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Impact of abiotic stress and micronutrient supplementation on the 2-acetyl-1-pyrroline content in KDML105 rice (*Oryza sativa* L.)

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ABSTRACT

This study investigated the effects of abiotic stress and mineral supplementation on 2-acetyl-1-pyrroline (2-AP) content in Thai jasmine rice (*Oryza sativa* L. cv. 'Khao Dawk Mali 105'; KDML105). Rice aroma quality fundamentally influences the market value of aromatic rice. Therefore, enhancing the aromatic compound, 2-acetyl-1-pyrroline (2-AP) could improve rice aroma quality. In this study, KDML105 rice plants at the reproductive stage were used to investigate the effects of abiotic stress (drought, salinity and combined salt-drought stress) and mineral supplementation (Ca, Cu, Mg, Fe, and Zn) on 2-AP content compared to the control plants which were not treated with abiotic stress and exogenous minerals supplementation. The results found that proline levels in plants which were treated with salt stress or combined salt-drought stress accumulated higher leaf proline content (7.7-fold and 9.7-fold increases) compared to those of control plants (30.15 µg/g FW). Although the proline content increased, there were no significant differences in 2-AP levels of the plants. In contrast, the proline content of plants subjected to minerals did not increase while the 2-AP content increased compared to the control plants (3.37 ppm), particularly in plants supplemented with Ca and Cu (6.10 and 5.26 ppm, respectively). These findings indicated that mineral supplements did not stimulate proline production. However, it could induce or suppress other compounds in 2-AP synthesis pathway, resulting in high 2-AP accumulation. This was probably due to the downregulation of the betaine aldehyde dehydrogenase 2 gene (*BADH2*). Not only gene regulations in the pathway, but also external factors, such as the drying process after harvesting, affect 2-AP content. The results revealed that the solar-dried grain method caused higher 2-AP content compared to hot-air-dried and undried grains. Thus, targeted mineral supplementation and appropriate post-harvest processes are involved in enhancing aromatic quality in jasmine rice. In addition, supplemented plants with Ca significantly enhanced FRAP activity accompanied by remarkable increases in both total phenolic and total flavonoid contents.

Keywords: 2-acetyl-1-pyrroline, Aromatic rice, Abiotic stress, Antioxidant capacity, Nutrient supplementation

INTRODUCTION

Khao Dawk Mali 105 (KDML105) rice, internationally recognized as "jasmine rice", holds a distinguished position in Thailand cuisine and the global rice market due to its exceptional physical appearance, cooking quality, and distinctive aroma [1, 2]. The 12th World Rice Conference 2020 reported that jasmine rice dominated the global market [3]. However, Jasmine Rice still faces the challenge of high competition with other rice varieties such as Malys Angkor from Cambodia and ST24 from Vietnam [4]. The significant price premium commanded by KDML105, approximately double that of other rice cultivars in international markets [5], which raises the critical importance of maintaining and enhancing its quality characteristics.

Rice aroma quality fundamentally influences market value [6]. KDML105's distinctive fragrance stems from the accumulation of 2-acetyl-1-pyrroline (2-AP), a volatile compound whose synthesis correlates with proline content [7]. Multiple environmental and physiological factors modulate 2-AP synthesis and degradation, resulting in substantial variations in grain aromatic content [5, 8].

The 2-AP biosynthesis in aromatic rice is composed of complex networks. It is activated by the reformation of glutamate, proline, and ornithine to Δ1-pyrroline-5-carboxylate (P5C) by proline dehydrogenase (PDH), Δ1-pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT), respectively. The pathway culminates in a non-enzymatic reaction between P5C/Δ1-pyrroline and methylglyoxal to form 2-AP [9, 10]. Previous studies examining 2-AP accumulation in various

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above-ground tissues of rice plants have demonstrated that the flag leaf exhibits the highest 2-AP content. Consequently, utilizing this specific tissue for analyzing the relationship and gene expression patterns associated with aroma compound biosynthesis could enhance our understanding of the underlying mechanisms of 2-AP formation [11].

