Applied Soil Ecology 119 (2017) 396–406

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Microbial communities in the cysts of soybean cyst nematode affected by tillage and biocide in a suppressive soil

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ARTICLE INFO

Keywords:
Microbial community
Next generation sequencing
Soybean cyst nematode
Suppressive soil
Biological control

ABSTRACT

Suppressive soil harbors biological agents with potential for managing plant diseases. However, given the rich and complex composition of suppressive factors, the microbes involved in disease suppression have been difficult to identify. We conducted amplicon-based metagenomic analysis of microbial communities in the cysts of soybean cyst nematode (SCN, *Heterodera glycines*) from an SCN-suppressive field to study bacteria and fungi involved in SCN suppression. The experiment was a split-plot design with conventional tillage and no-till as main treatments, and formaldehyde as a biocide and no-formaldehyde (control) as sub-treatments. All plots were planted with SCN-susceptible soybean from 2009 to 2013. Tillage had little effect on SCN, while formaldehyde increased SCN population density, suggesting biological factors are involved in SCN suppression. SCN cysts were collected at planting and midseason in 2013 for bacterial 16S rRNA and fungal ITS1 sequencing. Tillage did not affect bacterial and fungal diversity, composition, or relative abundance of taxa. However, formaldehyde lowered bacterial community diversity, and changed the bacterial and fungal community composition when compared to the control. Formaldehyde reduced the bacterial genera *Lysobacter* and *Actinocorallia*, which are frequently isolated from cysts, but increased the relative abundance of *Pseudomonas* in the cysts. *Streptomyces* were found to be more dominant at planting than at midseason. The fungi important in regulating SCN population such as *Pochonia*, *Exophiala,* and *Clonostachys* had lower relative abundance, whereas *Trichoderma* and *Phoma* had higher relative abundance under formaldehyde treatment than control. Our study suggests that both bacteria and fungi played important roles in suppression of the SCN.

1. Introduction

Soil borne pathogens and pests persist in a complex soil environment. Plant diseases caused by soil borne pathogens, including diseases caused by nematodes, result in substantial losses to agricultural production (Rivoal et al., 2009; Wrather et al., 2003) and roots infected by nematodes are prone to attack by other soil borne pathogens (Xing and Westphal, 2006). The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the major pathogen of soybean (*Glycine max* (L.) Merr.) in the Midwestern United States (Wrather and Koenning, 2006; Wrather et al., 2003). Annual yield suppression due to SCN in the United States alone in 2003 to 2005 was estimated at approximately $1.5 billion (Wrather and Koenning, 2006). The eggs of SCN can persist in soil under a wide range of temperatures and may be dormant for over 10 years when the environment is not suitable for development, making the pathogen difficult to control by short term non-host crop rotations (Chen, 2011; Porter et al., 2001). Moreover, management strategies such as application of nematicides and use of resistant cultivars have limitations because most effective nematicides have been restricted due to their harm to the environment or cost (Rich et al., 2004), while genetic resistance in host cultivars can be overcome rapidly (Zheng and Chen, 2011). Enhancement of soil-based natural suppression could be an effective option to manage the disease or as a part of an integrated disease management program.

The biological control of nematodes has been considered an alternative to nematicides for decades. There are numerous organisms regulating nematode populations, but bacteria and fungi are the most extensively studied (Chen, 2011; Chen and Dickson, 2012). For example, *Pasteuria penetrans*, which is an obligate parasitic bacterium of nematodes (Chen and Dickson, 2012, 1998), has been extensively studied for

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http://dx.doi.org/10.1016/j.apsoil.2017.07.018
Received 13 May 2017; Received in revised form 4 July 2017; Accepted 11 July 2017

managing root-knot nematodes, *Meloidogyne* spp. Distinct species of *Pasteria* parasitizing SCN have been reported in different countries (Lee et al., 1998; Noel and Stanger, 1994; Sayre et al., 1991). *Pasteuria nishizawae* was very effective in reducing SCN population when mixed with soil in a field trial (Noel et al., 2010, 2005), and has been developed to commercial products for management of SCN. Bacteria that produce antibiotic substances or nematicidal compounds are also candidates of nematode biological control agents (Chen and Dickson, 2012). For example, *Streptomyces avermitilis* produces avermectins, which are highly toxic to nematodes (Egerton et al., 1979); there are many products developed based on this bacterium and used worldwide (Chen and Dickson, 2012). In addition, some antibiotic-producing bacteria are used in commercial products to control plant-parasitic nematodes, such as *Pseudomonas fluorescens*, *Bacillus chitinosporus*, and *B. firmus* (Chen and Dickson, 2012).

Fungi are also important in nematode management in natural and agricultural ecosystems, but have been less well studied than bacteria. The fungi that are most frequently used in biological control products are generally egg-parasitic fungi such as *Purpureocillium lilacinum*, *Pochonia chlamydosporia*, and *Trichoderma* spp. (Chen and Dickson, 1996, 2012; Chen et al., 2000). *Hirsutella minnesotensis* and *H. rhossiliense*s are nematode-endoparasitic fungi, which parasitize and are frequently isolated from the second-stage juveniles (J2) of SCN in soybean fields (Chen and Reese, 1999; Liu and Chen, 2000; Ma et al., 2005). Greenhouse studies showed that they were very effective in parasitizing SCN J2 (Liu and Chen, 2001). However, to date, no commercial products have been developed from those fungi. Research has shown that combinations of biocontrol agents had enhanced, reduced or no effect when compared to application of individual agents (Meyer and Roberts, 2002). The mechanism by which biocontrol agents manage nematodes in soil is poorly understood. An improved exploitation of biological control will greatly benefit from a thorough understanding of natural mechanisms that regulate nematode population densities.

Disease-suppressive soil is defined as the soil in which the pathogen cannot establish or is able to establish but is maintained at a low level (Baker and Cook, 1974). Soils suppressive to SCN have been reported in a number of locations in the USA and other regions of the world (Bao et al., 2011; Carris et al., 1989; Chen, 2007a; Chen et al., 1996b; Sun and Liu, 2000). Suppressive soils are often developed under monoculture of a susceptible host (Chen, 2007a; Gair et al., 1969). This suppression has been attributed to diverse microbes, including bacteria and fungi. An extensively studied nematode suppressive soil is a sugar beet cyst nematode (*Heteradera schachtii*) suppressive soil in California (Westphal and Becker, 1999). In order to determine whether biotic or abiotic factors were involved in the suppression, they fumigated the soil with metam sodium, methyl bromide, methyl iodide, formaldehyde and aerated steam (Westphal and Becker, 1999). The fumigated soil had higher nematode population density in the greenhouse. The suppressiveness was also able to be established by transferring untreated soil into fumigated soil (Westphal and Becker, 2000). Further research showed that *Dactylella oviparasitica* and *Paecilomyces lilacinus* (*Purpureocillium lilacinum*) were frequently isolated from infected cysts (Westphal and Becker, 2001). Although suppressive soils for different plant-parasitic nematodes such as *Heterodera avenae*, *H. schachtii*, *H. glycines*, *Meloidogyne* spp., and *Criconemella xenoplax* have been discovered (Chen, 2007a; Gair et al., 1969; Kluepfel et al., 1993; Weibelzahl-Fulton et al., 1996; Westphal and Becker, 1999), and a few microbial organisms have been associated with nematode suppression, the roles of soil microbial communities in suppression of nematode populations remains poorly understood.

