Modelling folates reaction kinetics during cowpea seed germination in comparison with soaking

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Abstract

Folate is a fundamental vitamin for metabolism in plants and humans. A modelling approach has been developed to characterize the reactivity of folates in cowpea seeds during germination at 30 °C, using a water-to-seed ratio of 1:1 (w/w). For this purpose, the concentrations of folic acid, 10-formylfolic acid, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and tetrahydrofolate were determined in seeds during germination times up to 96 h. Two reaction models were sequentially built and adjusted to experimental data to describe changes in concentration in cowpea seed during two germination phases: before 14 h and after 48 h. Results showed intense enzymatic interconversion of all folate vitamers into 5-methyltetrahydrofolate before 14 h of germination and high enzymatic production of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and tetrahydrofolate after 48 h of germination. This study suggests that a long germination process could be more beneficial than soaking to increase the production of bioavailable folates within the seed for human consumption.

Keywords

Folates; legume; germination; interconversion; production.

1. Introduction

Today the consumption of legume seeds is increasingly recommended due to their high nutritional value (Iqbal et al., 2006), but also because of their environmental benefits and sustainable production in general (Graham & Vance, 2003). Legumes such as cowpea seeds are rich in proteins, essential amino acids along with starch and minerals (El-Adawy, 2002). In particular, legumes are a good source of vitamins belonging to the folates group (vitamin B9) that are essential for humans (Gonçalves et al., 2016). Indeed, legumes generally contain between 300 and 400 µg/100 g (db) of folates (Ringling & Rychlik, 2012). The main folate vitamers in legume seeds are polyglutamated, methylated or formylated forms (Rébeillé et al., 2006; Scott, Rébeillé, & Fletcher, 2000). Folates play an important role in DNA synthesis and amino acid metabolism (Gorelova, Ambach, Rébeillé, Stove, & Van Der Straeten, 2017; Scott et al., 2000). The recommended daily intake of natural folates for adults ranges between 300 µg and 400 µg (Krawinkel et al., 2014), 5-CH_3-H_4folate and PteGlu showing similar bioavailability in humans (Pentieva et al., 2004; Pietrzik et al., 2010).

The folate biosynthesis and interconversion pathways are well described in the literature. They involve several intermediaries in cytosol, mitochondrion and chloroplast (Rébeillé et al., 2006). These reactions result in the production of H_4folate from the hydroxymethyldihydropterin, *p*ABA and glutamate (Rébeillé et al., 2006). From tetrahydrofolate, several enzymatic reactions and intermediaries can yield 5-CHO-H_4folate and 5-CH_3-H_4folate (Rébeillé et al., 2006). Enzymatic interconversions between the different folate forms can also occur within the seed (Jägerstad & Jastrebova, 2013). For example, 5-CH_3-H_4folate can be used by the seed metabolism as a methyl donor for biosynthesis of methionine (Gorelova et al., 2017).

Prior to consumption, legumes seeds require to be processed. Several methods can be used alone or in combination: dehulling, soaking, cooking (boiling or steaming), frying and germination. Some antinutritional factors being present in the seed such as phytates (Galiotou-Panayotou et al., 2008) or enzyme inhibitors (Gonçalves et al., 2016) can be reduced using soaking-cooking process

(Ibrahim et al., 2002). However heat due to cooking also damages sensitive molecules such as vitamins (Kon, 1979), in particular folates (Coffigniez et al., 2019 ; Delchier, Ringling, Maingonnat, Rychlik, & Renard, 2014; Dang, Arcot, & Shrestha, 2000). The most commonly used method to prepare legume seeds in West Africa is soaking at room temperature and then boiling (Madodé, 2012). To increase folate content, a germination step before cooking appears to be the most appropriate method (M. Hefni & Witthöft, 2014), and several authors observed folate production during germination. For instance, Shohag et al., (2012) showed that the folate content in legume seeds increased fourfold after 96 h of germination at room temperature. Similarly, Hefni & Witthöft, 2014 found a three-fold increase in folates in beans after 72 h of germination at 25 °C. In addition, Hefni & Witthöft (2014) showed a 50% increase in folate content in *faba* beans after 12 h of soaking at room temperature.

However, to the best of our knowledge, there is no reaction kinetic model available in the literature that mechanistically describes the reactivity of folates in legumes during germination. The development of such a model could be helpful to identify optimized germination pathways that increase folate content in sprouted legumes. Besides, none comparison between soaking and germination processes has been performed concerning the reactivity of folates in legumes seeds.

A modelling approach has been recently developed to describe folates diffusion and chemical reactivity during cowpea soaking process using a water-to-seed ratio of 4:1 (w/w) (Coffigniez et al., 2019). This approach highlighted the high apparent diffusivity of folates at high soaking temperatures whereas intense folate vitamers interconversions were observed at lower temperatures. This present study went further by upgrading the latter model so that folate vitamers reactivity (degradation and production) could also be evaluated in the case of germination at 30 °C, with an update of the folates' reaction scheme. Then, the results of germination model adjustment were discussed in terms of folates production capacity.

2. Materials and methods

2.1. Materials

The cowpea variety studied here was the *Wankoun* brownish cultivar obtained from IITA (International Institute for Tropical Agriculture) in Benin in September 2017. This cultivar, which is largely cropped and consumed in Benin, germinates easily. The seeds were placed in a vacuum pack and stored at 4 °C in the dark until use.

All the solutions used in the present study were the same as those used by Coffigniez et al. (2019). 2-(N-morpholino)-ethanesulfonic acid (MES) (purity ≥ 99.5%), DL-Dithiothreitol (DTT) (purity ≥ 98%), and sodium acetate trihydrate (purity ≥ 99.0%) were purchased from Sigma-Aldrich (Steinheim, Germany). Disodium hydrogen phosphate dehydrate (purity ≥ 99.5%) was bought from Merck (Darmstadt, Germany) and potassium dihydrogen phosphate (purity ≥ 98 %) from AppliChem (Darmstadt, Germany). L(+)-Ascorbic acid (purity ≥ 99.1%), sodium chloride (purity ≥ 99.9%), acetonitrile (LC-MS/MS grade) and Ultrapure water were provided from VWR (Ismaning, Germany). Lyophilized chicken pancreas powder was purchased from Pel-Freez Biologicals (Rogers, USA), and rat serum from Biozol (Eching, Germany). The unlabelled reference compounds: Folic acid; 10-formylfolic acid; (6*R*,*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-formyl-tetrahydrolfolate); (6*R*,*S*)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-methyl-tetrahydrofolate); (6*S*)-5,6,7,8-tetrahydrofolic acid (tetrahydrofolate) and the internal standards [$^{13}C_5$]-folic acid; [$^{13}C_5$]-(6*S*)-tetrahydrofolate; [$^{13}C_5$]-(6*S*)-5methyl-tetrahydrofolate, calcium salt; [$^{13}C_5$]-(6*S*)-5-formyl-tetrahydrofolate, calcium salt were all purchased from Schircks Laboratories (Jona, Switzerland).

