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Stimulation of bacteria and protists in rhizosphere of glyphosate-treated barley

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ABSTRACT

Glyphosate is extensively used for weed control and to ripen crops. Despite a number of studies on the direct effect of glyphosate on plants and soil organisms, only little is known about indirect effect of glyphosate on rhizosphere microbial communities, following the accelerated turnover of the fast-dying root biomass. In microcosms we studied the indirect effect of glyphosate on the microbial community in the rhizosphere of barley with phyllosphere application of glyphosate in comparison to leaving the plant intact or cutting off the shoot. Attempting to link the response of bacterial and protist communities to foliar application of glyphosate, we measured bacterial and protist abundance, diversity and physiological status, as well as soil organic carbon. Foliar application of glyphosate doubled bacterial abundance of the culturable fraction present in the rhizosphere compared to the other treatments with no effect on total abundance. Also the abundance of culturable protists increased as an effect of glyphosate and the bacterial genetic diversity as revealed by 16S rDNA DGGE analysis was affected. Overall, the results indicate that when barley leaves are treated with glyphosate, the availability of organic carbon in the rhizosphere of the dying roots is altered, which in turn may alter the bacterial and protist communities and their interactions. This can have implications for general soil carbon turnover processes and CO_2 release in arable systems.

1. Introduction

Glyphosate (*N*-[phosphonomethyl]glycine) is the most widely used herbicide globally in terms of treated area as well as total amount used (Coupe et al., 2012). It is the active compound of RoundUp® and, besides being applied in agriculture as weed control agent, glyphosate is also used to treat winter cover crops in order to obtain a better establishment of the subsequent spring crop and to artificially accelerate and synchronize ripening of various crops (Duke and Powles, 2009; Helander et al., 2012; Landbrugsrådgivning Syd, 2012). In addition, global glyphosate market includes applications in non-agricultural areas, such as in home and garden, industrial and railroads (Woodburn, 2000).

From 2004 to 2008, the global glyphosate usage has reached about 600,000 t, mainly due to an increase of planting areas dedicated to glyphosate-resistant crops (up to 75 million hectares) and to crops for bio-energy production (Yin, 2011). Although the Danish Environmental Protection Agency reports a reduction in glyphosate volumes sold (more than 700 t less from 2008 to 2009), glyphosate still accounts for 35% of all pesticides used in agricultural purposes in Denmark (Miljøstyrelsen, 2010) and further to ensure even ripening of crops. In fact, due to weather conditions all the northern European agricultural practice adopts pre-harvest management, such as glyphosate ripening synchronization, to maximize the crop yields (Monsanto, 2010; Friends of the earth Europe, 2013; Glyphosate Facts, 2014). The Home-Grown Cereals Authority (HGCA, 2009) estimated that in UK 78% of the glyphosate is used as ripening agent, although the use varies greatly between countries. Applied at field conditions, glyphosate is taken up by the plant shoots and accumulates in phloem sap. The compound is acting systemically and is within 24 h translocated to all metabolically active tissues including seeds, nodules and roots (Bromilow et al., 1993). The presence in the entire plant also makes it possibly that microorganism in the rhizosphere are exposed to

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this compound via the decaying roots (Helander et al., 2012). Because glyphosate is not degraded within the plant it may be transported via the root system to the deeper soil layers (Helander et al., 2012). Moreover, Laitinen et al. (2007) showed that, after 8 h, up to 10% of the foliar applied glyphosate (application rate of 720 g ha^{-1}) may be found in the rhizosphere of quinoa (*Chenopodium quinoa*, Willd) plants due to active translocation within the plant.

The application of glyphosate poses risks to the environment by polluting groundwater (Borggaard and Gimsing, 2008) or by impacting non-target soil organisms. Several studies report the detrimental effect of glyphosate on terrestrial ecosystems: decrease in root colonization and spore formation by arbuscular mycorrhizal fungal, decrease of earthworm activity, increased root colonization by *Fusarium* and other fungal pathogens, and indirect modification of the interaction between fungi and other microorganisms (Araújo et al., 2003; Druille et al., 2015; Kremer and Means, 2009; Zobiole et al., 2011; Schafer et al., 2012, 2014; Zaller et al., 2014). Because of its mode of action (blocking shikimic acid pathway), glyphosate can affect not only plants but also fungi, bacteria, and at least one member of the Amoebozoa, namely *A. castellanii* (Maeda and Dudareva, 2012; Lu et al., 2013; Henriquez et al., 2015). Glyphosate in soil may affect the microbial communities by increasing the number of glyphosate-degrading microorganisms which can utilize the compound as a carbon substrate and by decreasing the number of glyphosate-sensitive species (Araújo et al., 2003; Johal and Huber, 2009; Zobiole et al., 2011; Schafer et al., 2014). Despite the large interest in the topic, the data on glyphosate impact on soil microorganisms are not consistent, which probably reflects field site and concentration-specific response of the microbial community to glyphosate (Busse et al., 2001; Ratcliff et al., 2006; Kremer and Means, 2009; Means et al., 2007; Zobiole et al., 2011; Lane et al., 2012a,b; Schafer et al., 2014). Few studies have studied the effect of glyphosate on protists, with a focus on glyphosate toxicity in aquatic environment (Tsui and Chu, 2003; Pérez et al., 2012). Hence, the continuous use of glyphosate raises concerns regarding side-effects on many key soil organisms and the ecosystem services they support (Borggaard and Gimsing, 2008; Helander et al., 2012; Santos et al., 2012). Accordingly, Andréa et al. (2003) concluded that repeated multiple applications of glyphosate caused a decrease in its mineralization rates in soil, indicating an impact on the soil microbial activity.

