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Effects of Ultrasonic Stimulation on Resistance to Sheath Blight (*Rhizoctonia solani*) in Rice

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Plant pathogen can cause a series of defensive responses, such as changes of defense enzymes in plant. The effects of ultrasound on resistance to sheath blight in rice (Tetep and Wanhui 88) were examined in the present study. The results showed that ultrasound can increase germination capacity and the content of chlorophyll and activities of superoxide dismutase (SOD) and polyphenol oxidase (PPO), decrease disease index and the content of malondialdehyde (MDA) in response to *Rhizoctonia solani* in rice. It was concluded that ultrasound may enhance the resistance of rice against sheath blight through improving the activity of some defence related metabolisms.

Key Words: Ultrasound, Sheath blight, Rice, Germination capacity, Chlorophyll content, Defence enzymes.

INTRODUCTION

Rice sheath blight is one of the three major diseases in rice (*Oryza sativa* L.)[1]. Since 1980s, with the extensive planting of short-stalked varieties and the increase of fertilization level and planting density, the harm of rice sheath blight is becoming more and more serious. Rice sheath blight becomes a serious barrier of rice high-yield, stable yield and high quality.

Plant pathogen can cause a series of defensive responses in plant, such as the release of reactive oxygen species[2,3] (ROS) and variation in antioxidative enzyme activities[4-7]. In host-pathogen interactions, the formation and scavenging of reactive oxygen species are closely correlated with disease resistance in plants. The excessive production of reactive oxygen species attack membrane system in plant and cause the peroxidation of membrane lipid. Malondialdehyde (MDA) is an important indicator measured the peroxidation of membrane lipid. In plants, the scavenging of reactive oxygen species is correlated with defense enzymes, such as superoxide dismutase (SOD) and peroxidase (POD).

Recently, some studies showed that mechanical stimulation can induce plant system to acquire resistance. Wang *et al.*[8] investigated the resistance of cucumber to disease of *cladosporium cucumerinum via* mechanical stress loading. Results indicated that the appropriate mechanical stimulation could significantly improve plant resistance and alter the activity of resistance-related enzymes. Gus-Mayer *et al.*[9] use a needle, which has the same diameter as a fungal hypha, to stimulate the suspension cell of parsley by local mechanical stimulation. Results showed that plant cells can perceive mechanical signals and cause defense effect.

The appropriate ultrasonic stimulation could promote the growth and proliferation of *Oryza sativa* Nipponbare callus cells[10]. But the study on the effects of ultrasound on the resistance of rice to sheath blight has not been reported. The effects of ultrasound on the content of chlorophyll and malondialdehyde and the activity of superoxide dismutase, polyphenol oxidase (PPO) and peroxidase in the rice leaves were examined in the present study. Thereby, this paper explored the groundwork of physiology and biochemistry on the resistance of rice to sheath blight induced by ultrasound.

EXPERIMENTAL

The seeds of rice cultivar Tetep used in the experiments were provided by the Fuling Agricultural Science Research Institute in Chongqing of China. The seeds of rice cultivar Wanhui 88 used in the experiments were provided by the Chongqing Three-gorge Agricultural Science Research Institute in Chongqing of China. The rice (Tetep and Wanhui 88) seeds were disinfected for 10 min with 0.1 % mercuric chloride and rinsed with water and primed for 12 h in moisture at 28 ºC. Then ultrasound (Output power: 280 W, frequency: 25 kHz, ultrasound processing time: 8 min) stimulated rice seeds in water-tank of ultrasonic cleaner. The rice seeds were cultivated for 20 days in the light incubator at 28 ºC and the 20 day-old rice seedlings were transplanted to the big pots stuffed the prepared soil (Loess: vermiculite: nutrition soil = 3:2:1). The rice seedlings, according to distance between hills of 5 cm × 5 cm, was planted in the light 15000 lux of 12 h per day at 28 ºC.

