Applied Soil Ecology 154 (2020) 103599

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Protist-enhanced survival of a plant growth promoting rhizobacteria, *Azospirillum* sp. B510, and the growth of rice (*Oryza sativa* L.) plants

Rasit Asiloglu[a,*], Keiko Shiroishi[b], Kazuki Suzuki[c], Oguz Can Turgay[d], Jun Murase[e], Naoki Harada[f]

[a] Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan
[b] Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan
[c] Institute for Research Promotion, Niigata University, Niigata 950-2181, Japan
[d] Faculty of Agriculture, Ankara University, Ankara 06560, Turkey
[e] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
[f] Institute of Science and Technology, Niigata University, Niigata 950-2181, Japan

ARTICLE INFO

Keywords:
Protozoa
Biofertilizer
Rhizosphere
Amplicon sequencings
Predator-prey
Microbial survival

ABSTRACT

Application of plant growth promoting rhizo-bacteria (PGPR) is a promising method for sustainably increasing the plant growth; however, survival of PGPR is the most important factor limiting its efficiency. We studied the effects of heterotrophic protists on the survival of introduced PGPR, *Azospirillum* sp. B510, on indigenous bacterial community structure, and on the growth of the rice (*Oryza sativa* L.) plant in the early stages. Sterile rice seedlings grown in sterilized soil were inoculated with *Azospirillum* sp. B510, four protist isolates, and an indigenous protist-free bacterial community. Impact of protists on bacterial community structure was investigated by high throughput sequencing. Heterotrophic protists significantly increased survival of *Azospirillum* sp. B510. Heterotrophic protists and *Azospirillum* sp. B510 significantly increased early plant growth and nitrogen uptake, and their simultaneous inoculation had the largest impact on plant biomass (155.1% increase) and nitrogen uptake (226.0% increase). Protists altered the community composition of bacteria. In particular, *Sphingobacteriia*, *Azospirillum*, *Rodospirillales*, *Massilia*, *Caloramator*, and *Agrobacterium* benefited from the presence of protists, while *Sphingomonadales*, *Ralstonia*, *Burkholderia*, and *Rhodoferax* decreased in the protist-inoculated microcosms. Impact of different protist isolates was differed on early plant growth, nitrogen uptake and bacterial community structure. Our results suggest that protists could be as beneficial as PGPR for early growth in rice plants, and simultaneous inoculation with protist and PGPR may be a key solution for chemical-free sustainable agriculture.

1. Introduction

Plant growth promoting rhizo-bacteria (PGPR) play a crucial role in the rhizosphere due to their ability to stimulate plants growth in several ways including solubilization, mineralization, fixation of nutrients, phytohormone production, and pathogen suppression (Gupta et al., 2015). The effect of PGPR on plant growth is species-specific and depends on their survival in the introduced environment. Among all PGPR, the *Azospirillum* genus is the most widely used commercial biofertilizer and is considered as one of the most important genera due to its ability to colonize over a hundred plant species and significantly improve the growth, development, and productivity of a wide variety of crops including rice (Cassán and Diaz-Zorita, 2016).

Studies investigating the effect of *Azospirillum* spp. on rice plant growth have been increasing in the last few decades. *Azospirillum* spp. mostly colonize on the rice root surface (Steenhoudt and Vanderleyden, 2000), while some strains display endophytic colonization in the interior of roots (Chamam et al., 2013) and shoot of rice plants (Elbeltagy et al., 2001). Nitrogen fixation by *Azospirillum* spp. in rice rhizosphere has been reported (Razie and Anas, 2008). The ability of several *Azospirillum* spp. to produce phytohormones has been shown for upland plants (Fukami et al., 2018), and it was suggested that *Azospirillum* sp. enhances the growth of rice plants by producing phytohormones (Banayo et al., 2012); however, to our knowledge, no direct evidence is currently available for phytohormone-mediated rice plant growth by *Azospirillum* spp. Recent studies showed that *Azospirillum* spp. promotes early tillering and reproductive growth of rice (Watanabe and Lin, 1984). One of the most studied *Azospirillum* strains is B510, which has

* Corresponding author at: Faculty of Agriculture, Niigata University, 8050 Ikarashi 2-no-cho, Nishi-ku, Niigata City, Niigata 950-2181, Japan.
E-mail address: asiloglu@agr.niigata-u.ac.jp (R. Asiloglu).

https://doi.org/10.1016/j.apsoil.2020.103599
Received 4 July 2019; Received in revised form 23 March 2020; Accepted 24 March 2020
Available online 01 May 2020
0929-1393/

the entire genome published (Kaneko et al., 2010). *Azospirillum* sp. B510 was shown to increase early rice plant growth (Chamam et al., 2013), rice plant biomass and grain yield (Isawa et al., 2010), and to enhance the resistance of rice plants to rice blast and rice blight diseases (Yasuda et al., 2009). Currently, crop growth highly depends on the large-scale use of chemical fertilizers, especially nitrogenous compounds, which pose an environmental hazard for rice-producing areas (Ghosh and Bhat, 1998). The consistent beneficial results make *Azospirillum* a widely used biofertilizer, which may help to decrease the usage of chemical fertilizers (Vessey, 2003). However, when used as biofertilizers, the survival of *Azospirillum* spp., as in the case of the other PGPR species, is under demand, which is the most important factor limiting its efficiency. Therefore, increasing the survival and efficiency of PGPR is one of the priority research areas and is necessary for chemical-free sustainable plant growth.

Heterotrophic protists are one of the major microeukaryotic groups in the rice rhizosphere (Asiloglu et al., 2015; Asiloglu and Murase, 2016) that densely inhabit the rice roots (Asiloglu and Murase, 2017), and are able to enhance rice plant growth (Herdler et al., 2008; Kreuzer et al., 2006). It has been suggested that heterotrophic protists increase the activity and survival of PGPR (Jousset, 2017). For example, presence of heterotrophic protists enhances bacterial activities like nitrogen fixation, mineralization, and nitrification (Alphei et al., 1996; Cutler and Bal, 1926; Hervey and Greaves, 1941; Kuikman et al., 1991; Nasir, 1923), stimulates the production of siderophores (Levrat et al., 1992), cyclic lipopeptides (Mazzola et al., 2009), and 2,4-diacetylphloroglucinol (Jousset and Bonkowski, 2010). Indirect effect of protists on plant hormonal balance by altering bacterial communities have been suggested (Gao et al., 2019), which can be linked to changes in plant metabolome (Kuppardt et al., 2018). Although the mechanism is not yet fully understood, it has been suggested that the selective predation activity of protists increases root growth by a 'hormonal effect', whereby this selective predation supports indole-3-acetic acid producing and nitrifying bacteria, most likely by consuming their competitors (Bonkowski, 2004; Bonkowski and Brandt, 2002; Krome et al., 2010). Increased bacterial traits, i.e., nitrogen fixation, IAA and siderophore production, is likely due to increased survival of beneficial bacteria (Gao et al., 2019; Jousset et al., 2008). For instance, increased activity and survival of a *Pseudomonas* spp., in the presence of protists has been shown previously (Flues et al., 2017; Jousset et al., 2006; Weidner et al., 2017). However, little is known about protist-PGPR interactions and, to our knowledge, nothing is known about interaction between protists and genus *Azospirillum*. Here, we studied protist–*Azospirillum* sp. B510 interaction in a rice rhizosphere to reveal the potential importance of protists on the survival of introduced *Azospirillum* sp. B510 and on early rice plant growth.

Considering that different protist groups may differentially impact bacterial community structure, nitrogen turnover and mineralization of crop residue (Hünninghaus et al., 2017; Rønn et al., 2002), we chose to study four commonly detected rhizosphere protists. The four protists were different from each other in morphotype (a ciliate, an amoeboflagellate, a flagellate and an amoeba), phylogeny and size. We hypothesized that: 1) introduction of heterotrophic protists enhances the survival of inoculated *Azospirillum* sp. B510 and its positive effect on plant growth; 2) heterotrophic protists by themselves, without *Azospirillum* sp. B510, also enhance early rice plant growth; and 3) heterotrophic protists have different effects on survival of PGPR, early rice plant growth, and bacterial community structure. The impact of protists on bacterial community structure was examined using high throughput sequencing methods. To our knowledge, this is the first study to use amplicon sequencing to examine the impact of protists on bacterial communities in a rhizosphere.

2. Materials and methods

2.1. Preparation of microorganisms

Heterotrophic protists were isolated in October 2017 from a rhizosphere soil of rice (*Oryza sativa* L. Koshihikari) plants taken from a paddy field under sub-drained conditions at Shindori Station in the Field Center for Sustainable Agriculture and Forestry, Niigata University, Niigata, Japan (N37.86, E138.96) on July 7, 2017. Detailed methods of isolation and molecular identification are provided in the Supplementary Information. The isolates were assigned to the following species using sequencing analysis (Table S1) and morphological and locomotive features under the microscope: *Vermamoeba vermiformis* (formerly known as *Hartmannella vermiformis*, Amoebozoa; Tubulinea) (~20 μm), *Naegleria* sp. (Excavata, Heterolobosea) (~25 μm), *Colpoda steinii* (Chromalveolata; Alveolata) (~30 μm), *Heteromita globosa* (Rhizaria; Cercozoa) (~10 μm). Approximate size of the protist isolates was estimated as average length of cells (n = 10). Prior to inoculation, the isolates were washed twice with sterile ultra-pure H_2O at 1000 rpm for 5 min and then resuspended in sterile ultra-pure H_2O.

