



Research article

Modulating γ -aminobutyric acid content across the vegetative and generative stages of black glutinous rice (*Oryza sativa* L.) via drought stress

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Abstract: γ -aminobutyric acid (GABA) is a non-protein amino acid naturally present in rice with health benefits. GABA is synthesized through glutamate decarboxylation, facilitated by glutamate decarboxylase, due to drought stress in the environment. This study assesses the effects of drought stress on GABA content in black glutinous rice during vegetative and generative stages, followed by an investigation of rewatering effects at each growth stage. Drought stress was simulated in vivo using PEG-6000 concentrations of 0% and 10%, applied at vegetative and generative stages. In the generative phase, providing 10% PEG-6000 increased GABA content, H_2O_2 , and MDA levels, while also causing photosynthetic disruption and chlorophyll reduction. In contrast, due to the rewatering process, GABA production decreased, along with upregulation of GABA-T gene expression during both vegetative and generative stages. These findings suggest that increased GABA production may contribute to tolerance to PEG-induced osmotic stress in black glutinous rice, based on metabolic adjustment, gene expression, and antioxidant activity. Increased GABA accumulation was associated with enhanced physiological and biochemical response to PEG-induced osmotic stress, suggesting that GABA metabolism may contribute to the adaptive response of black glutinous rice under osmotic stress.

Keywords: GABA; drought stress; black Glutinous rice; gene expression; photosynthetic system

1. Introduction

Rice (*Oryza sativa* L.) is a crucial food crop that functions as the primary dietary source for the major Indonesian population [1]. Rice demand escalates alongside population growth, with per capita rice consumption reaching 111.58 kg per person each year [2]. However, anthropogenic activities have played a role in global warming, shifting climates, and extreme weather events, such as prolonged droughts and heavy rainfall in Indonesia. According to Abebaw [3], climate change disrupts planting schedules and causes crop failures, specifically under drought conditions. As an abiotic stressor, drought reduces plant growth and significantly decreases rice productivity up to 15%–20% [4].

As a global-scaled primary limiting factor in crop production, drought stress occurs when plants experience inadequate water supply, which disrupts their optimal growth and development [5]. Panda et al. [6] clarified that drought stress induces oxidative stress, due to photosynthetic activity reduction that leads to the accumulation of reactive oxygen species (ROS). These ROS can disturb the oxidative compounds and the antioxidant balance in plants. Therefore, drought stress affects physiological processes and crop yield production [7]. In response to drought conditions, plants often modify their root system architecture, alongside primary and secondary metabolites. A primary metabolite in rice, like γ -aminobutyric acid (GABA), has been reported to elevate under drought conditions [8].

GABA is a non-protein amino acid synthesized from glutamate via the decarboxylation process [9]. As a neurotransmitter within the human brain, GABA has been massively popular in various health products [10]. GABA reduces blood pressure and suppresses the proliferation of cancer cells. Insufficient GABA levels in the body have been associated with mental health disorders and neurological issues, such as anxiety, depression, insomnia, and epilepsy [11]. Therefore, further studies are necessary to enhance GABA levels in pigmented rice. Increasing GABA in plants may mitigate ROS levels, which are detrimental radicals that can impair plant cellular functions.

Rice contains numerous bioactive compounds that may provide health benefits [12]. These benefits have been associated with reducing with cancer, coronary heart disease, and hypertension [13]. Pigmented rice, including black rice, has been shown to possess greater nutritional value. Mbanjo et al. [14] and Jaksomsak et al. [15] demonstrate that pigmented rice contains higher nutrient levels compared to polished white rice. Nevertheless, pigmented rice often exhibits a more intricate texture, which may reduce market acceptance.

Inducing stress treatments can enhance the nutritional and sensory qualities of rice. Pigmented rice, such as brown, red, and black rice, contains different levels of bioactive compounds, including GABA [16]. Kang et al. [17] demonstrated that black rice had the highest GABA content (9.39 mg/100 g dry weight), compared to red and brown rice. To mimic drought conditions, dehydration stress can be simulated using polyethylene glycol (PEG-6000), which reduces water availability in the growth medium by creating osmotic pressure [18,19]. However, the application of PEG-6000 to black glutinous rice to induce dehydration stress and increase GABA levels in vivo has not yet been investigated further. Therefore, this study aimed to evaluate the physiological, biochemical, and molecular responses of black glutinous rice to PEG-induced osmotic stress during the vegetative and generative stages, with particular emphasis on GABA accumulation, oxidative stress markers (H_2O_2 and MDA), antioxidant capacity, and GABA-T gene expression before and after rewatering.

2. Materials and methods

2.1. Plant materials and experimental conditions

This study was carried out at the Center of Excellence on Crop Industrial Biotechnology (PUI-PT BioTIn) and the Agrotechnopark Jubung, Universitas Jember (8°11'12.2"S 113°38'08.6"E). Homogeneous and healthy local black glutinous rice seeds were used as the plant material and were grown under the same conditions. Black glutinous rice seeds were sown individually in plastic pots (25 cm diameter × 30 cm height) containing 8 kg of planting medium consisting of topsoil, compost, and sand (2:1:1, w/w/w). Plants were maintained under greenhouse conditions at the Agrotechnopark Jubung, University of Jember, with a temperature of 28 ± 2 °C, relative humidity of 70%–80%, and a natural photoperiod of approximately 11 h light/13 h dark. Plants were irrigated daily to maintain field capacity until the initiation of PEG-induced osmotic treatment.

The experiments involved two treatment factors with five replications. The treatment factors included PEG-6000 concentration (0% and 10%, w/v) and application stage (vegetative and generative). Drought was simulated by applying 0% and 10% of PEG-6000 to the planting medium [20]. For the vegetative stage, PEG-6000 was applied 20 days after sowing for two weeks. For the generative stage, PEG-6000 was applied at panicle emergence for two weeks [21]. During the stress treatment, no additional irrigation was provided to the PEG-treated pots other than the designated PEG solution. Following the two-week stress period, plants were rewatered daily with tap water for seven days until soil moisture returned to approximately 80%–90% of field capacity. Rewatering was evaluated by regular watering for a week after the PEG-induced osmotic stress. The rewatering process was determined to indicate the plant's response to tolerance to PEG-induced osmotic stress. Visual signs such as re-greening leaves were used to assess the rewatering process.

