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INCORPORATION OF EXOGENOUS TYROSINE AND PHENYLALANINE INTO C-GLYCOSYLFLAVONES IN GLYPHOSATE-TREATED BARLEY SEEDLINGS

Although phenylalanine ammonia lyase (PAL) which is responsible for channelling phenylalanine units into phenylpropanoid pathway has usually shown some tyrosine ammonia lyase (TAL) activity as well, exogenous L-tyrosine has been a poor precursor of phenolic compounds in most plants except grasses where the activity of TAL is often comparable to that of PAL (lor reviews see Young et al., 1966; Camm, Towers, 1973). Our earlier experiments with barley seedlings revealed a considerable activity of TAL (4.9 nmol/h per primary leaf) that was more than were needed for maximum synthesis of all their flavonoids (C-glycosylflavones) under most favourable conditions. The incorporation of exogenous L-tyrosine (from a $2 \cdot 10^{-3}$ M solution) into barley C-glycosylflavones appeared to be only twice less than that of exogenous L-phenylalanine of the same concentration (Лаанест, 1981).

In searching for possible ways to increase the utilization of tyrosine for phenolic biosynthesis under experimental conditions, we paid attention to an inhibitor of the shikimic acid pathway, glyphosate (N-phosphonomethylglycine). The primary inhibitory action of the herbicide is located at the reaction which leads to 3-enolpyruvylshikimate-5-phosphate and results in reducing the endogenous supply of L-phenylalanine and other aromatic amino acids (Amrhein et al., 1980). It may be assumed that in a situation where the resources of the principal polyphenol precursor, phenylalanine, are limited, exogenous tyrosine might be more effectively used for flavonoid biosynthesis. In a recent work of our laboratory with buckwheat seedlings (Margna et al., 1985) it has been demonstrated that at a high glyphosate concentration (10^{-2} M) the synthesis of flavonoids from exogenous L-tyrosine did indeed show an absolute increase as compared with lower glyphosate concentrations, and there occurred a significant rise in the relative labelling of flavonoids. Still the effect was too slight to consider L-tyrosine a true alternative precursor of flavonoids in buckwheat seedlings. In the hope that barley seedlings would be a more suitable material to study the problem of the precursor role of tyrosine, we designed tracer experiments with that material using both phenylalanine and tyrosine on a glyphosate background.

Material and methods

The experiments were carried out with isolated primary leaves of barley (*Hordeum vulgare* L. cv. Astoria) excised from 80 h old etiolated seedlings grown in tap water. The excised material was incubated for 40 h in light (illumination from white fluorescent tubes, light intensity

28 W m^{-2}; temperature 25 °C) on filter paper moistened with distilled water, with solutions of [1-^{14}C]-L-tyrosine (concentration 10^{-3} M, specific activity 8.4 Ci/mol) and [1-^{14}C]-L-phenylalanine (10^{-3} and 10^{-2} M, specific activities 5.8 and 0.57 Ci/mol, respectively), or with a mixture of glyphosate (10^{-4}, 10^{-3}, or 10^{-2} M) and an amino acid.

After incubation the plant material was washed with water in order to remove external label and was then assayed. Content of C-glycosylflavones was determined spectrophotometrically (Laanest, 1978) after their separation by two-dimensional paper chromatography in a mixture of isoamyl alcohol—petrol ether—acetic acid—water, 3 : 1 : 3 : 3, organic phase (I) and 10 per cent acetic acid (II). The content of individual flavonoids was expressed in nmols per primary leaf. The radioactivity of flavonoids was assayed in the ethanolic eluates with the use of a Beckman LS-100C liquid-scintillation counter.

