Microbial Ecology

Effects of Arbuscular Mycorrhizas on Ammonia Oxidizing Bacteria in an Organic Farm Soil

T. R. Cavagnaro[2], L. E. Jackson[1], K. M. Scow[1] and K. R. Hristova[1]

(1) Department of Land, Air and Water Resources, University of California Davis, One Shields Avenue, Davis, CA 95616-8627, USA
(2) School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia

Received: 11 January 2007 / Accepted: 17 January 2007 / Online publication: 16 February 2007

Abstract

Arbuscular mycorrhizal fungi (AMF) are potentially important in nutrient cycling in agricultural soils and particularly in soils managed for organic production; little is known, however, about the interrelationships between AMF and other members of soil microbial communities. Ammonia oxidizing bacteria (AOB) are a trophic group of bacteria having an enormous impact on nitrogen availability in soils and are expected to be influenced by the presence of AMF. In a field study, we utilized a unique genetic system comprised of a mycorrhiza defective tomato mutant (named *rmc*) and its mycorrhiza wild-type progenitor (named 76RMYC+). We examined the effect of AMF by comparing AOB community composition and populations in soil containing roots of the two tomato genotypes in an organically managed soil. Responses of AOB to soil N and P amendments were also studied in the same experiment. Phylogenetic analysis of cloned AOB sequences, derived from excised denaturing gradient gel electrophoresis (DGGE) bands, revealed that the organic farm soil supported a diverse yet stable AOB community, which was neither influenced by mycorrhizal colonization of roots nor by N and P addition to the soil. Real-time *Taq*Man polymerase chain reaction (PCR) was used to quantify AOB population sizes and showed no difference between any of the treatments. An alternative real-time PCR protocol for quantification of AOB utilizing SYBR green yielded similar results as the *Taq*Man real-time PCR method, although with slightly lower resolution. This alternative method is advantageous in not requiring the detailed background information about AOB community composition required for adaptation of the *Taq*Man system for a new soil.

Correspondence to: T. R. Cavagnaro; E-mail: tim.cavagnaro@sci.monash.edu.au

Introduction

Hyphae of arbuscular mycorrhizal fungi (AMF) colonize roots, increasing the absorptive surface area of the plant root system [49] and enhancing plant N capture from both organic and inorganic sources [15, 18, 21, 28]. AMF can also impact the wider soil community, including bacteria involved in N transformations [1], rhizosphere bacteria [34, 42], nematodes [6], collembolans [16], manganese oxidizers [2, 31], and plant pathogenic fungi [44].

Interactions between the ammonia oxidizing bacteria (AOB) and AMF are expected to be important given that both groups play important roles in soil N cycling [18]. AOB control the rate limiting step of nitrification, the conversion of ammonia $\left(NH_4^+\right)$ to nitrite $\left(NO_2^-\right)$ [45]. The root zones of plants are altered by AMF, and in turn, may support different communities and population sizes of AOB compared to non-mycorrhizal plants. For example, AMF can decrease pH of the rhizosphere [33, 49], increase rates of N mineralization [25], and/or increase available C. Some studies have begun to address linkages between AOB and AMF; however, clear patterns are not yet evident. Amora-Lazcano *et al.* [1] report AOB population densities (based on most probable number (MPN)) are greater in soil containing mycorrhizal than non-mycorrhizal roots. Conversely, Klopatek and Klopatek [30] report lower and higher densities of AOB (MPN) and AMF (spore counts), respectively, under tree canopies than interspaces. Linkages between nitrification and AMF are also unclear [18]. This also needs to be considered in the context of recent research that suggests that the Crenarchaeote may play a role in nitrification [51], including arable soils [33].

A major challenge in studying the ecology of AMF in the field is the establishment of appropriate controls. A mycorrhiza defective tomato mutant, named *rmc*, and its mycorrhizal wild-type progenitor, named 76RMYC+, have been used to study the impacts of AMF on plant

DOI: 10.1007/s00248-007-9212-7 • Volume 54, 618–626 (2007)

growth and nutrition [17], plant competition [11], and the wider soil community [10]. This approach avoids the unknown side effects of establishing non-mycorrhizal controls (e.g., by nonspecific fumigation) or growth of constitutively mycorrhizal and non-mycorrhizal plant species in a single experiment. The functional gene, *amoA*, a key gene in ammonia oxidation, has been used to study the population dynamics of AOB in agricultural soils [40]. Combined with denaturing gradient gel electrophoresis (DGGE) that targets the same gene [47], these methods provide ways to examine community composition and population size of AOB, instead of 16S rRNA approaches. In addition, signature regions of 16S rRNA and *amoA* genes have been successfully applied in phylogenetic analyses of AOB with a comparable resolution [46, 47].

At an organic farm, where AMF are known to increase plant N uptake [10], interactions between AMF and AOB were investigated in a field experiment using the mycorrhiza defective tomato mutant (*rmc*) and its mycorrhizal wild-type progenitor (76RMYC+), with and without nutrient addition, in conjunction with the real-time *Taq*Man polymerase chain reaction (PCR) assay for *amoA* quantification [40] and DGGE, cloning, and phylogenetic analysis for *amoA* community composition. An organic farm was the context for this study because lack of fungicide and P fertilizers increase mycorrhizal colonization, and nitrification rates are higher compared to conventional vegetable production [8, 36]. The specific aims of this study were: (1) to compare the community composition and abundance of AOB in an organically managed agricultural soil containing tomato roots colonized by AMF or with greatly reduced colonization and (2) to assess the impact of N and P addition to soil at the beginning of the growing season on the community composition and abundance of AOB at the time of tomato harvest. In addition, a real-time PCR protocol for quantification of AOB utilizing SYBR green was developed, and the results were compared to those using the *Taq*Man real-time PCR method.

Methods

Bacterial Cultures. Strains of *Nitrosospira multiformis* and *Nitrosospira tenuis* were obtained from Jeanette Norton, Utah State University, and cultured in American type culture collection (ATCC) liquid medium 929. All strains were cultured at 28°C in the dark with periodic pH adjustment.

