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Degradation of the phosphonate herbicide glyphosate in soil: evidence for a possible involvement of unculturable microorganisms

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Abstract

The properties of microbial strains responsible for the rapid mineralization of the herbicide glyphosate in soil were investigated in soil–water mixtures supplemented with 10 mmol l^{-1} active ingredient. Over 2 weeks degradation kinetics were linear, as expected in the case of non-growth-linked metabolization, and the rate of utilization was not enhanced following repeated treatment of the soil with increasing herbicide doses. The availability of exceeding phosphorus, nitrogen and carbon sources did not affect the rate of glyphosate utilization, that was maximal under conditions of neutral pH, high oxygen and low osmolarity. The screening of 1200 bacterial strains isolated on a rich medium in the absence of the herbicide failed to identify any strain able to cleave the glyphosate molecule. When antibiotics with different mode of action were added to the mixtures, while some inhibitors of protein synthesis exerted considerable effects, those that are active only against actively-proliferating cells were scarcely effective. An MPN analysis was performed to enumerate degrading microorganisms, but in no dilution the same extent of utilization measured in the original mixture could be found. Results suggest that at least the first steps in herbicide degradation could be accomplished by some microbial species unable to grow in vitro and form visible colonies on plates.

1. Introduction

During recent years an intensive use of herbicides has raised increasing concern mainly due to their massive pollution of the environment. To address this problem, the herbicide glyphosate (*N*-[phosphonomethyl]glycine) might be very promising. It acts by interfering with the enzyme that catalyses the sixth step in the shikimate pathway, 5-*enol*-pyruvyl-shikimate-3-phosphate (EPSP) synthase (Cole, 1985), has little or no chronic and neuro-toxic effects, and no obvious carcinogenic and mutagenic activity as well, and is thus to be considered ineffective against man and animals (Atkinson, 1985). Moreover, even though the rate of utilization has been reported to vary considerably between different soils, it is rapidly and completely degraded by soil microorganisms to water, carbon dioxide and phosphate. Following adsorption through the phosphoric acid moiety, mineralization proceeds without any lag phase, and seems to be a co-metabolic process that occurs under both aerobic and anaerobic conditions (Torstensson, 1985). The first step in the predominant degradation pathway is the cleavage to glyoxylate and amino–methyl–phosphonic acid (AMPA), that is also biologically degradable (Rueppel et al., 1977). A slower degradation of AMPA than glyphosate has been reported in some instances, possibly reflecting tighter binding to soil (Torstensson, 1985).

However, the use of glyphosate is limited by the lack of distinguishing between weeds and crops, so that the availability of herbicide-resistant crops would

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be of greatest agronomical value (Kishore et al., 1992). This goal seemed to be achieved when bacterial genes encoding for glyphosate-resistant EPSP synthases were cloned, endowed with chloroplast transit signals and used to transform plants (Della-Cioppa et al., 1987). Despite these results, with the only exception of soybean Roundup Ready™ (Monsanto), commercial level tolerance to glyphosate in crops has not been achieved to date, possibly due to the occurrence of secondary targets of the herbicide in plant metabolism which could result in a yield penalty (Racchi et al., 1995).

Such a drawback might be overcome by transforming plants with genes of microbial origin encoding for cleaving enzymes, yet in spite of many efforts the microbial species responsible for the rapid mineralization of glyphosate in the soil have not been identified. Several bacterial strains were isolated that were able to convert glyphosate to sarcosine and inorganic phosphate under phosphorus-limiting conditions, but such ability was lost when other P sources were made available (Dekker and Duke, 1995). A strain of *Arthrobacter atrocyaneus* was indeed reported to metabolize glyphosate to AMPA (Pipke and Amrhein, 1988). However, in this case also, inorganic phosphate completely inhibited (or repressed) herbicide degradation. Only an *Achromobacter* strain taken from a glyphosate waste stream treatment facility was found to efficiently cleave glyphosate to AMPA and glyoxylate (Barry et al., 1992). A gene encoding for a glyphosate oxido–reductase was cloned from this microbe, and its expression in plants was found to impart herbicide resistance (Padgette et al., 1996). However, the enzyme was not isolated, and little information about the properties of such strain was made available.