2.2 Soaking experiments

The soaking experiments were performed at 30 °C (which corresponds to room temperature in Benin) (Madodé, 2012) in a Erlenmeyer placed in a closed thermo-regulated water bath (WB22,

Memmert, Hannover, Germany) in the dark, with an initial volume of water corresponding to a water-to-seed ratio of 4:1 (w/w, soaking conditions traditionally used in Benin) (Coffigniez et al., (2019)). After soaking, the seeds were rapidly removed from the soaking-cooking water and centrifuged at 7 g for 5 min at 25 °C to eliminate residual water.

2.3 Germination experiments

Unlike soaking, germination process was performed using a lower water-to-seed ratio of 1:1 (w/w) and the same equipment as for soaking experiments. As for soaking experiments, the water bath was closed to maintain a moisture-saturated air around the seeds in order to facilitate germination process. After 3 h of germination incubation, both seeds and Erlenmeyer were rinsed three times a day with deionized water, to prevent microbial growth. The investigated germination times were 6 h, 14 h, 24 h, 48 h, 72 h, and 96 h. After 3 h of germination, all the available water was absorbed by the seeds. After germination, the seeds were freeze-dried and stored at − 80 °C for a maximum of two weeks before folates analysis. Each germination experiment was performed in duplicate (around 120 seeds per experiment). The germination rate was calculated as the percentage of germinated seeds, based on the emergence of the radicle.

2.4 Seed water content determination

Seed water content was measured as the difference between weight mass and dry mass Water content was measured for each experimental condition (i.e. each time using twenty-five seeds. The difference between weight mass and dry mass (obtained after freeze-drying at − 80 °C for 48 h). allowed to calculate water content on a dry basis.

2.5 Folates quantification

Folates extraction and quantification by liquid chromatography coupled with tandem mass spectrometry were performed using the procedure of Striegel et al., (2018) with slight modifications as proposed by Coffigniez et al., (2019).

After grinding, folates were heat extracted (10 min) from 100 mg of cowpea seeds flour into 10 mL MES buffer with an addition of internal standards solutions in each sample in amounts to fall within the given range of calibration. To deconjugate all folates, the samples were incubated overnight at 37 °C with 2 mL of chicken pancreas suspension and 400 µL of rat serum. After inactivating the enzyme by boiling samples (10 min), acetonitrile was added and the samples were centrifuged for 20 min at 4000 rpm and 4 °C. The supernatant was purified by solid phase extraction (SPE) on strong anion exchange (SAX, quaternary amine, 500 mg, 3 mL) (Phenomenex, Aschaffenburg, Germany) and concentrated in 2 mL of elution buffer. The samples were filtered through a 0.22 µm membrane filter, then folates were separated by liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS). The separation was carried out on a Shimadzu Nexera X2 UHPLC system with a model DGU-20ASR degassing unit (Shimadzu, Kyoto, Japan). Folates were separated at 30 °C on a 2.7 µm, 100 × 2.1 mm C18-LC column (Restek, Bad Homburg, Germany), coupled with a PDA Nexera SPD-M30A diode array detector (Shimadzu, Kyoto, Japan). The concentration of unlabeled analytes was quantified before each extraction using a calibration curve with external standards. Separation was performed by High-performance Liquid Chromatography (HPLC), coupled with a SPA-M20A diode array detector (Schimadzu, Kyoto, Japan).

3. Multi-response germination modelling

3.1 Model assumptions

In the model, a single cowpea seed (Ω_S) was assumed to have a 2D-axisymmetric pseudo-ellipsoidal shape as described by Coffigniez et al., (2018). The model described the initial 3h of soaking followed by germination. As shown in figure 1, folates' reaction scheme considers both the interconversion and metabolization of folic acid, 5-formyl-H_4folate, 10-formyl-folic acid and 5-methyl-H_4folate during cowpea seed germination. In our study, the folates' reaction scheme was

simplified with the aim to focus on folates active forms only. Indeed, we assumed for example that PteGlu, 10-CHO-PteGlu and 5-CHO-H_4folate were converted into 5-CH_3-H_4folate without taking any intermediary products into account. In fact, PteGlu can be reduced into H_4folate in two steps thanks to dihydrofolate reductase (Jägerstad & Jastrebova, 2013). Then, the serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate reductase can convert the H_4folate into 5-CH_3-H_4folate (Rébeillé et al., 2006). H_4folate can also be converted into 5-CH_3-H_4folate via the intermediate production of 10-CHO-H_4folate by 10-formyltetrahydrofolate synthase (Rébeillé et al., 2006) followed by the intermediate production of 5,10-methenyl-H_4folate and 5,10-CH_2-H_4folate by 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate reductase (Rébeillé et al., 2006; Jägerstad & Jastrebova, 2013). In the model, all the reactions were considered as being apparently irreversible to have an order of magnitude of net productions, conversions and degradations, despite most of them can be reversible. For the model, we considered the following assumptions:

(A1) Mass transfer between the seeds and its environment was neglected since germination process was mainly (from $t \geq 3$ h) performed with seeds being not in contact with soaking water.
(A2) The folates concentrations were considered to be homogeneous inside the seed.
(A3) The seed volume remained constant during the germination process (no swelling).
(A4) The folates interconversions were assumed to follow first-order kinetics.
(A5) Both 5-formyl-H_4folate and 5-methyl-H_4folate were produced from the tetrahydrofolate form.
(A6) Tetrahydrofolate (H_4folate) production was assumed to follow zero-order kinetics after 48 h of germination. However, the concentration of tetrahydrofolate remained low in comparison with the other forms before 48 h.

The five state variables studied here were folic acid [PteGlu] ([X_1]), 10-formyl-H_4folate [10-CHO-PteGlu] ([X_2]), 5-formyl-H_4folate [5-CHO-H_4-folate] ([X_3]), 5-methyl-H_4folate [5-CH3-H_4-folate] ([X_4]) and H_4folate ([X_5]) concentrations all expressed in kg.m^{-3} in seeds.

