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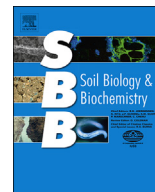
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Evolution of the amino acid fingerprint in the unsterilized rhizosphere of a legume in relation to plant maturity



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ABSTRACT

The exudation of low-molecular-weight compounds, such as amino acids, contributes to the structure of rhizospheric microbial communities and plays a crucial role in nutrient mobility throughout the vegetative cycle of the plant. Due to their low concentration and their constant removal by microorganisms, the study of amino acids is difficult in unsterilized rhizospheric soil. Our aim was to investigate the dynamics of amino acids in unsterilized soil.

Since there was currently no available method to estimate accurately amino acid exudation in soil, a methodology was developed to assess amino-acid fingerprints in the rhizosphere. This proved a viable approach for understanding the dynamics of amino-acid release into the rhizosphere. The method was tested on *Medicago truncatula*, with the hypothesis that the fingerprint of amino acids in the rhizosphere should reflect amino acid exudation from leguminous plants, subject to the influence of microbiological components of the soil, and should be correlated with the stage of plant growth. *M. truncatula* plants were grown in either unsterilized or sterilized substrate. Amino acids in the substrate samples were identified and quantified by gas chromatography-mass spectrometry (GC-MS) and microbial biomass in the soil samples was also assessed.

The total content of amino acids in the sterilized rhizospheric substrate increased substantially during plant growth with the highest content observed at the end of the vegetative stage. Despite the probable continual removal of amino acids by microorganisms, we also observed a significant quantitative and qualitative plant-dependent effect on the amino acid fingerprint in unsterilized soil. A clear chronological differentiation of this fingerprint in the rhizosphere of unsterilized and sterilized substrate was revealed by principal component analysis. We observed predominantly serine and glycine in the sterilized rhizosphere, whereas asparagine became prominent before flowering in the unsterilized rhizosphere soil. Root amino acid metabolic pathways and exudation appear to differ in unsterilized soil compared to in sterilized substrate, indicating an adaptation to the rhizospheric environment.

The established method could be used in other applications, for example, for future ecophysiological studies aiming at following root exudation of amino acids in response to changing environmental conditions.

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1. Introduction

During the last decades, the massive use of chemical nitrogen

fertilizers has contributed markedly to the increase in the circulation of anthropogenic reactive nitrogen (Nr) in the Earth's atmosphere, hydrosphere and biosphere (Galloway et al., 2003). Environmental consequences for both natural and agricultural ecosystems are various (e.g. greenhouse gas emissions, eutrophication) and are magnified as Nr moves along biochemical pathways, a phenomenon that has been named “the N cascade”.

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Practices promoting positive biological interactions within cropping systems are now regarded as a promising way to reduce inputs (Powelson et al., 2011; de Vries and Bardgett, 2012). Biological alternatives, such as the use of diazotrophic prokaryotes, may help to reduce mineral N fertilization and limit the environmental impact of agricultural practices without any associated dramatic drop in yield (Garg and Geetanjali, 2007). Within this context, interactions between plant roots and rhizosphere microbial communities via rhizodeposition are of increasing interest (Schenck zu Schweinsberg-Mickan et al., 2012; el Zahar Haichar et al., 2014). For instance, the presence of roots can enhance soil organic matter decomposition by stimulating the growth of soil microorganisms (Helal and Sauerbeck, 1984), thus influencing nutrient availability for plants in sub-optimal agronomic conditions (Dakora and Phillips, 2002; Paterson, 2003).

Since legumes have the capacity to conduct biological nitrogen fixation due to their endophytic rhizobacteria, they can constitute a substantial source of N in cropping systems (Jensen, 1996; Khan et al., 2002; Paynel and Cliquet, 2003; Corre-Hellou and Crozat, 2005; Paterson et al., 2005; Lesuffleur et al., 2007; Mahieu et al., 2007; Wichern et al., 2007, 2008; Fustec et al., 2010; Nyfeler et al., 2011; Oberson et al., 2013). N is then transferred to the soil via rhizodeposition, during which a large range of organic compounds are released from actively growing roots at the soil-root interface (Farrar et al., 2003; Badri and Vivanco, 2009; Fustec et al., 2010), including root debris, decomposed root material, cell lysates, mucilage and exudates (Lynch and Whipps, 1990). Exudates include organic acids, sugars, amino acids (AAs), specialized metabolites, peptides, proteins, ureides and lipids (Walker et al., 2003; Bais et al., 2006). The composition of rhizodeposits is a determining factor for the structure of the rhizosphere microbial communities, generally enhancing the range and number of microorganisms and their activity (Söderberg and Bååth, 1998; Henry et al., 2005). Experiments using various methods have indicated that significant amounts of N can be released to the soil by growing legumes (Wichern et al., 2008; Fustec et al., 2010). In particular, exudation of soluble N compounds is of great importance for C and N cycling in soil, where they constitute an N source for rhizosphere microorganisms (Grayston et al., 1998; Kuzyakov, 2002). However, little is known about the composition of N-rich rhizodeposits and the relationships with parameters which directly influence their destiny in the N cycle in soil. Among low-molecular-weight soluble compounds, exudation of AAs is one of the main N source for the soil community (Kelley and Stevenson, 1996; Merbach et al., 1999; Yu et al., 2002; Fischer et al., 2007). In annual crops, exudation of organic compounds by the plant roots is known to be low at the seeding stage, thereafter increasing to reach a maximum at flowering before dropping off towards seed maturity (Aulakh et al., 2001). Different factors are known to strongly affect AA release by the roots: notably, the plant genotype (Ivarson et al., 1970; Lesuffleur et al., 2007; Sauheitl et al., 2010), the growth stage (Aulakh et al., 2001), abiotic factors such as water and temperature (Jones et al., 2009; Farrell et al., 2014), or biotic factors such as microbial community structure (Bais et al., 2006; Moe, 2013). However, relationships between plant physiology and exudated compounds remain poorly understood, particularly regarding AAs.

Amino acid exudation in the soil is difficult to assess, particularly due to two methodological limitations. First, AAs released by the roots are quickly consumed by microbial communities (Stevenson, 1982; Owen and Jones, 2001; Jones and Willett, 2006). Second, AAs are difficult to extract properly from the soil because they are mainly present as zwitterions and, via their cationic function, are adsorbed to anionic mineral particles and humic substances in colloids (Dashman and Stotzky, 1982; Wang and Lee, 1993; Sposito, 2008). To overcome these difficulties, most studies

on root exudates have been performed either in hydroponic systems and/or using sterilized substrates. The principal convenience of these methods is the easy recovering of the root exudates (Meharg, 1994; Grayston et al., 1997; Todorovic et al., 2001; Kuijken et al., 2015). However, this advantage is offset by the loss of the plant–soil particle interaction in hydroponic systems and the plant–microorganism interaction when sterilized soil is used. Both these deficiencies are cause for concern, as a number of studies have shown that root exudation is strongly affected by soil type (Mimmo et al., 2011) and microbial activity (Meharg and Killham, 1991; Grayston et al., 1998; Moe, 2013).

