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Research Article

Influence of germination time on free amino acids, phenolic compounds and γ-aminobutyric acid in pigeon pea (*Cajanus cajan* (L.) Huth) seeds

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Abstract

This research studied the influence of phytochemical compounds on the germination time of pigeon pea (*Cajanus cajan* (L.) Huth). Over the 72 hours germination period, the contents of total and most individual free amino acids, particularly histidine, showed substantial increases. The content of γ-aminobutyric acid (GABA) was also greatly increased, from 0.24 μg/g to 2.7 μg/g, representing a nearly ninefold increase. The total phenolic content (TPC) and total flavonoid content (TFC) were positively related with germination time, with TPC highest at 62.49 mg GAE/100 g DW at 72 h, and TFC increased from 18.87 mg RE/100 g DW to 30.05 mg RE/100g.DW at 72 hours. The contents of individual phenolic acids and flavonoids, such as, protocatechuic acid, p-coumaric acid, ferulic acid and rutin, experienced noticeable increases. However, vanillic and catechin were only detectable at 72 hours, respectively. Antioxidant capacity measured by DPPH and FRAP assays increased from 102.30 to 128.95 mgTE/100g DW and 694.13 to 836.93 mg $FeSO_4$/100g DW mmol/g, respectively, and therefore highlighting the enhanced antioxidant potential of germinated pigeon pea. The results from Fourier-transform infrared spectroscopy (FTIR) supported these findings. The study emphasizes the potential of germination to enhance the phytochemical compounds of pigeon pea seeds.

Keywords: antioxidant capacity; bean; bioactive compounds; GABA; phenolics

Introduction

Cajanus cajan (L.) Huth, more commonly known as pigeon pea, is a vital dietary legume renowned for its remarkable nutritional and health properties. Particularly significant is its high protein content and comprehensive range of essential amino acids, making it a crucial food source in regions where protein malnutrition is common (Kuraz Abebe, 2022). Pigeon pea is also abundant in bioactive phytochemicals, such as phenolic acids, flavonoids, and isoflavones which are associated with various health benefits (Talari and

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Shakappa, 2018; Tungmunnithum *et al*., 2021) Another intriguing feature is its high level of γ-aminobutyric acid (GABA), a non-protein amino acid known for lowering blood pressure, enhancing cognitive function, and reducing anxiety (Ngo and Vo, 2019; Oketch-Rabah *et al*., 2021) Previous studies suggested that the germination process significantly alters the nutritional and bioactive compound composition of legumes, often enhancing their health-promoting properties (Ghavidel and Prakash, 2007; Hassan *et al*., 2020; Chinma *et al*., 2021). Germination, in particular, has been associated with the reduction of a-amino adipic acid and an increase of GABA (Kuo *et al*., 2004). The amino acids present in the pigeon pea do not only contribute to its protein content, but also influence its overall nutritional value and potential health benefits. Some of these free amino acids, such as lysine, aspartic acid, and glutamic acid are found in higher quantities in pigeon peas (Talari and Shakappa, 2018). Others, such as the sulfur-containing amino acids cysteine and methionine, are crucial for promoting health and wellbeing (Townsend *et al*., 2004). Amino acids are critical for immune system function, muscle growth, and repair. They are also important for brain health, since they affect mood and sleep patterns. They are essential for the synthesis of hormones, the creation of energy, and the control of metabolism. They additionally perform a critical role in preserving general health and muscle mass, particularly in older populations. Furthermore, by scavenging free radicals and promoting the production of important antioxidants like glutathione, free amino acids like taurine, methionine, and cysteine directly demonstrate antioxidant qualities. Additionally supporting antioxidant defenses are histidine, lysine, and arginine via their metabolites or by scavenging free radicals. These amino acids are essential for lowering oxidative stress, which protects the tissues and cells from harm and promotes general health. The phenolic acids found in pigeon pea include gallic, caffeic, and p-coumaric, among others. Meanwhile, flavonoids, such as quercetin, kaempferol, and their glycosides, are also present (Sreerama *et al*., 2012). The content and composition of these compounds can vary significantly depending on plant genetic factors, growing conditions, and post-harvest processing techniques (García-Díaz *et al*., 2018; Nicolás-García *et al*., 2021). Germination, a critical physiological process, can substantially influence the nutritional and bioactive profile of legumes like pigeon pea. This process is known to enhance the biosynthesis of amino acids (Ghavidel and Prakash, 2007). For instance, GABA has been shown to increase during the germination process (Xu and Chang, 2007; Orak *et al*., 2016). Despite these findings, the specific impact of germination on the nutritional and bioactive components in *C. cajan* remains insufficiently studied, and the optimal germination time to exploit these benefits is still uncertain.

This study aims to investigate the influence of different germination times on the amino acids, GABA, and bioactive compounds such as phenolic acids and flavonoid compounds in pigeon pea seeds associated with antioxidant capacity. The objective of this study is to provide important knowledge for wider utilization of these pigeon peas. In addition, evaluating these bioactive chemicals and antioxidant capacity at different stages of germination offers important insights that might improve the usage of pigeon pea as a food source that promotes health.

