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REGULAR ARTICLE

Crop diversity facilitates soil aggregation in relation to soil microbial community composition driven by intercropping

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Abstract

Background and Aims Studies verify that intercropping increases soil macro-aggregates but the mechanism underlying the increase is poorly understood.

Methods Three long-term field experiments were conducted starting in 2009 at three sites in an oasis in northwest China. The first was a split-plot design: *Rhizobium* (with or without inoculation) and three cropping systems (faba bean/maize intercropping and corresponding monocultures). The second and third experiments were both single-factorial randomized block designs with nine cropping systems (maize intercropped with faba bean, chickpea, soybean, or oilseed rape, and the corresponding monocultures). Soil aggregates were determined by the wet sieving method. Microbial biomass and community composition in 2015 and 2016 were determined by phospholipid fatty acid (PLFA) and high throughput sequencing analysis of 16S *rRNA*.

Results Soil macro-aggregates (> 2 mm) in intercropping systems increased by 15.5–58.6% across three sites and two years, an effect derived partly from increased relative abundance of soil *Sordariales*, from enhanced arbuscular mycorrhizal fungi biomass, or from reduced relative abundance of *Nitrospirae*, depending on soil type.

Conclusions Intercropping alters soil microbial community composition and further facilitates soil aggregation. These findings provide insights into the mechanisms underlying the maintenance of biodiversity in ecosystem functioning.

Keywords Arbuscular mycorrhizal fungi · Crop diversity · Intercropping · Legumes · Long-term field experiments · Maize · *Nitrospirae* · Soil aggregates · *Sordariales*

Abbreviations

AMF	Arbuscular mycorrhizal fungi
C	Carbon
FAMEs	Fatty acid methyl esters
FB	Fungal biomass
K	Potassium
N	Nitrogen

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non-AMF	non-arbuscular mycorrhizal fungi
P	Phosphorus
PLFA	Phospholipid fatty acid
PLS-DA	Partial least squares discriminant analysis
SEM	Structural equation model

Introduction

Intensive farming systems have been characterized as high input systems with chemical fertilizers, pesticides and simplified crop diversity, thus leading to potential problems regarding risks of soil degradation, environmental pollution, and low resilience. Intercropping systems in which at least two crops are grown in the same field at the same time are able to both enhance crop diversity at the field scale and maintain multiple ecosystem functions such as efficient use of natural resources (water, soil, light and heat (Vandermeer 1989)) and control of crop diseases (Zhu et al. 2000), and are therefore considered to be one management solution for problems associated with modern farming systems.

Intercropping has been widely adopted in many countries including China (Francis 1986; Li et al. 2013a). Maize/bean in Kenya and banana/pineapple in Mozambique are grown in south and east Africa (Mucheru-Muna et al. 2010; Rusinamhodzi et al. 2012), wheat/maize, faba bean/maize, and agroforestry systems are found in Indonesia, China and India in Asia (Li et al. 2007; Zomer et al. 2009), maize/soybean, pea/barley, and wheat/canola are grown in Canada and Brazil in the Americas (Li et al. 2013b), and potato/cabbage and vegetables/vegetables are grown in Denmark, the United Kingdom, Spain, and Germany in Europe (Hauggaard-Nielsen et al. 2009; Zomer et al. 2009; Li et al. 2013a). Most previous studies have demonstrated that intercropping can enhance crop productivity (Willey 1979; Ghosh et al. 2006; Li et al. 2007; Dahmardeh et al. 2010; Mucheru-Muna et al. 2010; Snapp et al. 2010), crop nitrogen (N) (Senaratne et al. 1995; Chu et al. 2004; Xiao et al. 2004; Garland et al. 2017) and phosphorus (P) acquisition and utilization (Li et al. 2003, 2007; Hauggaard-Nielsen et al. 2009; Dissanayaka et al. 2015), and biological control of crop diseases (Zhu et al. 2000). As a result, overyielding in intercropping usually leads to greater removal of nutrients from soils and contributes to sustainability in terms of soil fertility. Previous work indicates that intercropping is able to maintain soil fertility in terms of soil organic matter (SOM) content, total N, Olsen P, exchangeable potassium (K) and soil enzyme activities (Wang et al. 2014, 2015). However, soil physical properties under intercropping, especially over the long term, have been little studied.

Soil aggregates represent a key soil physical property and a specific indicator of high soil quality (Tisdall and Oades 1982; Six et al. 2000; Diaz-Zorita et al. 2002) and environmental health (Siddiky et al. 2012b). For instance, a soil with well-developed soil aggregates facilitates root growth (Rillig et al. 2014), regulates soil water and gaseous interactions (Oades 1984; Six et al. 2004; Spohn and Giani 2011), augments soil carbon (C) sequestration (Mardhiah et al. 2014), and increases resistance to soil erosion (Six et al. 2006; Siddiky et al. 2012b). Agricultural practices such as tillage (Wang et al. 2010; Kihara et al. 2012; Plaza-Bonilla et al. 2013), fertilization (Plaza-Bonilla et al. 2012), and land use (Jagadamma et al. 2014) together with soil properties such as soil texture (Amézketa 1999; Denef et al. 2002) have effects on soil aggregates. In addition, previous studies show that cropping systems can change the content and distribution of soil aggregates. For example, soil water stable aggregates were higher in a soybean-wheat-corn-cover crop rotation system than in a corn monoculture (Tiemann et al. 2015) or in a corn-soybean-wheat-alfalfa rotation system (Corbin et al. 2010). Soil aggregates increased by 50% under walnut/perennial kura clover intercropping (Kremer and Kussman 2009), were 4.5 times higher in wheat/alfalfa intercropping (Corbin et al. 2010), and increased by 52% in maize/pigeon pea intercropping compared to the corresponding monocultures (Garland et al. 2017). However, the mechanisms underlying enhanced soil aggregation remain poorly understood and studies using long-term field experiments are rare.

Soil aggregates can be influenced by a range of soil biota (Tisdall and Oades 1982; Miller and Jastrow 2000; Rillig 2004; Siddiky et al. 2012a). Five main factors influencing soil aggregation can be summarized as soil microorganisms (Tisdall and Oades 1982), plant roots (Degens 1997), soil fauna (Brown et al. 2000), inorganic binding agents (Six et al. 2002) and environmental variables (Denef et al. 2001).

Soil microorganisms are closely associated with the formation and stabilization of soil aggregates (Tisdall and Oades 1982; Gupta and Germida 1988, 2015; Bossuyt et al. 2001; Six et al. 2004; Rillig and Mummey 2006; Bach and Hofmockel 2014; Mardhiah

et al. 2014). Fungal hyphae play an important role in both nutrient acquisition (Jakobsen and Rosendahl 1990) and the formation and stabilization of soil aggregates (Tisdall 1994; Gupta and Germida 1988). The formation of soil aggregates is positively correlated with the activity and biomass of soil fungi but not with bacterial activity (Bossuyt et al. 2001). Studies show that arbuscular mycorrhizal fungi (AMF), fungal hyphae and certain bacteria play major roles in promoting an increase in soil aggregates (Gupta and Germida 1988). Soil fungal and bacterial community composition changes with increasing cultivation time and changing cropping system (Duchicela et al. 2013). For instance, intercropping altered the bacterial community composition in the rhizosphere of wheat/maize, wheat/faba bean and faba bean/maize intercropping systems (Song et al. 2007a, b), and the numbers of fungi, bacteria and actinobacteria in the soil increased by 115.5, 43.6 and 57.3% in a soybean/sugarcane intercropping system compared to the corresponding monocultures (Li et al. 2013a), and also increased in a watermelon/pepper intercropping system (Sheng et al. 2012).

We therefore hypothesize that intercropping alters the composition of the soil microbial community and this drives soil aggregation. The hypothesis was tested by exploring three questions, namely (1) whether intercropping increases soil macro-aggregates (> 2 mm) in long-term field experiments, (2) whether intercropping alters soil microbial biomass and microbial community composition and (3) which microorganisms are associated with increasing soil macro-aggregates (> 2 mm), based on our long-term intercropping field experiments.

Materials and methods

Study sites

Three long term experimental sites were established in 2009, i.e. Hongsipu site of Ningxia Academy of Agriculture and Forestry Sciences, Ningxia Hui Autonomous Region (38°37'N, 104°40′E, 1450 m a.s.l., Expt. 1), Jingtan site of the Institute of Soils, Fertilizers and Water-Saving Agriculture, Gansu Academy of Agricultural Sciences, Gansu Province (37°05′N, 104°40′E, 1645 m a.s.l., Expt. 2) and Baiyun site of the Institute of Soils, Fertilizers and Water-Saving Agriculture, Gansu Academy of Agricultural Sciences, Gansu Province (38°37'N, 102°40′E, 1504 m a.s.l., Expt. 3). The soils had been reclaimed for 2, 20 and > 100 years in Expt. 1, Expt. 2 and Expt. 3. Before the experiments started the cropping systems were monocropped maize at the Expt. 1 and Expt. 2 sites and wheat/maize intercropping at the Expt. 3 site. The original vegetation was temperate needlegrass arid steppe in Expt. 1, temperate semi-shrub and dwarf semi-shrub desert in Expt. 2, and one crop annually of cold-resistant economic crops in Expt. 3 (http://www.nsii.org.cn).

The mean annual temperature and annual precipitation of the three experimental sites were 8.9 °C, 185.4 mm y^{-1}, 6.6 °C, 200 mm y^{-1} and 7.7 °C, 150 mm y^{-1}, respectively. The soils were sandy soil (83% sand + 15% silt + 2% clay) in Expt. 1, sandy loam (51% sand + 45% silt + 4% clay) in Expt. 2 and sandy loam (57% sand + 39% silt + 4% clay) in Expt. 3 (International Society of Soil Science). The soils at the three experimental sites were a calcareous (pH (2.5:1 $CaCl_2$) 7.4) Entisols (Sierozems), a calcareous (pH (1:2.5 soil: DI water) 7.6) Aridisol and a calcareous (pH (1:2.5 soil: DI water) 7.7) Aridisol, respectively. The SOM contents at the three experimental sites were 5.7, 15.9 and 19.1 g kg^{-1}, respectively, and the soil total N contents were 0.27, 0.83 and 1.1 g, with 2.4, 9.3 and 20.3 mg available P (Olsen P) and 109, 205 and 233 mg available K kg^{-1} dry soil in the top 20 cm of the soil profile.

