Letters in Applied Microbiology 1995, **20**, 349–352

Effects of glyphosate on nitrogen fixation of free-living heterotrophic bacteria

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DST/65: received 12 December 1994 and accepted 13 December 1994

A. SANTOS AND M. FLORES. 1995. The effect of the herbicide glyphosate (*N*-(phosphonomethyl)glycine) on the growth, respiration and nitrogen fixation of *Azotobacter chroococcum* and *A. vinelandii* was studied. *Azotobacter vinelandii* was more sensitive to glyphosate toxicity than *A. chroococcum*. Recommended dosages of glyphosate did not affect growth rates. More than 4 kg ha^{-1} is needed to find some inhibitory effect. Specific respiration rates were 19·17 mmol O_2 h^{-1} g^{-1} dry weight for *A. chroococcum* and 12·09 mmol h^{-1} g^{-1} for *A. vinelandii*. When 20 kg ha^{-1} was used with *A. vinelandii*, respiration rates were inhibited 60%, the similar percentage inhibition *A. chroococcum* showed at 28 kg ha^{-1}. Nitrogen fixation dropped drastically 80% with 20 kg ha^{-1} in *A. vinelandii* and 98% with 28 kg ha^{-1} in *A. chroococcum*. Cell size as determined by electron microscopy decreased in the presence of glyphosate, probably because glyphosate induces amino acid depletion and reduces or stops protein synthesis.

INTRODUCTION

Members of the genus *Azotobacter* play an important role in nitrogen soil cycle (Ab-del-Malek 1971). They have been found in soils throughout the world, the predominant species depending upon the pH and moisture content of the soil. Soil of the rhizosphere of certain plants may contain larger numbers of azotobacters (10^6–10^7 g^{-1} of soil) than soils without roots. Cyst formation contributes to maintenance of this bacteria under suboptimal conditions. *Azotobacter chroococcum* appears to be the most widespread species, occurring mainly in neutral and alkaline soils. An organism that can reduce dinitrogen (N_2) to ammonia (NH_3) has the task of replenishing the biologically unavailable nitrogen on the planet and the reward of being able to overcome the limitation imposed by a nitrogen-deficient environment. In addition, ecological studies show that free-living nitrogen fixation micro-organisms fix N_2 in all of the seasons of the year, giving a considerable amount of organic nitrogen to agricultural soils. Usually, an estimate of 10–15 kg N ha^{-1} $annum^{-1}$ is given.

Many factors affect soil ecology, such as herbicides and other chemicals used in agriculture. One of these herbicides is glyphosate (*N*-(phosphonomethyl)glycine) which is a broad spectrum herbicide that is very effective on deep-rooted perennial species, annual and biennial species of grasses, sedges and broadleaved weeds.

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Mechanisms of action are not well known but a disruption of phenolic metabolism has been implicated. Glyphosate appears to inhibit the aromatic amino acid biosynthetic pathway, and accumulation of chlorophylls and carotenoids producing ultrastructural alterations and damages. The damage observed was a partial disruption of the chloroplast envelope and swelling of the rough endoplasmic reticulum (RER) (Cañal *et al.* 1985; Kitchen *et al.* 1981).

Glyphosate is degraded by micro-organisms in soils, therefore, various metabolites or degradation products of glyphosate have been identified. Aminomethylphosphonic acid is the principal product of glyphosate degradation in soils. Sarcosine, glycine and even CO_2 are possible non-phytotoxic products of glyphosate degradation in soils (Moshier and Penner 1978; Pipke and Amrhein 1988; Fitzgibbon and Braymer 1988; Liu *et al.* 1991).

MATERIALS AND METHODS

Organisms, medium and herbicide

Azotobacter chroococcum (CECT* 203) and *Azotobacter vinelandii* (CECT 204) were grown in a modified Burk culture media, containing (g l^{-1}): glucose, 20; K_2HPO_4, 0·64; KH_2PO_4, 0·16; $MgSO_4 . 7H_2O$, 0·2; $FeSO_4$, 0·003; distilled water, 1000 ml; pH 7·2.

Isopropylamine salt of glyphosate with 59·4% (w/v) of active product was used. The remaining inactive carrier was distilled water. Trade mark: Roundup (Monsanto)™.

