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Effects of components in culture medium on glutamate decarboxylase activity and γ-aminobutyric acid accumulation in foxtail millet (*Setaria italica* L.) during germination

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ABSTRACT

The effects of glutamic acid (Glu), pyridoxal-5-phosphate (PLP) and calcium chloride ($CaCl_2$) in culture medium on glutamate decarboxylase (GAD) activity and γ-aminobutyric acid (GABA) accumulation in foxtail millet (*Setaria italica* L.) during germination were investigated in this study. The components in culture medium for GABA accumulation were optimised using response surface methodology (RSM). Results showed that GAD activity and GABA yield were dependent on the addition of Glu, PLP and $CaCl_2$ into the culture medium. Box–Behnken design indicated that the optimal culture components for GABA accumulation were: Glu at a concentration of 1.2 mg/ml, PLP at a concentration of 50 μM and $CaCl_2$ at a concentration of 2.5 mM. Under the optimal conditions, the maximal production of GABA (42.9 mg/100 g FW) was obtained. Analysis of variance for the regression model suggested that the model can quite exactly predict GABA accumulation in millet during germination.

1. Introduction

Nowadays, there is a growing interest in natural, minimally processed, nutritional and healthful foods. A plant-based diet, focussing mainly on whole grains, has become one of the most important guidelines for lowering the risk of human diseases (Lawrence & Machlin, 1995). Public interest in nutritious foods has led to investigations by biochemical technology to enhance nutritious compositions in cereals, such as in brown rice (Komatsuzaki et al., 2007), maize (Young, Juvik, & DeMason, 1997) and sorghum (Elmaki, Babiker, & Tinay, 1999). Germination, as a biochemical technique, has been used to meet this objective (Chavan & Kadam, 1989; Sangronis & Machado, 2007). There are significant changes during seed germination, including interconversion and production of new compounds (Kumar & Chauhan, 1993). Especially, germination can increase the contents of functional components of cereals and enhance their healthful effects. For example, the yield of hydroxyl radical-inhibiting water-soluble protein from millet was increased by germination (Li, Chen, Yao, & Xu, 2007).

Millet (*Setaria italica* L.) is an important cereal and nutritious food in traditional diets, especially for people on the Eurasian continent and Africa. The main components of millet include starch, protein, lipid, vitamins and minerals (Usha, Sripriya, & Chandra, 1996). There have been reports on improving the nutritive value in millet by germination (Mbithi-Mwikya, Van-Camp, Yiru, & Huyghebaert, 2000; Sripriya, Usha, & Chandra, 1997). *In vitro*, protein digestibility significantly increased and phytic acid decreased in finger millet during sprouting (Mbithi-Mwikya et al., 2000). Mineral content increased in foxtail millet during germination (Mbithi-Mwikya et al., 2000; Sripriya et al., 1997; Usha et al., 1996). However, to the best of our knowledge, there is a lack of information on γ-aminobutyric acid (GABA) accumulation in germinated foxtail millet.

GABA acts as an inhibitory neurotransmitter in the brain and spinal cord of mammals (Manyam, Katz, Hare, Kanifefski, & Tremblay, 1981) and shows a series of functions, such as regulation of blood pressure and heart rate, and alleviation of pain and anxiety (Mody, Dekoninck, Otis, & Soltesz, 1994). Recent studies show that GABA is also a strong secretagogue of insulin from the pancreas, and effectively prevents diabetes (Adeghate & Ponery, 2002; Hagiwara, Seki, & Ariga, 2004). GABA is so important for people's health that methods to enrich GABA in functional foods have been tried, e.g. GABA enrichment in green tea produced by anaerobic treatment (Tsushida & Murai, 1987), GABA accumulation in rice germ by soaking in water (Sadami, Satoshi, Kazuhito, & Isao, 2000), and GABA enrichment in brown rice by soaking and gaseous treatment (Komatsuzaki et al., 2007).

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GABA is produced primarily by decarboxylation of glutamic acid (Glu), catalysed by glutamate decarboxylase (GAD, EC4.1.1.15). It has been reported that GABA synthesis in plants is promoted by various environmental stresses, e.g. mechanical stimulus, heat or cold shock and hypoxia (Bown & Shelp, 1997). GABA accumulation is mediated primarily via GAD (Bown & Shelp, 1997). Therefore, promoting GAD activity is helpful in stimulating GABA accumulation (Shelp, Bown, & Mclean, 1999). GAD is a pyridoxal-5-phosphate (PLP)-dependent enzyme, and its activity could be promoted by PLP (Shelp et al., 1999). In addition, GAD in plants is a Ca^{2+}/CaM-binding protein and its activity is stimulated by Ca^{2+}/CaM (Baum, Chen, Arazi, Takatsuji, & Fromm, 1993).

