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ORIGINAL PAPER

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EFFECT OF SOAKING AND ADDING GLUTAMIC ACID AND PHYTASE ENZYME SIMULTANEOUSLY ON IMPROVING GABA CONTENT AND REDUCING PHYTIC ACID CONTENT OF GERMINATED HUYET RONG BROWN RICE (VIETNAM)

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ABSTRACT

Background. Germination can be used to improve the nutritional value of brown rice. The present study investigated treatment methods to enhance the content of a bioactive compound, γ-aminobutyric acid (GABA), and reduce the level of an anti-nutritional factor, phytic acid, in Huyet Rong germinated rice.

Material and methods. The study investigated the effect of temperature (20–40°C), rice:water ratio (1:1 to 1:3), and time (6–24 h) when soaking on the moisture percentage, phytase activity, GABA content, and phytic acid content of brown rice during germination (72 h). Glutamic acid (0–8 mM) and phytase enzyme (0–800 IU/L) were added to the soaking water for GABA synthesis and phytic acid hydrolysis.

Results. Brown rice initially contained 17.32 mg/100 g dry weight (DW) of GABA and 14.14 mg/g DW of phytic acid. It was soaked in distilled water at 30°C with a rice:water ratio of 1:2 (w/v) for 12 h. After that, the grains were germinated in an incubator at 30°C for 24 h. After germination, the GABA content increased to 167.63 mg/100 g DW (an increase of more than 9 times), and the phytic acid level decreased to 9.07 mg/g DW (a decrease of 35.86%). When adding glutamic acid (5 mM) and phytase (600 IU/L) simultaneously to the soaking water, the GABA content after germination increased to 200.41 mg/100 g DW, and the phytic acid level decreased to 6.79 mg/g DW.

Conclusion. Huyet Rong brown rice soaked with glutamic acid and phytase enzyme had higher GABA content (increased nearly 12 times) and less phytic acid content (decreased by about 52%) than untreated brown rice after germination. This method might be applied to improve the biological value of brown rice varieties and to further develop food products from germinated rice.

Keywords: GABA, rice germination, glutamic acid, Huyet Rong, phytase, phytic acid

INTRODUCTION

Rice is a major cereal for billions of people all around the world, especially in Vietnam (Khanh et al., 2021). Rice grain usually goes through a milling process with two stages. The first step is to remove the husk from the paddy grain to obtain a whole brown rice grain with a brown bran layer around it. The second step is to remove the bran layer and even the embryo from the brown rice grain to obtain a white rice grain. The

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bran layer is composed of pericarp, aleurone, and subaleurone layers. Both the bran layer and the embryo contain large amounts of nutrients and phytochemical components (Kaur et al., 2016). Therefore, more nutrients (protein, fat, vitamin, mineral, fiber) and bioactive compounds (γ-aminobutyric acid – GABA, γ-oryzanol, flavonoids, phenolic compounds) which can maintain and improve human health are found in brown rice than in white rice (Saleh et al., 2019).

However, brown rice is not usually consumed as a staple grain due to its dark color and longer cooking time (relative to white rice), and the grassy flavor and hard texture are unpopular with consumers (Wu et al., 2013). The germination of brown rice not only improves its palatability but also further enhances its nutritional value and health benefits (Nguyen et al., 2020). The water is rapidly absorbed during the grain soaking process and thereby promotes a series of biochemical reactions that lead to softening of the kernel structure, degradation of high molecular weight polymers into simple and easily digestible components, and synthesis of some bioactive compounds (Munarko et al., 2021).

GABA is one of the significant phytochemical components in germinated brown rice. It is already present in brown rice in relatively low amounts, but its content increases substantially during germination (Cáceres et al., 2017). GABA acts as a major inhibitory neurotransmitter, thus playing an important role in supporting several physiological functions including insomnia, depression, and autonomic nervous system disorder during menopause (Munarko et al., 2021). GABA also has many other health benefits, such as reducing hypertension, regulating blood cholesterol levels, preventing cancer cell proliferation, and inhibiting chronic alcohol-related diseases (Ngo and Vo, 2019). Both glutamate and glutamic acid are substrates for GABA synthesis. However, compared with glutamate, glutamic acid has a lower solubility and the substitution of Na^+ by H^+ ion on the carboxyl group can reduce glutamate-induced inhibition, thereby improving the efficiency of GABA production (Wang et al., 2018).

