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Influence of insecticidal toxins from *Bacillus thuringiensis* subsp. *kurstaki* on the degradation of glyphosate and glufosinate-ammonium in soil samples

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Abstract

Investigations dealing with the persistence in soil of glyphosate [*N*-(phosphonomethyl)glycine] (GLYP) and glufosinate-ammonium [the ammonium salt of DL-homoalanin-4-yl(methyl)phosphinic acid] (GLUF) herbicides and of insecticidal toxins produced by *Bacillus thuringiensis* subsp. *kurstaki* (Berliner) are largely reported in the literature. However, no information on the influence of these insecticidal toxins on the persistence in soil of herbicides is available. Preliminary results regarding the influence of insecticidal toxins extracted from a commercial formulation of *B. thuringiensis* subsp. *kurstaki* (Berliner) (Btk) on the degradation of the herbicides glyphosate and glufosinate-ammonium in a loam and a sandy loam soil, under laboratory conditions, were obtained. Soil microbial carbon (SMC) and insecticidal activity of incubated soil samples were also estimated. In both soil types, persistence of GLYP was significantly higher with respect to GLUF. Average GLYP and GLUF half-life was 14.4 and 8.0 days, respectively. Addition of Btk toxins lead to a significant increase of GLYP and GLUP persistence in both soil types. More specifically, average GLYP and GLUF half-life in soil samples receiving the Btk treatment was 24.3 and 14.2 days, respectively. In contrast to herbicide persistence in soil, Btk toxins did not influence microbial carbon content of incubated soil samples. The insecticidal activity of Btk toxins in soil rapidly decreased during the 28-day incubation time. Considering that degradation of GLYP and GLUF was mainly a microbial process, the absence of effects of Btk toxins on the soil microbial carbon and the rapid decrease of insecticidal activity of Btk toxins in the soil suggest a possible effect of the Btk toxins on other soil properties and/or mechanisms influencing herbicide degradation. The present preliminary investigation permitted to highlight the possibility of the Btk toxins to enhance the persistence of GLYP and GLUF in soil, under laboratory conditions. However, further studies are necessary to investigate whether or not the effects observed in this study under artificial and controlled conditions can be extrapolated to field conditions.

Keywords: Glyphosate; Glufosinate-ammonium; *Bacillus thuringiensis* subsp. *kurstaki* toxins; Herbicide degradation

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] (GLYP) and glufosinate-ammonium [the ammonium salt of DL-homoalanin-4-yl(methyl)phosphinic acid] (GLUF) are non-selective herbicides widely used in weed management of several agricultural crops. The importance of these two herbicides has increased in the last years due to the increased demand for herbicide-tolerant (HT) crops (Shaner, 2000). Crops tolerant to GLYP and GLUF are the most cultivated genetically modified (GM) crops and environmental issues concerning

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the cultivation of crops tolerant to GLYP and GLUF are of current interest (Engel et al., 2002). The impact of GLYP and GLUF tolerant crops on soil and water quality is supposed to result from the patterns in herbicide use and the consequent impact of these herbicides (Moorman and Keller, 1996).

GLYP and GLUF are non-residual herbicides that degrade readily from soil, with estimated half-lives ranging from 7 to 60 days and from 1 to 25 days, respectively (Giesy et al., 2000). Degradation by soil micro-organisms is the predominant way by which GLYP and GLUF are metabolised in soil (Tebbe and Reber, 1988; Giesy et al., 2000). More specifically, degradation of GLYP and GLUF in soil is considered a co-metabolic process (Torstensson, 1985). GLYP and GLUF are strongly adsorbed to soil and consequently are characterised by low mobility through the soil profile (Smith, 1988; Zaranyika and Nyandoro, 1993). No detrimental effects were observed on soil microbial activity and biomass when GLYP and GLUF were applied at normal agricultural rates under laboratory and field conditions (Wardle and Parkinson, 1992; Accinelli et al., 2002). According to Wauchope et al. (2001), GLYP and GLUF replace herbicides that are in general more persistent in soil and adsorb less to soil particles.

