Original Research

Root Radial Oxygen Loss and the Effects on Rhizosphere Microarea of Two Submerged Plants

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Abstract

Radial oxygen loss (ROL) has been suggested to be a major process to protect plants exposed to the anaerobic by-products of soil anaerobiosis. The aim of the present study was to test the effects of root ROL from two submerged plants (*Hydrilla verticillata* and *Vallisneria spiralis*) on the rhizosphere oxygen profile and rhizosphere microarea. Phospholipid fatty acids (PLFAs) of sediment samples were used to characterize and quantify the microbial community. The results showed clearly that there were significant differences between the two plants in radial oxygen loss, which affected rhizosphere physicochemical parameters and the microbial community. Rhizosphere total biomass, bacteria, gram-positive bacteria, actinomycetes, and microbial diversity of *V. spiralis* were significantly higher than those of *H. verticillata*. The present study highlights root ROL as a key parameter affecting the microbial community of the rhizosphere microarea.

Keywords: root radial oxygen loss, phospholipid fatty acids, rhizosphere, microbial community, submerged plant

Introduction

Submerged plants are essential constituents and primary manufacturers in the water ecological system, and play a regulatory role in the ecological system of material circulation and energy [1]. Since flooded soils are generally oxygen deficient and dominated by anaerobic microflora, they are a potentially hostile environment for the roots of submerged plants [2]. However, due to the presence of aerenchyma in roots and stems [3], oxygen is transported to roots and some of this leaks into the anaerobic sediment. This radial oxygen loss (ROL) is one of the most important functional characteristics of submerged plants, which may relate to their adaptability to the wetland substrate [4] and to their ability to remove nutrients in anoxic conditions [2]. When submerged plant roots release oxygen into the sediment, an aerobic-anaerobic gradient with some distance away from the roots is formed, where aerobic, anaerobic, and facultative anaerobic microorganisms can co-exist [2, 5].

The rhizosphere is where physicochemical and microbial processes take place that are induced by the interaction of plants, microorganisms, sediment, and pollutants [4]. The role of ROL in wetland microbial ecology is probably essential because it has the potential to establish steep redox gradients that specific groups of microbes exploit to oxidize methane, NH_4^+, Fe^{2+}, or organic matter [6, 7], therefore affecting the rhizosphere microbial community. Root ROL and the rhizosphere microbial population promote plant growth and health by facilitating the breakdown of organic

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matter, mineralizing nutrients [8, 9] and protecting from phytotoxins such as S_2^- and Fe^{2+} [7, 10, 11]. So the study of microbial communities associated with plant ROL is important for understanding their ecological role in natural environments [12].

Hydrilla verticillata (*H. verticillata*) and V*allisneria spiralis* (*V. spiralis*) (Hydrocharitaceae) are two perennial submersed plants widely used in lake ecological restoration. They tolerate a wide range of environmental conditions, making them suitable pioneer species for inclusion in restoration projects to reverse the eutrophication of lakes [13, 14].

The objectives of this study were to study ROL from the root of *H. verticillata* and *V. spiralis* and analyze the possible influences on the microbial community structure and diversity and on chemical transformations in the sediment. The microbial community was assessed by analyzing sediment for characteristic phospholipid fatty acids (PLFAs) [15-17]. The effects of ROL on sediment and rhizosphere oxygen and redox levels were revealed using microelectrodes, while ROL from whole root systems was determined using titanium citrate [18].

Materials and Methods

Experiment Plants and Sediment

H. verticillata used in the present experiment was collected from Lake Caohai, China. *V. spiralis* was collected from Lake Donghu, China. All samples were placed in separate air-sealed plastic bags and transported to a laboratory as soon as possible, and each sample was rinsed with tap water several times and then inoculated indoors at room temperature.

Sediment was collected from Donghu Lake, Wuhan Province, China. The sediment was ground to pass through an 80-mesh sieve, and analyzed for the following parameters prior to the plant growth experiment: total nitrogen (TN 2.34 ± 0.01 mg·g⁻¹) using the Kjeldahl method; total phosphorus (TP 2.05 ± 0.07 mg·g⁻¹) using the molybdenum blue method [19]; total Fe (21.74 ± 0.41 mg·g⁻¹) were determined using the modified 2, 2,-bipyridyl spectrophotometric method [20]; organic content ($12.78\pm0.13\%$) as loss on ignition at 550ºC; and sediment porosity (0.20 ± 0.03) and pH using a pH microelectrode (Unisense, 6.97 ± 0.22).