Brown rice also contains a non-nutritive component called phytic acid (myo-inositol hexakisphosphate), the major storage form of phosphorus accumulating in the aleurone layer of cereal grains (Fukushima et al., 2020). Phytic acid accounts for about 75% of the total P in rice grains. It forms complexes with micronutrients including Fe, Zn, and Mg making them non-absorbable by humans (Raboy, 2003). Due to the natural occurrence of phytase enzyme in grains, phytic acid is hydrolyzed to lower myo-inositol phosphate and inorganic phosphate. In particular, the enzyme phytase is produced more significantly during germination (Kumar et al., 2010). However, the decomposition of phytic acid has not yet occurred completely by only natural germination.

Therefore, it is very important to investigate germination treatment that combines the addition of glutamic acid and phytase enzyme to increase GABA content and decrease phytic acid content, thereby improving the nutritional and biological value of brown rice.

MATERIAL AND METHODS

Material and chemical

Huyet Rong brown rice was purchased from Phu Minh Tam Trading Service Co., Ltd. (Ho Chi Minh city, Vietnam). The phytase enzyme was derived from the Tien Phong Biotechnology Joint Stock Company (Ho Chi Minh City, Vietnam). This is a crude enzyme which is granular, ivory white, and water-soluble with an activity of 2,000 IU/g and produced from *Aspergillus niger*. L-glutamic acid, sodium hypochlorite (99%, Merck, Germany).

Experimental design of soaking and germination

Investigate the effect of soaking conditions. 500 g of brown rice was used per sample. Brown rice was washed three times with tap water to remove external impurities and then sterilized in 1% NaClO solution for 20 min with a rice: solution ratio (w/v) of 1:2 (Liyezi et al., 2020). The grains were then soaked in distilled water with a rice:water ratio (w/v) of 1:1, 1:2, and 1:3. The mixtures was placed in an incubator (Mir-262, Sanyo, Japan) at adjustable temperatures (20, 30, 40°C) for 6, 12, 18, and 24 h. Brown rice was separated by a sieve and then placed in plastic boxes (26 cm long × 19 cm wide × 6 cm high) with a drainage net at the bottom and covered with a thin layer of fabric for germination in the incubator at 30°C for 72 h (Lee et al., 2013). Samples were taken from the incubator every 12 h. During germination, samples were irrigated every 24 h with 250 mL of water to allow this water to flow down to the box tray below the net and maintain the necessary moisture for grains during germination.

After soaking, the surface of the rice grain was drained using a centrifugal rotator for moisture percentage analysis. After germination, samples were stored at –80°C for further analysis of phytase activity, GABA content, and phytic acid content.

Investigate the effect of glutamic acid and phytase enzyme added during soaking. The steps were similar to those described above with the best parameters selected, and the soaking water was supplemented with glutamic acid at concentrations of 0, 2, 5, and 8 mM and phytase with levels of 0, 400, 600, and 800 IU/L.

Analytical methods

Moisture percentage. The moisture percentage was determined by drying 5 g of sample in an air oven (UM700, Memmert, Germany) at 105°C to a constant weight.

Phytase activity. The phytase activity was determined using the method of Fukushima et al. (2020). Germinated brown rice powder (1 g) was homogenized with 1 mL of 0.5 M sodium acetate buffer and then centrifuged at 15,000×g and 4°C for 10 min (Z323K, Hermle Labourtechnik GmbH, Germany). The mixed buffer solution consisted of 350 µL of 0.1 M sodium acetate buffer (pH 5.0), and 100 µL of 2 mM sodium phytate was pre-incubated at 40°C for 10 min in a water bath (Rex C-90, Memmert, Germany). The enzyme reaction was started by adding 100 µL of the crude enzyme into the mixed buffer. After incubation at 40°C for 30 min, 1.5 mL of a solution of acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1 v/v) and 100 µL of 1.0 M citric acid were added. The mixture was then centrifuged at 15,300×g and 4°C for 10 min. The absorbance of the clear solution was recorded at 655 nm by a UV-VIS Spectrophotometer (722N, Inesa, China). A mixed buffer solution without crude enzyme was used as a blank. To determine the enzyme activity, a calibration curve was prepared with 0.03 M KH_2PO_4. Enzyme activity (U) was expressed as 1 µmol of phosphate released per minute.

