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Article Title: Assessing the effect of organic residue quality on active decomposing fungi in a tropical Vertisol using 15N-DNA stable isotope probing
Year of publication: 2011
Link to published article: http://dx.doi.org/10.1016/j.funeco.2010.09.005

Publisher statement: España, M. et al. (2011). Assessing the effect of organic residue quality on active decomposing fungi in a tropical Vertisol using 15N-DNA stable isotope probing. Fungal Ecology, Vol. 4(1), pp. 115-119

Short communication

Assessing the effect of organic residue quality on active decomposing fungi in a tropical Vertisol using ^{15}N-DNA stable isotope probing

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Running title: Residue quality controls active decomposing soil fungi.

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Abstract (150 words)

^{15}N-DNA stable isotope probing (^{15}N-DNA-SIP) combined with 18S rRNA gene-based community analysis was used to identify active fungi involved in decomposition of ^{15}N-labeled maize and soybean litter in a tropical Vertisol. Phylogenetic analysis of ^{15}N-labeled DNA subjected to 18S rRNA gene-based community fingerprinting showed that organic residue quality promoted either slow (i.e. *Penicillium* sp., *Aspergillus* sp.) or fast growing (i.e. *Fusarium* sp., *Mortierella* sp.) fungal decomposers in soils treated with maize or soybean residues, respectively, whereas *Chaetomium* sp. were found as dominant decomposers in both residue treatments. Therefore, we have clear evidence that specific members of the fungal community used ^{15}N derived from the two different organic resources for growth and stimulated early decomposition of maize or soybean decomposition. In conclusion, our study showed that ^{15}N-DNA-SIP-based community analyses cannot only follow the flow of N from organic resources into bacteria, but also into the actively decomposing fungal communities of soils.

Keywords

^{15}N-DNA stable isotope probing, active decomposing fungi, tropical soil, plant residue quality.

Entire text (1486 words)

Soil fungi represent a major proportion of soil microbial biomass and have been acknowledged to be predominant decomposers of organic matter in tropical soils (Rillig *et al*. 2001; Yang & Insam 1991; Lodge 1985). However, the relationship between fungal community dynamics and fungal decomposition processes in tropical soils remains is relatively unexplored and thus needs further investigation to improve our current understanding of the specific contributions of fungi to organic residue decomposition and nutrient cycling in soils (Gomes *et al*. 2003). Although there has been progress in understanding the nature of bacterial communities contributing to crop residue decomposition (Bernard *et al.* 2007), knowledge of fungal communities is limited, and generally restricted to cultivation-dependent studies (e.g. Robinson *et al.* 1994).

The development of nucleic acid-based, cultivation-independent approaches has opened new avenues for the sensitive detection of microbial communities in terrestrial ecosystems (Theron & Cloete, 2000), but these have rarely been used to study fungal communities in tropical soil ecosystems (Gomes *et al*. 2003). Since the advent of combining molecular techniques with stable isotopes (e.g. ^{13}C, and ^{15}N), DNA-based stable isotope probing (DNA-SIP, Radajewski *et al*. 2000) has became a powerful tool to achieve better understanding of microbial processes through identifying relevant organisms that determine nutrient cycling and metabolize plant-derived carbonaceous compounds (Rasche *et al*, 2009; Bernard *et al*, 2007; el Zahar Haichar *et al*. 2007). While ^{13}C-based DNA-SIP has focused on the C cycle, ^{15}N-SIP is particularly attractive to trace microbial processes involved in N cycling (Buckley *et al*. 2008; Buckley *et al*. 2007; Cupples *et al*. 2007). However, ^{15}N-DNA-SIP has to our knowledge not been used to investigate the role of soil fungi involved in the decomposition of complex plant residues.

In a previous study, we have proven that ^{15}N-DNA-SIP combined with 16S rRNA gene-based community fingerprinting was appropriate to detect active bacteria involved in the decomposition of complex, ^{15}N-labeled plant residues of different quality (i.e. maize and soybean) in a tropical Vertisol (España *et al*. in revision). Based on this preliminary study, we hypothesized that ^{15}N-DNA-SIP along with 18S rRNA gene-based community fingerprinting was suited to assess the effect of organic residue quality on active members of the decomposing fungal community in the previously assayed tropical soil.

