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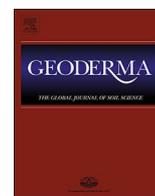
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Effects of glyphosate application and nitrogen fertilization on the soil and the consequences on aboveground and belowground interactions



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ABSTRACT

The application of nitrogen (N) and herbicides are commonly used to fertilize crops and protect them against weed development, but are also considered as soil and environment pollutants. Even so, the individual and combined non-target effects of N fertilizers and herbicides on multitrophic interactions within agrosystems are not well known. From soil samples collected in the field, we examined the effects of the direct application of glyphosate and/or N fertilization on microbial activities and soil nutrient status. In addition, we investigated the increase in biomass and, nutrient acquisition of the bean (*Phaseolus vulgaris*) and the consequences of the applications of N and glyphosate on the performance of the herbivore aphid (*Aphis fabae*). From soils that did (N+) or did not receive (N0) synthetic N fertilization over a 6-year period, we assessed the effects of glyphosate (CK, without glyphosate; FR, field rate of glyphosate) and N fertilization (N+, with N fertilization; N0, without N fertilization) applications in a mesocosm experiment for 75-days. Following the 75 day treatment, the biological and physiological consequences, both belowground and aboveground were determined. The growth of arbuscular mycorrhizal fungi (AMF) and dehydrogenase activity, were negatively affected following N+ fertilization and the application of the FR of glyphosate, while in the absence of glyphosate, alkaline phosphatase (AIP) activity was reduced. Functional microbial responses were unaffected by both N and glyphosate, even when applied in combination. Conversely, the N fertilization significantly increased the nitrate content (NO₃⁻) in the CK soils and the total N in the FR soils, compared to CK/N0 and FR/N0 soils. The combined effects of glyphosate and nitrogen fertilization (FR/N+) significantly decreased the soil C:N ratio, but significantly increased nitrification compared to CK/N0 and FR/N0 soils. The FR/N+ treatments positively affected plant performance, improving the total chlorophyll, sucrose, ammonium, amino acid content, and pod biomass, compared to the CK/N0 and FR/N0 soils. Unlike glyphosate, which did not appear to exert an effect when applied alone or in combination, N fertilization significantly increased aphid nymph survival. The non-metric multidimensional scale allowed us to establish belowground and aboveground interactions with glyphosate and N fertilization. We conclude that glyphosate and N fertilization have negative effects on soil microflora and potential pests, but do not necessarily affect belowground and aboveground interactions, and may offer equal or superior benefits to crop productivity.

1. Introduction

Within a framework focused on sustainable agriculture, linking belowground and aboveground organisms in agrosystems and their response to high anthropic pressure induced by agricultural practices is a major concern. Recently, profound effects of belowground communities on aboveground insects through plant-mediated interactions have been highlighted (Bardgett and Wardle, 2010). Among the most

commonly applied inputs within agrosystems are fertilizers and herbicides. Many studies report their intentional and unintended effects on trophic interactions at the aerial and soil levels within agrosystems (Birkhofer et al., 2008).

Mineral fertilizers, especially nitrogen (N), have been a major contributor to the impressive crop yield increases realized since the 1950s (Robertson and Vitousek, 2009). N may also be considered as a limiting factor for the growth of both plant and soil organisms (Mattson, 1980),

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including belowground microbial communities (Demoling et al., 2007) and the N content of a plant is one of the characteristics that is vitally important to herbivores (Mattson, 1980) in terrestrial ecosystems. In a similar manner to mineral fertilizers, pesticides may induce changes in belowground communities, modifying crop nutrient acquisition and thus relationships between plants and aboveground communities through a bottom-up effect (Saska et al., 2016).

Glyphosate (*N*-phosphonomethylglycine) is a systematic non-selective herbicide, which is the most widely used in the world to control weeds in crops (Helander et al., 2012). Glyphosate acts by inhibiting the activity of 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme found in plants, bacteria and fungi (Padgett et al., 1995), some of which play key roles in soil nutrient cycling (Feng et al., 2005). Glyphosate has been shown to be rapidly decomposed by microorganisms in soil (Giesy et al., 2000) and risks of glyphosate toxicity to non-target organisms are controversial. Degradation of glyphosate depends on the composition and properties of the soil (Gimsing et al., 2007), climate conditions (Helander et al., 2012), and management practices of phosphate fertilizers (Bott et al., 2011). The primary product formed from glyphosate metabolism is aminomethylphosphonic acid (AMPA) which has great environmental persistence and mobility in soil (Kjær et al., 2005), and is also toxic to non-target organisms (Damin and Trivelin, 2011).

Soil processes related to phosphorus (P), carbon (C) and N cycles and soil quality indicators such as arbuscular mycorrhizal fungi (AMF), community-level physiological profiles (CLPP), alkaline phosphatase (AIP) and dehydrogenase (DH) activities, have been found to be very sensitive to the presence of agrochemicals (Ahtiainen et al., 2003; Goverde et al., 2000).

AMF are obligate symbionts, often working in cooperation, based on the exchange of C from the plant and P delivered by the fungi (Smith and Smith, 2011). AMF may be able to modulate the resilience of ecosystems to abiotic stresses such as nutrient deficiency, drought (Garrido et al., 2010), and biotic stresses such as plant herbivores (Shah et al., 2008; Koricheva et al., 2009). AMF are thus important ecosystem drivers (Veresoglou et al., 2012) that can improve plant growth, N uptake (Verzeaux et al., 2016), and P uptake (Goverde et al., 2000). Recent studies have shown conflicting results about the effect of glyphosate on AMF root colonization, ranging from increases to decreases or neutral effects (Malty et al., 2006; Ronco et al., 2008; Druille et al., 2013). Overall, glyphosate and N fertilization can affect soil microbial activities (such as AMF growth and colonization), which are known to create changes in foliar chemistry (increased plant P content, but reduced N) and may thus influence plant-herbivore interactions (Wurst et al., 2004).

In addition, studies have shown that interactions between belowground microbial activity and aboveground herbivore performance can occur (Tao and Hunter, 2012). Short-term changes in the nutrient quality of crop plants induced by the application of fertilizers can influence herbivore populations (Garratt et al., 2010; Tao and Hunter, 2012), and changing the ratio of macronutrients in plants can affect herbivore feeding and performance (Sterner and Elser, 2002). Indirectly, colonization by AMF positively (Gange et al., 1999) or negatively (Koricheva et al., 2009) affects herbivore performances, depending on both the herbivore and fungal species present.

A variety of carbon-containing compounds including accumulated herbicides are available for soil microorganisms, thus influencing the transformations of plant nutrients in the soil (Das et al., 2003). The community level physiological profile (CLPP) has been used in a variety of environments to assess the catabolic capacities among microbial communities (Lowit et al., 2000; Gomez et al., 2004). The responses of the CLPP and some soil enzymes can vary widely depending on the level of N fertilization. For example, several researchers have shown that high levels of N fertilization increased (Olander and Vitousek, 2000; Kalembasa and Symanowicz, 2012), or decreased (Shen et al., 2010; Kalembasa and Symanowicz, 2012) both DH and AIP activities and

microbial functional diversity (Sarathchandra et al., 2001). Additionally, optimum applications of N fertilizer had a neutral effect on microbial functional activities (Lupwayi et al., 2012).

The potential perturbation of soil microbial communities and their processes has attracted interest because of the mode of action of glyphosate (Carlisle and Trevors, 1986). Glyphosate having a low C:N ratio (3:1), the excessive organic N compared to microbial demand may be readily mineralized by heterotrophic microorganisms, thus enhancing microbial activity (Haney et al., 2000). However glyphosate can be toxic for microorganisms, such as certain strains of nitrogen-fixing bacteria (Damin and Trivelin, 2011). It has been shown that glyphosate has no significant effect on soil microbial activity at the recommended field rate as compared to a 100 fold higher dose (Ratcliff et al., 2006). Moreover, glyphosate is traditionally considered to be a herbicide with relatively low ecological and toxicological side effects on terrestrial ecosystems (Giesy et al., 2000) but the impact of the application of glyphosate-based herbicides on insect herbivores, such as aphids, has been studied only rarely (Saska et al., 2016).