Microcosm Experiments

The experiment contained seven microcosms (Fig. 1), each of which was comprised of an aquarium (inner size: 40×28×15 cm) with thirty cylindrical plastic cups (5 cm diameter and 8 cm height). Three replicate microcosms were used throughout the experiment for each plant, while a microcosm without submerged plants served as a control. About 5 cm depth sediment was filled in and outside the cups. Three plant seedlings (about 10 cm) were then planted in each cup. The microcosms were placed in a greenhouse and incubated at 25 ± 1ºC under a 12/12 h light/dark cycle. The illuminations were about 1,500 lux during the day and 0 in darkness. In addition, tap water was added daily to replenish water lost from evaporation. The experiment lasted 60 days with sampling first at 20 days and every 10 days thereafter.

Sediment and Plant Sampling

The characters of sediment and plant were traced after 20 days when *H. verticillata* seedlings began rhizogenesis. At each sampling, six cups in each microcosm were randomly selected. Firstly, one of which was used for the *in situ* measurement of oxygen and redox potential (Eh) profiles and *in vitro* measurement of root inner oxygen 60 days after replanting.

Previous studies had defined the sediment within a 4 mm distance around the root as the rhizosphere sediment [9, 21]. After the plants were carefully extracted, the sediment attached to the roots was collected and deemed as rhizosphere sediment in this study. Control sediment was defined as sediment without plants, and was sampled at 3-4 cm depth. Sediment samples were analyzed for chemical parameters and PLFAs. Before PLFA analysis, fresh sediment samples were immediately freeze-dried, ground and passed through an 80 mesh sieve and stored at -20ºC.
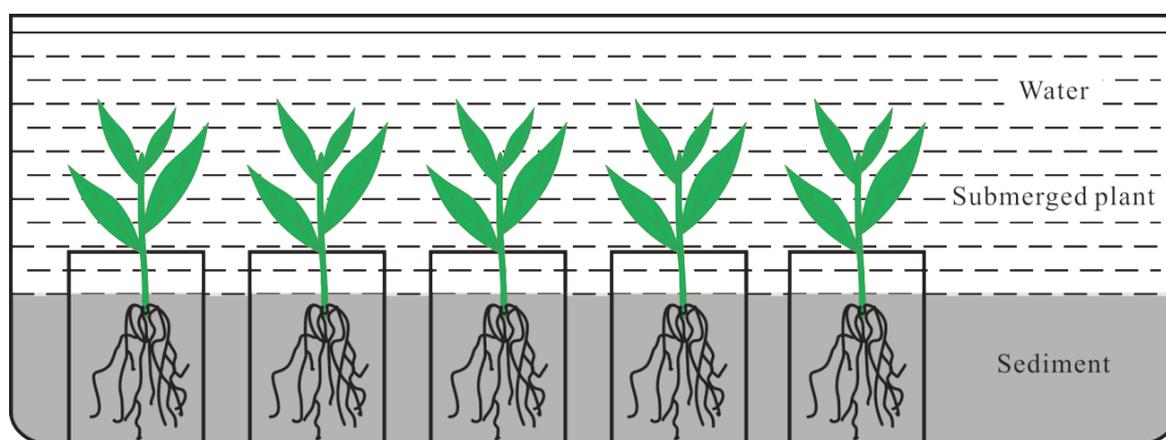

Fig. 1. Diagram of the microcosm experiment.

The plant samples were also collected and rinsed thoroughly with deionized water for the measurement of root ROL. After ROL measurement, each plant sample was washed carefully with deionized water and then separated into roots and shoots (including leaves and stems). Some fresh roots were used for porosity determination, and the remaining roots and shoots were weighted after drying (24 h at 80ºC) to a constant weight to obtain the final dry mass (dw).

Analytical Methods

The oxygen and Eh profiles of the water-sediment interface were measured by microelectrodes (OXY25, ø=25 µm, RO100, ø=100 µm, Unisense, Denmark). The oxygen electrode is connected to an OXY-meter (a high-sensitivity picoampere amplifier, Unisense) and the cathode is polarized against the internal reference. The reference electrode of Eh electrode is a simple open-ended Ag-AgCl electrode with a gel-stabilized electrolyte (Unisense) and is used to establish a reference potential against the redox microelectrode. A motor controller (MC-232, Unisense) with a micromanipulator (MM 33, Unisense) was connected with microelectrodes and a computer. The measurements of water-sediment oxygen and Eh profiles were performed in the center of the range at a distance of about 2 mm from the plants. The spatial resolutions of all measurements were 500 µm in depth. The periods for "wait before measure" and "measure" were both set as 3 s for O_2 and 10 s for Eh.