2.2. Chlorophyll content

The chlorophyll content was determined using a method developed by Lichtenthaler and Babani [22]. A centrifuged-leaf extract sample (0.10 g of fresh leaf) was taken and mixed with methanol (p.a.) to 0.3 mL. The mixture was then placed into a microtube and re-centrifuged at 10,000 rpm for 10 min at 4 °C. Next, 200 µL of the supernatant was mixed with 800 µL of methanol (p.a.) and transferred to a microtube. The mixture absorbance was measured using a spectrophotometer at wavelengths of 470 nm, 652.4 nm, and 665.2 nm. The concentration of total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids were calculated according to Lichtenthaler and Babani [22] using the following equations (Eqs (1)–(4)):

$$\text{Total chlorophyll (mg/g)} = [(1.44 \times A_{665.2}) + (24.93 \times A_{652.4})] \times ((V/1000) \times W) \quad (1)$$

$$\text{Chlorophyll a (mg/g)} = [(16.72 \times A_{665.2}) - (9.16 \times A_{652.4})] \times (V/1000) \times W \quad (2)$$

$$\text{Chlorophyll b (mg/g)} = [(34.09 \times A_{652.4}) - (15.28 \times A_{665.2})] \times (V/1000) \times W \quad (3)$$

$$\begin{aligned} \text{Total carotenoids (mg/g)} = & [(1000 \times A_{470}) - (1.63 \times \text{Chlorophyll a}) \\ & - (104.96 \times \text{Chlorophyll b}) / 221] \times (V/1000) \times W \end{aligned} \quad (4)$$

where A is the absorbance at α , V is the total volume of sample extract (after centrifugation), and W is

the sample weight. Pigment concentrations were subsequently converted to mg/g fresh weight (FW) using the total extract volume and fresh sample weight. Total chlorophyll was calculated independently from the absorbance values using the equation of Lichtenthaler and Babani rather than by summing chlorophyll a and chlorophyll b.

2.3. GABA content

The GABA levels extraction process followed the methodology by Karladee and Suriyong [23]. The rice samples were first ground into a fine powder using a mortar. Approximately 300 mg of leaf tissue (dry weight basis/DW) was used for determining leaf GABA content, and finely ground rice flour (dry weight basis/DW) was used for determining grain GABA content. It was then transferred to a test tube and dissolved in 80% ethanol, vortexed, and filtered through filter paper. The filtrate was placed in a water bath at 80 °C to evaporate the ethanol. After evaporation, 0.5 mL of distilled water was added, and the mixture was centrifuged at 10,000 rpm for 10 min for layered separation. After centrifugation, the upper floating layer was removed. Subsequently, 0.2 mL of 0.2 M borate buffer and 1.0 mL of 6% phenol were added to the remaining solution. For standard solution preparation, various concentration (0.1–0.6 mg/mL) of GABA were added to test tubes, followed by 0.2 mL of borate buffer and 1.0 mL of phenol reagent.

The rice flour extract was thoroughly mixed and placed in a cooling bath for 5 min. The 0.4 mL of NaOCl solution (either 10% or 15%) was added to the extract, and mixed vigorously for 1 min. The mixture was then returned to a cooling bath for another 5 min. In the final step, the solution was boiled in a water bath at 100 °C for 10 min. The extract was transferred to a second cooling bath while the water bath was heated up and boiled the solution. Once cooled, the solution absorbance was measured using UV-visible spectrophotometry at 630 nm. A GABA stock solution (1 mg/mL) was prepared by dissolving analytical-grade γ -aminobutyric acid in distilled water. Working standards (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL) were prepared by diluting the stock solution with distilled water. The calibration curve was constructed by plotting absorbance against GABA concentration, and sample concentrations were calculated from the resulting linear regression equation. The absorbance values (x) are plotted against a GABA standard curve ($y = 10.14x + 0.05$, $R^2 = 0.98$). The blank solution consisted of 2.0 mL of 80% ethanol. GABA concentration was calculated from the regression equation and expressed as mg/100 g dry weight.

2.4. Determination of H_2O_2 , and MDA contents

Reactive oxygen species (ROS)-related parameters were analyzed to evaluate oxidative damage and cellular toxicity under stress conditions. Hydrogen peroxide (H_2O_2) content was determined following the method of Jira-Anunkul and Pattanagul [24], which involved mixing the extract with 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide, followed by absorbance measurement at 390 nm. Malondialdehyde (MDA) content was determined according to Miftahuddin et al. [25]. Samples were mixed with 20% trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (TBA), incubated at 95 °C for 30 min, and subsequently measured at 532 and 600 nm.

2.5. Determination of free amino acid and total soluble protein

The analytical procedure for the total amino acid determination was adapted from Noviyanti et al. [26]. Approximately 0.10 g of fresh leaf tissue was homogenized in 0.3 mL of 50 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The resulting clear supernatant was collected and used as the crude extract for the determination of free amino acids and total soluble protein. Twenty-five microliters of supernatant were taken, followed by the addition of 400 µL of 0.2 M potassium phosphate buffer (pH 8). Then, 200 µL of 0.1% TNBS was added, vortexed, and incubated at 50 °C for 30 min. The 400 µL of 0.1 M Na₂SO₃ was added, vortexed, and incubated for 15 min at room temperature, after the absorbance was measured at 420 nm. Total amino acid content (mg/mL) was calculated using the standard equation $y = ax + b$, where x indicates the absorbance value. A modified Bradford method was used to determine total soluble protein content. Total soluble protein was determined according to the Bradford method [27] using Bradford reagent prepared in the laboratory. Briefly, 50 µL of the crude extract was mixed with 950 µL of freshly prepared Bradford reagent to obtain a final reaction volume of 1.0 mL. After incubation for 10 min at room temperature, the absorbance was recorded at 595 nm. Total dissolved protein concentrations were determined using a bovine serum albumin (BSA) standard and were reported in mg BSA per gram of wet weight sample.

2.6. Antioxidant capacity

Antioxidant capacity was determined based on free radicals neutralization by antioxidants. This capacity was assessed through various techniques, including ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), ABTS in conjunction with hydroxyl, and ABTS alongside superoxide.

2.6.1. 2,2 azinobis 3-ethylbenzothiazole-6-6sulfonic acid (ABTS)

The procedure of Re et al. [28] was used for performing the ABTS method. Antioxidant activity with ABTS was evaluated by preparing ABTS and phosphate-buffered saline (PBS) stock solutions. The ABTS stock solution was prepared by dissolving 0.38 g of ABTS in 50 mL of water, followed by adding 0.666 g of potassium persulfate. The 0.2 M PBS stock solution was prepared by dissolving 3.556 g of NaH₂PO₂ and 26.08 g of NaH₂PO₄·2H₂O in 500 mL of distilled water, followed by adding 5.4 g of NaCl to achieve a pH of 7.4. The working solution was prepared until the absorbance reached 0.70 ± 0.02 at 734 nm. Blank controls were prepared using similarly, but without the addition of samples.

The mixed sample with ABTS reagent was homogenized by vortexing for 30 sec and incubated in a dark condition for 6 min. The percentage of ABTS radical test was calculated using the following Eq (5):

$$\text{Antioxidant capacity} = (\text{Abs Blank} - \text{Abs Sample}) / (\text{Abs Blank}) \times 100\% \quad (5)$$

where Abs Blank is the absorbance value without sample addition and Abs Sample is the absorbance value with sample addition.

