown that upon increasing the doses of 2,4-D a "saturation level" will set in quite early, above which upon further increases of the dose inner concentration does not increase further (Chmielewsky et al. 1966). The authors did not report more detailed investigations.

The fact that transport processes are going on mainly at the plasmatic level suggests, that several authors have exaggerated the importance of translocation (Crafts 1953, 1961, 1964, Luckwill and Lloyd-Jones 1960a, Slife et al. 1962). Similar conclusions have been drown from experiments carried out with intact plants (Linscott and Mc Carty 1962).

Formely the ratio of hydrophylic to hydrophobic molecules was regarded as the most important factor in the auxin-auxinherbicide effect (Weldstra 1947, 1956, Veldstra and Booij 1949). Leopold et al. (1960) have tested several ^{14}C labeled phenoxyacetic acid derivatives from this point of view. The results obtained did not confirm the hypothesis that there was a correlation between the degree of lipophylic character and the efficiency of the molecule. Their finding that phenoxyacetic acid is very highly water soluble whereas 2,3-D, 2,5-D and 3,4-D are nearly as water soluble as 2,4-D, is remarkable. When equimolar mixtures of 2,4-D and its analogues or phenoxyacetic acid were used in our experiments accumulation was strongly inhibited by the compounds in question. This indicates that the ratio H : L cannot be responsible for this phenomenon at least as far as phenoxyacetic acid is concerned. It is well possible, however, that the inhibitory effect of phenoxyacetic acid and that of 2,3-D, 2,5-D and 3,4-D have quite different reasons. In connection with this problem one has to bear in mind a hypothesis according to which phenoxyacetic acid possesses a very high diffusion rate and therefore its diffusion into the tissues and therefrom back into the medium is so intense as to prevent 2,4-D from reaching its site of action (Wedding and Blackman 1961).

The only reason why this theory cannot be accepted for the interpretation of our results is, that it does not explain the quantitative aspects of the agreement between the inhibitory effect of phenoxyacetic acid and that of 2,3-D, 2,5-D and 3,4-D. It may be suggested that the competition is connected rather with the distribution of electrostatic charges or the coplanarity of the ring and the side chain.

This hypothesis is supported to some extent by our finding that β-indolylacetic acid stimulates rather than inhibits the accumulation of 2,4-D and that this inhibition of accumulation sometimes does and sometimes does not run in step with the inhibition of growth.

In some other experiments the presence or lack of the growth stimulating action of 2,4-D and of its analogues could be interpreted by the position of the substituents (F a l u d i 1964, F a l u d i et al. 1965a). On the basis of these findings an attempt will now be made to interpret the difference between inhibition of accumulation and inhibition of growth. As shown in the present paper inhibition of accumulation was accompanied by inhibition of growth when 2,5-D and 3,4-D were added together with 2,4-D. In both of these substituted compounds the substituents, just as 2,4-D, decrease the positivity of the center around carbon atom 1 in the benzene ring. This may account for the competition. This hypothesis is in line also with the finding that neither diortho-2,6-D nor dimeta-3,4-D have a higher inhibitory effect on growth than the combination 2,4-D* + 2,4-D. The stimulatory effect of β-indolylacetic acid on accumulation and its fluctuations may be well explained by supposing that accumulation is compensated by two growth processes which differ to some extent in their mechanisms and that this ensures a higher saturation level.

The stimulatory effect of β-indolylacetic acid on accumulation and its fluctuations can be interpreted by the fact that the stimulatory effect of high concentrations of IAA on growth works together with that of 2,4-D (F a l u d i et al. 1964). In this way compensation of accumulation by growth may set in at a higher saturation level.

The results of our laboratory experiments carried out on tissue cultures fully support the data of field trials which report significant differences in sensitivity to auxin herbicides between different varieties of the same species (D e r s c h e i d 1952, D e r s c h e i d et al. 1953, W i l l i a m s 1954). The genetical stability of these differences (W i l l i a m s 1953) is substantiated by their dependence on cytoplasmic structure and metabolism, as suggested in the present paper. It has been namely pointed out that both in decarboxylation (Table 5) and in the metabolization of the 2,4-D accumulated (Fig. 3) there are varietal differences. This is clearly shown by the chromatographic differences between the sensitive and the resistant varieties with respect to the alcohol soluble fractions of tissue extracts treated with 2,4-D labeled either in the ring or in the carboxyl side clain. Our data also indicate, however, that decarboxylation is not the most essential factor as supposed by some workers (L u c k w i l l and L l o y d-J o n e s 1960b, E d g e r t o n 1961, E d g e r t o n and H o f f m a n 1961), instead, the difference may have several other causes. This hypothesis is supported by the fact that it is the sensitive variety in which decarboxilation goes on very actively while in the resistent variety no significant decaroboxylation process can be detected.

All our experimental data strongly favour the view that although accumulation plays the most important role in transport processes from the point of view of auxin effect, yet it is but a necessary and not a sufficient prerequisite.

Summary

The accumulation of the auxin herbicide 2,4-D has been studied in tissue cultures made from 2,4-D sensitive and resistant potato varieties and exhibiting "auxin-type" growth. "Auxin-type" growth was shown to be the case by changes in the water content, dry matter content and total nitrogen content of the tissue cultures (Fig. 1 and Table 1).

The accumulation of the radioactivity deriving from labeled 2,4-D was most characteristically represented in the alcoholic fraction of tissue extracts. By using culture media containing 2,4-D in concentrations of 5×10^{-5} M, 10^{-4} M and 2×10^{-4}, respectively, the concentration rate was uniformly 2,2. When, however, the culture medium contained 2,4-D in a concentration of 10^{-3} M, which inhibits growth, the concentration rate was somewhat below 1. This phenomenon is considered to be due to compensation of accumulation by growth (Table 2). The time curves representing growth and accumulation, respectively, support this idea (Fig. 2).

When 2,4-D was added together with phenoxyacetic acid or with substituted dichloro compounds it was found that only 3,4-D, 2,5-D 3,4-D and phenoxyacetic acid inhibited accumulation by more than what would be expected with a twofold concentration of 2,4-D. The inhibitory effect of the dichloro-derivatives mentioned above could by explained by their lypophilic character similar to that of 2,4-D. The ability of phenoxyacetic acid to inhibit accumulation to the same extent, however, seems to refute this hypothesis. No direct correlation was found between inhibition of accumulation and inhibition of growth. Inhibition of growth may be connected with the fact that substitutions in carbon atoms 3,4- or 3,5-have the same influence on the positivity of carbon atom 1 in the benzene ring as the substitution in carbon atoms 2,4- has.

As far as varietal differences are concerned it has been shown that the 2,4-D sensitive variety accumulated more 2,4-D in the tissues than the resistant one did, but the rate of decarboxylation was also higher. Consequently, no significant difference in accumulation could be detected (Table 5). On the contrary, there was a striking difference between the two varieties in the metabolization of 2,4-D. From this point of view decarboxylation does not play an important role (Fig. 3).

Of the transport factors the accumulation of radioactivity deriving from labeled 2,4-D is of major importance, from the point of view of auxin effect, however, this has to be regarded only as a necessary factor rather than a sufficient one.

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