Oxygen microelectrodes are nowadays widely used in plant science to measure oxygen concentrations in tissues such as rice roots [9]. Root inner oxygen contents were measured by oxygen microelectrode (OXY25, ø=25 µm, Unisense, Denmark). After the *in situ* measurement of O_2 and Eh profiles at 60 days, plants were transferred to a stagnant solution containing 1% (w/v) agar in Hoagland's solution [22] and was deoxygenated by N_2 gas before use, followed immediately by covering the solution surface with 0.5 cm of paraffin liquid to prevent atmospheric O_2 [9] (Fig. 2). After 2 h, the inner oxygen produced by individual roots was measured by microelectrode 1 cm from the root apex.
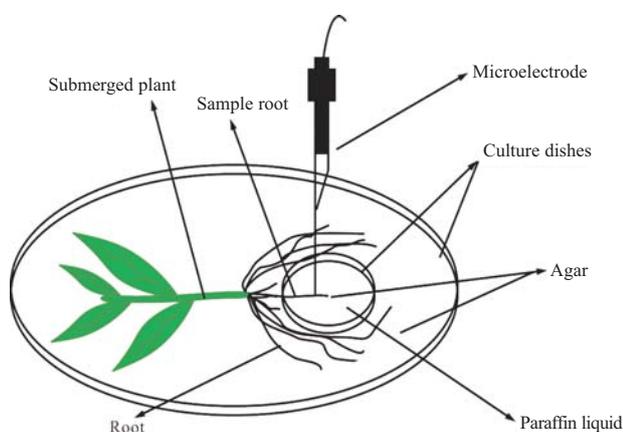

Fig. 2. Diagram of the measurement of the inner oxygen produced by individual roots in an agar microcosm.

Root ROL of each plant was quantified by the Ti^{3+}-citrate method [18]. Briefly, 30 mL $TiCl_3$ (1.16 M) was added to a 200 mM deoxygenated sodium citrate (300 mL) to form a Ti^{3+}-citrate solution. pH was adjusted to 5.6 with saturated $NaHCO_3$ solution. Plant samples were washed carefully and then inserted into 50 mL tubes with roots completely immersed in 40 mL deoxygenated 10% Hoagland's solution [22]. Subsequently, Ti^{3+}-citrate solution was added to the tubes, followed immediately by covering the solution surface with 2 cm of paraffin oil to prevent oxidation by atmospheric O_2. The test tubes were incubated at 25ºC under 1,500 lux illumination. After 6 h, all tubes were shaken gently and the solutions were measured at 527 nm using a UV-visible spectrophotometer. The amount of released O_2 was determined based on Ti^{3+}-citrate oxidation. Root ROL was calculated according to the following formula:

$$ROL = 1000 \ c(y − z)/g/T$$

...where c is the initial volume of Ti^{3+}-citrate added to each tube (L), y is the concentration of Ti^{3+}-citrate solution without plants (mM), z is the concentration of Ti^{3+}-citrate solution with plants (mM), g is root dry weight (g), and T is response time (h).

Root porosity was measured according to the method described by Kludze et al. [18]. About 0.6 g of fresh roots (1-3 cm from the root apex) was used for porosity determination. Porosity was calculated using the following equation:

$$Porosity \ (\%) = [(Pvr − Pr)/(r + P − Pr)] × 100$$

...where Pvr is the mass of pycnometer with vacuumed roots and water (g), Pr is the mass of pycnometer with water and fresh roots (g), r is the mass of fresh roots (g), and P is the mass of pycnometer with water (g).

Fresh sediment samples were extracted with 2 mol·L⁻¹ KCl and saturated $CaSO_4$ (sediment:solution ratio 1:10), and the extracts were measured respectively for NH_4^+ and NO_3^- [23]. Total Fe and Fe^{2+} in the extract were determined using a modified 2, 2,-bipyridyl spectrophotometric method [20] after digestion with HCl (3 M) for 16 h, and analyzed at 520 nm.