Materials and Methods

Chemicals and reagents

All standard compounds, inclusive of phenolic acids like protocatechuic acid, gallic acid, p-coumaric acid, p-hydroxybenzoic acid, caffeic acid, vanillic acid, chlorogenic acid, syringic acid, ferulic acid and sinapic acid; flavonoids: rutin, quercetin, apigenin, catechin, myricetin and kaempferol; amino acids, such as aspartic acid, glutamic acid, serine, asparagine, threonine, glutamine, tyrosine, glycine, proline, alanine, methionine, valine, phenylalanine, isoleucine, leucine, tryptophan, cysteine, histidine, lysine, and arginine, as well as γ-aminobutyric acid (GABA) and vanillin were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Additionally, the reagents required for assessing the antioxidant capacity, namely Folin–Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid

(Trolox) and 2,4,6-tripyridyl-S-triazine (TPTZ) were also sourced from Sigma-Aldrich Co. (USA). For high-performance liquid chromatography (HPLC) analysis, all the required solvents and reagents were secured from Merck (Darmstadt, Germany). It should be noted that all the chemicals and reagents incorporated in this research study were of an analytical grade.

Plant identification and sample preparation of pigeon pea seed

During June-July 2021, dry pigeon pea seeds were collected from the northeastern region of Thailand, where the average temperature was 27.7 °C and the humidity level stood at 77%. Expert plant taxonomists confirmed the identity of the samples, and we archived them in the herbarium with the voucher specimen number TC072021. Before germination, the seeds were cleansed of impurities and stored at 4 °C.

Germination

The germination process, as detailed in a previous study (Chinma *et al*., 2021), was carried out three times. In summary, 250 g of cleaned pigeon pea seeds were sterilized with a solution of food-grade sodium hypochlorite (0.07 g/L) from Sigma Aldrich Co (USA), for half an hour. After draining, the seeds were soaked in sterile distilled water for 6 h at a temperature of 28 °C. The soaked seeds were then allowed to germinate for periods of 12, 24, 36, 48, and 72 h at the same temperature (28 °C) and relative humidity of 40-60%. The uniformly germinated pigeon pea seeds (as shown in Figure 1) were selected and dried using a freeze dryer (Scanvac CoolSafe, 100-9 Pro, LaboGene ApS, Lillerød, Denmark) until their moisture content fell below 7 g/100 g. The dried samples were then ground, passed through a 40-mesh sieve wire, and stored at −20 °C for future analysis.

Figure 1. Appearance of pigeon pea seeds at different germination times

Free amino acid profile determination

The method previously described was used to analyze free amino acids (Saensouk *et al*., 2022). The Liquid Chromatography-Mass Spectrometer (LC/MS/MS) was executed using a Shimadzu LCMS-8030 triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), with the spectrometer operating in electrospray ionization (ESI) mode. Amino acid analysis was conducted via isocratic elution on an InertSustain® C18 column (2.1×150 mm, 3 µm, GL Sciences Inc., Tokyo, Japan), paired with a guard column, utilizing a mobile phase comprised of solvent A (0.1%, v/v formic acid in water) and solvent B (0.1% v/v formic acid in water/methanol, 50:50, v/v) used consisted of 50:50 (A: B), at a flow rate of 0.2 mL/min and a column temperature of 40 °C. The MS/MS parameters included an interface nebulizing gas flow rate of 3 L/min, desolvation line (DL) temperature of 250 °C, heat block temperature of 400 °C, and a drying gas flow rate of 15 L/min. The MS/MS was carried out according to with previously described (Saensouk *et al*., 2022). As an amino acid in dried weight of the sample in µg/g DW ± SD (n = 3), the obtained data were provided. A summary of the auto-optimizations may be found in Table S1 of the Supplementary Materials.

γ-Aminobutyric acid determination

The extraction and quantification of GABA followed the protocol by (Lee and Hwang, 2017). Briefly, 0.5 g of the dried germinated pigeon pea seeds were mixed with 10 mL of 3% trichloroacetic acid and subjected

to ultrasonication at 40 °C for 1 h. Post centrifugation, the supernatants was extracted three times, combined, and the volume adjusted to 25 mL with 3% trichloroacetic acid. The solution was filtered using a 0.22 µm nylon filter before LC/MS/MS analysis. The analysis was performed using a Shimadzu LCMS-8030 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) with electrospray ionization, coupled with an HPLC system. The condition of analysis according to previously published (Saensouk *et al*., 2022). The auto-optimizations are shown in the Supplementary Materials (Table S1). The GABA concentrations were determined using external standards.

Preparation of extracts for determination of total phenolic content, total flavonoid content and antioxidant capacity

In accordance with the methodology detailed by Chumroenphat *et al*. (2023), 1-g of a dried and finely ground germinated pigeon pea seeds were mixed with a methanol and water mixture (80:20, v/v) and shaken on an orbital shaker at 150 rpm at 35 °C for 12 h. After filtration, the residues were subsequently re-extracted with the same solvent, and the remaining supernatants were gathered. The total volume was adjusted to 20 mL using the extraction solvent. The resulting extract was then utilized for the assessment of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity.