Inputs are often studied separately in agrosystems without considering whether they can act individually or together. In addition, studies have mainly focused on the effects of inputs either on the belowground or aboveground compartment (Barnard et al., 2006). The objective of this study was to assess the individual and combined effects of N fertilization and glyphosate applications on belowground and aboveground parameters of plants grown under laboratory conditions. Using this approach, we hypothesize that: 1) N fertilization and treatment with glyphosate can have synergistic and/or antagonistic effects on certain soil microbial activities (e.g. AMF, CLPP, soil enzymes) and the nutrient status of the soil: 2) The changes in belowground microbial activities may modulate both plant nutrient acquisition, plant biomass, and the performance of aboveground herbivores through bottom-up processes.

2. Materials and methods

2.1. Field experiment and soil sampling

Sampling was conducted in November 2015. The soil was collected from the experimental site “La Woestyne” in Northern France (50°44'N, 2°22'E, 40 m above sea level). Prior to the establishment of the experiment in 2010, the field was prepared using a chisel plough and a rotary power system, fertilized conventionally, and cultivated with wheat (*Triticum aestivum* L.). In 2010, winter cover crops (including *Vicia sativa*, *Vicia faba*, *Trifolium alexandrinum*, *Phacelia tanacetifolia*, *Avena sativa* and *Linum usitatissimum*) were sown directly, and the experimental field was split into two N fertilization regimes (without or with N fertilizer). Between 2010 and 2015, the crop rotation in each plot included green pea (*Pisum sativum* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), flax (*Linum usitatissimum* L.), beet (*Beta vulgaris* L.) and wheat. All crops were sown by using a no-till system and following a winter cover crop except wheat, which was sown directly after the maize harvest. The plot without N measured 7 × 8 m while the plot with N measured 14 × 8 m. A 7-m wide corridor separated the two plots to avoid N contamination. N fertilization in the field was determined according to the N budget method (Machet et al., 1990), and the fertilizer used consisted of 50% urea, 25% ammonia and 25% nitrate. Since 2010, a cumulative amount of 650 kg N ha⁻¹ was added to the plot with N, while the plot without N was not fertilized. Since the beginning of the experiment, both plots were frequently treated with glyphosate applications for weed control. The soil is classified as a silt loam with the following properties: 66.8% silt, 21.2% clay, 12% sand. Fifty 20-cm depth soil cores were randomly sampled in each plot by using a 7-cm diameter auger. Fresh soils sampled in each plot were mixed and sieved through a 5-mm mesh. A sub-sample was then separated out, air-dried and sieved (2 mm) for chemical analyses, which were performed before the beginning of the mesocosm experiment (T0).

2.2. Establishment of the mesocosm experiment

A 75-day mesocosm experiment was carried out to assess the aboveground and belowground effects of glyphosate and N fertilization applications on soil nutrients content, microbial activities in the soil, plant performance (*Phaseolus vulgaris*), and aphid nymph survival (*Aphis fabae*) in soil without (N0) or with nitrogen fertilizer (N+). For the mesocosm experiment, 1600 g of fresh homogenized soil with a 75–80% water holding capacity (WHC) was placed into plastic pots (2.2 L volume, 15.5 cm high). The 12 pots were completely randomized, and three replicates were used for each treatment combination. At T0, glyphosate, in the isopropylamine salt form, was applied to the soil surface in the following doses: zero control (CK) and 0.96 mg active ingredient per kg dry weight soil (a.i./dw; FR), prepared with deionized water. FR corresponds to the conventional recommended field application rate (720 g active ingredient ha⁻¹) for soil with a bulk density of 1.45 g cm⁻³. CK received equal to the amount of deionized water added to the glyphosate treatments. The pots were incubated in a dark room at 22 ± 1 °C for 15 days. At T7 (7 days) and T14 (14 days), 150 g of fresh soil samples were manually collected from throughout the pots for chemical analysis (Supplementary Fig. 1). At T15 following glyphosate application and just before sowing of the beans, 798.75 mg KNO₃ kg⁻¹ of soil were applied in the liquid form in the N+ pots. *Phaseolus vulgaris* seeds were sown on day T15 and a long cycle of light (16 h light at 380 μmol m⁻² s⁻¹) was set up to reproduce summer days. Watering was adjusted to water uptake by the plants to ensure similar moisture conditions in microcosms with fast growing plants. At T75, aphids, plants (including root system), and soils were collected. From each replicated pot, the living aphids were put into Eppendorf tubes and frozen. The root system was kept intact as much as possible. Plant roots and plant aboveground biomass were weighed and dried separately before the measurement of C and N contents. The central leaf of the intermediate stage from each plant was stored at –80 °C in the dark until biochemical analyses (chlorophyll, sugars, starch, ammonium and total amino acids). The soil samples were kept at 4 °C one week prior to analysis. The moisture content, pH, total organic carbon (TOC), total nitrogen (TN), extractable inorganic N (NO₃⁻), available phosphorus (PO₄³⁻), cation exchange capacity (CEC), and microbial enzyme activities (dehydrogenase (DH), alkaline phosphatase (ALP), and community level physiological profile (CLPP)) of the soil were measured.

2.3. Measurements of plant parameters

2.3.1. Percentage of root colonization

The AMF colonization of the beans was monitored in 30 root subsamples of 1 cm length per plant and the roots were stained with trypan blue according to Koske and Gemma (1989). Mycorrhizal infection was quantified using the method of McGonigle et al. (1990), with 150 intersections counted for each sample.

2.3.2. Analyses of aboveground biomass

The aboveground biomass was collected manually from each pot. The leaf samples were immediately stored at –80 °C in the dark until analysis, and then the leaf metabolites were extracted by mashing with liquid nitrogen and divided with three analytical replicates. Chlorophyll of leaves was extracted in the dark with 3 mL of acetone (80%) at 0 °C. The chlorophyll content was calculated using spectrophotometric measurements (Eon spectrophotometer, BioTek Instruments Inc., USA) at wavelengths of 470, 645, and 663 nm. Acetone (80%) was used as a control and shown as 1 mg of chlorophyll per 1 g of fresh leaves. The method to determine the starch, and sugar contents was based on that described by Ni et al. (2009), with some modifications. Total starch in each sample was quantified using 30 μL of the insoluble carbohydrate fraction using a kit from Boehringer Mannheim (R-Biopharm, Darmstadt, Germany). To quantify soluble sugars,

amino acids, and starch, about 100 mg of leaves were extracted with 80% ethanol (v/v) (2 h, 4 °C, with agitation), and centrifuged at 12,000g (2 min, 4 °C). The supernatant was extracted and then a second and third extraction were carried out on the pellet with 60% ethanol and water (2 h, 4 °C, with agitation), respectively. After centrifugation, the combined supernatants that included all the soluble molecules were collected for quantification of total soluble and reducing sugars, and stored at –20 °C for amino acid and ammonium determinations. The sugar concentrations were determined enzymatically using the D-Glucose/D-fructose/Sucrose kit (R Biopharm, Mannheim, Germany; Bergmeyer et al., 1974) and were expressed as mg of sugar per g of dry leaves. The total amino acids were determined using the method described by Rosen (1957). The precipitates were stored at –20 °C before the determinations of starch. The starch content was measured by drying the pellet at 100 °C to remove the residual ethanol and placed in water at 100 °C to release enzymes, amylose and amylopectin. The starch concentration was determined enzymatically using α-amylase and amyloglucosidase with acetate buffer according to the published protocol of Smith and Zeeman, 2006. The fresh biomass was weighed and a representative subsamples were dried at 65 °C for 96 h to determine the dry matter content. Metabolite concentrations were calculated on the basis of dry weight.

Freeze-dried, above-ground biomass samples were ground to a powder and approximately 1 mg was weighed into tin capsules. C and N were measured using an elemental analyzer (Flash EA 1112, Thermo Electron, Germany).

