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Effects of soaking duration and incubation conditions on GABA biosynthesis in MangBuk brown rice of Vietnam

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ABSTRACT

Introduction: Many people are currently interested in improving and maintaining their health status by changing their dietary habits, like eating more natural foods; thus sprout products are becoming increasingly popular. In this context, sprouted brown rice grains are an excellent example of functional food, because besides their nutritive value, they also lower the risk of various diseases and/or exert health-promoting effects. In this paper, we focused on the bioactive compound γ-aminobutyric acid (GABA) in germinated brown rice. GABA is known as an important amino acid that can help reduce hypertension and inhibit cancer cells development. **Methods:** We investigated the hydration characteristics of brown rice by drying them in a moisture analyser at 130°C until reaching a constant weight. The effects of soaking (duration and pH of soaking solution), as well as incubation conditions (temperature and time) on GABA biosynthesis in MangBuk brown rice of Vietnam were measured. Quantification of GABA was measured using a spectrophotometer. **Results:** GABA content in MangBuk type 1 brown rice was higher than in type 2. GABA content reached its highest value at 691.88 µg/g for type 1 rice and 596.48 µg/g for type 2 rice when MangBuk brown rice was soaked in a pH 7 water at 30°C for 12 hours, and then incubated at 35°C for 30 hours in aerobic condition. **Conclusion:** Germination conditions modified the content of biologically active compounds in MangBuk soft and hard rice varieties. GABA was synthesised during germination based on three factors, namely time of incubation, temperature of incubation, and pH of solution.

Keywords: gamma-amino butyric acid, germinated grains, hydrolytic enzymes, MangBuk brown rice, soaking duration

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INTRODUCTION

MangBuk red rice (brown rice) is a traditional rice of the Xe Dang people in the upland commune of MangBuk, Kon Tum province, Vietnam, which has been preserved from generation to generation. This rice has only two varieties: soft rice and hard rice. Every year in April, the Xe Dang people start to plant rice and wait until October to harvest it. Strangely, red rice plants like to live on arid land, without watering, and enjoy only rainwater from the beginning to the end of the season. The planted rice grows and develops wildly by itself.

Brown rice is an intact whole rice grain obtained after peeling off its rice husk. It contains more nutritional components, such as dietary fibre, phytic acid, vitamin E, and vitamin B than ordinary milled rice. These bio-functional components exist mainly in the germ and bran layers, most of which are removed by polishing or milling. In germinated grains, hydrolytic enzymes are activated and they decompose starch, non-starch polysaccharides, and proteins, which leads to an increase in oligosaccharides and amino acids (Patil & Khan, 2011; Albarracin *et al.*, 2019). The decomposition of high molecular weight polymers during germination leads to the generation of bio-functional substances and the improvement of organoleptic qualities due to the softening of texture and increase in flavour. Germinated rice is produced by soaking it in water at an appropriate temperature and for the right duration. The result yields a 0.5-1 mm long sprout from the brown rice grain; at this stage nutrient accumulation in the grain is at its maximum. Manufactured germinated rice is mostly sold in dried form (the drying does not affect the superior nutritional value accumulated from germination), which looks very similar to ordinary brown rice. The goal of the drying process is to prolong the shelf life of germinated rice (Albarracin *et al.,* 2019). Compared to other rice varieties, germinated brown rice offers considerable health benefits, thanks to its increased amounts of γ-aminobutyric acid (GABA), dietary fibre, inositols, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc, γ-oryzanol, and prolylendopeptidase inhibitor. Additionally, the process of germination enhances the bio-availability of nutrients by neutralising phytic acid and releasing proteins, vitamins, and enzymes that allow important nutrients to be absorbed during digestion (Patil & Khan, 2011; Albarracin *et al.,* 2019). The germination of brown rice also helps free its bound minerals, making them more absorbable by the body; besides, the rice also becomes more tender and tastier (Kayahara & Tsukahara, 2000).