In this study, we have firstly developed a method for the extraction and quantification of AA exudates present in soil. Then, we have used this to carry out a comparison of changes in the AA exudate fingerprint in the unsterilized rhizosphere versus the sterilized rhizosphere at different stages of plant growth. We assume that the AA fingerprint is the balance between plant exudation and sequestration by microorganisms (Fig. 1).

2. Material and methods

2.1. Plant material and experimental design

Seeds of *Medicago truncatula* Gaertn. cv. Jemalong (Leguminosae), line A17 were scarified, sterilized (50% H₂SO₄, 10 min) and germinated on moist filter paper for 24 h. Fifteen germinated seeds were transferred into small pots (5 cm height × 5.5 cm diameter; one seed per pot) filled with unsterilized soil removed from a field (81.8% sand, 9.2% silt, 8.2% clay, 0.7% organic matter, pH 6.0 (CaCl₂)). The pot size was chosen to ensure a rapid and dense colonization of

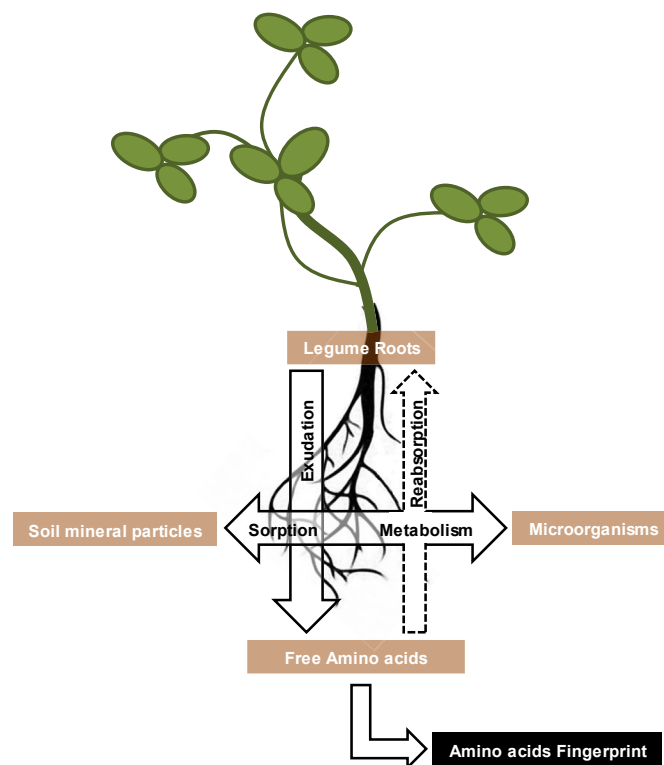


Fig. 1. Schematic of the factors that determine the amino-acid fingerprint at the root-soil interface, as the result of interactions between root exudation and soil microflora activity. At typical soil pH, amino acids are in their zwitterionic form and may be adsorbed to mineral particles mainly in the cationic form, metabolized by microorganisms and/or reabsorbed by the plant.

the soil by the roots. The soil was covered with a thin layer of sterilized sand (Fig. 2). In parallel, 15 germinated *M. truncatula* seeds were transferred in the same manner into small pots filled with sterile sand prepared by washing with deionized water and autoclaving (120 °C, 20 min). Plants were kept in a growth chamber (16 h photoperiod, 30 °C) and watered with a nutrient solution (modified from Murashige and Skoog, 1962) (mM): 1 CaCl₂, 1 MgSO₄, 2 KH₂PO₄, 2 K₂HPO₄, 1 NH₄NO₃, 4 KNO₃, 0.1 MnSO₄, 0.03 ZnSO₄, 1.10⁻³ Na₂Mo₄, 0.1 H₃BO₃, 5.10⁻³ KCl, 1.10⁻⁴ CoCl₂, 25.10⁻³ FeSO₄, 25.10⁻³ Na₂ EDTA, pH 5.8). The water content was adjusted to 150 g kg⁻¹ (60% of the maximum water holding capacity of soil) by injecting the solution via a leaky plastic cone inserted into the substrate (Fig. 2). Pots of control soil or sterilized sand without plants (hereafter called 'controls') were prepared in the same way as the pots containing a plant. *M. truncatula* and substrates were harvested and sorted at the 1st ramification emerging (29 days after sowing (DAS)), the 2nd ramification emerging (45 DAS), and just before flowering (59 DAS). Samples of soil from 4 independent plants were taken at each harvesting time in order to have a good representation of the soil AA fingerprint. Controls (soil and sterilized sand) were also sampled at each stage (n = 4).

2.2. Harvest and sampling

By 29 DAS (first harvest), in pots containing a plant, the whole soil/sand volume was completely colonized by the roots, and so was considered to be rhizospheric. Plants were cut at the stem base and above-ground parts were sampled. The soil substrate around roots was broken carefully by hand into aggregates and sorted to remove all remaining roots (Jones and Willett, 2006). Roots were then rinsed with 15 mL of 2 mM formic acid to recover the adherent substrate. Including pots without plants, four different kinds of substrates were thus sampled: *i*) rhizospheric unsterilized soil (hereafter called RO), *ii*) rhizospheric sterilized sand (RD), *iii*) control with unsterilized soil (CO), and *iv*) control with sterilized sand (CD). For each category, a 40 g subsample was transferred into 185 mL of 2 mM formic acid and pooled with the 15 mL containing

adherent soil (w/v 1:5) in 250 mL polypropylene bottles (Jones and Willett, 2006). The suspension was shaken (1 h, 200 rpm, 20 °C) to liberate AAs adsorbed ionically to mineral particles. Then, the suspension was centrifuged (10 min, 8000 rpm) and the supernatant was filtered at 0.22 µm to remove particulate matter (HMWC) and living microorganisms (Jones and Darrah, 1994; Barber, 1995; Qualls and Richardson, 2003; Jones et al., 2005). The filtrate was stored frozen (-20 °C) in polypropylene bottles and 1 g of substrate was frozen in cryotubes (-80 °C) for the analysis of total microbial biomass.

2.3. Extraction of amino acids

The solution recovered from 0.22 µm filtration was purified by passage through a 6 mL cation-exchange column in H⁺ form (DOWEX 50W-X8, 200–400 mesh) to remove interfering organic components and metal ions recovered from the soil matrix. The column was prewashed by rinsing with 5 mL 10 mM HCl, then with 25 mL deionized water. After passing the filtrate, the column was washed with 25 mL deionized water. The AA fraction retained by the resin was recovered in 15 mL 6 M NH₄OH solution. The eluate was dried on a rotary evaporator (200 rpm, 45 °C), the residue dissolved in 4 mL 70% methanol, and stored frozen (-20 °C) in hemolysis plastic tubes until required.

