Factor Affecting Accumulation of Gamma-Aminobutyric Acid (GABA) in Rice Germ (Khao Dawk Mali 105)

Sriwiang Tipkanon*
Department of Agro-Industry Product Development, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi 25230, Thailand

Ahmed Moursy Abdallah
Department of Food and Dairy Sciences and Technology, Faculty of Environmental Agricultural Science, Suez Canal University, El-Arish, Egypt

* Corresponding author. E-mail: swtn@kmutnb.ac.th
Received: 24 April 2014; Accepted: 16 June 2014; Published online: 2 July 2014
DOI: 10.14416/j.ijast.2014.06.001

Abstract
GABA content in rice germ is affected by many factors. The objective of this study was to screen significant factors which affect GABA content. Four factors (pH, $CaCl_2$ concentration, soaking temperature, and soaking time) were tested using the eight-run Plackett-Burman design. Factors were considered to have significant effects on response at $\alpha = 0.05$. The results revealed that soaking temperature and soaking time most significantly affected GABA content (positive effect). Subsequently, effect of soaking temperature and time were conducted by soaking the rice germ in 0.5 mmol/L $CaCl_2$, pH 6 at 30, 40, and 50°C for 1, 2, 4, 8, and 16 h. The results indicated that rice germ soaked at 40°C for 8 h was the highest GABA content (307.1 mg/100 g of rice germ, dry basis).

Keywords: *Gamma-aminobutyric acid, GABA, Rice germ, Plackett-Burman design*

1 Introduction

Rice (*Oryza sativa* Linn.) is the staple food for nearly one-half of the world's population especially for Asian countries. Rice germ is a by-product of the rice milling process which is included in rice bran [1]. Rice germ serves as rich sources of hypoallergenic protein, oil, dietary fiber and nutrient essential for life: such as vitamin B and E, beta-carotene and gamma oryzanol. Besides, rice germ also contains a functional compound called gamma-aminobutyric acid or GABA. GABA is an amino acid that is produced by decarboxylation of glutamic acid in living organisms. It is known that GABA plays an important role in the central nervous system as a neurotransmitter and function by lowering the blood pressure in human brain [2]. GABA levels in rice germ are influenced by many factors, including the duration of incubation of seeds. GABA in rice is synthesized from glutamic acid by glutamate decarboxylase, GAD, and the activity of GAD shows high correlation with the germination ratio [3]. The GABA content in the rice germ has been extensively investigated. Studies on the effect of water soaking on GABA accumulation in fragrance rice showed that GABA in rice germ increased with increasing soaking time [3-5].

During water soaking, the contents in rice grains could be changed by enzymes responsible for scarification, proteolysis, and amino acid metabolism. Saikusa et al. [6] reported that soaking rice in water brought about remarkable change in the component and content of free amino acid in the rice kernel; the most significant of these changes was an increase of γ-aminobutyric acid content. Haiminori is rice cultivar with giant embryo that was originally developed by chemical mutation [7]. After rice grains were soaked in water for 4 h, the amount of accumulated GABA in brown rice of Haiminori was ≈3-4 times of dry rice grains [8]. Water soaking can enrich GABA content in the germ of all Thai rice varieties (Khao Dawk Mali 105, Pathum Thani 1, Chai Nat 1, Supan Buri 1, Leuang Pratew 123, and Plai Ngahm). The GABA

accumulation differed among rice varieties after soaking for different times. GABA content in most of Thai rice varieties increased during 4 h of incubation at 40°C [5]. Based on this finding, an efficient and simple method via water soaking has been developed for production of GABA from rice germ.

Besides the possible contribution to the taste of rice, GABA in rice is worth further investigating since it has been proved to be effective for lowering the blood pressures of mammals including dogs, rabbits, pigs, and cats [9]. Ohmori et al. [10] clearly showed that green tea enriched with GABA by anaerobic treatment [11] worked effectively to keep the blood pressure of spontaneously hypertensive rats (SHR) at a normal level. In Japan, GABA-enriched tea has been produced on a commercial basis for people with hypertension [12].

During soaking, GABA content in rice germ is affected by many factors. Thus, the objective of this research was to study factor affecting accumulation of gamma-aminobutyric acid (GABA) in rice germ (Khao Dawk Mali 105) during soaking. This research will provide a basis for commercial production of GABA-enriched rice germ.