With the ultimate aim of isolating degrading strains and obtaining tolerant crop plants based on herbicide detoxification, we evaluated the kinetics of glyphosate metabolization in both untreated soils and samples that had been previously treated with increasing rates of the herbicide. The activity of utilizing microorganisms was measured in soil–water mixtures either in response to nutrient amendments or under different conditions of pH, osmolarity and oxygen availability.

2. Materials and methods

2.1. Determination of glyphosate degradation in soil–water mixtures

Commercially available potting mixtures were purchased at local markets. Chemical characteristics are presented in Table 1. Soil samples (5 g) were transferred into 120 ml Erlenmeyer flasks and resuspended in 20 ml of sterile water, supplemented when appropriate with different compounds. Technical grade glyphosate (≥99% by HPLC, kindly provided by Sipcam Ltd, Salerano, adjusted to pH 7.0 with potassium hydroxyde and filter-sterilized) was added to a final concentration of 10 mmol l^{-1}. Incubation was in the dark on a rotary shaker (150 rpm) at 30 ± 1°C. At increasing time after inoculation (every day over two weeks, but for d 6 and d 13) 500 µl aliquots were withdrawn from each mixture, centrifuged for 10 min at 8000*g*, and the residual concentration of glyphosate in the supernatant was determined by RP-HPLC following pre-column derivatization 5 min at 50°C with 130 mM *p*-toluene-sulphonyl chloride in acetonitrile (1:1 by volume; Tomita et al., 1991). Derivatized samples (20 µl) were injected onto a 4.6×250 mm Zorbax

Table 1
Rates of glyphosate degradation in different commercial potting mixtures

Trademark	pH (H_2O)	Organic matter (%)	Total C (%)	Total N (%)	Free NH_4^+ (µmol g^{-1})	NO_2^- (µmol g^{-1})	Cl^- (µmol g^{-1})	Available P (µmol g^{-1})	Glyphosate degradation (µmol g^{-1} d^{-1})
Fertiflora	7.6	35.5	20.6	2.0	1.06	0.40	31.9	0.16	0.30 ± 0.04
Greenworld, acid	4.2	80.8	48.0	0.9	1.35	0.14	11.9	4.25	0.14 ± 0.07
Greenworld, neutral	6.2	78.3	47.9	0.9	0.05	0.12	2.2	1.92	2.37 ± 0.08
Hobbyflora	8.5	20.3	11.6	0.5	0.79	0.13	32.6	0.25	0.69 ± 0.03
Radicom	7.0	36.2	21.2	0.5	0.23	0.09	5.3	0.78	1.81 ± 0.02
Tercom	7.1	41.8	23.9	1.2	0.06	0.09	3.0	0.35	1.26 ± 0.11
Tercompost	7.3	20.6	12.7	0.5	0.04	0.10	13.5	0.19	0.92 ± 0.02
Terflor	6.9	49.5	30.5	1.0	0.03	0.07	10.2	0.32	1.81 ± 0.12
Tergarden	6.9	36.1	24.0	0.5	0.03	0.11	5.3	0.82	1.42 ± 0.06

Samples (5 g) of each soil were transferred into 120 ml flasks and resuspended in 20 ml of sterile water containing 10 mmol glyphosate l^{-1}. At increasing time after the inoculum the residual concentration of the herbicide in the supernatant was determined by RP-HPLC. The rate of glyphosate degradation was estimated utilizing the linear regression equation of residual amount plotted against the time of incubation. Each treatment was run in triplicate; mean values ± S.E.M. are reported.

ODS column (DuPont) equilibrated with 50 mM sodium phosphate, pH 2.3, containing 15% (v/v) acetonitrile. Isocratic elution proceeded at a flow rate of 1 ml min^{-1}, monitoring the eluate at 280 nm. Under these conditions, AMPA eluted at 7.2 ± 0.1 min, and glyphosate at 8.8 ± 0.2 min. Following isocratic elution, the flushing of 70% (v/v) methanol into the column allowed release, detection and quantitation of the derivatization product of sarcosine (not shown). Peaks were automatically integrated (Kontron Data System D450) and quantitated by comparison with calibration runs carried out with authentic standards (Sigma Chemical Co.). Detection limit was about 0.5 mmol l^{-1} for all compounds. The S.E. for the predicted *y*-value in the regression was 2.3 and 2.1% for glyphosate and AMPA, respectively. The rate of herbicide degradation was estimated utilizing the linear regression equation of residual glyphosate concentration plotted against the time of incubation, and was expressed as μmol (g of soil)$^{-1}$ d^{-1}. Each treatment was run at least in triplicate; mean values $\pm$ S.E. are reported. The release of inorganic phosphate was determined by the malachite green dye method as described by Forlani (1997).

