Received Date : 03-Jun-2012
Revised Date : 01-Aug-2012
Accepted Date : 23-Sep-2012
Article type : Research Paper
Editor : Angela Sessitsch

Acidobacterial community responses to agricultural management of soybean in Amazon forest soils

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ABSTRACT

This study focused on the impact of land-use changes and agricultural management of soybean in Amazon forest soils on the abundance and composition of the acidobacterial community. Quantitative real-time PCR (qPCR) assays and pyrosequencing of 16S rRNA gene were applied to study the acidobacterial community in bulk soil samples from soybean croplands and adjacent native forests, and mesocosm soil samples from soybean rhizosphere. Based on qPCR measurements, *Acidobacteria* accounted for 23 % in forest soils, 18 % in cropland soils and 14 % in soybean rhizosphere of the total bacterial signals. From the 16S rRNA gene sequences of *Bacteria* domain, the phylum *Acidobacteria* represented 28 % of the sequences from forest soils, 16 % from cropland soils and 17 % from soybean rhizosphere. *Acidobacteria* subgroups 1-8, 10, 11, 13, 17, 18, 22 and 25 were detected with subgroup 1 as dominant among them. Subgroups 4, 6 and 7 were significantly higher in cropland soils than in forest soils, which subgroups responded to decrease of soil Aluminium. Subgroups 6 and 7 responded to high content of soil Ca, Mg, Mn and B. These results showed a differential response of the *Acidobacteria* subgroups to abiotic soil factors, and open the possibilities to explore acidobacterial subgroups as early-warning bio-indicators of agricultural soil management effects in the Amazon area.

Keywords: soil microbiology; soil factors; land-use changes; tropical rainforest; 16S rRNA gene.

1. Introduction

Soil bacterial communities in the Amazon area have been analyzed in different types of soils (Borneman & Triplett, 1997; Kim *et al*., 2007; O'Neill *et al*., 2009; Cenciani *et al*., 2009; Jesus *et al*., 2009; Navarrete *et al*., 2010). Based on these studies, the bacterial community composition

was revealed in soils from different Amazon regions. The *Acidobacteria* phylum has been described as dominant in soils from Western Amazon (Kim *et al*., 2007; Jesus *et al*., 2009) and Central Amazon (Navarrete *et al*., 2010). However, the role of this dominant group in the bacterial community of Amazon soils is largely unknown.

Acidobacteria have consistently been detected in many different habitats around the globe by 16S rRNA gene-based molecular surveys, including soil and rhizosphere niches (Chow *et al*., 2002; Kuske *et al*., 2002; Gremion *et al*., 2003; Quaiser *et al*., 2003; Fierer *et al*., 2005; Stafford *et al*., 2005; Janssen 2006; Sanguin *et al*., 2006; De Cárcer *et al*., 2007; Singh *et al*., 2007; Kielak *et al*., 2009; DeAngelis *et al*., 2009). These observations have revealed that *Acidobacteria* are ubiquitous and among the most abundant bacteria phyla in soil. In spite of their high abundance, little information is available on their ecology, which is mainly due to the lack of culturable representatives in bacterial collections (Kishimoto *et al*., 1991; Liesack *et al*., 1994; Coates *et al*., 1999; Bryant *et al*., 2007; Eichorst *et al*., 2007; Fukunaga *et al*., 2008; Koch *et al*., 2008; Lee *et al*., 2008; Nunes da Rocha *et al*., 2009; Ward *et al*., 2009; Kulichevskaya *et al*., 2010; Pankratov & Dedysh, 2010; Eichorst *et al*., 2011; Männistö *et al*., 2011; Pankratov *et al*., 2011).

Land-use changes is one of the greatest threats to biodiversity worldwide, and one of the most devastating land-use changes, especially in the tropics, is the conversion of intact forests into cultivation fields (Morton *et al*., 2006). The Amazon is a vast, yet vulnerable, hotspot of biodiversity, and the maintenance of soil fertility is critical to sustaining these diverse ecosystems. With this in mind, increased attention has recently been paid to belowground biodiversity in the Amazonian region, and a number of cultivation-independent studies have sought to assess the impact of land-use changes on microbial communities resident to Amazon soils (Borneman & Triplett, 1997; Cenciani *et al*., 2009; Jesus *et al*., 2009; Navarrete *et al*., 2010, 2011). However,

despite this increased appreciation of belowground microbial diversity in the Amazonian region, little is still known about bacterial taxa responses to alterations in soil chemical properties and fertility in consequence to deforestation and agricultural management of Amazon forest soils.

Because of the substantial effects that land-use changes may have on the chemical and physical characteristics of former tropical forest soils, and the high abundance and presumed importance of *Acidobacteria* for the functioning of soil systems, we would like to obtain better insight into the ecological characteristics of *Acidobacteria* in Amazon soils and in soils recently converted into cultivation. For this purpose, we applied quantitative real-time PCR and pyrosequencing spanning the V4 region of the 16S rRNA gene to analyze the abundance and the composition of the acidobacterial community inhabiting bulk soil from soybean croplands and adjacent forests collected in an agricultural zone located in the Southeastern Brazilian Amazon as well as soybean rhizosphere soil from mesocosm experiment. The relative abundances of *Acidobacteria* at the phylum and subgroup taxonomic levels in soil samples were correlated with soil factors in order to explore responses to alterations in soil characteristics due land-use changes and agriculture management of Amazon forest soils.

2. Materials and methods

2.1. Site description and soil sampling

Bulk soil samples were collected in two different field locations in the Southeastern Brazilian Amazon, in the state of Mato Grosso, Brazil in the Porto dos Gaúchos municipality (-15°13'39" S and -54°04'31" W) and the Ipiranga do Norte municipality (-13°21'57" S and -54°54'24" W) (Figure 1). Oxisol is the predominant soil order in the sampling sites (SEPLAN, 2001), and the

climate in the region is classified as Am (Koppen's classification), with annual average temperature of 28 ^{o}C and average precipitation of 2000 mm.

The field locations were considered replicates and the sampling sites were selected according to the vegetation cover, soil use and management practices. In the Porto dos Gaúchos municipality, areas covered with native tropical rainforest were cleared in 2008 and subsequently converted into agricultural land. Since 2004, forest conversion to agricultural use occurred in areas located in Ipiranga do Norte municipality. In both field locations, forest conversion to agricultural use followed annually the rotational production order: millet – soybean – maize, under no-tillage. After deforestation, fertilizers, pesticides and a liming treatment were applied to the cropland fields of both locations. The cropland fields received different amounts of lime to increase soil pH to 5 and 6.