Formaldehyde is an organic compound toxic in general to organisms including humans, and its effect on nematodes was evaluated previously (Giblin-Davis et al., 1988). However, sensitivity to formaldehyde differs among different organisms. Formaldehyde was used to suppress the biological antagonists of plant pathogens to illustrate natural suppression of diseases and pathogens by biological factors. For example, when it was applied to a field with severe cereal cyst nematode (*Heterodera avenae*) and take-all (*Gaeumannomyces graminis*) damage, the cereal yield was increased by formaldehyde, but the cereal cyst nematode population increased as well (Williams, 1969). This implied that formaldehyde might eliminate the fungi rather than the nematodes and as a result nematode-antagonistic microbial organisms were reduced and nematode populations increased (Warcup, 1952). This phenomenon was observed in another study in which application of 3000 l/ha formalin (38% formaldehyde) resulted in increased cereal cyst nematode populations and reduced parasitism of female nematodes because of the decline of *Pochonia chlamydosporia* and *Nematophthora gynophila* (Kerry et al., 1980). Formaldehyde is not a very effective nematicide at the level that can kill microbial organisms, which makes it an excellent biocide to investigate nematode suppressive soils.

Inconsistent effects of tillage on SCN densities have been reported from different geographical locations in the USA (Chen, 2007b; Crookston et al., 1991; Howard et al., 1998; West et al., 1996). Soybean cyst nematode population density was reduced by no-till in western Kentucky and the southern USA (Edwards et al., 1988; Hershman and Bachi, 1995), but not in Illinois (Niblack et al., 1995) or Minnesota (Chen, 2007b), where soybean yields increased in fields with conventional tillage practices (Chen, 2007b). Conventional or reduced tillage practices have been reported to reduce the bacterial and fungal communities in soil compared to no till (Vargas Gil et al., 2011; Yin et al., 2010). However, there are few studies regarding the microbial populations associated with the SCN as affected by tillage. Bernard et al. (1997) found that soybean-wheat double cropping and tillage affected the fungi that parasitize the females and eggs of SCN. Using a culture-dependent method, Chen and Liu (2007) found that during soybean growth fungal parasitism of SCN juveniles was lower in conventional tillage than no-till.

Using a next generation sequencing approach, it is possible to fully identify the bacterial and fungal communities in cysts that might be affected by tillage practices and formaldehyde treatment, which will provide essential information for SCN management under modern agricultural systems. This study was conducted in a research field with a history of long term no-till and soybean monoculture. The nematode egg population density in this field in the fall season preceding this experiment was below 10,000 eggs/100 cm^3 soil, with a 10-year average of 6000 eggs/100 cm^3 soil, which was lower than egg densities in nearby fields (Chen, 2007a). The nematode-susceptible soybean yield of this field is similar to the average soybean yield in the state. In addition, the SCN J2 endoparasitic fungus *H. rhossiliense*s was frequently isolated from this field in previous studies (Chen, 2007a). Autoclaved and formaldehyde treated soil from this field used in greenhouse studies had significantly higher SCN egg population densities than that in untreated soil (Bao et al., 2011; Chen, 2007a). Furthermore, 90% formaldehyde-treated or autoclaved soil mixed with 10% untreated soil had similar egg population densities as untreated soil (Chen, 2007a). Such research indicated that this field was an SCN-suppressive field, and biotic factors were involved in the suppression. These discoveries encouraged us to further examine the changes in diversity and composition of the bacterial and fungal community in SCN cysts in response to tillage and formaldehyde treatments with the goal of discovering the bacterial and fungal taxa that play important roles in nematode suppression.

2. Materials and methods

2.1. Field experimental design and maintenance

The experimental field was located at the University of Minnesota Southern Research and Outreach Center research farm (44° 04′ 21″ N, 93° 31′ 24″ W), Waseca, Minnesota, USA. This field had been planted to soybean continuously for 41 years with no-till management during the 16 years prior to a study initiated in 2008. The soil is a Nicollet clay

loam (fine loamy, mixed, mesic Aquic Hapludoll), and suppressive to the SCN (Chen, 2007a). The experiment was a split-plot design with no-till and conventional tillage as main plots, and formaldehyde treatment and without formaldehyde treatment as subplots with four replicates. The formaldehyde treatment was intended to act as a biocide to reduce bacterial and fungal populations, reduce soil suppressiveness, and identify potential microbial taxa involved in suppression. Each experimental unit (subplot) was 7.6 m long and 4.57 m wide with six rows of soybean. For the formaldehyde treatment, 6.8 liters of 38% formaldehyde (formalin) in 180 liters of water was applied by irrigation in the four central rows (3 m wide) of each subplot three weeks before planting soybean. The conventional tillage treatment consisted of fall chisel plowing in 2008, 2010, and 2012 and moldboard plowing in 2009 and 2011 after harvesting soybean. All plots were planted with the soybean cyst nematode susceptible cultivar Pioneer Hi-Bred 92B13 all years and the soybean was drill-planted at 2.5 cm deep using a 6 row John Deere 7240 regular seed planter. No fertilizer was applied.

2.2. SCN population density measurement

A bulk soil sample consisting of 40 soil cores (2 cm diam., 20 cm deep) was collected from each subplot in a systematic pattern across the two central rows at planting (June), at midseason (August), and at harvest (October) from 2009 to 2013. A total of 16 composite bulk soil samples were obtained at each sampling time point. The soil samples were stored in a cold room (4 °C) before being processed. The soil was passed through a 5-mm-aperture sieve and mixed thoroughly. A subsample of 100 cm^3 soil was used for SCN egg population density determination. Cysts were extracted from the subsample with a semi-automatic elutriator (Byrd et al., 1976), and separated from soil particles and debris with centrifugation in a 63% (w/v) sucrose solution. Eggs were released from the cysts mechanically (Niblack et al., 1993), and collected in a 50-ml tube in 25 ml water. The number of eggs was counted in 0.5 to 2.0 ml of the egg suspension depending on the total number of eggs, and the total number of eggs in 100 cm^3 of soil was calculated. The SCN egg population density was expressed as number of eggs/100 cm^3 soil.

2.3. Collection of cysts for sequencing

In 2013, SCN cysts were extracted from soil collected from the 2nd and 4th rows of each subplot using a small trowel at planting and midseason after the soil sampling for egg population. Cysts were extracted with a modified hand-decanting method (Chen et al., 2001a). About 4 kg soil was soaked for 1 h and stirred with an electric drill stirrer to break soil aggregates, and then processed. The soil suspension created by spraying a strong jet of water on the sample to float the cysts was poured through an 850-μm-aperture sieve nested on a 250-μm-aperture sieve. This procedure was repeated at least three times for each bucket of soil to collect the cysts in the soil sample. Cysts with debris and soil particles on the 250-μm-aperture sieve were collected, and the cysts were separated from the debris and soil particles with a sucrose flotation and centrifugation method (Jenkins, 1964). A subsample of 200 intact cysts was hand-picked randomly with forceps under an inverted microscope and treated with 0.5% NaOCl for 3 min to surface sterilize the cysts, and rinsed with sterile deionized water three times and then stored at −80 °C.

2.4. DNA extraction, amplification and sequencing

The surface sterilized cysts were crushed using a small pestle in a 1.5-ml microfuge tube, and DNA was isolated from the mixture using a modified proteinase K method as described previously by Subbotin et al. (1999). RNase was added after the proteinase K incubation step, and protein was precipitated in 5 M ammonium acetate. The DNA was precipitated in isopropanol followed by ethanol precipitation, and the final pellets were resuspended in nuclease-free water. The extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientific) and diluted to 5 ng/μl for use as PCR templates.

