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Short communication

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

This study was carried out to understand the interplay of plant Nitrogen Utilizing Efficiency (NUE) with protease activity and microbial proteolytic community composition in the rhizosphere and bulk soils. Protease activity, diversity and abundance of protease genes (using DGGE and qPCR respectively of two key bacterial protease encoding genes: alkaline metallo-peptidase (*apr*) and neutral-metallopeptidases (*npr*)) were monitored in both rhizosphere and bulk soils from two maize in-bred lines L05 and T250 with higher and lower NUE respectively, using a rhizobox approach. Illumina sequencing was employed to assess the diversity of proteolytic communities encoding for the above-mentioned protease genes. Our results show higher enzyme activity, higher abundance and diversity of proteolytic genes in L05 maize rhizosphere, with higher NUE than in T250 maize rhizosphere.

NUE is an inherent plant characteristic, regulated by complex genetic and metabolic factors (Xu et al., 2012; Ngezimana and Agenbag, 2014; Zamboni et al., 2014). There are increasing evidences that plant NUE depends on microbial activity in the rhizosphere, particularly on the activity of the proteolytic communities (Mooshammer et al., 2014). Studies have indicated that metalloproteases of bacterial origin mainly contribute to the measured soil protease activity (Hayano et al., 1987; Bach and Munch, 2000; Kamimura 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,b). Although it has been reported that the release of root exudates increases N mineralizing activities (Renella et al., 2007), information on the interactions between the proteolytic microbial communities of the rhizosphere and the plant NUE is unknown. Next generation sequencing technologies (NGS) provide advanced tools to analyze microbial genes in soil. As far as we know, this approach has not been applied yet for assessing the abundance and diversity of proteolytic genes in soil. We hypothesized that plants with different NUE select different proteolytic microbial communities characterized by different levels of proteolytic activity in the rhizosphere. To test our hypothesis, we studied the composition of the proteolytic microbial communities and proteolytic activities in the rhizosphere and bulk soil of the L05 and T250 maize lines, characterized respectively by high and low NUE.

A sandy clay loam Eutric Cambisol (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 was sieved through a 2 mm sieve, at field moisture after removing visible plant materials; 600 g soil were used to prepare rhizoboxes in which L05 and T250 maize lines were grown in 5 replicates each, and watered, grown and sampled as reported

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by Pathan et al. (2015). Rhizosphere (R) and bulk soil (B) samples of the L05 and T250 maize lines were named as L05R, L05B, T250R and T250B, respectively. Enzyme assays were performed immediately after sampling and for nucleic acids samples were preserved at −20 °C till extraction. The N-benzoyl-L-argininamide (BAA) and Casein-hydrolyzing activities (protease activities) were determined according to Ladd and Butler (1972) and Nannipieri et al. (1974), respectively. DNA was extracted as per Ascher et al. (2009) using FastDNA spin kit for soil (MP Biomedicals, USA) and only intracellular DNA was used for further studies.

Primers FaprI/RaprII (amplicon length 194 bp) for *apr* gene and FnprI/RnprII (amplicon length 233 bp) for *npr* were used as mentioned in Bach et al. (2001) and employed for PCR-DGGE, qPCR and Illumina analyses. The PCR-DGGE conditions for *apr* and *npr* amplicons were adapted from Sakurai et al. (2007). qPCR and sequencing amplicons were prepared as mentioned by Bach et al. (2001). Sequencing was performed on an Illumina HiSeq 2000, in paired-end 150 × 2 bp at the Beijing Genomics Institute. For Illumina sequencing, five replicates of each soil type were pooled together for an in depth analysis of all gene sequences. Enzyme activities data, qPCR data and diversity data based on DGGE were analyzed by ANOVA. The significance of differences between mean values were determined by the Fisher PLSD.

With the exception of the Casein-hydrolyzing activity, other enzymatic activity (BAA-hydrolyzing activity) and microbial biomass were higher in the rhizosphere of the L05 maize as compared to T250 maize line, indicating that the high NUE L05 maize line has a higher N mineralization rate in the rhizosphere than the low NUE T250 maize line (Supplementary Fig. 1). L05 rhizosphere displayed as well a higher BAA-hydrolyzing activity whereas the rhizosphere of the T250 had a higher Casein-hydrolyzing activity.