Azospirillum sp. B510 that were isolated from rice (*O. sativa* L. Nipponbare) plants (Elbeltagy et al., 2001) were obtained from the Japan Collection of Microorganisms, RIKEN BioResource Research Center, Ibaraki, Japan. Freeze-dried cells were rehydrated and cultured at 30 °C for 5 days in a sterile growth media consisting of 5.0 g/L peptone and 3.0 g/L beef extract. The cells were harvested by centrifugation (3000 ×g, 15 min, 4 °C) and washed with 10 mM $MgCl_2$, followed by one more wash with sterile ultra-pure H_2O. The bacterial pellet was resuspended in sterile ultra-pure H_2O.

The protist-free bacterial inoculum was obtained from soil taken from the same rice field of Shindori station on April 12, 2018. Briefly, 100 g of the plow layer soil was shaken with 200 mL ultra-pure H_2O for an hour at 170 rpm min^{-1} and then filtered (< 500 μm) to eliminate the soil particles. Since preliminary observations showed the presence of small flagellates (~1 μm), the resulting suspension was filtered through 0.8 μm pore size mixed cellulose ester membrane filters (Advantec, Tokyo, Japan) in order to eliminate all protists. The 50 μL of protist-free bacterial inoculum was cultured in 100 μL of the amoeba saline solution (Page, 1988) at 25 °C in 96-well microtiter plates for 5 days to check for absence of protists (n = 96).

2.2. Sterile rice seedlings and experimental set-up

The outer layer of the rice (*O. sativa* L. Nipponbare) seeds was removed and the seeds were disinfected with 0.2 M sodium hypochlorite (NaClO) by shaking for 20 min at 90 rpm. Next, the seeds were washed two times with sterile ultra-pure H_2O in a new sterile tube for 5 min at 90 rpm. After washing, the seeds were sowed in 0.6% agar media consisting of Kasugai's nutrient solution (per liter: 0.04 g $[NH_4]_2SO_4$, 0.02 g $Na_2HPO_4{\cdot}12H_2O$, 0.03 g KCl, 0.004 g $CaCl_2$, 0.006 g $MgCl_2{\cdot}2H_2O$, 0.005 g $FeCl_3$) in a sterile growth bottle. The rice seeds were grown for 14 days in a growth chamber at 24 °C with a day length of 16 h (250 μmol m^{-2} s^{-1}).

Soil was taken from the same rice field under drained conditions at the Shindori station on April 12, 2018. The soil was air-dried, sieved (< 2 mm), and then stored at 4 °C. Prior to the experiment, the soil was sterilized by autoclaving at 121 °C for 30 min. This procedure was repeated 3 times, and 1000-times diluted soil solution was microscopically checked for growth of microorganisms.

Centrifugation tubes (50 mL) were filled with 40 g of the sterile soil. Protist-free bacterial inoculum (4 mL) and Kasugai's nutrient solution were added to all microcosms, and the microcosms were incubated under submerged conditions. After 1 day of incubation, a total 500 protists cells/g soil and/or 10^7 *Azospirillum* sp. B510 cells/g soil were inoculated into the microcosms as a single species or in combination (Table 1), while control microcosms received the same amount of

Table 1
Experimental set-up.

Microorganisms	Treatments (n = 3)							
	Ctrl	Azo	Pro mix	Pro mix + Azo	Ver + Azo	Nae + Azo	Col + Azo	Het + Azo
Protists-free bacterial inoculum	O	O	O	O	O	O	O	O
Azospirillum sp. B510		O		O	O	O	O	O
Vermamoeba vermiformis			O	O	O			
Naegleria sp.			O	O		O		
Colpoda steinii			O	O			O	
Heteromita globasa			O	O				O

sterile ultra-pure H_2O. In order to reveal the differences of mixture of four protist isolates from their individual impact on *Azospirillum* sp. B510 and plant growth, single species of protist isolates and *Azospirillum* sp. B510 were combined in four treatments. Experimental set-up and the treatment names are shown in Table 1. Similar sized 14-day-old sterile rice seedlings were selected, and were individually transplanted to each microcosm. Before transplantation, the agar particles remaining around the roots were gently washed-off using sterile ultra-pure H_2O. The microcosms were kept submerged in a growth chamber at 25/30 °C (day/night) with a day length of 16 h (250 μmol m^{-2} s^{-1}).

2.3. Sampling and determination of nitrogen

The microcosms were destructively sampled after 25 days of incubation. The surface water of microcosms was removed, and the plants with soil were transferred from the microcosms into a 300 mL sterilized beaker. The shoots were cut off and shoot length was measured. The shoot biomass was measured after drying at 60 °C for 72 h. Then the shoots were cut into pieces (< 5 mm) and mixed thoroughly for determination of nitrogen. The rice roots were cut (< 5 mm) with sterilized scissors and mixed with the soil; rice roots were sampled together with the soil in the microcosms (hereafter called rhizosphere). Immediately after mixing, 0.5 g of rhizosphere samples were placed into 2 mL DNA extraction tubes and stored at −80 °C until use for nucleic acid extraction. DNA was extracted using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction and eluted in TE buffer (50 μL). Rhizosphere samples (5 g) were placed into sterilized 100 mL Erlenmeyer flasks for enumeration of protists. Total nitrogen in the soil and plant samples were analyzed after drying at 105 °C for 24 h using an MT-700 Mark 2 CN analyzer (Yanaco, Kyoto, Japan).

2.4. Enumeration of protists and gene abundance of Azospirillum sp. B510

The number of soil protists was estimated using a slight modification of most probable number (MPN) method (Darbyshire et al., 1974). Briefly, 5 g of soil was mixed with 45 mL of AMS solution on the sampling day to obtain ten times diluted soil solution. This mixture was used in a threefold dilution series as final concentration of diluted soil solutions were ranged from $3^1 \times 10$ to $3^8 \times 10$. Fifty microliters of the diluted suspensions ($n = 8$) were added with 100 μL food bacteria (*Escherichia coli* MG1655; final concentration ~10^7 cells mL^{-1}) and cultured in 96-well microtiter plates at 25 °C in dark. After 1 week, the growth of amoeba, flagellates and ciliates in the wells was observed at ×200 and ×400 magnifications using an inverted microscope (Nikon Eclipse TE2000-S, Tokyo, Japan). The wells in which the growth of protists were seen was scored as positive and the MPN was estimated using a MS™ Excel sheet provided by (Briones et al., 1999).

Colonization of *Azospirillum* sp. B510 in the rhizosphere was evaluated by a semi-quantitative nested real time PCR (Wakelin et al., 2008) using strain B510-specific primers (Isawa et al., 2010). Despite methodological traits of semi-quantitative nested real time PCR limiting us from making comparisons with data from related literature, it allows us to estimate the relative differences among the treatments within a specific experiment (Wakelin et al., 2008). Hence, no attempt was made to generate standard curves (Gentle et al., 2001). The first step of nested PCR reaction was performed by modifying the protocol by Isawa et al. (2010). The PCR mixture (25 μL) contained 10 μL DNA sample, 10 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate, 0.5 U ExTaq polymerase (TaKaRa, Otsu, Japan) and 0.1 volume of the 10 × PCR buffer provided with the enzyme. The primary PCR program included an initial denaturation step of 30 s at 94 °C, followed by 35 cycles of denaturation (30 s, 94 °C), primer annealing (30 s, 69 °C), and primer extension (30 s, 72 °C). Two μL of 10 × diluted primary PCR reaction products was used as template DNA in qPCR analysis. The qPCR reaction (10 μL) contained 10 pmol of each primer and 5 μL of SsoFast™ EvaGreen supermix (Bio-Rad, Hercules, CA, USA) by using CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). The qPCR program started with an initial denaturation step of 120 s at 98 °C, followed by 39 cycles of denaturation (2 s, 98 °C) and primer annealing (5 s, 69 °C) with a final step of primer extension of 10 s at 75 °C. Data were calculated using the comparative 2-$\Delta\Delta^{CT}$ method and are presented as the fold change relative to the control treatment (Ctrl) (Pfaffl, 2001).

2.5. Illumina library preparation and bioinformatics

The V4 region of the 16S rRNA gene was amplified from the extracted DNA using the universal primers (515F and 806R) tailed with Illumina barcoded adapters (Caporaso et al., 2012). After purification with Agencourt XP Ampure Beads (Beckam Coulter Inc., Brea, CA, USA), the PCR products were tagged with sequencing adapters using Nextera XT Index Kit v2 SetA (Illumina Inc., San Diego, CA, USA) index kit. Samples containing equal DNA concentrations were loaded onto a MiSeq reagent cartridge (MiSeq Kit V_2 300 cycles) and sequenced using 2 × 300 paired-end reads on an Illumina MiSeq sequencing platform (Illumina Inc.).

After sequencing, primary analysis of raw FASTQ data was processed using the QIIME2 pipeline (version 2018.11, https://qiime2.org) (Caporaso et al., 2010). Briefly, DADA2 (Callahan et al., 2016) was used for error-correction, removal of forward and reverse primers, quality filtering, doubleton removal, and chimera removal of the Illumina amplicon sequences, with reads truncated at 200 bp for each single end read, corresponding to a quality score > 30, and allowing forward and reverse sequences to overlap > 50 bp. QIIME2's q2-feature-classifier plugin was used for taxonomy assignment against the GreenGenes reference database (13_8 release) at 99% OTUs. To compare bacterial communities between the treatments, sequence read numbers were normalized to the minimum sequence number (30,000 reads) by random subsampling. The alpha-diversity was estimated according to microbial diversity metrics: Shannon index, Faith's phylogenetic distance, evenness, and observed OTUs. Beta-diversity was estimated by coupling principal coordinate analysis (PCoA) with distance matrixes that take the abundance of sequences into account (Bray–Curtis) or not (Jaccard). Linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011) was performed using the Galaxy server (http://

huttenhower.sph.harvard.edu/galaxy/).

