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Response of Phytic Acid to Nitrogen Application and Its Relation to Protein Content in Rice Grain

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Abstract: Nitrogen (N) fertilization has been recognized as improving the grain beneficial micronutrients, including Zn and Fe, in rice. However, only a few studies have explicitly focused on N-induced variation in anti-nutritional components such as phytic acid (PA), PA synthesis-related gene expression, and variation in grain protein fractions. Therefore, in this study, two culture systems (hydroponic and detached panicle culture systems) were used to elucidate the influence of N application on PA concentration and its relation to the grain protein fractions, such as albumin, globulin, prolamin, and glutelin, and total protein in rice. Results showed that N application generally decreased the grain PA concentration in brown rice and down-regulated the PA synthesis-related genes in the lipid-independent pathway. In contrast, total grain protein and its fractions concentrations increased significantly. For grain positional distribution, PA and protein concentration were generally higher in the aleurone fraction than in the milled rice, regardless of N application. However, higher N application decreased the PA in both aleurone fraction and milled rice, while increased the grain protein fractions mainly in milled rice. These findings imply that N application could substantially improve the rice nutrition by reducing the PA while increasing the protein concentration. Hence, these findings may provide critical bases for rice nutritional improvement through optimal N management.

Keywords: rice (*Oryza sativa*); nitrogen; phytic acid; grain protein; *RINO1*

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1. Introduction

Rice (*Oryza sativa* L.) is one of the largest-growing cereals globally and plays a crucial role in food security, particularly in South and East Asia [1]. Over the last few decades, spectacular progress has been made to maximize the grain yield and quality of rice for the growing populations worldwide by either conventional breeding methods such as cross-breeding or by molecular tools (gene knockout/CRISPR/Cas9) [2–4], but it remains a challenging task because of climate, limitation in water, and pathogens [5,6]. Nitrogen (N) is one of the significant elements for sustaining higher rice productivity. Various studies have been conducted to improve the N-use efficiency by genetic manipulation, such as overexpressed *PERMEASE1*, an amino acid transporter gene [7]. In addition, conventional N management practices (N application rate and time, field N fertilization/foliar spraying) also have a substantial impact on plant morphology, agronomic performance, stress defense, physiological traits, and yield potential [8–10]. For example, the heavy panicle fertilizer management characterized by decreasing basal N input but increasing topdressing level at the panicle initiation period may prolong the grain-filling period and yield increment [11].

Ning et al. (2009) further analyzed the yield components and concluded that N-induced high yield by the increment of panicle number and grain number per panicle was under genetic control and controlled by N level [12]. Nevertheless, the empirical knowledge based on rice nutritional improvement through optimal N management is limited. N is also essential for improving grain quality. It generally improves the milling and protein quality, but it may negatively affect the appearance, cooking, and eating quality of rice grain [13,14]. It has also been reported that N application has a significant positive impact on grain mineral concentration and proteins [15,16]. Based on solubility in water, salt, alcohol, and alkali, the rice grain protein can be classified into four fractions: albumin, globulin, prolamin, and glutelin. Glutelin has a better nutritional value than prolamin for the human diet due to its higher lysine concentration and easy digestibility in the gastrointestinal tract [12]. A knockdown line of rice 13-kD prolamin were observed with a 56% increase in lysine content [17]. Using an in vitro digestive system, Wang et al. (2016) found that glutelin also exerted a higher antioxidant capacity as compared with prolamin [18]. Thus, a high glutelin/prolamin ratio indicates a high protein nutritional quality. However, the response of those protein fractions to exogenous N regulation is still insufficient, especially with a wide range of N gradients. Furthermore, the real absorption of minerals in the human digestive tract, also known as their dietary bio-availabilities, depends not only on their quantity but also on anti-nutritional factors such as phytic acid (PA, IP_6, *myo*-inositol-1,2,3,4,5,6-hexa*kis* phosphoric acid, $C_6H_{18}O_{24}P_6$).

PA and the higher phosphorylated inositol phosphates derivative, including inositol pentaphosphate (IP_5) are known as the most abundant storage form of phosphorus (P) in plant seeds [19]. Human and monogastric animals cannot hydrolyze these higher phosphorylated inositol phosphates, and these phosphate compounds easily bind with proteins and minerals and form insoluble and non-digestible complexes such as phytate (phytic acid salt) that cause a significant reduction in the bioavailability of minerals such as Fe and Zn [20]. Thereby, the higher grain PA, to some extent, is believed to exacerbate the occurrence of malnutrition for people who mainly live on plant-based food such as rice, which is characterized as inherently low in essential micronutrients, the poorest source of grain protein, and having abundant PA [21]. Zinc-regulated and iron-regulated transporter-like proteins (ZRT, IRT-like proteins) are closely linked with and involved in the transport of Zn and Fe. Optimized N application upregulates these transporter proteins involved in grain mineral allocation [22]. Many studies also explained the significant positive correlations between protein and mineral [23], suggesting the possibility of simultaneous enhancement for both minerals and proteins in grains.

So far, various studies have shown that P application promotes grain PA accumulation [24]. This is not surprising as PA is the principal storage form of grain P and accounts for 65–85% of grain total P [19]. However, the available information regarding the effect of N application on grain PA concentration is still insufficient and inconsistent. For instance, Simić et al. (2020) reported that N application changed grain Pi, but did not alter grain PA concentration in maize [25]. Kaplan et al. (2019) observed that N application increased maize grain PA when it was increased from 100 to 300 kg N ha^{-1} [26]. On the contrary, other research showed that N application decreased grain PA concentration in rice and wheat [12,27]. Meanwhile, energy-dispersive X-ray fluorescence and quantitative analysis have demonstrated that grain nutrients were unevenly distributed within an individual rice grain [28,29]. However, little attention has been given to understanding how the N application may influence grain's positional PA concentration and its transcriptomic profiling. In the present study, two rice genotypes, XS110 and its low phytic acid (*lpa*) mutant HIPJ, were used to elucidate the effect of N application in two culture systems, namely, hydroponic and detached panicle culture systems. The hydroponic culture was selected to exclude the complexity of field conditions. The detached panicle culture with a wide range of N levels was used to exclude the possible translocation of N from leaf or other plant tissue to the developing rice grain. The key objectives of this study were: (i) to quantify the effect of N supply on grain PA and protein concentrations and their positional

distribution; (ii) to clarify the transcript profiling of PA biosynthetic genes in response to N application. These findings could provide critical bases for rice nutritional improvement through optimal N management.

2. Materials and Methods

2.1. Plant Materials

The field experiments were conducted during rice growing seasons (April to October) at Zhejiang University, Hangzhou, China (30°18′ N, 120°04′ E) in 2015–2016. Two rice genotypes were used in the present study, XS110, and its corresponding *lpa* mutant HIPJ. XS110 was a *japonica* cultivar widely cultivated in south China, whereas the nonlethal *lpa* was originally isolated by gamma-irradiated XS110 mature grains, as reported previously by Liu et al. (2007) [30].

2.2. Hydroponic Culture

A hydroponic culture experiment was conducted in a greenhouse with natural light and temperature. The hydroponic culture experiment was laid in a randomized blocks design with two genotypes and three N levels, and five replications. Specifically, 1-month-old rice seedlings were transplanted to culture pots filled with 3.5 L 1/2 strength IRRI (International Rice Research Institute) nutrient solution. Each pot contained three uniform seedlings. After their initial adaptation to the solution medium, all rice plants were randomly divided into three groups for different N applications. The three N levels were as followed; 1.45 mM (low N, LN), 2.90 mM (medium N, MN), and 5.80 mM (high N, HN) and 4 replicates. MN was the standard N concentration according to the IRRI solution, with the 1/2 and 2 × of standard N concentration being set for LN and HN. The other essential elements, except for N, in the solution were kept constant for three N treatments. N was applied in the form of NH_4NO_3. The growth solution was changed once a week, and the pH was adjusted to 5.8. Pots were rotated periodically (2–3 times a week) to diminish the positional effect. At maturity (35 days after flowering), grains from each pot were harvested individually for subsequent 1000-grain weight (measured in paddy rice) and chemical analysis.