The present study investigated the influence of adding $CaCl_2$, PLP and Glu (to culture solution) on GAD activity and GABA accumulation when millet seeds were germinated under hypoxia. Response surface methodology (RSM) was applied to optimise the compositions of culture solution for GABA accumulation. The objective of this work is to provide a scientific basis for industrialised production of GABA-enriched foxtail millet.

2. Materials and methods

2.1. Materials

Seeds of foxtail millet (Jingu-34) were kindly supplied by Shanxi Academy of Agricultural Science (Taiyuan, China). The seeds were sanitised manually and stored at 4 °C prior to use.

GABA, PLP and phenylisothiocyanate (PITC) were purchased from Sigma Chemicals Co. (St. Louis, USA). Acetonitrile was of high performance liquid chromatography (HPLC) grade; other chemicals and reagents to be used were of analytical grade, which were all purchased from Shanghai Institute of Biochemistry (Shanghai, China).

2.2. Seeds germination

Dry seeds (20 g) were surface-sterilised with 1% (v/v) sodium hypochlorite solution for 30 min, thoroughly rinsed with distilled water, and then steeped in distilled water at 25 ± 1 °C for 8 h. After that, the steeped grains were drained and placed in cultivated pots with lids (5.5 × 6 cm) where they were germinated with 100 ml of culture medium at 32 °C in darkness. The culture solution was aerated by a pump at an air flow rate of 1.5 l/min, which was controlled by a flowmeter (Yuyao Jintai Meter Co., Ltd. Zhejiang, China). The germinated millet seeds were carefully washed with distilled water at the end of germination, dried on a filter paper, and then flash-frozen in liquid nitrogen for further experiments.

2.3. Experimental design

2.3.1. Effects of Glu, PLP and $CaCl_2$ on GAD activity and GABA accumulation

In order to determine the proper scope of Glu, PLP and $CaCl_2$ for GAD activity and GABA accumulation in millet during germination, different cultivating solutions were prepared. These were various Glu concentrations, ranging from 0 to 2.5 mg/ml, PLP, at a range of concentrations from 0 to 500 μM, and $CaCl_2$ solutions at different concentrations, ranging from 0 to 10 mM. All solutions were prepared using 0.01 M citrate buffer solution and the initial pH was adjusted to 5.8.

2.3.2. Optimisation of components for GABA accumulation

On the basis of single factor experiments, the components in culture medium, namely Glu (X_1), PLP (X_2) and $CaCl_2$ (X_3), for GABA yield (Y) in millet were optimised using RSM (Design–Expert version 6.0.10 Trial, Delaware, USA Echip, 1993). The factors and levels investigated in Box–Behnken design are shown in Table 1. Seventeen combinations, including five replicates of the centre points were employed to evaluate the combined effects of variables on GABA yield. The experimental results were analysed by quadratic stepwise regression to fit the second-order equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{i<j}^{3} B_{ij} X_i X_j \quad (1)$$

where Y denotes response observed for treatment combination $X = (x_1, x_2, \ldots, x_p)$ for p factors, β_0 represents the intercept, and the parameters of B_i, B_{ii} and B_{ij} represent the regression coefficients of variables for linear, quadratic and interaction regression terms, respectively. An analysis of variance (ANOVA) table is generated to determine individual linear, quadratic and interaction regression coefficients. The significances of polynomial relations were tested using Fisher's F-test. The regression coefficients were used for statistical analyses to generate contour maps of the regression models.

2.4. Determination of GABA

Germinated foxtail millet (1.00 g) was mulled with 6 ml of 4% (v/v) acetic acid. The homogenate was deposited for 1 h for extracting GABA sufficiently, and then centrifuged at 6037g for 15 min.

Table 1
Box–Behnken design and the responses for GABA yield in germinated foxtail millet.