DNA Extraction from Soil. Before DNA extraction from soils, humic acid, a PCR inhibitor, was removed from soil as follows: 2 g of soil was incubated in 4 ml of 0.1% $Na_4P_2O_7$ in 10 mM Tris–HCl buffer (pH 8.0)–1 mM ethylenediaminetetraacetic acid (EDTA), at room temperature for 10 min and then centrifuged at 6000×*g* for 10 min at room temperature. The supernatant was discarded and DNA extracted from 0.5 g of the remaining soil using a Bio 101 FastDNA SPIN kit for soil as described by the manufacturer (Bio 101, Inc., CA, USA). The quantity, relative to a DNA size marker and quality of the extracted DNA, were analyzed by electrophoresis on a 1.5% agarose gel and by spectroscopic analysis. DNA concentrations are expressed on a dry soil basis.

DGGE with AmoA Primers. For DGGE analysis, a GC clamp was attached to primer *AmoA*-1F (5′-GGGGT TTCTACTGGTGGT-3′) [47]. Two different reverse primers *amoA*-2R-TG (5′-CCCCTCTGGAAAGCCTTCTTC-3′), a perfect match for the *N. tenuis amoA* sequence, and *amoA*-2R-GC (5′-CCCCTCGGCAAAGCCTTCTTC-3′) a perfect match for the *N. multiformis amoA* sequence, were used as described previously [40] (see [3]). PCRs were performed in a PE thermocycler with a 1:20 dilution of the DNA extracts (giving 2.5 ng/µl) as a template in a total volume of 25 µl with *Ex Taq* DNA polymerase (Takara Bio Inc, Shiga, Japan) in the supplied buffer. PCR using the *AmoA*-1F/*amoA*-2R-TG primer pair was performed using the thermocycler conditions as described previously [40]. PCR using the *AmoA*-1F/*amoA*-2R-GC primer pair was performed using the following thermocycler conditions: after initial denaturation for 3 min at 95°C, amplification proceeded for 35 cycles consisting of 1 min at 95°C, 1 min at 56°C, and 45 s at 72°C, followed by a final 5-min extension at 72°C. Genomic DNA extracted from pure cultures of *N. multiformis* or *N. tenuis* using the Ultra Clean Microbial DNA Isolation Kit (MoBio, CA, USA), were included as positive controls in the PCR reactions using the *amoA*-2R-GC and *amoA*-2R-TG reverse primers, respectively. PCR products were subjected to DGGE as described previously [39, 40] and visualized by UV transillumination after staining with SYBR green. Excised DGGE bands were cloned using a Topo TA kit (Invitrogen, CA) as described previously [27]. Selected clones (three to five per excised DGGE band) were sequenced with an ABI Prism automatic sequencer (Applied Biosystems) at Davis Sequencing Inc., Davis, CA (URL: http://www.davissequencing.com/). Cloned sequences were compared to sequences in the GenBank database using BLAST. Phylogenetic inferences (including bootstrap values) were made using the cloned sequences, their closest database matches and *amoA* sequences of known AOB and the Crenarchaeote, using the program ClustalW (V.1.8; [50] http://www.ddbj.nig.ac.jp/search/clustalw-e.html]) and a neighbor joining tree constructed using the program NJPlot ([43] http://pbil.univ-lyon1.fr/software/njplot.html]). Sequence data obtained in this study were deposited in the GenBank database under accession numbers DQ103321–DQ103331.

AOB Population Size Determination: Real-Time Quantitative TaqMan PCR. Population sizes of AOB were determined using the real-time quantitative PCR (*Taq*Man) method. Extracted DNA was diluted to *ca.* 1 ng/μl, and 5 μl of this dilution was used in the real-time PCR. Real-time PCR was performed in a 25-μl reaction mixture that consisted of *ca.* 5 ng of template DNA (5 μl of soil DNA diluted to *ca.* 1 ng/μl), 12.5 μl of *Taq*Man Universal Master (Applied Biosystems, NJ, USA), H_2O, and primers and probe. The A189 forward primer [26], the *amoA*-2R′ reverse primer (modification of primer *AmoA*-2R designed by Rotthauwe *et al.* [47], see Okano *et al.* [40]), and the A337 probe [40], were used at concentrations of 300, 900, and 100 nM, respectively. The PCR protocol for *amoA* quantification was as described previously [40]. The fluorescence signal was used to calculate C_T (threshold cycle) values, using the thermocyclers internal software (7300 System SDS software, V1.2.2, Applied Biosystems, CA, USA). Copy numbers of the *amoA* gene in environmental samples were determined using an external standard curve prepared by cloning the gene in a plasmid [40]. The equation describing the relationship is $C_T = -3.96 \times \mathrm{Log}_{10}(amoA) + 44.7$, and $R^2 = 0.99$. The final DNA concentration in each reaction was taken into account in all calculations of the *amoA* copy numbers after spectrophotometric quantification of DNA concentration.

AOB Population Size Determination: SYBR Green Real-Time Quantitative PCR. Population sizes of AOB were also determined using a SYBR green real-time quantitative PCR chemistry. Extracted DNA was diluted to *ca.* 1 ng/μl, and 1 μl of this dilution was used in the real-time PCR. Real-time PCR was performed in a 10-μl reaction mixture that consisted of *ca.* 1 ng of template DNA, 5 μl of SYBR® *Premix Ex Taq* TM (2×), 0.3 μl of the A189 forward primer (10 μM), 0.9 ul of the *amoA*-2R′ reverse primer (10 μM), 0.2 μl of ROX™ Reference Dye (50×), and 2.6 μl H_2O (SYBR green reagents were supplied by Takara Bio Inc, Shiga, Japan). The PCR protocol for *amoA* quantification was as follows: 15 s at 95°C, and then 40 cycles consisting of 15 s at 95°C, 30 s at 55°C, and 31 s at 72°C, followed by a dissociation stage of 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C, using the 7300 Real-time PCR system (Applied Biosystems, CA, USA). The fluorescence signal was used to calculate C_T (threshold cycle) values, using the thermocyclers internal software (7300 System SDS software, V1.2.2, Applied Biosystems, CA, USA). Copy numbers of the *amoA* gene in three randomly selected environmental samples were determined using an external standard curve prepared by cloning the gene in a plasmid (as above) and by comparing with the results obtained by the *Taq*Man assay.