It is reported that when aromatic rice grows under unappreciated growth conditions such as rain-fed, drought, or dry soil, it results in an increase in 2-AP content in its grains [7]. Moreover, high 2-AP levels in rice grains may depend on farming system. [5] presented that aromatic rice plants grown under organic farming systems had high 2-AP levels which were caused by high levels of nitrogen, humic acid and microbial populations found in the system. Not only are growth conditions and farming systems involved in 2-AP accumulation in grains, but mineral elements that plants absorb also influence 2-AP content. Each mineral element contributed to variation in 2-AP accumulation in rice plants depending on the growth stage of rice plants. For example, boron could induce high accumulated 2-AP during the heading stage [12]. Previous studies have showed that 2-AP increases under various stress conditions, which are the same conditions that activate the antioxidant system. Furthermore, the biosynthesis of 2-AP is associated with the metabolic pathways of proline and other precursors, which are directly linked to the plant's antioxidant system [13, 14].

The objective of this work was to induce high 2-AP accumulation in rice grains by optimizing growth conditions and evaluating the effects of individual micronutrient supplementation on 2-AP accumulation in rice grains. This included studying how individual micronutrient supplementation affects 2-AP accumulation in rice grains. Post-harvesting processes were also determined to compare the best way to preserve 2-AP in rice grains. The effects of micronutrient supplementation on antioxidant compounds were also investigated.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Seeds of rice (Oryza sativa L. cv. 'KDML105') used in this study were provided by the Surin Rice Research Center, Thailand. Preliminary experiments tested salt concentrations of 25, 50, 75, 100, 125 and 150 mM, and water deficit periods of 3, 5, 7, 9 and 11 days. The concentration of 100 mM NaCl and 7-day water deficit were selected as they enabled rice growth to the reproductive stage with maximum proline accumulation. The rice seeds were soaked in water for 2 days following by germinating in water-soaked tissues for 7 days. Then, four seedlings were transferred to a plastic pot containing 5 kg of soil and grown under natural sunlight in a greenhouse. There were five replications per group. When the growth of rice plants was in the booting stage (approximately 60 days after transplantation), plants were divided into 9 groups as follows: control, no abiotic stress and no minerals supplementation, drought stress (water withheld for 7 days), then rewatering until the heading stage, salt stress (plants were watered with 100 mM NaCl for 10 days), then watered with water without NaCl solution until they reached the heading stage. For the combined stress treatment, rice plants were watered with 100 mM NaCl solution for 7 days followed by 7 days of water withholding. For the exogenous mineral applications that have been modified from Yoshida's rice nutrient solution formula [15], calcium 0.8 M ($CaCl_2.2H_20$), copper 1.24 mM ($CuSO_4.5H_2O$), magnesium 1.3 M ($MgSO_4.7H_2O$), iron 28.5 mM ($FeCl_3.6H_2O$) and zinc 1.22 mM ($ZnSO_4.7H_2O$). Each pot was supplied with 5 L per day for 10 days until the plants reached the heading stage. The plants in these groups were then allowed to grow until grain formation stage. There were five replications per group.

2-Acetyl-1-pyrroline (2-AP) content analysis

To analyze 2-acetyl-1-pyrroline (2-AP), first configure the Gas Chromatography (GC) system by setting the Nitrogen-Phosphorus Detector (NPD) to 250 °C, the column oven to 200 °C, and the Helium carrier gas flow to 3 ml/min, while maintaining detector gas flows of 80 ml/min for Zero Air and 4 ml/min for Hydrogen. Next, set the Headspace (HS) parameters with an oven temperature of 120 °C, an injector at 130 °C, and a transfer line at 140 °C. Once both systems reach their setpoint temperatures and the NPD baseline signal is stable, load the prepared sample vials into the HS autosampler tray and initiate the sequence by clicking "Start Run" to analyze all samples sequentially. Finally, quantify the 2-AP concentration by comparing the sample peak area against a 2-AP standard calibration curve [16].

RNA extraction and gene expression analysis

Total RNA was extracted from rice leaf tissues (0.1 g) using TRI reagent (Thermo Fisher Scientific, Waltham, MA, USA). DNase treatment was performed to eliminate genomic DNA contamination by RQ1 RNase-Free DNase kit, Promega. RNA quantity was measured with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.) following protocols described by [17]. Then, the expressions levels of genes associated with 2-AP biosynthesis, ornithine aminotransferase (OAT), proline dehydrogenase (PDH), Δ1-pyrroline-5-carboxylate synthetase (P5CS), and betaine aldehyde dehydrogenase 2 (BADH2), were determined. The actin gene was used for calculating relative expression level.