The microbial communities of rhizosphere and control sediment at 60 days were characterized using PLFA analysis according to Bossio and Scow [24]. Briefly, lipids were extracted twice from freeze-dried sediment using phosphate buffer (50 mM, pH 7.4), chloroform, and methanol at a ratio of 0.8:1:2. Supernatant from the second extraction was added to the first. Samples were shaken and the phases were allowed to separate overnight. The organic phase was decanted and evaporated under N_2 at 30ºC. The resulting lipid material was fractionated into neutral lipids, glycolipids, and polar lipids on a silica gel SPE column (Agilent SPE 5982-2265, USA) by elution with chloroform, acetone, and methanol, respectively. Methanol was evaporated under N_2 at 30ºC. After mild methanolic KOH to form fatty acid methyl esters (FAMEs), samples were then dissolved in hexane-containing methyl nonadecanoate fatty acid (19:0) as an internal standard and analyzed in an Agilent 6890N gas chromatograph (Wilmington, USA) with MIDI

Table 1. Differences in morphological parameters and ROL of two different plants.

Time (d)	Species	Root biomass (mg dw)	Shoot biomass (mg dw)	Root inner oxygen (µmol·L⁻¹)	Root porosity (%)	ROL (µmol O₂·h⁻¹·g⁻¹ dw root)
20	*H. verticillata*	2.52±0.83 [a]	36.37±15.15 [a]	-	-	35.61±4.46 [a]
	V. spiralis	3.10±0.41 [a]	26.88±2.89 [a]	-	-	39.72±5.62 [a]
30	*H. verticillata*	4.76±0.72 [a]	55.49±14.95 [a]	-	-	41.76±8.49 [a]
	V. spiralis	4.95±0.75 [a]	69.19±13.02 [a]	-	-	66.24±5.18 [b]
40	*H. verticillata*	6.81±0.90 [a]	73.89±10.34 [a]	-	-	45.69±8.93 [a]
	V. spiralis	7.44±0.77 [a]	69.19±13.02 [a]	-	-	68.03±4.32 [b]
50	*H. verticillata*	8.09±0.33 [a]	134.32±20.66 [a]	-	-	55.69±2.71 [a]
	V. spiralis	14.73±1.64 [b]	149.36±28.03 [a]	-	-	69.30±5.79 [b]
60	*H. verticillata*	12.23±3.82 [a]	209.00±7.32 [a]	79±1 [a]	22.56±1.26 [a]	66.61±8.10 [a]
	V. spiralis	15.60±1.80 [a]	211.40±14.74 [a]	103±10 [b]	26.76±2.26 [b]	78.51±6.65 [b]

means±SD with three replicates, small letters in the same column represent significant differences between two plants, P<0.05

peak identification software (version 6.2, MIDI, Newark, USA) and an Agilent 19091B-102 (25.0 m×200 µm×0.33 µm) capillary column.

The total amount of PLFAs was used to indicate the total microbial biomass, and the amounts of the different PLFA biomarkers were used to represent the different groups of sediment microorganisms. The sum of the following PLFA biomarkers were considered to represent bacterial origin (Gram-positive bacteria by i14:0, i15:0, a15:0, i16:0, a16:0, a17:0, Gram-negative bacteria by 18:1ω5c, 18:1ω7c, bacteria were represented by the sum of the two) [25]. Biomarkers 18:1ω9c, 18:3ω6c, and 16:1ω5c were used for indicating fungi, and 10Me16:0 and 10Me17:0 were used as a marker for actinomycetes [16, 26]. Diversity indices were calculated as:

$$\text{Shanon index (H)} = -\Sigma p_i \ln(p_i)$$

...where p_i is the relative abundance of PLFA.

Statistical Analysis

Quantitative data was expressed as means ±SD. Data were analyzed using SPSS 19.0. One-way analysis of variance (ANOVA) followed by *post-hoc* least significant difference (LSD) testing was used to assess the effect of plant species on physicochemical parameters, total biomass, and PLFA biomarkers. Statistical significance was set at the P<0.05 level.

Results

Characteristics of Plant Growth and Root ROL

Root biomass, shoot biomass and root ROL increased with time (Table 1). Major external morphological parameters such as root and shoot biomass for *V. spiralis* were greater than those for *H. verticillata*, but were not statistically significant (P>0.05), except for the differences in the root dry weights at 50 days after replanting (P<0.05). As measured by the Ti³⁺-citrate method, ROL of *V. spiralis* was significantly higher than that of *H. verticillata* (except at 20 days).