Experimental design and crop management

Experiment 1

A split-split-plot experimental design with three replicates was adopted in Expt. 1 starting from 2009. The main plot factor was inoculation, either inoculated with *Rhizobium* sp. strain NM353 suspension (provided by the Biology College of China Agricultural University, Beijing) or uninoculated. The sub-plot factor was five N application rates (0, 75, 150, 225 and 300 kg N ha^{-1} y^{-1}) as urea ($CO(NH_2)_2$, 46% N). The sub-sub-plot factor was three cropping systems, comprising one intercropping system, faba bean (*Vicia faba* L. cv. Lincan no. 5)/maize (*Zea mays* L. cv. Xianyu no. 335) and two monocultures (faba bean and maize). In our experiment we collected soil samples from only one N level (225 kg N ha^{-1} y^{-1}), so our experimental design became a split-plot design. The main plot factor was inoculation with *Rhizobium* or uninoculated, and the sub-plot factor was the three cropping systems.

All treatments received 52 kg P ha^{-1} $year^{-1}$ as triple superphosphate ($Ca(H_2PO_4)_2•H_2O$, 20% P) in accordance with conventional agricultural practice in the region based on recommended application rates. All plots received 50 kg K ha^{-1} $year^{-1}$ as potassium sulfate (K_2SO_4, 46% K) in 2016, and all P and K fertilizers were evenly broadcast and incorporated into the soil before the faba bean was sown. Maize received half the N application rate (as urea), and the other half was applied with irrigation in two equal portions at the stem elongation and pre-tasseling stages. Faba bean received 50% of the total N rate of maize, and this was evenly broadcast and incorporated into the soil along with basal fertilizer K before the faba bean was sown.

There were a total of 90 plots in Expt. 1. Each plot was 3.0 m long × 1.2 m wide (faba bean monocropping), 3.0 m × 2.4 m (maize monocropping), or 6.0 m × 3.6 m (intercropping) (Supplementary Fig. 1a). Each intercropping plot contained three strips, each 1.2 m wide, and the three strips comprised 6 rows of maize and 6 rows of faba bean. Two rows of maize (0.4 m inter-row distance) and two rows of faba bean (0.2 m inter-row distance) were planted alternately in each intercropping strip and the spacing between the maize and adjacent faba bean was 0.3 m. The inter-plant spacing (within the same row) of maize and faba bean was 0.3 m and 0.2 m, respectively, in both the monocropping and intercropping plots. The between-plot spacing was 0.5 m within each block and the blocks were separated by a 0.5-m-wide irrigation furrow and 0.5-m ridges. The densities of monocropped maize and faba bean were 8.3 and 25 plants m^{-2}, respectively. The densities of intercropped maize and faba bean were also 8.3 and 25 plant m^{-2}, respectively. Thus, the overall proportional density of each crop species was equal in the monocropping and intercropping systems. Two-thirds of each intercropped area was occupied by maize and one-third by faba bean. The positions of the two species in the strips remained the same each year. Faba bean (2 rows) and maize (2 rows) were intercropped continuously on the same strips of land. All intercropped and monocropped plots were planted in a south–north row orientation.

Dates of sowing were 19 to 21 March for faba bean and 17 to 19 April for maize in 2015 and 2016. Dates of harvesting were 25 to 27 July for faba bean and 30 September to 2 October for maize in 2015 and 2016. Grain yields and aboveground biomass of faba bean and maize were determined on samples collected by harvesting two rows of maize and two rows of faba bean in the intercropping and monocropping plots, respectively, when the crops were at maturity. After harvesting, the above-ground parts of the crops were removed from the plots. All plots were irrigated during the growing season according to local farming practice at the same time every year. Seven applications of irrigation of 150 mm (1500 m^3 ha^{-1}) each were carried out on 8 to 10 April, 1 to 5 May, 18 to 20 May, 1 to 5 June, 15 to 20 June, 20 July, and 1 to 5 August. No organic fertilizers or herbicides were used during the growing season.

Experiments 2 and 3

A split-plot experimental design with three replicates was adopted in Expts 2 and 3, and the design has remained the same since 2009. The main-plot factor was three P application rates (0, 40, and 80 kg P ha^{-1} y^{-1}) as diammonium phosphate ($(NH_4)_2HPO_4$, 18% N and 20% P). The sub-plot factor was nine cropping systems comprising four intercropping system: faba bean/maize, soybean (*Glycine max* L.cv. Zhonghuang No. 30)/maize, chickpea (*Cicer arietimum* L. cv. Longying No. 10)/maize, oilseed rape (*Brassica campestris* L. cv. Gannan No. 4)/maize and five monocultures (faba bean, soybean, chickpea, oilseed rape and maize). Soil samples were collected from only one P level (40 kg N ha^{-1} y^{-1}), so the experimental design became a single-factorial randomized block. The crop combinations and species were averaged as replicates.

All plots of intercropped or monocropped maize received 75 kg N ha^{-1} $year^{-1}$ as basal fertilizer either as urea ($CO(NH_2)_2$, 46% N) in zero P application plots or as urea plus diammonium phosphate ($(NH_4)_2HPO_4$, 18% N and 20% P). 150 kg N ha^{-1} was divided into two equal amounts and applied with irrigation at the stem elongation and pre-tasseling stages. Faba bean, soybean, chickpea and oilseed rape received 50% of the total N rate applied to maize and this was evenly broadcast and incorporated into the soil along with fertilizer P and K before sowing, in accordance with conventional agricultural practice in the region based on recommended application rates. All plots received 50 kg K ha^{-1} $year^{-1}$ as potassium sulfate (K_2SO_4, 46% K) in 2016 only.

There were 81 plots in Expts 2 and 3. Each plot was 6.0 m × 4.2 m in Expt. 2 and 5.5 m × 4.0 m (faba bean, soybean, chickpea, or oilseed rape monocropping), 5.5 m × 5.0 m (maize monocropping), or 5.6 m × 5.5 m (intercropping systems) in Expt. 3

(Supplementary Fig. 1b, c). Intercropping plots contained three strips (Expt. 2) or four strips (Expt. 3), each 1.4 m wide. Two rows of maize (0.4 m inter-row distance) and three rows of faba bean, soybean, chickpea or oilseed rape (0.2 m inter-row distance) were planted alternately in each intercropping strip, and the spacing between maize and the adjacent crops was 0.3 m. The inter-plant spacing (within the same row) of maize and associated crops was 0.3 m and 0.2 m, respectively, in both the monocropping and intercropping plots. The between-plot spacing was 0.5 m within each block and the blocks were separated by a 0.5-m-wide irrigation furrow and 0.5-m ridges. Maize rows occupied 0.8 m/1.4 m = 4/7 of the intercropped area, and the associated crops occupied 0.6 m/1.4 m = 3/7 of the intercropped area. To compare intercropping with monocropping, the densities of intercropped maize and associated crops were designed to be equal to that of monocropped maize and associated crops with 8.3 and 25 plants m^{-2} in the case of maize and associated crops, respectively. The positions of the two species in the strips were alternated between years. For instance, three rows of faba bean and two rows of maize were rotationally intercropped with one crop strip in one year and the other crop strips in the subsequent year in the faba bean/maize intercropping. All intercropped and monocropped plots were planted in a south–north row orientation in Expt. 2 and east–west row orientation in Expt. 3.

Faba bean, chickpea and oilseed rape were sown on 18 to 22 March and the crops were harvested on 25 July to 31 July. Maize and soybean were sown on 18 to 20 April and harvested on 20 September to 5 October in 2015 and 2016. At maturity, grain yields and above-ground biomass of associated crops and maize were determined on samples collected by harvesting two rows of maize and three rows of associated crops in the intercropping system and two rows of maize in the monocropped maize, three rows of the associated crop in the monocropped associated crops. After harvest, the above-ground parts of the crops were removed from the plots.

All plots were irrigated during the growing season according to local farming practice at the same times each year. Five applications of irrigation of 150 mm (1500 m^3 ha^{-1}) each were made on 1 May to 10 May, 1 June to 5 June, 10 July to 20 July, 10 August, and 25 to 31 August in Expt. 2. Six applications of irrigation of 150 mm (1500 m^3 ha^{-1}) each were made on 10 to 20 May, 1 to 5 June, 20 to 25 June, 5 July, 25 to 30 July, and 14 to 20 August in both years in Expt. 3. No organic fertilizers or herbicides were used during the growing season.

Soil sampling

Soil samples for microbial community analysis were collected from the top 20 cm of the soil profile using an auger (35 mm diameter) in plots fertilized with 225 kg N ha^{-1} y^{-1} in Expt. 1 and 40 kg P ha^{-1} y^{-1} in Expts 2 and 3 on 18 to 20 June in both 2015 and 2016. Four soil cores were collected from each monocropping plot and mixed together to give one composite sample per plot. Similarly, four soil cores were collected from each crop strip in the intercropping plots to give two composite samples from each intercropping plot (Supplementary Fig. 2). The composite samples were sieved through a 2-mm mesh and transferred to polyethylene bags for storage at −80 (microbial analysis) or 4 °C.

Soil samples for soil aggregate analysis were collected from plots in situ which were taken from the top 20 cm of the soil profile using an aluminum box (80 mm diameter) in plots fertilized with 225 kg N ha^{-1} y^{-1} in Expt. 1 and 40 kg P ha^{-1} y^{-1} in Expts 2 and 3 after the maize harvest in both 2015 and 2016. Crop residues, surface impurities and dry soil layers were removed before sampling. There was one soil sample from each monocropping plot and from each species strip in the intercropping plots giving two samples for each intercropping plot (Supplementary Fig. 2). The samples were then air-dried.

Separation of soil aggregates

Soil aggregates were separated by the wet sieving method using a Model TTF-100 soil aggregate analyzer (Luda Machinery Instrument Co. Ltd., Shangyu city, Zhejiang province, China). Air dried soil samples (100 g) were placed in the soil aggregate analyzer (five sieves). Soil samples were pre-immersed in water for 5 min, shaken vertically for 2 min for each sieve at a frequency of 40 times per minute, and washed from each sieve into the corresponding aluminum box. The soil samples were divided into four fractions: macro-aggregates (> 2 mm), macro-aggregates (2–0.25 mm), micro-

aggregates (0.25–0.106 mm) and primary particles (< 0.106 mm).