Grain drying process

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To evaluate the effect of drying methods on 2-AP content, rice grains were treated under three drying conditions: (1) solar drying for 3 days, (2) hot air drying at 60°C for 3 days, and (3) no drying (control). Following the drying treatments, grain samples were analyzed for 2-AP content using the previously described analytical method to determine the influence of post-harvest processing on aromatic compound retention.

Total phenolics content, total flavonoid content and antioxidant capacity in rice grains

To determine bioactive compounds and antioxidant properties, two grams of dehusked rice seeds were used to quantify total phenolic compounds (TP) and total flavonoid compounds (TF). In addition, ferric reducing antioxidant power (FRAP) was measured as an indicator of antioxidant capacity using the method described by [18, 19].

Proline content determination

Fresh rice leaves (0.1 g) and roots (0.2 g) were cut into small pieces, placed in a mortar, and ground thoroughly. Five milliliters of 3% sulphosalicylic acid were added and grinding continued until the tissue turned brown. The mixture was filtered through filter paper, and the filtrate volume was measured. Two milliliters of filtrate were transferred to a screw-cap test tube, mixed with 2 mL glacial acetic acid and 2 mL ninhydrin using a vortex mixer, then boiled in water for 1 hour. The reaction was stopped by cooling in ice water. Four milliliters of toluene were added and mixed using a vortex mixer. After allowing air bubbles to dissipate, the pink-colored toluene layer was collected and absorbance was measured at 520 nm. Proline content was determined using a standard curve and calculated using the formula (Eg.1):

$$\text{Proline content (mg/g tissue)} = [V \times \text{proline from standard curve (mg)}] / (2 \times W) \quad (1)$$

where V = total volume of extract (mL)
and W = leaf weight (g) [20].

Statistical analysis

The experiment employed a randomized complete block design (RCBD) with five replications for each treatment. Statistical significance among treatment means was determined using analysis of variance (ANOVA), with significance determined at $P < 0.05$. Multiple comparisons were performed using Duncan's multiple range test (DMRT) to identify specific differences between treatment groups.

RESULTS AND DISCUSSION

The effects of abiotic stress (drought, salt, and combined drought and salt stress) and mineral supplementation (Ca, Cu, Mg, Fe, and Zn) on proline and 2-AP contents were systematically investigated in this study. It was found that abiotic stress treatments caused a mark increase in proline accumulation in rice leaves at the booting stage (Table 1). The highest proline content was observed in combined salt and drought stress (291.54 µg/g FW), followed by those exposed to salt stress alone (231.43 µg/g FW). Notably, there was no significant difference between those groups. In contrast, mineral supplementation treatments resulted in proline concentrations ranging from 25.81 to 32.37 µg/g FW, with no significant differences observed among treatments.

These findings indicated that exposure of rice plants to abiotic stress, particularly salt stress or combination between salt and drought stresses, induced significant accumulation of proline, while mineral supplementation did not significantly alter proline levels compared to the control.

Conversely, 2-AP content in rice grains harvested from plants grown under abiotic stress treatment did not show any significant difference. These findings are inconsistent with previous studies that presented that high 2-AP content was found in rice grains collected from plants subjected to salt or drought stress [21, 22]. This may be caused by differences between NaCl concentrations and stress duration employed in this experiment.

Nevertheless, the highest 2-AP content (3.97 ppm) was found in grains collected from salt-stressed plants compared to other abiotic stress treatments which were range approximately (3.22 – 3.37 ppm). Under the mineral supplementation, exogenous Ca was able to enhance the highest 2-AP content (6.10 ppm) followed by supplementation with copper (5.26 ppm) (Table 1).

It is generally known that a marked increase in proline level is found when plants grow under inappropriate conditions, such as abiotic stress, to protect plant cells from abiotic stress [23]. In this case, it acts as an osmoprotectant that is why the proline level observed in this experiment was higher in plants treated with drought, NaCl, and combined drought and salt stress. According to the result, it suggested that high proline accumulation in the booting stage did not associate with high 2-AP accumulation in rice grains, though proline is the molecule involved in 2-AP synthesis. [24] suggested that forming 2-AP in grains is associated with the balance of carbon and nitrogen, grain filling conditions, and the expression level of the *BADH2* gene, which occurred later.

