Effect of glyphosate on the activity of DAHP synthase isozymes in callus cultures of groundnut (Arachis hypogaea L.) selected in vitro

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Two isozymes of 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHP synthase or DS, EC 4.1.2.15) from the callus cultures of Arachis hypogaea L. were resolved by DEAE cellulose column chromatography and characterized using selective assays based on divalent cation requirements and regulation by the end-product amino acids. The total extractable activity of DAHP synthase did not show any change in the glyphosate sensitive and tolerant cell lines when compared to 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EC 2.5.1.19), the known target of inhibition by glyphosate. However, the activities of both, DAHP synthase and EPSP synthase were inhibited by glyphosate in vitro. The inhibitory effects of glyphosate on the activities of (a) EPSP synthase and DAHP synthase, and (b) isozymes of DAHP synthase, have been compared. The data indicate that the Co²⁺ dependent isozyme of DAHP synthase is a putative target for the action of glyphosate in A. hypogaea.

Materials and Methods
All the chemicals used were obtained from Sigma Chemical Co., St. Louis, MO, USA and were of analytical grade. The barium salt of shikimate-3-phosphate was prepared according to Coggins et al.

In vitro cultures
Proliferative callus cultures of Arachis hypogaea L. cv. JL-24 were initiated from the leaf explants on semi-solid MS (Murashige and Skoog) medium fortified with 2.5% (w/v) sucrose, 100 mg myo-inositol and 1 mg l¹ each of benzylaminopurine and naphthalene acetic acid. Selection and maintenance of glyphosate tolerant cell lines of Arachis has been described. In essence, the glyphosate tolerant cell line (referred to as GR2 in the text), was selected through a step-wise selection protocol by challenging the callus at a sublethal dose of 0.3 mM glyphosate (i.e. <ID₉₀=0.5 mM glyphosate). After about six months, giberellic acid was also included in the medium at a concentration of 1 mg l¹. This helped in maintaining a healthy green colour of the callus under selection. The selection pressure was increased slowly over a period of two years. Presently GR2 growing at 12 mM glyphosate, shows ca. 20-fold increase in tolerance to the herbicide, and grows at the rate comparable to the control, unselected cell line (data not shown).

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Isopropylamine salt of glyphosate was used for all experiments. The stock solution was prepared by titrating with 1 N NaOH until it dissolved completely and making up the final volume with double distilled water. The solution was adjusted to neutral pH, passed twice through 0.45 μm Whatman filters and added to the autoclaved medium to the desired concentration.

**Fractionation of DAHP synthase isozymes**

The callus (10 g) was frozen in liquid nitrogen and ground to a very fine powder and extracted with two volumes of buffer A [100 mM EPPS, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 5 mM sodium metabisulphite, 1 mM MnCl₂, 1 mM tryptophan and 10% (v/v) glycerol]. The extract was filtered through four layers of cheese cloth and centrifuged at 0 °C. The supernatant was desalted on a Sephadex G-25 column (2.5 x 40 cm) and fractionated on a DE-52 ion exchange column (2 x 35 cm). The isozymes were eluted using a linear gradient of 0-3 M NaCl prepared in buffer B [50 mM KH₂PO₄ buffer, pH 7.5, 1 mM each of phenylmethylsulfonyl fluoride and dithiothreitol, 5 mM sodium metabisulfite, 0.1 mM each of MgCl₂ and MnCl₂, 0.5 mM phosphoenolpyruvate, 100 μM tryptophan and 10% (v/v) glycerol]. The fractions containing the highest enzyme activity were pooled and proteins precipitated with 0-75% (w/v) ammonium sulfate. The protein precipitate was dialysed overnight against three changes of buffer B and stored at -15 °C.

DAHP synthase activity was assayed and characterized on the basis of divalent cation requirement and end-product feedback regulation according to Sharma et al. The isozyme peak showing maximum activity in presence of Co²⁺ was designated as DS-Co and the one yielding maximum activity in presence of Mn²⁺ was designated as DS-Mn.