Functional genes putatively involved in biological nitrogen fixation were predicted using PICRUSt (Langille et al., 2013) software (version 1.1.3, normalize_by_copy_number.py), which applies 16S rRNA genes to predict the abundances of functional genes by matching sample OTUs with reference genomes. Briefly, the sequences were assigned to OTUs using QIIME2 (v 2018.11) according to PICRUSt online 'closed-reference' OTU picking protocol against the Greengenes reference database (gg_13_5_otus) at 97% identity, followed by the metagenome contributions.py script based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) database and the -l option to specify KEGG orthologs for the N-fixation. The specific KEGG orthologs (K02588 + K02586 + K02591 − K00531) were selected as defined by the N-fixation pathway module (M00175). The data for the abundance of N-fixation genes were normalized to the highest values of individual processes, which were set to one.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using R version 3.5.2 (https://www.r-project.org/). Comparison between means were performed with Tukey's honestly significant difference (HSD) test at 0.05 level, which was carried out using R version 3.5.2 (https://www.r-project.org/) with 'glht' function in the 'multcomp' package (Hothorn et al., 2008). ANOVA and Tukey's HSD tests were carried out separately for growth of introduced microorganisms, plant growth parameters, nitrogen content of soil and plant, relative gene abundances of putative nitrogen fixation, and the alpha diversity of bacterial communities. The beta diversities analyzed with permutational multi-variate analysis of variance (PERMANOVA) with 999 random permutations ($p < 0.05$), which was conducted on all principal coordinates obtained during PCoA based on Bray-Curtis dissimilarities and Jaccard index with the adonis function of the *vegan* package. Significant biological consistency and effect relevance of the treatment-specific bacterial groups were analyzed by LEfSe as follows: Firstly, the non-parametric factorial Kruskal-Wallis sum-rank test ($p < 0.05$) was conducted to detect features with significant differential abundances. After this step, linear discriminant analysis (LDA), in which the logarithmic score was set to 2.0, were conducted to estimate the effect size of each differentially abundant feature. The data for significant increases or decreases for each differentially abundant feature was provided by LEfSe (data not shown).

2.7. Accession numbers

The raw sequence data obtained in this study have been deposited in the NCBI database under the BioProject ID PRJNA550019.

3. Results

3.1. Growth of introduced microorganisms

At the end of the experiment, the introduced protists (500 cells g^{-1} soil) successfully populated the microcosms ($> 10^4$ cells g^{-1} soil) in all protist-inoculated treatments, while the non-protist microcosms did not contain detectable levels of protists (Fig. 1A). All the four protist species were able to grow in combination with each other in Pro mix and Pro mix + Azo treatments, indicating that either the protist-protist predation did not occur or it did not have a strong effect. Presence of *Azospirillum* sp. B510 did not have a significant effect on the number of protists (Tukey's HSD, $p < 0.05$). Protist numbers in the Pro mix (5.3×10^4) and Pro mix + Azo (5.6×10^4) treatments were similar and we did not find a significant difference between two treatments (Tukey's HSD, $p < 0.05$). In treatments with a single protist species, the number of each protist reached over 10^4, with only the introduced protists being observed in the representative microcosms.

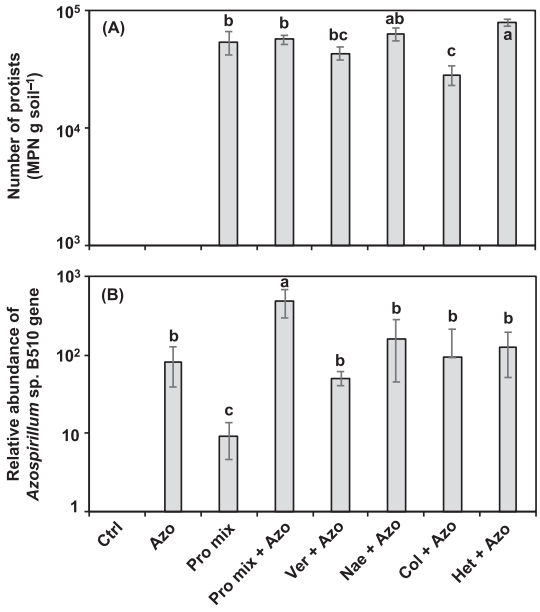


Fig. 1. Growth of introduced heterotrophic protists estimated by MPN count (A), and abundance of *Azospirillum* sp. B510-specific 16S rRNA gene relative to the control (Ctrl) treatment (B) at the end of the experiment (25 DAT). Error bars represent standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$, Tukey's HSD). Ctrl, control; Azo, *Azospirillum* sp. B510; Pro mix, mixture of the four protists; Pro mix + Azo, *Azospirillum* sp. B510 and mixture of the four protists; Ver + Azo, *Vermamoeba vermiformis* + *Azospirillum* sp. B510; Nae + Azo, *Naegleria* sp., + *Azospirillum* sp. B510; Col + Azo, *Colpoda steinii* + *Azospirillum* sp. B510; Het + Azo, *Heteromita globasa* + *Azospirillum* sp. B510 (See Table 1 for details).

Successful colonization of *Azospirillum* sp. B510 in the inoculated microcosms was detected using strain-specific primers by semi-quantitative nested real time PCR (Fig. 1B). The relative gene abundance to the control (Ctrl) treatment showed that mixed inoculation with the four protists significantly increased the gene abundance of *Azospirillum* sp. B510, while single protist treatments had no significant effect (Tukey's HSD, $p < 0.05$). Although semi-quantitative nested real time PCR was not able to detect *Azospirillum* sp. B510 in the initial indigenous bacterial community, amplicon sequencing analysis for the initial indigenous bacterial community showed that relative abundance of *Azospirillum* sp. was 1.95 ± 0.07% (data not shown). Most likely *Azospirillum* sp. B510 detected in the Ctrl and Pro mix treatments, where it was not inoculated, was originated from the initial indigenous bacterial community and the relative gene abundance was significantly higher in the Pro mix treatment compared to the Ctrl treatment.

3.2. Rice plant growth

The rice plants in all microcosms grew healthily and both protists and *Azospirillum* sp. B510 had a positive impact on plant growth after 25 days compared to the control (Fig. 2). While protists and *Azospirillum* sp. B510 increased the shoot biomass by 59.1% and 60.1%, respectively, their combination (Pro mix + Azo) had the greatest effect on shoot biomass (155.1% increase) (Fig. 2A). The combination of *Azospirillum* sp. B510 with a single type of protist also increased shoot biomass; each protist type had a different effect, ranging from 39.5% (*Colpoda steinii*) to 95.5% (*Naegleria* sp.). Similar effects of introduced microorganisms on rice shoot height were observed (Fig. 2B). Compared to the control, vertical growth of rice shoot was significantly increased in the Azo (15.6%) and Pro mix (14.6%) treatments.

Besides shoot biomass and shoot height, all of the introduced microorganisms significantly (Tukey's HSD, $p < 0.05$) increased the number of leaves in 25 days (Fig. 2C) compared to control treatment;

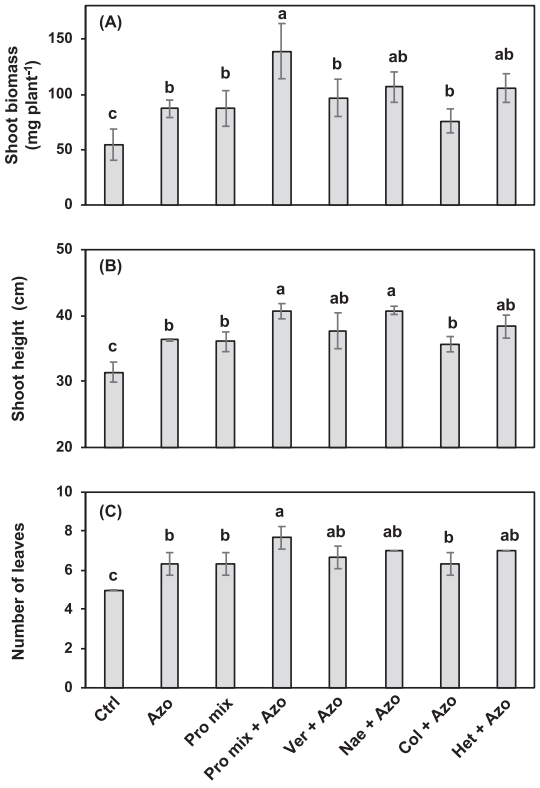


Fig. 2. Effect of introduced microorganisms on early rice plant growth (25 DAT). (A) Shoot biomass; (B) shoot height; and (C) number of emerged leaves. Error bars represent standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$, Tukey's HSD). Ctrl, control; Azo, *Azospirillum* sp. B510; Pro mix, mixture of the four protists; Pro mix + Azo, *Azospirillum* sp. B510 and mixture of the four protists; Ver + Azo, *Vermamoeba vermiformis* + *Azospirillum* sp. B510; Nae + Azo, *Naegleria* sp., + *Azospirillum* sp. B510; Col + Azo, *Colpoda steinii* + *Azospirillum* sp. B510; Het + Azo, *Heteromita globasa* + *Azospirillum* sp. B510 (See Table 1 for details).

this ranged from 6.3 to 7.6, as opposed to 5 in case of control. The maximum number was observed in the Pro mix + Azo treatment, which was significantly higher from the effect of *Azospirillum* sp. B510 and mixed protists (Pro mix) on the total leaf number. The number of leaves in the Pro mix + Azo treatment was not significantly (Tukey's HSD, $p < 0.05$) different from those in the Ver + Azo and Nae + Azo treatments, while it was significantly (Tukey's HSD, $p < 0.05$) higher than those in the Col + Azo and Het + Azo treatments.