2 Materials and Methods

2.1 *Material*

Germ from Thai rice variety (cv. Khao Dawk Mali 105) was obtained from Jibtong Surin Rice Mill, Thailand. This variety was chosen because it is commercially available and on the basis of our preliminary study, it contains a high level of GABA content compared to Plai Ngahm Prachinburi, Chai Nat 1, and Khao Ban Na 432.

2.2 *Reagent*

Gamma-amino-n-butyric acid (≥ 99%) was purchased from Sigma Aldrich Chemical (Hamburg Germany). Acetic acids, sodium bicarbonate, and calcium chloride were purchased from Merck (Darmstadt, Germany). 4-Dimethyl-aminoazobenzene-4-sulfonyl chloride (DABSYL-CL) analytical grade was obtained from Fluka Chemical and acetonitrile (HPLC grade) was obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.3 *Preparation of rice germ*

Rice germ was sieved through a 32 mesh sieve, collected, packed in plastic (HDPE) bags, and stored in cardboard boxes at -18°C until further analysis. Two separated batches of rice germ were prepared.

2.4 *Initial screening of significant factors by the Plackett-Burman design*

The eight-run Plackett-Burman design was employed to screen/select the two most critical factors (pH, $CaCl_2$ concentration, soaking temperature and/or soaking time) for GABA accumulation as shown in Table 1.

Table 1: Plackett-Burman design for selection of significant factors affecting concentration of GABA

Treatments	Sampling	Factors and their levels[a]						
		A	B	C	D	E	F	G
1	5	+6	+2	+50	-4	+	-	-
2	3	+6	+2	-30	+12	-	-	+
3	6	+6	-0.5	+50	-4	-	+	+
4	2	-4	+2	-30	-4	+	+	+
5	1	+6	-0.5	-30	+12	+	+	-
6	7	-4	-0.5	+50	+12	+	-	+
7	8	-4	+2	+50	+12	-	+	-
8	4	-4	-0.5	-30	-4	-	-	-

Note: [a](A) pH value: low level, 4; high level, 6. (B) Concentration of $CaCl_2$ (mmol/L): low level 0.5; high level, 2. (C) Soaking temperature (°C): low level, 30; high level, 50. (D) Soaking time (h): low level, 4; high level, 12. (E), (F), and (G) are dummy variables: - indicates a low level: +indicates a high level.

2.5 *Determination of GABA in rice germ*

One-fifth gram or one-half gram of rice germ (0.2-0.5 g.) was weighed in plastic tube; Then 2 mL of $CaCl_2$ solution [0.5 mmol/L (low level) or 2 mmol/L (high level)] at pH 4 or 6 was added. The samples were incubated at temperature and time while shaking following Table 1. Each suspension was removed from the water bath, thereafter it was centrifuged at 4500 rpm for 10 min and the supernatant was separated. Quantitative analysis of GABA content was performed by using HPLC. One mL of supernatant was pipetted and added with 200 µL of 0.4 mol/L $NaHCO_3$ and 400 µL of 6 mmol/L DABSYL-CL acetronitrile solution. The reaction was performed at 70°C for 20 min. After derivatization, the sample was filtered (0.45 µm membrane filter) to vial and 10 µL of sample was

injected into HPLC unit (Agilent 1100 series, Agilent Technologies, USA) equipped with a column (Supelcosil-LC-DABS 3 μm, 150×4.6 mm i.d.). Acetronitrile was used as the mobile phase with a flow rate of 1 mL/min with the column temperature being 40°C and the ultraviolet detector set at 315 nm. [5,13].

2.6 *Statistical analysis*

The Plackett-Burman design was employed to screen/select critical factors. The main effects of each factor on GABA content were estimated as the difference between average of measurements made at the higher level and at the lower level. The significance of each factor was determined via a t-test. Preliminary results (Table 2) revealed that soaking temperature and soaking time most significantly affected GABA concentration. Therefore, these two factors were selected for further study.

A full 3 (30, 40, and 50°C) × 5 (1, 2, 4, 8, and 16 h) factorial design was employed to attain the effect of soaking temperature and time conditions at a given pH (6) and a level of $CaCl_2$ concentration (0.5 mmol/L) for maximum GABA concentration. Rice germ without soaking was used as a control. The experiments were carried out in triplicate. All data were analysed by analysis of variance (ANOVA) using the SPSS software (Statistical Software Package for Social Sciences; SPSS Inc., and IBM Co., Chicago, IL, USA). The post hoc comparisons were performed by the Duncan Multiple Range Test (DMRT) when ANOVA had indicated that the model and treatment effects were significant ($p < 0.05$).