2.4. Measurements of soil parameters

2.4.1. Chemical analyses

Soils were passed through a 2 mm sieve and oven-dried at 45 °C for 48 h. Moisture was measured after drying samples at 105 °C for 24 h. The pH was measured after shaking the dried soil with deionized water (1:5 dry mass ratio) for 45 min. The mixture was then left for 5 min and the pH was recorded using a FE20-FiveEasy™ pH meter (Mettler Toledo, Switzerland). CEC was quantified by shaking the soil samples for 1 h with a solution of hexammine cobalt chloride (1.66 mol L⁻¹) (1:10 mass ratio) (Ciesielski et al., 1997). After filtration, absorbance was measured at two wavelengths (475 and 380 nm) using an Eon spectrophotometer (BioTek Instruments Inc., USA). For TOC, TN and PO₄³⁻, sieved soil was finely ground by using a ball-mill (Retsch, MM400). TOC and TN was analyzed using a CN elemental analyzer (Flash EA 1112, Thermo Electron, Germany). Since initial analyses revealed that the soil was free of carbonate, the soil total C was assumed to be equal to the TOC. The NO₃⁻ content of the soils was determined by extracting the soil samples with 2 M KCl (1:5 fresh mass ratio) for 1 h on a rotary shaker. The extracts were centrifuged for 10 min at 4000 rpm and the supernatants were analyzed by a continuous flow analytical system (Alpkem flow solution IV power base, OI Analytical, USA). Taking the NO₃-N contents (NO₃⁻) before the incubation (T0) as baselines, the net nitrification rate (NIT) was calculated as follows (Eq. (1)):

$$NIT = (Nt - N0)/t \quad (1)$$

where, *N0* and *Nt* are the NO₃⁻-N (including NO₂⁻-N) contents of the soil at time 0 and *t* after incubation (Man and Zucong, 2009).

The PO₄³⁻ content was determined by extracting the soil samples with 0.5 M NaHCO₃ pH 8.5 (1:20 dry mass ratio) and was measured in the extract using the colorimetric method at 882 nm (Olsen et al., 1954).

The analysis of glyphosate and AMPA (aminomethylphosphonic acid - the major degradation product of glyphosate) concentrations in soils was performed by Eurofins laboratories (France, www.eurofins.fr/env) using liquid chromatography coupled to tandem mass spectrometry.

2.4.2. Microbial analyses

2.4.2.1. Determination of enzyme activities. Dehydrogenase activity (DH) based on the reduction of the 2,3,5-triphenyltetrazoliumchloride (TTC) to triphenyl tetrazolium formazan (TPF) was determined according to the method initially described by Casida et al. (1964) with some modifications (Nivelles et al., 2016). Soil samples prepared with CaCO₃ (100:1 fresh mass ratio) were mixed both in a 2,3,5-triphenyltetrazoliumchloride (TTC) solution (3%) and in deionized water, and then incubated at 37 °C for 24 h in a dark room. After incubation, TPF was extracted with a solution of pure methanol. After filtration, TPF concentrations were determined by spectrophotometry at 485 nm. Alkaline phosphatase activity (ALP) was determined according to the method described by Tabatabai and Bremner (1969). Fresh soil samples were pre-incubated at 27 °C for 48 h. After pre-incubation, deionized water was added to soil samples (1:1 mass ratio) and mixed. A part of the soil samples was used, and substrate *p*-nitrophenyl phosphate solution (pNPP, 1%) and borate buffer (pH 9) were added. After incubation at 37 °C for 1 h, CaCl₂ (0.5 N) and NaOH (1 N) were added to stop the reaction. Finally, all samples were centrifuged at 10,000g for 5 min. The released *p*-nitrophenol (*p*-NP) was measured at 405 nm.

2.4.2.2. Community level of physiological profiles. CLPP were assessed using Biolog EcoPlates™ (BIOLOG, Hayward, USA) as described by Govaerts et al. (2007). The analysis of CLPP was started within 24 h after sampling. Briefly, 10 g of fresh soil samples were shaken for 60 min with 90 mL of sterilized saline solution (0.85% NaCl, w/v) and brought to a 10⁻³ final dilution. Each well of the Biolog EcoPlates was inoculated with 150 µL from each extraction that included three replicates. The plates were incubated at 25 °C in the dark for 196 h and read at 590 nm every 24 h. Data recorded at the exponential phase (120 h) were used to calculate the average well color development (AWCD) and Shannon index (H'). AWCD and H' represent the soil functional activity and diversity respectively.

2.5. Measurements of aphid parameters

A colony of *Aphis fabae* was initiated from a single apterous parthenogenetic female (provided in 2016 by the National Agricultural Research Institute of Colmar, France). The insects were maintained on faba bean (*Vicia faba*) inside ventilated plastic cages (360 × 240 × 110 mm) in growth chambers under controlled conditions (20 ± 1 °C, 60 ± 5% relative humidity, and a 16L:8D photoperiod at 2 klux) to induce parthenogenesis. Twenty-four hours before the start of the aphid experiment, adult aphids were placed on *Vicia faba* leaves in 9 cm diameter Petri dishes containing agar. At T68 (week 10), nine aphid nymphs of *A. fabae* of < 12 h taken from the aphid culture were enclosed in 7 small clip cages (made from ventilated 3.5 cm diameter Petri dishes, attached to either side of a hair clip) placed on intermediately-aged leaves of each *P. vulgaris* plant. Every 24 h, the number of living and dead aphids was counted during one week. At T75 (week 11), the living aphids were collected, stored at -80 °C and then lyophilized for C and N analyses (Flash EA 1112, Thermo Electron, Germany).

2.6. Data analysis

All statistical analyses were performed using R software v. 3.1.2 (R Development Core Team, <http://www.R-project.org>, 2014). For statistical analyses, the 31 Biolog substrates were both non-grouped into AWCD and H' and grouped into (1) phosphate carbon (2) amines, (3) amino acids, (4) polymers, (5) carboxylic acids, and (6) carbohydrates. In figures and tables, differences between treatments were performed by a non-parametric Kruskal-Wallis test followed by a Conover post-hoc test ($p < 0.05$). Tests were performed using the PMCMR package (Pohlert, 2016). A Tukey test was performed using the “agricolae”

package (De Mendiburu, 2016). Values presented in figures and tables correspond to the average of three data ($n = 3$) ± standard error (SE). The Cox model was used to compare the effects of treatments on the survival rate of aphid nymphs (*Aphis fabae*), depending on the number of days following bean (*Phaseolus vulgaris*) infestation, according to an ANOVA test. Values are means ($n = 162$).

Correlations between environmental variables (soil parameters), plant and aphid performances were computed using Spearman's rank correlation coefficient (“Hmisc” package). A non-metric multi-dimensional scaling (NMDS) of the plot × plant and aphid responses matrix was employed to visualize the differences in the performance of the plant and aphid performances among treatments, in addition to the Bray-Curtis distance and the “vegan” package in R. To see how these compositional differences were related to environmental variables, the significant impacting variables were subsequently fitted onto the NMDS ordination using the ‘envfit’ function in the “vegan” package (Oksanen et al., 2015). Soil, plant and aphid parameter data were analyzed using a two-way ANOVA with (i) glyphosate and (ii) fertilizer as factors.

3. Results

3.1. Negative effects of glyphosate and/or N fertilization on soil enzymes and arbuscular mycorrhizal fungi

Soil enzyme activities and AMF were significantly affected by the different treatments (Fig. 1). For the field application rate of glyphosate (FR), we observed a significant increase ($p = 0.038$) in the percentage of root length colonized by AMF following growth of the bean plants in nitrogen-free soil (FR/N0), compared to the root length colonized following growth in fertilized (FR/N+) soil (Fig. 1A). There was a significant increase ($p = 0.040$) in the DH activity determined in untreated soil (CK/N0) compared to the activity in N fertilized soil, which had not been treated with glyphosate (CK/N+) (Fig. 1B). There was a significant increase ($p = 0.024$) in ALP activity determined in non-fertilized soils that had received a FR dose of glyphosate, compared to N fertilized soils, irrespective of whether glyphosate had been applied. Also ALP activity was significantly greater in non-fertilized soils, which had not received treatment with glyphosate (CK/N0), compared to soils receiving both N fertilization and glyphosate (FR/N+) (Fig. 1C).