Growth

One ml of cultures with an O.D. of 1·0 at λ 500 nm was transferred to 250 ml Erlenmeyer flasks containing 100 ml of Burks media with concentrations of glyphosate between 4 kg ha^{-1} ($5{\cdot}9 \times 10^{-4}$ mol l^{-1}) and 40 kg ha^{-1} (59×10^{-4} mol l^{-1}). Incubation was in a rotary bed shaker (200 rev min^{-1}) at 28°C. Growth was measured spectrophotometrically at λ 500 nm. Biomass was measured at O.D. of 1·0; 100 ml of these cultures were centrifuged (5000 rev min^{-1}, 10 min) and pellets were dehydrated at 60°C for 24 h. Biomass was used to calculate the specific respiration and nitrogen fixation rates, as mmol O_2 h^{-1} g^{-1} of dry weight and mmol ethylene h^{-1} g^{-1} of dry weight.

Respiration activity

Respiration activity was measured directly on 2·5 ml culture samples (O.D. 1·0) in Warburg manometers, containing 0·2 ml of 20% (w/v) KOH in the centre well, shaken at 150 rev min^{-1} at 28°C.

Nitrogen fixation activity

Nitrogen fixation was measured as acetylene reduction activity. Samples of cultures (10 ml, O.D. 1·0) were transferred to 60 ml conical flasks, sealed and 5 ml of acetylene (freshly prepared from CaC_2 and water) was injected. Flasks were shaken at 28°C. Gas samples (100 μl) were taken by syringe for gas chromatography in 15 min intervals. Ethylene was detected by flame-ionization in a GDC Pye Unicam gas chromatograph with a 186 cm Poropak R column, 3 mm i.d. Peak surface was taken as being proportional to ethylene concentration (Postgate 1974). The relation between peak surface and ethylene concentration was: $Y = 2{\cdot}087 \times X + 0{\cdot}558$; Y: μmol ethylene; X = peak area (cm^2).

Scanning electron microscopy

Culture samples (1·0 ml) were centrifuged (5000 rev min^{-1}, 10 min) twice to remove polysaccharides of the cells, then samples were fixed with glutaraldehyde (2% (v/v)) on 0·1 mol l^{-1}, pH 7·0 phosphate buffer. Samples were dehydrated with acetone in critical point with CO_2.

RESULTS AND DISCUSSION

Growth curves show that *A. chroococcum* and *A. vinelandii* have a different sensitivity to glyphosate. Glyphosate lowered growth of *A. vinelandii* using rates of 4 kg ha^{-1} to 20 kg ha^{-1}. *Azotobacter chroococcum* resisted concentrations until 28 kg ha^{-1}. Concentrations of 4–16 kg ha^{-1} did not affect growth of *A. chroococcum*. Specific growth rates (μ) were also inhibited (Table 1). The presence of this herbicide in culture medium at and above recommended dosage rates (0·2–4 kg ha^{-1}) allows growth of every strain. These results prove that *Azotobacter* is a genus quite resistant to the presence of glyphosate. In all cases the authors found similar final optical density on cultures, and viable counts (Table 2) showed that all differences found at the beginning did not disappear at the end of the growth curves. Viable counts made at the end of the exponential phase show that glyphosate reduces the number of viable cells on cultures, similar to the results that Richardson *et al.* (1979) obtain for other kinds of micro-organisms. Similar O.D. (500 nm) at the end of the exponential phase may be explained as an increase of production of extracellular polysaccharides (data not shown). It is accepted that glyphosate is implicated in the inhibition of aromatic amino acid biosynthesis. The enzyme 5-enolpyruvylshikimate-3-phosphate synthase is inhibited by physiological concentrations of glyphosate and is the most sensitive site of action for glyphosate in reducing aromatic amino acid levels. Aromatic amino acid depletion reduces protein synthesis,

Table 1 Effect of glyphosate on growth rate at different concentrations

Glyphosate rate (kg ha^{-1})	Specific growth rate (h^{-1}) ± S.E.							
	Control	4	8	12	16	20	24	28
A. chroococcum	0·295 ±0·015	0·262 ±0·017	0·262 ±0·020	0·252 ±0·012	0·250 ±0·017	0·249 ±0·015	0·238 ±0·013	0·235 ±0·008
A. vinelandii	0·147 ±0·012	0·120 ±0·021	0·112 ±0·011	0·104 ±0·010	0·102 ±0·011	0·099 ±0·009		

Azotobacter vinelandii was more sensitive and all concentrations reduced specific growth rates.
S.E., Standard error.