Response surface methodology (RSM) was employed to attain an optimal soaking temperature and time conditions at a given pH (6) and a level of $CaCl_2$ concentration (0.5 mmol/L). The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

where Y is the predicted response, b_0 is the value of the fitted response at the center point of the design [point (0,0)], b_1 and b_2 are linear regression term, b_{11} and b_{22} are quadratic regression terms, and b_{12} is the cross product regression term. The SPSS software (SPSS Inc., and IBM Co., Chicago, IL, USA) was used for regression analysis of the experimental data and STATISTICA (StatSoft, Inc., Tulsa, OK, USA) was used to plot the response surface graph. The quality of fit of the second-order polynomial model equation was expressed in the form of contour plot, in order to illustrate the relationship between the response and the experimental levels of the variables.

3 Results and Discussion

3.1 *Initial screening of significant factors using the Plackett-Burman design*

A total of four factors (pH, $CaCl_2$ concentration, soaking temperature and/or soaking time) were screened for effective accumulation of GABA using the eight-run Plackett-Burman design. The concentrations (mg/100g dry basis) of GABA at various processing conditions are shown in Table 2. Factors were considered to have a significant effect on the response (i.e., GABA concentration) when the calculated t value was greater than 3.182 at $\alpha = 0.05$ (Table 2). According to Table 2, pH (a low level at 4; a high level at 6) and $CaCl_2$ concentration (a low level at 0.5 mmol/L; a high level at 2 mmol/L) did not significantly affect GABA content. In contrast, soaking temperature (a low level at 30°C; a high level at 50°C) and soaking time (a low level at 4 h; a high level at 12 h) inserted a significant positive effect on GABA content (Table 2). Therefore, the effect of these two factors (soaking temperature and time) was further investigated by factorial design.

Table 2: t value and effect of pH, $CaCl_2$ concentration, soaking temperature and time on GABA concentration from the Plackett-Burman design

GABA content (mg/100g)	Factors[a]			
	A	B	C	D
t value	0.574	0.427	3.216[b]	4.314[b]
Low level	207.30	208.57	184.28[b]	174.71[b]
High level	217.29	216.02	240.31[b]	249.88[b]

Note: [a](A) pH value: low level, 4; high level, 6. (B) Concentration of $CaCl_2$ (mmol/L): low level 0.5; high level, 2. (C) Soaking temperature (°C): low level, 30; high level, 50. (D) Soaking time (h): low level, 4; high level, 12. [b]Significant positive effect at $p < 0.05$ using at t test ($t_{\alpha = 0.05 \text{ at df} = 3} = 3.182$).

Similar results reported by Sunte et al. [14] were that the amount of GABA in brown rice (Khao Dawk

Mali 105) under a condition with adjusted 4, 5, and 5.5 pH were not significantly different ($p < 0.05$). Its high accumulation under a lightly acidic condition and low accumulation under a basic condition are consistent with pH-activity response reported [15] for glutamate decarboxylase (optimum pH, 5.9) and Gaba-pyruvate transaminase (optimum pH, 8.9) from radish leave. For the effect of $CaCl_2$ concentration on GABA content, Liu et al. [3] reported that 0.5 mmol/L Ca^{2+} markedly elevated the GAD activity, and increased GABA content in rice germ (Haiminori) when soaked rice germ in $CaCl_2$ solution (0.5 mmol/L) was compared with control (soaking rice germ in water).