The bacterial V4 hypervariable region of the 16S rRNA gene was amplified with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Peiffer et al., 2013). The fungal ITS1 region was amplified with primers ITS1F (5′-TTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). Additional sequences were added to primers to allow for barcoding amplicons (Smith and Peay, 2014): forward primer = Illumina adapter/10-base pad/2-base linker/forward gene primer, and reverse primer = Reverse complete 3′ Illumina adapter/12-base barcode/10-base pad/2-base liner/reverse primer. Amplifications were carried out in a total volume of 20 μl using 25 ng template DNA, 10 μl of HotStart Taq plus Master Mix (Qiagen, USA), and 1 μl forward and reverse primer each at 0.2 μM. Thermal cycling consisted of polymerase heat-activation and initial denaturation of 10 min at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C (53 °C, 55 °C, 58 °C for fungi) for 20 s, and elongation at 72 °C for 60 s, with a final elongation at 72 °C for 8 min. For samples in which low amounts of PCR amplicons were produced, the final $MgCl_2$ concentration was increased to 2 mM. Negative control samples were treated similarly with the exclusion of template DNA. For bacterial 16S amplification, three independent PCRs were performed and pooled together. For fungal ITS1 amplification, amplicons from reactions with three different annealing temperatures were performed and pooled. The pooled amplicons were purified with Ampure magnetic purification beads (Agencourt) and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) on a Qubit® Fluorometer. The amplicons of 32 samples of bacteria or fungi were each combined in equimolar ratios, and sequenced in separate lanes. A mock fungal community, which had 21 known fungal species, was included in the fungal sequencing, as well as the negative controls. Paired-end sequencing (2 × 250 bp) was carried out on one quarter lane of an Illumina MiSeq platform at the University of Minnesota Genomics Center (Saint Paul, MN, USA).

2.5. Quality control of sequences

We received 3,450,509 and 1,180,535 sequences from the Illumina platform for bacterial 16S and fungal ITS1 amplicons, respectively. The quality of the read2 fungal sequences was poor based on the mock community sample, so only the read1 sequences were used. The bacterial and fungal data were treated differently. The 16S sequences were paired with Mothur using default parameters (Schloss et al., 2009). The low quality sequences were filtered out if they had less than 150 bp overlap, the sequences had more than eight homopolymers, or any ambiguous base. The remaining sequences were trimmed to 245 bp and aligned to the bacterial V4 region, keeping all sequences that started with position 1968 and ended before position 11550. For the fungal sequences, the first 10 bases and the last 15 bases were trimmed to discard poor quality reads at the beginning and end of the sequences. All read1 sequences were further trimmed if the quality score in a window size of 50 was below 28, and only the sequences between 200 bp and 251 bp were retained for downstream analysis. The sequences that had more than one ambiguous base and eight homopolymers were also discarded. After processing in Mothur, bacterial and fungal sequences were moved independently to Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010). Chimera checking was performed using the Usearch series of scripts (Edgar, 2010). De novo and reference-based chimera checking was performed with the UCHIME algorithm and sequences that were characterized as chimeric by either method were removed (Edgar et al., 2011). We obtained 2,734,783 and 850,089 high quality sequences for bacteria and fungi, respectively. Sequences were clustered into OTUs using de novo OTU picking with the Usearch pipeline in QIIME with a 97% threshold. The reference database for taxonomy assignment of bacteria was SILVA

release 128 and for fungi the dynamic version of UNITE release 2016-8-22 was used. Taxonomy was subsequently assigned to each representative OTU against the corresponding database using Blast.

2.6. Analyses of SCN egg population density

Analysis of variance was performed in R v.3.2.3 (R Core Team, 2016). Homogeneity of variance was tested for each treatment before ANOVA and an interaction was detected between sampling time points; therefore, each sampling time point was analyzed separately. The SCN was evaluated for normality, and data that were not normal were log transformed before performing ANOVA. Means of egg population density with tillage and biocide were compared using the least significant difference (LSD) test at $P < 0.05$.

2.7. Data analysis based on OTUs

Rarefaction curves were generated in QIIME. The OTU tables generated by QIIME were transferred to R v.3.2.3 for further statistical analysis. The OTUs that had less than 10 counts per sample were filtered out of the OTU table prior to downstream analysis in R. Observed species and the inverse Simpson diversity index were calculated and plotted in R using the packages 'Phyloseq' (McMurdie and Holmes, 2013) and "ggplot2" (Wickham, 2009), respectively. The values of observed species and inverse Simpson index were transformed using $\log_2$. Nonparametric Wilcoxon test and *t*-test were used to detect significant differences of observed species and inverse Simpson index between treatment plots. The OTU counts were transformed to relative abundance of each sample, and normalized by using $\log_2$ transformation. The OTUs with a relative abundance < 0.05% were excluded from the OTU table. A Bray-Curtis dissimilarity matrix was calculated using the R package "Vegan" (Oksanen et al., 2011), and the significance of dissimilarity between treatments was tested by the Adonis function in Vegan. In order to detect which order or genera were affected by the treatments, the relative abundance of genera was calculated and fitted in the "negative binomial" model in the R package "mvabund" (Wang et al., 2012). A multivariate test was used to detect the interaction between sample time points. Because there was significant interaction between sample time points, the relative abundance of genera was tested separately at each time point. The significance of relative abundance of orders or genera within tillage and formaldehyde treatments was tested using the *t*-test and the *P* value was adjusted with a Bonferroni correction to control for false positives.

Sequence data related to this project was deposited in GenBank BioProject under accession number PRJNA308986 and PRJNA309307. The raw sequences are available through the Sequence Read Archive database (SRP068618) and (SRP068853), and the data will be available at time of publication.

3. Results

3.1. SCN egg population density

The overall mean initial SCN egg population density at planting in 2009 was 4326 eggs/100 cm^3 soil. No effect of tillage on the egg population density was observed in most of the 15 sampling time points over the 5 years, except that no-till slightly increased egg population density at planting in 2012 and 2013 as compared with conventional tillage (Fig. 1). Formaldehyde consistently increased egg population density from planting in 2010 to the end of 2013 (Fig. 1).

3.2. Overall bacterial and fungal communities in cysts

After excluding OTUs with fewer than 10 sequences, we obtained a total of 2,715,794 sequences for bacterial and 837,981 sequences for fungal amplicons. The cyst samples from planting and midseason had overall bacterial OTU counts from 48,863 to 128,794 per sample, and also had the lowest (9571) and highest (59,774) fungal OTU counts per sample. The rarefaction curves indicated that the bacterial species observed did not increase after 40,000 sequences (Fig. S1A), and fungal observed species increased slowly after 10,000 sequences (Fig. S1B), suggesting the sequencing coverage was adequate to capture the majority of taxa. The most abundant phyla for bacteria were: *Proteobacteria* (57.48%) and *Actinobacteria* (31.04%); and the less abundant phyla included: *Bacteroidetes* (5.82%), *Verrucomicrobia* (2.25%), *Firmicutes* (1.54%), *Planctomycetes* (0.72%), *Chloroflexi* (0.37%), *Acidobacteria* (0.31%), and *Cyanobacteria* (0.25%). The phylum that dominated in the fungal community was *Ascomycota* (72.05%); and the less abundant groups included *Zygomycota* (9.32%), *Basidiomycota* (7.62%), unidentified fungi (7.17%), unassigned non-fungal taxa (3.79%), *Glomeromycota* (0.047%), and *Rozellomycota* (0.0025%).

