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Research Article

RESEARCH ARTICLE

The effects of plant nutritional strategy on soil microbial denitrification activity through rhizosphere primary metabolites

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One sentence summary: Plant nutritional strategies affect composition of primary metabolite exudates and denitrification activity in root adhering-soil.
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ABSTRACT

The aim of this study was to determine (i) whether plant nutritional strategy affects the composition of primary metabolites exuded into the rhizosphere and (ii) the impact of exuded metabolites on denitrification activity in soil. We answered this question by analysing primary metabolite content extracted from the root-adhering soil (RAS) and the roots of three grasses representing different nutrient management strategies: conservative (*Festuca paniculata*), intermediate (*Bromus erectus*) and exploitative (*Dactylis glomerata*). We also investigated the impact of primary metabolites on soil microbial denitrification enzyme activity without carbon addition, comparing for each plant RAS and bulk soils. Our data show that plant nutritional strategy impacts on primary metabolite composition of root extracts or RAS. Further we show, for the first time, that RAS-extracted primary metabolites are probably better indicators to explain plant nutrient strategy than root-extracted ones. In addition, our results show that some primary metabolites present in the RAS were well correlated with soil microbial denitrification activity with positive relationships found between denitrification and the presence of some organic acids and negative ones with the presence of xylose. We demonstrated that the analysis of primary metabolites extracted from the RAS is probably more pertinent to evaluate the impact of plant on soil microbial community functioning.

Keywords: primary metabolites; plant nutrient management strategies; rhizosphere; root adhering-soil; root extract; denitrification enzyme activity without carbon addition

INTRODUCTION

Soil exploration by roots induces physical and chemical changes at the soil–root interface that differentiate the rhizosphere from bulk soil (Bais *et al.* 2006; Badri and Vivanco 2009; Haichar *et al.* 2014). These changes are mainly orchestrated by plant roots releasing a variety of compounds called rhizodeposits, including lysates from sloughed-off root caps and border cells, as well as root exudates released from intact root tissue (Haichar *et al.* 2014). Root exudates are believed to represent about 5–21% of net assimilated carbon (Nguyen 2003; Derrien, Marol and Balesdent 2004). Root exudates are often divided into two classes of compounds: low-molecular-weight compounds include primary

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metabolites such as amino acids, organic acids and sugars and also secondary metabolites and account for much of the root exudate diversity, whereas high-molecular-weight compounds, such as mucilage and proteins, account for a large proportion of root exudates in terms of mass (Bais *et al.* 2006; Haichar *et al.* 2014). Root exudates can be passively released into the soil due to the concentration gradient between root cells and the soil solution or can be actively secreted in response to environmental changes (Marschner 1995; Jones, Hodge and Kuzyakov 2004; Bais *et al.* 2006).

As root exudates are only found in a narrow zone of soil around the roots and are rapidly assimilated by soil microbes or adsorbed into soil, they are some of the most poorly characterised components of the belowground carbon cycle (Wardle 2002; Paterson 2003; Phillips *et al.* 2008). This has substantially limited our understanding of their ecological significance. Until recently, root exudate studies were mainly performed by growing plants in sterile hydroponic culture and collecting the carbon accumulated in the external medium (Strehmel *et al.* 2013). However, this method may affect root physiology and exudate recovery (for review, see Nguyen 2003). In some cases, roots have been temporarily removed from the soil to be studied, which may stress or injure the root and disrupt mycorrhizal networks (Neumann and Romheld 2001). Hence, the collection of root exudates from soil-grown plants under natural conditions remains challenging.

Root exudates are believed to play an important role in mediating plant–microbe interactions. Indeed, plants are able to shape soil microbial community structure and functioning through root exudation, as already demonstrated by Haichar *et al.* (2008) and Haichar, Roncato and Achouak (2012). It is well established that plant species adapted to particular habitat conditions can have different functional traits (Grime 1979) and it is becoming clear that these traits can also determine plant nutrient management strategies (Grime 1979; Tilman 1990), ecosystem properties (Grime 2001; Garnier *et al.* 2004) and, more recently, plant–microbe interactions (de Vries *et al.* 2012; Grigulis *et al.* 2013; Cantarel *et al.* 2015).

Fast-growing plant species with a higher photosynthetic capacity and rapid rates of N acquisition are called exploitative (competitive, N-rich), and are characterised by a high specific leaf area and specific root length but a low leaf and root dry matter content. In contrast, species with lower biomass N concentrations, specific leaf area and specific root length, and longer lifespan, higher leaf and root dry matter content and slower growth are called conservatives (stress tolerant, N-poor) (Hobbie 1992; Aerts and Chapin 1999; Craine, Fargione and Sugita 2005). Moreover, exploitative grasses seem to have a higher NO_3^- and NH_4^+ uptake ability than conservatives (Grassein *et al.* 2015). For example, *Dactylis glomerata*, an exploitative grass, had a higher affinity for ammonium and nitrate than *Sesleria caerulea*, a conservative one (Grassein *et al.* 2015). In addition, exploitative plants are thought to invest more in root exudation than their conservative counterparts due to their larger photosynthetic capacity (Personeni and Loiseau 2004; De Deyn, Cornelissen and Bardgett 2008). Nevertheless, plant exudation is poorly understood relative to other processes in the terrestrial carbon cycle (Jones, Hodge and Kuzyakov 2004). Recently, Kaštovská *et al.* (2014) showed that differences in soil N cycling associated with exploitative *versus* conservative plants were closely connected to their different investments in root exudation, governing the coupling of plant–microbe interactions in time and space.

The aim of this study was (i) to compare the effect of plant nutritional strategy on the composition of primary metabolites extracted from the root-adhering soil (RAS) or from the roots and (ii) to determine the impact of primary metabolites present in the RAS on soil denitrifier activity. The hypothesis was that the denitrification enzyme activity follows the trend bulk soil < conservative < intermediate < exploitative grasses due to greater abundance and diversity of root exudates. For this, a laboratory experiment was performed with a panel of three perennial C3 grass species distributed along a gradient of plant nutrient management strategies: one conservative (*Festuca paniculata*), one intermediate (*Bromus erectus*) and one exploitative (*Dactylis glomerata*). After 11 weeks of growth, the primary metabolites were extracted from the RAS and from the roots of each plant species and analysed. In addition, microbial denitrification activity was measured in each plant rhizosphere without adding carbon sources.