Bulk soil samples were collected from soybean production fields before sowing the seeds (October 2009) and after soybean (*Glycine max* [L.] Merrill cultivar M-SOY 8866) harvest (April 2010) in order to consider an expected variation in soil characteristics during the soybean cultivation (Figure 1). Soil samples were also collected at the same time from adjacent forests to represent the native soil–plant conditions (Figure 1). At each sampling site, the soil samples were collected from five points. One central sampling point and other four sampling points (at least 50 m apart from the central point) directed towards the north, south, east and west of the central point. Soil samples were taken from the 0 to 20 cm topsoil layer (tilled zone). First, the litter layer was removed, and then the soil sample was collected using a 5 cm (diameter) aseptic cylindrical core. A total of 40 bulk soil samples were collected in field (2 field locations x 2 sampling sites per field location x 2 sampling periods x 5 sampling points per site). Samples were transported to the laboratory under ice, stored at −20 °C until processing within 72 h after sampling.

2.2. Abiotic soil factors

For soil physicochemical analysis, soil sample consisted of a composite sample for each sampling site and period by mixing five sub-samples collected from the sampling points. From each composite sample, a sub-sample was removed, air dried and passed through a 2 mm mesh sieve, and then analyzed according to EMBRAPA (1997) at the Department of Soil Science at the University of São Paulo (ESALQ-USP) for the soil fertility properties. Soil pH was measured in a 1:2.5 soil:water suspension. Exchangeable Al, Ca and Mg were extracted with KCl 1 mol L^{-1}. Calcium and Mg were determined by atomic absorption spectrometry and Al by acid-base titration. Phosphorous and K were extracted by ion exchange resin. Potential acidity (H + Al) was estimated by an equation based on the pH determined in SMP buffer solution (pH SMP). Available micronutrients (Fe, Mn, Zn, and Cu) were extracted by Mehlich 1 and determined by atomic absorption spectrometry. Boron was extracted with hot water and determined by spectrophotometry with azomethine-H at 420 nm. Some of the results allowed the calculation of other parameters–such as: exchangeable bases (SB), the sum of Ca, Mg and K; cation exchange capacity (CEC), the sum of Ca, Mg, K, Al, and H; base saturation (V), the percentual relation of SB and CEC; and Al saturation (m%), the percentual relation of exchangeable Al and CEC. Soil texture was determined with Bouyoucos densimeter after shaken the soil vigorously with NaOH 1 mol L^{-1} as dispersant.

2.3. Statistical analysis for soil factors

Analyses of similarity (ANOSIM) of soil physicochemical properties were performed considering the differences in cropland and forest soils. Distance matrix (Euclidean metric) was constructed with non-transformed data. ANOSIM was carried out using Primer six (version 6.1.5, Primer-E

Ltd., Plymouth, UK). Correlation analysis was performed in R (version 2.6.0, The R Foundation for Statistical Computing). Spearman's rank coefficients were calculated to investigate the correlation among the soil factors.

2.4. Soybean rhizosphere soil

In order to consider the abundance and composition of acidobacterial community in the soybean rhizosphere niche, normalizing the influence of environmental parameters (such as moisture regime and temperature) on the growth conditions for the plants, soybean plants were grown in mesocosms in the greenhouse at CENA-USP, Piracicaba, São Paulo. Soil samples were taken at the same five sampling points used for bulk soil collecting in soybean production fields before sowing the seeds. Each soil sample consisted of a composite sample by mixing five sub-samples collected from the 0 to 20 cm topsoil layer. Twenty mesocosms in ceramic pots (30 cm high x 20 cm diameter) were filled with the soils. Duplicate pots were filled with 8 kg of soil and disposed over a bottom of 5-cm layer filled with washed stones. Seeds of the same soybean cultivar (M-SOY 8866) used in field were germinated in the mesocosms at a regime of 12/12 h light/dark cycle and average temperatures 28 ^{o}C at day and 19 ^{o}C at night. The moisture content of the pots was regularly adjusted by an external visual controller installed in each pot. The temperature and moisture regimes were chosen close to natural conditions to create optimal growth conditions for the plants. The plants were grown from November 2009 until January 2010 (84 days). A total of 20 mesocosm samples from soybean rhizosphere soil were taken in late January, prior to plant maturity (soil from 2 soybean production fields x 5 sampling points per field x 2 ceramic pots per sampling point). Roots and associated soil were transported to the laboratory on ice and then

processed to obtain the rhizosphere soil. The roots were shaken to remove the loose soil, and the tightly attached soil including small aggregates (<0.5 cm) was used for DNA extraction.

2.5. Isolation of DNA from soil and quantitative real-time PCR assay

DNA was extracted from 250 mg of bulk soil samples from croplands and adjacent native forests, and mesocosm samples from soybean rhizosphere soil using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. DNA extraction was performed in triplicate for each soil sample and stored at −20 °C until use. Quantitative real-time PCR (qPCR) using the 16S rRNA gene as biomarker was performed to assess the abundance of the acidobacterial and total bacterial communities in each of 40 bulk soil samples and 20 mesocosm samples from soybean rhizosphere soil. As standard, amplicons of *Acidobacteria capsulatum* (DSMZ 11244) and bacterial clone from an environmental sample were obtained by PCR using primers pA-F (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards *et al*., 1989) and 1378R (5'-CGGTGTGTACAAGGCCCGGGAAGG-3') (Heuer *et al*., 1997), purified (QIA-quick PCR purification kit, Qiagen, Venlo, the Netherlands) and ligated into the pGEM-T vector (Promega, Leiden, the Netherlands). Ligation products were transformed with *E.coli* JM109 competent cells (Promega, Leiden, the Netherlands). Cloned inserts were re-amplified using primers SP6 and T7, and plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Venlo, the Netherlands) from appropriate clones (i.e. belonging to the desired target group). DNA standard curves were generated by dilution series of 10^3 to 10^8 copies μL^{-1} using duplicate 10-fold dilutions of isolated plasmid DNA. For qPCR of 16S rRNA gene fragments from *Acidobacteria* and total bacteria: Acid31 (5'-GATCCTGGCTCAGAATC-3') (Barns *et al*., 1999)/Eub518 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer *et al*., 1993) and Eub338 (5'-

ACTCCTACGGGAGGCAGCAG-3') (Lane, 1991)/Eub518 primer pairs were used, respectively. Each 25 μL reaction contained 12.5 μL absolute qPCR SYBR green 2 x reaction mix (Abgene, Epsom, UK), 1.25 μL of each primer (30 μM), 2.5 μL bovine serum albumin (BSA; 10 mg ml^{-1}) and 50 ng template DNA. All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). PCR conditions for *Acidobacteria* and total bacteria were carried out as described by Fierer *et al*. (2005) with the modification of annealing temperature to 49 ^{o}C for *Acidobacteria*. PCR amplifications and product quantification were performed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Automated analyses of PCR amplicon quality (for example, PCR baseline subtraction, Ct-threshold setting to the linear amplification phase) and quantity were performed with ROTOR-GENE 6 software (Corbett Research, Sydney, Australia). Statistical analyses of qPCR data were performed using the STATISTICA 9 package (StatSoft Inc, Tulsa, OK, USA). One-way ANOVA was used to determine significance of the differences between all soil samples. The comparison of soil samples was based on *post hoc* analysis using Tukey's HSD test. Correlations were calculated to test the relationships between acidobacterial relative abundance and soil factors by using the "multtest" package in R (version 2.6.0, The R Foundation for Statistical Computing).