2.4. Amino acids analysis

To reduce the polarity, and to improve the volatility and separation of AAs, the AA extract was derivatized by silylation (Kataoka, 1996). A reagent mix was prepared with acetonitrile (AcN), *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MBDSTFA) and triethylamine (TEA) in a 15:15:1 ratio (v/v/v, respectively). 2 mL of the AA extract were transferred to a 2 mL microtube, 4 µL of a 1.25 mM solution of α -aminobutyric acid (α -ABA) was added as internal standard and the sample taken to dryness using a Speed-Vac[®] evaporator. 50 µL reagent mix was added to the dried AA extract, the microtube was thoroughly vortexed, centrifuged, and incubated in a heated block (95 °C, 30 min). Following cooling, 25 µL of the derivatized extract were transferred to a GC vial containing an insert for GC-MS analysis.

Analyses were carried out with a 436-GC coupled to a Simple Quadruple (SQ) SCION MS (Bruker). Compounds were separated on a BR5MS column (Bruker; 5% diphenyl/95% dimethyl polysiloxane, 30 m × 0.23 mm i.d, 0.25 µm film thickness). The GC was operated in constant pressure mode with helium as carrier gas (1 mL min⁻¹). Injection (0.25 µL) was performed in split mode-4. The elution profile was: 60 °C for 1 min, linear gradient of 30 °C min⁻¹ to 120 °C, linear gradient of 8 °C min⁻¹ to 300 °C, 300 °C for 5 min, resulting in a total run time of 39 min. The injector, MS transfer line and source temperatures were respectively 280 °C, 290 °C and 220 °C. Mass spectra were collected using electron impact (EI) ionization at 70 eV. The GC-MS was operated in the SIM detection mode at mass range m/z 50–650.

2.5. Microbial biomass

Substrate samples (0.5 g of fresh soil or 1 g of fresh sand) were subjected to DNA extraction using a NucleoSpin[®] Kit (MACHEREY-NAGEL GmbH & Co. KG) as described by the manufacturer. The DNA extract was re-suspended in 50 µL ES buffer (5 mM Tris/HCl, pH 8.5) and was quantified using a Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, MA, USA).

The bacterial and fungal DNA in the soil samples was amplified by using bacteria-specific and fungus-specific primers, respectively

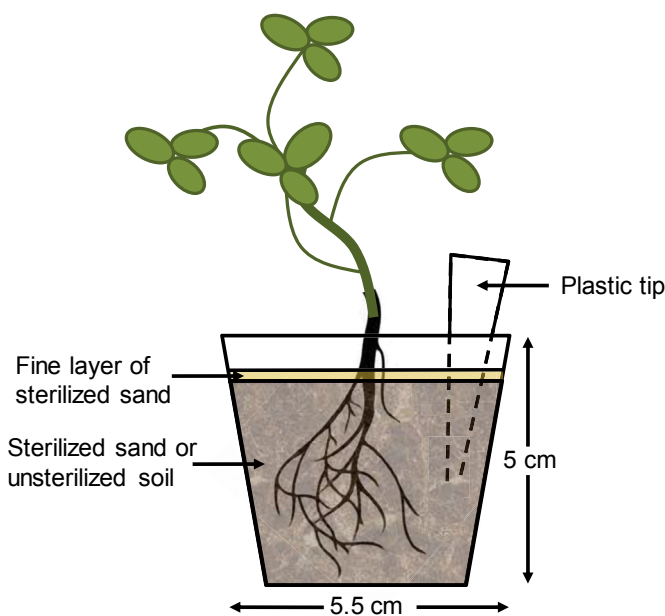


Fig. 2. Schematic of *Medicago truncatula* in 100 mL plastic pots containing either unsterilized soil or sand, overlaid with a fine layer of sterilized sand. Plants were watered through a plastic tip planted into the substrate.

(Table 1). Quantities of bacterial and fungal DNA in the sample were measured separately with real time PCR, by fluorescence using an external standard curve generated from serial dilutions of bacterial and fungal DNA from pure culture (*Escherichia coli* and *Fusarium graminearum*, respectively). Each PCR mixture contained 10 μ L of Supermix Sso Fast™ Eva Green® (©Biotium, Inc.), 1 μ L of each (forward and reverse) primers, 1 μ L DNA and was brought to 25 μ L with sterile deionized water. After denaturation at 98 °C for 2 min, the PCR products were amplified for 40 cycles: at 98 °C for 30 s and 50 °C for 3 s for bacteria or 60 °C for 3 s for fungi. PCR amplification efficiency was established by the means of calibration curves, with a minimum of 0.85 (85%).

2.6. Statistical analysis

Statistical analysis was done with R software version 3.1.2 (R foundation for Statistical Computing). All data were normally distributed performing the Shapiro Wilk's test for normality. Significant changes of the AA content in samples were tested using a one-way ANOVA (analysis of variance) and/or the Tukey's test. P-values < 0.05 were considered statistically significant. Principal component analysis (PCA) was performed with the PCA function from the FactoMineR package. Default values were normalized (scale.unit = TRUE), and, no individual was supplemented (quanti.sup = NULL and quali.sup = NULL). Confidence ellipses were drawn using the function coord.ellipse from the FactoMineR package, around the barycenter of all the variables (bary = TRUE), with a confidence level of 0.95.

3. Results

3.1. The total amino acid content in the rhizosphere

During the experiment, the total AA content measured in control substrates without plant (CO and CD) did not change significantly ($p > 0.05$). At 29 DAS, in both substrates (soil and sand), there was no effect of the presence/absence of a plant on the total AA content recovered from the rhizospheric compartment ($p > 0.05$; Fig. 3). At 45 DAS, in contrast, the presence of a plant significantly increased the total AA content in the rhizospheric compartment of the soil ($p < 0.05$) and of the sterilized sand ($p < 0.05$) compared with their respective control substrates without plant (CO and CD). At this stage, however, there was no quantitative difference between the AA content recovered from RO and RD ($p > 0.05$). By 59 DAS, the total AA content in RO had increased markedly and become significantly higher than in CO ($p < 0.05$). Similarly, between 45 and 59 DAS, the AA content in RD increased markedly, reaching a 3-fold higher level than in RO ($p < 0.001$).