Soil microbial biomass: Phospholipid fatty acid (PLFA) analysis

The PLFA method was used to determine soil microbial biomass (Bligh and Dyer 1959; Frostegård et al. 1991). Briefly, the phospholipids were extracted from 5 g fresh soil with citrate buffer (pH 4): chloroform: methanol (0.8:1:2) solvent mixture. The phospholipids were separated with solid-phase extraction silica gel columns (Bonna-Agela Technologies Co., Ltd., Tianjin, China). The phospholipids collected were methylated to phospholipid fatty acid methyl esters (FAMEs) using 1 mL methanol-toluene (1:1) and 0.2 mol L^{-1} KOH-methanol solvent. The FAMEs were then dissolved in hexane and detected by gas chromatography-mass spectrometry (Model N6890, Agilent, Santa Clara, CA). Standard 19:0 phospholipid was added before detection and the peak was used as the standard peak. The 18:1ω9c and 18:2ω6c characteristic fatty acids were used as the fungal biomarkers, and i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, 17:0, cy17:0, 18:1ω7c, and 16:1ω7c were used as bacterial biomarkers. The AMF biomarker was 16:1ω5c and the saprobic fungal biomarker was 18:2ω6c (Frostegård and Bååth 1996; Moeskops et al. 2010, 2012).

Microbial community composition: High throughput sequencing

Fungal and bacterial community composition was analyzed by Illumina MiSeq sequencing of 16S *rRNA* (Miseq PE300) gene and 18S *rRNA* (PE250) gene amplicons. DNA was extracted from 0.5 g soil samples using the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA). Amplification of 16S and 18S *rRNA* gene fragments was performed using bar-coded primers according to Fan et al. (2014) and Rousk et al. (2010). Briefly, universal primers 338F (5′- ACTCCTACGGGAGGCAGCA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′) were used to amplify 16S *rRNA* gene fragments and SSU1196R (5'-TTAGCATGGAATAATRRAATAGGA-3′) and SSU0817F (5'-TCTGGACCTGGTGAGTTTCC-3′) were used to amplify the 18S *rRNA* gene. After gel-purification, PCR products were quantified with the QuantiFluo - ST blue fluorescence quantitative system (Promega Inc., Madison, WI). Sequencing of 18S and 16S *rRNA* gene amplicons was performed on Miseq PE250 and Miseq PE300 platforms, respectively, by Shanghai Majorbio Bioinformatics Technology Co., Ltd., China. The paired-end (PE) raw sequence reads were quality-filtered using the FLASH software package on the Quantitative Insights into Microbial Ecology platform (QIIME, http://qiime.org/scripts/assign_taxonomy.html) (Caporaso et al. 2010). Briefly, low quality sequences were filtered (< 50 bp in length with an average quality value of 20), then the paired reads were spliced into a single sequence, minimum overlap length of 10 bp barcode. The high-quality sequences were then clustered into the operational taxonomic units (OTUs, > 97% sequence identity) using the Usearch (version 7.1, http://drive5.com/uparse/). Representative sequences of each OTU were classified using the Ribosomal Database Project (RDP, version 2.2, http://sourceforge.net/projects/rdp-classifier/) classifier (Wang et al. 2007).

Calculations

Soil properties (soil aggregates and soil microbial parameters) of faba bean/maize, soybean/maize, chickpea/maize and oilseed rape/maize intercropping systems were compared directly with those in the monocropping systems. Crop combinations and crop species were not considered.

The averages of soil aggregates in monocropping and intercropping systems were calculated as follows:

$$ASA_M = (A_{mono_a} + A_{mono_b})/2 \quad (1)$$

$$ASA_I = (A_{inter_a} + A_{inter_b})/2 \quad (2)$$

Where A_{monoa} and A_{monob} are the soil aggregates from monocropped plot a and monocropped plot b used in the monocultures, and A_{intera} and A_{interb} are soil aggregates from intercropped plot a and intercropped plot b in one intercropping strip. ASA_M, average of soil aggregate in monocultures; ASA_I, average of soil aggregate in intercropping system.

The above equations were also used to calculate the averages of soil microbial biomass and relative abundance in monoculture or intercropping.

Statistical analysis

The overall effect of cropping system on soil aggregates and soil microbial biomass was evaluated using linear

mixed-effects models with the "nlme" package (Pinheiro et al. 2013), R version 3.4.0 (public web access: http://cran.r-project.org/). In the statistical analysis, soil aggregates (> 2 mm, 2–0.25 mm, 0.25–0.106 mm, < 0.106 mm) and soil microbial biomass (FB, F/B, non-AMF, non-AMF/B, AMF, SFB, BB, ACT) were treated as fixed factors, while experimental site was treated as a random factor at the 5% probability level.

In Expt. 1 a split-plot design of ANOVA (procedure shown in Supplementary Table 5) was used to analyze the effects of inoculation with rhizobium or not, cropping system (monocropping vs. intercropping) and their interactions on the soil aggregates (> 2 mm, 2–0.25 mm, 0.25–0.106 mm, < 0.106 mm) and soil microbial biomass (FB, F/B, non-AMF, non-AMF/B, AMF, SFB, BB, ACT) in two separate years. Fisher Protected Least Significant Difference (LSD) test was used for pairwise comparisons to determine the differences among inoculation, cropping system and their interactions. The liner model was $y_{jkl} = \mu + \beta_j + A_k + \delta_{jk} + B_l + (AB)_{kl} + \varepsilon_{jkl}$ (β_j: block, A_k: main-plot treatment, δ_{jk}: main-plot error, B_l: sub-plot, $(AB)_{kl}$: interaction of main-plot and sub-plot, ε_{jkl}: sub-plot error). In Expts 2 and 3 a randomized block design of ANOVA (procedure shown in Supplementary Table 6) was used to analyze the effects of cropping system (monocropping vs. intercropping) on the soil aggregates (> 2 mm, 2–0.25 mm, 0.25–0.106 mm, < 0.106 mm) and soil microbial biomass (FB, F/B, non-AMF, non-AMF/B, AMF, SFB, BB, ACT) in two separate years. Fisher Protected LSD test was used for pairwise comparisons to determine the differences between the two cropping systems. The model was $y_{ij} = \mu + \beta_j + \tau_i + \varepsilon_{ij}$ (β_j: block, τ_i: treatment, ε_{ij}: error). Significant differences between treatments were examined at the 5% level (soil aggregates) or 10% level (soil microbial biomass). All analyses were conducted using SAS software (SAS Deployment Wizard 8.0).

Principal co-ordinates analysis (PCoA), based on Bray Curtis distance, was used to distinguish the separation of soil fungal and bacterial community composition among the three experimental sites. Bray-Curtis distance is a normalization method to distinguish the dissimilarity in species composition between two different sites or blocks andwas developed by Bray and Curtis (1957). It is currently used in biological studies to quantify the community compositional dissimilarity between two different microbial groups. The value of the Bray-Curtis distance is between zero and one, with zero indicating that the two sites or microbial groups have the same composition and one indicating that the species in the two sites or microbial groups are completely different from each other (Bloom 1981). The Bray-Curtis distance in the PCoA was used to describe the differences in species richness among different groups according to the calculation of uniformity abundance tables.

Partial least square discriminant analysis (PLS-DA) was used to detect the differences explaining the soil fungal and bacterial community composition of intercropping and monocropping at each of the three experimental sites. The Wilcoxon rank-sum test was used to evaluate the relative abundance of *Sordariales* influenced by cropping system (Haynes 2013) with False Discovery Rate (fdr) (a multiple test correction) and confidence intervals (CI). All above analyses were calculated on the platform of Shanghai Majorbio Bioinformatics Technology Co., Ltd., China (http://www.i-sanger.com/). Statistical testing of variation in microbial community composition was conducted using analysis of similarity (ANOSIM) and was conducted using 999 permutations on the Galaxy platform (public web access: http://www.freebioinfo.org/).

Structural equation modeling (SEM) is a method of establishing, estimating, and testing causality models, including a diverse set of mathematical models, computer algorithms, and statistical methods together to fit data (Kline 2011). SEM is an important tool for analyzing the relationships between observed variables and latent variables based on multivariate data analysis, covariance analysis, path analysis, factor analysis, etc. (Schermelleh-Engel et al. 2003). The SEM was used in our work to explore the pathways of how species numbers (monocropping vs. intercropping) influenced soil macro-aggregates (> 2 mm) through soil fungal and bacterial community structure using the packages "lavaan" (Rosseel 2012) in R 3.4.0 (procedure shown in Supplementary Table 7). The SEM in Fig. 7 and supplementary Fig. 3 were both outputs of the statistical package according to Supplementary Table 7. We first considered a full model that included all possible pathways and then sequentially eliminated nonsignificant pathways until we attained a stable and significant model at three separate experimental sites (Supplementary Fig. 3). Then we combined the data from the three experimental sites to obtain a general result which explained the increase in soil macro-aggregates (> 2 mm)

under intercropping systems (species numbers = 2) regardless of the experimental site background. We used the χ^2 test, Akaike information criteria and the root mean square error of approximation to evaluate the fit of the model.

Results

Grain yields and aboveground biomass

We do not present or consider grain yield or aboveground biomass in this paper but, as would be expected, overyielding was found in the intercropping systems compared to the monocultures. For example, faba bean/maize intercropping increased grain yields by 13.3% on average over the two years in Expt. 1. In Expt. 2 the grain yields of faba bean/maize, soybean/maize and chickpea/maize increased by 30.3, 19.2, and 25.7% on average (unpublished data).

Overall effect of intercropping on soil aggregates and microbes

Irrespective of experimental site, intercropping systems significantly increased soil macro-aggregates (> 2 mm) ($P = 0.013$, $P = 0.039$), soil FB ($P = 0.011$, $P < 0.001$), non-AMF ($P = 0.004$, $P < 0.001$), the ratio of fungal to bacterial biomass ($P < 0.001$, $P = 0.002$), and the ratio of non-AMF to bacterial biomass ($P < 0.001$, $P = 0.004$) compared to corresponding monocrops in both 2015 and 2016 (Supplementary Table 1). Soil AMF ($P = 0.041$) and saprobic fungal biomass ($P = 0.005$) increased in 2016 only (Supplementary Table 1). Soil primary particles (< 0.106 mm) ($P = 0.018$, $P = 0.009$) decreased in the intercropping systems compared to the corresponding monocropping systems in both 2015 and 2016 (Supplementary Table 1). Soil macro-aggregates (2–0.25 mm), bacterial biomass and actinobacterial biomass were not influenced by cropping system in either year (Supplementary Table 1).