Field Experiment Design. Samples were collected as part of a larger study of the impact of mycorrhizal colonization of roots and nutrient addition on the soil ecology and productivity of fresh market organic tomato production in Yolo County California, USA. Detailed discussion of experimental design, field site description, and results of the larger study, including field site and management, plant growth and nutrition, soil aggregation, soil nematode communities, total microbial communities with phospholipid fatty acid (PLFA) analysis, and mycorrhizal colonization are reported elsewhere [10].

The field site was a tomato field on an organically managed farm, Jim & Deborah Durst Farming, in Esparto, Yolo County, California. A tomato/cover crop/alfalfa rotation is employed, and the farm has been organically managed since 1988. No composts or manures are added. The soil was a Zamora loam, a fine-silty, mixed thermic, Mollic Haploxeralfs. A bed (1 m wide) and furrow system is employed on this farm, and the plants are subsurface drip-irrigated. The experiment was established at the same time as the field was planted to commercial tomato production.

The mycorrhiza defective tomato (*Solanum lycopersicum* L.) mutant, *rmc*, and its mycorrhizal wild-type progenitor, 76RMYC+, were planted in the field on May 20th, 2003. One week after planting, in-growth cores containing 505 g of soil (collected from the top 15 cm in the area immediately surrounding the experimental plots) were installed between the experimental plants at a depth of 20–22.5 cm. The ends of the in-growth cores were covered with mesh with 1 × 2 mm openings, which allowed growth of roots into the in-growth cores. The soil added to the in-growth cores was amended with P and N as follows: low P referred to as P0 (no added P) or High P referred to as P50 [50 μg P g dry soil^{-1}, applied as $Ca(H_2PO_4)_2$], and Low N referred to as N0 (no added N), or High N referred to as N100 [100 μg N g dry soil^{-1}, applied as $(NH_4)_2SO_4$)]. The N and P treatments were applied in combination; thus, the final nutrient treatments were: P0/N0, P0/N100, P50/N0, and P50/N100.

A total of 32 soil samples were analyzed across eight treatments: genotype (two levels: 76RMYC+ or *rmc*) and nutrient addition (four levels: P0N0, P50N0, P0N100, and P50N100). Two (of four) experimental blocks (I and II) were included in the present work, with each treatment appearing twice in each block.

The experiment was destructively harvested on the 30th July 2003. Soil was removed from the in-growth cores, mixed by hand for 30 s, after which uniform subsamples were taken and soil analyses performed. Samples for DNA extraction were taken first; aggregates (5–10 mm in diameter) were collected and transferred to tubes for storage at −80°C. Triplicate soil samples (30 g moist soil) were taken, extracted with 2 M KCl, and inorganic N content determined colorimetrically using a modification

of Miranda *et al.* [37] for NO_3^- (plus NO_2^-) and Forster [14] for NH_4^+. Gravimetric moisture contents were determined after drying approximately 50 g moist soil at 105°C for 48 h. Roots were extracted from soil by wet sieving. These roots were then cleared with 10% KOH (W/V), stained with Trypan blue and colonization of roots by AM fungi determined at 20× magnification [10].

Data Analysis. For each field replicate, NH_4^+ and NO_3^- concentrations were measured in triplicate, as were DNA samples used in *Taq*Man real-time PCR quantification of *amoA* gene copy numbers. Data were analyzed using generalized linear models (GLM). Before analysis, *amoA* copy numbers were converted to copies g dry soil^{-1}. The *Taq*Man and SYBR green standard curves were compared by testing for inequalities of slopes in GLM. The *amoA* copy numbers quantified using the *Taq*Man and SYBR green methods were compared using one-way analysis of variance (ANOVA). All analysis was performed using SAS statistical software (version 8.02, SAS Institute, Cary, North Carolina).

Results

Impact of AMF and Nutrients on AOB Community Composition and Abundance. AOB community composition did not change, based on DGGE banding patterns and number of cloned sequences, in response to neither mycorrhizal colonization of roots nor to N and/or P addition to the soil (Fig. 1). DGGE analysis of amplified PCR products, using the *amoA* 1F/*amoA* 2R-GC and *amoA* 1F/*amoA* 2R-TG primer sets targeting *N. multiformis* and *N. tenuis* sequence types, identified six and five distinct bands, respectively. These bands were successfully cloned,