3.2. The evolution of the AA fingerprint

At 29 DAS, the presence/absence of a plant had no significant effect on the AA fingerprints in the rhizospheres compared to the related control ($p > 0.05$; Fig. 4), with the notable exception of γ -amino-butyric acid (GABA) which was very much less abundant in

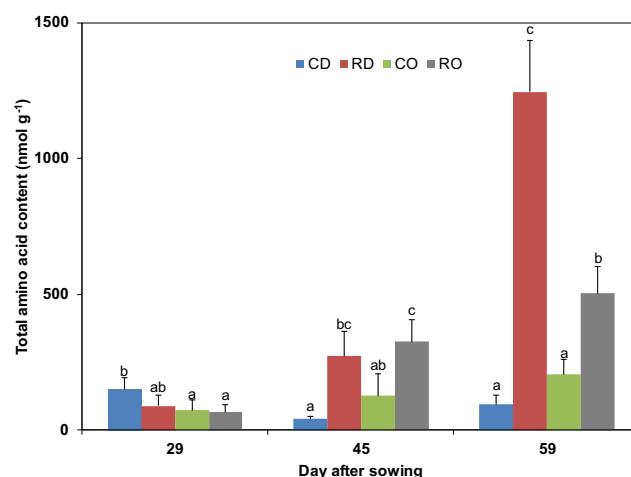


Fig. 3. Total amino acid content summed for 21 compounds measured in the rhizosphere of *Medicago truncatula* at 29 days after sowing (DAS), 45 DAS and 59 DAS in dried sterilized sand (RD) and in dried unsterilized soil (RO). Control unsterilized soil (CO) and control sterilized sand (CD) (without plants) are also represented for comparison. Mean values are given with error bars for \pm SD ($n = 4$). Different letters above the vertical bars indicate significant differences of the total amino acid content for each condition, based on ANOVA test at the 5% significance level.

RD than in CD ($p < 0.001$) and also significantly less abundant in RO than in CO ($p < 0.05$). In RD, there was significantly less proline than in CD ($p < 0.01$).

At 45 DAS, although the total AA content was similar in RO and RD, the AA fingerprints were different. Throughout plant growth in RD, there was a significant increase of all AAs, and notably of alanine, glycine, isoleucine, leucine, lysine, phenylalanine and valine ($p < 0.001$; Fig. 4, a and b). Asparagine and serine reached the highest concentration (60.8 ± 34.5 nmol g^{-1} and 57.2 ± 15.7 nmol g^{-1} dried sterilized sand, respectively), which represented more than 20% of the total AA content (Table 2). At this stage, asparagine (125.3 ± 11.74 nmol g^{-1} dried unsterilized soil; $p < 0.001$), glycine, isoleucine and valine (all $p < 0.05$) showed levels that were significantly different between RO and CO (Fig. 4, c and d). Similar result was obtained for glutamine ($p < 0.05$, data not shown). Asparagine represented 38.6% of the total AA content in RO (Table 2).

At 59 DAS, the contents of all AAs were higher in RD than that in CD ($p < 0.01$, Fig. 4, a and b). Serine showed the highest content among the entire AA fingerprint in RD (312.4 ± 47.1 nmol g^{-1} sterilized sand) and represented 21.1% of the total AA content (Table 2). We also found concentrations superior to 100 nmol g^{-1} for glycine, asparagine and glutamate. In RO, at the same stage (Fig. 4, c and d), concentrations of the following AAs were higher than those in CO: asparagine and lysine (data not shown) (both $p < 0.001$), aspartate, tyrosine, methionine (data not shown), phenylalanine and threonine (all $p < 0.05$). At 59 DAS, as at 49 DAS, asparagine was still the major AA in RO, representing 38.4% of the total AA content (Table 2). Additionally, its concentration in RO was higher than in RD ($p < 0.05$; Fig. 4).

Table 1
Amplification primers used for bacterial and fungal rRNA fragments.

Primer	Sequence	Target	Source of reference
ITS1	TCCGTAGGTGAACCTGCCG	rRNA fungi	(White et al., 1990)
ITS2	GCTRCGTTCTTCATCGATGC	rRNA fungi	(White et al., 1990)
NadF	TCCTACGGGAGGCAGCAGT	rRNA bacteria	(Nadkarni et al., 2002)
NadR	GGACTACCAGGTTATCTAATCCTGTT	rRNA bacteria	(Nadkarni et al., 2002)

