Effects of Glyphosate on Shikimic Acid Accumulation in Tobacco Cell Cultures with Low and High Yields of Cinnamoyl Putrescines

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To study the flow of carbon through the shikimate pathway in tobacco cell cultures with low and high yields of cinnamoyl putrescines, the cell cultures were treated with glyphosate. In the presence of glyphosate the levels of free shikimic acid were increased more than 300-fold by both cell lines. Despite of a normally 10-fold higher level of cinnamoyl putrescines, the high yielding cell line accumulated only 25% more free shikimic acid than the low yielding cell line. This result together with earlier observations indicated that the increased formation of cinnamoyl putrescines was rather limited by the activity of phenylalanine ammonia lyase than by increased substrate supply caused by alterations in the shikimate pathway.

Introduction

The search for biochemically orientated selection systems for cell lines with high yields of secondary metabolites made us compare a p-fluorophenylalanine resistant cell line (TX4) overproducing cinnamoyl putrescines with low yielding wild type cells (TX1). The biochemical characterization revealed that the overproduction was mainly due to increased activities of enzymes involved in that secondary pathway (*e.g.* phenylalanine ammonia lyase (PAL), trans-cinnamate 4-monooxygenase, 4-coumaroyl-CoA synthetase, ornithine and arginine decarboxylases) [1–3]. Increased production of phenylpropanoids is generally thought to be controlled by PAL activity [4]. However, Margna provided some evidence that the substrate supply rather than PAL activity is the limiting factor in phenylpropanoid biosynthesis [5]. The question was therefore whether alterations in the shikimate pathway had also occurred in the overproducing cell line to provide the increased demand for phenylalanine for the biosynthesis of cinnamoyl putrescines.

Our first approach was to inhibit PAL of both cell lines by the competitive inhibitor α-aminooxy-β-phenylpropionic acid (AOPP) [6]. Under such conditions the levels of free phenylalanine increased 6-fold by TX1 and 17-fold by TX4 cells [6]. In the end, however, the pool sizes of phenylalanine were the same in both cell lines. Therefore, it was concluded that the flux through the shikimate pathway was similar by TX1 and TX4 cells [6].

In the meantime the elucidation of the mode of action of the herbicide glyphosate by Amrhein's group [7, 8] provided us with an even better inhibitor to verify our conclusions from the AOPP experiments [6]. It has been shown that glyphosate inhibited very specifically the 5-enolpyruvylshikimate-3-phosphate synthase which made the cells accumulate shikimic acid in buckwheat seedlings [8, 9]. Glyphosate was also found to be a very potent inhibitor of the shikimate pathway in our tobacco cell cultures. The increases in free shikimic acid in the presence of glyphosate by TX1 and TX4 cells were in good agreement with our earlier conclusions that the overproduction of cinnamoyl putrescines by TX4 cells was not necessarily dependent upon an alteration in the shikimate pathway [6].

Abbreviations: AOPP, L-α-aminooxy-β-phenylpropionic acid; PAL, phenylalanine ammonia lyase; PFP, *p*-fluorophenylalanine; Glyphosate, N-(phosphonomethyl)glycine; MCW, MeOH:$CHCl_3$:H_2O.

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Material and Methods

Plant material

Maintenance and characteristics of the sensitive cell line TX1 and the PFP-resistant cell line TX4 (*Nicotiana tabacum* L. cv. Xanthi) have been described previously [1, 3, 6].

Growth experiments

TX1 and TX4 cells were supplemented with the indicated sterile filtered solutions of glyphosate and the aromatic amino acids. The cells were grown for 10 days before they were harvested by vacuum filtration for fresh weight determination and extraction of metabolites.

Feeding of [^{14}C]shikimic acid

Cells of four-day-old cultures of TX1 and TX4 [2.0 g fr. wt./20 ml MX-medium (6)] were incubated for 24 h with 5×10^{-3} M [≙ 16.9 mg] glyphosate before 1 μCi [2,3,4,5*n*-^{14}C]shikimic acid was added for another 48 h. The cells were harvested by vacuum filtration and extracted with MCW as described [6]. The residue was hydrolyzed by 6 N HCl in an autoclave for determining incorporation into protein [6]. Distribution of soluble radioactivity was measured after removal of lipophilic compounds [6]. One part of the extract containing all hydrophilic components was chromatographed on Polygram-Ionex 25-SA-Na sheets with 1.5 M CH_3COONa (L1) which separated shikimic acid, the aromatic amino acids and cinnamoyl putrescines from each other. Separation of phenylalanine and tyrosine was achieved on cellulose TLC sheets by *n*-propanol: NH_3 7:3 (L2).