DS-Co fraction was further clarified by centrifugation and applied to a hydroxyapatite column (1.5 x 12 cm) pre-equilibrated with buffer C [0.1 M KH₂PO₄ buffer, pH 7.5, 1 mM dithiothreitol, 0.1 mM each of MgCl₂ and MnCl₂, 0.5 mM phosphoenolpyruvate, 100 μM tryptophan and 10% (v/v) glycerol]. The bound protein was eluted using a linear gradient of 0-1-0.5 M KH₂PO₄ buffer, pH 7.5, 1 mM dithiothreitol, 0.1 mM each of MgCl₂ and MnCl₂, 100 μM tryptophan and 10% (v/v) glycerol], at 15 ml/hr and 2 ml fractions were collected. The fractions with DS-Co activity were pooled in and concentrated with 35-40% ammonium sulfate precipitation. The protein precipitate was recovered, resuspended in buffer C and dialysed overnight against excess of the same buffer.

**Assay of DAHP synthase**

DAHP synthase activity was assayed spectrophotometrically by a modified procedure of Rubin and Jensen and Morris et al., as follows: the reaction mixture contained 2 mM phosphoenolpyruvate, 4 mM erythrose 4-phosphate, 50 mM EPPS buffer (pH 8.0), 1 mM MgCl₂, MnCl₂ or CoCl₂ (as required) in a final volume of 250 μl. For end-product regulation, 1 mM of phenylalanine, tryptophan or tyrosine was included. The reaction was initiated by the addition of 150 μl enzyme, incubated at 37°C for 30 min, and terminated with 400 μl of 10% (w/v) TCA, followed by centrifugation at 3000 g for 3 min. To 250 μl supernatant an equal volume of periodic acid was added and incubated at 37°C for 45 min, followed by addition of 500 μl sodium arsenite. The brown colour thus obtained was allowed to disappear at room temperature, 2 ml thiothriitric acid was added, and the mixture was incubated at 100°C for 10 min. The absorbance of the pink coloured complex (due to formation of DAHP) was read at 549 nm against protein blanks. The 0 min reading was taken as control. For assaying DAHP synthase in column fractions, 0.5 mM Mn²⁺ was included. Preliminary standardisation was done by the differential assay procedure of Ganson et al. The protein was estimated according to the procedure of Bradford using BSA as the standard.

**Assay of EPSP synthase**

Phosphatase activity was assayed by measuring the formation of p-nitrophenol from p-nitrophenyl phosphate, as described by Malamy and Horecker. The background, non-specific phosphatase activity was determined for correction of the total extractable EPSP synthase activity, since the procedure described measures the rate of appearance of P₃ detected spectrophotometrically by the Malachite Green binding assay. EPSP synthase was assayed by the method of Fornani et al. with slight modification. The protein was extracted by crushing 1 g callus under liquid nitrogen and suspending in 2 ml of ice cold 50 mM Hepes NaOH (pH 7.0) containing 10% (w/v) glycerol, 5 mM reduced glutathione, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA and 5.0 μM sodium meta-vanadate and 10% (w/v) polyvinylpyrrolidone. The extract was filtered.
through four layers of cheese cloth and centrifuged at 10,000 g for 30 min. The reaction was initiated by adding 50 µl protein extract in a final volume of 100 µl containing 100 mM Hapes NaOH (pH 7.4), 1 mM each of shikimate-3-phosphate and phosphoenolpyruvate, 5.0 µM sodium meta-vanadate, and incubated at 37°C for 30 min. The reaction was stopped by adding 1 ml of a solution containing 9.2 mM Malachite Green dye, 5.0 µM sodium meta-vanadate and 0.2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) to stabilise the colour development, followed, after exactly one min, by 34% (w/v) sodium citrate. After 15 min at room temperature, the samples were read at 660 nm against the unincubated blanks. Under these conditions, the molar absorption coefficient is 75,000 M⁻¹ cm⁻¹. The enzyme activity is expressed as nkat mg⁻¹ protein (where 352 nkat mg⁻¹ protein=21.1 µmole s P; released min⁻¹ mg⁻¹ protein).