Soil aggregates

Soil macro-aggregates (> 2 mm) were significantly higher in the intercropping systems than the corresponding monocultures. In general, soil macro-aggregates (> 2 mm) under intercropping systems increased by 17.8% ($P < 0.001$), 58.6% ($P < 0.001$) in Expt. 1 (Fig. 1a, Table 1), 15.5% ($P = 0.007$), 53.2% ($P = 0.038$) in Expt. 2 (Fig. 1b, Table 1), and 34.4% ($P = 0.017$) and 33.1% ($P = 0.031$) in Expt. 3 (Fig. 1c, Table 1) in 2015 and 2016 compared with the corresponding monocultures. Soil macro-aggregates (2–0.25 mm) in intercropping systems decreased by 13.4% ($P = 0.032$) in Expt. 1 (Fig. 1d, Table 1) and 16.2% ($P = 0.009$), 14.5% ($P = 0.046$) in Expt. 3 (Fig. 1f, Table 1) compared with the corresponding monocultures in 2015 and 2016, with the exception of Expt. 1 in 2015. Micro-aggregates (0.25–0.106 mm) in the intercropping systems decreased by 16.7% ($P = 0.030$) in Expt. 1 (Fig. 1g, Table 1), 25.9% ($P = 0.008$) in Expt. 2 (Fig. 1h, Table 1) and 20.5% ($P = 0.048$), 23.3% ($P = 0.021$) in Expt. 3 (Fig. 1i, Table 1) compared with the corresponding monocultures in both years, with the exception of Expt. 1 in 2015 and Expt. 2 in 2016. Primary particles (< 0.106 mm) under the intercropping systems decreased by 56.6% ($P < 0.001$) in Expt. 1 (Fig. 1j, Table 1), 24.3% ($P = 0.012$) in Expt. 2 (Fig. 1k, Table 1), and 14.2% ($P = 0.031$), 32.2% ($P = 0.032$) in Expt. 3 (Fig. 1l, Table 1) in 2015 and 2016 compared with the corresponding monocultures, with the exception of Expt. 1 in 2015 and Expt. 2 in 2016. In addition, inoculation with rhizobium decreased soil macro-aggregates (> 2 mm) by 5.5% ($P = 0.012$, Table 1) and 15.0% ($P < 0.001$, Table 1) in 2015 and 2016, and increased micro-aggregates (0.25–0.106 mm) by 19.3% ($P = 0.034$, Table 1) in 2016 in Expt. 1, but had no significantly influence on other fractions of soil aggregates.

Soil microbial biomass

Inoculation with rhizobium increased soil non-AMF, the ratio of fungal biomass and non-AMF biomass to bacterial biomass (Supplementary Table 2) and AMF biomass (Supplementary Table 2) in Expt. 1 in 2015, but had no significantly influence on other soil microbial biomass parameters.

Soil total FB increased in intercropping systems over the monocultures by 13.4% ($P = 0.094$), 28.3% ($P = 0.019$) in Expt. 2 (Fig. 2b, Table 1) in 2015 and 2016. The ratio of fungal biomass to bacterial biomass in intercropping increased by 25.1% ($P = 0.047$) in Expt. 3 in 2015 compared to the corresponding monocultures (Fig. 2f).

There were similar trends in soil non-AMF biomass (Fig. 2h, i, Table 1). Soil non-AMF biomass in intercropping systems increased by 23.7% ($P = 0.094$),

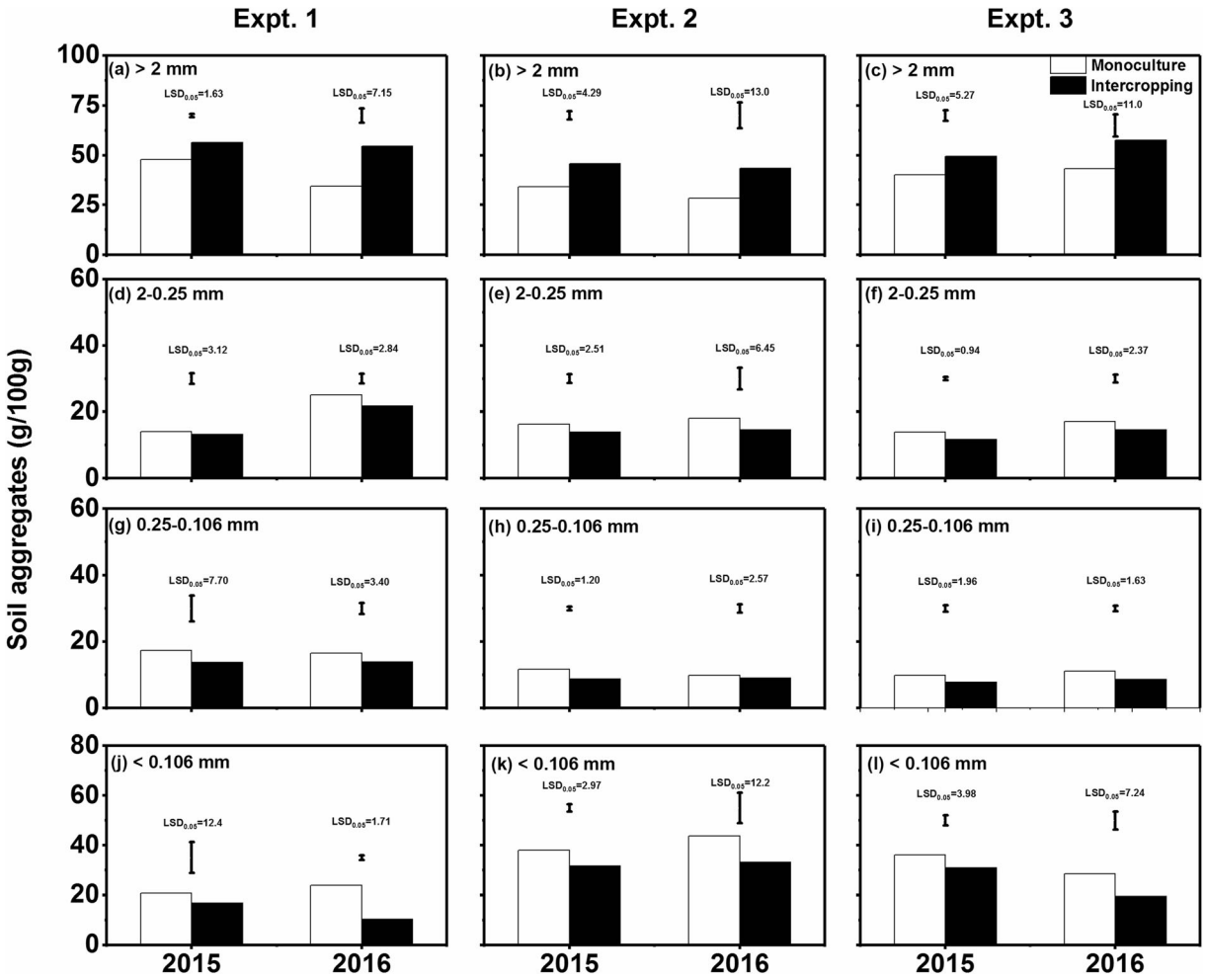

Fig. 1 Soil aggregates (g (100 g)$^{-1}$) of (**a**, **b**, **c**) > 2 mm, (**d**, **e**, **f**) 2–0.25 mm, (**g**, **h**, **i**) 0.25–0.106 mm, and (**j**, **k**, **l**) < 0.106 mm as affected by cropping system (averaged monocropping vs. intercropping) in Expts 1, 2, and 3 in 2015 and 2016. The Fisher protected least significant difference (LSD) test was used for pairwise comparisons to determine the differences between cropping systems in Expt. 1, Expt. 2 and Expt. 3. Lines indicate the standard value of the difference between monocropping and intercropping systems at the same experimental site using LSD at $P < 0.05$. Data show mean values with $n = 12$ in Expt. 1 and $n = 24$ in Expts. 2 and 3

37.7% ($P = 0.050$) in Expt. 2 (Fig. 2h) and 36.1% ($P = 0.108$), 25.1% ($P = 0.103$) in Expt. 3 (Fig. 2i) in 2015 and 2016 compared to the respective monocultures. The ratio of non-AMF biomass to bacterial biomass increased by 35.2% ($P = 0.055$) in Expt. 3 in 2015 (Fig. 2l) in intercropping systems over corresponding monocultures.

Saprobic fungal biomass increased by 9.4% in Expt. 2 in 2016 (Fig. 3e, Table 1) compared with the corresponding monocultures. Bacterial biomass decreased by 6.7% in Expt. 2 in 2015 (Fig. 3h, Table 1). However, there were no significant differences in soil AMF or actinobacterial biomass between the intercropping and monocropping systems (Fig. 3, Table 1).