γ-aminobutyric acid (GABA) content. The GABA content was analyzed by a combination of its derivatization using 1-fluoro-2,4-dinitrobenzene (FDNB) with high-performance liquid chromatography (HPLC) (IshiKawa et al., 2009). Germinated brown rice powder (2 g) was extracted twice with 200 mL of hot water (80°C) for 1 h and then the mixture was centrifuged at 15,000×g and 4°C for 10 min. The extract was mixed and evaporated to 40 mL at low pressure (70°C). Next, 0.2 mL of the sample containing GABA was added to 2 mL of 500 mM $NaHCO_3$ and mixed with 0.4 mL of ethanol solution containing 5% FDNB (1-fluoro-2,4-dinitrobenzene). The mixture was incubated at 37°C for 1 h with stirring. GABA dinitrophenyl derivatives solution (DNP-GABA) was adjusted to pH 2.0 with 0.4 mL of 6 M HCl, then extracted twice with 6 mL of ether. The extract was evaporated by drying it at 37°C at low pressure. The yellow residue after drying containing DNP-GABA was dissolved in 3 mL of 0.1 M NaOH and diluted 40 times with 10 mM Tris-HCl buffer (pH 6.0). The mixture was filtered through a 0.2 µm filter membrane (Millipore, Japan) and 50 µL of the filtrate was subjected to an HPLC system (UltiMate 3000). The extract was passed through an AtlantisTM dC18 column (3.9 × 150 mm) with the mobile phase being 10 mM Tris-HCl buffer (pH 6.0). The column derivatives were then eluted with methanol (0–80%) for 80 min. The flow rate was 0.5 mL/min and the absorbance was measured at 400 nm. The determination of DNP derivative was performed by comparing the retention time with the N(-2,4-dinitrophenyl)-L-2-aminobutyric butyric acid standard. The standard curve prepared at concentrations of 0, 2, 4, 6, 8, and 10 ppm was used to determine the amount of GABA in the sample.

Phytic acid content. Phytic acid content was determined using a modified colorimetric method (Gao et al., 2007). Germinated brown rice powder (0.5 g) was mixed with 10 mL of 2.4% HCl in a 14 mL Falcon tube. The mixture was shaken at 220 rpm for 16 h (SK-600, Jeiotech, Korea) and centrifuged at 15,000×g and 4°C for 10 min. The extract was transferred to a 14 mL Falcon tube containing 1 g of NaCl. The mixture was shaken at 350 rpm for 20 min to dissolve the salt and kept at 4°C for 60 min. The mixture was then centrifuged at 15,000×g and 4°C for 10 min. The supernatant (1 mL) was diluted 25-fold in a 50 mL Falcon tube with distilled water. The diluted solution (3 mL) was mixed with 1 mL of Wage reagent which included 0.03% $FeCl_3.6H_2O$ and 0.3% sulfosalicylic acid in a 14 mL Falcon tube. The centrifugation was then

carried out at 15,000×g and 4°C for 10 min. A series of calibration standards containing 0; 1.12; 2.24; 3.36; 5.6; 7.84 and 11.2 mg/l P was prepared from sodium phytate with the P content at 18.38%. The absorbance of color reaction products for both samples and standards was measured at 500 nm. The P content in the samples was determined based on the calibration curve and then the phytic acid content was calculated.

Data analysis

The obtained results were the average of three replications. The data was graphed using Microsoft Excel software and statistically analyzed by Portable Statgraphics Centurion software (version 15.2.11.0, USA) using One-Way ANOVA to test for a significant difference between the treatment means through LSD at a 95% confidence level ($p = 0.05$).

RESULTS AND DISCUSSION

Nutritional composition of Huyet Rong brown rice

The analysis results in Table 1 showed that the content of the two components, GABA and phytic acid, was also consistent with that reported in other studies. According to Kittibunchakul et al. (2017), the GABA content of brown rice from 32 Thai rice varieties ranged from 7.60 to 28.05 dry weight (DW); in Vietnamese Huyet Rong brown rice, GABA accounted for 17.32 ±0.52 mg/100 g DW. The phytic acid content (14.14 mg/g DW) was similar to the results of Kumar et al. (2017), with the phytic acid content of 6 brown rice varieties ranging from 9.09 to 29.55 mg/g DW.