^{15}N-enriched leaf residues (90 atom-%), i.e. maize (*Zea mays* L.) (total nitrogen (N_t) content 1.21%; C-to-N_t ratio: 32; cellulose content: 24.9%) and soybean (*Glycine max* L. Merr.) (2.71%; 15; 15.5%), and an unlabeled control of both residues were incubated (1%) under controlled conditions (40% water holding capacity, 25°C) in topsoil (0-20 cm) of a Vertisol taken from a long-term field experiment in Venezuela (Rodriguez *et al.*, 2004). After 15 days incubation, DNA was extracted from 0.3 g fresh soil (FastDNA Spin Kit for soil, MP Biomedicals, Solon, USA), and quantified (BioPhotometer 6131, Eppendorf, Hamburg, Germany). Isopycnic fractionation (SIP) of labeled and unlabeled DNA was performed according to Cadisch *et al*. (2005) and Hutchens *et al*. (2004). DNA density gradients were purified (Sambrook *et al*., 1989), and DNA from density-resolved SIP fractions was quantified as described above. ^{15}N-enrichment of DNA fractions was determined according to España et al. (in revision). Fungal 18S rRNA genes were amplified by a semi-nested PCR protocol using a ready-to-use PCR mix (Biomix, Bioline, Luckenwalde, Germany). The first PCR (PCR 1) was performed in 25 µl reactions containing 12.5 µl ready-to-use PCR mix, 30 ng DNA of each fraction, oligonucleotides (0.2 mM each) NS1 and EF3 (Oros-Sichler *et al*. 2006; Fisher Scientific, Schwerte, Germany) and ultra-pure PCR water (Roth, Karlsruhe, Germany). PCR 1 was performed with 94°C for 5 min; 25 cycles at 94°C for 30 s, 47°C for 45 s and 72°C for 3 min; and a final extension at 72°C for 10 min. Two µl of PCR 1 were

used as template for the second amplification (PCR 2) with oligonucleotides NS1 (Oros-Sichler *et al*. 2006) and NS2-GC (Marschner *et al*. 2002) (0.2 mM each, Fisher Scientific). PCR 2 was run with 94°C for 5 min, 80°C for 10 min, 35 cycles at 94°C for 30 s, 47°C for 45 s and 72°C for 3 min, and a final extension at 72°C for 10 min. Amplicons were checked in 2% Sybr® Green stained agarose gels (Sigma-Aldrich, Munich, Germany) and subjected to denaturing gradient gel electrophoresis (DGGE) analysis according to España et al. (in revision). Three independent DGGE analyses per fraction were performed for each sample to verify the method reproducibility. Four bands revealing distinct changes of their relative intensity along the gradient (from 'light' unlabeled to 'heavy' ^{15}N-labeled DNA fractions) were selected for cloning and sequencing analysis (Fig. 1; bands: maize (M): M1 to M4; soybean (S): S1 to S4). DGGE gel bands were purified and used for generating the 18S rRNA gene libraries according to España et al. (in revision). From each library, four positive clones were partially sequenced (GATC Biotech, Konstanz, Germany) with reverse M13 primer and sequence information (approximately 550 bases) was subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database. Sequences were deposited in Genbank under accession numbers HM475173 to HM475268.

Our study showed that ^{15}N-DNA-SIP was suited to asses active decomposing fungi in both ^{15}N-labeled plant residues evaluated and proved that ^{15}N-DNA-SIP was useful for fungal decomposition studies using organic materials with contrasting biochemical composition. A prerequisite to the successful application of ^{15}N-DNA-SIP is the use of highly ^{15}N-enriched (at least 90 atom%) residues allowing a sufficient labeling of soil DNA to obtain reliable, ^{15}N-enriched SIP fractions (Fig. 1) (Cadisch *et al.* 2005) and to identify the active decomposing community, either fungi (this study) or bacteria, as was shown by España *et al*. (in revision). However, the use of unlabeled residues as control is mandatory to eliminate the effect of different G+C contents (Buckley *et al.* 2007; Cupples *et al.* 2007; Neufeld *et al.* 2007).

Residue incorporation induced remarkable differences in the community structure by inducing new bands in the 18S rRNA gene DGGE gels (patterns labeled with "M" for maize, and "S" for soybean), which were not present in the control (C) treatment (without residue) (Fig. 1). In both residue treatments, unlabeled control treatments revealed only small community changes compared to those of the ^{15}N-labeled treatments. Several bands increased (e.g. M1, M2, M4, S1, S2, and S4) in relative intensity from 'light' (fraction #5, 2.4 atom-% (soybean), and 0.06 atom-% (maize), Fig. 1) to 'heavy' (fraction #1, 87.1 atom-% (soybean), and 57.8 atom-% (maize), Fig. 1) DNA fractions, or remained constant (M3, and S3).