2.3. Detached Panicle Culture System

A detached panicle culture system was used with a wider range of N levels. It was also laid in a randomized blocks design with the two genotypes, five N levels, and five replicates. The detached panicle culture was performed at the full heading stage in a paddy field. The rice panicles in field with similar anthesis periods and uniform length were collected to impose different N treatments. NH_4NO_3 was used as N source, and five N levels were designed, 0 (N_0), 0.1% (N_1, M/V), 0.2% (N_2, M/V), 0.3% (N_3, M/V), and 0.6% (N_4, M/V). All other basic nutrient concentrations were kept the same except for N, namely 1.50 mM $CaCl_2$, 2.66 mM K_2SO_4, 0.75 mM $MgSO_4{\cdot}7H_2O$, 0.75 mM $MgSO_4$, 2.94 mM KH_2PO_4, 5.00 μM KI, 100.27 μM H_3BO_3, 100.00 μM $MnSO_4{\cdot}4H_2O$, 29.91 μM $ZnSO_4{\cdot}7H_2O$, 1.03 μM $Na_2MoO_4{\cdot}2H_2O$, 0.10 μM $CuSO_4{\cdot}5H_2O$, 0.11 μM $CoCl_2{\cdot}6H_2O$, 45.27 μM Na_2EDTA, 45.99 μM $FeSO_4{\cdot}7H_2O$, 4.06 μM nicotinic acid, 26.64 μM glycine, 1.48 mM pyridoxine hydrochloride, 0.59 μM thiamine hydrochloride, 0.075% filtered sulfurous acid and 131.47 mM sucrose. The solution was replaced every 5 days. A detailed procedure was followed as described by Su et al. (2018) [31].

Fresh grain samples of XS110 in 2016 were collected in the middle position of panicle from each N treatment at the 5th and 10th day after the initial culture. Their grains were frozen in liquid N and then stored at −80 °C for subsequent transcriptional expression analysis. The other panicles of the two genotypes were harvested individually after 20 days of different concentration N treatments in the solution, and 1000-grain weight (measured in paddy rice) and chemical analysis were performed.

2.4. Chemical Analyses

At maturity, harvested rice grains were firstly rinsed with ultrapure water (18.2Ω) to remove dust, and then air-dried. Grains used for determining grain protein, PA, and Pi analysis were dehusked by a laboratory de-husker (Model: JLGJ China) and ground using a portable grinder (Model JFSJ100, China) fitted with a 0.25 mm screen. For further investigating the influence of different N levels on PA and protein's grain positional distribution, the aleurone fractions and milled rice of XS110 in 2016 were further separated using a milled rice polisher (Model JNMJ3, Taizhou Grain Industry Instrument Crop, Zhejiang, China) based on the degree of milling (DOM, defined as the weight percentage of grain fractions removed) [31,32]. In the current study, rice grain with DOM $\leq$ 6.5% and >12% is considered as aleurone fraction and milled rice. Brown rice is the unmilled rice with aleurone fraction. Triplicate measurements were performed for each treatment.

The four protein fractions were analyzed spectrophotometrically based on Liu et al. protocol (2005) [33]. Total protein concentration was calculated as the sum of four protein fractions (albumin, globulin, prolamin, glutelin). PA concentration was quantified spectrophotometrically as described by Su et al. (2018) [31]. Inorganic phosphorus (Pi) concentration was determined as described by Jiang et al. (2019) [34], and total grain P was estimated as the sum of phytic acid-phosphorus (PA-P) and Pi. All these tested parameters have three replicates.

2.5. Quantitative RT-PCR

Total RNA was extracted from the grains using Trizol reagent kit (Invitrogen) with its recommended protocol, then treated with DNasel and reverse transcribed using the ReverTra Acc qPCR Reverse Transcriptase Kit (Toyobo, Oscak, Japan). Gene expression was quantified by real-time PCR using the SYBR Real-time PCR Master Mix Reagent Kit (Toyobo, Oscak, Japan). The primer set *OsAct* for the actin gene was used as an internal control. All primers used in this study were synthesized in Sangon biological engineer technology Co., Ltd. (Shanghai, China). For primers sequences see Table 1.

Table 1. Sequence of primers for PA synthesis-related genes.

Gene Name	Accession Number	Primer (Forward, 5′-3′)	Primer (Reverse, 5′-3′)	Tm
OsActin	AK100267	TGCGACGTGGATATTAGGAA	TGCCAGGGAACATAGTGGTA	59–73
RINO1	AK103501	AAACATTCCGATCCAAGGAG	CAAGCTCATAGAGGATGGCA	59–81
ITP5/6K-3	AK067068	GCTGAACTTCCTCCAAGACC	GAACCGATCTCTTGTCCCAT	59–117
ITP5/6K-6	AK102571	TTGGGTTTGATGTCGTTGTT	ATGACTGCTTGATTGCATCC	59–132
IPK1	AK102842	TGCCAAAGATTGGGTTTACA	TAGAAGGTGACGAGCCAGTG	59–74
IPK2	AK072296	GGAGCAAACCCTGTACCACT	ACCAGCTTCACCCTTACACC	59–114
MRP5	AK121451	TTGTCGCCGTGATAACATTT	CAGAAAGAACACCTCCAGCA	59–72

2.6. Statistical Analysis

The results were presented as mean ± standard deviation (SD, n = 3). Data were subjected to analysis of variance (ANOVA) using IBM SPSS statistics 21, and the differences between means were compared by Tukey's multiple comparison test at $p < 0.05$.

3. Results

3.1. Alterations in Grain Weight and Protein Concentration under Different N Levels

N application substantially increased the grain total protein and its four fractions (albumin, globulin, prolamin, glutelin) concentrations under hydroponic and detached panicle culture systems.

In hydroponic culture, N application increased the grain weight under MN and HN treatments compared to LN (Table 2). Rice grain protein was primarily constituted of glutelin, which accounted for 85.6% of grain total protein, prolamin with 7.2%, and least were globulin (5.4%) and albumin (5.2%). Similar to grain weight, grain total protein,

including albumin, globulin, prolamin, and glutelin in MN and HN, were significantly higher as compared to LN (except for globulin of HIPJ, in 2015, and prolamin of HIPJ, in 2016). In terms of glutelin/total protein, no significant differences were observed across N levels for HIPJ in both years, whereas significant decrement were observed as the N level increased for XS110 in both years. Grain glutelin/prolamin generally decreased for both XS110 and HIPJ in 2015, whereas the difference was insignificant for HIPJ in 2016. Both the increment of grain weight and protein concentration resulted in significantly higher grain protein content (mg grain^{-1}) in MN or/and HN as compared with LN. Whole rice grain (brown rice) was further separated into aleurone fraction and milled rice (inner endosperm fraction). Results showed that N application led to a significant rise in protein content in milled rice. In contrast, no significant variation was observed in aleurone fractions (except for glutelin in MN) (Figure 1).