Since the introduction of glyphosate in 1974 the direct ecosystem effects have been studied, while the indirect effects of glyphosate (e.g. by accelerated availability of nutrients from decaying roots of treated glyphosate-sensitive plants) on rhizosphere bacterial and protist communities and their ecological interactions have received little attention. Hence, the aim of the present study was to examine if foliar treatment of non-resistant plants with glyphosate would indirectly affect the rhizosphere bacterial and protist communities, with emphasis on the microbial turnover of root-derived carbon. As an effect of the devastating impact of glyphosate on roots, we hypothesized that foliar application of glyphosate would cause a transient increase in availability of readily degradable substrates in the rhizosphere due to enhanced turnover of the rapidly dying roots. Accordingly, glyphosate might enhance microbial biomass and alter the activity and structure of the community which, in turn, might lead to an increased abundance of bacterivorous protists as shown by Winding et al. (1997). When using glyphosate on winter crops to enhance the establishment of the following spring crops or to artificially synchronize the ripening of crops, plants will die in a few days. Whereas the use of glyphosate will increase the turnover of root biomass, roots of non-treated and manually harvested plants will be left in the soil to degrade at a more moderate rate.

These hypotheses were tested in soil–sand microcosms with glyphosate-sensitive barley plants treated with glyphosate in the phyllosphere. The effect of glyphosate on the content of dissolved organic carbon in rhizosphere soil, the abundance of rhizosphere bacteria and protist, and changes in genetic diversity of bacterial communities were measured. Assessment of such non-target effects of the foliar application of glyphosate, is relevant for risk assessment of herbicides, but also contributes to the understanding of the ecological interactions between bacteria and protists in the rhizosphere and resulting effects on carbon turnover.

2. Materials and methods

2.1. Soil type and location

The soil used for the microcosms was collected in an experimental field plot at Risø, Denmark (N 55.685279, E 12.098343), sieved through a 2-mm sieve and stored at 4 °C until used for experimentation. The soil was a sandy loam with 11% clay, 14% silt, 49% fine sand and 25% coarse sand, with total N and total C of 0.13% and 1.5%, respectively, and with a pH of 7.0 (measured in water) which is representative of the eastern part of Denmark.

2.2. Experimental design

Seeds of barley (*Hordeum vulgare* cv. Asano) were surface sterilized in 70% ethanol for 3 min. The seeds were rinsed three times with deionized sterile water, transferred to sterile wet paper filters and incubated in a sterile petri dish for 48 h at 25 °C in darkness for pre-germination. To avoid compaction and collapse of the agricultural soil during incubation and to reduce concentration of organic carbon, the soil used for plant growth was mixed with quartz sand at a ratio of 1:2. The sand (0.6–2 mm) was washed with tap water and oven-dried over night at 105 °C before mixing with the soil.

Two barley seedlings of similar height were then placed in each glass tube (25 mm diameter by 200 mm length) filled with 100 g of the soil–sand mixture. The microcosms were placed at 16 °C with a day/night cycle of 16/8 h and 70% humidity for 30 days. Soil moisture status was checked gravimetrically and maintained by alternating between adding sterile water and Hoagland solution (Hoagland and Arnon, 1950) every second day. After 16 days of growth, watering of plants was interrupted for one week to mimic conditions of moderate water stress as farmers often choose to harvests during a dry period.

2.3. Experimental treatments

The microcosms were divided in three treatment groups of five replicates each: (1) foliar treatment with glyphosate (indicated as '*Glyphosate*'); (2) plants with the aerial part cut off (indicated as '*Cut*'); and (3) untreated plants (indicated as '*Untreated*'). The glyphosate used was a commercial RoundUp® formulation produced by Monsanto (11.2% glyphosate; isopropylamine salt of glyphosate as active ingredient). In the glyphosate treatment a daily dose of approx. 2.4 mL of RoundUp® (9.6 g L^{-1} glyphosate) was applied directly onto the leaves of each plant using a brush. The glyphosate was applied twice a day for three consecutive days (day 25–27 after planting). In this way direct glyphosate contamination of the soil was avoided and glyphosate translocation through the plant to the soil was considered minimal in our experimental set up. In the *Cut*, the shoots were removed at ground level 25 days after planting, to mimic conventional harvest leaving the roots in the soil, while the plants in the *Untreated* were left without any further treatment until harvesting. Prior to this experiment, pilot experiments were made with barley plants