Total phenolic contents (TPC) determination

The Total phenolic contents assay was done following the Folin-Ciocalteau method used in a previous study (Chumroenphat *et al*., 2021). After preparing 10% (v/v) of the Folin-Ciocalteu reagent in distilled water, 100 µl of the reagent was combined with 20 µl of each extract and pipetted into the appropriate well of a 96-well plate. After 4 min of incubation, 75 µl of a 10% (w/v) sodium carbonate solution was added to the mixture. The combination was then allowed to stand for two hours at room temperature. A Thermo Fisher Scientific, USA Varioskan Lux Multimode microplate reader was used to measure the solution absorbance at 725 nm. In milligrams of gallic acid equivalents (mg GAE/100g DW), the findings were reported.

Total flavonoid contents (TFC) determination

Total flavonoid contents were det ermined using the colorimetric method described by Chumroenphat *et al*. (2021). In brief, 10 µL of a 5% $NaNO_2$ solution was added to the 96-well plate after 25 µL of extract and 100 µL of filtered water were combined. Before adding 50 µL of 1 M NaOH and 50 µL of purified water, the mixture was shaken for 6 min with 15 µL of a 10% $AlCl_3$ $6H_2O$ solution added after 5 minutes of shanking. A Thermo Fisher Scientific, USA Varioskan Lux Multimode microplate reader was used to measure the combination instantaneously at 510 nm. The findings were presented as milligrams of rutin equivalents (mg RE/100 g DW) for each 100-gram sample.

Phenolic acid and flavonoid profile determination

The extraction of dried unprocessed and germinated pigeon peas was performed in line with the method proposed in prior studies (Saensouk *et al*., 2022). Each 1 g dried germinated pigeon pea seeds powder was extraction with 20 mL of HCl/methanol (1:100, v/v) for 12 h at 37 °C, while being agitated at 150 rpm in a dark environment. Following the initial filtration, the residue was re-extracted, and the combined filtrates were evaporated to dryness under a 40 °C vacuum. The residual matter was then redissolved in a 5 mL methanol/water solution (50:50, v/v) and filtered through a 0.45-µm pore size nylon membrane filter. The subsequent HPLC analysis was conducted using an InertSustain® C18 column (4.6×250 mm, 5 µm, GL Sciences Inc., Japan) with a guard column, with the temperature of the column oven at 38 °C. The procedure outlined in previous studies (Saensouk *et al*., 2022) was followed for the analysis of these compounds, with detection wavelengths set at 280 nm and 320 nm for phenolic acids and 370 nm for flavonoids. The calculated

content of phenolic acids and flavonoids were identified according to the retention time and use of external standards.

Antioxidant capacity determination

The assessment of DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) of the germinated pigeon peas was conducted following the methodologies published by Seansouk *et al*. (2022). The DPPH assay involved mixing 20 µL of the extract or control (deionized water) with 180 µL of a 60 µM DPPH solution (dissolved in methanol), followed by a 30-min incubation in the dark at room temperature. The absorbance was then measured at 517 nm using a Varioskan Lux microplate reader (Thermo Fisher Scientific, USA), with results expressed as mg Trolox equivalents (TE) per 100 g of dried sample (mg TE/100 g DW). Concurrently, the FRAP assay was performed by combining 5 µL of each extract with 180 µL of FRAP reagent in a 96-well plate. The FRAP reagent was prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 (v/v/v) ratio and was incubated at 37 °C for 2 h before use. After shaking the reaction mixture for 1 min and incubating at 37 °C for 15 min, and the absorbance was measured at 593 nm against a control. FRAP values were then presented as mg $FeSO_4$ per 100 g of dried weight (mg $FeSO_4$/100g DW).

Fourier-transform infrared spectroscopy (FTIR) measurements

The Fourier-transform infrared spectroscopy (FTIR) spectra of pigeon pea seed powder samples were obtained with a Frontier FTIR instrument equipped with a universal attenuated total reflection (UATR) accessory and a Diamond/KRS-5 crystal composite (Perkin Elmer, Waltham, MA, USA). This enabled immediate evaluation of the dry powder without requiring further processing. The spectral data, which ranged from 4000-400 cm^{-1}, were collected from 32 scans with a resolution of 4 cm^{-1}, with the background spectrum automatically subtracted.

Statistical analysis

All data are shown as the mean and standard deviation (SD) of three replicates. The data was evaluated utilizing a one-way analysis of variance (ANOVA) and the least significant difference (LSD) test, with $p<0.05$ considered significant using SPSS Statistics software.

Results and Discussion

Changes in the free amino acid composition of C. cajan seeds during their germination

C. cajan, commonly known as pigeon pea, is a significant source of both essential and non-essential amino acids. Essential amino acids, which cannot be synthesized by the human body and must be obtained through diet, are present in considerable quantities in pigeon pea seeds. These include lysine, methionine, and tryptophan. Non-essential amino acids, which the body can produce, such as alanine, aspartic acid, and glutamic acid, are also found in these seeds. The presence of free amino acids in *C. cajan* were shown in Table 1.