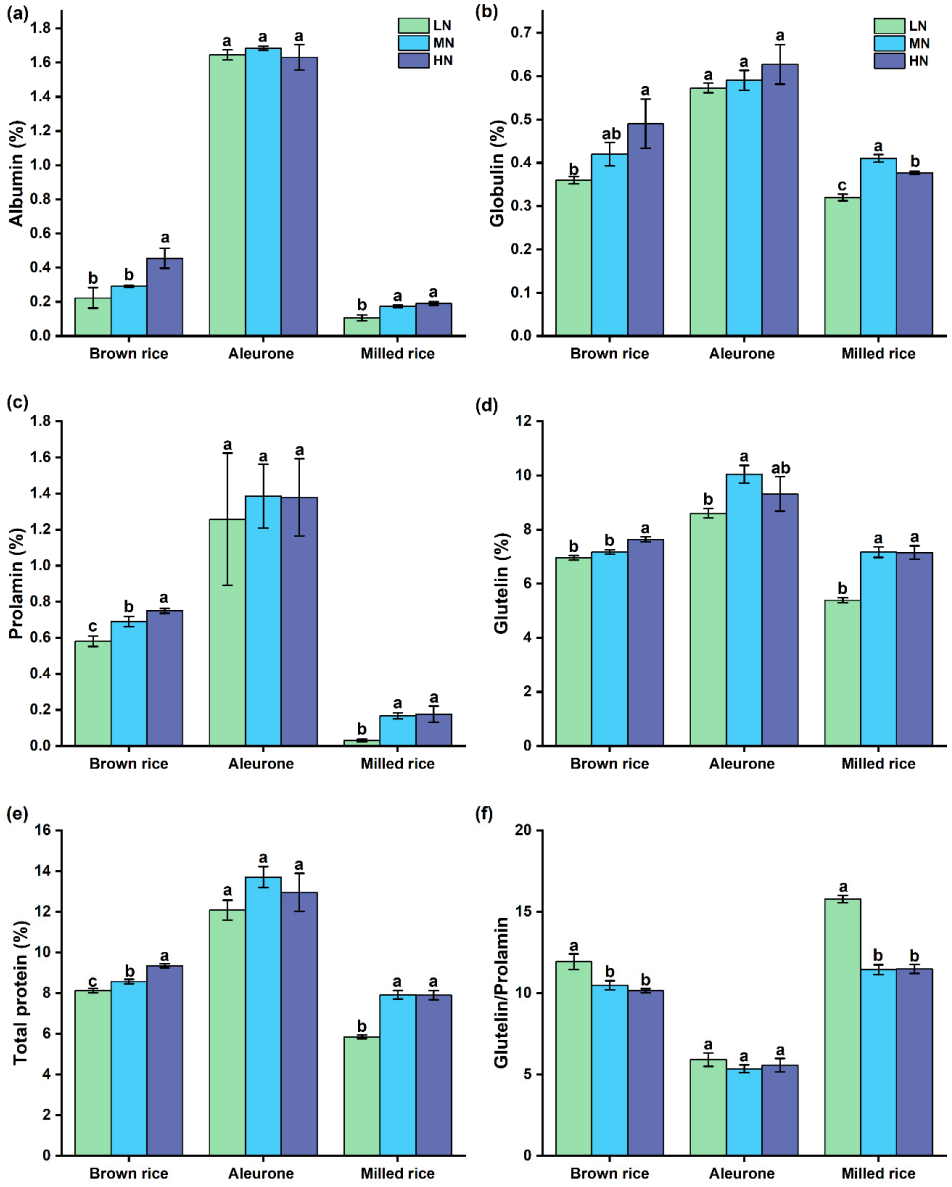

Figure 1. Effect of N levels on (**a**) Albumin (%), (**b**) Globulin (%), (**c**) Prolamin (%), (**d**) Glutelin (%), (**e**) Total protein (%), (**f**) Glutelin/Prolamin in different rice grain fractions, including brown rice, milled rice, and aleurone (XS110 of 2016 in hydroponic culture). LN: low nitrogen level; MN: medium nitrogen level; HN: high nitrogen level. Bars indicate the mean ± SE. Different letters for every three N levels (LN, MH, HN) of each grain fraction denoted significant differences according to a one-way ANOVA analysis followed by Tukey's multiple comparison test.

Table 2. Effect of N levels on grain dry weight, albumin, globulin, prolamin, glutelin, total protein concentration, glutelin/total protein, protein content in rice (Hydroponic culture).

Year	Genotype	N Level	1000-Grain Weight (g)	Albumin (%)	Globulin (%)	Prolamin (%)	Glutelin (%)	Total Protein (%)	Glutelin /Total Protein	Protein (mg Grain^{-1})	Glutelin/Prolamin
2015	XS110	LN	23.8 ± 0.2 ^{b}	0.327 ± 0.030 ^{c}	0.380 ± 0.053 ^{b}	0.611 ± 0.092 ^{b}	7.09 ± 0.16 ^{c}	8.41 ± 0.18 ^{c}	84.3 ± 1.5 ^{a}	2.00 ± 0.04 ^{b}	11.76 ± 1.65 ^{a}
		MN	26.5 ± 0.2 ^{a}	0.454 ± 0.010 ^{b}	0.444 ± 0.072 ^{ab}	0.759 ± 0.029 ^{ab}	7.62 ± 0.06 ^{b}	9.28 ± 0.11 ^{b}	82.1 ± 0.8 ^{ab}	2.46 ± 0.04 ^{a}	10.05 ± 0.31 ^{b}
		HN	24.2 ± 0.1 ^{b}	0.518 ± 0.008 ^{a}	0.522 ± 0.013 ^{a}	0.786 ± 0.042 ^{a}	8.04 ± 0.22 ^{a}	9.86 ± 0.17 ^{a}	81.5 ± 0.8 ^{b}	2.38 ± 0.03 ^{a}	10.23 ± 0.37 ^{ab}
	HIPJ	LN	23.8 ± 0.5 ^{b}	0.627 ± 0.035 ^{a}	0.533 ± 0.035 ^{a}	0.167 ± 0.031 ^{b}	6.69 ± 0.39 ^{b}	8.01 ± 0.46 ^{b}	83.4 ± 0.4 ^{a}	1.91 ± 0.11 ^{b}	40.73 ± 4.99 ^{a}
		MN	25.3 ± 0.0 ^{a}	0.537 ± 0.015 ^{b}	0.500 ± 0.010 ^{a}	0.300 ± 0.072 ^{a}	7.47 ± 0.55 ^{ab}	8.80 ± 0.52 ^{ab}	84.8 ± 1.5 ^{a}	2.22 ± 0.13 ^{ab}	25.67 ± 4.9 ^{b}
		HN	25.1 ± 0.3 ^{a}	0.653 ± 0.021 ^{a}	0.517 ± 0.029 ^{a}	0.240 ± 0.036 ^{ab}	8.61 ± 0.75 ^{a}	10.02 ± 0.70 ^{a}	85.9 ± 1.4 ^{a}	2.51 ± 0.21 ^{a}	36.18 ± 3.73 ^{b}
2016	XS110	LN	23.7 ± 0.1 ^{b}	0.223 ± 0.060 ^{b}	0.356 ± 0.009 ^{b}	0.584 ± 0.029 ^{c}	6.96 ± 0.09 ^{b}	8.12 ± 0.17 ^{c}	85.7 ± 0.8 ^{a}	1.93 ± 0.03 ^{c}	11.93 ± 0.48 ^{a}
		MN	24.4 ± 0.5 ^{ab}	0.291 ± 0.005 ^{b}	0.418 ± 0.027 ^{ab}	0.685 ± 0.028 ^{b}	7.18 ± 0.12 ^{b}	8.57 ± 0.11 ^{b}	83.7 ± 0.3 ^{b}	2.09 ± 0.02 ^{b}	10.48 ± 0.28 ^{b}
		HN	24.9 ± 0.0 ^{a}	0.455 ± 0.059 ^{a}	0.493 ± 0.057 ^{a}	0.754 ± 0.014 ^{a}	7.64 ± 0.05 ^{a}	9.35 ± 0.12 ^{a}	81.8 ± 0.5 ^{c}	2.33 ± 0.03 ^{a}	10.14 ± 0.13 ^{b}
	HIPJ	LN	22.7 ± 0.1 ^{b}	0.326 ± 0.038 ^{b}	0.353 ± 0.053 ^{b}	0.708 ± 0.042 ^{a}	6.40 ± 0.45 ^{b}	7.79 ± 0.37 ^{b}	82.1 ± 1.9 ^{a}	1.77 ± 0.08 ^{c}	9.04 ± 0.33 ^{a}
		MN	24.7 ± 0.2 ^{a}	0.488 ± 0.022 ^{a}	0.512 ± 0.021 ^{a}	0.828 ± 0.056 ^{a}	7.37 ± 0.37 ^{a}	9.20 ± 0.32 ^{a}	80.1 ± 1.3 ^{a}	2.27 ± 0.08 ^{b}	8.91 ± 0.27 ^{a}
		HN	24.6 ± 0.3 ^{a}	0.494 ± 0.025 ^{a}	0.580 ± 0.036 ^{a}	1.086 ± 0.287 ^{a}	8.03 ± 0.23 ^{a}	10.19 ± 0.51 ^{a}	78.9 ± 2.0 ^{a}	2.51 ± 0.12 ^{a}	7.68 ± 1.62 ^{a}
		CV	3.9	29.6	16.1	42.8	8.3	9.0	2.0	11.6	71.4

LN: low nitrogen level; MN: medium nitrogen level; HN: high nitrogen level. All data were presented as mean ± SE. CV: the coefficient of variation. Different letters for every three N levels (LN, MH, HN) in each year denoted significant differences according to a one-way ANOVA analysis followed by Tukey's multiple comparison test.