Results
Partial purification of DAHP synthase isozymes
Two peaks of DAHP synthase were resolved upon DE-52 cellulose chromatography from the callus extract of A. hypogaea (Fig. 1). These were characterized on the basis of their divalent cation requirement and regulation by the end-product aromatic amino acids. The peaks were pooled separately and concentrated as described earlier in Materials and Methods. The peak eluting first (peak A, specific activity 0.53±0.08 nmole mg⁻¹ protein min⁻¹) was identified as the DAHP synthase-Co dependent isozyme (DS-Co) since it yielded maximum activity in presence of Co²⁺ as the cofactor (2.37±0.05 nmole mg⁻¹ protein min⁻¹). The trailing peak (peak B, specific activity 0.48±0.03 nmole mg⁻¹ protein min⁻¹), eluting at a higher salt concentration, was characterized as DAHP synthase-Mn dependent isozyme (DS-Mn) as it showed maximum specific activity with Mn²⁺ (1.89±0.01 nmole mg⁻¹ protein min⁻¹). Further, DS-Co proved insensitive to regulation by the end-product aromatic amino acids phenylalanine, tyrosine or tryptophan. In contrast, DS-Mn was activated ca. 2.3-fold (3.76±0.03 nmole mg⁻¹ protein min⁻¹) in presence of tyrosine and inhibited by ca. 50% (0.83±0.03 nmole mg⁻¹ protein min⁻¹) in presence of tryptophan, as compared to the control (1.68±0.00 nmole mg⁻¹ protein min⁻¹). The feedback insensitive isozymes of aromatic biosynthesis pathway have been shown to be cytosol localised and are involved in biosynthesis of aromatic amino acids for secondary metabolism by a simple

![Fig. 1 — Resolution of two activity peaks of DAHP synthase from callus cultures of Arachis hypogaea after elution with 0-0.3 M NaCl from a DEAE cellulose (DE-52) column. (The protein was extracted under liquid nitrogen and desalted through a Sephadex G-25 column. Crude extract was chromatographed on DE-52 column, and the enzyme activity assayed in 3 ml fractions as described in Materials and Methods. DS-Co and DS-Mn were characterized on the basis of Co²⁺ or Mn²⁺ requirement for maximum activity and regulation by the end-product aromatic amino acids).](image-url)
overflow mechanism. However, the feedback regulated isozymes are present in the chloroplasts and are responsible for the stringently regulated biosynthesis of amino acids required for primary metabolic pathways. Partial purification of DAHP synthase was desired, not only to resolve isozymes, but also to avoid contamination by phenolics, proteases and phosphatases.

**Effect of glyphosate selection on the activities of EPSP synthase and DAHP synthase**

The specific activities of DAHP synthase and EPSP synthase were compared in glyphosate sensitive and tolerant cell line GR2 (Table 1). These two cell lines grow at similar rates and have been described in detail in an earlier report. The amount of total extractable protein was comparable between the two cell lines, with or without glyphosate selection. The cell line GR2 showed ca. 1.7-fold increase in the activity of EPSP synthase (specific activity 0.32±0.04 nkat mg⁻¹ protein) when compared to glyphosate sensitive control cell line (specific activity 0.19±0.06 nkat mg⁻¹ protein). However, no significant difference in the total extractable activity of DAHP synthase was discernible between the two cell lines (specific activity 1.20±0.09 and 1.25±0.10 nmole mg⁻¹ protein min⁻¹, respectively). Similarly the activities of DAHP synthase isozymes, DS-Co and DS-Mn, were also comparable in the two cell lines. The specific activities of DS-Co in the glyphosate sensitive cell line and tolerant, GR2 cell line were 0.65±0.07 and 0.72±0.01 nmole mg⁻¹ protein min⁻¹, respectively. The specific activity of DS-Mn was 0.52±0.05 and 0.49±0.06 nmole mg⁻¹ protein min⁻¹, in the control and GR2 cell line, respectively. The data indicate that EPSP synthase is the target for glyphosate action in *A. hypogaea*, that is over-expressed in the GR2 cell line, and that the expression of other enzymes of shikimic acid pathway are not affected by glyphosate. These findings are consistent with our earlier report.

Earlier Pinto et al. reported increase in the DAHP synthase activity, when potato (*Solanum tuberosum*) cells grown in suspension cultures were treated with sublethal doses of glyphosate. The relative increase in the activity was a reflection of glyphosate concentration, and glyphosate had no effect on DAHP synthase activity *in vitro*. However, our results indicate that glyphosate inhibits the activity of EPSP synthase as well as DAHP synthase *in vitro* by 50% of the control in presence of 2 µM and 1 mM glyphosate under standard assay conditions of substrate saturation (Fig. 2). In contrast to its effect on EPSP synthase, the glyphosate concentration required for 50% inhibition of DAHP synthase activity is 1000 fold higher.