3.2. The unsteady state reaction model

The mass balance equations for folates species in cowpea seeds (Eq. (1)) can be written as:

$$\left.\begin{aligned} \frac{\partial[X_1]}{\partial t} &= -k_1[X_1] \\ \frac{\partial[X_2]}{\partial t} &= -k_2[X_2] \\ \frac{\partial[X_3]}{\partial t} &= -k_3[X_3] + k_{p3}[X_5] \\ \frac{\partial[X_4]}{\partial t} &= -k_4[X_4] + k_1[X_1] + k_2[X_2] + k_3[X_3] + k_{p4}[X_5] \end{aligned}\right\} \quad (1)$$

where k_i and k_{pi} (i=1,…4) are the degradation and production rate constants of species i (s^{-1}). Model adjustments were performed twice and sequentially, considering experimental kinetics database. The first and second model adjustments were performed for the germination time intervals of [0 h–14 h] and [48 h–96 h]. H_4folate concentration ([X_5], $kg.m^{-3}$) was not considered during the first 48 h of germination whereas its production was modelled in the second step ([48 h–96 h]), assuming zero-order kinetics (assumption A6) as:

$$\frac{\partial[X_5]}{\partial t} = k_5 \quad (2)$$

where k_5 is expressed in $kg.m^{-3}.s^{-1}$. Equations (1) and (2) have the following initial and boundary conditions:

$$[X_i] = [X_i]_0 \text{ for } t = 0 \quad (3)$$

$$\vec{\nabla}[X_i].\vec{n} = 0 \quad (4)$$

Model input parameters are given in table 1. A mass balance analysis of the reaction processes involving folate species i was performed by calculating the produced ($m_{X_{i,prod}}$), degraded ($m_{X_{i,degr}}$) and residual ($m_{X_{i,res}}$) mass fractions all expressed in relation to the initial mass ($m_{X_{i,0}}$) inside the seed as:

$$\frac{m_{X_{i,prod}}}{m_{X_{i,0}}} = \int_0^t \left(\iint^V k_{pi} [X_i] dV + k_i [X_i] dV \right) dt \quad (5)$$

$$\frac{m_{X_{i,degr}}}{m_{X_{i,0}}} = \int_0^t \left(\iint^V -k_i [X_i] dV \right) dt \quad (6)$$

$$\frac{m_{X_{i,res}}}{m_{X_{i,0}}} = \frac{m_{X_{i,0}} + m_{X_{i,prod}} - m_{X_{i,degr}}}{m_{X_{i,0}}} \quad (7)$$

3.3. Numerical solution

The system of five partial differential equations (Eqs. (1) and (2)) was solved using the FEM-based commercial Comsol Multiphysics™ (version 5.2a, Comsol Inc., Stockholm, Sweden) with the initial conditions given by Eq. (3), and the boundary conditions given by Eq. (4). A 10 000-element mesh was created in Comsol. Lagrange polynomials (second order function) were the interpolation functions. As described by Coffigniez et al., (2018), the linearized problem was solved by the MUMPS time-dependent solver (Multifrontal Massively Parallel Solver), which implements a parallel distributed LU factorization of large sparse matrixes. The maximum time step was 0.05 s and the Jacobian was updated for each iteration. The typical simulation time was 5 min using a 3.25 Gb free memory (RAM) and 3-GHz Intel core Duo CPU computer (32 bits).

3.4. Parameter identification

In this multi-response approach, the rate constants (k_i and k_{pi}) were identified by regression analysis using a Bayesian approach, with the minimization of the determinant of dispersion of the matrix C with the following elements:

$$C_{i,j} = \sum_{u=1}^{n} \left(\frac{\tilde{X}_u^i - \hat{X}_u^i}{max\{\tilde{X}^i\}} \right) \left(\frac{\tilde{X}_u^j - \hat{X}_u^j}{max\{\tilde{X}^j\}} \right) \quad (8)$$

where u is the index of experimental runs (u = 1,⋯,6) corresponding to the experimental sampling times, $\tilde{X}_u$ are the experimental data points for experimental run u, $\hat{X}_u$ are the values predicted by the

model and (i, j) are the indexes of the concentration responses. As shown in Eq. (8), the residuals are evaluated according to the relative difference between the experimental and predicted values. In this approach, not only the sum of squares for each response is taken into account (diagonal elements of matrix C) but also the cross products of the responses (covariance). As described by Coffigniez et al., (2018), the model parameters were iteratively adjusted to the goodness-of-merit *min(det*(C)) using a minimization procedure of the Nelder-Mead simplex with the "fminsearch" function of Matlab software. The standard deviation of each adjusted parameter was determined via Monte Carlo simulations with 200 random draws.

$$X_{noise} = \tilde{X} + \sigma_y \delta \quad (9)$$

where σ_y is the experimental standard deviation estimated for each experimental datum and δ is a random number between 0 and 1 that is normally distributed. The adjusted parameters follow a normal distribution. The mutual correlation coefficients between adjusted model parameters were also estimated based on the Monte-Carlo results.

4. Results and discussion

4.1. Folates kinetics during germination

The germination in a limited water-to-seed ratio (1:1 (w/w)) induced the starting of seed germination at 14 h (Germination rate of 14%) (Figures 2 and supplementary figure 1). During germination, two distinct time periods were observed in terms of folates behaviour, especially for H_4folate, 5-CHO-H_4folate and 5-CH_3-H_4folate: the first period (called G1) up to 14 h and the second period (called G2) after 48 h. These two time periods were hence modelled separately and the time gap between 14 h and 48 h was subjected to mathematical interpolation.

4.1.1. Experimental changes in folates during germination

Figure 3 shows folates concentration (expressed in µg/100g db) kinetics in the seed during germination at 30 °C. The initial concentration of total folate in cowpea seeds was 420.4 ± 33.0 µg/100g, with 5-CHO-H_4folate, 5-CH_3-H_4folate, PteGlu, 10-CHO-PteGlu and H_4folate representing 54.2%, 11.4%, 15.6%, 15.6% and 3.2% of this amount respectively. Close to these results, Hoppner & Lampi (1993) reported a total folate concentration of 367.1 ± 28.6 µg/100 g (db) in cowpea seeds and 399,1 ± 31.5 µg/100 g (db) in black eyed peas. Ringling & Rychlik (2012) also showed that 5-CHO-H_4folate was the most abundant form in chickpea (42%), while Delchier et al. (2016) reported a most abundant 5-CH_3-H_4folate form in cowpea, pea, green pea, lentils, faba bean, mung bean and soybean. Moreover, in this present study, a lower H_4folate concentration and a higher PteGlu concentration in cowpea raw seed was observed in comparison with values described by Delchier et al. (2016). This might be due to H4folate oxidization into PteGlu during cowpea seed transport and/or storage.