Soil fungal and bacterial community composition in the three experiments

Soil fungal and bacterial community composition showed significant differences among the three experiments (Fig. 4). In the case of the fungal community composition, the first and second principal components (PC1, PC2) accounted for 36.1% (Fig. 4a, ANOSIM, $r = 0.48$, $P = 0.001$) and 25.8% (Fig. 4b, ANOSIM, $r = 0.46$, $P = 0.001$) of the variation in species level in 2015

Table 1 Split-plot design of ANOVA in Expt. 1 and randomized block design of ANOVA in Expts. 2 and 3 for the effects of inoculation with rhizobium (I), cropping system (Cs) and their interactions or cropping system (Cs) on soil aggregates (> 2 mm, 2–0.25 mm, 0.25–0.106 mm, < 0.106 mm) and soil microbial biomass parameters (FB, F/B, non-AMF, non-AMF/B, AMF, SFB, BB, ACT) using Fisher protected LSD

Experimental site	Year	Source	d.f.	> 2 mm		2-0.25 mm		0.25–0.106 mm		< 0.106 mm		F/B		FB	
				Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio
Expt. 1	2015	Main plots													
		I	1	25.802	**18.77***	3.723	1.840	37.715	4.830	13.356	0.770	2.386	7.110	0.114	**1024.76*****
		Subplots													
		Cs	1	215.765	**156.97*****	2.343	0.640	40.826	5.230	45.818	2.630	0.544	1.620	0.064	**577.3*****
		I*Cs	1	0.0156	0.010	0.174	0.050	0.191	0.020	0.533	0.030	3.309	**9.86***	0.046	**416.61*****
	2016	Main plots													
		I	1	155.356	**10.77***	24.052	7.490	21.390	**10.06***	23.912	1.560	0.067	0.080	0.010	2.290
		Subplots													
		Cs	1	1215.463	**84.22*****	33.753	**10.51***	23.011	**10.82***	550.937	**36.05***	1.774	2.090	0.004	0.910
		I*Cs	1	2.825	0.200	0.529	0.160	5.940	2.790	6.847	0.450	0.008	0.010	0.002	0.510
Expt. 2	2015	Cs	1	205.588	**137.58****	7.995	15.660	13.730	**116.96****	10.925	**85.49***	0.577	**9.15***	0.011	6.210
	2016	Cs	1	339.791	**24.88***	18.433	5.470	1.234	2.310	169.761	14.030	0.512	**50.36***	0.004	1.740
Expt. 3	2015	Cs	1	132.657	**58.94***	7.671	**107.38****	6.102	**19.53***	39.411	**30.74***	1.676	5.510	0.003	19.91*
	2016	Cs	1	306.032	**30.97***	9.160	**20.2***	10.123	**47.09***	127.367	**29.98***	0.422	5.720	0.0001	3.690

Experimental site	non-AMF		non-AMF/B		AMF		SFB		BB		ACT	
	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio	Mean squares	*F* ratio
Expt. 1	Main plots											
	0.203	**8.400***	0.0095	**20.65***	3.983	**8.75***	0.102	0.820	15.632	2.140	1.550	1.080
	Subplots											
	0.133	**5.500***	0.0033	**7.06***	1.217	2.670	0.086	0.700	32.202	4.410	5.771	4.010
	0.230	**9.47***	0.0015	3.240	1.795	3.940	0.187	1.510	0.440	0.060	0.0033	0.000
	Main plots											
	0.063	0.130	0.0067	2.590	0.0001	0.000	0.006	0.120	3.426	0.800	0.0004	0.000
	Subplots											
	1.011	2.000	0.0023	0.890	0.1067	2.320	0.104	2.030	5.338	1.240	0.063	2.040
	0.001	0.000	0.0009	0.360	0.0030	0.070	0.0002	0.000	1.399	0.330	0.346	1.130
Expt. 2	0.698	**9.2***	0.0089	5.250	0.0058	**9.75***	0.0199	1.380	2.653	**375.15****	0.095	**13.38***
	0.393	**18.42***	0.0025	1.210	0.0079	1.250	0.0026	**24.82***	0.016	0.010	0.0002	0.060
Expt. 3	1.498	7.820	0.0030	**16.68***	0.0050	0.340	0.0297	2.000	0.593	0.270	0.0051	0.020
	0.312	8.270	0.0007	5.740	0.0084	1.090	0.0147	5.320	1.343	1.010	0.0007	0.260

Inoculated rhizobium has two levels, inoculation with rhizobium and uninoculated. Cropping system has two levels, monocropping and intercropping systems. Fungal biomass, FB; ratio of fungal to bacterial biomass, F/B; non-arbuscular mycorrhizal fungal biomass, non-AMF; ratio of non-AMF to bacterial biomass, non-AMF/B; arbuscular mycorrhizal fungal biomass, AMF; saprobic fungal biomass, SFB; bacterial biomass, BB; and actinobacterial biomass, ACT. Significant effects ($P < 0.05$ or $P < 0.10$) of treatments are indicated in **bold**

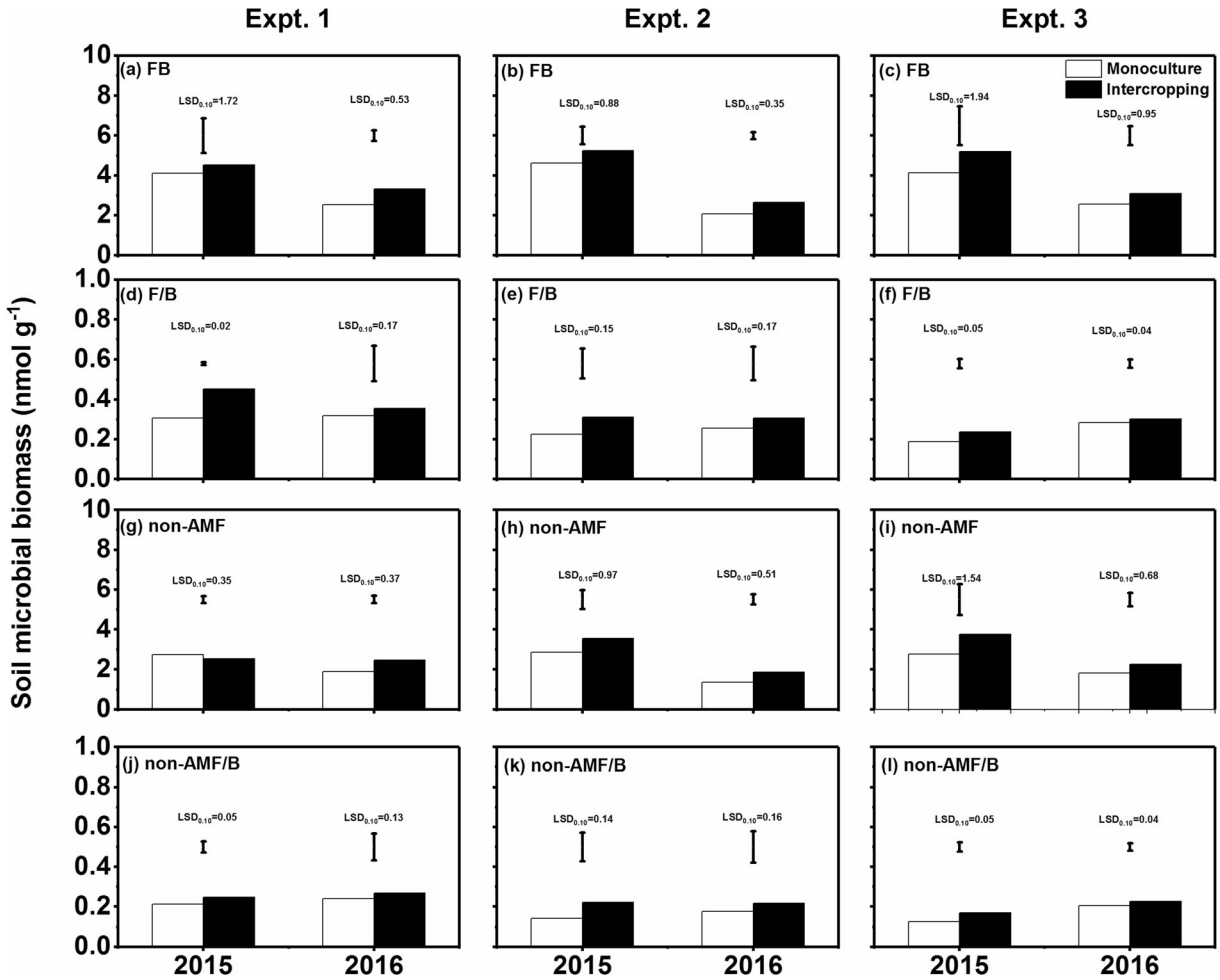

Fig. 2 Soil microbial biomass (nmol g^{-1}) of (**a**, **b**, **c**) fungal biomass (FB), (**d**, **e**, **f**) ratio of fungal biomass to bacterial biomass (F/B), (**g**, **h**, **i**) non arbuscular mycorrhizal fungal (non-AMF) biomass, and (**j**, **k**, **l**) ratio of non-arbuscular mycorrhizal fungal biomass to bacterial biomass (non-AMF/B) as affected by cropping system (averaged monocropping vs. intercropping) in Expts 1, 2, and 3 in 2015 and 2016. The Fisher protected least significant difference (LSD) test was used for pairwise comparisons to determine the differences between cropping systems in Expt. 1, Expt. 2 and Expt. 3. Lines indicate the standard value of the difference between monocropping and intercropping system at the same experimental site using LSD at $P < 0.10$. Data show mean values with $n = 12$ in Expt. 1 and n = 24 in Expts 2 and 3

and 2016. PC1 differentiated Expt. 3 from the other two experimental sites in 2015 and PC2 separated Expt. 2 from Expt. 1 (Fig. 4a). In the second year, PC1 separated Expt. 1 from Expts 2 and 3 and PC2 separated Expt. 2 from Expt. 3 (Fig. 4b). High loadings by *Cryptococcus* and *Fusarium* (2015) and *Pterocystis* and *Salpingoeca* (2016) contributed to the separation of Expt. 3 from Expt. 1 along the PC1 axis.

Similarly, in the case of the bacterial community composition, PC1 and PC2 accounted for 41.9% (Fig. 4c, ANOSIM, $r = 0.74$, $P = 0.001$) and 39.8% (Fig. 4d, ANOSIM, $r = 0.76$, $P = 0.001$) of the variation in species level in 2015 and 2016. PC1 differentiated Expt. 3 from Expts 1 and 2 and PC2 separated Expt. 2 from Expt. 1 in 2015 and 2016 (Fig. 4c, d). High positive loadings by *Gaiella* and *Amaricoccus* (2015) and *Microlunatus* and *Pseudonocardia* (2016) contributed to the separation of Expt. 3 from Expts 1 and 2 along the PC1 axis.

