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Optimisation of temperature and time for the dark germination bioprocess of *Moringa oleifera* seeds to boost nutritional value, total phenolic content and antioxidant activity

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Abstract

The aim of the present work was to determine the best dark germination conditions to obtain germinated moringa (*Moringa oleifera*) seeds with increased nutritional and antioxidant properties. Response surface methodology (RSM), based on a central composite rotatable design, was performed to optimise the germination temperature (25-40°C) and germination time (24-360 h) in order to maximise crude protein content (CPC), total phenolic compounds (TPC) and antioxidant activity (AoxA) of moringa seeds. According to regression models, higher values of CPC, TPC and AoxA were observed at 30°C and 336 h of germination. Dark germination of moringa seeds under the abovementioned conditions did not affect CPC (25.05 ± 0.50 g/100 g (dw)) but increased TPC (209.64 to 271.67 mg GAE/100 g (dw)) and AoxA (8759.64 to 10972.19 μmol TE/100 g (dw)), while some antinutritional components significantly decreased when compared with unprocessed moringa seeds. The optimised germinated moringa seeds could be used as a source of natural antioxidants and protein in the formulation of functional foods.

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Introduction

Moringa (*Moringa oleifera* Lam.) is a perennial foliaged tree native to sub-Himalayan tracts of Northern India, introduced in most tropical and subtropical countries, and widely cultivated due to its high adaptability to climatic conditions and dry soils (Teixeira *et al*., 2014; Leone *et al*., 2016). In addition to these interesting agronomic characteristics, all plant parts of moringa, including leaves, stems, roots, flowers, and seeds, are suitable for human and animal consumption, and possess relevant nutritional, nutraceutical and industrial features (De la Mora-López *et al*., 2018). Moringa seeds in particular, represent a good source of protein and oil which is composed mostly of unsaturated fatty acids (Sánchez-Machado *et al*., 2010), contain both macro and microminerals, vitamins (Okiki *et al*., 2015), and have a diverse profile of phenolic compounds (Singh *et al*., 2013). These last compounds are related to many biological activities, being able to act as antioxidant, antimicrobial and hypoglycaemic agents (Singh *et al*., 2013; Wang *et al*., 2017), and exert antiproliferative effects on some cancer cell lines (Adebayo *et al*., 2017).

Despite the apparent high nutritional and nutraceutical value of these seeds, the presence of some antinutritional factors such as saponins, phytic acid and other phytochemical constituents that interfere with protein digestion and normal metabolism, negatively affect the body weight and growth performance when used as feed in animal models (Igwilo *et al*., 2010; Annongu *et al*., 2014).

Nutritional properties, as well as the level and activity of bioactive compounds present in leguminous plants, may be modified by applying technological and biotechnological processes, among which germination is one of the cheapest and

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most efficient alternatives (Świeca and Baraniak, 2014). Generally, the germination process changes the nutritive value of seeds (Rumiyati *et al*., 2012), reduces some antinutritional factors (Paucar-Menacho *et al*., 2010), and increases the content of compounds correlated with antioxidant properties, such as phenolic compounds (Domínguez-Arispuro *et al*., 2017). In some seeds, germination not only increases the content of phenolic compounds that exert antioxidant activity, but also promotes the increment of tannins, chemical compounds that are traditionally considered antinutritional because of their capacity to bind and precipitate proteins and other organic compounds (Li *et al*., 2014). Changes in composition and antioxidant properties in germinated seeds can vary greatly depending on the germination conditions (Dawood *et al*., 2013).

Currently, the search for unconventional foods or ingredients with high protein content accompanied by other bioactive compounds, such as polyphenols, has increased, particularly in developing countries, where the average intake of macro and micronutrients is lower than required. Given the high protein content and bioactive compounds in moringa seeds, it becomes quite necessary to carry out in-depth research on the processing of seeds into edible products with high nutritional and nutraceutical value. The objective of the present work was therefore to optimise the conditions (germination temperature and germination time) in order to maximize the crude protein content (CPC), total phenolic content (TPC) and antioxidant activity (AoxA) of moringa seeds during dark germination.

Materials and methods

Materials

Moringa seeds used in the present work were obtained from mature pods of trees harvested in Obregon, Sonora, Mexico. Sample seeds were cleaned, milled and stored in sealed plastic bags at 4°C for future chemical determinations and germination assays.