Trials	X_1	X_2	X_3	GABA yield (mg/100 g FW)	
	Glu (mg/ml)	PLP (μM)	$CaCl_2$ (mM)	Observed value	Predicted value
1	0 (1.5)	0 (50)	0 (2.5)	40.3 ± 1.28	41.4
2	0 (1.5)	0 (50)	0 (2.5)	39.9 ± 0.35	41.4
3	−1 (0.5)	−1 (0)	0 (2.5)	29.8 ± 1.46	31.2
4	0 (1.5)	+1 (100)	+1 (4.5)	29.0 ± 2.02	29.0
5	−1 (0.5)	0 (50)	+1 (4.5)	34.1 ± 2.05	33.7
6	0 (1.5)	0 (50)	0 (2.5)	42.8 ± 0.40	41.4
7	+1 (2.5)	0 (50)	+1 (4.5)	24.5 ± 0.87	26.0
8	0 (0.5)	+1 (100)	0 (2.5)	32.8 ± 3.22	33.4
9	+1 (2.5)	+1 (100)	0 (2.5)	27.4 ± 1.97	25.7
10	0 (1.5)	−1 (0)	+1 (4.5)	35.8 ± 2.56	34.7
11	0 (1.5)	+1 (100)	−1 (0.5)	34.8 ± 0.74	35.9
12	−1 (0.5)	0 (50)	−1 (0.5)	34.4 ± 2.98	32.8
13	+1 (2.5)	0 (50)	−1 (0.5)	24.7 ± 1.17	25.1
14	0 (1.5)	−1 (0)	−1 (0.5)	25.9 ± 2.63	25.9
15	0 (1.5)	0 (50)	0 (2.5)	43.0 ± 0.18	41.4
16	+1 (2.5)	−1 (0)	0 (2.5)	23.9 ± 1.54	23.6
17	0 (1.5)	0 (50)	0 (2.5)	41.0 ± 3.03	41.4

The supernatant was collected and treated with 4 ml of ethanol to remove macro-molecular polymers and then centrifuged at 16770*g* for 20 min. The purified supernatant was evaporated (0.1 MPa, 45 °C) to volatilise the acetic acid and ethanol. The residues were dissolved with 0.5 ml of distilled water and centrifuged at 2683*g* for 10 min.

The centrifugal suspension was filtered through a 0.45 μm membrane filter. 100 μl of the filtered supernatant was analysed by HPLC (Agilent 1200, USA) with a Prodigy C_{18} reversed-phase column (5 μm), 4.6 × 250 mm i.d., as described by Rossetti and Lombard (1996). The standard solution of GABA and sample were determined by pre-column derivatization of phenylthiocarbamyl–GABA (PTC–GABA) from PITC. Mobile phase A consisted of 70 mM sodium acetate buffer solution (pH 5.8) treated with 0.5 ml of triethylamine per litre of buffer; and mobile phase B was acetonitrile. The elution system involved 92% of mobile phase A and 8% of mobile phase B at a flow rate of 0.5 ml/min during the entire run. Twenty microlitres of each sample were injected, detected at 254 nm, at a column temperature of 27 °C.

2.5. Determination of GAD activity

One gramme of germinated millet was mixed, on an ice bath, with 5 ml of potassium phosphate buffer (1/15 M, pH 5.8), which contained 2 mM β-mercaptoethanol, 2 mM EDTA and 0.2 mM PLP. Then, the homogenate was centrifuged at 15652*g* for 20 min in a refrigerated high speed centrifuge (GL-20G-II, Anting Science Instrument Factory, Shanghai, China). The supernatant was the crude GAD. The reaction mixture consisted of 200 μl of crude enzyme liquid and 100 μl of substrate (1% of Glu, pH 5.8). The reaction solution was incubated at 40 °C for 2 h and then terminated at 90 °C for 5 min. The centrifugal suspension was filtered through a 0.45 μm membrane filter. The filtrate was analysed for GABA content by the GABA determination method, as mentioned above. One unit of enzyme activity was defined as the release of 1 μmol of GABA produced per 1 h at 40 °C (Zhang, Yao, Chen, & Wang, 2007).

2.6. Statistical analysis

Average values and standard deviations were computed for experimental data. Statistical analysis was performed using Fisher's *F*-test. $P < 0.05$ or 0.01 was taken as significant.