Table 1. Nutritional composition of Huyet Rong brown rice (mean ±SD)

Composition	Value
Moisture percentage, %	12.08 ±0.24
GABA content, mg/100 g DW	17.32 ±0.52
Phytic acid content, mg/g DW	14.14 ±0.35

SD – standard deviation.

Effect of soaking process on moisture percentage, phytase activity, GABA and phytic acid content

The moisture percentage. The Huyet Rong brown rice had an initial moisture percentage of 12.08%. The longer the soaking time, the higher the grain moisture percentage became (Fig. 1). However, the moisture percentage increased the most in the first 6 h, then rose more slowly when the soaking time was extended to 18 h and increased almost insignificantly in the final soaking period up to 24 h. There are two main mechanisms that lead to water absorption by the grain. The first is the penetration of water into the pores of grain structure through the embryo attachment site and ventral side (Tong et al., 2017). The second is the hydration of dry matter ingredients, especially starches that can absorb water equivalent to up to 10 times their weight (Lucas et al., 2007).

The soaking temperature had a great influence on the water absorption of the grain ($p < 0.05$). When soaking grains at 20°C, the moisture percentage increased

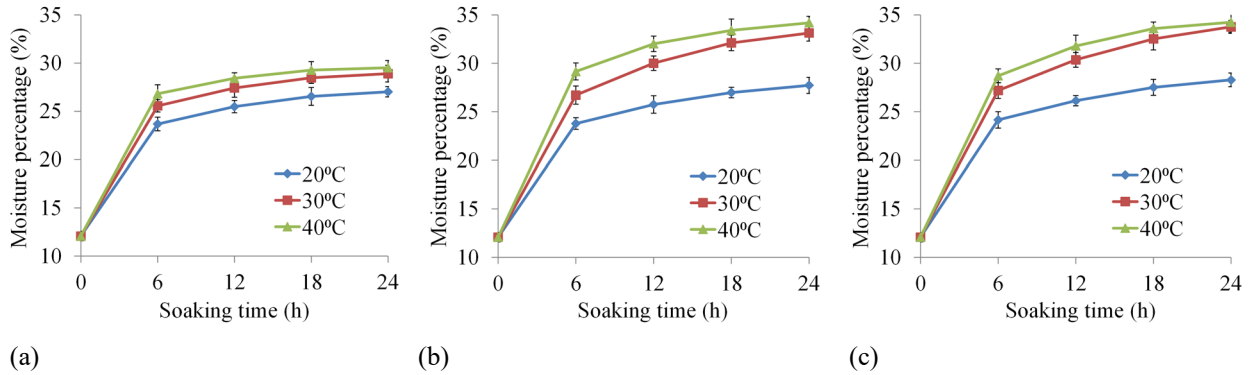

Fig. 1. The change of moisture percentage during the soaking time at different temperatures with brown rice:water ratios of 1:1 (a), 1:2 (b), and 1:3 (c)

slowly with soaking time and the final moisture percentage only reached 28.29% after 24 h when the ratio of rice to water was 1:3. When increasing the soaking temperature to 30°C and 40°C, the moisture absorption rate was quite fast and there was no significant difference between these two temperatures. The moisture percentage achieved was 33.76% and 34.24%, respectively, under the same conditions. The dependence of the water uptake on temperature is due to the rise in the diffusion coefficient of water with increasing temperature (Saleh et al., 2018).

The moisture percentage of the grain also depended on the ratio of rice to soaking water ($p < 0.05$). At a ratio of 1:1, the moisture absorption rate of the grain was low. After 24 h of soaking at 40°C, the moisture percentage was only 29.52%. When the amount of water was doubled (1:2), the water uptake rate of the grain was greater at 30°C and 40°C, while at 20°C, even if the ratio was increased from 1:1 to 1:2, the moisture percentage did not increase significantly due to the limited ability of grains to absorb water at very low temperatures. At a ratio of 1:3, an increase in the moisture percentage of the grain was not clearly observed compared to the ratio of 1:2 (the moisture percentage reached 34.18% and 34.24%, respectively, at the end of soaking at 40°C).