3.3. Nitrogen content of soil and rice plant

Compared to the control, there was an increase in total N uptake (2.53 to 4.83 mg plant^{-1}) and N concentration (25.9 to 34.4 mg g^{-1}) in the shoot of rice plants exposed to protists and/or *Azospirillum* sp. B510 (Tukey's HSD, $p < 0.05$) (Fig. 3A and B). Total N and N concentration were the highest when both *Azospirillum* sp. B510 and the mix culture of protists were present. Compared to the control, total N was increased by 226% and shoot N concentration was increased by 88.4% in the Pro mix + Azo treatment. The positive impact of *Azospirillum* sp. B510 and mixed culture of protists on total N and N concentration were similar. The combination of *Azospirillum* sp. B510 with a single type of protist also significantly affected total N and N concentration. The amount of soil nitrogen ranged between 1.3 and 3.4 mg g dw^{-1} soil (Fig. 3C). The concentration of soil N did not increase with time; in fact, in the Pro mix treatment, it was significantly lower (Tukey's HSD, $p < 0.05$) than that of the initial soil.

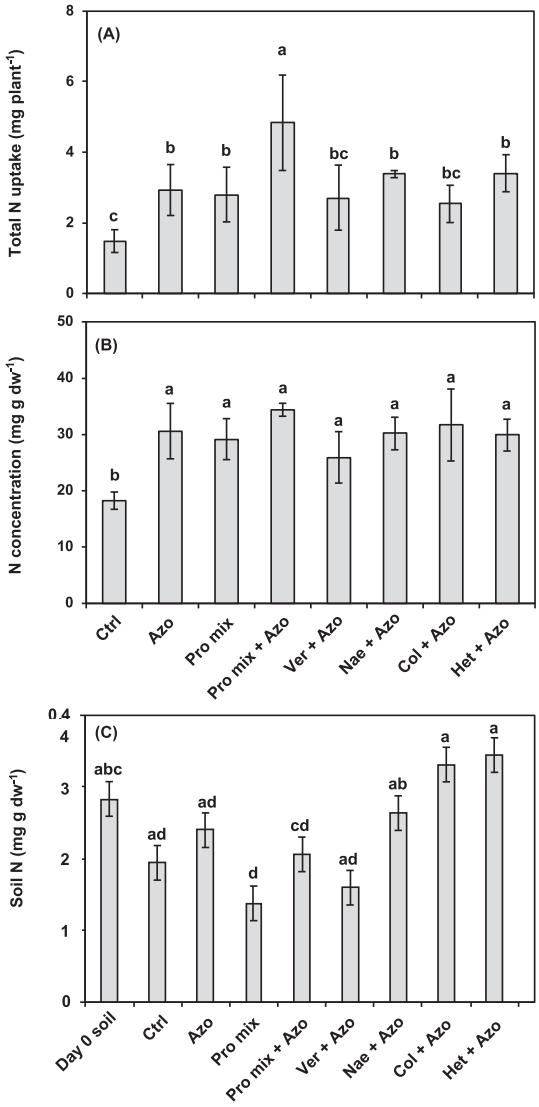


Fig. 3. Total nitrogen uptake (A) and nitrogen concentration of rice shoots (A) and total nitrogen in the soil (B) at the end of the experiment (25 DAT). Error bars represent standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$, Tukey's HSD). Day 0 soil, soil sample collected on day 0; Ctrl, control; Azo, *Azospirillum* sp. B510; Pro mix, mixture of the four protists; Pro mix + Azo, *Azospirillum* sp. B510 and mixture of the four protists; Ver + Azo, *Vermamoeba vermiformis* + *Azospirillum* sp. B510; Nae + Azo, *Naegleria* sp., + *Azospirillum* sp. B510; Col + Azo, *Colpoda steinii* + *Azospirillum* sp. B510; Het + Azo, *Heteromita globasa* + *Azospirillum* sp. B510 (See Table 1 for details).

3.4. Bacterial community structure

A total of 994,858 quality sequences were obtained after chimeric and doubleton removal, with 30,358 to 48,176 sequences for each microcosm (median frequency of 41,252). The average read length of the sequences was 253 ± 5 bp and sequences were assigned to 90 to 157 OTUs at 99% similarity level. Alpha diversity indices are shown in Table S2. There was no significant difference among the treatments for observed OTUs, Shannon index, and evenness. Faith's phylogenetic diversity were significantly higher in the protist-inoculated treatments except Col + Azo and Het + Azo (Tukey's HSD, $p < 0.05$). Fig. 4 shows the PCoA analysis based on Bray–Curtis dissimilarity (Fig. 4A) and Jaccard index (Fig. 4B). The circles indicate the protist inoculated (blue) and non-inoculated (Black) treatments. No significant differences were found for overall bacterial community composition among the

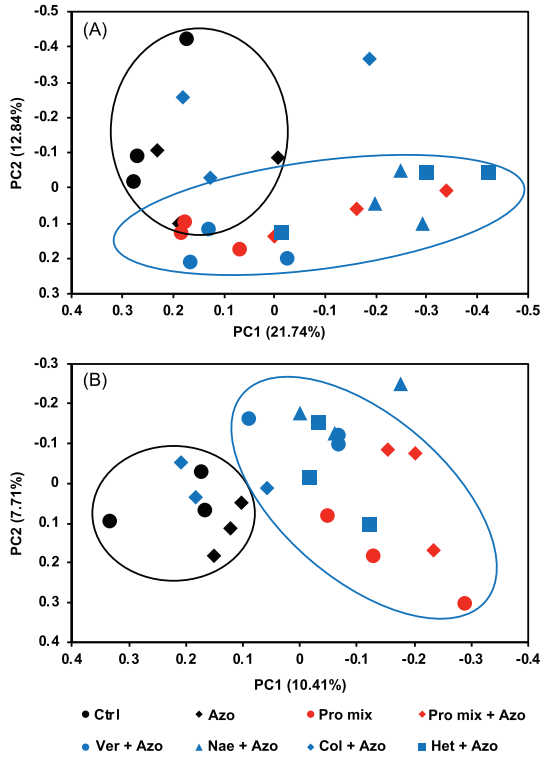


Fig. 4. Principal coordinate analysis (PCoA) based on the distance matrixes of Bray–Curtis dissimilarities (A) and Jaccard index (B) comparing rhizosphere bacterial communities among all treatments. Black color indicates non-protist treatments without (circle) and with (diamond) *Azospirillum* sp. B510, red color indicates mix community of the four protists without (circle) and with *Azospirillum* sp. B510 (diamond), and blue color indicates single protist treatments with *Azospirillum* sp. B510 (*Vermamoeba vermiformis*, circle; *Naegleria* sp., triangle; *Colpoda steinii*, diamond; and *Heteromita globosa*, square). Ctrl, control; Azo, *Azospirillum* sp. B510; Pro mix, mixture of the four protists; Pro mix + Azo, *Azospirillum* sp. B510 and mixture of the four protists; Ver + Azo, *Vermamoeba vermiformis* + *Azospirillum* sp. B510; Nae + Azo, *Naegleria* sp., + *Azospirillum* sp. B510; Col + Azo, *Colpoda steinii* + *Azospirillum* sp. B510; Het + Azo, *Heteromita globasa* + *Azospirillum* sp. B510 (See Table 1 for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatments (PERMANOVA, data not shown). However, the PCoA analysis showed a slight shift in bacterial community composition (Fig. 4).

The major phyla in all treatments were comprised of Firmicutes (46.6 ± 9.2%), Proteobacteria (31.6 ± 5.6%), and Bacteriodetes (15.4 ± 5.3%). At the class level relative abundance, the top ten classes represented over 95% of the communities (Fig. S1). The relative abundance of Clostridia and Bacilli (Firmicutes) was 25.3 ± 9.5% and 21.4 ± 7.1%, respectively. The third dominant class, Saprospirae (14.1 ± 5.5%), belongs to phylum Bacteroidetes. The abundant classes of the phylum Proteobacteria included Betaproteobacteria (13.5 ± 3.4%), Alphaproteobacteria (12.4 ± 4.0%), Deltaproteobacteria (3.4 ± 3.1%), and Gammaproteobacteria (2.3 ± 1.3%). There was no significant impact of treatment on the relative abundance of the top ten classes.

