Methane oxidation enhancement of rice roots with stimulus to its shoots

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ABSTRACT

This study aimed to know whether the methane oxidation of rice roots was enhanced when rice shoots was stimulated. Stimulus was applied using a mechanical device which continuously touched the rice shoots with strength of 5, 30 and 60 min/day. In 5 min/day and 30 min/day stimuli treatments, methane oxidation rates of rice roots at 6 h were both significantly improved. Rice height was decreased significantly in 30 min/day stimuli treatment. Root biomass and root activities were significantly improved in 5 min/day and 30 min/day stimuli treatments. Length, surface area and volume of rice roots in 30 min/day stimuli treatment were improved significantly. Stomata areas were found to be significantly higher in 30 min/day and 60 min/day stimuli treatments. Continuous stimuli of 5 min/day and 30 min/day on rice shoots led to methane oxidation capacity enhancement of rice roots and response of rice at the physiological and morphological level. This study ascertained the importance of biological activity in paddy fields on the methane release process.

Keywords: *Oryza sativa* L.; morphological traits; roots activity; radial oxygen loss; stomata

Methane is the second largest contributor to global warming, which is mainly produced by diverse methanogenic bacteria during the anaerobic metabolism (Garcia et al. 2000, Itoh et al. 2011). Flooded paddy fields are one of the major sources of methane emission into the atmosphere. Generally, methane emission was greatly influenced by the balance between methane production and methane oxidation in paddy fields (Yang and Chang 2001), and the latter always occurred at the soil-water interface, the rice roots, and the rice rhizosphere (Bosse and Frenzel 1997). Like other wetlands plants, rice evolved aerenchyma to supply the atmospheric oxygen for respiration of roots by facilitating radial oxygen loss (ROL) (Armstrong 1967). In rhizosphere, methane emitted out from the soil was oxidized when it encountered the oxygen transferring from the aerenchyma of rice. In South China, methane emission of paddy fields applying rice-duck farming by raising ducks in

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paddy fields was found to be significantly lower than that of conventional paddy fields. As ducks running everywhere in paddy fields, water and surface soil were both disturbed and mudded by them. The dissolved oxygen content in water and surface soil was increased, and the redox potential state of soil was improved. As a result, methane emission from paddy fields was reduced to some extent (Huang et al. 2005, Zhan et al. 2011). Meanwhile, stem length, stem diameter and roots biomass of rice in such system were significantly changed (Wang et al. 2008). So the possible mechanism of methane reduction was related to the stimuli from ducks in grazing weeds and preying on insects. Now no studies were performed on the relationship between methane oxidation and rice under continuous stimuli. This study aimed to know the change of methane oxidation of rice roots under quantitative stimulus, and also to discover the mechanism related in this process by means of analyzing physiological response and methane oxidation preferences. As a result, this study would provide a new viewpoint to clarify the effect of biological activities on methane emission in paddy fields.

MATERIAL AND METHODS

Plant material and pot experiment conditions. Seeds of rice (*Oryza sativa* L.) cv. Huanghuazhan were first surface-sterilized with 0.01% potassium permanganate for 30 min, and then washed with purified water three times and soaked in purified water for 1 day in dark place. Seeds were grown on filter paper in a sterile petri dish in a growth chamber with 16 h light (27°C) and 8 h dark (25°C) for 6 days. Seedlings were then transferred into the soil in pot experiment in the growth chamber as above. The soil collected from paddy fields of South China Agricultural University Farm (23°14'N, 113°37'E) was air-dried and ground to pass a 5 mm sieve before used. Organic matter of soil (pH 6.35) was 20 g/kg. Nitrogen ($CO(NH_2)_2$, 0.325 g/kg), phosphate ($Ca(H_2PO_4)_2 \cdot H_2O$, 0.243 g/kg) and potassium (KCl, 0.241 g/kg) were added into the soil and mixed properly at 3 days before transplanting. Two seedlings were planted in a plastic cup (15 cm in height, 12 cm in diameter) containing 400 g soil covered with 2 cm surface water in depth. Seedlings were cultivated for 28 days before its use.