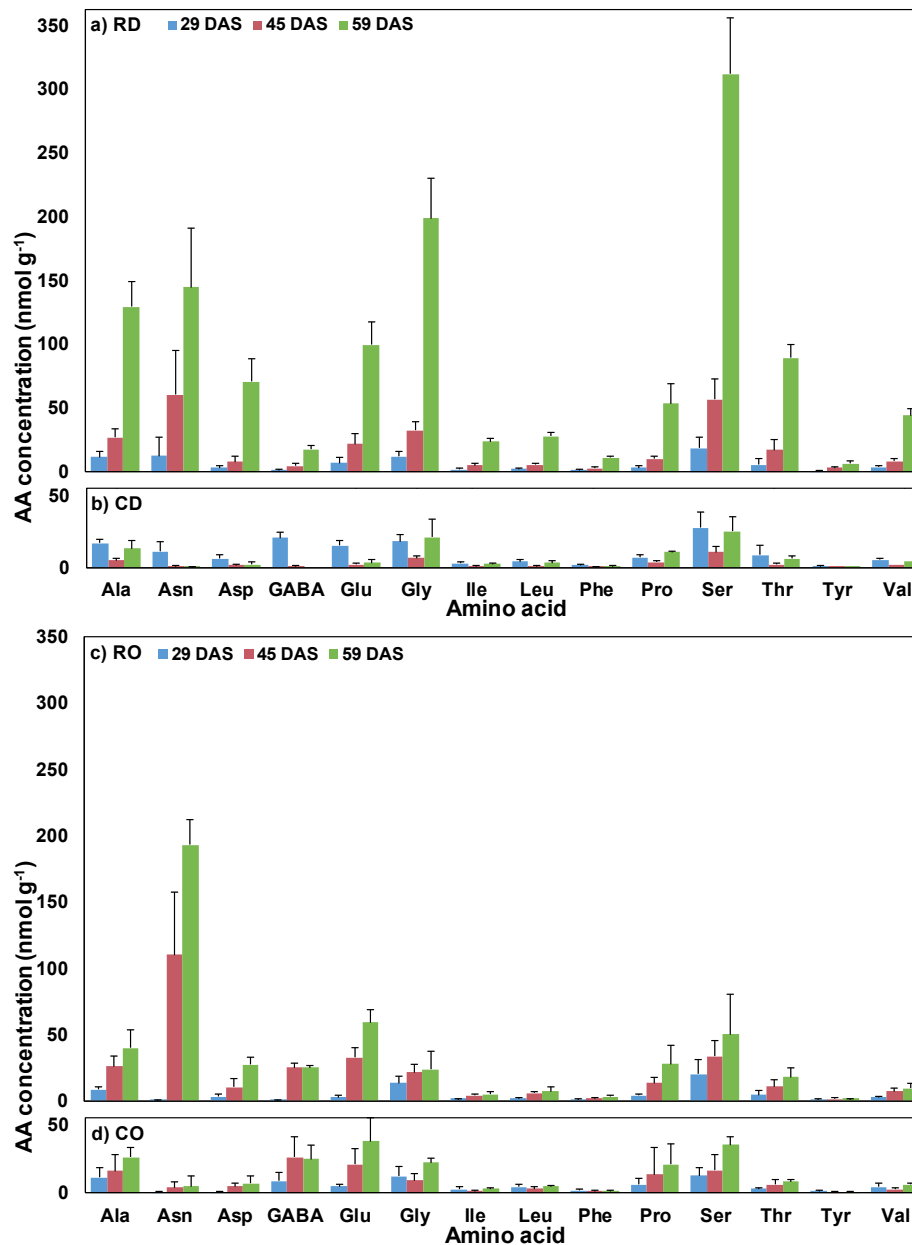


Fig. 4. Concentration of 21 amino acids in the rhizosphere of *Medicago truncatula* at 29 days after sowing (DAS), 45 DAS and 59 DAS in: dried sterilized sand (a, RD) and in dried unsterilized soil (c, RO). Control sterilized sand (b, CD) and control unsterilized soil (d, CO) (without plant) are also represented for comparison. Mean values are given with error bars for \pm SD ($n = 4$). Ala, Alanine; Asn, Asparagine; Asp, Aspartate; GABA, γ -aminobutyric acid; Glu, Glutamate; Gly, Glycine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Pro, Proline; Ser, Serine; Thr, Threonine; Tyr, Tyrosine; Val, Valine. For clarity, the amino acids present at only low concentration in the rhizosphere ($<1.50 \text{ nmol g}^{-1}$) – arginine, cysteine, histidine, glutamine, methionine, lysine and tryptophan – are not illustrated.

3.3. AA diversity in the rhizosphere

In order to define the major AAs causing variation in the fingerprints, Principal Components Analysis (PCA) was performed on RO and RD using as variables the concentrations of the 21 AAs present (Fig. 5). The first two axes accounted for 89.28% of total inertia, so 89.28% of the total variability of the dataset is represented on the first plane. Position of variables (vectors) in the plane highlights a positive correlation between the first principal component and all the variables which are well represented in the plane (Fig. 5, a). Some of them are also highly correlated: *i.e.* asparagine (0.67, 0.68), lysine (0.56, 0.80) and glutamine (0.61, 0.70) have closed coordinates in the plane (Table 3). The second axis is

highly related to the presence of histidine (correlation = 0.93). In parallel RO and RD showed a remarkable disposition: the second axis contrasts substrates collected at 29 and 45 DAS to those harvested at 59 DAS (Fig. 5, b). So, on the second axis AAs distribution depends on plant growth stage, and is mainly linked to the abundance of histidine.

At 45 and 59 DAS, the first axis contrasts RD and RO. In detail, this first component is particularly linked to alanine, glycine, valine, leucine, isoleucine, serine, threonine and phenylalanine (correlation coefficient on average 0.96, Table 3). In addition, in view of their closed coordinates in the plane (Table 3), alanine (0.98, -0.17) glycine (0.93, -0.34), valine (0.96, -0.27), leucine (0.96, -0.24), isoleucine (0.95, -0.29), serine (0.94, -0.32),

Table 2

Recovery of the principle 8 amino acids (AAs) as a percentage of total AAs in the rhizosphere of *Medicago truncatula*, at 29 days after sowing (DAS), 45 DAS and 59 DAS in sterilized sand and unsterilized soil. Control unsterilized soil and control sterilized sand are presented for comparison. Values are means ($n = 4$). Ala, Alanine; Asn, Asparagine; GABA, γ -aminobutyric acid; Glu, Glutamate; Gly, Glycine; Pro, Proline; Ser, Serine; Thr, Threonine. For clarity, amino acids which represent individually 6% or less of the total AA content – arginine, asparagine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine and valine – are not represented. Different letters (a, b, c) indicate significant differences within rows (ANOVA test at the 5% significance level).

		Ala	Asn	GABA	Glu	Gly	Pro	Ser	Thr
29 DAS	Control sand	11.2ac	7.4ab	13.7d	10.2bc	12.2ad	4.9ab	18.5bc	5.8a
	Rhizospheric sand	13.5bc	14.3bc	1.8ab	7.8ac	13.9bcd	3.9ab	21.4cd	6.3a
	Control soil	15.3c	0.5ab	11.8cd	6.2ac	16.7cd	8.0ab	17.1ac	4.1a
	Rhizospheric soil	12.5bc	0.7ab	1.0a	4.3a	20.3d	6.0ab	30.5d	6.6a
45 DAS	Control sand	12.9bc	2.1ab	2.0ab	5.3ab	17.1cd	9.4ab	26.9d	5.6a
	Rhizospheric sand	10.0ac	22.4c	1.8ab	8.2ac	12.0ad	3.9a	21.1cd	6.3a
	Control soil at 45	12.5ac	3.0ab	20.7e	16.6d	7.0ab	10.6ab	13.0ab	4.7a
	Rhizospheric soil	8.0a	38.6d	7.7bd	10.1bc	6.6ab	4.1a	10.3ab	3.4a
59 DAS	Control sand	14.3a	0.1a	0.0a	3.4a	22.4d	11.6b	26.7d	6.6a
	Rhizospheric sand	10.4bc	11.6ac	1.4a	8.0ac	16.0cd	4.3a	25.1cd	7.2a
	Control soil	12.6ab	2.4ab	11.9d	18.4d	10.7ac	10.2ab	17.1ac	4.1a
	Rhizospheric soil	7.9a	38.4d	5.0abc	11.7c	4.7a	5.5ab	10.0a	3.5a

Scale:



threonine (0.95, -0.27) and phenylalanine (0.96, -0.25) are highly correlated. Such results are consistent with the significant differences in the abundances of these AAs measured in RO and RD at 45 and 59 DAS.

3.4. Microbial biomass in soil

At each growth stage, fungal and bacterial biomasses were clearly higher in unsterilized soil than in sterilized sand (Fig. 6). This difference attests to the effectiveness of sterilization for diminishing microbial biomass and limiting recolonization. Bacterial biomass in unsterilized soil did not differ between RO and CO for a given stage, but increased between 25 and 59 DAS ($p < 0.01$; Figs. 6, 2a). A similar observation was made with fungal biomass, which increased in RO ($p < 0.01$; Figs. 6, 2b) and CO ($p < 0.01$) between 25 and 59 DAS with no difference between RO and control unsterilized soil at each growth stage ($p > 0.05$).