Table 2 Viable count of *Azotobacter vinelandii* and *A. chroococcum* at the end of the exponential phase, when O.D. was 1·0 at 500 nm

Glyphosate rate (kg ha^{-1})	Viable counts × 10^6 ml^{-1} ± S.E.							
	Control	4	8	12	16	20	24	28
A. chroococcum	4·5 ±0·29	4·6 ±0·28	4·5 ±0·26	4·4 ±0·25	4·0 ±0·22	2·3 ±0·19	1·1 ±0·11	0·75 ±0·03
A. vinelandii	4·0 ±0·25	3·8 ±0·19	2·8 ±0·22	3·0 ±0·23	2·1 ±0·17	1·1 ±0·09		

S.E., Standard error.

causing a lower growth and eventually cellular death. This mechanism of action may explain growth results, i.e. that low protein synthesis levels may cause cessation of growth. Glyphosate divalent metal cation chelation properties may also be important in biochemical interactions. Aminophosphonic acids such as glyphosate chelate metal cations in aqueous medium (Sandberg *et al.* 1978; Stalhman and Phillips 1979; Buhler and Burnside 1983). This property affects bacterial or plant physiology at any of the many points dependent on metal cations. Metal cations such as Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+} on its role like cofactors for enzymes of many pathways, may explain the scarce growth at high concentrations.

It may also explain inhibition rates for nitrogen fixation found for *A. vinelandii* and *A. chroococcum* (80% and 98%), which indicates that nitrogen fixation was the parameter most affected by glyphosate because nitrofixation depends on another process, like respiration, which is affected by glyphosate. This is supported by this group's results and the results of others (Foley *et al.* 1983). Respiration rates evaluated by the Warburg method are shown in Fig. 1. Respiration rate of *A. chroococcum* was 19·07 mmol O_2 g^{-1} h^{-1} in control cultures. This respiration level dropped from 16 kg ha^{-1} of glyphosate. Though all concentrations used with *A. vinelandii* reduced respiration activity, 20 kg ha^{-1} of glyphosate made an inhibition of 60%, similar to the percentage that 28 kg ha^{-1} made in *A. chroococcum*.

Respiration gives ATP for nitrogen fixation and protects the nitrogenase enzyme from oxygen. This may be the reason nitrogen fixation is more affected than other parameters studied. Conversely, glyphosate plays another role as a cation chelation agent and reduces the existence of Ca^{2+}. Ca^{2+} is necessary for respiration protection of nitrogenase (Castillo 1987). According to Wills and McWorther (1985), glyphosate increases its toxicity in the presence of cations such as K^+ or Na^+. Therefore, this may also explain the results obtained, as the Burk culture medium has these kinds of monovalent cations.

Azotobacter vinelandii and *A. chroococcum* showed a similar nitrogen fixation activity (about 40 mmol ethylene