Nowadays, genetically modified crop varieties with two biotech traits (stacked trait crops) are commercially available and currently cultivated in several countries. Stacked trait products are mainly represented by plants that have been genetically modified to exhibit tolerance to GLYP or GLUF and resistance to lepidopteran pests. Resistance to lepidopteran pests has been achieved by insertion of genes from different strains of the bacterium *Bacillus thuringiensis* subsp. *kurstaki* (Berliner) (Btk) encoding for specific insecticidal crystal toxins (Betz et al., 2000). Btk toxins are active against the European corn borer (*Ostrinia nubilalis* Hübner) and other lepidopteran pests species. Major benefits exhibited by Btk-protected crops comprise decreased pest management costs, increased yield and preservation of population of beneficial insects (Betz et al., 2000). Btk toxins are highly specific against target insect species and non toxic to vertebrates (Betz et al., 2000). However, Btk-protected crops release insecticidal toxins into the soil by root exudates and when crop residues are incorporated in the soil (Palm et al., 1994; Saxena et al., 2002). It is consequently likely to assume that cultivation of GLYP or GLUF tolerant/lepidopteran resistant stacked trait crops or use of GLYP or GLUF in the weed management of Btk-protected crops may lead to the joint presence in the soil environment of GLYP or GLUF herbicides and Btk insectidal toxins. Several authors reported short persistence and no risk of bioaccumulation of Btk proteins in soil, under field conditions (Herman et al., 2001; Head et al., 2002). Conversely, laboratory investigations showed that Btk toxin, extracted from a commercial formulation of Btk, was rapidly and tightly adsorbed to soil constituents, persisting in soil for more than 6 months, the longest time studied (Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998).

To date, no information regarding the effect of Btk toxins on persistence of GLYP and GLUF in soil is available. Considering the growing interest in stacked trait crops, especially of GLYP or GLUF tolerant/lepidopteran resistant stacked trait crops in current agriculture, and the wide use of GLYP and GLUF in weed management of Btk-protected crops, more information is needed.

The objective of the present preliminary investigation was to estimate the influence of Btk toxins, extracted from a commercial formulation of Btk, on the degradation of GLYP and GLUF in incubated soil samples collected in two locations of the Po Valley (Italy). Efforts to relate soil microbial carbon (SMC) and residual insecticidal activity of incubated soil samples with herbicide persistence are also described.

2. Materials and methods

2.1. Soils

Surface (0–20 cm) soil samples were taken from two agricultural areas of the Po Valley (Italy), Cadriano (44°35′N, 11°27′E) and Ozzano (44°28′N, 11°28′E). Cadriano soil was classified as a loam (Udic Ustochrepts, fine silty, mixed, mesic) and Ozzano soil as a sandy loam (Udertic Ustochrepts, fine, mixed, mesic). In both the locations, soils were collected from fields with no pesticide application during the last 5 years. Some physico-chemical properties of the two employed soils are given in Table 1. Five grams of soil (air-dried basis) were weighed in sterile

Table 1
Properties of Cadriano and Ozzano soils

Location	Textural class	Soil texture			pH (H_2O) (1:2.5)	Organic C (%)	CEC (meq. 100 g^{-1})
		Sand (%)	Silt (%)	Clay (%)			
Cadriano	Loam	35.5	40.0	24.5	7.90	0.92	30.2
Ozzano	Sandy loam	63.4	22.6	14.0	8.11	0.70	28.3

culture tubes. Before the beginning of the experiment, soil moisture was adjusted to the gravimetric content at −33 kPa using ultrapure water. Soil samples were kept in the dark in a climatic chamber at 25 °C ± 0.5 for 10 days. The conditioning period of 10 days allowed the soil to establish a steady-state level of microbial activity (Franzluebbers et al., 1996).

2.2. Soil treatments and samples incubation

Conditioned soil samples were treated with water solutions of the commercial formulation of Roundup Bioflow (Monsanto Agricoltura Italia S.p.A., Lodi, Italy; isopropylamine salt of GLYP, suspension concentrate, containing 360 g active ingredient, a.i., l^{-1} formulation), Basta (Bayer CropScience S.r.l., Milano, Italy; ammonium salt of GLUF, suspension concentrate, containing 120 g a.i. l^{-1} formulation) and Btk toxins extracted and purified from the microbial insecticide Dipel 2× (Sipcam S.p.a.; Pero, Italy; wettable powder, containing 32,000 International Unit mg^{-1} formulation). The two herbicides were applied at 10 μg a.i. g^{-1} soil (air-dried basis). Application rate was calculated considering a normal agricultural rate and a soil layer of 1 cm (Accinelli et al., 2002). Considering the preliminary characteristic of the present investigation, a high rate of Btk toxin was adopted: 10 μg a.i. g^{-1} soil (air-dried basis). The two herbicides and the Btk toxins were added to soil samples as water solutions using a 1 ml syringe. Treated samples were incubated in the dark in a climatic chamber at 25 °C ± 0.5. The moisture level of soil samples was checked at a 4-day interval and adjusted to the imposed initial value, corresponding to the gravimetric content at −33 kPa. For herbicide half-life estimation, sampling times were 0, 3, 7, 14, 21 and 28 days after treatment (DAT). At each sampling time, samples were removed and stored at −20 °C until analysis. Herbicide half-lives were calculated by the slopes of the straight lines obtained plotting the natural logarithm of the percentage of herbicide remaining against the time.