Fungal material: The rice sheath blight pathogen (*Rhizoctonia solani* RH-9), used for inoculation, is a strong

TABLE-1
EFFECTS OF ULTRASONIC STIMULATION ON GERMINATION CAPACITY AND DISEASE INDEX IN RICE

Testing variety	Germination capacity		Disease index	
	U0R0	U1R0	U0R1	U1R1
Wanhui 88	77.33 ± 2.42	85.67 ± 4.27	32.00 ± 0.11	27.70 ± 0.28
Tetep	37.33 ± 1.75	41.56 ± 1.76	17.89 ± 0.24	15.03 ± 0.18

U0R0, no ultrasonic stimulation and non-inoculated with *R. solani*, U0R1, no ultrasonic stimulation but inoculated with *R. solani*, U1R0, ultrasonic stimulation but non-inoculated with *R. solani*, U1R1, ultrasonic stimulation and inoculated with *R. solani*

pathogenic strain provided by the Plant Protection and Pathology Department of Yangzhou University in Jiangsu of China. Small wooden toothpicks were divided into the length of 0.8 cm to 1.0 cm and placed at the bottom of petri-dish. The appropriate potato dextrose agar (PDA) sterilized was infunded to the sterilized petri-dish with small toothpicks. Before inoculation, the *R. solani* was activated on the potato dextrose agar culture medium in dark at 28 ºC for 4 d until plentiful brown spores appeared. The inoculation period is the spike formation stage of rice. The wooden toothpicks of 0.8 cm to 1.0 cm were inserted into the medial sheath of the second leaf and the third leaf inverted in rice. The leaves inoculated with *R. solani* were marked with a small red label.

Experimental design: The experimental plants were divided into 4 groups: no ultrasonic stimulation and non-inoculated with *R. solani* (U0R0), no ultrasonic stimulation but inoculated with *R. solani* (U0R1), ultrasonic stimulation but non-inoculated with *R. solani* (U1R0), ultrasonic stimulation and inoculated with *R. solani* (U1R1).

The indexes, which included the content of chlorophyll and malondialdehyde and the activity of superoxide dismutase, polyphenol oxidase and peroxidase in the marked leaves selected randomly of different rice cultivars (Tetep and Wanhui 88) and groups (U0R0, U0R1, U1R0 and U1R1), were measured respectively at 0 d before inoculation with *R. solani* and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 days after inoculation with *R. solani*. The test samples were obtained from at least five different plants. Each treatment took 3 samples. Each sample was analyzed three times.

Measurements: The germination capacity was measured on 15th day after ultrasonic treatment of rice seeds and sowing. Each treatment took six samples. Each sample, which included sowing fifty rice seeds was analyzed. The disease index was measured on 15th day after inoculation with *R. solani*.

The chlorophyll content of rice leaves were measured according to the method of ethanol extraction. In brief, the chlorophyll was extracted from 0.3 g of fresh weight (FW) tissue using ethanol (95 %). The extracts were measured spectrophotometrically at 665, 649 and 470 nm.

The lipid peroxidation level was determined in terms of the malondialdehyde content as described by Guo *et al.*[11]. The samples taken from 1.0 g of fresh weight tissue in rice leaves were homogenized in a mortar with 5.0 mL of 50 mM phosphate buffer (pH 7.0). The uniform homogenate was divided equally into two centrifuge tubes. Then the two tubes were immediately centrifuged at 15000 rpm for 25 min at 4 ºC. The supernatant was used for assay of the lipid peroxidation level. The malondialdehyde content (nmol g^{-1} FW) was obtained from the conversion of malondialdehyde concentration (μmol L^{-1}).

To extract peroxidase, polyphenol oxidase and superoxide dismutase enzymes[12], the samples taken from 1 g of fresh rice leaves were homogenized in a mortar with 3 mL of 50 mM cool phosphate buffer containing 1 % (w/v) polyvinylpyrrolidone (pH 7). The uniform homogenate was immediately centrifuged at 15000 rpm for 20 min at 4 ºC. The supernatant was used for assay of peroxidase, polyphenol oxidase and superoxide dismutase activities. The activities of peroxidase, polyphenol oxidase and superoxide dismutase were measured using the methods of Lee and Lin[13], Constabel and Ryan[14] and Spychalla and Desborough[15], respectively. 1 Unit (U) of peroxidase activity was defined as an absorbance change of 0.01 units per min. The peroxidase activity was represented for U mg^{-1} protein min^{-1}. 1 Unit (U) of polyphenol oxidase activity was defined as an absorbance change of 0.01 units per min. The polyphenol oxidase activity was represented for U mg^{-1} protein min^{-1}. 1 Unit (U) of superoxide dismutase activity was defined as the amount of enzyme that would inhibit 50 % of nitro blue tetrazolium (NBT) photoreduction. The superoxide dismutase activity was represented for U mg^{-1} protein.