2.6. Amplification and sequencing of 16S rRNA gene fragments

Primers targeting the V4 region of bacterial 16S rRNA gene were used for the amplification. Adapter sequence was added to the primers as recommended by Roche; barcodes of 8 bp and AC linker (added to forward primers only); and the primer sequence 5'-AYTGGGYDTAAAGNG-3'

for the forward primer and 5'-CCGTCAATTCMTTTRAGT-3' for the reverse primer (http://pyro.cme.msu.edu/). DNA template in PCR reactions consisted of pools by mixing aliquots from 15 DNA sub-samples from bulk soil (three replicates of soil DNA extraction for each of five soil samples) for each sampling site and period or 15 DNA sub-samples (three replicates of soil DNA extraction for each of five soybean rhizosphere soil samples) for each replicate of mesocosm experiment. PCR reaction was performed in duplicate for each pooled DNA sample (samples were described in Figure 1). The reactions contained 2.5 × reaction buffer, 0.2 mM of each dNTP, 1 μM of each primer (Alpha DNA, Montreal, Canada), 10 ng of template DNA, and 0.056 U of FastStart High-Fidelity PCR System enzyme blend (Roche Applied Sciences, Indianapolis, IN, USA). The following conditions were chosen for amplification after optimization: initial denaturation for 5 min at 95 °C; 27 cycles of 45 s at 95 °C, 45 s at 57 °C, and 1.5 min at 72 °C; and final extension for 10 min at 72 °C. After gel analysis, amplicons from 10 reactions were combined and purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). PicoGreen base quantification allowed the normalization for construction of multiplex amplicon pools (Harris *et al*., 2010). The samples were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

2.7. Sequence analysis

Sequences and quality information were extracted from the Standard Flowgram Format (SFF) files using the SFF converter tool in the Galaxy interface (Goecks *et al*. 2010). Sequences were analyzed with the Qiime version 1.2.1 scripts (Caporaso *et al*. 2010), which were made available in the Galaxy interface. Firstly, all the sequences that had a perfect match to the primer sequences contained no homopolymer run exceeding six nucleotides and without ambiguous characters were assigned to samples by matching to barcode sequences. Reverse complementary sequences were

put in the forward orientation and assigned to samples by partially matching to barcode sequences. This step was confirmed by V-REVCOMP tool using Hidden Markov Models (HMMs) (Hartmann *et al*. 2011). Secondly, sequence errors introduced during pyrosequencing were detected using the option for titanium data in the Denoiser 0.91 program (Reeder & Knight, 2010). The sequences were also checked for PCR chimeras using UCHIME version 4.2.40 (Edgar *et al*., 2011). Only sequences with a length between 200 and 350 base-pairs were accepted and bases with a quality score lower than 20 were trimmed. The low quality sequences were discarded. The obtained high quality sequences were clustered into Operational Taxonomic Units (OTUs) using UCLUST version 1.2.21 (Edgar, 2010) with a minimum sequence identity cutoff of 97 %. For each OTU the most abundant sequence was selected as a representative for all sequences within an OTU. Taxonomy was assigned to the representative sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4), with a minimum support threshold of 60 %. Finally, the OTU table was filtered for specific taxonomic terms using scripts provided by Qiime. Relative abundances of *Acidobacteria* were estimated in each bacterial community by comparing the number of sequences classified as belonging to the *Acidobacteria* versus the number of classified bacterial sequences per sample. The relative abundance of *Acidobacteria* subgroups across all individual samples, in turn, was estimated by comparing the number of sequences classified as belonging to each *Acidobacteria* subgroup versus the number of classified acidobacterial sequences. The classified acidobacterial sequences were deposited in MG-RAST under accession numbers 4497240.3 to 4497251.3 (list of public access codes can be found in Supplementary Table 1). Explicit relationship between relative abundance of *Acidobacteria* subgroups and bulk soils factors from cropland and forest sites was examined by constrained ordination generated by redundancy analysis (RDA) performed using CANOCO 4.5 (ter Braak & Šmilauer, 2002). Spearman's rank

correlation coefficients were calculated to explore the relationship between relative abundance of *Acidobacteria* subgroups and soil factors referent to different sampling sites by using the "multtest" package in R (version 2.6.0, The R Foundation for Statistical Computing). *P* values were corrected for multiple testing, using the false discovery rate controlling procedure (Benjamini & Hochberg, 1995).

3. Results

3.1. Bulk soil physicochemical factors

The soils contained 43–50 % sand, 4–6 % silt and 46–53 % clay in all sites. Based on ANOSIM of the physicochemical factors, bulk soil from cropland and adjacent forests formed distinct groups, which groups were confirmed by significant *R*-value (R=0.955, $P<0.0001$). Soil pH ranged from 3.7 to 4.7 in forest soils and from 5.0 to 5.7 in the majority of the cropland soils. The detected levels of Al, Al saturation and Fe were respectively 10, 8 and 3 times higher in forest soils than in cropland soils, except for the bulk soil from cropland BS-C1 (Figure 1; Supplementary Table 2). Sum of bases (SB) and base saturation (V) were higher in cropland soils than forest soils (Supplementary Table 2). Results showed that soil pH was negatively correlated ($P<0.05$) with Al saturation, potential acidity, Al, Fe and Mn (Table 1). Both SB and V were negatively correlated with Al ($P<0.05$), potential acidity ($P<0.05$) and Al saturation ($P<0.0005$) (Table 1).

3.2. Acidobacterial community abundance

ANOVA analyses were carried out on qPCR data targeting 16S rRNA gene fragment abundances for total bacteria as well as for *Acidobacteria*. These analyses showed significant differences between bulk soils from cropland and adjacent forest ($P<0.005$) both for the relative and absolute

acidobacterial abundance (Table 2). *Acidobacteria* accounted, on average, for 23 % in forest soils, 18 % cropland soils and 14 % in soybean rhizosphere of the total bacterial signals. Although the highest *Acidobacteria* 16S rRNA gene content was observed in the bulk soils, the overall abundance of *Acidobacteria* gene copies was not statistically different between bulk soils from cropland and mesocosm samples from soybean rhizosphere (Table 2). The abundance of *Acidobacteria* relative to total bacteria 16S rRNA gene copy numbers was significantly correlated with soil organic matter (R=0.627, *P*=0.00001), Al (R=0.413, *P*=0.0004) and pH (R=-0.407, *P*=0.008) (Figure 2). *Acidobacteria* represented the largest portion of the bacterial community in soils with high organic matter content, Al content and acidity. Soil Ca levels (R=-0.482, *P*=0.001) and base saturation (R=-0.494, *P*=0.001) were negatively and significantly correlated with the relative abundance of *Acidobacteria*.

