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# Molecular physiological mechanism of increased weed suppression ability of allelopathic rice mediated by low phosphorus stress

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## ABSTRACT

To explore the inducible mechanism of weed-suppressive potential in allelopatic rice accession mediated by phosphorus nutrient deficiency, we studied the molecular physiological properties of allelopathic rice regulated by different phosphorus concentrations in hydroponics. We found that in hydroponics, the weed-suppressive potential of allelopathic rice PI312777 was enhanced under low phosphorus treatment, showing higher inhibitory effects on the dry weight of barnyard grass in presence of root exudates of allelopathic rice PI312777 than control (normal nutrient condition). However, reverse was true in non-allelopathic rice Lemont. The expression of four genes encoded the key enzymes involved in phenolic metabolism pathway were all up-regulated in allelopathic rice PI312777, but down-regulated in non-allelopathic rice Lemont, except slightly up-regulated phenylalanine ammonia-lyase. The HPLC analysis showed that some phenolic acids (Cinnamic acid, caffeic acid, 4-hydroxybenzoic acid, syringic acid and ferulic acid) were enhanced in the leaves, roots and root exudates of allelopathic rice PI312777 under low phosphorus treatment compared to control. But they were not influenced in non-allelopathic rice Lemont. Thus low phosphorus stress increased the allelopathic potential of allelopathic rice PI312777 to prevent the competitor to absorb the nutrients in hydroponics. The enhanced weed-suppressive potential in allelopathic rice mediated by low phosphorus stress was due to the up-regulation of the genes associated with phenolic metabolism, which led to the accumulation of phenolic acids in hydroponic solution from the roots of donor plant.

**Key words:** Allelopathic, gene expression, low phosphorus stress, *Oryza sativa* L., phenolic acids, rice.

## INTRODUCTION

Allelopathy is defined as "any direct or indirect, harmful or beneficial effect by one plant on another through the production and excretion of chemical compounds into the environment" (21). The use of allelopathy to control weeds in food crops is a bioengineering technology for the sustainable development of agriculture in 21st century (12). Allelopathy in crops might act as a biological weed control in the agroecosystem, to reduce dependence on synthetic herbicides, decrease the cost of crop production and also

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to protect the environment and biodiversity (6,21,23). Rice is a staple food crop heavily infested by weeds, leading to increased use of herbicides for their control, consequently, resulting in eco-environmental problems viz., (i) increased weed resistance to herbicides, (ii) serious environmental pollution and (iii) very harmful to human health (14). So the study of allelopathy and its application to control the weeds have become major theme.

As a quantitative trait, crop allelopathy is significantly affected by both genetic effects and environmental conditions (3,8,18,19,28). Our previous studies showed that environmental stresses (nitrogen or phosphorus deficiency) significantly increases the allelopathic potential in rice (22,28) through increased phenolic compounds (22,23). Although progress has been made in rice allelopathy, the molecular mechanism of rice allelopathy in response to nutrient stress is still unknown. In this study, we determined the molecular mechanism of changes in rice allelopathic effects on barnyard grass in mixture of rice/weed under low phosphorus treatment.

## MATERIALS AND METHODS

Allelopathic rice accession PI312777 and Lemont were used as donor plants and the barnyard grass (*Echinochloa crus-galli* L.) was used as receptor. The experiment was conducted in a greenhouse.