Total protein content in enzyme extracts was determined according to the method of Bradford[16]. Protein content was calculated by comparison to a standard curve made with bovine serum albumin as a standard.

Statistical analysis: The results of the data are the means of three measurements. The significance of differences among means of treatments was tested by independent-sample t-test. All these statistical analyses were conducted on SPSS13.0 software (USA). The figures were produced using Origin7.5 software.

RESULTS AND DISCUSSION

Change of germination capacity and disease index: As seen from Table-1, the germination capacity in group U1R0 was higher obviously than that in U0R0 in rice (Tetep and Wanhui 88). However, the disease index in group U1R1 was lower than that in U0R1 in rice.

Change of chlorophyll content: As seen from Fig. 1, the content of chlorophyll in group U1R0 and U1R1 was higher on the whole respectively than that in U0R0 and U0R1 in the measurement period in rice (Tetep and Wanhui 88). With the persistence of inoculation time, the content of chlorophyll in U0R1 and U1R1 showed the trend of decrease. The results indicated that ultrasound can increase the content of chlorophyll and enhance the level of photosynthesis. After infected by pathogen, the tissue or cells were damaged. Thereby, the content of chlorophyll showed the trend of decrease.

Change of malondialdehyde content: Malondialdehyde, often as lipid peroxidation indicators, is the final product of

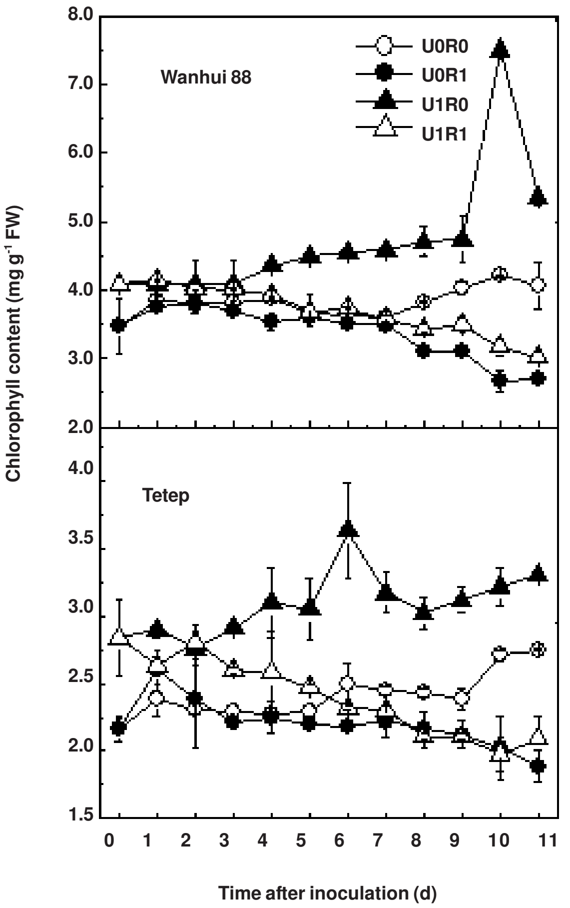

Fig. 1. Effects of ultrasonic stimulation and inoculation with *R. solani* on chlorophyll content in rice leaves

