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Biochemical Bases for a Widespread Tolerance of Cyanobacteria to the Phosphonate Herbicide Glyphosate

Giuseppe Forlani [1,*], Mauro Pavan [1], Magdalena Gramek [1,2], Paweł Kafarski [2,3] and Jacek Lipok [2]

[1] *Department of Biology and Evolution, University of Ferrara, Italy*
[2] *Department of Chemistry, University of Opole, Poland*
[3] *Department of Bioorganic Chemistry, Wroclaw University of Technology, Poland*

Possible non-target effects of the widely used, non-selective herbicide glyphosate were examined in six cyanobacterial strains, and the basis of their resistance was investigated. All cyanobacteria showed a remarkable tolerance to the herbicide up to millimolar levels. Two of them were found to possess an insensitive form of glyphosate target, the shikimate pathway enzyme 5-*enol*-pyruvyl-shikimate-3-phosphate synthase. Four strains were able to use the phosphonate as the only phosphorus source. Low uptake rates were measured only under phosphorus deprivation. Experimental evidence for glyphosate metabolism was also obtained in strains apparently unable to use the phosphonate. Results suggest that various mechanisms may concur in providing cyanobacterial strains with herbicide tolerance. The data also account for their widespread ability to metabolize the phosphonate. However, such a capability seems limited by low cell permeability to glyphosate, and is rapidly repressed when inorganic phosphate is available.

Keywords: Cyanobacteria — EPSP synthase — Glyphosate — Herbicide tolerance — Phosphonate/phosphate uptake — Target enzyme-based resistance — Xenobiotic metabolism.

Abbreviations: AMPA, aminomethylphosphonic acid; EPSP, 5-*enol*-pyruvyl-shikimate-3-phosphate; IC_{50}, concentration causing 50% inhibition of enzyme activity; NMR, nuclear magnetic resonance; PEP, phospho-*enol*-pyruvate; S3P, shikimate-3-phosphate.

Introduction

Since it is rapidly and completely degraded by soil-borne microorganisms to water, carbon dioxide and inorganic phosphate (Ternan et al. 1998, Kafarski et al. 2001), the broad-spectrum herbicide glyphosate (*N*-[phosphonomethyl]glycine) may be considered to be an environmentally friendly pesticide. Although additional effects have been reported in plants (e.g. De Maria et al. 2006), it acts mainly through the inhibition of 5-*enol*-pyruvyl-shikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) (Funke et al. 2006), the enzyme that catalyzes the penultimate step in the common pre-chorismate pathway, the addition of the carboxyvinyl group of phospho-*enol*-pyruvate (PEP) to shikimate-3-phosphate (S3P) (Herrmann and Weaver 1999). Being endowed with little or no acute or chronic toxicity, and also no apparent carcinogenic and mutagenic activity (Williams et al. 2000), it is considered safe to mammals, which do not possess a functional shikimate pathway and thus need a daily dietary intake of aromatic amino acis. Formerly, the inability of glyphosate to distinguish between weeds and crops severely limited its use (Duke 2005). However, bacterial genes encoding glyphosate-resistant EPSP synthases were subsequently cloned, endowed with chloroplast transit signals and used to transform plants (Dill 2005). During recent years, herbicide-tolerant seeds have become available for several species of greatest agronomical value (Gianessi 2005). This steadily increased its use, and nowadays glyphosate represents the most successful agrochemical ever, with worldwide sales that approach US$2 billion (Woodburn 2000).

Even though the rate of utilization has been reported to vary considerably between different soils (e.g. Strange-Hansen et al. 2004), in most cases glyphosate mineralization proceeds without any lag phase under both aerobic and anaerobic conditions, and is believed to be a co-metabolic process (Torstensson 1985). Despite numerous attempts, with the only exception of an *Achromobacter* strain isolated from a glyphosate waste stream treatment facility (Barry et al. 1992), and a *Streptomyces* sp. taken from a municipal sewage treatment plant (Obojska et al. 1999), in no case has the identification of a microbial strain able to utilize the herbicide constitutively been reported to date. Some evidence that at least the first step(s) in glyphosate degradation may be accomplished by bacterial strains which are quiescent, or proliferate so slowly as to be unable to form visible colonies on agar plates, has been provided which may explain this apparent inconsistency (Forlani et al. 1999). On the other hand, numerous bacterial strains have been described that are capable of utilizing this phosphonate as a source of phosphorus. However, as for most phosphonates, inorganic phosphate (P_i) was inhibitory

*Corresponding author: E-mail, flg@unife.it; Fax, +39-0532-249761.

Table 1 Cyanobacterial strains used in the present work, and their doubling time under the growth conditions employed

Strain [section]	ATCC medium	Generation time
Anabaena sp. ATCC 27347 (PCC 7120) [IV]	616	6.2 ± 0.4
Arthrospira fusiformis CCALA 023 [III]	1679	7.2 ± 0.6
Leptolyngbya boryana ATCC 27894 (PCC 6306) [III]	616	5.1 ± 0.3
Microcystis aeruginosa PCC 7941 (CCALA 106) [I]	616	5.5 ± 0.2
Nostoc punctiforme ATCC 29133 (PCC 73102) [IV]	616	6.2 ± 0.3
Spirulina platensis, strain C1 (PCC 9438) [III]	1679	6.6 ± 0.8

Generation times, expressed in days, were calculated during the logarithmic phase of growth. Data are means ± SD over four independent replications.

to glyphosate breakdown, which proceeds only under conditions of phosphorus starvation (Ternan et al. 1998). Two main pathways for glyphosate degradation were identified, both leading to the breakage of the carbon–phosphorus bond (Kafarski et al. 2001, Karpouzas and Singh, 2006). In the first one, glyphosate is converted to aminomethylphosphonic acid (AMPA) and glyoxalate by a glyphosate oxidoreductase. AMPA is then either directly metabolized to methylamine and orthophosphate, or undergoes acetylation prior to the cleavage of the C–P bond. Alternatively, the initial cleavage by an as yet not well characterized C–P lyase complex (Huang et al. 2005) yields sarcosine, which is further converted to glycine and a C_1-unit, that is in turn incorporated into purines and some amino acids.

The application of herbicides in agricultural systems may exert side effects on the soil microflora and, following their leaching from the root zone into drainage water or groundwater, on water ecosystems as well, including a possible shift in microbial or algal community structure. This may be particularly true in the case of compounds which interfere with amino acid biosynthesis, thus potentially also being effective on microbial metabolism. This possibility has been investigated quite extensively for glyphosate. At the recommended rate for field usage, because of its rapid decomposition by soil microorganisms, its effect on microbial population dynamics was thought to be negligible (Grossbard 1985). However, increasing data suggest that, at least under certain circumstances, glyphosate may exert non-target effects in either the soil or water environment (Cedergreen and Streibig 2005, Ratcliff et al. 2005, Vereecken 2005). Also in the case of transgenic plants owing their tolerance to Round-up Ready™ technology, despite full resistance to the herbicide, adverse effects might derive from the consequent inhibition of beneficial rhizobacterial strains, facilitating pathogen attack (Meriles et al. 2006).

Being the only group of prokaryotes capable of oxygenic photosynthesis, cyanobacteria, also referred to as blue-green algae, are widely distributed even in strikingly different habitats. They play a major role in both carbon and nitrogen global cycling, since several strains, either symbiotic or free-living, can accomplish biological N_2 fixation. Possible negative side effects that the use of glyphosate may have for cyanobacteria in cultivated soils and in natural environments have not been adequately investigated to date. Conflicting data were reported as to their sensitivity in vivo to the herbicide (Peterson et al. 1994). Some strains showed severe growth inhibition when glyphosate was applied at micromolar concentrations (Ravi and Balakumar 1998, Issa 1999). In contrast, a few species were found to exhibit a remarkable natural tolerance to this herbicide (Powell et al. 1991). In the case of *Anabaena variabilis*, herbicide tolerance was shown to reflect an insensitive form of EPSP synthase, whereas the occurrence of glyphosate metabolism was ruled out (Powell et al. 1992). However, a glyphosate-resistant form of EPSP synthase is not a common feature of cyanobacteria, the enzyme purified from *Spirulina platensis* being completely inhibited by the herbicide at micromolar levels (Forlani and Campani 2001). A claim that an *Anabaena* strain would be able to use the herbicide as a phosphorus source by means of an extracellular alkaline phosphatase (Ravi and Balakumar 1998) was not supported further.