GABA is a non-protein amino acid widely distributed in nature that is a neurotransmitter in the brain and spinal cord of mammals (Bown *et al.,* 1999). GABA health benefits include: diuretic effects, tranquillising effects (Jakobs *et al.,* 1993), Alzheimer's disease prevention (Ito & Ishikawa, 2004), regulation of blood pressure and heart rate, pain and anxiety relief (Kono & Himeno, 2000), improved insulin secretion to prevent diabetes (Huang *et al.,* 2007), or inhibition of cancer cell proliferation (Oh & Oh, 2004). GABA is produced primarily by the decarboxylation of L-glutamic acid, catalysed by the glutamate decarboxylase enzyme (GAD) during the germination process of brown rice (Bown *et al.,* 1999). The amount of GABA in germinated brown rice in Japan was noticed to be ten times more as compared to milled white rice and two times more than that of brown rice (Kayahara & Tsukahara, 2000). Roohinejad *et al.* (2009) reported that GABA content after germination in Malaysian brown rice seeds ranged between 0.01 and 0.1 mg/g. Based on that, we studied in this

paper what are the conditions that give the highest GABA content in MangBuk red rice.

MATERIALS AND METHODS

Materials

The two varieties of MangBuk brown rice were taken from Mang Den village, DakLong commune, Kon Plong district, Kon Tum province, Vietnam. The raw rice materials were two types of brown rice of the same origin, but different in characteristics. Type 1 brown rice was soft rice, while type 2 brown rice was hard rice.

Analysis of hydration characteristics of brown rice

Brown rice was washed with fresh water to rinse out contaminants and then thoroughly rinsed with distilled water. Type 1 and type 2 brown rice were divided into five portions (20 g/sample) for each type. Brown rice samples were soaked in distilled water (pH 7) with a ratio of 1:2 (w/v) in an incubator (Wise Cube, DAIHAN-Korea) at 30°C for 6, 8, 10, 12, and 14 hours. At each time interval (duration), the moisture content of the brown rice samples was analysed by drying them in a moisture analyser (AND MX-50, Japan) at 130°C until reaching a constant weight.

Analysis of GABA content in germinated rice at different pH of soaking solution

The brown rice was soaked for the durations mentioned above. Type 1 and type 2 brown rice were divided into three portions (20 g/sample) for each type. Brown rice samples were soaked in water (1:2) (w/v) with adjusted pH levels of 5, 6, and 7 in an incubator at 30°C with the soaking duration chosen based on the results of the previous experiment (12 hours). For each pH, the GABA content of brown rice samples was analysed using the absorption spectrophotometry method (UV-VIS 2502, LaboMed, Inc).

Analysis of GABA content in germinated rice at different incubation durations

The brown rice was soaked in water with a pH chosen based upon the results of the previous experiment. Type 1 and type 2 brown rice were divided into six portions (20 g/sample) for each type. After being soaked in water at 30°C for 12 hours (duration chosen from the previous experiment), brown rice samples were stored in Erlenmeyer flasks. They were then incubated with water (1:0.5) (w/v) at pH 7 without lids (aerobic) and with lids (anaerobic) in an incubator set at 30°C for 18, 24, and 30 hours of duration.

With lids, the Erlenmeyer flasks were airtight and thus the samples lacked oxygen. Without lids, the samples had access to oxygen throughout the incubation duration. At each incubation time interval, GABA content in brown rice samples was analysed by absorption spectrophotometry (Karladee & Suriyong, 2012).

Analysis of GABA content in germinated rice at different incubation temperatures

The brown rice was incubated for a duration chosen based on the results of the previous experiment. Type 1 and type 2 brown rice were divided into 6 portions (20 g/sample) for each type. After being soaked in water with adjusted pH 7 at 30°C for 12 hours, brown rice samples were stored in Erlenmeyer flasks, then incubated with water (1:0.5) (w/v) at pH 7 without lids and with lids in an incubator set at each tested temperature (30, 35, and 40°C) for 30 hours. For each incubation temperature, GABA content of the samples was analysed by absorption spectrophotometry.

Determination of GABA content

Determination of GABA content was based on the assay of Karladee and Suriyong (Karladee & Suriyong, 2012). Whenever measurement was needed to be taken, germinated rice was grounded to fine powder after soaking and incubation. The powder was diluted with distilled water with a ratio of 1:1. Then, 10 ml of the sample and 0.53 g of Na_2CO_3 were added in a 50 ml falcon tube. After that, the mixture solution was centrifuged at a rotational speed of 5000 rpm for 15 minutes. Following that, all residues and other components of the centrifuged mixture were removed by a filter paper. The supernatants were then collected and taken to quantify GABA content as follows: 0.6 ml of supernatants was mixed with 0.4 ml borate buffer (pH 0.9) and 2 ml phenol 6.0% (w/v). Next, the mixture was shaken vigorously for one minute and cooled in water at 20°C for five minutes. 1.6 ml NaOCl 8% was added and boiled at 100°C for ten minutes, then cooled in water for five minutes. Finally, GABA content was measured with an absorbance of 630 nm wavelength on a spectrophotometer.