In contrast, supplementing plants with micronutrients did not induce stress and resulted in low proline accumulation. As mentioned earlier, 2-AP formation in grains depends on several factors. Exogenous Ca may stimulate supporting factors such as carbon and nitrogen assimilation and sugar metabolism and enhance enzyme activities leading

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to high 2-AP accumulation [25, 26]. These results were aligned with previous research reporting that appropriate concentrations of mineral elements such as nitrogen, phosphorus, calcium, manganese, zinc, boron, copper, and selenium can promote 2-AP content in aromatic rice grains [27]. The research by [28] pointed out that nano fertilizers containing calcium (and phosphorus; hydroxyapatite nanoparticles) improved 2-AP content in rice grains, suggesting a beneficial role for calcium in stimulating 2-AP production.

Table 1 Proline content in leaves at the booting stage and 2-AP content in rice grains following drought treatment, salt treatment, combined salt and drought stresses and various mineral supplementation treatments.

Treatment	**Leaf of booting stage rice Proline (µg / gFW)**	**Rice grains 2-AP (ppm)**
Control	30.15±1.65^{b}	3.37±0.48^{c}
Drought (7days)	50.85±6.45^{b}	3.22±0.28^{c}
NaCl (100 mM)	231.43±61.15^{a}	3.97±0.10^{bc}
Drought + NaCl	291.54±53.63^{a}	3.37±0.54^{c}
Ca	25.81±3.66^{b}	6.10±0.40^{a}
Cu	26.91±3.74^{b}	5.26±1.46^{ab}
Mg	32.37±4.85^{b}	3.94±0.05^{bc}
Fe	27.16±2.05^{b}	3.65±0.82^{bc}
Zn	26.87±5.17^{b}	4.28±0.71^{bc}

Different letters in the same column indicate significant differences at the 95% confidence level ($p < 0.05$) using Duncan's Multiple Range Test (DMRT).