4. Discussion

4.1. Advances in the methodology for the recovery of rhizospheric amino acids and determination of the amino-acid fingerprint

In order to avoid the continual removal of material by soil microorganisms, most studies on root exudates have been performed in axenic conditions (Kuijken et al., 2015) and/or in an inert substrate (Lesuffleur et al., 2007). However, this approach disregards the fact that soil is a complex assemblage of mineral and organic particles, hosting a large range of biological organisms that can affect both the architecture of roots system and the rhizodeposition (Neumann et al., 2009; Schreiter et al., 2015). Hence, the qualitative and quantitative composition of root exudates is expected to vary, affected by various environmental factors including soil type and microbial activity (Meharg and Killham, 1991; Mimmo et al., 2011). As a consequence, this approach is very likely to lead to an underestimation of total exudation. As no suitable method existed to follow net AA exudation in soil, we proposed to study the AA

fingerprint in the rhizosphere of *M. truncatula* growing in unsterilized conditions in order to define the net result of interactions between AA exudation and the rhizospheric environment. To estimate the extend of plant exudation, we compared plants grown in unsterilized soil with plants grown in a sterilized substrate (limited microbial effects). However, the techniques available for soil sterilization (autoclaving, gamma-irradiation) mostly have unwanted effects on soil structure and the dynamics of major nutrients such as nitrogenous compounds (Tanaka et al., 2003; Buchan et al., 2012). Sterilization can cause an accumulation of ammonium ions, probably derived from the decomposition of organic matter (dead microorganisms) (Tanaka et al., 2003). Also, cation and anion exchange capacities of the soil are modified as a consequence of sterilization, and this affects the extractability of ammonia and organic nitrogen (McNamara et al., 2003). The soil selected for our experiment was a sandy soil (81.8% sand), therefore autoclaved sand was chosen as sterilized substrate. Autoclaved sand is a suitable sterilized substrate for comparison with unsterilized soil, as it allows both the study of the microbial effect on the AA exuded by roots and limits the sterilization-related bias.

Soil sampling strategy and methodology have a significant impact on the recovery of AAs from the rhizosphere. One dominant approach to sampling rhizospheric soil in both field and pot experiments, is to take soil aggregates adhering to the roots (Hinsinger et al., 2005). Considering that there is a concentration gradient of exudated molecules in the rhizosphere, and that not all exudate-containing soil will adhere to the roots, this method underestimates the rhizosphere effect (Hinsinger et al., 2005). We have developed an alternative approach, which is to grow *M. truncatula* in a small volume of soil (100 mL), which allows us to consider the total amount of the soil present as rhizospheric since it is completely colonized by the root system. Pots with the same amount of soil and the same fertilization but without plants were used as control.

To minimize overestimation of N rhizodeposition due to small amounts of root debris remaining in the soil (Jones et al., 2009), visible roots were removed quickly without any further treatment

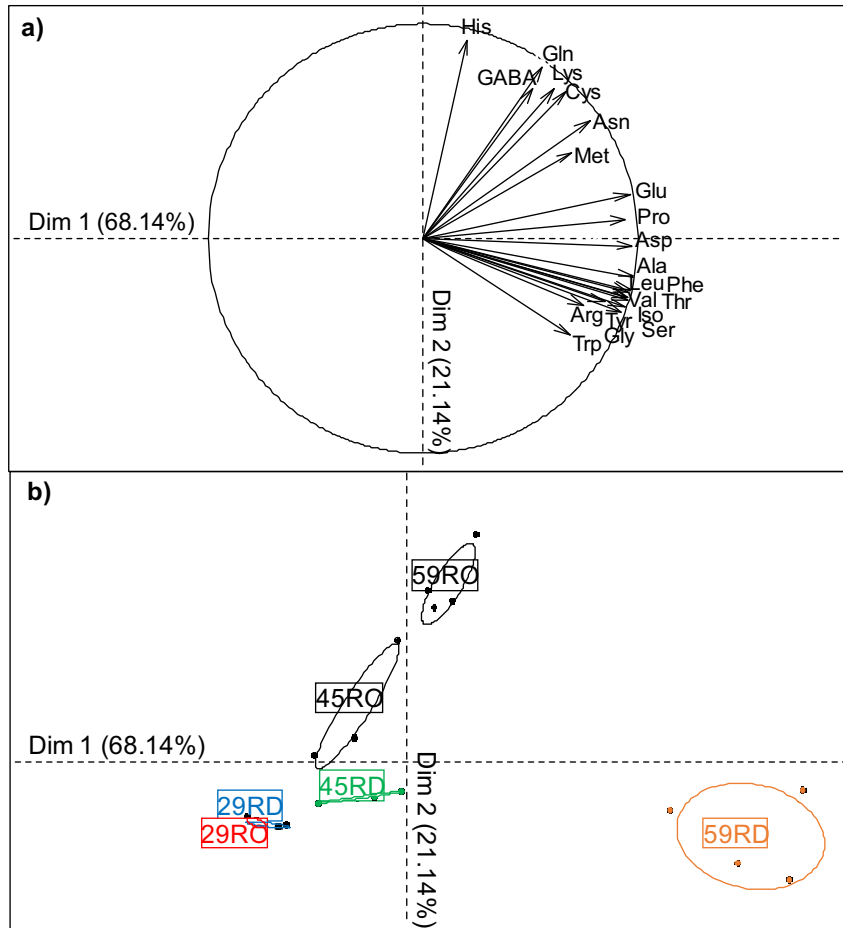


Fig. 5. Principal component analysis in the 1–2 plan (68.14%–21.14%) of the diversity of 21 amino acids in the rhizosphere of *Medicago truncatula* at 29 days after sowing (DAS), 45 DAS and 59 DAS in: dried sterilized sand (RD) and in dried unsterilized soil (RO). a) Variables graph (correlation circle). b) Individual graph. Contained confidence ellipses are given at 95%. Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartate; Cys, Cysteine; GABA, γ -aminobutyric acid; Gln, Glutamine; Glu, Glutamate; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Phe, Phenylalanine; Pro, Proline; Ser, Serine; Thr, Threonine; Trp, Tryptophan; Tyr, Tyrosine; Val, Valine.

Table 3

Coordinates, cosine-square, correlation coefficient and *P* value, allocated to each amino acid for the first axes of the PCA performed on rhizospheric soil and rhizospheric sand at 29, 45 and 59 DAS. The first two axes represent 89.28% of the total inertia.

Amino acid	Coordinates		Correlation		<i>P</i> Value	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Alanine	0.98	−0.17	0.98	−	6.02×10^{-17}	−
Glycine	0.93	−0.34	0.97	−	9.36×10^{-16}	−
Valine	0.96	−0.27	0.96	−	3.38×10^{-14}	−
Leucine	0.96	−0.24	0.96	−	5.90×10^{-14}	−
Isoleucine	0.95	−0.29	0.96	−	1.53×10^{-13}	−
GABA	0.51	0.26	0.96	0.70	1.93×10^{-13}	1.49×10^{-04}
Methionine	0.78	0.55	0.95	0.55	5.06×10^{-13}	5.05×10^{-03}
Serine	0.94	−0.32	0.95	−	1.06×10^{-12}	−
Threonine	0.95	−0.27	0.94	−	6.81×10^{-12}	−
Phenylalanine	0.96	−0.25	0.94	−	9.52×10^{-12}	−
Aspartate	0.97	−0.04	0.93	−	9.04×10^{-11}	−
Cysteine	0.69	0.40	0.85	0.40	1.85×10^{-07}	4.96×10^{-02}
Glutamate	0.96	0.21	0.78	−	7.09×10^{-06}	−
Asparagine	0.67	0.68	0.75	0.68	2.89×10^{-05}	2.37×10^{-04}
Lysine	0.56	0.80	0.69	0.80	1.89×10^{-04}	2.72×10^{-06}
Glutamine	0.61	0.70	0.69	0.70	2.17×10^{-04}	1.58×10^{-04}
Arginine	0.75	−0.31	0.67	−	3.86×10^{-04}	−
Histidine	0.20	0.93	0.61	0.93	1.45×10^{-03}	7.93×10^{-11}
Tyrosine	0.85	−0.30	0.56	−	4.66×10^{-03}	−
Tryptophan	0.69	−0.45	0.51	−0.45	1.03×10^{-02}	2.83×10^{-02}
Proline	0.94	0.09	0.98	−	6.02×10^{-17}	−

so that the extraction of AAs could be done within less than 1 h after removal of the soil from the pot. Treatments such as sieving or air-drying cause an increase of dissolved organic nitrogen (Jones and Willett, 2006), presumably due to root tissue breakdown.