Here we report the results of a screening of six collection cyanobacterial strains for glyphosate sensitivity. Their growth was found to be unaffected by the presence of the herbicide up to millimolar concentrations. The basis of such a resistance was investigated throughout, showing that cyanobacteria may owe their tolerance to various mechanisms at the cellular level.

Results

Effect of increasing glyphosate concentrations on cyanobacterial growth

A small group of six cyanobacterial strains belonging to different orders (Chroococcales [I], Oscillatoriales [III] and Nostocales [IV]; Table 1) were chosen for testing possible

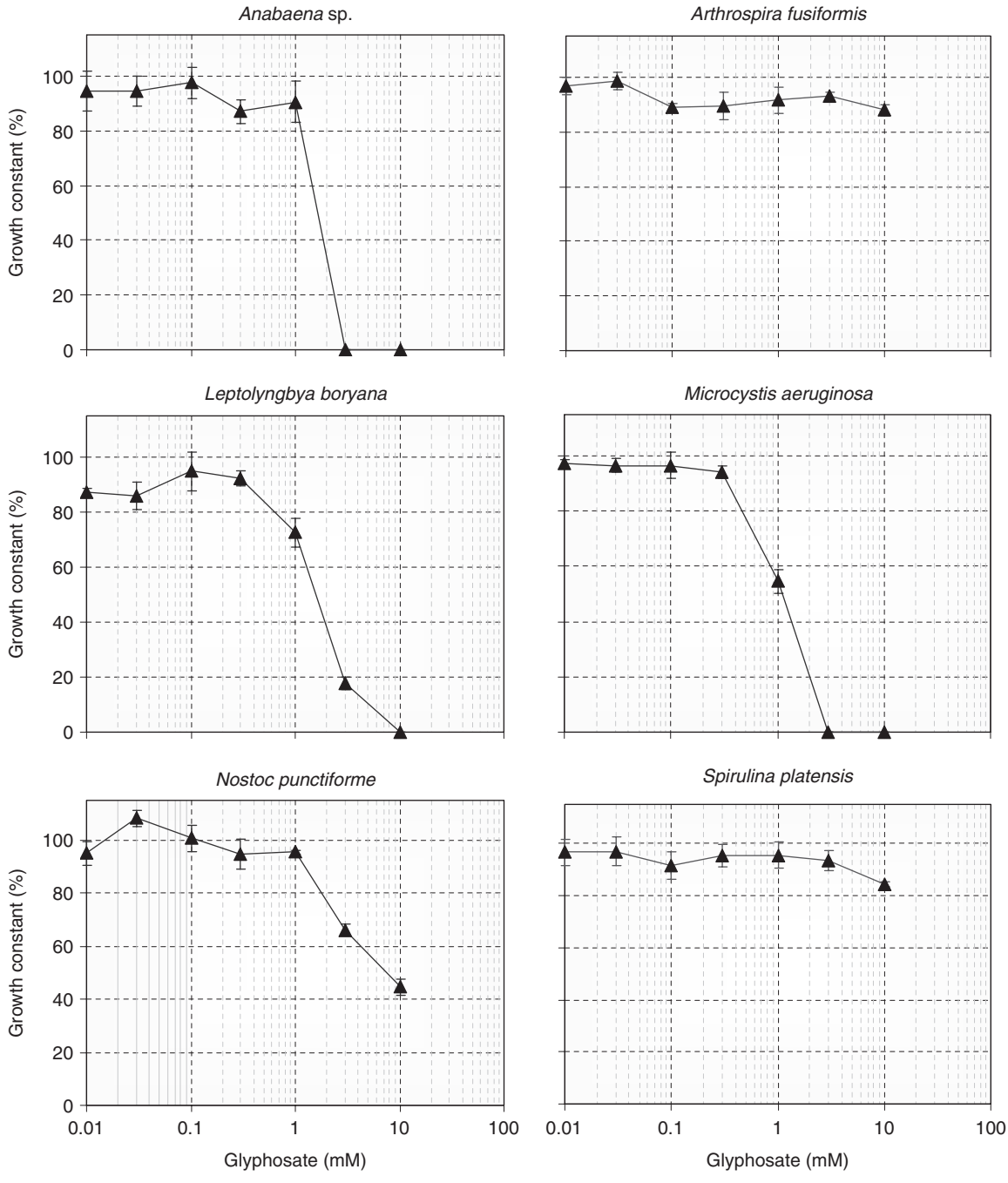

Fig. 1 Effect of glyphosate on cyanobacterial growth. Increasing concentrations of the phosphonate, ranging from 10 µM to 10 mM, were added to the culture medium. The resulting growth rates were then determined by following the biomass increase over a 21 d incubation. For a given strain, a completely randomized design with three replications (four for controls) was set up. The results, expressed as a percentage of untreated controls, are the mean ± SD over replicates.

non-target effects of the widely used herbicide glyphosate. Under the standard growth conditions adopted, these strains showed a comparable generation time (Table 1). Their growth constant was also determined following the addition to the culture medium of the active ingredient at increasing concentrations. Interestingly, as summarized in Fig. 1, an overall, remarkable tolerance was evident. In all cases, cell proliferation was completely unaffected by the herbicide in the micromolar to millimolar range. Only at concentrations exceeding 0.3 mM did glyphosate inhibit the growth of some strains. A noteworthy resistance was found in the case of the two hypersaline pond species, *A. fusiformis*

and *S. platensis*, that did not show any significant increase in their doubling time even at the highest dose tested (10 mM). The result was quite unexpected for the latter strain, whose EPSP synthase had been shown to be completely inhibited by the herbicide at 0.1 mM (Forlani and Campani 2001). Moreover, with the only exception of *Nostoc*, that in the millimolar range indeed showed a gradual reduction of its growth constant, for freshwater strains an unusually rapid loss of cell viability was evident when a critical threshold was overcome. This was also true for *Anabaena* sp., in spite of being expected to possess a resistant form of the target enzyme (Powell et al. 1992). The different behavior between the two halophilic strains and the other isolates might derive indirectly from the use of a different culture medium. In fact, ATCC media 616 and 1679 have a strikingly dissimilar P_i content (0.23 vs. 2.9 mM K_2HPO_4, respectively) that could influence herbicide uptake. In order to rule out this possibility, the experiment was repeated by lowering the P_i content in medium 1679 to 1, 0.5 or 0.2 mM. Neither the absolute growth rate nor the glyphosate sensitivity of both *Arthrospira* and *Spirulina* strains was significantly affected (data not shown).

Table 2 Properties of cyanobacterial EPSP synthases potentially related to glyphosate tolerance

Strain	Specific activity (pkat mg^{-1} protein)	Glyphosate IC_{50} (mM)
Anabaena sp.	61.3 ± 18.7	8.8 ± 0.1
Arthrospira fusiformis	63.0 ± 9.4	0.048 ± 0.005
Leptolyngbya boryana	61.8 ± 13.0	0.023 ± 0.003
Microcystis aeruginosa	58.8 ± 22.3	0.037 ± 0.003
Nostoc punctiforme	76.6 ± 25.4	>50
Spirulina platensis	34.7 ± 4.6	0.029 ± 0.005

Specific activity levels in actively proliferating cells were determined on desalted crude extracts following ammonium sulfate fractionation. Data are the means ± SD over five independent measurements. The concentrations of glyphosate causing 50% inhibition of enzyme activity (IC_{50}) were evaluated on pooled active fractions following anion-exchange chromatography, by utilizing the linear regression equation of enzyme activity values, expressed as a percentage of untreated controls, plotted against the logarithm of inhibitor concentration (Fig. 2). Confidence values were computed according to Snedecor and Cochran (1989).