### Bioassay

Treatments followed Xiong *et al*. (27) and Song *et al.* (23) with minor modifications. Seeds of two rice accessions and barnyard grass were germinated and then sown in separate seedling trays. When the seedlings reached 3-leaf (rice) and 2-leaf (barnyard grass) stages, 40 uniform seedlings of each were selected and transplanted into a Styrofoam plate holes (spaced at 5×6 $cm^2$), and the seedlings were stabilized with a cotton plug inserted into each hole. The Styrofoam plate was floated on a pot (45×35×15 $cm^3$) filled with 10 l Hoagland nutrient solution (2,5,9,27). After 7d of recovery in the Hoagland nutrient solution, 35 rice seedlings and 5 weed seedlings were transplanted into a Styrofoam plate to form a hydroponic rice-weed mixture with the barnyard grass seedlings in the centre surrounded by the rice seedlings under different phosphorus treatments in the hydroponics. This setting could induce rice allelopathy (2,9,23,27). Two phosphorus treatments: normal phosphorus dose (6 $mg \bullet L^{-1}$) and low phosphorus dose (0.5$mg \bullet L^{-1}$) were used. A monoculture of barnyard grass under the same conditions was used as control (CK) for each treatment. In all treatment solutions, pH 5.5 was maintained throughout the experiment with1M NaOH or 0.5M $H_2SO_4$ (9,23). The barnyard grass dry weight (snap-killed in an oven at 105 °C for 20 min, and then dried at 70 °C until constant weight). Dry weight was recorded at 7 days after treatment. The roots and leaves of rice were also collected to determine the differential expressions of genes encoding the key enzymes involved in phenylpropanoid metabolic pathway by *qRT-PCR* (25,28). Simultaneously the rice roots, leaves, and co-culture solutions of rice/weed were collected to determine the phenolic acids content using High performance liquid chromatography (HPLC).

The experimental treatments were replicated thrice. The inhibition rate of dry weight of barnyard grass over control was calculated and used as the index to evaluate the allelopathy potential in two rice accessions (17,23,27). The inhibition rate (IR) was calculated as under:

$$IR = (CK - Treatment)/CK \times 100\%,$$

Where, inhibition: IR > 0, promotion: IR < 0.

**Analysis of gene expressions by *qRT-PCR***

Total RNA was extracted from 0.5 g samples (leaves and roots) by TRIZOOL reagent (Invitrogen). The concentration of RNA was assessed by UV-Visible spectrophotometer (VARIAN), and the structural integrity was detected with 1% non-denaturing agarose gel. The minim genome DNA was wiped off using DNaseI (Rnase free) and Reverse-transcription. Real-time quantitative PCR were performed according to ExScriptTMRT reagent Kit and SYBR Premix Ex Taq$^{TM}$ kit (TaKaRa) by CFD3120 MiniOpticon (BIO-RAD). The results were analyzed using Opticon Monitor3 software (25). The actin gene was employed as control (5-TGTAAGCAACTGGGATGA-3 and 5-CCTTCGTAGATTGGGACT-3). Gene specific primers were designed as per Table 1. All genes were obtained from rice (*O. sativa* L.) and the experiments were the experiments repeated 6-times. The specificity of amplification was verified by melting curve at the end of the PCR cycle. Fluorescence was read at every temperature increment of 0.2°C with a hold time of 2 sec. The relative quantification (ratio) of a target gene was calculated with the following formula: Ratio = $2^{-\Delta\Delta Ct}$ (20). Three biological replicates were carried out for each set of experiments. To calculate PCR efficiencies for each gene, 10-folds of serial dilutions of templates were used in reaction and $R^2$ values were computed (13).

**Sequence Analysis**

The products of *qRT-PCR* were selected and sent to Shanghai Sangon Biological Engineering Technology and Service Co., Ltd for sequencing. DNA sequences were analyzed at NCBI (the National Center for Biotechnology Information, USA, www.ncbi.nlm.nih.gov) using Blastn and Blastx (Table 3).

**Determination of phenolic allelochemicals**

The collected co-culture solutions of 1000 ml from rice/weed were used to determine the phenolic allelochemicals by HPLC. After filtration, the root exudates were adjusted to pH 2.6 by 2 mM HCl and evaporated to 10 ml. The concentrated solution was eluted by 80% methanol (pH 2.6) and evaporated to dry, later re-dissolved in 100% methanol for HPLC analysis (8,10,16). Simultaneously, 5 g roots and leaves of rice under each treatments were triturated in 5 ml 80% methanol (LC grade and adjusted to pH 2.6 with 2 N HCl), shaken in 35 $^{o}$C, 225 rpm/min for 4 h, repeated three times. Later the extravasate was also dried and re-dissolved in 100% methanol to analyse the phenolic acids. Five phenolic acids were mixed as standard compounds were dissolved by methanol, the concentration of each phenolic acid was: cinnamon acid $4\times10^{-3}$ mmol·L$^{-1}$, caffeic acid $5\times10^{-4}$ mmol·L$^{-1}$, 4-Hydroxybenzoic acid $6\times10^{-4}$ mmol·L$^{-1}$, syringic acid $1\times10^{-3}$ mmol·L$^{-1}$, ferulic acid $6\times10^{-3}$ mmol·L$^{-1}$. Before analysis, all liquids were further purified by microfiltering through a 0.45μm glass fibre. The model of HPLC was waters 1525, dual λ