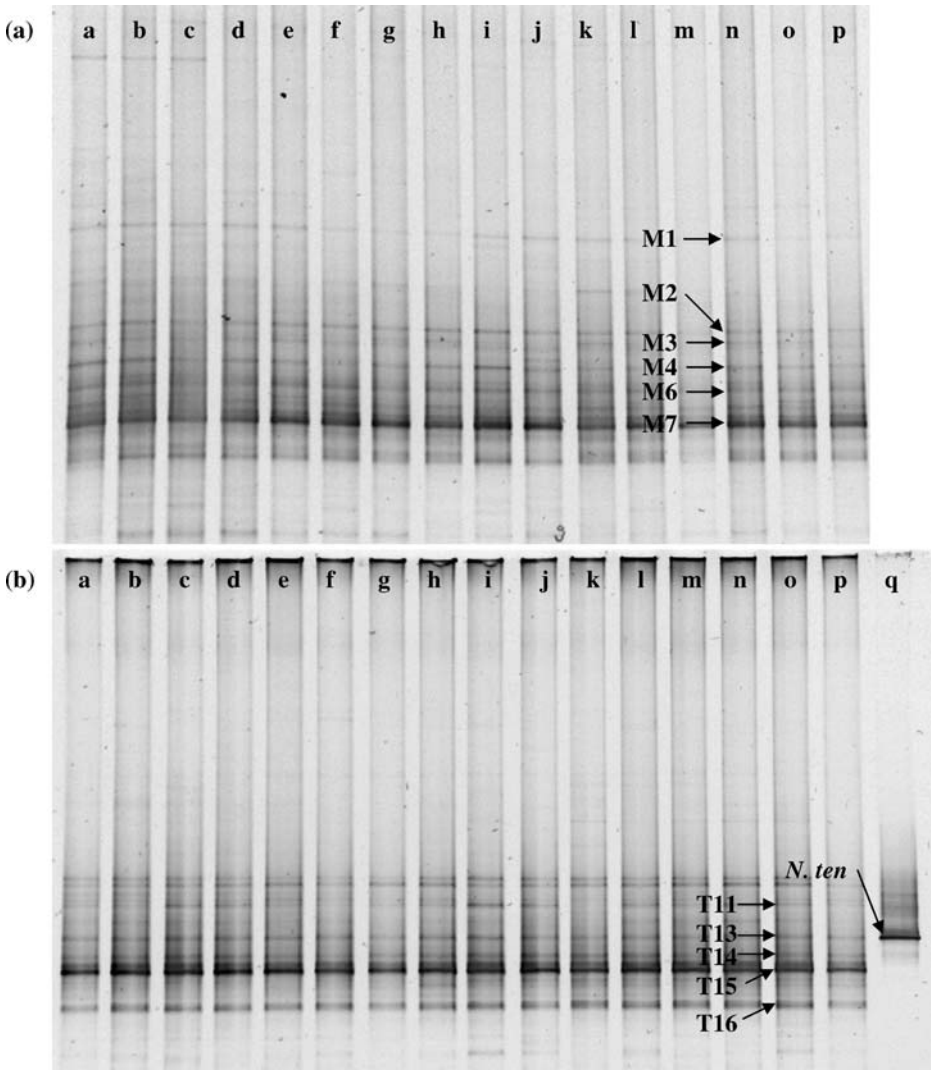

Figure 1. Detection of *amoA* bands in DNA amplified (*block I*) with **a** *AmoA*-1F/*amoA*-2R-GC primers, and **b** *AmoA*-1F/*amoA*-2R-TG primers. *Lanes* are as follows: *a* P0/N0 76RMYC+ sample 1, *b* P0/N0 76RMYC+ sample 2, *c* P50/N0 76RMYC+ sample 1, *d* P50/N0 76RMYC+ sample 2, *e* P0/N0 *rmc* sample 1, *f* P0/N0 *rmc* sample 2, *g* P50/N100 *rmc* sample 1, *h* P50/N100 *rmc* sample 2, *i* P50/N0 *rmc* sample 1, *j* P50/N0 *rmc* sample 2, *k* P50/N100 76RMYC+ sample 1, *l* P50/N100 76RMYC+ sample 2, *m* P0/N100 *rmc* sample 1, *n* P0/N100 *rmc* sample 2, *o* P0/N100 76RMYC+ sample 1, *p* P0/N100 76RMYC+ sample 2, *q N. tenuis* pure culture DNA positive control. *Arrows* and *labels* indicate bands that were sequenced.