There was no effect of the different treatments on functional responses (AWCD, $p = 0.48$; H', $p = 0.37$) (data not shown).

3.2. Positive effects of glyphosate and/or N fertilization on soil nutrient content, bean growth, and nutrient status of plant

There were significantly higher levels of total nitrogen (TN) ($p = 0.015$) in soils receiving both fertilizer and glyphosate (FR/N+) compared to non-fertilized (N0) soils irrespective of whether glyphosate had been applied (CK or FR), and higher levels of N in CK/N+ soils compared with FR/N0 soils (Fig. 2A). Fig. 2B shows exactly the inverse of Fig. 2A concerning the C:N ratio ($p = 0.014$). There were significantly higher levels of nitrate ($p = 0.024$) in soils that had been fertilized with N (CK/N+ and FR/N+), as compared to unfertilized soils (CK/N0 and FR/N0) (Fig. 2C). There was a significant increase ($p = 0.024$) in the average net nitrification rate in fertilized soils (N+) compared to non-fertilized soils (N0), irrespective of whether glyphosate had been applied (CK or FR) (Fig. 2D).

Overall, plants that received both glyphosate and N fertilizer had significantly higher levels of total chlorophyll (Fig. 3A, $p = 0.022$), amino acids (Fig. 3B, $p = 0.025$), and ammonium (Fig. 3C, $p = 0.033$) compared to non-fertilized (N0) plants without or with the application of glyphosate (CK or FR). Significantly higher levels of sucrose (Fig. 3D, $p = 0.047$) were found in plants that had received the N+ treatments (CK/N+ and FR/N+) compared to plants that did not receive either fertilization or glyphosate (CK/N0). Higher dry weights of bean pods were harvested from plants that had received nitrogen fertilization (CK/

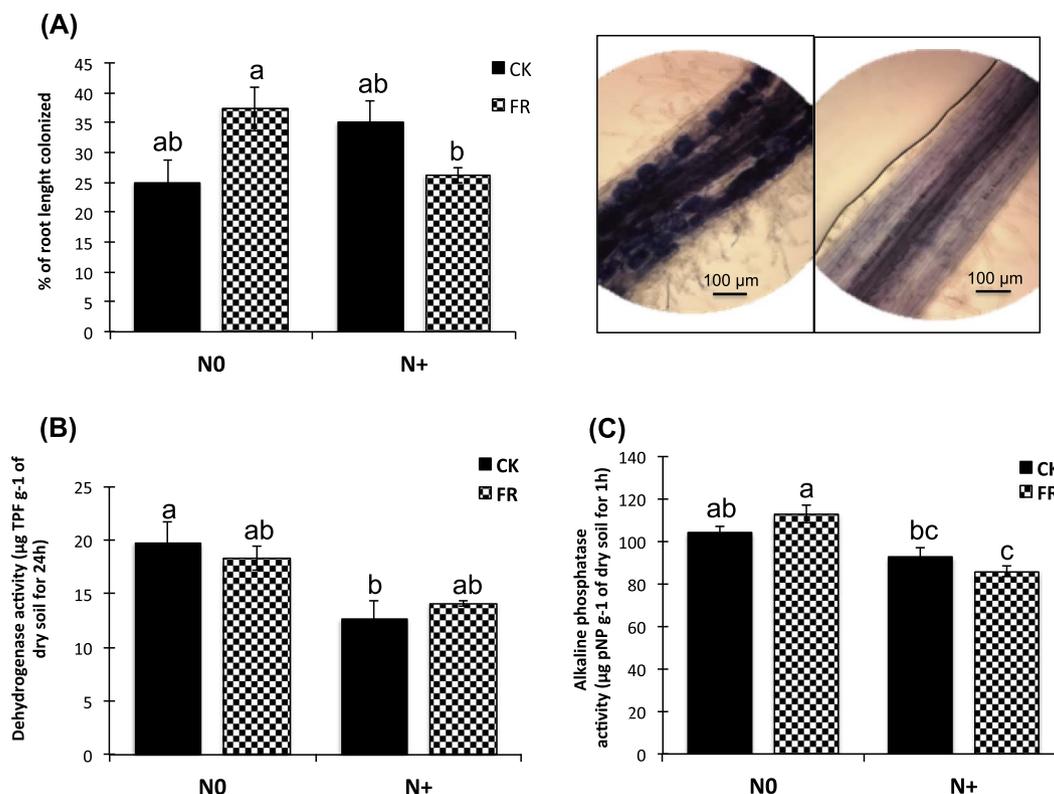


Fig. 1. After 11 weeks (T75), effects of the glyphosate and nitrogen fertilizer applications on (A) percentage of root length colonized by arbuscular mycorrhizal fungi of soil collected from mesocosms without (control, CK) or with (field rate, FR) glyphosate herbicide and without (N-) or with (N+) nitrogen fertilizer application (inset shows a common bean root colonized (left photo) or not (right photo) by arbuscular mycorrhizal fungi), soil (B) dehydrogenase activity and, (C) alkaline phosphatase activity. Vertical bar represent mean values ($n = 3$) \pm SE. The vertical bars with different lower-case letters are significantly different from each other at $p < 0.05$ according to Conover test posthoc.

N+ and FR/N+) compared to non-fertilized plants (CK/N0 and FR/N0).

3.3. Positive effects of N fertilization on survival rate of nymphs

The Cox model showed the significant difference between N0 and N+ fertilization ($p = 0.008$) (Fig. 4). The results showed no significant effect of glyphosate alone ($p = 0.820$) nor of a coupled effect of glyphosate and N fertilizer ($p = 0.750$) on the survival of the nymph aphids.

3.4. Linking above-ground and below-ground interactions

Significant effects of glyphosate were found on the C:N ratio of the soil, the TN, and the ammonium content of the leaves (Table 1). Similarly, values of NIT, ALP, DH, C:N ratio of the soil, TN, CEC, NO_3^- and all above-ground parameters were significantly affected by N fertilization. Finally, significant effects of glyphosate and N fertilizer were determined on the microbial degradation of carboxylic acids, C:N ratio of the soil, TN, and AMF.

Overall, DH was positively correlated with ALP ($r^2 = 0.70$, $p < 0.01$) and C:N in soil ($r^2 = 0.75$, $p < 0.01$), and negatively correlated with NO_3^- ($r^2 = -0.90$, $p < 0.001$), NIT ($r^2 = -0.89$, $p < 0.001$), sucrose content ($r^2 = -0.74$, $p < 0.01$), and nymph survival ($r^2 = -0.77$, $p < 0.01$), while ALP was negatively correlated with pod biomass ($r^2 = -0.77$, $p < 0.01$), starch ($r^2 = -0.68$, $p < 0.05$), chlorophyll ($r^2 = -0.71$, $p < 0.01$), ammonium ($r^2 = -0.72$, $p < 0.01$), and amino acid content ($r^2 = -0.71$, $p < 0.01$) of plants (data not shown). Consistently, CEC was negatively correlated with AWCD ($r^2 = -0.66$, $p < 0.05$) and with the carboxylic acid degradation ($r^2 = -0.67$, $p < 0.05$), while amine degradation was positively correlated with TOC ($r^2 = 0.75$, $p < 0.01$). H'

was negatively correlated with total sugars ($r^2 = -0.66$, $p < 0.05$) and sucrose content ($r^2 = -0.71$, $p < 0.01$). TOC was only positively correlated with plant C ($r^2 = 0.68$, $p < 0.05$), aboveground biomass ($r^2 = 0.69$, $p < 0.01$), root biomass ($r^2 = 0.65$, $p < 0.05$), and total biomass ($r^2 = 0.67$, $p < 0.05$). Nitrate content, nitrification rate, and total nitrogen were positively correlated with plant nutrient status (plant C, plant N, sucrose, ammonium, amino acids) and yield of plant (pod biomass, aboveground biomass, root biomass), and negatively correlated with the C:N ratio in both the plant and the soil. The survival of aphid nymphs was positively correlated with NO_3^- ($r^2 = 0.75$, $p < 0.01$), NIT ($r^2 = 0.73$, $p < 0.01$), and negatively correlated with DH activities ($r^2 = -0.77$, $p < 0.01$).

