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Physiological and molecular features of allelopathic and non-allelopathic rice in response to weed competition

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ABSTRACT

To determine the physiological costs of rice against weeds, a field study was done to evaluate the physiological and molecular traits of allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE). Results showed that the allelopathic rice variety caused >70 % inhibition in paddy weeds than non-allelopathic rice variety. The protective enzymatic activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), was higher in the roots and leaves of PI compared with LE, at the 3-, 5-, and 7-leaf stages, both with and without weed-removal treatment. The photosynthetic indices of PI leaves were significantly higher than those of LE, in the non-weeded treatment. Levels of ribulose-1,5-bisphosphate carboxylase, phosphoenolpyruvate carboxylase and glycolate oxidase were higher and gene expression was up-regulated in the leaves of both PI and LE, when grown in non-weeded field plots compared with weed-removal treatment. At the same leaf stages, the enzyme levels and gene expression were significantly higher in PI leaves compared with LE leaves. Similarly, at the same growth stages expression of six genes related to nutrients (N, P and K) absorption and utilization was higher in PI than in LE in both roots and leaves. The heat values of leaves, shoots and roots at all three leaf stages were significantly higher in PI compared with LE. These results clarified the question about physiological costs of rice against paddy weeds, suggested that under weeds stress, the allelopathic rice PI have higher nutrients absorption/utilization and photosynthetic efficiency by up-regulating those relative genes to obtain greater organic substances.

Key words: Allelopathic rice, allelopathy, CAT, enzymes, inhibition, molecular response, non-allelopathic rice, *Oryza sativa* L., physiological trait, PDO, rice, SOD, weed competition,

INTRODUCTION

Allelopathy is defined as “any process involving secondary metabolites produced by plants, algae, bacteria, or fungi that influences the growth and development of biological and agricultural systems” (1). Putnam and Duke (19) suggested that incorporation of an allelopathic characteristic into a rice cultivar could improve its competitive ability against paddy weeds. Rice allelopathy was first observed by Dilday *et al*. (2), who found that in a field test, 3–5% of the selected rice accessions had > 10 cm radius of allelopathic activity compared with a non-allelopathic rice accession. Allelopathy has important agronomic application as an alternative to synthetic herbicides for weed control in rice production (13). Allelopathic activity in rice is an inheritable trait (10) and the degree of allelopathic activity can also be influenced by stresses (nutritional deficiency, water shortage, UV irradiation, and the density of neighboring plants) (3,8,12,27). Most of

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the rice allelochemicals reported previously are plant secondary metabolites, such as phenolic acids, terpenoids, flavonoids, and steroids (4,9,11,15,21,22). The enzymes are also involved in rice allelopathy, including phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, ferulic acid 5-hydroxylase, caffeic acid *O*-methyltransferases, and diterpene cyclase (9,23,24,29).

Allelopathic rice plants exhibits the weed suppression activity at 3- to 7-leaf stages with maximum allelopathic potential at 5- to 6-leaf stages (7,14) and the allelopathic characteristic disappears at maturity (25,31,32). Grain yields of rice are significantly decreased, if not weeded during the early stages of growth (26). Most studies have focused on the secondary metabolism of allelopathic rice accessions. hence, little is known about the potential differences in primary metabolism (physiological and nutrients metabolism) between allelopathic and non-allelopathic plants (10,17). However, Olofsdotter (16) addressed a question that the physiological cost of the naturally occurring weed-defense mechanisms in allelopathic plants needs to be confirmed, because the synthesis of allelochemicals might require energy and organic resources that would otherwise be used for grain production. Furthermore, investigation of allelopathic traits in rice accessions have been conducted primarily in laboratory and glasshouse conditions, whereas such experiments need to be conducted under field conditions. In this paper, an allelopathic rice accession and a non-allelopathic rice accession were planted in field plots under weeded and non-weeded conditions, to compare the physiological and photosynthetic indices and enzymes related to photosynthesis and nutrients absorption and utilization. This paper aimed to verify the physiological costs of rice against weeds in rice crop field.

MATERIALS AND METHODS

A highly allelopathic rice accession PI312777 (PI) and a non-allelopathic rice accession Lemont (LE), identified previously (3), were used as test accessions. Experiments were conducted in summer 2013 in the Research Farm, Agroecological Institute, Fujian Agriculture and Forestry University, Fuzhou, China. The paddy soil of the test fields was sandy loam, pH : 5.4 and available nitrogen, phosphorus, and potassium contents were 92.4, 37.9 and 36.8 mg·kg^{-1}. The night and daytime temperatures ranged from 22 to 38 °C during the experimental period.