Phylogenetic assignment of cloned bands revealed that the majority of sequences obtained from both maize and soybean treatments were affiliated with *Chaetomium* sp. (bands M3, M4, S3, and S4), while sequences obtained from bands S1 and S2 were related to *Fusarium* sp. and *Mortierella* sp., respectively. These active species belong to *Ascomycota*, known as key players in organic residue decomposition (Thorn & Lynch 2007; Kjøller & Struwe 2002; Montgomery *et al*. 2000). *Chaetomium* sp. and *Fusarium* sp. are known to degrade a range of contrasting and complex organic materials (e.g. maize and soybean residues) (Shaheen *et al*. 2008; Katapodis *et al*. 2007), while *Mortierella* sp. has been shown to utilize mainly easy degradable C compounds (Thorn & Lynch 2007). Interestingly, *Penicillium* sp. and *Aspergillus* sp., slow growing fungi, and important in the initial cellulose degradation (Horwath 2007), were identified only in the maize treatment (bands M1 and M2) evincing almost twice as much cellulose than soybean residue. Contrastingly, *Fusarium* sp. and *Mortierella* sp., which were only found in the soybean treatment, are considered to be fast growing fungi (Thorn & Lynch 2007; Kjøller & Struwe 2002; Montgomery *et al*. 2000). We assumed that the fast fungal response to soybean residue addition was due to the development of opportunistic fungi feeding on relatively easily degradable plant residue constituents such as glucose and proteins rather than cellulous components. Although it has been shown that

readily decomposable compounds are mainly utilized by bacteria, fast growing opportunistic fungi have recently been found to also be stimulated by easy accessible C sources and high N availability (Poll *et al.* 2010; Rousk & Bååth 2007; van der Wal *et al*. 2006).

In conclusion, ^{15}N-DNA-SIP along with 18S rRNA gene-based community profiling was a powerful approach to follow the flow of N from contrasting organic resources into the fungal communities actively decomposing complex maize and soybean residues in a tropical Vertisol. Indications were provided that certain fungi were essentially involved in early stages of organic matter decomposition. However, we investigated only some prominent DGGE bands in more detail and therefore recommend for future research to extend community profiling approaches with studying total fungal communities to assess their richness and abundance as well as relevant functional genes to better understand the essential contribution of soil fungi in specific decomposition processes and nutrient cycling.

Acknowledgements

Mingrelia España thanks the National Foundation for Science, Technology, Innovation and the National Institute of Agricultural Research in Venezuela for financial support.