Table 1. The primers used in *qRT-PCR*

| The key enzyme | Accession number | Source | Primer sequence (5'-3') |
|---|---|---|---|
| Phenylalanine ammonia-lyase | AK068993 | *O. sativa* | S-CCGTGCTCTTTGAGGCTAAC<br>A-GCTTGTGAGTCAGGTGGTCG |
| Cinnamate-4-hydroxylase | AAV44089 | *O. sativa* | S-ACCGCAGCGTCTCCTTC<br>A-ACCACCCGAGCATCCAG |
| Hydroxylase | AK069765 | *O. sativa* | S-CCGCCTTCAACGACAA<br>A-CGCCCATACGACGATT |
| *O*-methyltransferases | ABB90678 | *O. sativa* | S-TGTCCTGTGAAATGGGTG<br>A-CCTCGGAACAAGAACTG |

Table 2. Dry weight of barnyard grass in co-culture with two rice accessions under different phosphorus rates

| Treatment | Control (g/plant) | Normal phosphorus dose (g/plant) | Inhibition rate (%) | Low phosphorus dose (g/plant) | Inhibition rate (%) |
|---|---|---|---|---|---|
| Allelopathic rice* + Barnyardgrass | 0. 102±0.11 | 0.068±0.005 | 33.33 | 0.058±0.006 | 43.14 |
| Non-Allelopathicrice**+ Barnyardgrass | 0. 102±0.11 | 0.091±0.004 | 10.78 | 0.094±0.002 | 7.84 |

*PI312777, ** Lemont

Table 3. Similarity comparison of sequences from *qRT-PCR* products to genomic library

| Clone | Accession No. | Length (bp) | Best homologue in database | Score | %ID/E value | Source |
|---|---|---|---|---|---|---|
| Phenylalanine ammonialyase | ref\|NM_001054016\| | 177 | Phenylalanine ammonialyase | 322 | 100%/3e-85 | *O. sativa* |
| Cinnamate-4-hydroxylase | ref\|NM_001061725\| | 127 | Cinnamon acid 4-hydroxylase | 235 | 100%/e-59 | *O. sativa* |
| Hydroxylase | ref\|NM_001068556\| | 132 | E-class P450 | 244 | 100%/5e-62 | *O. sativa* |
| *O*-methyltransferases | ref\|NM_001068677\| | 220 | Caffeoyl-CoA O-methyltrans-ferase | 407 | 100%/1e-110 | *O. sativa* |

absorbance detector is waters 2487 and a 300mm × 3.9 mm ID column, filled with uBondapak 18 °C was placed. Linear gradient elution was done at a flow rate of 1.0 ml · $min^{-1}$. Solvent A was 5% acetic acid in distilled water and solvent B was methanol. For 0~6min, solvent A : solvent B was 2 : 3, as for 6~20min, solvent A : solvent B was 3 : 7. Identification of phenolic compounds was done by comparing their retention times with those of standard compounds. Simultaneously, peak area of each phenolic compound was registered to analyze the different contents of phenolic compounds. The ratio of each phenolic acids under different phosphorus treatment was calculated as under:

Rate (phenolic acid) = Area of phenolic acid (detected by HPLC) under lower phosphorus dose / Area of phenolic acid (detected by HPLC) under normal phosphorus dose

**Statistical analysis**

All the experimental data were processed by Microsoft Excel 2000. The analysis of multivariate regression and the stepwise linear regression was conducted by using the statistical programs of DPS (24).