The effect of pH upon the activity of utilizing microorganisms was evaluated by resuspending the soil into equimolar mixtures of MES (2-[*N*-morpholino]ethanesulfonic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) and AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid) at an overall concentration of 200 mM, adjusted to different pH values with potassium hydroxide. The effect of decreased oxygen availability was determined by reducing flask shaking, and the corresponding oxygen level was measured with a commercial detection kit (Hannah Instruments).

2.2. Soil treatment with increasing glyphosate concentrations

To evaluate whether a repeated treatment with glyphosate would enrich the soil for herbicide-utilizing strains, a 20 l container was filled with a mixture (0.1:1 by weight) of sand:loam (Greenworld neutral), and seeds of maize and various weeds were sown 2 cm deep in the soil. Plantlets were grown in a growth chamber, and were periodically watered as required with tap water. One month after planting, 250 ml of an aqueous dilution of a glyphosate commercial formulation (Round-up®, Monsanto), corresponding to a 1 mM solution of the active ingredient, was applied to the surface of the pot. This was equivalent to a herbicide application of 2.4 kg ha^{-1}. The treatment was repeated every 2 weeks by increasing the herbicide rate to 4.8, 9.6, 24, 48 and 96 kg a.i. ha^{-1}.

2.3. Microbial strains isolation, screening for glyphosate degradation and MPN analysis

Microbial strains were isolated from potting loam (Greenworld neutral) following shaking for 16 h with 5 ml g^{-1} of 10 mmol l^{-1} sodium pyrophosphate solution, a treatment that was found to yield maximal recovery (not shown). Serial dilutions were plated onto NMMP agarized medium containing per liter 5 g casein, acid hydrolyzed, 2 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 2.61 g K_2HPO_4, 2.07 g NaH_2PO_4, and 1 mg each of $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$ and $CaCl_2$ (Hopwood et al., 1985) and incubated for up to 7 d at 30°C. In the attempt to isolate microbial strains able to utilize glyphosate as the only N or C source, serial dilutions were plated onto a modified N- or C-free minimal medium (Davies and Mingioli, 1950) containing 1 and 20 mM glyphosate, respectively. Plates also containing 0.1 g l^{-1} yeast extract were also used, to allow possible growth of auxotrophs.

To evaluate glyphosate-utilizing activity, isolates were grown overnight in liquid medium; preinocula were adjusted with fresh medium to an O.D. of 1.0 (600 nm), and 0.1 ml aliquots were used to start cultures (20 ml of NMMP medium in 120 ml Erlenmeyer flasks) in the presence of 10 mmol l^{-1} glyphosate. Ten strains were co-inoculated in the same flask. After 1, 4 and 14 d of incubation at 150 rpm at 30°C, 500 μl aliquots were withdrawn, and the residual concentration of the herbicide was estimated as above. MPN analyses were performed in both water–soil mixtures and samples in which soil (Greenworld neutral) had been resuspended in liquid NMMP medium. Dilutions from 10^0 to 10^{-3} were carried out, with 10 replications. Glyphosate degradation was evaluated as indicated 7, 14 and 21 d after inoculation.

3. Results

A preliminary screening was performed to evaluate the ability of microorganisms from different soils to metabolize the herbicide glyphosate. Soil–water mixtures were supplemented with 10 mmol glyphosate l^{-1} and incubated for up to 14 d at 30°C: results, summarized in Table 1, showed a widespread presence of microbial strains able to metabolize this compound. In autoclaved control samples, after an initial decrease to 9.3–9.2 mmol l^{-1}, most likely due to a partial adsorption to clay and organic matter through the phosphonic acid moiety (Sprankle et al., 1975a), no further reduction of glyphosate concentration was evident in the mixture supernatant. On the contrary, in non-sterilized samples of all nine soils tested the concentration of the herbicide was found to be progressively reduced.

