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Cover letter for submission of manuscript

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Dear Editor,

I am enclosing herewith a manuscript entitled "**Protease encoding microbial communities and protease activity of the rhizosphere and bulk soils of two maize lines with different N uptake efficiency**", for publication as a research article in **Soil Biology and Biochemistry**. The research reported in this manuscript is funded by Ministryfor Education under Research project 'PRIN 2009MWY5F9' and main author DivyashriBaraniya was supported by the Marie Curie ITN action'TRAINBIODIVERSE', grant N° 289949.

This article reports the study on proteolyticactivity and proteolyticcommunities associated with rhizosphere and bulk soil from 2 maize plants with different nitrogen utilizing efficiencies. We hypothesized that plants differing in the N use efficiency (NUE) also induce changes in the soil microbial communities, diversity of genes encoding proteases, and activity of protease, urease and chitinase.

Understanding the relationship between soil microbial communities expressing protease activity and nitrogen in the rhizosphere is still a challenge, due to difficulties in sampling the rhizosphere microenvironment. We tested our assumptions by studying changes in the biochemical activity and the microbial community structure in the rhizosphere of inbred maize (*Zeamais* L.) lines L05 and T250, characterized by high and low NUE respectively, using rhizobox experiments.

The adopted experimental approach allowed to understand the relative plant induced changes on relevant microbial groups and specific enzyme activities in the rhizosphere of maize plants with different NUE.

Our work is important because it can provide useful information on responses of soil microbial communities under the influence of plants with different NUEs.

With the submission of this manuscript I would like to undertake that the above mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and all co-authors are fully aware of this submission. The authors have no conflicts of interest to declare.

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Highlights

- It is the first ever work that shows the response of microbial communities involved in proteolysis, to maize plants with different Nitrogen Utilizing Efficiencies (NUE).
- This study shows the so far unknown nature of protease encoding bacterial genes by Illumina sequencing.
- Out of the two proteases genes studied here (*apr* and *npr*), it has been found that most of the *npr* gene pool is formed by sequences belonging to unknown uncultured bacterial species.
- Plants with different NUEs have shown to harbor different proteolytic communities.

Protease encoding microbial communities and protease activity of the rhizosphere and bulk soils of two maize lines with different N uptake efficiency

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Abstract

Present study was carried out to understand the interplay of plant Nitrogen utilizing efficiency (NUE) with potential proteolytic activity and proteolytic community composition of the rhizosphere and bulk soils, sampled from rhizoboxes with two inbred maize lines, L05 and T250, with higher and lower NUErespectively. Microbial biomass was estimated as ATP content and two key bacterial protease encoding genes: alkaline metallo-peptidases (*apr*)and neutral-metallopeptidases(*npr*) were characterized by DGGE and Illumina sequencing of amplicons. Higher protease activity and microbial biomass were observed in rhizosphere soil of the plant line with higher NUE (L05), which also had higher values for Shannon-Weiner diversity indices (H) for DGGE band pattern, with *npr*gene showing higher overall diversity in rhizosphere soil than in the lower NUE plant (T250) rhizosphere. Stronger root effects were observed for *apr* gene than *npr*. Illumina sequencing showed differences in the composition of proteolytic microbial communities inrhizosphere and bulk soils for both L05 and T250, and many unknown *apr* and *npr* gene sequences were also reported. Furthermore, Illumina sequencing results agreed with DGGE data in highlighting higher overall diversity for npr (1,520,600 unique sequences) than for apr (934,598 unique sequences). Different members of *Bacillus sp.* were identified as most abundant contributors to *npr* gene pool whereas

*apr*gene pool was dominated by genes from *Pseudomonas sp*. This research suggests that plants with different NUE select different bacterial populations with protease encoding genes, which may affect the protease activity of the rhizosphere soil.

Highlights

- It is the first ever work that shows the response of microbial communities involved in proteolysis, to maize plants with different Nitrogen Utilizing Efficiencies (NUE).
- This study shows the so far unknown nature of protease encoding bacterial genes by Illumina sequencing.
- Out of the two proteases genes studied here (*apr* and *npr*), it has been found that most of the *npr* gene pool is formed by sequences belonging to unknown uncultured bacterial species.
- Plants with different NUEs have shown to harbor different proteolytic communities.

Keywords: Nitrogen use efficiency; rhizosphere; protease activity; protease encoding genes; Illumina sequencing; PCR-DGGE

1. Introduction

Genetic and physiological mechanisms of N acquisition by important cereal plants are increasingly known (Hirel et al., 2007) but currently, at field scale, the Nitrogen Use Efficiency (NUE) in cereal production is still lower than 40% (Raun and

Johnson, 1999). This is because although the NUE is an inherent plant characteristic, regulated by complex genetic and metabolic factors (Xu et al. 2012, Ngezimana and Agenbag, 2014, Zamboni et al., 2015), the N acquisition by crop plants is also limited by N losses by volatilization, runoff and leaching, and by microbial N immobilization. Moreover, there are increasing evidences that plant NUE also depends on microbial activity in the rhizosphere, particularly on activity of the proteolytic communities (Mooshammer et al. 2004). This is linked to the fact that the most of soil N is of peptide or protein origin, as 96-99% of soil total N is organic and after acid hydrolysis, amino acidic N accounts for 30-50% of the N in soil (Nannipieri and Paul, 2009). The N phyto-availability in soil also depends on the hydrolysis of other organic N forms, such as urea and chitin catalyzed by the ureases and chitinases, the latter being produced by fungi and bacteria (Metcalfe et al., 2002). Chitinase is,therefore, a key soil enzyme, regulating the release of low molecular weight N-sugars from which N is rapidly mineralized to inorganic N (Gooday 1994). Proteins in soil originate from plants, animals and microorganisms, either through active excretion or passive release, and therefore a high proportion of protein N in the rhizosphere is expected. In soil environment, protein N is released after protein hydrolysis by extracellular proteases of plants, animals and microbial origin (Adamczyk et al., 2010, Godlewski and Adamczyk, 2007;

Hayano 1993; Watanabe, 2009), and previous studies indicated that metalloproteases of bacterial origin mainly contribute to the measured soil protease activity (Hayano et al. 1987, Bach and Munch, 2000, Kammimura and Hayano, 2000).