3. Results and discussion

3.1. Effects of Glu on GAD activity and GABA yield of germinated foxtail millet

The effects of Glu addition, in culture medium, on GAD activity and GABA yield of germinated millet are shown in Fig. 1. There was a gradual increase in GABA yield with increasing substrate concentration, but a sharp decrease occurred when Glu concentration exceeded 1.5 mg/ml. GABA content was 35.7 mg/100 g FW when Glu concentration in the culture medium was 1.5 mg/ml. GAD activity was also enhanced with Glu addition, but it showed no significant difference ($P < 0.05$) when Glu concentration was between 1.0 and 2.0 mg/ml. The maximal increase of GAD activity was 35%, compared with the control.

Recently, the regulation of GAD activity by glutamate availability has been investigated *in situ* (Scott-Taggart, Cauwenberghe, Mclean, & Shelp, 2002). The activity of GAD of yeast showed a linear increase with increasing substrate–glutamate concentration (Tong et al., 2002). The GAD activity of rice germ was dependent on the substrate concentration and reached a maximal at 50–100 mM glutamate (Sadami et al., 2000). Elevating glutamate levels stimulated GABA synthesis in isolated *Asparagus* mesophyll cells (Cholewa, Andrzej, Shelp, Snedden, & Bown, 1997; Chung, Bown, & Shelp, 1992). These increases of GABA were probably caused by increases of the substrate in the vicinity of the cytosolic GAD, which suggests that GAD activity and GABA content were regulated by glutamate addition (Komatsuzaki, Shima, Kawamoto, Momose, & Kimura, 2005; Shelp et al., 1999).

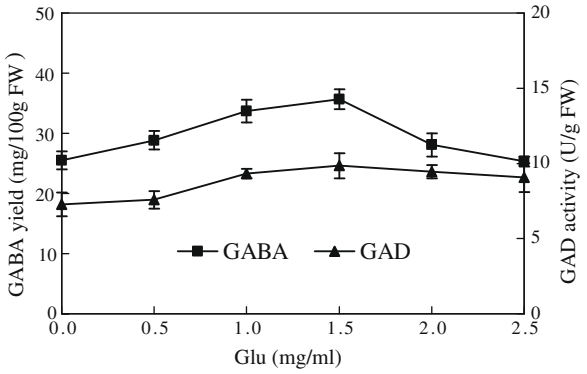

Fig. 1. Effects of Glu concentration on GAD activity and GABA accumulation in germinated millet. Soaked millet grains were cultivated in darkness at 32 °C for 48 h; the culture medium was aerated at an air flow rate of 1.5 l/min. Data were measured and expressed as the means ± SD of triplicates.

3.2. Effects of PLP addition on GAD activity and GABA yield in germinated foxtail millet

As expected, PLP addition effectively increased GABA content and enhanced GAD activity (Fig. 2). GABA yield increased rapidly with the increase of PLP, but it gradually decreased when the concentration of PLP was above 50 μM. GAD activity was increased sharply by addition of PLP. The optimal concentration of PLP was 50 μM. GAD activity increased by 32% compared with the control.

Previous studies showed that PLP plays an important role in stimulating GAD activity as a cofactor of the enzyme (Tong et al., 2002), and that addition of PLP to the culture medium might influence GABA production (Komatsuzaki et al., 2005). GAD activity in the spore of *Aspergillus oryzae* was increased 40-fold by addition of PLP (Noriyoshi & Katanori, 2002). An increase of PLP concentration also favoured activity of GABA-transaminase, which was also dependent on PLP concentration and converted GABA (Satyanarayan & Nair, 1990). Our data suggest that addition of PLP to culture medium enhanced GABA accumulation and GAD activity in germi-

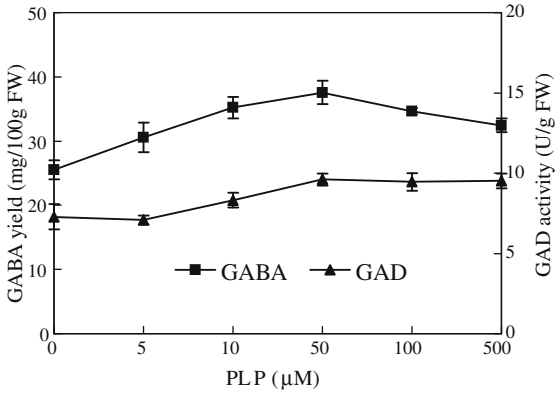

Fig. 2. Effects of PLP concentration on GAD activity and GABA accumulation in germinated millet. Soaked millet grains were cultivated in darkness at 32 °C for 48 h; the culture medium was aerated at an air flow rate of 1.5 l/min. Data were measured and expressed as the means ± SD of triplicates.

