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Control of *Cortaderia selloana* with a glyphosate-based herbicide led to a short-term stimulation of soil fungal communities

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Abstract In the north of Spain, *Cortaderia selloana* plants have invaded ecosystems of high ecological value. Control of this species is carried out with the application of glyphosate-based formulations. The aim of this work was to determine, under microcosm conditions, the short-term (2 months) effects of the application of a glyphosate-based herbicide (Roundup®) on *C. selloana* rhizosphere microbial communities. To this purpose, before and after the application of Roundup®, several parameters that provide information on the biomass, activity and diversity of rhizosphere fungal and bacterial communities (enzyme activities, basal and substrate-induced respiration, potentially mineralizable nitrogen, nitrification potential rate, ergosterol content and community-level profiles with Biolog™ plates and ARISA) were determined. We observed a stimulation of some microbial parameters, in particular those related to fungal communities. Further research is needed to determine the long-term consequences of this short-term fungal stimulation for soil functioning.

Keywords Exotic species · Invasive species · Microbial properties · Roundup® · Soil health

Introduction

In the north of Spain, *Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn. plants (commonly known as pampas grass) have invaded both degraded and high ecological value ecosystems. Control of this species is carried out with the application of glyphosate, a systemic herbicide that inhibits the shikimic acid pathway, which is used by plants and microorganisms for the synthesis of aromatic amino acids (Duke and Powles 2008). Then, soil microorganisms (e.g. fungi, bacteria) may be susceptible to the application of glyphosate-based herbicides. Glyphosate applied to plant leaves is translocated throughout the plant and can then be released into soil from roots (Kremer et al. 2005). In addition, one of the surfactants commonly included in glyphosate-based products, polyoxyethylene amine, has been reported to be potentially toxic to microorganisms (Tsui and Chu 2003). In any case, research on the effects of glyphosate on soil microorganisms has yielded conflicting results (Accinelli et al. 2005; Ratcliff et al. 2006; Lupwayi et al. 2007; Gomez et al. 2009; Mijangos et al. 2009; Powell et al. 2009). These inconsistent observations are most likely due to differences in edaphoclimatic and environmental conditions across studies, as well as to

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differences in the specific microbial parameters and techniques used in those studies. Not surprisingly, there are also inconsistencies between short-term and long-term studies. Short-term studies reflect immediate toxicity effects on soil microbial communities, with consequences for the ulterior, more environmentally-relevant, long-term effects. Well-designed short-term toxicity studies can provide information on the amount of the substance that can be tolerated, without toxic effects, by soil microbial communities under the conditions of the study, as well as useful information for the design of long-term, chronic toxicity studies. Relevantly, there is a need to study these effects at different time-points to check for temporal fluctuations in the soil microbial properties.

The aim of this work was to determine, under microcosm conditions, the short-term (2 months) effects of the control of *C. selloana* plants with a glyphosate-based herbicide, Roundup®, on rhizosphere microbial communities. To this purpose, before and after the application of Roundup®, several parameters that provide information on the biomass, activity and diversity of rhizosphere microbial communities were determined.

Materials and methods

Soil was sampled from the top layer (0–10 cm) of a local natural grassland with no known history of glyphosate exposure, left to air-dry for 1 week, sieved to <2 mm and subjected to physicochemical characterization (MAPA 1994). The soil was silty clay, with a cation exchange capacity of 15 mEq 100 g^{-1}, an organic matter (OM) content of 3.5 %, a pH of 6.3, a nitrogen (N) content of 0.25 %, a phosphorus (P) content of 8.1 mg kg^{-1}, and a potassium (K^+) content of 84 mg kg^{-1}. Sixteen pots were filled with 2-kg dry weight (DW) soil each and then planted with *C. selloana* seedlings. Plants were grown for 2 months (i.e. until roots occupied the whole pot) under the following conditions: 25/18 °C temperature, 50/60 % relative humidity, 14 h photoperiod, and 250 μmol photon m^{-2} s^{-1} minimum light intensity. Plants were watered with tap water twice a week. After these 2 months (sampling time=S1; just before Roundup® application), 4 pots were destructively sampled for the determination of soil microbial parameters (see below). The remaining 12 pots were treated with a foliar application of Roundup® (10 ml of 2 % Roundup®). One month (sampling time=S2) and 2 months (sampling time=S3) after Roundup® application, another 4 pots were destructively sampled, respectively. The remaining 4 pots were planted with *Festuca arundinacea* seeds and incubated for 4 months under the same conditions described above. In our region, *F. arundinacea* (commonly known as tall fescue) is a good candidate for revegetation of grasslands after glyphosate control of *C. selloana*.