In no case substantial amounts (≥0.5 mmol l^{-1}) of the two major alternative metabolites, sarcosine and AMPA (Torstensson, 1985), could be detected. Degradation kinetics were linear, with a nearly constant rate that began to slow down when the residual concentration of the herbicide dropped below 2 mmol l^{-1} (data not shown). However, significant differences were found as to the extent of glyphosate utilization, with rates ranging from 0.14 to 2.37 μmol g^{-1} d^{-1} (1.4–23.7% of the initial concentration). No relationship was evident between this trait and the chemical characteristics of the soils (Table 1). Only the potting mixture which had shown the maximal rate of glyphosate breakdown (Greenworld neutral) was used in further determinations, the results of which are reported below.

In soil samples, the herbicide-metabolizing population was found to be stable with time. Experiments performed after a 10 month storage of the soil at room temperature showed no significant decrease in the rate of glyphosate degradation (2.39 ± 0.10 μmol g^{-1} d^{-1}, $n = 15$). In a representative trial, the results are depicted in Fig. 1, half of the herbicide added to the mixture was metabolized during the first 7–8 d of incubation. In the meantime, lower but proportional amounts of inorganic phosphate, most likely derived from the breakdown of the compound, were released in the medium. Attempts to isolate microbial strains onto agarized minimal media containing the herbicide moiety as the only N or C source failed. A repeated treatment of the soil with increasing doses of the herbicide with the aim to enrich it for utilizing strains also failed: treated samples showed rates of glyphosate degradation even lower than those of untreated soil (1.75 ± 0.13 vs. 2.49 ± 0.26 μmol g^{-1} d^{-1}, $n = 4$, $P < 0.05$).

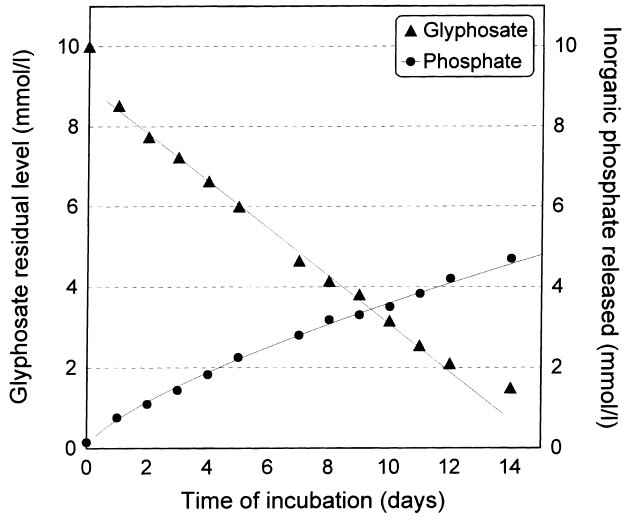

Fig. 1. Glyphosate degradation in a water–soil mixture. Soil (5 g) was transferred into a 120 ml flask and resuspended in 20 ml of sterile water containing 10 mmol l^{-1} of technical grade glyphosate. At increasing time after the inoculum the residual concentration of the herbicide in the supernatant was determined by RP-HPLC. The concentration of inorganic phosphate in the same samples was measured colorimetrically by the malachite green dye assay.

Table 2
Effect of nutrient availability on glyphosate degradation in soil–water mixtures

Addition	Rate of glyphosate degradation (μmol g^{-1} d^{-1})
Water–soil mixture	2.47 ± 0.23
+ phosphate 1 mM	2.73 ± 0.08
+ phosphate 10 mM	2.76 ± 0.07
+ nitrate 5 mM + ammonium 1 mM	2.49 ± 0.12
+ glucose 5.5 mM	2.43 ± 0.07
+ PCA medium	$0.34 \pm 0.03^{***}$
+ LB medium	$0.15 \pm 0.05^{***}$
+ NMMP medium	$4.84 \pm 0.25^{***}$

Soil samples (5 g) were transferred into 120 ml flasks and resuspended in 20 ml of sterile water containing 10 mmol glyphosate l^{-1}, supplemented as appropriate, or directly in 20 ml of the indicated culture medium. The rate of glyphosate degradation was estimated utilizing the linear regression equation of residual amount plotted against the time of incubation. Each treatment was run in triplicate; mean values ± S.E.M. are reported. Significant differences to the control (water–soil mixture without amendments), calculated by using standard statistical procedures for analysis of variance and t test according to Glantz (1988), are indicated ($^{***}P < 0.01$, $^{**}P < 0.05$, $^{*}P < 0.10$).