2.3. Herbicide extraction and analysis

GLYP, GLUF and aminomethylphosphonic acid (AMPA), the main metabolite of GLYP (Sprankle et al., 1975; Malik et al., 1989), were extracted from the soil samples using 250 ml of a 0.1 M potassium phosphate monobasic solution, with pH adjusted to 8. The mixture was shaken for 12 h using an orbital shaker. After centrifugation at 5000 × *g* for 20 min, the supernatant (10 ml) was filtered through a 0.2 μm size filter and analysed following a multiresidue approach. More specifically, the three compounds were analysed by RP-HPLC following pre-column derivatisation using the methodology proposed by Miles et al. (1986). Briefly, derivatisation was achieved by adding to 0.1 ml extract, 0.9 ml borate buffer, 0.9 ml acetone and 0.1 ml of 0.01 M 9-fluorenylmethyl chloroformate (FMOC-Cl) in acetone. After a 20 min reaction at 20 °C, the mixture was extracted with three 1 ml washes of ethyl ether. Derivatised samples were analysed by a chromatograph system that consisted of a Waters 501 pump (Waters Corp., St. Milford, MD), a Rheodyne 7125 injector (50 μm loop) (Rheodyne Europe GmbH, Bensheim, Germany), a 0.46 cm × 25 cm Luna NH_2 column (Phenomenex Inc.), and a RF-10AXL spectrofluorometric detector (Shimadzu Italia S.r.l., Milano, Italy). Isocratic elution was carried out at room temperature and the eluent flow was set at 1.5 ml min^{-1} with acetonitrile/0.05 M potassium phosphate monobasic (pH 6.0) solution (35/65, v/v). Detection of the three compounds was achieved by setting the detector at excitation and emission wavelengths of 285 and 370 nm, respectively. All the employed reagents were analytical grade and supplied by Carlo Erba Reagenti S.p.a. (Milano, Italy).

Herbicide standard solutions were prepared using analytical grade GLYP (98%), GLUF (94.5%) and AMPA (99%) provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The adopted analytical procedure permitted to achieve a clear separation of GLYP, GLUF and AMPA.

Efficiency of the employed extraction method was validated by analysing sterilised soil samples separately treated with GLYP, GLUF and AMPA, incubated at the same conditions adopted for the herbicide degradation experiment and sampled at 0.5, 3, 7, 14, 21 and 28 days. Soils of the two locations were sterilised by autoclaving two times for 1 h at 121 °C and 103 kPa. Non-sterilised control soil was included. Soil samples were separately treated with GLYP, GLUF and AMPA at 10 μg a.i. g^{-1} soil (air-dried basis). The test was conducted in triplicate.

2.4. Btk toxins extraction and purification

The insecticidal crystal proteins contained in the microbial insecticide Dipel 2× were extracted and purified according to the method described in Tapp and Stotzky (1998). Briefly, Dipel 2× was initially washed with 1 M sodium chloride and subsequently with deionised water. Each washing was repeated two times. Obtained washed sediment was extracted with a MOPS buffer (0.1 M 3-*N*-morpholinopropane-sulfonic acid, pH 7.8) containing 0.5 M dithiothreitol and 1 M potassium thiocyanate. The extract was dialysed for 8 h against deionised water and precipitated using ammonium sulphate. The precipitate was centrifuged at 27,000 × *g* and resuspended in deionised water. Finally, the pellet was dialysed and lyophilised. Purified toxins were analysed by SDS-PAGE as described in Venkateswerlu and Stotzky (1990). Concentration of the stock solutions used for soil treatments was calculated after quantification of the Btk toxins using the Lowry method.