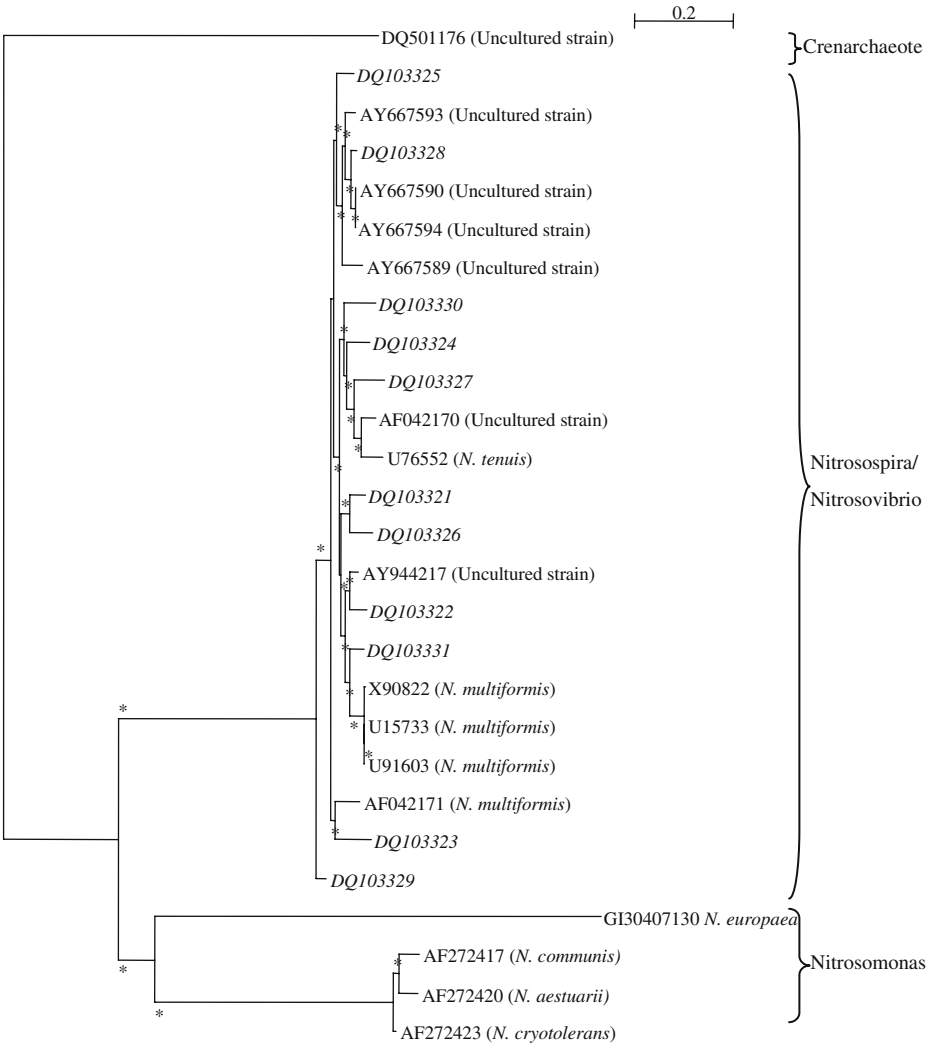

Figure 2. Phylogenetic positions of cloned *amoA* gene sequences. This tree was constructed using the neighbor-joining method. Bootstrap values greater than 75 are indicated [*asterisks* (*)] at the nodes. *Latin binomials* are sequences from bacterial isolates obtained from the GenBank database, *cloned environmental gene sequences* also obtained from the GenBank database are indicated (uncultured strain). Sequences generated in the present study are labeled *DQ103321–DQ103331*.

sequenced, and revealed 11 new and distinct *amoA* sequences belonging to the *Nitrosospira/Nitrosovibrio* subgroup (Fig. 2). These environmental clones of AOB were closely related to *N. multiformis* and *N. tenuis*, but not to *Nitrosomonas* spp. The *N. tenuis* positive control included in the DGGE analysis (amplified using the amoA-1F and and-2R-TG primer set) yielded a single distinct band (Fig. 1b) that was later cloned, sequenced, and its identity confirmed (Fig. 2).

The *amoA* copy numbers/g dry soil did not differ ($P > 0.05$) neither in the presence or absence of mycorrhizal tomato roots nor as a function of N level or P level alone or in combination (Fig. 3). The mean *amoA* copy numbers across all samples was 6.7×10^6 ($\pm 3.7 \times 10^5$)/g dry soil, which is equivalent to a mean of 2.7×10^6 ($\pm 1.5 \times 10^5$) AOB cells/g dry soil (assuming an average *amoA* copy number of 2.5/AOB cell [40]). There was, however, a significant block effect, indicating spatial differences in *amoA* copy numbers (block I = $5.7 \times 10^6 \pm 3.9 \times 10^5$, block II = $7.7 \times 10^6 \pm 5.1 \times 10^5$).

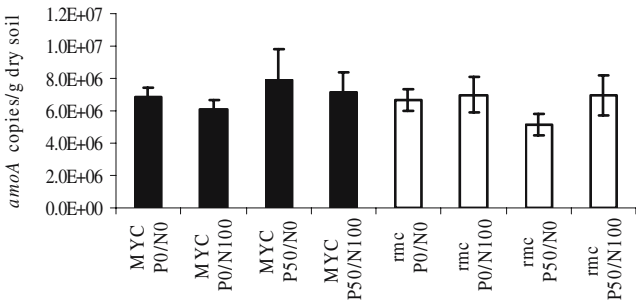

Figure 3. *amoA* copy number/g dry soil in response to mycorrhizal colonization of roots and N and P addition. *N.B.* No significant differences were detected ($P > 0.05$), *MYC* mycorrhizal 76RMYC+ plants, *rmc* mycorrhiza defective tomato mutant. Nutrient addition treatments to the in-growth cores (P level/N level) were: P0 = 0 μg P g dry soil^{-1}, P50 = 50 μg P g dry soil^{-1}, and N0 = 0 μg N g dry soil^{-1}, N100 = 100 μg N g dry soil^{-1}. Values are mean (±SE). $n = 4$.

Mycorrhizal Colonization and Inorganic Soil N. The percentage (arcsine transformed) of the root length was significantly higher in the 76RMYC+ (0.24 ± 0.02) than *rmc* plants (0.04 ± 0.01). Concentrations of NH_4^+ and NO_3^- (plus NO_2^-) were in the range of 0.5–1.3 and 2–6 μg N g, respectively (Fig. 4). At harvest, NO_3^- concentrations were significantly higher in soils containing non-mycorrhizal roots (pooled over N and P addition treatments), whereas the reverse was true of NH_4^+ (pooled over N and P addition treatments), but the differences were only <4 μg/g for NO_3^- and <1 μg/g for NH_4^+. Addition of nutrients at the beginning of the growing season was not reflected in differences in NH_4^+ and NO_3^- concentrations at the time of harvest.

SYBR Green System for Detection of AOB. The SYBR green system was able to detect the *amoA* gene in the samples analyzed with similar precision to the *Taq*Man assay. A standard curve relating the C_T value and AOB was generated with a 10-fold dilution series of a plasmid containing the *amoA* gene. There was a strong linear ($R^2 = 0.99$) inverse relationship between C_T and the $\log_{10}$ number of *amoA* copies over 7 orders of magnitude (Fig. 5). The equation describing the relationship is $C_T = -3.62 \times \mathrm{Log}_{10}(amoA) + 35.3$. When compared directly to the standard curve used in the *Taq*Man real-time PCR system, the two standard curves have significantly different slopes ($P < 0.0001$). The SYBR green and *Taq*Man methods estimated 8.6×10^6 ($\pm 3.4 \times 10^6$) and 4.6×10^6 ($\pm 6.5 \times 10^5$) *amoA* copies/g soil, respectively, for the same set of samples ($n = 3$); the difference in estimated copy numbers was not significant ($P = 0.3$).