After 14 h of germination, the experimental 10-CHO-PteGlu and 5-CHO-H_4folate concentrations in seeds were reduced by 26% and 82%, whereas 5-CH_3-H_4folate concentration increased by 308%. These changes can be explained by the conversion of both 10-CHO-PteGlu and 5-CHO-H_4folate storage form into the 5-CH_3-H_4folate active form (Gorelova et al., 2017). This folate conversion was achieved during the first 8 h of germination (Figure 3), before the emergence of the radicle that appeared between 6h and 14 h of germination (Figure 2 and supplementary figure 1). The experimental PteGlu concentration remained almost the same after 14 h of germination. Overall, the net total folate concentration in seeds decreased by 5% in 14 h, probably due to the use of folates by the seeds to trigger germination. These results are similar to those found in the literature. Indeed, Coffigniez et al., (2019) soaked cowpea seeds for 14 h at 30 °C, using a water-to seed ratio of 4:1 (w/w) and observed a 29% and 87% decrease in 10-CHO-PteGlu and 5-CHO-H_4folate concentrations, principally due to their interconversion into 5-CH_3-H_4folate (increase of

344% for the latter molecule). It is noteworthy to mention that no germination was observed during soaking (water-to-seed ratio of 4:1 (w/w)) (Coffigniez et al., 2019). Moreover, Hefni & Witthöft, (2014) performed germination of faba beans and chickpeas with a first soaking phase using a water-to-seed ratio of 5:1 (w/w) for 12 h. These authors observed after this process a 25% and 93% decrease in 10-CHO-PteGlu, and a 172% and 93% increase in 5-CHO-H_4folate concentration in faba beans and chickpeas seeds respectively. Faba beans being soaked at 30 °C using a water-to-seed ratio of 4:1 (w/w) induced similar results, with a decrease of 73% and 14% in 10-CHO-PteGlu and 5-CHO-H_4folate concentrations and an increase of 112% in 5-CHO-H_4folate (M. E. Hefni et al., 2015).

From 14 h to 48 h, the experimental concentration of all vitamers in the seeds remained constant (except a slight decrease of the 10-CHO-PteGlu) (Figure 3). This is the reason why we didn't model this phase between 14 h and 48 h. During this period, the seeds germination was initiated, the germination rate reaching its maximum at 48 h (Figure 2).

Between 48 h and 96 h of germination, experimental PteGlu and 10-CHO-PteGlu concentrations in the seeds decreased by 15% and 21% (or 13.8% and 8.7% of the initial concentration of each vitamers), whereas the experimental concentrations of H_4folate, 5-CHO-H_4folate, and 5-CH_3-H_4folate increased by 58%, 124% and 171% (or increase of 113%, and 695% of the initial concentration of H_4folate and 5-CH_3-H_4folate and a decrease of 36.8% of the initial concentration of 5-CHO-H_4folate) (Figure 3). The decrease in both PteGlu and 10-CHO-PteGlu again appears to be due to interconversion into 5-CH_3-H_4folate. However, the total concentration of folates doubled between 48 h and 96 h (and also between 0 h and 96 h) of germination due to the production of H_4folate, 5-CHO-H_4folate, and 5-CH_3-H_4folate. In this context, we assumed that 5-CHO-H_4folate was also converted into 5-CH_3-H_4folate. The high level of total folate production after 48 h of germination was strongly depending on germination rate. Indeed, the latter increased from 14% to 78% when germination time increased from 14 h to 48 h (Figure 2). After 48 h, root and shoot elongations took place, enhancing cell division and DNA duplication. These last biological

modifications require a high level of folates, especially the 5-CH_3-H_4folate at the origin of all methylation reactions (Gorelova et al., 2017), explaining the high production of the 5-CH_3-H_4folate and its main precursor, the H_4folate, observed in figure 3. The quite significant increase of 5-CHO-H_4folate could reflect the buildup of new storage, or requirement for more direct regulatory roles (Rébeillé et al., 2006). In the literature, other authors also reported a significant amount of folate production during legume and cereal germination. For instance, Shohag et al. (2012) showed that the folate content increased about 3.5 to 4.3 times during germination of soybean and mungbean at room temperature until a maximum was reached after 4 days. As for this present study, this increase was mainly due to the production of 5-CH_3-H_4folate, and to a lesser extent to H_4folate and 5-CHO-H_4folate production. Hefni & Witthöft (2014) also found a three-fold increase in folate concentration in faba beans and chickpeas after 3 days of germination at 25 °C, due to an increase in 5-CH_3-H_4folate concentration (455% for faba beans and 297% for chickpeas). The same increase of folate content was also reported by Kariluoto et al., (2006) after 4 days of rice germination at 25 °C.

4.1.2 Modelling the enzyme rate constants involved in conversion and production of folates

The estimated conversion rate constants (k) were adjusted to experimental data assuming first-order kinetics (Table 2) for the two germination periods: G1 (before 14 h) and G2 (after 48 h). This parameter decreased during G2 to one fifth in the case of 10-CHO-PteGlu and 56 time for 5-CHO-H_4folate in comparison with G1. Concerning the PteGlu vitamer, the conversion constant was almost the same than the one of 10-CHO-PteGlu during G2, but was not identifiable during G1 and supposed null, due to low measurement accuracy for this vitamer. The interconversions of PteGlu, and 5-CHO-H_4folate into 5-CH_3-H_4folate are already described in the literature and involve a pool of endogenous enzymes in seeds (Rébeillé et al., 2006). However, this scheme was simplified to the purpose of modelling, as described in Figure 1. The estimated conversion values during G1 were close to the one found by Coffigniez et al., (2019) for cowpea soaked at 30 °C in water to seed ratio

of 4:1 (w/w), with a parameter values of 0.81×10^{-5} s^{-1} for 10-CHO-PteGlu and 7.95×10^{-5} s^{-1} for 5-CHO-H_4folate.

The estimated consumption of 5-CH_3-H_4folate during G1 was modelled assuming first-order kinetics and reported in table 2. Coffigniez et al., (2019) showed a similar rate constant of 5-CH_3-H_4folate during a cowpea soaking at 30 °C in water-to-seed ratio of 4:1 (w/w). The 5- CH_3-H_4folate consumption was probably due to its use by seeds in methylation reactions (Gorelova et al., 2017; Scott et al., 2000). Due to the net observed production of 5-CH_3-H_4folate during the G2 (Figure 3), its consumption was not estimated and fixed as null. The overall net production was just taken into account, and therefore was probably underestimated.