A variety of laboratory procedures have been used to extract and quantify AAs in soil, but no standard protocols have been validated. Compounds are recovered through the percolation of distilled water or nutrient solution (micro-lysimeter) or by aspiration of the soil solution (micro-suction cups) with no root alteration (Paynel et al., 2001; Mimmo et al., 2011; Oburger et al., 2013). However only a small proportion of AAs are present free in the soil: rather they are largely adsorbed on complex particles (Sposito, 2008). While, they are potentially recoverable in good yield with a sufficiently concentrated solution of eluate, too concentrated a solution is likely to be stressful and damaging to the roots. We have therefore opted for a strategy in which the roots are quickly removed before recovering the AAs present in the rhizosphere.

Recovering exudate without contamination can be problematic. As a general rule, the soil is immersed in distilled water or a concentrated salt solution, shaken for a short period of time (1–3 h), centrifuged, filtered and the filtrate analyzed (Grayston et al., 1997; Jones et al., 2002; Jones and Willett, 2006; Fischer et al., 2007; Sauheitt et al., 2010; Farrell et al., 2014). AA recovery from the soil is significantly greater when extraction uses a concentrated salt solution rather than distilled water (Jones and

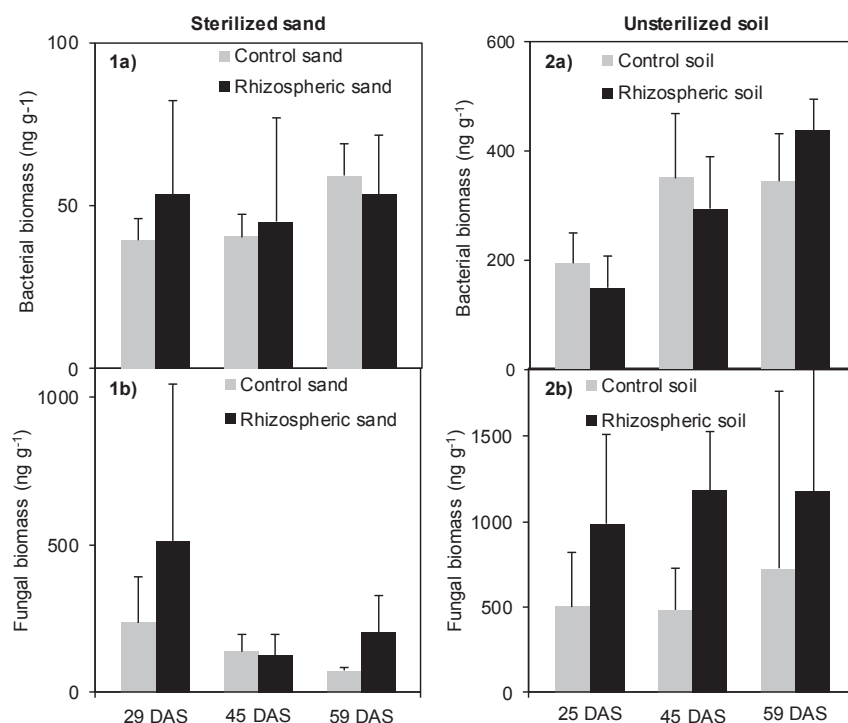


Fig. 6. Bacterial and fungal biomasses in the rhizosphere of *Medicago truncatula* at 29 days after sowing (DAS), 45 DAS and 59 DAS in: sterilized sand (1a and 1b, respectively) and in unsterilized soil (2a and 2b, respectively). Control unsterilized soil and control sterilized sand (without plants) are also represented for comparison. Mean values are given with error bars for \pm SD ($n = 4$).

Willett, 2006; Vranova et al., 2013). However, a concentrated salt solution (1 M or more) is commonly used to break soil microbial cells, in order to extract DNA (Schneegurt et al., 2003). Hence, too concentrated a salt solution can lyse microbial cells with a concomitant impact on AA recoveries. In the present work we have therefore used a dilute, slightly acidic solution: 2 mM formic acid. This methodology, aimed at suppressing the anionic function of the target molecules, allows their recovery and separation from neutral and anionic compounds by cation exchange. Using this methodology, we were able to assess free AAs usually found as dominant in water-soluble root exudates of plants, such as alanine, serine, arginine, glutamine, glutamate, aspartate, glycine, proline, cysteine, lysine and GABA (Hütsch et al., 2002; Phillips et al., 2004; Lesuffleur et al., 2007; Vranová et al., 2014).

4.2. Changes in the amino acid fingerprint in relation to growth of *Medicago truncatula*

It was found that the amount of AAs exudated increased dramatically during growth of *M. truncatula*, a finding consistent with that of Hamlen et al. (1972) for alfalfa and Chaparro et al. (2013) for *Arabidopsis thaliana*. Exudation is expected to increase with the emergence and growth of lateral roots (McDougall and Rovira, 1970; Jones, 1998; Darwent et al., 2003). Up to 40% of the total AAs recovered in the rhizosphere of *M. truncatula* grown on sterilized sand was composed of serine and glycine. The high abundance of serine and glycine is consistent with the results obtained in *Trifolium repens* grown in sterilized sand (Paynel et al., 2001; Lesuffleur et al., 2007). In *T. repens*, glycine constitutes less than 5% of the free root AAs but is the major AA in exudates. In contrast, asparagine was abundant in root tissues of *T. repens* but not in water-soluble exudates (<1%) (Paynel et al., 2001; Lesuffleur et al., 2007). While this might suggest that glycine exudation may

be selective, in our study of *M. truncatula* substantial amounts of asparagine were found in rhizospheric sand. Asparagine is known to be involved in the control and assimilation of N by the roots (Gaufichon et al., 2013) and to be prominent in N transport to the whole plant in xylem in non-N fixing as well as in N-fixing legumes (Do Amarante et al., 2006).