Statistical analysis

Each treatment was repeated three times. All parameters were analysed by analysis of variance (ANOVA) using IBM SPSS Statistics for Window, version 20.0 (IBM Corp, Armonk, New York, USA).

RESULTS AND DISCUSSION

Hydration characteristics of brown rice

Soaking duration affected the germination ability of brown rice. The study results in Figure 1 show that for both types of brown rice, during the first six hours of soaking, seeds absorbed moisture very quickly, increasing the moisture content from 11% to 30% due to the difference in moisture content inside and outside of brown rice. In addition, during this stage, there was

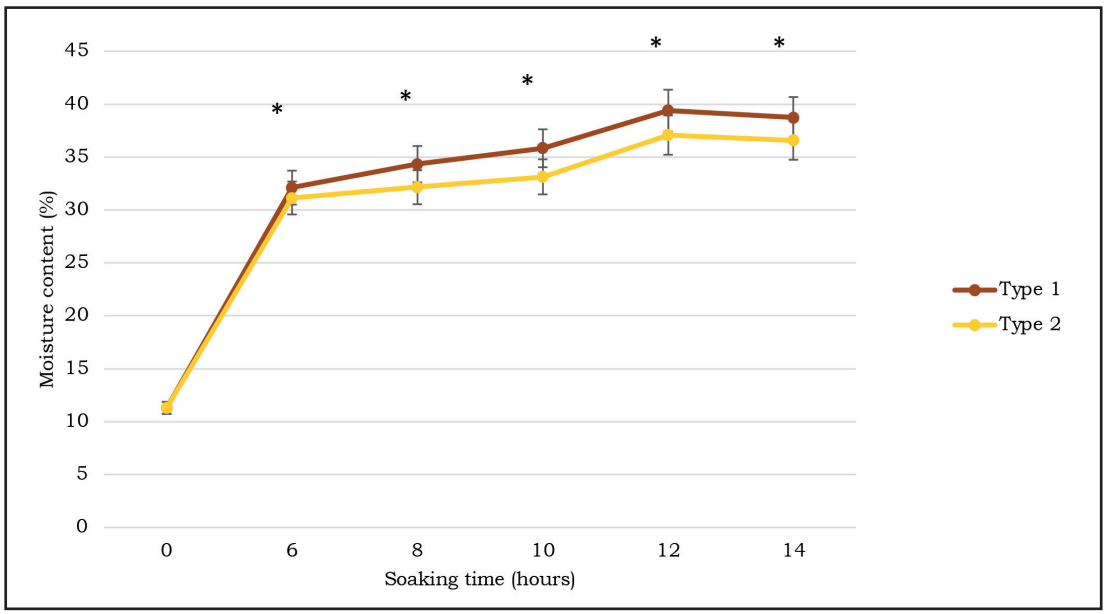

Figure 1. Hydration characteristics of MangBuk soft rice (Type 1) and MangBuk hard rice (Type 2) during soaking at 30°C

*Statistically significant differences between different times of incubation (p<0.05)

Table 1. GABA content in two varieties of germinated brown rice (MangBuk type 1 and 2) in different soaking solutions

Type of brown rice	*pH*	*GABA content (µg/g)*
Type 1	Brown rice (control)	20.76±0.72^{f}
	pH 5	155.03±14.31^{d}
	pH 6	216.85±8.02^{b}
	pH 7	284.60±3.77^{a}
Type 2	Brown rice (control)	16.96±2.56^{f}
	pH 5	40.84±10.31^{e}
	pH 6	183.02±8.92^{c}
	pH 7	227.38±9.60^{b}

Values represent *means±SEM* from 3 different experiments.
^{abcdef} Different letters in the column indicate significant differences between control and treatment at $p<0.05$.

a quick absorption into the embryo of the kernel (Bello, Tolaba & Suarez, 2004). After that, the dehumidification process slowed down and reached a saturation state after 12 hours for both rice types. When the soaking duration was increased from 12 to 14 hours, the absorption of water in the grain changed insignificantly. In this phase, water was absorbed in a constant linear rate until mass transfer reduction, when equilibrium was reached, correlating with grain metabolism and the visible onset of germination. This phase can be correlated to the filling of voids in the grains by water molecules and the lack of chemical links between grain constituents and water (Borges *et al.,* 2017). Many previous studies have shown that a humidity of about 35% - 40% (w/w) would ensure normal seed germination (Cung *et al.,* 2013; Komatsuzaki *et al.,* 2007). Therefore, the soaking process should end after 12 hours for both rice types. The moisture content at this moment was 39.4% for type 1 brown rice and 37.0 % for type 2 brown rice.