Soil management and environmental factors influence the abundance and distribution of microbial genes encoding for neutral metallo-peptidases (*npr*), alkaline metallo-peptidases (*apr*) and serine peptidases (*sub*) (Bach et al. 2001, 2002, Fuka et al. 2008a, 2008b, 2009, Rasche et al. 2014, Sakurai et al. 2007; Tsuboi et al. 2014,). Proteases catalyze the hydrolysis of the terminal amino acid of polypeptide chains (exopeptidases) or of internal peptide bond (endopeptidases) on one or few related substrates, with the majority of proteases acting on several substrates. However, the number of assays for soil protease activity is limited to few substrates and optimal pH values. Increased N mineralizing activities in response to the release of root exudates has been reported (Renella et al., 2007), but in spite of their importance in determining N availability to plants, studies on the link between the diversity of protease encoding genes and protease activities in the rhizosphere are still scarce(Nannipieri et al., 2012). Little information is also available on the relations between the proteolytic microbial community of the rhizosphere and the plant NUE.Next generation sequencing technologies (NGS) provide advanced tools to analyze microbial genes in soil: this

129 approach has been applied for the analyses of PCR amplicons
130 of 16S rRNA (Vasileiadis et al. 2013), ITS (Internal
131 Transcribed Spacer) (McHugh and Schwartz, 2015) and
132 ammonia monooxygenases (Pester et al., 2012), but not yet for
133 assessing the abundance and diversity of proteases genes in
134 soil.
135 We hypothesized that plants with different NUE select different
136 proteolytic microbial communities characterized by different
137 levels of proteolytic activity in the rhizosphere. To test our
138 hypotheses, we studied the composition of the proteolytic
139 microbial communities and proteolytic activities in the
140 rhizosphere and bulk soil of the L05 and T250 maize lines,
141 characterized by high and low NUE, respectively. Previous
142 work showed that these two maize lines have different genetic
143 responses to N availability (Zamboni et al., 2014) and also host
144 different microbial communities in their rhizosphere (Pathan et
145 al., 2015). Furthermore, we have also applied a NGS
146 assessment of neutral metallo-peptidases (*npr*) and alkaline
147 metallo-peptidases (*apr*) PCR amplicons, in order to unravel
148 the diversity of these genes in the bulk and rhizosphere soils of
149 the two maize lines. We also measured the urease and chitinase
150 activities to understand their contribution to N availability in
151 the maize rhizosphere. Results of thisresearch can improve our
152 understanding of the effects of microbial selection in the

rhizosphere of maize plants with different plant NUE on the turnover of protein-N in the rhizosphere.

2. Materials and methods

2.1 Soil properties and rhizobox set up

A sandy clay loam EutricCambisol (World Reference Base for Soil Resources, 2006), under conventional maize crop regime, located at Cesa (Tuscany, Central Italy), was sampled from the Ap horizon (0-25 cm). Soil had a pH value (in H_2O) of 7.1, contained 32.1% sand, 42.2% silt, 25.7% clay, 10.8 g kg^{-1} total organic C (TOC), 1.12 g kg^{-1} total N and 6.45 g kg^{-1} total P. The soil was sieved at field moisture (< 2 mm), after removing visible plant material. After sieving, 600 g of soil was placed in the soil compartment of the rhizoboxes as reported by Pathan et al. (2015). The L05 and T250 maize lines were grown for 21 and 28 days, respectively, a suitable growth period to allow the full colonization of the plant compartment by plant roots and prevent nutrient starvation. Plants were regularly watered with distilled sterile H_2O and no fertilizers were applied during the plant growth. Full details on the maize growth conditions were reported by Pathan et al. (2015). Five rhizobox replicates for each maize lines were prepared. The used rhizoboxes allowed precise sampling of the rhizosphere due to the presence of fixed sampling groves at precise gradual distances from the surface of the plant compartment. Rhizosphere (R) and bulk soil (B)

178 samples of the L05 and T250 maize lines were named as L05
179 R, L05 B, T250 R and T250 B, respectively. Rhizosphere and
180 bulk samples were kept separate after sampling, and
181 immediately analyzed for the enzyme activities or stored at -
182 80°C before ATP determination or DNA extraction.
183
184 2.2 Soil microbial biomass and enzymatic activities
185 Soil microbial biomass was estimated by determining the ATP
186 content according to Ciardi and Nannipieri (1990). The N-
187 benzoyl-L-argininamide (BAA) and casein hydrolyzing
188 activities (protease activities) were determined according to
189 Ladd and Butler (1972) and Nannipieri et al (1974),
190 respectively. Concentrations of NH_4^+-N and tyrosine released
191 by the assays with BAA or Na-caseinate, respectively, were
192 spectrophotometrically quantified (Perkin Elmer Lambda 2)
193 from calibration curves obtained using standards after reaction
194 with the Nessler or Folin reagents after subtracting the
195 absorbance of controls.Urease activity was determined using
196 6% urea solution as substrate according to Nannipieri et al.
197 (1980), and NH_4^+-N concentration was determined as above
198 described for the for the protease assay. Toaccount for fixation
199 of NH_4^+-N released by BAAase and urease activities, NH_4^+-N
200 solutions withconcentrations in the range of thosereleased by
201 urease andprotease activitieswere incubated with the same soil,
202 and recovery ofNH_4^+-N were in the range 95-98%.Chitinase

activity was determined by the hydrolysis of 4-nitrophenyl-β-D-glucosaminidine (SIGMA) in 0.1 acetate buffer at pH 5.2, for 1 h at 50°C using 1 g d.w. soil. The p-nitrophenol released by the chitinase activity was spectrophotometrically quantified using calibration curves, after subtracting of the absorbance of controls. Preliminary experiments have showed that 50°C was the optimal temperature for soil chitinase activity.

2.3 Nucleic acids extraction and PCR-DGGE analysis

DNA was extracted by sequential extraction method from 0.5 g soil as described by Ascher et al. (2009) using the FastDNA spin kit for soil (MP Biomedicals, USA), andthe intracellular DNA fraction was used in this study. The DNA yield and purity were analysed with a Qubit 2.0 fluorometer (Life Technologies, USA) using Quant-iTdsDNA *HS* kitaccording to the manufacturer's instructions, and stored at -20°C till prior to analysis.

The primersFaprI/RaprII for *apr* gene and FnprI/RnprII for *npr*as mentioned in Bach et al. (2001) were used for PCR and were amplified according to conditions as used by Bach et al. (2001). The DGGE conditions for the fingerprinting of the *apr* and *npr*ampliconswere those previously used by Sakurai et al. (2007). The DGGE fingerprints were performed using aINGENY PhorU System (Ingeny International BV, Netherlands), theDGGE gels were stained with SybrGreen I

(FMC Bio Products, Rockland, ME, USA), and the banding patterns were analysed by a Gel Doc system (Bio-Rad, USA).

