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The herbicidally active compound *N*-2-(5-chloro-pyridyl) aminomethylene bisphosphonic acid acts by inhibiting both glutamine and aromatic amino acid biosynthesis

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Abstract. The effect of the herbicidally active compound *N*-2-(5-chloro-pyridyl)aminomethylene bisphosphonic acid (Cl-pyr-AMBPA), previously found *in vitro* to inhibit the activity of the first enzyme in the shikimate pathway 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, was investigated *in vivo* on suspension cultured cells of *Nicotiana plumbaginifolia* Viviani. Amino acid pool measurement showed an actual reduction of tyrosine, tryptophan and phenylalanine level following the addition of the compound to the growth medium. However, an even stronger effect was noticed for other amino acids, mainly glutamine. When the activity of the enzymes involved in the glutamate cycle was measured in the presence of Cl-pyr-AMBPA, glutamate synthase was unaffected, while glutamine synthetase was significantly inhibited. Contrary to the herbicide phosphinothricin, the inhibitor bound reversibly to the enzyme. Kinetic analysis accounted for an inhibition of uncompetitive type with respect to ammonium, glutamate and ATP, with K_i values of 113, 97 and 39 μM, respectively. Only the exogenous supply of a mixture of glutamine and aromatic amino acids relieved cell growth inhibition, suggesting that the phytotoxic properties of Cl-pyr-AMBPA are due to inhibition of key enzymes in both the corresponding pathways.

Keywords: amino acid synthesis inhibition, DAHP synthase, free amino acid content, glutamine synthetase, glyphosate, phosphinothricin.

Introduction

Aminoalkylphosphonic acids are structural analogues of amino acids in which the carboxylic group is replaced by phosphonic or related functions. During recent years increasing evidence has been shown for the ability of many aminophosphonates to interact with enzymes involved in amino acid metabolism. In spite of significant differences including size, shape (flat CO_2H *vs* tetrahedral PO_3H_2) and acidity (pK difference of at least 3 units), several enzymes do not differentiate between carboxylic and phosphonic moiety as concerns the binding to active sites. The structural antagonism between amino acids or intermediates in their synthesis and the phosphonic counterparts results in several instances in enzyme activity inhibition. The most remarkable examples are provided by the inhibition of the key enzyme in ammonia assimilation, glutamine synthetase (glutamine-ammonia ligase, GS, EC 6.3.1.2) by a naturally occurring analogue of glutamic acid, phosphinothricin (L-homoalanine-4-yl-[methyl]phosphinic acid; Köcher 1989), or other synthetic phosphonates (Farrington *et al.* 1987; Miliszkiewicz *et al.* 1992), and that of the pre-chorismate pathway enzyme 5-*enol*-pyruvyl-shikimate-3-phosphate synthase (EPSPS, 3-phosphoshikimate 1-carboxyvinyl transferase, EC 2.5.1.19) by the phosphonate herbicide glyphosate ([*N*-phosphonomethyl]glycine; Cole 1985).

Inhibitors of amino acid biosynthesis have been helpful research tools in elucidating plant nitrogen metabolism. Beyond their significance in basic biochemical and physiological studies, several of them have found practical application as herbicides (Kishore and Shah 1988), mainly due to their effectiveness and low environmental impact. In fact, most amino-phosphonates show low toxicity to mammals and a favorable ecological fate. For instance, both glyphosate and phosphinothricin are completely degraded by the soil microflora within a few weeks after field application (Kafarski *et al.* 2000). However, despite these desirable

Abbreviations used: aaa, aromatic amino acids; Cl-pyr-AMBPA, *N*-2-(5-chloro-pyridyl)aminomethylene bisphosphonic acid; DAHPS, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase; E4P, erhythrose-4-phosphate; EPSPS, 5-*enol*-pyruvyl-shikimate-3-phosphate synthase; GS, glutamine synthetase; M-pyr-AMBPA, *N*-2-(6-methyl-pyridyl)aminomethylene bisphosphonic acid.

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environmental features, their use is limited to date because of their lack of plant selectivity.