After 48 h of germination (G2), the estimated production rate constants k_{pi} were adjusted to the experimental data assuming first-order kinetics (table 2). The production rate constant k_{pi} was found to be 2.6 times higher for 5-CH_3-H_4folate (active folate form) than for 5-CHO-H_4folate. Moreover, after 48 h of germination (G2), the production rate constants were 10 times higher than the conversion rate constants k_i. The production and enzymatic conversion reactions are usually represented by Michaelis-Menten laws (Neuburger et al., 1996). In our study, due to the complexity of the real folates' reaction scheme, it was preferable to use first-order kinetics instead. This equation depends on the substrate that was assumed to be H_4folate in the case of folates production. In practice, the production of 5-CH_3-H_4folate and 5-CHO-H_4folate involves numerous steps and several chemical intermediates. H_4folate is one of the intermediates and is the first folate vitamer to be produced during folates biosynthesis. Moreover, the production of 5-CH_3-H_4folate and 5-CHO-H_4folate from H_4folate creates other intermediates such as 10-formyltetrahydrofolate (Rébeillé et al., 2006) that were not taken into consideration in our model. However, due to the lack of information about substrate concentration responsible for H_4folate production, changes in the concentration of

H_4folate were modelled assuming zero-order kinetics, with a resulting rate constant of $k = 1.01 \pm 0.02 \times 10^{-3}$ m^2s^{-1} (data not shown).

As the vitamer concentrations did not significantly change between 14 h and 48 h of germination, the production, consumption and conversion rate constants were neglected during this period. However, this constant state may reflect a balance between production and degradation of different vitamers.

From the model, it can be hypothesized that the degradation of 5-CH_3-H_4folate was negligible during G2 ($t \geq 48$ h), but the seeds may have used this vitamer in methylation reaction pathways. The correlation coefficient between the 5-CHO-H_4folate conversion and production rate constants was found to be 0.32 for the G2 model. However, the correlation coefficient between the different adjusted parameters was always less than 0.17 for the G1 model.

During G1, the model provided a good fitting performance, with an overall RMSE of less than 10% (db). After 48 h of germination (G2), the model described the interconversion of PteGlu, 10-CHO- PteGlu and 5-CHO-H_4folate into 5-CH_3-H_4folate but also the production of 5-CHO-H_4folate and 5-CH_3-H_4folate from H_4folate with an overall RMSE of 12.4% (db). The gap between model and experimental data was due to the simplifying hypotheses. The identification of the vitamer rate constants was made possible thanks to the model proposed here. This wouldn't be the case if only experimental data was considered alone.

4.2. Folates: predicted degradation vs. predicted production rates during germination

Figure 4 shows changes in the predicted degraded (through interconversion), produced (by both enzymatic reactions from H_4folate and interconversion from the other forms in the case of 5-CH_3-H_4folate) and the residual folates' mass fractions in cowpea seeds during germination at 30 °C.

During G1, the degraded fractions of 10-CHO-PteGlu and 5-CHO-H_4folate represented 26.4% and 93.9%, after 14 h of germination. These forms were interconverted into 5-CH_3-H_4folate with a produced fraction of the latter of 479.2%. The degraded fraction of 5-CH_3-H_4folate was 173.0% after 14 h of germination, resulting to a reduction of 20.5% of the total amount of folates. These results are close to those described by Coffigniez et al. (2019). Indeed, after soaking at 30 °C using a water-to-seed ratio of 4:1 (w/w), the degraded fractions of 10-CHO-PteGlu, 5-CHO-H_4folate represented 31.7% and 93.3% and the produced fraction of 5-CH_3-H_4folate represented 572.8%. The degraded fraction of 5-CH_3-H_4folate was 218.4%, corresponding to a degradation of 25.9% of the total amount of folate in seeds. However, due to higher water-to-seed ratio (4:1 (w/w)), the authors observed and modelled vitamers diffusion that accounted for 9.9% of the total amount of folate after 14 h, and more specifically 4% for both the PteGlu and 5-CHO-H_4folate, and less than 25% for the 10-CHO-PteGlu and 5-CH_3-H_4folate.

Between 48 h and 96 h of germination (G2), the degraded fractions were 21.8%, 11.4%; and 21.1% for PteGlu, 10-CHO-PteGlu and 5-CHO-H_4folate, which was lower (except for PteGlu) than during G1. However, 5-CHO-H_4folate and 5-CH_3-H_4folate were both produced during G2, with produced fractions of 55.8% and 685.0% respectively. This high production rate doubled total folate content in the seeds after 96 h of germination.

4.3. Process recommended to maximize folate content in the seed

In Benin, cowpea seeds are usually soaked overnight before being boiled for 25 min (Madodé, 2012). This present study showed that a soaking step at 30 °C using a water-to-seed ratio of 1:1 (w/w) conducted to the seeds germination. The model predicted that applied 8 h (around one night) of this process induced a loss of 11.3% of total folate content in seeds. This is lower than the 23% loss of total folate content observed during a soaking step at 30 °C using a water-to-seed ratio of 4:1 (w/w) for 8 h (Coffigniez et al., 2019). Moreover, when the present germination model was coupled

to the cooking model described in our previously work (Coffigniez et al., 2019), the results showed that applying a germination step of 8 h followed by cooking for 25 min at 95 °C led to a remaining concentration of total folate of 66% (279 µg/100g (db)). This folate concentration is higher than the 57% (242 µg/100g bs) remaining concentration in the case of soaking for 8h and using a water-to-seed ratio of 4:1 (w/w), followed by the same cooking step (Coffigniez et al., 2019). The daily folate requirement for healthy adults is 300-400 µg (Krawinkel et al., 2014). One hundred grams (on a dry basis) of cooked cowpea seeds could hence provide 90% of the daily folate requirements after applying this germination-cooking scenario.

However, Brouwer et al. (2001) highlighted that only 50% of total folate content from a food matrix is bioavailable for humans. Indeed the polyglutamate forms are 33% less available than monoglutamate forms (Wei et al., 1996), while the predominated polyglutames forms of folates are in a range of three to six glutamates in plants (Rébeillé et al., 2006; Delchier et al., 2016). For example, the mature winged bean contains essentially folates linked to five or six glutamates (Luo et al., 2017). Furthermore, in humans, the different vitamers are converted into the 5-CH_3-H_4folate active form by intestinal conjugase. This enzyme has a limited conversion capacity, which reduces the availability of folates (Scott et al., 2000). An early estimation of the folate bioavailability from lima beans was 70 % (Tamura & Stokstad, 1973), but has to be questioned due to the limited accuracy of the respective folate assays. Moreover, the degree of bioavailability has been shown to vary strongly between single foods, and also within one food item (Mönch et al., 2015). In view of this literature data, it seems essential to increase the consumption of folates to assure its daily requirements, as using a germination step before cooking legumes.