3.2 *The effect of soaking temperature and time on GABA concentration*

To investigate the accumulation of GABA, the rice germ was soaked in $CaCl_2$ solution (0.5 mmol/L) and pH 6 at 30°C, 40°C, and 50°C for 0 (control), 1, 2, 4, 8, and 16 h. Then the changes of GABA content were analyzed. The result revealed that the GABA content was affected by soaking temperature and time. As shown in Table 3 and Figure 1, soaking at temperatures of 40°C and 50°C resulted in a greater GABA content of rice germ than 30°C. The amount of GABA accumulated in rice germ during water soaking at 50°C (172.27-279.07 mg/100 g of rice germ, dry basis) was less than the GABA amount at 40°C (192.10-307.10 mg/100 g of rice germ, dry basis). The soaking temperature is also one of the major factors affecting GABA content. The temperature not only affects the biocatalyst activity and stability but also has an effect on the thermodynamic equilibrium of a reaction [16]. Thus, the decrease in GABA content of the water-soaked germ at 50°C caused by heat temperature is presumably due to inactivation of glutamate decarboxylase and the proteolytic enzymes. In addition, GABA content in rice germ was the highest when the rice germ was soaked in 0.5 mmol/L $CaCl_2$ solution at pH 6, temperature at 40°C for 8 h (307.10 mg/100g of rice germ, dry basis).

GABA content was low but detectable in dry rice germ (42.30 mg/100 g of rice germ, dry basis). The remarkable accumulation of GABA in rice germ was most clearly demonstrated after soaking (Figure 1). GABA accumulation in rice germ proceeded rapidly at an early stage of incubation. The GABA content increase in rice germ after soaking was ≈ 2.5-7.5 times in comparison with rice germ before soaking (control). GABA has been reported to be accumulated in many plants under stresses, it is said that glutamate decarboxylase is an important for the accumulation of GABA [15, 17-18]. Saikusa et al. [6] noticed that GABA accumulation in rice germ proceed very rapidly at an early stage of incubation, accompanied by the parallel loss of the glutamate concentration.

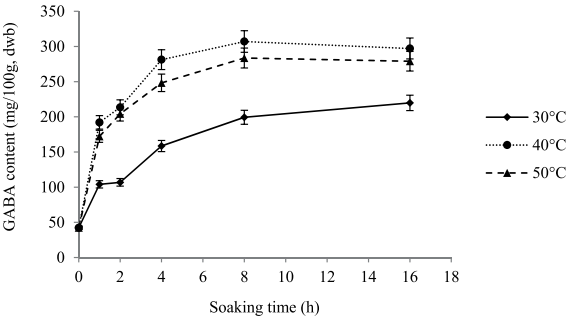

Figure 1: Accumulation of GABA in the rice germ during soaking in 0.5 mmol/L $CaCl_2$ solution, pH 6 at various temperature (30°C, 40°C, 50°C) and time (1, 2, 4, 8, 16 h) comparing with control (before soaking). The vertical bar represent the standard deviations (S.D., n=3).

Table 3: Effects of soaking rice germ in 0.5 mmol/L $CaCl_2$, pH 6 at the temperature of 30°C, 40°C, 50°C and soaking time of 1, 2, 4, 8, and 16 h on GABA concentration comparing with control (before soaking)

Temperature (°C)	Time (h)	GABA content (mg/100g dry basis)
Control (before soaking: 0 h)	0	$42.30^{h} \pm 1.15$
30	1	$104.03^{g} \pm 8.69$
	2	$106.87^{g} \pm 9.25$
	4	$158.47^{f} \pm 7.19$
	8	$199.37^{de} \pm 20.77$
	16	$219.80^{cd} \pm 22.63$
40	1	$192.10^{def} \pm 10.60$
	2	$213.53^{d} \pm 11.17$
	4	$281.30^{ab} \pm 19.37$
	8	$307.10^{a} \pm 35.07$
	16	$297.13^{a} \pm 25.02$
50	1	$172.27^{ef} \pm 22.79$
	2	$204.27^{de} \pm 24.13$
	4	$248.27^{bc} \pm 22.61$
	8	$283.60^{a} \pm 24.63$
	16	$279.07^{ab} \pm 15.55$

Note: Mean values in column followed by different letters are significantly different ($p < 0.05$).