3.3. Phylum and subgroup-levels relative abundance of *Acidobacteria*

Sequence data were examined in soils to estimate the phylum and subgroup relative abundance of *Acidobacteria*. Across the twelve soil samples (samples described in Figure 1), *Acidobacteria* represented 19 % of all classified bacterial sequences detected (32,637 sequences out of 175,434 sequences). The relative abundance of acidobacterial sequences within an individual soil bacterial community represented on average 28 % in forest soils, 16 % in cropland soil and 17 % in soybean rhizosphere.

We further classified *Acidobacteria* DNA sequences into acidobacterial subgroups using the RDP 2 classifier (http://rdp.cme.msu.edu/). The average relative abundances of *Acidobacteria* subgroups for bulk soil from forest and cropland, and soybean rhizosphere soil samples are listed in Table 3 as well as the numbers of OTUs assembled using a 97 % similarity for each

Acidobacteria subgroup. *Acidobacteria* subgroups 1 and 3 were the most abundant in all soils analyzed. Redundancy analysis (RDA) of the relative abundance of the *Acidobacteria* subgroups (1-7, 10 and 13) showed that some subgroups (4-7, 10 and 13) were more closely related to cropland soils (Figure 3). *Acidobacteria* subgroups 1 and 2 were more closely related to forest soils characteristics (Figure 3). Statistically significant differences were found for the abundances of the acidobacterial subgroups 4 ($P<0.05$), 6 ($P<0.05$) and 7 ($P<0.005$) relative to all *Acidobacteria* in cropland soils versus the adjacent forest soils (Table 3). Interestingly, the relative abundances of these acidobacterial subgroups (4, 6 and 7) were statistically correlated with different soil factors, specially those linked to soil acidity such as Al and Al saturation, sum of bases and base saturation (Table 4; Figure 4). Significant correlations were also found between these subgroups (4, 6 and 7) and soil Ca and Mg. The dominant *Acidobacteria* subgroup 1 was positively correlated to potential acidity and Aluminium saturation while subgroup 3 was negatively correlated to soil pH (Table 4). *Acidobacteria* subgroups 10 and 13 were detected in all three environments studied. Subgroup 10 was correlated to soil factors linked to soil acidity such as pH, Al and Al saturation. Moreover, subgroup 13 was correlated to soil P, B and Zn. Most subgroups appear to be minor constituents of soil acidobacterial communities and often occurring in one or two of the sampled soils; subgroup 11 was detected only in bulk soil from cropland; subgroup 17 was detected in bulk soil from forest and cropland, but not in soybean rhizosphere soil; subgroups 18 and 22 were detected in bulk soil from cropland and soybean rhizosphere soil, but not in forest soils; and subgroup 25 was detected only in soybean rhizosphere soil. No sequences affiliated to *Acidobacteria* subgroups 9, 12, 14-16, 19-21, 23, 24 and 26 were detected in any soil sample (Table 3).

4. Discussion

The present study assessed the soil acidobacterial community in a Brazilian Amazon region characterized by high rate of deforestation due to the expansion of cropland (mainly soybean) into areas previously covered by native forest. Our results based on qPCR assays and pyrosequencing analyses show that the overall abundance of *Acidobacteria* (relative to all bacteria) differed between soybean cropland and adjacent native forest soils, and they revealed that the differences in soils factors were the major predictors of the variability in relative abundance of *Acidobacteria* subgroups found in these Amazon soils.

Although the overall abundance of *Acidobacteria* significantly differed (P<0.005) between soybean cropland and adjacent forest soils, the pyrosequencing data revealed that not all *Acidobacteria* subgroups responded to this change in land-use. Recent studies have demonstrated that changes in soil microbial communities across space are often correlated with differences in soil chemistry (Frey *et al*., 2004; Nilsson *et al*., 2007; Lauber *et al*., 2008; Jenkins *et al*., 2009; Kuramae *et al*., 2011). In particular, it has been shown that the composition, and in some cases diversity, of soil bacterial community is correlated with soil pH (Sait *et al*., 2006; Eichorst *et al*., 2007; Hartman *et al*., 2008; Jenkins *et al*., 2009; Lauber *et al*., 2009) and other soil factors than pH, such as Ca/Mg ratio, and Al and phosphorous content (Faoro *et al*., 2010). This pattern holds both for overall bacterial community composition (Fierer & Jackson, 2006; Baker *et al*., 2009; Lauber *et al*., 2009; Kuramae *et al*., 2010) and for the composition of individual bacterial groups (Nicol *et al*., 2008; Davis *et al*., 2009; Jenkins *et al*., 2009; Jones *et al*., 2009).

Although previous studies have indicated the effect of soil pH on the abundance of *Acidobacteria* in different soil types (Jones *et al*., 2009; Rousk *et al*., 2010) and spatial scales (Lauber *et al*., 2009), the present study shows differential responses of the *Acidobacteria*

subgroups to other soil characteristics (including soil factors not measured in previous studies, such as Al, Ca, Mg, K, B and micronutrients) than soil pH altered probably by liming practice required in the agriculture management into Amazon forest soils. In the soil environment, pH is a master variable and it is related to changes in other soil factors, such as Al concentration and nutrient availability (McBride, 1994). Aluminum toxicity has long been known to affect microbes as well as plants in tropical soils (Wood, 1995; Joner e*t al*., 2005). The soil in the region where our samples were collected are rich in Al, in particular the forest soils. Hence, it is not surprising that Al and pH would covary and correlate with acidobacterial community in these native soils. In addition, bacterial communities dominated by *Acidobacteria* phylum changed significantly along gradients of base saturation, Al and pH in Western Amazon soils (Jesus *et al.,* 2009).

Liming practice in agriculture is based on calculation of the necessity of Ca and Mg by the plant, tolerancy to Aluminium and clay content. In acid tropical soils, changing in soil chemical properties due lime requirement for agricultural practices includes a decrease in H^+ activity, a decrease in Al, Al saturation and Mn toxicities, an increase in Ca and Mg availability, and benefits associated with Ca as a complementary ion on the cation exchange complex (Abruňa *et al*., 1964; Amedee, 1976; Oliveira & Pavan, 1996). This practice appears to have direct effect on *Acidobacteria* subgroups 4, 6 and 7 since the abundance of these subgroups were negatively correlated with Al and Al saturation. However for subgroup 6, Ca and saturation of bases were additional factors, while for subgroup 7, Mg and sum of bases (K, Ca, Mg) were additional factors explaining their high abundance in cropland soils. Such elements (Mg, K, Ca) are required for the growth of all living organisms. Magnesium ions are required by large number of enzymes for their catalytic action, including all enzymes utilizing or synthesizing ATP, or those that use other nucleotides to synthesize DNA and RNA. However, the ionic magnesium can not directly be

uptaken by the biological membranes because they are impermeable to magnesium (and other ions), so transport proteins must facilitate the flow of magnesium and other ions, both into and out of cells (Bevenbach, 1990). Future studies on *Acidobacteria* subgroups 4, 6 and 7 functions certainly will elucidate the role of those subgroups in soil.