Table 2. GABA content of brown rice incubated in aerobic and anaerobic conditions for different durations

Incubation duration (hours)	*GABA content (µg/g)*	
	Aerobic incubation	*Anaerobic incubation*
Type 1 brown rice		
Brown rice (control)	20.76±0.73^{g}	20.76±0.73^{g}
18	420.73±9.55^{d}	397.31±2.62^{e}
24	482.90±9.48^{b}	437.57±9.39^{c}
30	538.19±5.91^{a}	517.04±7.58^{a}
Type 2 brown rice		
Brown rice (control)	16.96±2.56^{g}	16.96±2.56^{g}
18	351.66±8.91^{e}	310.75±9.97^{f}
24	437.00±6.70^{c}	412.53±7.53^{d}
30	478.44±12.12^{b}	462.87±6.15^{b}

Values represent means±*SEM* from 3 different experiments.
^{abcdefg} Different letters indicate significant differences between control and treatment at $p<0.05$.

Effect of pH of soaking solution on GABA content in rice germination

The GABA content of germinated brown rice with various soaking solutions is shown in Table 2. The GABA content of type 1 brown rice (not soaked) was 20.76 µg/g and it ranged from 155.03 to 284.60 µg/g after being soaked in solutions with different pH (Table 1). The GABA content of type 2 brown rice (not soaked) was 16.96 µg/g and it ranged from 40.84 to 227.38 µg/g after being soaked. The highest value of GABA content for type 1 brown rice (284.60 µg/ g) was found in samples soaked in water at pH 7, while the production of GABA was lower at pH 5 and pH 6 with respective values of 155.3 µg and 216.85 µg. Similarly, the highest value of GABA content for type 2 brown rice (227.38 µg/ g) was observed in samples soaked in water at pH 7, while the production of GABA was lower at pH 5 and pH 6 with respective values of 40.84 µg and 183.02 µg. GABA contents were significantly different based on pH levels (p<0.05), but also based on the type of brown rice.

The results showed that higher GABA content was obtained with a soaking solution at pH 7. These results were similar to those reported by Zhang *et al.* (2014) who suggested that soaking Indica and Japonica germinated brown rice at 30°C in distilled water with pH 7 resulted in the highest GABA. However, Sunte *et al.* (2007) found that Thai Hommali 105 rice soaked in a pH 5 buffer solution had the highest GABA content, while Watchraparpaiboon *et al.* (2010) measured the highest GABA content in Thai Khao Dawk Mali 105 and Chainat 1 rices when soaked in a pH 6 water, and Thai Sangyod Muang Phatthalung rice soaked in a pH 3 water for 36 hours (Banchuen *et al.,* 2009). Another study on Thai Phitsanulok 2 rice soaked at 33°C for 300 minutes in a pH 5.7 water provided the highest GABA content (Singh *et al.,* 2017). The increase of GABA content during soaking is due to the action of glutamate decarboxylase (GAD) that gradually increases during water soaking and transforms glutamate into carbon dioxide and GABA (Komatsuzaki *et al.*, 2007). During germination, biomolecules, such as lipids and proteins, increase. Soaking water acts as a hydrolytic enzyme to digest the rice seed proteins and lipids, causing an increase in permeability that allows proteins and lipids to migrate into the rice seed (Raj & Singaravadival, 1979).

Table 3. GABA content of brown rice incubated in aerobic and anaerobic conditions at different temperatures

Incubation temperature (°C)	*GABA content (µg/g)*	
	Aerobic incubation	*Anaerobic incubation*
Type 1 brown rice		
Brown rice (control)	20.76±0.73^{e}	20.76±0.73^{e}
30	536.62±2.25^{c}	494.31±14.20^{c}
35	691.88±2.30^{a}	590.50±5.95^{b}
40	597.93±12.92^{b}	508.87±10.63^{c}
Type 2 brown rice		
Brown rice (control)	16.96±2.56^{e}	16.96±2.56^{e}
30	511.11±5.02^{d}	464.17±5.13^{d}
35	596.48±6.53^{b}	557.67±11.52^{b}
40	542.32±1.86^{c}	462.98±9.97^{d}

Values represent means±*SEM* from 3 different experiments.
^{abcd} Different letters indicate significant differences between control and treatment at p<0.05.