The NMDS showed that only nitrogen fertilization had an overall effect on all parameters ($p = 0.003$). Specifically, the N fertilized soils irrespective of the glyphosate treatments (CK/N+ and FR/N+) were characterized by an increase in the aboveground yield of the beans (total biomass, above-ground biomass, and pod biomass), and the concentrations of some plant nutrients (total sugar, starch, sucrose, chlorophyll, ammonium, amino acids, plant nitrogen, and plant carbon) and of nymph survival. In contrast, CK/N0 and FR/N0 were strongly separated from other treatments, and were characterized by an increase in root biomass, photosynthesis, plant and aphid C:N ratio. The first axis clearly divided N0 and N+ treated soils, probably initially due to the different responses of plants, AMF, and aphids between these treatments, and secondly due to a positive correlation with ALP ($p < 0.05$) activity, DH activity, soil C:N ratio ($p < 0.01$). Conversely, N+ treatment was correlated positively with TN and NO_3^- content ($p < 0.01$) (Fig. 5).

4. Discussion

Overall, the results show that in soils that have not been subjected

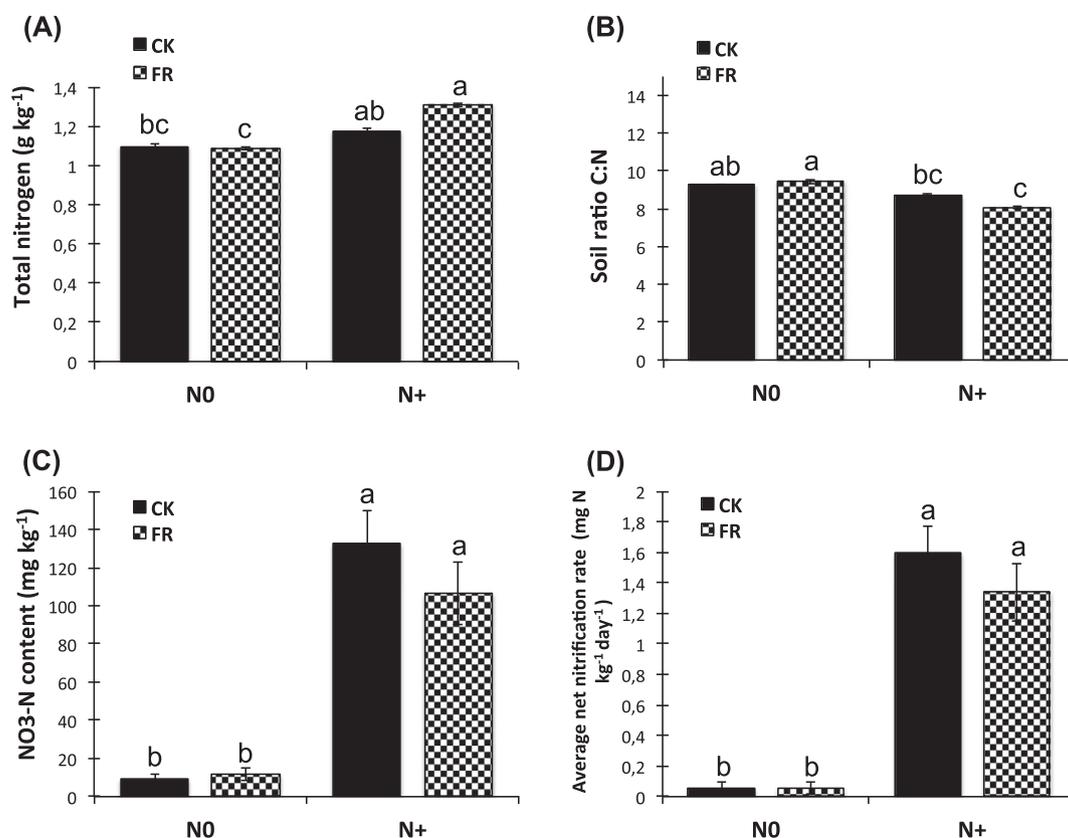


Fig. 2. (A) Total nitrogen content, (B) ratio soil C:N, (C) nitrate content and (D) average nitrification rate measured in soils collected from mesocosms without (control, CK) or with (field rate, FR) glyphosate herbicide and without (N⁻) or with (N⁺) nitrogen fertilizer application. Vertical bar represent mean values ($n = 3$) \pm SE. The vertical bars with different lower-case letters are significantly different from each other at $p < 0.05$ according to Conover test posthoc.

to N fertilization, there were increases in microbial activities, regardless of the application of glyphosate. However when N fertilization and glyphosate were combined, there was a reduced effect on belowground activities. Conversely, in soils that had been fertilized with N, there was an increase in the nitrogen nutrients in the soil, the primary metabolites of the plants and also the survival of the aphid larvae. Glyphosate combined with N fertilization had a neutral or synergistic effect on the wide range of belowground and aboveground parameters discussed below.

4.1. Effects of chemical inputs on below- and above-ground parameters

4.1.1. Effects of nitrogen fertilization

As shown in Table 1 and Fig. 1, nitrogen (N) fertilization did not affect AMF. These results disagree with other studies which have shown that N fertilization can reduce root colonization by AMF (Egerton-Warburton and Allen, 2000; Verzeaux et al., 2016), but this depends on several factors especially host specificity, and soil N:P ratio (Johnson et al., 2003). By contrast, soil enzyme activities (dehydrogenase (DH) and alkaline phosphatase (ALP)) were negatively affected by N fertilization. In agreement with other research, N fertilization has been shown to inhibit both the DH activity (Shen et al., 2010) and the ALP activity (Ajwa et al., 1999; Shen et al., 2010; Kalembsa and Symanowicz, 2012). These results suggest that these two enzymes can be used as indicators of ecological changes caused by N fertilization and management practices, as supported by research by Ajwa et al. (1999).

This study demonstrated the positive effect of N fertilization on nitrification, soil nutrient content such as nitrates (NO₃⁻), total nitrogen (TN) (Fig. 2), plant nutrient status (amino acids, total sugars, sucrose, chlorophyll, plant carbon (C), plant N, starch, ammonium), and plant yield (total biomass, pod biomass, aboveground biomass) (Fig. 3). The increase in nutrient levels in leaves by the addition of

nitrogen may increase carbon flow to the roots and promote chemorganotrophs capable of degrading the organic matter of the soils (Moreira and Siqueira, 2006). In addition, N fertilization stimulated an increase in the NO₃ content of the soil, which has been shown to be a major pathway of N nutrition for soybeans (Nelson et al., 1984).

There was a positive effect of N fertilization on the survival of aphid nymphs (Fig. 4). These results are consistent with some studies showing that the response of aphids to an increase in N was positive. Other studies are consistent with our results and have shown that N fertilizer use can have an impact on insect populations directly through at least two pathways in crop plants: nutritional status and plant defense. An improved nutritional status of plants can enhance insect population growth (Awmack and Leather, 2002) because N is a macronutrient known to be limiting for phytophagous insects (Behmer, 2009).