3.3. Alpha diversity affected by treatments and season

Cysts extracted from soil with conventional tillage had slightly higher bacterial diversity than no-till at both planting and midseason (Fig. S2A & B), although this differences was not significant at $P < 0.05$. According to observed species and the inverse Simpson diversity index, cysts from the formaldehyde treated soil had significantly lower bacterial alpha diversity than cysts from the control treatment at both time points (Fig. 2A & B). Bacterial diversity did not differ between planting and midseason ($P > 0.05$) (Fig. 2A & B). Tillage did not affect fungal alpha diversity associated with cysts at either sampling time point (Fig. S3A & B). Formaldehyde also did not affect fungal diversity associated with cysts at either planting or midseason (Fig. 3A & B), but fungal diversity was higher ($P < 0.05$) at midseason than planting according to the inverse Simpson index but not by the observed species count (Fig. 3B).

3.4. Bacterial and fungal community composition affected by treatments and season

Tillage did not affect bacterial community composition in cysts at planting or midseason according to the Adonis analysis using the Bray Curtis distance matrix (Fig. S4). However, formaldehyde treatment changed bacterial community composition in cysts ($P < 0.001$) at both sampling time points (Fig. 4A & B). No difference in bacterial community composition was detected between planting and midseason (Fig. 4C).

The fungal community composition in cysts was different ($P < 0.1$) between conventional tillage and no-till at planting but not at midseason (Fig. S5). Formaldehyde treatment changed fungal community composition at both sampling time points ($P < 0.05$) (Fig. 5A & B), and the fungal community composition was different ($P < 0.05$) between planting and midseason (Fig. 5C).

3.5. Bacterial taxa affected by treatments and season

The relative abundance of bacterial taxa associated with cysts was not significantly different between tillage treatments at all taxonomic levels. There was an interaction between formaldehyde treatment and sampling time points, so the formaldehyde treatment effect on bacterial community was analyzed separately at planting and midseason. At planting, the orders that had higher relative abundance in cysts after formaldehyde treatment compared to control were *Sphingobacteriales*, *Xanthomonadales*, *Rhizobiales*, *Streptosporangiales*, while *Pseudomonadales* and *Enterobacteriales* had lower relative abundance in samples after formaldehyde treatment compared to the control (Fig. 6A & B). In midseason, *Sphingobacteriales*, *Richettsiales*, *Xanthomonadales*, *Pseudonocardiales* had lower relative abundance in the formaldehyde treatment compared to the control, and *Pseudomonadales*, *Enterobacteriales* had higher relative abundance in cysts under

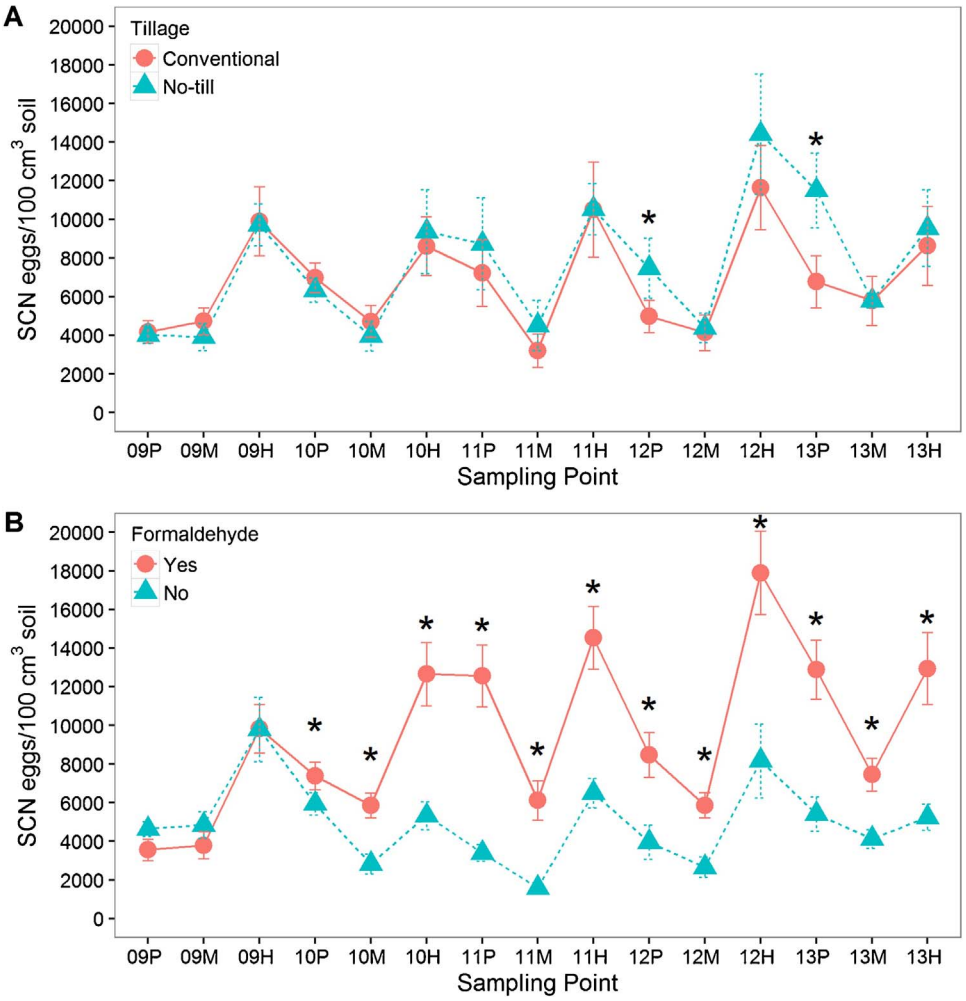

Fig. 1. Soybean cyst nematode egg population affected by tillage (A) and formaldehyde (B) from 2009 to 2013. P = Planting, M = Midseason, H = Harvest, and the numbers in front of the letters are years from 2009 to 2013. Data are means of eight replicates with standard error. The * on top of the error bar means there are significantly differences at $P < 0.05$ between treatments within each sampling time point according to LSD.

formaldehyde treatment compared to the control (Fig. 6C & D).

The relative abundance of some taxa was significantly different between sampling time points. The abundant orders such as *Streptomycetales*, *Burkholderiales* and *Erysipelotrichales* were significantly decreased in midseason, while *Enterobacteriales*, *Sphingobacteriales*, *Rickettsiales*, *Xanthomonadales* and *Verrucomicrobiales* were increased. At the genus level, bacteria that have been isolated frequently from the cysts of SCN including *Lysobacter* and *Actinocorallia,* had lower relative abundance under formaldehyde treatment than the control at both sampling time points. Formaldehyde had an inconsistent effect on *Variovorax* (Table S2). The relative abundance of *Streptomyces* was significantly lower at midseason compared to at planting (Table S2). The relative abundance of some rhizobacteria was also changed by formaldehyde.

3.6. Fungal taxa affected by treatments and season

Similar to bacterial taxa results, tillage did not significantly change the relative abundance of fungi at any taxonomic levels, and there was an interaction between formaldehyde treatment and sampling time points, so the effect of formaldehyde on the relative abundance of fungal orders or genera was also analyzed separately at planting and midseason. At planting, orders that had significantly lower relative abundance in cysts after formaldehyde treatment included *Helotiales*, unidentified *Orbiliomycetes*, *Chaetothyriales*, unidentified *Ascomycota*, and unidentified fungi. *Hypocreales* and *Cantharellales* were the only orders that had higher relative abundance after formaldehyde treatment than the control (Fig. 7A & B). In midseason, *Helotiales*, *Orbiliomycetes*, *Chaetothyriales*, *Mortierellales*, and unidentified *Ascomycota* had lower relative abundance under formaldehyde treatment compared to the control. Only *Eurotiales* had significantly higher abundance under formaldehyde treatment when compared with control (Fig. 7C & D).