Differential responses of *Acidobacteria* subgroups to abiotic soil factors are probably due the lifestyles of these microorganisms in soils (Ward *et al*., 2009). When these responses are related to specific factors that are involved in soil management they may be used to develop early-warning bio-indicators for soil effects for instance due to agricultural management of Amazon forest soils into soybean cultivation. According to McCarty & Munkittrick (1996), bio-indicator is an anthropogenically-induced response in biomolecular, biochemical, or physiological parameters that has been causally linked to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization. Our findings about differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes. However, there are still not many studies focusing on responses of acidobacterial subgroups to environmental factors. Ultimately, a better understanding of how agricultural management affects soil microbial ecology will support development of more productive, sustainable systems.

In this study, biological effects at the taxonomical acidobacterial subgroup level were detected using primer set "universal" targeting the V4 region of bacterial 16S rRNA gene. In our soil samples were detected 15 different acidobacterial subgroups among the 26 acidobacterial subgroups classified – subgroups 1-8 according to Hugenholtz *et al*. (1998); subgroups 9-11 according to Zimmermann *et al*. (2005) and subgroups 12-26 according to Barns *et al*. (2007). Many researchers have pointed out that not all acidobacterial subgroups could be detected using

group-specific primers, such as Acid31F (Barns *et al*., 1999) and ACIDO (Lee & Cho, 2011) designed for the selective amplification and detection of members of the phylum *Acidobacteria*. According to Barns *et al*. (2007), George *et al*. (2009), Jones *et al*. (2009), Kielak *et al*. (2009), Sait *et al*. (2006), Lee & Cho (2011), the two group-specific primers (Acid31F and ACIDO) do not detect the subgroups 2, 22 and 25, which subgroups were accounted in this study by using the "universal" V4 region primers. In our soil samples, acidobacterial subgroup 2 was one of the most dominant. The acidobacterial subgroups 1-8 identified in this study were also ubiquitous and abundant members of soil acidobacterial communities in soil surveys worldwide (Barns *et al*., 1999; Janssen *et al*., 2006; Jones *et al*., 2009; Rousk *et al*., 2010). In our analysis, acidobacterial subgroups 4, 6 and 7 showed preponderant responses to abiotic soil factors which were clearly affected by the changes in soil use from native forests into soybean production fields, such as Al, Ca, Mg, Mn and B.

In conclusion, the acidobacterial community responds to agricultural management of Amazon forest soils into soybean production sites, through the effects on various soil factors, not only those related to soil acidity. The differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes in Amazon soils recently converted into cultivation and open the possibilities to explore acidobacterial subgroups as bio-indicators for agricultural soil management effects in the wide Amazon area.

5. Acknowledgments

This study was supported by a grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Wageningen - 2238/10-1), Conselho Nacional de Desenvolvimento Científico

(CNPq - 152084/2011-8) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - 08/58114-3). Publication number xxxxx of the NIOO-KNAW, Netherlands Institute of Ecology.

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Table 1. Spearman's rank correlation matrix for soil chemical attributes of the 0 to 20 cm topsoil layer at different sampling sites.

Soil attributes	pH	OM	Al	H+Al	m	P	K	Ca	Mg	SB	CEC	V	B	Fe	Mn	Zn	Cu
pH	-																
OM		-															
Al	-0.850*		-														
H+Al	-0.778*	0.785*	0.857*	-													
m	-0.634*		0.785*	0.785*	-												
P						-											
K			-			0.754*	-										
Ca			-0.785*	-0.785*	-1***	0.850*		-									
Mg			-0.778*	-0.742*	-0.958***	0.801*		0.958***	-								
SB			-0.785*	-0.785*	-1***	0.850*		1***	0.958***	-							
CEC											-						
V			-0.761*	-0.833*	-0.976***	0.826*		0.976***	0.934***	0.976***		-					
B	-0.876*		0.932**	0.748*									-				
Fe	-0.814*		0.904**										0.969**	-			
Mn	-0.757*		0.855*										0.938**	0.988***	-		
Zn																-	
Cu																	-

Significant levels for the Spearman's rank coefficients are indicated at the *P<0.05; **P<0.005; ***P<0.0005 level.

Table 2. Absolute and relative abundance of *Acidobacteria* in cropland and adjacent forest bulk soils and soybean rhizosphere niche.

	Bulk soil - Forest (BS-F)				Bulk soil - Cropland (BS-C)				Soybean rhizosphere (SR)				Statistics	
	BS-F1	BS-F2	BS-F3	BS-F4	BS-C1	BS-C2	BS-C3	BS-C4	SR-I	SR-II	SR-III	SR-IV	BS-F vs. BS-C	BS-C vs. SR
Absolute abundance (qPCR analysis) (10^7 16S rDNA copies g soil)														
Total bacteria	3.48a[1] (0.17)[2]	2.90a (0.08)	6.0A (0.23)	4.59A (0.14)	1.64a (0.01)	2.96a (0.13)	7.12A (0.29)	4.55A (0.15)	2.28a (0.09)	2.89a (0.10)	7.27A (0.22)	6.08A (0.12)	ns[3] (1-2)	ns (1-2)
													ns (3-4)	ns (3-4)
Acidobacteria	0.96a (0.07)[2]	0.84a (0.02)	1.07A (0.06)	0.85A (0.01)	0.49a (0.05)	0.56a (0.02)	0.74A (0.01)	0.50A (0.005)	0.44a (0.01)	0.45a (0.007)	0.78A (0.008)	0.72A (0.03)	** (1-2)	ns (1-2)
													** (3-4)	ns (3-4)
Relative abundance (%)														
Acidobacteria (*qPCR assays*)	27.58a (2.61)[2]	28.96a (1.83)	17.83A (2.72)	18.51A (1.76)	29.87a (2.8)	18.92b (0.12)	10.39A (2.11)	10.98A (1.18)	19.30a (1.10)	15.57a (1.64)	10.72A (1.13)	11.84A (2.53)	* (1-2)	ns (1-2)
													**(3-4)	ns (3-4)
Acidobacteria (*Pyrosequencing analysis*)	19.92a (4057)[4]	22.06a (3614)	28.78A (4967)	22.97A (2771)	25.0a (2704)	15.56b (2435)	9.58A (991)	15.16A (1316)	10.43a (1848)	15.03a (3391)	7.90A (2925)	8.72A (1618)	ns (1-2)	* (1-2)
													** (3-4)	ns (3-4)

[1] Values with the same lower or upper-case letters were not significantly different ($P<0.05$) based upon on a Tukey's HSD test.

[2] Standard deviation of the average for each of five replicates soil.

[3] Tukey's HSD test was performed separately for field location and sampling period (samples 1-2 and 3-4). Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.005$; ***$P<0.0005$.

[4] Total number of acidobacterial reads obtained by barcoded pyrosequencing. Sequences taxonomy assignments were made using Ribosomal Database Project 2 classifier (release 10.4).

