Rhizosphere 4 (2017) 82–88

Contents lists available at ScienceDirect

Rhizosphere

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

Microhabitat segregation of heterotrophic protists in the rice (*Oryza sativa* L.) rhizosphere

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ARTICLE INFO

Keywords:
Community structure
Flagellate
Protist
Protozoa
Rice root

ABSTRACT

The rice rhizosphere is a hotspot for microorganisms, where heterotrophic protists are the major group of microeukaryotes and probably function as microbial predators. In this work, the microscale distribution of soil protists in a rice rhizosphere was studied in a microcosm experiment. Rice seedlings (*Oryza sativa* L. Nipponbare) were grown in "mini-rhizoboxes" made of thin glass plates and containing a nutrient solution and a diluted suspension of a rice-field soil in soft agar. This enabled nondestructive microscopic observations and the enumeration of heterotrophic protists in the rhizosphere. Different morphotypes of protists (ciliates, flagellates, and amoebae) densely populated the rhizosphere within 3 days after root development with an extended area at the root tips. There was a clear boundary between the rhizosphere and non-rhizosphere in terms of protistan density. Flagellates had the highest frequency and abundance in the colonization. Different protist morphotypes differed in their microscale distribution patterns. Flagellates including amoeboflagellates dominated near the roots, while amoebae inhabited sites adjacent to the flagellates, at a slight distance from the roots and with a clearly segregated habitat. Ciliates were exclusively observed at the tips of the rice roots. Denaturing gradient gel electrophoresis demonstrated that the microeukaryotic community in the rice rhizosphere was distinct from that in the non-rhizosphere. Protistan species inhabiting the rice rhizosphere were affiliated with Cercozoa (*Nucleocercomonas* sp. and *Gymnophrys* sp.), Amoebozoa (*Echinamoeba* sp.), and Ciliophora (*Oxytricha* sp.). Our results demonstrate that the rhizosphere effect of rice roots on heterotrophic protists differs at a microscale depending on the root segment. They also suggest a close relationship between Cercozoa flagellates and the roots.

1. Introduction

All nutrients absorbed by plants must pass through the rhizosphere, where roots, microorganisms, and animals intensively interact. Plants allocate a substantial part of their photosynthesized carbon to root-infecting symbionts and free-living rhizobacteria. Heterotrophic protists are an essential element of the microbial loop in the rhizosphere, where they function as grazers of rhizobacteria. Protistan grazing releases nutrients immobilized in bacterial biomass (Clarholm, 1985; Griffiths, 1986; Kuikman and Van Veen, 1989; Kuikman et al., 1991; Alphei et al., 1996; Murase et al., 2006) and shapes the bacterial community (Kreuzer et al., 2006; Krome et al., 2009; Rosenberg et al., 2009). Interactions between bacteria and protists in the rhizosphere may also promote non-nutritional plant growth (Jentschke et al., 1995) and alter the architecture of plant roots (Kreuzer et al., 2006; Rosenberg et al., 2009; Krome et al., 2010). The hormonal effect of protistan grazing on plant growth has been suggested (Bonkowski and Brandt, 2002; Bonkowski, 2004; Krome et al., 2010), but counter examples have also been reported (Vestergård et al., 2007; Ekelund et al., 2009).

Moisture availability is a key environmental factor controlling the soil protistan community (Geisen et al., 2014). Wetland rice field soil is often characterized in terms of the dominant heterotrophic protists, especially under organically enriched conditions (Murase et al., 2014; Murase et al., 2015). The rice rhizosphere is also enriched with heterotrophic protists as a major microeukaryotic group (Asiloglu et al., 2015; Asiloglu and Murase, 2016).

In a flooded soil, rice roots supply oxygen (Gotō and Tai, 1956; Ando et al., 1983) and organic matter to the rhizosphere, which supports active microbial metabolism (Kimura et al., 1979). The amounts of exudates and oxygen released from rice roots vary depending on the root segment. The root tip is the most active part of the root, delivering biologically active root exudates but also mucilages, and border cells to the rhizosphere (Chaboud and Rougier, 1984), the latter may influence the activity of amoebae in the rice rhizosphere (Somasundaram et al.,

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http://dx.doi.org/10.1016/j.rhisph.2017.08.001
Received 29 June 2017; Received in revised form 5 August 2017; Accepted 10 August 2017
Available online 24 August 2017

2008). Oxygen availability also controls the community structure and activity of heterotrophic protists in rice field soil (Murase et al., 2014; Takenouchi et al., 2016). Under submerged conditions, the radial loss of oxygen by rice roots is highest at the tip (Armstrong, 1971; Colmer and Pedersen, 2008). Based on these observations, we hypothesized that the spatial heterogeneity of the chemical environments in the rice rhizosphere provides various micro-niches that support soil protists differing in their growth requirements.

The study of heterotrophic protists in soil has been complicated by difficulties in observing their distribution, sites of activity, and mutual interactions. An agar medium containing isolated bacteria and protists offers an alternative method of identifying the interactions between plant roots, bacteria, and protists (Zwart et al., 1994; Kreuzer et al., 2006). However, previous studies using this approach focused only on a few model protists, such that information on the spatial distribution of the natural community of soil protists in the rice rhizosphere is still lacking. In the present work, we conducted a mini-rhizobox experiment to study the colonization of the rhizosphere of newly developed rice roots by a natural assemblage of soil protists. The microscale distribution of different morphotypes of protists was determined by microscopic observations, while the developed protistan community was explored through a molecular analysis targeting 18S rRNA genes.