Treatments and mechanical stimuli device. Seedlings of 28 days with similar height were used. The stimuli strengths of 5, 30 and 60 min/day were selected, and the whole stimuli duration lasted 14 days. Untreated seedlings cultivated in the same procedure were used as the control. In previous studies, mechanical stimuli was defined as actions plants were possibly exposed to in natural environment such as blowing, touching, rubbing and flexing (Jaffe and Forbes 1993, Anten et al. 2005, 2010). In rice-duck farming system, it was inevitable that ducks touched the rice during their activities of grazing weeds and preying on insects, which belonged to mechanical stimulus. The shaker moved at a constant speed and was suitable to be used as a drive force to apply steady mechanical stimuli to plants. In order to apply mechanical stimuli to the shoots of seedlings, a mechanical stimuli device was designed. It was composed of shaker (KYC 100C Shaker, Shanghai Fuma Laboratory Equipment CO. Ltd., Shanghai, China), wood framework, and iron ring with cotton (Figure 1). The iron ring was constructed by two moveable semicircles with a connection pole.

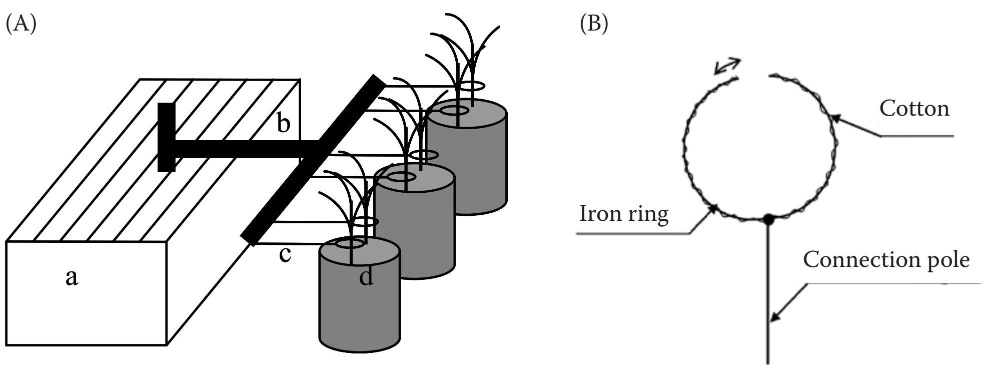

Figure 1. The mechanical stimulus device (A) used in the experiment; (a) shaker; (b) framework; (c) iron ring; (d) seedlings grown in soil culture and the iron ring with cotton (B)

The ring was open to let the seedlings in and then closed to loop it exactly. A framework was bound on the shaker. After the seedlings were closed in the ring, ring with cotton was driven by the shaker with the reciprocation motion at 60 rpm to continuously touch the shoots of the seedlings. Stimuli position of seedling was 2 cm distant from the surface water.

Determination of rice morphological traits. Height and stem diameter were determined using a plastic ruler and a caliper. Whole soil in plastic cup was put into purified water in a basin. Rice plant was obtained by gently shaking the basin to dissolve the soil adhered. Whole plant was then washed with purified water three times. After drying at 105°C for 2 h and 80°C for 24 h in dry oven, the dry weight (DW) of aboveground biomass and roots were determined. Root to aboveground biomass ratio was then calculated. Fresh roots was measured using a root analysis instrument WinRhizo-STD4800 (Regent Instruments Inc., Quebec, Canada) according to the method Zhang et al. (2009). Root activity was determined using the triphenyl tetrazolium chloride (TTC) method (Aibibu et al. 2010).

Determination of radial oxygen loss (ROL) from rice roots. Cleaned rice roots were immersed in the Hoagland's nutrient solution (40 mL) in a tube covered with black plastic films to determine ROL (Sasikala et al. 2009). Dissolved oxygen in solution was removed by aerating with N_2. A layer of paraffin oil was added on top of the solution. Basal part of shoot was wrapped with plastic film. A 5 mL aliquot of Ti^{3+} citrate solution (pH 5.6) was injected into the nutrient solution. The tube was then gently rocked. Blank treatment without plant was prepared similarly. The tubes were cultivated for 12 h in a growth chamber at 27°C. A 5 mL aliquot was extracted and measured at 527 nm on a spectrophotometer (UV-1750, Shimadzu, Japan) for the concentration of Ti^{3+}.