Specific activity levels and glyphosate sensitivity of cyanobacterial EPSP synthases

In order to obtain more information about the molecular basis of such a general tolerance to glyphosate, some of the properties of the herbicide target, the shikimate pathway enzyme EPSP synthase, were determined in partially purified preparations. Specific activity levels were measured in desalted extracts from cells harvested in the exponential phase of growth. Data were on the whole quite uniform, with mean values ranging from 59 to 77 pkat mg^{-1} protein (Table 2). Only in the case of *S. platensis* was a significantly lower result obtained. However, no relationship was evident between enzyme levels and the tolerance in vivo to the herbicide. To assess enzyme sensitivity, EPSP synthase was further enriched by anion-exhange chromatography. The final preparations showed a 4- to 6-fold enzyme purification, depending on the strain (results not presented). The activity in such preparations was evaluated in the presence of a wide range of glyphosate concentrations, from 2 μM to 50 mM. Striking differences were found in this case among strains (Fig. 2). As expected, the enzyme from *S. platensis* was steadily inhibited as the phosphonate increased from 10 to 100 μM. Similar patterns were obtained for *Arthrospira*, *Leptolyngbya* and *Microcystis*. The estimated concentrations causing 50% inhibition of enzyme activity (IC_{50}), reported in Table 2, place these EPSP synthases into the most sensitive of the three categories characterized among the bacterial enzymes (Shultz et al. 1985). A completely different result was obtained with the other two strains. Consistent with previous data (Powell et al. 1992), the enzyme from *Anabaena* sp. was unaffected by micromolar concentrations of the herbicide. Only at levels exceeding 1 mM was a significant inhibition evident, with an IC_{50} of about 9 mM. Even more striking was the tolerance shown by *Nostoc* EPSP synthase, that was substantially uninhibited in the whole range of concentrations tested. The data thus strongly support the possibility that these two strains may owe their tolerance at the whole-cell level to the presence of these glyphosate-insensitive forms of the enzyme. In contrast, the even more remarkable resistance found with the other cyanobacterial strains, that possess highly sensitive EPSP synthases, implies that other tolerance mechanism(s) do exist.

Ability of cyanobacteria to use the phosphonate moiety as the only phosphorus source

In the presence of a sensitive target, herbicide tolerance might rely upon either cell impermeability, or the occurrence of degradative pathways or detoxifying enzymes. To discriminate among these possibilities, the ability of the six cyanobacterial strains to grow in modified media containing glyphosate as the only phosphorus source was evaluated. The resulting growth rates were compared with those obtained in standard media, and in media devoid of any phosphorus source. The data, outlined in Table 3, show that in most cases the significant reduction of cell proliferation observed under phosphorus starvation was almost completely relieved in the presence of the herbicide. However, in this case also, the two halophilic strains behave

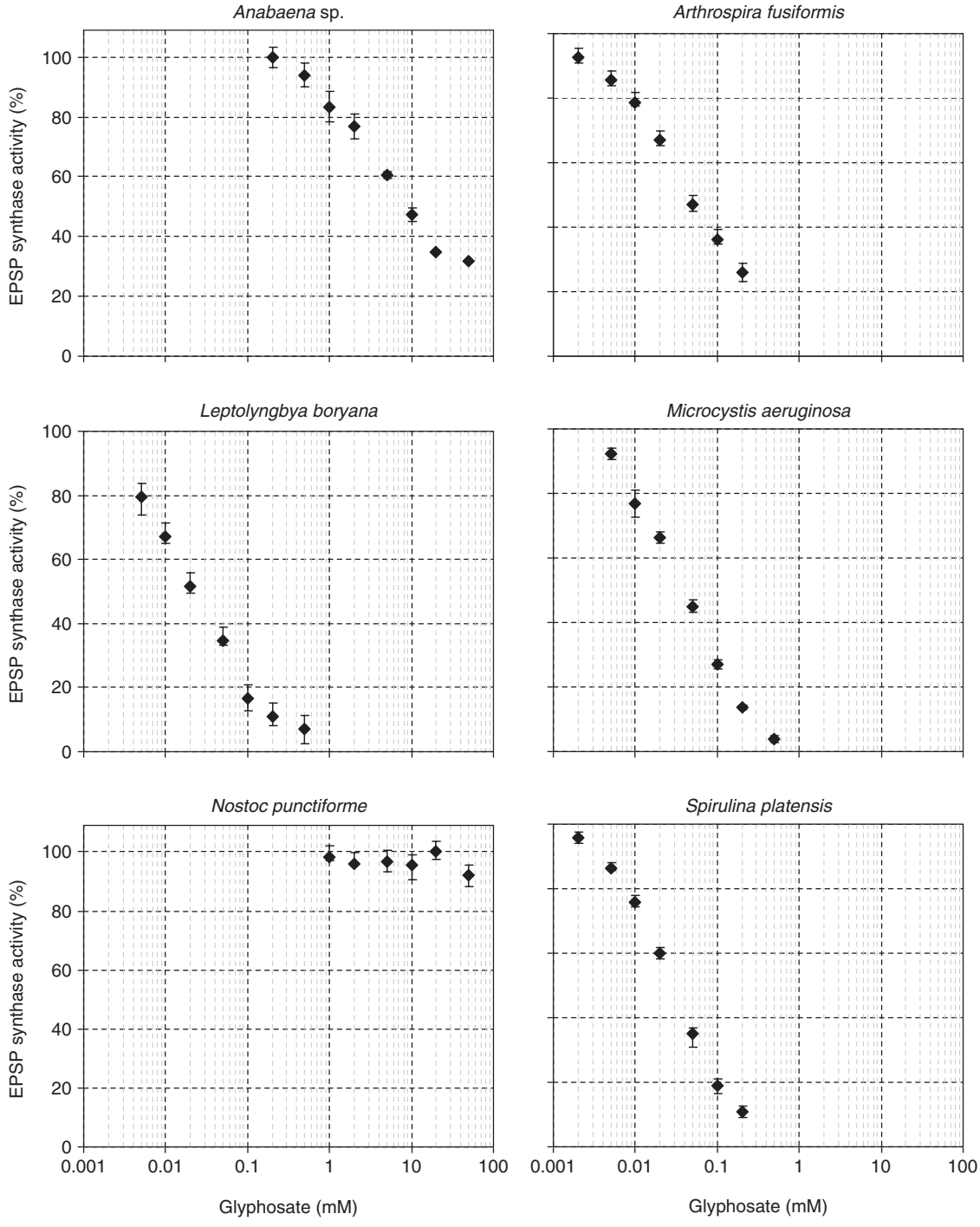

Fig. 2 Sensitivity of cyanobacterial EPSP synthase to glyphosate. The enzyme was partially purified by anion-exchange chromatography, and the activity was determined in the presence of increasing concentrations of the herbicide, ranging from 1×10^{-6} to 5×10^{-2} M. At least three replications were carried out for each dose. The results were expressed as a percentage of activity in untreated controls, and are the mean ± SD over replicates.

differently from the other species, being apparently unable to use the phosphonate as a source of phosphorus. Because a lower but significant growth rate was also found in this case in medium devoid of any phosphorus source, the experiment was carried out again by growing *S. platensis* cultures in Erlenmeyer flasks over a prolonged period. The results, depicted in Fig. $3A_1$, showed that during the first 2 weeks after the inoculum the growth

Table 3 Ability of cyanobacteria to use glyphosate as the only source of phosphorus

Strain	Inorganic phosphate	+	−	−	+
	Glyphosate	−	−	+	+
Anabaena sp.		100.0 ± 4.6	46.1 ± 2.1	101.8 ± 2.1*	97.0 ± 6.0
Arthrospira fusiformis		100.0 ± 3.0	43.1 ± 2.5	41.6 ± 2.0	104.4 ± 2.0
Leptolyngbya boryana		100.0 ± 4.8	49.7 ± 1.2	85.8 ± 4.8*	118.2 ± 4.5
Microcystis aeruginosa		100.0 ± 1.8	29.3 ± 5.7	81.2 ± 1.9*	96.5 ± 2.8
Nostoc punctiforme		100.0 ± 1.0	15.4 ± 3.7	88.8 ± 3.8*	110.9 ± 7.1
Spirulina platensis		100.0 ± 4.0	52.6 ± 1.2	40.8 ± 0.6*	105.0 ± 2.8

The inorganic source of phosphorus in ATCC media 616 and 1679 (K_2HPO_4 0.23 and 2.9 mM, respectively) was either omitted, or replaced, or added with the same concentration of the herbicide. The resulting growth rates were evaluated as described, and expressed as a percentage of those obtained with standard medium. Values are the means ± SD over four replications. In all cases the growth in the absence of any phosphorus source was significantly ($P < 0.01$) different from that in standard medium. Significant differences between growth in glyphosate-containing medium and in medium devoid of any phosphorus source are indicated with an asterisk.

in phosphorus-lacking medium was indeed indistinguishable from that in standard medium. Most probably, despite the repeated cell washing to remove the external medium, the amount of phosphate inside the cells is enough to sustain at least a couple of cell divisions. In fact, the occurrence of phosphorus storage compounds such as polyphosphates has been reported for many cyanobacteria (Kromkamp 1987). The experiment was thus repeated in the case of *Spirulina* and *Leptolyngbya* by using phosphorus-starved cells as the inoculum. Moreover, for the former strain, a lower level of glyphosate was used, in order to reduce the possibility of a P_i supply deriving from even a negligible chemical hydrolysis of the phosphonate. The results, depicted in Fig. $3A_2$ and B, confirmed previous data.