2.4 Quantification of protease encoding genes

Quantitative PCR (qPCR) was conducted on a CFX Connect Real-Time PCR Detection System (Bio-rad Laboratories) to determine the abundance of *apr* and *npr* genes, using the primer sets FP *apr*I/ RP *apr*II for the *apr* gene and FP *npr*I/ RP *npr*II for the *npr* gene, according to Bach et al. (2001). *Pseudomonas fluorescence* (isolated from an agricultural soil) and *Bacillus cereus* (DSM31)were used aspositive controls for *apr*and*npr*genes respectively. Each qPCR assay was conducted in a 96-well plate and included three replicates for each standard, negative controls, and sample. Amplification was performed using the iTaq Universal SYBR Green Supermix (Bio-rad Laboratories), adding to each reaction mixture forward and reverse primers for both genes at concentration of 0.6 µM, 3% of bovine serum albumin (BSA), 20 ng DNA template for *apr* gene and 30 ng DNA template for *npr*. The PCR runs for both genes started with an enzyme activation step at 95°C for 3 min, followed by 42 cycles of denaturation at 94°C for 25 s. Annealing conditions were 54°C for 30 s for the *apr* and at 53°C for 30 s for the *npr*gene, respectively, followed by extension at 72°C for 30 s. The specificity of amplification products were confirmed by melting curve analysis and

expected sizes of amplified fragments were checked by running the ampliconson a 2% agarose gel stained with ethidium bromide for 90 mins at 100 V.

2.5 Illumina sequencing of *apr* and *npr* genes

Alkaline metallo-peptidase (*apr*) and neutral metallopeptidase (*npr*) genes were targeted by PCR as previously described (Bach et al. 2001), using primers pairs FP aprI/RP aprII for *apr* (amplicon length 194 bp) and FP nprI/RP nprIIfor the *npr*gene (amplicon length 233 bp), respectively. The PCR reactions were carried out on a Biometra T Professional thermocycler (Biometra BiomedizinischeAnalytik GmbH, Germany). For both *apr* and *npr*genes the reaction mixture contained 0.8 µM of forward and reverse primers, 20 ng of template DNA, 0.3% BSA, 0.2 mMdNTP mix, 2.5 µl of 10X DreamTaq Buffer having 20 mM $MgCl_2$ and 1 unit of Dream Taq Polymerase (Thermo Fisher Scientific, USA). The PCR programs consisted of a hot start step for 5 min at 95°C, followed by 80 °C for 5 min during which Taq polymerase was added. Thirty-five cycles of denaturation at 94°C for 30 s, annealing at 55°C for *npr* and 58°C for *apr*, respectively, followed by an extension step at 72°C for 30s and a final extension step at 72°C for 7 mins. After PCR, amplicons were run on a 2% agaorose gel for 90 mins, single bands were excised and purified from gel using Nucleospin Gel and PCR cleanup kit (MACHERY-NAGEL

GmbH and Co. KG, Germany), according to the manufacturer's instructions. Purified amplicons were quantified on Qubit 2.0 fluorometer using Quant-iTdsDNA *HS* reagent as per manufacturer's instructions and sequenced using anIlluminaHiSeq 2000 in paired-end 150x2 bp at the Beijing Genomics Institute. For Illumina sequencing the five replicates of each plant were pooled together for an in depth analysis of all gene sequences, according to the Illumina sample preparation guide (http://supportres.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). The obtained Illumina sequences of *apr* and *npr*ampliconswere processed separately, using the USEARCH and UPARSE pipelines (Edgar, 2010, 2013). Paired reads from each sample were firstly assembled with the fastq_mergepairs command. Assembled reads were then filtered allowing a maximum expected error of 0.5 and discarding reads with length <190 and <230 bp for *apr*and*npr* gene, respectively. Barcode labels were then added to the sequences, and 4 samples available for each gene were merged with the cat command. Unique sequences were then identified, sorted by abundance, and singletons were discarded. Gene sequences were assigned to operational taxonomical units (OTUs)at minimum identity levels of 97% or 95%, according to the UPARSE algorithm. The OTUs were further filtered for

303 the presence of chimeras with the UCHIME tool, andreads
304 were finally mapped back to obtain OTUs abundance.For each
305 gene, sequences were pooled together and dereplicated in order
306 to identify and count the unique sequences.
307
308 2.6 Data analyses
309 Microbial biomass and enzyme activities data were analyzed by
310 ANOVA. The significance of differences between mean values
311 were determined by the Fisher PLSD. For PCR-DGGE
312 analysis, bands were identified and their intensities were
313 measured after normalizing lanes and background subtraction
314 using Quantity-One® software (Bio-Rad Laboratories, USA).
315 Band intensities were used to calculate the Shannon-Weaver
316 diversity index H (Shannon and Weaver, 1963) according to the
317 eq. 1, using the PAST software (Hammer et al. 2001),
318
319 (eq. 1) $$H = -\sum \left(\frac{n_i}{N}\right) \log(ni/N)$$
320
321 where n_i is the relative intensity of each DGGE band, S is the
322 number of DGGE bands for each lane and N is the sum of
323 intensities for all bands in a given sample (or lane).The DGGE
324 banding pattern was clustered to UPGAMA dendrograms based
325 on Raup and Crick similarity indices (Raup and Crick, 1979)
326 using the PAST software. A principal component analysis
327 (PCA) for enzyme activity data and Shannon-Wiener diversity

index were carried out based on correlation matrix and results were displayed as biplot using PAST.

2.7 Analysis of the Illuminasequencing data

Mothur v. 1.32.1 was used for calculating diversity indexes and rarefaction curves from the OTU data (Schloss et al., 2009). The OTUs fasta sequences were analysed and annotated on NCBI with blastx and blastnusing the Blast2go software (Conesa et al., 2005). Phylogenetic trees were constructed on the aligned sequences with the PhyML (Phylogeny Maximum Likelihood) approach (Guindon and Gascuel, 2003) by applying the Shimodaira–Hasegawa [SH]-aLRT test, and alignments and tree generation were carried out using the SeaView software (Gouy et al., 2010).

3 Results

3.1 Microbial biomass and enzyme activities

Microbial biomass based on ATP content was significantly higher in the rhizosphere of the L05 maize line, as compared to its bulk soil, whereas no significant differences were observed between rhizosphere and bulk soil of the T250 maize line (Figure 1A). BAAhydrolyzingactivity was significantly higher in the rhizosphere of both L05 and T250 maize line, as compared to their respective bulk soils (Figure 1B). Moreover, BAAhydrolyzingactivity was significantly higher in the rhizosphere of the L05 than in the T250 maize line rhizosphere

(Figure 1B). Caseinasehydrolyzing activity was only enzyme activity that was significantly lower in the rhizosphere of both L05 and T250 maize line, as compared to their respective bulk soils (Figure 1C), andalso it was only enzyme activity that was significantly lower in the rhizosphere of the L05 than in the T250 maize line rhizosphere (Figure 1C). Chitinase activity was significantly higher in the rhizosphere of the L05 than its respective bulk soil, whereas there was no significant difference between rhizosphere and bulk soil of the T250 maize line (Figure 1D). Moreover, the chitinase activity was significantly higher in the rhizosphere of the L05 than in the T250 maize line rhizosphere (Figure 1D).Urease activity was significantly higher in the rhizosphere of both L05 and T250 maize line, as compared to their respective bulk soils (Figure 1E). Moreover, the urease activity was significantly higher in the rhizosphere of the L05 than in the T250 maize line rhizosphere (Figure 1D). The PCA analysis showed that ATP, Urease, caseinase hydrolysing and chitinase activities were related to each other, but not related to BAA hydrolysing activity (Figure 2).