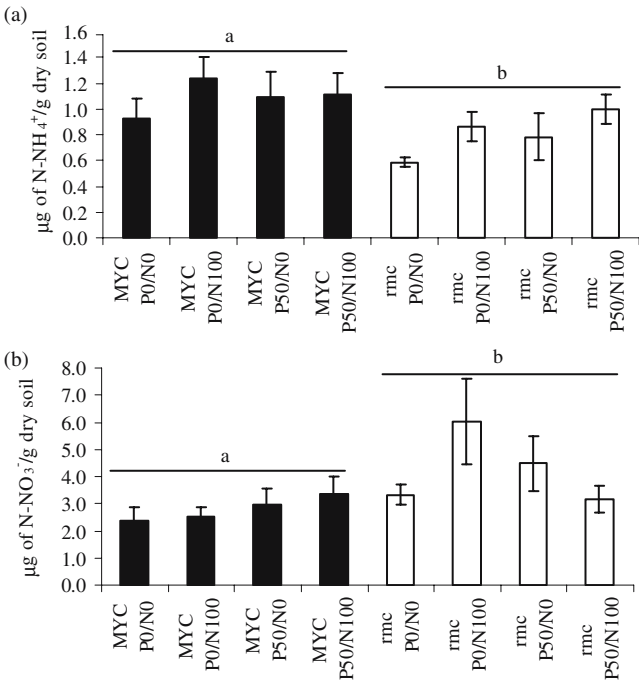

Figure 4. Concentrations of (a) $N - NH_4^+$ and (b) $N - NO_3^-$, at harvest. *MYC* Mycorrhizal 76RMYC+ plants, *rmc* mycorrhiza defective tomato mutant. Significant differences between genotypes (pooled over N and P addition treatments) were found, and are indicated in the figure. Nutrient addition treatments to the in-growth cores (P level/N level) were: P0 = 0 μg P g dry soil^{-1}, P50 = 50 μg P g dry soil^{-1}, and N0 = 0 μg N g dry soil^{-1}, N100 = 100 μg N g dry soil^{-1}. Values are mean (±SE). $n = 4$.

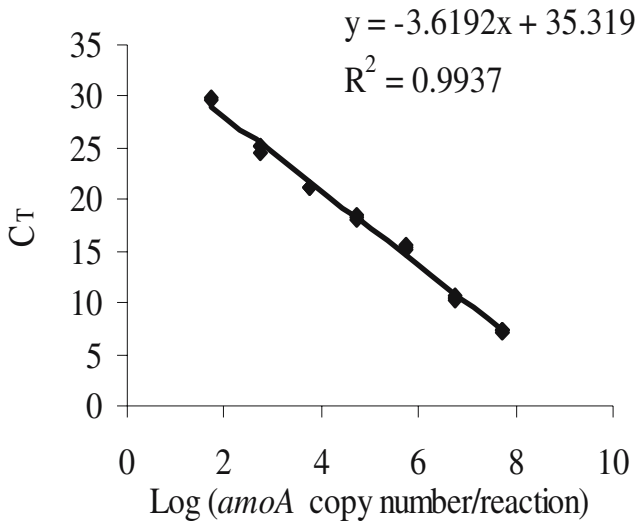

Figure 5. Standard curve for *amoA* SYBR green real-time PCR.

Discussion

AOB community composition was similar in the presence and absence of mycorrhizal roots and at all levels of N and/or P addition. Although the response of AOB to the presence of AMF at the community level has not been studied previously, AOB community composition is known to shift in response to changes in grazing intensity and topography [41], soil liming [4, 5], and along an atmospheric N deposition gradient [29]. The lack of difference in AOB communities among treatments may, in part, be a reflection of the short time course studied, a single growing season. However, differences in total bacterial diversity in the rhizosphere of mycorrhizal and non-mycorrhizal plant species have been shown to be evident over an even shorter period of time [34]. The apparent lack of a mycorrhizal effect on AOB community composition is consistent with our observation that the wider soil community (including microbial biomass carbon, PLFA profiles, and nematode communities) of this long-term organic farm, also did not differ in response to the same experimental treatments [10]. AMF make up a very small fraction of the biodiversity present in this active and frequently disturbed organic farm soil [10]. Thus, AMF may have only small or transient effects on the wider communities of this soil, explaining the lack of observed differences.

AOB population sizes at harvest were similar in soils containing roots, irrespective of their colonization by AMF. A previous study showed that large differences in initial concentrations of soil NH_4^+ are needed for changes in AOB population size to be resolved [40]. Amora-Lazcano *et al.* [1] reported an order of magnitude higher AOB population densities in soil containing roots colonized by AMF; however, soil NH_4^+ concentrations at harvest were not reported. The concentrations of NH_4^+ and NO_3^- in soil containing 76RMYC+ roots were marginally higher and lower, respectively, than those containing *rmc* roots, but these differences were only on the order of 0.5–4 μg N g soil. The significance of these small differences is uncertain given the rapid mineralization and turnover of NH_4^+ in organically managed soils in California tomato production and the fact that NO_3^- concentrations often exceed 20 μg N g soil [8, 9]. While colonization of roots by AMF did not have a significant impact on AOB population sizes in this study, this does not preclude such effects under other circumstances such as if NH_4^+ concentrations are higher or if AMF alter N mineralization [25].

The AOB identified in this soil were all closely related to *N. multiformis* and *N. tenuis*, consistent with previous findings that soils are dominated by strains belonging to the genera *Nitrosospira*, rather than *Nitrosomonas* as previously thought [20, 24, 35]. None of the *amoA* sequences amplified from this soil were closely related to those from the Crenarcheote, as was expected with the primer sets used here (see below also). Eleven unique sequences of the *amoA* gene were identified, representing greater AOB sequence diversity than the three stains found, using a similar DGGE (AmoA-1F/AmoA-2R-TG primer set, targeting sequences with no mismatch to the amplified portion of the *N. tenuis amoA* gene) and sequencing approach in a nearby organically managed field on a similar soil type [40]. This degree of variation in AOB community composition is, however, within the range found using DGGE analysis of AOB 16S rRNA gene in grassland soils at high and low grazing intensities and topographic positions [41]. Conversely, it is considerably less than what has been found using phylogenetic analysis of AOB 16S rRNA PCR products amplified and cloned from soil DNA extracts, along a transect of Southern Californian mountain ridge tops receiving differing levels of atmospheric N deposition [29].

