Modulating Rice Grain Phenylalanine: Effects of Foliar Biostimulant Application

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Abstract

Phenylketonuria (PKU) requires a strict low-phenylalanine diet to prevent harmful accumulation in the body. Current dietary options, largely reliant on processed foods, are often unpalatable, expensive, and inaccessible in developing countries. This study aimed to naturally develop a rice strain with reduced phenylalanine levels, avoiding genetic modification, andoptimize biological stimulants for modulating phenylalanine biosynthesis. Field trials involved foliar application of cinnamic acid (0, 0.5, 1, and 1.5 g/L) and phenylalanine (0, 0.25, 0.5, and 1 g/L) on two local rice cultivars (Helal and Keshvari) during seed formation and filling, using a randomized complete block design with three replications. Key molecular traits measured included total protein content, phenylalanine ammonia-lyase (PAL) enzyme activity, and expression of genes involved in phenylalanine biosynthesis and catabolism (*phenylpyruvate aminotransferase*, *arogenate dehydratase*, and *PAL*). Results showed that phenylalanine application increased *PAL* gene expression, while cinnamic acid suppressed it. Although both stimulants significantly downregulated phenylalanine biosynthesis genes and reduced total protein content for both treatments, a decrease in total protein content occurred at the highest applied concentration. Notably, cinnamic acid significantly decreased phenylalanine levels in Keshvari (1 and 1.5 g/L), while increasing tyrosine and tryptophan concentrations at higher dosages. In addition, phenylalanine treatments at concentrations of 0.5 and 1 g/L led to decreased expression of the *arogenate dehydratase* gene. Moreover, the application of phenylalanine at 0.5 and 1 g/L reduced the expression of the *phenylpyruvate aminotransferase* gene in both rice cultivars studied. Cinnamic acid treatment can reduce phenylalanine in rice, offering a natural strategy for PKU-friendly diets.

Keywords Biostimulants, Phenylketonuria, Phenylalanine, Cinnamic acid, Rice

Introduction

Rice (*Oryza sativa L.*) is one of the oldest and most widely consumed cereal crops worldwide. It serves not only as a staple food for more than half of the global pop-

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ulation but also as a key contributor to the economies of rice-producing countries. With the global population projected to reach 9.7 billion by 2050, the demand for rice and other staple crops is expected to rise substantially. To address this challenge, various strategies, including modern biotechnological approaches and breeding programs, are being developed to enhance rice productivity and sustainability (Mohidem, 2022). Beyond its nutritional and economic value, rice also holds significant cultural importance; in many societies, it symbolizes prosperity, abundance, and well-being, and is commonly featured in traditional ceremonies and rituals. Owing to its adaptability to diverse environments and its central role in food security, rice remains an indispensable crop, particularly in Asia and other developing regions (Mohidem, 2022).

From another aspect, phenylketonuria (PKU) is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase gene, impairing the metabolism of phenylalanine (Phe) and leading to its accumulation in the liver and urine (Van Spronsen, 2021). Phe, an essential amino acid, is also a precursor for tyrosine synthesis. In PKU patients, defective hydroxylation of Phe results in low tyrosine levels, causing symptoms like developmental delays, vomiting, poor growth, hypopigmentation, and seizures. As they age, affected individuals may show a smaller head size compared to peers, hyperactivity, attention deficits, repetitive movements, and intellectual disability (Blau, 2016; Ashe, 2019; Ali, 2021). Since Phe is an essential amino acid that must be obtained from food, PKU patients require a strict diet low in Phe, under medical supervision, to prevent adverse effects. Due to the importance, complexity, and cost of producing specialized foods for PKU, their diet is particularly expensive, especially in underdeveloped countries. Currently, the main approach involves hydrolyzing food and combining it with pseudo-foods such as stimulants and vitamins to create semi-medicated products (Soltanizadeh, 2014). However, this method has major drawbacks, including high costs, reduced food quality due to hydrolysis, limited food variety, high perishability, spoilage, and the frequent use of artificial sweeteners like aspartame to improve taste (Chattopadhyay, 2014). Despite current drawbacks, alternative methods are needed to safely and naturally reduce Phe content in foods for PKU patients. A simpler, cost-effective approach that maintains food quality and avoids hydrolysis is essential. A recent study by Ghalamboran (Ghalamboran, 2023) introduced a novel strategy using chitosan nanoparticles as elicitors during rice growth. This method enhances phenylalanine ammonia lyase (PAL) activity and reduces total protein content, leading to lower Phe levels in rice grains. Previous studies on various plants, including soybean, *Lactuca sativa L*., and rice, using elicitors like phenolic acids, cinnamic acid (CA), and chitosan have reported increased PAL activity and reduced Phe and total protein content (Baziramakenga, 1997; Hussain, 2011; Cheng, 2015; Ghalamboran, 2023). The current study examined the impact of spraying biological elicitors on developing rice grains to regulate Phe metabolism. Given its high nutritional value and wide-

spread consumption, especially in Eastern countries, rice is a dietary staple for PKU patients (Huke, 1982). The Phe biosynthetic pathway in plants begins with the shikimic acid pathway, which produces chorismate, a precursor for aromatic amino acids like Phe, tyrosine, and tryptophan. These amino acids are essential for synthesizing secondary metabolites involved in plant biological functions. Enzymes in the shikimic acid pathway can be inhibited, potentially blocking chorismate production and disrupting the organism's biological processes (Zulet-González, 2020). Chorismate, the final product of the shikimic acid pathway, enters the phenylpropanoid pathway and acts as the main precursor for Phe production in both the plastid and cytosol of plant cells. In plastids, chorismate is converted to prephenate by chorismate mutase (CM1), then to arogenate via prephenate aminotransferase. Arogenate can either be converted to tyrosine by arogenate dehydrogenase (ADH) or to Phe by arogenate dehydratase (ADT); thus, *ADT* gene expression indicates Phe synthesis in plastids. In the cytosol, chorismate is also converted to prephenate by CM2. Prephenate is then transformed into phenylpyruvate by prephenate dehydratase and finally into Phe by phenylpyruvate aminotransferase (PPY-AT). Measuring *PPY-AT* gene expression helps assess the effects of foliar elicitor sprays on Phe production (Qian, 2019).