2.6.2. Hydroxyl radical scavenging activity

An antioxidant test using the hydroxyl method was carried out according to Siswoyo et al. [29].

The test was performed on each sample using a solution of 28 mM deoxyribose (50 μ L), 1 mM EDTA (100 μ L), 10 mM FeCl₃ (100 μ L), 1 mM H₂O₂ (50 μ L), 1 mM ascorbic acid (50 μ L), and 28 mM phosphate buffer at pH 7.4 (150 μ L). The sample, combined with the solution, was incubated at 37 °C for 1 h. After incubation, the mixture was cooled at room temperature, and 400 μ L of 1% TBA along with 400 μ L of 2.8% TCA were added to develop a pink chromogen. The mixture was then incubated again for 30 min at 80 °C in an oven. Afterward, the absorbance was measured at 532 nm.

Antioxidant activity through hydroxyl radical (OH[•]) scavenging activity was expressed as a percentage (%) of inhibition of hydroxyl radicals. The hydroxyl radical scavenging activity was calculated using the following Eq (6):

$$\text{Antioxidant capacity} = (\text{Abs Blank} - \text{Abs Sample}) / (\text{Abs Blank}) \times 100\% \quad (6)$$

where Abs Blank is the absorbance value without sample addition and Abs Sample is the absorbance value with sample addition.

2.7. Relative expression of GABA-T

The 0.1 g fresh leaf tissue was ground into a fine powder in liquid nitrogen. The powder was then homogenized in 1 mL of AccuZol™, a reagent used for total RNA isolation. Subsequently, 200 μ L of chloroform was added, mixed by shaking for 15 sec, and allowed to incubate on ice for 5 min. The mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. The upper phase was transferred to an equal volume of isopropyl alcohol and re-centrifuged at 12,000 rpm at 4 °C for 10 min. After discarding the supernatant, 80% ethanol was added to the pellet, followed by re-centrifugation at 12,000 rpm at 4 °C for 5 min. The pellet was collected and dissolved in RNase-free water. RNA concentration was measured in absorbance at 260 and 280 nm. About 1 μ g of RNA was used to synthesize the first-strand cDNA using the AccuPower® CycleScript™ RT Premix (dT₂₀) solution kit. The final volume was adjusted to 20 μ L with DEPC-treated water. cDNA synthesis was executed using a PCR machine with the specified conditions: Annealing, cDNA synthesis, and a second melting phase. The reaction was held at 95 °C for 5 min. The cDNA product was amplified using PCR, with primers targeting GABA-T. The primer sequences used for PCR amplification were adopted from Kim et al [30]: GABA-T (forward: 5'-AAGGTTCCAGGAGGGAGTCA and reverse: 5'-CTGGGTGAATGCATGATCGC), yielding an expected amplicon size of 535 bp, and Actin (forward: 5'-TCCATCTTGGCATCTCTCAG and reverse: 5'-GTACCCGCATCAGGCATCTG), yielding an expected amplicon size of 335 bp. The PCR products were separated on a 1.5% agarose gel at 80 V for 50 min. A 100 bp DNA ladder (PROMEGA) loaded at 3 μ L was served as the molecular weight marker. The electrophoresis results were visualized with a gel-doc system and quantified using ImageJ software for relative gene expression analysis.

2.8. Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD) from independent replicates. Statistical differences among treatments were evaluated using one-way analysis of variance (ANOVA). When significant differences were detected ($p < 0.05$), mean comparisons were performed using Duncan's multiple range test (DMRT) at a 5% significance level. Differences among treatments were considered statistically significant at $p < 0.05$, and means followed by different superscript letters indicate statistically significant differences at $p < 0.05$. All statistical analyses were performed using IBM SPSS

Statistics software 25.

3. Results

3.1. Morphological characters

In Figure 1, PEG-induced osmotic stress significantly influences black glutinous rice's growth, based on the morphological traits of the plants. Inhibited growth is presented from plants experiencing drought stress at 10% PEG-6000, resulting in a shorter height. The rewatering process could repair the plants, resulting in a fresher appearance and enhancements in all morphological aspects.

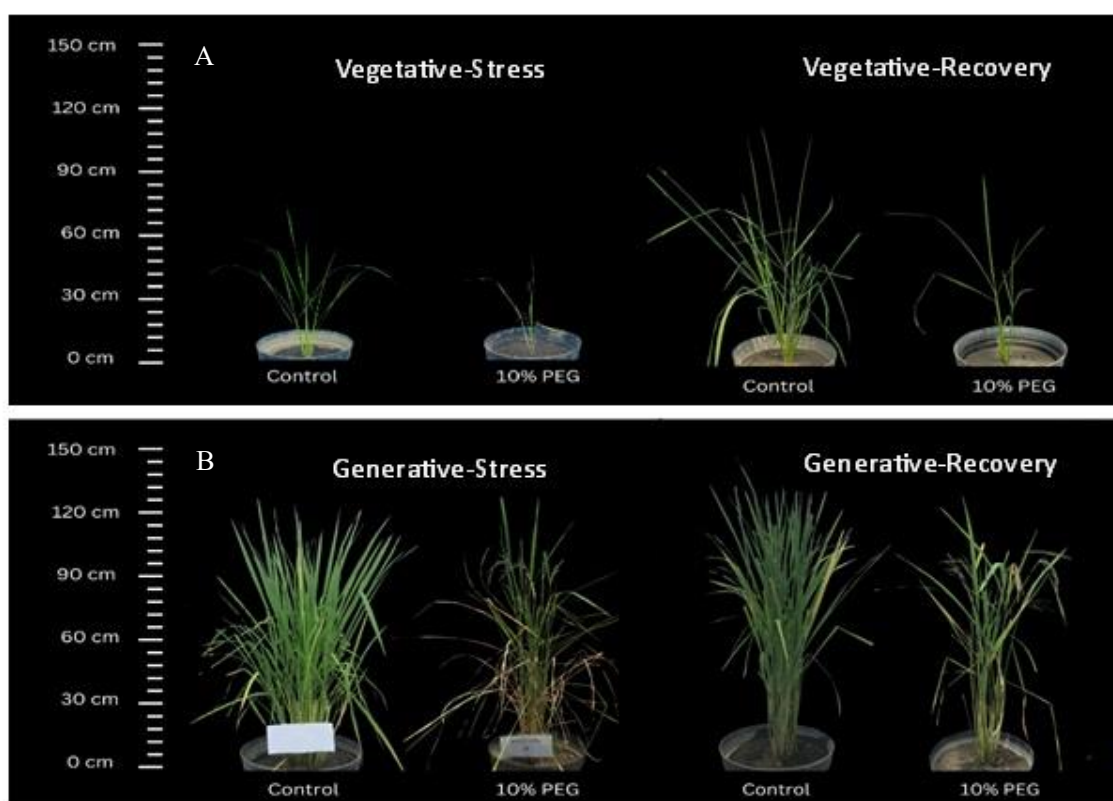


Figure 1. Morphology of black glutinous rice after PEG-induced osmotic stress during the vegetative (A) and generative stage (B).