The content of total free amino acids of ungerminated pigeon peas was 102.36 µg/g DW and gradually increased in the seeds with the germination time, reaching about 2.3 times higher value after 72 h of the process (268.43 µg/g). These results indicated that the amino acid content of pigeon peas increased with germination time, corresponding with a previous study of germinated soybeans, which similarly revealed a significant increase in the concentration of free and total amino acids during germination. (Qi *et al*., 2022). The free amino acids are produced during the hydrolysis of stored proteins, and these amino acids contribute in the germination process by promoting protein synthesis in endosperm and embryo (Ali and Elozeiri, 2017).

According to Yang *et al*. (2019), the seedlings exhibit high levels of free protein amino acid content, which facilitates the synthesis of proteins and the growth of the plant.

Arginine showed the highest content among individual amino acids in all samples. The content of some individual amino acids greatly increased with the time of germination, particularly histidine, which increased by about 16 times between 0 h and 72 h. The contents of glutamic acid, asparagine, threonine, glutamine, tyrosine, alanine, arginine, and tryptophan were also significantly higher after 72 h of germination compared to their levels before germination. In contrast, contents of glycine and phenylalanine decreased as germination time increased. Certain free amino acids, such as aspartic acid, serine, proline, lysine, leucine, and isoleucine, did not show a constant content of free amino acids with germination time. These results are similar to those previously reported for changes in amino acids due to the globular structural alterations that occur during germination, thereby resulting in enhanced accessibility, which can be attributed to the rise in amino acid concentrations (Chinma *et al*., 2021; Ma *et al*., 2017). In more detail, some amino acid content in pigeon peas was reduced after germination. These findings may be explained by the possibility that some amino acids present in the seed can be used to synthesize new proteins, leading to a potential decrease in their content in germinated seeds (Guo *et al*., 2021).

Table 1. Content of free amino acids in pigeon pea seeds at different germination times

Amino acid	Content (μg/gDW)/Germination times						
	0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
Alanine	3.24±0.08 e	7.88±0.95 b	5.08±0.16 d	11.77±0.19 c	8.55±0.20 e	7.49±0.22 g	17.09±0.10 f
Arginine	30.20±0.09 a	37.75±0.09 a	38.38±0.06 a	39.92±0.77 b	46.16±0.06 a	47.43±0.64 a	53.65±0.58 a
Asparagine	4.36±0.08 e	4.17±0.06 e	3.36±0.02 i	3.74±0.05 e	8.59±0.04 e	3.59±0.04 k	16.83±0.34 g
Aspartic acid	1.30±0.09 l	4.48±0.21 e	4.11±0.17 f	2.46±0.05 h	0.44±0.01 m	1.97±0.16 d	4.18±0.09 b
Cysteine	ND	ND	ND	ND	ND	ND	ND
Glutamic acid	6.73±0.53 c	4.75±0.07 d	20.28±0.50 c	1.89±0.03 h	2.97±0.21 h	19.14±0.80 d	46.37±0.20 b
Glutamine	1.48±0.01 l	2.48±0.10 g	2.12±0.02 k	0.82±0.01 i	1.70±0.76 j	6.17±0.22 h	3.24±0.06 n
Glycine	2.27±0.01 j	2.05±0.34 g	0.75±0.03 m	0.61±0.01 k	0.57±0.01 l	0.47±0.03 n	0.73±0.03 q
Histidine	1.98±0.06 k	3.13±0.09 f	3.67±0.23 f	4.77±0.08 e	31.70±0.21 c	40.43±0.77 b	31.59±0.08 d
Isoleucine	3.32±0.09 h	3.02±0.01 f	4.35±0.06 e	3.24±0.04 g	5.99±0.05 f	5.72±0.08 i	4.17±0.11 l
Leucine	3.92±0.09 g	1.68±0.05 h	0.71±0.01 m	0.71±0.01 j	0.71±0.02 k	9.10±0.28 f	2.70±0.41 o
Lysine	2.85±0.04 j	2.52±0.11 g	2.15±0.02 k	1.86±0.06 h	2.04±0.03 i	2.00±0.01 m	2.01±0.01 p
Methionine	3.59±0.03 h	2.83±0.23 f	25.16±0.10 b	50.15±0.23 a	42.82±0.11 b	10.27±0.50 e	9.35±0.19 h
Phenylalanine	12.13±0.31 b	5.61±0.01 c	4.26±0.51 f	4.21±0.36 e	2.63±0.07 h	2.45±0.04 l	3.99±0.02 m
Proline	3.46±0.19 f	4.96±0.07 d	2.65±0.04 j	2.94±0.08 h	1.57±0.12 j	4.84±0.12 j	2.94±0.05 o
Threonine	3.14±0.16 f	2.83±0.28 f	3.62±0.03 g	3.98±0.16 e	4.59±0.14 g	3.66±0.33 k	7.97±0.02 i
Tryptophan	3.89±0.03 g	4.92±0.22 c	0.86±0.01 l	8.22±0.89 d	20.05±0.58 d	7.28±0.70 g	18.79±0.36 c
Tyrosine	5.04±0.04 d	6.92±0.15 b	6.19±0.10 d	7.55±0.15 d	2.36±0.19 h	28.16±0.69 c	33.38±0.30 c
Serine	4.85±0.16 e	4.35±0.08 e	3.44±0.02 h	3.39±0.05 f	2.80±0.09 h	2.54±0.01 l	4.43±0.21 k
Valine	4.61±0.03 e	4.97±0.20 d	5.05±0.05 d	4.41±0.07 e	2.04±0.26 i	2.50±0.13 l	5.02±0.04 j
Total amino acids	102.36±4.14 g	111.30±3.32 f	136.19±2.14 e	156.64±3.29 d	188.28±3.18 c	205.21±5.77 b	268.43±3.20 a