In detached panicle culture, the effect of N application on grain protein concentration was more prominent, especially at high N levels (Table 3). With the increase in N levels, grain protein and their fractions showed a significant and progressive increment, but high N levels (N4) did not result in an additional contribution to the increase in grain weight; instead, it was decreased significantly. Take XS110 in 2015 as an example, from N_0 to N_4, the increased extent of albumin, prolamin, glutelin, and total protein concentration was 68.0%, 278.0%, 287.0%, and 380.9%, respectively. Between both genotypes in two years, the highest increased extent of total protein content (mg grain^{-1}) was recorded for XS110 in 2016 (124.79%). Additionally, we observed that the ratio of glutelin/total protein increased along with N levels, which was different from the hydroponic culture. Grain glutelin/prolamin generally increased first then decreased under high N level, whereas XS110 in 2016 characterized as the progressively increasing trend with N application.

Under N application, the highest CV of four protein fractions was observed for prolamin in both culture systems (Table 2), but the lowest CV was culture system dependent (glutelin in hydroponic culture, albumin for detached panicle culture system). Additionally, we also observed a higher CV of glutelin/prolamin as compared with glutelin/total protein in both culture systems (Tables 2 and 3).

3.2. Alterations in Grain Phytic Acid with N Application

Both genotypes and nitrogen levels significantly influenced grain PA. Grain PA of HIPJ (*lpa*) was consistently lower than that of XS110 (wild type), irrespective of N levels (Figure 2, Table 4). N application generally decreased grain PA in both genotypes and both years.

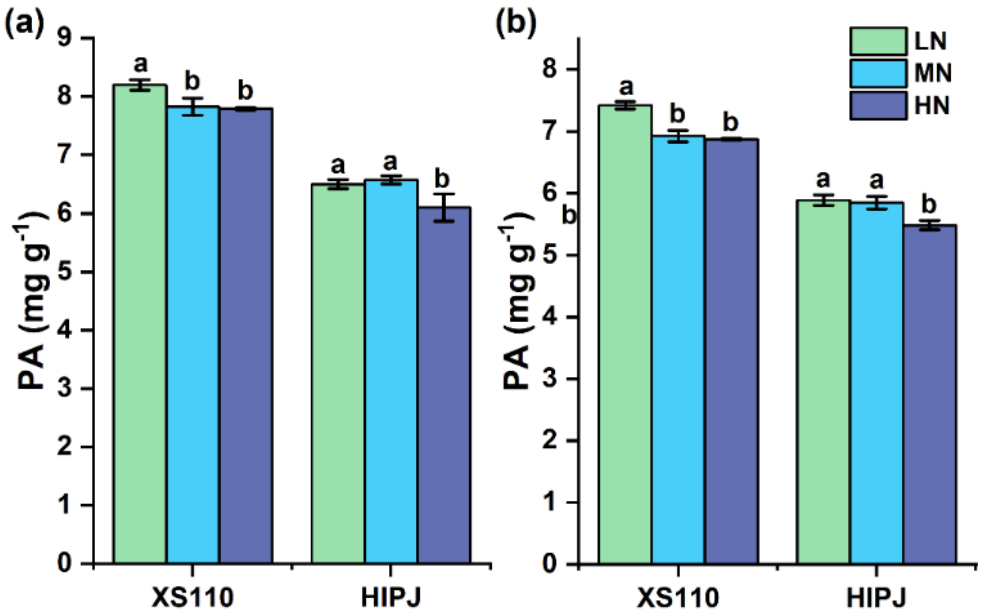

Figure 2. Effect of N levels on grain phytic acid (PA) concentration on rice grain under hydroponic culture; (**a**) 2015; (**b**) 2016. LN: low nitrogen level; MN: medium nitrogen level; HN: high nitrogen level. Bars indicate the mean ± SE. Different letters for every three N levels (LN, MH, HN) denoted significant differences according to one-way ANOVA analysis followed by Tukey's multiple comparison test.

Table 3. Effect of N levels on grain dry weight, albumin, globulin, prolamin, glutelin, total protein concentration, glutelin/total protein, glutelin/prolamin and protein content in rice (detached panicle culture system).

Year	Genotype	N Level	1000-Grain Weight (g)	Albumin (%)	Globulin (%)	Prolamin (%)	Glutelin (%)	Total Protein (%)	Glutelin/Total Protein	Protein (mg Grain^{-1})	Glutelin/Prolamin
2015	XS110	N_0	16.33 ± 0.17 ^{c}	0.350 ± 0.012 ^{e}	0.156 ± 0.005 ^{e}	0.180 ± 0.003 ^{e}	2.24 ± 0.12 ^{e}	2.92 ± 0.14 ^{e}	76.5 ± 0.7 ^{c}	0.48 ± 0.03 ^{e}	12.43 ± 0.51 ^{d}
		N_1	18.81 ± 0.06 ^{b}	0.397 ± 0.007 ^{d}	0.358 ± 0.005 ^{d}	0.266 ± 0.003 ^{d}	4.45 ± 0.11 ^{d}	5.47 ± 0.10 ^{d}	81.3 ± 0.4 ^{b}	1.03 ± 0.02 ^{d}	16.74 ± 0.22 ^{b}
		N_2	18.84 ± 0.11 ^{b}	0.424 ± 0.004 ^{c}	0.458 ± 0.005 ^{c}	0.376 ± 0.014 ^{c}	6.99 ± 0.08 ^{c}	8.24 ± 0.06 ^{c}	84.7 ± 0.3 ^{a}	1.55 ± 0.02 ^{c}	18.58 ± 0.62 ^{a}
		N_3	20.57 ± 0.08 ^{a}	0.456 ± 0.011 ^{b}	0.499 ± 0.003 ^{b}	0.507 ± 0.022 ^{b}	8.87 ± 0.17 ^{b}	10.33 ± 0.17 ^{b}	85.8 ± 0.3 ^{a}	2.12 ± 0.03 ^{a}	17.49 ± 0.43 ^{ab}
		N_4	15.43 ± 0.35 ^{d}	0.588 ± 0.008 ^{a}	0.589 ± 0.015 ^{a}	0.696 ± 0.021 ^{a}	10.75 ± 0.03 ^{a}	12.62 ± 0.06 ^{a}	85.2 ± 0.2 ^{a}	1.95 ± 0.04 ^{b}	15.46 ± 0.43 ^{c}
	HIPJ	N_0	17.36 ± 0.35 ^{ab}	0.352 ± 0.007 ^{d}	0.413 ± 0.002 ^{c}	0.207 ± 0.038 ^{c}	4.67 ± 0.30 ^{d}	5.65 ± 0.28 ^{e}	82.7 ± 1.3 ^{b}	0.98 ± 0.03 ^{d}	22.9 ± 2.55 ^{ab}
		N_1	16.67 ± 1.13 ^{bc}	0.424 ± 0.033 ^{c}	0.516 ± 0.012 ^{b}	0.256 ± 0.023 ^{c}	6.87 ± 0.27 ^{c}	8.07 ± 0.27 ^{d}	85.2 ± 0.5 ^{a}	1.34 ± 0.06 ^{c}	26.89 ± 1.36 ^{a}
		N_2	18.92 ± 0.45 ^{a}	0.508 ± 0.034 ^{b}	0.610 ± 0.008 ^{a}	0.368 ± 0.042 ^{b}	7.39 ± 0.13 ^{c}	8.87 ± 0.15 ^{c}	83.3 ± 0.5 ^{ab}	1.68 ± 0.03 ^{b}	20.23 ± 2.01 ^{bc}
		N_3	17.54 ± 0.59 ^{ab}	0.510 ± 0.019 ^{b}	0.635 ± 0.010 ^{a}	0.528 ± 0.013 ^{a}	8.61 ± 0.51 ^{b}	10.28 ± 0.52 ^{b}	83.7 ± 0.8 ^{ab}	1.80 ± 0.03 ^{a}	16.29 ± 0.58 ^{c}
		N_4	15.21 ± 0.49 ^{c}	0.577 ± 0.005 ^{a}	0.622 ± 0.019 ^{a}	0.536 ± 0.030 ^{a}	9.56 ± 0.10 ^{a}	11.30 ± 0.09 ^{a}	84.6 ± 0.2 ^{ab}	1.72 ± 0.04 ^{ab}	17.88 ± 0.8 ^{c}
2016	XS110	N_0	17.22 ± 0.11 ^{cd}	0.403 ± 0.006 ^{d}	0.181 ± 0.010 ^{d}	0.431 ± 0.011 ^{d}	4.19 ± 0.09 ^{e}	5.21 ± 0.09 ^{e}	80.5 ± 0.3 ^{c}	0.90 ± 0.01 ^{d}	9.73 ± 0.05 ^{c}
		N_1	17.71 ± 0.26 ^{c}	0.443 ± 0.006 ^{c}	0.292 ± 0.010 ^{c}	0.532 ± 0.036 ^{c}	6.01 ± 0.21 ^{d}	7.28 ± 0.23 ^{d}	82.6 ± 0.4 ^{b}	1.29 ± 0.03 ^{c}	11.32 ± 0.44 ^{b}
		N_2	18.74 ± 0.57 ^{b}	0.447 ± 0.006 ^{bc}	0.382 ± 0.018 ^{b}	0.631 ± 0.039 ^{b}	7.10 ± 0.04 ^{c}	8.56 ± 0.09 ^{c}	82.9 ± 0.6 ^{b}	1.60 ± 0.03 ^{b}	11.28 ± 0.64 ^{b}
		N_3	20.18 ± 0.42 ^{a}	0.470 ± 0.010 ^{a}	0.401 ± 0.010 ^{b}	0.640 ± 0.031 ^{b}	8.48 ± 0.68 ^{b}	10.00 ± 0.65 ^{b}	84.8 ± 1.3 ^{a}	2.02 ± 0.09 ^{a}	13.25 ± 0.67 ^{a}
		N_4	16.52 ± 0.24 ^{d}	0.467 ± 0.012 ^{ab}	0.453 ± 0.005 ^{a}	0.764 ± 0.035 ^{a}	10.01 ± 0.09 ^{a}	11.70 ± 0.11 ^{a}	85.6 ± 0.2 ^{a}	1.93 ± 0.01 ^{a}	13.12 ± 0.49 ^{a}
	HIPJ	N_0	15.71 ± 0.27 ^{d}	0.403 ± 0.006 ^{c}	0.246 ± 0.005 ^{d}	0.546 ± 0.026 ^{d}	3.71 ± 0.05 ^{d}	4.91 ± 0.06 ^{d}	75.7 ± 0.5 ^{b}	0.77 ± 0.01 ^{e}	6.81 ± 0.25 ^{c}
		N_1	16.51 ± 0.12 ^{c}	0.440 ± 0.010 ^{b}	0.424 ± 0.007 ^{c}	0.653 ± 0.032 ^{c}	6.64 ± 0.09 ^{c}	8.15 ± 0.05 ^{c}	81.4 ± 0.6 ^{a}	1.35 ± 0.01 ^{d}	10.18 ± 0.4 ^{a}
		N_2	19.21 ± 0.28 ^{b}	0.453 ± 0.006 ^{b}	0.474 ± 0.013 ^{b}	0.744 ± 0.016 ^{c}	7.67 ± 0.04 ^{b}	9.34 ± 0.07 ^{b}	82.1 ± 0.2 ^{a}	1.79 ± 0.01 ^{c}	10.3 ± 0.17 ^{a}
		N_3	20.58 ± 0.14 ^{a}	0.477 ± 0.006 ^{a}	0.519 ± 0.012 ^{b}	0.877 ± 0.061 ^{b}	8.51 ± 0.28 ^{b}	10.39 ± 0.22 ^{b}	82 ± 0.9 ^{a}	2.14 ± 0.06 ^{a}	9.73 ± 0.54 ^{ab}
		N_4	16.12 ± 0.43 ^{cd}	0.480 ± 0.010 ^{a}	0.588 ± 0.003 ^{a}	1.102 ± 0.020 ^{a}	9.77 ± 0.33 ^{a}	11.94 ± 0.31 ^{a}	81.8 ± 0.7 ^{a}	1.92 ± 0.01 ^{b}	8.86 ± 0.14 ^{b}
		CV	9.53	13.61	32.17	43.22	32.63	31.03	3.31	31.78	35.59