MATERIALS AND METHODS

Plant culture

A laboratory experiment was performed using three perennial C3 grass species distributed along a gradient of plant nutrient management strategies (Gross *et al.* 2009; Maire *et al.* 2009; Cantarel *et al.* 2015): one conservative (*Festuca paniculata*, FP), one intermediate (*Bromus erectus*, BE) and one exploitative (*Dactylis glomerata*, DG). Grass species were collected in the French Alps (Lautaret Pass; 45°2′5.1″ N, 6°22′43.5″ E; elevation: 2000 m asl). Species were sampled in the field and separated into individual tillers. For each plant population, seven tillers (i.e. seven clones by species) with three young mature leaves and the root system clipped to 3 cm were grown under controlled conditions to standardise plant growth. Plants were cultivated on luvisol with no added nitrogen source (WRB 2006) collected at La Côte Saint-André (Isère, France) and which is continuously cropped with maize. It is a loamy soil comprising 16.2% clay, 45.4% loam and 28.4% sand, with total nitrogen content of 1.9 $g.kg^{-1}$. The soil pH (7.7) was measured following ISO 10390, and was not modified by the presence of plants. The soil was sieved (2 mm mesh size) and 170 g was placed into plastic pots. The seven individuals from each plant population were grown in a greenhouse (13 h day, 22°C/11 h night, 18°C) with light intensity of about 8–10 klux for 11 weeks. Plants were moistened by immersion in water every 3 days. Four pots with bulk soils (i.e. without plants) were incubated as controls under the same conditions.

Plant harvesting

Three individuals from each plant population were used to study rhizosphere primary metabolites, with the other four used for denitrification activity measurements.

Immediately following plant harvesting, the RAS and the root system of each plant were recovered. Following Czarnes, Dexter and Bartoli (2000), the RAS is considered as the remaining soil adhering to the roots after shaking. The roots of each plant were manually separated from the RAS, washed carefully with distilled water, frozen in liquid N_2 and stored at –80°C. The RAS was carefully separated from any remaining fine roots and then lyophilised and stored at –80°C. Both the dry RAS and root systems were used to characterise primary metabolites.

The RAS of the last four individuals from each plant population was collected and stored at 4°C overnight prior to measuring denitrification activity.

Primary metabolite extraction

From root-adhering soil

To extract primary metabolites from the RAS, 20 g of lyophilised soil was crushed and extracted using an accelerated solvent extraction system (ASE®200, Dionex, Salt Lake City, UT, USA). Fontainebleau sand (inert substrate) was added to fill the cylinder containing the RAS. Ultra-pure (UP) water was used as solvent to extract primary metabolites. The extraction was performed in two cycles: the cylinder was heated at 50°C for 5 min at a pressure of 103.4 bars with UP water, followed by 15 min of static phase and 1 min of flush to collect the extract. This cycle was repeated a second time and 15–20 mL of a solution of primary metabolites was obtained. Extracts were frozen with liquid nitrogen, lyophilised, weighed and stored at –20°C.

From roots

The extraction of primary metabolites from roots was based on the methodology described by Lisec *et al.* (2006). Briefly, 20 mg of lyophilised roots was crushed and put in a 2 mL tube, then 700 μL of a methanol:chloroform solution (4:3) was added and the tube vortexed. Subsequently, 150 μL of UP water was added, vortexed and the tube placed in an ultrasonic bath for 10 min. A further 200 μL of ultra-pure water was then added and the tube vortexed and centrifuged for 5 min. From the supernatant, 600 μL was transferred to 2 mL tubes and kept to one side. The remaining material was resuspended with 500 μL of a methanol:chloroform solution (1:1), followed by vortexing and centrifuging for 5 min. From the supernatant, 400 μL was then mixed with the previously collected upper phase. The combined supernatants (1 mL) were then dried in a CentriVap® (CentriVap Concentrator Labconco®), weighed and stored at –20°C.

Derivatisation of primary metabolites and GC-MS analysis

Trimethylsilyl (TMS) derivatives were obtained by manual derivatisation based on the methodology described by Ribeiro *et al.* (2014). Before the derivatisation, 1.5 mg of root extract or RAS extract was placed for 20 min in a CentriVap® concentrator to remove residual humidity. First, 62.5 μL of *O*-methylhydroxylamine hydrochloride (20 mg mL^{-1} in pyridine) was added and incubated for 30 min at 40°C. Then, 87.5 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added and left for 60 min at 40°C. Derivatised extracts were transferred to a vial with insert prior to analysis.

Sample aliquots of 1 μL were injected (Agilent 7683 Series Injector) at 290°C (splitness mode) in a Hewlett Packard 6890A gas chromatograph (Agilent Technologies) coupled to a Hewlett Packard 5973 mass spectrometer (Agilent Technologies), using a DB-5MS capillary column (Agilent; 60 m × 0.25 mm × 0.25 μm) with helium as carrier gas at a column flow rate of 2.3 mL.min^{-1}. The oven temperature program was 5 min at 70°C, followed by a 4°C.min^{-1} ramp up to 306°C. The transfer line temperature was set at 300°C. The column effluent was ionised by electron impact at 70 eV. Mass spectra were recorded at 1.29 scans.s^{-1} within a mass-to-charge ratio range of 50–600 *m/z* at a source temperature of 230°C. A solvent delay of 7 min was set.

Identification of compounds was done by comparing mass spectra with public databases (NIST/ CNRS/WILEY275) or by using derivatised standards (41 compounds including 13 sugars, 19 amino acids and 9 organic acids).

Denitrification enzyme activity without carbon source addition

The denitrification enzyme activity (DEA) of the microbial community present in the RAS was measured in each plant rhizosphere and in the bulk soil in four replicates. According to Patra *et al.* (2005), 10 g dry mass equivalent of RAS from each plant or bulk soil was placed in a 150 mL airtight plasma-flask sealed by a rubber stopper. In each flask, soils were moistened at 100% water holding soil capacity to obtain optimal conditions for denitrification and air was removed and replaced with a He:C_2H_2 mixture (90:10 v/v) to create anoxic conditions and inhibit N_2O-reductase. It should be noticed that in our conditions microbial communities do not use C_2H_2 as carbon source as its concentration remained constant for each experiment. Potassium nitrate (50 μg of N-KNO_3.g^{-1} dry soil) was added to each flask to saturate the soil with only a nitrate source in order to feed the microbial communities. However, no carbon source was added as the RAS already contained rhizodeposits. After 2 h of incubation at 28°C, the amount of N_2O was measured each hour for 5 h from a 0.53 mL gas sample. The slope of the linear regression was used to estimate the DEA in the form of the N_2O produced (μg^{-1}.h^{-1}.g dry soil^{-1}). N_2O was measured with a gas chromatograph coupled to a micro-catharometer detector (μGC-R3000, SRA instruments, Marcy l'Etoile, France).