To improve the weed management potential of such non-selective herbicides, the possibility for introduction of herbicide-tolerant genes into crops has been widely exploited (Dekker and Duke 1995). Even though these efforts have been successful in several cases, in Europe an extensive use of transgenic plants in agriculture is encountering environmentalists' hostility and public disfavor. Another useful approach may be represented by the synthesis and screening of analogues of the active molecules for selective forms. Structural analogues similar to phosphinothricin and glyphosate have been explored exhaustively, and hundreds of derivatives have been synthesized and tested for herbicidal activity. Whereas no selective substances sharing the same biochemical targets have been identified to date, such research resulted in the discovery of new classes of active compounds, among which are aminomethylene bisphosphonic acids. Their target at the cellular level is still poorly understood, but recent evidence that accounts for the ability of different bisphosphonates to inhibit farnesyl pyrophosphate synthase (Cromartie and Fisher 1995), squalene synthase (Biller *et al.* 1996), mitochondrial H^+-pyrophosphatase (Vianello *et al.* 1997) and geranyl-geranyl diphosphate synthase (Oberhauser *et al.* 1998) suggests that they should be considered a heterogeneous class of compounds with various modes of action.

We previously evaluated the biological activity of a series of *N*-pyridyl-derivatives of aminomethylene bisphosphonic acid, which possess two strongly acidic residues and one positively charged amino group, and thus closely resemble glyphosate. Most of them exerted a remarkable phytotoxic effect at both the plant and the cell culture level, and depressed anthocyanin biosynthesis *in vivo* (Lejczak *et al.* 1996). Thus the occurrence of a target in plant aromatic metabolism was investigated. While *in vitro* activity of EPSPS was found to be unaffected (Forlani *et al.* 1997), that of the plastidic and Mn^{++}-stimulated isoform of the first enzyme in the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS, EC 4.1.2.15), was markedly reduced. A kinetic analysis indicated that the most effective compound *N*-2-(5-chloro-pyridyl)aminomethylene bisphosphonic acid (Cl-pyr-AMBPA) inhibits enzyme activity competitively with respect to the substrate erythrose-4-phosphate (E4P), ruling out the possibility of an inhibition simply based upon its metal-chelating properties (Forlani *et al.* 1996). Another compound, *N*-2-(6-methyl-pyridyl)aminomethylene bisphosphonic acid (M-pyr-AMBPA), showed non-competitive inhibition with respect to both phospho*enol*pyruvate and E4P. Amino acid pool measurements of tobacco cells grown in the presence of M-pyr-AMBPA pointed to an actual reduction of free aromatic amino acids, showing that DAHPS inhibition takes place *in vivo*, and suggesting that the interference of this aminophosphonate with plant aromatic biosynthesis may account for a large part of its phytotoxicity. However, exogenous supply of a mixture of Phe, Tyr and Trp failed to achieve full reversal of cell growth inhibition (Forlani *et al.* 1999).

To ascertain whether DAHPS activity could represent the main target of Cl-pyr-AMPBA *in vivo*, we studied the effect of sublethal concentrations of the inhibitor on cultured *Nicotiana plumbaginifolia* cells. Here we report experimental evidence that supports a multiple mode of action, and suggests that the herbicidal properties of this compound may be ascribed to the block of the biosynthesis of both aromatic amino acids and glutamine.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemicals, St Louis, MO, USA. Analytical grade glyphosate and phosphinothricin were from Riedel-de Haën, Seelze, Germany. Cl-pyr-AMBPA and M-pyr-AMBPA were synthesized as previously described (Lejczak *et al.* 1996).

Cell culture

Cell suspension cultures of *N. plumbaginifolia* Viviani were grown in Erlenmeyer flasks in MS medium (Murashige and Skoog 1962) containing 0.3% (w/v) sucrose and 0.5 mg L^{-1} of both 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Incubation was under dim light at 26 ± 1°C on a rotary shaker (120 rpm). Subcultures were made every 2 weeks by transferring 20-mL aliquots to 100 mL of fresh medium. The effect of Cl-pyr-AMBPA, phosphinothricin and glyphosate on exponentially growing cells was measured as described previously (Forlani *et al.* 1999). Briefly, cell samples withdrawn from the stock cultures in the late exponential phase of growth were used to inoculate 100-mL culture flasks to a density of 1.0–1.2 mg mL^{-1} (dry weight) in a final volume of 25 mL. Filter-sterilized compounds (brought to pH 6.0 with KOH) were added just after the density of cell population reached 2.0 mg mL^{-1} (dry weight). After a further 6 d of incubation, when untreated controls reached the early stationary phase of growth, cells were harvested by vacuum filtration, and the dry weight increase was determined on each sample following oven drying at 90°C for 48 h. The same protocol was adopted for reversal experiments, where inhibitors and amino acid supplements were added to the culture medium simultaneously.