2.5. Persistence of insecticidal activity of Btk toxins in soil

Insecticidal activity of the Btk toxins in soil samples was estimated by insect bioassay employing second-instar larvae of the European corn borer (*O. nubilalis* Hübner). Sampling times were 0 and 28 DAT. Larvae used in the bioassay were obtained from a laboratory colony established from insects collected at Cadriano (Bologna, Italy). Larvae, artificial diet and test trays were provided by DiSTA (Division of Entomology, University of Bologna, Italy). Distilled water was added to single 5 g soil samples to achieve a 10 ml volume. The slurry was shaken for 2 h in a orbital shaker and finally was uniformly mixed to the insect diet (500 ml) and allowed to dry. Six dilutions of the soil slurry-diet mixture were prepared by two-fold serial dilution of the initial mixture. Portions (3 ml) of the soil slurry-diet mixture obtained from each sample were added to single cells of a 100-cell bioassay tray. Reference dose–response relationship was estimated using mortality data obtained by mixing the soil slurry with the insect diet in order to obtain a concentration range from 0 to 35 μg Btk toxins g^{-1} diet. One second-instar *O. nubilalis* was added per cell. For each sampling time and dilution, 15 larvae were employed. The test was conducted on soil samples treated with Btk toxins and Btk toxins plus each herbicide. The test tray was closed with a perforated plastic lid and placed in a climatic chamber at 27 °C ± 0.5 and a photoperiod of 16 h light:8 h dark. The test was conducted in triplicate. Insect mortality was scored after 7 days, and the results were analysed by probit analysis for the estimation of median lethal concentrations (LC_{50}), after correction for mortality with Abbot's formula (Abbot, 1925). Control mortality was ≤10%.

2.6. Soil microbial carbon

SMC was estimated in soil samples using the fumigation extraction method (Vance et al., 1987). Sampling times were 0 and 28 DAT. Briefly, for the fumigation treatment, soil samples were placed in 5 l desiccators containing a 25 ml glass beaker with 25 ml ethanol-free chloroform. After incubation for 24 h in the dark at 25 °C, the chloroform was removed by repeated evacuation. Fumigated and unfumigated soil samples were extracted with 0.5 M potassium sulphate for 60 min in a rotary shaker and then filtered through a glass-fibre filter (Whatman GF/A, Maidstone, United Kingdom). Organic C in the extract was determined by dichromate digestion as reported in Vance et al. (1987). SMC was calculated by the expression $SMC = E_c/k_{ec}$, where E_c = (organic C extracted from fumigated soils) − (organic C extracted

from unfumigated soils). A correction factor (k_{ec}) of 2.64 was adopted (Vance et al., 1987).

2.7. Experimental design and statistical analysis

For the herbicide degradation experiment, a factorial design with chemical input (GLYP, GLUF, GLYP + GLUF, in presence and absence of Btk toxins) and soil type (Cadriano and Ozzano soils) as variables was employed. Each chemical input and soil type combination were replicated three times. SMC and LC_{50} were determined in soil samples incubated for 0 and 28 DAT. For LC_{50} analysis, only chemical input combinations including the Btk toxins treatment were included. Herbicide half-life, SMC and LC_{50} data were subjected to analysis of variance and significant differences ($P < 0.05$) determined by a least significant difference (LSD) test using the software Statistica release 6.1 (Statsoft Italia S.p.a., Milano, Italy). LC_{50} was estimated using the software SAS release 6.11 (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Herbicide extraction and analysis

Recovery data for GLYP, GLUF and AMPA applied to sterile soil samples and incubated at different time intervals are reported in Table 2. As showed in Fig. 1, no significant decrease ($P > 0.05$) of GLYP concentrations were observed within the 28 days incubation period, suggesting that sterilised conditions were achieved and maintained during the whole incubation period. Similar results were obtained with GLUF and AMPA (data not shown). These findings were further supported by differences of AMPA concentrations, the main metabolite of GLYP, between sterilised and non sterilised control soil samples (Fig. 1).

GLYP, GLUF and AMPA are strongly adsorbed by the soil and difficulties to efficiently extract these compounds have been reported by Piccolo et al. (1996). In the present experiment, the moderate clay and organic C content of the two studied soils (Table 1) permitted the method to achieve recovery values ≥87, 82 and 84% of the applied GLYP, GLUF and AMPA, respectively (Table 2). The analytical procedure adopted in the present experiment (pre-column derivatisation with FMOC-Cl) has been extensively used by several authors (Glass, 1983; Miles et al., 1986) and permitted to accurately detect the studied compounds. The detection limit of GLYP, GLUF and AMPA were 0.2, 0.1 and 0.05 μg a.i. g^{-1} soil (air-dried basis), respectively. Although the use of ^{14}C-labelled compounds can be preferred in some circumstances (i.e. adsorption/desorption studies), the employment of ^{14}C-labelled GLYP or GLUF to study their degradation process, by measuring the evolved $^{14}CO_2$, may not correctly reflect the degradation of the parent compounds. In this case, it should be assumed that $^{14}CO_2$ is liberated concurrently with the decomposition of the herbicides (Torstensson, 1985).