The 30 most abundant genera are shown in Fig. S2. Different genera were dominant in each treatment: *Bacillales* in Ctrl (15.0 ± 5.4%), *Symbiobacterium* in Azo (27.2 ± 23.9%), *Sediminibacterium* in Pro mix (15.9 ± 2.5%) and Ver + Azo (18.3 ± 3.3%), *Alicyclobacillus* in Pro mix + Azo (12.3 ± 10.6%), and Nae + Azo (21.7 ± 2.2%) and Het + Azo (17.5 ± 15.0%), and *Clostridium* in Col + Azo (15.7 ± 8.4). Presence of each protist significantly affected relative abundance of several bacteria at the genus level (Fig. S3) (Tukey's HSD, $p < 0.05$).

3.5. Differential bacterial composition and putative N-fixation functionality

Significantly distinct bacterial groups at multiple taxonomic levels among the treatments were characterized by LEfSe (Fig. 5 and Fig. S4). Mix culture of the four protists significantly affected the relative abundance of several bacteria from class to genus taxonomic levels (Fig. 5A and B). The abundancy of 19 bacterial taxa (11 genus, 3 family, 3 order, and 2 class levels) including *Sphingobacteriia* (class level), *Rodospirillales* (order level), and *Massilia*, *Caloramator*, *Agrobacterium* (genus levels) significantly increased ($p < 0.05$), while the relative abundance of 16 bacterial taxa (7 genus, 5 family, and 4 order levels) such as *Sphingomonadales* (order level), *Sphingomonadaceae* (family level), and *Ralstonia*, *Burkholderia*, and *Rhodoferax* (genus levels) significantly decreased ($p < 0.05$) in the presence of heterotrophic protists. Although *Azospirillum* sp. B510 did not significantly change the bacterial community itself (data not shown), the combination of protists with *Azospirillum* sp. B510 (Pro mix + Azo treatment) influenced more bacterial groups (26 positive and 19 negative) than treatment with only protists (Fig. 5C and D). Each of the single protist species, except *Colpoda steinii*, affected the relative abundance of bacterial taxa differently (Fig. S4A-C).

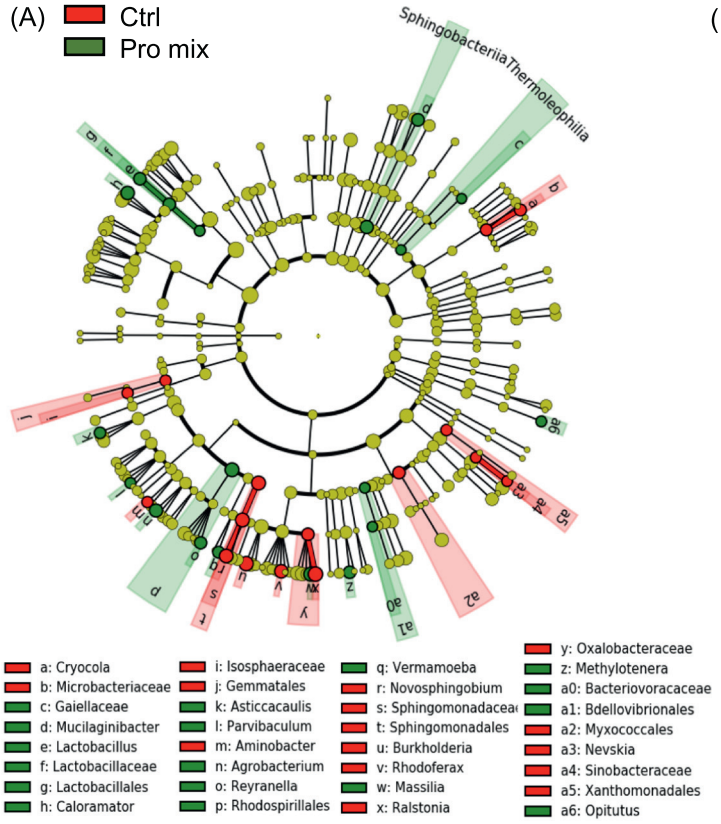
Although there was no significant difference in the expression of functional genes putatively involved in nitrogen fixation among the treatments (Fig. S5A), the bacterial classes that were abundant in each treatment were different (Fig. S5B). The most abundant taxon involved in N-fixation in the Pro mix + Azo, Nae + Azo, and Het + Azo treatments was Alphaproteobacteria, while Deltaproteobacteria was dominant in other treatments. There was increased relative abundance of Clostridia in all protist-inoculated treatments.

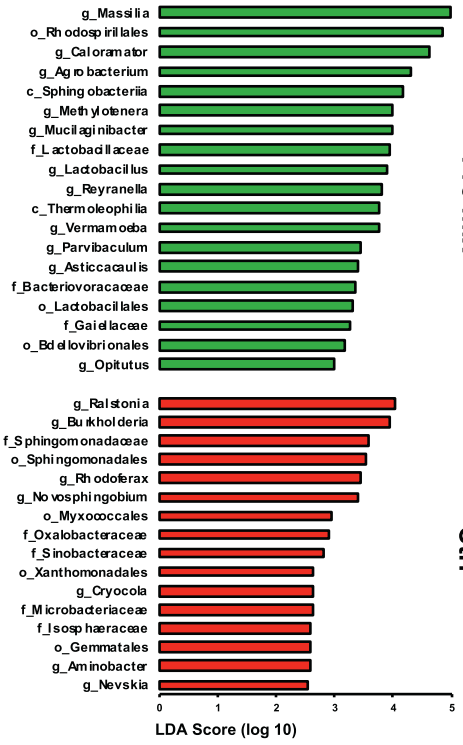
4. Discussion

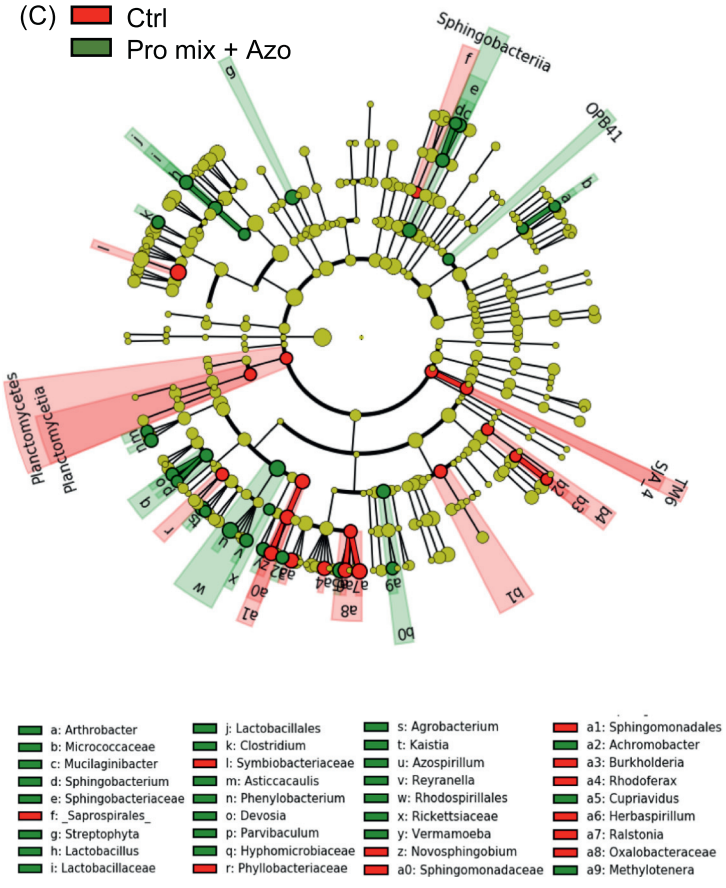
In this study, we demonstrated protist-enhanced survival of introduced *Azospirillum* sp. B510 and an increased growth and nitrogen uptake in rice plants. Our results, in agreement with previous studies, showed an increased rice plant growth and nitrogen uptake by heterotrophic protists (Herdler et al., 2008; Kreuzer et al., 2006) and by *Azospirillum* sp. B510 (Chamam et al., 2013; Isawa et al., 2010; Sasaki et al., 2010). Increased population and activity of introduced PGPR in the presence of protists have been shown for several PGPR species (Jousset, 2017); to the best of our knowledge, this is the first study using protists to increase the survival and thus efficiency of introduced *Azospirillum* in a planted microcosm. Investigation of the soil bacterial community structure further enabled us to reveal the importance of prey-predator interactions in the rhizosphere for early rice plant growth.