The ANOVA indicates that different PEG-6000 concentrations and stages can influence rice plant growth. In Table 1, plant heights at vegetative stage are shown as 67.20 ± 3.70 cm (0% PEG-6000) and 40.50 ± 3.13 cm (10% PEG-6000), while values at 122.30 ± 2.24 cm (0% PEG-6000) and 116.10 ± 3.12 cm (10% PEG-6000) are shown in generative stage. Similarly, the number of leaves in rice plants treated with 10% PEG-6000 was 69.00 ± 2.00 , which was lower than the 88.00 ± 4.00 in the 0% PEG-6000 treatment. After rewatering, no significant difference was present in the number of leaves between the 0% and 10% PEG-6000 treatments.

Table 1. The effect of PEG-induced osmotic stress on the morphology of black glutinous rice plants.

Growth Stage	PEG-6000 (%)	Plant Height (cm)		Number of Leaves	
		Treatment	Rewatering	Treatment	Rewatering
Vegetative	0	67.20 ± 3.70 ^c	116.20 ± 3.26 ^c	12.00 ± 2.00 ^c	26.00 ± 2.00 ^c
	10	40.50 ± 3.13 ^c	101.30 ± 3.11 ^c	6.00 ± 1.00 ^c	10.00 ± 1.00 ^c
Generative	0	122.30 ± 2.24 ^a	144.50 ± 2.15 ^a	88.00 ± 4.00 ^a	68.00 ± 1.00 ^a
	10	116.10 ± 3.12 ^a	141.00 ± 2.02 ^a	69.00 ± 2.00 ^a	63.00 ± 3.00 ^a

*Note: Values are expressed as mean ± SD (n = 5). Means within the same column followed by different superscript letters are significantly different according to one-way ANOVA followed by DMRT at $p < 0.05$.

3.2. Photosynthetic pigment profiles

The chlorophyll-a and -b contents during the vegetative phase under 0% PEG-6000 treatment were higher than those under 10% PEG-6000 treatment, with values of 5.05 ± 0.09 mg/g FW and 4.30 ± 0.12 mg/g FW, respectively. Similarly, chlorophyll-a and -b contents under the 0% PEG-6000 treatment during the generative phase were higher than those under the 10% PEG-600, measuring 9.60 ± 0.08 mg/g FW and 8.52 ± 0.11 mg/g FW, respectively. In contrast, the total carotenoid levels at 10% PEG-6000 were found to be higher than those at 0% PEG, reaching 3.17 ± 0.07 mg/g FW. These findings highlight the significant role of rewatering to repair chlorophyll content in black glutinous rice leaves, based on the increased chlorophyll-a and -b contents, coupled with a decrease in total carotenoid content. The following condition also suggests that PEG-induced osmotic stress has a strong impact on the chlorophyll levels in black glutinous rice leaves. Table 2 presents that different concentrations of PEG-6000 treatment significantly affect the chlorophyll content in rice leaves. Consequently, a DMRT was performed at a 5% significance level.

Table 2. The effect of PEG-induced osmotic stress on photosynthetic pigment profiles in rice plant leaves.

Growth Stage	PEG-6000 (%)	Chl. a	Chl.b	Ttl Chl (mg/g FW)	Ttl Carotenoid
		(mg/g FW)	(mg/g FW)		(mg/g FW)
Vegetative	0	5.05 ± 0.09 ^c	4.30 ± 0.12 ^c	9.35 ± 0.05 ^c	1.58 ± 0.04 ^d
	10	4.15 ± 0.07 ^d	3.80 ± 0.06 ^d	7.96 ± 0.04 ^d	3.17 ± 0.07 ^c
Generative	0	9.60 ± 0.08 ^a	8.52 ± 0.11 ^a	18.12 ± 0.05 ^a	3.72 ± 0.06 ^b
	10	7.69 ± 0.05 ^b	7.27 ± 0.05 ^b	14.96 ± 0.04 ^b	6.54 ± 0.09 ^a

*Note: Values are expressed as mean ± SD (n = 5). Means within the same column followed by different superscript letters are significantly different according to one-way ANOVA followed by DMRT at $p < 0.05$.

Figure 2(A) presents the ratio of chlorophyll a/b that emphasizes the influence of PEG-induced osmotic stress on chlorophyll contents in black glutinous rice. PEG-induced osmotic stress occurs when a lower chlorophyll a/b ratio is present. In vegetative and generative stages, the ratio for 0% PEG-6000 was higher than that for 10% PEG (1.18 vs 1.09, respectively). After the rewatering process, a highly significant difference in the chlorophyll a/b ratio between the treatments of 0 and 10% PEG-6000 confirmed that drought stress was present under the 10% PEG-6000 condition, despite the

difference observed being less distinct. The vegetative stage demonstrated a more rapid rewatering than the generative phase.

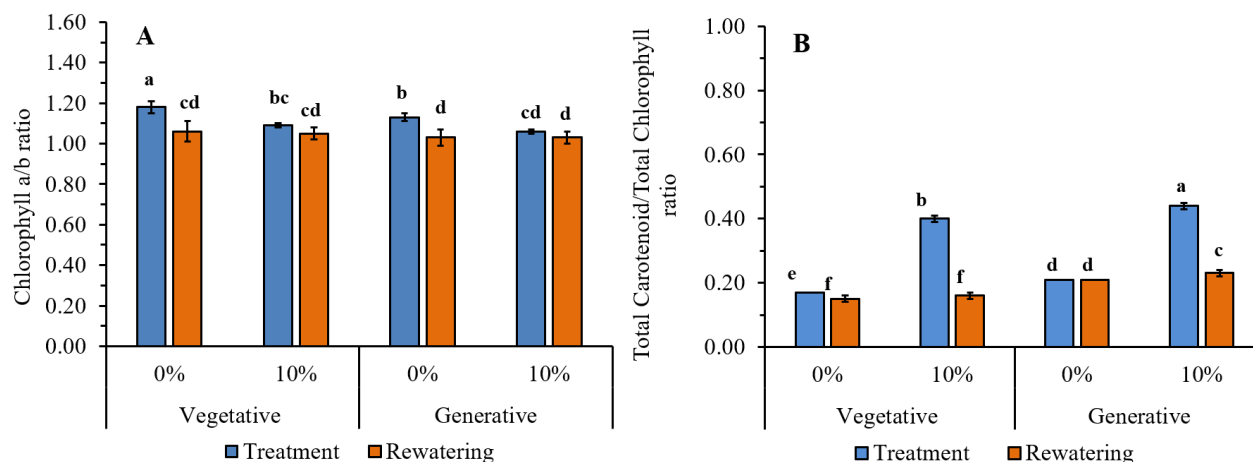


Figure 2. The effect of PEG-induced osmotic stress on the ratio of chlorophyll a/b ratio (A) and ratio of total carotenoid-to- total chlorophyll in rice leaves (B). Different superscript letters indicate statistical differences between treatments in rice plants. Numbers with the same superscript letter indicate no significant difference ($p < 0.05$).