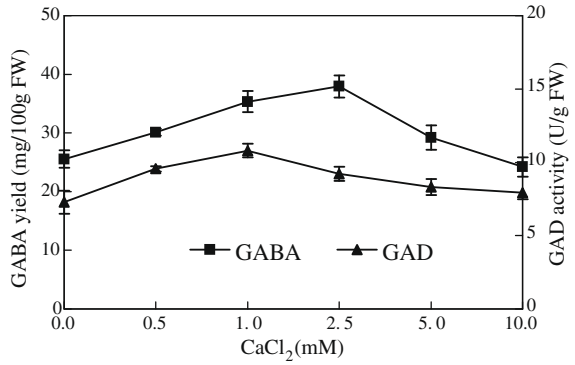

Fig. 3. Effects of $CaCl_2$ concentrations on GAD activity and GABA accumulation in germinated millet. Soaked millet grains were cultivated in darkness at 32 °C for 48 h; the culture medium was aerated at an air flow rate of 1.5 l/min. Data were measured and expressed as the means ± SD of triplicates.

nated foxtail millet, which is in accordance with that of rice germ, which also required the optimal concentration of PLP for GAD activity to be 50 μM (Sadami et al., 2000).

3.3. Effects of $CaCl_2$ on GAD activity and GABA yield in germinated foxtail millet

The effects of increasing exogenous $CaCl_2$ concentrations on GAD activity and GABA yield are presented in Fig. 3. Both GABA content and GAD activity first increased and then decreased. The maximal GABA content was 37.9 mg/100 g FW at the $CaCl_2$ concentration of 2.5 mM. A steep increase of GAD activity was observed with $CaCl_2$ concentrations of 0–1.0 mM, the maximal value of activity was 48% higher than that of the control.

Researches have indicated that plants GAD was stimulated by Ca^{2+}/CaM (Baum et al., 1993). GAD activity was increased 2–8 fold in various soybean tissues when treated with 500 μM Ca^{2+} plus 200 nM CaM. The data support a model of Ca^{2+}/CaM-mediated activation of GAD (Snedden, Arazi, Fromm, & Shelp, 1995). Our findings showed that increasing Ca^{2+} stimulated GAD activity and promoted GABA synthesis in millet, which also confirmed that GAD was regulated by a Ca^{2+}/CaM signal transduction pathway.

3.4. Optimisation of components in culture medium for GABA accumulation

3.4.1. Analysis of Box–Behnken experiments

The Box–Behnken design and the corresponding experimental data are shown in Table 1. Multiple regression analysis of the data demonstrated that the RSM design model was consistent with the second-order polynomial equation referred to as Eq. (1). The second-order polynomial model describing the correlation between GABA content and the three variables in this study was that obtained in Eq. (2) below:

$$Y = 11.23 + 18.50X_1 + 339.15X_2 + 7.88X_3 - 7.44X_1^2 - 2192.27X_2^2 - 1.14X_3^2 - 39.22X_1X_3. \quad (2)$$

The statistical analysis (Table 2) indicated that the proposed model was adequate and had a satisfactory value of R^2 (0.9699). The closer the value R^2 is to unity, the better does the empirical model fit the actual data. An F-value of 41.42, obtained by ANOVA, implied that the model was very significant ($P < 0.0001$), and an adequate precision of 16.904 showed an adequate signal. The model also possessed no significant lack of fit ($F = 1.27$, $P = 0.4207$). These results proved the validity of the experimental model.

3.4.2. Effects of Glu, PLP and $CaCl_2$ on GABA accumulation

Response surfaces plots were used to illustrate the interactive effects of Glu, PLP and $CaCl_2$ concentrations on GABA accumulation. Response surface plots for GABA yield are presented in Fig. 4.

Fig. 4a shows the effects of Glu and PLP on GABA accumulation during millet germination at a constant $CaCl_2$ concentration of 2.5 mM. Glu had the most significantly linear and quadratic effects ($P < 0.0001$) on GABA accumulation; PLP had the most significant quadratic effect ($P < 0.0001$). However, Glu and PLP did not interact significantly ($P > 0.05$) (Table 2). At a fixed PLP concentration, Glu addition led to a gradual increase of GABA accumulation but it declined later. When Glu concentration was 1.2 mg/ml, the yield reached its peak, indicating that too much Glu would decrease GABA yield. When Glu addition was set, GABA yield sharply increased with the addition of PLP and the optimal concentration was 50 μM.

