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Original article

Dynamics of cultivable arylsulfatase-producing bacterial and fungal communities along the phenology of field-grown rape

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ABSTRACT

The dynamics of bacterial and fungal communities possessing arylsulfatase (ARS) activity were followed in the rhizosphere of field-grown rape from stem elongation to seed maturity. Soil ARS activity as well as density, structure and diversity of both cultivable ARS bacterial (ARS-BC) and fungal (ARS-FC) communities were determined. ARS activities evolved along the phenology of rape and were correlated at reproductive stages with the densities of ARS-BC and ARS-FC communities. However, at vegetative stage, ARS activities were only significantly correlated with the sizes of ARS-FC but not with those of ARS-BC. Significant temporal shifts in the structure of ARS-BC and ARS-FC were observed. Furthermore, the more pronounced shifts observed in ARS-BC than in ARS-FC, suggests that ARS bacterial communities had a higher dynamic throughout plant development. Concerning the specific taxonomic groups, *Actinobacteria*, *Trichoderma* sp. and *Eupenicillium* sp. were dominant whatever the developmental stage of rape whereas *Proteobacteria* such as *Pseudomonas* sp., *Klebsiella* sp. and *Raoultella* sp. were presented at the later reproductive stages. This study suggests that arylsulfatase expressing bacterial and fungal communities were affected differently during rape development.

1. Introduction

Sulfur (S) is an essential nutrient for plants [18,27,28], mainly available as sulfates in the soil and S dioxide in the atmosphere. Over the last three decades, drastic restrictions on emissions of S dioxide pollutants have resulted in S deficiency in some agricultural soils with concomitant reductions of agronomic performances for many crops such as rape [56]. Plants are therefore more dependent on soil sulfates supply for their growth and to maintain both yields and harvest quality. In most aerobic agricultural soils, S is tied up in different organic forms, including C-bonded S and sulfate esters (C–O–S) [21,38,45,57]. This fraction is considered as the most labile form of organic S and could be important for plant S nutrition [20,30]. Mobilization of sulfates from such polymers is assumed to be performed mainly by microbial sulfatases (sulfohydrolase, E.C. 3.1.5.6) [19]. Amongst this highly homologous family of enzymes, arylsulfatase (ARS) is considered as a key functional marker of S mineralization [26]. As suggested by Kertesz and Mirleau [25], a specialized soil microflora could be implicated in sulfate esters mineralisation, but it is not clear yet which microorganisms are responsible of these transformations.

Until the study of Crégut et al. [13], very little was known about soil microorganisms which were able to mineralize sulfate esters *via* ARS activity. These authors found that the density of ARS bacterial community (ARS-BC), mainly composed of *Actinobacteria* and *Pseudomonads*, contributed to explain shifts in soil potential ARS activity under field-grown rape and barley. However, the correlation coefficients obtained were relatively low, suggesting that ARS-BC represents only one part of the total ARS microbial community. There is now increasing evidence that bacteria but also fungi together dominate organic matter decomposition and nutrient cycling in terrestrial ecosystems [16]. Due to fundamental differences in bacterial and fungal physiology and ecology, edaphic factors such as pH [37] nutrients [2] and C availability [41,50] may influenced the bacterial and fungal communities, with consequences on the dynamic of nutrients as S.

Then, the overall objective of this study was to gain a better understanding of the relative contribution of ARS bacterial (ARS-BC) and fungal soil (ARS-FC) communities in the mineralization of organic S under field-grown rape, a plant with high S requirements. We studied four rape developmental stages, from stem elongation to seed maturity, since changes of S demand during these stages was believed to modify soil ARS activity and to influence ARS

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microorganisms. We used a cultivation-based approach to estimate the density and to characterize the genetic structure of the ARS-BC and ARS-FC in parallel with an estimation of soil ARS activity. Even if cultivation methods can be seen as a limitation in microbial diversity studies [8,9], the number of sequences for the ARS gene available in GenBank is relatively small, limiting the use of direct molecular approaches to target the ARS microbial communities, as previously done for other microbial communities [43]. Therefore, we assumed that cultivation methods are still useful in providing for a better understanding of the diversity of microorganisms important for soil S biogeochemistry.

2. Materials and methods

2.1. Experimental field design and sampling

Experimental field was located in the "Institut National de Recherche Agronomique (INRA)" in Dijon (France). The main soil (Cambic Fluvisol) chemical characteristics were: 13.5 g kg^{-1} organic C, 1.14 g kg^{-1} organic N, 270 mg kg^{-1} organic S and pH water: 6.7. This soil contained 329, 608 and 63 g kg^{-1} soil clay, silt and sand, respectively. A winter cultivar Campala of *Brassica napus* L. was growing in a conventional crop system. In the field experimental design, four sub-plots of 24 m^2 (3 × 8 m) were considered. Rape was sown on August 19 (2005) and five randomly rape plants with root adhering soil were taken from each sub-plot at stem elongation, flowering, seed development and seed maturity, which corresponds respectively to 195, 217, 242 and 293 Days After Sowing (DAS). Rape received 130 and 70 kg ha^{-1} of N and S mineral fertilizers, respectively. N fertilizer was applied 206, 222 and 229 DAS and S fertilizer was applied 222 DAS. No organic amendment was realised before the sowing of rape. At the laboratory, the roots were shaken vigorously to separate the soil not tightly adhering to roots. The root adhering soil was then immediately and manually separated. Rhizosphere soil samples were sieved to pass through a 5-mm sieve. Samples were then stored at 4 °C before processing (within the week after the sampling).