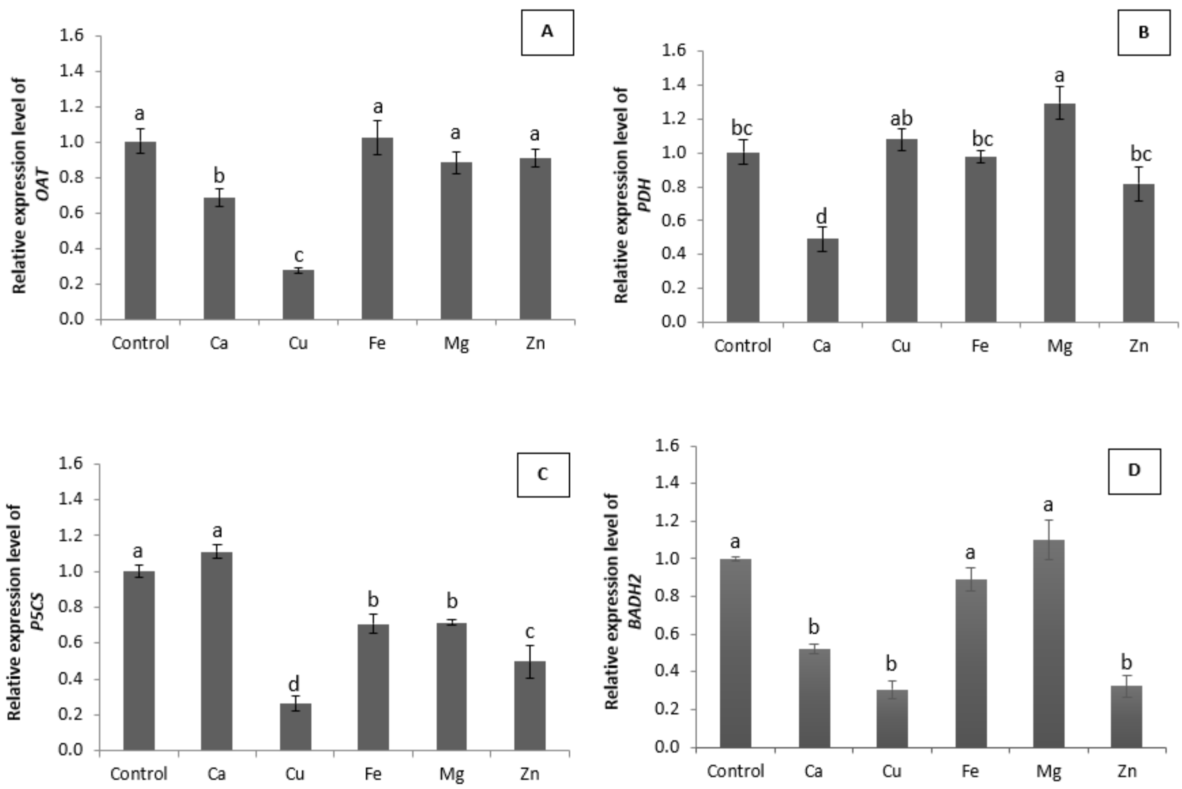

Figure 1 Relative gene expression levels of genes involved in the 2-AP biosynthesis pathway (a) *OAT*, (b) *PDH*, (c) *P5CS*, and (d) *BADH2* in the leaf of the booting stage of rice plants supplemented with no micronutrient supplementation (control), Ca, Cu, Fe, Mg, and Zn. The histogram shows the relative abundance of mRNA for each gene and each treatment after normalization with *OsActin*. Different letters in the bar were significantly different at the level of 95% ($p<0.05$) using Duncan's New Multiple Range Test (DMRT).

Since mineral supplementation led to higher 2-AP accumulation than abiotic stress treatments, we examined the expression of genes associated with the 2-AP biosynthesis pathway. There are several amino acid precursors (proline, ornithine, and glutamate) and enzymes (proline dehydrogenase (PDH), ∆1-pyrroline-5-carboxylic acid synthase (P5CS), and ornithine aminotransferase (OAT)) involved in this

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pathway. Among these, inactivation of betaine aldehyde dehydrogenase (BADH2) gene expression is considered to play an important role in contributing to 2-AP production [29, 30].

In this study the transcription levels of ornithine aminotransferase (*OAT*) were significantly decreased when plants were supplied with exogenous Cu and Ca by 3.6- and 1.5-fold, respectively, compared to the control (Figure 1a). For proline dehydrogenase (*PDH*), the highest expression was found in plants applied with exogenous Mg (1.3-fold higher than the control) whereas its expression was suppressed approximately 2.0-fold when plants were treated with exogenous Ca (Figure 1b). The expression of ∆1-pyrroline-5-carboxylate synthetase (P5CS) was noticeably down regulated in response to Mg, Zn, Cu, and Fe treatments compared to the control group. The Cu treatment resulted in the most significant decrease in P5CS transcript levels, which were 3.8-fold lower than the control (Figure 1c). Betaine aldehyde dehydrogenase 2 (BADH2) expression was markedly reduced in plants supplied with Ca, Cu, and Zn compared to the control, with *BADH2* transcript levels 3.1-fold lower in Cu and Zn treatments. No significant differences in *BADH2* expressions were observed.

Although supplementation with Ca, Cu, and Zn resulted in varying expression levels of OAT, PDH, and P5CS, BADH2 expression was significantly downregulated in these treatment groups compared to the control (Figure 1). Previous studies have established that *BADH2* expression is negatively associated with 2-AP accumulation in scented rice cultivars [29, 31]. Therefore, the observed reduction in *BADH2* gene expression may contribute to the elevated 2-AP content in these treatment groups (Table 1).

Furthermore, [32] reported that the highest 2-AP content occurs in mature rice grains, which typically exhibit low proline levels and reduced P5CS expression. This suggests that proline produced in leaves might be directly translocated to rice grains and subsequently converted into 2-AP. Two mechanisms have been proposed for 2-AP accumulation in mature grains: (1) 2-AP synthesized in leaves and stem sheaths is transported to grains, and (2) proline is translocated from leaves to grains, where 2-AP synthesis subsequently occurs [33, 34]. The lack of significant upregulation of the three genes involved in the initial steps of 2-AP synthesis in leaf tissue supports these translocation hypotheses.

Table 2 Total phenolic content, total flavonoid content and FRAP value in grains of rice plants under various minerals treatments.