Statistical analysis

Using retention times and peak areas obtained from GC-MS chromatograms of studied extracts, data matrices were built in order to carry out hierarchical cluster analysis (HCA). Peak areas were normalised by using relative values, i.e. percentages of each compound in the chromatogram. Between-class analysis (BCA, i.e. discriminant principal component analysis (PCA)) was performed using R studio software (version 0.98.1103). The 'ade4', 'mixOmics' and 'RVAideMemoire' packages were uploaded on R studio. Differences between compounds that explained variations in RAS and root extracts (obtained with the BCA) were analysed using one-way analysis of variance (ANOVA1), followed by a Tukey's HSD test in R studio. A hierarchical heatmap was created using R statistical software 3.1.2 and the 'gplots' package.

For each plant, ANOVA1 and *post hoc* Tukey's HSD test were performed to test differences between DEA in the RAS of the different grass species. Effects of rhizosphere primary metabolites in RAS on the DEA rate were determined using simple linear regressions between primary metabolite concentrations in RAS and the DEA averaged for each plant and for the control bulk soil. All tests were performed in conformity with the assumptions of normality and homogeneity of variance (Shapiro and Bartlett tests were used, respectively).

RESULTS

Plant primary metabolite profiles

Metabolite profiling was performed on the RAS and root extracts of each plant and on the control bulk soil extract. Representative base peak chromatograms obtained from RAS and root extracts of *Dactylis glomerata* (DG) and from the bulk soil are shown in Supplementary Fig. S1. Over 63 peaks were detected in RAS extracts, of which 43 could be annotated, while in root extracts over 75 peaks were detected, of which 49 were annotated (Supplementary Fig. S1). GC-MS chromatograms obtained

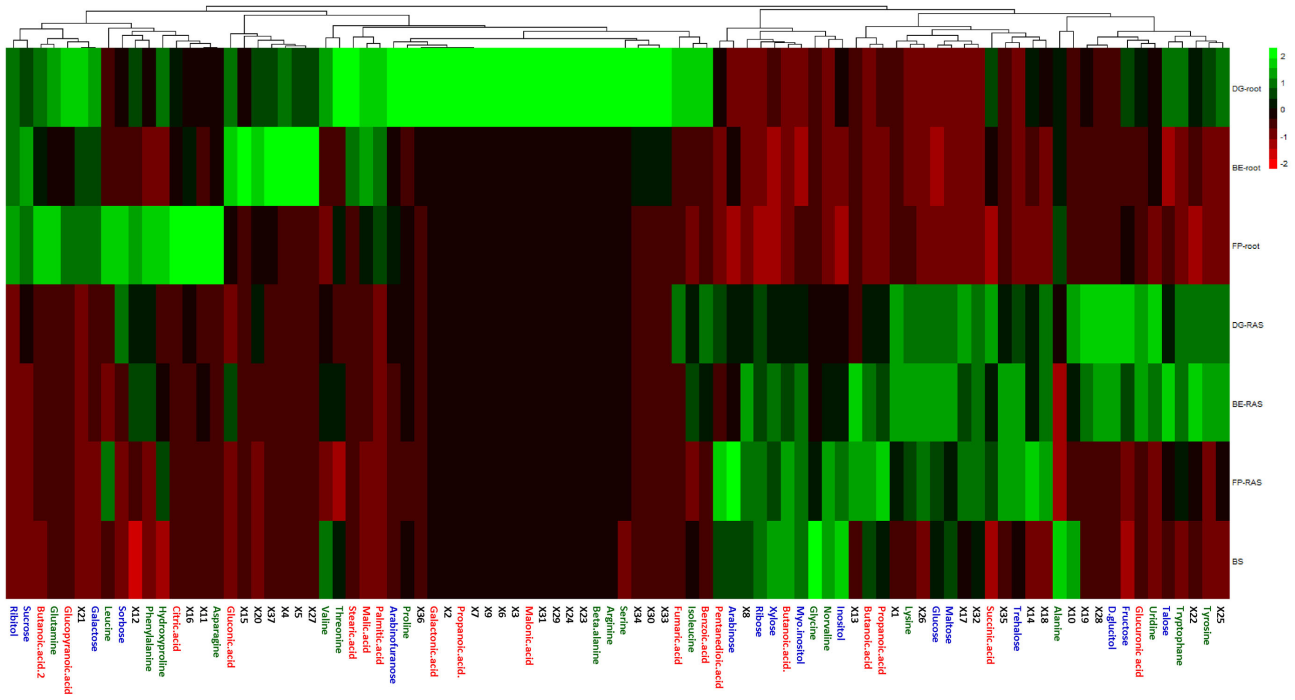

Figure 1. Hierarchical cluster analysis. Heat map representing the diversity and relative abundance of primary metabolites present in the RAS and root extracts of *Festuca paniculata* (FP, conservative), *Bromus erectus* (BE, intermediate), *Dactylis glomerata* (DG, exploitative) and in control bulk soil (BS). Red represents an absence of primary metabolites in root or RAS extracts, and green represents a high relative abundance of primary metabolites in root or RAS extracts. Molecules coloured red, blue, green and black correspond to organic acids, sugars, amino acids and non-identified compounds, respectively.

from the bulk soil extract showed the presence of a few residual molecules such as glucose and glycine. In addition, we noticed differences between profiles obtained from the RAS and from root extracts of the same plant species (Supplementary Fig. S1). Experiments were performed in triplicate for each compartment (soil and root) and similar profiles were observed for each sample, demonstrating good reproducibility (data not shown).

A total of 16 sugars, 18 organic acids and 19 amino acids were identified from RAS and root extracts (Fig. 1). Among the compounds that were reproducibly detected in both types of extract, 37 could not be annotated using standards or databases. This analysis shows that some primary metabolites are more specific to the root compartment (e.g. ribitol, sucrose) or to the soil compartment (e.g. xylose, ribose) allowing us to discriminate both type of extract as the main clustering vector in the HCA.