Amino acid extraction and analysis

Plant material was harvested, resuspended in 1 mL g^{-1} of a 3% (w/v) 5-sulfosalicylic acid solution and homogenized in a Teflon-in-glass Potter homogenizer by 3 × 15 strokes. After centrifugation for 5 min at 12 000 *g*, 1-mL aliquots of the supernatant were dried at room temperature in a Speed-Vac Concentrator (Savant Instr., Hicksville, NY, USA). Samples were reconstituted in 0.1 mL of 2.5 N NaOH, resulting in a pH value of 10.2 ± 0.2, and immediately analysed. Aliquots (10 µL) were mixed with the same volume of *o*-phthaldialdehyde solution (0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 0.5 M β-mercaptoethanol and 10% (v/v) methanol). After exactly 60 s, derivatized samples were injected onto a 4.6 × 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE, USA) equilibrated with 60% solvent A (50 mM sodium phosphate + 50 mM sodium acetate buffer, pH 7.5, containing 2% (v/v) of methanol and tetrahydrofuran) and 40% solvent B (65% methanol). Elution proceeded at a flow rate of 1 mL min^{-1} using a computer-controlled (Data System 450; Kontron, München, Germany) complex gradient from 40 to 100% solvent B,

monitoring the eluate at 340 nm as described (Svedas *et al.* 1980). This procedure allowed complete resolution of equimolar mixtures of derivatizable amino acids (all the 20 protein amino acids but Pro and Cys), with a detection limit of about 0.1 nmol. Peaks were integrated by area, with variation coefficients ranging from 0.8 to 3.2%.

Enzyme assays

GS activity in partially purified preparations was measured at 35°C by a hemibiosynthetic assay as previously described (Forlani 2000). The activity of the purified enzyme was measured by a biosynthetic assay. Briefly, the mixture contained 50 mM Hepes–NaOH buffer pH 7.4, 50 mM L-Glu, 5 mM ATP, 20 mM $MgCl_2$, 1 mM NH_4Cl, and a limiting amount of enzyme (10–25 pkat) in a final volume of 0.1 mL. After up to 20 min at 35°C, the P_i released was quantified by a modification of the malachite green assay method (Forlani 2000). NADH-dependent glutamate synthase activity was extracted and assayed as described (Lacuesta *et al.* 1990), while replacing Tris–HCl with 100 mM potassium phosphate, and adding 1 mM phenylmethylsulfonyl fluoride to the extraction buffer. Protein concentration was determined by the method of Bradford (1976), using BSA as the standard.

Measurement of Cl-pyr-AMBPA uptake

Cl-pyr-AMBPA was added to a final concentration of 200 μM to exponentially growing cultures (5.0–5.5 mg dw mL^{-1}) either in the presence or in the absence of amino acid supplement. At the indicated time after the addition, 2-mL aliquots were withdrawn and cells were sedimented by centrifugation 3 min at 8000 *g*. The supernatants were read at 340 nm against exact blanks from which Cl-pyr-AMBPA had been omitted, and the residual concentration of the bisphosphonate was calculated on the basis of the molar absorption coefficient 3750 M^{-1} cm^{-1}. Treatments were carried out in triplicate, and results were normalized on a dry weight basis as above.

Statistical analysis and evaluation of kinetic constants

Data were analysed by using standard statistical procedures for analysis of variance and *t*-test. Confidence limits were calculated according to Snedecor and Cochran (1967). Where differences are reported, they are at the 99, 95 or 90% confidence level (***$P \leq 0.01$, **$P \leq 0.05$, *$P \leq 0.10$, respectively). The concentrations causing 50% inhibition of dry weight increase or enzyme activity were estimated utilizing the linear regression equation of values plotted against the logarithm of inhibitor concentration. Inhibition types and K_i values were evaluated according to Webb (1963).

Results

In vivo *effects of Cl-pyr-AMBPA on* N. plumbaginifolia *exponentially growing cells*

The addition of Cl-pyr-AMBPA in the range 10^{-5}–10^{-3} M to the culture medium was found to cause severe growth inhibition, which was comparable to that exerted by the broad-spectrum herbicide glyphosate (Fig. 1). Under the experimental conditions employed, the concentrations of Cl-pyr-AMBPA and glyphosate causing a 50% reduction of dry weight increase were 260 ± 39 and 117 ± 14 μM, respectively. To investigate whether the interference of Cl-pyr-AMBPA with the activity of the first enzyme in the pre-chorismate pathway, DAHPS (Forlani *et al.* 1996), could account for such an effect, amino acid pools were quantified in extracts from cells treated with sublethal doses of the compound, and compared to those of untreated controls.