In parallel, 20 control pots (unplanted/Roundup®-untreated) were filled with 2-kg DW soil each. Four control pots were destructively sampled for the determination of soil microbial parameters at S0 (just before *C. selloana* planting), S1, S2 and S3 sampling times. The remaining 4 control pots were planted with *F. arundinacea* seeds and incubated for 4 months under the same conditions described above. After this 4-month period, *F. arundinacea* shoots were harvested, oven-dried for 48 h at 70 °C and their dry weights were recorded.

Soil samples were sieved (2 mm mesh) and stored at 4 °C for the determination of microbial parameters (at −20 °C for molecular analyses). Alkaline (Alk-PHO) and acid phosphatase (Ac-PHO), β-glucosidase (GLU) and arylsulphatase (SUL) enzyme activities were determined following Dick et al. (1996) and Taylor et al. (2002). Dehydrogenase (DEH) and arginine deaminase (ARG) enzyme activities were determined following Mers and Schinner (1991) and Alef and Kleiner (1996), respectively. These enzyme activities take part in the release of phosphates (Alk-PHO, Ac-PHO), glucose (GLU), sulphate (SUL) and ammonium (ARG), making these nutrients available for soil biota and plants. DEH activity is a good indicator of overall microbial oxidative activity in soil (Muñoz-Leoz et al. 2013).

Potentially mineralizable nitrogen (PMN) and potential nitrification rate (PNR) were measured following Powers (1980) and ISO 15685 Norm (2004), respectively. Basal respiration (BR) and substrate-induced respiration (SIR) were measured following ISO 16072 Norm (2002) and ISO 17155 Norm (2002), respectively. Basal respiration and SIR are measured to determine soil microbial activity and potentially active microbial biomass, respectively. Ergosterol content was determined following Gong et al. (2001). Community-level physiological profiles of bacteria and fungi were determined with Biolog Ecoplates™ (Insam 1997) and Biolog FF Microplates™ (Shugeng et al. 2009), respectively,

Table 1 Soil microbial parameters in *C. selloana* pots at S1 (Roundup®-untreated), S2 (1 month after Roundup®-treatment) and S3 (2 months after Roundup®-treatment) sampling times

	S1	S2	S3
Dehydrogenase	114 ± 11	182 ± 17**	137 ± 9*
β-glucosidase	121 ± 2**	99 ± 6	99 ± 2
Arylsulphatase	106 ± 3	131 ± 3***	120 ± 2**
Acid phosphatase	107 ± 2	78 ± 3**	125 ± 2**
Alkaline phosphatase	106 ± 1	100 ± 5	108 ± 1
Arginine deaminase	104 ± 2	91 ± 5	83 ± 11
Nitrification potential rate	103 ± 5	117 ± 5	101 ± 6
Potentially mineralizable nitrogen	72 ± 6*	127 ± 9*	98 ± 6
Basal respiration	110 ± 3	102 ± 8	99 ± 5
AWCD from Biolog EcoPlates™	94 ± 2	77 ± 11	101 ± 7
AWCD from Biolog FF MicroPlates™	121 ± 1***	97 ± 13	122 ± 1*
Substrate-induced respiration	118 ± 2*	114 ± 2	108 ± 3
Ergosterol content	113 ± 7	134 ± 10*	109 ± 9
NUS from Biolog EcoPlates™	133 ± 4	96 ± 10	120 ± 4
NUS from Biolog FF MicroPlates™	90 ± 6**	72 ± 9	102 ± 13*
Number of bacterial peaks from ARISA	98 ± 15	90 ± 17	45 ± 5
Number of fungal peaks from ARISA	106 ± 10	110 ± 16	195 ± 12***