3.2 PCR-DGGE microbial community composition

The DGGE analysis showed complex banding patterns for both *npr* and *apr* genes. The UPGAMA based on Raupand Crick's similarity index for *npr* and *apr* genes showed that the

rhizosphere and bulk soils of the L05 and T250 maize lines clustered separately, although the separation between clusters was not significant (Figure 3).The Shannon-Wiener diversity indices for the apr gene showed a significantly greater ($P<$ 0.05) diversity in the rhizosphere of both maize lines, as compared to their respective bulk soils: the diversity indices for the *npr* gene could be ranked as : T250B, > L05R > L05B > T250R (Table 1).

3.3 Protease gene quantification

The qPCR analysis showed a significantly ($P<$ 0.05) higher number of *apr gene* copies in the rhizosphere and bulk soil of the L05 as compared to the T250 maize line, whereas for the *npr* gene there were no significant differences between the copy numbers regardless of the maize line and soil type (Table 1). The PCA carried out on qPCR and, ATP data and enzyme activities showed that the rhizosphere of the high NUE L05 maize line clustered separately from the respective bulk soil and from the T250 rhizosphere and bulk soil (Figure 2). The first two axes explained 52.89% of the total variance. The PCA also showed that both *apr* and *npr* gene abundances clustered together, with higher correspondence to the BAA-hydrolazing activity than to the caseinasehydrolyzing activity (Figure 2).

3.4 Proteases high throughput sequencing analyses

Assembly of paired-reads was correctly performed for more than 99% of sequences for each sample (Supplementary Table 1). After discarding ambiguous sequences and sequences shorter than target length for *apr* gene (190 bp) and *npr*(230 bp) amplicons, the retained sequences were 49.2 % and 72.4 % for the *apr* gene and *npr* genes, respectively. A total of 9,34,598and 1,520,600 unique sequenceswere obtained for the *apr* and for the *npr* gene respectively (Table 2 a). Clustering of these sequences at 97% similarity resulted in 1767 and 1308average OTUs for the *apr* and *npr* gene, respectively(Table 2 a). Blastx results at 97% identity showed that many OTUs gave the same hits, albeit their nucleotidic sequences were different ; for this reason analyses were also performed with OTUs at 95% similarity, in this case the number of detected OTUs per samples were as expected lower, varying between 631 for T250 rhizosphere and 765 for L05 bulk sample (Table 2 b). Results herewith presented refer to the analyses of OTUs with 95% minimum identity.

The rarefaction curves (Supplemetary figure 1) indicated a representative and deep sampling of total diversity for both *apr* and *npr* genes, with Good's coverage values that were always >99.99% (Table 2 b). OTUsanalyses were conducted on the first 50 most abundant OTUs covering 74% of total OTUs diversity for *apr* and 85.4% for *npr* (Supplementary Table 1 a and 1 b). The most abundant *apr* OTUs revealed high

phylogenetic similarity with *Pseudomonas*sp, followed by *Caulobacter*sp.and *Dickeya*sp.(Figure 4A). Both hierarchical clustering (Supplementary figure 6 and 7) and PCA (Figure 5A, B) analyses indicated that the protease gene diversity was influenced by the maize line, less from the rhizosphere or bulk soil, particularly for the T250 maize line (Figure 5A, B).

Analysis of the most abundant *npr* OTUs revealed that majority of OTUs assigned to uncultured bacteria; most of the others showed highphylogenetic similarity with members *of Bacillus sp.*(Supplementary Table 1b). Based on their sequences, the*npr* OTUs were more diverse than *apr* OTUs (Figure 4).

Multivariate analyses were conducted to explore the discrimination between samples, and to identify OTUs mostly responsible for differences. Both hierarchical clustering (Supplementary figure 1 and 3) and PCA (Figure 5) analyses indicated that the samples are grouped mostly according to the plant variety, and not to the soil sampling position (bulk or rhizosphere). For the T250 variety, bulk and rhizosphere samples are closely grouped, while for L05 variety differences between rhizosphere and bulk soil *apr* OTUs patterns were higher. PCA also highlighted a number of OTUs that were more related to samples, especially for L05 bulk and rhizosphere (Figure 5).

4 Discussion

With the exception of the casein hydrolyzing activity, all enzymatic activities and microbial biomass were found to be higher in the rhizosphere of the L05 maize as compared to T250 maize line, indicating that the high NUE L05maize line has a higher N mineralization rate in the rhizospherethan the low NUE T250 maize line. These results are in agreement with previous reports on the greater capability of the L05 maize line to acquire N from the rhizosphere (Zamboni et al., 2014; Pathan et al., 2015). The rhizosphere of the L05 and T250 maize lines also differed for the type of protease activities, as the L05 rhizosphere displayed a higher BAA-hydrolyzing activity whereas the rhizosphere of the T250 had a higher caseinhydrolyzing activity (Figure 1). It is important to note that casein hydrolysing activities probably measured protease activity acting on high molecular weight substrates that generally are associated to microbial death events or release of extracellular enzymes degrading organic polymers (Nannipieri et al., 2012). These differences could depend on different factors including genetic diversity of the protease encoding genes, molecular integrity and extracellular stabilization of different proteases by the rhizosphere organic matter (Bonmati et al., 2009, Overall these results indicated that in the rhizosphere of the two maize lines the protein N mineralization depended on different proteolytic mechanisms.

Analysis of the DGGE fingerprints indicated highercomplexity of the proteolytic communities in the rhizosphere of the L05 than thoseof the T 250 maize, showing that the two plant lines selected different proteolytic populations during the plant growth. These results are in line with those of Sakurai et al. (2007) who also reported rhizosphere effects on the diversity of the *apr* gene.Gene copy numbers were also significantly affected for the *apr* gene. These results support overall positive rhizosphere effect of high NUE on the *apr* as compared to the *npr*, as shown by the significantly higher apr abundance in rhizosphere of L05 than T250 (Table 1).Previous studies on Maize rhizosphere by Aira et al. (2010), revealed that different genotypes modifies the structure of rhizospheric microbial communities, but not their abundance and no significant changes in biomass of main microbial groups were reported. But in our studies we have noticed significant changes in copy numbers of *apr* gene, but no significant changes in abundance of *npr* gene.