Seed germination and sample preparation

Moringa seeds were manually cleaned to remove broken seeds and foreign matters. A portion of 50 g of seeds was surface disinfected with 0.5% w/v sodium hypochlorite for 10 min, and then rinsed with water before being soaked in distilled water (1:10 w/v) for 24 h at room temperature (25°C). The water was then drained off, and imbibed seeds were germinated by layering them over a moistened filter paper in germination trays in an incubator chamber equipped with temperature control. Germination was carried out over a range of temperatures from 25 to 40°C and over a broad range of times (24-360 h) in the absence of light. A relative humidity of 80–90% was maintained within the incubator chamber. Whole germinated seeds obtained under different germination conditions (Table 1) were freeze-dried, milled into a powder and passed through a sieve (80-mesh screen). Additionally, raw moringa seeds were ground and sieved to obtain unprocessed moringa flour (UM), which was used as a control. All flours were packed and stored at -20°C until analysis.

Table 1. Experimental design[1] used to obtain different combinations of germination process variables for producing germinated moringa seeds, and experimental results for response variables.

Assay[2]	Process variables		Response variables		
	Germination temperature (°C)	Germination time (h)	CPC[3]	TPC[4]	AoxA[5]
1	27.2	73	23.5	197.3	8126.3
2	37.8	73	25.6	284.4	6280.5
3	27.2	310	24.1	273.9	8960.9
4	37.8	310	23.7	256.0	8591.6
5	25	192	23.0	227.7	7921.6
6	40	192	24.9	269.9	6576.1
7	32.5	24	25.2	294.4	9056.1
8	32.5	360	25.5	256.0	11592.8
9	32.5	192	21.9	276.0	9392.9
10	32.5	192	22.4	284.2	9052.5
11	32.5	192	22.9	272.3	9445.8
12	32.5	192	21.5	297.6	9817.1
13	32.5	192	22.8	295.2	10280.1

[1]Central composite rotatable design with two factors and five levels; 13 assays; [2]Does not correspond to order of processing. [3]g/100 g (dw) [4]mg gallic acid equivalents (GAE)/100 g (dw); [5]µmol Trolox equivalents (TE)/100 g (dw).

Total phenolic extraction

Free and bound phenolic compounds were extracted using 80% aqueous methanol following the procedure described by Mora-Rochín *et al*. (2010). Free phenolics were obtained by mixing 0.5 g sample with 10 mL chilled ethanol-water (80:20, v/v) for 10 min in a shaker at 50 rpm. The blends were centrifuged (3,000 g, 10 min), and the supernatant was recovered and concentrated to 2 mL at 45°C using a vacuum evaporator, and stored at −20°C until use. Bound phenolic compounds were released and recovered from the leftover pellet following the method reported by Adom and Liu (2002) with minor modifications as suggested by Mora-Rochín *et al*. (2010).

Determination of total phenolic content

The contents of free and bound phenolic compounds of unprocessed and germinated moringa seed samples were determined using the colorimetric method as described by Singleton *et al*. (1999). A calibration curve was prepared using gallic acid as a standard, and the total phenolic content (TPC) was expressed as mg gallic acid equivalents (GAE)/100 g dry weight (dw). TPC was calculated as the sum of free and bound phenolic compounds.

Antioxidant activity

The antioxidant activity (AoxA) of free and bound phenolic extracts was determined by the oxygen radical absorbance capacity (ORAC) assay using fluorescein as a probe as described by Ou *et al*. (2001). Trolox was used as a standard reference, and the results were expressed as µmol Trolox equivalents (TE)/100 g (dw). All measurements were made in triplicate. Total AoxA was calculated as the sum of AoxA from free and bound phenolic extracts.

Proximate chemical composition

Chemical composition analyses were conducted according to the standard methods (AOAC, 1999): drying at 130°C to determine moisture (method 925.09 B); defatting in a Soxhlet apparatus with petroleum ether for lipid analyses (method 920.39 C); and micro-Kjeldahl for crude protein content (CPC) quantification (N × 6.25) (method 960.52). The carbohydrate content was estimated by difference using Eq. 1:

$$Carbohydrates[\text{g}/100\ g] = 100\text{-}CPC\ [g/100\ g] - Lipids\ [g/100\ g] - Ash\ [g/100\ g] \quad \text{(Eq. 1)}$$

Determination of antinutritional components

Phytic acid

The phytic acid content of the samples was determined following the colorimetric procedure described by Vaintraub and Lapteva (1988). Phytic acid was extracted with 3.5% (w/v) HCl. Suitable aliquots were diluted with distilled water to make 3 mL, and then used for the assay. The results were expressed as mg phytic acid equivalents (PAE)/100 g (dw) using phytic acid as a standard (León-López *et al*., 2013).