To ascertain whether the availability of other P, N and C sources could affect the rate of glyphosate degradation, as previously reported in the case of several bacterial strains which were capable of metabolizing the herbicide in pure cultures only under conditions of phosphate starvation (Dekker and Duke, 1995), soil–water mixtures were supplemented with exceeding concentrations of various nutrients. Results, outlined in Table 2, accounted for a constitutive expression of microbial activities responsible for glyphosate degradation, since in no case a significant reduction or increase in the rate of utilization was found. Contrasting results were obtained when soil samples were resuspended in some commonly used culture media: undefined rich media as PCA and LB caused a severe inhibition, while a medium specifically developed for *Streptomyces* strains (NMMP, Hopwood et al., 1985) strongly enhanced the rate of glyphosate metabolization. As the inhibitory effects exerted by the above additions could result indirectly from the proliferation of other microbial strains leading to variation in chemical or physical factors such as pH and oxygen availability, the rate of herbicide degradation in soil–water mixtures was evaluated also by varying such factors.

Table 3
Effect of pH, NaCl and oxygen availability on glyphosate degradation

Addition	Rate of glyphosate degradation (μmol g^{-1} d^{-1})
Water–soil mixture	2.51 ± 0.25
buffered at pH 5.0	$0.70 \pm 0.07^{***}$
buffered at pH 6.0	$1.62 \pm 0.14^{**}$
buffered at pH 7.0	2.40 ± 0.06
buffered at pH 8.0	$1.23 \pm 0.09^{***}$
buffered at pH 9.0	$0.12 \pm 0.02^{***}$
+ NaCl 100 mM	$1.70 \pm 0.07^{**}$
+ NaCl 200 mM	$0.55 \pm 0.04^{***}$
+ NaCl 400 mM	$0.18 \pm 0.04^{***}$
0.62 mmol O_2 l^{-1}	2.20 ± 0.15
0.50 mmol O_2 l^{-1}	$0.91 \pm 0.06^{***}$
0.25 mmol O_2 l^{-1}	$0.34 \pm 0.10^{***}$

Soil samples (5 g) were transferred into 120 ml flasks and resuspended in 20 ml of sterile water containing 10 mmol glyphosate l^{-1}, supplemented as appropriated. Oxygen availability was varied by varying flask shaking. The value for the control was 0.87 mmol l^{-1} O_2. Each treatment was run in triplicate; mean values $\pm$ S.E.M. are reported. Statistical analysis as in Table 2.

Results, reported in Table 3, showed maximal activity of degrading strains under conditions of neutral pH and high oxygen content. Moreover, the supplementation with NaCl, a compound that is included at high concentration in several media (e.g. LB), was found to severely affect glyphosate mineralization (Table 3). The overall results were carefully taken into account in the attempt to recover metabolizing strains under pure culture, for which all the conditions that had been found to maximize the rate of herbicide breakdown were used. About 1200 isolates obtained by plating serial dilution of an active soil–water mixture onto NMMP medium were examined. Not one of the isolates grew on glyphosate as the only N or C source, or even utilized the herbicide by co-metabolism. To rule out the hypothesis that degrading strains could strictly adhere to soil particles and thus may be negatively selected during serial dilutions, the soil was soaked overnight in the presence of various detergents prior to isolation (Kepner and Pratt, 1994). Microbial recovery was in fact found to be improved by such treatments, with maximal yield obtained after soaking with 10 mM sodium pyro-phosphate (data not shown). However, also in this case none of the strains capable of forming visible colonies on NMMP was found to catalyze the breakdown of the glyphosate moiety when grown in liquid medium.