Our results based on the composition of the proteolytic community of the rhizosphere and bulk soil of the two maize lines indicate asignificantly higher richness for *npr* than *apr* gene, and significant differences betweenrhizosphere of L05 and T250 maize lines. Analysis of OTUs confirmedresults by Watanabe and Hayano (1994a, 1994b) that *Bacillus* spp. are the main source of *npr* genes in soil. However, several unknown

metallo-peptidase *npr*gene sequences outnumbered other known OTUs in both rhizosphere and bulk of the studied maize lines. This is indeed the first work dealing with the high-throughput assessment of protease genes in bulk and rhizosphere soils. Results indicate a high diversity of these genes in soil, as shown by the number of unique sequences and OTUs. However, together with the high number of unassigned sequences suggest that our current knowledge on the abundance and distribution of the protease encoding genes in soil is still very limited. Taken together, the genetic and biochemical analysis of the rhizosphere of the both maize lines indicated that the L05 maize line with higher NUE selected more strongly the proteolytic microbial communities in the rhizosphere as compared to the low NUE T250 maize line, with potential influence on the predominant protease mechanism. In fact, while the BAAhydrolyzing activity has a trypsin-like protease activity, the casein hydrolyzing activity is less specific serine proteases (Ladd, 1972). It can't be excluded that a more specialized proteolytic community may contribute to the observed higher NUE of the L05that the T250 maize line.

For *apr*, the most abundant OTUs were reported to belong to different members of *Pseudomonas sp.*;this confirms previous studies reporting high *Proteobacteria* populations in maize rhizopshere(Peiffer et al., 2013). Furthermore these OTUs were significantly more abundant in the rhizosphere of the L05 than

in the T250rhizosphere. Other abundant*apr*OTUs detected in the maize rhizosphere such as *S.griseus* and *Caulobacter sp*., *N. watsoni* and *Clostridium* sp., *Brevibacillus*sp. and *Thermoactinomyces* sp.play important roles in maize growth, being involved in chitinase activity, plant pathogen biocontrol, non-symbiotic N fixationNO_3^--N reduction, or N and P mineralization (Jackson et al., 1997; Philippot et al. 2002; Bressan and Figueiredo, 2008; Peiffer et al., 2013; Yadav et al., 2013;Li et al., 2014). Interestingly, another dominant OTU was identified as a protease of *Dickeya*sp., a plant pathogen, also detected in maize rhizosphere (Chaparro et al., 2014); this may be related to the past use of the soil for maize cultivation.

Very interestingly many identified organisms contributing to both *apr* and *npr* OTUs, like *Bacillus*sp, *Paenibacillus*sp, *Clostridium* sp., *Pseudomonas* sp., *Azoarcus* sp., are genera encompassing several plant growth promoting rhizobacteria (PGPR) (Hurek and Reinhold-Hurek, 2003, Kumar et al., 2011, Goswami et al., 2015, Kefela et al., 2015). Certain plant growth promoting microbes have been found to enhance N uptake from soil, primarily by nutrient mobilization and increase plant NUE (Parra-Cota et al., 2014). Present results also support the hypothesis that most soil proteolytic communities may also play important roles as PGPRs, thus supporting their involvement in soil fertility.

5. Conclusion

Our work showed that maize lines differing for NUE, host different microbial communities and select different protease encoding genes in their rhizosphere. In particular, the two maize lines mainly influenced the abundance and diversity of the *apr* gene than *npr* gene. Though *npr* gene was less affected by rhizosphere and plant properties , it has been unraveled that most *npr* OTUs were from unknown organisms and this suggests the need for a future research identifying hidden players behind *npr* gene pool. NUE-dependent selective effect also results in differences in the functional potential of the rhizosphere microbial communities and apparently in the mechanisms responsible for the protein N mineralization. Future research should also characterize the N forms in the rhizosphere of the two maize lines and the maize root exudate profiles to further clarify the link between the protease gene diversity and the protein N fate in the rhizosphere of the studied maize lines.

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Table 1. Gene copy numbers and valuesof the Shannon-Weiner indices for DGGE bands for the npr and apr genes in the rhizosphere and bulk soil of the L05 and T250 maize lines. Values are shown as mean (n = 5) and standard deviation, and different superscripts indicate significant differences ($P < 0.05$) of values within each column.

Soil	apr gene		npr gene	
	Copy numbers and standard deviation			
L05R	$2.7 \cdot 10^{5\,a}$	$\pm 1.5 \cdot 10^{5}$	$7.1 \cdot 10^{7\,a}$	$\pm 1.1 \cdot 10^{7}$
L05B	$1.8 \cdot 10^{5\,a}$	$\pm 1.4 \cdot 10^{5}$	$5.9 \cdot 10^{7\,a}$	$\pm 1.1 \cdot 10^{7}$
T250R	$1.2 \cdot 10^{5\,d}$	$\pm 8.8 \cdot 10^{4}$	$4.2 \cdot 10^{7\,a}$	$\pm 1.2 \cdot 10^{7}$
T250B	$1.4 \cdot 10^{5\,c}$	$\pm 6.5 \cdot 10^{4}$	$3.0 \cdot 10^{7\,a}$	$\pm 5.3 \cdot 10^{6}$
	Shannon-Weiner diversity index(H) and standard deviation			
L05R	1.36^{b}	±0.006	2.03^{a}	±0.018
L05B	1.33^{c}	±0.020	1.73^{c}	±0.009
T250R	0.69^{d}	±0.001	2.04^{a}	±0.004
T250B	1.55^{a}	±0.021	1.92^{b}	±0.003

Table 2(a). Preprocessing and OTUs clustering of *apr* and *npr* Illumina reads.

Label	Gene	Sample	n of paired sequences assembled (%)	n of sequences passing filter[a]	total n of unique sequences	final n of OTUs at 97%	final n of OTUs at 95%
D1	apr	L05_rhizo	1,435,410 (99.8%)	867,314 (60.4%)	934,598	1763	1136
D2	apr	L05_bulk	1,215,724 (100%)	879,698 (72.4%)		1844	1201
D3	apr	T250_rhizo	1,110,522 (99.9%)	766,169 (69.3%)		1797	1183
D4	apr	T250_bulk	1,185,430 (99.9%)	677,685 (57.2%)		1664	1041
D5	npr	L05_rhizo	1,480,616 (99.8%)	728,431 (49.2%)	1,520,600	1331	712
D6	npr	L05_bulk	1,367,344 (99.8%)	938,675 (68.6%)		1421	765
D7	npr	T250_rhizo	1,399,313 (99.9%)	806,304(57.6%)		1242	631
D8	npr	T250_bulk	1,185,430 (99.9%)	804,063 (63.7%)		1239	639

[a]maximum error 0.5, length > 190 bp for apr, > 230 bp for npr

Table 2(b). Coverage, diversity and richness indexes in the analyzed *apr* and *npr* Illumina reads.