Table 3. Abundance of *Acidobacteria* subgroups relative to all *Acidobacteria* and numbers of OTUs of acidobacterial subgroups in cropland and adjacent forest bulk soils and soybean rhizosphere niche.

	% of total acidobacterial sequences (range)			OTUs (range)			Statistics (% of total acidobacterial sequences)		Statistics (OTUs)	
	BS-F	BS-C	SR	BS-F	BS-C	SR	BS-F vs. BS-C	BS-C vs. SR	BS-F vs. BS-C	BS-C vs. SR
1	51.4 (40.1-65.2)[1]	32.8 (30.9-36.5)	41.0 (29.5-60.7)	42 (41-43)	41 (34-48)	45 (41-49)	ns[2]	ns	ns	ns
2	11.2 (8.0-13.9)	7.2 (6.7-7.6)	5.3 (2.9-7.8)	16 (12-23)	16 (10-23)	12 (8-15)	ns	ns	ns	ns
3	29.0 (20.1-51.2)	29.6 (17.7-51.2)	30.8 (22.8-40.3)	27 (19-34)	32 (25-41)	39 (23-54)	ns	ns	ns	ns
4	0.1 (0.0-0.4)	4.6 (0.58-7.3)	5.4 (0.7-10.5)	2 (0-4)	18 (7-27)	11 (3-18)	* (1-2) / * (3-4)	ns	* (1-2) / * (3-4)	ns
5	4.0 (3.0-5.3)	3.8 (1.0-6.0)	1.2 (0.4-1.6)	5 (4-7)	6 (4-8)	5 (4-6)	ns	ns	ns	ns
6	2.5 (1.6-3.1)	16.6 (5.4-23.1)	13.1 (5.6-20.3)	6 (5-7)	10 (6-13)	13 (7-19)	* (1-2) / * (3-4)	ns	* (1-2) / * (3-4)	ns
7	<0.1 (0.0-0.04)	1.8 (0.5-3.3)	1.4 (0.1-2.8)	1 (0-1)	6 (4-7)	4 (1-6)	** (1-2) / * (3-4)	ns	** (1-2) / * (3-4)	ns
8	<0.1 (0.0-0.02)	<0.1 (0.0-0.07)	<0.1 (0.0-0.05)	1 (0-1)	1 (0-1)	1 (0-2)	ns		ns	
9	ND	ND	ND	ND	ND	ND				
10	0.1 (0.04-0.2)	0.3 (0.2-0.4)	0.3 (0.0-0.9)	3 (1-5)	3 (1-5)	3 (0-7)	ns	ns	ns	ns
11	ND	<0.1 (0.0-0.1)	ND	ND	1 (0-2)	ND				
12	ND	ND	ND	ND	ND	ND				
13	1.3 (0.9-1.7)	2.3 (1.5-3.3)	1.3 (0.8-1.9)	7 (5-9)	6 (5-8)	4 (3-7)	ns	ns	ns	ns
14	ND	ND	ND	ND	ND	ND				
15	ND	ND	ND	ND	ND	ND				
16	ND	ND	ND	ND	ND	ND				
17	<0.1 (0.0-0.2)	0.5 (0.0-1.3)	ND	1 (0-2)	4 (0-7)	ND	ns		ns	
18	ND	<0.1 (0.0-0.1)	<0.1 (0.0-0.2)	ND	1 (0-2)	1 (0-3)		ns		ns
19	ND	ND	ND	ND	ND	ND	ns		ns	
20	ND	ND	ND	ND	ND	ND	ns		ns	
21	ND	ND	ND	ND	ND	ND	ns		ns	
22	ND	0.1 (0.09-0.5)	<0.1 (0.0-0.02)	ND	1 (0-4)	1 (0-1)		ns		ns
23	ND	ND	ND	ND	ND	ND				
24	ND	ND	ND	ND	ND	ND				
25	ND	ND	<0.1 (0.0-0.05)	ND	ND	1 (0-1)				
26	ND	ND	ND	ND	ND	ND				

BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere.

[1]Range (%) of the average for each of four replicates soil.

[2]Tukey's HSD test was performed separately for field location and sampling period (samples 1-2 and 3-4). SR. Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.005$.

Table 4. Spearman's rank correlation coefficients and statistical significance between abundance of *Acidobacteria* subgroups (1-7, 10 and 13) relative to all *Acidobacteria* and soil factors.

Soil factors	*Acidobacteria* subgroups								
	1	2	3	4	5	6	7	10	13
[illegible]	-0.304	-0.437	-0.707*	0.804*	0.375	0.815*	0.847**	0.710*	0.484
OM	0.347	0.558	-0.130	-0.427	0.221	-0.290	-0.375	-0.706	-0.143
Al	0.619	0.691	0.446	**-0.920****	-0.223	**-0.952*****	**-0.920*****	-0.807*	-0.669
[illegible]	0.737*	0.610	0.117	-0.810*	-0.134	-0.769*	-0.826*	-0.841*	-0.465
m	0.737*	0.675	0.344	**-0.933****	-0.259	**-0.972*****	**-0.939*****	-0.723*	-0.666
[illegible]	-0.587	-0.553	-0.114	0.541	-0.069	0.705	0.478	0.352	0.802*
[illegible]	-0.410	-0.482	0.011	0.274	-0.198	0.489	0.305	-0.066	0.477
Ca	-0.530	-0.503	-0.459	0.767*	0.265	**0.882****	**0.984*****	0.543	0.330
Mg	-0.433	-0.455	-0.485	0.685	0.247	0.822*	**0.957*****	0.451	0.208
SB	0.496	-0.493	-0.469	0.736*	0.251	0.866*	**0.978*****	0.499	0.288
CEC	0.342	0.187	-0.547	-0.124	0.179	0.142	0.226	-0.540	-0.281
V	-0.608	-0.575	-0.399	0.845*	0.280	**0.897****	**0.979*****	0.653	0.402
[illegible]	0.448	0.642	0.395	-0.690	0.032	-797*	-0.666	-0.683	-0.750*
Fe	0.256	0.736*	0.327	-0.597	0.194	-0.606	-0.538	-0.686	-0.516
[illegible]	0.293	0.748*	0.589	-0.796*	-0.155	-0.792*	-0.673	-0.660	-0.653
Zn	-0.670	-0.326	-0.008	0.455	0.046	0.644	0.427	0.391	0.810*
Cu	-0.160	0.614	0.234	-0.242	0.356	-0.664	-0.158	-0.033	-0.038

Significant levels for the Spearman's rank coefficients are indicated at the *P<0.05; **P<0.005; ***P<0.0005 level.

Bold values indicate a significant difference using Bonferroni correction.

Coefficient of determination (R^2) for significant correlations can be found in Supplementary Table 3.

