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Restoration of nitrogen cycling community in grapevine soil by a decade of organic fertilization

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ABSTRACT

Traditional intensive agricultural practices negatively impact soil quality, leading to increased erosion in the Mediterranean region. In contrast, sustainable management practices that have utilized organic fertilization over a decade improved soil quality in the Spanish "Pago Casa Gran" vineyard, Valencia. Grape prunings, combined with sheep manure or leguminous cover, have improved soil chemical and biological parameters, such as organic carbon, nitrogen content, respiration, PLFA, total DNA and aggregation, however, there has been a lack of information on their effect on specific functional microbial groups. The nitrogen cycling community of the soil was investigated by quantification of genes involved in key pathways, in particular nitrogen fixation, denitrification and nitrification. The abundances of bacterial *nifH*, *nosZ*, *nirS* and *nirK* genes significantly increased under a decade of organic fertilization when compared to inorganic fertilization, and was linked to increased soil organic carbon. The abundance of *nifH* was lower where fertilizers rich in ammonia and nitrate were used and its increase under organic fertilization was more related to the availability of organic carbon than to the nature of the organic amendment. Archaeal *amoA* abundance did not correlate with the type of fertilization and the bacterial *amoA* abundance was more dependent on the availability of soluble nitrogen than on the type of management. An increase in *nosZ* under organic fertilization may suggest a greater abundance of denitrifiers with the ability to reduce nitrous oxide to nitrogen gas, reducing greenhouse gas emission. Our results indicate that soil microbial communities involved in biological nitrogen cycling, in particular nitrogen fixation and denitrification, are more abundant under management practices that include organic fertilization when compared to traditional agricultural practices. It may therefore be recommended that organic fertilization be utilized in agricultural systems to assist with sustaining healthy soils.

1. Introduction

Soil degradation worldwide is accelerated as a result of urbanization and intensified farming practices utilizing extensive irrigation, with increased use of agrochemicals and heavy machinery (Tilman et al., 2002; Zalidis et al., 2002; Li et al., 2015). While intensive arable farming degrades the physicochemical, biochemical and microbiological properties of soil (Caravaca et al., 2002; Bellamy et al., 2005), sustainable agricultural management practices, which include soil enrichment with various sources of organic matter, such as manure, compost and oat straw, can enhance soil organic carbon, aggregate stability, water retention, and improve soil function and fertility (Pérez-Piqueres et al., 2006; Johnston et al., 2009; García-Orenes et al., 2010; 2013; Morugán-Coronado et al., 2015; Prosdocimi et al., 2016). Understanding the influence of agricultural land management practices on soil health and microbial communities is particularly important under semi-arid conditions (García-Orenes et al., 2013), where intensive arable agriculture causes a progressive decline in soil organic matter and associated soil fertility (Caravaca et al., 2002). Organic, rather then conventional, fertilization recovered microbial communities in grapevine soils to levels observed in nearby forests in Chile (Castañeda et al., 2015) and has been implemented to increase plant productivity, reduce chemical inputs, and increase the sustainability of such agroecosystems (Macci et al., 2013).

Microbial biomass, enzymatic activity, respiration rate as well as microbial carbon and nitrogen (N) content are all related to the mineralization and immobilization rates of N (Alef et al., 1988; Hart et al., 1994;Barrett and Burke, 2000; Bengtsson et al., 2003). The major natural input of available N into the biosphere is through prokaryotic N fixation by diazotrophs. The assimilation of inorganic N by soil

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microorganisms is critical for N retention in soil and for reducing the loss of fertilizer N from the environment (Vinten et al., 2002; Tahovská et al., 2013), particularly in degraded agricultural land. In addition, N is often applied in forms that are less efficient or unavailable for uptake by plants, such as urea (Witte, 2011) or organically bound N, making microbial N cycling a key process required for N transformation in soils (Fitter et al., 2005; Wallenstein and Vilgalys, 2005; He et al., 2010). Nitrification and denitrification are major pathways in the soil N cycle, involving ammonia oxidation and nitrate and nitrite reduction to N_2O and N_2 (reviewed by Teixeira and Yergeau, 2012), respectively. Elucidating the impact of N application practices on microbial diversity and community structure in general, and on N cyclers in particular, is pivotal to identifying agricultural practices that enhance the soil potential for microbial immobilization and transformation of fertilizer N.

Organic fertilization has been tested for the last decade in Mediterranean grapevine production in the Valencia province, Spain, and has resulted in improved soil fertility and quality indicators, with increased microbial diversity (determined by phospholipid fatty acid analysis; PLFA) and enhanced biological activity, when compared to conventional agrochemical inorganic fertilization (García-Orenes et al., 2016). However, no information could be deduced about the functional microbial groups involved in biogeochemical processes, importantly, the N cycle. The main aim of this study was to determine the impact of a decade of organic fertilization practices on the N-cycling microbial community in this grapevine soil.

Genes associated with the N cycle have been measured using quantitative polymerase chain reaction (qPCR), to make inferences about the function of soil microbial communities associated with soil geomorphology and land-use (Colloff et al., 2008), pasture management (Wakelin et al., 2009), N-fertilisers (Okano et al., 2004; Cavagnaro et al., 2008) and tillage (Cavagnaro et al., 2008). Functional N genes, including the N fixation gene *nifH*, the nitrification *amoA* genes (in both bacteria and archaea) and, in particular, the denitrification genes *nir*K, *nir*S and *nos*Z, have been used as targets in molecular analysis of soil N cycling potential (reviewed in Teixeira and Yergeau, 2012). The influence of organic fertilization on the populations of soil N cyclers was investigated by measuring the abundance of these N-cycling genes, with the aim of determining the best sustainable management strategy to enhance soil potential for N cycling.