Label	Gene	Sample	Coverage	Simpson eveness	Inverted Simpson	Chao	Shannon eveness	non parametric Shannon
D1	apr	L05_rhizo	99.99%	0.039	44.66	1292.3	0.645	4.53
D2	apr	L05_bulk	99.99%	0.031	37.82	1318.7	0.626	4.44
D3	apr	T250_rhizo	99.99%	0.036	43.45	1297.1	0.649	4.59
D4	apr	T250_bulk	99.99%	0.034	35.99	1117.0	0.634	4.41
D5	npr	L05_rhizo	99.99%	0.028	19.95	727.0	0.609	4.01
D6	npr	L05_bulk	99.99%	0.025	18.96	776.3	0.597	3.97
D7	npr	T250_rhizo	99.99%	0.022	14.02	658.4	0.536	3.46
D8	npr	T250_bulk	99.99%	0.022	14.71	679.6	0.542	3.51

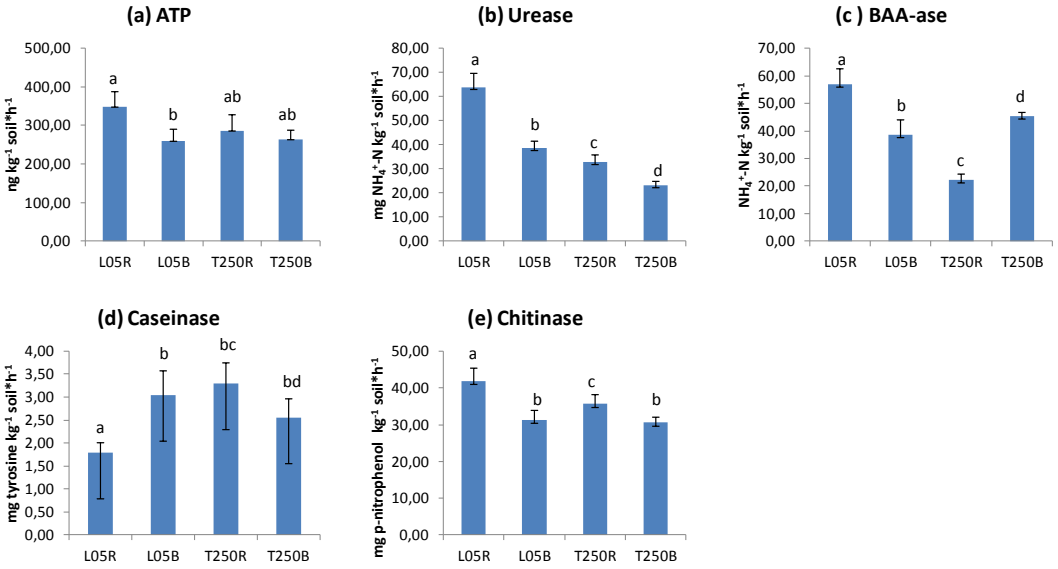


Figure 1. Activity results (a) ATP content,(b) urease activity,(c) BAA-ase activity,(d) caseinase activity,(e) chitinase activity, of the rhizosphere and bulk soil of the L05 and T250 maize lines. Values are the mean of five replicates and the error bars represent the standard deviation of the mean values. Significant differences are shown by different alphabetic letters over error bars.

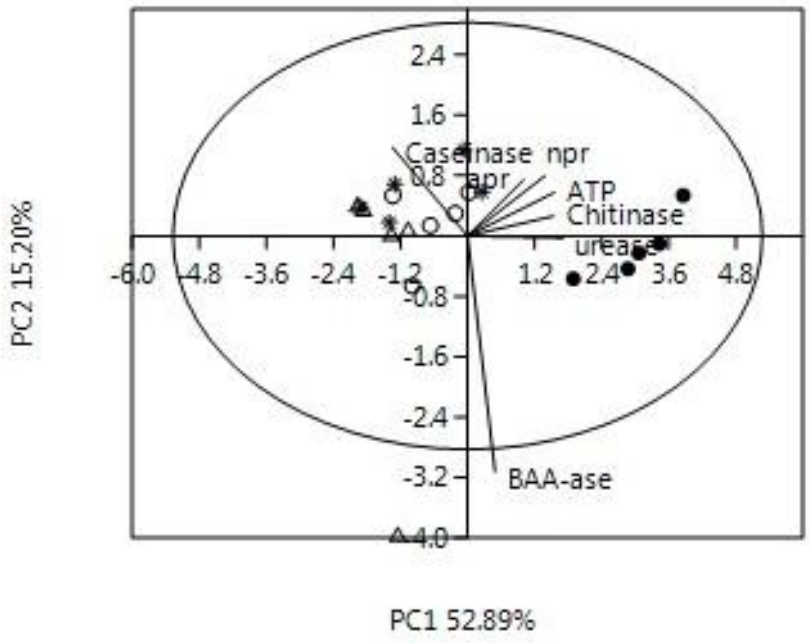
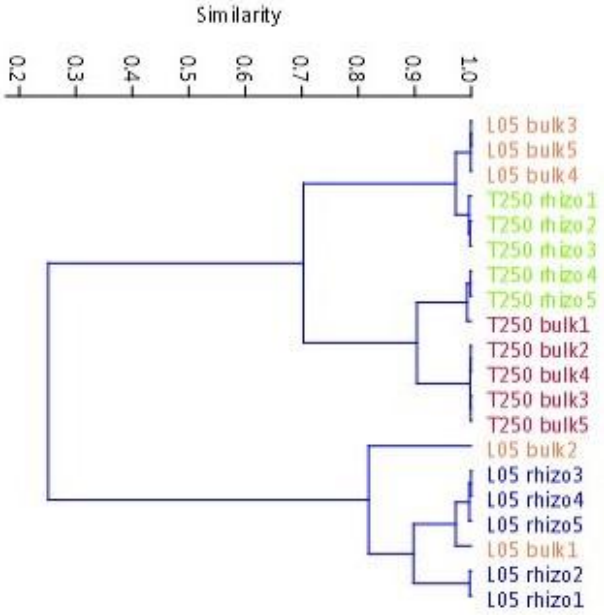


Figure 2. PCA on ATP, Urease, BAA-ase, Caesinase,Chitinase, npr gene copy numbers and apr gene copy numbers . Solid sky blue squares reperesents L05 rhizosphere samples, blue boxes represent L05 bulk samples, solid orange dots represent T250 rhizosphere samples and brown circles represent T250 bulk samples

(a)



(b)