If extended and controlled (to prevent microbiological growth), the germination step could be a solution to greatly enhance the nutritional value of cowpea, as already observed for other legumes and seeds (Shohag et al., 2012; Koehler et al., 2007). Indeed, after 4 days of germination, the undetected vitamin C in raw soybean and mungbean sharply increased (Shohag et al., 2012) and the

vitamin B2 content increased between 1,5 and 2,5-fold in beans, lentils and peas seeds (Vidal-Valverde et al., 2002). Moreover, a germination of 48 h induced a decrease of 54%, 23%, and 26% in trypsin inhibitor, phytate and tannins in cowpea(Ibrahim et al., 2002). The sensory of four-day-germinated lentil and three-day-germinated mung bean seeds were judged to be 84% and 90% acceptable by consumers (Kavas & El, 1991), while cowpeas germinated seeds were judged acceptable only until 24 h of germination (Uwaegbute et al., 2000). Therefore, complementary experimental investigations should be undertaken to evaluate the possibility to use germinated cowpea flour and to prepare some traditional African meals as *akara* or *moin-moin* with a high nutritional values and acceptable sensory.

5. Conclusion

The present study revealed contrasted chemical behaviour of folate vitamers in cowpea seeds during germination at 30 °C, using a water-to-seed ratio of 1:1 (w/w). A 2D axi-symmetric cowpea seed model was built that takes into account both the conversion and production of folates, with satisfactory fitting performance. The results revealed significant interconversion of 10-CHO-PteGlu and 5-CHO-H_4folate into 5-CH_3-H_4folate before 14 h of germination, making the folates more bioavailable. The extent of interconversion was in the same order of magnitude as that observed during soaking at 30 °C using a water-to-seed ratio 4:1 (w/w). After 48 h of germination, an extremely high rate of folates biosynthesis, more specifically 5-CH_3-H_4folate, was induced. A long germination (water-to-seed ratio of 1:1 (w/w)) process is a possible alternative to soaking (water-to-seed ratio of 4:1 (w/w)) to significantly increase folate concentration in cowpea seeds for human consumption. This model could help identifying optimized germination pathways to produce new folate-enriched cowpea-based products such as flours, by limiting experimental tests. Such a model is now being used together with a multicriteria optimization approach to identify germination

processing methods that increase vital nutrient concentrations while decreasing the content in antinutrients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions

Figure 1: Folate reaction scheme occurring during germination process. Blue lines, green dotted lines and orange dotted lines represent the enzymatic interconversion, the enzymatic production and the folate use by seed metabolism.

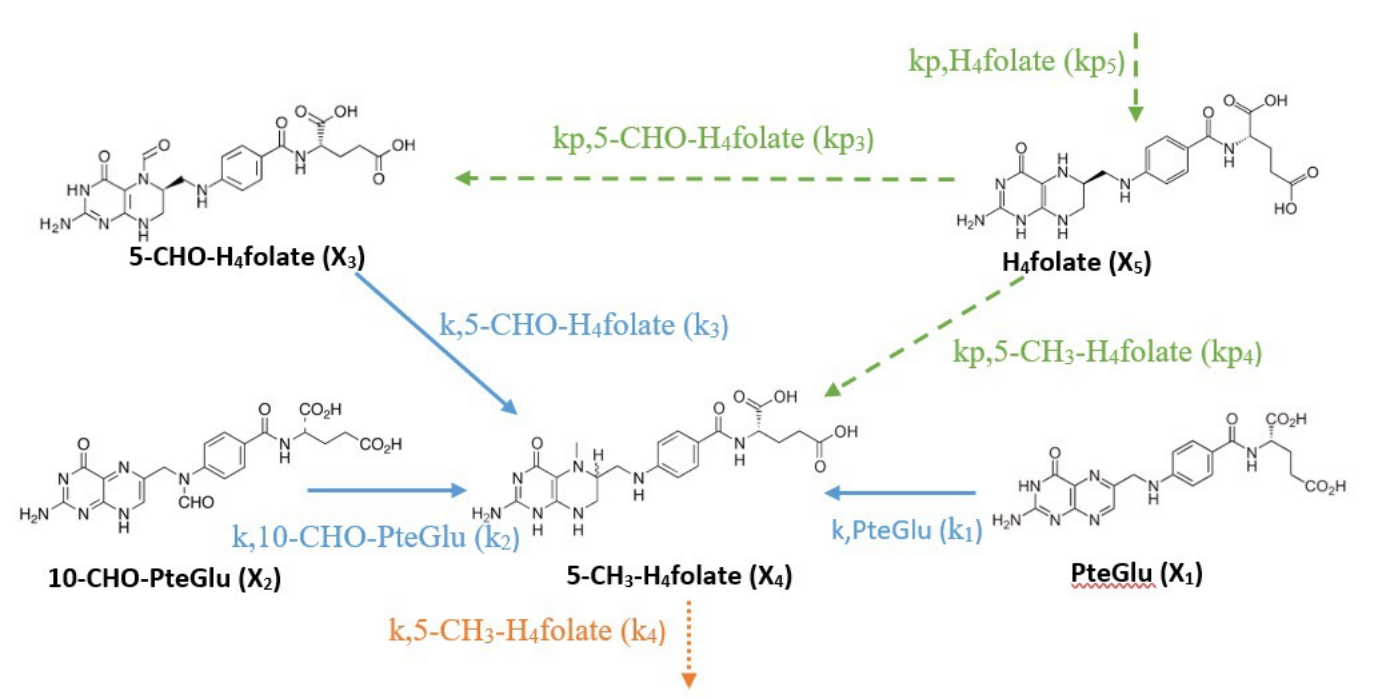
Figure 2: Germination rate (%) as a function of time (h). A seed was considered having germinated when the roots appeared outside the testa ($n = 2$).