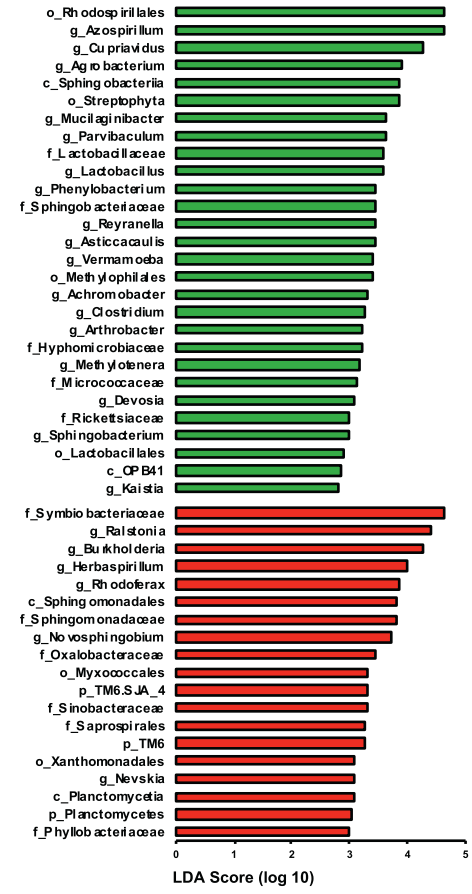
4.1. Protist–Azospirillum sp. B510 interaction

In the present study, results of qPCR and amplicon sequencing showed that mixed culture of protists, *Naegleria* sp., and *H. globosa*, increased gene abundance of *Azospirillum* sp. B510 in the rhizosphere, but only the effect of mixed culture was significant. So far, only a few studies have revealed the impact of protists on survival of PGPR. Protists provide a growth advantage to *Pseudomonas*, a toxic producing bio-control PGPR, by feeding on their nontoxic competitors (Jousset et al., 2006, 2009). Gluconic acid, which has a role in bacterial mineralization of phosphorus, can protect bacteria from protist predation (Gómez et al., 2010). However, as *Azospirillum* spp. is not known to produce any toxic compounds, a distinct mechanism might be involved in its enhanced survival in the presence of protists. Competition for nutrients between *Azospirillum* spp. and soil bacteria is an important factor affecting *Azospirillum* survival (Bashan and Levanony, 1990). It is likely that protists may stimulate *Azospirillum*'s survival by feeding on their competitors and providing nutrients. However, bacterial networks and interactions are very complex, and further information on









(caption on next page)

Fig. 5. A linear discriminant analysis effect size (LEfSe) method identifies the significantly different ($p < 0.05$, Kruskal-Wallis test) bacteria at multiple taxonomic levels by comparing community composition of Ctrl with Protists (A and B) and Mix (C and D) treatments. Cladograms illustrating the taxonomic groups that explain the most variation among rhizobacterial communities (A and C). The taxa with significantly different abundances between treatments are represented by colored dots, and from the center outward, they represent the kingdom, phylum, class, order, family, and genus levels. The colored shadows represent trends of the significantly differed taxa. Histograms of LDA scores showed significant bacterial differences within the treatments (B and D). The first letter indicates the taxonomic level: p, phylum; c, class; o, order; f, family; and g, genus. Ctrl, control; Pro mix, mixture of the four protists; Pro mix + Azo, *Azospirillum* sp. B510 and mixture of the four protists (see Table 1 for details).

interaction between introduced *Azospirillum* and the indigenous bacterial community should give more insight into their potential survival.

Although we did not analyze root growth in this study, it is well documented that protists shape the root architecture of plants, for example, increased lateral root branching and root volume in rice (Herdler et al., 2008; Kreuzer et al., 2006) and other plants (Bonkowski, 2004; Bonkowski et al., 2001; Jentschke et al., 1995; Krome et al., 2010). The sites of primary root colonization of *Azospirillum* sp. are the points of lateral root emergence and the root hair zones (Steenhoudt and Vanderleyden, 2000). Chamam et al. (2013) observed *Azospirillum* sp. B510 cells both at the surface and inside the root of *O. sativa* L. Nipponbare. Therefore, it is possible that protists potentially increased the habitat of *Azospirillum* sp. B510, resulting in higher populations of this bacteria in protist-inoculated plants than in non-protist inoculated plants. Further investigation should focus on the potential survival mechanisms of *Azospirillum* sp. B510 from protist predation and the effect of protists on *Azospirillum* sp. B510 colonization at the surface and inside of rice roots.

4.2. Heterotrophic protists and rice plant growth

Nitrogen is one of the most important nutrients for the early growth of rice plants. The microbial loop concept explains the nutrient turnover by protists preying on bacteria (Bonkowski, 2004; Clarholm, 1985); plants can take up considerable amounts of nitrogen released from consumed bacterial biomasses as a result of protist predation (Bonkowski et al., 2000; Griffiths, 1994). Protists influence nitrogen mineralization (Murase et al., 2006) and significantly increase rice shoot biomass (Herdler et al., 2008) and shoot nitrogen content (Kreuzer et al., 2006). Similarly, our results showed that both shoot biomass and nitrogen uptake were significantly higher in all protist-inoculated microcosms with and without *Azospirillum* sp. B510. Probably early plant growth is triggered by nitrogen available in the rhizosphere, which is likely generated by protists predation on bacteria and/or protist-enhanced possible nitrogen fixation by strain B510 (Yasuda et al., 2009). We did not find any significant difference in the number of functional genes putatively involved in biological nitrogen fixation among the treatments; however, the DNA-based results of putative assignments of low-resolution 16S rRNA gene information to functionality may not provide sufficient information on actively N-fixing bacteria.

Production of plant hormones or analogues by PGPR stimulates plant growth (Gupta et al., 2015). Bonkowski and Brandt (2002) showed that predation of amoeba increased the IAA-producing bacterial community. Concentrations of bioactive-free auxins in *Lepidium sativium* shoots were strongly increased by the predation activity of protists on bacteria (Krome et al., 2010). In our study, besides the introduced *Azospirillum* sp. B510, the relative abundance of several potential plant hormone-producing bacteria, from order Rhodospirillales, class Sphingobacteria, genus *Arthobacter*, and genus *Clostridium*, was increased in protist-inoculated treatments. Further evaluation of phytohormone-producing bacteria and protist interaction should be conducted to better understand protist-enhanced plant growth.

The effects of the four protist isolates on shoot biomass, shoot height, number of leaves, and nitrogen uptake varied. Protists can display different predation selectivity on bacteria (Murase et al., 2006; Rønn et al., 2002), and our results also support this. Therefore, the impact of protists on bacterial functionality and nitrogen turnover may vary (Rønn et al., 2002). Compared to Azo treatment, *Naegleria* sp. and *H. globosa* impacted the plant biomass. Current knowledge on increased rice plant growth by protists was derived exclusively from laboratory studies of *Acanthamoeba* sp. Our previous studies showed that *Naegleria* sp. and small flagellates are actively present in the rice rhizosphere (Asiloglu and Murase, 2016, 2017). This study suggests that amoeboflagellates and flagellates could play an important role in the early growth of rice plants.

Addition of *Azospirillum* sp. B510 and protists may have increased nutrients, which may have an impact on the increased N uptake of rice plants. Although we could not estimate the amount of biomass N of the introduced microbes, microbial biomass N usually contains around 0.05 $mg.kg^{-1}$ (Guo et al., 2019; Xu et al., 2018). This represents < 1% of total nitrogen uptake by rice plants in our study. Considering that N uptake in the inoculated treatments increased over 1.32 mg $plant^{-1}$ compared to control, the impact of biomass N of introduced microbes on our results was most likely insignificant.

4.3. Azospirillum sp. B510 and rice plant growth

Azospirillum sp. B510 successfully colonized in the microcosms under submerged conditions and significantly increased rice plant growth. *Azospirillum* spp. prefers and moves toward favorable nutrient conditions like root exudates by chemotaxis (Bashan, 1986; Heinrich and Hess, 1985), and toward optimal oxygen concentrations, called aerotaxis (Bashan, 1986; Steenhoudt and Vanderleyden, 2000). Considering oxygen (Ando et al., 1983; Gotō and Tai, 1956) and organic matter supplements (Kimura et al., 1979) by rice roots during early plant growth, rice roots potentially provide a favorable habitat for *Azospirillum*. Our results are in agreement with previous studies, which showed that strain B510 successfully colonized in microcosms within 10 days (Chamam et al., 2013) and significantly increased rice plant growth (Chamam et al., 2013; Isawa et al., 2010; Sasaki et al., 2010).

The *Azospirillum* genus influences plant growth in several ways. Nitrogen-fixing ability and production of phytohormones are considered as the most important features of *Azospirillum* for influencing plant growth (Fukami et al., 2018). Our results showed that presence of *Azospirillum* sp. B510 increased nitrogen uptake and concentration in the shoots. We assume that the effect of strain B510 on rice plant growth is related to its N-fixation ability (Kaneko et al., 2010), and phytohormone production may have also played an important role.

4.4. Impact of protists on bacterial community structure

The bacterial community structure was top-down regulated by the presence of protists in this study. Although the results of bacterial abundances obtained by amplicon sequencing do not necessarily represent the bacterial population, differences in the relative abundances of bacterial groups in the protist-inoculated treatments are indicative of protist predation activity. We should also consider the possibility that some bacteria, which were exclusively detected in the protist-inoculated treatments, may enter within protist isolates, as heterotrophic protists can be hosts of some bacteria especially members of Alphaproteobacteria (Gourabathini et al., 2008; Snelling et al., 2006). For instance, *Reyranella* sp. that can grow within food vacuoles of amoeba (Pagnier et al., 2011) was exclusively detected in amoeba-

inoculated treatments in this study. However, the similar patterns of bacteria among the treatments indicate that the majority of sequences originated from the indigenous soil bacteria.

Protist predation influenced several potential PGPR, including *Massilia*, *Agrobacterium*, *Clostridium*, and *Sphingobacterium*. Previous studies showed that, in addition to the direct effect of protists on nutrient turnover, protists indirectly increase plant growth by shifting the bacterial community structure toward beneficial bacteria (Bonkowski, 2004; Bonkowski and Brandt, 2002). However, not all potential PGPR species benefit from protist predation. In this study, the relative abundance of *Herbaspirillum* (known as a PGPR) was significantly decreased in all protist-inoculated treatments. The relative abundance of *Agrobacterium* significantly increased in the Ver + Azo and Het + Azo treatments, while it was slightly decreased in the Nae + Azo treatment. Further studies are needed to assess which protist species can be linked to enhancement of the indigenous soil PGPR species for better plant growth.