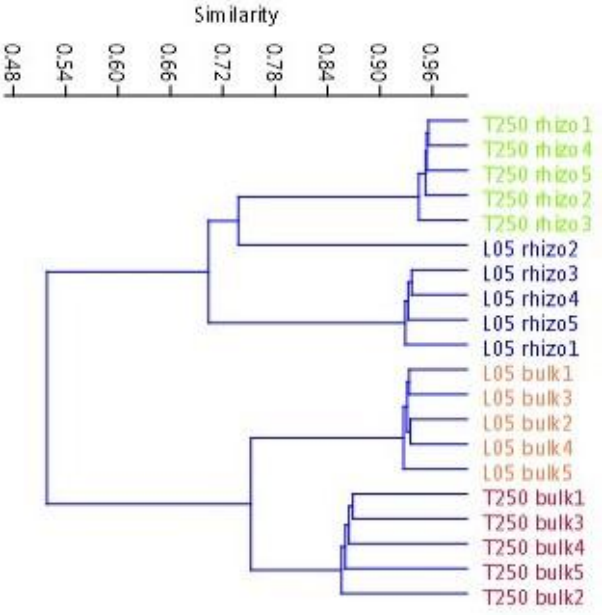
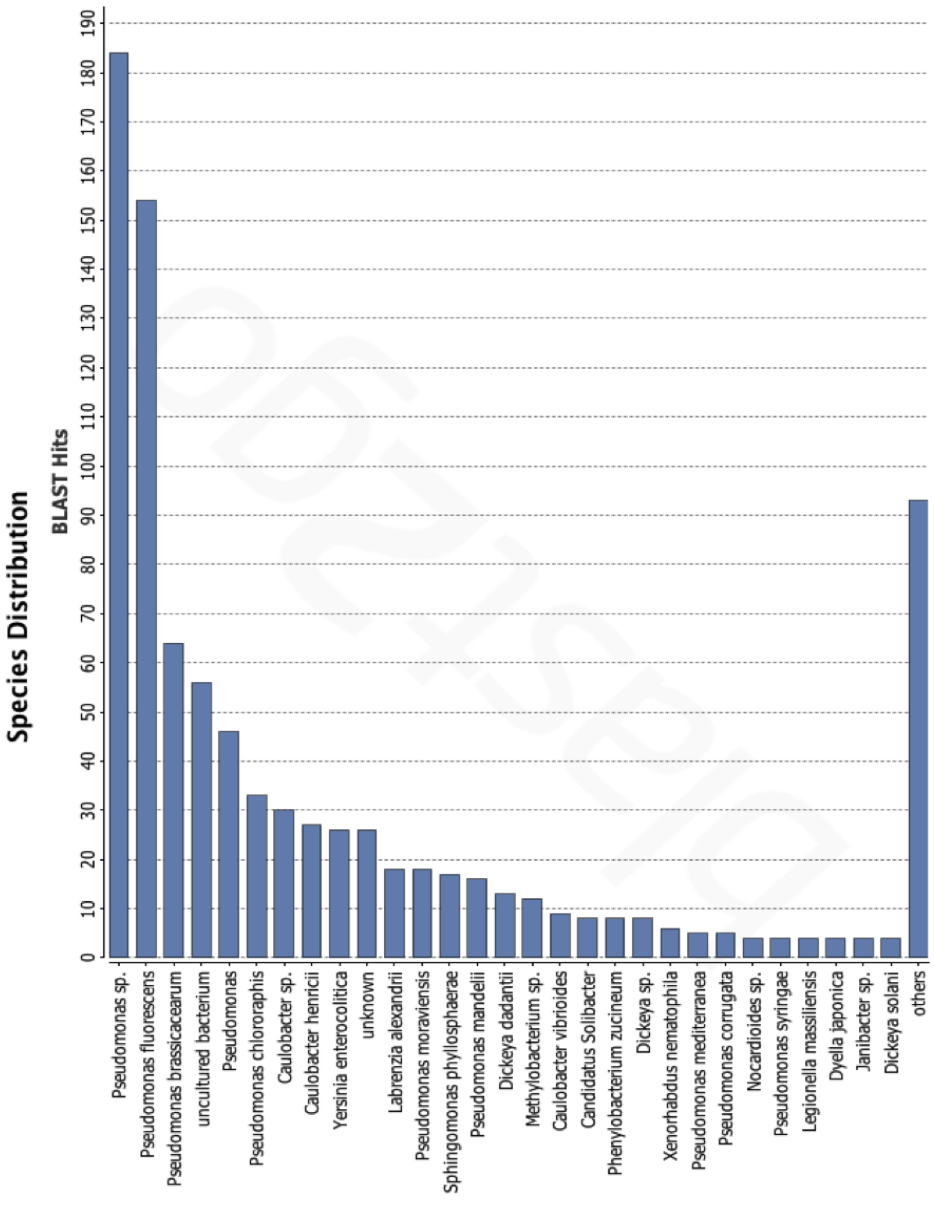
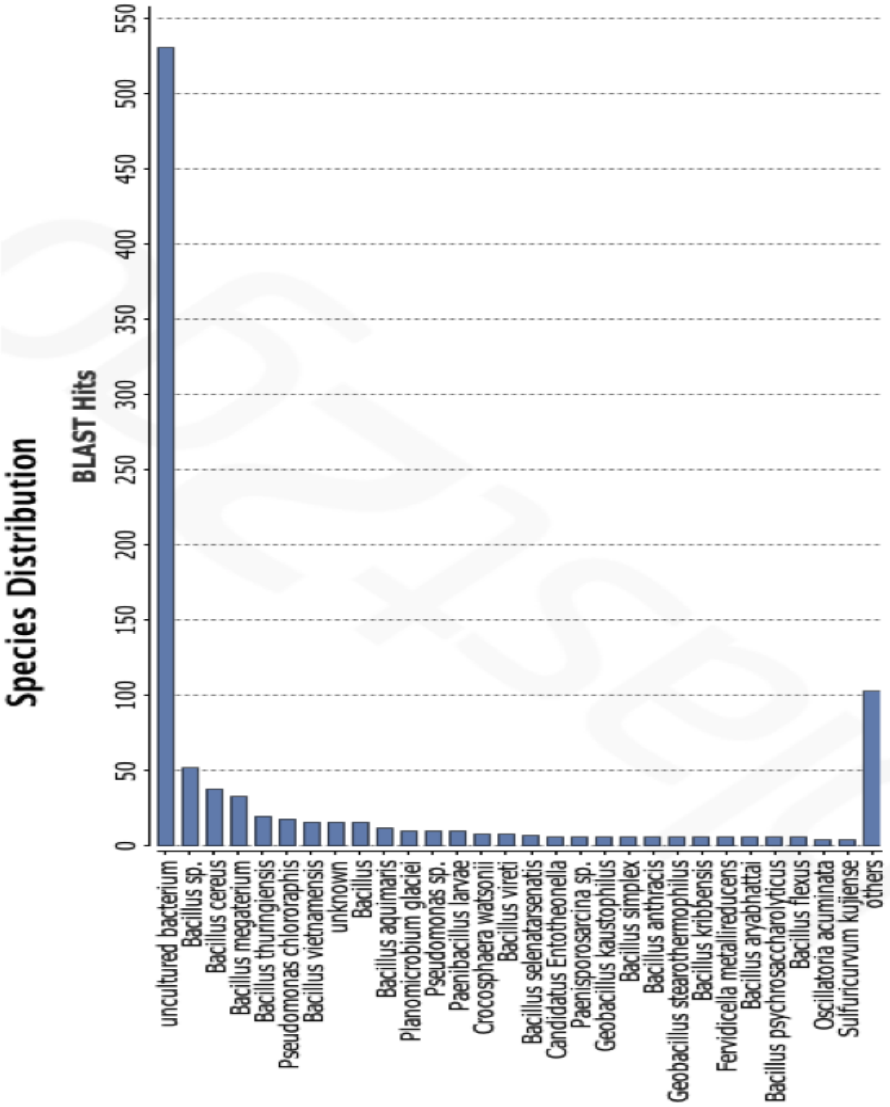