Principal Component Analysis (PCA) on enzyme activities, DGGE and qPCR data 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 (Fig. 1). The PCA also showed that both *apr* and *npr* gene abundances and diversity clustered together, with higher correspondence to the BAA-hydrolyzing activity than to the Casein-hydrolyzing activity (Fig. 1). In fact, while the BAA hydrolyzing activity has a trypsin-like protease activity, the Casein-hydrolyzing activity is a less specific

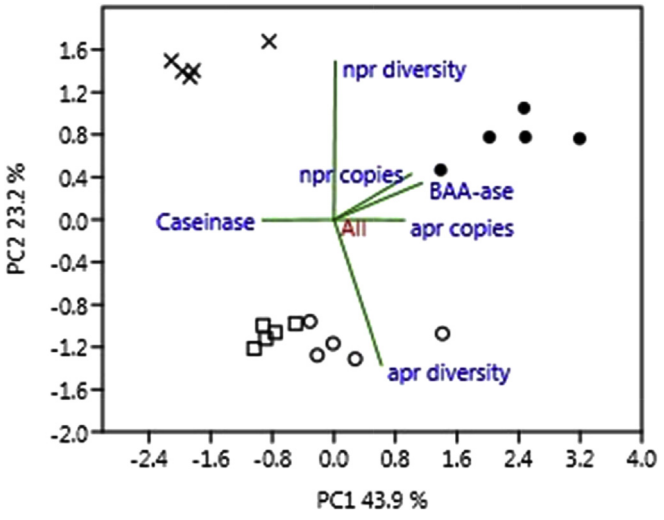

Fig. 1. PCA on BAA-ase, Caesinase, npr gene copy numbers, apr gene copy numbers and diversity of apr and npr genes based on Shannon–Weiner indices of DGGE bands. Solid dots represent L05 rhizosphere samples, circles represent L05 bulk samples, cross represent T250 rhizosphere samples and squares represent T250 bulk samples.

serine protease (Ladd and Butler, 1972). It can't be excluded that a more specialized proteolytic community may contribute to the observed higher NUE of the L05 than the T250 maize line.

The DGGE analysis showed complex banding patterns for both *npr* and *apr* genes. 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 ranked T250B > L05R > L05B > T250R (Table 1). These results are in line with those of Sakurai et al. (2007) who also reported rhizosphere effects on the diversity of the *apr* gene. 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). Both DGGE and qPCR results support overall positive rhizosphere effect of higher NUE plant on the *apr* as compared to the *npr*. However, it cannot be ruled out that different (one week) length of growth time of the two maize-lines prior to analysis may have been also responsible for differences in enzyme activities and gene abundance. Previous studies on Maize rhizosphere by Aira et al. (2010), reported effect on composition of rhizospheric microbial communities, but not on their abundance and on biomass of main microbial groups. But, in our studies we have noticed significant changes, also in abundance of *apr* gene.

Illumina data were analyzed with USEARCH and UPARSE pipelines (Edgar, 2010, 2013), and resulted in an average of good quality assembled sequences of 797,716 for apr and 819,368 for npr. Number of unique sequences was very high, respectively 934,598 for apr and 1,520,600 for apr. Rarefaction curves showed that the sequencing covered the vast majority of the observed diversity, with Good's coverage >99.9% for both genes. Species distribution of 50 most abundant OTUs based on number of BLAST hits were calculated using blast2go software (Conesa et al., 2005).

OTUs clustering at 95% similarity level revealed that the most abundant *apr* OTUs had high phylogenetic similarity with *Pseudomonas* sp., whereas most *npr* OTUs were from unknown sources and most others showed phylogenetic similarity with *Bacillus* spp. (Fig. 2(a) and (b)). The functional annotation showed that the most abundant apr OTU were annotated as unassigned peptidases of subfamily M10B, whereas a number of npr OTUS were identified as thermolysins and stearolysins from different *Bacillus* species (Supplementary Table 1(a) and (b)).

Illumina OTUs analyses are in agreement with results by Watanabe and Hayano (1994a,b), who reported that *Bacillus* spp. are the main source of *npr* genes in soil. However, several unknown metallo-peptidase *npr* gene sequences outnumbered other known

Table 1
Gene copy numbers and values of 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 for $P < 0.05$ (one way ANOVA and Fisher's PLSD test), of values within each column.

Soil type	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	$1.2 \cdot 10^{5\ b}$	$\pm 8.8 \cdot 10^{4}$	$4.2 \cdot 10^{7\ a}$	$\pm 1.2 \cdot 10^{7}$
T250B	$1.4 \cdot 10^{5\ b}$	$\pm 6.5 \cdot 10^{4}$	$3.0 \cdot 10^{7\ b}$	$\pm 5.3 \cdot 10^{6}$
Shannon–Weiner diversity index (H) and standard deviation				
L05R	1.359^{a}	±0.006	2.032^{a}	±0.018
L05B	1.328^{a}	±0.020	1.729^{b}	±0.009
T250R	0.691^{b}	±0.001	2.040^{a}	±0.004
T250B	1.554^{c}	±0.021	1.919^{c}	±0.003