Soil microbial community composition under cropping systems in the three experiments

Cropping system significantly influenced soil fungal and bacterial community composition in both years (Fig. 5). In the case of fungal community

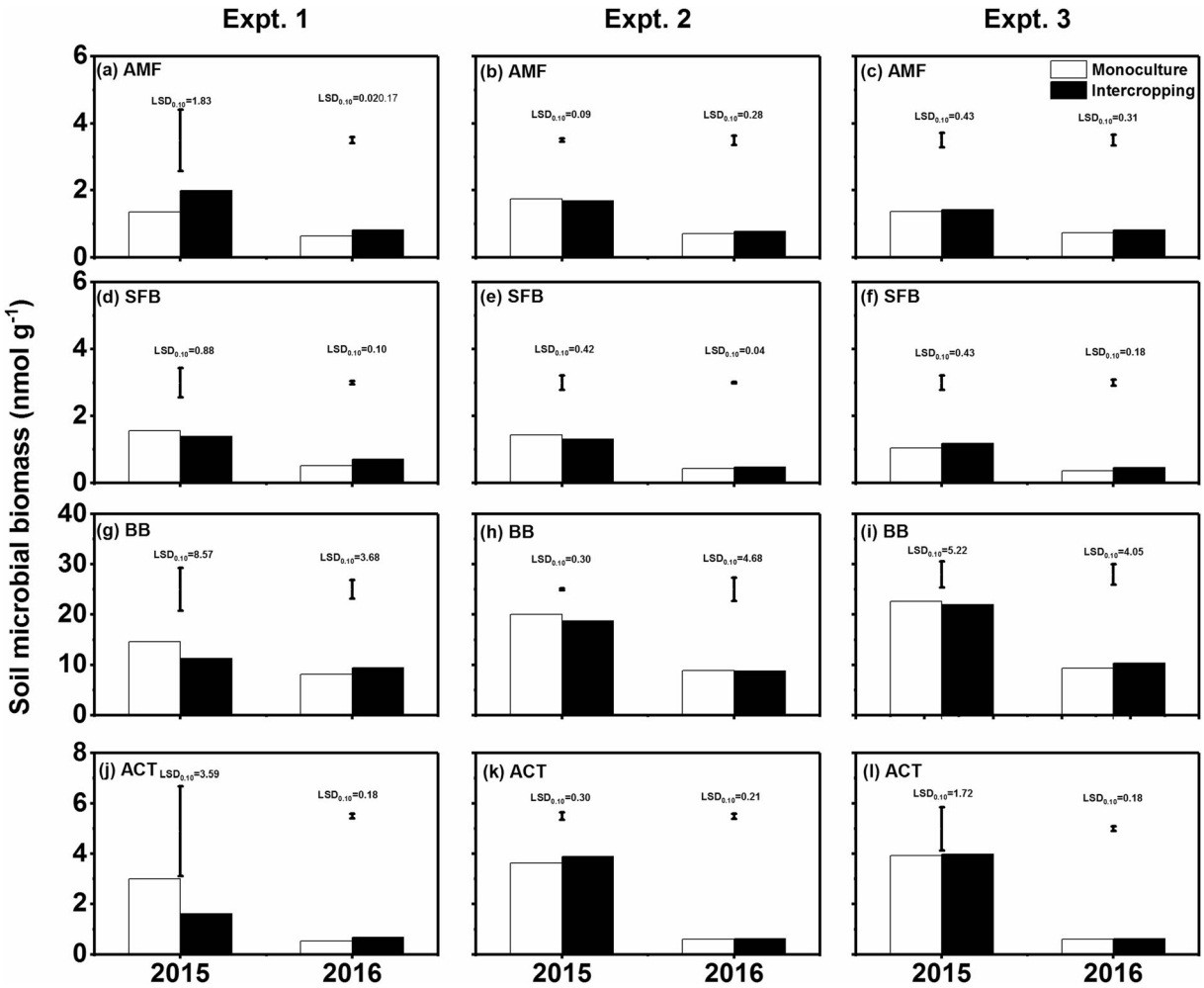

Fig. 3 Soil microbial biomass (nmol g^{-1}) of (**a**, **b**, **c**) arbuscular mycorrhizal fungal (AMF) biomass, (**d**, **e**, **f**) saprobic fungal biomass (SFB), (**g**, **h**, **i**) bacterial biomass (BB), and (**j**, **k**, **l**) actinobacterial (ACT) biomass as affected by cropping system (averaged monocropping vs. intercropping) in Expts 1, 2, and 3 in 2015 and 2016. The Fisher protected least significant difference (LSD) test was used for pairwise comparisons to determine the differences between cropping systems in Expt. 1, Expt. 2 and Expt. 3. Lines indicate the standard value of the difference between monocropping and intercropping system at the same site using LSD at $P < 0.10$. Data show mean values with $n = 12$ in Expt. 1 and n = 24 in Expts 2 and 3

composition, intercropping systems were clearly set apart from the monocultures along the first component axis in Expts 2 and 3 in both years (Fig. 5e, f, g, h, i and j). The first and second principal components (COMP1, COMP2) in Expts 2 and 3 accounted for 14.6% (Fig. 5e, ANOSIM, $r = 0.12$, $P = 0.034$) and 14.8% (Fig. 5i, ANOSIM, $r = 0.11$, $P = 0.032$) of the variation in species level in 2015 and 13.7% (Fig. 5f, ANOSIM, $r = 0.17$, $P = 0.008$) and 12.3% (Fig. 5j, ANOSIM, $r = 0.10$, $P = 0.035$) in 2016. High loadings by *Acanthamoeba* and *Stygamoeba* (Expt. 2) and *Pseudallescheria* and *Schizoplasmodium* (Expt. 3) in 2015 and by *Pterocystis* and *MPE1–14* (Expt. 2) and *Pterocystis* and *Zea* (Expt. 3) in 2016 contributed to separating the intercropping systems from the monocultures along the COMP1 axis.

In terms of bacterial community composition the intercropping systems were also clearly set apart from the monocultures along the COMP1 axis in Expt. 3 in 2015 (Fig. 5k). COMP1 and COMP2 in Expt. 3 accounted for 13.4% (Fig. 5k, ANOSIM, r = 0.11, $P = 0.032$) of the variation in species level in 2015. High loadings by *Candidatus_Nitrotoga* and *Smaragdicoccus* contributed to separating the intercropping systems

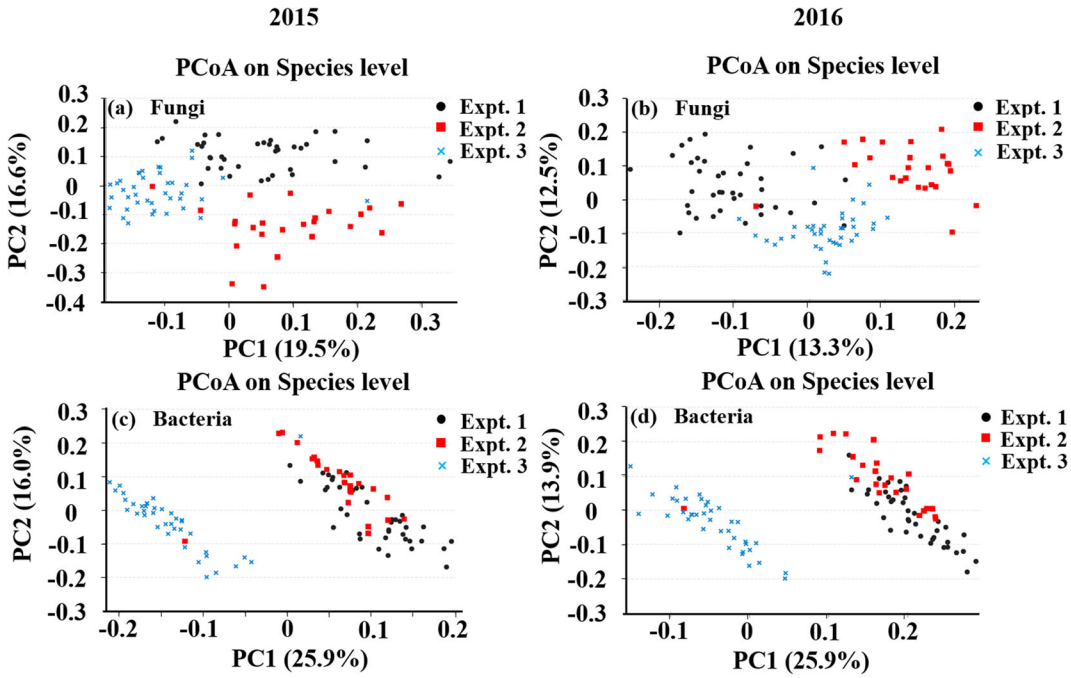

Fig. 4 (**a**, **b**) Soil fungal community composition and (**c**, **d**) bacterial community composition as affected by three experimental sites, i.e. Expts. 1, 2, and 3, at species level in 2015 and 2016. Principal coordinates analysis (PCoA) analyses was used to show variation in the composition (Bray Curtis distance). Black circles indicate Expt. 1, red squares Expt. 2 and blue multiple signs Expt. 3

from the monocropping along the COMP1 axis in Expt. 3 in 2015.

The results show that the fungal community composition was significantly influenced by cropping system, especially in Expts 2 and 3. In contrast, bacterial community composition was influenced by cropping system in Expt. 3 only.

Intercropping increased the relative abundance of soil *Sordariales* (*Sordariomycetes)* by 14.4% ($P = 0.290$) in Expt. 1, 23.9% ($P = 0.020$) in Expt. 2 and 18.7% ($P = 0.020$) in Expt. 3 (Fig. 6a) in 2015. In the second year the relative abundance of *Sordariales* increased by 17.2% ($P = 0.150$) in Expt. 1, 43.5% ($P = 0.003$) in Expt. 2 and 6.9% ($P = 0.090$) in Expt. 3 in the intercropping systems over the monocropping systems (Fig. 6b).