The oxygen value decreased rapidly in the agar and reached near zero at a distance of approximately 500 µm from the agar surface (data not shown). When the microelectrode reached within the root, oxygen concentrations were about 79 and 103 µmol·L⁻¹ for *H. verticillata* and *V. spiralis*. These results indicated that root internal morphological parameters such as aerenchyma might promote oxygen release from the root to the rhizosphere sediment. The root porosities were 22.56% and 26.76% for *H. verticillata* and *V. spiralis*, respectively.

Characteristics of the Rhizosphere Oxygen and Eh with Different Plants

To investigate the real status of the rhizosphere oxygen distribution in rhizosphere sediment, the oxygen concentration profiles were detected *in situ* for both *H. verticillata* and *V. spiralis*. The oxygen dissolved in the water layer remained stable at approximately 224 µmol·L⁻¹ for *H. verticillata* and 250 µmol·L⁻¹ for *V. spiralis*, compared with 203 µmol·L⁻¹ in the control sediment (Fig. 3). The content of oxygen decreased rapidly in the sediment and reached to 0 at a distance of approximately 3.5-8.5 mm from the water-sediment interface. The thickness of aerobic layer decreased in the order *V. spiralis* > *H. verticillata* > control sediment, suggesting that plants can improve rhizosphere sediment oxygen concentrations and the amount of dissolved oxygen in the water layer. When the microelectrode was in contact with the root surface, a peak appeared at about 15 mm under the water-sediment interface (Fig. 3a). Because the root inner oxygen concentrations (Table 1) were significantly higher than this peak, it could also veri-
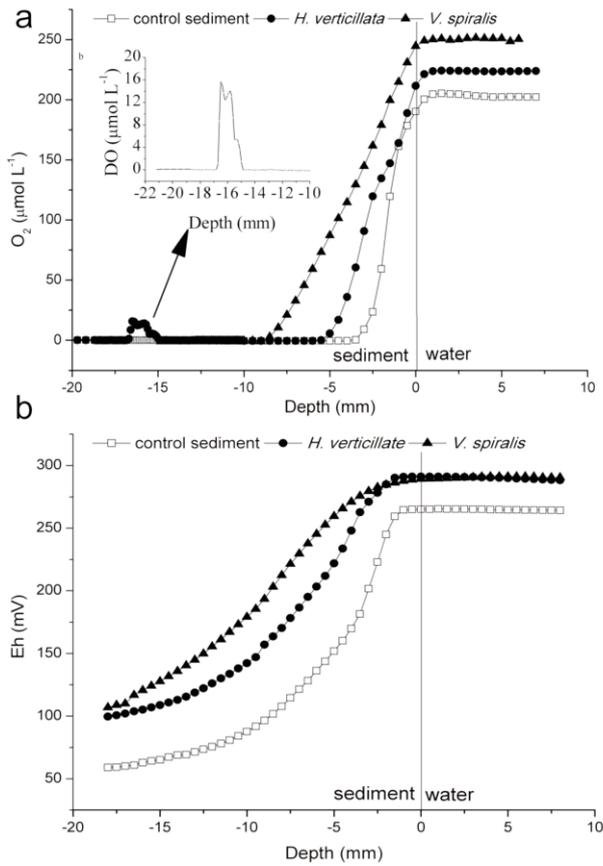
Fig. 3. Oxygen and redox potential profiles of the rhizosphere sediment (2 mm away from the root surface) and the control (without plants) 60 days after planting in Lake Donghu sediment with *H. verticillata* and *V. spirals*.

fy the existence of root ROL, which made a microarea "oxidation ring" around the roots.

Eh values underwent similar trends (Fig. 3b), with a clear distinction between water and sediment. Eh values of planted sediments were higher than those of control sediment, especially for *V. spiralis* with higher ROL, which meant the potential for loss of oxygen to sediment through root ROL that could improve sediment Eh conditions.

Changes to Chemical Parameters

NH_4^+ concentrations in the rhizosphere sediment were significantly lower than those in control sediment (Fig. 4a), suggesting that, in some way, the presence of roots was responsible. The average NH_4^+ concentration of the sediment in the control was 3.19 mg·kg⁻¹, while the rhizosphere sediments were 1.54 and 1.29 mg·kg⁻¹ for *H. verticillata* and *V. spiralis*, respectively.