Population densities of AOB in our study soil were in the same range as densities reported in similar soil types using the same methods [40] and in Swedish arable soils using a *Taq*Man PCR method targeting 16S rRNA gene [22]. However, AOB population sizes reported here are higher than in a transect along Californian mountain ridge tops [29] using the methods of Hermansson and Lindgren [22], and in previous studies using MPN [19, 38, 48] or fluorescence *in situ* hybridization (FISH) [7, 12, 13, 23]. While the population sizes reported here are similar to studies using similar methods, they are still higher than those using other methods, highlighting the need for caution when making comparisons between studies.

We expanded and improved the quantitative PCR method previously developed by our research group to enumerate AOB based on the *amoA* gene [40]. The new SYBR green method developed in this study estimated similar copy numbers, although with slightly lower resolution as evidenced by higher standard errors, as the *Taq*Man method. The SYBR green method could be advantageous when it is not feasible to perform the sequencing and phylogenetic analysis of AOB community composition needed to ensure that the *Taq*Man probe can detect all AOB present. Thus, SYBR green would be useful in exploring new soil types and/or environments, when comparing a variety of soils, or when studying soils that have novel *amoA* sequences. The SYBR green assay also has the advantage of relying on the specificity of the PCR primers only, not on the *Taq*Man probe, and it may be further optimized based on the growing database of *amoA* sequences, including recent discovery of ammonium monooxygenase in the Crenarchaeote [32, 51], which may play an important role in nitrification in arable soils [32].

The main aim of the work presented here was to compare the community composition and abundance of AOB in an organically managed agricultural soil containing tomato roots colonized by AMF or with greatly reduced colonization. No such interactions were observed in this study. Similar studies in a wide range of soils and ecosystems are required before more general conclusions about interactions between AM and AOB can be drawn. Finer scale sampling of the rhizosphere/mycorrhizosphere may also be necessary to identify any such interactions. The effects of N and P addition to the soil at the beginning of the growing season on AOB community composition and population sizes were also assessed. Again, no significant impacts were found on the AOB. Future research may need to include studies over shorter time courses and/or assess N turnover rates and changes in mineral N pool sizes in this context.

Acknowledgement

This work would not have been possible without the ongoing support of our farmer cooperators Jim and Deborah Durst. We wish to thank Professor Sally Smith (University of Adelaide) and Dr. Susan Barker (University of Western Australia), for allowing us to use the *rmc* tomato mutant/wild-type system. We thank Mr. Binyam Gebreyesus and Dr. Kevin Feris for valuable discussions and the four anonymous referees of this manuscript for

their valuable comments. Our research is funded by the California Department of Food and Agriculture Specialty Crops Program (SA6674), the United States Department of Agriculture National Research Initiative Soils and Soil Biology Program (2004-03329), and grant number 5 P42 ES004699 from the National Institute of Environmental Health Sciences (NIEHS), NIH.