![Fig. 2 - *In vitro* inhibition of (a) EPSP synthase and (b) DAHP synthase activities by glyphosate. The control values for EPSP synthase and DAHP synthase were 0.22±0.01 nkat mg⁻¹ protein and 1.35±0.03 nmole mg⁻¹ protein min⁻¹, respectively.](image-url)
Glyphosate induced inhibition of Co-dependent isozyme of DAHP synthase

The inhibitory action of glyphosate (1 mM) on the two DAHP synthase isozymes recovered from the DE-52 column was tested. The Co-dependent isozyme (peak A, DS-Co) was much more strongly inhibited (56%) by glyphosate than the Mn-dependent isozyme (peak B, DS-Mn) (12%) (Table 2). Inclusion of 1 mM Co²⁺ (for peak A, DS-Co) or Mn²⁺ (for peak B, DS-Mn) resulted in elevation of DS-Co and DS-Mn activities by ca. 330% and 278%, respectively. However, for peak A, the residual enzyme activity upon addition of 1 mM glyphosate dropped from ca. 44% (control A) to 26% (control B). Thus, in the presence of glyphosate, the extent of activation of DS-Co by Co²⁺ was significantly reduced from ca. 330% (control A) to ca. 200% (control B). The inhibitory effects of glyphosate on the activity of DS-Mn (peak B) were less dramatic both in presence and in absence of Mn²⁺. In view of the metal chelating properties of glyphosate¹⁶, the effect of EDTA was also investigated to preclude the possibility of sequestration or chelation of the activating cation by glyphosate. The activity of peak A (DS-Co) was inhibited ca. 15% by 1 mM EDTA as compared to ca. 74% loss in presence of glyphosate (control B). Further, the in vitro inhibition of DS-Co activity by EDTA was also seen in the case of DS-Mn, and evident irrespective of the presence of either Co³⁺ or Mn²⁺ as the activating cation.

The kinetics of the Co-dependent isozyme recovered from the hydroxylapatite column, free of contaminating phosphatase activity, was studied (Fig. 3). From the Lineweaver-Burk plot, glyphosate was found to be a competitive inhibitor of DS-Co with respect to erythrose 4-phosphate. The inhibition of phosphoenolpyruvate by glyphosate was non-competitive. Earlier, Co³⁺ activated isozymes of DAHP synthase from mung bean (Vigna radiata)¹⁷ and tobacco (Nicotiana sylvestris)¹⁸ had been reported to be inhibited by glyphosate. The inhibition of DAHP synthase in Nicotiana sylvestris was competitive with respect to erythrose 4-phosphate.

Discussion

Glyphosate is an effective broad spectrum herbicide as well as a potent inhibitor of microbial growth. Our results indicate that EPSP synthase is the prime target for the phytotoxic action of glyphosate in groundnut consistent with earlier reports⁶. However, considering the vast diversity in the isozymes of

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**Table 2 — Inhibitory effect of glyphosate on the activities of DAHP synthase isozymes in vitro, in glyphosate tolerant cell line GR2, in presence and absence of divalent cations.** (Reaction mixtures contained 2 mM phosphoenolpyruvate, 4 mM erythrose 4-phosphate and 50 mM EPPS buffer (pH 8.0), and 1 mM glyphosate and/or Co²⁺ or Mn²⁺ as indicated. Control A was without glyphosate or any divalent cation; Control B and C included either Co³⁺ or Mn²⁺ respectively. The enzyme activity is expressed as nmol mg⁻¹ protein min⁻¹. The values in parentheses denote the % enzyme activity).