3.2. Degradation of GLYP and GLUF

Degradation of GLYP and GLUF in soil samples receiving the different chemical inputs was correctly described by the first order kinetic model, as

Table 2
Mean percent recovery±S.E. (standard error of the mean) of glyphosate (GLYP), glufosinate-ammonium (GLUF) and aminomethylphosphonic acid (AMPA) for sterilised soils from Cadriano and Ozzano

Incubation time (days)	Recovery					
	GLYP		GLUF		AMPA	
	Cadriano (%)	Ozzano (%)	Cadriano (%)	Ozzano (%)	Cadriano (%)	Ozzano (%)
0	99 ± 0.8	100 ± 0.3	102 ± 1.4	95 ± 1.8	98 ± 1.6	103 ± 2.5
3	98 ± 0.4	97 ± 0.7	94 ± 2.3	88 ± 2.7	91 ± 2.0	93 ± 2.4
7	99 ± 0.5	96 ± 0.3	82 ± 2.1	97 ± 1.3	91 ± 1.1	84 ± 2.2
14	104 ± 0.9	99 ± 0.3	91 ± 0.9	91 ± 2.1	99 ± 2.0	93 ± 1.9
21	87 ± 0.4	89 ± 0.8	92 ± 2.1	93 ± 2.2	91 ± 1.4	97 ± 2.0
28	91 ± 0.7	92 ± 0.2	88 ± 1.5	90 ± 2.5	89 ± 2.2	92 ± 1.9

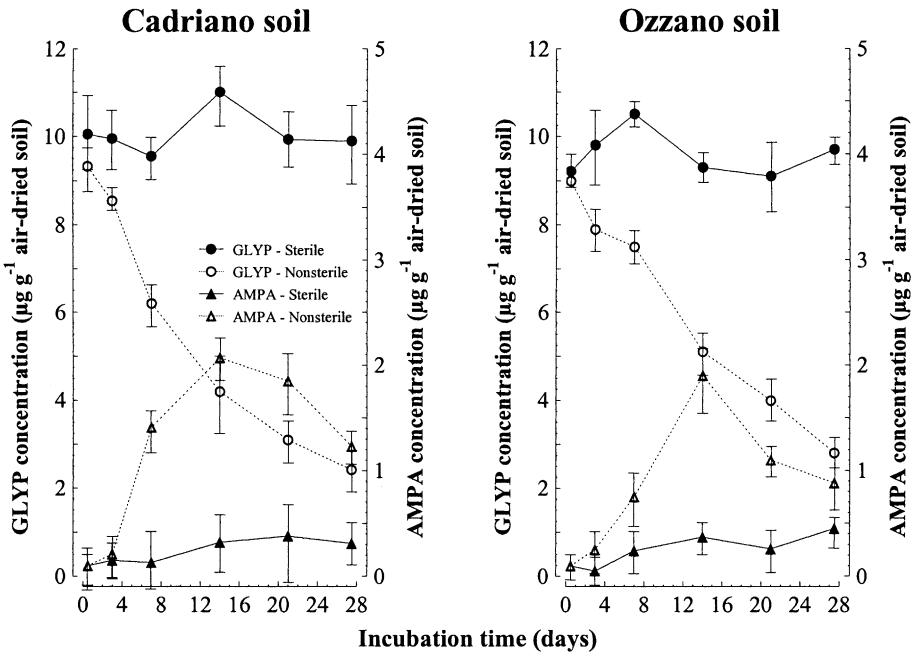

Fig. 1. Concentration of glyphosate (GLYP) and aminomethylphosponic acid (AMPA) in incubated sterilised and non-sterilised samples of Cadriano and Ozzano soil. Bars represent standard errors of the means.

indicated by the calculated coefficients of determination (Table 3). Degradation rates of GLYP and GLUF in accord to the first order kinetic model has been reported by Smith (1988). However, in some cases, degradation of GLYP and GLUF do not obey this model. Some authors reported that degradation of GLYP in soil is initially rapid but slows over time to a quasi-steady-state (Nomura and Hilton, 1977; Torstensson, 1985; Eberbach, 1999). Major parts of these studies have been carried out using ^{14}C-labelled GLYP and consequently cannot be compared directly with results discussed in the present investigation. Using non-labelled GLYP, Eberbach (1999) reported that initially rapid degradation was ascribed