Values are expressed as mean ± standard deviation (n=3). Means with different letters (a–g) within the row are significantly different at $p<0.05$; DW, dry weight.

Impact of germination time on γ-aminobutyric acid content in C. cajan seeds

The germination of *C. cajan* seeds has been found to significantly influence the levels of GABA (Figure 2). Determination of sample taken at the start of germination (0 h) indicated a GABA content of 5 μg/gDW. As the germination process progressed, a notable increase in GABA content was observed. At 12 hours, the content had risen to 14 μg/g, and by 24 h, it had reached 16 μg/gDW. A significant spike was noted at 36 h, with the content increasing to 35 μg/gDW. By 48 h, the GABA content had further increased to 41 μg/gDW. A dramatic surge was observed at 60 h, with the GABA content reaching 64 μg/gDW. Finally, at the end of the 72-h germination period, the GABA content reached 72 μg/gDW. These results highlight a strong relationship between the germination time of *C. cajan* seeds and the enhancement of GABA content. This is consistent with previous research reporting an increasing GABA content in bean seeds with increased germination time

of bean seeds (Vann *et al*., 2020). The substantial increase in GABA content over the 72-h germination period can be attributed to the activation of enzymes that convert glutamate into GABA during germination. Glutamate decarboxylase (GAD) is the enzyme that removes a carboxyl group from glutamate, resulting in the formation of GABA (Yogeswara *et al*., 2020).

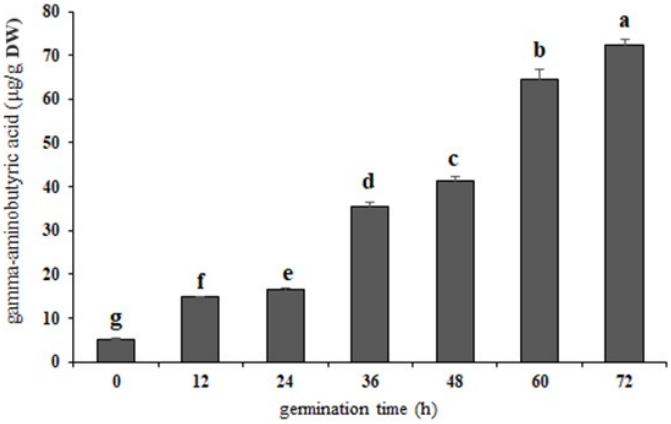

Figure 2. γ-Aminobutyric acid (GABA) content in pigeon pea seeds at different germination times
Different letters above bars indicate significant differences at $p<0.05$.

Influence of germination time on total phenolic content and total flavonoid content in pigeon peas

The TPC and TFC of pigeon peas after different seed germination times are shown in Table 2. The TPC showed a generally increasing trend with respect to germination time. The lowest TPC was at 0 h (47.52 mg GAE/100 g DW), while the highest levels were observed at 60 h and 72 h and were not significantly different from each other at $p<0.05$ (62.33-62.49 mg GAE/100 g DW). This could suggest that the phenolic biosynthesis during germination may be influenced by factors beyond time alone, perhaps environmental, or physiological, which warrants further investigation. The TFC displayed a consistently increasing trend throughout the germination process, starting from 18.87 mg RE/100 g DW at 0 h and reaching 30.05 mg RE/100 g DW at 72 h (Table 2). This could imply that the biosynthesis of flavonoids is positively correlated with the length of germination, indicating that the germination process could potentially be utilized to enhance the flavonoid content.

The increase in TPC and TFC during the germination of pigeon pea found in our study, can be related to several factors. (I) Enzyme activation: The germination process triggers the activation of various enzymes related to the phenolic (including flavonoid) biosynthesis pathways, such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). An increase in the activity of enzyme typically results in increased production of phenolic compounds (Lepiniec *et al*., 2006). (II) Stress response: The germination process imposes certain stress conditions on the seeds, such as oxidative stress. In response, plants often increase the synthesis of phenolic compounds including flavonoids, which are known to possess antioxidant properties and help in mitigating stress (de Camargo *et al*., 2017). (III) Degradation: some phenolic compounds can be stored in a bound form in seeds and are released during germination, contributing to the overall increase (Dueñas *et al*., 2007). (IV) Genetic factors: The genetic makeup of the seed itself plays a crucial role in changes the content of total phenolic compounds and total flavonoids during germination. Variations in the genes involved in the biosynthetic pathways can lead to differences in the content of these compounds (Dixon and Pasinetti, 2010).