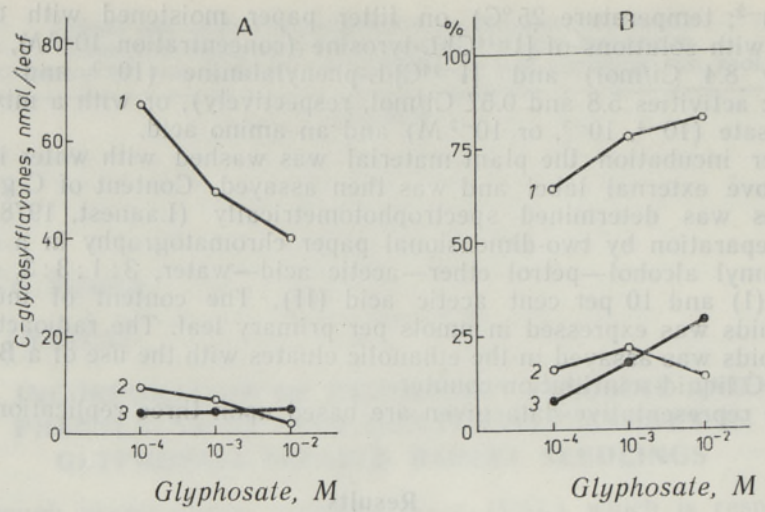
The representative data given are based upon three replications.

Results

As can be seen from the Table, a single primary leaf of barley incubated in water for 40 h accumulated approximately 160 nmols of C-glycosylflavones. Addition of aromatic amino acids to the incubation medium resulted in a certain reduction of flavonoid formation. At the concentration of 10^{-3} M, the efficiency of exogenous phenylalanine, as judged by incorporation of label into C-glycosylflavones, appeared to be 1.5 times higher than that of tyrosine (18.0 and 11.9 nmol per leaf, respectively). Glyphosate had a high inhibitory effect on C-glycosylflavone formation in spite of the presence of aromatic amino acids. Moreover, even at its lowest concentration, the herbicide strongly suppressed the incorporation of exogenous substrates into flavonoids. Nevertheless, L-phenyl-

Formation of C-glycosylflavones from exogenous L-tyrosine and L-phenylalanine in glyphosate-treated barley leaves (nmol/leaf) during a 40 h incubation period

Background	Glyphosate, M	Saponarin		Lutonarin		Lutonarin 3′-methyl ether	
		total	formed from exogenous substrate	total	formed from exogenous substrate	total	formed from exogenous substrate
Water	—	116	—	21.7	—	21.6	—
L-tyrosine, 10^{-3} M	—	94.9	8.94	14.2	1.42	17.0	1.57
	10^{-4}	40.4	3.01	5.06	0.437	8.89	0.664
	10^{-3}	17.8	3.43	1.63	0.291	4.99	0.581
	10^{-2}	12.4	3.66	1.30	0.419	3.10	0.733
L-phenylalanine, 10^{-3} M	—	109	12.7	17.0	2.72	16.6	2.55
	10^{-4}	43.1	5.73	6.58	1.80	9.56	1.88
	10^{-3}	24.2	4.05	2.69	1.12	6.21	1.87
	10^{-2}	12.0	1.23	0.990	0.277	2.92	0.644
L-phenylalanine, 10^{-2} M	—	108	59.3	21.1	14.1	20.0	13.0
	10^{-4}	75.9	45.0	14.1	10.9	17.1	12.8
	10^{-3}	44.5	31.9	8.58	8.21	10.6	9.21
	10^{-2}	33.4	25.6	5.47	6.20	9.81	8.32



Amount of C-glycosylflavones synthesized from exogenous 10^{-2} M (*1*) or 10^{-3} M (*2*) L-phenylalanine and 10^{-3} M L-tyrosine (*3*) in glyphosate-treated barley leaves. *A* — absolute amount, nmol/leaf; *B* — percentage of the total production of C-glycosylflavones.

alanine from a 10^{-2} M solution was readily channelled for C-glycosylflavone formation at any glyphosate level (Fig. *A*, curve *1*) while the incorporation of the same precursor from a 10^{-3} M solution (curve *2*) appeared to be much less. Both processes were progressively inhibited by increasing glyphosate concentrations.

