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Effects of nicosulfuron on the abundance and diversity of arbuscular mycorrhizal fungi used as indicators of pesticide soil microbial toxicity

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ABSTRACT

The key role of arbuscular mycorrhizal (AM) fungi in maintaining soil fertility and ecosystem functioning and their general sensitivity to pesticides make them good candidate bioindicators in pesticide soil microbial toxicity assessment. We investigated the impact of the herbicide nicosulfuron on mycorrhizal colonization and community structure of AM fungi *via* a pot-to-field experimental approach. This allowed the assessment of nicosulfuron toxicity (i) at extreme exposure schemes (pot experiment, Tier I) invoked by the repeated application of a range of dose rates (x0, x10, x100, x1000 the recommended dose) and (ii) under realistic exposure scenarios (x0, x1, x2, x5 the recommended dose) in the field (Tier II). In the pot experiment, the x100 and x1000 dose rates significantly reduced plant biomass, mycorrhizal colonization and AM fungal richness as determined by DGGE. This coincided with the progressive accumulation of herbicide concentrations in soil. In contrast, no effects on AM fungi were observed at the nicosulfuron dose rates tested in the field. Clone libraries showed that the majority of AM fungi belonged to the *Glomus* group and were sensitive to the high levels of nicosulfuron accumulated in soil at the latter culture cycles. In contrast, a Paraglomeraceae and a *Glomus etunicatum* ribotype were present in maize roots in all cycles and dose rates implying a tolerance to nicosulfuron-induced stress. Overall, the deleterious effects of nicosulfuron on AM fungi induced by the highest dose rates in the pot experiment could be attributed either to fungal-driven toxicity or to plant-driven effects which have subsequent implications for mycorrhizal symbiosis. We suggest that the tiered pot-to-field experimental approach followed in our study combined with classic and standardized molecular tools could provide a realistic assessment of the toxicity of pesticides onto AM fungi as potential bioindicators.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are obligate symbiotic microorganisms living in association with the vast majority of higher plant species providing increasing supply of P and other minerals, drought tolerance and resistance to pests and diseases (Kiers et al., 2011). In agricultural systems AM fungi are exposed to diverse inputs including fertilizers and pesticides. The generally negative effect of phosphorus fertilizers on AM fungi is well documented (Smith et al., 2011). However, less is known regarding their interactions with pesticides. Previous studies showed that AM fungi respond in various ways to pesticide exposure. Fungicides like fenpropimorph and fenhexamid had a clear inhibitory effect on AM fungi (Zocco et al., 2011). In contrast, insecticides like aldicarb, fenamiphos and dimethoate had no inhibitory effect (Nemec, 1981; Schweiger and Jakobsen, 1998) or even stimulated AM fungal colonization and P uptake (Spokes et al., 1981). Finally herbicides could impact AM fungi either directly (Pasaribu et al., 2011; Li et al., 2013) or indirectly by exerting phytotoxicity to their plant hosts (Druille et al., 2012). All the above suggests that AM fungi are generally

Abbreviations: AM fungi, arbuscular mycorrhizal fungi; ALS, acetolactate synthase; HPLC, high performance liquid chromatography; DGGE, denaturating gradient gel electrophoresis; FOK, first order kinetics.

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responsive to pesticide exposure. This combined with their key role on plant diversity and functioning of above ground ecosystems (Van der Heijden et al., 1998) makes them good candidate bioindicators for assessing the soil microbial toxicity of pesticides (Wan and Rahe, 1998; Giovannetti et al., 2006). This is in line with the establishment by the International Standard Organization of the ISO10832 standard 'Effects of pollutants on mycorrhizal fungi'. Despite that the utilization of appropriate experimental protocols and well-standardized methods are necessary for a comprehensive assessment of the toxicity of pesticides onto non-target soil microbes like AM fungi, especially in view of the revision of the relevant regulatory framework at EU level (Martin-Laurent et al., 2013).

In the past most studies have used plant growth, AM fungal colonization and/or P uptake to demonstrate potential toxicity of pesticides on AM fungi (Schweiger and Jakobsen, 1998; Druille et al., 2012). *In vitro* tests in Ri T-DNA-transferred carrot roots have been also used to assess the toxicity of pesticides on AM fungi (Wan and Rahe, 1998; Li et al., 2013). However, these tests do not provide any information regarding the impact of pesticides on the diversity and community structure of AM fungi. The fast development of molecular tools during the past 10 years (Rosendahl, 2008) allowed us to go beyond the simple observation of the indirect effects of pesticides on the performance of mycorrhizal plants, and further explore the direct effect of pesticides on abundance, diversity and function of the micro-symbiotic partner.