Table 2. Total phenolic content (TPC) and total flavonoid content (TFC) of pigeon pea seeds at different germination times

Germination time (h)	TPC (mg GAE/100 g DW)	TFC (mg RE/100g DW)
0	47.52±0.74^{d}	18.87±0.43^{f}
12	51.33±1.29^{c}	20.56±1.43^{e}
24	53.38±0.31^{b}	22.21±1.41^{d}
36	55.38±1.61^{b}	23.13±1.90^{d}
48	55.52±1.25^{b}	25.03±0.76^{c}
60	62.33±1.20^{a}	27.64±0.74^{b}
72	62.49±0.59^{a}	30.05±1.40^{a}

Values are expressed as mean ± standard deviation (n=3). Means with different letters (a–f) within the column are significantly different at $p<0.05$. GAE, gallic acid equivalent; RE, rutin equivalent; DW, dry weight.

Influence of germination time on profile of phenolic acids and flavonoids of pigeon peas

This investigation into the changes in phenolic acid content during the germination of pigeon pea (*C. cajan*) has provided intriguing findings. It was observed that content of several phenolic acids, including gallic, protocatechuic, p-coumaric, and ferulic acids, showed an incremental increase as seed germination progressed (Table 3). Notably, vanillic and chlorogenic acids were only detectable after 72 and 48 h of germination, respectively, thereby suggesting that they might be synthesized or released in substantial quantities at specific germination stages. This aligns with previous studies suggesting that the synthesis of certain phenolic compounds might be triggered at specific phases of germination (Hyder Rizvi *et al*., 2022; Tarzi *et al*., 2012). On the other hand, no evidence of p-hydroxybenzoic acid, caffeic acid, syringic acid, or sinapic acid was found, indicating that their synthesis or release was not influenced by the germination process in this instance. Interestingly, the total phenolic acid content exhibited a substantial increase throughout the germination period, from 47.34 µg/g DW at 0 h to 86.02 µg/g DW at 72 h. This consistent increase was in line with previous research indicating that germination can lead to a significant enhancement in the content of phenolic compounds (Ariviani *et al*., 2021). The findings provide valuable insights into the potential of phenolic compounds useful for the health benefits of germinated pigeon pea, which could be of significant interest to the food and health industry. They also underline the value of germination as a simple and effective method for enhancing the phenolic acid content and thus the health-promoting properties of pigeon pea, corroborating similar results observed in other legume species, which seem to be Fabaceae family (Singh *et al*., 2017).

The present study sheds light on the involved subtleties of flavonoids in the germination process of *C. cajan*. Throughout the germination timeline, changes in the content of rutin, quercetin, catechin and myricetin were observed, while apigenin, and kaempferol were not detected in *C. cajan* (Table 3). Rutin content exhibited a significant increase, from an initial content of 15.41 µg/g DW to a peak of 42.94 µg/g DW at the 60 h of germination, before slightly but statistically significantly decreasing ($p<0.05$) to 40.89 µg/g at 72 h. This fluctuation could be indicative of active biosynthesis and subsequent utilization during the later stages of germination. Known for its antioxidative properties (Shahidi and Yeo, 2018), the escalating rutin content implies the potential enhancement of the nutraceutical value of germinated pigeon pea. In contrast, quercetin content, initially the dominant flavonoid, followed an intriguing trajectory, increasing gradually until 36 h and then decreasing. The gradual drop post 36 h could possibly be attributed to its bioconversion into other metabolites or use in protective physiological processes during germination (Panche *et al*., 2016). Myricetin content displayed a consistent decrement trend throughout germination, a process likely related to its role in germination-induced metabolic activities. Despite this decrease, myricetin's presence as a potent antioxidant still contributes to the overall health-promoting profile of germinated pigeon pea (Semwal *et al*., 2016). The total flavonoid content exhibited an overall increase from the start (57.74 µg/g DW) to the end of germination (86.53 µg/g DW), thereby indicating the positive impact of germination on enhancing flavonoid content in

pigeon pea. Future studies could leverage these insights to optimize germination conditions for flavonoid enhancement in pigeon peas, with potential implications for functional food and nutraceutical applications.