Phe levels in plants are influenced not only

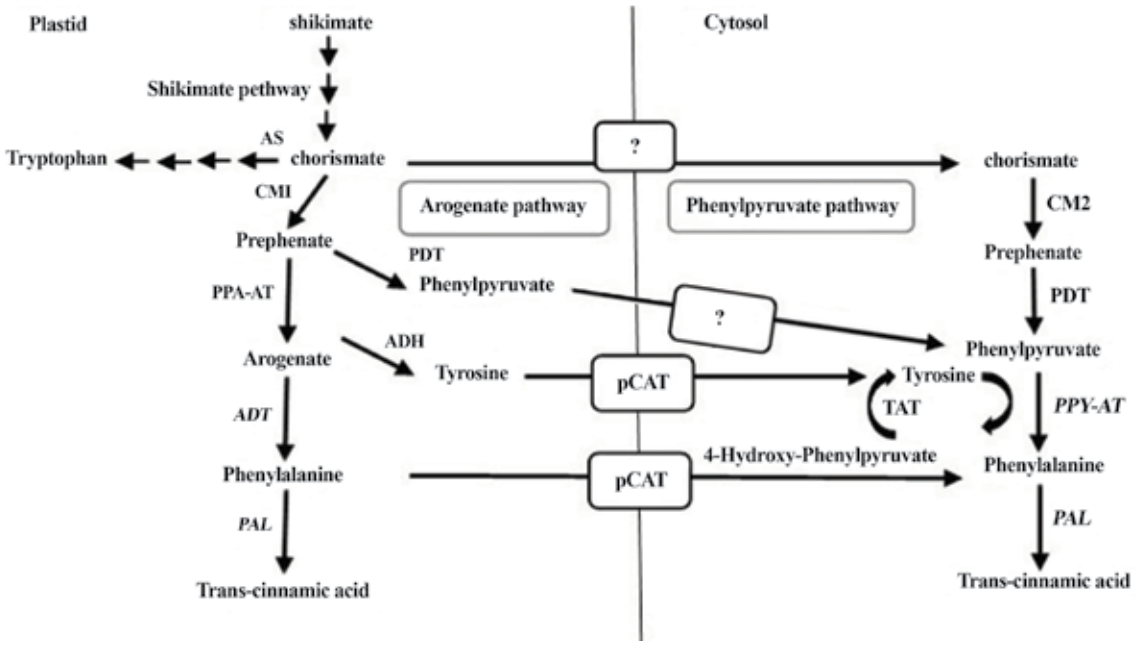


Fig. 1. Schema of Phe biosynthetic pathways in plant cells. Enzymes whose gene expression was measured are bolded. Paths and enzymes whose processes are unknown are grayed out. Abbreviations: AS - anthranilate synthesis, ADH - arogenate dehydrogenase, *ADT* - *arogenate dehydratase*, pCAT - plastidial cationic amino acid transporter, CM - chorismate mutase, PDT - prephenate dehydratase, PPA-AT - prephenate aminotransferase, *PPY-AT- phenylpyruvate aminotransferase*, TAT - tyrosine aminotransferase, *PAL - phenylalanine ammonia lyase* (Qian, 2019).

by the expression of biosynthetic genes and related enzyme activity but also by its catabolism. PAL is the key enzyme in Phe catabolism, converting Phe into CA, a precursor of various secondary metabolites such as flavonoids, isoflavonoids, tannins, anthocyanins, coumarins, lignin, and flavonols. Measuring *PAL* gene expression and enzyme activity provides insight into the extent of Phe catabolism and its conversion into secondary metabolites in plant cells. This study focused on two physiological strategies, inhibition and stimulation, to control Phe production in rice. The goal was to reduce Phe content in rice grains while maintaining other key qualities. The effectiveness of these strategies in altering gene expression and their impact on Phe biosynthesis and catabolism were investigated. Two local rice cultivars, Helal and Keshvari, were used to explore the genetic potential for Phe reduction. Foliar spraying with two biological elicitors, Phe and CA, at different concentrations was tested during four growth stages. Key variables such as total protein, PAL enzyme activity, and the gene expression of *PPY-AT*, *ADT*, and *PAL* were examined. This approach highlights the potential of science and technology in addressing medical and food-related challenges in the future.

Material and methods

Preparation of rice seeds

The rice seeds (*Oryza sativa L*., cultivars Helal and Keshvari) were obtained from Amol Rice Research Institute (Amol, Iran). The cultivars Helal and Keshvari were selected because rice cultivation in our region is traditionally based on local landraces, and it is essential to utilize the genetic potential of these regionally adapted varieties. At the same time, the methodology employed in this study is not limited to these specific cultivars; rather, it can be applied to any rice variety worldwide, making the findings broadly relevant and transferable.

Experimental design

The data presented is a composite analysis derived from three consecutive years, i.e., 2019, 2020, and 2021. The method used for planting rice in this research was a local method. The rice seeds of both cultivars were soaked in water for 3 days. Then, the germinated seeds (Figure 2A) were placed in a flooded soil bed soaked in a large amount of water and covered with nylon (Figure 2B). After 31 days, the seedlings (Figure 2C) were transplanted by hand to the rice paddies (plots) (Figure 2D). In the main field, the division was done in such a way that the cultivated land was divided into plots with an area of 5 x 6 square meters. To prevent interference between treatments, a distance of 50 cm was maintained between the plots. Each plot included 16 planting holes, in which 4 seedlings were planted. The distance between the holes was 30 cm. The water in the test plots was kept at a level of 5 cm during the plant growth period, and the soil bed was dried 10 days before rice harvesting. The project area has the coordinates of latitude 36 degrees and 28 minutes east and longitude 52 degrees and 23 minutes north, with an elevation of 29.8 meters above sea level.

Preparation of experimental treatments

Elicitors of CA and Phe were prepared using Merck brand (Germany) at various concen-

trations: 0, 0.5, 1, and 1.5 g/L for CA, and 0, 0.25, 0.5, and 1 g/L for Phe. Foliar spraying of rice spikes was done in 4 periods of rice physiological growth (spike onset, pre-flowering, flowering onset, and milky onset of grain) during sunset on the aerial parts (Figure 2D). Safety measures were taken during foliar application to avoid interference with the efficacy of different concentrations of functional elicitors. The selection of CA and Phe concentrations was guided by three considerations. First, previous studies indicated that within this concentration range, these compounds influence the biosynthetic pathway of Phe in rice and other cereal grains. Second, the chosen concentrations were optimized to activate the biosynthesis pathway while avoiding toxic or adverse effects on grain development, thereby ensuring plant health and maintaining optimal yield. Third, employing multiple concentration levels enables the evaluation of dose–response relationships and facilitates the identification of the most effective concentration for enhancing Phe biosynthesis (Fatahi Siahkamary, 2025).

Sampling time

Sampling was done after complete ripening of rice seeds (Helal variety after 67 days and Keshvari variety after 97 days). Furthermore, samples were taken from the sampling line, which was the bushes in the middle of the plots, in order to minimize testing errors. Rice seeds, along with their hulls, were placed in liquid nitrogen upon harvesting from the clusters and transferred to a -80°C freezer for further analysis.

Gene expression analysis

In order to study the expression levels of genes that are effective in Phe biosynthesis