All data were presented as mean ± SE. CV: the coefficient of variation. Different letters for every five N levels in each genotype and year denoted significant difference according to one-way ANOVA analysis followed by Tukey's multiple comparison test.

Table 4. Effect of N levels on grain P components (phytic acid, inorganic phosphorus, total phosphorus, and PA-P/TP) in the detached panicle culture system.

Year	Genotype	N Level	PA Concentration mg kg^{-1}	Pi Concentration mg g^{-1}	TP Concentration mg kg^{-1}	PA-P/TP
2015	XS110	N_0	7.87 ± 0.18 ^{a}	0.28 ± 0.01 ^{c}	2.50 ± 0.06 ^{b}	88.6 ± 0.2 ^{a}
		N_1	7.42 ± 0.11 ^{ab}	0.29 ± 0.02 ^{c}	2.39 ± 0.05 ^{bc}	87.6 ± 0.8 ^{a}
		N_2	7.19 ± 0.07 ^{bc}	0.33 ± 0.01 ^{b}	2.36 ± 0.02 ^{bc}	85.7 ± 0.3 ^{b}
		N_3	6.83 ± 0.31 ^{c}	0.37 ± 0.01 ^{b}	2.29 ± 0.09 ^{c}	83.7 ± 0.5 ^{c}
		N_4	7.68 ± 0.12 ^{a}	0.59 ± 0.01 ^{a}	2.75 ± 0.04 ^{a}	78.6 ± 0.3 ^{d}
	HIPJ	N_0	6.12 ± 0.24 ^{a}	1.04 ± 0.03 ^{b}	2.76 ± 0.09 ^{b}	62.4 ± 0.7 ^{ab}
		N_1	6.06 ± 0.38 ^{a}	1.00 ± 0.06 ^{b}	2.71 ± 0.05 ^{b}	62.9 ± 2.9 ^{a}
		N_2	4.95 ± 0.24 ^{b}	1.02 ± 0.07 ^{b}	2.41 ± 0.14 ^{c}	57.8 ± 0.6 ^{abc}
		N_3	4.45 ± 0.05 ^{b}	1.09 ± 0.13 ^{b}	2.35 ± 0.12 ^{c}	53.5 ± 3.3 ^{c}
		N_4	6.20 ± 0.18 ^{a}	1.32 ± 0.02 ^{a}	3.07 ± 0.05 ^{a}	56.9 ± 1 ^{bc}
2016	XS110	N_0	7.67 ± 0.22 ^{a}	0.36 ± 0.01 ^{c}	2.52 ± 0.06 ^{ab}	85.8 ± 0.6 ^{a}
		N_1	7.91 ± 0.21 ^{a}	0.35 ± 0.01 ^{c}	2.58 ± 0.06 ^{a}	86.5 ± 0.5 ^{a}
		N_2	6.78 ± 0.16 ^{b}	0.36 ± 0.01 ^{c}	2.27 ± 0.04 ^{c}	84.2 ± 0.7 ^{b}
		N_3	6.56 ± 0.09 ^{b}	0.42 ± 0.01 ^{b}	2.26 ± 0.03 ^{c}	81.5 ± 0.3 ^{c}
		N_4	6.71 ± 0.33 ^{b}	0.47 ± 0.01 ^{a}	2.36 ± 0.10 ^{bc}	80.1 ± 0.6 ^{c}
	HIPJ	N_0	6.31 ± 0.31 ^{a}	0.68 ± 0.01 ^{d}	2.46 ± 0.08 ^{b}	72.3 ± 1.2 ^{a}
		N_1	5.84 ± 0.13 ^{b}	0.87 ± 0.03 ^{c}	2.51 ± 0.04 ^{b}	65.5 ± 1.1 ^{b}
		N_2	5.64 ± 0.03 ^{bc}	0.94 ± 0.02 ^{bc}	2.53 ± 0.03 ^{b}	62.9 ± 0.4 ^{b}
		N_3	4.32 ± 0.11 ^{d}	1.03 ± 0.11 ^{b}	2.25 ± 0.13 ^{c}	54.2 ± 2 ^{c}
		N_4	5.38 ± 0.03 ^{c}	1.27 ± 0.02 ^{a}	2.79 ± 0.03 ^{a}	54.3 ± 0.4 ^{c}
		CV	16.91	51.12	8.63	18.2

Note: Pi: inorganic phosphors; TP: total phosphorus, PA-P/TP: the ratio of phytic acid phosphorus to total phosphorus. All data were presented as mean ± SE. CV: the coefficient of variation. Different letters for every five N levels denoted significant differences according to one-way ANOVA analysis followed by Tukey's multiple comparison test.