References

1. Amora-Lazcano, E, Vázquez, MM, Azcón, R (1998) Response of nitrogen-transforming microorganisms to arbuscular mycorrhizal fungi. Biol Fertil Soils 27: 65–70
2. Arines, J, Vilarino, A, Sainz, M (1989) Effect of different inocula of vesicular–arbuscular mycorrhizal fungi on manganese content and concentration in red clover (*Trifolium pretense* L.) plants. New Phytol 37: 699–705
3. Avrahami, S, Liesack, W, Conrad, R (2003) Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Environ Microbiol 5: 691–705
4. Backman, JSK, Hermansson, A, Tebbe, CC, Lindgren, PE (2003) Liming induces growth of a diverse flora of ammonia-oxidising bacteria in acid spruce forest soil as determined by SSCP and DGGE. Soil Biol Biochem 35: 1337–1347
5. Backman, JSK, Klemedtsson, AK, Klemedtsson, L, Lindgren, PE (2004) Clear-cutting affects the ammonia-oxidising community differently in limed and non-limed coniferous forest soils. Biol Fertil Soils 40: 260–267
6. Bakhtiar, Y, Miller, D, Cavagnaro, TR, Smith, SE (2001) Interactions between two arbuscular mycorrhizal fungi and fungivorous nematodes and control of the nematode with fenamifos. Appl Soil Ecol 17: 107–117
7. Biesterfeld, S, Figueroa, L, Hernandez, M, Russell, P (2001) Quantification of nitrifying bacterial populations in a full-scale nitrifying trickling filter using fluorescent *in situ* hybridization. Water Environ Res 73: 329–338
8. Burger, M, Jackson, LE (2003) Microbial immobilization of ammonium and nitrate in relation to ammonification and nitrification rates in organic and conventional cropping systems. Soil Biol Biochem 35: 29–36
9. Burger, M, Jackson, LE (2004) Plant and microbial nitrogen use and turnover: rapid conversion of nitrate to ammonium in soil with roots. Plant Soil 266: 289–301
10. Cavagnaro, TR, Jackson, LE, Six, J, Ferris, H, Goyal, S, Asami, D, Scow, KM (2006) Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. Plant Soil 282: 209–225
11. Cavagnaro, TR, Smith, FA, Hay, G, Carne-Cavagnaro, VL, Smith, SE (2004) Inoculum type does not affect overall resistance of an arbuscular mycorrhiza-defective tomato mutant to colonisation but inoculation does change competitive interactions with wild-type tomato. New Phytol 161: 485–494
12. Daims, H, Ramsing, NB, Schleifer, KH, Wagner, M (2001b) Cultivation- independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence *in situ* hybridization. Appl Environ Microbiol 67: 5810–5818
13. Daims, H, Purkhold, U, Bjerrum, L, Arnold, E, Wilderer, PA, Wagner, M (2001a) Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. Water Sci Technol 43: 9–18
14. Forster, JC (1995) Soil nitrogen. In: Alef, K, Nannipiero, P (Eds.) Methods in Applied Soil Microbiology and Biochemistry, Academic Press, San Diego, pp 79–87
15. Frey, B, Schuepp, H (1993) Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. New Phytol 124: 221–230
16. Gange, AC (2000) Arbuscular mycorrhizal fungi, collembola and plant growth. Trends Ecol Evol 15: 369–372
17. Gao, LL (2002) Control of arbuscular mycorrhizal colonisation. Studies of a mycorrhiza-defective tomato mutant. Ph.D. Thesis. The University of Adelaide, Adelaide
18. Hamel, C (2004) Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone. Can J Soil Sci 84: 383–395
19. Hashimoto, T, Hattori, T (1987) Length of incubation for the estimation of the most probable number of nitrifying bacteria in soil. Soil Sci Plant Nutr 33: 507–510
20. Hastings, RC, Saunders, JR, Hall, GH, Pickup, RW, Mc-Carthy, AJ (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl Environ Microbiol 64: 3674–3682
21. Hawkins, HJ, Johansen, A, George, E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226: 275–285
22. Hermansson, A, Lindgren, PE (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Appl Environ Microbiol 67: 972–976
23. Hesselsoe, M, Brandt, KK, Sorensen, J (2001) Quantification of ammonia oxidizing bacteria in soil using microcolony technique combined with fluorescence *in situ* hybridization (MCFU-FISH). FEMS Microbiol Ecol 38: 87–95
24. Hiorins, WD, Hastings, RC, Head, IM, McCarthy, AJ, Saunders, JR, Pickup, RW, Hall, GH (1995) Amplification of 16s ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology 141: 2793–2800
25. Hodge, A, Campbell, CD, Fitter, AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413: 297–299
26. Holmes, AJ, Costello, A, Lidstrom, ME, Murrell, JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett 132: 203–208
27. Hristova, K, Gebreyesus, B, Mackay, D, Scow, KM (2003) Naturally occurring bacteria similar to the methyl tert-butyl ether (MTBE)-degrading strain PM1 are present in MTBE-contaminated groundwater. Appl Environ Microbiol 69: 2616–2623
28. Johansen, A, Jakobsen, I, Jensen, ES (1993) External hyphae of vesicular–arbuscular mycorrhizal fungi associated with *Trifolium subterraneum*. 3. Hyphal transport of 32P and 15N. New Phytol 124: 61–68
29. Jordan, FL, Cantera, JJL, Fenn, ME, Stein, LY (2005) Autotrophic ammonia-oxidizing bacteria contribute minimally to nitrification in a nitrogen-impacted forested ecosystem. Appl Environ Microbiol 71: 197–206
30. Klopatek, CC, Klopatek, JM (1997) Nitrifiers and mycorrhizae in pristine and grazed Pinyon–Juniper ecosystems. Arid Soil Res and Rehabil 11: 331–342
31. Kothari, SK, Marschner, H, Romheld, V (1991) Effect of vesicular–arbuscular mycorrhizal fungus and rhizosphere microorganisms on manganese reduction in the rhizosphere and manganese concentrations in maize (*Zea mays* L). New Phytol 117: 649–655
32. Leininger, S, Urich, T, Schloter, M, Schwark, L, Qi, J, Nicol, GW, Prosser, JI, Schuster, SC, Schleper, C (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442: 806–809
33. Li, X-L, George, E, Marschner, H (1991) Extension of the phosphorus depletion zone in VA-mycorrhizal white clover in a calcareous soil. Plant Soil 136: 41–48

34. Marschner, P, Crowley, DE, Lieberei, R (2001) Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11: 297–302
35. Mendum, TA, Sockett, RE, Hirsch, PR (1999) Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the subdivision of the class Proteobacteria in arable soils to nitrogen fertilizer. Appl Environ Microbiol 65: 4155–4162
36. Miller, RL, Jackson, LE (1997) Survey of vesicular–arbuscular mycorrhizae in lettuce production in relation to management and soil factors. J Agric Sci 130: 173–182.
37. Miranda, KM, Espey, MG, Wink, DA (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5: 62–71
38. Morrill, LG, Dawson, JE (1967) Patterns observed for the oxidation of ammonium to nitrate by soil organisms. Soil Sci Soc Am J 31: 757–760
39. Nicolaisen, MH, Ramsing, NB (2002) Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. Microbiol Methods 50: 189–203
40. Okano, Y, Hristova, KR, Leutenegger, CM, Jackson, LE, Denison, RF, Gebreyesus, B, LeBauer, D, Scow, KM (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl Environ Microbiol 70: 1008–1016
41. Patra, AK, Abbadie, L, Clays-Josserand, A, Degrange, V, Grayston, SJ, Loiseau, P, Louault, F, Mahmood, S, Nazeret, S, Philippot, L, Poly, F, Prosser, JI, Richaume, A, LeRoux, X (2005) Effects of grazing on microbial functional groups involved in soil N dynamics. Ecol Monogr 75: 65–80
42. Paulitz, TC, Linderman, RG (1989) Interactions between fluorescent pseudomonads and VA mycorrhizal fungi. New Phytol 113: 37–45
43. Perrière, G, Gouy, M (1996) WWW—query: an on-line retrieval system for biological sequence banks. Biochimie 78: 364–369
44. Perrin, R (1990) Interactions between mycorrhizae and diseases caused by soil-borne fungi. Soil Use Manage 6: 189–195
45. Prosser, JI (2002) Molecular and functional diversity in soil microorganisms. Plant Soil 244: 9–17
46. Purkhold, U, Pommerening-Roser, A, Juretschko, S, Schmid, MC, Koops, H-P, Wagner, M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66: 5368–5382
47. Rotthauwe, JH, Witzel, KP, Liesack, W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63: 4704–4712
48. Sarathchandra, SU (1979) A simplified method for estimating ammonium oxidizing bacteria. Plant Soil 52: 305–309
49. Smith, SE, Read, DJ (1997) Mycorrhizal Symbiosis, 2nd ed. Academic Press Ltd., Cambridge, UK
50. Thompson, JD, Higgins, DG, Gibson, TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680
51. Venter, JC, Remington, K, Heidelberg, JF, Halpern, AL, Rusch, DD (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304: 66–74