Fungal genera that have been reported to be important in regulating nematode populations such as *Exophiala*, *Pochonia*, *Purpureocillium*, *Penicillium*, *Fusarium*, *Phoma, Trichoderma*, and *Clonostachys* were detected (Table S3). Based on the Bonferroni corrected *P* value of the *t*-test, *Exophiala, Pochonia* and *Clonostachys* had significantly lower relative abundance under the formaldehyde treatment than in the control treatment. *Penicillium* and *Fusarium* were not affected by formaldehyde at either sampling time points. Additionally, the relative abundance of *Phoma* was significantly higher at planting than in midseason (Table S3). Notably, the relative abundance of *Trichoderma* was extremely high under formaldehyde treatment compared to the control at both sampling time points (Table S3).

4. Discussion

4.1. The minor effect of tillage on SCN, and associated bacteria and fungi

Over five years in a field which had a long-term history of soybean monoculture and no-till, we found that tillage did not affect the SCN egg population density consistently; SCN egg population densities were slightly reduced by conventional tillage only at planting in 2012 and 2013. The results were similar to the research conducted previously in this area. In a soybean-corn annual rotation field in New Richland, MN,

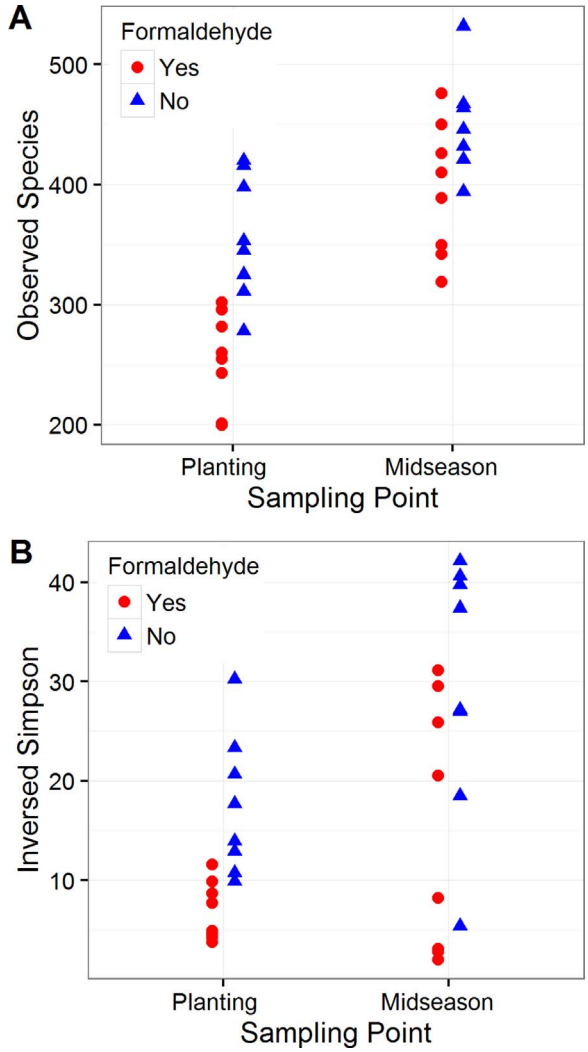

Fig. 2. Bacterial community alpha diversity as affected by formaldehyde.

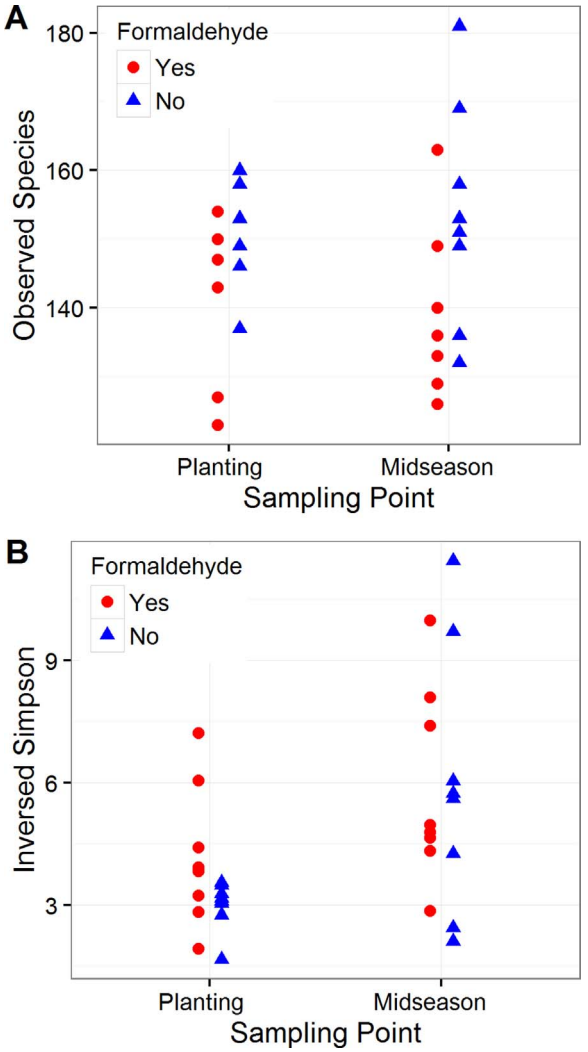

Fig. 3. Fungal community alpha diversity as affected by formaldehyde.

which is in the same county as the current research, there was no tillage effect on SCN egg population density (Chen et al., 2001b). Later research in a field in Waseca showed that tillage had only minimal and inconsistent effects on SCN egg population density (Chen, 2007b). The mechanism of the slight reduction of SCN population density in 2012 and 2013 under conventional tillage is unclear. The involvement of microbial activities associated with conventional tillage cannot be ruled out in this study. It is also possible that the soil tillage before winter might cause more mortality of SCN during the winter because it brought the SCN cysts to the soil surface. The significant reduction of SCN egg population density at planting in 2012 and 2013 could also be due to colder winter temperatures as compared with the previous three years (https://sroc.cfans.umn.edu/weather-sroc/historic-reports). Alternatively, the tilled soil might favor SCN J2 hatch, and thus reduce egg population density before planting.

The microbes affected by tillage have not been reported widely at the community level. In a study of multi-crop sequences in Ohio, conventional tillage reduced the relative abundance of dominant bacterial species compared with long term no-till, and most of the bacterial species in tilled soil had low relative abundance (less than 1%) (Sengupta and Dick, 2015). Another study in Brazil suggested that nitrogen-fixing *Rhizobiales* had higher abundance in no-till than conventionally tilled plots (Souza et al., 2013). However, we did not find that tillage either strongly affected the diversity or composition of the bacteria and fungi in the cysts at either sampling time point. In addition, the relative abundance of bacteria and fungi of every taxonomic rank was not different between the two tillage treatments. Our results indicate that the bacteria and fungi colonizing the cysts were stable and not altered by tillage.