In hydroponic culture (Figure 2), the genotypic difference in grain PA was firstly observed, and the average grain PA concentration of HIPJ (*lpa*) rice across three N levels was 18.9–19.5% lower than that of wild type (XS110) in two years. Because of N application, the grain PA concentration was lower with N application (MN and HN) compared with LN treatment, and the decreased extent was 4.95–6.15% in 2015 and 6.84–7.33% in 2016.

When we further separated the whole rice grain (brown rice) into aleurone fraction and milled rice, results showed that rice grain PA was observed with large positional variation, milled rice contained the lowest grain PA as expected, whereas most grain PA was found in the aleurone fraction. Relative to LN, HN treatment significantly decreased PA in aleurone fractions, whereas the lowest PA in milled rice was observed at the MN level (Figure 3).

In the detached panicle culture system, accompanied by the significant increment of grain weight (Table 3), grain PA and total P generally decreased from N0–N3 levels (Table 4), but their concentrations significantly increased in N4 as compared with the N3 level. On the contrary, grain Pi significantly increased as the N level increased. As the dominant grain P fraction, the average of PA-P/total P across the five N levels was 72.2%.

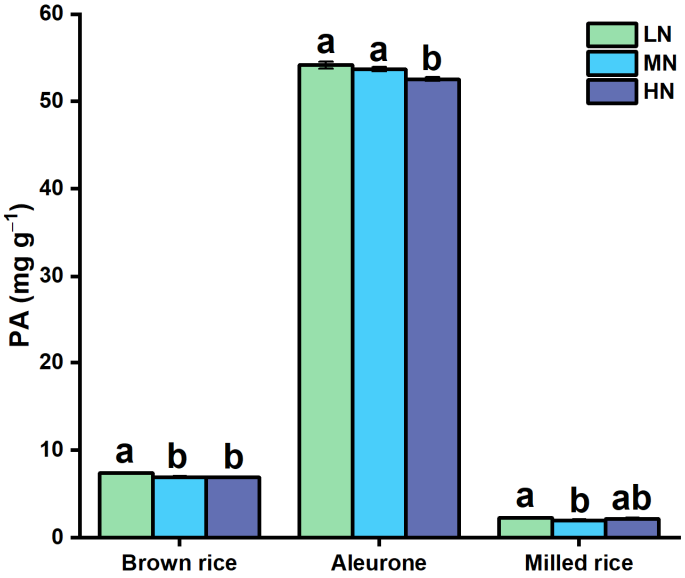

Figure 3. Effect of N levels on phytic acid (PA) concentration in different rice grain fractions (brown rice, milled rice, and aleurone) (XS110 of 2016 in hydroponic culture). LN: low nitrogen level; MN: medium nitrogen level; HN: high nitrogen level; PA: phytic acid. Bars indicate the mean ± SE. Different letters for every 3 N levels (LN, MH, HN) denoted significant differences according to one-way ANOVA analysis followed by Tukey's multiple comparison test.

3.3. Transcript Profiling of PA under N Application

We further analyze the transcriptional variation of six PA synthesis-related genes in response to N application (Figure 4). Results showed that *RION1* was the highly expressed, whereas the *IPK2* was the least. All genes, except for *IPT5/6K-6*, were relatively highly expressed on the 10th day compared with the 5th day after the initial culture. N application generally induced a down-regulated effect on the transcriptional expression of inositol phosphate-related genes. For the 5th day, N_1 treatment showed the transcriptional plateau for *RION1*, *IPT5/6K-3*, *IPK1*, and *MRP*, and then generally decreased as the N level increased. Grain *IPT5/6K-6* showed a progressive decrement as responsive to increasing N level, but for the *IPK2* gene, the trend was just the opposite. Grains cultured on the 10th day showed a similar variation. Specifically, the N_4 treatment showed the lowest transcriptional expression of *RION1*, *IPT5/6K-3* compared with other N treatments. For *IPT5/6K-6* and MRP, N_0 and/or N_1 showed the highest transcriptional expression, N_4 was observed with the least. *IPK1* was highest expressed at the N_0 level, but with other N levels insignificant, whereas *IPK2* expression generally increased as the N level increased.

3.4. Correlation Analysis

The correlations among tested nutritional components in both culture systems were analyzed (Figure 5). In hydroponic culture (Figure 5a,b), no significant correlation was observed between PA and grain total protein in both genotypes, PA was insignificantly correlated with protein fractions for XS110, but significantly and negatively correlated with prolamin for HIPJ. In the detached panicle culture system (Figure 5c,d), grain PA was significantly and negatively correlated with total protein and three protein fractions (globulin, prolamin, and glutelin), regardless genotypes.

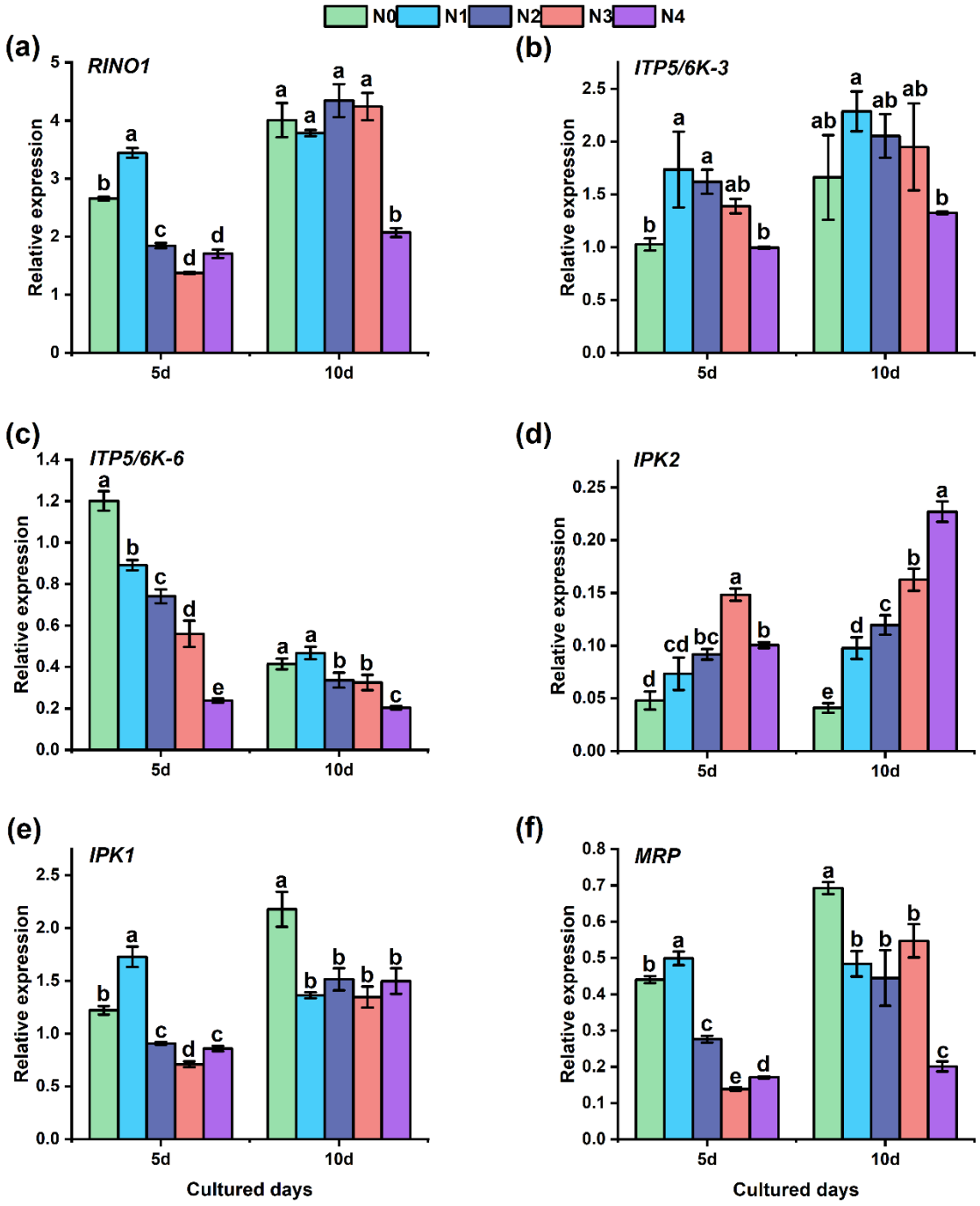

Figure 4. N-induced phytic acid (PA) synthesis-related genes expression in the grains; (**a**) *RINO1*, (**b**) *ITP5/6K-3*, (**c**) *ITP5/6K-6*, (**d**) *IPK2*, (**e**) *IPK1*, (**f**) *MRP* under detached panicle culture for XS110. Bars indicate the mean ± SE. Different letters for every five N levels denoted significant difference according to one-way ANOVA analysis followed by Tukey's multiple comparison test.