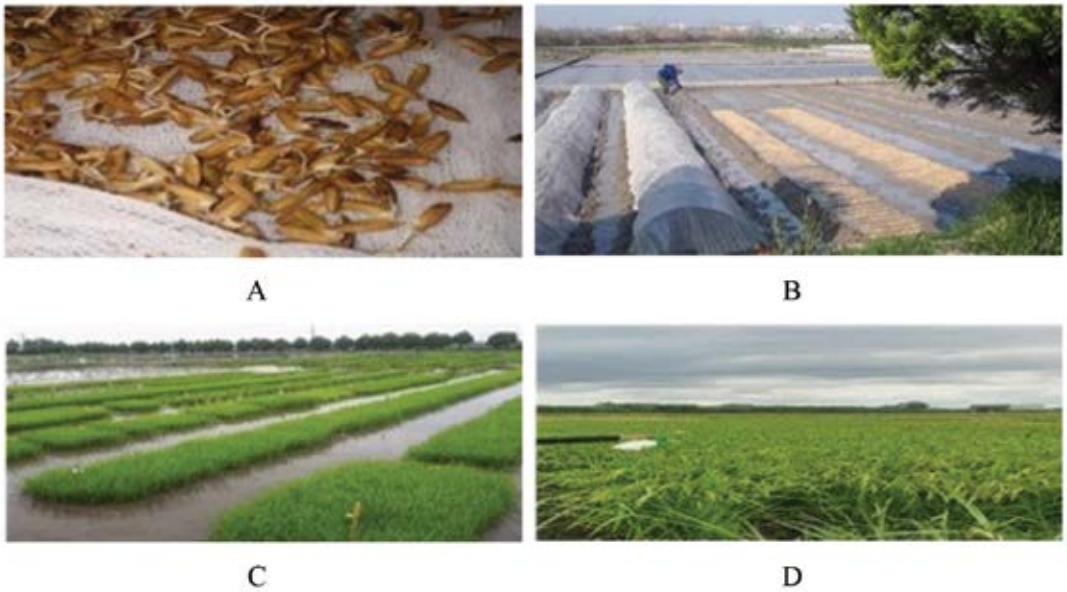


Fig. 2. A. The stage of soaking the seeds before placing them in the main field. B- Transferring the germinated seeds to the main field. C- Growing the germinated seeds in beds (a special farm that is very small and very fertile, suitable for seedling growth) and preparing for transfer to the main land. D- Spraying the growing bushes at predetermined stages.

and catabolism, the quantitative real-time PCR (qRT-PCR) technique was used. RNA extraction from rice seeds stored at -80°C was performed using the RNSOL Reagent kit (Rojetechnologies, Iran) with 3 repetitions for each sample. The qualitative analysis of the extracted RNAs was conducted using gel electrophoresis. cDNA synthesis was performed using the Pars Tous kit (Pars Tous, Iran). The sequences of the studied genes (*Arogenate dehydratase* (*ADT*), *Phenylalanine ammonia-lyase* (*PAL*), *Phenylpyruvate aminotransferase* (*PPY-AT*), *Ubiquitin 10*) were obtained from the NCBI website. The primers for the target genes were designed using Oligo 7 software (Table1). Furthermore, the Ubiquitin 10 gene was also considered as an internal reference gene in the current study. Quantitative expression of genes was performed using the qRT-PCR technique and an ABI device (Step One Plus system, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to a specific temperature and time schedule (95°C for 5 minutes, followed by 35 cycles at 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes). The cycle threshold (CT) samples were examined by Step One software, and CTs with better melt curves and amplification plots were selected. The relative expression of each gene was analyzed using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Determination of amino acids

To determine amino acids in rice kernels, a modified method by Yang Zhang (Yang, 2016) was employed. To process each experimental treatment, 100 mg of powdered rice kernel was first measured and mixed with 1 ml of 80% ethanol. The samples were then stored at 4°C. They were subsequently placed in a thermomixer for one hour at 80°C and 700 rpm before allowing them to cool down to ambient temperature. The samples were then centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatant was carefully transferred into a new tube. These tubes were sealed with paraffin and placed in a freeze-dryer overnight for complete lyophilization. Upon completion, the samples were stored at -20°C. The next step involved adding 1 ml of distilled water to each tube and filtering the contents through 0.22 µm syringe filters. Afterwards, 250 µl of the samples were transferred to a new tube, followed by the addition of 200 µl of borate buffer and 100 µl of ortho-phthalate dihydrate (OPD). The samples were vortexed for 120 s to ensure proper mixing. Subsequently, 50 µl of 0.5 mM HCl was added to each sample and vortexed for an additional 15 s to dissolve the mixture. Finally, the samples were loaded into the HPLC instrument equipped with a HALO 5 µm, C18 column for further analysis. The chromatographic separation

Table 1 Primers used for qRT-PCR in this study

Gene	Acc. No	Forward primer(5`to3`)	Reverse primer(5` to 3`)
ADT	NM_001403279.1	GAGCTATCCTACCGATGTCAG	CTGCTATCGGTGCTTCCAAG
PAL	NM_001401906.1	ACTGCCTCAAGGAGTGGAAC	CTCCTCTCCTCCTCGATGA
Ubiquitin 10	AK101547.1	GACTACAACATCCAGAAGGAG	CAGGCACATCGGCAGCTC
PPY-AT	AC087182.12	GAAGCTGCGTTGGTTGCGT	CAGTAAGCACCTGAAGGATGA

was performed using a reversed-phase C18 column (150 × 4.6 mm, 5 μm) maintained at 30 ± 2 °C. The mobile phases consisted of mobile phase A: 10 mM ammonium acetate (pH adjusted to 4.8 with acetic acid) and mobile phase B: HPLC-grade acetonitrile. The flow rate was set to 1.0 mL·min^{-1}, and the injection volume was 10 μL. Detection was carried out using a UV detector at a primary wavelength of 258 nm. A calibration curve was generated using phenylalanine standards prepared by serial dilution to obtain seven concentration levels (1, 5, 10, 25, 50, 100, and 200 μM), which were used to ensure accurate quantification. The total run time for each sample was 12–18 min (Haghighi, 2015).

Determination of the PAL

The PAL activity was determined according to Aydas' method (Aydas, 2013). The PAL activity was measured by the rate of conversion of Phe to trans-CA. One unit of PAL activity is equivalent to one micromole of CA produced per minute.

Determination of the total protein

The total protein in rice kernel was measured by standard Bradford essay (Kruger, 2009), and bovine serum albumin was used as a standard material to check the protein content in each extract.

Statistical analysis

The effect of treatments (CA and Phe and cultivars) was investigated in a factorial experiment (4×4×2) using a randomized complete block design (RCBD) with three replications. Each treatment had 3 plots with dimensions of 5 × 6 square meters. The experimental data obtained from different methods were normalized using the Kolmogorov-Smirnov test and then statistically analyzed using SPSS software version 2016. Comparisons of treatment averages were performed with Duncan's test at a 95% probability level, and the data were also plotted using Excel.

Assessment of yield and grain quality parameters

Spikelet fertility

Spikelet fertility was calculated as the ratio of filled spikelets to the total number of spikelets per panicle, expressed as a percentage. At maturity, five representative panicles from each treatment were sampled, and the numbers of filled and unfilled spikelets were recorded. The following formula was used:

$$\text{Spikelet fertility (\%)} = \frac{\text{Number of filled spikelets}}{\text{Total number of spikelets}} \times 100$$

This method follows the standard procedure described by the International Rice Research Institute (IRRI, 1996).

Thousand grain weight

Thousand grain weight was measured by counting and weighing 1,000 fully matured and air-dried grains from each treatment. The grains were cleaned, dehulled, and adjusted to a standard moisture content of 14% before weighing to ensure accuracy. The average weight was expressed in grams (g). The procedure followed the guidelines described in the Standard Evaluation System for Rice (IRRI, 1996).

Milling recovery

Milling recovery was determined as the percentage of total milled rice obtained from a given weight of rough rice after dehusking and polishing. Approximately 150 g of

paddy rice from each treatment were dehulled using a laboratory husker, and the resulting brown rice was polished using a rice polisher. The weight of the milled rice was recorded and expressed as a percentage of the original paddy weight. This procedure was performed according to the method described by the International Rice Research Institute (IRRI, 1996).

Determination of grain yield

Grain yield was determined at physiological maturity by harvesting the central rows of each plot to avoid border effects. The harvested panicles were threshed, cleaned, and air-dried to a constant weight, and the yield was expressed as the weight of dehulled rice per plant (or per square meter) after adjusting to 14% moisture content. The procedure followed the standard guidelines provided by the International Rice Research Institute (IRRI, 1996).