Primary metabolites from the RAS extracts

Between class analysis (BCA) performed on chromatographic profiles from RAS extracts of conservative (FP), intermediate (BE) and exploitative (DG) plants and from the bulk soil (BS) showed significant differences between planted and bulk soils (Fig. 2A), suggesting the presence of plant root exudates of different nature in the rhizosphere compared with the bulk. In addition, the planted soils differed significantly between each other, suggesting that plant nutrient management strategy affects the composition of the primary metabolites present in the RAS. Most of the variability in the data could be explained by component 1 (60.9%), which made it possible to discriminate most of the different groups, while component 2 accounted for 25.8% and mainly discriminated plant species (Fig. 2A). Furthermore it is interesting to notice that the profile of FP RAS extract was the closest to that of BS according to axis 1, whereas DG RAS was the most distant, BE showing an intermediate position between FP and DG.

BCA and HCA of RAS and bulk extracts made it possible to highlight most discriminant primary metabolites responsible for the differences between soil types (Supplementary Fig. S2A, and Figs 1 and 3A). Most of these discriminant metabolites showed variations in their relative abundance and a few of them were even absent in some plant species. For example, there was a three-fold greater abundance of hydroxyproline in the RAS of FP than in the RAS of DG or BE, whereas fructose was 10 times more abundant in DG and BE than in FP RAS (Fig. 3A). Butanoic acid was more abundant in BE and FP RAS compared with DG RAS, which was equal to bulk soil. In addition, fumaric acid, glutamine and sorbose were detected only in DG RAS (Figs 1 and 3A).

Primary metabolites from root extracts

BCA performed on chromatographic profiles from the root extracts of conservative (FP), intermediate (BE) and exploitative (DG) plants showed significant differences between plant nutrient strategies (Fig. 2B): the data variability explained by the main and second axis represented, respectively, 68.6% and 31.4% of the total variability (Fig. 2B). Certain discriminant compounds showed variations in their relative abundance and others in their presence or absence in some plant species (Supplementary Fig. S2B, and Figs 1 and 3B). This was the case for malic acid and trehalose, which were more abundant in DG and BE root extracts than in FP root extracts (Fig. 3B). Fructose and galactose levels did not differ significantly between FP and BE root extracts ($P > 0.05$), while they were higher in DG root extracts ($P < 0.05$). In contrast, leucine and citric acid levels were not significantly different between DG and BE root extracts ($P > 0.05$), whereas these molecules were more abundant in FP root extracts ($P < 0.05$).

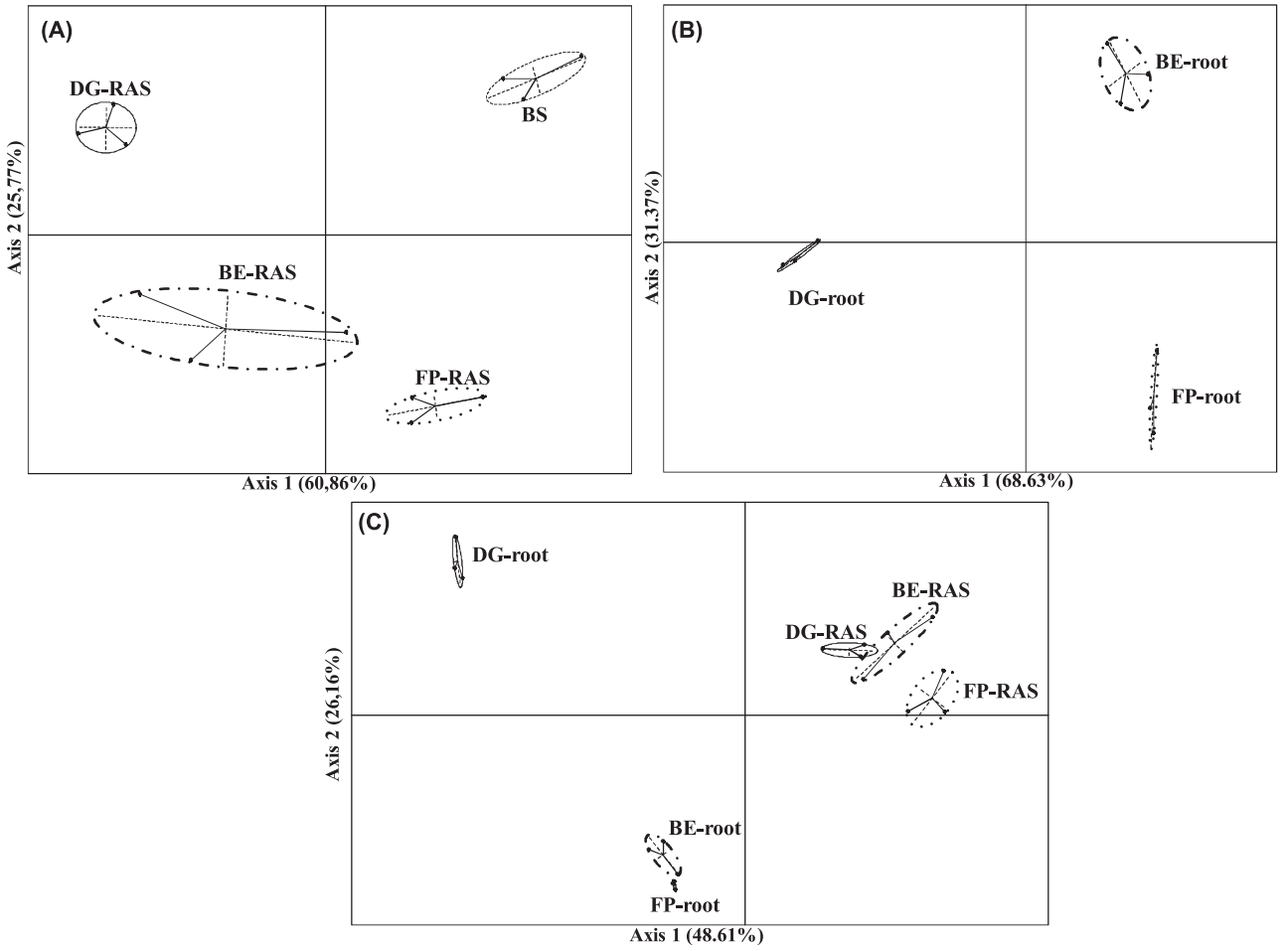

Figure 2. Between class analysis (BCA) performed on GC-MS data obtained from RAS and root extracts of *Festuca paniculata* (FP, conservative), *Bromus erectus* (BE, intermediate), *Dactylis glomerata* (DG, exploitative) and from control bulk soil (BS). Analyses were based on peak areas and retention times. (**A**) RAS extracts and bulk soil; (**B**) root extracts; (**C**) RAS and root extracts. The value for each axis is shown.