Quantitation of cinnamoyl putrescines was determined by their absorption at 318 nm in acidic MCW (ε_{318} caffeoyl putrescine = 18 600, ε_{318} feruloyl putrescine = 19 600). For quantitative determination of shikimic acid the lipophilic-free extract was passed through a Dowex-50-H^+ column (5 × 1,2 cm) and washed with 25 ml water.

In some cases the water eluate of the Dowex-50-H^+ column was further purified on a Dowex-1-$HCOO^-$ column. Shikimic acid was quantitatively eluted by 1 N HCOOH. The solutions were freeze dried before being prepared for GC and GC/MS. In extracts purified on the anion exchange resin, shikimic acid was the only prominent peak of GC chromatograms in glyphosate treated cells.

GC and GC/MS determination of shikimic acid

The samples were silylated with MSTFA and pyridine 1:1 at 80 °C for 30 min. GC separation was achieved on a 15 m × 0.25 mm i.d. fused silica capillary column coated with SE 30 employing a Perkin-Elmer gaschromatograph F 22. Conditions used: flame ionization detection; carrier gas helium 0.65 bar; split ratio 1:30; temperature program 150–210 °C 6 °C/min, then 30 °C/min to 300 °C. For GC/MS the same chromatographic system was coupled with the mass spectrometer AEI-MS 30 via a jet separator. The spectra were recorded at an electron energy of 24 eV in combination with the data system AEI-DS 50. Quantitative values were calculated from a calibration curve of shikimic acid. All values were corrected for the loss of added labeled shikimic acid during the sample preparations.

Results and Discussion

Effects of glyphosate on growth and cinnamoyl putrescine accumulation

In most growth experiments TX1 cells were a little more sensitive to glyphosate than TX4 cells (Fig. 1). The sensitivity of TX1 cells agreed well with the inhibiton pattern observed by Haderlie *et al.* [10]. As pointed out there [10], the inhibition pattern seemed to be dependent upon the culture age of the inoculated cells. Thus, we also noted different sensitivities to glyphosate in separate experiments. At concentrations above 0.5 mM [5.9 mg glyphosate/0.8 g inoculum fr. wt.] growth of both cell lines was inhibited to the same extent.

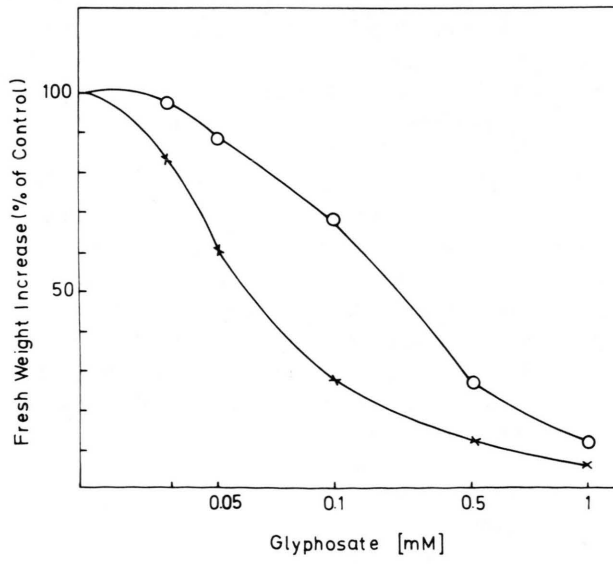

Fig. 1. Inhibition of cell growth of TX1 (×) and TX4 (○) by glyphosate. The inoculumn size was 0.8 g fr. wt./70 ml. The cells were harvested after 10 days. The controls (no glyphosate) were 13.4 g for TX1 and 19.2 g for TX4.

The growth inhibitory effect of glyphosate seems to be due to the depletion of aromatic amino acids by the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase [7]. Therefore, growth inhibition was found to be reversible in several systems by the addition of the aromatic amino acids or casein hydrolysate [10–12]. Addition of 5×10^{-4} M tryptophan, tyrosine and phenylalanine to TX1 and TX4 cell cultures, however, alleviated only slightly the growth inhibition caused by 0.25 and 0.5 mM glyphosate. In TX1 cells growth inhibition by 0.5 mM glyphosate recovered from 18.2% to 33.9% in the presence of the aromatic amino acids (TX4:29.1% to 34.5%). It has been noticed that feeding of aromatic amino acids does not always reverse growth inhibition caused by glyphosate [13]. This may be due to other disturbances caused by glyphosate [14].