Linking soil aggregates with soil microbial composition and biomass by the structural equation model (SEM)

We used SEM methods to determine the effects of different pathways of microbial biomass and microbial community composition on the soil macro-aggregates (> 2 mm) in the intercropping and monocropping systems (Fig. 7). Across both years and all three experiments the soil macro-aggregate contents (> 2 mm) were facilitated by the indirect pathway (i.e. increased relative abundance of *Sordariales* and AMF biomass) and the direct pathway. Both AMF biomass and *Sordaiales* relative abundance had positive effects on soil macro-aggregates (> 2 mm) (Fig. 7). Both actinobacterial biomass and *Proteobacteria* relative abundance had negative effects on soil macro-aggregates (> 2 mm) (Fig. 7). Interestingly, looking at individual experiments, in Expt. 1 the SEM shows that the relative abundance of *Nitrospirae* reduced soil macro-aggregates (> 2 mm). Intercropping decreased the relative abundance of soil *Nitrospirae*. The indirect intercropping effects mediated by *Nitrospirae* were negative, reinforcing the direct intercropping effect on macro-aggregates (> 2 mm) (Supplementary Fig. 3a). In Expt. 2 the SEM shows that AMF biomass enhanced soil macro-aggregates (> 2 mm) but bacterial biomass reduced them. Intercropping increased soil AMF biomass and the relative abundance of soil *Sordariales*. The indirect intercropping effects mediated by AMF biomass were positive, reinforcing the direct intercropping effect on macro-aggregates (> 2 mm) (Supplementary Fig. 3b). In Expt. 3 the

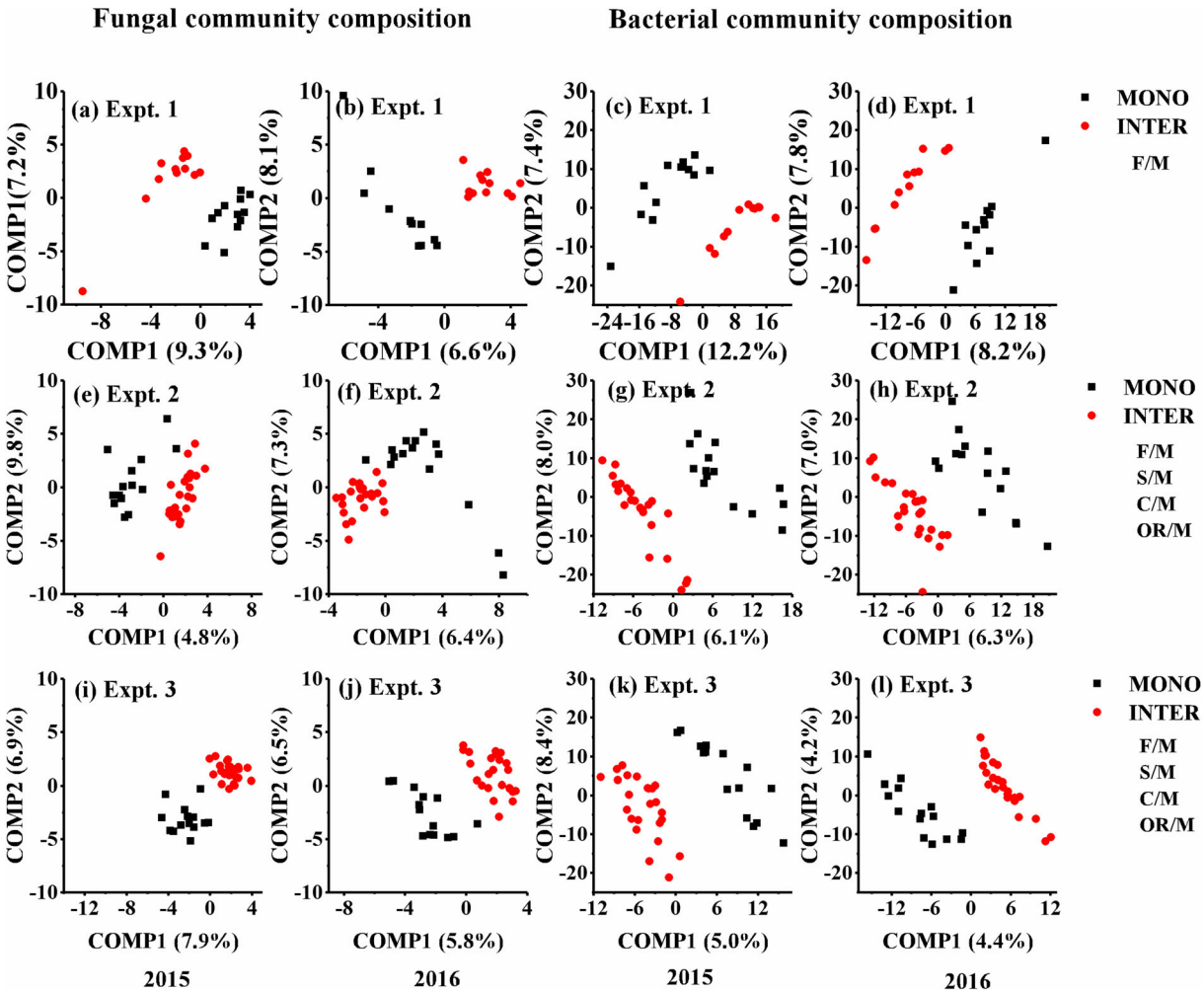

Fig. 5 Soil fungal and bacterial community composition as affected by cropping system (monocropping vs. intercropping) in (**a**, **b**, **c**, **d**) Expt. 1, (**e**, **f**, **g**, **h**) Expt. 2, and (**i**, **j**, **k**, **l**) Expt. 3 at species level in 2015 and 2016. The method of partial least squares discriminant analysis (PLS-DA) was used to analyze the variation in the composition. (**a**), (**b**), (**e**), (**f**), (**i**), (**j**) refer to the PLS-DA of the fungal community composition and (**c**), (**d**), (**g**), (**h**), (**k**), (**l**) refer to bacterial community composition. In Expt. 1 there is one crop combination, that is F/M intercropping system. In Expts 2 and 3 there are four crop combinations, namely F/M, S/M, C/M and OR/M. F/M, faba bean/maize intercropping; S/M, soybean/maize intercropping; C/M, chickpea/maize intercropping; and OR/M, oilseed rape/maize intercropping. Black squares indicate MONO, red circles indicate INTER. MONO indicates monocropping and INTER indicates intercropping. COMP1 and COMP2 indicate the scores of the first two components. Statistical testing among variation in microbial community composition was conducted using analysis of similarity (ANOSIM)

SEM shows that both the relative abundance of *Sordariales* and the SOM content had positive effects on the soil macro-aggregates (> 2 mm). Interestingly, the enhanced relative abundance of soil *Sordariales* and SOM were driven by intercropping. In other words, the positive effect of intercropping on soil macro-aggregates (> 2 mm) occurred mainly through its positive effect on the relative abundance of soil *Sordariales* and on SOM content (Supplementary Fig. 3c).

Discussion

Crop diversity altered below-ground soil microbial communities

Our results support the hypothesis that intercropping alters below-ground microbial community and composition. Intercropping for 7–8 years increased soil total FB, non-AMF biomass and the ratio of fungal biomass to bacterial biomass (Fig. 2). Soil fungal and bacterial

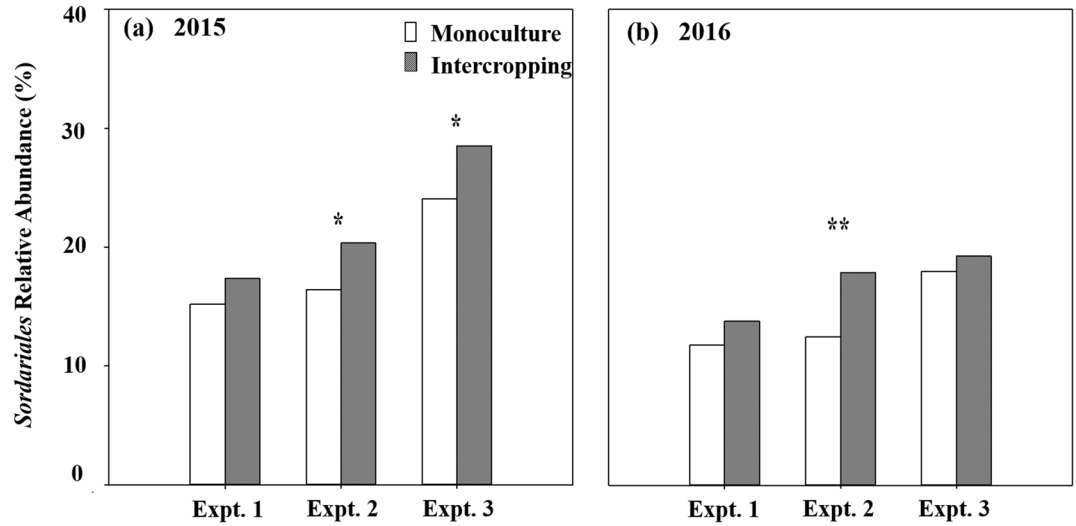

Fig. 6 Relative abundance of the soil fungi *Sordariales* at order level in Expts 1, 2 and 3 as affected by cropping system (monocropping vs. intercropping) in (**a**) 2015 and (**b**) 2016. The Wilcoxon rank-sum test was used to evaluate the relative abundance of *Sordariales* as influenced by cropping system (monocropping vs. intercropping) with False Discovery Rate (fdr) and confidence intervals (CI). Values ($n = 3$) with asterisks (*, **, ***) refer to significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$) between monoculture and intercropping. Monoculture refers to the monocropping systems and Intercropping indicates the intercropping systems

community composition under intercropping were separated distinctly from the monocropping systems and the relative abundance of *Sordariales* was enhanced by intercropping compared to the corresponding monocultures (Figs. 5 and 6). A previous study shows that intercropping changed the rhizosphere bacterial community composition in wheat/maize, wheat/faba bean and faba bean/maize intercropping systems (Song et al. 2007a, b) but the soil fungal community was not examined. In sugarcane/soybean and watermelon/pepper intercropping systems the numbers of rhizosphere bacteria and actinobacteria determined using a plate count technique increased in one year in greenhouse pot experiments (Li et al. 2013a; Sheng et al. 2012). Rotational cropping systems represent a type of crop temporal diversity practice and have been considered to increase the diversity of soil microbial communities and the ratio of fungi to bacteria (Alvey et al. 2003; Bünemann et al. 2004; Yin et al. 2010; Suzuki et al. 2012). Tiemann et al. (2015) found that soil bacterial and non-AMF biomass increased with increasing number of crop rotations (McDaniel et al. 2014). After seven years in an experimental grassland system in North America, soil microbial community biomass, respiration, and fungal abundance increased significantly with greater plant diversity (Zak et al. 2003). In an experiment at Jena in Germany, Eisenhauer et al. (2010) found that plant species were unique in their contribution to the functioning of the belowground system. To our knowledge, few studies have focused on soil fungal, bacterial and actinobacterial community composition and biomass together in field intercropping systems over time scales of several years. Our results are in accordance with the results from experimental grassland systems. Furthermore, our data support the hypothesis that crop diversity alters general soil (not only rhizosphere soil) microbial community composition and biomass in agricultural intercropping systems under field conditions over a longer time scale.