Significant differences in butanoic acid levels were detected between plant species, with a greater abundance in FP root extracts ($P < 0.001$). Moreover, according to Fig. 1, malonic, propanoic and galactonic acids as well as arginine and β-alanine were present only in DG root extracts, while citric acid and asparagine were found only in FP root extracts.

Comparison between primary metabolites recovered from RAS and root extracts

Figure 1 shows the disparity of plant primary metabolites between RAS and root extracts. The levels of some primary metabolites were higher in root extracts than in RAS extracts (Fig. 1). This is the case for some sugars such as ribitol or sucrose and also organic acids such as palmitic and malic acids (Fig. 1). On the other hand, fewer molecules were found in higher levels in all RAS extracts and not in BS (the case of trehalose and glucose). These results suggest that some molecules present in the roots are not exuded into the soil; these compounds could also be immediately assimilated and transformed by soil microbes or complexed with soil organic matter.

BCA performed on chromatographic profiles from both RAS and root extracts showed that root extracts differ significantly from RAS extracts for all the plant species tested (Fig. 2C). Axis 1 explaining 48.6% of variance separated the two distinct areas. Even if methods of extraction differed for both matrices (RAS or root tissues), they were optimised to allow the best extraction of primary metabolites according to the matrix used. It is also important to notice that the same type of metabolite was found to be predominant for a given plant in its roots and RAS enabling their discrimination by multivariate analysis (Supplementary Fig. S2): for example sucrose was found to be significantly more abundant in DG root and RAS extracts ($P < 0.001$) compared with the other species and the same observations could be made for butanoic acid and FP and for trehalose and BE (Fig. 3). This shows that although matrix and extraction protocol differed, the same tendency for compound accumulation in roots and RAS could be highlighted.

Denitrification activity in the root adhering soil of plant species

In order to determine the effect of plant rhizosphere primary metabolites on denitrifying activity, we investigated, for the first time, the DEA without carbon addition of microbial communities inhabiting the RAS without adding carbon sources and by considering rhizodeposits as the only carbon source. DEA was found to vary in the RAS in relation to plant nutritional

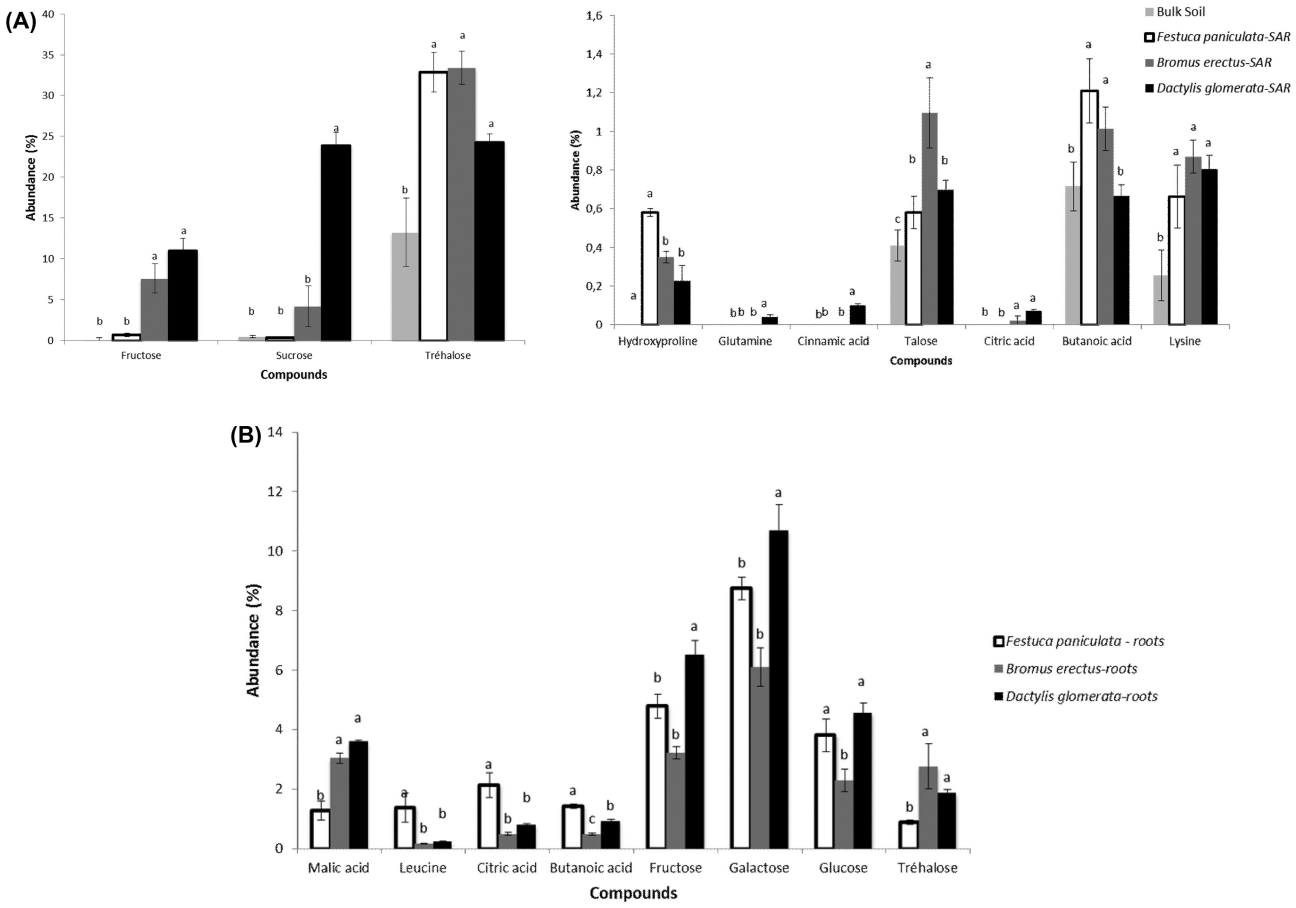

Figure 3. Relative abundance of the most discriminant primary metabolites explaining metabolic differences in the RAS (**A**) and root (**B**) extracts of *Festuca paniculata* (FP, conservative), *Bromus erectus* (BE, intermediate), *Dactylis glomerata* (DG, exploitative) and control bulk soil (BS). Statistical differences between treatments are indicated using letters a–c (Tukey's HSD test, $P < 0.05$). Means and standard error are represented (n = 4).

strategy ($F_5 = 5.5$, $P < 0.05$) (Fig. 4). Indeed, DEA in the control bulk soil was 1.3 μg N-N_2O^{-1}.h^{-1}.g dry $soil^{-1}$, whereas for the RAS of FP (conservative) or BE (intermediate), it was 1.8 and 2.0 μg N-N_2O^{-1}.h^{-1}.g dry $soil^{-1}$, respectively, and 2.5 μg N-N_2O^{-1}.h^{-1}.g dry $soil^{-1}$ in the RAS of DG (exploitative). Figure 4 shows the three statistical groups, the first formed by the bulk soil, the second by FP and BE (the conservative and the intermediate grasses, respectively) and the last by DG, the exploitative grass. So, denitrification increased in the RAS of FP and BE in comparison with the bulk soil, and was significantly higher in the RAS of DG (Tukey's HSD test, $P < 0.01$). This suggests that the increased denitrification activity in the RAS of plants is related to root exudation.