Results

Foliar elicitors alter Phe pathway gene Expression in rice cultivars

The data illustrated in Figure 3 clearly demonstrate that foliar application of Phe and CA on immature rice spikes significantly influenced the expression of *PAL*, *ADT*, and *PPY-AT* genes in rice seeds. According to the bar chart, CA treatment in the Helal variety (at all three concentrations) led to a substantial reduction in *PAL* gene expression. However, in the Keshvari variety, a decrease in *PAL* expression was only observed at the 1.5 g/L concentration (Figure 3A). In contrast, across both cultivars, *PAL* gene expression markedly increased in response to all Phe concentrations (Figure 3B). *ADT* gene expression showed a consistent and significant decrease in both varieties under all concentrations of CA foliar spray. Notably, this reduction appeared to be concentration-independent in the Keshvari variety (Figure 3C). Additionally, Phe application resulted in a significant reduction of *ADT* expression in the Helal variety at 0.5 and 1 g/L, with no notable effect observed at 0.25 g/L. In contrast, all three concentrations led to a marked decline in *ADT* expression in the Keshvari variety (Figure 3D).

Furthermore, CA exerted a pronounced impact on *PPY-AT* gene expression, precipitating a steep decline in both cultivars (Figure 3F). While the lowest concentration applied (0.5 g/L) did not cause any significant change compared to the control, the higher concentrations (1 and 1.5 g/L) notably widened the gap between treated and control samples. Indeed, the modulation of *PPY-AT* gene expression was clearly dependent on the applied concentration (Figure 3F). On the other hand, Phe treatment also led to a decrease in *PPY-AT* gene expression. In this case, both cultivars exhibited reduced gene expression at higher concentrations (1 and 0.5 g/L) (Figure 3F). Overall, the CA elicitor at a concentration of 1 g/L appeared to be optimal, particularly in the Keshvari variety. Not only did it maintain *PAL* gene expression, but it also effectively reduced the expression of the biosynthetic genes *ADT* and *PPY-AT* in both cultivars.

The grouping of treatments in the graphs was based on cultivars rather than treatment concentrations within a single cultivar in order to facilitate the comparison of genotypic responses. Since the objective of this

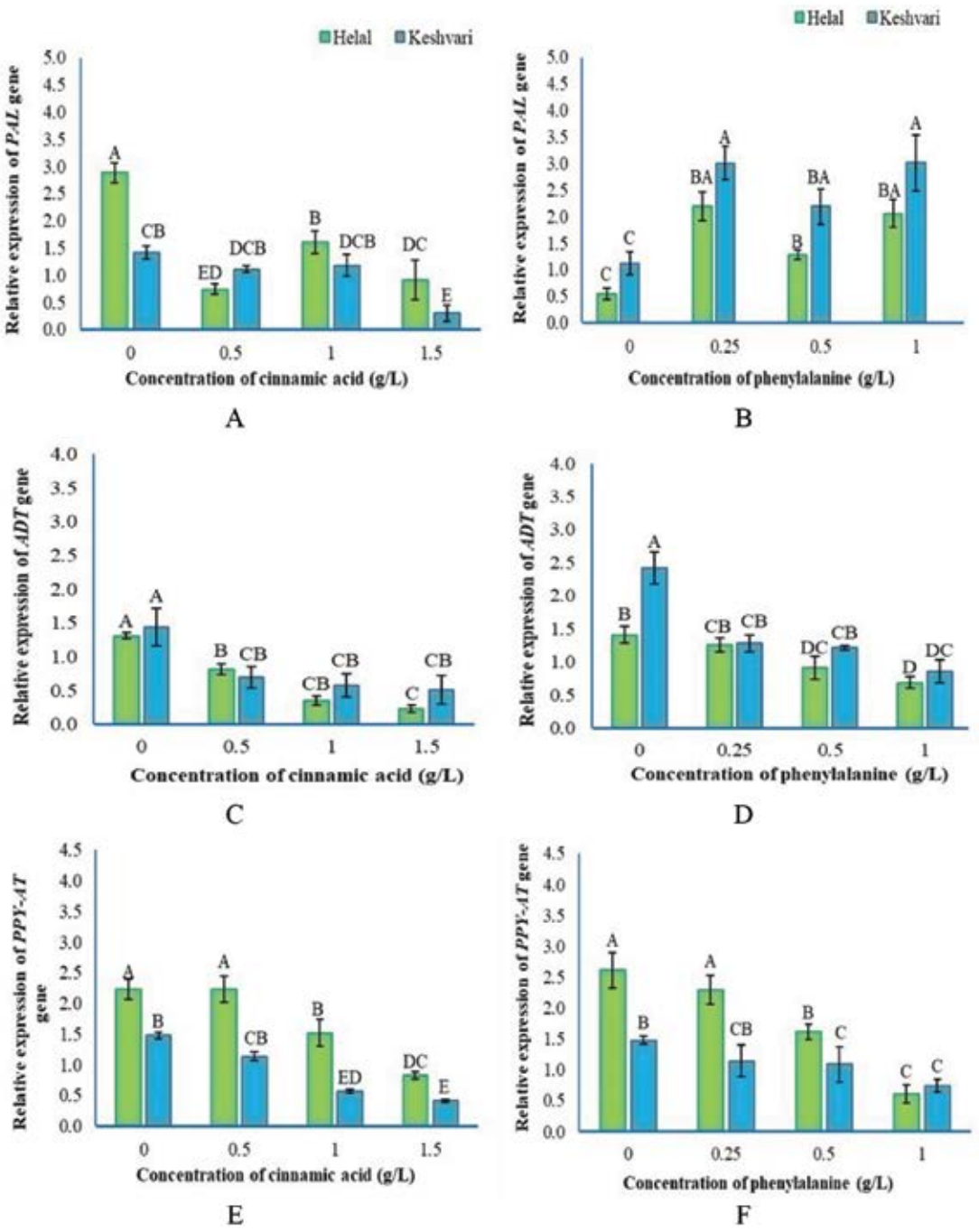


Fig. 3. Relative expression levels of enzymes involved in Phe amino acid biosynthesis and catabolism in rice kernels of the Helal (Green) and Keshvari (Blue) cultivars, treated with various concentrations of CA and Phe spraying. The figure in divided into the following panels: (A) Comparison of PAL gene expression under different CA concentrations, (B) Comparison of PAL gene expression under different Phe concentrations, (C) Comparison of ADT gene expression under different CA concentrations, (D) Comparison of ADT gene expression under different Phe concentrations, (E) Comparison of PPY-AT gene expression under different CA concentrations, (F) Comparison of PPY-AT gene expression under different Phe concentrations. Data are presented as mean values from at least three replicates ± standard error. Significant differences between means are indicated by alphabet mismatches at the probability level of $P \leq 0.05$, according to Duncan's test.

study was not only to assess the effects of Phe and CA treatments but also to evaluate the variability in response between the local rice cultivars, presenting the data by cultivar allowed for a clearer visualization of inter-cultivar differences. Nevertheless, the statistical analyses were performed across both factors (cultivar and treatment concentration), ensuring that the effects of treatment levels within each cultivar were also considered.