Saponin

The saponin content was determined following the spectrophotometric method described by Hiai *et al*. (1976). Saponin was first extracted with 80% methanol. The concentration of saponin was interpolated from a standard curve of different levels of diosgenin in 80% methanol, and expressed as mg diosgenin equivalents (DE)/100 g (dw) (León-López *et al*., 2013).

Tannin

Tannin was extracted with 80% methanol (Xu and Chang, 2007) and assayed colorimetrically following the modified vanillin method of Price *et al*. (1978), and expressed as mg catechin equivalents (CE)/100 g (dw).

Trypsin inhibitors

Quantitative trypsin inhibitor measurements were performed, and trypsin inhibitor units (TIU) were defined according to the enzymatic assay described by Welham and Domoney (2000) using a-N-benzoyl-DL-arginine-pnitroanilidehydrochloride (BAPNA) as a trypsin substrate. Trypsin inhibitor activity expressed as trypsin inhibitor units TIU/mg (dw) was calculated from the absorbance read at 410 nm against a reagent blank.

Experimental design and statistical analysis

The effects of germination temperature (GT) and germination time (Gt) were analysed using response surface methodology (RSM) based on a central composite rotational design as shown in Table 1. Crude protein content (CPC), total phenolic content (TPC) and antioxidant activity (AoxA) of germinated seeds were considered response variables. The second-order polynomial equation was applied to estimate the response variables (Eq. 2):

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad \text{(Eq. 2)}$$

Applying the stepwise regression procedure, nonsignificant terms ($p > 0.1$) were deleted from the equation, and a new polynomial was used to obtain a predictive model for each response variable. Predictive models were used to graphically represent the system. The conventional graphical method was applied as an optimisation technique to obtain maximum values of the response variables. Contour plots of each of the response variables were superimposed to obtain a contour plot for observation and selection of the best (optimal) combination of germination temperature (GT) and germination time (Gt) for the production of optimised germinated moringa seeds (OGM) through a dark germination

bioprocess. The statistical software Design Expert Version 7.0.0 (Stat-Ease, Minneapolis, MN, USA) was used for analysis and evaluation.

The chemical composition and antinutritional components of UM and OGM were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test comparisons using a significance level of 95% ($p < 0.05$).

Results and discussion

Predictive models for crude protein content (CPC), total phenolic content (TPC) and antioxidant activity (AoxA)

The CPC, TPC, and AoxA of germinated moringa seeds depended on the germination conditions (Table 1). CPC varied from 21.5 to 25.6 g/100 g (dw), while TPC varied from 197.3 to 297.7 mg GAE /100 g (dw), and AoxA varied from 6280.5 to 11592.8 µmol TE/100 g (dw). Polynomial regression models showing the relationships among response variables (CPC, TPC, and AoxA) and process variables (GT; germination temperature, Gt; germination time) were obtained. The predictive models explained 90.13% of the total variability in CPC ($p = 0.0021$), 92.37% in TPC ($p = 0.0009$), and 88.09% in AoxA ($p = 0.0002$). The lack of fit was not significant ($p > 0.1$), and the relative dispersion of the experimental points from the predictions of the models (CV) was found to be < 10%, indicating that the experimental models obtained were adequate and reproducible. Predictive models included all terms of the second-order polynomial equation that were significant ($p \leq 0.05$). The second-order adjusted models using uncoded variables for CPC, TPC, and AoxA are presented in Eq. 3, 4, and 5, respectively.

$$CPC = 44.0095 - 1.3833\,GT - 0.0083Gt - 0.0010(GT*Gt) + 0.0258\,GT^2 + 0.0001\,tG^2 \quad \text{(Eq. 3)}$$

$$TPC = -787.5369 + 51.7385\,GT + 1.8409\,Gt - 0.0417(GT*Gt) - 0.6261\,GT^2 - 0.0011\,Gt^2 \quad \text{(Eq. 4)}$$

$$AoxA = -40885.2942 + 3127.0284\,GT + 7.0848\,Gt - 49.6014\,GT^2 \quad \text{(Eq. 5)}$$

Germination temperature and time effects on response variables and optimisation

According to the predictive models shown above, a contour plot for each response variable was generated. Figure 1 (A) shows the contour plot of CPC depending on the effects of GT and Gt. Minimum values of CPC were observed at moderate germination time (100 – 200 h) when the germination temperature was in the interval from 26 to 34°C. CPC slightly increased at short germination times and high germination temperatures. Combinations of long germination times with a germination temperature of approximately 25°C also promoted an increase in CPC.