Figure 1. Location of the sampling sites in the Southeastern Brazilian Amazon and time-location scheme for soil sampling (bulk soil from field and soybean rhizosphere from greenhouse mesocosm experiment). Sampling points were located under cropland and adjacent forest in both the Porto dos Gaúchos municipality (-15°13'39" S and -54°04'31" W) and the Ipiranga do Norte municipality (-13°21'57" S and -54°54'24" W), state of Mato Grosso, Brazil. Bulk soil sampling was performed before sowing soybean seeds (October 2009) and after soybean harvest (April 2010) in cropland sites. Concomitantly, bulk soil samples were taken in adjacent forests. In the time-location scheme for soil sampling codes indicate the name attributed to the pooled DNA samples used for pyrosequencing analysis (BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere). The roman numerals I, II, III and IV indicate replication of greenhouse mesocosm experiment.

Figure 2. Effect of (A) organic matter, (B) Aluminium (Al) and (C) soil pH on abundance of *Acidobacteria* relative to total bacteria as indicated by the number of 16S ribosomal (DNA) (rDNA) copies measured using real-time quantitative PCR assays. Spearman's rank correlations and their statistical significance are depicted. Coefficient of determination (R^2) was calculated.

Figure 3. Constrained ordination diagram for sample plots (● = bulk soil from forest; ■ = bulk soil from cropland) in the first two redundancy analysis (RDA) axes based on the soil chemical characteristics of the different sampling sites and their relationship with the relative abundance of *Acidobacteria* subgroups (1-7, 10 and 13). Each vector points to the direction of increase for a given acidobacterial subgroup (Gp) and its length indicates the strength of the correlation between this variable and the ordination scores. Bulk soil (BS) samples were taken before sowing soybean seeds (BS-C1 and BS-C3) and after soybean harvest (BS-C2 and BS-C4) in cropland sites.

Concomitantly, bulk soil samples were taken in adjacent forests (BS-F1 and BS-F3) and (BS-F2 and BS-F4).

Figure 4. Effect of soil pH, Al, Al saturation (m), base saturation (V), Ca and Mg on abundance of *Acidobacteria* subgroups (4, 6 and 7) relative to all *Acidobacteria* using pyrosequencing data. Triangles (grey) represent *Acidobacteria* subgroup 4; Squares (no filled) represent *Acidobacteria* subgroup 6; circles (black) represent *Acidobacteria* subgroup 7. Spearman's rank correlations between subgroup abundance and each soil factor are depicted. **P*<0.05; ***P*<0.005; ****P*<0.0005.