Moreover, soaking could lead suspension cells to become adapted to water stress. Such stress may also contribute to the accumulation of GABA by reducing the oxidation of succinic semialdehyde to succinate. However, GABA accumulation in germinated brown rice at different pH levels of soaking solution is dependent on the variety and origin of rice.

Effect of incubation duration on GABA content in rice germination

As grains are being soaked, imbibition begins, respiration is accelerated, which stimulates the metabolism of amino acids, resulting in the formation of enzyme systems. GABA synthesis is usually initiated because of the activation of GAD enzyme during the soaking process, and enzyme activity increases with germination time (Karladee & Suriyong, 2012). In our study, the rice germination in aerobic incubation gave higher GABA content than anaerobic incubation in both types of brown rice and for all times of incubation. The germination duration also clearly influenced GABA content. The GABA content of type 1 brown rice had increased a lot after 18 hours, and it reached its highest value after 30 hours of incubation at 30°C (538.19 µg/g), which was 25.92 times higher than that of the control (no incubation, 20.76 µg/g) in aerobic condition. The GABA content of type 2 brown rice had also increased a lot after 18 hours and reached the highest value after 30 hours of incubation at 30°C (478.44 µg/g), which was 28.21 times higher than that of the control (no incubation, 16.96 µg/g) in aerobic condition. The results were similar in anaerobic condition for both types of Mang Buk brown rice. The results also showed that GABA content of type 1 brown rice was higher than type 2 in both conditions of incubation and for all times of incubation.

Previous studies have also shown that incubation conditions and duration have a direct impact on the metabolism of glutamic acid leading to the alteration of GABA content in sprouted brown rice. Karladee & Suriyong (2012) reported that GABA content reached its highest level of 17.87 mg/100 g of dry matter after 24 hours of incubation in 21 Thailand rice varieties (11 purple rice landraces and 10 modern white rice varieties). Patil & Khan (2011) also reported that Korean giant embryo brown rice (Keunnunbyeo) incubated for 21 hours had the highest GABA content. Similar result was also recently found by Zhang *et al.* (2022), who showed that Chinese brown rice germinated for 24 hours with pulsed light exposure gave the highest GABA content. However, working on three varieties of Southern Thai brown rice, Banchuen *et al.* (2010) suggested that the optimum conditions for producing the highest GABA content were to germinate them in a closed vessel for 36 hours for Sangyod Phatthalung rice and Chiang Phatthalung rice, and for 48 hours for Niaw Dam Peak Dam rice. GABA content was highest after 72 hours of germination for Chinese red rice (Ding *et al.*, 2018) and Indica rice (MTU 1010 and KNM 118) (Mohmmed *et al.,* 2021), or after 40 hours at 35°C for high yielding variety rice (cv. Jhelum) (Hussain *et al.*, 2020) and Brazilian red rice (Müller *et al.*, 2021). Taken together, we think genetic differences among rice varieties regulate the synthesis of GABA in grains, as genetic diversity is a basic prerequisite for successful exploitation of desirable traits through plant breeding (Hussain *et al.*, 2020).

Effect of incubation temperature on GABA content

Based on the results obtained from the changes in environment pH and incubation time, we continued to monitor GABA production during MangBuk rice germination based on incubation temperature. The effect of different

Table 4. Current reports (last fifteen years) about the optimal germination conditions for GABA enrichment in brown rice