5. Conclusion

Overall our results showed that the mixed culture of heterotrophic protists significantly increased rice plant growth during the early stage by increasing the 1) number of introduced PGPR, *Azospirillum* sp. B510; 2) relative abundance of indigenous PGPR; and 3) nitrogen uptake by most likely accelerating the turnover of nitrogen. We also showed that the impact of protists on rice plant growth was comparable with the introduced PGPR. This is an intriguing finding since the known mechanisms of protists and *Azospirillum* in supporting plant growth are distinct. Further studies focusing on the inner response of rice plants to protists and PGPR at the molecular level should provide a better understanding. Additionally, the ability of protists to carry digestion-resistant bacteria within the cell can be used for a targeted delivery of PGPR species to the plant roots.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are thankful to staff from Shindori station (Niigata University) for providing the soil and plant samples, to Prof. Kimiko Itoh and Dr. Murat Aycan (Graduate School of Science and Technology, Niigata University) for their support on real time qPCR analysis, and to Prof. Kazushi Suzuki (Graduate School of Science and Technology, Niigata University) for providing *E. coli* MG1655. This work was supported by a research grant from The Yanmar Environmental Sustainability Support Association (K30059) to R.A.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2020.103599.

References

Alphei, J., Bonkowski, M., Scheu, S., 1996. Protozoa, Nematoda and Lumbricidae in the rhizosphere of Hordelymus europeaus (Poaceae): faunal interactions, response of microorganisms and effects on plant growth. Oecologia 106, 111–126. https://doi.org/10.1007/BF00334413.

Ando, T., Yoshida, S., Nishiyama, I., 1983. Nature of oxidizing power of rice roots. Plant Soil 72, 57–71. https://doi.org/10.1007/BF02185094.

Asiloglu, R., Murase, J., 2016. Active community structure of microeukaryotes in a rice (Oryza sativa L.) rhizosphere revealed by RNA-based PCR-DGGE. Soil Science and Plant Nutrition 62, 440–446. https://doi.org/10.1080/00380768.2016.1238281.

Asiloglu, R., Murase, J., 2017. Microhabitat segregation of heterotrophic protists in the rice (Oryza sativa L.) rhizosphere. Rhizosphere 4, 82–88. https://doi.org/10.1016/j.rhisph.2017.08.001.

Asiloglu, R., Honjo, H., Saka, N., Asakawa, S., Murase, J., 2015. Community structure of microeukaryotes in a rice rhizosphere revealed by DNA-based PCR-DGGE. Soil Science and Plant Nutrition 61, 761–768. https://doi.org/10.1080/00380768.2015.1049931.

Banayo, N.P.M., Cruz, P.C.S., Aguilar, E.A., Badayos, R.B., Haefele, S.M., 2012. Evaluation of biofertilizers in irrigated rice: effects on grain yield at different fertilizer rates. Agriculture 2, 73–86. https://doi.org/10.3390/agriculture2010073.

Bashan, Y., 1986. Migration of the rhizosphere bacteria Azospirillum brusilense and Pseudomonas fluorescens towards wheat roots in the soil. Microbiology 132, 3407–3414. https://doi.org/10.1099/00221287-132-12-3407.

Bashan, Y., Levanony, H., 1990. Current status of Azospirillum inoculation technology: Azospirillum as a challenge for agriculture. Can. J. Microbiol. 36, 591–608. https://doi.org/10.1139/m90-105.

Bonkowski, M., 2004. Protozoa and plant growth: the microbial loop in soil revisited. New Phytol. 162, 617–631. https://doi.org/10.1111/j.1469-8137.2004.01066.x.

Bonkowski, M., Brandt, F., 2002. Do soil protozoa enhance plant growth by hormonal effects? Soil Biol. Biochem. 34, 1709–1715. https://doi.org/10.1016/S0038-0717(02)00157-8.

Bonkowski, M., Griffiths, B., Scrimgeour, C., 2000. Substrate heterogeneity and microfauna in soil organic 'hotspots' as determinants of nitrogen capture and growth of ryegrass. Appl. Soil Ecol. 14, 37–53. https://doi.org/10.1016/S0929-1393(99)00047-5.

Bonkowski, M., Jentschke, G., Scheu, S., 2001. Contrasting effects of microbial partners in the rhizosphere: interactions between Norway spruce seedlings (Picea abies Karst.), mycorrhiza (Paxillus involutus (Batsch) Fr.) and naked amoebae (protozoa). Appl. Soil Ecol. 18, 193–204. https://doi.org/10.1016/S0929-1393(01)00165-2.

Briones, A.M., Reichardt, W., Briones Jr., A.M., W, R., 1999. Estimating microbial population counts by "most probable number" using Microsoft Excel®. J. Microbiol. Methods 35, 157–161. https://doi.org/10.1016/S0167-7012(98)00111-0.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8.

Cassán, F., Diaz-Zorita, M., 2016. Azospirillum sp. in current agriculture: from the laboratory to the field. Soil Biol. Biochem. 103, 117–130. https://doi.org/10.1016/j.soilbio.2016.08.020.

Chamam, A., Sanguin, H., Bellvert, F., Meiffren, G., Comte, G., Wisniewski-Dyé, F., Bertrand, C., Prigent-Combaret, C., 2013. Plant secondary metabolite profiling evidences strain-dependent effect in the Azospirillum-Oryza sativa association. Phytochemistry 87, 65–77. https://doi.org/10.1016/j.phytochem.2012.11.009.

Clarholm, M., 1985. Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol. Biochem. 17, 181–187. https://doi.org/10.1016/0038-0717(85)90113-0.

Cutler, D.W., Bal, D.V., 1926. Influence of protozoa on the process of nitrogen fixation by Azotobacter chroococcum. Ann. Appl. Biol. 13, 516–534. https://doi.org/10.1111/j.1744-7348.1926.tb04295.x.

Darbyshire, J.F., Wheatley, R.E., Greaves, M.P., Inkson, R.H.E., 1974. A rapid micro-method for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11, 465–475.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H., Minamisawa, K., 2001. Endophytic colonization and in planta nitrogen fixation by a Herbaspirillum sp. Appl. Environ. Microbiol. 67, 5285–5293. https://doi.org/10.1128/AEM.67.11.5285.

Flues, S., Bass, D., Bonkowski, M., 2017. Grazing of leaf-associated cercomonads (Protists: Rhizaria: Cercozoa) structures bacterial community composition and function. Environ. Microbiol. 19, 3297–3309. https://doi.org/10.1111/1462-2920.13824.

Fukami, J., Cerezini, P., Hungria, M., 2018. Azospirillum: benefits that go far beyond biological nitrogen fixation. AMB Express 8, 1–12. https://doi.org/10.1186/s13568-018-0608-1.

Gao, Z., Karlsson, I., Geisen, S., Kowalchuk, G., Jousset, A., 2019. Protists: puppet masters of the rhizosphere microbiome. Trends Plant Sci. 24, 165–176. https://doi.org/10.1016/j.tplants.2018.10.011.

Gentle, A., Anastasopoulos, F., McBrien, N.A., 2001. High-resolution semi-quantitative real-time PCR without the use of a standard curve. BioTechniques 31, 502–508. https://doi.org/10.2144/01313st03.

Ghosh, B.C., Bhat, R., 1998. Environmental hazards of nitrogen loading in wetland rice fields. Environ. Pollut. 102, 123–126. https://doi.org/10.1016/S0269-7491(98)80024-9.

Gómez, W., Buela, L., Castro, L.T., Chaparro, V., Ball, M.M., Yarzábal, L.A., 2010. Evidence for gluconic acid production by Enterobacter intermedium as an efficient strategy to avoid protozoan grazing. Soil Biol. Biochem. 42, 822–830. https://doi.org/10.1016/j.soilbio.2010.01.019.