Determination of methane oxidation rate by rice roots. After the stimuli ended, the methane oxidation rates of rice roots were determined according to the method of Bosse and Frenzel (1997). Different parts involved in the determination were all sterilized at 121°C for 20 min or under the ultraviolet radiation. The widemouth bottle (250 mL) sealed with rubber stopper and vaseline was used as a container. A tube with 5 mL NaOH (1 mol/L) was placed on the bottom to adsorb the carbon dioxide. Fresh rice roots cleaned with sterilized purified water were placed in the widemouth bottle. Rubber stopper was equipped with gas injection inlet and a glass tube linked with a U-shape tube to supply the oxygen by facilitating the electrolysis of NaOH solution. The gas tight was confirmed by observing the level change in U-shape tube. Pure methane (1 mL) was injected into the bottle. After 10 min, the initial gas sample of 0.5 mL was drawn out from the bottle using the syringe. At 6, 12, 24, 36 and 48 h, gas sample was achieved following the same way. Methane oxidation rate (r) was calculated as:

$$r = \frac{(C_O - C_t)}{(W_t \times t)}$$

Where: r – methane oxidation rate; t – time duration (h); C_0 – initial concentration of methane (ppmV); C_t – concentration (ppmV) at the moment of t, and W_d – weight of dry roots (g). Methane was determined using gas chromatography Agilent 6890N equipped with column HP-PLOT/Q (Beijing, China).

Analysis of stomata of leaf sheath of rice under stimuli. After the stimuli ended, a small part of the sheath (0.5 cm width × 0.5 cm length) of rice randomly selected in different treatments and the control were cut at the position rightly rubbed by the ring. Sheath were put in 2.5% (w/v) glutaraldehyde and rinsed for 4 h. Then sheath was washed with phosphate buffer solution for three times. The sheath was then washed in 30, 50, 70, 90, 100% ethanol and methylbutyl acetate. Sheaths dried at critical point under vacuum were placed onto a copper platform prior to analysis using a Scanning Electron Microscopy (SEM, XL-30-ESEM, Philips, Amsterdam, the Netherlands). Stomata area was calculated from the width (μm) and length (μm) determined using the scale from the SEM.

Statistical analyses. Data were analyzed using the Excel 2003 (Seattle, USA) and SPSS 13.0 software (New York, USA). Results were expressed as $\bar{x}$ ± S.E. ANOVA was applied to analyze differences between treatments and the control.

RESULTS

Change of morphological traits of rice in treatments was compared in Table 1. Rice height in 30 min/day and 60 min/day stimuli treatment were significantly decreased compared to that in the control ($P < 0.05$). Rice height in 5 min/day stimuli treatment was not significantly different from the

Table 1. Morphological traits of rice in the control, 5, 30 and 60 min/day treatment

Trait	Control	5	30	60
Height (cm)	45.48 ± 1.10^{a}	44.05 ± 1.28^{ab}	40.50 ± 2.25^{bc}	37.33 ± 1.16^{c}
Stem diameter (cm)	0.36 ± 0.01^{b}	0.39 ± 0.01^{a}	0.40 ± 0.01^{a}	0.33 ± 0.02^{b}
Aboveground biomass (g)	0.43 ± 0.01^{a}	0.42 ± 0.02^{a}	0.39 ± 0.02^{a}	0.32 ± 0.01^{b}
Root biomass (g)	0.08 ± 0.01^{b}	0.11 ± 0.01^{a}	0.12 ± 0.01^{a}	0.09 ± 0.01^{b}
Root to aboveground biomass ratio	0.20 ± 0.01^{c}	0.25 ± 0.02^{b}	0.31 ± 0.01^{a}	0.28 ± 0.01^{ab}
Whole biomass (g)	0.51 ± 0.01^{a}	0.53 ± 0.03^{a}	0.51 ± 0.03^{a}	0.41 ± 0.01^{b}

Different letters indicate significant differences

control. Stem diameter of rice in 5 min/day and 30 min/day stimuli treatment were both significantly higher than those in the control (P < 0.01), while the stem diameter of rice in 60 min/day treatment was not significantly different from that of the control. Compared to the control, root biomass in 5 min/day and 30 min/day stimuli treatment were significantly improved (P < 0.01). Root biomass in 60 min/day treatment was not different from that in the control. Meanwhile, aboveground biomass was discovered to be not significantly different in 5 min/day and 30 min/day stimuli treatment but decreased in 60 min/day stimuli treatment (P < 0.01). Root to aboveground biomass ratio was significantly improved in the three stimuli treatments (P < 0.01). Whole-plant biomass of rice in 5 min/day and 30 min/day stimuli treatment was not significantly different from that of the control. However, the whole-plant biomass was decreased greatly in 60 min/day stimuli treatment (P < 0.01).