Exogenous tyrosine did not show any preferential utilization in absolute units, and even the lowest glyphosate concentration decreased its incorporation three times. However, in contrast to phenylalanine, the incorporation of exogenous tyrosine was not further inhibited by higher glyphosate doses. As the total flavonoid content continued to decrease within glyphosate concentrations 10^{-4}—10^{-2} M, the share of C-glycosylflavones synthesized from exogenous tyrosine increased from 7.6 to 28.6 per cent of their total amount (Fig. *B*). Correspondingly, it resulted in a substantial rise of the relative efficiency of tyrosine as compared with phenylalanine of the same concentration, the ratio of exogenous tyrosine/phenylalanine label being 0.44 at 10^{-4} M glyphosate, 0.61 at 10^{-3} M, and 2.2 at 10^{-2} M.

Discussion

There have been reports on partial or complete reversion of glyphosate-caused growth inhibition by aromatic amino acids (see surveys by Duke, Hoagland, 1981; Hoagland, Duke, 1982). As for phenylpropanoid synthesis, L-phenylalanine completely reversed glyphosate-induced inhibition of anthocyanin synthesis in buckwheat seedlings (Holländer, Amrhein, 1980; Тохвер, Мядамюрк, 1984); aromatic amino acids increased levels of hydroxyphenolic compounds above those in glyphosate-treated soybean seedlings (Duke, Hoagland, 1981), and a partial reversion of phytoalexin formation in soybean leaves was obtained by feeding phenylalanine and tyrosine (Holliday, Keen, 1982).

In the present study exogenous aromatic amino acids fed to glyphosate-treated barley seedlings were not able to substitute their diminished endogenous pools available for polyphenol synthesis. Theoretically, this

might occur if the catalytic potential of corresponding enzymes were lowered by glyphosate treatment.

As a rule, glyphosate increases PAL activity. The effect is evidently a secondary one and results from decreased feedback control due to decreased substrate supply and, thus, to decreased amount of the product (Hoagland, Duke, 1982). Studies of our laboratory showed that in barley, both PAL and TAL activities increased under glyphosate treatment making it possible to synthesize 215 to 265 (TAL) or 2200 to 2500 (PAL) nmols of phenylpropanoid units during the 40 h incubation period. At the same time, even in nontreated seedlings their actual requirement for C-glycosylflavone formation did not exceed 160 nmols. That excludes the possible limiting role of ammonia lyases. One might assume that the starvation for all three aromatic amino acids which undoubtedly leads to a suppression of general protein synthesis results in a deficiency of some other enzymes of the phenylpropanoid pathway responsible for flavonoid formation. Still, comparison of phenylalanine uptake from 10^{-2} and 10^{-3} M solutions clearly shows its dependence on the concentration of exogenous substrate, and even at the highest glyphosate dose a single primary leaf was able to incorporate 40 nmols of exogenous phenylalanine into C-glycosylflavones (Fig. *A*, curve *1*). Therefore we cannot point to any particular site in flavonoid biosynthesis which could be responsible for the suppression of the aromatic amino acid incorporation into barley C-glycosylflavones under our experimental conditions. Moreover, considering that not only tyrosine but phenylalanine as well give rise to *p*-hydroxycinnamic acid, and flavonoid synthesis will further proceed along a common route, the eventual inhibition of some later enzymatic steps of the pathway seems unlikely to result in the different glyphosate-dependency of phenylalanine and tyrosine incorporation into C-glycosylflavones.