The total carotenoids-to-total chlorophyll ratio indicates the relative levels of total carotenoids in comparison to total chlorophyll in plants (Figure 2(B)). This ratio distinctly illustrates the influence of PEG-induced osmotic stress on black glutinous rice. The following ratios during the vegetative and generative stages under 10% PEG-6000 treatment were observed at 0.40 and 0.44, respectively, indicating that the ratios for the 10% PEG-6000 treatment were higher than that of the 0% PEG-6000 treatment. After the rewatering process, a highly significant statistical difference continued to exist between the ratio values for the 0% and 10% PEG-6000 treatments, confirming that the 10% PEG-6000 group was under drought stress. After rewatering, a reduction was found in chlorophyll a/b ratio values, with significant differences between the 0% and 10% PEG-6000 groups. Also, the ratio for the 0% PEG-6000 remained statistically significant, but was greatly diminished due to rewatering.

3.3. Free amino acid and total soluble protein

The ANOVA results showed that PEG-6000 concentration significantly affected free amino acid levels in rice plant leaves. Therefore, a DMRT analysis at a 5% significance level was conducted (Table 3). PEG-induced osmotic stress significantly affected free amino acid levels in black glutinous rice leaves. The ANOVA revealed a significant difference between growth stages and PEG-6000 concentrations on total amino acid levels in rice leaves. The total free amino acid levels increased during the vegetative and generative stages under 10% PEG, reaching 4.02 ± 0.01 mg/g FW and 3.90 ± 0.19 mg/g FW, respectively. The application of 10% PEG-6000 also resulted in higher amino acid content compared to controls with 0% PEG.

Moreover, PEG-induced osmotic stress significantly influenced the total soluble protein content in the leaves of black glutinous rice. ANOVA revealed a significant difference between the growth stages and PEG-6000 concentrations regarding soluble protein content. The total soluble protein increased during vegetative and generative stages when exposed to 10% PEG, with values

of 3.26 ± 0.01 mg/g FW and 2.03 ± 0.03 mg/g FW, respectively. These findings underscore the significant effect of PEG-induced osmotic stress on the total soluble protein content in black glutinous rice leaves, with more extreme conditions in the vegetative stage than the generative stage.

Table 3. The influence of PEG-induced osmotic stress on the free amino acid levels in rice leaves.

Growth Stage	PEG-6000 (%)	Free Amino Acids (mg/g FW)		Total Soluble Protein (mg/g FW)	
		Treatment	Rewatering	Treatment	Rewatering
Vegetative	0	2.79 ± 0.01^b	1.13 ± 0.02^{ab}	2.51 ± 0.01^c	1.96 ± 0.38^{bc}
	10	4.02 ± 0.01^c	1.57 ± 0.02^c	3.26 ± 0.01^d	2.22 ± 0.70^d
Generative	0	1.79 ± 0.00^a	1.12 ± 0.04^a	1.84 ± 0.01^a	1.71 ± 0.01^a
	10	3.90 ± 0.19^c	1.55 ± 0.03^c	2.03 ± 0.03^b	1.90 ± 0.06^b

*Note: Values are expressed as mean $\pm$ SD ($n = 5$). Means within the same column followed by different superscript letters are significantly different according to one-way ANOVA followed by DMRT at $p < 0.05$. Means sharing one or more common letters (e.g., ab and bc) are not significantly different.

3.4. H_2O_2 and MDA contents

The 10% PEG-6000 significantly increased H_2O_2 and MDA levels at both growth stages compared to the control (Table 4). At the vegetative stage, H_2O_2 increased from 9.20 to 25.10 $\mu\text{mol/g}$ FW during stress and decreased to 18.10 $\mu\text{mol/g}$ FW after rewatering. MDA followed a similar trend, increasing from 3.53 to 6.77 $\mu\text{mol/g}$ FW during treatment and declining to 2.96 $\mu\text{mol/g}$ FW during rewatering. At the generative stage, the oxidative response was markedly stronger. H_2O_2 increased sharply from 8.80 to 63.90 $\mu\text{mol/g}$ FW and remained high (58.60 $\mu\text{mol/g}$ FW) during rewatering. MDA also rose significantly from 4.06 to 8.19 $\mu\text{mol/g}$ FW and only slightly decreased to 6.19 $\mu\text{mol/g}$ FW after rewatering.

Table 4. The effect of PEG-induced osmotic stress on H_2O_2 and MDA contents in rice leaves.

Growth Stage	PEG-6000 (%)	H_2O_2 ($\mu\text{mol/g}$ FW)		MDA ($\mu\text{mol/g}$ FW)	
		Treatment	Rewatering	Treatment	Rewatering
Vegetative	0	9.20 ± 0.10^c	9.40 ± 0.15^c	3.53 ± 0.81^c	3.69 ± 0.51^{bc}
	10	25.10 ± 0.05^b	18.10 ± 0.08^b	6.77 ± 0.05^b	2.96 ± 0.18^c
Generative	0	8.80 ± 1.21^c	10.30 ± 1.05^c	4.06 ± 0.51^c	4.20 ± 0.12^b
	10	63.90 ± 0.02^a	58.60 ± 0.08^a	8.19 ± 0.42^a	6.19 ± 0.04^a

*Note: Values are expressed as mean $\pm$ SD ($n = 5$). Means within the same column followed by different superscript letters are significantly different according to one-way ANOVA followed by DMRT at $p < 0.05$. Means sharing one or more common letters (e.g., bc) are not significantly different.

3.5. Antioxidant capacity and hydroxyl radical scavenging

As ANOVA revealed that the PEG-6000 concentration significantly affected the antioxidant capacity of rice plants, a further analysis with DMRT at a 5% significance level was conducted (Table 5). Based on the ANOVA results, PEG-induced osmotic stress also had a significant effect on antioxidant capacity

and hydroxyl radical scavenging activity in black glutinous rice leaves.

Table 5. The effect of PEG-induced osmotic stress on antioxidant capacity and hydroxyl radical scavenging activity in rice leaves.