Unlike NH_4^+, the NO_3^- concentrations in the rhizosphere were higher than those in the control sediment during the different sampling dates (Fig. 4b). The average NO_3^- concentrations of the sediment in the rhizosphere sediment were 0.68 and 0.73 mg·kg⁻¹ of the two plants, while the control was 0.63 mg·kg⁻¹.

Total Fe did not change with time among rhizosphere and control sediment (Fig. 4c). Fe^{2+} was oxidized by root ROL and changed to Fe^{3+}. Fe^{2+} in control sediment was significantly higher than the rhizospheres of *H. verticillata* and *V. spiralis*.

Microbial Community Structure Assessed by PLFAs

The PLFA profiles of the whole microbial community structure showed that the samples collected from sediment
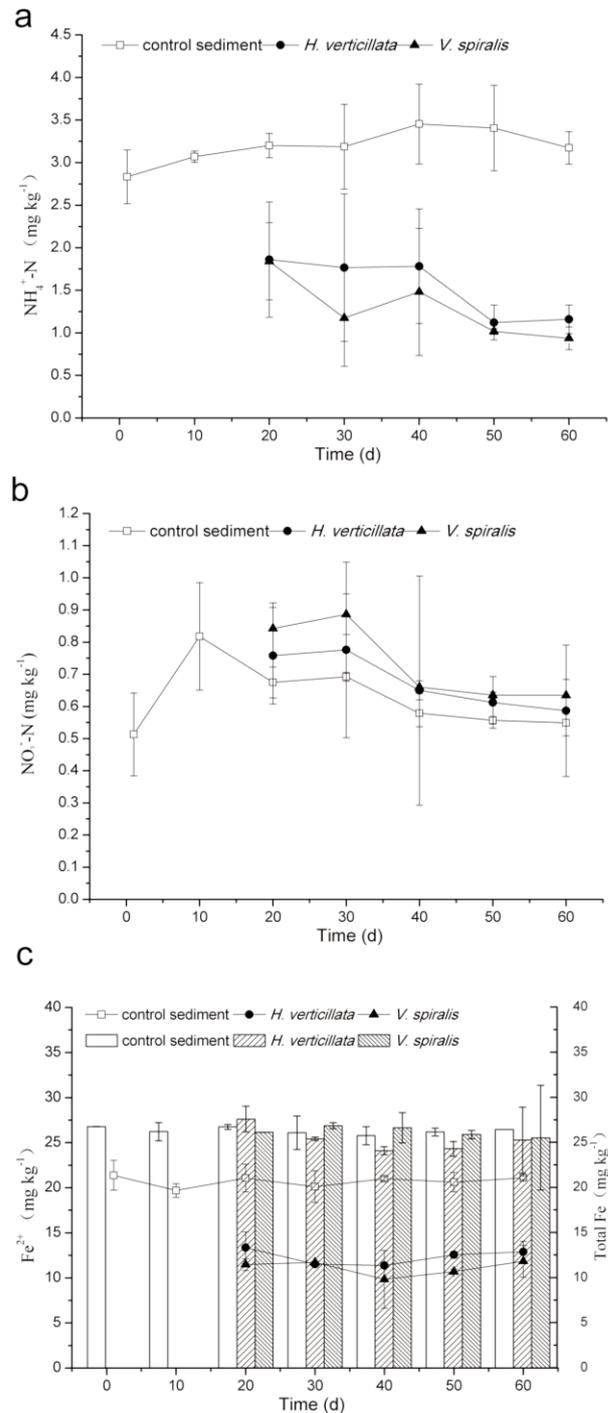

Fig. 4. Changes of chemical parameters in control and *H. verticillata* and *V. spiralis* rhizosphere sediment.

Table 2. Contents of PLFAs and index of Shanon in sediment of the two plants.