To see under which conditions the formation of phenylalanine was suppressed, the cells of the growth experiments (Fig. 1) were extracted with MCW to determine the effect of glyphosate on the accumulation of cinnamoyl putrescines. This seemed to be a better indicator to demonstrate inhibition of the shikimate pathway than phenylalanine itself, because pool sizes of phenylalanine were very low compared to the phenylalanine derived cinnamoyl putrescines [6] and free phenylalanine was not reduced in glyphosate treated tobacco cells [10]. On the other hand phenylalanine derived secondary compounds were often found in much lower amounts in glyphosate treated cells [7, 9, 15]. In the presence of 1.0 mM glyphosate (Fig. 1) the content of cinnamoyl putrescines was reduced from 0.4% to 0.15% on dry weight basis in TX1 cells and from 4.2% to 0.9% in TX4 cells. Since the specific content of cinnamoyl putrescines in TX1 and TX4 remained normally quite constant on dry weight basis during the experimental period, one has to assume that the new cells had even lower contents of cinnamoyl putrescines. If one considers the low growth rates at 1 mM (Fig. 1) one would conclude that at least parts of cinnamoyl putrescines inoculated with the cell material had been "degraded".

Feeding of [2,3,4,5n-^{14}C]shikimic acid to glyphosate treated cells

The results from the previous experiments suggested a preincubation with 5 mM glyphosate [16.9 mg glyphosate/2.0 g cells fr. wt./20 ml] for 24 h before feeding labeled shikimic acid for 48 h. The distribution of radioactivity revealed a complete uptake of shikimic acid in glyphosate treated and untreated cells (Table I). While in untreated cells a substantial amount of the radioactivity was incorporated into protein as phenylalanine and tyrosine, only traces of radioactivity were associated with the MeOH:$CHCl_3$:H_2O unsoluble protein fraction in the presence of glyphosate (Table I). The good incorporation of shikimic acid into protein agreed well with the results published recently [6]. In the presence of glyphosate the incorporation of shikimic acid into phenylalanine, tyrosine and the cinnamoyl putrescines was completely inhibited and all radioactivity accumulated as shikimic acid (Fig. 2). This result entirely supports the proposed mode of action of glyphosate [8, 9].

Table I. Feeding of [2,3,4,5n-^{14}C]shikimic acid to glyphosate treated and untreated TX1 and TX4 cells. Four-day-old cultures [2.0 g fr. wt./20 ml] were incubated with 5 mM glyphosate [≙ 16.9 mg] for 24 h before 1 μCi shikimic acid was added for 48 h. The percentage of radioactivity found in the metabolites was calculated from chromatograms of MCW-extracts in the systems L1 and L2.

Glyphosate	TX1		TX4	
	–	+	–	+
	% Radioactivity			
Medium	4.6	4.3	3.4	3.9
MCW-extract	29.8	91.8	56.2	93.1
Shikimic acid *	3	93	5	93
Phenylalanine + tyrosine *	56	< 1	3	< 1
Cinnamoyl putrescines *	22	< 1	66	< 1
Residue (protein)	65.7	3.9	40.4	3.0

* Denote percent distribution of radioactivity of MCW-extracts.

Accumulation of shikimic acid in glyphosate treated cells

The cells from the feeding experiments were analyzed for their shikimic acid and their cinnamoyl putrescine content. Shikimic acid in untreated TX1 and TX4 cells was not unequivocally to determine by GC and GC/MS. Levels of free shikimic acid in glyphosate treated cells were increased more than 300-fold reaching levels between 0.7–0.9% shikimic acid on dry weight basis (Table II). Similar amounts of free shikimic acid (600–900 μg/100 mg dry weight) were also found when TX1 and TX4 cells were grown in the presence of glyphosate (0.25–0.5 mM) as in the growth experiments (Fig. 1). At lower con-