2. Materials and methods

2.1. Growth of rice in a mini-rhizobox

Soil was taken from the plow layer (0–10 cm depth) of a rice field (site D2) under drained conditions at Anjo Research and Extension Center, Anjo, Aichi, Japan (20 m above sea level; 34°48'N, 137°30'E) in May 2013. The field site and soil properties have been described elsewhere (Lu et al., 2002). Prior to use in the experiment, the soil was sieved (< 2 mm) and then stored at 4 °C.

The mini-rhizobox consisted of two glass slides (76 × 100 mm) inserted into a 5-mm-thick, U-shaped rubber frame to which they were fastened by stainless steel clips to seal the bottom and sides of the microcosm (Supplementary data, Fig. S1). The 22-day-old rice seedlings (*Oryza sativa* L., Nipponbare) were grown in pots with submerged soil, gently washed with sterilized water—this treatment did not necessarily sterilize the roots—, and individually transferred to a rhizobox. The microcosms were filled with 0.7% agar medium containing 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 $FeCl_3$), 0.001% (w/v) of methylene blue as a redox indicator, and a soil suspension at a final concentration of 0.33 g L^{-1} that included ~10^2 cells of heterotrophic protists per microcosm (estimated by the most probable number method, data not shown). A rhizobox with no plants was prepared as the control. The glass slides of the rhizobox were covered with black nylon screens to block light penetration from the sides. Eighteen rhizoboxes (9 planted and 9 unplanted) were prepared and kept submerged in Kasugai's nutrient solution during their 3-week incubation in a growth chamber at 30/25 °C (day/night) with a day length of 16 h (150 μmol m^{-2} s^{-1}). The nutrient solution was changed weekly to avoid algal growth.

2.2. Microscopic observation and enumeration of protists

The presence and distribution patterns of protists in the mini-rhizobox were nondestructively monitored by observing the microcosms from the sides every 3 days at ×20 magnification using an inverted microscope (Olympus IX70) equipped with phase-contrast optics. The frequency of protistan colonization was calculated based on the presence of flagellates, amoebae, and ciliates in the crown roots that developed after transplantation of the seedlings.

A preliminary experiment showed that the protists densely and evenly populated a specific area near the roots but were no longer detectable at a certain distance from the roots. This clearly indicated the formation of a boundary between the rhizosphere and non-rhizosphere and thus we suggest that a rhizosphere effect on protists is likely. Although the dimensions of the area inhabited by protists varied for each root, the distribution pattern was the same, which allowed an approximate estimate of the area inhabited by protists for each root. This area could be divided into four zones: 1. the root tip, approximated to a semicircular-shaped area; 2. the tip sides, approximated to a rectangular-shaped area; 3. the sides of the root between the tip and the base, approximated to trapezoid-shaped area; and 4. a narrow area at the base, at the beginning of the root, approximated to a rectangular-shaped area (Supplementary data, Fig. S2A). Flagellates always inhabited sites close to the roots, especially at the tip, while amoebae always colonized the area adjacent to flagellates and at a slight distance from the roots. Therefore, the areas of amoeba and flagellate distribution were calculated separately for each zone. First, the total area inhabited by flagellates and amoebae was determined according to the dimension of each zone. The area inhabited by flagellates alone was then calculated in the same way, and that by amoebae by subtracting the area of flagellates from the total inhabited area. The area of ciliate distribution was calculated for zones 1 and 2, where ciliates were exclusively observed. More than 10 photographs of protists (size: 420 × 600 μm) were taken for each zone of an individual root and the number of protists was counted as follows: Flagellates and amoebae were counted in a 100 × 100 μm area, and ciliates, due to their larger size and fewer numbers, in a 420 × 600 μm area. The average value was determined and multiplied by the total area inhabited by protists. The data are expressed as number per root.

The spatial change in protistan density was determined in the radial direction from the main root (tip and base segments) and in the longitudinal direction along the root (from tip to base) by microscopically observing the rhizosphere of a selected root in triplicate rhizoboxes developed 10 days after transplantation (DAT). For the radial change, consecutive images were taken at a radial distance of 1 cm from the surface of the root tip and the base; for observation, the base was fixed approximately 3 cm from the tip (Supplementary data, Fig. S2B). For the longitudinal change along the root, the area along a radial distance of 0–400 μm from the root surface was scanned from the tip to the base. Flagellates and amoebae detected in the images were counted separately. Their number over the radial distances from the root tip and base were counted for five replicates of a 0.01-mm^2 area (100 × 100 μm) per image. The number of flagellates and amoebae in the longitudinal direction was counted in a 0.01-mm^2 area at radial distances of 0–100 μm, 100–200 μm, 200–300 μm, and 300–400 μm from the root surface. The density was expressed as cells per 0.01 mm^2. Because of their fast swimming speed and large body size, ciliates were not counted in the microscale analysis.

2.3. Molecular analysis of the protistan community in the rhizobox

The protistan communities in the rhizosphere and non-rhizosphere areas of the microcosms were determined 7, 14, and 21 DAT by sacrificing triplicate rhizoboxes at each time point. Roots that developed after transplantation and the surrounding agar medium at a 1-cm radial distance from the roots were collected as the rhizosphere sample. The roots were cut into pieces (< 5 mm) and mixed with rhizosphere agar medium. The rest of the agar medium in the planted microcosms, lacking roots and not colonized by protists, was sampled as the non-rhizosphere sample. Roots that developed before transplantation and the surrounding agar in the planted microcosms were carefully excluded. The washed roots of the seedlings before transplantation and the agar media of the unplanted microcosms were also collected.

DNA was extracted from 500 μL of the samples by bead-mill homogenization using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instruction and then eluted in 100 μL of TE buffer.