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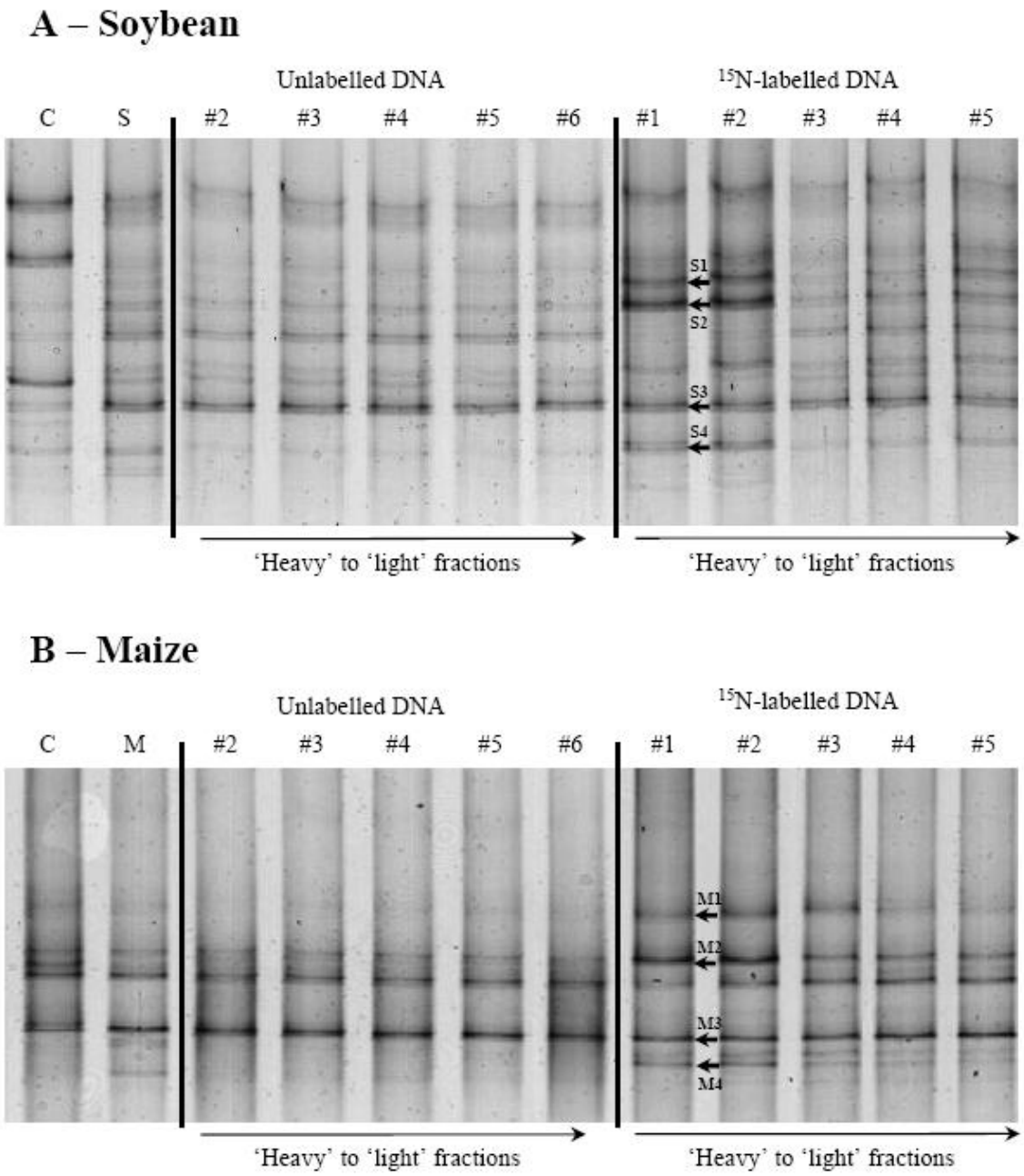
Figure caption

Figure 1

Denaturing gradient gel electrophoresis (DGGE) patterns of 18S rRNA genes obtained from DNA fractions along the caesium chloride (CsCl) density gradient (fraction #1 ('heavy') to fraction #6 ('light') from unlabeled control and ^{15}N-labeled soybean (A) and maize (B) residue treatments. ^{15}N-enrichment in labeled fractions: soybean (fraction #1 (87.1 atom-%), #2 (44.6 atom-%), #3 (20.4 atom-%), #4 (3.02 atom-%), and #5 (2.4 atom-%)), and maize (fraction #1 (57.8 atom-%), #2 (22.0 atom-%), #3 (19.2 atom-%), #4 (0.24 atom-%), and #5 (0.06 atom-%)). DGGE pattern coding: C = DGGE pattern of control without any residue treatment; M/S = DGGE pattern of non-CsCl-fractionated soil DNA from soybean (S) and maize (M) residue treatment. Arrows indicate the DGGE-bands which have been selected for cloning and sequencing analysis. Affiliation of sequenced clones (12 clones per band): *Fusarium* sp. (Ascomycetes, band S1; 9 clones with associated Genbank accession number (closest NCBI match): EJ613599, homology: 98-100%; 2 clones: AB110910, 99-100%; 1 clone: GQ166777, 98%), *Mortierella* sp. (Zygomycetes; band S2; 4 clones: AY129549, 99%; 3 clones: EU736291, 98%; 2 clones: AY550125, 100%; 2 clones: AF113425, 99-100%; 1 clone: AY546098, 99%), *Penicillium* sp. (Ascomycetes; band M1; 4 clones: AF245241, 98-99%; 4 clones: AF245268; 99-100%; 2 clones: AF245267, 98-99%; 1 clone: GU190185, 99%; 1 clone: AF245245, 99%), *Aspergillus* sp. (Ascomycetes; band M2; 5 clones: EF033516, 98-99%; 2 clones: AP007173, 98-99%; 2 clones: FJ393420, 99-100%; 2 clones: EU884135, 98-100%; 1 clone: AB048285, 100%), and *Chaetomium* sp. (Ascomycetes; bands S3, S4, M3, M4; all clones: FJ393436, 98-100%). Note: from fractions #1 of the unlabeled control treatments and fractions #6 of the ^{15}N-labeled treatments, no 18S rRNA gene amplicon could be achieved.



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266 **Figure 1**