In order to get more information about the nature of utilizing microbial strains, the rate of glyphosate degradation was measured in soil–water mixtures in the presence of several antibiotics with different mode of action. Results are summarized in Table 4. The substantial ineffectiveness of cycloheximide along with the

Table 4
Effect of various antibiotics on glyphosate degradation by soil microorganisms

Addition	Rate of glyphosate degradation (μmol g^{-1} d^{-1})
NMMP medium	4.61 ± 0.11
+ cycloheximide	$4.01 \pm 0.21^{*}$
+ ampicillin	$1.82 \pm 0.31^{***}$
+ bacitracin	4.39 ± 0.06
+ carbenicillin	4.43 ± 0.30
+ cefotaxime	$2.20 \pm 0.35^{***}$
+ penicillin	4.22 ± 0.39
+ chloramphenicol	$0.12 \pm 0.07^{***}$
+ nalidixic acid	$0.03 \pm 0.03^{***}$
+ rifampicin	$0.15 \pm 0.07^{***}$
+ streptomycin	$0.06 \pm 0.03^{***}$

Soil samples (5 g) were transferred into 120 ml flasks and resuspended in 20 ml of NMMP medium containing 10 mmol glyphosate l^{-1}. Filter-sterilized antibiotics were added to a final concentration of 100 μg ml^{-1}. Each treatment was run in triplicate; mean values $\pm$ S.E.M. are reported. Statistical analysis as in Table 2.

potent inhibitory effect exerted by streptomycin and nalidixic acid strongly suggested that procaryotes are responsible for at least the first steps in herbicide degradation. Moreover, all those compounds that act by interfering with some activity required for cell division resulted barely effective or had no effect, while most substances which impair cell functions strictly required also by not-proliferating cells (as inhibitors of protein synthesis) were found to exert considerable results. Finally, MPN experiments were performed in order to enumerate degrading microorganisms. The possibility that such strains may represent a very restricted fraction of total soil inhabitants could in fact account for the failure of the screening performed with $\times 1000$ isolates in pure culture. Since glyphosate had been found to not sustain bacterial growth per se, the assay was carried out both in soil–water mixtures and in co-metabolism by serially diluting a mixture with NMMP liquid medium. Even if the incubation was maintained for up to 3 weeks, not in one of 10^{-1} dilutions was the same extent of glyphosate degradation found as that in the primary mixture, and in all 10^{-2}-dilutions herbicide utilization, if any, was not discernible from the inaccuracy (about 2.2%) of the HPLC method employed to quantify the residual concentration of glyphosate.

4. Discussion

While plants metabolize the herbicide glyphosate to little, if any extent (Stock et al., 1991), microbial activities were early found to lead to a rapid and complete mineralization of the compound in soil (Sprankle et al., 1975b; Torstensson, 1985). However, with the