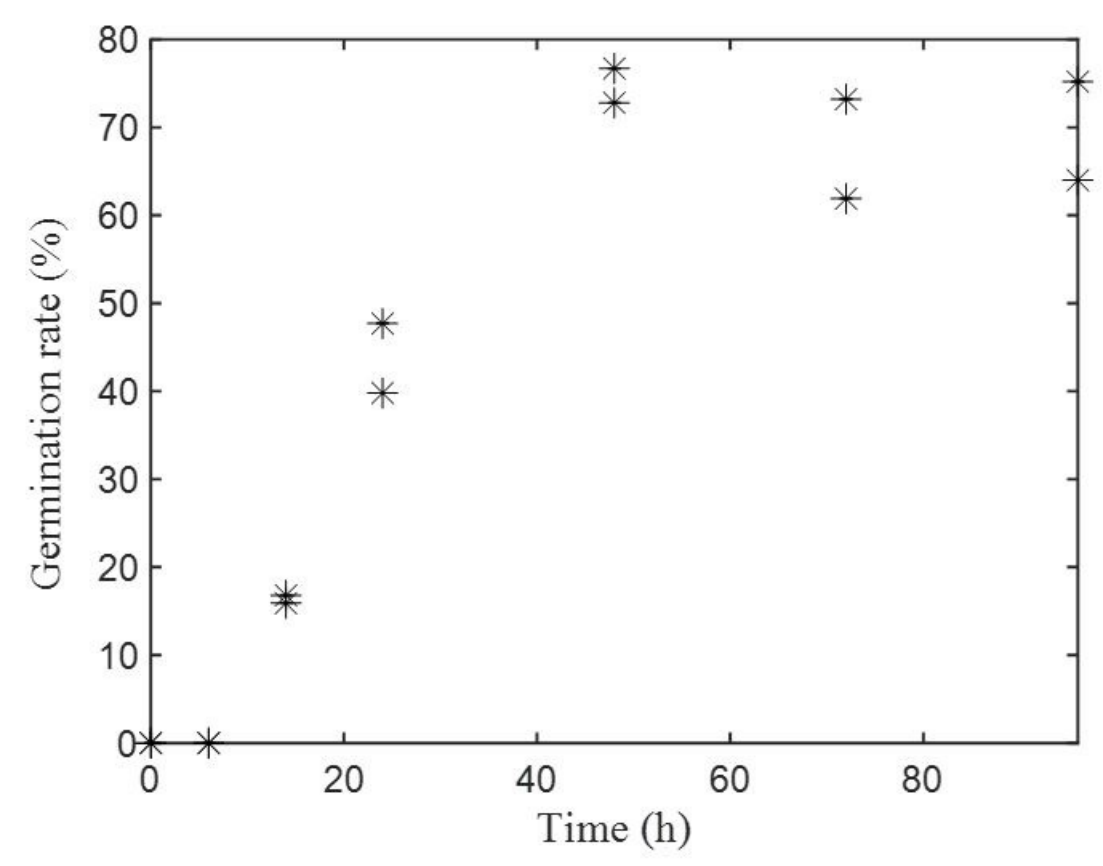
Figure 3: Predicted (lines) and experimental data (symbols) for concentrations of folic acid [PteGlu] (X_1), 10-formylfolic acid [10-CHO-PteGlu] (X_2), 5-formyltetrahydrofolate [5-CHO-H_4folate] (X_3), 5-methyltetrahydrofolate [5-CH_3-H_4folate] (X_4), tetrahydrofolate [H_4folate] (X_5) and total folate [Total] in cowpea seeds (expressed as µg/100g on a dry basis) during the germination process at 30 °C (including the 3 h of soaking). Error bars represent standard deviations ($n = 4$).

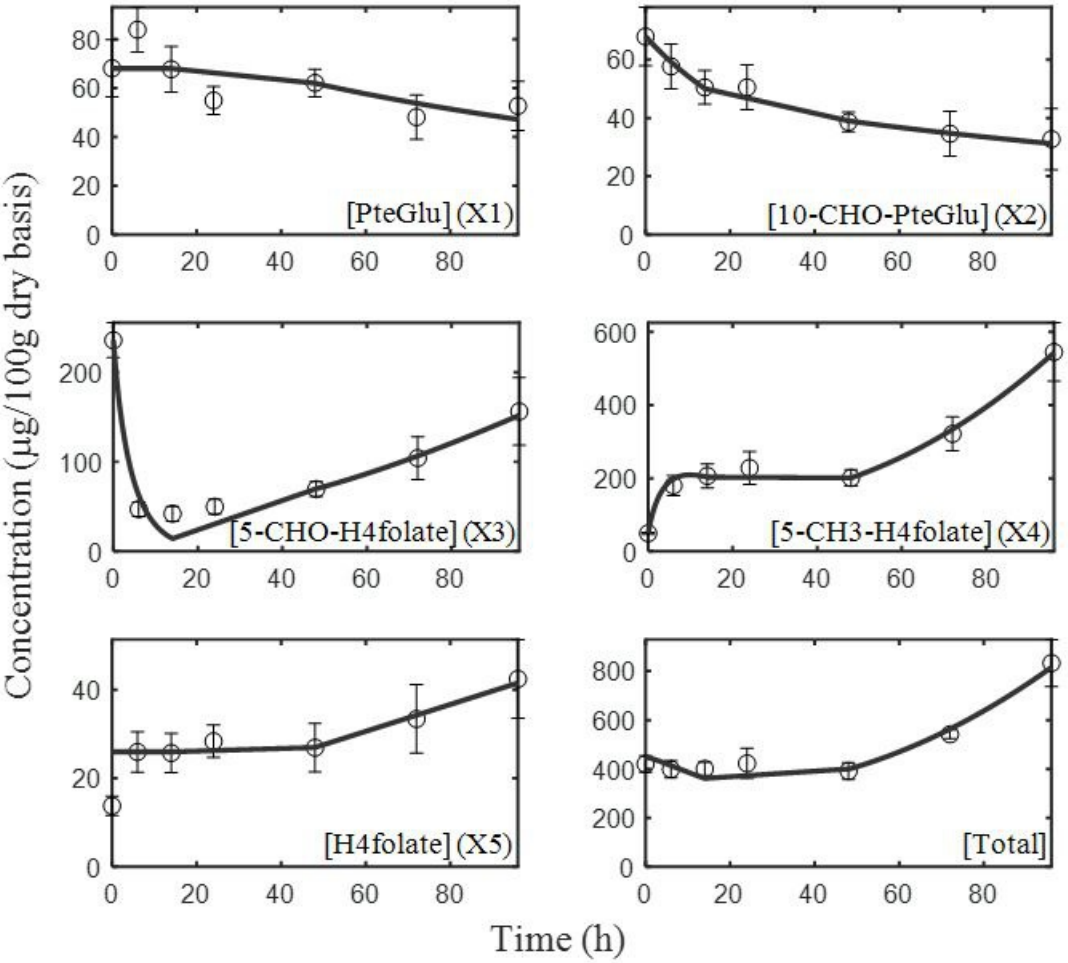
Figure 4: Predicted algebraic residual, produced and degraded mass fraction kinetics for folic acid [PteGlu] (X_1), 10-formylfolic acid [10-CHO-PteGlu] (X_2), 5-formyltetrahydrofolate [5-CHO-H_4folate] (X_3), 5-methyltetrahydrofolate [5-CH_3-H_4folate] (X_4) and total folate [Total] in cowpea seed during germination at 30 °C (including the 3 h of soaking).