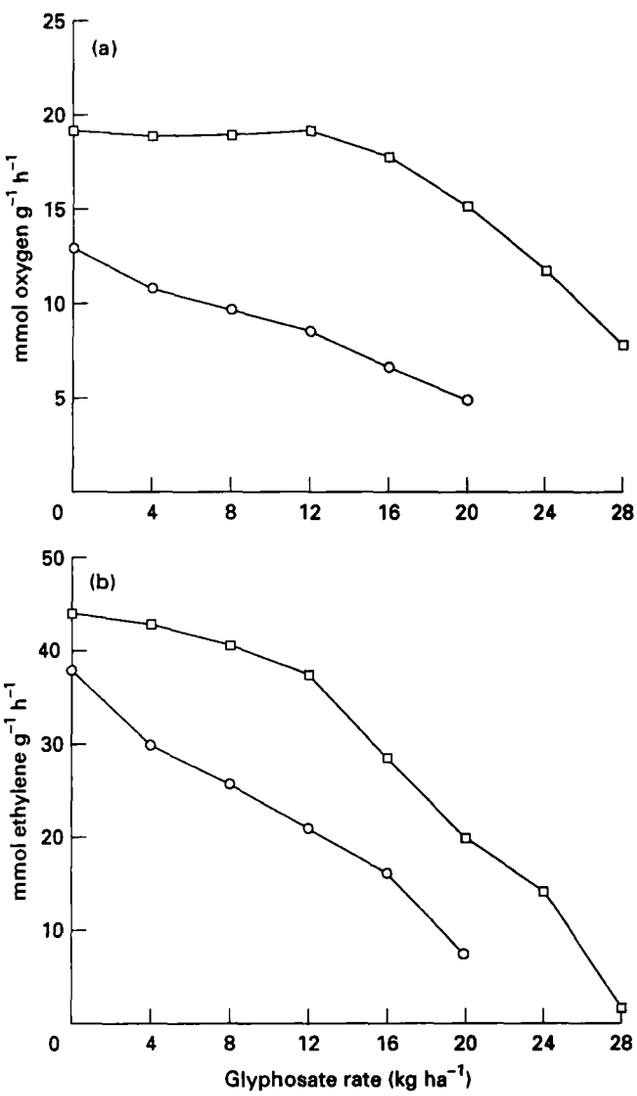

Fig. 1 Specific respiration rates and its relation with glyphosate concentration, and effects of glyphosate on nitrogenase activity. High dosages of glyphosate inhibited nitrogenase activity by about 80–90%. *Azotobacter vinelandii* was more sensitive to glyphosate than *A. chroococcum*. ○, *A. vinelandii*; □, *A. chroococcum*

Table 3 Effects of glyphosate on cellular size of *Azotobacter chroococcum*

Glyphosate rate (kg ha^{-1})	Cellular size (μm) ± S.E.		
	Control	16	28
Vegetative cells	3·15 ± 0·269	2·39 ± 0·252	2·11 ± 0·213
	×	×	×
	1·29 ± 0·093	0·93 ± 0·087	0·73 ± 0·029
Cystic cells	1·48 ± 0·035	1·44 ± 0·024	0·76 ± 0·015
	×	×	×
	1·46 ± 0·025	1·43 ± 0·019	0·73 ± 0·012

S.E., Standard error.

h^{-1} g^{-1} dry weight). Herbicide treatment did not affect this parameter at low concentrations in *A. chroococcum* but 28 kg ha^{-1} inhibited nitrogen fixation by 96%. *Azotobacter vinelandii* was more sensitive to glyphosate, 20 kg ha^{-1} of glyphosate dropped nitrogen fixation by 80% (Fig. 1).

Glyphosate treatment, however, did reduce cell size of *A. vinelandii* and *A. chroococcum*. Control series showed large cells (3·1 × 1·3 μm on *A. chroococcum* and 2·1 × 0·73 μm on *A. vinelandii*). Bacteria from high concentration cultures were smaller than control cultures in *A. chroococcum* (Table 3). Cells of *A. vinelandii* had little changes in cell size. Cysts of *A. chroococcum* and *A. vinelandii* were induced in the presence of glyphosate. When higher dosages in vegetative forms of *A. vinelandii* were used, cysts formed because of the high concentration of glyphosate. Cyst size was reduced at high concentrations.

The scanning microscopy can explain respiration and nitrogen fixation results as well. When high dosages of glyphosate were used, photographs of vegetative forms of *A. vinelandii* showed the presence of cysts; this kind of cell, as a resistant form, has got little metabolic activity. A low number of vegetative forms could therefore mean that nitrofixation was also affected as well due to the fact that the vegetative cell is the only form that has nitrogen fixation activity. The same studies confirm the results of viable count. The number of recognized cells dropped in all cases in the presence of glyphosate, in addition to a minor cellular size. It was also determined that glyphosate affects ultrastructural composition of vegetative or cyst cells, similar to the results that Campbell *et al.* (1976) found for plant cells.

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