4.2. Biotic factors involved in suppression as indicated by formaldehyde, and cysts were the ideal model to study biotic factors

Formaldehyde consistently increased the egg population density from the second year of application. The increase of SCN population density by formaldehyde treatment was most likely not due to its effect on soybean growth because there was no difference in soybean yield between the formaldehyde treatment and control (unpublished data), but was probably due to the reduction of SCN suppressiveness by the formaldehyde application. This result also indicates that the soil in the field was suppressive to SCN and biotic factors were involved, corroborating the results of greenhouse tests of the soil from this field (Bao et al., 2011; Chen, 2007a). Previous studies of the SCN-suppression factors mainly focused on isolation and culturing of bacteria or fungi directly from the nematodes (Atibalentja et al., 2000; Chen and Chen, 2002; Chen et al., 1994, 1996a; Liu and Chen, 2000). The bacteria and fungi that are relevant in the suppression are not all readily isolated in culture. Thus, using high through-put sequencing in this study, we characterized the comprehensive bacterial and fungal communities in the cysts of SCN, which has expanded our knowledge of cyst- or egg-

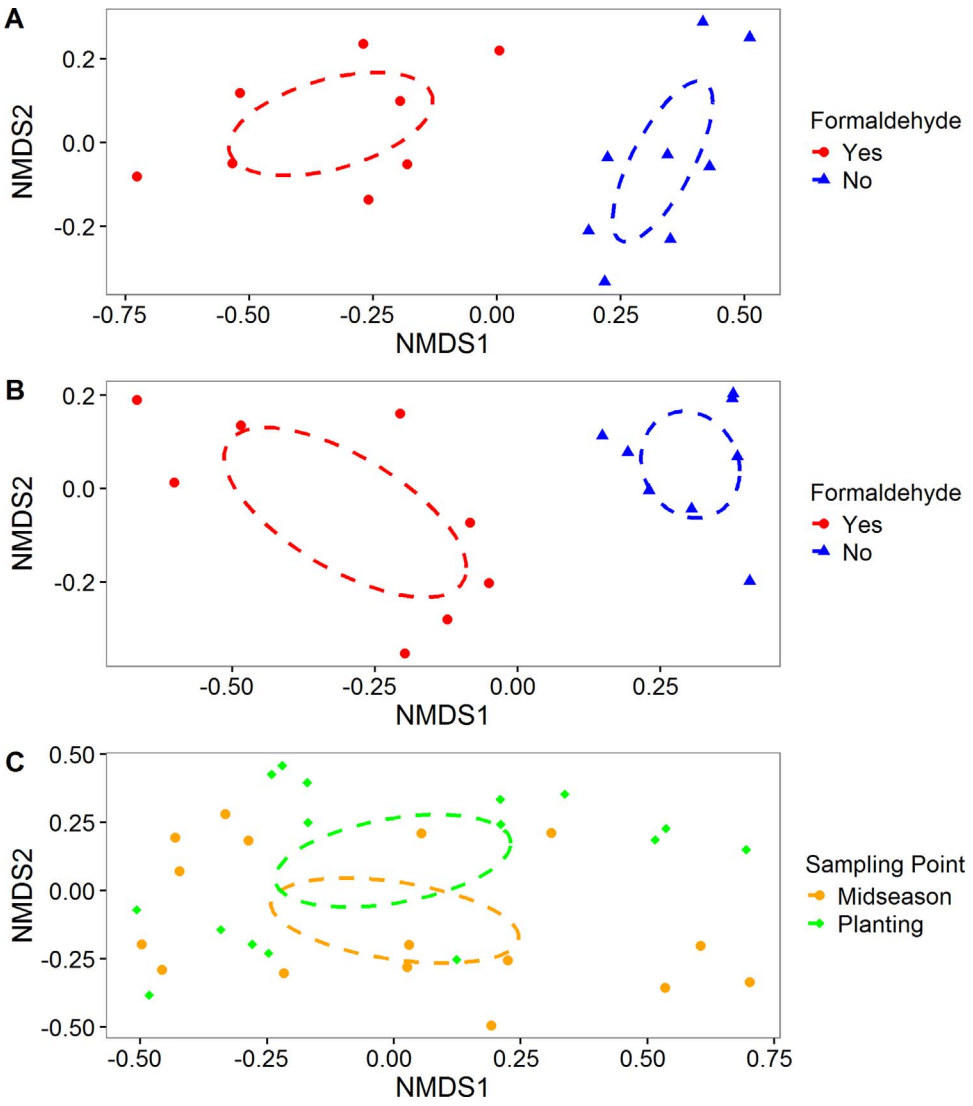

Fig. 4. Bacterial NMDS plot based on Bray-Curtis distance matrix affected by formaldehyde within planting (A) and midseason (B), and between seasons (C). Ellipses were drawn at a confidence level of 0.95.

colonizing microbes, and found some biological control taxa that might be important in the suppression of SCN populations in this field.

Nematode cysts are comprised of the dead melanized body of the nematode females and can exist in soil for an extended period of time. Presumably, fungi and bacteria can invade the cysts either through natural openings (e.g., the mouth, anus, and the vulva) or disrupted cuticle (Kerry and Crump, 1977). Some parasitic fungi may be able to enter females or cysts by penetrating their cuticular walls (Chen and Dickson, 1996). Early studies showed that fungi were detected in young females as soon as they were exposed to the soil rhizosphere (Gintis and Morgan Jones, 1982). However, mature females had a higher frequency of fungal colonization than younger females (Gintis et al., 1983). A study on cereal cyst nematode suppressive soil found that there was high fungal colonization of cysts but nearly no fungal infection of young females in a suppressive soil (Westphal and Becker, 2001). Such research suggested that the fungi colonizing late stages of females or cysts can be important biotic suppressive factors. Parasitic bacteria such as *Pasteuria* were able to infect juvenile stages of soybean cyst nematodes, and reproduce throughout maturation stages of the cyst nematode (Atibalentja et al., 2004). Additionally, some bacteria can be symbionts in soybean cyst nematodes at different stages, including juvenile stages (Atibalentja and Noel, 2008; Endo, 1979). Thus, in this study, investigation of the microbes in mature brown cysts should identify the bacteria and fungi that are closely involved in nematode suppression.

A recent study investigating the bacterial and fungal communities in the rhizosphere soil of soybeans grown in SCN-suppressive soils in the greenhouse found that some fungal genera that potentially regulate SCN populations were more abundant in soils from fields that had been in long term rather than short term soybean monoculture (Hamid et al., 2017). In this study we identified the bacterial and fungal communities directly associated with the cysts of SCN. A number of biological control agents potentially involved in the suppression of SCN in the field, such as *Pseudomonas*, *Trichoderma*, *Pochonia*, and *Purperocillium*, were present in cysts.

4.3. Biological management of SCN

The most widely studied bacteria that have biological control potential of plant-parasitic nematodes are *Pasteuria*, *Pseudomonas*, and *Bacillus* (Tian et al., 2007). We did not find *Pasteuria* and *Bacillus*, but *Pseudomonas* was significantly higher under the formaldehyde treatment than the control treatment at both sampling time points (Table S1). Previous studies found that some species of *Pseudomonas* are resistant to formaldehyde in soil (Sondossi et al., 1989). Some strains of *Pseudomonas* can inhibit nematode egg hatch or invasion ability (Aalten et al., 1998; Westcott and Kluepfel, 1993). A study on *Pseudomonas fluorescens* mutants suggested that the extracellular protease AprA is important in inhibiting root-knot nematode egg hatch and inducing juvenile mortality (Siddiqui et al., 2005). Other rhizobacteria that have been reported to control nematodes include *Enterobacter* (Hu et al.,