PAL activity in rice cultivars is regulated by CA and Phe concentrations

A detailed analysis of the data reveals a profound impact on PAL enzyme activity following the foliar application of biological stimulants (Figure 4). The 1.5 g/L concentration of CA induced the most significant metabolic response in the applied cultivars. Specifically, PAL activity decreased across all concentrations of CA in the Helal variety, whereas in the Keshvari variety, a decrease was observed only at the 1.5 g/L concentration of this stimulant (Figure 4A). In contrast, foliar application of Phe at all three concentrations led to a notable increase in PAL activity in both cultivars (Figure 4B).

Regulatory effects of CA and Phe on protein accumulation in rice seeds

As shown in Figure 5, the total protein content in rice kernels was affected by the CA and Phe foliar spray applications. A downward trend in total protein content was observed under the CA foliar application, with a notable decrease in both cultivars (Helal and Keshvari). Specifically, this indicator significantly decreased at a concentration of 1.5 g/L of the CA stimulant. However, other concentrations of this stimulant did not cause noteworthy changes in the desired factor and acted as an inert substance (Figure 5A). Hence, the concentration of 1 g/L of CA stands out as optimal, as it not only preserved total protein content, gene ex-

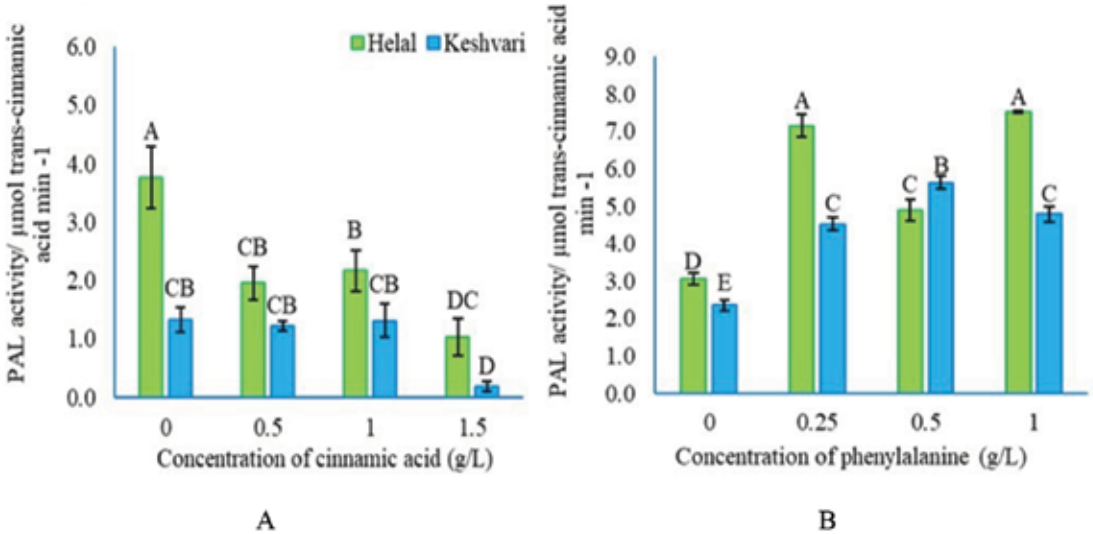


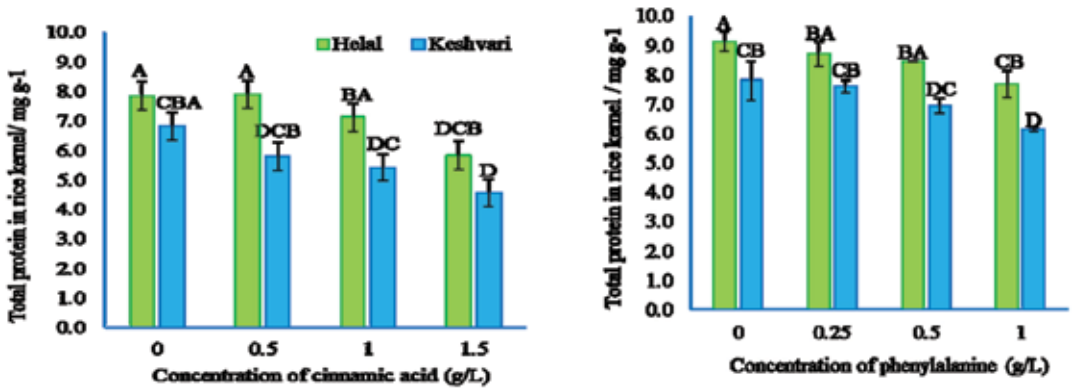
Figure 4. Effect of various concentrations of CA and Phe on PAL enzyme activity in rice kernels from the Helal (Green) and Keshvari (Blue) cultivars. Panels are as follows: (A) PAL enzyme activity under different concentrations of CA, (B) PAL enzyme activity under different concentrations of Phe. Data are presented as mean values from at least three replicates ± standard error. Significant differences between means are indicated by different letters ($P \leq 0.05$), according to Duncan's test.

pression, and PAL enzyme activity but also exerted a suppressive effect on Phe biosynthetic genes (*ADT* and *PPY-AT*). The stimulatory effect of Phe was also significant at the highest applied concentration (1 g/L), resulting in a decrease in total protein. Indeed, the trend remained steady up to the 1 g/L concentration of Phe (Figure 5B). Therefore, the significant consequence observed at this concentration is consistent in both cultivars.

The Phe content in rice Kernel

The lack of a detectable effect of Phe on the analyzed amino acids in Figure 6 can is related to the fact that, although Phe altered the expression of genes associated with its biosynthetic and catabolic pathways, these transcriptional changes did not lead to statistically significant variations in the concentrations of Phe, tryptophan, or tyrosine when compared with the control. In other words, the observed gene expression shifts were not reflected in measurable changes in amino acid levels. Moreover, neither of the rice cultivars displayed a significant alteration in amino acid content under Phe treatment. Likewise, treatment with CA in the Helal cultivar did not produce statistically meaningful differences in amino acid levels. Given that the results were not statistically significant, the related data and graphs were excluded from Figure 6 for the sake of emphasizing on statistically relevant outcomes. Additionally, in this study, Phe content was measured in dehulled and polished rice grains (milled rice) rather than whole kernels with husk and bran, as the aim was to assess the nutritional and biochemical status of the edible portion commonly consumed.

Regarding the figure allocated to the Phe content, it is clear that spraying with the CA elicitor led to a significant response and a substantial reduction in Phe amino acid lev-



A B

Fig. 5. Impact of varying concentrations of CA and Phe on total protein content in rice kernels from Helal (Green) and Keshvari (Blue) cultivars. The figure is divided into: (A) Total protein content in response to different concentrations of CA, (B) Total protein content in response to different concentrations of Phe. Data are expressed as mean values from a minimum of three replicates ± standard error. Significant differences between means are denoted by different letters ($P \leq 0.05$), as determined by Duncan's test.

els (Figure 6A). Ironically, all concentrations (0.5, 1, and 1.5 g/L) resulted in a significant decrease in Phe levels. Interestingly, while the lowest concentration (0.5 g/L) did not cause a noteworthy increase in tryptophan and tyrosine amino acids, higher concentrations (1 and 1.5 g/L) showed a remarkable decline in Phe levels, along with a notable rise in tryptophan and tyrosine. Therefore, as Phe levels decreased with higher concentrations, an increase in other amino acids occurred. There was no substantial difference between the increase in tyrosine levels at concentrations of 1 and 1.5 g/L, indicating that both concentrations led to the same increase in tyrosine, with a similar experience in tryptophan.