Experimental design

The test field was divided into 15 plots (each measuring 2 m × 2 m), and furrows (50 cms wide × 50 cms deep) were dug around each plot. Six plots were planted with PI rice seedlings, 6-plots with LE rice seedlings and 3-control plots (without rice), to determine the inhibitory effects of each rice accession (each treatment or control) on the paddy weeds. The treatments were replicated thrice in randomized block design. Six rice seedlings were transplanted in each row, thus per plot (7 rows and 9 lines) had 63 seedlings. The plant to plant distance was 16 cm and rows were 20 cms apart (Fig. 1). Three plots of PI and 3 of LE, were hand-weeded daily and served as controls to compare the gene expression of each accession under weed suppression (i.e. non-competitive conditions). Plots were irrigated regularly to maintain soil moisture. When rice seedlings reached the 3-, 5-, and 7-leaf stages, five rice plants were sampled from each plot, as per 'the five diagonal point sampling method' (Fig. 2). Each treatment and control had three biological replications for analyses of enzyme activity, gene expression, and heat value. In rice seedlings at 7-leaf stage the aboveground parts of paddy weeds in each plot, without weeding and without rice seedlings, was cut and oven-dried at 80 °C for 48 h. Thereafter weeds dry weight (g per m^2) was determined.

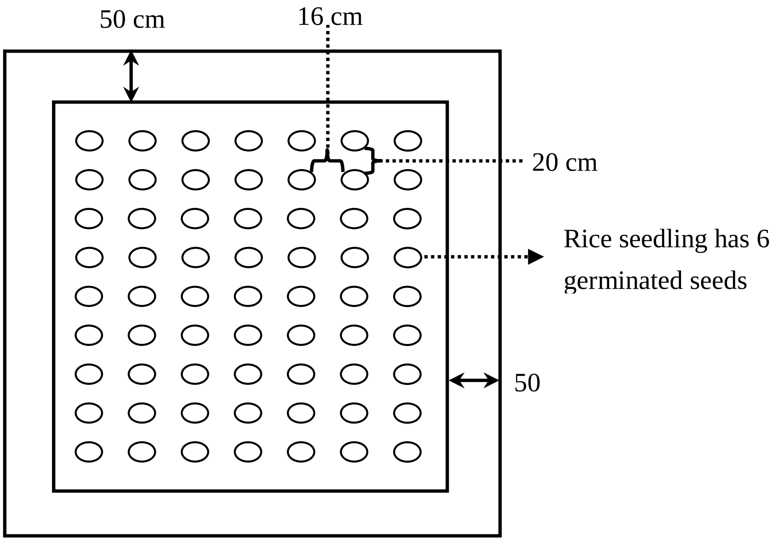

Figure 1. Experimental design of rice culture in test fields

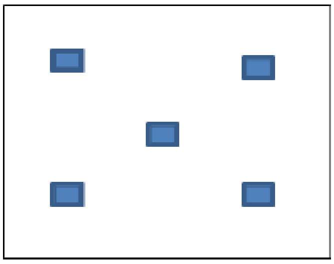

Figure 2. Five Diagonal Points Sampling Method

Protective enzyme activity of rice leaves and roots

The second internode leaf (functional leaf) and healthy roots of rice seedlings were analyzed for enzymatic activity as per Ye *et al.* (30). The enzyme activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were determined as described in Book *Principles and Techniques of Plant Physiological Biochemical Experiment* (28). All measurements were done in triplicate.

Photosynthetic indices of rice leaves

The second internode leaf was also used to determine the photosynthetic indices as per Ye *et al.* (30). Net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were measured using a LI-6400XT Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). Chlorophyll content was measured using a SPAD-502 PLUS (Konica Minolta Camera Co. Ltd., Japan). Five leaves per experimental plot were measured at 9–11 a.m. on a sunny day.

Expression of genes for photosynthesis and nutrition in rice accessions

The second internode leaf and the root tip of 1.0 cm were used to determine the gene expression. Expression of genes related to photosynthesis and nutrition in two rice accessions were analyzed by a quantitative real-time polymerase chain reaction (*q*RT-PCR) as described previously (9). The gene-specific primers were designed referencing to NCBI database, as shown in Table 1. Reactions with 10-folds serial dilutions of templates were used to calculate the PCR efficiencies and the R^2 values as described previously (18). The relative quantification (ratio) of each target gene was calculated using the following formula: Ratio = $2^{-\Delta\Delta Ct}$ (5). Three biological replicates were analyzed for each experimental plot, and six replicate PCR reactions were done for each biological sample. The results were analyzed using Opticon Monitor 3 software.

Table 1. The *q*RT-PCR primer sequences of genes relate to photosynthesis and nutrition of rice (*Oryza sativa*).