Soil aggregation was enhanced by crop diversity

Our results also support the hypothesis that intercropping promotes soil aggregation. The percentages of soil macro-aggregates (> 2 mm) were higher in the intercropping systems than in the monocultures across the three field experiments and both years in the present study (Fig. 1), an effect also reported from other intercropping systems (Kremer and Kussman 2009; Corbin et al. 2010; Kihara et al. 2012). For instance, previous studies show that soil aggregates increased by 50% in walnut/perennial kura clover (Kremer and

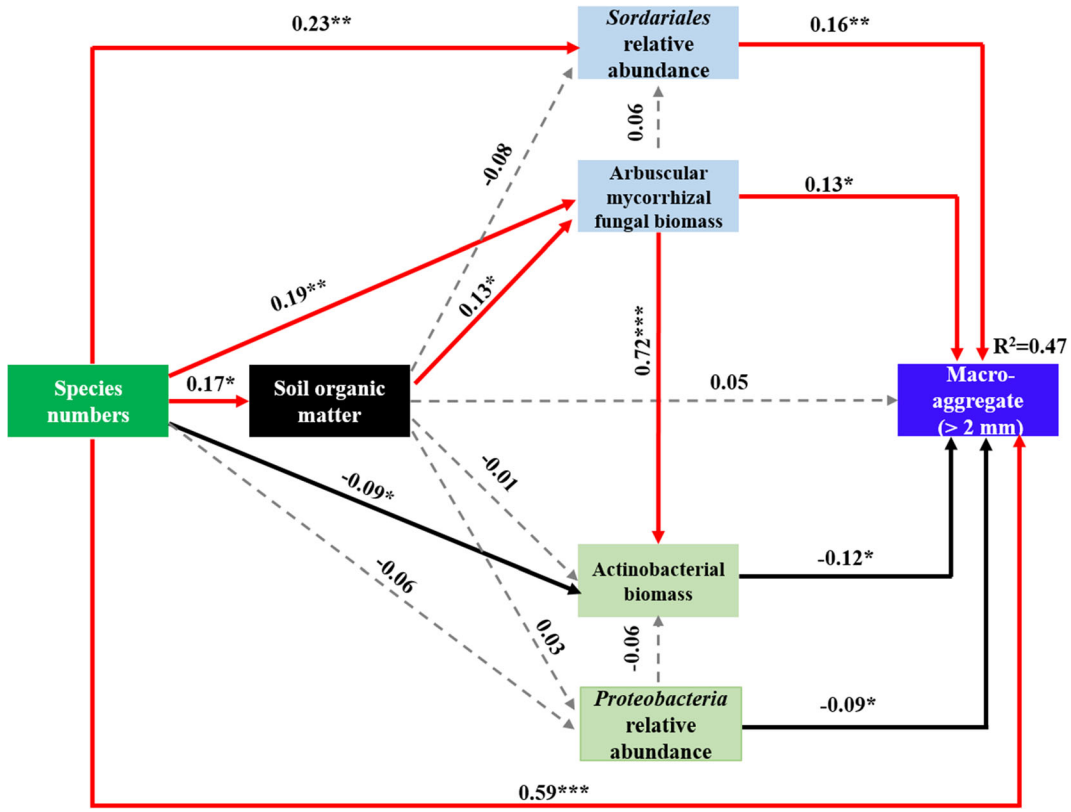

Fig. 7 The final structural equation model (SEM) describing the effects of soil microbial biomass and relative abundance on soil macro-aggregates (> 2 mm) in intercropping and monoculture systems. The SEM concluded significant pathways between species numbers (monocropping vs. intercropping) and macro-aggregates (> 2 mm) through soil microbial biomass and relative abundance across the three experiments. Red and black solid arrows refer to the pathways that were significantly positive and negative, respectively, and grey dashed arrows indicate the non-significant pathways. The numbers represent the standard path coefficients. R^2 denote the proportion of variance explained in the model. *P < 0.10, **P < 0.01, ***P < 0.001. We used the χ^2 test, Akaike information criteria and the root mean square error of approximation to evaluate the fit of the model. Species numbers refer to monocropping systems (1 species) and intercropping systems (2 species)

Kussman 2009), 4.5 times in wheat/alfalfa (Corbin et al. 2010) and 12–36% in watermelon/pepper intercropping systems (Kihara et al. 2012) compared to the respective monocultures. The complex aggregation of soil which is affected by a range of biotic and abiotic factors represents a key aspect of soil quality and leads to a good soil structure (Gupta and Germida 1988, 2015; Wang et al. 2001; Barthes and Roose 2002; Rillig 2004; Bronick and Lal 2005; Rillig and Mummey 2006; Rillig et al. 2014). Intercropping practices may therefore be of benefit in improving and stabilizing soil structure in agricultural ecosystems due to the increase in soil aggregates. However, few studies have focused on the mechanisms underlying the enhancement of soil aggregates by intercropping systems.

Altered microbial composition and biomass are related to enhanced soil macro-aggregates (> 2 mm) by intercropping

SEM analysis indicates that enhanced soil macro-aggregates (> 2 mm) were closely correlated with AMF biomass, the relative abundance of *Sordariales* (Fig. 7), or reduced relative abundance of *Nitrospirae* (Supplementary Fig. 3a), depending on soil type. As mentioned above, we also found that the percentage of soil macro-aggregates (> 2 mm) and soil FB increased in the intercropping systems. These results suggest that the increase in soil macro-aggregates (> 2 mm) in our intercropping systems was related to soil fungi (Tisdall and Oades 1982; Six et al. 2004; Rillig and Mummey 2006; Mardhiah et al. 2014). Soil microorganisms play

important roles in the formation of soil aggregates (Tisdall and Oades 1982; Bossuyt et al. 2001; Rillig and Mummey 2006). Numerous studies show that soil fungi are vital in promoting soil aggregate formation in three ways. First, fungi secrete polysaccharides and phenolic acids which will be released into the soil environment and combine with soil clay materials in new ways to promote soil micro- and macro-aggregate formation and stabilization (Tisdall et al. 1997; Caesar-TonThat and Cochran 2000). Second, hydrophobic proteins produced by filamentous fungi can decrease the water pressure on the surface of the fungi and change soil polarity to increase fungal survival and adaptation to adverse environmental conditions and promote soil micro-aggregate formation (Linder et al. 2005; Rillig and Mummey 2006). Finally, fungal hyphae entangle and enmesh primary particles, micro-aggregates or SOM to form a larger fraction of macro-aggregates (Bossuyt et al. 2001; Piotrowski et al. 2004; Rillig 2004; Wang et al. 2010).

In contrast, soil bacteria are important in determining soil aggregates (< 2 mm) (Leifheit et al. 2014). In the present study soil micro-aggregates decreased in the intercropping systems but soil bacterial biomass did not decrease (Figs. 1 and 3). Soil actinobacterial biomass did significantly reduce soil macro-aggregates (> 2 mm) and the relative abundance of soil *Proteobacteria* also decreased soil aggregation (Fig. 7). Soil bacteria secrete mucus or decomposition products of organic substances such as saccharides and phenolic acids through metabolism (Six et al. 2004). These substances adsorb on the surfaces of the primary particles and clump them into micro-aggregates (Tisdall 1994). In addition, bacteria and actinobacteria are widely active in regulating the metabolism of soil fauna such as earthworms and termites, enhancing the stabilization of soil micro-aggregates but not of soil macro-aggregates (> 2 mm) (Gupta and Germida 1988; Oades and Waters 1991). Thus, increasing bacterial biomass and relative abundance of *Proteobacteria* may accelerate the disintegration of soil macro-aggregates (> 2 mm). Other studies show that mineral N additions had a predominant but negative influence on aggregates (Guillou et al. 2011). Nitrifying bacteria such as *Nitrospirae* reduce available soil N by about 50% by the processes of nitrification and denitrification (Singh and Gupta 2018). Thus, a decrease in the relative abundance of *Nitrospirae* may promote soil aggregation.

Saprobic fungi are widely distributed in soils and are notable for their high species richness and abundance (Buee et al. 2009; Tedersoo et al. 2014; Lehmann and Rillig 2015). Recent studies have indicated an important role of soil saprobic fungi in the formation and stabilization of soil aggregates (Caesar-TonThat and Cochran 2000; Tisdall et al. 2012; Lehmann and Rillig 2015). Tisdall et al. (2012) used six saprotrophic fungi to prevent soil erosion and found that fungal hyphae, together with metabolites on their surfaces, stabilized the soil against wind erosion by cross linkage and entanglement of soil primary particles (Tisdall et al. 2012). In the present study the relative abundance of soil *Sordariales* showed a significant positive relationship with soil macro-aggregates (> 2 mm) (Fig. 7). Soil saprobic fungi appear likely to be key soil microorganisms regulating the formation and stabilization of soil macro-aggregates (> 2 mm). *Sordariales* are part of the saprobic fungal community and are able to decompose plant stems, leaves or bark (Lehmann and Rillig 2015). Our SEM shows that enhanced relative abundance of *Sordariales* driven by intercropping is likely to be a key driver promoting soil aggregation.

Conclusions

Our data support the notion that increasing soil macro-aggregates (> 2 mm) under intercropping systems is related to soil microbial community composition. Firstly, intercropping improved soil structure with increasing soil macro-aggregates (> 2 mm) in the agricultural ecosystems but soil aggregates of size < 0.106 mm declined. Secondly, intercropping enhanced soil total FB and non-AMF biomass, yet showed no effects on soil bacterial biomass. Finally, SEM shows that the relative abundance of soil *Sordariales* was enhanced by intercropping in all three experiments and had a significant positive effect on soil macro-aggregates (> 2 mm) in Expt. 3. *Sordariales* may therefore be one of the key drivers promoting soil aggregates in intercropping systems. Further studies may focus on which particular genera and species of *Sordariales* or of saprobic fungi may be involved in regulating the processes of soil aggregation in intercropping systems.

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