Phytase activity. Along with the prolongation of soaking and germination time, the physiological activity of the grain was activated as shown by the increased phytase activity (Fig. 2). However, in the samples soaked at 40°C, although water uptake was the greatest, the high temperature inhibited the sprouts, leading to lower phytase activity compared to the samples soaked at 20°C. In addition, when soaking grains at a high temperature, there was a significant loss of dry matter (data not shown), and germination took place slowly, resulting in low enzyme activity. Temperature acts to regulate germination in three main ways. Firstly, it may be involved in the removal of either primary or secondary dormancy. Secondly, temperatures outside of the normal limits for germination may cause the establishment of secondary dormancy in grain when conditions are not favorable for seedling establishment. Finally, the temperature at which seeds are incubated determines their capacity for germination and the rate at which this occurs. Each species has a range of temperatures over which germination may proceed. There are three cardinal temperatures for germination, namely the maximum, minimum and optimum germination temperatures. The maximum and minimum temperatures represent the extremes of the range of temperatures over which the seed is able to germinate. The optimum germination temperature may be defined as that temperature at which the highest percentage of germination may be obtained in the shortest possible period of time. These temperatures vary considerably between species, but may also vary between cultivars of the same species. Within the temperature range of a species, the rate of germination usually increases as the temperature rises. However, as the temperature approaches the maximum for germination, the germination rate begins to slow. And temperatures greater than the maximum favorable temperature for germination

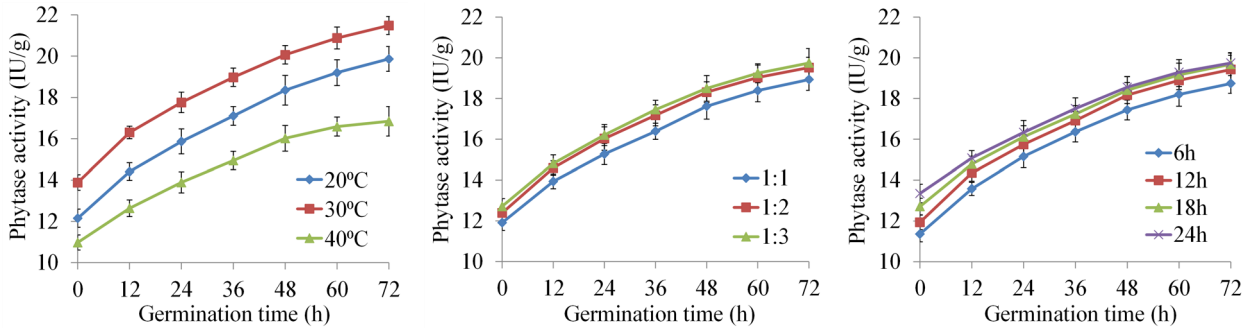

Fig. 2. The change of phytase activity during the germination time at different temperatures (a), ratios of brown rice and water (b), and soaking times (c)

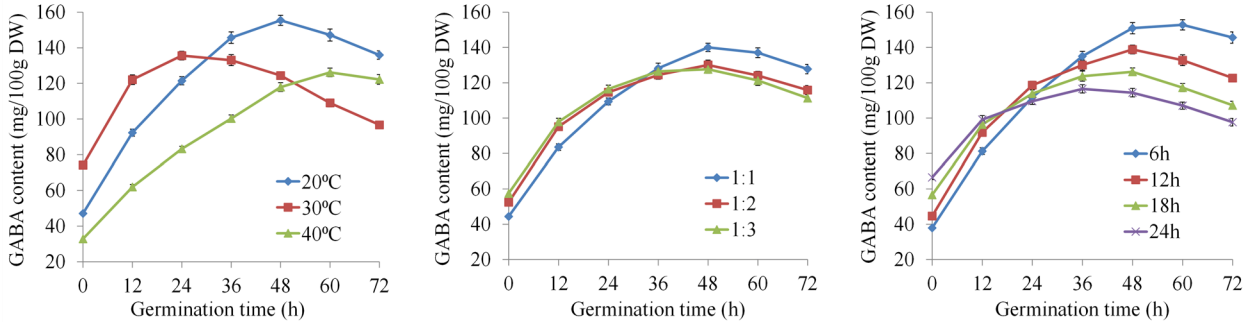

Fig. 3. The change of GABA content during the germination time at different temperatures (a), ratios of brown rice and water (b), and soaking times (c)

will result in the suspension of germination (Hills and Van Staden, 2003). According to Cabrera et al. (2020), the germination of *Megathyrsus maximus* did not even occur at 10°C and 42°C. Similarly, no cultivar of Jalapeño and Cayenne pepper had more than 1.0% germination at 40°C (Carter and Vavrina, 2001).