Values are expressed as % of the average of control (unplanted/Roundup®-untreated) pots. *, ** and *** represent statistically significant differences at $p < 0.05$, 0.01 and 0.001, respectively, with respect to unplanted controls. Mean values ($n = 4$) ± standard errors

AWCD average well colour development, *NUS* number of utilized substrates

following Epelde et al. (2008). Average well colour development (AWCD) and number of utilized substrates (NUS) were determined after 42 and 95 h of incubation for bacteria and fungi, respectively. DNA was extracted from soil (0.25-g soil) using the Power Soil DNA Isolation Kit (MO Bio Laboratories, CA). Community-level genetic profiles were obtained with ARISA following Cardinale et al. (2004) for bacteria [ITSF (GTCGTAACAAGGTAGCCGTA)/ITSReub (GCCAAGGCATCCACC) primers] and Ranjard et al. (2000) for fungi [2234C (GTTTCCGTAGGTGAACCTGC)/3126T (ATATGCTTAAGTTCAGCGGGT) primers]. Data were analysed with GeneMarker Software (Softgenetics LCC, State College, PA) (Welkie et al. 2010).

Differences between planted/Roundup®-treated and control (unplanted/Roundup®-untreated) pots were analysed with one-way ANOVA using Microsoft Stat View Software (SAS Institute 1998). A Principal Response Curve (PRC) analysis was performed using Canoco 5 (ter Braak and Šmilauer 2012), to examine temporal responses of the microbial parameters.

Results and discussion

At S1, just before Roundup® application, *C. selloana* growth resulted in an increase in GLU, SIR and AWCD from Biolog FF MicroPlates™, as well as a decrease in PMN and NUS from Biolog FF MicroPlates™, compared to control pots (Table 1). *C. selloana* plants appear to stimulate processes related to the C cycle and to compete with soil microorganisms for organic N, as indicated by the increase in GLU and SIR and the decrease in PMN, respectively. Vegetated soils have been reported to have higher rates of microbial activity, compared to bare soil (Barrutia et al. 2011; Yin et al. 2014).

After Roundup® treatment, *C. selloana* plants showed phytotoxic symptoms (e.g. photochemical efficiency was reduced by 55 % after 20 days; data not shown); plants were already dead at S2. At S2, Roundup®-treated pots showed higher values of DEH, SUL, PMN and ergosterol content (and lower values of Ac-PHO), compared to control pots. At S3, Roundup®-treated pots showed higher values of