With the only exception of Glu, the level of which resulted substantially steady, the intracellular concentration of free amino acids was found to vary greatly during the culture growth cycle (Forlani *et al.* 1999). This behaviour made it difficult to distinguish in treated samples between specific effects and an apparent reduction simply due to the lowering in cell growth rate. However, as for M-pyr-AMBPA (Forlani *et al.* 1999), the comparison of data obtained with cells harvested at different times but at the same cellular density shows an actual reduction of Phe, Tyr and Trp levels (data not presented), suggesting that the inhibition brought about on DAHP synthesis *in vitro* may occur also *in vivo*. This reduction took place only 3–4 d after the addition of the inhibitor, when a dramatic increase in aromatic amino acid level was found in untreated controls, and was significantly less severe than the corresponding lowering in cell growth rate. Moreover, contrary to both M-pyr-AMBPA and glyphosate, an even more remarkable effect was evident for other amino acids, mainly Gln, Glu, Asn, Arg and Gly (Table 1). This seemed to indicate the occurrence of further target(s) for Cl-pyr-AMBPA in the plant cell nitrogen metabolism, which could play a not negligible role in determining its phytotoxicity.

Inhibition of GS activity by Cl-pyr-AMBPA

As the inhibitory effect was particularly relevant for Gln and Glu, which altogether accounted for more than 60% of total free amino acid content of untreated controls, and very similar to that caused by the addition to the culture medium of the GS inhibitor phosphinothricin (Table 1), the activity of the two enzymes of the glutamate cycle was determined in

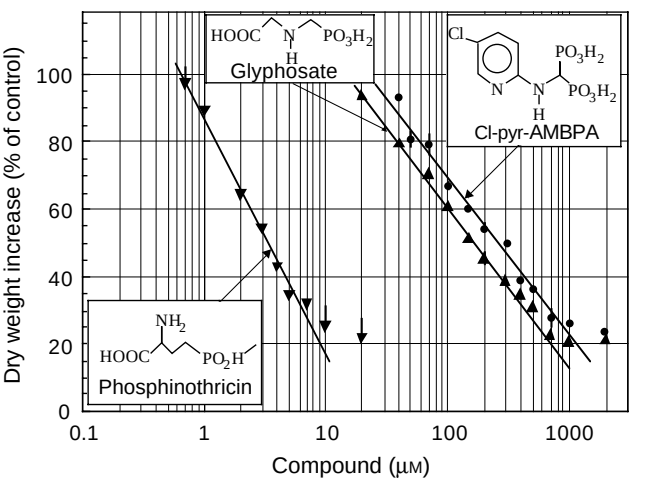

Fig. 1. Comparison among the inhibitory effect of Cl-pyr-AMBPA and those of the widely-used herbicides glyphosate and phosphinothricin on exponentially growing cells of *N. plumbaginifolia*. Treatment and analysis as described in 'Materials and methods'. Data, expressed as percent of untreated control (8.92 ± 0.17 mg mL^{-1}) are means ± s.d. of the results obtained in three independent experiments, in which each dose was run in duplicate.

Table 1. Amino acid pools in cells of *N. plumbaginifolia* 4 d after the addition of various inhibitors to the culture medium

Following the addition of the inhibitors at concentrations causing 50%-inhibition of dry weight increase, 25-mL aliquots were withdrawn, and cells were extracted as described in 'Materials and methods'. Amino acids were derivatized with *o*-phthaldialdehyde and quantitated by RP-HPLC. Results, expressed as μmol g^{-1} dry weight, are means ± s.e. of data obtained in two experiments, in which each treatment was carried out with three replicates. Pro and Cys were not detectable with the method employed, while His co-eluted with Gln. The corresponding cellular densities and growth rates are also indicated