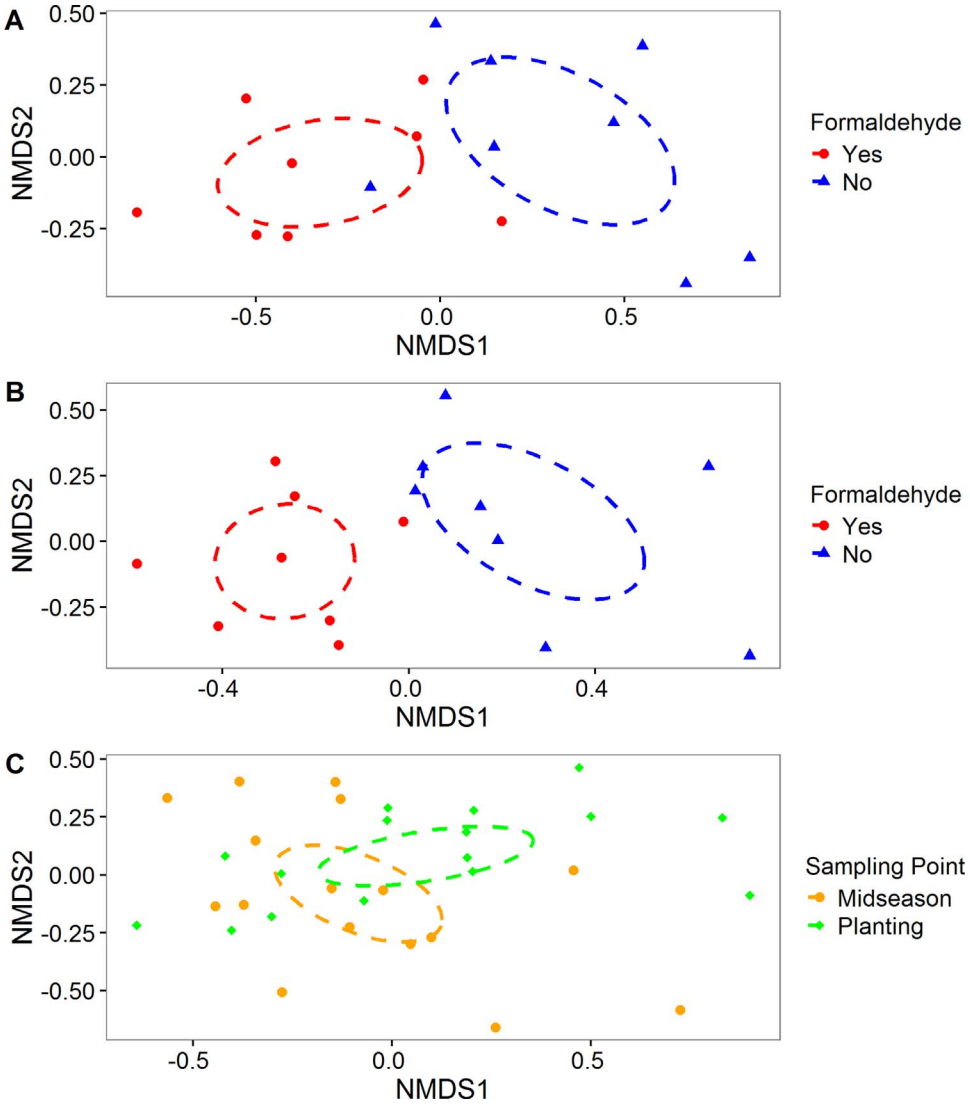

Fig. 5. Fungal NMDS plot based on Bray-Curtis distance matrix affected by formaldehyde within planting (A) and midseason (B), and between seasons (C). Ellipses were drawn at a confidence level of 0.95.

1999) and *Rhizobium* (Hallmann et al., 2001). *Enterobacter* were frequently detected from the cysts in this field, but like *Pseudomonas,* it is unlikely that they play an important role in suppressing SCN populations because their relative abundance was greater in the formaldehyde treatment than in no-formaldehyde treatment. *Actinobacteria* is a phylum that is well-known for antibiotic producing species such as *Streptomyces*, which was highly abundant at planting with a relative abundance of 25%. Abundance dropped to 5% at midseason and was not affected by formaldehyde. The change between sampling time points may have been due to temperature changes of the soil, or an increase of other bacterial species at midseason.

We detected the presence of previously reported nematode biological control fungi in cysts, especially those in *Hypocreales*. Species within *Exophiala*, *Fusarium*, *Nectria, Pochonia*, and *Clonostachys* have the potential for nematode biocontrol, and the relative abundance of those genera were generally lower under formaldehyde treatment than in the control. We speculate that these genera, especially *Pochonia* and *Exophiala*, were important in the suppression of SCN populations in this field because these genera were reported frequently to have nematode management potential. In contrast, the well-known nematode biological control fungus *Purpureocillum* had higher abundance under the formaldehyde treatment at planting. This does not necessarily indicate *Purpureocillum* did not reduce SCN population densities in the field. It is possible that with the suppression of other saprophytic competitive fungi, *Purpureocillum*, which is a fast-growing species, could restore its population fast after formaldehyde treatment, resulting in higher abundance than the control. *Trichoderma* also had significantly higher relative abundance under formaldehyde than the control (Table S2). Some species of *Trichoderma* have been reported to produce a non-enzymatic factor that inhibits egg hatch (Meyer et al., 2000), and some species are also beneficial for plant growth (Harman et al., 2004). Because *Trichoderma* are ubiquitous hyphomycete soil fungi, the massive increase also could due to the decrease of other competitive fungi by formaldehyde. The increase of *Purpureocillum* and *Trichoderma* in the formaldehyde treatment did not result in lower SCN population density probably due to the suppression of many other SCN-antagonistic fungal and bacterial species. This result may suggest that formaldehyde may not be a suitable agent for study of suppressive soil if *Purpureocillum* and/or *Trichoderma* play a major role in the suppression.

4.4. Future research directions

A portion of the OTUs could not be assigned to a specific taxa. Those OTUs should not be ignored, because most of the known biological control fungi were isolated using culture-dependent method and identified based on morphology, and there are still some cultured fungi isolated from SCN that have not been identified and do not have ITS sequences in the databases. We detected a portion of fungi in