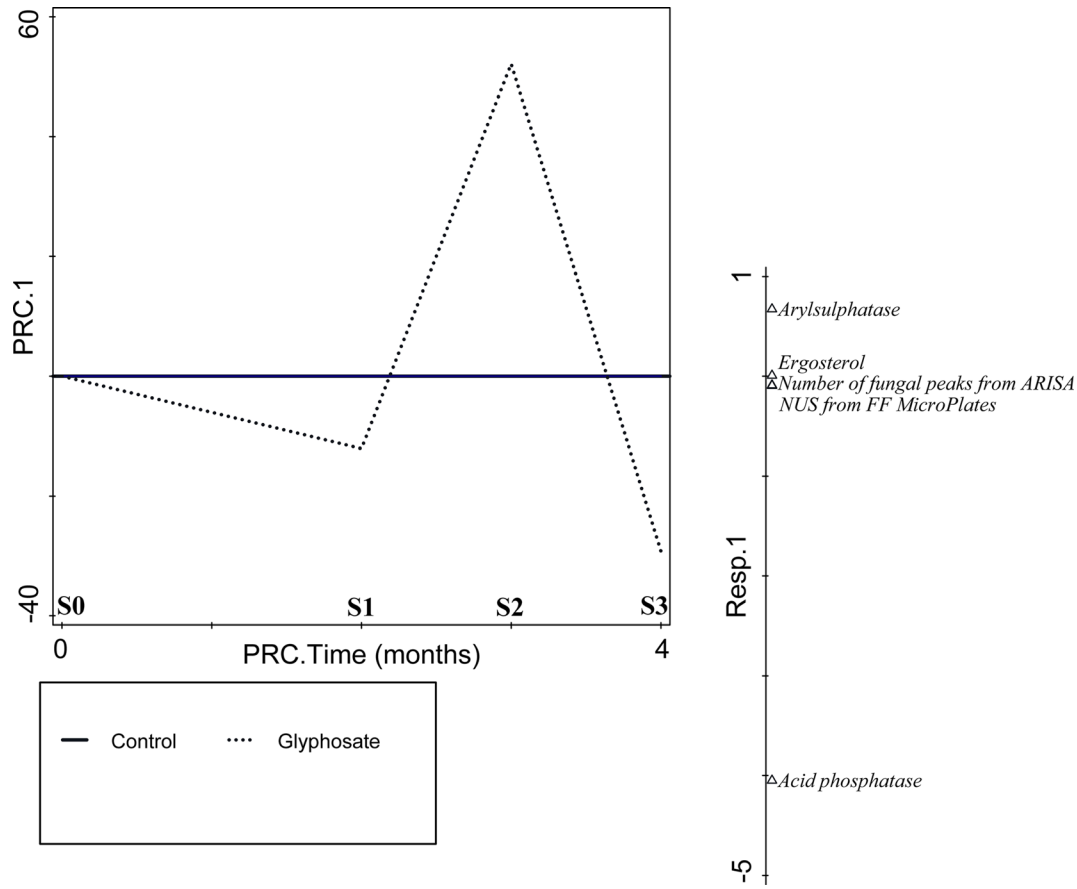

Fig. 1 Principal response curve carried out to examine temporal responses of the microbial parameters (only the five microbial parameters showing the best fit are included in the figure), as a result of the control of *C. selloana* plants with Roundup®. Glyphosate curve-S0: unplanted, Roundup®-untreated; glyphosate curve-S1: 2 months after planting, Roundup®-treated; glyphosate curve-S2: 3 months after planting, 1 month after Roundup®-treatment; glyphosate curve-S3: 4 months after planting, 2 months after Roundup®-treatment; Control curve-S0, S1, S2 and S3: unplanted, Roundup®-untreated. The effect of the treatment factor is significant according to Monte Carlo permutation test (F = 33.9, p = 0.022).

DEH, SUL, Ac-PHO, AWCD and NUS from Biolog FF MicroPlates™, and number of fungal peaks from ARISA (Table 1). Then, after Roundup® application, Ac-PHO activity decreased at S2 to then increase at S3. Glyphosate is an organic P compound that can compete with soil P for binding sites (Gimsing et al. 2003); in consequence, the decrease in Ac-PHO at S2 could be attributed to feedback inhibition of this activity by available P.

As a result of Roundup® application, the values of some parameters related to fungal activity, biomass and biodiversity (i.e. SUL, ergosterol content, AWCD and NUS from Biolog FF MicroPlates™ and number of fungal peaks from ARISA) increased, particularly at S3. SUL activity has been suggested to be an indicator of fungi, as only fungi contain ester sulphate, the substrate of SUL activity (Bandick and Dick 1999). Other studies also found a glyphosate-induced increase in fungal activity and population size (Araújo et al. 2003), and fungi:bacteria ratio (Powell et al. 2009). In contrast, Ratcliff et al. (2006) observed an early and transient (1 month) increase in soil bacterial parameters, compared to those of soil fungi. Our data provide a plausible explanation for conflicting reports on the impact of glyphosate on soil bacteria and fungi: bacteria could be favoured immediately after glyphosate treatment (probably, as a result of glyphosate degradation and/or the presence of labile plant organic compounds), whereas fungi could be favoured later on when more complex substrates (e.g. those resulting from the decomposition of herbicide-killed plant tissues) are present in the rhizosphere. In any event, some soil-borne fungi

have been reported to grow on glyphosate (Krzysko-Lupicka and Sudol 2008), suggesting that fungi can play an important role in the biodegradation of organophosphonates in soil. Further research is needed to determine the long-term consequences of this short-term fungal stimulation for soil functioning.