<table>
<thead>
<tr>
<th></th>
<th>Peak A (DS-Co)</th>
<th>Peak B (DS-Mn)</th>
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<tbody>
<tr>
<td>Control A (no ion)</td>
<td>0.62 ± 0.07 (100)</td>
<td>0.51 ± 0.02 (100)</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>0.27 ± 0.03 (43.5)</td>
<td>0.45 ± 0.02 (88.2)</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.60 ± 0.02 (96.8)</td>
<td>0.49 ± 0.01 (96.1)</td>
</tr>
<tr>
<td>Control B (+ Co²⁺)</td>
<td>2.05 ± 0.08 (100)</td>
<td>0.85 ± 0.00 (100)</td>
</tr>
<tr>
<td>Glyphosate + Co²⁺</td>
<td>0.54 ± 0.03 (26.3)</td>
<td>0.43 ± 0.01 (50.6)</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.74 ± 0.04 (84.9)</td>
<td>0.73 ± 0.04 (85.9)</td>
</tr>
<tr>
<td>Control C (+ Mn²⁺)</td>
<td>1.09 ± 0.03 (100)</td>
<td>1.42 ± 0.01 (100)</td>
</tr>
<tr>
<td>Glyphosate + Mn²⁺</td>
<td>0.30 ± 0.01 (27.5)</td>
<td>1.25 ± 0.05 (88.0)</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.94 ± 0.03 (86.2)</td>
<td>1.22 ± 0.02 (85.9)</td>
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Fig. 3 — Double reciprocal plots Co-dependent isozyme of DAHP synthase (DS-Co) with (a) erythrose 4-phosphate and (b) phosphoenolpyruvate as the variable substrates. [Partially purified enzyme fraction recovered from the hydroxylapatite column was assayed as described in Materials and Methods in absence (●) or presence (○) of 1 mM glyphosate].
aromatic pathway enzymes and their regulation, the extraordinary effectiveness of glyphosate against both plants and microorganisms warrants a detailed examination. We are reporting the resolution of two isozymes of DAHP synthase, viz. DS-Co and DS-Mn, from the callus cultures of A. hypogaea, for the first time. The detailed purification protocol and kinetic analysis of these isozymes will be presented elsewhere (manuscript under preparation). The isozymes of DAHP synthase have earlier been characterized in leaves of Vigna radiata, Daucus carota, Spinacia oleracea, Brassica oleracea, Glycine max, Medicago sativa, Cucurbita pepo, Triticum aestivum and Secale cereale, in suspension cultures of Nicotiana sylvestris, in tubers of Solanum tuberosum and in leaves and callus cultures of Brassica juncea. Enzymes needed for the production of aromatic amino acids have been demonstrated within the plastids of these plant species. The cytosolic isozymes, being unregulated, are assumed to provide the precursors for secondary metabolic pathways by a simple overflow mechanism. The resolution of DAHP synthase isozymes in the cytosolic and plastidic compartments in a number of plant species has been instrumental in formulating the "dual pathway hypothesis of aromatic biosynthesis". A co-ordinate regulation between members of isozyme pairs for the key enzymes in the synthesis of primary and secondary metabolites is indicated.

Our data show that the Co specific isozyme of DAHP synthase is a possible target of glyphosate inhibition in groundnut. Although glyphosate is a metal chelator, the inhibition of DS-Co can not be a result of cobalt chelation. Since cobalt stimulates activity of DS-Co, the sequestration of activating cation should result in non-competitive inhibition by glyphosate with both erythrose 4-phosphate and phosphoenolpyruvate as substrates. However, in the present study, the inhibition of DS-Co is competitive with respect to erythrose 4-phosphate. General inhibition of DAHP synthase activity by glyphosate in vivo seems improbable since accumulation of high levels of shikimate-3-phosphate and shikimic acid has been reported in a variety of species. Since the Mn stimulated isozyme, DS-Mn, is not susceptible to glyphosate, in vivo inhibition of only DS-Co, may not be deleterious, and the glyphosate action at the DS-Co step may be bypassed through the activity of DS-Mn isozyme. The activity of DS-Mn, however, may allow futile loss of energy rich substrates erythrose 4-phosphate and phosphoenolpyruvate into the aromatic pathway, resulting in a possible energy drain. In plants like V. radiata, DS-Mn is feed back inhibited by the pathway intermediate L-aroegenate rather than the end-products of the pathway. Glyphosate induced inhibition of EPSP synthase depletes cellular levels of L-aroegenate, which normally inhibits the activity of DS-Mn. The distinctly incomplete ability of aromatic amino acids to reverse glyphosate induced growth inhibition in groundnut and many other systems supports this hypothesis.

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