Growth Stage	PEG-6000 (%)	ABTS IC ₅₀ (µg/µL)		Hydroxyl Radical Scavenging IC ₅₀ (µg/µL)	
		Treatment	Rewatering	Treatment	Rewatering
Vegetative	0	6.23 ± 0.02 ^c	6.57 ± 0.02 ^b	6.48 ± 0.03 ^d	6.23 ± 0.01 ^c
	10	5.53 ± 0.02 ^a	6.21 ± 0.04 ^a	5.48 ± 0.04 ^a	6.07 ± 0.04 ^a
Generative	0	7.13 ± 0.03 ^d	7.21 ± 0.03 ^c	6.30 ± 0.02 ^c	6.22 ± 0.01 ^c
	10	5.69 ± 0.02 ^b	7.29 ± 0.15 ^c	5.73 ± 0.02 ^b	6.12 ± 0.02 ^{ab}

*Note: Values are expressed as mean ± SD (n = 5). Means within the same column followed by different superscript letters are significantly different according to one-way ANOVA followed by DMRT at $p < 0.05$. Means sharing one or more common letters (e.g., ab) are not significantly different.

Antioxidant¹ capacity during vegetative and generative stages increased after applying 10% PEG-induced drought stress, with values of 5.53 ± 0.02 µg/µL and 5.69 ± 0.02 µg/µL, respectively. Similarly, the hydroxyl radical scavenging activity also increased under 10% PEG-6000 treatment during both stages, showing values of 5.48 ± 0.04 µg/µL and 5.73 ± 0.02 µg/µL, respectively. Lower IC₅₀ values indicate an increased antioxidant capacity through the mechanisms of ABTS and hydroxyl radical scavenging activity. The application of 10% PEG-6000 treatment consistently resulted in higher values than those observed in the 0% PEG-6000 control. These outcomes suggest that drought stress plays a crucial role in affecting the antioxidant capacity of black glutinous rice. During the vegetative phase, drought stress significantly elevated antioxidant capacity, as the vegetative phase demonstrated the most pronounced antioxidant activity.

3.6. GABA content

PEG-induced osmotic stress significantly influenced the GABA concentration in black glutinous rice leaves. The ANOVA results showed a significant difference in GABA levels in the rice leaves based on treatment phases and PEG-6000 concentration (Figure 3). For example, during the vegetative phase, the application of 10% PEG-6000 resulted in a higher GABA concentration (8.19 mg/100 g DW) compared to the control group (0% PEG). Similarly, in the generative phase, the 10% PEG-6000 treatment produced a significantly higher GABA level (11.89 mg/100 g DW) than the control. These findings suggest that drought stress increases GABA levels in black glutinous rice plants. Furthermore, the 10% PEG-6000 treatment in the generative stage led to a greater GABA concentration (11.89 mg/100 g DW) than in the vegetative stage, indicating that the vegetative phase may not respond as quickly to drought stress and thus may accumulate more GABA than the generative phase of the black glutinous rice plants.

The rewatering process was performed to indicate that drought stress can cause GABA to build up in black glutinous rice plants. This was confirmed by the decline in GABA levels after the rewatering process. The ANOVA revealed a significant difference between treatment stages and PEG-6000 concentrations on the GABA content in black glutinous rice leaves. During the vegetative stage, the 10% PEG-6000 resulted in a higher GABA content of 4.10 mg/100 g DW compared to the 0% PEG-6000 treatment. During the generative stage, GABA levels under 10% PEG-6000 was also greater than those in the control group at 5.74 mg/100 g DW.

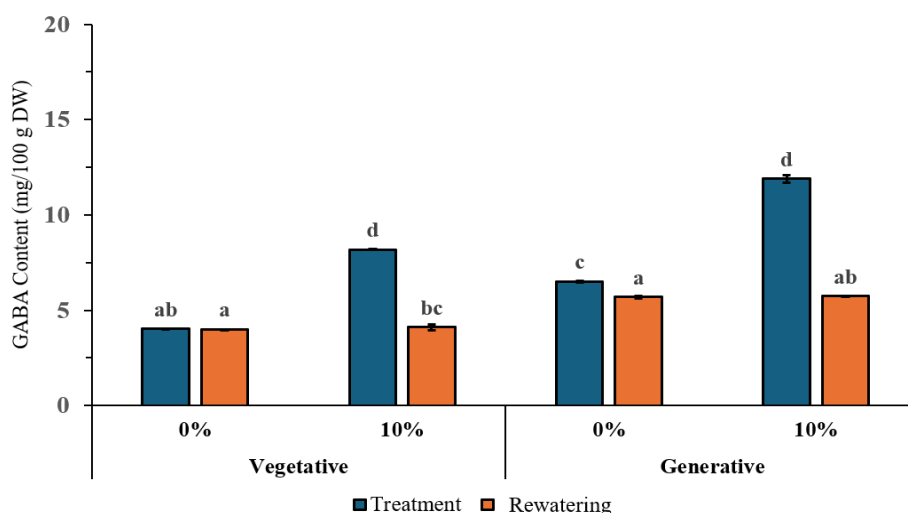


Figure 3. GABA accumulation in black glutinous rice leaves under PEG-induced osmotic stress. Different superscript letters indicate statistical differences between treatments in rice plants. Numbers with the same superscript letter indicate no significant difference ($p < 0.05$).

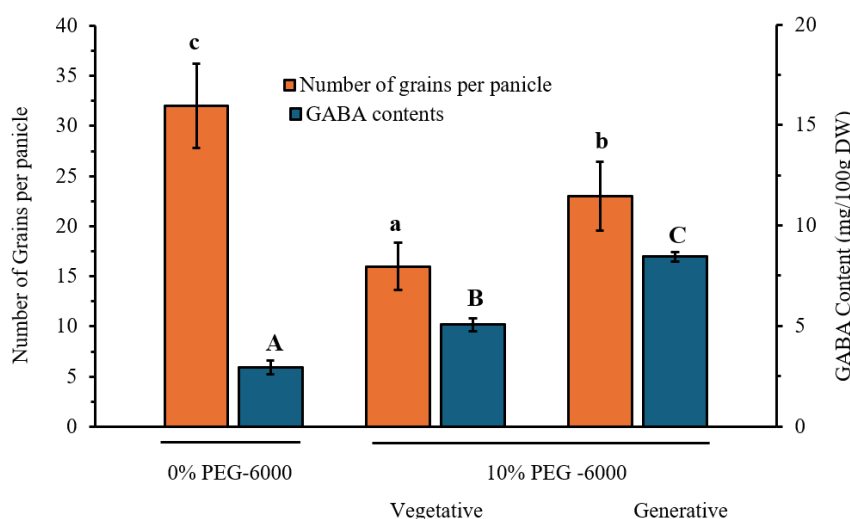


Figure 4. GABA accumulation in black glutinous rice seed under PEG-induced osmotic stress. Different superscript letters indicate statistical differences between treatments in rice plants. Numbers with the same superscript letter indicate no significant difference ($p < 0.05$).