Table 3
Half-lives ($t_{1/2}$) ± S.E. (standard error of the mean) and average coefficients of determination (r^2) of glyphosate (GLYP) and glufosinate-ammonium (GLUF) in Cadriano and Ozzano soils as a function of different chemical inputs

Chemical input	$t_{1/2}$ ± S.E. (days)		r^2	
	Cadriano	Ozzano	Cadriano	Ozzano
Glyphosate				
GLYP (control)	12.3 ± 0.3	17.4 ± 0.1	0.95	0.97
GLYP + GLUF	11.9 ± 0.1	16.0 ± 0.1	0.95	0.93
GLYP + Btk	22.1 ± 0.2	29.7 ± 0.2	0.93	0.94
GLYP + GLUF + Btk	19.3 ± 0.2	26.0 ± 0.3	0.99	0.92
Glufosinate-ammonium				
GLUF (control)	6.2 ± 0.1	9.2 ± 0.3	0.98	0.99
GLYP + GLUF	6.6 ± 0.2	10.2 ± 0.2	0.98	0.98
GLUF + Btk	11.2 ± 0.2	16.2 ± 0.1	0.89	0.91
GLYP + GLUF + Btk	12.2 ± 0.1	17.3 ± 0.2	0.99	0.89

to adsorption of the herbicide into a nonextractable form.

Estimated half-lives of GLYP and GLUF in the loam (Cadriano) and in the sandy loam (Ozzano) soil, by using the first-order kinetic model, are reported in Table 3. Persistence of GLYP in soil samples receiving the different chemical inputs was significantly lower ($P < 0.05$) in the loam soil with respect to the sandy loam soil. Based on the results obtained with sterilised soil samples and above discussed, degradation of GLYP in the two soils was a microbial process. According to Torstensson (1985), degradation of GLYP is considered fairly correlated with SMC and microbial activity of soil. As described below, SMC was higher in Cadriano soil than in Ozzano soil and was not significantly influenced ($P > 0.05$) by the different chemical input and incubation period (Fig. 2). Consequently, the measured differences of GLYP persistence between the two soils are supported by differences in SMC. In contrast to GLYP, persistence of GLUF was not significantly different ($P > 0.05$) in the two soils. These findings suggest that GLUF degradation in soil was more influenced by other soil properties rather than SMC. Persistence of GLYP and GLUF in soil samples treated with single herbicides was not significantly different ($P > 0.05$) with respect to samples treated with the two herbicides. Average half-life was 14.4 and 8.0 days for GLYP and GLUF, respectively. In contrast, persistence of the two herbicides was significantly increased ($P < 0.05$) by the addition of the Btk toxins. In both Cadriano and Ozzano soils, half-life of GLYP and GLUF was approximately 2-fold higher than in non-Btk-treated samples. Similar results were obtained in soil samples receiving the highest chemical input (GLYP + GLUF + Btk toxins), thus confirming the role of the Btk toxins in the increase of the herbicide persistence.

Btk toxins are known as crystal (Cry) proteins (Vaeck et al., 1987). No data on the specific effect of such compounds on the degradation of GLYP and GLUF is available in the literature. Working with GLYP, Moshier and Penner (1978) observed that herbicide degradation was only slightly influenced by the addition of high rates of readily metabolised substrates (glucose, sarcosine, glycine and lucern tissue). Various organic compounds have been shown to adversely affect herbicide degradation in soil (Alvey and Crowley, 1995). More specifically, Abdelhafid et al. (2000)