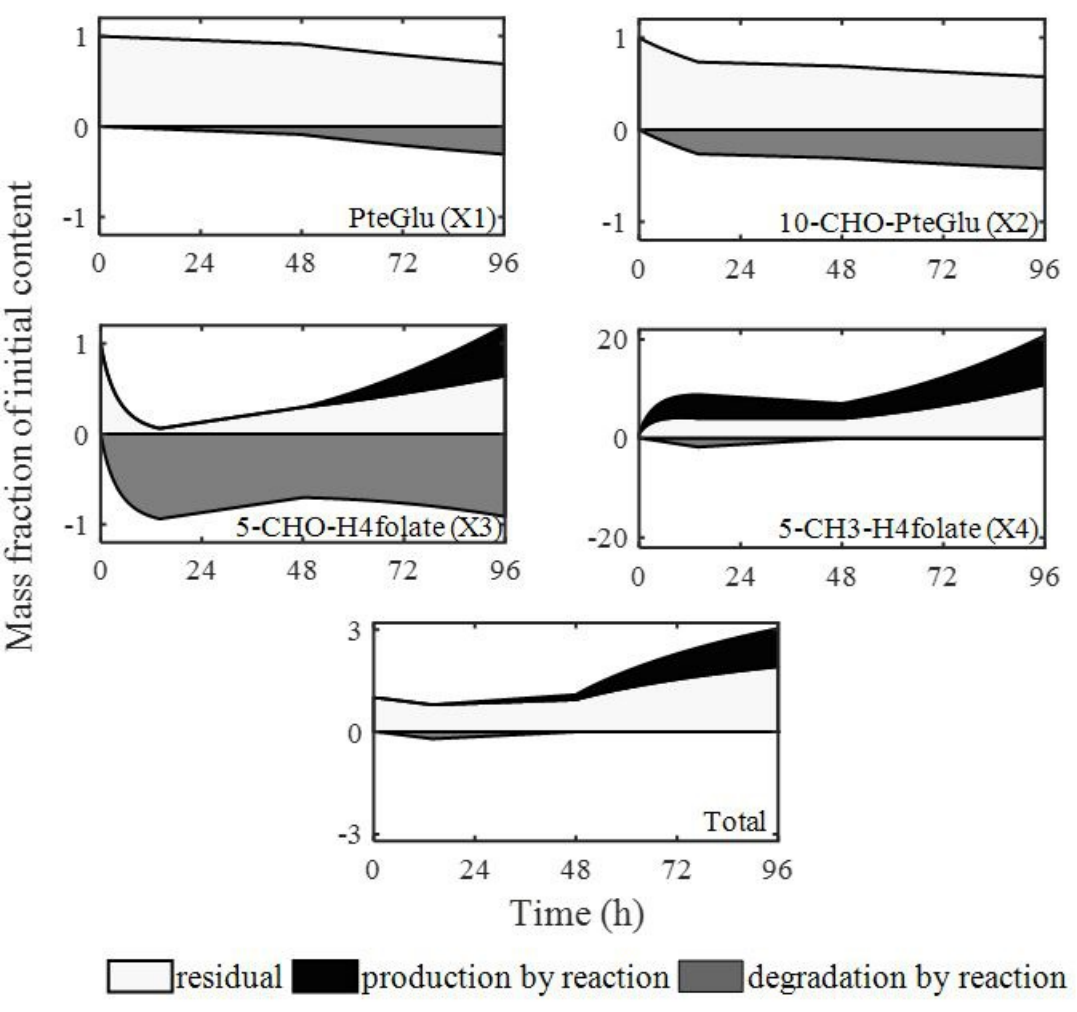
Table 1. Input parameters used in the 2D-axisymmetric reaction model.

Table 2. Estimated conversion rate constants (k_X) and production rate constants (kp_X) for each folates in cowpea seeds during the first 14 h of germination at 30 °C (G1) (including the 3 h of soaking) and after 48 h of germination at 30 °C (G2) (mean values ± standard deviations determined with Monte-Carlo simulations: 200 sets).


kp,H4folate (kp5)
kp,5-CHO-H4folate (kp3)
5-CHO-H4folate (X3)
H4folate (X5)
k,5-CHO-H4folate (k3)
kp,5-CH3-H4folate (kp4)
k,10-CHO-PteGlu (k2)
k,PteGlu (k1)
10-CHO-PteGlu (X2)
5-CH3-H4folate (X4)
PteGlu (X1)
k,5-CH3-H4folate (k4)
Enzymatic conversion
Enzymatic production
Use in metabolism


Germination rate (%)
Time (h)
0
10
20
30
40
50
60
70
80
0
20
40
60
80


Concentration (µg/100g dry basis)
[PteGlu] (X1)
[10-CHO-PteGlu] (X2)
[5-CHO-H4folate] (X3)
[5-CH3-H4folate] (X4)
[H4folate] (X5)
[Total]
Time (h)


Mass fraction of initial content
PteGlu (X1)
10-CHO-PteGlu (X2)
5-CHO-H4folate (X3)
5-CH3-H4folate (X4)
Total
Time (h)
residual
production by reaction
degradation by reaction

parameter	value	unit
$[PteGlu]_0$ ($[X_1]_0$)	0.79×10^{-3}	$kg.m^{-3}$
$[10-CHO-PteGlu]_0$ ($[X_2]_0$)	0.79×10^{-3}	$kg.m^{-3}$
$[5-CHO-H_4folate]_0$ ($[X_3]_0$)	2.76×10^{-3}	$kg.m^{-3}$
$[5-CH_3-H_4folate]_0$ ($[X_4]_0$)	0.58×10^{-3}	$kg.m^{-3}$
$[PteGlu]_{48}$ ($[X_1]_{48}$)	0.72×10^{-3}	$kg.m^{-3}$
$[10-CHO-PteGlu]_{48}$ ($[X_2]_{48}$)	0.45×10^{-3}	$kg.m^{-3}$
$[5-CHO-H_4folate]_{48}$ ($[X_3]_{48}$)	0.81×10^{-3}	$kg.m^{-3}$
$[5-CH_3-H_4folate]_{48}$ ($[X_4]_{48}$)	2.34×10^{-3}	$kg.m^{-3}$
$[H_4folate]_{48}$ ($[X_5]_{48}$)	0.31×10^{-3}	$kg.m^{-3}$
ρ_{DM}^0	1302	$kg.m^{-3}$
V_{Ω_S}	$1.09*10^{-7}$	m^3

Species X	*Process*	$k_{X,\Omega_S} \times 10^5 (s^{-1})$	$k_{pX,\Omega_S} \times 10^5 (s^{-1})$	RMSE*
				In seed (S)
Folic acid (X_1)	G1	0	0	12.5
	G2	0.16 ± 0.01	0	8.8
10formyl-folic acid (X_2)	G1	0.63 ± 0.02	0	7.6
	G2	0.13 ± 0.01	0	6.7
5formyl-H_4folate (X_3)	G1	6.18 ± 0.23	0	22.6
	G2	0.11 ± 0.01	1.72 ± 0.04	21.4
5methyl-H_4folate (X_4)	G1	1.02 ± 0.03	0	21.5
	G2	0	4.53 ± 0.09	50.0

*RMSE: Root mean square error between experimental and predicted concentrations $(mg/100 kg\ db)$.

G1 = germination ≤ 14 h (including the 3 h of soaking); and G2 = germination ≥ 48 h