GABA content. The results in Figure 3 showed that GABA content increased significantly with germination time, but after a certain limit, the amount of GABA decreased. GABA is produced from the decarboxylation of glutamic acid under the action of glutamate decarboxylase (Yogeswara et al., 2020). However, when the germination process intensifies, the respiratory intensity increases; at this point, although the synthesis of GABA still takes place, GABA continues to be metabolized to produce the energy needed for enzymes to function and for sprouts to grow (Che-Othman et al., 2020). In the early stages, when glutamic acid is sufficient, GABA synthesis dominates. However, over time, the amount of glutamic acid gradually decreases and when there is very little remaining glutamic acid, GABA decomposition dominates.

Phytic acid content. During soaking and germination, the phytic acid content decreased significantly (Fig. 4). Soaking reduces phytic acid via two processes. One is hydrolysis by phytase produced naturally from the grain and the other is diffusion of soluble phytates into the soaking medium (Feizollahi et al., 2021). The decrease in

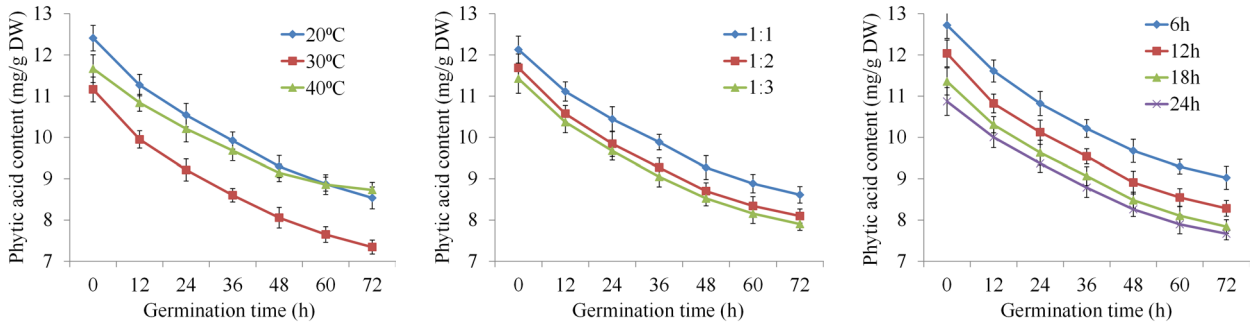

Fig. 4. The change in phytic acid content during germination at different temperatures (a), ratios of brown rice and water (b), and soaking times (c)

phytic acid content during germination was mainly due to the strong phytase enzyme activity (Kumar et al., 2010).

It could be seen that the sample 30–1:2–12–24 (the sample soaked at 30°C with a rice:water ratio of 1:2 for 12 h, then germinated for 24 h) produced the optimum results (Table 2). After germination, the GABA content increased significantly from 17.32 to 167.63 mg/100 g DW (an increase of about 9 times), while the phytic acid content decreased from 14.14 to 9.07 mg/g DW (decreased about 35.8%).

Table 2. GABA and phytic acid content of optimal samples

Samples Soaking temperature, °C – Ratio of rice and water – Soaking time, h – Incubation time, h	GABA content after germination mg/100 g DW	Phytic acid content after germination mg/g DW
20–1:1–12–48	163.72^{a}	9.67^{def}
20–1:3–12–48	164.43^{ab}	9.31^{abc}
20–1:1–18–48	164.51^{ab}	9.19^{ab}
30–1:2–06–48	164.67^{abc}	9.27^{ab}
20–1:1–12–60	165.34^{abcd}	9.31^{abc}
20–1:2–06–72	166.19^{abcd}	9.43^{bcd}
20–1:1–06–72	166.81^{abcde}	9.62^{cdef}
20–1:2–06–60	167.26^{bcde}	9.79^{ef}
30–1:2–12–24	167.63^{bcde}	9.07^{a}
30–1:1–06–48	167.85^{cde}	9.66^{def}
20–1:1–06–60	168.38^{de}	9.95^{f}
20–1:3–06–60	168.40^{de}	9.61^{cde}
20–1:2–12–48	169.42^{e}	9.43^{bcd}

Values with different superscripts within a column were significantly different at 5% significance level ($p < 0.05$).