Evaluation of agronomic and grain Quality traits

The analysis of spikelet fertility under CA treatment revealed no statistically significant differences among the tested rice cultivars compared with the control. Although a slight decreasing trend was observed in the Helal cultivar at higher concentration (1.5 g/L), this change was not statistically significant (Fig 7, A). Similarly, the response of Helal and Keshvari cultivars to Phe treatment indicated a relative increase in spikelet fertility; however, these variations were not significant when compared to the control (Fig 7, B).

Examination of Figure 7(C, D) indicates that CA and Phe treatments did not cause any statistically significant increase or decrease in the thousand grain weight of the Helal and Keshvari cultivars, and the inherent grain quality of these cultivars was maintained. The analysis of milling recovery showed that the slight reduction observed under CA treatment at 0.5 g/L in the Keshvari cultivar was not statistically significant compared with the control (figure 7, E). Likewise, foliar application of the elicitor Phe did not induce any significant response in this trait (figure 7, F). Finally, the evaluation of He-

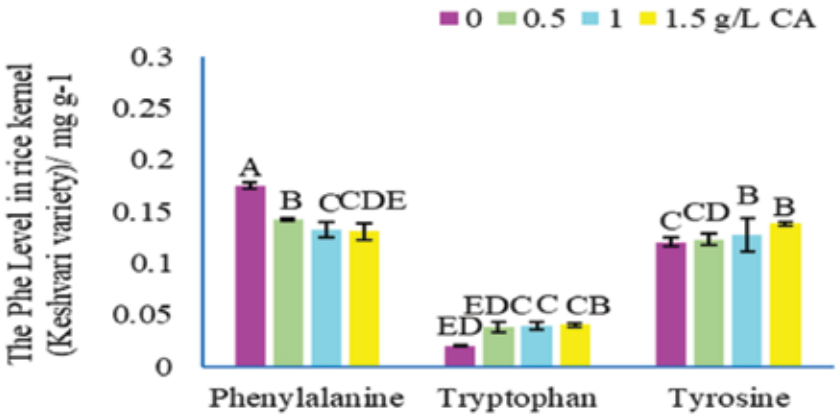


Fig. 6. Impact of varying concentrations of CA on Phe content in rice kernels from Keshvari cultivar. Figure (A) Phe content in response to different concentrations of CA. Data are expressed as mean values from a minimum of three replicates ± standard error. Significant differences between means are denoted by different letters ($P \leq 0.05$), as determined by Duncan's test.

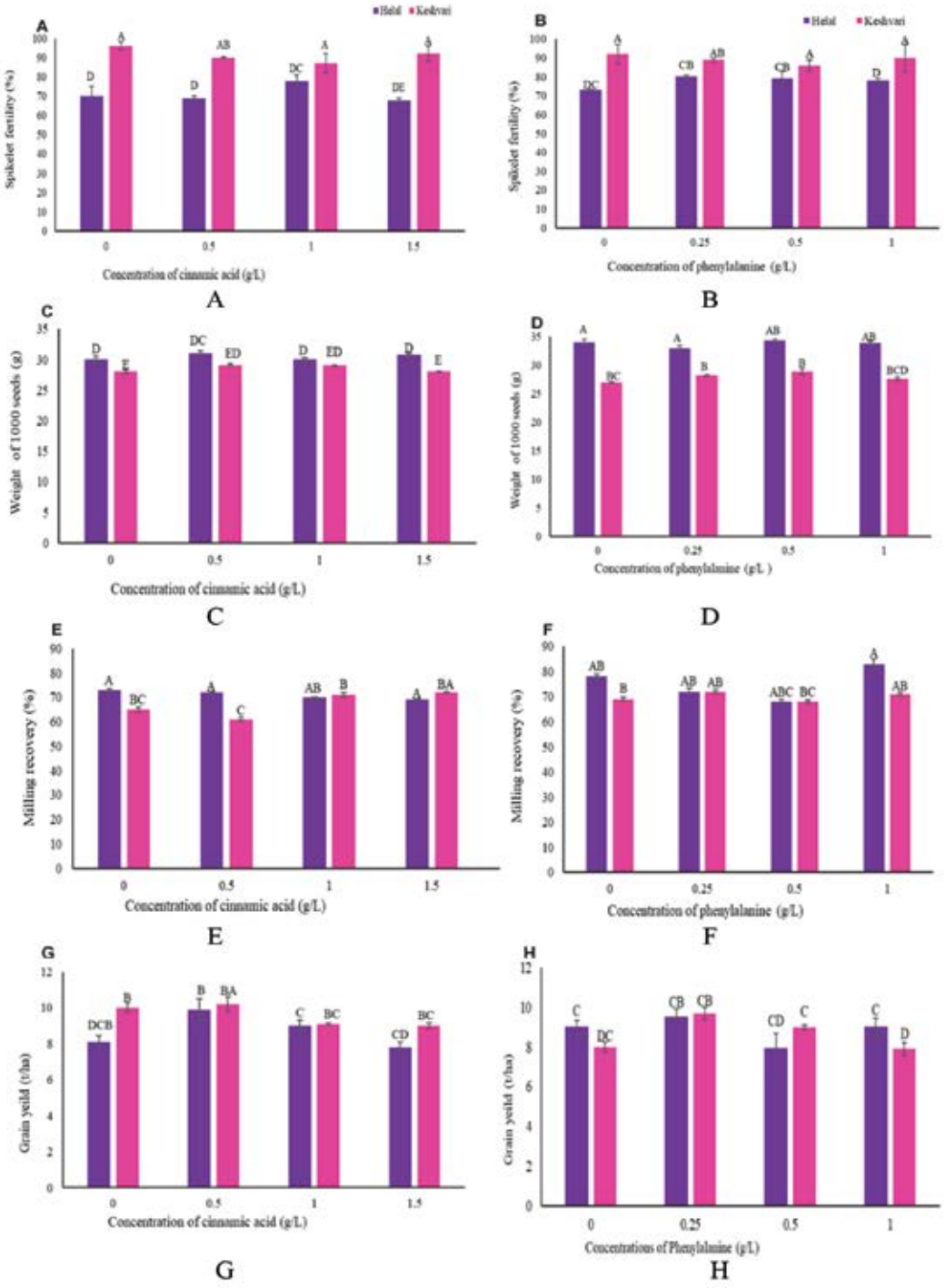


Fig. 7. Evaluation of agronomic and grain quality traits in Helal and Keshvari cultivars under the foliar application of CA and Phe biostimulants; (A) percentage of spikelet fertility under different concentrations of CA, (B) percentage of spikelet fertility under different concentrations of Phe, (C) comparison of thousand-seed weight in consumption varieties treated with CA, (D) comparison of thousand-seed weight in consumption varieties treated with Phe, (E) comparison of milling recovery percentage in Helal and Keshvari cultivars under foliar spraying of CA, (F) comparison of milling recovery percentage in Helal and Keshvari cultivars under foliar spraying of Phe, (G) investigation of grain yield index under CA foliar spraying, (H) investigation of grain yield index under Phe foliar spraying. Data are presented as mean values from at least three replicates ± standard error. Significant differences between means are indicated by alphabet mismatches at the probability level of $P \leq 0.05$, according to Duncan's test.

lal and Keshvari cultivars under the applied treatments indicated that foliar application of these elicitors did not result in any significant increase or decrease in grain yield (figure 7 G, H).