4.1.2. Effects of glyphosate

After 11 weeks (T75), AMF, which are ecologically and physiologically important to many plant communities, were not affected by the application of glyphosate (Table 1). These results are inconsistent with Druille et al. (2013) who have shown that individually, glyphosate can reduce root colonization by AMF. These contrary results can be explained by several factors including application rates, growth stage of the crop and application site of glyphosate (Malty et al., 2006). Indeed Druille et al., 2013 have shown that the direct application of glyphosate at the field rate to the soil results in a lower reduction of the arbuscules compared to an indirect application of glyphosate to the foliage of plants, and it is the direct application that was carried out in this study. These results also show that glyphosate applied alone had no effect on plant C metabolites, thus limiting the potential of modifying plant-fungal communication. In addition, enzymatic activities were not affected by the application of glyphosate. In agreement with other studies, we suggest that the field rate used is insufficient to have an effect

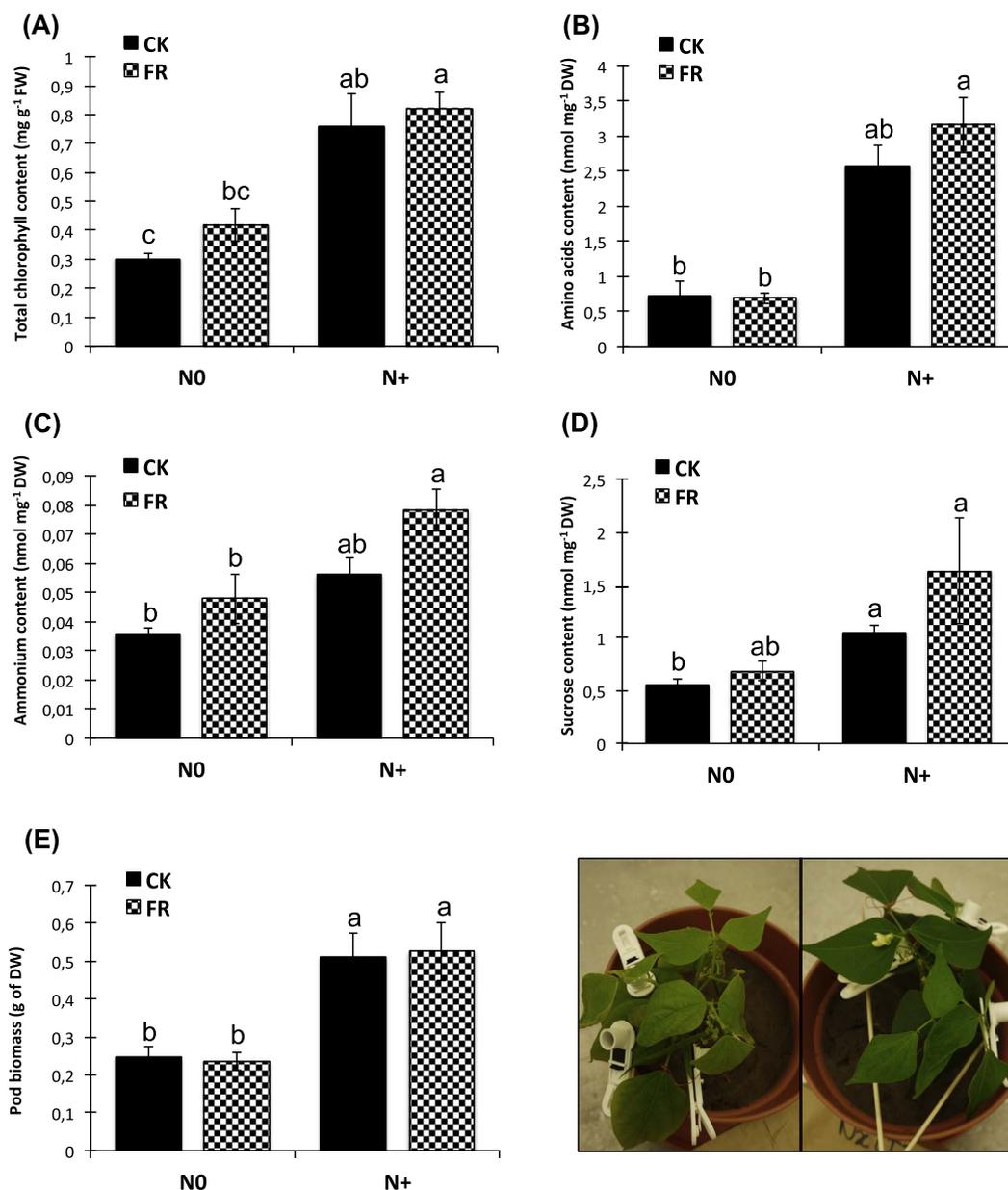


Fig. 3. (A) Total chlorophyll (Chlorophyll a + b; mg g^{-1} FW), (B) amino acids, (C) ammonium, (D) sucrose, and (E) pod biomass measured in common bean collected from mesocosms without (control, CK) or with (field rate, FR) glyphosate herbicide and without (N⁻) or with (N⁺) nitrogen fertilizer application. Vertical bar represent mean values ($n = 3$) \pm SE. The vertical bars with different lower-case letters are significantly different from each other at $p < 0.05$ according to Conover test posthoc. Inset shows two bean leaves with more (right photo) or less (left photo) of total chlorophyll content pigments.

on the activities of DH and AIP (Ratcliff et al., 2006).

The application of glyphosate affected the TN content and the C:N ratio of the soil, as well as the ammonium (NH_4^+) content of the plants (Table 1). Glyphosate can affect processes of the N cycle that are mediated by the microbiota and can change the metabolism of N inside the plant, depending on the transformations of nitrogen by soil microbes (Grossbard and Atkinson, 1985; Haney et al., 2002). For instance, it was shown that the herbicide ammonium glufosinate inhibits the incorporation of NH_4^+ in organic compounds and causes a build-up of ammonium content in plant tissue. A part of the accumulated NH_4^+ can be exuded through the roots (Damin and Trivelin, 2011). Our results agree with the literature, which is why we suggest that glyphosate increased the TN content of the soil leading to a decrease of the incorporation of NH_4^+ -N into amino acids in the plant tissues. Then, a greater exudation of this ion by the roots may decrease the soil C:N ratio. The overall decrease in TN and TOC (total organic carbon)

observed in the Supplementary Fig. 1 between 14 and 75 days following herbicide application may be explained by an increased mineralization of organic material under high temperature and frequent soil humidification in the mesocosm (Mikha et al., 2005; Fontaine et al., 2011).

There was no effect of glyphosate on biomass and yield of beans and on aphid performance. Our results are in disagreement with Lipok (2009) who reported that *Aphis fabae* development was reduced in the presence of glyphosate. The previous disagreements may be explained by the contrasted glyphosate application patterns. In our study, aphids were placed on leaves of *Phaseolus vulgaris* 68 days after application of glyphosate to the soil surface. In the study of Lipok (2009), aphids were placed on leaves of *Vicia faba* 24 h after a glyphosate addition via glass capillary or were directly reared on glyphosate-enriched artificial diet. Therefore, the available literature does not provide sufficient data for understanding the indirect consequences of glyphosate on pests.