Morphological characteristics and activity of roots under different stimuli were explored (Table 2). Length, surface area and volume of rice roots in 30 min/day stimuli treatment were all improved significantly compared to those of the control (P < 0.01). In 60 min/day stimuli treatment, root length and surface area of rice were both decreased significantly in the process of stimuli compared to those in the control (P < 0.01), and the root volume of rice was similar to that in the control. No significant change of average diameters was found in all stimuli treatments compared to the control. When stimuli duration was 5 min/day, root activity was significantly higher than those in the control and in 60 min/day stimuli treatment (P < 0.01). Root activity of rice in 60 min/day treatment was significantly lower than that of the control (P < 0.01). Also, a difference of root activity between 5 min/day and 30 min/day stimuli treatments was not significant.

ROLs of rice roots of different stimuli durations were determined (Figure 2). 5 min/day and 30 min/day stimuli duration improved ROL of rice significantly (P < 0.05). Meanwhile, the ROL of rice in 30 min/day stimuli treatment was significantly higher than that of rice in 60 min/day stimuli treatment (P < 0.05). As the stimuli duration prolonged to be 60 min/day, ROL of rice was not significantly different from that of the control. Based on the SEM photos of sheath stomata, the areas of stomata were calculated (Figures 3 and 4). Stomata areas were significantly higher in the 30 min/day and 60 min/day stimuli treatment compared to the control (P < 0.01).

Table 2. Traits of rice roots in the control, 5, 30 and 60 min/day treatment

Trait	Control	5	30	60
Root length (cm)	503.70 ± 21.65^{b}	595.30 ± 41.77^{b}	719.41 ± 49.30^{a}	305.57 ± 28.42^{c}
Root surface area (cm^2)	83.79 ± 6.09^{b}	92.50 ± 4.94^{b}	125.05 ± 14.33^{a}	50.68 ± 5.29^{c}
Root volume (cm^3)	1.11 ± 0.11^{b}	1.15 ± 0.05^{b}	1.74 ± 0.29^{a}	0.67 ± 0.08^{b}
Average diameter (mm)	0.53 ± 0.02^{a}	0.50 ± 0.01^{a}	0.55 ± 0.02^{a}	0.53 ± 0.02^{a}
Root activity (μg/g/h)	141.78 ± 6.19^{b}	188.34 ± 4.06^{a}	170.75 ± 6.66^{a}	117.40 ± 11.98^{c}

Different letters indicate significant differences

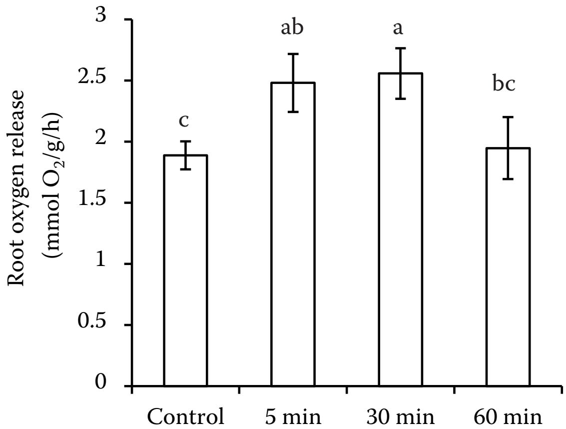

Figure 2. Root oxygen release of rice roots in the control, 5, 30 and 60 min/day treatment

Methane oxidation rates of roots under different stimuli were determined (Figure 5). At 6 h, the methane oxidation rates of rice roots in the treatment of 5 min/day and 30 min/day stimuli duration were significantly improved compared to the control (P < 0.01). However, the methane oxidation rate of rice roots in 60 min stimuli treatment was not significantly different from those of the control. At 12 h, although the methane oxidation rates in 5 min/day and 30 min/day stimuli treatments began to decrease, they were still higher than that of the control. The methane oxidation rates of rice roots in 60 min/day stimuli treatments were similar to those of the control. The methane oxidation rates of rice roots in 5 min/day and 30 min/day stimuli treatments continued to descend after 6 h. From 24 h to 48 h, the differences of methane oxidation in all treatments diminished quickly and no significant difference was observed at this period.