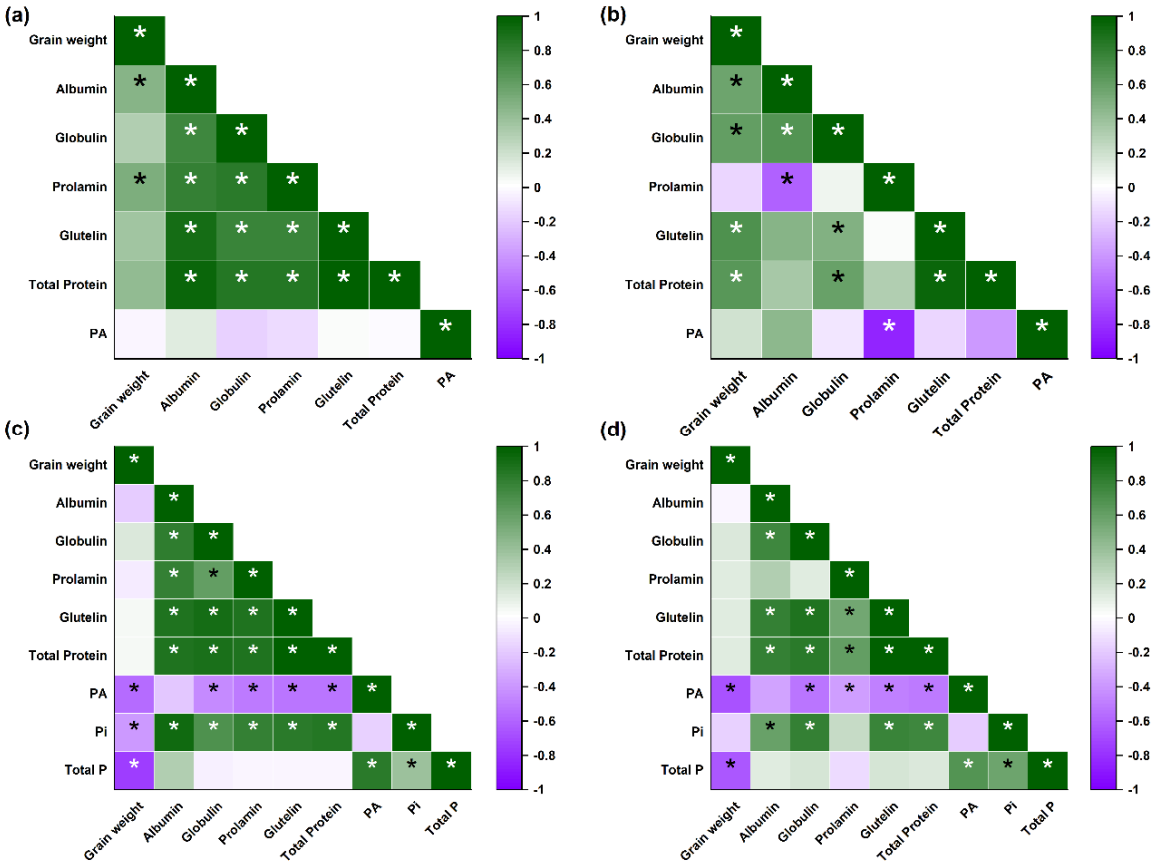

Figure 5. Pearson correlation among grain phytic acid (PA) and protein fractions for the following: (**a**) XS110 for hydroponic culture; (**b**) HIPJ for hydroponic culture; (**c**) XS110 for detached panicle culture; (**d**) HIPJ for detached panicle culture. The symbol * represents significance at the level of $p < 0.05$.

Grain PA concentration at maturity was significantly and positively correlated with gene expression level of *RINO1*, *ITP5/6K-6*, *IPK1*, and *MRP*, but significantly and negatively correlated with *IPK2* when detached panicles were cultured for 5 days. Grain PA concentration at maturity was only significantly and positively correlated with *ITP5/6K-6* gene expression level but significantly and negatively correlated with *IPK2* when grain was cultured for 10 days (Figure 6).

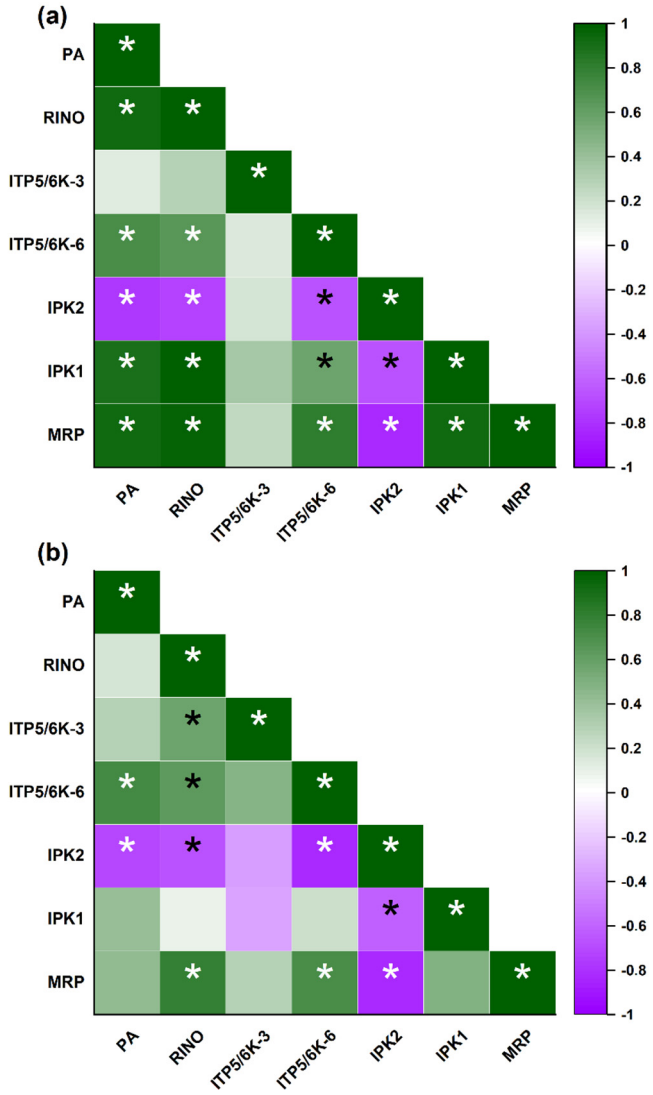

Figure 6. Pearson correlation between PA-related genes expressions ((**a**) for 5 days after culture, (**b**) for 10 days after culture) and grain phytic acid (PA) concentrations. (XS110 for detached panicle culture). The symbol * represents significance at the level of $p < 0.05$.

4. Discussion

N-fertilization is an effective and one of the most critical agronomic practices for maximizing the grain yield, quality, and biofortification potential [35–37]. Hence, agronomic measures aiming at improving the nutritional quality, such as protein and minerals content, while reducing the anti-nutritional components such as PA, have become a matter of great interest [38–40]. Various studies have demonstrated that N-fertilization substantially improves the beneficial grain micronutrients, including Zn and Fe in rice. In contrast, N-induced variation in anti-nutritional components such as PA, PA synthesis-related gene expression, and grain protein fraction variation has been overlooked or received less attention. The current results suggest it may be possible to achieve quality optimization with N regulation (Figure 5).