Discussion

There are two schools of thought when it comes to reducing Phe levels in plants. One approach involves inhibiting the expression of genes responsible for its biosynthesis, such as *ADT* and *PPY-AT*, while the other focuses on stimulating catabolic pathways by enhancing *PAL* gene expression and activity. In line with these perspectives, the present study investigated the expression of genes involved in Phe biosynthesis using both inhibitory and stimulatory strategies. Inhibition targeted the suppression of Phe biosynthesis enzymes (*ADT*, *PPY-AT*) in the plastids and cytosol, while stimulation aimed to enhance *PAL* gene expression and activity. In this regard, it was assumed that supplementing with Phe led to its accumulation in plant cells, particularly during specific growth stages, resulting in higher Phe levels for limit interval. Additionally, Phe influenced the synthesis of various compounds, redirecting the pathway to boost tyrosine and tryptophan production. Ultimately, the Phe foliar spray may elevate substrate levels, shifting the arogenate pathway to enhance tyrosine synthesis. The increase in tyrosine production results from altering Phe's metabolic pathway. Elevated substrate levels stimulate PAL activity and enhance its gene expression, while also redirecting the biosynthetic pathway towards tryptophan production. Foliar spraying of Phe acts as a stimulant, reducing Phe content in plant cells and making the plant less dependent on Phe synthesis during growth. This independence is driven by increased substrate levels. After Phe catabolism during seed filling and aging, when amino acid production slows, the amino acids in the seeds are hydrolyzed and used for various metabolic processes. Thus, foliar-applied Phe is catabolized during plant growth, especially during senescence, contributing to metabolic needs. In contrast, in this study, Phe spraying, despite significantly reducing the expression of the *ADT* and *PPY-AT* genes, did not lead to a decrease in Phe content in any of the examined cultivars. Analysis of this factor revealed no significant differences between treated and control samples across any of the consumption varieties over three consecutive years.

The study highlights the effectiveness of foliar Phe application during rice seed growth, reducing Phe biosynthetic gene expression (*ADT* and *PPY-AT*) while significantly increasing *PAL* gene expression, approximately fourfold in Helal and threefold in Keshvari. In comparison, Feduraev (Feduraev, 2020) reported a twofold increase in PAL enzyme activity and a slight rise in gene expression in wheat following external Phe application. Similarly, Peng (Peng, 2023) observed a 53% increase in PAL activity and gene expression in Tartary buckwheat, while Wen (Wen, 2005) found a 1.87-fold increase in PAL activity and gene expression in grape berry after salicylic acid treatment. These findings align with other studies, highlighting the impact of elicitor treatments on PAL regulation. Foliar application of Phe during

the growth stage, before grain ripening, led to a significant reduction in *ADT* gene expression in both rice cultivars. This reduction became more pronounced with higher Phe concentrations. The observed decrease in gene expression, and the potential impact on Phe content, may be influenced by factors such as the timing and concentration of elicitor application, as well as the genetic potential of the cultivars used. In Keshvari, Phe application led to a 1.5-fold reduction in *ADT* expression, while in Helal, *ADT* expression dropped by nearly 50%. This reduction may be due to increased Phe content in the plastid, the primary site of Phe biosynthesis, which can overwhelm its export capacity and cause intermittent accumulation. This feedback regulation reduces carbon flow toward the plastid Phe pathway, inhibiting *ADT* gene expression. Previous studies (Cho et al., 2007; Yamada et al., 2008) suggest that Phe content regulates *ADT* expression, and feedback mechanisms, potentially controlled by redox processes in photosynthesis, also contribute to downregulating biosynthetic genes like *ADT* (Wakasa, 2009).

CA, used as a pseudo-hormone and elicitor, can have dual effects in plants. It was expected to increase PAL activity and gene expression while downregulating Phe biosynthesis genes (*PPY-AT*, *ADT*). However, CA treatment resulted in a surprising decrease in both *PAL* gene expression and activity, as well as reduced expression of Phe biosynthesizing genes. This outcome suggests that CA did not enhance PAL performance as anticipated. However, despite not enhancing gene expression or PAL enzyme activity, it significantly reduced Phe content in the Keshvari cultivar. Additionally, its impact on reducing total protein content and nitrate reductase activity indicates that reduced nitrogen levels may decrease enzyme activities, including those involved in amino acid biosynthesis (Singh, 2013). However, in the present study, CA increased the levels of the amino acids tryptophan and tyrosine. This suggests that it may have acted specifically by enhancing the expression of genes encoding enzymes involved in the biosynthesis of these amino acids, leading to their elevated levels in the harvested samples. The lowered expression of *PPY-AT* and *ADT* genes suggests a reduction in Phe biosynthesis in both plastids and cytosol. Additionally, the decrease in PAL activity may be linked to reduced Phe levels, its substrate. What is more, the effects of CA during the spike filling period may vary due to factors such as genetic potential, elicitor efficacy, and application site. CA and Phe influence Phe catabolism by disrupting biosynthetic pathways and altering key enzymatic processes. Differences in responses across cultivars may result from genetic variation, environmental factors (pH, soil moisture, temperature), plant growth stage, and stimulant concentration.

In addition, this study investigated the cytosolic pathway of Phe biosynthesis, mediated by the *PPY-AT* gene, which serves as an alternative to the plastid pathway (MacDonald 2007). The flux of this pathway increases when entry into the arogenate pathway is restricted (Yoo, 2013; Corea, 2012; Maeda, 2010). Data from qRT-PCR analysis showed that stimulant consumption affected

PPY-AT expression, with higher concentrations eliciting stronger responses. In the Helal cultivar, Phe and CA treatments led to nearly identical metabolic responses, reducing *PPY-AT* expression by about 75%, while Keshvari showed a 50% decrease. The cytosolic chorismate mutase directs carbon flux toward cytosolic Phe production through the phenylpyruvate pathway (Qian, 2019).

Another finding of this study is the significant increase in PAL activity in rice seeds following Phe stimulation. However, this increase was not sufficient to reduce the overall Phe content. Data analysis revealed a strong correlation between *PAL* gene expression and enzyme activity across both cultivars and all tested Phe concentrations. These results are consistent with previous studies, such as (Singh, 2010), which reported enhanced PAL activity in crops like *Pisum sativum* following foliar application of Phe. Plants accumulate phenolic compounds as a defense mechanism or in response to stress or stimulants (Singh, 2003; Singh, 2002; Vermerriset, 2006), with Phe content playing a central role in regulating this accumulation. What is more, PAL activity can be influenced by secondary metabolite content (Jan, 2021; Waterman, 2019). Studies show that foliar spraying with amino acids, such as Phe, boosts defense enzyme activity and resistance to pathogens in plants like Arabidopsis, tomato, and petunia (Oliva, 2020). Moreover, Bahadur (Bahadur, 2012) reported a near doubling of PAL activity in pea leaves with Phe spray, while Ghalamboran (Ghalamboran, 2023) observed a 2.5-fold increase in rice grains using chitosan nanoparticles. In this study, Phe foliar spray resulted in a 4.5-fold increase in PAL activity in the Helal variety and a nearly 2.5-fold increase in Keshvari, compared to controls.