DISCUSSION

Stem elongation inhibition was thought to be one kind of classical syndromes in plant responses to stimuli including touching, rubbing and flexing (Coutand 2010). Mechanically-stressed tobacco plants produced shorter stem than control plants did (Anten et al. 2005). Here similar phenomenon of height lowering was observed. Rice height in 5 min/day treatment was not decreased significantly. Plant height lowering was related to threshold of stimuli intensity. Stem diameter in 30 min/day stimuli treatment was significantly enhanced.

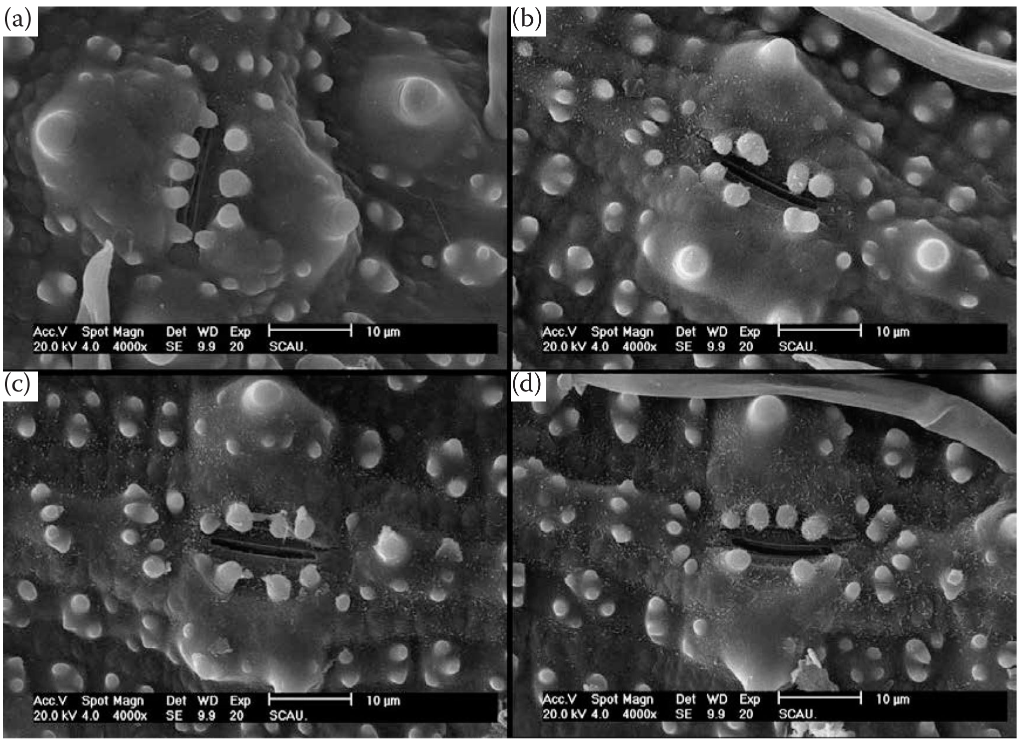

Figure 3. Scanning electron microscopy photos of stomata of rice leaf sheath in the control (a); 5 min/day (b); 30 min/day (c) and 60 min/day (d) treatment

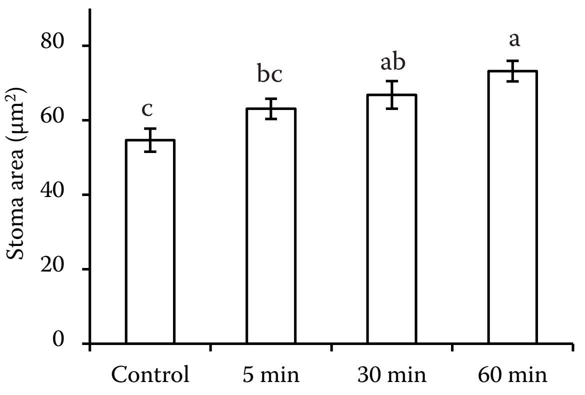

Figure 4. Stomata area of rice leaf sheath in the control, 5, 30 and 60 min/day treatment