Simple linear regressions were performed on all the primary metabolites extracted from the RAS (90 in total) and denitrification activity measured in each plant rhizosphere. Figure 5 illustrate primary metabolites that present a positive or negative correlation with DEA. DEA was positively related to succinic acid (Fig. 5A; $P < 0.05$, $F_{1,3} = 39.9$) and butanoic acid (Fig. 5B; $P < 0.1$, $F_{1,3} = 10.5$) concentrations in RAS. These findings suggest that organic acids stimulate the denitrification rate more than sugars. Moreover, the greatest denitrification rate link was found with xylose (Fig. 5C; simple linear regression, $P < 0.001$, $F_{1,3} = 10.67$). This negative relationship between the DEA rate and xylose levels in soil could suggest an inhibition of denitrification.

DISCUSSION

In the rhizosphere, root exudation is of central importance in C transfer from the plant to the soil. The application of ^{14}C and ^{13}C isotopes in rhizosphere studies has led to significant progress in understanding C flow in the rhizosphere (Haichar *et al.* 2008, 2016). The consensus estimate is that 16–50% of the net photosynthesised C is allocated to the roots (Kuzyakov and Domanski 2000), with 40–90% of this fraction entering the soil as rhizodeposits (Lynch and Whipps 1991). Actually, researchers agree with the fact that organic molecules found in the rhizosphere mostly originate from plant root exudation, which is thought to influence microbial populations in a plant-specific manner (Costa *et al.* 2006).

In this study we focused on root exudation as a plant functional trait and aimed to determine the effect of plant nutritional strategies on the composition of primary metabolites exuded and the impact that this exudation had on soil microbial denitrification activity.

Impact of plant nutritional strategy on root exudates' primary metabolite composition

The plant nutrient strategies adopted by fast- and slow-growing plant species (i.e. exploitative and conservative species, respectively) are known to contribute, more or less depending on the

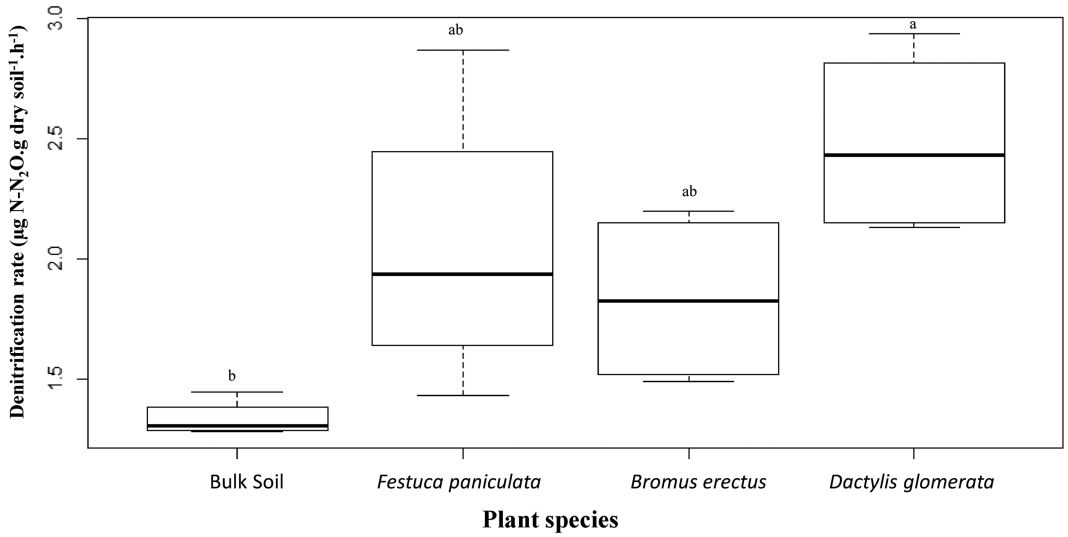

Figure 4. Comparison of semi-real denitrification rates in the RAS of three grasses with different nutrient management strategies, *Festuca paniculata* (FP, conservative), *Bromus erectus* (BE, intermediate) and *Dactylis glomerata* (DG, exploitative), and from control bulk soil (BS). Means and standard error are represented ($n = 4$). For each sample, box plots labelled with the same letter do not differ at $P < 0.05$.

strategy under study, to soil carbon cycling. Exploitative species may impact soil carbon pools by adding large amounts of carbon to the soil, potentially via greater root exudation and high specific root length, whereas conservative species contribute by adding low-quality plant material, i.e. highly concentrated carbon forms in nutrient-poor tissues (De Deyn, Cornelissen and Bardgett 2008).

Significant differences were observed between planted (DG, BE and FP) and control BS in terms of primary metabolite profiles, showing differences in the carbon sources exuded by plant roots in the rhizosphere. Plant exudation in the root area induces the 'rhizosphere effect', resulting in an increase in microbial abundance and activity, as demonstrated early on by Hiltner (1904). Data from the present study show significant differences between plant species of different nutrient management strategy, suggesting that this plant trait significantly affects the composition of primary metabolites transferred into the soil. A possible difference in root exudation between exploitative and conservative plants has already been suggested in the literature due to differences in plant photosynthetic capacity and relative growth rate (Personeni and Loiseau 2004; De Deyn, Cornelissen and Bardgett 2008). Indeed, using $^{13}CO_2$ pulse labelling, De Deyn, Cornelissen and Bardgett (2008) showed that exploitative plants showed the highest carbon assimilation rate, whereas, conservative ones showed the lowest. After 24 h of pulse labelling, ^{13}C enrichment in aboveground tissue had declined sharply in exploitative plants, whereas conservative ones remained enriched. Recently, Kaštovská *et al.* (2014) demonstrated that the exploitative *Glyceria* sp., in spite of its smaller belowground carbon allocation, invested significantly more of its net carbon fixation to exudation than the conservative *Carex* sp.