		Addition			
	Control	Cl-pyr-AMBPA 200 μM	M-pyr-AMBPA 400 μM	Glyphosate 100 μM	Phosphinothricin 3 μM
Dry weight (mg mL^{-1})	6.74 ± 0.18	4.34 ± 0.17	4.47 ± 0.05	4.57 ± 0.08	5.03 ± 0.21
Growth rate (% of control)	100.0	54.5	52.7	56.7	61.0
Amino acid:					
Asp	5.94 ± 0.89	8.74 ± 1.84	6.21 ± 0.17	10.06 ± 1.41**	6.31 ± 0.21
Glu	24.46 ± 0.15	16.37 ± 1.14***	18.61 ± 0.92***	36.98 ± 1.47***	31.75 ± 2.26**
Asn	6.84 ± 0.99	2.03 ± 0.26***	6.97 ± 0.50	9.55 ± 0.47**	1.08 ± 0.04***
Ser	12.69 ± 0.59	14.47 ± 0.88	22.59 ± 1.45***	10.18 ± 2.70	3.30 ± 0.64***
Gln	84.94 ± 7.03	15.43 ± 1.01***	101.25 ± 1.50**	148.72 ± 21.40**	22.49 ± 0.69***
Arg	2.49 ± 0.36	0.53 ± 0.08***	2.61 ± 0.20	1.93 ± 0.55	0.24 ± 0.02***
Gly	2.92 ± 0.97	0.44 ± 0.14**	2.88 ± 0.11	1.91 ± 0.31	0.49 ± 0.13**
Thr	4.75 ± 0.62	4.12 ± 0.49	5.34 ± 0.67	4.16 ± 0.67	4.08 ± 0.15
Ala	10.68 ± 3.93	8.42 ± 1.30	7.83 ± 1.21	14.18 ± 6.44	4.41 ± 0.20
Tyr	4.06 ± 0.27	1.07 ± 0.14***	2.31 ± 0.62**	1.66 ± 0.91**	1.38 ± 0.31***
Trp	1.43 ± 0.08	0.63 ± 0.02***	1.27 ± 0.13	1.24 ± 0.10	1.28 ± 0.21
Met	0.16 ± 0.01	0.12 ± 0.01**	0.19 ± 0.01*	0.15 ± 0.02	0.14 ± 0.01
Val	1.34 ± 0.27	0.84 ± 0.02*	1.38 ± 0.05	1.13 ± 0.12	1.48 ± 0.09
Phe	6.79 ± 1.41	1.99 ± 0.20***	2.88 ± 0.31**	1.53 ± 0.61***	7.06 ± 0.33
Ile	0.76 ± 0.08	0.92 ± 0.04	0.94 ± 0.01*	0.47 ± 0.06**	1.48 ± 0.21***
Leu	1.66 ± 0.17	1.91 ± 0.04	1.46 ± 0.03	1.28 ± 0.19	1.36 ± 0.26
Lys	0.64 ± 0.11	0.39 ± 0.13	0.66 ± 0.05	0.59 ± 0.37	0.63 ± 0.24
All	172.55 ± 14.55	78.42 ± 5.76***	185.38 ± 2.98	245.72 ± 17.09***	88.96 ± 12.50***

partially purified preparations obtained by anion-exchange chromatography of extracts from mid-log-grown cultured cells (Forlani 2000). In the presence of 0.5 mM Cl-pyr-AMBPA the activity of NADH-dependent glutamate synthase (EC 1.4.1.14) appeared unaffected (98 ± 3% of untreated control, which was 0.104 ± 0.018 nkat mg^{-1} as measured in three independent determinations), while that of GS, evaluated by the hemibiosynthetic assay, was found to be drastically reduced. Under standard assay conditions, the activity of the enzyme in the untreated control was found to be linear with time for at least 20 min. Contrary to phosphinothricin, which inactivates GS irreversibly (Köcher 1989), the same characteristic was evident in the presence of increasing concentrations of the bisphosphonate even though, as an obvious consequence, the catalytic rate was correspondingly lower (Fig. 2*A*). Moreover, the Cl-pyr-AMBPA-treated enzyme was found to be fully reactivated by dialysis while, as expected, phosphinothricin-treated samples were not (Fig. 2*B*).

As kinetic analysis with impure enzymes could be a source of experimental artefacts, GS was purified from cells harvested in the middle exponential phase of growth (Forlani 2000). The availability of highly enriched preparations allowed the use of a biosynthetic assay that more accurately measures the physiological reaction. Enzyme activity was thus evaluated by varying one of the three substrates in the presence of various concentrations of the inhibitor. Hanes–Woolf plots of the data, as well as Lineweaver–Burk and