Glyphosate uptake by cyanobacterial strains

The inability of *S. platensis* and *A. fusiformis* to use glyphosate as the only source of phosphorus suggests that their tolerance may depend upon a negligible uptake of the phosphonate. However, the same phenotype may rely upon the presence of detoxifying enzymes, since whatsoever modification of the herbicide molecule would abolish its biological activity without providing the cell with P_i for growth. To obtain evidence for herbicide uptake, its residual concentration in the culture supernatant was determined at increasing times after inoculation in media containing glyphosate or a combination of glyphosate and P_i as the phosphorus source. This analysis was performed with three strains that were representative of the different properties found (resistant or sensitive EPSP synthase, ability or inability of using glyphosate as a phosphorus source). Preliminary trials showed very low uptake rates for both compounds, rates that made it very difficult to distinguish between true incorporation into the cells and experimental variability among samples. Thus the experiments were carried out with lower substrate concentrations. The results, reported in Fig. 4B, showed a decrease over time of glyphosate concentration only in the case of *Nostoc* cultures. A lowering of the residual concentration of the herbicide was also evident in the case of *Leptolyngbya* (Fig. 4A), although the differences were not statistically significant. In contrast, no apparent decrease in glyphosate level was found in the exhausted medium of *Spirulina* (Fig. 4C). In all samples, the presence of inorganic phosphate was below the sensitivity of the analytical method used (about 2 nmol ml^{-1}), thus no phosphate seems to be released in the medium from glyphosate. A different pattern was found for P_i. In this case, the rate of uptake was strikingly higher, and during 3–4 weeks all the phosphate initially present in the medium (0.12 or 0.25 μmol ml^{-1} depending on the strain) was incorporated into the cell biomass. Interestingly, the minor decrease in glyphosate residual levels previously found for *Nostoc* and *Leptolyngbya* was completely abolished if P_i was available in the meantime (lower panels in Fig. 4).

To gain more information, short-term uptake kinetics were determined. To increase the sensitivity of the method, cells were harvested by centrifugation, washed twice with medium devoid of any phosphorus source, resuspended at high density in the same medium and finally added with factorial combinations of P_i and glyphosate. No significant decrease of both substrates was found within a few hours with phosphorus-fed cells (data not shown). In contrast, when *Nostoc* and *Leptolyngbya* cultures were starved for 2 weeks before the experiment, a rapid and quantitative incorporation of inorganic phosphate was evident, that reached a plateau 8–10 h after the addition. However, under the same experimental conditions, the uptake of glyphosate was very low, near the sensitivity level of the method (Fig. 5A, B). This notwithstanding, a result that is consistent with low but coherent rates of glyphosate uptake in *Leptolyngbya* was obtained by assessing the effect of the concurrent presence of the phosphonate upon P_i uptake. When the same concentration of the herbicide was added

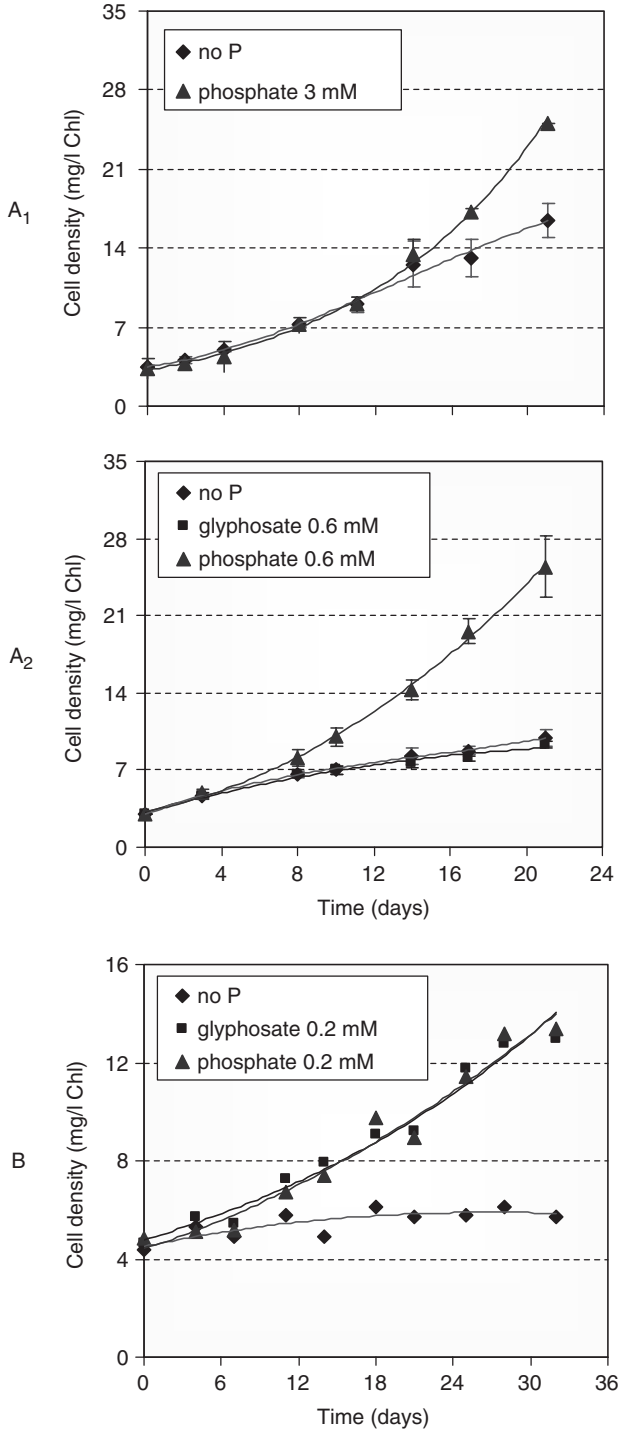

Fig. 3 Growth kinetics of *Spirulina platensis* (A) and *Leptolyngbya boryana* (B) in media with P_i or glyphosate as the only phosphorus source, compared with those in medium lacking any phosphorus supply. If *Spirulina* cultures were started with phosphorus-fed cells, during the first 2 weeks the growth in the absence of any phosphorus source was indistinguishable from that in P_i-containing medium (A_1). The experiment was thus repeated by using cells starved for 14 d in media lacking any phosphorus source as the inoculum (A_2 and B).

in the meantime, a small but consistent decrease of phosphate incorporation by starved cells was indeed evident (Fig. 5D). Interestingly, a different picture was obtained with *Spirulina*, where a low decrease in the external concentration of phosphate was also found in the case of starved cells (Fig. 5C).

Finally, in order to obtain a positive control that could serve as a means of comparison, the same protocol was applied to a glyphosate-sensitive plant cell culture. Also in this case several attempts to show a significant decrease in the external concentration of the phosphonate failed when phosphorus-fed cells were used, even though the adsorption medium described by Hetherington et al. (1998) was used instead of the standard MS medium (not shown). In contrast, when *Nicotiana plumbaginifolia* suspension-cultured cells were grown for 5 d in a modified MS medium lacking any phosphorus source prior to use, a rapid and quantitative uptake of P_i was found (Fig. 5E). In spite of this, if glyphosate was instead added to the medium, its concentration decreased only slightly with time, with a pattern (Fig. 5F) resembling those obtained with *Nostoc* and *Leptolyngbya*.

Evidence supporting the capability of Spirulina to cleave the C–P bond in the herbicide molecule

Because cell impermeability could represent a mechanism contributing significantly to the high tolerance shown in vivo by *S. platensis*, other experiments were performed either by increasing the glyphosate level or by using commercial formulations. When the active principle was added at concentrations exceeding 20 mM, a rapid loss of cell viability was found. Moreover, the strain showed a remarkably higher sensitivity toward the commercial product, that contains surfactants, as well as toward the isopropylamine salt (data not presented). This is consistent with previous results (Powell et al. 1991), and further strengthens the hypothesis that herbicide uptake may be a critical point for its toxicity in some cyanobacteria. Above 1 mM, glyphosate is in fact expected to diffuse through the cell membrane, without the need for carrier involvement (Denis and Delrot 1993).