Nicosulfuron is a low-dose, high-potency herbicide of the sulfonylureas group which is considered the most important group of new era herbicides currently available on the global market (Sarmah and Sabadie, 2002). It is used for the post-emergence control of annual grass and broad-leaf weeds in maize (Hinz and Owen, 1996). In plants, nicosulfuron acts by inhibiting acetolactate synthase (ALS) which catalyzes the first common step in the biosynthesis of the branched-chain amino acids leucine, valine, and isoleucine (Babczinski and Zelinski, 1991). This enzyme can be also found in bacteria and fungi. Previous *in vitro* studies have showed that ALS-inhibitors like nicosulfuron could inhibit this enzyme in microbes (Falco and Dumas, 1985; LaRossa and Schloss, 1984). Despite that, little is known regarding the effect of nicosulfuron on non-target soil microbes. In the only study currently available, Seghers et al. (2005) showed that nicosulfuron did not induce significant changes on the abundance and function of soil methanotrophs, whereas transient changes on community structure were evident.

The main aim of the current study was to assess the soil microbial ecotoxicity of nicosulfuron using AM fungi as bio-indicators. This was done through a pot-to-field tiered approach which allowed us to monitor the potential side effects of the herbicide on the abundance and diversity of AM fungi at both extreme (pot, Tier I) and realistic (field, Tier II) exposure scenarios. Possible effects of nicosulfuron on plant growth, colonization capacity and intraradical AM fungal community were investigated with a combination of classical and molecular methods allowing the identification of members of the AM fungal community that are either sensitive or tolerant to nicosulfuron exposure. In parallel, the dissipation of nicosulfuron in soil was determined to identify possible correlations between pesticide residues and toxicity.

2. Materials and methods

2.1. Experimental setup – pot experiment

A pot experiment was established to assess the impact of nicosulfuron on AM fungi under extreme exposure conditions. The soil used was obtained from the field site in Serbia, Novi Sad where the field experiment described below was established. The field site did not have a recent history of previous nicosulfuron use. The soil was characterized as loamy clay (36.8% sand; 35.8% silt; 27.4% clay) with pH 6.75, organic carbon content 2.59%, total N 1.92 g kg^{-1}, $CaCO_3$ 1.4 g kg^{-1}, P_2O_5 0.084 g kg^{-1} and K_2O 0.273 g kg^{-1}. The soil was initially sieved to pass through a 2 mm mesh and was distributed into 20 plastic pots (4.5 kg of soil dry weight per pot). The soil in the pots were watered to adjust the moisture content of the soil to 70% of its maximum water holding capacity and was pre-incubated at room temperature for a week. Each pot was seeded with 10 maize seeds which after emergence were thinned to four seedlings per pot. When maize plants reached the 3–4 leaves stage (at approximately 10 days after sowing) the pots were separated into four groups comprising five pots each. The pots in the first three groups were treated uniformly with different dose rates of nicosulfuron corresponding to x10, x100 and x1000 the recommended dose (60 g a.i. ha^{-1} corresponding to 0.047 mg kg^{-1} soil dry weight). These dose rates are particularly high compared to the recommended dose and they were chosen to evaluate toxicity under extreme exposure schemes simulating a tier I toxicity assessment. In all cases, the commercial formulation of the herbicide ACCENT® (750 mg kg^{-1}) provided by DuPont™ was used. The final set of pots received the same amount of sterile water without pesticide to serve as non-treated controls. Plants were grown under controlled temperature (23–25 °C), at 11-h day period and 60% relative humidity. Soil water content was maintained by daily watering (w/v). The plants were harvested after 6 wks by cutting at the base of the stem and the root system was carefully removed, washed free of soil and the shoot and root dry weight (60 °C oven for 2 days) was determined. Root samples were taken for estimation of AM fungal root colonization, while root fragments were also stored at −20 °C for subsequent molecular analysis. In addition, soil subsamples were collected from each pot for analysis of nicosulfuron residues in the soil at the end of each culture cycle. Upon harvest the soil in each pot was re-treated with the same dose rate of nicosulfuron and handled as described above. In total five culture cycles were performed. The repeated application scheme followed in the pot experiment allowed us to determine the potential toxicity of nicosulfuron on AM fungi on a long-term exposure basis. This is in line with previous studies which suggested that single application approaches do not provide a realistic picture of the potential soil microbial toxicity of pesticides and long-term studies are required to establish a real pesticide-stress condition for the terrestrial microbiota (Zabaloy et al., 2012).

2.2. Experimental set up – field experiment

A field experiment was conducted in a site in the area of Rimski Sancevi (19° 51,321 to 45° 19,928) Novi Sad, Serbia to assess the impact of nicosulfuron on AM fungi under realistic exposure conditions. A randomized complete block design was followed with four replicate micro-plots (6 m × 5 m) per nicosulfuron dose rate. The soil of the field site was on maize cultivation for the last few years. The field was seeded with maize (*Zea mays* variety NS640) in 28 April 2011 at distances of 25 cm within rows and 75 cm between rows using a pneumatic seeder. A 75 cm-wide margin was left between plots to minimize any cross contamination between treatments. Maize emerged a week later and nicosulfuron (ACCENT®, 750 mg kg^{-1}) was applied to the different plots at three dose levels (x1, x2 and x5 the recommended dose) in 4 June 2011 using a backpack sprayer. These dose rates represent a realistic exposure scenario simulating a tier II toxicity assessment. For comparison purposes, a treatment where no herbicide was applied was included. Before treatment and at 2, 7, 14, 28, 56 and 116 days post application soil samples were collected from each plot (five samples collected from the top 10 cm of each plot were homogenized