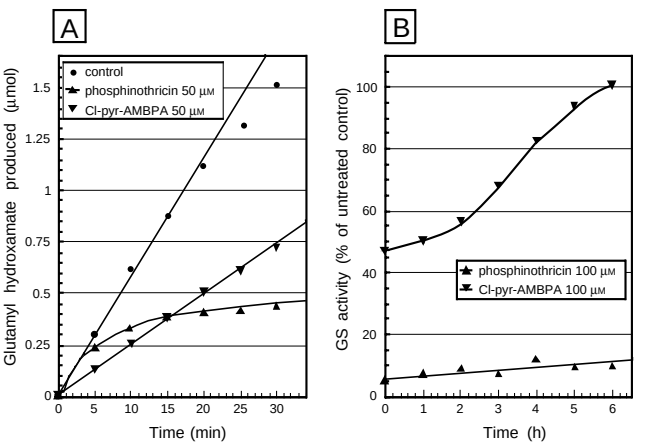

Fig. 2. Reversibility of the inhibition of GS by Cl-pyr-AMBPA in comparison with that by phosphinothricin. Treatment and analysis as described in 'Materials and methods'. (*A*) Kinetics of control and inhibited enzyme. (*B*) Re-activation of GS by dialysis following incubation for 5 min at 35°C in the presence of the substrates and either the inhibitors. Results, expressed as percent of uninhibited dialysed controls, are means of two replicates, with s.d. < 5%.

Dixon plots, showed uncompetitive inhibition with respect to all substrates (Fig. 3). The calculated K_i values ranged from 39 (against ATP) to 113 μM (against ammonia).

Reversal of Cl-pyr-AMBPA toxicity by exogenously supplied glutamine and aromatic amino acids

To elucidate whether the inhibition brought about by Cl-pyr-AMBPA on GS activity could play a major role in its phytotoxicity, and to investigate the respective weight of such inhibition and that of DAHPS, reversal experiments were performed. Cultured cells were fed with exogenous Gln and/or aromatic amino acids, added to the culture medium at the same time as the compound. As a term of comparison, the same procedure was adopted also for phosphinothricin and glyphosate, which indeed inhibit the synthesis of Gln and Phe-Tyr-Trp, respectively (Lea *et al.* 1984; Cole 1985). All three substances were used at concentrations causing 65% inhibition of dry weight increase. Results are presented in Fig. 4. When supplied singly, either Gln or the mixture of aromatic amino acids slightly decreased the inhibitory effect of Cl-pyr-AMBPA, but full reversion was not achieved. On the contrary, when added together they succeeded in coun-

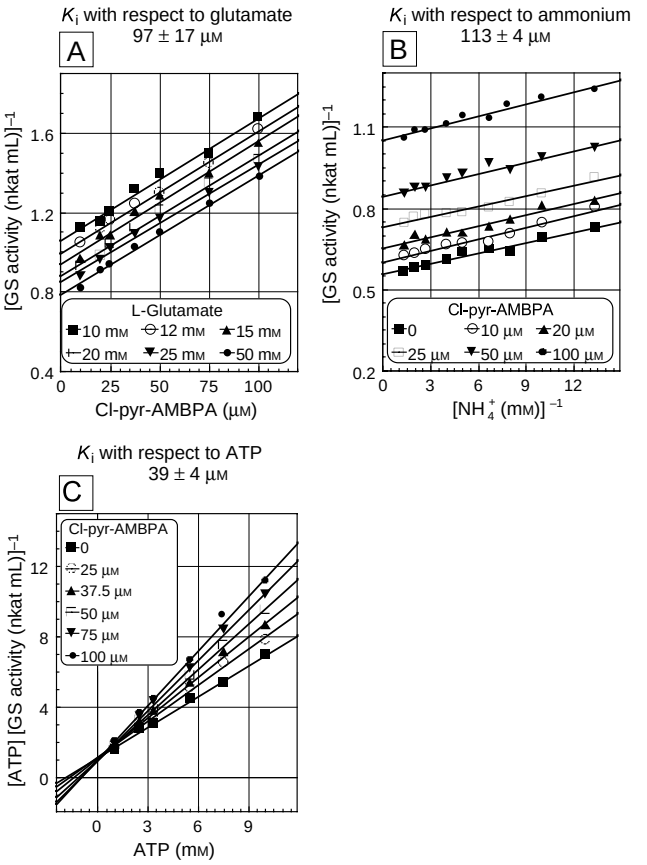

Fig. 3. Kinetic analysis of GS inhibition by Cl-pyr-AMBPA. (*A*) Dixon plot of activity with Glu as the variable substrate. (*B*) Lineweaver–Burk plot of data obtained with different NH_4^+ concentrations. (*C*) Hanes–Woolf plot of activity at varying ATP level. The concentrations of the invariable substrates were fixed at 50, 5 and 1 mM for Glu, ATP and NH_4^+, respectively. All patterns account for an inhibition of uncompetitive type.