Type of rice	*Optimal germination conditions for GABA enrichment*					*References*
	Temperature and times of soaking	*pH of soaking solution*	*Temperature of germination*	*Times of germination*	*Highest GABA content (mg/ 100g)*	
Thai Hommali 105 rice		pH 5			21.93	Sunte *et al.*, 2007
Thai Sangyod Muang Phatthalung rice		pH 3		36h	44.53	Banchuen *et al.*, 2009
Thai Khao Dawk Mali 105 and Chainat 1 rice	35°C for 24h	pH 6			16.48 for Khao Dawk Mali 105 and 14.50 for Chainat 1	Watchraparpaiboon *et al.*, 2010
Thai Sangyod Phatthalung rice and Chiang Phatthalung rice		pH 3		36h in closed vessel	44.53 for Sangyod Phatthalung and 29.25 for Chiang Phatthalung	Banchuen *et al.*, 2010
Thai Niaw Dam Peak Dam rice				48h in closed vessel	20.92	Banchuen *et al.*, 2010
Thai red Jasmine brown rice	35°C for 24h				41.02	Wichamanee & Teerarat, 2012
21 Thai rice varieties (11 landraces purple rice and 10 modern white varieties)				24h	13.65 - 23.6	Karladee & Suriyong, 2012
Japonica brown rice	3h		35°C	21h	24.9	Komatsuzaki *et al.*, 2007; Cung *et al.*, 2013.
Indica and Japonica brown rice	30°C	pH 7	35°C	36h	131 for Indica rice and 138 for Japonica rice	Zhang *et al.*, 2014
Chinese Dongnong 419 rice	30°C for 12h		25°C	40h	28.14	Cao et al., 2015
Thai Phitsanulok 2 rice	33°C for 300 min	pH 5.7			18.67	Singh *et al.*, 2017
Chinese red rice				72h	44.8	Ding *et al.*, 2018
Indica High yielding variety rice (cv. Jhelum)	5.76 h		35°C.	40h	48.18	Hussain *et al.*, 2020
Indica MTU 1010 and KNM 118 rice	28±2°C for 12h			72h	98.53 for MTU 1010 and 71.77 for KNM 118	Mohmmed *et al.*, 2021
Indonesian brown rice (var. Inpari 43)	120h			24h	126.55	Munarko *et al.*, 2021
Vietnamese MangBuk soft rice and MangBuk hard rice	30°C for 12h	7	35°C	30 hours in aerobic condition	69.188 for soft rice and 59.648 for hard rice	This study

incubation temperatures in aerobic and anaerobic conditions on GABA content are shown in Table 3.

The GABA content was highest at 35°C with both types of rice in both conditions of incubation. However, the GABA content of samples in aerobic condition was higher than that in anaerobic condition, and the GABA content in type 1 germinated brown rice was higher than in type 2 germinated brown rice. The highest GABA content of type 1 brown rice was observed at 35°C of incubation in aerobic condition (691.88 µg/g), while that of type 2 brown rice was 596.48 µg/g under the same conditions. The GABA contents of non-incubated brown rice were measured at 20.76 µg/g and 16.96 µg/g for type 1 brown rice and type 2 brown rice, respectively. After 30 minutes of incubation at 35°C, the GABA contents increased by 33.33 to 35.17 times compared to the initial contents. Therefore, 35°C was the best incubation temperature to increase GABA content during germination.

A similar result was reported by Wichamanee & Teerarat (2012) with a GABA content of 41.02 mg/100 g in red rice grain after soaking at 35°C, as well as Watchraparpaiboon *et al.* (2010) who reported that GABA contents of brown Thai rice were the highest after soaking rice grains in water at 35°C for 24 hours. Komatsuzaki *et al.* (2007) also soaked brown rice at 35°C to make GABA content reached 24.9 mg/100 g, higher than the conventional method (10.1 mg/100 g). Thus, Cung *et al.* (2013) suggested that the optimal incubation temperature of germinated rice should be 35°C. Zhang *et al.* (2014) reported that the highest GABA content was obtained by a germination at 35°C for 36 hours. However, the maximum GABA synthesised during germination was recorded in Indica Jhelum rice and Indica Tangdhar Zag rice after 5.76 hours of soaking and 40 hours of germination at 35°C (Hussain *et al.,* 2020), and in Indonesian Brown Rice (var. Inpari 43) after 120 hours of soaking and 24 hours of germination (Munarko *et al.,* 2021). Therefore, we believe that different rice varieties of different origins need different soaking temperatures, durations, and germination conditions to reach their highest GABA content (Table 4).

CONCLUSION

Germination conditions modified the content of biologically active compounds in MangBuk soft and hard rice varieties. GABA was synthesised during germination based on three factors: time of incubation, temperature of incubation, and pH of solution. The GABA content reached its highest value at 691.88 µg/g for type 1 rice and 596.48 µg/g for type 2 rice when MangBuk brown rice of Vietnam was soaked in a pH 7 water at 30°C for 12 hours, and then continually incubated at 35°C for 30 more hours in aerobic condition. These results showed that germination is an important process to preserve brown rice bioactive compounds. We also revealed, for the first time, the effectiveness of optimal conditions for maximising GABA accumulation in MangBuk brown rice in Vietnam.

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Authors' contributions

HTNT, experiments realisation and data analysis; PPH, principal investigator, study conceptualisation and design, data analysis and interpretation, manuscript draft preparation; TMDN, data analysis and interpretation, manuscript draft preparation and review.

Conflict of interest

The authors declare no conflict of interest.

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