In order to obtain further evidence supporting the ability of cyanobacterial strains to metabolize glyphosate once it has entered the cell, herbicide degradation was finally studied in vivo using a whole-cell system. The time course of glyphosate metabolism in high density cultures was followed by means of ^{31}P-nuclear magnetic resonance (NMR) spectroscopy. However, the percentage of the herbicide that is apparently used by the cultures when it represents the only source of phosphorus (Fig. 4) was too low to be detectable with such a technique. Moreover, at least in the case of the other strains, both the amount of glyphosate that is taken up and the final biomass were not

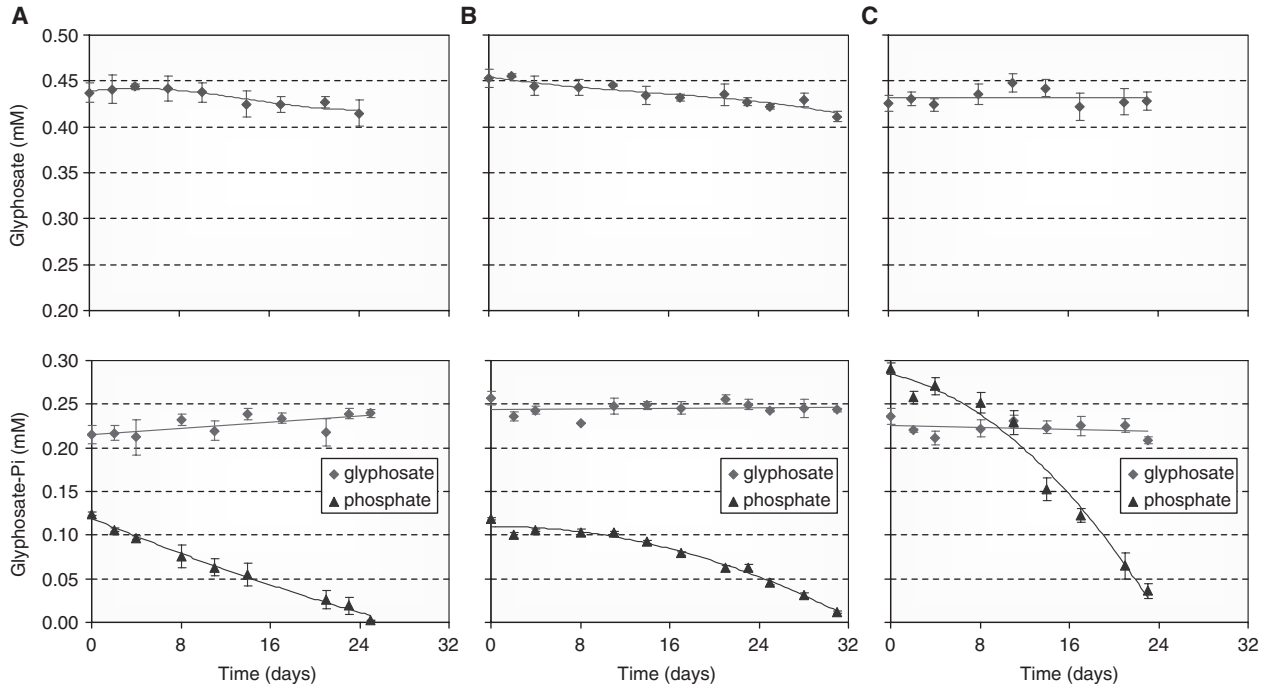

Fig. 4 Time course of phosphate and/or glyphosate uptake by selected cyanobacterial strains. Modified media containing either 0.5 mM glyphosate as the only phosphorus source (upper panels) or a combination of the phosphonate and P_i (lower panels) were inoculated with *Leptolyngbya*, *Nostoc* and *Spirulina* cells (A, B and C, respectively). At increasing times after the inoculum, the residual concentration of the herbicide in the supernatant was determined by reverse-phase HPLC following derivatization with 2,4-dinitrofluorobenzene. The concentration of inorganic phosphate in the same samples was measured colorimetrically by the malachite green dye assay. No P_i release was detected in the flasks containing the phosphonate as the only phosphorus source. The results are the mean ± SD over three independent replications.

enough to provide a reliable signal if cells were analyzed. To overcome such drawbacks, an experimental system was set up in which *S. platensis* cells grown in standard medium and then starved for 2 weeks in medium devoid of phosphorus sources were directly transferred at high density into NMR tubes, and then added with glyphosate. Under such conditions, cells maintained viability, and a significant increase in the biomass was detected over time (not shown). Following the addition of the phosphonate at 10 mM, a previously unrecorded peak became evident at about 7 p.p.m. (Fig. 6A). During further incubation inside NMR tubes, this peak at first decreased, then disappeared, concomitantly with the transient appearance of additional peaks at about 1–3 p.p.m., the phosphate region. An analysis performed 14 d after herbicide addition showed only a weak signal in the latter range, whereas glyphosate was below the signal noise ($\leq$0.5 mM). Interestingly, when the same experiment was repeated at a lower initial concentration of the herbicide (1 mM), a strikingly different pattern was obtained (Fig. 6B). In this case the signal corresponding to the phosphonate showed only a very minor decrease (about 0.13 mmol l^{-1} over 2 weeks), and that for orthophosphate monoester was detectable only 14 d after the addition. These results further strengthen the possibility that in this strain a negligible carrier-mediated uptake of glyphosate does occur when supplied at micromolar levels, thus contributing to the remarkable tolerance to the herbicide. Only at millimolar levels does a concentration-driven diffusion through the membrane take place, which is proportional to the external level of the phosphonate, and is limiting for its utilization.

Discussion

Despite a large number of studies on the behavior of glyphosate in the soil, to date the sensitivity of cyanobacteria to this successful herbicide has been evaluated only sporadically. Some species have been included in wider screening of microorganisms for glyphosate sensitivity (Peterson et al. 1994), or single strains have been characterized with respect to herbicide susceptibility (Powell et al. 1991, Issa 1999). As a consequence, inconsistent results have been reported on both the occurrence of non-target effects in water environments and the ability of cyanobacteria to metabolize the phosphonic moiety (e.g. Powell et al. 1991, vs. Ravi and