GABA content and number of grains per panicle were significantly affected by PEG-induced osmotic stress conditions, as confirmed by one-way ANOVA followed by DMRT ($p < 0.05$) (Figure 4). GABA content increased significantly from the control (2.94 ± 0.34 mg/100 g DW) to the vegetative stage (5.06 ± 0.31 mg/100 g DW), and reached the highest value at the generative stage (8.44 ± 0.22 mg/100 g DW). Each treatment was significantly different from one another, indicating a progressive accumulation of GABA with increasing stress intensity and developmental stage. In contrast, the number of grains per panicle showed an opposite trend. The control plants had the highest grain number (32 ± 4.2), which decreased significantly at the vegetative stage (16 ± 2.4). At the generative stage, the grain number (23 ± 3.4) was higher than the vegetative stage but still significantly

lower than the control.

3.7. Relative expression of *GABA-T*

In Figure 5, the mRNA expression of *GABA-T* was generally higher during the rewatering phase compared to the stress phase across all growth phase treatments. This condition indicates the upregulation of *GABA-T* that helps alter *GABA* into valuable metabolites after stress has been alleviated. The excess *GABA* was metabolized during rewatering to restore normal cellular homeostasis. *GABA-T* plays a role by altering *GABA* into succinic semialdehyde, which can be dehydrogenated by succinic semialdehyde dehydrogenase to produce succinate and NADH, entering the TCA cycle and acting as an electron donor in the electron transport chain. Increased transcription of *GABA-T* ensures rapid alteration of *GABA*, supporting metabolic recovery and energy production. *GABA-T* helps reconnect the *GABA* to the TCA cycle, ensuring a supply of carbon skeletons and energy necessary for repair and growth.

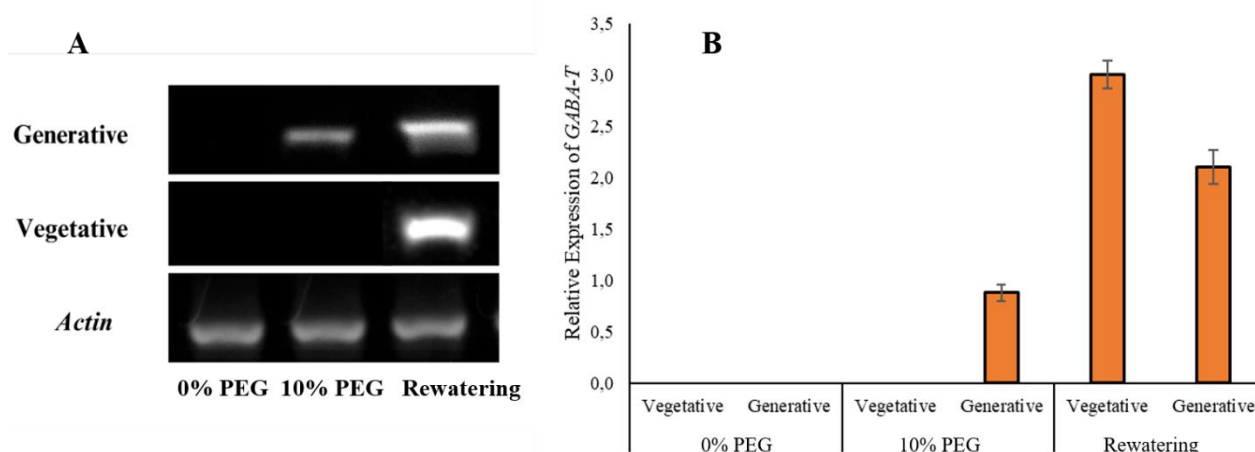


Figure 5. *GABA-T* expression at vegetative and generative stages under control (0% PEG), 10% PEG, and rewatering conditions. (A) Semi-quantitative RT-PCR analysis with actin as an internal control. (B) Relative expression levels of normalized *GABA-T* to actin (mean $\pm$ SD).

This study revealed that *GABA-T* was relatively upregulated during the rewatering stage. However, rewatering has different effects at each growth stage. Rewatering during the vegetative stage shows higher relative expression of *GABA-T* than during the generative stage. The vegetative organs are vital for the restoration of energy and resource flow before reproduction takes place. Unlike vegetative tissues, rewatering plants in generative stage is limited because reproductive structures may have already been lost or compromised during stress. If drought stress coincides with flowering or grain filling during generative stage, a permanent effect on yield will occur, even if the vegetative tissues recover.

4. Discussion

PEG-induced drought stress consistently affected black glutinous rice at morphological, physiological, and biochemical levels, with various responses according to different growth stages. Reduced morphological traits observed under 10% PEG reflects impaired cell expansion and biomass

accumulation due to decreased water availability and turgor pressure. Growth inhibition was more pronounced during the generative stage, indicating that reproductive development is particularly sensitive to osmotic imbalance due to higher metabolic demand and assimilate requirement [31].

Alterations in photosynthetic pigments further confirmed stress-induced physiological disruption. Declines in total chlorophyll under osmotic stress condition induced by PEG-6000 indicate photosynthetic apparatus impairment and reduced carbon assimilation capacity [32,33]. Changes in chlorophyll-b may represent a compensatory mechanism to optimize light harvesting under stress [34]. Plants that were suffered from drought stress showed reduced chlorophyll-a, -b, and total chlorophyll, along with increased total carotenoids under 10% PEG. Consequently, drought stress had a notable effect on the chlorophyll content in black glutinous rice leaves. Therefore, rewatering provides considerable effect on the chlorophyll content in black glutinous rice leaves. The ANOVA results indicated a significant difference in chlorophyll content ($p < 0.05$), which varied depending on different growth stages and PEG concentration. Increased total carotenoid accumulation likely contributed to photoprotection and ROS quenching [35]. Nevertheless, reduced pigment concentration overall reflects stress-induced degradation and accelerated senescence [36], which may partly explain the observed growth suppression.

Drought stress also caused the upregulation of total free amino acids, particularly during the vegetative stage. The amino acids accumulate to support osmotic adjustment, maintain cellular water potential, and provide structural stability. In short-term drought, enhanced nitrogen assimilation and amino acid biosynthesis have previously been reported to show no major changes in total protein content [37]. This condition suggests metabolic reprogramming toward protective solutes, which further supports their role in osmo-protection and cellular stabilization. However, the magnitude of amino acid accumulation differs between growth stages, indicating stage-specific metabolic plasticity.