Figure 3. UPGAMA custers based on Raup Crick similarity for (a) npr gene and (b) apr gene

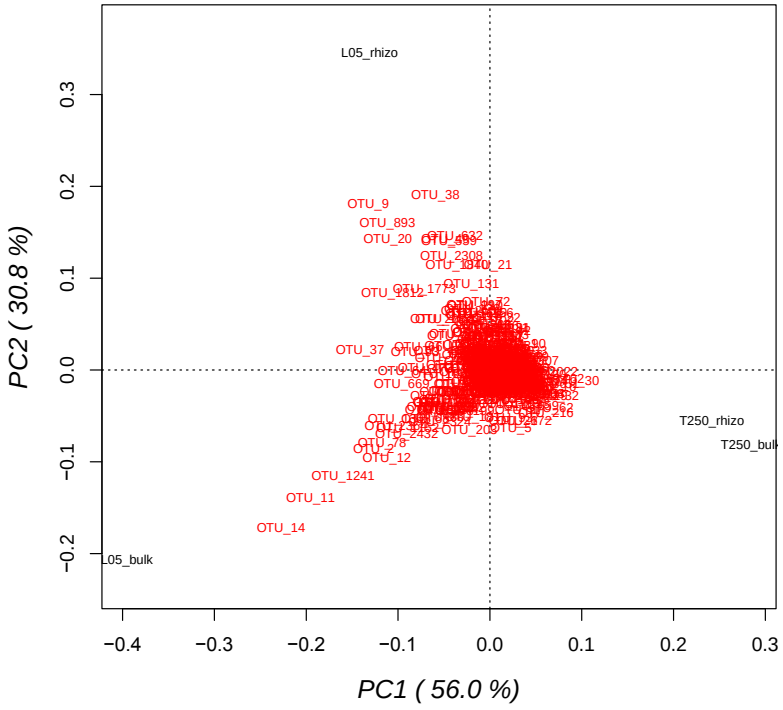

Species Distribution
BLAST Hits
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
Pseudomonas sp.
Pseudomonas fluorescens
Pseudomonas brassicacearum
uncultured bacterium
Pseudomonas
Pseudomonas chlororaphis
Caulobacter sp.
Caulobacter henricii
Yersinia enterocolitica
unknown
Labrenzia alexandrii
Pseudomonas moraviensis
Sphingomonas phyllosphaerae
Pseudomonas mandelii
Dickeya dadantii
Methylobacterium sp.
Caulobacter vibrioides
Candidatus Solibacter
Phenylobacterium zucineum
Dickeya sp.
Xenorhabdus nematophila
Pseudomonas mediterranea
Pseudomonas corrugata
Nocardioides sp.
Pseudomonas syringae
Legionella massiliensis
Dyella japonica
Janibacter sp.
Dickeya solani
others

(a)

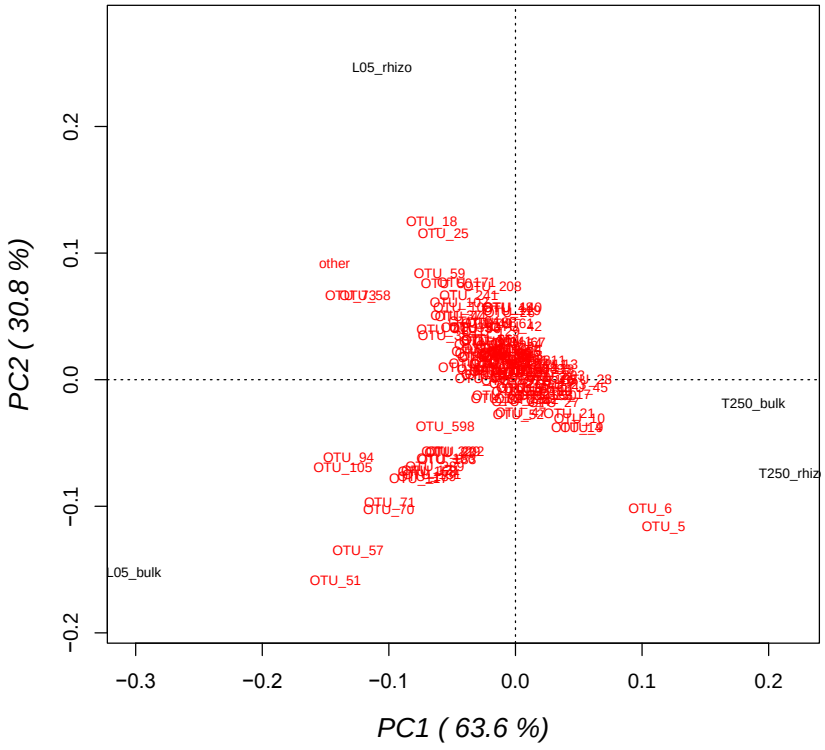


(b)

Figure 4. Species distribution of the hits of the 50 most abundant **(a)** *apr* OTUs and (b) npr OTUs


L05_rhizo
OTU_9
OTU_38
OTU_893
OTU_20
OTU_2308
OTU_21
OTU_131
OTU_37
OTU_12
OTU_1241
OTU_11
OTU_14
L05_bulk
T250_rhizo
T250_bulk
PC2 (30.8 %)
PC1 (56.0 %)
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3

(a)


L05_rhizo
OTU_18
OTU_25
other
OTU_59
OTU_598
OTU_94
OTU_105
OTU_71
OTU_70
OTU_57
OTU_51
OTU_6
OTU_5
L05_bulk
T250_bulk
T250_rhiz
PC2 (30.8 %)
PC1 (63.6 %)
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2

(b)

Figure 5 Principal Component Analysis of (a) *apr* OTU data and (b) npr OUT data covering the 99.9% of total diversity. Samples grouping is reported, together with ordiplot of OTUs scores.

Supplementary Material for online publication only

Click here to download Supplementary Material for online publication only: supplementary materials_3_sep_15.docx