In the Biolog FF MicroPlates™, the following significant differences in absorbance values (reflecting C substrate utilization rates) were observed at (S1) higher values for α-D-glucose, glucuronamide and glycerol in *C. selloana* pots vs. control and lower values for D-ribose; (S2) higher values for Tween 80, D-arabitol, D-cellobiose, D-galactose and gentibiose in Roundup®-treated pots vs. control and lower values for dextrin, D-gluconic acid, 2-keto-D-gluconic acid, D-raffinose, turanose and succinic acid; (S3) higher values for α-cyclodextrin, i-erythritol, L-fucose, α-methyl-D-galactoside, stachyose, D-tagatose, L-malic acid, D-saccharic acid, succinamic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid and L-serine in Roundup®-treated pots vs. control and lower values for D-cellobiose and L-sorbose. No significant differences were observed in the Biolog EcoPlates™. Then, interestingly, 19 substrates showed higher absorbance values in the Biolog FF MicroPlates™ at S2 or S3 for Roundup®-treated pots vs. control. The utilization of two of these substrates (i.e. Tween 80 and L-serine) might be explained by the stimulation of fungal populations due to Roundup® application: (i) this commercial formulation includes surfactants like Tween 80 (ii) and serine is an intermediate compound formed in the degradation of glyphosate (Kishore and Jacob 1987). A similar effect (i.e. stimulation of the capacity to use C substrates) was reported at shorter times in soil bacteria (Mijangos et al. 2009).

According to the PRC (Fig. 1), at S2, a large variation between Roundup®-treated and control was observed, mainly towards higher SUL values; at S3, this variation was also high but, in this case, in the opposite direction (as reflected by the Ac-PHO values).

F. arundinacea growth was significantly lower in Roundup®-treated (5.9 ± 0.5 g DW) vs. control (7.4 ± 0.1 g DW) pots. This reduction of *F. arundinacea* growth might be due to the following: (i) phytotoxicity of residual glyphosate, its degradation products (e.g. aminomethylphosphonic acid), ingredients present in Roundup® (e.g. surfactants) or the accumulation of shikimate pathway intermediates (De Maria et al. 2006); (ii) alteration of nutrient cycling or sequestration due to changes in soil microbial communities and/or (iii) depletion of essential nutrients such as N, as a result of the previous growth of *C. selloana* plants in the same pots. It must be highlighted that PMN was not affected at S3 (Table 1) and that, although the difference was not statistically significant, the mean value of soil ammonium content was higher in Roundup®-treated (2.4 ± 0.9 mg N-NH_4^+ kg^{-1} DW soil) than control (1.5 ± 0.4 mg N-NH_4^+ kg^{-1} DW soil) pots. In a similar study (Mijangos et al. 2009), we found higher contents of soil ammonium as a result of Roundup® application. Glyphosate released into soil from roots can increase the amino acid content in root exudates (Kremer et al. 2005). We speculate that the 20 % reduction in *F. arundinacea* growth observed in Roundup®-treated pots is probably caused by a residual phytotoxic effect due to Roundup® application and/or a consequence of changes in rhizosphere microbial communities altering nutrient cycling or sequestration, not by nutrient deficiency.

We concluded that the control of *C. selloana* with Roundup® leads to a short-term stimulation of rhizosphere fungal communities. Further research is needed to determine the long-term consequences of this short-term fungal stimulation for soil functioning.

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