## RESULTS AND DISCUSSION

**Suppression of barnyard grass**

The dry weight of barnyard grass grown in the pots containing different putative allelochemicals released from the roots of the two rice cultivars under different phosphorus supplies were significantly variable (Table 2, Figure 1), indicating that allelopahic rice PI312777 had higher ability to suppress the target weed compared to Lemont, especially under low phosphorus supply. We also found that the allelopathic potential of PI312777 was markedly enhanced to suppress the target plants as phosphorus supply was decreased, the reverse was true in Lemont, showing the decrease in allelopathic potential with declining phosphorus supply. Previous reports showed that the allelopathy in rice was quantitatively inherited (3,11). The gene mapping results confirmed that rice allelopathy was a polygenic character which could be affected by environmental factors (7,28). Therefore, it had become a major issue in the field to further understand the physioecological properties and the mechanism of allelopathy in response to environmental stress. In present study, we found that low phosphorus stress could effectively activate the weed-suppressive potential of allelopathic rice, showing the increased inhibitory effects on the accompanying weeds, which was confirmed by the inhibition rate of barnyard grass dry weight (Figure 1). The result was completely consistent with our previous studies that low nitrogen stress highly induced weed-suppressive ability of allelopathic rice accession to suppress the accompanying weed, barnyard grass in hydroponic mixture of rice/weed (23,27).

**Genes analysis**

This study also showed that the increased weed-suppressive potential of allelopathic rice accession resulted from the enhanced phenolic allelochemials, which were attributed to the up-regulation of the relevant genes encoding the key enzymes involved in phenolic metabolism. *qRT-PCR* approach was used to analyse differential expressions of

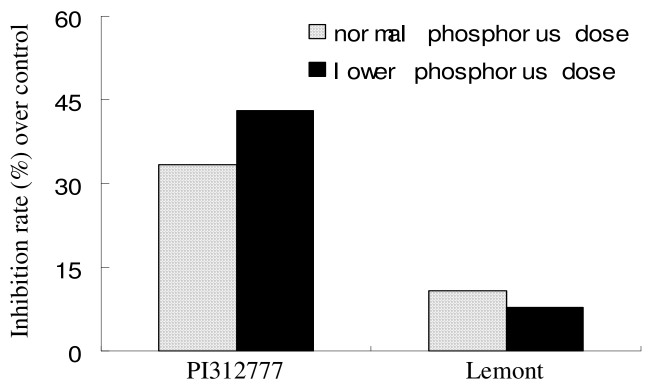


Figure 1 . Inhibitory effects of phosphorus doses and rice varieties on fry matter accumulation in barnyard grass

relevant genes associated with phenolic metabolism pathway and the similarity analysis of sequences (clones) from *qRT-PCR* products to database search was used to validate the results of *qRTPCR* analysis. Result showed that all sequences were 100% in ID value and their E values were e-59 to 5e-62, which confirmed the accuracy of *qRTPCR* analysis (Table 3). The results of *qRT-PCR* showed that the differential expression of 4 genes encoding the key enzymes involved in phenolic metabolism were all up-regulated in roots and leaves of allelopathic rice PI312777, especially in gene coding for PAL (Table 4) under low phosphorus treatment, showing 5.74±0.51 and 6.79±0.47 fold up-regulation in roots and leaves of allelopathic rice PI312777, respectively. However, reverse was true in non allelopathic rice Lemont except for *PAL*, showing 1.87±0.14 and 2.13±0.18 fold up-regulation in roots and leaves, respectively. These results suggested that phenolics biosynthesis in PI312777 was enhanced, when exposed to low phosphorus treatment, due to the increased expressions of these key enzymatic genes in phenolics metabolic pathway, which might be the important molecular ecological machinery of allelopathy in the suppression of target weed mediated by different nutrient conditions.