4.3. Relationship of the amino acid fingerprint to the substrate type and the growth stage

It has been shown that roots release substantial amounts of AAs and that the plant is the main source of low-molecular-weight soluble molecules in the soil (Nguyen, 2003; Jones et al., 2004; Fischer et al., 2007). However, the apparent rate of AA exudation in unsterilized conditions is affected by the soil microbiota (Moe, 2013). Consistently, in our study, concentrations of AAs in unsterilized rhizospheric soil were lower than in sterilized rhizospheric sand, strongly suggesting continual sequestration of AAs by the soil microbial community (Jones et al., 2005). Since microorganisms can be either stimulated or inhibited by components of root exudates, microbial biomass was estimated in rhizospheric unsterilized soil and compared with microbial biomass in control soil (without plant) (Hartmann et al., 2009). We expected that microbial biomass would be higher in pots with plants. However, irrespective of the presence or absence of the plant, bacterial biomass increased significantly over time and showed no difference between rhizospheric and bulk soil. This unexpected result could be explained by the availability of nitrogen due to N fertilization, which moderated the effect of exudates and reduced competition for N between the root and microorganisms (Hartmann et al., 2009). In addition, the quantification of total microbial biomass may not be pertinent (Chichester et al., 2013), as it quantifies both active and dead microorganisms in soil, whereas only active microorganisms are

involved in ongoing processes in the rhizosphere. However, in our opinion this should not rule out the influence of root exudates on microbial diversity, as the microbial communities of soil were shown to be highly dependent on *M. truncatula* root exudates (Zancarini et al., 2012). Many AA transport systems in microorganisms are believed to be similar to those of plants and most of these microorganisms are adapted to use AAs as source of both C and N (Jones and Hodge, 1999). Soil microbes preferentially degrade AAs with high C:N ratios, such as isoleucine, leucine or valine and can also be very competitive for lysine and glycine (Lipson et al., 1999; Owen and Jones, 2001). Furthermore, most of these microorganisms have the enzyme machinery for the deamination of AAs such as glutamate, and can use it to produce carbon skeletons for sugar or fatty acid synthesis (Apostel et al., 2013). This selective sequestration may explain why in unsterilized soil we found only small concentrations of acidic and high C:N ratio AAs, such as glutamate (C:N = 5), tyrosine (C:N = 9), GABA, leucine and isoleucine (C:N = 6), compared with sterilized rhizospheric sand. In addition, GABA is suspected to serve as a sole C and N source for colonizing *Pseudomonas putida* KT2440 (Ramos-González et al., 2005).

With time, however, a notable effect of plant presence/absence on the soil AA fingerprint was found, with significantly more AAs recovered from unsterilized rhizospheric soil than from controls without plant at 59 DAS: alanine, asparagine, aspartate, glutamine, histidine, lysine and methionine all increased in abundance. In a study performed on *A. thaliana*, a high correlation was demonstrated between AA pathways in root exudation and the activation of functional genes corresponding to AA metabolism specifically expressed at vegetative developmental time points (Chaparro et al., 2013). For example, glycine was positively correlated with sarcosine oxidase functional genes, involved in the glycine, serine, and threonine metabolism pathway. Also, 41 genes involved in AA metabolism were negatively correlated with asparagine accumulation.

By applying principal components analysis, the relative abundance of individual AAs could be correlated to the growth stage of *M. truncatula*, the type of substrate and to the presence of others AAs, according to their biosynthetic pathways. For instance, methionine is linked to the presence of cysteine in unsterilized rhizospheric soil at 59 DAS. This result is consistent with the role of cysteine as sulfur atom donor in the *de novo* synthesis of methionine (Ravanel et al., 1998). Also, serine is linked to glycine: both are derived from 3-phosphoglycerate generated during glycolysis. In sterilized rhizospheric sand, serine, isoleucine and threonine were highly correlated, probably because they all belong to the family of AAs synthesized from the same precursor, aspartate (Ireland, 1997; Azevedo et al., 2006). In plants, aspartate is also converted to asparagine, which was found at high levels in unsterilized rhizospheric soil after 45 DAS. This abundance may well relate to the key role of asparagine in the transport and storage of N in plants. Certainly, asparagine both dominated the AA pool in established plants and showed the largest increase. This role could potentially be competing with the synthesis of lysine, threonine, methionine and leucine (Ferreira et al., 2005). An experiment on microbial diversity in an arable soil treated with a single AA (asparagine, gluconic acid, glutamic acid or urocanic acid) showed that only the asparagine treatments increased the microbial biomass (Degens, 1998). Based on this observation, depending on the growth stage, it may be suggested that individual AAs may play a specific role in structuring microbial communities and/or microorganisms may enhance net AA exudation from root.

In this study, only a small amount of serine was recovered in rhizospheric soil whereas it has previously been reported as a major AA exudated by plants (Shepherd and Davies, 1994; Murray

et al., 1996; Paynel et al., 2001). However, Lipson et al. (1999) reported that microorganisms have a lower affinity for serine as a sole C-source than for other AAs, which would potentially result in its accumulation. Also, high proportions of serine have been observed in rhizospheric soil extracts (Kielland, 1995). Lesuffleur et al. (2007) found a clearly higher concentration of serine in root exudates than in the tissues and suggested a selective exudation. These authors reported that serine efflux was much higher in exudates of plants grown in sterilized axenic condition (hydroponic system) than in exudates of plants grown on sterilized sand. Hence, independently of the presence of microorganisms, these results suggested that the growth substrate could have an important impact on serine root efflux and accumulation in the rhizosphere.

5. Conclusions

Taken overall, the results herein presented suggest that the differences in the AA fingerprint between unsterilized soil and sterilized sand is not only due to uptake and sequestration by microorganism in the soil, but also because plants growing in sterilized substrate completely modify the metabolism and exudation of AA in the root compared to plants growing in unsterilized soil. This has considerable consequences for experimental design. Critically, it indicates that growing plants in non-sterile growth systems is inappropriate for detecting substantial amounts of exudates. But, reducing and standardizing the environment may also lead to quantitative and qualitative alterations in the profile of AAs exudated. Sampling procedure is critical, as root exudation highly depends on the rhizospheric environment. The methodology herein proposed offers an easy means to sample and analyze amino acid fingerprints in unsterilized rhizospheric soil and to relate these to plant growth. Despite the apparent continual removal of exudated compounds by microorganisms, we were able to show that *M. truncatula* growth is associated with an overall increased AA exudation rate during vegetative growth in unsterilized soil. This study highlights a clear impact of the substrate type and the growth stage on the AA fingerprint in the rhizosphere and indicates that AA metabolic pathways and exudation can differ in unsterilized soil compared to those in a sterilized substrate, apparently an adaption to the rhizospheric environment. The recovery of AAs from unsterilized soil is valuable in that it provides the most representative exudation pattern with respect to C and N input and output. However, the fingerprint observed will depend on the microbial communities present and a study aimed at establishing a better understanding of the relationship between rhizospheric microorganisms and the AA exudation fingerprint is required.

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