Table 3. Content of phenolic acids and flavonoids in pigeon pea seeds (μg/g DW) at different germination time

Parameter	Germination time (h)						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Phenolic acids content (μg/gDW)							
Gallic acid	1.21±0.01 g	1.32±0.01 f	1.42±0.01 e	1.64±0.01 d	1.73±0.01 c	1.93±0.01 b	2.20±0.01 a
Protocatechuic acid	41.51±0.46 e	51.53±1.31 d	51.68±0.19 cd	52.13±0.56 bc	52.70±1.10 b	52.84±0.18 b	56.05±0.31 a
P-hydroxybenzoic acid	ND	ND	ND	ND	ND	ND	ND
Vanillic acid	ND	ND	ND	ND	ND	ND	10.26±0.33 a
Chlorogenic acid	ND	ND	ND	ND	6.67±0.26 b	6.70±0.24 b	8.40±0.18 a
Caffeic acid	ND	ND	ND	ND	ND	ND	ND
Syringic acid	ND	ND	ND	ND	ND	ND	ND
P-coumaric acid	1.63±0.01 f	2.20±0.01 e	2.26±0.01 d	2.32±0.01 c	2.36±0.01 c	2.62±0.01 b	3.10±0.01 a
Ferulic acid	2.99±0.02 f	3.43±0.01 e	3.43±0.01 e	3.60±0.01 d	4.46±0.01 c	5.05±0.13 b	6.01±0.03 a
Sinapic	ND	ND	ND	ND	ND	ND	ND
Total phenolic acids	47.34±0.50 d	58.48±1.34 c	58.79±0.59 c	59.69±0.59 c	67.92±1.39 b	68.73±0.57 b	86.02±0.87 a
Flavonoid compounds content (μg/gDW)							
Rutin	15.41±0.04 e	16.64±0.17 d	16.66±0.02 d	19.20±0.04 c	19.34±0.01 c	42.94±0.27 a	40.89±0.61 b
Quercetin	39.45±0.96 d	41.53±0.23 c	42.03±0.46 b	43.31±0.06a	34.49±0.58 e	34.29±0.87 e	33.26±0.30 f
Apigenin	ND	ND	ND	ND	ND	ND	ND
Catechin	ND	ND	ND	ND	ND	ND	10.10±0.01 a
Myricetin	2.88±0.04 a	2.78±0.03 b	2.68±0.03 c	2.58±0.03 d	2.48±0.01 e	2.38±0.03 f	2.28±0.03 g
Kaempferol	ND	ND	ND	ND	ND	ND	ND
Total flavonoids	57.74±1.04 f	60.95±0.43 e	61.37±0.51 d	65.09±0.13 c	56.21±0.60 f	79.71±1.17 b	86.53±0.95 a

Values are expressed as mean ± standard deviation (n=3). ND: Not detected. Means with different letters (a–g) within the row are significantly different at $p<0.05$.; DW, dry weight.

Influence of germination time on antioxidant capacity of pigeon peas

The antioxidant capacity of unprocessed and germinated pigeon peas at different germination times was determined by DPPH and FRAP assays and results are shown in Table 4. In the DPPH assay, a consistent increase in antioxidant capacity during germination from 102.3 mg TE/100 g DW (0 h) to 129.0 mg TE/100 g DW (72 h) were observed. This increasing trend reflects the growing biosynthesis of antioxidant compounds during the germination process, possibly triggered by the oxidative stress response during germination, as documented in studies such as by Bailly et al. (2008). In our study, the enhanced antioxidant activity might be attributed to the increased phenolic acids and flavonoid contents. Similarly, the FRAP assay data also demonstrated an increasing pattern in antioxidant capacity during germination from 694 mg $FeSO_4$/100 g DW (0 h) to 837 mg $FeSO_4$/100 g DW (72 h). The trend can be explained by the generation of antioxidant compounds during germination, which leads to increased ferric reducing capacity. FRAP values have been reported to correlate well with the content of phenolics and other antioxidant substances in numerous studies (Thaipong *et al*., 2006; Benzie and Strain, 1996). In conclusion, the consistent increase in antioxidant capacity with germination time, as demonstrated by both DPPH and FRAP assays, indicates that germination is an effective process to enhance the antioxidant potential of pigeon peas. Moreover, prior reports indicated a positive association between bioactive compounds and their antioxidant capacity (Fendri *et al*., 2022).

Table 4. Antioxidant capacity determined as DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) of pigeon pea seeds at different germination times

Germination time (h)	DPPH assay (mg TE/100 g DW)	FRAP (mg FeSO4/100 g DW)
0	102.30±1.04 g	694.13±16.40 e
12	108.72±0.88 f	760.27±24.21 d
24	112.42±0.58 e	746.93±1.40 d
36	115.31±0.79 d	793.33±15.13 d
48	119.51±0.76 c	800.93±23.81 c
60	123.81±2.28 b	805.00±35.63 b
72	128.95±4.51 a	836.93±17.26 a

Values are expressed as mean ± standard deviation (n=3). Means with different letters (a–g) within the column are significantly different at p<0.05. TE, Trolox equivalent; DW, dry weight.