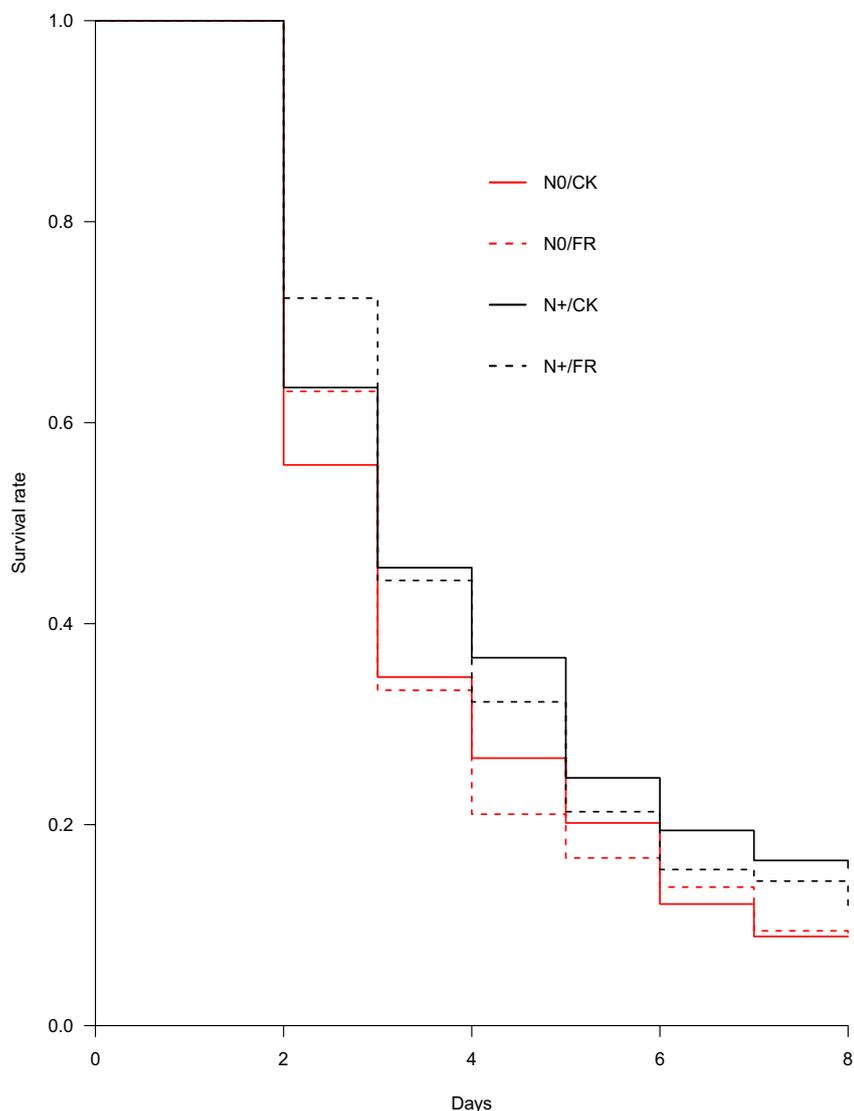


Fig. 4. Effect of glyphosate and/or N fertilization on survival rate of aphid (*Aphis fabae*) nymph depending on the number of days following infestation of bean (*Phaseolus vulgaris*). In legend: black full line: CK/N +: without glyphosate/with nitrogen fertilization; Red full line: CK/N0: without glyphosate/without nitrogen fertilization; Black dotted line: FR/N +: with glyphosate/with nitrogen fertilization; Red dotted line: FR/N0: with glyphosate/without nitrogen fertilization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Analysis of variance for soil (microbial and chemical), plant and aphid properties as affected by glyphosate (G), or fertilizer (F) and their interactions (G × F).

Stars represent significance level according to ANOVA (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001). NIT: average nitrification rate, AIP: alkaline phosphatase activity, DH: dehydrogenase activity, CARB. ACIDS: microbial degradation of carboxylic acids, SOIL C:N: ratio soil C:N, TN: total nitrogen, CEC: cation exchange capacity, NO₃⁻: soil nitrate content, AMF: arbuscular mycorrhizal fungi, NYMPH SURV: survival rate of nymph aphid, T. BIOM: plant total biomass, AA: leaf amino acid content, AMMONIUM: leaf ammonium content, STARCH: leaf starch content, T.SUGARS: leaf total sugar content, SAC: leaf sucrose content, CHL: leaf chlorophyll content, PLANTC:N: ratio plant C:N, PLANT N: plant nitrogen content, PLANT C: plant carbon content.

Below-ground parameters										
Treatments	NIT	AIP	DH	CARB. ACIDS	SOIL C:N	TN	CEC	NO ₃ ⁻	AMF	
G	0.385	0.838	0.943	0.984	0.0290*	0.000449***	0.0771	0.358	0.728	
F	5.67 × 10 ⁻⁷ ***	0.000323***	0.00195**	0.681	1.04 × 10 ⁻⁶ ***	1.04 × 10 ⁻⁶ ***	0.0453*	1.95 × 10 ⁻⁵ ***	0.712	
G × F	0.415	0.052	0.303	0.0234*	0.00196**	0.000345***	0.152	0.152	0.00651**	

Above-ground parameters												
	NYMPH SURV	T. BIOM	POD BIOM	AA	AMMONIUM	STARCH	T. SUGARS	SAC	CHL.	PLANT C:N	PLANT N	PLANT C
G	0.760	0.669	0.968	0.345	0.0267*	0.0722	0.154	0.280	0.246	0.267	0.423	0.575
F	0.0453*	0.0297*	0.00114**	5.11 × 10 ⁻⁵ ***	0.00272**	0.0183*	0.0342*	0.044*	0.000152***	0.00162**	0.00930**	0.0177*
G × F	0.139	0.304	0.815	0.339	0.469	0.0871	0.571	0.505	0.692	0.940	0.432	0.330

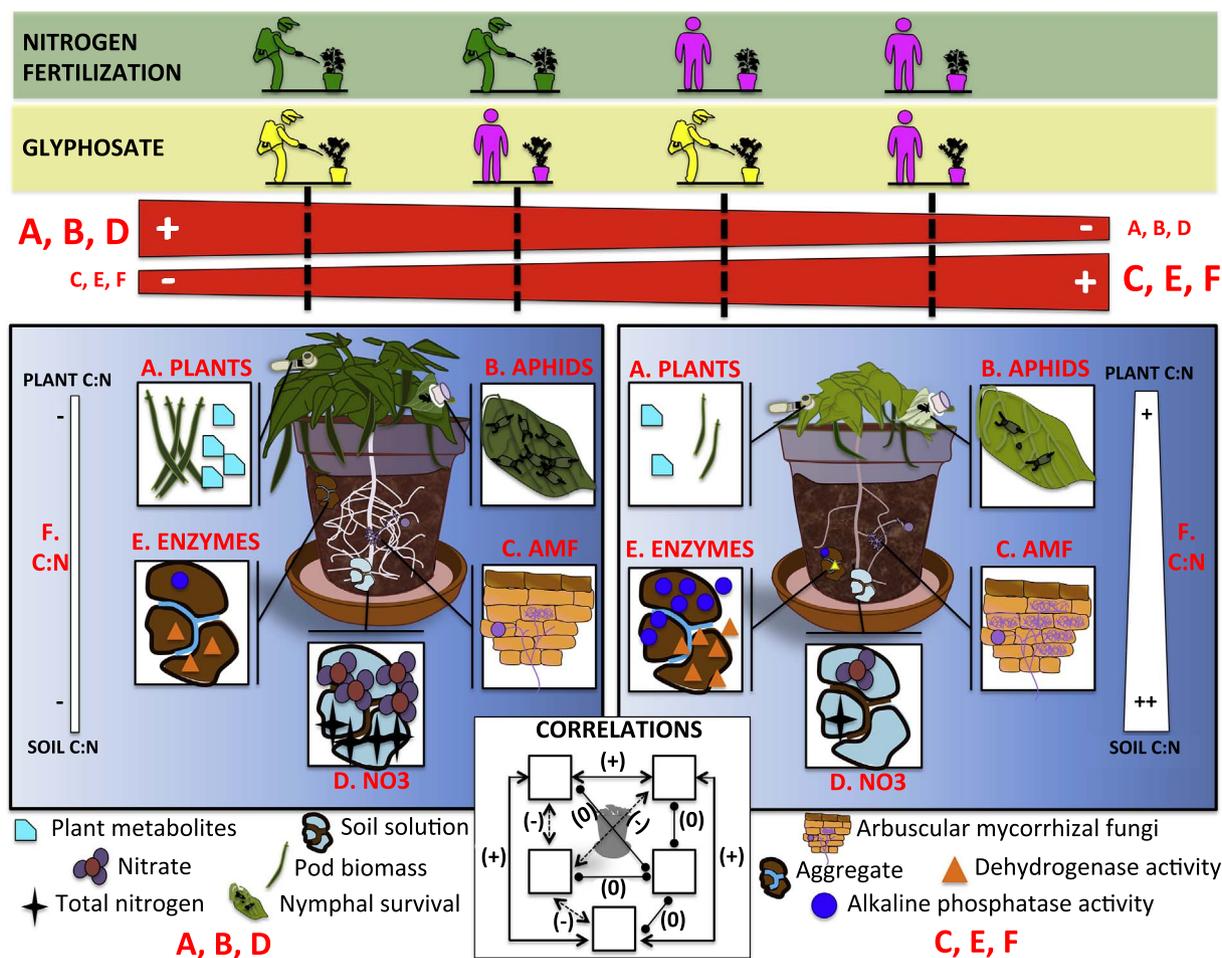


Fig. 6. The two rectangles summarize the effects of glyphosate and/or nitrogen fertilization on soil, plant and aphid parameters. The square in the bottom center of the figure represents the links between the aboveground and belowground interaction: (0) no correlation; (+) positive correlation; (–) negative correlation. In legend: the color pictograms representing personages and plants symbolize the treatments as follow: yellow color: glyphosate, green color: nitrogen fertilization, pink color: water. Abbreviations in figure: P: Phosphorus, C: carbon, N: nitrogen, C/N –: low C:N ratio, C/N +: high C:N ratio, C/N ++: highest C:N ratio, AMF: arbuscular mycorrhizal fungi. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Belowground and aboveground linkages with nitrogen fertilization and glyphosate applications