2.2. Preparation of soil suspensions, enumeration and isolation of bacteria and fungi possessing ARS activity

Rhizosphere soils (3 g) were suspended in 30 ml of sterile Phosphate Buffered Saline (PBS), pH 7.2 on an orbital shaker (120-rev min^{-1}) for 30 min. Cultivable bacteria having arylsulfatase (ARS) activity were quantified by plating 10^{-3} soil suspension dilutions on the modified M9 mineral medium containing the ARS chromogenic substrate, 5-bromo-4-chloro-3-indolyl sulfate (X-Sulf, Sigma, France), as sole sulfur source [13]. Cultivable fungi having ARS activity were quantified similarly by plating 10^{-2} soil suspension dilutions on a modified Czapec mineral based medium (glucose 2 g l^{-1}, NH_4NO_3 2 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgCl_2$, $6H_2O$ 0.4 g l^{-1}, $FeCl_2$, $4H_2O$ 7 mg l^{-1}, KCl 0.5 g l^{-1}, $ZnCl_2$ 0.5 mg l^{-1}, $MnCl_2$ 0.1 mg l^{-1}, $CuCl_2$ 0.025 mg l^{-1}, $AlCl_3$ 0.03 mg l^{-1}, $NiCl_2$ 0.03 mg l^{-1}, KI 0.01 mg l^{-1}, boric acid 1 mg l^{-1}, neomycin 0.1 g l^{-1}, 0.1 g l^{-1} of X-Sulf and 20 g l^{-1} of agar). Three replicates per dilution were prepared and incubated at 28 °C. The number of microbial colonies possessing ARS activity, detected by their blue color following the hydrolysis of X-sulf, was expressed as log CFU g^{-1} of dry soil. Colonies were purified by repeated sub-culturing and single colonies were picked for subsequent analysis.

2.3. Bacterial and fungal rRNA gene amplification

PCR amplification targeting 16S rRNA was performed using 27f and 1492r primers [23] while the EF4 and Fung5 primers were used for fungal partial 18S rRNA gene amplification [44]. Prior amplification, fungal DNA from a single colony was extracted as described by [35] and purified using the GENECLEAN *Turbo* Nucleic Acid Purification kit (MP Biomedicals, France). 2.5 μl 10x *Taq* Polymerase buffer (MP Biomedicals, France), 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM of each bacterial or fungal primer, 0.625 U of *Taq* Polymerase (MP Biomedicals, France), one single bacterial colony or 25 ng of fungal DNA were combined in a final volume of 25 μl. DNA amplification was carried out in an i-Cycler (BioRad) in the following conditions: 4 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, plus an additional 15 min cycle at 72 °C. PCR products were separated on a 1% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light.

2.4. RFLP analysis and sequencing

After PCR amplification, 10 μl of each amplicon were digested for 18 h at 37 °C with 2.5 U of *Alu*I (MP Biomedicals, France) for 16S rRNA genes and with 2.5 U of *Rsa*I and *Hinf*I (MP Biomedicals, France) for partial 18S rRNA genes. Restriction fragments were separated in 6% acrylamide gel electrophoresis in 1x TBE run for 6 h at 20 mA. Gels were stained with SYBR green II (1/10000, Roche Molecular). Restriction fragment lengths were estimated and compared. Each different restriction pattern was defined as an operational taxonomical unit (OTU). Two of each different representative OTUs were commercially sequenced (GATC Biotech, Konstanz, Deutschland). DNA sequences were edited and screened against those in the GenBank database using BLASTn (http://www.ncbi.nlm.nih.gov/). The sequences have been deposited in GenBank under accession numbers JN387713–JN387718, corresponding to OTUs B1–B6, JN387719–JN387722 corresponding to OTUs B13–B16, JN3877223 corresponding to OTU B23, JN387724–JN387725 corresponding to OTU B28–B29 and JN387726–JN387736 corresponding to OTU F1–F11.

2.5. Soil arylsulfatase activity

Arylsulfatase (ARS) potential activity was determined according to Tabatabai and Bremner [46]. ARS activity was expressed as μg *p*-nitrophenol g^{-1} of dry soil h^{-1}.

2.6. Statistical analysis

The log-transformed CFU data and the ARS activities values were analysed by analysis of variance (one-way ANOVA, StatBox ver.6, software, Grimmersoft, Paris, France, http://www.grimmersoft.com). One matrix per cultivable bacterial and fungal communities (along rape phenology), where columns take into account the relative frequency of the different representative OTUs, was constructed respectively. Matrices were analyzed using Principal Components Analysis (PCA, StatBox ver.6, software, Grimmersoft, Paris, France) in order to estimate the temporal evolution of the structure of microbial communities having ARS activity.

3. Results

3.1. Quantification of ARS-BC, ARS-FC and soil potential ARS along rape phenology

The average density of cultured arylsulfatase (ARS) bacterial communities (ARS-BC) was significantly higher than that of ARS fungal communities (ARS-FC) (6.7 log CFU g^{-1} dry soil and 4.5 log CFU g^{-1} dry soil, respectively) (Table 1). The densities of both ARS-BC and ARS-FC changed significantly ($P < 0.05$) along phenology of rape. The lowest density of ARS-BC was detected at 195 DAS (stem