2. Materials and methods

2.1. Study site

This research was conducted at “Pago Casa Gran” vineyard located in Moixent municipality in Valencia Province (eastern Spain) (coordinates 38° 49´ 24´´N, 0° 48´ 17´´W). The climate is typically Mediterranean, semiarid, with a mean annual precipitation of 413 mm and a mean annual temperature of 16.3 °C. The experimental area is cultivated with grapevine (*Vitis vinifera* L.). The soil was classified as Haploxerept (Soil Survey Staff, 2014) with a clay-loam texture (44% sand, 20% clay and 43% silt) and initial values of total organic C of 9.3 g kg^{-1}, total N of 0.35 g kg^{-1} and $CaCO_3$ of 35.6% (García-Orenes et al., 2016).

2.2. Experimental design

Three plots of 3.4 ha each, with different agricultural management practices, were established in a vineyard in 2005: 1) OPM (organic using pruning + manure): annual application of prunings from disease-free grapevine (performed in January, consisting of chipped pruned branches and weeds from a previous harvest with C/N ratio of approx. 80) as well as manure from sheep (0.63% N, 0.27% P_2O_5 and 0.81% K_2O), at a rate of 20 Mg ha^{-1} yr^{-1} with subsequent shallow ploughing (total N added in manure is estimated as 126 kg ha^{-1} yr^{-1}), 2) OPL (organic using pruning + legume cover crop): annual application of prunings from visually disease-free grapevine, shallow ploughing and sowing of a native leguminous crop cover (vetch, *Vicia villosa*) in October at a rate of 50 kg ha^{-1} yr^{-1} (total N added as leguminous is estimated as 15 kg ha^{-1} yr^{-1}) and 3) IF (inorganic fertilization): the prevalent local management system, fertilization with NPK 8/4/12 at a rate of 250 kg ha^{-1} yr^{-1} (in this type of fertilizer the N is applied as ammonium nitrate (NH_4NO_3) to reach a total of 8% of N), application of herbicide glyphosate 5 times per year and ploughing (total N added as fertilizer is estimated as 20 kg ha^{-1} yr^{-1}).

2.3. Soil sampling

In March 2014, six soil samples from each of the three vineyard plots (OPM, OPL and IF) were collected by García-Orenes et al. (2016) in a randomised manner. The soil samples within each plot were 10 m apart and 0–15 cm deep and were collected and treated as described by García-Orenes et al. (2016). An aliquot of each fresh field-moist soil sample was frozen at −20 °C and stored for molecular analysis (DNA extraction and qPCR). The biological, chemical and physical properties of the soil were reported by García-Orenes et al. (2016).

2.4. DNA extraction and quantitative PCR (qPCR) gene analysis

A subsample of 0.25 g soil from each soil sample (6 samples per treatment) was used for total DNA extraction using a DNA PowerSoil kit (Mo Bio, Carlsbad, USA) according to the manufacturers instructions with the following modifications: soil was vortexed at 300 rpm on an STD 3500 Shaker VWR for 20 min, and DNA eluted in a final volume of 65 uL elution buffer. The required modifications were determined by testing these parameters prior to final DNA extractions and measuring the DNA yield using a nanodrop (ND-1000 spectrophotometer, NanoDrop Technologies) as well as testing the final quality of the DNA using agarose gel electrophoresis for high molecular weight, intact, total DNA.

Quantitative PCR (qPCR) analyses were used to quantify the total bacterial functional gene pools, related to the N cycle, in the soil. Bacterial denitrifiers were assessed by amplifying nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*) genes. N fixers were assessed by amplifying the nitrogenase (*nifH*) gene and archaeal and bacterial nitrifiers (ammonium-oxidizing) were assessed by amplification of the archaeal or bacterial *amoA* gene (referred to as *amoA*-arch or *amoA*-B respectively). Amplification of the *16S rRNA* gene was used to quantify total bacteria in samples. Details of the primer sets utilized, and qPCR cycling conditions for each primer set, are summarized in Table 1.

For qPCR analysis each sample was assayed in triplicate (three technical replicates of each of the six independent replicates = 18 qPCR reactions per gene per treatment) on a CFX96 Touch Real-Time PCR detection system (Bio-Rad laboratories, CA, USA). The qPCR reaction mixture contained 12.5 µL of 2x iQ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA), 1 µL each forward and reverse primer to a final concentration of 400 nM, 1.25 µL 50 mg mL^{-1} ultrapure BSA (Invitrogen, CA, USA), 0.5 µL PCR grade DMSO (Sigma-Aldrich, MO, USA), 1 µL of DNA template and RNase/DNase-free water to a final volume of 25 µL.

Standards containing 10^7 – 10^1 copies of each target sequence were produced by cloning PCR products from soil DNA amplification products into pGEM-T using a pGEM-T Easy Vector System II (Promega, WI, USA); plasmids were sequenced to confirm successful cloning and transformation of the target genes. In all qPCR assays, all samples were amplified in parallel with a triplicate serial dilution (10^1-10^7 gene copies per reaction) of these standards. The efficiencies of qPCR assays were determined by amplification of a serial dilution of soil DNA (5 fold dilution series, from 5 to 0.008 µL DNA per reaction), to give standard curves with efficiencies ranging from 68.9 to 88.7%, with R^2 values ranging from 0.988 to 0.997. The efficiencies and standard curves from