Gene	Primer sequence (5′-3′)	Accession number
Actin	F-TGTAAGCAACTGGGATGA R-CCTTCGTAGATTGGGACT	NM_197297
Genes related to photosynthesis		
Ribulose-1,5-bisphosphate carboxylase	F-CAGCAATGGCGGAAGGAT R-CCGATGATACGGATAAAGG	D00644
Phosphoenolpyruvate carboxylase	F-AGCAAGTCCTGAGGCACC R-CACAGCAGCACTCCCATT	Ab234234
Glycolate oxidase	F-CATAATGATTGCTCCCAGTG R-GTTCTTGATGTCAGTTTCCCT	Af022740
Genes related to nutrition		
Nitrate reductase	F-GGAGAAGCCCACCAAG R-CCCCATGAGATTCCAGAT	AK121810
Glutamate synthase	F-TCACGAATAAAAGAAAGGC R-GACACGTCCATGCTCATC	AJ441304
Nitrate transporter protein	F-GCACGGTGGCGATGAAA R-GGAGATGGTGGTGAAGGAC	AB008519
Ammonium transporter protein	F-GTGGTGCTCGTCCATTTCC R-CGTATTGCTGGTCTCGGTC	AF289478
Phosphate transporter protein	F-TGGTTCCTCCTTGACATCG R-GCCCATCGCCAGCAT	AF356960
Potassium transporter protein	F-AGGGAGCAGCAGCAAGA R-AGGGAGTAGAGGGCGAAGGT	AJ427972

Heat values of rice tissues

Combustion heat is the total energy released as heat, when a substance undergoes complete combustion with oxygen under standard conditions. Heat value can therefore be used as a quantitative index for assessing the total organic substances of rice plants. The heat value of rice tissues was measured according to the National Standards of People's Republic of China method (6) in an oxygen bomb calorimeter (WZR21T, Shanghai Shuoguang Science & Technology Ltd, Co. Shanghai, China). In brief, the leaves, shoots, and roots of rice seedlings at the 3-, 5-, and 7-leaf stage were separated and dried at 80 °C for 48 h, then ground and sieved through 25 mm mesh sieve. From each sample, 0.3-g powder sample was used to test the heat of combustion. This determination was done in triplicate for each sample.

Statistical analysis of data

All experimental data are presented as means ± SE (standard error), and subjected to one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) at $p < 0.05$. The statistical analysis was performed using the SPSS 20.0 program.

RESULTS AND DISCUSSION

Paddy weeds biomass

Rice allelopathy may confer competitive advantage on paddy weeds, reducing the need for synthetic herbicides. However, allelopathy may have an energetic cost, as these secondary metabolites are ultimately biosynthesized from primary metabolites (16). Primary metabolism, which begins with carbon dioxide and photosynthesis, is fundamental and vital for living organisms. Relatively few papers have explored the potential physiological costs associated with production of allelochemicals in allelopathic rice. Qiu *et al*. (20) reported that in a hydroponic system under nitrogen stress, allelopathic rice PI had a higher ability to utilize nitrogen compared with non-allelopathic LE. Similar results were observed when PI was grown under low phosphorus levels in a hydroponic system (27). In these field tests, the PI significantly


Allelopathic rice 'PI312777' Non-allelopathic rice 'Lemont'

Figure 3. Paddy weeds in field plots grown with allelopathic rice PI312777 (PI) and non-allelopathic rice.

inhibited the paddy weeds in test fields, compared with LE test fields (Fig. 3). Dry weight of the aboveground parts of the paddy weeds was 85.82 % lower in allelopathic rice (PI) plots and 14.49 % lower in non-allelopathic rice (LE) plots compared to control (Fig. 4).

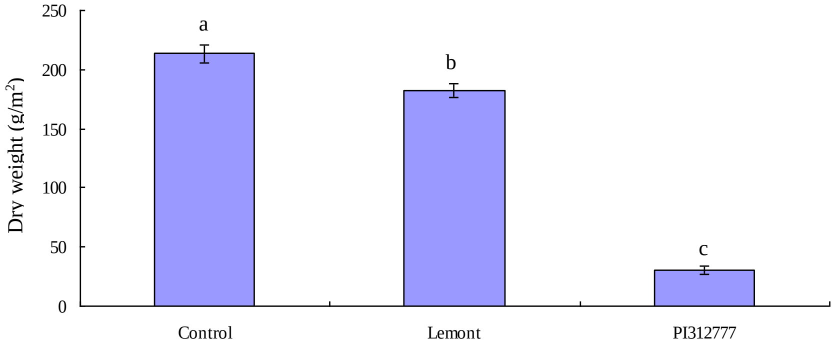

Figure 4. Dry-weight of paddy weeds in field plots grown with allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE). The bars represent the standard errors of the mean (n=3). Different letters indicate significant differences at $P < 0.05$.