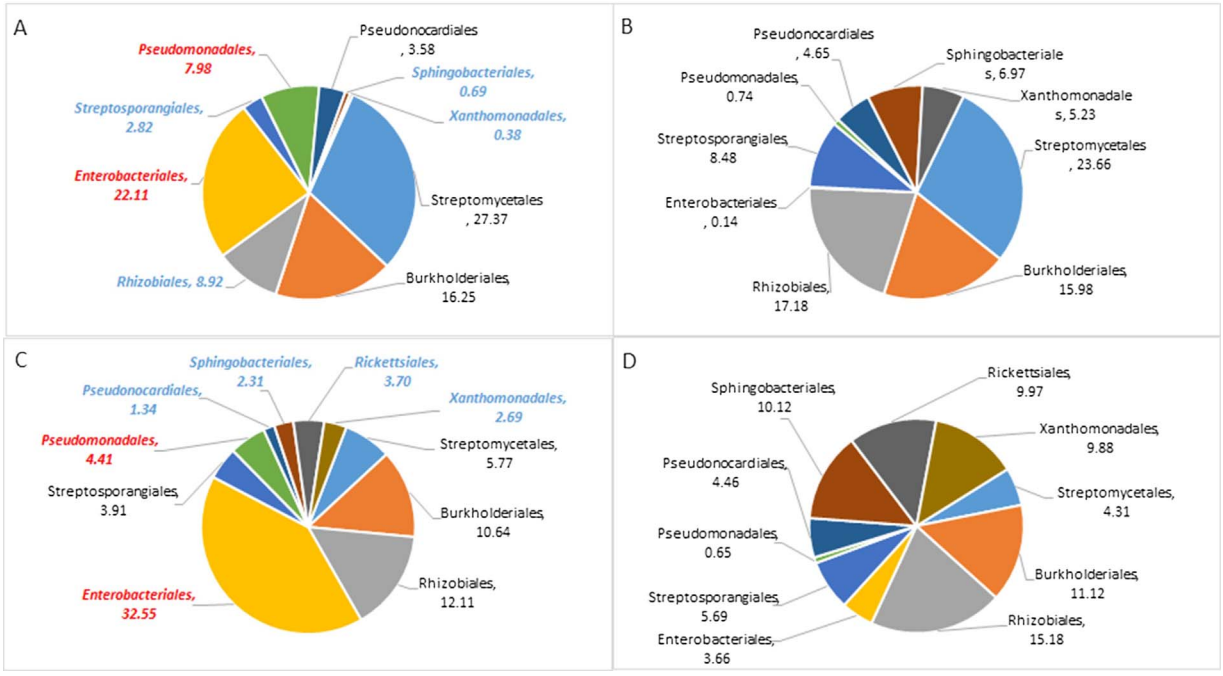

Fig. 6. The relative abundance of top bacterial orders affected by formaldehyde treatment sampled at planting and midseason. A: Formaldehyde treatment sampled at planting, B: No formaldehyde treatment (control) sampled at planting, C: Formaldehyde treatment sampled at midseason, D: No formaldehyde treatment (control) sampled at midseason. Orders with blue text indicate formaldehyde significantly decreased the relative abundance compared with the control treatment within each season; red text indicates an increase according to the Bonferroni adjusted *P* value at $P < 0.05$.

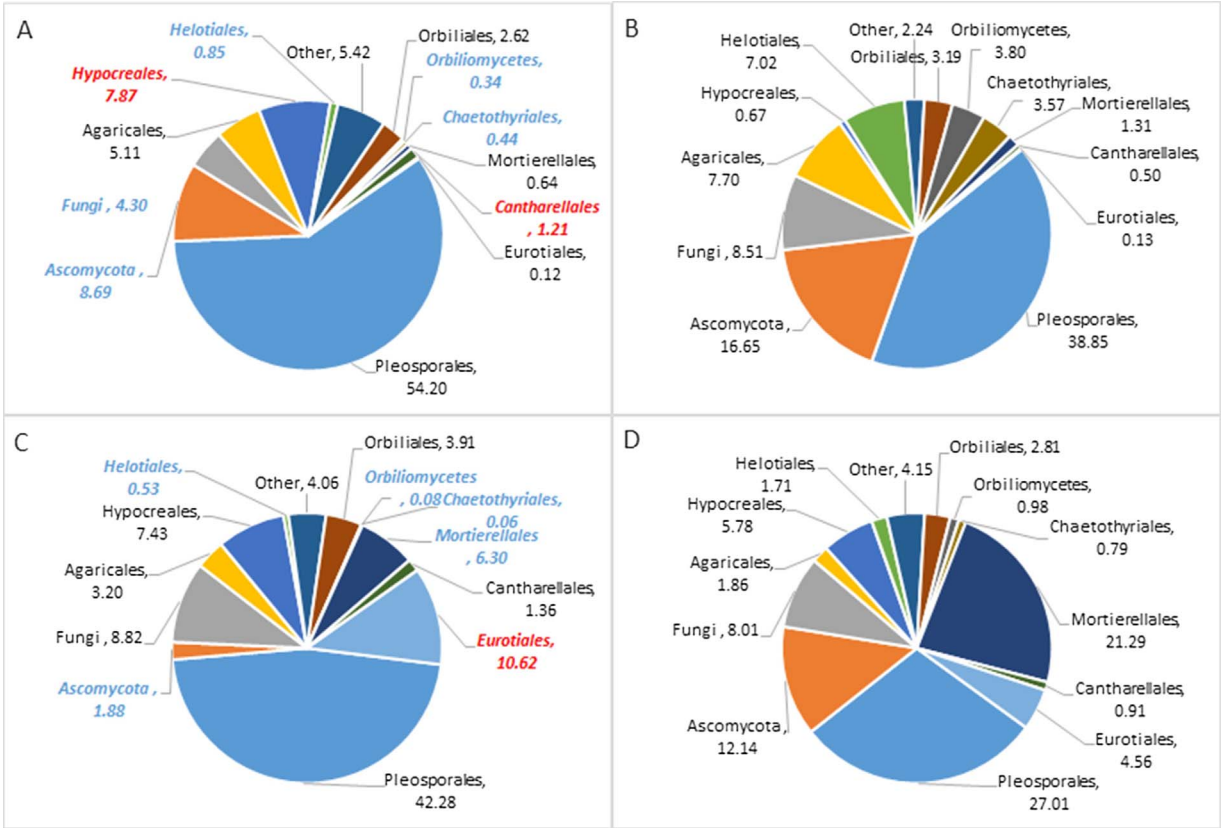

Fig. 7. The relative abundance of top fungal orders affected by formaldehyde treatment sampled at planting and midseason. A: Formaldehyde treatment sampled at planting, B: No formaldehyde treatment (control) sampled at planting, C: Formaldehyde treatment sampled at midseason, D: No formaldehyde treatment (control) sampled at midseason. Orders with blue text indicates formaldehyde treatment significantly decreased the relative abundance compared with control treatment within each sampling time point; red text indicates an increase according to the Bonferroni adjusted *P* value at $P < 0.05$.

Ascomycota that could not be identified to a lower taxonomic rank, and fungi in the family *Orbiliaceae* that were decreased by formaldehyde. There is an urgent need to improve our fungal databases to minimize numbers of unidentified OTUs. These unidentified fungi, especially those in *Orbiliaceae*, to which many nematophous fungi belong, could be important in regulating SCN populations in this field.

Biocontrol would greatly benefit from a thorough understanding of the function of the microbes and the mechanisms of SCN suppression in the field. The information uncovered in this study is based on gene amplification of the 16S and ITS region, and is not comprehensive enough to reveal potential mechanisms, but has characterized for the first time using culture-independent approaches the composition of microbial communities in SCN cysts. Future research should be conducted on the candidate biocontrol microbes we have shown to decrease in response to formaldehyde treatment to better understanding mechanisms of suppression, including identification and functional analysis of genes or metabolites that are involved in nematode suppression, factors that affect their growth in soil, and the inhibition of other microbial taxa by biological control organisms.

5. Conclusion

In this study, we investigated the microbial communities colonizing the cysts of SCN in a suppressive soil under tillage and formaldehyde treatments. The formaldehyde treatment changed the SCN suppression level of the soil, and revealed the differences of microbial communities in the SCN cysts. There was not much difference for bacterial and fungal communities between conventional tillage and no-till. However, the bacterial and fungal community compositions were significantly different between formaldehyde treatment and control. Formaldehyde changed the relative abundance of biological control bacteria *Lysobacter*, *Actinocorallia* and *Pseudomonas*; and biological control fungal taxa such as *Exophiala*, *Phochonia*, *Clonostachys*, *Trichoderma*, *Purpureocillium* and *Phoma*. Our study suggests that both bacteria and fungi might play important roles in soil suppression of SCN.

Acknowledgements

The authors thank C. Johnson, W. Gottschalk, and J. Ballman for field sample collection, and M. Dornbusch and C. Klatt for technical support. This study was funded by the National Research Initiative (NRI) Arthropod and Nematode Biology and Management Program of the USDA National Institute of Food and Agriculture Grant No. 2009-35302-05261.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.07.018.

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