Gotō, Y., Tai, K., 1956. On the differences of oxidizing power of paddy rice seedling roots among some varieties. Soil Science and Plant Nutrition 2, 198–200. https://doi.org/10.1080/00380768.1956.10431883.
Gourabathini, P., Brandl, M.T., Redding, K.S., Gunderson, J.H., Berk, S.G., 2008. Interactions between food-borne pathogens and Protozoa isolated from lettuce and spinach. Appl. Environ. Microbiol. 74, 2518–2525. https://doi.org/10.1128/AEM.02709-07.
Griffiths, B.S., 1994. Microbial-feeding nematodes and protozoa in soil: their effectson microbial activity and nitrogen mineralization in decomposition hotspots and the rhizosphere. Plant Soil. https://doi.org/10.1007/BF00010107.
Guo, Z., Han, J., Li, J., Xu, Y., Wang, X., 2019. Effects of long-term fertilization on soil organic carbon mineralization and microbial community structure. PLoS One. https://doi.org/10.1371/journal.pone.0211163.
Gupta, G., Parihar, S., Ahirwar, N., Snehi, S., Singh, V., 2015. Plant growth promoting Rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. Journal of Microbial & Biochemical Technology 7, 96–102. https://doi.org/10.4172/1948-5948.1000188.
Heinrich, D., Hess, D., 1985. Chemotactic attraction of Azospirillum lipoferum by wheat roots and characterization of some attractants. Can. J. Microbiol. 31, 26–31. https://doi.org/10.1139/m85-007.
Herdler, S., Kreuzer, K., Scheu, S., Bonkowski, M., 2008. Interactions between arbuscular mycorrhizal fungi (Glomus intraradices, Glomeromycota) and amoebae (Acanthamoeba castellanii, Protozoa) in the rhizosphere of rice (Oryza sativa). Soil Biol. Biochem. 40, 660–668. https://doi.org/10.1016/j.soilbio.2007.09.026.
Hervey, R.J., Greaves, J.E., 1941. Nitrogen fixation by Azotobacter chroococcum in the presence of soil protozoa. Soil Sci. 51, 85–100.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363. https://doi.org/10.1002/bimj.200810425.
Hünninghaus, M., Koller, R., Kramer, S., Marhan, S., Kandeler, E., Bonkowski, M., 2017. Changes in bacterial community composition and soil respiration indicate rapid successions of protist grazers during mineralization of maize crop residues. Pedobiologia 62, 1–8. https://doi.org/10.1016/j.pedobi.2017.03.002.
Isawa, T., Yasuda, M., Awazaki, H., Minamisawa, K., Shinozaki, S., Nakashita, H., 2010. Azospirillum sp. strain B510 enhances rice growth and yield. Microbes Environ. 25, 58–61. https://doi.org/10.1264/jsme2.ME09174.
Jentschke, G., Bonkowski, M., Godbold, D.L., Scheu, S., 1995. Soil protozoa and forest tree growth: non-nutritional effects and interaction with mycorrhizae. Biol. Fertil. Soils. https://doi.org/10.1007/BF00336088.
Jousset, A., 2017. Application of protists to improve plant growth in sustainable agriculture. In: Mehnaz, S. (Ed.), Rhizotrophs: Plant Growth Promotion to Bioremediation. Springer, Singapore, Singapore, pp. 263–273. https://doi.org/10.1007/978-981-10-4862-3_13.
Jousset, A., Bonkowski, M., 2010. The model predator Acanthamoeba castellanii induces the production of 2,4, DAPG by the biocontrol strain Pseudomonas fluorescens Q2-87. Soil Biol. Biochem. 42, 1647–1649. https://doi.org/10.1016/j.soilbio.2010.05.018.
Jousset, A., Lara, E., Wall, L.G., Valverde, C., 2006. Secondary metabolites help biocontrol strain Pseudomonas fluorescens CHA0 to escape protozoan grazing. Appl. Environ. Microbiol. 72, 7083–7090. https://doi.org/10.1128/AEM.00557-06.
Jousset, A., Scheu, S., Bonkowski, M., 2008. Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. Funct. Ecol. 22, 714–719. https://doi.org/10.1111/j.1365-2435.2008.01411.x.
Jousset, A., Rochat, L., Péchy-Tarr, M., Keel, C., Scheu, S., Bonkowski, M., 2009. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. The ISME Journal 3, 666–674. https://doi.org/10.1038/ismej.2009.26.
Kaneko, T., Minamisawa, K., Isawa, T., Nakatsukasa, H., Mitsui, H., Kawaharada, Y., Nakamura, Y., Watanabe, A., Kawashima, K., Ono, A., Shimizu, Y., Takahashi, C., Minami, C., Fujishiro, T., Kohara, M., Katoh, M., Nakazaki, N., Nakayama, S., Yamada, M., Tabata, S., Sato, S., 2010. Complete genomic structure of the cultivated rice endophyte Azospirillum sp. B510. DNA Res. 17, 37–50. https://doi.org/10.1093/dnares/dsp026.
Kimura, M., Wada, H., Takai, Y., 1979. The studies on the rhizosphere of paddy rice. Soil Science and Plant Nutrition 25, 145–153. https://doi.org/10.1080/00380768.1979.10433155.
Kreuzer, K., Adamczyk, J., Iijima, M., Wagner, M., Scheu, S., Bonkowski, M., 2006. Grazing of a common species of soil protozoa (Acanthamoeba castellanii) affects rhizosphere bacterial community composition and root architecture of rice (Oryza sativa L.). Soil Biol. Biochem. 38, 1665–1672. https://doi.org/10.1016/j.soilbio.2005.11.027.
Krome, K., Rosenberg, K., Dickler, C., Kreuzer, K., Ludwig-Müller, J., Ullrich-Eberius, C., Scheu, S., Bonkowski, M., 2010. Soil bacteria and protozoa affect root branching via effects on the auxin and cytokinin balance in plants. Plant Soil 328, 191–201. https://doi.org/10.1007/s11104-009-0101-3.
Kuikman, P.J., Jansen, A.G., Van Veen, J.A., 1991. ^{15}N-nitrogen mineralization from bacteria by protozoan grazing at different soil moisture regimes. Soil Biol. Biochem. 23, 193–200. https://doi.org/10.1016/0038-0717(91)90134-6.
Kuppardt, A., Fester, T., Härtig, C., Chatzinotas, A., 2018. Rhizosphere protists change metabolite profiles in Zea mays. Front. Microbiol. 9, 1–11. https://doi.org/10.3389/fmicb.2018.00857.
Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821. https://doi.org/10.1038/nbt.2676.
Levrat, P., Pussard, M., Alabouvette, C., 1992. Enhanced bacterial metabolism of a Pseudomonas strain in response to the addition of culture filtrate of a bacteriophagous amoeba. Eur. J. Protistol. 28, 79–84. https://doi.org/10.1016/S0932-4739(11)80322-6.
Mazzola, M., de Bruijn, I., Cohen, M.F., Raaijmakers, J.M., 2009. Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for Pseudomonas fluorescens. Appl. Environ. Microbiol. 75, 6804–6811. https://doi.org/10.1128/AEM.01272-09.
Murase, J., Noll, M., Frenzel, P., 2006. Impact of protists on the activity and structure of the bacterial community in a rice field soil. Appl. Environ. Microbiol. 72, 5436–5444. https://doi.org/10.1128/AEM.00207-06.
Nasir, S.M., 1923. Some preliminary investigations on the relationship of protozoa to soil fertility with special reference to nitrogen fixation. Ann. Appl. Biol. 10, 122–133. https://doi.org/10.1111/j.1744-7348.1923.tb05658.x.
Page, F.C., 1988. A New Key to Freshwater and Soil Gymnamoebae: With Instructions for Culture. Freshwater Biological Association, Ambleside, pp. 121. https://doi.org/10.2307/3226333.
Pagnier, I., Raoult, D., La Scola, B., 2011. Isolation and characterization of Reyranella massiliensis gen. nov., sp. nov. from freshwater samples by using an amoeba coculture procedure. Int. J. Syst. Evol. Microbiol. 61, 2151–2154. https://doi.org/10.1099/ijs.0.025775-0.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45e–45. https://doi.org/10.1093/nar/29.9.e45.
Razie, F., Anas, I., 2008. Effect of azotobacter and azospirillum on growth and yield of rice grown on tidal swamp rice field in South Kalimantan. Journal of Soil Science and Environment 10, 41–45. https://doi.org/10.29244/jitl.10.2.41-45.
Rønn, R., McCaig, A.E., Griffiths, B.S., Prosser, J.I., 2002. Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl. Environ. Microbiol. 68, 6094–6105. https://doi.org/10.1128/AEM.68.12.6094-6105.2002.
Sasaki, K., Ikeda, S., Eda, S., Mitsui, H., Hanzawa, E., Kisara, C., Kazama, Y., Kushida, A., Shinano, T., Minamisawa, K., Sato, T., 2010. Impact of plant genotype and nitrogen level on rice growth response to inoculation with Azospirillum sp. strain B510 under paddy field conditions. Soil Science and Plant Nutrition 56, 636–644. https://doi.org/10.1111/j.1747-0765.2010.00499.x.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60. https://doi.org/10.1186/gb-2011-12-6-r60.
Snelling, W.J., Moore, J.E., McKenna, J.P., Lecky, D.M., Dooley, J.S.G., 2006. Bacterial–protozoa interactions; an update on the role these phenomena play towards human illness. Microbes Infect. 8, 578–587. https://doi.org/10.1016/j.micinf.2005.09.001.
Steenhoudt, O., Vanderleyden, J., 2000. Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24, 487–506. https://doi.org/10.1016/S0168-6445(00)00036-X.
Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586. https://doi.org/10.1023/a:1026037216893.
Wakelin, S.A., Warren, R.A., Kong, L., Harvey, P.R., 2008. Management factors affecting size and structure of soil Fusarium communities under irrigated maize in Australia. Appl. Soil Ecol. https://doi.org/10.1016/j.apsoil.2007.12.009.
Watanabe, I., Lin, C., 1984. Response of wetland rice to inoculation with azospirillum lipoferum and pseudomonas Sp. Soil Science and Plant Nutrition 30, 117–124. https://doi.org/10.1080/00380768.1984.10434675.
Weidner, S., Latz, E., Agaras, B., Valverde, C., Jousset, A., 2017. Protozoa stimulate the plant beneficial activity of rhizospheric pseudomonads. Plant Soil. https://doi.org/10.1007/s11104-016-3094-8.
Xu, Y., Tang, H., Xiao, X., Li, W., Li, C., Sun, G., Cheng, K., 2018. Effects of long-term fertilization management practices on soil microbial carbon and microbial biomass in paddy soil at various stages of rice growth. Revista Brasileira de Ciência Do Solo 42. https://doi.org/10.1590/18069657rbcs20170111.
Yasuda, M., Isawa, T., Sshinozaki, S., Minamisawa, K., Nakashita, H., 2009. Effects of colonization of a bacterial endophyte, Azospirillum sp. B510, on disease resistance in rice. Biosci. Biotechnol. Biochem. 73, 2595–2599. https://doi.org/10.1271/bbb.90402.