only exception of an *Achromobacter* strain taken from a glyphosate waste stream treatment facility (Barry et al., 1992), and despite the numerous attempts, in no case the isolation of a microbial strain able to utilize constitutively the herbicide in pure culture has been reported to date. A number of microorganisms, however, have been described that are capable of utilizing this phosphonate as a source of phosphorus when grown in media in which inorganic phosphate had been omitted, but the latter was inhibitory to glyphosate breakdown (e.g. Kishore and Jacob, 1987; Pipke and Amrhein, 1988). In a study aiming to investigate the occurrence of glyphosate-degrading activity in environmental isolates, a screening of 163 strains showed 26 isolates able to metabolize the herbicide to sarcosine and inorganic phosphate under condition of P starvation, but in no case evidence for its metabolism or co-metabolism to AMPA was obtained (Dick and Quinn, 1995). Since sufficient phosphate is usually present in most environments to satisfy the microbial demand, and the bulk of herbicide metabolism in the soil was shown to result in the conversion of glyphosate to AMPA, a compound in which the C–P bond is still conserved (Rueppel et al., 1977), these results conflict with the widespread ability of soil microbial populations to metabolize the glyphosate molecule, a fact that is confirmed also by our results. In an initial screening all the nine potting mixtures tested, to a different extent, were found to rapidly break down the herbicide. Chemical analysis of residues in the soil is both complex and time-consuming, and made difficult by the tendency of the phosphonate to adsorb to organic matter (Sprankle et al., 1975a,b). Thus most laboratory degradation studies have been conducted to date with ^{14}C-glyphosate, with the rate of $^{14}CO_2$ evolution being used as an indicator of herbicide breakdown (e.g. Smith and Aubin, 1993). To overcome such difficulties, a herbicide concentration largely exceeding those possibly deriving from field treatments was applied to the soil in this study. This might cause the results to be somewhat inconsistent with those obtainable in the field, as the activity of microbial strains whose EPSP synthase is inhibited by high concentrations of the herbicide might be impaired. Moreover, microorganisms may exhibit in vitro different physiological responses due to differing conditions of temperature, nutrient and oxygen availability. Even bearing in mind such criticisms, the results we obtained clearly indicate that microbial utilization of glyphosate in the soil also occurs at a high rate in the presence of other P, N and C sources. However, degradation kinetics were in all cases linear, and no colony was obtained by plating onto minimal media containing the herbicide as the sole C or N source. The possibility that herbicide degradation occurs by co-metabolism is also strengthened by the failure of repeated treatments of the soil to enhance the activity of utilizing microorganisms: as C is limiting, while usually sufficient N and P are present in most soils to accomplish the microbial requirement, there is no selective advantage for an organism unable to use the herbicide as the only C source. Conversely, the lack of detectable amount of both AMPA and sarcosine in soil–water mixtures accounts for a complete mineralization of the herbicide, and the continuous release of high amounts of inorganic phosphate into the medium as glyphosate breakdown proceeds rules out the possibility that the initial cleavage of the compound could be accomplished to supply microorganisms with P.

This notwithstanding, the analysis of >1000 colonies isolated from the same soil in which glyphosate was promptly metabolized failed to identify any microorganism able to degrade the herbicide. The analytical method employed is capable of detecting any modification in its moiety. Thus, even if the complete degradation of glyphosate would require the coordinated action of several microbial species, such a screening should allow the detection of at least the strains which bring about the first step in the sequence. The evaluation of the effect of several antibiotics on the rate of glyphosate utilization shed some light on such an apparent anomaly. Beyond providing evidence of a leading role for bacteria (even if a possible involvement of fungal strains in the overall process of glyphosate mineralization could not be excluded), the results strongly point at quiescent micro-organisms as the responsible for herbicide breakdown. Most compounds tested that inhibit only actively proliferating bacteria were substantially ineffective, while all those acting by interfering with RNA or protein synthesis almost completely hindered glyphosate metabolism. The possibility that at least the first steps in herbicide degradation may be accomplished by bacterial strains which are quiescent (or proliferate so slowly to be unable to form visible colonies on agar plates) could account for the failure of both the enrichment procedure based on repeated treatment of the soil with increasing doses of the herbicide and the screening of isolates for the ability of degrading the compound. This hypothesis is consistent with degradation kinetics in soil–water mixtures amended with exceeding concentrations of nutrients, that resulted in linear rates with time. It is also supported by the results of the MPN analysis performed to quantify glyphosate-utilizing microorganisms: in the absence of cell proliferation in no dilution the same extent of utilization can actually be expected as that in the original suspension.

Until very recently, microbial identification required the isolation of pure cultures followed by testing for multiple physiological and biochemical traits. This led to the so-termed *great plate count anomaly* (the discrepancy between direct microscopic count and viable

plate count), with an underestimation of the microbial diversity in most habitats. It is now generally accepted that even more than 99% of microbes are unculturable with the commonly established methods (Torsvik et al., 1990). In situ detection techniques are currently under development in order to allow the study of such, yet uncultivated microorganisms (Kepner and Pratt, 1994; Amann et al., 1995). Thus it is not surprising that glyphosate degradation might be accomplished by some of these species. Nonetheless, this might represent a drawback in obtaining glyphosate tolerant crop plants based on herbicide detoxification. However, direct DNA extraction from the soil could allow likewise the cloning and biotechnological use of bacterial activities responsible for the breakdown of the herbicide. Work is under way in our laboratory in order to prove the feasibility of such an approach.

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