The effects of PLP and $CaCl_2$, at a constant Glu concentration of 1.5 mg/ml, on GABA yield are illustrated in Fig. 4b. $CaCl_2$ had a significantly quadratic effect ($P = 0.0002$) on GABA yield. Besides, there was a significant interaction ($P = 0.0006$) between PLP and $CaCl_2$ (Table 2), which also influenced GABA accumulation. At the fixed $CaCl_2$ concentration, PLP addition sharply increased GABA yield. At the fixed PLP concentration, GABA yield increased rapidly with $CaCl_2$ addition at the beginning, but at a slower rate towards the end, which was the same as the increase of GABA yield with increasing PLP concentration. When $CaCl_2$ concentration was 2.5 mM, GABA yield reached its maximal level. Our results indicate that GABA accumulation in germinated foxtail millet was accelerated by Ca^{2+} and PLP, which agreed with previous reports (Baum et al., 1993; Tong et al., 2002).

Fig. 4c shows the effects of $CaCl_2$ and Glu on GABA yield in germinated foxtail millet at a constant PLP concentration of 50 μM.

Table 2
Analysis of variance (ANOVA) for the response surface regression model[a].

Source	Sum of squares	Degree of freedom	Mean squares	*F*-value	*P*-value
Model	686.04	7	98.01	41.42	<0.0001
X_1	116.96	1	116.96	49.43	<0.0001
X_2	9.56	1	9.56	4.04	0.0753
X_3	1.69	1	1.69	0.71	0.4200
$X_1$2	233.12	1	233.12	98.52	<0.0001
X_2^2	126.48	1	126.48	53.45	<0.0001
X_3^2	87.20	1	87.20	36.85	0.0002
X_2X_3	61.54	1	61.54	26.01	0.0006
Residual	21.30	9	2.37		
Lack of fit	13.06	5	2.61	1.27	*0.4207*
Pure error	8.24	4	2.06		
Cor total	707.34	16			

[a] $R^2 = 0.9699$, adj $R^2 = 0.9465$, pred $R^2 = 0.8643$, adequate precision = 16.904.