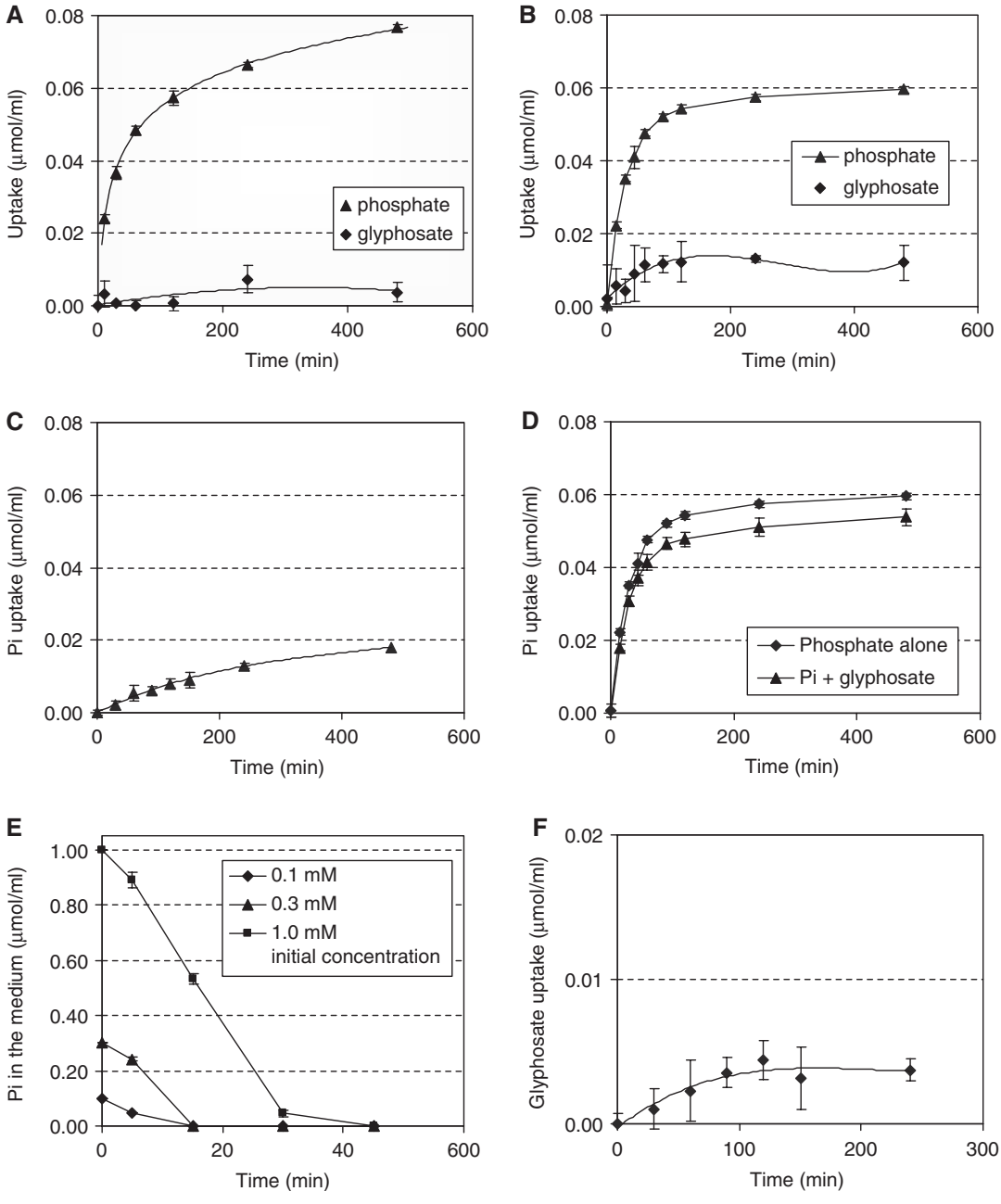

Fig. 5 Uptake of glyphosate and inorganic phosphate by phosphorus-starved cells. Late log-grown cells were starved of phosphorus for 2 weeks, then harvested by centrifugation. Pellets were resuspended with a small volume of a modified medium devoid of any phosphorus source, so as to obtain a high cell density (about 50 mg l^{-1} Chl), and the resulting suspension was distributed in parallel flasks. At increasing times following the addition of either P_i or glyphosate to a final concentration of 100 µM, aliquots were harvested axenically. Cells were rapidly filtered off, and the residual concentration of either of the compounds in the filtrate was determined. The amounts taken up by the cells were calculated by the difference from time 0 values. The results are the mean ± SD over three independent determinations. A, *Nostoc*; B, *Leptolyngbya*; C, *Spirulina*. In the latter case, no evidence for glyphosate uptake was obtained. In order to assess a possible competition of glyphosate and P_i for the same carrier, a third series of samples was carried out with *Leptolyngbya* by adding equimolar amounts of the two compounds in the meantime. The effect of the concurrent presence of the herbicide on the rate of P_i uptake is shown in D. As a positive control to strengthen the reliability of the indirect evaluation of glyphosate uptake, a similar protocol was also applied to herbicide-sensitive cultured cells of *Nicotiana plumbaginifolia*, following growth in a modified MS medium lacking KH_2PO_4. Because of the larger biomass, in this case P_i was rapidly and completely absorbed from the medium even if supplied at the highest doses (E). In contrast, in the case of glyphosate, the uptake kinetics strongly resembled those obtained with *Nostoc* and *Leptolyngbya* (F).

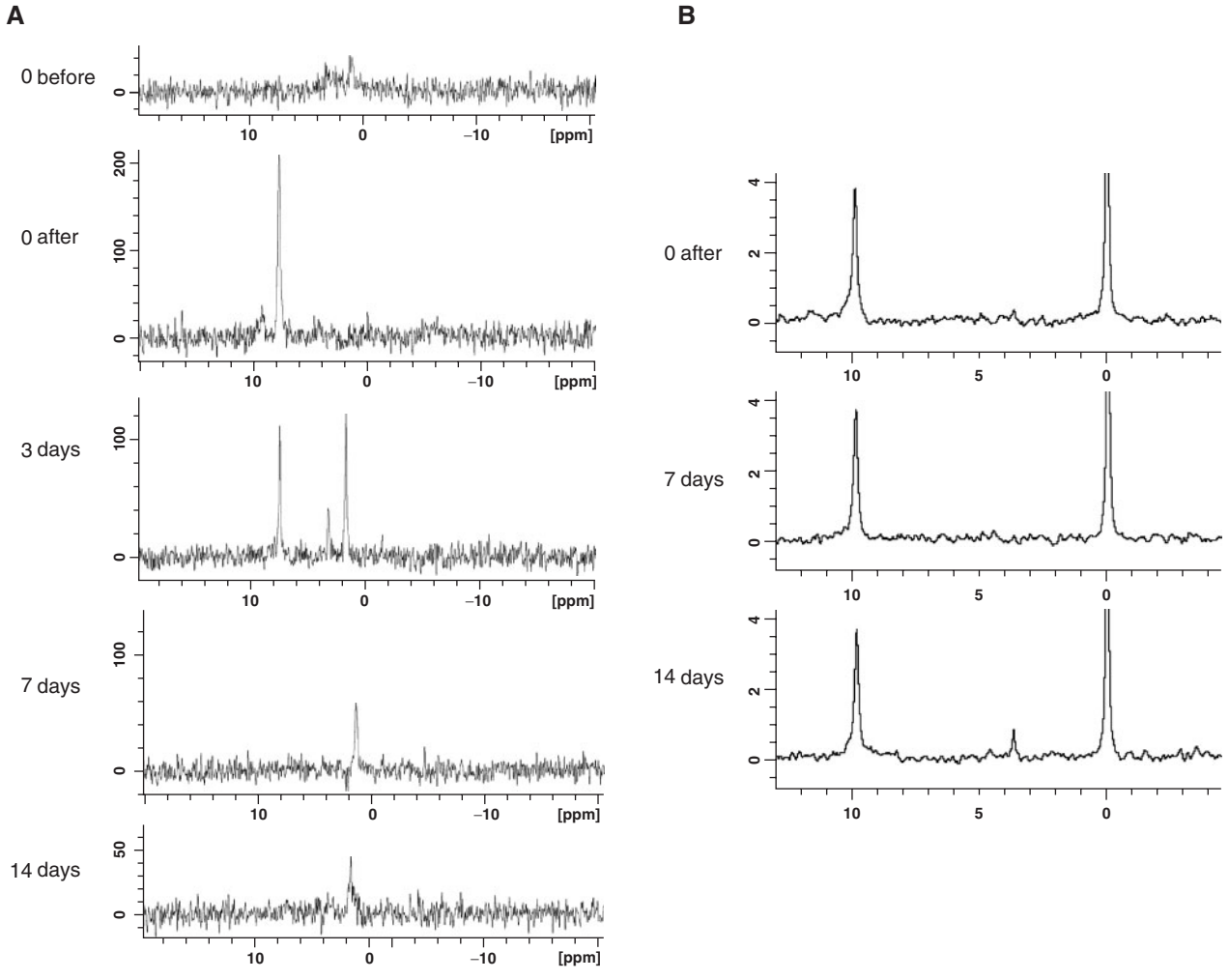

Fig. 6 NMR evidence for glyphosate metabolism in high density *Spirulina* cultures. Cells harvested in the late-log phase were grown for 2 weeks in medium devoid of any phosphorus source, then settled and resuspended in a small volume of the same medium, so as to obtain a high cell density (100–200 mg l^{-1} Chl). Aliquots (0.5 ml) of the resulting suspensions were placed into sterile NMR tubes, to which glyphosate was added, and incubated for a further 14 d under standard conditions. A significant increase of the biomass was evident after the transfer (not shown). ^{31}P-NMR measurements were performed just before and after the addition of glyphosate at 10 mM (A) or 1 mM (B), and at increasing times thereafter, as indicated. The signals in A were attributed to orthophosphate (1.7 p.p.m.), orthophosphate monoester (3.5 p.p.m.) and glyphosate (7.9 p.p.m.) according to the shifts shown by pure standards. Orthophosphoric acid was added to the samples in B as an internal standard (the signal at 0 p.p.m.) just before NMR analysis. In this case the signal at about 10 p.p.m. is attributable to glyphosate, and that at 3.5 p.p.m. to orthophosphate monoester.