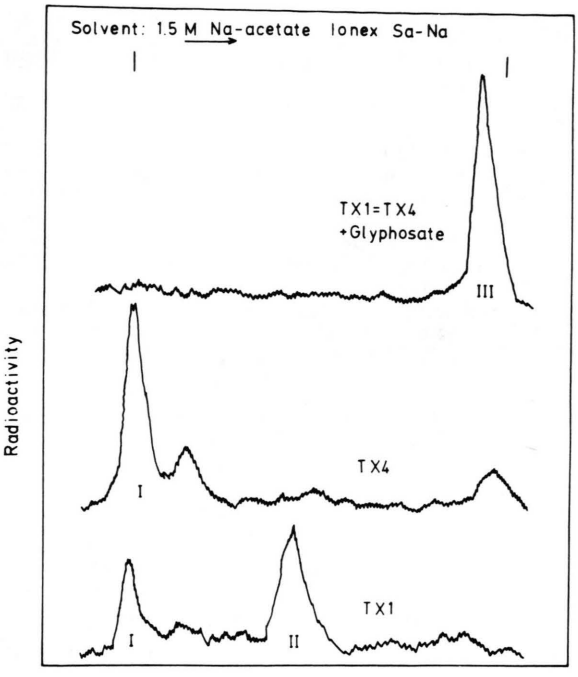

Fig. 2. Distribution of radioactivity of the MCW-extracts after feeding of [2,3,4,5*n*-^{14}C]shikimic acid for 48 h to glyphosate treated [5 mM] and untreated TX1 and TX4 cells. I = cinnamoyl putrescines, II = tyrosine + phenylalanine, III = shikimic acid.

centrations (0.025–0.1 mM) of glyphosate the accumulation of shikimic acid was decreasing with decreasing levels of the herbicide. Addition of 0.5 mM of the three aromatic amino acids did not increase the accumulation of shikimic acid in TX1 and TX4 cells.

The differences in the shikimic acid accumulation by TX1 and TX4 cells were not big enough to indicate a higher flux in the shikimate pathway by the cinnamoyl putrescines overproducing cell line. The similar accumulation of shikimic acid in TX1 and TX4 cells under conditions where the flow of carbon through the shikimate pathway was blocked at the 5-enolpyruvylshikimate-3-phosphate synthase supports our interpretation of the AOPP-experiments [6] that the overproduction of cinnamoyl putrescines was not due to an increased supply of the primary precursor phenylalanine. Our results are supporting the idea that the activity of phenylalanine ammonia lyase, the first enzyme connecting a primary with a secondary pathway, is indeed controlling the formation of phenylpropanoids. It becomes more and more evident that the activity of the first enzyme leading into a secondary pathway might play a very decisive role for the capability of cell cultures to synthesize secondary compounds. Thus, we very recently demonstrated that tryptophan decarboxylase activity was increased 12-fold under conditions favorable for indole alkaloid synthesis in cell cultures of *Catharanthus roseus* [16]. In cultures of *Lupinus* it was shown that the lack of lysine decarboxylase activity is mainly responsible for the low yields of quinolizidine alkaloid synthesis in dark grown cultures [17]. However, these results do not exclude the possibility that in other systems increased substrate supply might be a limiting factor, too. The inhibition of light induced phenylpropanoid synthesis in excised buckwheat seedlings by AOPP caused a higher accumulation of free phenylalanine than in dark controls [18]. This indicated a light induced higher carbon flux through the shikimate pathway and showed that increased substrate supply might be involved in light induced phenylpropanoid synthesis [18]. Consequently, they found different accumulation of free shikimic acid in glyphosate treated light and dark grown buckwheat seedlings [9].

Table II. Effect of glyphosate on growth and on shikimic acid and cinnamoyl putrescine accumulation by TX1 and TX4 cells. Four-day-old cultures [2.0 g fr.wt./20 ml] were incubated with 5 mM glyphosate [≙ 16.9 mg] for 72 h.

Glyphosate	TX1		TX4	
	–	+	–	+
Growth (g F.W./flask)	6.3	2.5	4.7	3.5
Shikimic acid (μg/100 mg dr.wt.)	< 2.0	680	< 2.0	972
Cinnamoyl putrescines (μg/100 mg dr.wt.)	0.46	0.36	4.02	2.99

The biochemical characterization of tobacco cell lines with low and high yields of cinnamoyl putrescines suggests that one might have a good chance to select for cell types overproducing certain secondary compounds by selecting for high activity of the first enzyme leading into that pathway [19].

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