4.2.1. Interactions between soil and plant physiology

The correlations between all parameters (data not shown) and NMDS (Fig. 5) highlight the important contribution of a N fertilizer compared to the contribution of glyphosate on certain soil variables (TN, NO₃, C:N ratio) as well as on soil enzyme activities (DH and ALP) and the differentiated consequences that this may have on the performance of plants.

The Figs. 2, 3, and 5 show that the addition of N fertilizer led, after 11 weeks (T75), to a significant increase in the levels of soil TN, NO₃, and nitrification rates. These increases stimulated a greater synthesis of nutrients (sucrose, starch, amino acids, chlorophyll, and ammonium) required for plant growth and biomass (aboveground, pod) production. In parallel with our results, Winter and Rostás (2010) showed that photosynthetic efficiency, leaf N, and soluble protein content were significantly decreased in N deficient plants, whereas root biomass was increased. Streeter (1972) showed that the activity of nitrate reductase increased during the pod-filling stages in connection with an increase of nitrate in the soil. In the N+ treated plants, NO₃-assimilation has constituted an important role in N nutrition by providing the N complement required by plant growth. Our results suggest that the increase in TN in fertilized soils is derived from the assimilation and metabolism of N into the microbial biomass (Kuzakov and Xu, 2013).

Interestingly, Fig. 5 showed that the absence of N fertilization led to increased enzyme activities (DH and ALP) and to an increase in the C:N ratio, parameters negatively correlated with nitrate levels, soil nitrification and certain physiological traits of the plant (pod biomass, amino acids, chlorophyll, and ammonium). These results are in agreement with Akmal et al. (2012) who showed that enzymatic activities were negatively correlated with nitrate levels in soil. Moreover, it has been shown that a decrease in the levels of inorganic N in soils may lead to greater interspecific competition between soil microorganisms and plant roots (Kuzakov and Xu, 2013), thus reducing the accessibility of plants to nutrient resources. In agreement with other studies, we showed that the increase in the C:N ratio could improve enzyme activities and promote nutrient retention, and thus reduces the availability of N for the plant (Heijboer et al., 2016), because C availability is known as a limiting factor for microbial growth (Wardle, 1992). Ajwa et al. (1999) showed a link between the activities of DH and the C input via root mass, which could be at the origin of the improvement of the microbial activities. Our results partially corroborate the study of Ajwa et al. (1999), as the data show a positive correlation between amine degradation by microorganisms and root biomass (Fig. 5; r² = 0.61). In addition, the absence of N fertilizer allowed for an expansion of the plant root system and an increase in the enzyme activities.

In connection with a study carried out by Kalembasa and Symanowicz (2012), we suggest that the high values of biochemical indicators (enzymes activities, AMF) in soils having received a very low

dose of N input (N0) may have maintained the trophic balance between microbial activities (soil enzymes, AMF) and plant roots. In the long term this balance could be favorable to the ecological stability of unfertilized agrosystems.

We can note that the low dose of glyphosate added > 90-days before the final measurements did not induce major significant differences between the different treatments. Even if we accept that the addition of glyphosate to unfertilized soils can promote the synthesis of some carbon-rich sugars (glucose, fructose) and chlorophyll, it is detrimental for other nutrients rich in nitrogen (amino acids, ammonium) and thus contributes to a significant loss of bean biomass (Roth, 2009; Zilli et al., 2009). These authors have demonstrated that glyphosate application has been shown to strongly reduce root nodulation and thus nitrogen acquisition of the bean plant. Contrary to our hypotheses, we have seen no positive effect of microbial activities (DH, AIP, AMF, CLPP) on plant nutrient acquisition, plant biomass, or soil N content.

4.2.2. Interactions between soil, plant physiology and aphid performance

N fertilization alone or with glyphosate leads to an increase in soil nutrient content and the immediate availability of nitrate for plants. The consequences were a decrease in microbial activities and an increase in aerial biomass and primary plant metabolites suggesting an increase in appetite and therefore pressure of herbivores. Numerous studies have shown that mycorrhizal fungi associated with plants may alter above-ground interactions between plants and herbivores (Vannette and Rasmann, 2012). In our study, the reduction in the amount of AMF did not affect the performance of the aphids but N fertilization altered the plant-aphid relationship independent of any indirect consequences on AMFs. Our results are consistent with Koschier et al. (2007), who showed that cucumber root colonization by AMF did not affect host acceptance and development of another piercing/sucking insect, the thrips species *Frankliniella occidentalis*.

The data presented in Fig. 5 show the interactions between plant physiology and nymph survival. Compared to unfertilized pots, the increase of soil TN and nitrification of the soil, as well as increased plant nutrient status in fertilized pots led to enhanced nymph survival. Other research has agreed with our results, as Mahdavi-Arab et al. (2014) found that aphid performance increased with higher plant biomass and depended upon plant growth conditions, while Bogaert et al. (2017) reported that N fertilization was related to increased aphid performance through improvement of plant nutrient status. The nutrient status can be related to the availability of dietary N (free amino acids) in the phloem, a limiting factor for aphids growth and reproduction (Dixon, 1998).

The result presented here show a bottom-up effect, as the increase in the C:N ratio of the soil is itself correlated with the increase in the C:N ratios of the plant and of the aphids. Thus limiting N in the soil (high C:N) may limit the acquisition and remobilization of N in the plant and the aphid that feeds on it (high C:N). Indeed, improved plant nutritional quality such as increased leaf N content (i.e. decreased C:N ratio) has been reported to induce higher herbivore development (Awmack and Leather, 2002; Zehnder and Hunter, 2008) and population growth rate (Gratton and Denno, 2003).

Thus, our results confirmed that N fertilization plays a central role in bottom-up relationships by creating an imbalance between the C and N levels of soil, which is detrimental to soil enzyme activities, but does not lead to a decrease in the performance of plants and aphids. There is no evidence of a link between soil enzyme activity and, subsequent aphid performance.

5. Conclusion

In agriculture, crop productivity enhancement through the use of synthetic fertilizers and pesticides has often been over-emphasized, neglecting the potential services ensured by bottom-up interactions from the soil. In the present study, we have observed: (1) no effects of N

fertilization on belowground microbial parameters such as soil functional activity (AWCD), functional diversity (H' index); (2) negative effects on enzyme activities (DH, AIP) and AMF, with neutral and antagonist effects when N fertilization were applied in combination with glyphosate. Conversely, our results demonstrated increased levels of belowground (total nitrogen, nitrate content) and aboveground (plant metabolites, yield and nymph survival of the herbivorous *Aphis fabae*) following N application to the soil, with neutral or synergetic effects when N was applied in combination with glyphosate. Thus, it seems that there exists both belowground and aboveground linkages between nitrogen fertilization and glyphosate applications. The diversity of organic inputs through mixed cover crop residues and the improvement of AMF cycle through the supply of permanent living roots must be assessed in further research to develop new agricultural practices that prevent soils from deleterious effects of intensive chemical inputs.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2017.10.002>.

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