Balakumar, 1998). Moreover, contrasting evidence has also been described regarding the sensitivity of the glyphosate target, the shikimate pathway enzyme EPSP synthase (Powell et al. 1992, Forlani and Campani 2001). At least to our knowledge, and although only a small set of strains has been considered, this is the first report in which all the aspects related to the cyanobacterial cell response to glyphosate have been investigated together.

A first conclusion that can be drawn on the basis of the results described herein is a general tolerance of blue-green algae to this phosphonate. The growth of all examined strains was unaffected by the herbicide in the micromolar range. Only millimolar concentrations in some cases resulted in a loss of cell viability. Data on *M. aeruginosa* are consistent with a recent report, in which cyanobacterial growth was inhibited at concentrations $\geq$0.7 mM (120 p.p.m.) (Lopez-Rodas et al. 2007). Glyphosate is applied to the field at a recommended dose of 2.2 kg a.i. ha^{-1}, resulting in normal exposure rates in the soil ranging from 20 to 30 p.p.m. (Stratton, 1990). Therefore, it is quite unlikely that, following further dilution by soil sorption and leaching from the root zone into drainage

water, under normal circumstances the herbicide could be present in water ecosystems at concentrations high enough to inhibit cyanobacterial growth.

What could be the reason for such a widespread tolerance? Two cyanobacteria, namely *Synechocystis* PCC 6803 and *A. variabilis* ATCC 29413, were previously shown to be highly resistant to glyphosate (Powell et al. 1991). For the latter strain, tolerance was related to the presence of a resistant form of the target enzyme (Powell et al. 1992), whereas the analysis of the putative EPSP synthase sequence of the former did not lead to an obvious explanation (Dallachiesa et al. 1994). The characterization of EPSP synthase from the six strains examined here confirmed the occurrence of heterogeneity among cyanobacteria as to their sensitivity to glyphosate. Besides *Anabaena*, *N. punctiforme* was also found to possess a resistant form of the enzyme, while that from the other species was highly sensitive. Although both these strains belong to Section IV, this ought not to be considered a common feature of *Nostocales*. Some other species of the same group were also analyzed (namely *Tolypothrix* and *Cylindrospermum* spp.), and shown to be endowed with a sensitive EPSP synthase (G. Forlani et al., manuscript in preparation). The presence of an insensitive variant of the target enzyme could quite obviously be considered to be the biochemical basis of in vivo tolerance. However, at very high glyphosate concentrations these two strains showed a similar, or even higher susceptibility in vivo than those strains possessing a sensitive enzyme. Interestingly, a significant growth inhibition was evident at levels at which enzyme activity was completely unaffected. Of course the effect in vivo may be higher than that in vitro, particularly in the case of competitive inhibition, where the intracellular concentration of the competing substrate, in this case PEP (Cole 1985), could be non-saturating. However, such a behavior is suggestive of the occurrence of secondary targets, that in the presence of a resistant EPSP synthase may thus play a significant role. For instance, in the millimolar range, the activity of the Co^{2+}-dependent isoform of the first enzyme in the shikimate pathway, 3-deoxy-*arabino*-heptulosonate-7-phosphate synthase (EC 4.1.2.15), was also found to be susceptible to glyphosate (Racchi et al. 1995). However, this also implies that the widespread tolerance to glyphosate shown by all the examined cyanobacteria may not be primarily related to the properties of the target enzyme. In fact, our data suggest that such a tolerance may be provided by several mechanisms in a cooperative manner.

In early studies, the presence of degradative pathways in glyphosate-resistant *Synechocystis* PCC 6803 and *A. variabilis* ATCC 29413 was excluded (Powell et al. 1991). However, four strains out of six were found here to grow in a modified medium that contained the herbicide as the only phosphorus source. No P_i release was detected; thus the expression of extracellular phosphatases able to hydrolyze the C–P bond, as claimed by Ravi and Balakumar (1998), seems unlikely. Other results consistently supported the occurrence of glyphosate metabolization. A minor but significant decrease of the residual concentration of the herbicide in the medium was found in the case of *Nostoc* and *Leptolyngbya*, similar to that in suspension-cultured plant cells. In previous studies, a labeled active ingredient never accumulated to a higher internal than external level (Powell et al. 1991), thus its steady decrease with time in exhausted media (Fig. 4) should correspond to its utilization. Also, mainly, ^{31}P-NMR evidence obtained with high density *Spirulina* cultures (Fig. 6) clearly showed a gradual disappearance of the signal corresponding to the herbicide molecule, concomitantly with the increase of those signals corresponding to orthophosphate and orthophosphate monoester. Of course such a metabolic capability has to be considered a minor one, otherwise it would confer full tolerance to the herbicide even at the highest external concentrations. The whole set of data thus seems to suggest that some hydrolytic enzyme(s) inside the cyanobacterial cells may be able to recognize the herbicide molecule as a substrate, even though with low efficiency. The resulting low rate of C–P bond hydrolysis could not be able to destroy high levels of glyphosate, but may be enough to provide the cell with the low amounts of phosphate that are required for growth (Fig. 4) in the absence of more physiological phosphorus sources.

On the other hand, the low permeability of the cell membrane to the phosphonate seems to represent another mechanism that allows some cyanobacterial strains to grow in the presence of the herbicide. At micromolar concentrations, those at which diffusion across the membrane does not work (Denis and Delrot 1993), glyphosate incorporation seems to proceed through a low affinity carrier, and low amounts of herbicide are incorporated into the cell (Fig. 5A, B). This is also true for glyphosate-sensitive plant cells (Fig. 5F), consistent with previous data (Hetherington et al. 1998), and suggests that such low levels may be sufficient for the expression of herbicide toxicity. However, low glyphosate levels may be rapidly destroyed by the above-discussed hydrolytic activiti(es), providing in the meantime the phosphate required for growth. In phosphorus-starved cells the rate of P_i uptake was found to increase greatly (Fig. 5). This could rely upon either starvation-induced expression of a high affinity transporter, as in enterobacteria (Ternan et al. 1998), or, according to Ray and co-workers (1991), could occur through higher expression of a constitutive, low affinity carrier. In either case, even in phosphorus-starved cells the uptake of glyphosate was almost negligible. This seems particularly true in the case of *Spirulina*, the growth of which is completely unaffected by external glyphosate concentrations up to 10 mM.

Consistent, with this finding, this strain was unable to use the phosphonate as a phosphorus source, although it apparently possesses metabolic pathway(s) for the herbicide (Fig. 6). Only at high concentrations, and in high density cultures, when transport across the membrane is driven by diffusion (Denis and Delrot, 1993), does significant uptake occur. If it exceeds the metabolic capability, growth is inhibited. Several findings accounting for a strikingly higher sensitivity of cyanobacteria to commercial formulations of glyphosate (that contain surfactants) than to the active principle (Powell et al. 1991, Issa 1999; J. Lipok et al. unpublished data) are consistent with such a general picture. However, some variability seems to exist regarding cell permebility to glyphosate, since other strains of *S. platensis* were indeed found to use the active principle as the only phosphorus source (Lipok et al. 2007).

Interestingly, in the presence of orthophosphate, the low uptake rates shown by some strains were completely abolished. Such an effect might rely upon either a direct competition for the same carrier, or a repression of either or both the low affinity transporter and the hydrolytic activity(ies) that recognize glyphosate as a substrate. All these possibilities have been reported in bacteria (Pipke et al. 1987, Ray et al. 1991, Wanner 1992, Ternan et al. 1998) and higher plants (Denis and Delrot 1993). Data described in Fig. 5D seem to strengthen the first hypothesis. Work is under way in our laboratories to elucidate these aspects further.

Materials and Methods

Strains and growth conditions

Collection cyanobacterial strains, as listed in Table 1, were grown at $25 \pm 1^{\circ}C$ under 16 h days (300 $\mu mol\, m^{-2}\, s^{-1}$ PAR in the case of *Spirulina* and *Arthrospira*, and 200 $\mu mol\, m^{-2}\, s^{-1}$ for the other strains) and 8 h nights in 250 ml Erlenmayer flasks containing 60 ml of minimal medium (ATCC medium 1679 and 616, respectively). Cells were subcultured every 3 weeks by transferring 10 ml aliquots to 50 ml of fresh medium. Growth was followed by destructive harvest: 0.5–1.2 ml aliquots were withdrawn, and cells were sedimented by centrifugation for 10 min at $14{,}000 \times g$. Pellets were resuspended with 1.0 ml of methanol, and solubilization was allowed to proceed for 30 min in the dark, with occasional mixing. Samples were then centrifuged as above, and the total chlorophyll content in the supernatant was determined spectrophotometrically on the basis of the Arnon formula.