lipid peroxidation in plant cell. As seen from Fig. 2, the content of malondialdehyde in U0R1 and U1R1 was significantly higher than that in U0R0 and U1R0 in the measurement period excepted for 11 d in rice (Tetep and Wanhui 88) ($P < 0.05$). After inoculation with *R. solani*, the change of malondialdehyde content in U1R1 and U0R1 rose rapidly and then declined gradually. The content of malondialdehyde in Tetep and Wanhui 88 reached its peak respectively on 5th day (61.53 ± 1.45) and the 4th day (66.91 ± 0.11) after inoculation with *R. solani* in U1R1. But the content of malondialdehyde in Tetep (65.09 ± 1.06) and Wanhui 88 (72.78 ± 0.11) reached its peak in the 4th day after inoculation with *R. solani* in U0R1. In general, the content of malondialdehyde in U1R1 was slightly lower than that in U0R1. But in the peak, the content of malondialdehyde in U1R1 was significantly lower than that in U0R1 ($P < 0.05$).

Change of superoxide dismutase activity: superoxide dismutase is a key enzyme in the first metabolic step of superoxide elimination. As seen from Fig. 3, the activity of superoxide dismutase in U0R1 and U1R1 was significantly higher than that in U0R0 and U1R0 in the measurement period in rice (Tetep and Wanhui 88) ($P < 0.05$). After inoculation with *R. solani*, the activity of superoxide dismutase in U1R1 rose rapidly and reached the first peak on 1st day and maintained the peak on 2nd day and declined rapidly on 3rd day and rose rapidly and reached the second peak on 4rth day and then declined gradually in Tetep and Wanhui 88. The activity of superoxide dismutase in U0R1 reached two peaks respectively on 1st day and on 5th day after inoculation with *R. solani* in Tetep and Wanhui 88. The superoxide dismutase activity in group U1R1 was higher on the whole than that in U0R1 in rice (Tetep and Wanhui 88).

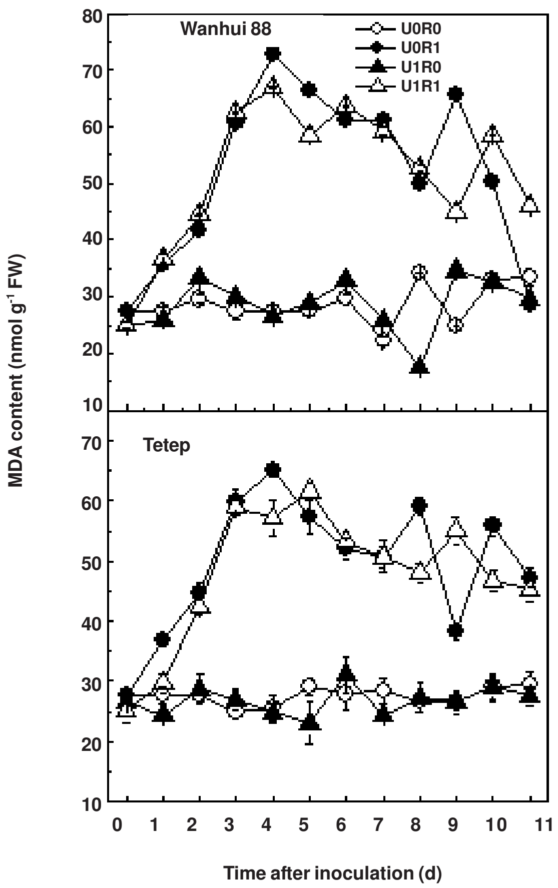

Fig. 2. Effects of ultrasonic stimulation and inoculation with *R. solani* on malondialdehyde content in rice leaves

Changes of peroxidase and polyphenol oxidase activities: peroxidase activity is strongly associated with disease resistance in plants. As seen from Fig. 4-A, the activity of peroxidase in U0R1 and U1R1 was significantly higher than that in U0R0 and U1R0 in the measurement period excepted for 11 d in rice (Tetep and Wanhui 88) ($P < 0.05$). After inoculation with *R. solani*, the change of peroxidase activity in U1R1 and U0R1 rose rapidly and then declined gradually. The activity of peroxidase in U0R1 and U1R1 reached its peak on 4th day after inoculation with *R. solani* in Tetep and Wanhui 88. The difference on the activity of peroxidase in U1R1 and U0R1 was generally not significant after inoculation with *R. solani* in Tetep. After inoculation with *R. solani* in Wanhui 88, the activity of peroxidase in U1R1 was significantly higher than that in U0R1 from the 1st day to 8th day ($P < 0.05$). It is indicated that the effects of ultrasound on the peroxidase activity of Tetep and Wanhui 88 are different.