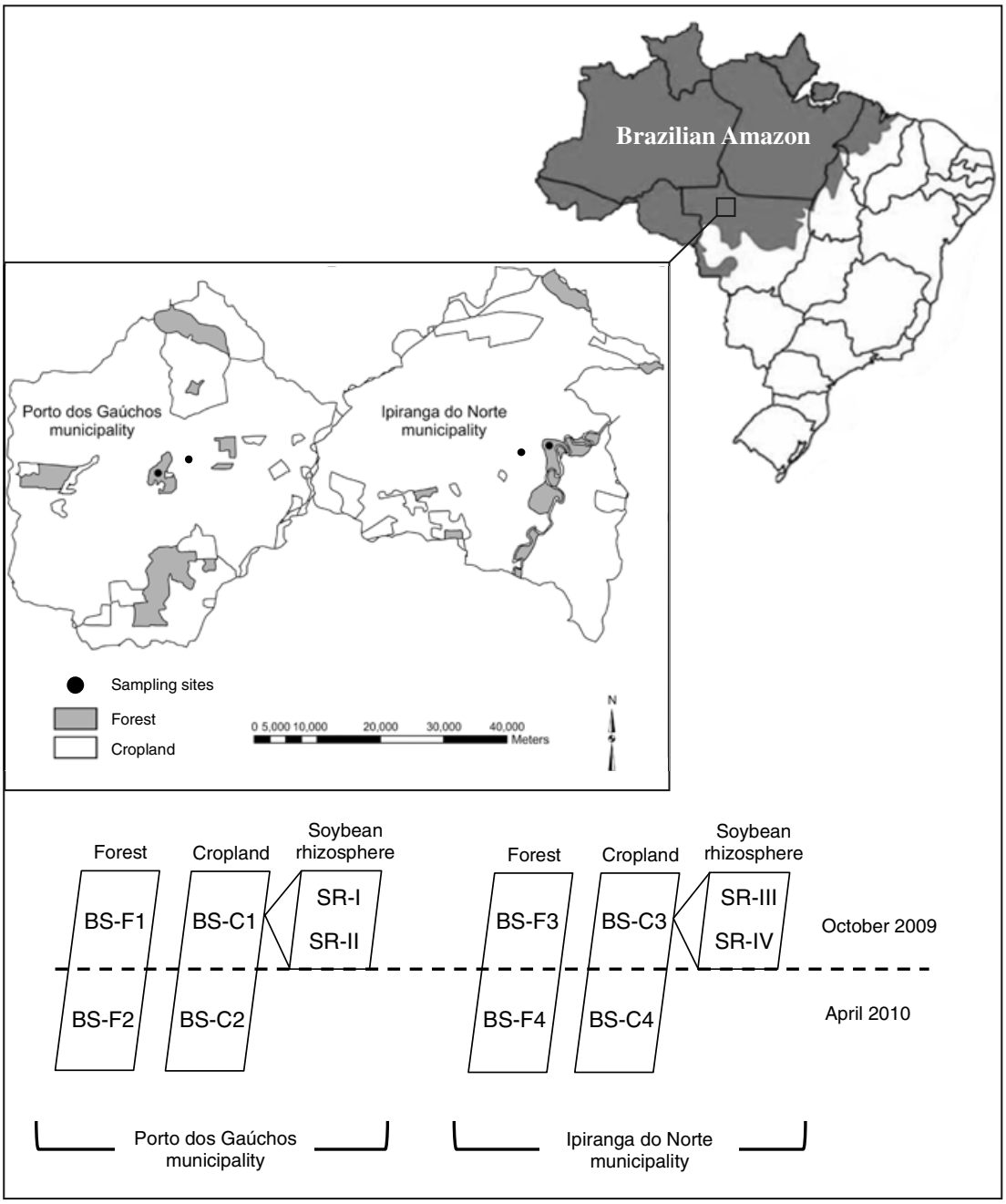

Figure 1

Figure 2

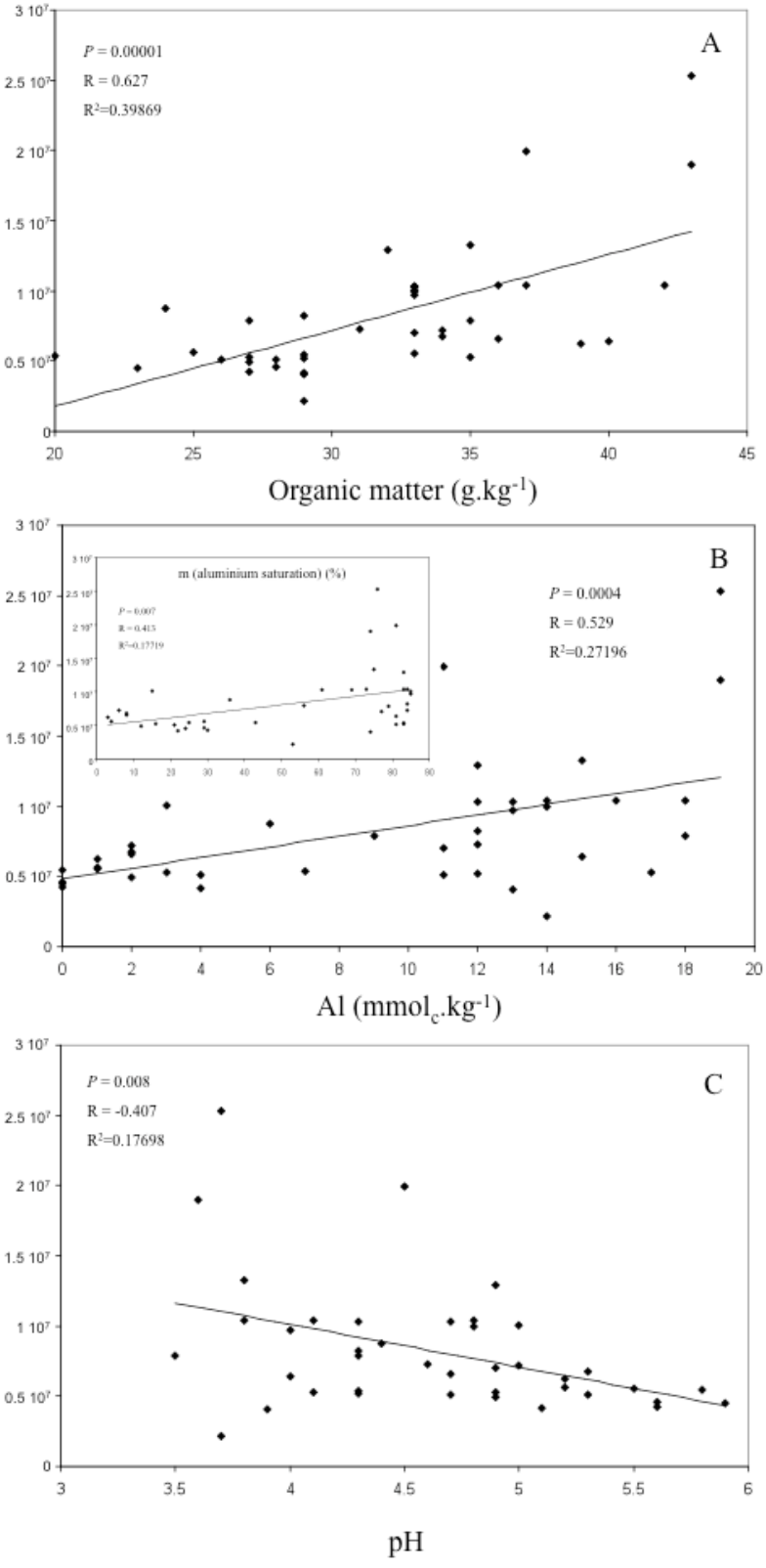

Figure 3

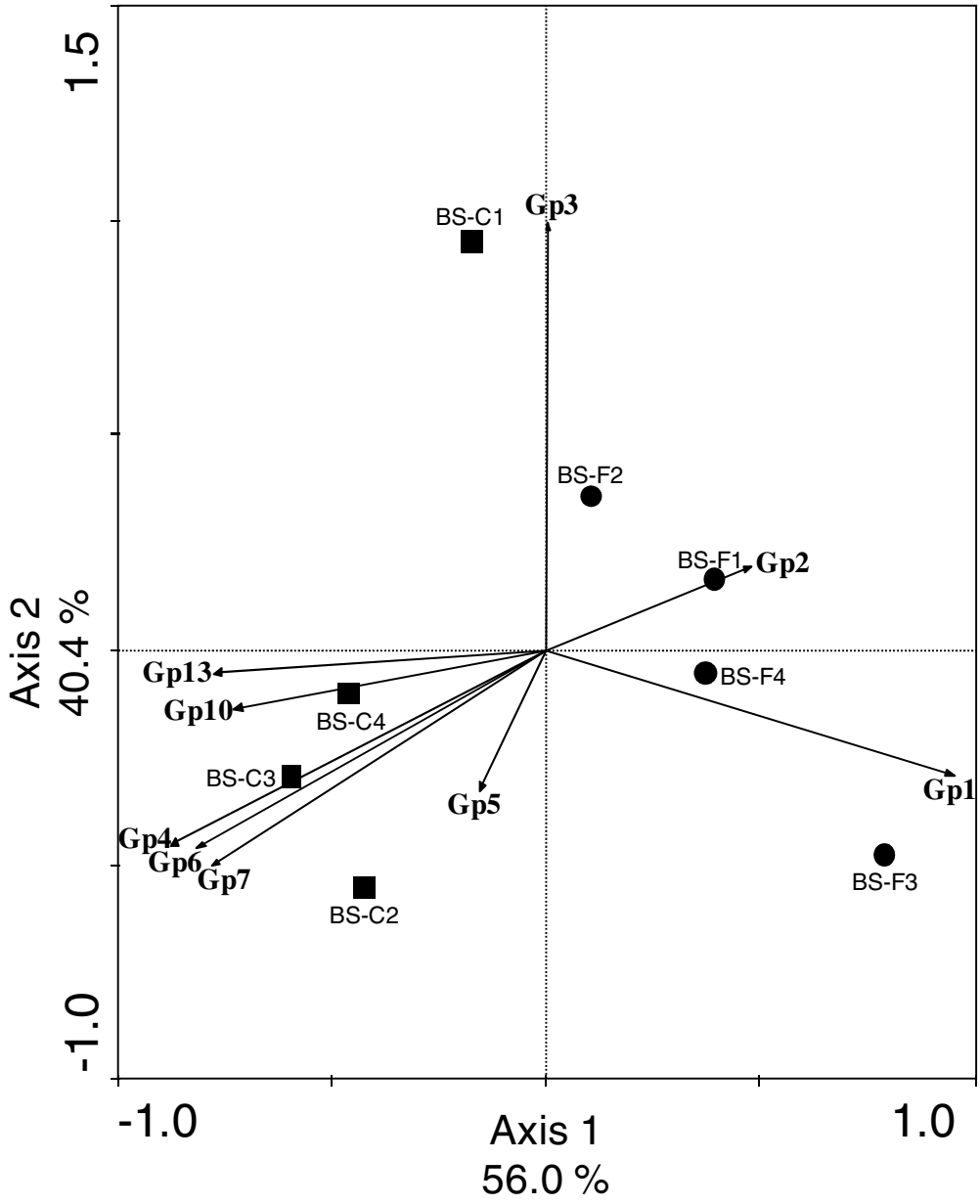

Figure 4