Interestingly, high germination temperature increased CPC in both short and long germination times. The apparent increase in CPC at long germination times may be attributed to a loss in dry matter, mainly sugars and lipids, through respiration during sprouting (Devi *et al*., 2015; Tesfay *et al*., 2016). Temperature is one of the most important factors during seed germination, having a significant effect on the germination percentage and germination rate (Yerima *et al*., 2016). Hassanein *et al*. (2017) observed that moringa seeds had a faster germination speed at high temperature (40°C). A higher germination speed would mean a greater loss in dry weight through respiration, and this fact allowed the apparent increase in CPC observed when moringa seeds were germinated at higher temperature and longer germination time (Devi *et al*., 2015). CPC also increased when germination was performed at medium to high temperatures (32-40°C) within a short period of time (<24 h), probably due to a quick reawakening of protein synthesis upon imbibition.

During germination, TPC significantly increased ($p < 0.05$), reaching values ranging from 197.3 to 297.7 mg GAE/100 g (dw), depending on germination temperature and time (Figure 1 (B)). TPC accumulation during dark germination of moringa seed was favoured at temperatures of approximately 32-40°C and short germination times. Plants activate several adaptive strategies in response to abiotic stress factors, including extreme temperatures. These adaptive mechanisms include changes in physiological and biochemical processes leading to the accumulation of several organic solutes such as sugars, polyols and phenolics (Tesfay *et al*., 2016). The biosynthesis of phenolics during germination is associated with a plant mechanism to adapt to temperature regimes through phenylalanine ammonia lyase (PAL) and catalase and superoxide dismutase activity, resulting in a synergistic biochemical composition, which helps

to maintain the cellular redox balance (Tesfay *et al*., 2016). Otherwise, a TPC-reducing trend observed for moringa seeds germinated at high temperatures during long germination times could be attributed to the activation of polyphenol oxidases, enzymes responsible for phenolic compound degradation during germination (Paucar-Menacho *et al*., 2017).

The contour plot for the AoxA of germinated moringa seed as a function of germination temperature and time is shown in Figure 1 (C). The germination process caused a gradual increase in AoxA when performed in the temperature range from 29 to 33°C over long periods of time. Navarro *et al*. (2016) reported that the best germination performance (high germination percentage and high germination rate) of moringa seeds was at 30°C. On the other hand, Tesfay *et al*. (2016) studied the effect of varying temperature regimes on moringa seed phytochemical compounds during germination, finding a significant impact on phenolic accumulation and early seed radicle emergence at the 30/20°C regime. Seed adaptation to harsh conditions impacts plant development and involves the mobilisation of different antioxidants, including phenolic compounds. As shown in Figure 1 (B), high germination temperatures (30-40°C) stimulated phenolic accumulation even at short germination times in moringa seeds during dark germination. Therefore, similar behaviour was expected for AoxA. However, the higher TPC does not always mean a higher AoxA because the antioxidant properties of compounds depend not only on TPC quantity but also on the chemical nature of the compounds present in the samples (Gharachorloo *et al*., 2013). Thus, germination at temperatures of approximately 30°C for a prolonged time probably induces the synthesis of phenolics with more effective antioxidant capacities. Moreover, moringa seeds contain other bioactive compounds in addition to TPC, such as saponins (Unuigbe *et al*., 2015), which might also behave as antioxidants. These compounds might also exert synergetic activities among TPC, which could be the main reason for the observed differences in antioxidant activity during germination at different temperature/time conditions.

Figure 1 (D) shows the superimposition of contour plots used to determine the best combination of process variables for the production of optimised germinated moringa seeds. The central point of the optimisation region (coloured in yellow) corresponded to the optimum combination of process variables (GT=30°C /Gt =336 h) for producing germinated moringa seeds with the highest CPC, TPC, and AoxA values. The experimental values of these variables differed by no more than 10% from their predicted values calculated using the predictive models mentioned above, indicating that the optimal conditions of the germination bioprocess were appropriate and reproducible.