To assess the effect of increasing glyphosate levels upon cell growth, late log-grown cells were used to inoculate 25-well square Petri dishes, 4.0 ml per well, to a density of 2.0 $mg\, l^{-1}$ Chl (4.0 in the case of *Spirulina* and *Arthrospira*). Suitable water dilutions of a filter-sterilized 1.0 M solution of the herbicide, brought to pH 7.0 with KOH, were added to obtain concentrations ranging from 0.01 to 10 mM. A completely randomized design was adopted, with three replications for each dose (four for untreated controls). In each well, cell growth was followed for 3 weeks by destructive harvest, as indicated. Following logarithmic transformation of data, growth constants and generation times were calculated from the linear portion of each curve. A similar protocol was used to evaluate the ability of growing in a medium containing the herbicide as the only source of phosphorus. Wells were filled with modified media in which P_i had been omitted. A factorial of various combinations of glyphosate and K_2HPO_4 (filter-sterilized) was added in a completely randomized design with four replicates. Before the inoculum, late log-grown cells were sedimented by centrifugation for 5 min at $4{,}000 \times g$, and washed twice with medium lacking any phosphorus source.

Some experiments were also performed with 1 liter or 100 ml Erlenmeyer flasks, but maintaining the same culture-to-flask volume ratio. When the aim was to evaluate the ability of a given strain to grow in the presence of glyphosate as the only phosphorus source, in some cases inocula were starved before use by growing for up to 14 d in media in which P_i had been omitted, as indicated.

Analytical methods

Phosphate determination. P_i was quantified colorimetrically by means of the green malachite acid dye assay, as described previously (Forlani 1997). Proper sample dilutions in a final volume of 100 µl, were added with 1.0 µl of the malachite green–molybdate–acid solution, followed, after exactly 1 min, by 0.1 ml of 34% (w/v) Na citrate. After 10 min at room temperature, adsorption at 660 nm was measured against exact blanks. Phosphate concentration was calculated on the basis of a molar absorption coefficient ranging from 45,000 to 60,000 $M^{-1}\, cm^{-1}$, evaluated experimentally for each batch of colorimetric solution. Reported values are means ± SD over at least three replicates.

Glyphosate determination. The concentration of the phosphonate in exhausted culture broths was quantified colorimetrically after its oxidation with hydrogen peroxide to orthophosphate as described by Glass (1981), with minor modifications. Briefly, spent medium was centrifuged for 10 min at $14{,}000 \times g$, and suitable dilutions in a final volume of 200 µl were added with the same volume of a 3% H_2O_2 solution. After incubation to dryness for 2 h at 140°C in a ventilated oven, residues were resuspended with 1.0 ml of 50 mM HCl, and the presence of P_i was measured as above. The herbicide concentration was estimated from the difference between the phosphate content before and after oxidation. Each sample was subjected to peroxide treatment in triplicate, and the evaluation of P_i levels in reconstituted samples was carried out on at least four different dilutions.

HPLC analysis. Glyphosate residual concentrations were also determined by reverse-phase HPLC following pre-column derivatization with Sanger's reagent. Sample dilutions in a final volume of 50 µl were sequentially added with 30 µl of a saturated sodium borate solution and 20 µl of a 25 mM 2,4-dinitrofluorobenzene solution in absolute ethanol. Following 60 min incubation at 55°C in the dark and centrifugation for 3 min at $14{,}000 \times g$, derivatized samples (20 µl) were injected onto a 4.6×250 mm Zorbax ODS column (Agilent Technologies) equipped with a 5 mm pre-column, previously equilibrated with 25 mM K phosphate, pH 3.2, containing 40% (v/v) methanol. Isocratic elution proceeded at a flow rate of 1 $ml\, min^{-1}$, monitoring the eluate at 383 nm (Kontron 450; Kontron, München, Germany). Under these conditions, the phosphonate eluted at 2.4 ± 0.1 min. Peaks were automatically integrated (Data System D450) and quantitated by comparison with calibration runs carried out with the standard. The detection limit was about 10 $\mu mol\, l^{-1}$, with an SEM of 0.7% for the predicted *y*-value in the range 50–500 µM.

NMR analysis. Herbicide metabolism was studied in vivo using a whole-cell system by means of ^{31}P-NMR. Following or not phosphorus starvation for 2 weeks, *S. platensis* cells were harvested by centrifugation, washed three times with phosphate-free medium, and resuspended in a minimal volume to obtain high density (100–200 mg Chl l^{-1}) cell cultures, similar to natural biomasses produced in ponds by algal cells floating to the water surface. Aliquots (0.5 ml) were then placed into sterile NMR tubes, supplemented with different phosphorus sources, and allowed to grow under standard conditions. ^{31}P-NMR measurements were performed 0, 3, 7 and 14 d after the transfer with a Bruker AvanceDRX 400 spectrometer operating at 161,976 MHz. All treatments were carried out in triplicate.

Enzyme extraction and assay

Cells in the late exponential phase of growth were harvested on filter papers by vacuum filtration (with the exception of *M. aeruginosa* cultures, collected by centrifugation), resuspended in ice-cold extraction buffer [50 mM HEPES-KOH buffer, pH 7.4, containing 10% (v/v) glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA and 10 μM ammonium molybdate], sedimented by centrifugation and washed twice with the same buffer. Material was then ground with alumina [3 g (g cells)$^{-1}$] in a pre-cooled mortar until a fine paste was obtained. All subsequent operations were carried out at 0– 4°C. The homogenate was resuspended with 10 ml g^{-1} of extraction buffer, and clarified for 10 min at 14,000×*g*. Solid ammonium sulfate was added to the supernatant to give 70% saturation. Precipitated proteins were collected by centrifugation, resuspended with column buffer (25 mM Tris–HCl, pH 7.5, containing 0.5 mM dithiothreitol) and desalted by passage through a Bio-Gel P6DG column (Bio-Rad) equilibrated with the same buffer.

EPSP synthase activity was measured in the forward direction at 35°C by determining the release of P_i using the malachite green dye assay method (Forlani 1997). The reaction mixture contained 50 mM HEPES-KOH, pH 7.4, 1 mM S3P, 1 mM PEP and a limiting amount of enzyme (10–25 pkat) in a final volume of 0.1 ml. After incubation for up to 30 min, the reaction was stopped by the addition of 1 ml of the malachite green colorimetric solution. The activity was calculated from the difference with respect to exact blanks in which S3P had been omitted. The ammonium salt of S3P was purified from the culture broth of *Klebsiella pneumoniae* strain ATCC 25597 and quantified as described previously (Forlani et al. 1992). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Enzyme purification

EPSP synthase was partially purified by anion-exchange chromatography. Desalted extracts were loaded at a constant flow rate of 40 ml h^{-1} onto a DEAE-Sephacel column (1.5 cm diameter, 12 ml bed volume) equilibrated with column buffer. Following extensive washing, proteins were eluted with a linear gradient from 0 to 250 mM NaCl (200 ml), while collecting 4 ml fractions. Active fractions were pooled, desalted as above against the same buffer, and immediately used for biochemical determinations.

Plant cell culture

For comparison, glyphosate uptake was also measured in *N. plumbaginifolia* cultured cells, that have been shown to be sensitive to the phosphonate at the micromolar level (LD_{50} 117 ± 14 μM; Forlani et al. 2000). Culture conditions were as reported previously. The rate of phosphonate uptake was determined on exponentially growing cells following or not phosphorus starvation for 5 d in a modified MS medium in which KH_2PO_4 had been omitted. Cells were adjusted to a density of 200 g l^{-1} (FW) and allowed to recover for 1 h prior of glyphosate addition. In order to increase the rate of herbicide uptake, the absorption medium described by Hetherington and co-workers (1998) was also used instead of MS medium.

Statistical analysis

Linear regression equations were computed by using an electronic worksheet. Data were analyzed by standard statistical procedures for analysis of variance and *t* test. Confidence limits were calculated according to Snedecor and Cochran (1989).

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