Activity of protective enzymes

For both allelopathic (PI) and non-allelopathic (LE) rice, SOD, POD, and CAT activity in roots at the 3-leaf stage was higher in the non-weeded treatment compared with the weed-removal treatment (Fig. 5). This difference was statistically significant in all comparisons except for SOD in the PI roots (Fig. 5). Enzyme activity was significantly higher in the non-weeded treatments than in weed-removal treatments, except for SOD and CAT in the LE roots at the 5-leaf stage. At the 7-leaf stage, only SOD and POD activity in PI roots were significantly differed between the non-weeded and weed-removal treatments. In general, the activity of these protective enzymes was higher in PI than in LE roots, regardless of weed treatment, this difference was significant in all comparisons except in SOD and POD at the 3-leaf stage in weed-removal treatment (Fig. 5).

For both allelopathic (PI) and non-allelopathic (LE) rice, SOD, POD, and CAT activity in rice leaves at the 3-, 5-, and 7-leaf stages was significantly higher in the non-weeded treatments than in weed-removal treatments, except for SOD activity at 3-leaf stage for PI and 5-leaf stage for LE, and CAT activity for PI and POD activity for LE at 7-leaf stage (Fig. 6). In general, the activity of these enzymes was significantly higher in PI than in LE leaves, except the SOD at the 3-leaf stage in the weed-removal treatment and at the 5-leaf stage in the non-weeded treatment.

Photosynthetic indices

The photosynthesis indices of PI leaves were significantly higher in non-weeded treatment than in weed-removal treatment, except the transpiration rate at 5-leaf stage and chlorophyll content at 3- and 7-leaf stages (Fig. 7). In contrast, these indices in LE

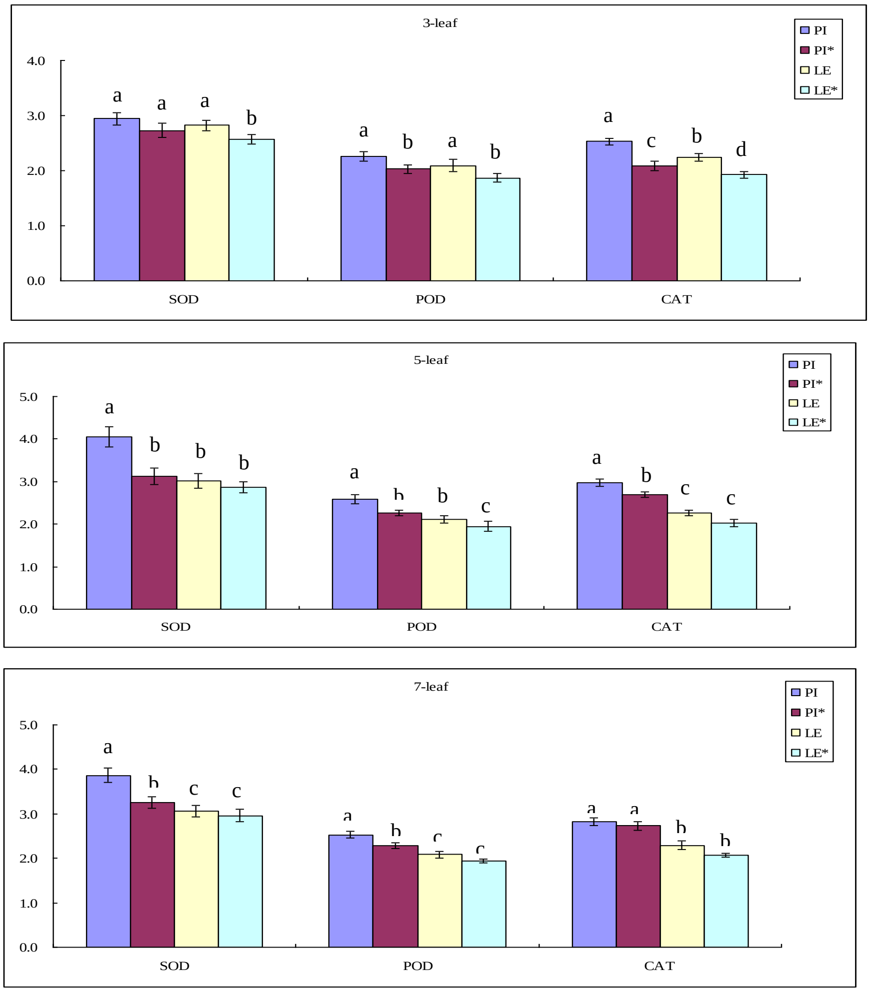

Figure 5. Protective enzymes in allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE) roots at different growth stages. The bars represent the standard errors of the mean (n=3). Different letters indicate significant differences at $P < 0.05$. Superoxide dismutase (SOD) is measured in unit·mg^{-1} pro; peroxidase (POD) is given as ΔOD470 min^{-1}·mg^{-1} pro; and catalase (CAT) is measured in mg (H_2O_2)·min^{-1}·mg^{-1} pro. The * indicates the weed-removal treatment.

leaves were significantly lower in non-weeded treatment than in weed-removal treatment, except the stomatal conductance at 7-leaf stage, intercellular CO_2 concentration at 5-leaf stage, and the transpiration rate and chlorophyll content at 3-, 5-, and 7-leaf stages. In general, photosynthesis indices did not vary significantly between PI and LE in the weed removal treatment (Fig. 7).