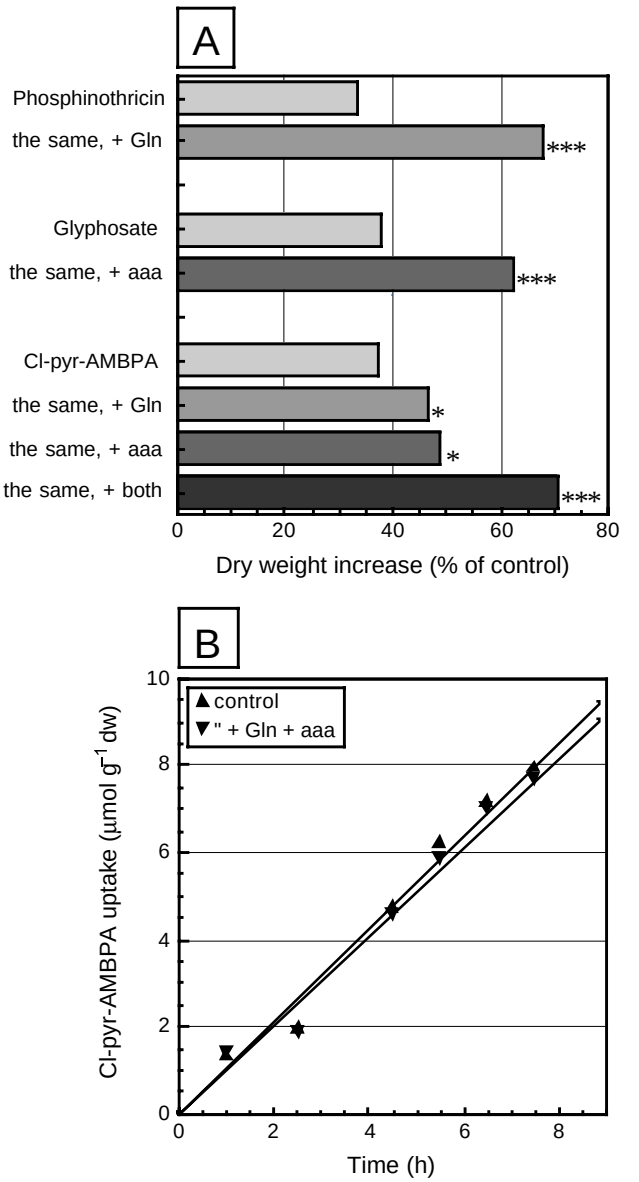

Fig. 4. Reversal of cell growth inhibition by exogenously supplied amino acids (Gln 2.5 mM and/or aaa: Phe and Tyr 0.25 mM, Trp 0.12 mM, *p*-hydroxy- and *p*-amino-benzoic acid 2.5 μM). Treatment and analysis as described in 'Materials and methods'. (*A*) Data are expressed as percent of that in untreated controls (MS medium, 8.87 ± 0.16 mg mL^{-1}; same, with aaa 8.87 ± 0.15 mg mL^{-1}; same, with Gln 9.44 ± 0.24 mg mL^{-1}; same, with both added 9.99 ± 0.26 mg mL^{-1}) and are means of the results obtained in two independent experiments, in which each treatment was performed in triplicate. To rule out the possibility that reversal may depend upon competition for membrane carrier(s), the rate of Cl-pyr-AMBPA uptake was evaluated at increasing time after its addition to the medium either supplemented or not with both aaa and Gln. (*B*) Results are mean of three replicates, with s.d. < 15%.

teracting the toxicity of the compound. The relief was very similar to that obtained in the case of phosphinothricin by Gln alone, or that in the case of glyphosate by aromatic amino acids (Fig. 4*A*). The rate of Cl-pyr-AMBPA uptake by amino acid-fed cells was not significantly different from that in untreated controls (1.04 against 1.06 μmol h^{-1} [g dw]$^{-1}$; Fig. 4*B*), ruling out the possibility that reversal could derive from a competition for the same transport system(s) across the membrane.