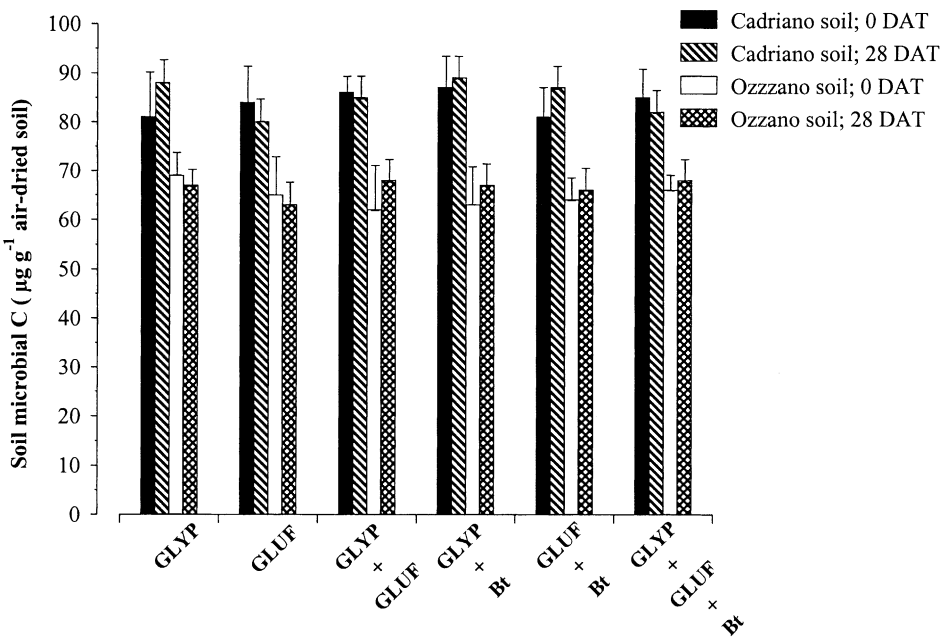

Fig. 2. Soil microbial carbon of incubated Cadriano and Ozzano soil samples subjected to different chemical input. Data are referred to 0 and 28 days after treatment (DAT). Bars represent standard errors of the means.

reported that organic N sources, including albumine, arginine, adenine and pyrazine led to a significant increase of atrazine persistence in soil. Considering the absence of effects on SMC, a possible involvement of Btk toxins on the nutrient *status* of the soil microbial community and/or on the bioavailability of the two herbicides can not be excluded. Some investigations showed that adsorption of GLYP and GLUF from soil can be significantly influenced by several compounds (i.e. Fe, Cu, Al, inorganic phosphates, etc.) which modify herbicide bioavailability (Nomura and Hilton, 1977; Gerritse et al., 1996). However, more specific investigations are needed to explain the role of Btk toxins in herbicide adsorption/desorption processes and in the nutrient status of the soil environment.

3.3. Btk toxins and persistence of Btk toxins in soil

According to the adopted methodology (Tapp and Stotzky, 1998), SDS-PAGE confirmed the presence of the toxin band with a molecular weight of 65–70 kDa (data not shown), which corresponded to the hydrolysed toxins, CryIAa, CryIAb and CryIAc, contained in the commercial formulation Dipel 2× (Van Frankenhuyzen, 1993; Kumar and Venkateswerlu, 1998).

Results from the bioassay against *O. nubilalis* larvae are reported in Table 4. As expected, artificial diet containing soil treated with Btk toxins showed detectable biocidal activity against the second instar larvae, thus confirming the high susceptibility of *O. nubilalis* to Btk toxins (Zwahlen et al., 2000). Preliminary investigations showed that the two herbicides, applied at the adopted rate (10 μg a.i. g^{-1} soil), did not lead to detrimental effects on *O. nubilalis* larvae (data not shown). Consequently, LC_{50} of GLYP and GLUF were not estimated. Differences in physico-chemical properties between the two employed soils did not influence insecticidal activity of the added Btk toxins. Average LC_{50} values were 1.1 and 29.9 μg g^{-1}, respectively, for soils sampled at 0 and 28 DAT. These findings are compatible with a rapid loss of the insecticidal activity of the Btk mixture toxins applied to the two soils. A rapid decrease of the insecticidal activity of crystal toxins from different strains of Bt has been also reported by other authors (Herman et al., 2001; Head et al., 2002). Working with a toxin obtained from a commercial formulation, Tapp and Stotzky (1998) observed a significant decrease in insect mortality of the toxin-treated soil within a 28-day incubation period. However, the same authors showed that persistence of the studied toxin was significantly influenced by soil type and in particular by soil pH values. In a soil type rich in kaolinite and characterized by low pH value, no decrease of insecticidal activity was observed during the incubation period.