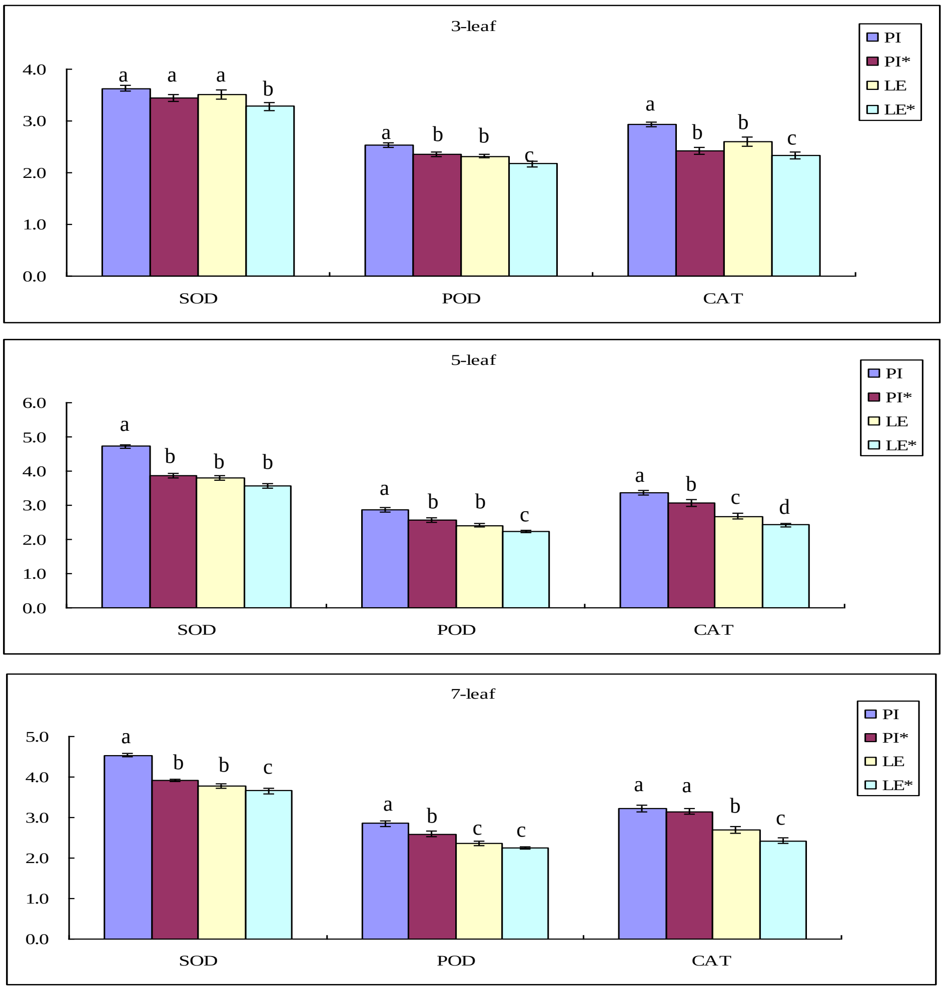

Figure 6. Protective enzymes in allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE) leaves at different growth stages. The bars represent the standard errors of the mean (n=3). Different letters indicate significant differences at $P < 0.05$. Superoxide dismutase (SOD) is measured in unit·mg^{-1} pro; peroxidase (POD) is given as ΔOD470 min^{-1}·mg^{-1} pro; and catalase (CAT) is measured in mg (H_2O_2)·min^{-1}·mg^{-1} pro. The * indicates the weed-removal treatment.

Photosynthesis and nutrition genes in allelopathic and non-allelopathic rice

Gene expression of three genes responsible for key photosynthesis enzymes ribulose-1,5-bisphosphate carboxylase, phosphoenolpyruvate carboxylase, and glycolate oxidase was analyzed using *q*RT-PCR. Gene expression of all three genes was up-regulated in both the PI and LE leaves at the 3-, 5-, and 7-leaf stage in the non-weeded treatment, than in weed-removal treatment (Table 2). The degrees of up-regulation were significantly higher (1.29 to 1.59 times) in PI leaves compared to LE leaves at the same growth stages. Under competitive conditions (non-weeded treatment), the photosynthetic capacity of PI leaves was significantly greater than LE leaves.