4.1. Response of Grain Protein and Its Fractions to N Application

Grain protein is a major quality determinant while evaluating grain nutrition and economic value. It has been found that wheat grain progressively accumulates N until maturity under a suitable planting environment [41], and post-flowering has been reported a critical influential period [42]. Extensive studies have investigated the N-induced vari-

ation of grain proteins and showed a promotional effect [12,40]. Consistently, our study under hydroponic culture also showed that N application had a favorable influence on grain protein concentration. By enlarging the N gradient from N deficiency to excess under the detached panicle culture system, this variation trend became more prominent, grain protein concentration increased even at higher N levels. This can be explained by the fact that grain protein content was inherited as a typical polygenic trait and thereby susceptible to environmental factors, especially for N, an essential component during grain protein synthesis [43]. On the other hand, grain weight under high N level showed a significantly decreased trend. Liang et al. (2017) explained that excess N decreased both sink strength and grain-filling rate, especially for the inferior grain [44], and thus a decreased grain weight was recorded in our study.

It shows that the quantity and proportion of protein fractions, such as glutelin/prolamin, determine the overall protein quality [18,45]. Therefore, further understanding is required concerning the variation of N-induced grain protein fractions. Ning et al. (2010) reported that grain albumin and globulin, as metabolic and structural grain protein patterns, were less affected by N application [46], whereas quantitative proteomics in wheat identified three globulin proteins upregulated in high-nitrogen plants [36]. Our results showed that all grain protein fractions, including albumin and globulin, increased as the N supply increased. The possible explanation of this inconsistency might be due to either species difference or the more N levels being used in our study [47]. Actually, by further enlarging the N gradient using a detached panicle system, this trend became more prominent, suggesting a detached panicle culture system is a useful culture system for source-sink regulation even in a relatively shorter duration. By using foliar N spraying at 10–20 days after anthesis, Souza et al. (1999) found that glutelin was most contributed to the grain protein increment [48]. We found prolamin was most affected by N application in both culture systems. Although glutelin/total protein showed a similar increasing trend as Ning et al. (2009) [12] (Table 3), while grain glutelin/prolamin decreased under excessive N levels (Tables 1 and 3). These findings imply that excessive N applied after heading enhances protein quantity but decreases the nutritional quality.

Histochemical studies show that rice caryopsis is composed of aleurone fractions, embryo, and starchy inner endosperm (milled rice) [49]. Traditionally, rice is consumed as milled rice, obtained by removing the embryo and aleurone fraction during the polishing process. Recently, brown rice or partially milled rice (with the combination of both brown rice and milled rice) has become more preferable for people who are health conscious. We therefore further analyzed positional protein distribution in rice grain. Our results demonstrated that grain protein, including the four fractions, was mostly distributed in the aleurone compared with the milled rice. It is consistent with Tong et al. (2019) [50], who also found that grain protein was mainly concentrated in the grain outer layer. Moreover, we also found that the effect of N application on grain protein concentration differed among the fractions. N application mainly increased grain protein, including its fractions in milled rice, rather than the most enriched aleurone fractions (except for glutelin in aleurone).

4.2. Response of Grain PA to N Application

Grain PA concentration is easily influenced by agricultural practices, including cultivation management (varieties, sowing time, soil properties, fertilization, irrigation, growth year and location, mixed cropping), climate (temperature, CO_2-induced global warming), and their interactions [19,31]. This fluctuation challenges the stable inheritance of *lpa* phenotype and provides the opportunity to decrease grain PA through appropriate agronomic management. Previous findings regarding N-induced grain PA variation showed inconsistent results. The possible reasons may be attributed to the planting environment's complexity and/or genetic PA inheritance. In this condition, two culture systems, hydroponic culture, and detached panicle culture systems, were used in the current study to exclude the interference of soil particles, unsuitable environment, and possible translocation of minerals (such as N, P) in the sink and source organs. Our results showed that

N application generally decreased grain PA concentration, which was agreed with the previous report in field rice trials [12]. In hydroponic culture designed with the relatively narrow N range to guarantee plant normal growth, grain PA reduction extent was 5.0–7.3% with N application (Figure 2), which is comparable to Xia et al. (2018) findings, where 5.3% of PA was recorded under field experiment [51]. In a detached culture system designed with a wide N gradient to fully explore the N-influence grain PA variation, results further showed that grain PA and total P concentration generally decreased with N application (except at N_4 level). Different from N-induced grain PA or total P variation, grain Pi concentration showed a generally increasing trend as the N level increased (Table 4), which is inconsistent with previous research by Bi et al. (2013), who reported no variation among different N rates [52]. The possible reasons for this discrepancy might be the combination of the wide N gradient range used (including the excessive N level), the difference in N–P interaction in culture solution and field soil, and/or the functional role of the root. Therefore, more research is required to elucidate further the role of different P fractions on rice grain development and PA synthesis with N application.

Compared with grain positional distribution, the present study showed that aleurone fraction contained the highest grain PA concentration as expected, whereas milled rice had the least. This was similar to those of Iwai et al. (2012), who used energy-dispersive X-ray microanalyses and observed that grain PA was mostly distributed in aleurone fractions [28]. Moreover, we further revealed that the N application had a significant distributional influence on grain PA concentration. Relatively to LN, N application decreased grain PA in both aleurone fraction and milled rice, but with N level dependent. It should be noted that in the detached panicle culture system, the significantly higher grain PA concentration under excessive-high N levels (N_4) seemed inconsistent with the general negative induction of the N effect. The lowest grain weight under the N_4 level also represented the higher proportion of the PA-enriched aleurone fraction, which can explain the grain PA increment under the N_4 level.

4.3. Transcript Profiling of PA under N Application

PA is synthesized by multiple inositol phosphate enzymes coded by a set of genes [53]. It is generally accepted that PA biosynthesis is followed by two separate pathways, lipid-independent and lipid-dependent pathways [21]. The lipid-independent pathway is a process of sequential and stepwise phosphorylation process from Ins(3)P_1 to IP_6 (PA), catalyzed by specific inositol phosphate kinases. Among them, *myo*-inositol-3-phosphate synthase (MIPS, a rate-limiting enzyme in the PA biosynthesis pathway) catalyzes the initial committed step in PA synthesis. Previous results showed that *RINO1* (*MIPS* coding gene in rice), *IPT5/6K-3*, *IPT5/6K-6* were key and highest expressed genes in this pathway [53]. Another alternative pathway is the lipid-dependent biosynthesis pathway, and the *IPK2* gene is reported to be mainly involved. Both pathways shared the same last step from Ins(1,3,4,5,6)P_5 to Ins(1,2,3,4,5,6)P_6 ($InsP_6$), catalyzed by inositol 1,3,4,5,6-penta*ki*sphosphate 2-kinase (IPK1). Synthesized cellular PA in cytosol still needs to transport to storage vacuoles as globoids, a multidrug resistance-associated protein (MPR), belong to the ATP-binding cassette (ABC), which is responsible for PA's transport and compartmentalization.

Our results from the detached panicle culture experiment also showed that *RINO1* was most highly expressed among six selected PA synthesis-related genes (Figure 4). In previous research, a strong emphasis was focused on the identification and characterization of genes necessary in PA synthesis [54], RNAi mediation in *lpa* generation [20,55], and transcriptome analysis of rice with various grain PA genetic backgrounds [56]. However, rare information was available about the transcription expression of PA-related genes in response to fertilizer or mineral regulation. Our results showed that N application generally decreased most PA synthesis-related genes, including *RINO1*, *IPT5/6K-3*, *IPT5/6K-6*, *IPK1*, and *MRP*, but increased *IPK2* gene expression. However, under N regulation, *RINO1*, *IPT5/6K-6*, *IPK1*, and *MRP* may be more important for PA biosynthesis (Figures 4 and 6). The trend was similar in both culture periods (5th and 10th days after the initial culture). Since the lipid-

independent pathway is the major route that contributes to grain PA accumulation, whereas the lipid-dependent pathway mainly involves signal transduction [21], the downregulation of PA synthesis-related genes treated by N application could be a possible partial reason for the grain PA reduction.

5. Conclusions

The present study reported the potential beneficial effect of N application on grain protein enhancement but anti-nutritional phytic acid (PA) reduction. Grain PA and protein, including albumin, globulin, prolamin, and glutelin, were primarily distributed in rice grains' aleurone fractions. Rational N application simultaneously enhanced grain protein quantity and quality but decreased grain PA and its essential regulatory genes. N application decreased grain PA in both aleurone and milled rice, but with N level dependent, whereas N application mainly increased protein, including the concentration of its fractions in milled rice. Optimized N application has the potential to promote the rice protein and mineral's bioavailability status.

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