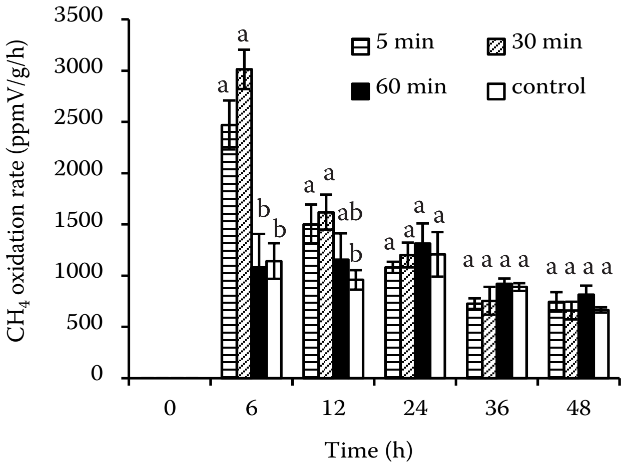

Figure 5. CH_4 oxidation rate of rice roots in the control, 5, 30 and 60 min/day treatment

Mechanical stress could induce the increase of stem diameter of plant, the diameter of trees swayed freely in the wind was found to be 36% greater at the stem base than the diameter of trees sheltered from the wind (Jacobs 1954). However, too much stimuli may have disturbed the process of matter accumulation on stem of rice in 60 min/day treatment and its stem diameter was not changed.

5 min/day and 30 min/day stimuli improved the roots activity significantly, implied that rice roots were in more metabolically active state than those in the control. Higher levels of indole glucosinolates were discovered in roots of plants exposed to foliar herbivory by *Pieris brassicae* L. (Soler et al. 2007). Stimuli on shoots of rice could be regarded as an environmental stress, enhancement of roots activity may be related with some signals production and response.

Short shoot length implied that the distance of the oxygen diffusion pathway was decreased, and higher oxygen could be sent to the root in same conditions. Height of rice in 30 min/day stimuli treatment was significantly lower, so ROL was improved. Higher root activity might result in more release of oxygen from plant roots (Kong et al. 2009). Here, root activities of rice in stimuli treatments of 5 min/day and 30 min/day were both improved, it brought the increase of ROL. Large stomata area could increase gas exchange on the sheath. However, it was also disadvantageous to the growth of plant by losing excessive water. So, the oxygen release from roots in 60 min/day stimuli treatment was not improved.

Methane oxidation capacity of the rice roots in 5 min/day and 30 min/day stimuli treatments was effectively increased. Firstly, root biomass, root surface area and root volume were all significantly improved. So the contact surface between roots and methane was enlarged, which was helpful to enhance the methane oxidation rates. Secondly, root activity of rice in 5 min/day and 30 min/day stimuli treatment was improved, which increased the oxidation capacity of roots. Excessive stimulus on aboveground rice was harmful to its growth. In 60 min/day stimuli treatment, the root surface area, root activity and oxygen release were all lower than those of the control but the root biomass. As the methane oxidation rates were similar to those of the control, it was very possible that methane oxidation capacity of rice roots was heavily influenced by biomass.

In conclusion, the continuous mechanical stimuli led to diverse responses of rice at the physiological and morphological level. Rice height was decreased significantly in 30 min/day stimuli treatments. Biomass, activities, ROL, methane oxidation rates (6 h) of rice roots were all significantly improved in 5 min/day and 30 min/day stimuli treatments. Length, surface area and volume of rice roots in 30 min/day stimuli treatment were all improved significantly. Stomata areas were significantly higher in 30 min/day and 60 min/day stimuli treatments than the control. As for the rice-duck farming system, this result confirmed the importance of the duck stimuli on rice and also supplied another favorable explanation to the methane emission reduction in the rice-duck farming system. Furthermore, this study emphasized the importance of animal activity such as raising duck and fish in paddy fields on the methane release process.

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