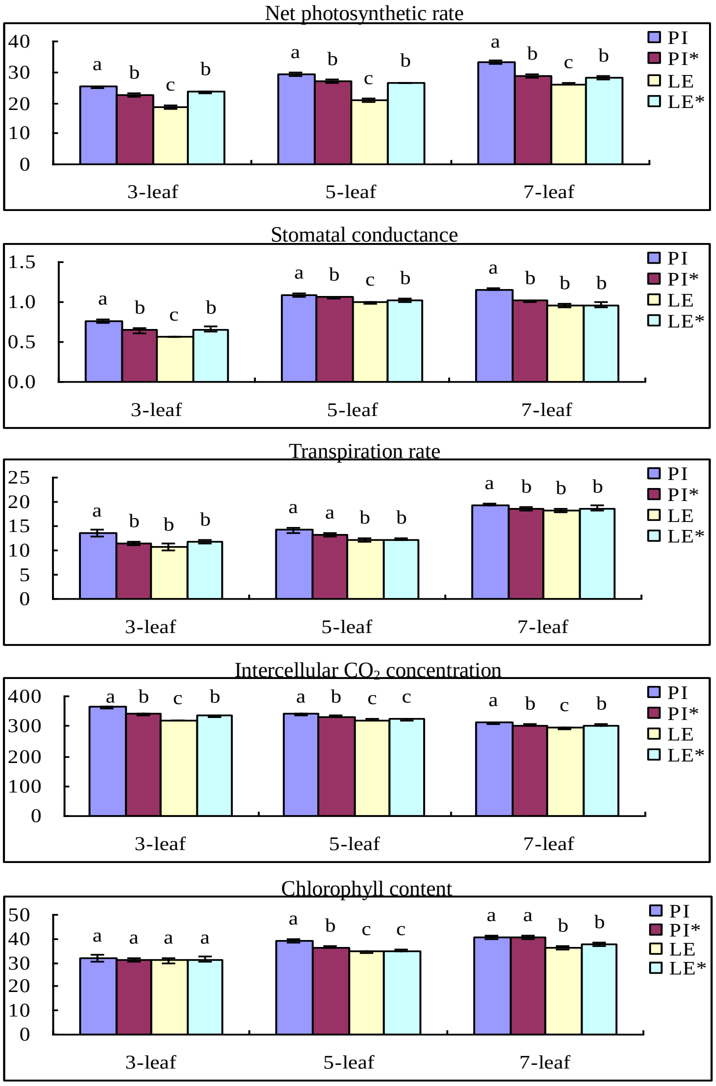

Figure 7. Photosynthetic indices of allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE) leaves at different growth stages. The bars represent the standard errors of the mean (n=5). Different letters indicate significant differences at $p < 0.05$. The * indicates the weed-removal treatment. Net photosynthetic rate (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$); Stomatal conductivity (mol $H_2O \cdot m^{-2} \cdot s^{-1}$), Intercellular CO_2 concentration (μmol $CO_2 \cdot mol^{-1}$), Transpiration rate (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$); Chlorophyll content (SPAD value).

Expression of six genes related to nutrients (N, P and K) absorption and utilization was up-regulated in both the roots and leaves of both rice accessions at the 3-, 5-, and 7-leaf stages in the non-weeded treatment than in weed-removal treatment (Table 3). As with the photosynthesis-related genes, the degrees of up-regulation of genes related to nutrition were significantly higher in PI compared with LE at the same growth stages. Gene expression was 1.41 to 2.15 times higher in the PI leaves compared with LE leaves, and 1.39 to 1.93 times higher in PI roots compared with LE roots. Under competitive conditions (non-weeded treatment), expression of genes related to nutrients absorption and utilization was greater in allelopathic PI relative to non-allelopathic LE.

In addition, the genes related to photosynthesis and nutrient absorption and utilization were up-regulated to a greater extent in PI compared with LE when plants grown in competition with weeds (Table 2 and Table 3), indicating that the conversion of photo-energy and nutrient absorption and utilization work more efficiently in allelopathic rice PI than that in non-allelopathic rice LE, especially when they are under weeds stress. Although the production of allelochemicals might require extra materials and energy, the high photosynthetic capacity, high synthesis of primary metabolites and high heat values in allelopathic rice may enable plants having a high competitive power when they are under stress (e.g. competition with weeds).