PLFA (nmol·g⁻¹ dry sediment)	Control sediment	*H. verticillata*	*V. spiralis*
Total biomass	22.40±2.29 [a]	25.39±3.68 [a]	27.63±0.63 [b]
Total bacteria	1.26±0.06 [a]	1.53±0.26 [a]	1.74±0.26 [b]
Gram-positive bacteria	0.95±0.04 [a]	1.10±0.23 [a]	1.29±0.13 [b]
Gram-negative bacteria	0.31±0.08 [a]	0.42±0.09 [a]	0.44±0.14 [a]
Fungi	0.16±0.01 [a]	0.24±0.10 [a]	0.27±0.04 [a]
Actinomycetes	0.18±0.01 [a]	0.18±0.01 [a]	0.28±0.01 [b]
Diversity index	1.22±0.02 [a]	1.33±0.05 [a]	1.46±0.10 [b]

means±SD with three replicates, small letters in the same row represent significant differences, P<0.05

contained a variety of PLFAs, which were composed of saturated, unsaturated, and methyl-branched fatty acids (Fig. 5). Irrespective of the plants, a13:0, i14:0, a17:0, 18:1ω5c, 18:0, and 20:2ω6,9c PLFAs showed higher contents in rhizosphere sediment compared to control sediment; whereas 12:0, 14:0, i15:0, 16:1ω5c, 17:1ω7c, 17:1ω5c, 17:0, and 20:1ω7c PLFAs were higher contents in control sediment compared to those of rhizosphere sediment (Fig. 5).

The contents of total biomass, total bacteria, gram-positive bacteria, gram-negative bacteria, fungi, actinomycetes, and diversity index in the rhizosphere of *H. verticillata* and *V. spiralis* were higher than those in the control sediment (Table 2). In addition, although the rhizosphere of *H. verticillata* and the control sediment differed substantially in their PLFA contents (Fig. 5), there were no significant differences in their total bacterial, gram-positive bacterial, gram-negative bacterial, fungal, actinomycic PLFA, and diversity index fractions (Table 2).

The results implied that the microbial community structure was similar. The proportion of fungal PLFAs was lower than that of bacterial PLFAs in rhizosphere sediment. The rhizosphere total biomass, bacteria, gram-positive bacteria, actinomycetes, and microbial diversity of *V. spiralis* were significantly higher than those of *H. verticillata* (Table 2).

Discussion

ROL from the roots of wetland and submerged plants, facilitated by extensive aerenchyma [3, 27], helps create oxidized rhizospheres. In the present study this is exemplified by the higher sub-surface Eh in the planted pots and the "oxidation ring" in Fig. 3a. Kludze et al. [18] showed that quantity of ROL to the rhizosphere was positively correlated with development of aerenchyma. The ability of ROL is strong when the aerenchyma is well developed [28]. The development of aerenchyma is considered the most important trait for a plant to tolerate sediment anaerobiosis [29]. In this experiment, *V. spiralis* had higher root porosity and inner oxygen than *H. verticillata* (Table 1). Similar results were also observed in the *in situ* measurement for the oxygen profile (Fig. 3a) by an oxygen microelectrode. Correspondingly, ROL of *V. spiralis* was greater than that of *H. verticillata*.

As oxygen is leaking from the roots, it is continuously consumed by microbial respiration and reoxidation of reduced compounds such as NH_4^+ and Fe^{2+} in sediment [30-32]. In this experiment, the rhizosphere of *H. verticillata* had higher NH_4^+ and Fe^{2+} than *V. spiralis*, which were both significantly lower than the control sediment (Fig. 4). *V. spiralis* with higher root ROL had higher Eh in rhizosphere sediment than *H. verticillata* (Fig. 3b). The Eh values of planted sediment presented were higher than those of control sediment. These results clearly suggested that root ROL oxidized rhizosphere sediments and made rhizosphere Eh increase. Correspondingly, NO_3^- of *H. verticillata* was
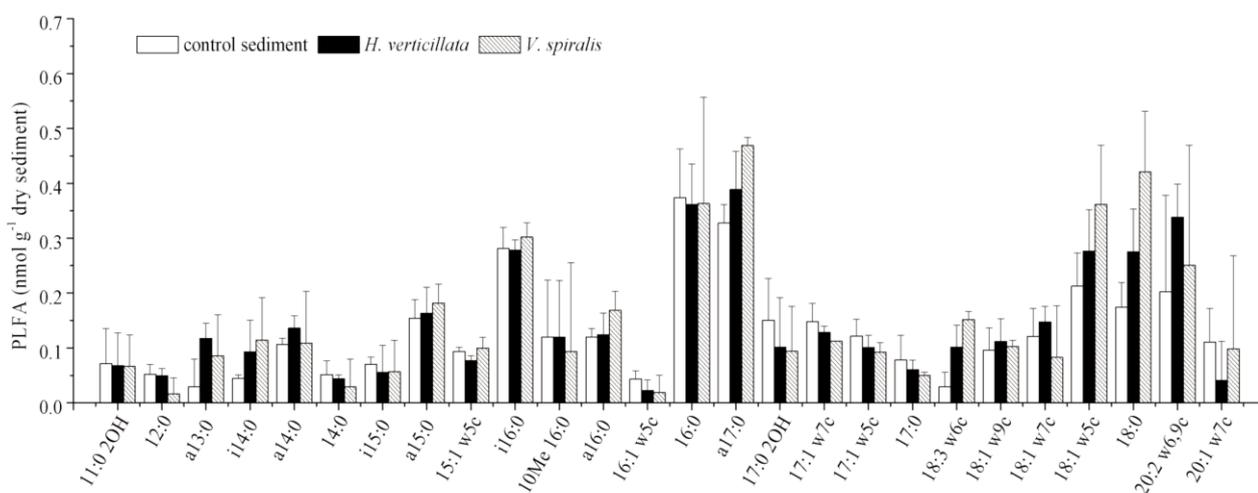