Table 4. Differential gene expression of key enzymes involved in phenolic metabolism in roots and leaves of allelopathic and non-allelopathic rice accessions under low phosphorus supply

| Key enzyme | Gene expression folds of I312777 | | Gene expression folds of Lemont | |
|---|---|---|---|---|
| | Root | Leaves | Root | Leaves |
| Phenylalanine ammonia-lyase | ↑, 5.74±0.51 | ↑, 6.79±0.47 | ↑, 1.87±0.14 | ↑, 2.13±0.18 |
| Cinnamate-4-hydroxylase | ↑, 3.27±0.23 | ↑, 3.86±0.29 | ↓, 1.39±0.17 | ↓, 1.02±0.07 |
| Hydroxylase | ↑, 2.79±0.21 | ↑, 3.67±0.16 | ↓, 1.30±0.13 | ↓, 0.84±0.08 |
| *O*-Methyltransferases | ↑, 2.19±0.14 | ↑, 3.59±0.27 | ↓, 2.39±0.12 | ↓, 1.02±0.12 |

Note: ↑ : Up-regulated; ↓ : Down-regulated

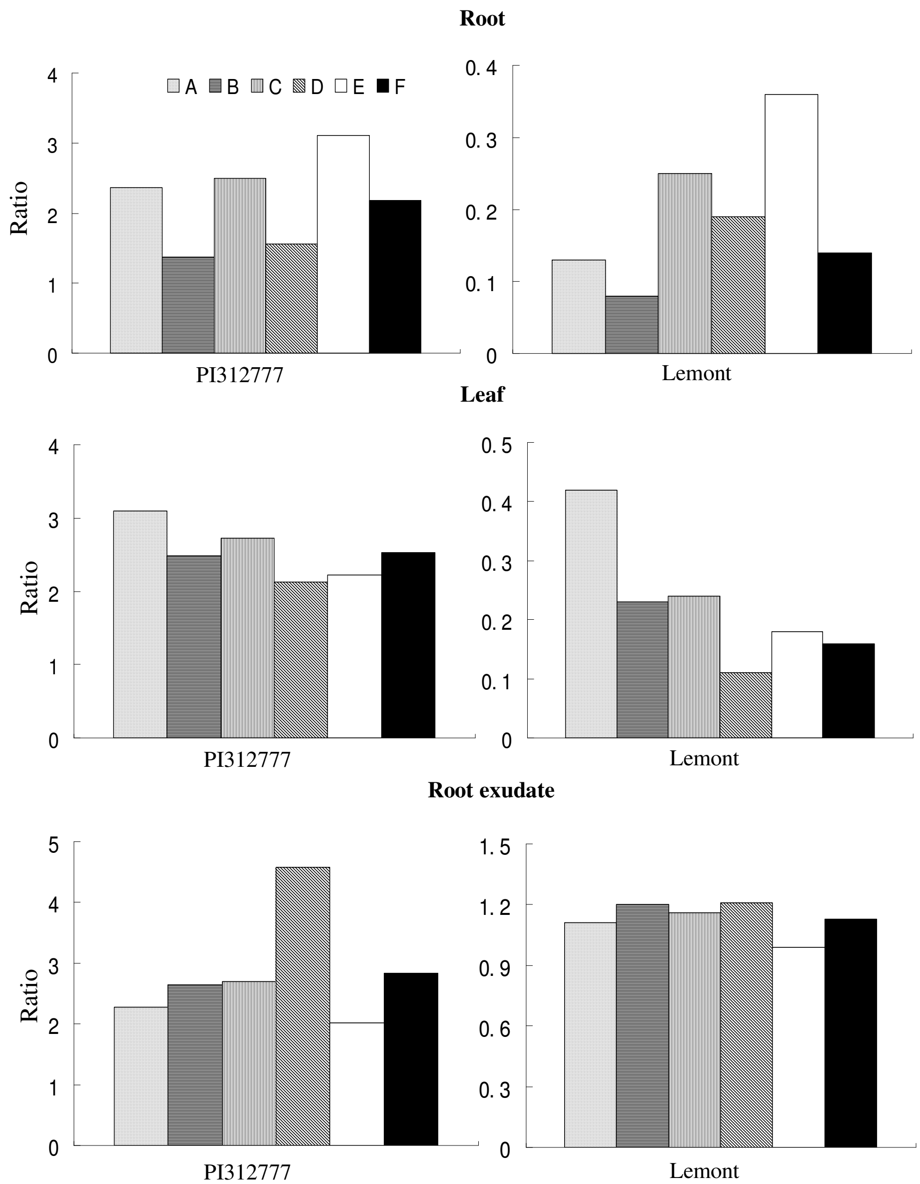


Figure 3. Effects of phosphorus doses on the concentration of Phenolic acids in root, leaf and root exudates of allelopathic (P1312777) and Non-allelopathic (Lemont) rice varieties. (A: Cinnamic acid; B: Caffeic acid; C: 4-Hydroxybenzoic acid; D: Syringic acid; E: Ferulic acid; F: Total phenolic acids)

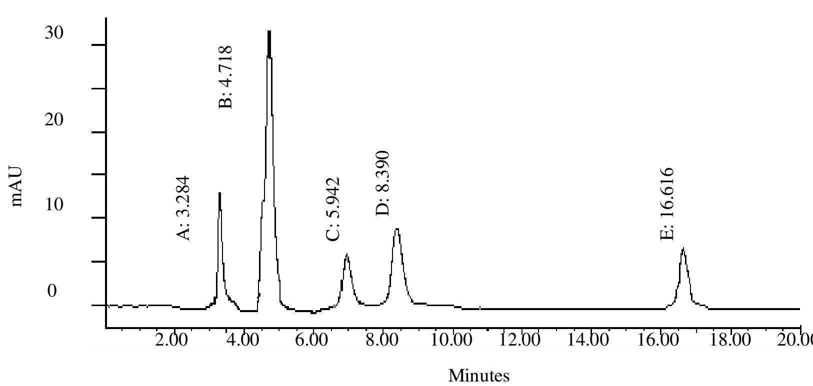


Figure 2. Chromatogram of 5- phenolic acids in mixture ( A: Caffeic acid; B: 4-Hydroxybenzoic acid; C: Vanillic acid; D: Ferulic acid; E: Cinnamic acid).

**Phenolic acids**

Further analysis (Figure 3) indicated that the total amount of 5 phenolic acid compounds in roots, leaves, and root exudates of allelopathic rice PI312777 co-cultured with barnyard grass were enhanced 2.53±0.14, 2.45±0.16, and 2.89±0.13 folds, respectively, under low phosphorus treatment over control. However, it was only enhanced 0.89±0.18, 0.95±0.07 and 1.17±0.13 folds, respectively, in non-allelopathic rice Lemont. It was also found that the increases of total phenolic acids in root