Fourier-transform infrared spectroscopy (FTIR) determination

FTIR is a valuable tool for studying the biochemical changes during germination, as it can identify functional groups in biological samples based on their characteristic absorption bands. The FTIR spectra of *C. cajan* seeds at different germination times are shown in Figure 3. The absorption peaks were similar for all samples regardless of the seed germination time. The phytochemicals in *C. cajan* seeds have been confirmed in the study, along with their changes to germination time and functional groups. The absorption peaks were found throughout the wavenumbers, including phenolic compounds showed its signature peaks at 3291 cm^{-1} (phenolic O-H stretching vibration), 2930 cm^{-1} (C-H stretching), 1639 cm^{-1} (aromatic moiety C=C stretching), 1539 cm^{-1} (C-O-C stretching) and 1050 cm^{-1} (C-O-C stretching) (Chumroenphat *et al*., 2021), The phenolic acids were found in all samples. This result agrees with the finding that increased germination time could increase the content of the phenolic acids, as shown in Table 3. The content of phenolic acids increased, such as gallic acid, protocatechuic acid, chlorogenic acid, p-coumaric acid, and ferulic acid, as shown in FTIR spectrum, where the absorption peaks of phenolic acid had the highest intensity. FTIR was employed to determine the functional groups of the components as well as the characterization structure of the composite.

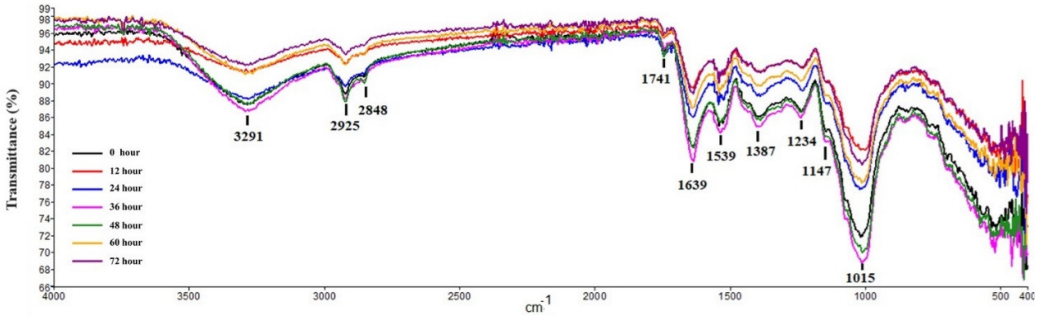

Figure 3. Fourier Transform Infrared Spectroscopy (FTIR) spectra of pigeon pea seeds at different germination times

Table 5. Characteristic absorption peaks of Fourier-transform infrared spectroscopy of unprocessed and germinated pigeon pea seeds and assigned functional groups

Wavenumber peak (cm^{-1})	Assignment	Compound	Reference
3291	O–H stretching vibration (Hydrogen-bonded)	Alcohols, phenols, and water	(Yang *et al*., 2019)
2925, 2848	C–H stretching vibrations	Alkanes and lipids	(Johnson *et al.,* 2020)
1741	C=O stretching vibration	Esters and carboxylic acids	(Johnson *et al*., 2020)
1639	Amide I (C=O stretching vibration), C=C stretching	Proteins and unsaturated fatty acids	(Mihály *et al*., 2017; Barth, 2007)
1539	Amide II (N–H bending and C–N stretching vibrations)	Proteins	(Mihály *et al*., 2017; Barth, 2007)
1387	C–H bending vibrations	CH_3 groups in alkanes or lipids	(Nzai and Proctor, 1998)
1234	C–O stretching vibration	Esters and phenols	(Johnson *et al*., 2020)
1147	C–O stretching vibration	Ethers and alcohols	(Johnson *et al*., 2020)
1015	C–O stretching vibration	Carbohydrates	(KacurÃ¡kovÃ¡, 2001)

Summarizes the target function groups in in *C. cajan* corresponding with the component, which are presented in Table 5. FTIR was performed to confirm the presence of amino acids and phenolic compounds and flavonoids determined in the presented research using other methods. However, this supports the findings of the previous study, where phytochemicals, such as amino acids, phenolic acids, and flavonoid compounds were found in *C. cajan* (Johnson *et al*., 2020).

Conclusions

This study examined the influence of germination time on the on free amino acids, phenolic compounds and γ-aminobutyric acid of *C. cajan*. The trend showed a significant increase in the concentration of phytochemical compounds during germination. It was found that the content of most of free essential and non-essential amino acids significantly increased during germination, with histidine showing the highest increase. There was also a notable increase in GABA content, as germination progressed. The TPC and TFC similarly displayed an increasing trend during germination. Contents of various phenolic acids increased, with the overall content significantly rising. The content of some flavonoids including rutin and quercetin also increased. The antioxidant capacity increased consistently during germination. Fourier-transform infrared spectroscopy (FTIR) confirmed the presence of the detected phytochemicals, underscoring germination's role in enhancing the antioxidant capacity of pigeon pea. Our reports demonstrate that this study is the first to offer a thorough examination of each bioactive compound's particular composition in germination pigeon pea seeds. However, further research is needed to elucidate the complete profile of bioactive compounds produced during germination and to optimize the germination conditions for the highest bioactive yield, along with in-vivo assays.

Authors' Contributions

TC performed the conception and design of study. TC and CP: analysis and/or interpretation of data designed. TC, CP and SS wrote the manuscript and reviewed the final manuscript for journal submission. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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