Effect of addition of glutamic acid and phytase enzyme in soaking water on GABA and phytic acid content

GABA content. The results in Table 3 indicate that when gradually increasing the concentration of glutamic acid from 0 mM to 5 mM, the GABA content after soaking and also after germination is also increased gradually but significantly ($p < 0.05$). However, if the glutamic acid concentration continued to increase to 8 mM, no further raise in GABA content was observed. Increasing the concentration of glutamic acid provides more substrate for the metabolic decarboxylase enzyme to produce more GABA (Yogeswara et al., 2020). However, the glutamic acid concentration is so high that it becomes redundant because in a certain period of time and with a certain amount of enzyme, it can only convert a certain amount of substrate.

Table 3. Effect of glutamic acid concentration on GABA content

Concentration of glutamic acid in soaking solution, mM	GABA content of brown rice after soaking mg/100 g DW	GABA content of brown rice after germination mg/100 g DW
0	132.25^{a}	165.32^{a}
2	145.90^{b}	184.19^{b}
5	161.94^{c}	200.41^{c}
8	162.08^{c}	200.26^{c}

Values with different superscripts within a column were significantly different at 5% significance level ($p < 0.05$).

Phytic acid content. When the grain is exposed to moisture, the endogenous phytase enzyme becomes active (Nkhata et al., 2018). However, the amount of endogenous enzyme is limited, so it only partially reduces it. Therefore, right after starting the soaking process, the endogenous phytase enzyme had not been activated, and the additional phytase enzyme-initiated hydrolysis of the phytic acid fraction was concentrated in the aleurone layer. In addition, a part of the phytase penetrated the inside of the grain and, together with the endogenous phytase, gradually hydrolyzed the phytic acid during soaking and subsequent germination. However, phytic acid was not fully hydrolyzed at this point because hydrolysis also required sufficient time, whereas soaking continued for only 12 h and incubation for only 24 h. Moreover, over the incubation time, the enzyme absorbed into the seeds during soaking might be gradually inactivated. It has been reported that malting of millet reduced phytic acid content by 23.9 % after 72 h and 45.3% after 96 h (Makokha et al., 2002; Coulibaly et al., 2011).

Table 4. Effect of phytase activity on phytic acid content

Phytase activity of soaking solution, IU/L	Phytic acid content of brown rice after soaking mg/g DW	Phytic acid content of brown rice after germination mg/g DW
0	11.50[c]	9.06[c]
2	10.07[b]	7.63[b]
5	9.23[a]	6.79[a]
8	9.26[a]	6.81[a]

IU – international unit.
Values with different superscripts within a column were significantly different at 5% significance level ($p < 0.05$).

From the above results, it was found that the addition of glutamic acid (5 mM) and phytase enzyme (600 IU/L) to the soaking water gave the best effect (Table 4). GABA content after germination increased 21.23% compared to that obtained without adding glutamic acid (from 165.32 to 200.41 mg/100 g DW). In contrast, phytic acid was significantly lower (24.83%) than it was in the absence of phytase enzyme (6.97 mg/g DW compared to 9.06 mg/g DW). In a similar study, Zhang et al. (2014) also soaked brown rice in 1 g/L glutamic acid solution at 30°C for 24 h and then germinated at 35°C for 48 h, resulting in a 20.2% increase in GABA content compared with soaking in neutral water. Liang et al. (2009) also investigated the effect of phytase on phytic acid content, soaking rice bran in 500 IU/L phytase solution at a ratio of 1:3 and at 50°C for only 30 min. The results showed that the phytase enzyme removed 92% of the phytic acid.

Rice is a popular food around the world and the main ingredient in the daily meals of Vietnamese people; therefore, increasing the GABA content and reducing the phytic acid content of brown rice will significantly improve the health of consumers.

CONCLUSION

The biological value of germinated brown rice can be enhanced by optimizing soaking and germination conditions. Simultaneously adding glutamic acid and phytase enzyme to the soaking water solution significantly improved the content of a bioactive compound, GABA, and significantly reduced the amount of an anti-nutritional factor, phytic acid. The results provide a theoretical basis for the further production of brown rice that has health benefits.

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