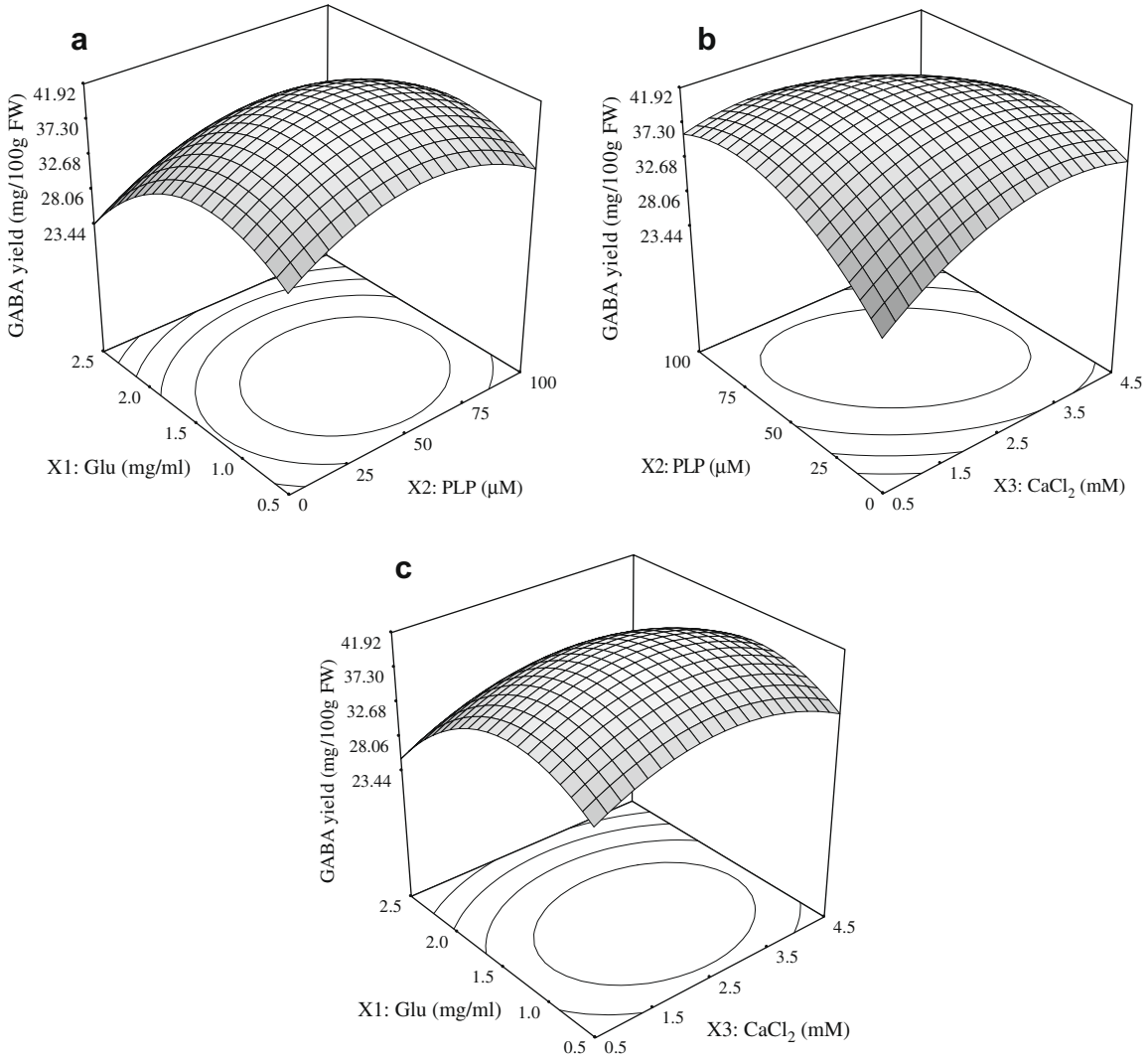

Fig. 4. Response surface plots showing effects of: (a) Glu and PLP, (b) PLP and $CaCl_2$, (c) $CaCl_2$ and Glu on GABA yield in germinated foxtail millet. (a) The $CaCl_2$ concentration was constant at 2.5 mM. (b) The Glu concentration was constant at 1.5 mg/ml. (c) The PLP concentration was constant at 50 μM.

The interaction between the two variables, in this model, on GABA accumulation was not significant ($P > 0.05$) (Table 2). The GABA yield increased when Glu addition increased from 0.5 to 1.2 mg/ml; thereafter it decreased when Glu was above 1.2 mg/ml. Adding $CaCl_2$ to culture solution might stimulate GAD activity and promote GABA accumulation in germinated foxtail millet. Our results also confirmed that GABA synthesis was related to Ca^{2+}, which was also reported in previous papers (Baum et al., 1993; Snedden et al., 1995).

3.4.3. Optimisation of components and verification of the model

According to the RSM test results, the optimal components of culture medium for GABA accumulation were 1.2 mg/ml of Glu, 50 μM PLP and 2.5 mM $CaCl_2$. Under the optimal conditions, maximal content of GABA in germinated millet was 41.9 mg/100 g FW.

Verification of the model Eq. (2) was performed under the optimum conditions for GABA accumulation. The observed content of GABA in millet germination under the optimal conditions was 42.9 mg/100 g FW, which agreed with the predicted value of 41.9 mg/100 g FW in the model. The experimental results proved that the model was valid. The GABA yield, under the optimal conditions, was 1.68 times higher than that of the control (whose GABA content was 25.5 mg/100 g FW). The present study showed that GABA content in germinated foxtail millet was comparable with its contents in germinated brown rice (Komatsuzaki et al., 2007).

4. Conclusions

The effects of Glu, PLP and $CaCl_2$ on GAD activity and GABA yield in germinated millet were studied. The results revealed that addition of Glu, PLP and $CaCl_2$ to culture medium influenced GAD activity and increased GABA content. ANOVA analysis in RSM demonstrated that the designed model was valid. Glu addition markedly influenced GABA yield, whilst the interaction between PLP and $CaCl_2$ was the most significant on GABA yield. The best combinations of culture components were 1.2 mg/ml of Glu, 50 μM PLP and 2.5 mM $CaCl_2$, using the contour plots. The highest GABA yield of 42.9 mg/100 g FW was obtained under the optimised conditions. All these results proved germinated foxtail millet to be a good source of GABA-enriched healthful foods.

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