Polyphenol oxidase is a terminal oxidase closely related to disease resistance in plant. After inoculation with *R. solani*, the change of polyphenol oxidase activity in U1R1 and U0R1 rose rapidly and then declined gradually. The activity of

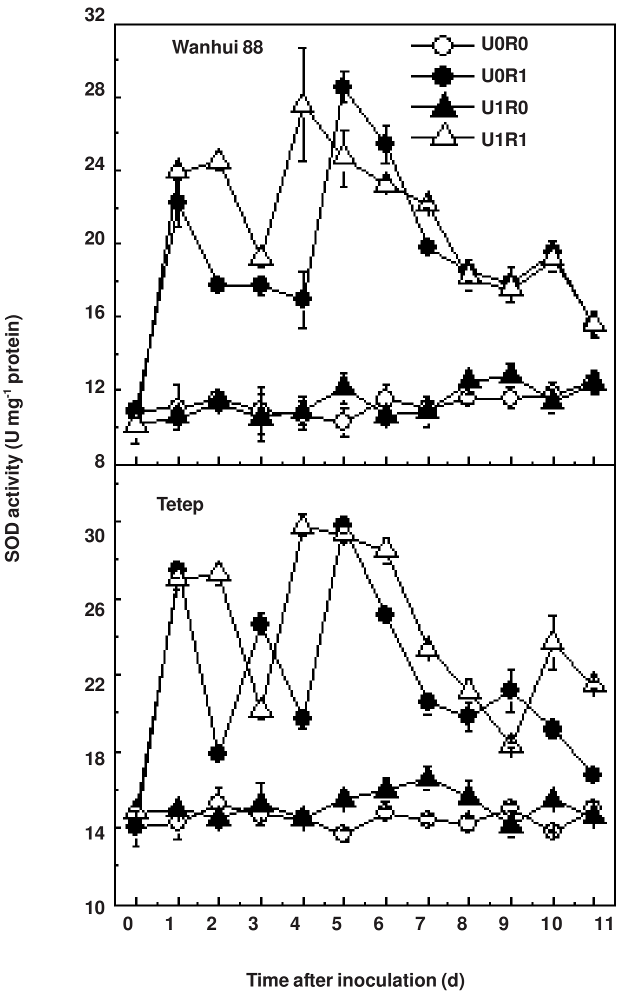

Fig. 3. Effects of ultrasonic stimulation and inoculation with *R. solani* on the activity of superoxide dismutase (SOD) in rice

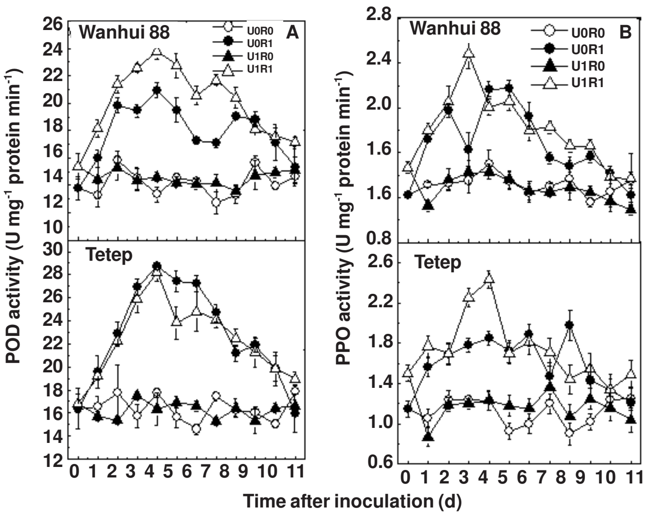

Fig. 4. Effects of ultrasonic stimulation and inoculation with *R. solani* on the activities of peroxidase (POD) and polyphenol oxidase (PPO) in rice