Discussion

Plant cell cultures have been widely adopted in screening for amino acid biosynthesis inhibitors and in the study of their mode of action, since the corresponding pathways are expressed also at the undifferentiated tissue level (Kishore and Shah 1988). To investigate whether *in vitro* inhibition of DAHPS by Cl-pyr-AMBPA (Forlani *et al.* 1996) occurred also *in vivo*, thereby providing the basis for phytotoxicity of the compound, free amino acid concentration was measured in *N. plumbaginifolia* cells at increasing time after the addition of the inhibitor to the culture medium. Sublethal doses, which gave rise to 50% inhibition of cell growth rate, were preferred in order to enhance specific effects and reduce variations simply due to the resulting cellular stress. From several previous studies on glyphosate it was noted that the block of the carbon flow in the shikimate pathway generally decreases Phe and Tyr levels, while increasing the total amount of free amino acids. In particular, increased synthesis of Glu and Gln was often found to occur in response to increases in ammonia also observed (Cole 1985; see also Table 1).

The results obtained with Cl-pyr-AMBPA show a strikingly different pattern. An actual reduction of aromatic amino acids level was found, as was for the close analogue M-pyr-AMBPA (Forlani *et al.* 1999) that occurred only after cultures more than doubled cellular density. However, total amino acid content was significantly reduced in treated cells, mainly due to a decrease in the level of Gln, Asn, Arg and Gly. Glu level also decreased, while that of Ser slightly increased. These results could occur due to inhibition of selected enzymes involved in nitrogen assimilation. Very similar effects on free amino acid pools were previously reported in the case of two other antimetabolites that act through the inhibition of GS, a key enzyme in ammonia metabolism: methionine sulfoximine (3-amino-3-carboxylpropylmethyl-sulfoximine; Goodchild and Sims 1990) and phosphinothricin (Shelp *et al.* 1992). As a matter of fact, the treatment of *N. plumbaginifolia* cultures with a sublethal dose of phosphinothricin resulted in a significant, comparable decrease of Gln, Asn, Arg and Gly (Table 1). When the two enzymes of the glutamate cycle were assayed in the presence of millimolar levels of Cl-pyr-AMBPA, whereas glutamate synthase was found to be unaffected, the activity of GS was actually markedly inhibited.

Kinetic analysis showed that inhibition of GS by Cl-pyr-AMBPA is uncompetitive with respect to all three substrates, suggesting that the inhibitor binding site is neither the same nor overlaps those for the substrates. This is an unusual feature, since all GS inhibitors described to date share the property of being glutamate analogues that compete for the same binding site as the natural substrate (Farrington *et al.* 1987; Köcher 1989; Logusch *et al.* 1991). Methionine sulfoximine, tabtoxinine-β-lactam (2-amino-4-[3-hydroxy-2-oxo-azacyclobutan-3-yl]butanoic acid) and phosphinothricin inactivate the enzyme irreversibly through the formation of phosphorylated intermediates that strictly bind to the active site (Köcher 1989). Unlike these, but similarly to substituted phosphinothricins (Logush *et al.* 1991) and other phosphonate inhibitors (Farrington *et al.* 1987), Cl-pyr-AMBPA inhibitory effect was reversed by dialysis, and the activity of the inhibited enzyme was linear with time (Fig. 2). This could account in part for the noteworthy lower effectiveness of the compound *in vivo*, as compared to that of phosphinothricin (Fig. 1).

The estimated K_i values for Cl-pyr-AMBPA were in the 10^{-5} to 10^{-4} M range, about an order of magnitude higher that those reported for phosphinothricins (Köcher 1989; Logush *et al.* 1991), but similar to those found for other GS inhibitors (e.g. 4-*N*-hydroxy-L-2,4-diaminobutyric acid; Fushiya *et al.* 1988) and for the inhibition of DAHPS activity by the same compound (Forlani *et al.* 1996). Data on free amino acid levels strongly support the possibility that the inhibition of GS activity may also occur *in vivo*. Reversal experiments were performed to ascertain whether GS and DAHPS activity might really represent the main targets of Cl-pyr-AMBPA. Even though the inhibition of GS activity could be regarded as the primary mode of inhibition when the consequent alterations of amino acid pools are weighed (Table 1), the addition of exogenous Gln failed to reverse the toxicity of the compound. The same addition significantly alleviated the inhibition of a comparable level of phosphinothricin. As expected, not even a mixture of aromatic amino acids could counteract the effect of Cl-pyr-AMBPA, whereas the same mixture alleviated inhibition by a similarly effective concentration of glyphosate. However, when Cl-pyr-AMBPA-treated cells were fed with both supplements, full reversal was obtained (Fig. 4). Thus, the data herein presented support the hypothesis that the inhibition of the synthesis of both Gln and aromatic amino acids might be the unusual, herbicidal mode of action for this *N*-substituted derivative of aminomethylene bisphosphonic acid.

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