Heat values

Heat values of leaves, shoots, and roots of the two rice accessions at all 3-tested leaf stages were significantly higher in the non-weeded treatment than in weed-removal treatment, except for LE leaves at the 3-leaf stage (Fig. 8). Heat values for leaves, shoots, and roots at all three of tested three leaf stages were significantly higher in PE compared with LE plants. In general, heat values followed the order : PI non-weeded > PI weeded > LE non-weeded > LE weeded. In general, the total organic substance was greater in allelopathic rice PI than in non-allelopathic rice LE, especially when plants were grown in competitive conditions (non-weeded treatments).

Table 2. Expression of genes relate to photosynthesis in rice (*Oryza sativa*) leaves

Gene	Leaf stage	PI312777	Lemont	PI : LE ratio
Ribulose-1,5-bisphosphate carboxylase	3-leaf	2.51 ± 0.12a	1.95 ± 0.06b	1.29
	5-leaf	3.12 ± 0.05a	2.07 ± 0.05b	1.51
	7-leaf	3.38 ± 0.13a	2.16 ± 0.08b	1.56
Phosphoenolpyruvate carboxylase	3-leaf	1.73 ± 0.11a	1.09 ± 0.02b	1.59
	5-leaf	2.09 ± 0.09a	1.32 ± 0.04b	1.58
	7-leaf	2.26 ± 0.05a	1.48 ± 0.03b	1.53
Glycolate oxidase	3-leaf	1.85 ± 0.03a	1.24 ± 0.06b	1.49
	5-leaf	1.92 ± 0.08a	1.36 ± 0.07b	1.41
	7-leaf	1.98 ± 0.07a	1.41 ± 0.03b	1.40

Means and standard errors (SE) from six replicates are shown for each determination. Different letters indicate significant differences ($P < 0.05$) between the two rice accessions.

Less than 5% of tested rice accessions were allelopathic and most of them belong to tropical *O. sativa* subspecies *Japonica* (2,7). It is suggested that most wild types of existing crops possess high allelopathic activity; however, this trait has been lost in the selective breeding for desirable agronomic characteristics, such as yield and disease resistance (2,19).

Table 3. Expression of genes relate to nutrition in rice (*Oryza sativa*) leaves and roots

Gene	Leaf stage	Leaves			Roots		
		PI312777	Lemont	PI : LE ratio	PI312777	Lemont	PI : LE ratio
Nitrate reductase	3-leaf	2.34 ± 0.08a	1.24 ± 0.03b	1.89	1.62 ± 0.02a	1.02 ± 0.03b	1.59
	5-leaf	2.58 ± 0.05a	1.37 ± 0.04b	1.88	1.79 ± 0.03a	1.19 ± 0.04b	1.50
	7-leaf	3.06 ± 0.06a	1.81 ± 0.01b	1.69	1.93 ± 0.05a	1.35 ± 0.02b	1.43
Glutamate synthase	3-leaf	1.89 ± 0.02a	1.25 ± 0.06b	1.51	1.84 ± 0.06a	1.17 ± 0.05b	1.57
	5-leaf	2.07 ± 0.06a	1.38 ± 0.05b	1.50	1.97 ± 0.04a	1.26 ± 0.02b	1.56
	7-leaf	2.39 ± 0.07a	1.69 ± 0.05b	1.41	2.26 ± 0.05a	1.43 ± 0.03b	1.58
Nitrate transporter protein	3-leaf	1.92 ± 0.04a	1.08 ± 0.02b	1.78	2.18 ± 0.07a	1.55 ± 0.06b	1.41
	5-leaf	2.07 ± 0.09a	1.32 ± 0.03b	1.57	2.35 ± 0.06a	1.69 ± 0.04b	1.39
	7-leaf	2.45 ± 0.11a	1.54 ± 0.07b	1.59	2.79 ± 0.05a	1.94 ± 0.06b	1.44
Ammonium transporter protein	3-leaf	2.04 ± 0.07a	1.26 ± 0.06b	1.62	2.42 ± 0.04a	1.48 ± 0.04b	1.64
	5-leaf	2.58 ± 0.05a	1.45 ± 0.05b	1.78	2.89 ± 0.03a	1.67 ± 0.03b	1.73
	7-leaf	2.77 ± 0.06a	1.53 ± 0.06b	1.81	3.07 ± 0.03a	1.94 ± 0.07b	1.58
Phosphate transporter	3-leaf	2.13 ± 0.08a	1.31 ± 0.04b	1.63	2.58 ± 0.05a	1.34 ± 0.05b	1.93
	5-leaf	2.88 ± 0.12a	1.53 ± 0.03b	1.88	3.14 ± 0.12a	1.82 ± 0.08b	1.73
	7-leaf	2.96 ± 0.08a	1.68 ± 0.07b	1.76	3.49 ± 0.13a	1.93 ± 0.07b	1.81
Potassium transporter protein	3-leaf	1.84 ± 0.07a	1.09 ± 0.05b	1.69	2.41 ± 0.08a	1.57 ± 0.09b	1.54
	5-leaf	2.36 ± 0.06a	1.24 ± 0.08b	1.90	2.96 ± 0.09a	1.84 ± 0.06b	1.61
	7-leaf	2.95 ± 0.04a	1.37 ± 0.04b	2.15	3.45 ± 0.13a	2.06 ± 0.10b	1.67

Means and standard errors (SE) of six replicates are shown for each determination. Different letters indicate significant differences ($P < 0.05$) between the two rice accessions.