polyphenol oxidase in U1R1 reached its peak on 4th day (2.43 ± 0.09) after inoculation with *R. solani* in Tetep. The activity of polyphenol oxidase in U0R1 reached its peak on 8th day (1.98 ± 0.15) after inoculation with *R. solani* in Tetep. On 3rd day after inoculation with *R. solani* in Wanhui 88, the activity of polyphenol oxidase in U1R1 (2.48 ± 0.13) reached its peak and was significantly higher than in U0R1 (1.63 ± 0.15) ($P < 0.05$). The activity of polyphenol oxidase in U0R1 reached its peak on 5th day (2.18 ± 0.07) after inoculation with *R. solani* in Wanhui 88 (Fig. 4B).

The effects of ultrasound on the resistance of rice to sheath blight were examined in the present study. As seen from Table -1, the ultrasonic stimulation could increase germination capacity and degrade disease in rice.

Chlorophyll is the main photosynthetic pigment in normal plant chloroplasts. The level of chlorophyll content will affect the level of plant photosynthesis and eventually the growth of plant. The test results showed that ultrasonic stimulation can increase the content of chlorophyll in rice (Tetep and Wanhui 88). The increase of chlorophyll content demonstrated that the damage of plant infected by pathogen was degraded and the resistance of rice to sheath blight was increased indirectly.

Various studies have proposed that the formation of reactive oxygen species should be an important early response in plant resistance[16,17]. The rapid release of reactive oxygen species is one of the fastest reactions for early identification of pathogen invasion and the start of defense mechanism in plant infected by pathogen. The excessive production of reactive oxygen species or the decrease of defense-enzyme role could cause the accumulation of reactive oxygen species and lipid peroxidation to form malondialdehyde. The test results showed that ultrasound can decrease the content of malondialdehyde. It is indicated that ultrasound can lighten the level of lipid peroxidation.

With the invasion of pathogens, the plant *in vivo* could cause a series of physiological and biochemical reactions. The studies on the resistance of rice against sheath blight have shown that the physiological and biochemical reactions were mainly reflected in the change of some resistance-related enzymes activities. The test results showed that ultrasound can improve the sensitivities of superoxide dismutase and polyphenol oxidase in response to *R. solani* in rice (Tetep and Wanhui 88). It is indicated that ultrasound can easily activate the signals of superoxide dismutase and polyphenol oxidase in rice leaves after invasion of pathogens and the rapidly increased enzymes enhanced the disease resistance of rice. There was differential effect of ultrasound on the activity of peroxidase in the two tested rice cultivars (Tetep and Wanhui 88) in response to *R. solani*. It was concluded that the effects of ultrasound on the activity of some defence-related metabolisms in plant are different.

In conclusion, ultrasound may enhance the resistance of rice against sheath blight through improving the activity of some defense related metabolisms. Zhou *et al.*[6] have studied the effect of mechanical stress on the elongation behaviour of immobilized plant cells. The result showed that the elongation direction of immobilized plant cells was still vertical with the direction of original stress after the abolition of stress outside. It is implicated that there was the function of memory in plant cells. Therefore, we speculated that the rice seeds were stimulated by ultrasound and then the plant cells may retain the signal of ultrasonic stimulation because of the memory of plant cells in the whole process of plant growth. The signal of ultrasonic stimulation may participate the metabolic process on the interactions of host-pathogen during the invasion of pathogens. Through a series of physiological and biochemical reactions and signal transduction, the signal of ultrasonic stimulation may activate the host defense gene and induce plant system to acquire resistance. Thereby, the disease resistance of host plant is enhanced.

The effects of ultrasound on the contents of chlorophyll and malondialdehyde and the activities of superoxide dismutase, polyphenol oxidase and peroxidase in response to *R. solani* in rice (Tetep and Wanhui 88) were examined in the present study. This established groundwork for the study of resistant mechanism on rice against sheath blight in physiology and biochemistry. The effects of ultrasound on resistance in different crop may vary. So it is necessary to further study their disease-resistant mechanism for fully understanding. Further research is needed to verify the molecular level, such as the detection of several enzymes above in molecular level. It is helpful to reveal the resistant mechanism on rice against sheath blight.

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