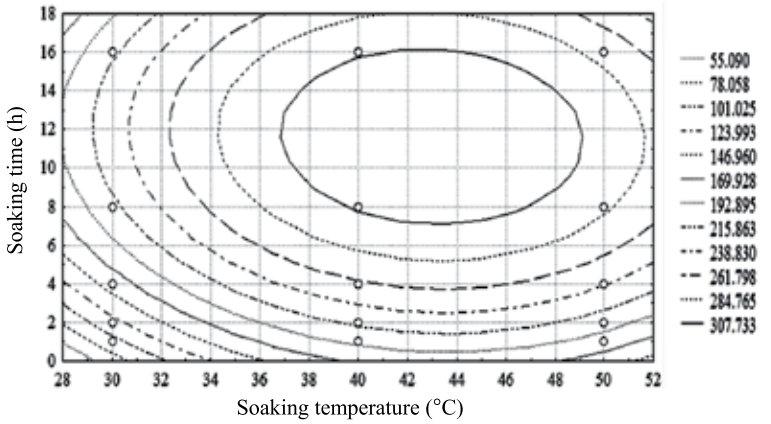

Figure 2: Contour plot for effect on GABA content (mg/100g rice germ, dry basis) of soaking temperature (°C) (X_1) and soaking time (h)(X_2) at a given pH (6) and the $CaCl_2$ concentration (0.5 mmol/L).

Stepwise regression analysis was performed on the experimental data. The best stepwise regression equation to predict GABA content is $Y = -976.017 + 52.973(X_1) + 29.017(X_2) - 0.606(X_1^2) - 1.105(X_2^2) - 0.077(X_1 X_2)$, where X_1 is soaking temperature (30-50°C), X_2 is soaking time (1-16 h). The coefficient of determination (r^2) value of equation was 0.917. The regression model effectively explains the variation in GABA content and the combination of both soaking temperature and soaking time.

Response surface methodology (RSM) was employed to attain an optimal soaking temperature and time condition that would yield maximal GABA content. According to Figure 2, the optimal soaking temperature and soaking time was around 37-49°C and 7-16 h, respectively, with the predicted maximal GABA content of ≥ 307.733 mg/100g rice germ (dry basis). Using the above equation for prediction, for instance at 40°C soaking temperature and 8 h soaking time, GABA was predicted to be 310.08 mg/100g rice germ (dry basis) (observed value = 307.10 mg/100g rice germ, dry basis).

Several researchers also demonstrated that GABA concentration in the germ increased remarkably with soaking under a slightly acidic condition [3,6,14,19]. The rice cultivar Haiminori has a giant embryo characteristic that is more favorable to GABA accumulation [8]. However, most rice is consumed as white, milled, and polished rice, and it contains mainly the endosperm part of the rice grain. The embryo of rice is a major component of rice bran and is usually a by-product of the milling process. Each year 60-70 million tons of rice bran is produced worldwide but it is rarely utilized for human food due to its low taste quality. More than 90% of the rice bran produced is used as animal feed [20]. Therefore, potential use of rice germ in health-promoting food should be further investigated.

4 Conclusions

In the experiment, GABA content in rice germ is affected by many factors. Soaking temperature and soaking time significantly affected GABA content (positive effect). Soaking can enrich the GABA content in the rice germ (Khao Dawk Mali 105). The GABA content increased with increasing soaking time. The optimal and most practical condition to produce the enrich GABA rice germ was $CaCl_2$ concentration (0.5 mmol/L), pH 6, and soaking temperature and time of 40°C and 8 h. At this optimal condition, the GABA content was 307.10 mg/100g rice germ (dry basis). This could be profitable for a rice germ (by-product of the rice milling process) for enhancing GABA. As GABA is a compound of bio-functionality, this could become a promising method of developing a GABA-enriched rice germ in health food product in further study.

Acknowledgements

This research was supported by grant from Science and Technology Research Institute, King Mongkut's University of Technology North Bangkok.

References

[1] I.H. Kim, C.J. Kim, J.M. You, K.W. Lee, C.T. Kim, S.H. Chung, and B.-S. Tae, "Effect of roasting temperature and time on the chemical composition of rice germ Oil," *JAOCS*., vol. 79, pp. 413-8, 2002.

[2] H. Mori, K. Kawabata, N. Yoshimi, T. Tanaka, T. Murakami, T. Okada, and H. Murai, "Chemopreventive effects of ferulic acid on oral and rice germ on large bowel carcinogenesis," *Anticancer Research*, vol. 19, pp. 3775-8, 1999.

[3] L.L. Liu, Q. Zhai, and J.M. Wan, "Accumulation of γ-aminobutyric acid in giant-embryo grain in relation to glutamate decarboxylase activity and it gene expression during water soaking," *Cereal Chemistry*, vol. 82(2), pp. 191-6, 2005.

[4] T. Saikusa, T. Horino, Y. Mori, "Accumulation of γ-aminobutyric acid (GABA) in the rice germ during water soaking," *Biosci. Biotech. Biochem*., vol. 58, pp. 2291-2, 1994.