Treatment	TP (mg GA E/100 g DW)	TF (mg Cat E/100 g DW)	FRAP (mmol Fe^{+2}/100 g DW)
Control	22.10 ±2.34^{bc}	18.27 ±6.64^{ab}	9.27 ±0.9^{b}
Ca	27.60 ±2.28^{a}	20.80 ±0.00^{a}	14.93 ±2.2^{a}
Cu	25.35 ±1.61^{ab}	17.82 ±2.84^{ab}	9.23 ±0.9^{b}
Mg	25.00 ±0.62^{abc}	14.50 ±3.93^{ab}	8.20 ±1.2^{b}
Fe	24.85 ±0.36^{abc}	12.60 ±2.90^{b}	8.10 ±1.2^{b}
Zn	22.58 ±1.62^{bc}	19.53 ±3.93^{ab}	8.16 ±1.1^{b}

TP: Total Phenolic content; TF: Total Flavonoid content; FRAP: Ferric Reducing Antioxidant Power.
Difference letters in the same column were significantly different at the level of 95% ($p<0.05$) using Duncan's New Multiple Range Test (DMRT).

The effects of different mineral treatments on bioactive compounds and antioxidant properties in rice grains are presented in Table 2. The antioxidant capacity and bioactive compounds in rice grains are primarily attributed to polyphenols [35, 36], which have been reported to confer health benefits against various diseases [37]. Calcium supplementation enhanced total phenolic content, total flavonoid content, and FRAP activity in rice grains (Table 2). Rice grains from plants treated with calcium exhibited the highest concentration of phenolic compounds (27.60 mg GAE/100 g DW), although no significant differences in phenolic content were observed among treatments. Similarly, calcium supplementation resulted in the highest flavonoid content in rice grains, though no statistically significant difference was detected between the Ca treatment and control. For FRAP, its activity was also highest in grains from plants treated with calcium (14.93 mmol Fe^{2+}/100 g DW), significantly exceeding values observed in other mineral treatments and the control group. These findings were consistent with previous studies demonstrating that calcium pretreatment positively influences polyphenol content and antioxidant capacity [38, 39]. These results indicate that calcium supplementation enhanced total phenolic content, total flavonoid content, and FRAP activity in rice grains (Table 2), consistent with previous studies demonstrating that calcium pretreatment positively influences polyphenol content and antioxidant capacity [38, 39]. Therefore, calcium supplementation during rice cultivation not only increases 2-AP content but also enhances the accumulation of bioactive compounds and improves antioxidant capacity.

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The association between drying methods and 2-AP content was shown in Table 3, solar-dried rice grains contained significantly higher 2-AP content compared to both oven-dried grains at 60°C and the non-dried control group. These findings indicate that the drying method significantly influences the retention and possibly the formation of 2-AP in rice grains during post-harvest processing.

It is reported that temperature significantly influences 2-AP level in rice grain by impacting enzyme activity, carbon availability, and the metabolic capacity of the grain [40]. This suggests that temperature is a critical factor affecting 2-AP content, with moderate temperatures being optimal. [41] reported that low temperatures (22/16°C) induced high accumulation of 2-AP in rice grains, which explains why hot air drying at 60°C might negatively affect 2-AP synthesis or retention.

The average temperature during solar drying in our study was 35-38°C, indicating that this moderate temperature range may be optimal for enhancing or preserving 2-AP content. However, it is important to note that the response of 2-AP content to temperature variations depends on rice varieties, geographical factors, and exposure duration.

Table 3 2-AP content in rice grains with different grains drying methods.

Drying methods (day)	2-AP (ppm)
No drying	3.22 ± 0.28^{b}
Solar drying for 3 days	5.40 ± 0.76^{a}
Heated air drying at 60 °C for 3 days	3.51 ± 0.79^{b}

Difference letters in the same column were significantly different at the level of 95% ($p<0.05$) using Duncan's New Multiple Range Test (DMRT).

CONCLUSIONS

The aromatic quality of rice grains, 2-AP level, after harvesting depends on many factors, for example abiotic stress induction, exogenous mineral application and post-harvest processes. The results of this study revealed that treating plants with abiotic stress did not provide any beneficial effects on 2-AP content. Conversely, supplementing plants with minerals especially, Ca had positive effects on 2-AP production since it could suppress the expression of BADH2, the key gene in the 2-AP synthesis pathway. Furthermore, exogenous Ca induced high accumulation of antioxidant capacity in rice grains. The process of drying grains after harvesting is also associated with 2-AP content. Among the methods used in this study, the solar drying method was the most effective for preserving aroma quality in rice grains.

DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors used Grammarly Premium for English grammar and used Claude.ai to create the graphical abstract. All scientific content, data analysis, and interpretation were performed solely by the authors without AI assistance.

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