Olofsdotter (16) questioned whether a high loss of yield caused by the physiological cost of allelopathy in rice which producing and releasing allelochemicals against paddy weeds in field test. Our results clarified this question because under weeds stress, the allelopathic rice PI has the high nutrient absorption/utilization and high photosynthetic efficiency by up-regulating those relative genes to gain more the total organic substance. If primary metabolism is enhanced without physiological cost, allelopathic rice may be able to out-compete paddy weeds without loss in yield. These features could be potentially used as markers of allelopathic rice accession.

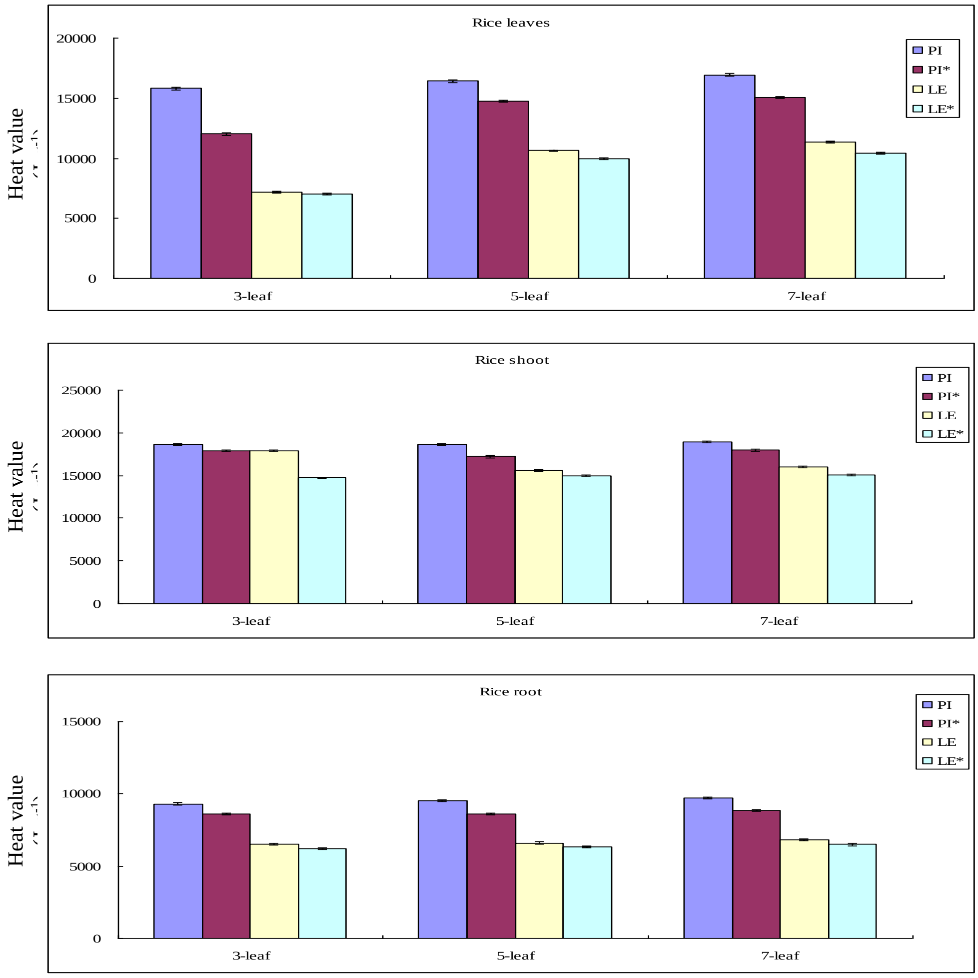

Figure 8. Heat values ($J \cdot g^{-1}$) of allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE) tissues at different growth stages. The bars represent the standard errors of the mean (n=3). Different letters indicate significant differences at $P < 0.05$. The * indicates the weed-removal treatment.

CONCLUSIONS

To determine the physiological costs of rice against weeds, a field study was done to evaluate the physiological and molecular traits of allelopathic rice PI312777 (PI) and non-allelopathic rice Lemont (LE). We found the physiological costs of rice against paddy weeds stress, the allelopathic rice PI have higher nutrients absorption/utilization and photosynthetic efficiency by up-regulating those relative genes to obtain greater organic substances.

ACKNOWLEDGEMENTS

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