Btk crystal toxins exhibit a high degree of specificity for the target insects (Betz et al., 2000). Upon ingestion by susceptible insects, crystals are solubilised in the gut where midgut protease process the toxins in the active form. Since available transgenic plants encode for the active Btk toxin, this latter process is not necessary. Active toxins bind to specific high affinity binding proteins on the surface of midgut epithelial cells (Nuñez-Valdez et al., 2001). These cells eventually swell and burst, causing a loss of gut integrity and resulting in larval death within 1–2 days (Knowles and Ellar, 1987). Binding of the Btk toxins to larva midgut receptors and pore formation are essential for toxicity (Van Frankenhuyzen, 1993). Different domains of the toxin molecule are responsible for the steps

Table 4
Comparative median lethal concentration (LC_{50}) ± S.E. (standard error of the mean) of Btk toxins at 0 and 28 days after treatment (DAT) in incubated Cadriano and Ozzano soil samples

Chemical input	LC_{50} (μg Btk toxins g^{-1} soil slurry-diet mixture)			
	Cadriano soil		Ozzano soil	
	0 DAT	28 DAT	0 DAT	28 DAT
Btk	1.3 ± 0.20	31.2 ± 0.08	1.8 ± 0.13	24.9 ± 0.08
Btk + GLYP	1.1 ± 0.06	28.7 ± 0.09	1.3 ± 0.08	30.4 ± 0.17
Btk + GLUF	2.1 ± 0.11	30.4 ± 0.05	1.5 ± 0.05	29.7 ± 0.07
Btk + GLYP + GLUF	1.9 ± 0.08	29.9 ± 0.12	1.2 ± 0.08	32.3 ± 0.04

of receptor recognition and pore formation (Convents et al., 1990). Some investigations showed that slight modifications of the crystal structure of the Btk toxins can lead to a significant alteration of the insecticidal activity (Grochulski et al., 1995; Nuñez-Valdez et al., 2001). Consequently, the measure of the insecticidal activity may not correctly describe the degradation of the Btk toxins in soil, suggesting that lost or decreased insecticidal activity does not necessarily implicate the complete degradation or inactivation of the toxins. Partially degraded Btk toxin residues with none or reduced biocide activity could be involved in processes occurring in the soil environment coupled to pesticide degradation. Other approaches to describe the persistence of Btk toxins in soil have been proposed. Palm et al. (1994) proposed to extract the toxins using an aqueous alkaline solution and analyse the extracted toxins by enzyme-linked immunosorbent assay. Although the aim of this approach was to directly estimate the fate of the toxins in soil, difficulties to achieve satisfactory recovery values were reported. In the present investigation, the significant influence of the Btk toxins mixture on the degradation of GLYP and GLUF suggests that the involvement of the Btk toxins could be more prolonged than indirectly predicted by the rapid loss of their insecticidal activity.

3.4. Soil microbial carbon

SMC of the two studied soils is reported in Fig. 2. Higher SMC was estimated in Cadriano soil with respect to Ozzano soil. The different chemical inputs did not significantly influence ($P > 0.05$) SMC of the two incubated soils. In a laboratory experiment, Accinelli et al. (2002) did not observe SMC variation in a sandy loam soil treated with different herbicides, including GLYP and GLUF, within an observation period comparable to that adopted in the present study. According to several authors, the size of the microbial biomass is a strong indicator of the pesticide degradation capacity of the soil (Walker et al., 1992). Differences in SMC between the two soils could partly support differences in GLYP persistence in the two soils. In contrast, degradation of GLUF was similar in both soils, suggesting that other mechanisms (i.e. bio-availability) and soil parameters (i.e. soil microbial activity) should be considered. Moreover, the present study confirmed the absence of adverse effects of the three chemicals, GLYP, GLUF and Btk toxins, on soil micro-organisms (Saxena and Stotzky, 2001; Accinelli et al., 2002).

4. Conclusions

On the basis of the obtained results the following conclusions may be drawn.

(i) The persistence of GLYP and GLUF was enhanced by the addition of a high rate of Btk insecticidal crystal toxins extracted and purified from the commercial formulation Dipel 2×. Since no influence of Btk toxins on SMC of the two soils was observed and a rapid decrease of the insecticidal activity of the added Btk toxins was estimated during the 28-day incubation period, the observed increase of GLYP and GLUF persistence was presumably due to the reduction of bio-availability of the two herbicides, modification of the soil nutritive *status* or other not measured properties, such as soil microbial activity.

(ii) The significant increase of the persistence of the two employed herbicides should be considered in investigations dealing with the environmental impact of GM crops.

(iii) In this preliminary investigation, a mixture of different Btk toxins was used. Considering that Btk-protected and GLYP or GLUF-tolerant/Btk-protected crops produce a single activated crystal toxin, results obtained in the present investigation can only roughly simulate a real agricultural situation. Moreover, the present experiment was carried out in the laboratory, under controlled conditions and consequently obtained results are not transferable to the field.

(iv) Others studies are necessary to better understand the interactions between these two groups of compounds. In addition, the definition of a dose–response relation of Btk toxins and GLYP and GLUF degradation in soil is a further important information to know.

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