The biochemical evidence of oxidative stress was clearly demonstrated by the extreme increase in H_2O_2 and MDA under 10% PEG treatment. Elevated H_2O_2 levels indicate excessive ROS generation, while increased MDA may contribute to membrane lipid peroxidation and structural damage [38]. The generative stage showed a dramatically higher accumulation of H_2O_2 and persistently high MDA content, even after the rewatering process. Although plant water status parameters were not directly measured, the marked increase in H_2O_2 and MDA together with chlorophyll degradation and growth inhibition are well-recognized physiological consequences of PEG-induced water deficit, indicating that the applied treatment successfully imposed osmotic drought stress. This suggests limited ROS detoxification capacity and incomplete membrane repair during the reproductive phase. In contrast, the vegetative stage exhibited partial rewatering of both parameters, reflecting more efficient redox regulation and cellular repair mechanisms. The reduction in MDA content observed after rewatering at the vegetative stage indicates partial recovery from PEG-induced osmotic stress. Rewatering restores cellular water status, which may reduce excessive ROS production and consequently limit membrane lipid peroxidation. Previous studies have shown that the recovery phase after water deficit is often accompanied by reactivation of antioxidant defense mechanisms and increased membrane stability, resulting in lower MDA accumulation [39,40]. Nevertheless, MDA levels remained higher than in well-watered controls, suggesting that rewatering resulted in partial recovery, not complete recovery, and that oxidative damage was only partially mitigated and complete physiological recovery was not achieved. Therefore, drought is highly sensitive at different growth stages, with reproductive tissues being more susceptible to oxidative injury [31].

Consistently, antioxidant capacity increased under 10% PEG treatment, as indicated by stronger ABTS and hydroxyl radical scavenging activity (lower IC_{50} values). This condition suggests activation of both enzymatic and non-enzymatic antioxidant systems due to elevated ROS. The high anthocyanin

content of black glutinous rice naturally contributed to radical scavenging capacity by neutralizing free radicals. The ABTS and hydroxyl radical scavenging assays demonstrated that extracts obtained from PEG-treated plants possessed greater in vitro radical-scavenging capacity than those from the control treatment. These assays reflect the antioxidant potential of metabolites present in the extracts but do not directly represent the in vivo oxidative status of plant tissues. In contrast, H_2O_2 and MDA are physiological indicators of reactive oxygen species accumulation and membrane lipid peroxidation within living tissues. Therefore, these measurements should be considered complementary rather than directly comparable. The concurrent increase in antioxidant capacity and oxidative stress markers suggests that PEG-induced osmotic stress triggered both enhanced antioxidant metabolite accumulation and oxidative damage. However, the present data do not establish whether the increased antioxidant capacity was sufficient to mitigate oxidative injury in vivo, particularly during the generative stage.

GABA content in leaves had contradictory results during PEG-induced drought and rewatering. Abiotic stress conditions, including drought, can stimulate GABA synthesis through cytosolic acidification and Ca^{2+} -mediated activation of glutamate decarboxylase [41]. Importantly, GABA content changes were accompanied by altered expression of the GABA-T gene, which encodes GABA transaminase, a key enzyme responsible for GABA catabolism within the GABA-shunt pathway.

The observed upregulation of GABA-T expression under PEG-induced osmotic stress may indicate enhanced GABA turnover through the GABA shunt. The GABA shunt has been reported to connect GABA metabolism with the TCA cycle by converting GABA into succinate, thereby contributing to carbon metabolism and cellular energy balance during abiotic stress [40–42]. In addition, previous studies have suggested that activation of the GABA shunt may contribute to redox homeostasis by supporting metabolic adjustment under stress conditions [41,42]. However, because the present study did not directly measure GABA-T enzymatic activity, succinate production, mitochondrial respiration, or TCA cycle intermediates, these mechanisms should be regarded as potential explanations, supported by previous literature, rather than as direct evidence from this study. Therefore, the increased GABA-T expression observed here is interpreted as being associated with metabolic adaptation to PEG-induced osmotic stress.

GABA metabolism responded strongly to drought stress, as reflected by increased GABA accumulation in rice grains under stress conditions. This accumulation is widely recognized as a rapid adaptive response triggered by cytosolic acidification and Ca^{2+} -mediated activation of glutamate decarboxylase, leading to enhanced GABA biosynthesis [43,44]. In developing grains, elevated GABA levels are associated with stress-induced metabolic reprogramming that supports osmotic adjustment and redox balance. The involvement of GABA-T further indicates active turnover through the GABA shunt, linking GABA metabolism with the TCA cycle to sustain energy production during stress [42,45]. Despite its protective role, increased GABA accumulation in grains was associated with reduced yield performance, suggesting a trade-off between stress tolerance and productivity. Under osmotic stress condition induced by PEG-6000, metabolic resources are preferentially allocated to survival mechanisms, including GABA-mediated stress responses, rather than grain filling and development [46,47].

One limitation of this study is that drought severity was not directly quantified using plant water status parameters such as relative water content (RWC), leaf water potential, or stomatal conductance. Nevertheless, the effectiveness of the PEG-induced osmotic treatment was consistently demonstrated by significant morphological inhibition, chlorophyll degradation, oxidative stress (H_2O_2 and MDA accumulation), enhanced antioxidant activity, and increased GABA accumulation. Future studies should integrate direct measurements of plant water status with biochemical analyses to provide a more comprehensive evaluation of drought intensity and plant adaptation. It should be noted that the PEG-

6000 treatment used in this study represents a controlled osmotic stress model rather than natural soil drought. Although PEG-induced osmotic stress is widely employed to simulate the water-deficit component of drought under controlled conditions, it cannot fully reproduce the complex physical, chemical, and biological processes associated with soil drying in field environments, such as changes in soil structure, root-soil interactions, and fluctuating environmental conditions. Therefore, the physiological and biochemical responses observed in this study should be interpreted as responses to PEG-induced osmotic stress, and further validation under soil-based osmotic stress condition induced by PEG-6000 would strengthen the applicability of these findings to field environments.

Compared with previous studies that primarily focused on GABA accumulation or oxidative stress responses separately, the present study integrates physiological, biochemical, and molecular analyses to evaluate the response of black glutinous rice to PEG-induced osmotic stress. By simultaneously assessing GABA accumulation, GABA-T gene expression, oxidative stress markers, antioxidant capacity, and recovery after rewatering at different developmental stages, this work provides a more comprehensive understanding of stress adaptation in black glutinous rice. These findings extend current knowledge by highlighting developmental stage-dependent differences in metabolic responses and recovery under controlled osmotic stress conditions.

5. Conclusions

This study demonstrates that PEG-induced osmotic stress is associated with coordinated physiological and biochemical responses in black glutinous rice, including changes in GABA accumulation, GABA-T gene expression, oxidative stress markers, antioxidant capacity, and chlorophyll content during both stress and post-rewatering recovery. The results indicate that the vegetative and generative stages differ in their responses to osmotic stress, with the generative stage exhibiting greater oxidative damage and less effective recovery. The principal contribution of this study is the integrated assessment of GABA metabolism, oxidative stress responses, and recovery following rewatering in black glutinous rice across different developmental stages. These findings provide new insight into the physiological adaptation of black glutinous rice to PEG-induced osmotic stress and establish a basis for future functional studies investigating the mechanistic role of GABA metabolism in stress tolerance under soil-based drought conditions.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest relevant to this research.

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