[5] W. Varanyanond, P. Tungtrakul, V. Surojanamatakul, L. Watanasiritham, and W. Luxiang, "Effect of water soaking on gamma-aminobutyric acid (GABA) in germ of different Thai rice varieties," *Kasetsart J.(Nat. Sci.)*, vol. 39, pp. 411-5, 2005.

[6] T. Saikusa, T. Horino, and Y. Mori, "Distribution of free amino acids in the rice kernel and kernel fractions and the effect of water soaking on the distribution," *J. Agri. Food Chem*., vol. 42, pp. 1122-5, 1994.

[7] H. Satoh and T. Omura. "New endosperm mutations induced by chemical mutagens in rice, *Oryza sativa* L," *Jpn. J. Breed.*, vol. 31, pp. 316-26, 1981.

[8] H. Maeda, H. Nemoto, S. Iida, T. Ishii, N. Nakagawa, T. Hoshino, M. Sakai, M. Okamoto, H. shinoda, and T. Yoshida. "A new rice variety with giant embryos, Haiminori," *Breeding Sci*., vol. 51, pp. 211-3, 2001.

[9] H.C. Stanton, "Mode of action of gamma aminobutyric acid on the cardiovascular system," *Arch. Int. Pharmacodyn*., vol. 143, pp. 195-200, 1963.

[10] M. Ohmori, T. Yano, J. Okamoto, T. Tsushida, T. Murai, and M. Hiuchi, "Effect of anaerobically treated tea (Gabaron tea) on blood pressure of spontaneously hypertensive rats," *Nippon Nogei Kagaku Kaishi*, vol. 61, pp. 1449-51, 1987.

[11] T. Tsushida, T. Murai, M. Ohmori, and J. Okamoto, "Production of a new type of tea containing a high level of γ-aminobutyric acid," *Nippon Nogei Kagaku Kaishi*, vol. 61, pp. 817-22, 1987.

[12] T. Okada, T. Sugishita, T. Murakami, H. Murai, T. Saikusa, T. Horino, A. Onoda, O. Kajimoto, R. Takahashi, and T. Takahashi, "Effect of the defatted rice germ enriched with GABA for sleeplessness, depression, autonomic disorder by oral administration," *Nippon Shokuhin Kagaku Kogaku Kaishi*, vol. 47(8), pp. 596-603, 2000.

[13] S.A. Cohen and D.P. Michaud, "Synthesis of a fluorescent derivatizing reagent, 6-Aminoquinolyl-*N*-hydroxysucinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high performance liquid chromatography," *Anal. Biochem*., vol. 211, pp. 279-87, 1993.

[14] J. Sunte, V. Srijesdaruk, and R. Tangwongchai, "Effects of soaking and germinating process on gamma-aminobutyric acid (GABA) content in germinated brown rice (Hom Mali 105)," *Agri. Sci. J.*, vol. 38(6)(Suppl), pp. 103-6, 2007

[15] L.G. Streeter and J.F. Thompson, "Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (Raphanus sativus L.)," *Plant Physiol*., vol. 49, pp. 572-8, 1972.

[16] R. Dhakal, V.K. Bajpai, and K.-H. Baek. "Production of GABA (γ-aminobutyric acid) by microorganisms: A review," *Braz. J. Microbiol*., vol. 43, pp 1230-41, 2012.

[17] J.V. Possingham, "The effect of mineral nutrition on the content of free amino acids and amides in tomato plants. II. A study of the effect of molybdenum nutrition," *Aust.J.Bot.Sci*., vol. 10, pp. 40-9, 1957.

[18] W. Wallace, J. secor, and L.E. Schrader. "Rapid accumulation of γ-aminobutyric acid and alanin in soybean leaves in response to an abrupt transfer to lower temperature, darkness or mechanical manipulation," *Plant Physiol*., vol. 75, pp. 170-5, 1984.

[19] D. Karladee and S. Suriyong, "γ-Aminobutyric acid (GABA) content in different varieties of brown rice during germination," *ScienceAsia*, vol. 38, pp.13-7, 2012.

[20] T.S. Kahlon and G.E. Smith, "Rice bran: A health-promoting ingredient," *Cereal Foods World*, vol. 49, pp. 188-94, 2003.