In search for a possible explanation to the effects observed we should consider changes in primary metabolism, first of all in protein synthesis. Deficiency of aromatic amino acids ultimately leads to a retardation of this process. Evidently, in glyphosate-treated plant material exogenous aromatic amino acids would preferentially be channelled to the sites of protein synthesis, and only a minor part of them could reach the centres of secondary biosyntheses. In a study on alkaloid synthesis, for instance, L. Nover (1979) showed that, in *Penicillium*, at high exogenous phenylalanine concentration practically all phenylalanine needed for protein synthesis came from this source while alkaloid formation got more than 90 per cent of its phenylalanine from endogenous pools, including supply from protein degradation. Protein synthesis requires individual amino acids in a certain proportion. J. B. Coates and D. D. Davies (1983) have found that total soluble protein from barley primary leaves contains 0.6 to 2.1 per cent of tyrosine and 3.7 to 4.8 per cent of phenylalanine. Our preliminary analyses have shown that the molar concentration of tyrosine and phenylalanine in total protein of the same material was 2.0 and 5.3, respectively. One could assume that at a certain low level of suppressed protein synthesis the process would become saturated with tyrosine while still needing some more phenylalanine, and at increasingly higher glyphosate concentrations more exogenous tyrosine would be left for secondary syntheses. The results of the present study are in accordance with the assumption (see the curves *2* and *3* in Fig. *A*). Besides that, at the highest glyphosate dose, incorporation of phenylalanine from the 10^{-2} M solution was unproportionally (almost 20 times) higher than that from the 10^{-3} M solution (curves *1* and *2*). It indicates that the rate of phenylalanine utilization for protein synthesis was evidently limited by the diminished supply of other aromatic amino acids, and, here again, a more substantial amount of the exogenous precursor became available for flavonoid formation. Unfortunately, poor solubility of tyrosine did not

allow us to apply it in higher concentrations to get comparable results with this compound. For the same reason, it seems to be difficult to find experimental conditions which would enable exogenous tyrosine to compete effectively with phenylalanine in polyphenol biosynthesis.

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EKSOGEENSE TÜROSIINI JA FENÜÜLALANIINI KASUTAMINE C-GLÜKOSÜÜLFLAVOONIDE SÜNTEESIKS GLÜFOSAADIGA TÖÖDELDUD ODRAIDANDITES

Artiklis kirjeldatud katsetes töödeldi odra esimesi pärislehti glüfosaadiga radioaktiivse L-türosiini või L-fenüülalaniini foonil, mis teoreetiliste kaalutluste põhjal oleks võinud osaliselt või täielkult kompenseerida glüfosaadi pidurdavat toimet flavonoidide sünteesile. Tegelikult aga pidurdas glüfosaat tugevasti mitte ainult C-glükosüülflavoonide moodustumist üldse, vaid ka eksogeensete substraatide kasutamist nende sünteesiks. Sealjuures ilmnesid inhibiitori dooside suurendamisel kvalitatiivsed erinevused türosiini ja fenüülalaniini lülitumises flavonoididesse, mille tagajärjel tunduvalt tõusis türosiini kui flavonoidide eellase suhteline efektiivsus. Tulemused viitavad polüfenoolide moodustumise substraatsele regulatsioonile ja valkude biosünteesi määravale osale selles.

Лембе ЛААНЕСТ

ВЛИЯНИЕ ГЛИФОСАТА НА ВКЛЮЧЕНИЕ ЭКЗОГЕННЫХ ТИРОЗИНА И ФЕНИЛАЛАНИНА В *C*-ГЛИКОЗИЛФЛАВОНЫ ПРОРОСТКОВ ЯЧМЕНЯ

Изолированные первичные листья ячменя обрабатывали разными дозами глифосата в присутствии меченых *L*-тирозина или *L*-фенилаланина, которые по теоретическим соображениям могли бы частично или даже полностью компенсировать действие глифосата на биосинтез флавоноидных соединений. Однако результаты показали, что глифосат сильно подавляет не только накопление *C*-гликозилфлавонов, но и исползование для их синтеза экзогенных субстратов. При этом под действием возрастающих доз ингибитора обнаруживались качественные различия между включением тирозина и фенилаланина, которые в конечном итоге приводили к заметному увеличению относительной эффективности тирозина как предшественника флавоноидов. Полученные результаты указывают на субстратную регуляцию накопления полифенольных соединений и на первичную роль биосинтеза белков в этом процессе.