Analysis reveals that reduced *ADT* and *PPY-AT* gene expression correlates with a decrease in total protein content, particularly at the highest concentrations of elicitors. At this concentration, both cultivars exhibited similar responses, thereby triggering a significant metabolic shift. This observation aligns with studies supporting the role of amino acid foliar sprays in enhancing photosynthesis and cell division, ultimately leading to improved plant growth (Levitt, 1980; Reham, 2016; Ping, 2023). Nevertheless, these benefits appear to plateau or reverse at excessive levels, as excessive amino acid uptake can reduce protein content. In support of this, research by Ghalamboran (Ghalamboran, 2023) demonstrated a 2.5-fold reduction in rice seed protein content following chitosan nanoparticle application, which was directly linked to decreased Phe levels.

As previously highlighted, CA application was expected to enhance *PAL* gene expression; however, contrary to expectations, it markedly reduced *PAL* expression in both Helal and Keshvari cultivars. This unexpected reduction may be attributed to suppressed nitrate reductase activity, which in turn leads to decreased nitrogen and amino acid content. Given that *PAL* expression is dependent on Phe availability, it is likely that the decline in substrate contributed to the observed reduction in gene expression. This is further supported by quantitative results, showing a six-fold reduction in *PAL* expression in He-

lal and a 75% decrease in Keshvari, thereby underscoring CA's strong inhibitory effect. In line with these findings, CA elicitor consumption reduces *PAL* gene expression and enzyme activity by disrupting Phecatabolism, as CA acts as a feedback modulator (Zhang, 2015). This inhibitory role is consistent with previous findings, such as those by Blount (Blount, 2000), who observed similar effects in tobacco, and Yuan (Yuan, 2023), who reported reduced PAL activity in taro treated with peppermint extracts rich in p-coumaric acid. In addition to affecting PAL, our results also show that CA reduces the expression of Phe biosynthesis genes, including *ADT* and *PPY-AT*. Interestingly, while CA decreased *ADT* expression in the Helal variety, the Keshvari variety did not exhibit a dose-dependent response, instead maintaining consistently reduced expression levels.

ADT gene expression in Arabidopsis thaliana is regulated by free Phe levels via an allosteric feedback mechanism (Chen, 2016). Though research is limited, evidence suggests a strong link between phenylpropanoid content and *ADT* expression. El-Azaz (El-Azaz, 2020) found a correlation between ADT regulation and lignin content in conifers, indicating secondary metabolites influence enzyme gene expression. Accordingly, CA may directly modulate *ADT* expression and promote lignin biosynthesis which is stems from Phe in monocots (Bubna, 2011). As a matter of fact, the study aimed to investigate gene expression levels related to Phe biosynthesis, focusing on both the plastidial and cytosolic pathways. As mentioned previously, the cytosolic pathway, an alternative to the arogenate (plastidial) pathway, increases in flux when the latter is limited (Yoo, 2021; Corea, 2012; Maeda, 2010). qRT-PCR data showed that certain concentrations of consumptive stimulants significantly affected gene expression. In the Helal variety, *PPY-AT* expression decreased, especially at higher concentrations. Unlike (Qian, 2019), this study found the cytosolic phenylpyruvate pathway is subject to feedback regulation. CA foliar application reduced both *PPY-AT* expression and PAL activity by limiting substrate availability. To further explain, the reduced expression of the *PPY-AT* gene, along with the decreased expression and activity of the PAL enzyme, is due to the availability of the product resulting from the catabolism of the amino acid Phe. This is because the presence of CA indicates an enhanced antioxidant system in the plant, reducing the need for further synthesis of Phe. Therefore, based on the data from amino acid content measurements, it can be concluded that due to the availability of CA, the cytosolic and plastidial pathways for Phe biosynthesis are redirected toward the production of tyrosine and tryptophan, which are required for the synthesis of pigments and plant hormones. This shift occurs as the precursor for many antioxidant compounds is already available to the plant. This aligns with findings by (Blount, 2000; Rajaeian, 2015; Bahadur, 2012; Liu, 2024; Jorrín, 1990). However, Mohagheghian (Mohagheghian, 2021) reported increased PAL activity in tobacco under salinity stress due to CA.

Additionally, Yang (Yang, 2022) confirmed reduced PAL activity in faba beans exposed

to CA. Several studies report that CA diminishes plant protein content by disrupting nitrogen uptake and nitrate reductase activity (López-González, 2023; Hussain, 2011). In lettuce, external CA application decreased protein content, consistent with our findings. Mohagheghian (Mohagheghian, 2021) observed a 12% protein reduction in tobacco under CA treatment, while Singh (Singh, 2013) reported similar declines in maize due to phenolic acids inhibiting amino acid binding during protein synthesis. Since nitrogen is vital for protein formation and tissue growth, its disruption by CA is significant. Kapoor (Kapoor, 2021) showed that CA suppresses nitrate reductase activity, reducing nitrogen and protein levels in *Pisum sativum*. Likewise, Hussain (Hussain, 2017; Wisetkomolmat, 2023) found that high CA concentrations reduce protein content in *Solanum lycopersicum* through decreased synthesis and increased degradation.

Conclusion

The epitome of these discussions is ultimately reflected in the Phe levels, as illustrated below:

Foliar application of Phe suppresses Phe biosynthetic genes but does not affect actual Phe levels in Helal and Keshvari cultivars. Conversely, CA application significantly enhances the tyrosine biosynthetic pathway, increasing tyrosine production. This phenomenon is cultivar-dependent, as only the Keshvari cultivar exhibits reduced Phe content under CA treatment. The Helal cultivar maintains Phe levels despite reduced gene expression. The observed increase in tryptophan levels further confirms the biosynthetic pathway shift, attributed to the chorismate compound favoring tryptophan production. Therefore, foliar application of CA proved effective in two key aspects. The foliar application of CA with higher concentrations induced a shift in the biosynthetic pathway, prioritizing tyrosine production and enhancing tryptophan synthesis. This observation suggests a strong correlation between CA concentration and the alteration of enzyme activity within the amino acid biosynthetic pathway. Notably, the effect varies across different rice varieties, indicating that the species and genome can influence CA's efficacy and its concentration-dependent effects. Precisely, the response of different plant varieties to various concentrations of CA plays a crucial role in determining amino acid production levels and modulating their biosynthetic pathways, with no discernible impact on the quantitative traits. The broader implications of this study highlight the potential of foliar elicitor treatments as a practical, non-GM approach for nutritional biofortification of rice. By demonstrating that CA and Phe can modulate Phe biosynthesis, our findings suggest a feasible strategy for producing rice with reduced Phe content, which could benefit individuals affected by phenylketonuria (PKU). Importantly, preliminary evaluations of agronomic traits (e.g., panicle length, thousand-grain weight, and spikelet fertility) indicated no adverse effects, supporting the practical applicability of this method. Beyond medical nutrition, such treatments may also improve amino acid balance in rice, potentially enhancing its value for general consumers. Considering the limited accessibility of specialized

low-Phe foods in developing countries, integrating this approach into existing rice cultivation systems could provide a cost-effective and scalable solution to address both health-related and agricultural needs.

Author contribution statement

P.D. and M. R. GH. designed and supervised the project. P.D. carried out the experiments and wrote the original draft of the manuscript. M.N. performed cultivation of rice, P.D. and M. R. GH. data analysis, and P.D. and S. B. H. performed gene expression. P.D. and M. R. GH. were responsible for editing the manuscript. All authors agreed on the final version of the manuscript.

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