It is interesting to notice that in our study the soil extract presenting the highest richness of primary metabolites in terms of diversity and quantity was that of the exploitative DG, whereas that of BS presented the poorest with intermediate values for FP and BE, so that component 1 of the BCA of RAS extracts allowed a good discrimination of plant gradient according to nutritional strategy. However, this is the first study to show that plant nutrient management strategy and root exudation composition are intimately linked.

The differences in primary metabolite composition according to plant nutritional strategy suggest variations in plant interaction with soil microbial communities, as root exudates are known to be the main way for plants to communicate with their environment (Bais *et al.* 2006; Philippot *et al.* 2013; Haichar *et al.* 2014). Indeed, in the rhizosphere, primary metabolites exuded by roots are expected to attract and stimulate the growth of rhizosphere microorganisms so as to provide them with carbon and nitrogen sources (Bais *et al.* 2006; Haichar *et al.* 2014) and hence to shape microbial community structure and function (Smalla *et al.* 2001; Costa *et al.* 2006; Haichar *et al.* 2008; Haichar, Roncato and Achouak 2012). For example, in our study, fumaric acid and glutamine were detected only in DG RAS. Fumaric acid exudation was also observed with barley (*Hordeum vulgare* cv. Barke) and was probably involved in tripartite interactions of beneficial microorganisms, plant and pathogens in the rhizosphere (Jousset *et al.* 2013). Liu *et al.* (2014) demonstrated that fumaric acid exudation is involved in inducing plant growth-promoting rhizobacterial colonisation by stimulating biofilm formation. These results suggest that the competitive DG could exude high levels of fumaric acid to specifically recruit beneficial bacteria. Glutamine exudation was also observed in grasses (Greenhill and Chibnall 1934; Curtis 1944; Raleigh 1946). It was shown that the exudation of glutamine is most noticeable in the early spring, when the absorption of ammonium salts by the root system is usually most active. Amino acids can provide both carbon skeletons and nitrogen to microorganisms (Jaeger, Dijkstra and Reetz 1999). These results suggest that exploitative plants could exude high levels of glutamine, as it is correlated with a high rate of nutrient uptake, as reviewed in Grime (2001), in order to enhance its interaction with soil microbial communities.

Comparison between roots extract content and RAS extract content

Significant differences were observed between the chromatographic profiles of root extracts of conservative, intermediate and exploitative plants, suggesting different plant nutrient strategies affect root extract content. In root extracts, among discriminant metabolites, high levels of butanoic acid, citric

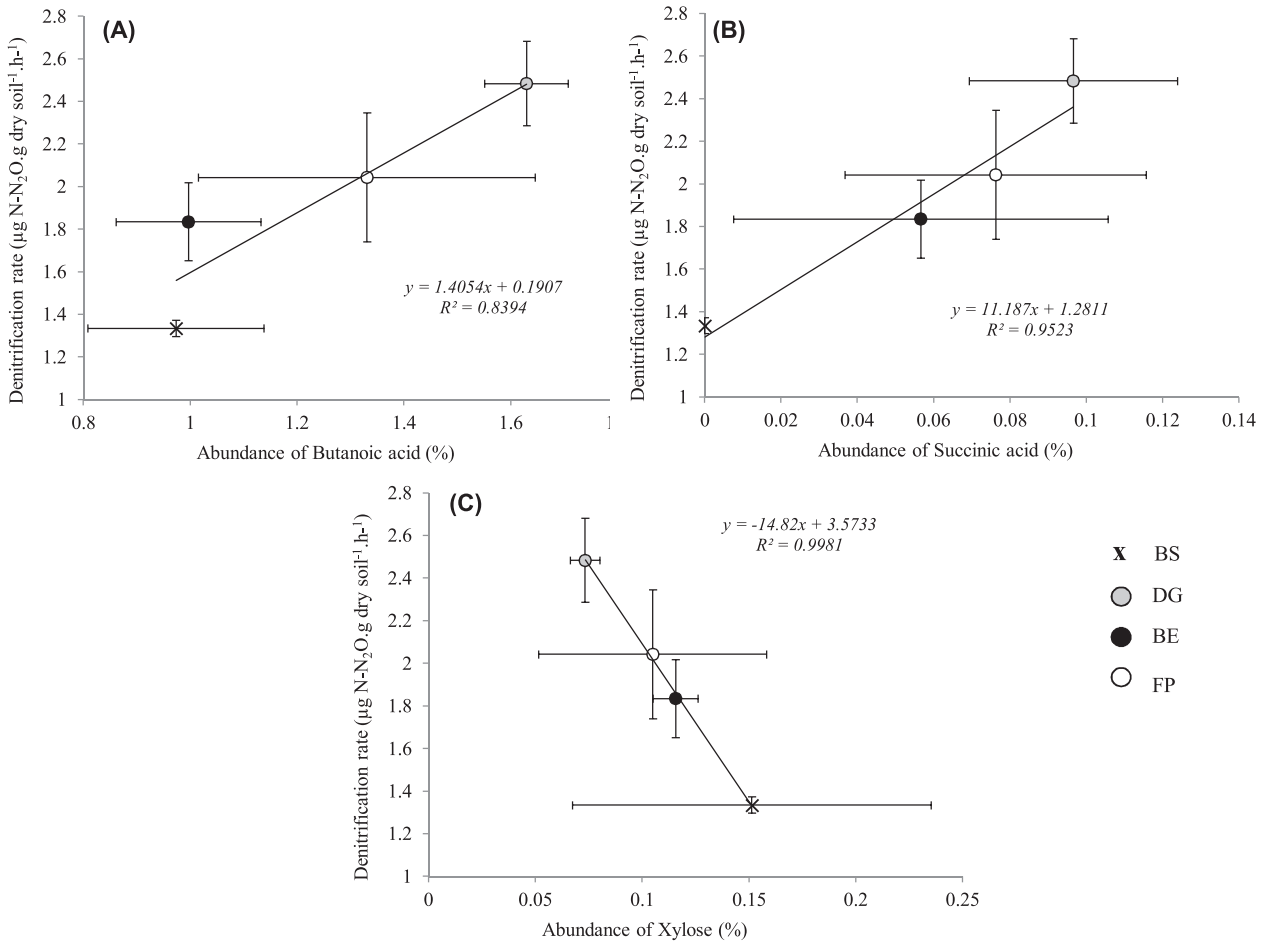

Figure 5. Relationships between the levels of primary metabolites and semi-real denitrification in the RAS of three grasses, *Festuca paniculata* (FP, conservative), *Bromus erectus* (BE, intermediate) and *Dactylis glomerata* (DG, exploitative), and from control bulk soil (BS). (**A**) succinic acid; (**B**) butanoic acid; (**C**) xylose. Significant simple linear regressions are shown ($P < 0.05$ for (A) and (C) and $P = 0.08$ for (B)). Means and standard error are represented ($n = 4$).

acid, phenylalanine, asparagine, glutamine and leucine were detected in FP root extracts, suggesting that the conservative plant FP keeps them more in the root and exudes less into the rhizosphere. These results are in agreement with the fact that conservative plants enhance nutrient conservation and invest heavily in high density tissues (Chapin 1980; Poorter and Garnier 1999).