exudates of allelopathic rice PI312777 were due to the enhancement of every single phenolic acid. These results suggest that allelopathic rice PI312777 could produce and release more phenolic acids, which were attributed to the up-regulation of relevant gene expressions to influence the companion weeds under low phosphorus treatment in mixture. This suggestion was further supported from the results differential analysis of relevant gene expressions.

These results showed that low phosphorus stress induced the enhancement in the weed-suppressive potential of allelopathic rice PI312777, which was attributed to the up-regulation of genes involved in phenolic metabolism, consequently enhancing the content of phenolic allelochemicals in the hydroponic system (released from the donor plants of allelopathic rice PI312777). We have demonstrated that rice allelopathy was an inducible genetic trait, which coupled with different response mechanisms involved in differential regulation of secondary metabolism pathway. It is therefore suggested that triggering a different induction by biotic factors including agricultural practices and environmental conditions not only could be effectively applied to elucidate the molecular genetics and enzymology of biosynthetic pathway, but also might pave the way for new trends in practical application of crop allelopathy. Hence, it is crucial in future lines of research to establish the specific management strategies to ensure a consistent weed-suppression in rice crop through allelopathy, for which further investigations are required to avoid down-regulation of key genes associated with allelopathic activity owing to environmental and production input factors (1,4).

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