Fig. 5. Main composition of phospholipid fatty acids in control and *H. verticillata* and *V. spiralis* rhizosphere sediment.

lower than that of *V. spiralis*, which were both higher than the control sediment. The results suggested that root ROL simultaneously enhanced rhizosphere nitrification [9]. In addition to nitrification, denitrification is another process in the soil N cycle. Kirk and Kronzucker [33] once established a model to estimate the potential for nitrification, denitrification, and nitrate uptake in the rhizosphere of wetland plants. The model showed that substantial quantities of NO_3^- can be produced in the rhizosphere of wetland plants through nitrification. The model also showed that the rates of denitrification and subsequent loss of N from the soil remain small when NO_3^- production and uptake were considerable. The calculation showed that wetland plants took up a large part of their N as NO_3^- formed from NH_4^+ in the rhizosphere, without excessive losses of N through denitrification [9].

These processes are mainly affected by the microbial process. Root ROL oxidizes rhizosphere sediment and affects the microbial process. The total amount of PLFA serves as an estimate for sediment microbial biomass [34]. Rhizosphere sediment contained higher microbial biomass compared to the control sediment, which was likely due to greater availability of substrates for microbial growth [35, 36]. A variety of microbial PLFAs representative of total bacteria, gram-positive bacteria, gram-negative bacteria, fungi, and actinomycetes were identified from the sediment (Fig. 5). The proportion of fungal PLFAs was lower than that of bacterial PLFAs in all sediment samples (Table 2).

The plant species effect on microbial community structure in rhizospheres is due to differences in root ROL and root architecture [10, 35, 37]. While *V. spiralis* expected a higher amount of total PLFAs, total bacterial, gram-positive bacterial, and actinomycetes, PLFAs because *V. spiralis* presented a higher root biomass and ROL (Table 1) than *H. verticillata*. The rhizosphere microbial diversity of *V. spiralis* was significantly higher than that of *H. verticillata* (Table 2), suggesting that root ROL not only affected the population of rhizosphere microorganisms but also affected microbial community diversity [37, 38].

Conclusion

Using a combination of microelectrodes and PLFA analysis, root ROL and its effects on rhizosphere microarea were identified. The results showed that root and shoot biomass and root ROL increased with time. ROL of *V. spiralis* was significantly higher than that of *H. verticillata*. The order of sediment oxygen penetration depth was *V. spirails* > *H. verticillata* > control sediment, and the order of the oxygen concentrations detected in the water layer and rhizosphere sediment was the same. The results showed that plants make the rhizosphere NH_4^+ and Fe^{2+} decrease and NO_3^- increase. ROL enhanced rhizosphere microbial activity and affected the microbial composition. The contents of total biomass, total bacteria, gram-positive bacteria, gram-negative bacteria, fungi, actinomycetes, and diversity index in the rhizosphere of *H. verticillata* and *V. spiralis* were

higher than those in the control sediment. *V. spiralis* might be expected to create significantly higher amounts of total PLFAs, total bacterial, gram-positive bacterial, and actinomycete PLFAs because of higher root biomass. The present study highlights that plant species with different root ROLs can change physicochemical parameters and microbial communities of the rhizosphere microarea.

Acknowledgements

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