Significant differences were observed between the chromatographic profiles of the RAS and root extracts of conservative, intermediate and exploitative plants, highlighting the difference between what is conserved in the root and what is exuded into the soil. For example, asparagine and citric acid were more abundant in the root extracts of FP than in the RAS extract. It was demonstrated that for certain grasses, any excess ammonia absorbed by the root system was largely stored in the root as asparagine (Greenhill and Chibnall 1934). This finding is in agreement with the fact that the conservative FP has a strategy of high resource conservation, as described by Grime (2001).

Until recently, due to limitations of the methodologies used for root exudate collection and characterisation, root extract content was often used as a proxy of root exudation composition. However, the collection of root exudates from the root-adhering soil, as done in this study, does not affect plant physiology or disrupt mycorrhizal networks as is the case for other methods (Nguyen 2003; Neumann and Romheld 2001). Hence, the collection of root exudates from the RAS should be used often for analysis of root exudate composition. In this study, we demonstrated that the analysis of primary metabolites extracted from the RAS is more relevant in explaining plant traits, such as nutritional strategy, and plant–microbe interactions.

Impact of rhizosphere primary metabolites on soil microbial denitrification activity

In order to determine the effect of plant rhizosphere primary metabolites on denitrifying activity, we investigated for the first time the denitrification activity of microbial communities colonising RAS without adding carbon sources. The increase in denitrification activity in the RAS compared with bulk soil could be related to root exudation, which is known to stimulate microbial activity (Grayston, Vaughan and Jones 1997) and denitrification (Henry *et al.* 2008). In our case, the higher rate of DEA found in DG RAS seems to be linked to a greater diversity of primary metabolites. DG was found to exude a greater diversity of organic acids (fumaric and citric acids, for example, were only found in DG), amino acids (e.g. glutamine) and higher quantities of main sugars such as sucrose and fructose, which could stimulate the denitrification rate in DG RAS.

In this study, plants were found to release, via root exudation, a large number of primary metabolites, i.e. sugars, organic acids and amino acids, representing potential carbon sources for microorganisms (Jones, Hodge and Kuzyakov 2004; Mounier *et al.* 2004; Singh *et al.* 2004). According to previous studies (Henry *et al.* 2008; Morley, Richardson and Baggs 2014), the nature of the carbon source was found to influence the denitrification rate (Figs 4 and 5). Indeed, DEA was positively related to succinic acid and butanoic acid levels in RAS, suggesting that organic acids stimulate the denitrification rate more than sugars, and was consistent with values described in Henry *et al.* (2008) and Morley, Richardson and Baggs (2014). The negative relationship between DEA rate and xylose levels in soil could suggest an inhibition of denitrification, but could also be the result of microbial degradation. Xylose is a pentose and is the second most abundant sugar present in plant lignocellulose after glucose, is frequently found in root exudates and is not synthesised by microorganisms (Jones, Hodge and Kuzyakov 2004; Vranova *et al.* 2013). In general, sugars are considered as promoters of microbial growth, as reviewed by Haichar *et al.* (2014). The fact that the highest xylose levels were found in bulk soil suggests that its origin is related to soil history (maize culture) and not to a recent input (i.e. plants from our experiment). In planted soils, xylose levels were low in the RAS of DG compared with that of FP or BE. Two hypotheses could be drawn from these observations: (i) xylose is taken up by plants and exploitative plants such as DG would have a higher uptake rate of this compound than other non-exploitative ones and thus xylose would be an inhibitor of denitrification since DG soils are the most active; and (ii) xylose is specifically metabolised by plant-associated microbes, and in DG, which, as an exploitative plant, exudes the richest and most diverse primary metabolites, this would lead to a greater diversity of microbes in its rhizosphere, resulting in a higher potential for metabolising xylose in these communities. Further work is required in this area to better understand how xylose and organic acids, such as succinic and butanoic acid, influence the denitrification rate.

In addition to the effect of root exudates on denitrification activity, it will be interesting to study this effect on the diversity of denitrifiers and on the expression of denitrification genes.

From this laboratory study, we can draw the conclusion that according to plant nutritional strategy, the quality of root exudates may shape differentially nitrogen cycling microbial communities leading to more or less N_2O emissions. Future studies are needed to confirm our results under field conditions with a broader range of plant strategies and by considering also other processes producing and reducing N_2O emissions (such as nitrification, for example).

In addition to carbon quality, it should be interesting to quantify the carbon exuded in the rhizospheric soil, as it is an important factor influencing denitrification as demonstrated by Henry *et al.* (2008). This study focused principally on primary metabolites, some of which remain unknown, and these could also play a role in plant–denitrifier interactions as shown by Bardon *et al.* (2014, 2016). Additional studies with the same type of methodological approach developed here are required for primary and secondary metabolite analyses to better explain the effect of plant–microbe interactions on ecosystem functioning.

At the time point when primary metabolites were analysed, the growth stage of plant species under study corresponded to the vegetative phase and it is well known that root exudation patterns vary during the life cycle of plants (Dunfield and Germida 2003; Hinsinger *et al.* 2005; Jones, Nguyen and Finlay 2009) suggesting the relationship between plant physiology and rhizodeposition. Therefore, we believe that time-course experiments will make it possible to capture this variability.

CONCLUSION

In conclusion, our study demonstrated (i) a coherent relationship between plant nutrient management strategy and primary metabolite composition, (ii) a strong positive relationship between denitrification rate and the levels of some organic acids in soil, and (iii) that RAS extraction is probably more explicative of plant–microbe interactions in the rhizosphere than root extract content.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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