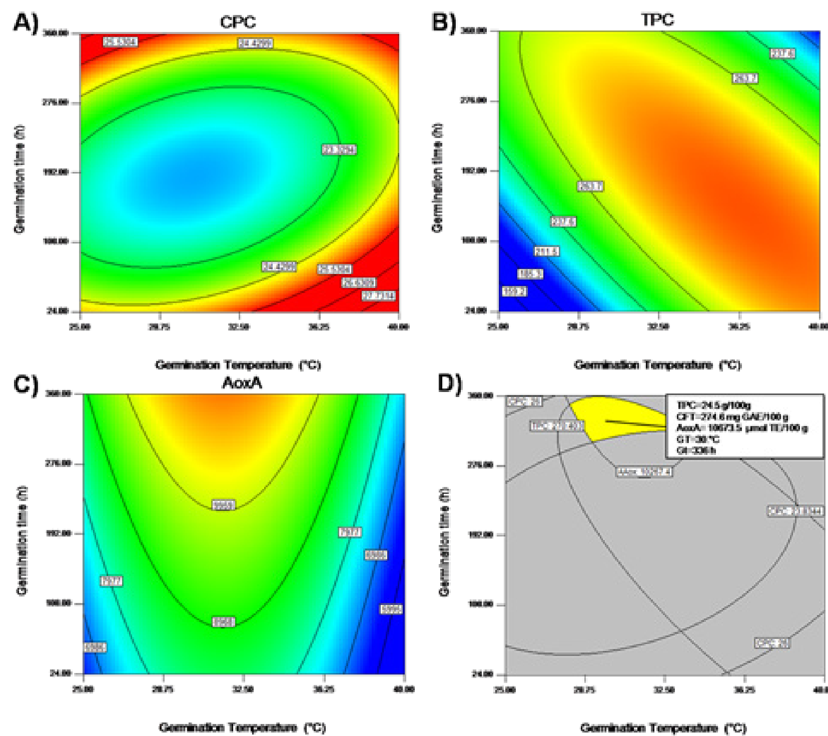

Figure 1. Contour plots showing germination temperature and time effects on (A) crude protein content, (B) total phenolic compounds, and (C) antioxidant activity of germinated moringa seeds. (D) The region with the best combination of process variables (GT; germination temperature, Gt; germination time) for production of optimised germinated moringa seeds.

Effect of optimal germination conditions on chemical composition and antinutritional components of moringa seeds

The proximate composition of unprocessed and germinated moringa seeds obtained under optimal conditions is presented in Table 2. The CPC in unprocessed moringa seeds (25.05 ± 0.50%) did not show a significant increase after dark germination processing (24.32 ± 0.87%). In the same way that occurred in other oilseeds (Dawood *et al*., 2013; Li *et al*., 2014), a significant reduction in lipid content was observed in moringa seeds by the germination process from 32.07 ± 0.04 to 17.39 ± 0.02%. In the germination process of seeds, lipids are used as a source of energy to start germination or for the synthesis of specific structural constituents in young seedlings (Dawood *et al*., 2013). The mobilisation of lipids with concomitant carbohydrate production was evidenced by a total carbohydrate increase from 41.23 ± 0.59 to 55.20 ± 0.94% (Table 2).

Table 2. Proximate composition of unprocessed and optimised germinated moringa seeds.

Component[1]	UM[2]	OGM[3]
Crude protein	25.05 ± 0.50[a]	24.32 ± 0.87[a]
Lipids	32.07 ± 0.04[a]	17.39 ± 0.02[b]
Ash	1.67 ± 0.05[b]	3.09 ± 0.05[a]
Carbohydrates	41.23 ± 0.59[b]	55.20 ± 0.94[a]

[1]g /100 g, dry weight sample; [2]Unprocessed moringa seeds; [3]Optimised germinated moringa seeds. Data are means of triplicate (n = 3) ± standard deviation. Means with different superscripts in the same row indicate significant differences ($p < 0.05$).

The effect of the optimised germination process on the antinutritional factors of moringa seeds is presented in Table 3. Germination processes significantly reduced the phytic acid content in optimised germinated moringa seeds when compared with unprocessed moringa seeds ($p < 0.05$). The reduction in phytic acid may be partially attributed to the leaching of phytate ions into soaking water during the soaking step performed before the germination process and may also have been affected by endogenous phytase, whose activity is increased during germination to promote the breakdown of phytic acid for its use as a source of inorganic phosphate (Azeke *et al*., 2011). In addition, the results clearly showed a reduction in the trypsin inhibitor activity from 2.87 ± 0.03 to 0.77± 0.01 TIU/mg (dw) as a consequence of the optimised germination process. The decrease in TIU could be due to the utilisation of a trypsin inhibitor as an energy source or degradation by hydrolytic enzymes during the process (Osman and Gassem, 2013). In contrast, germination of moringa significantly increased the tannin content from 62.58 ± 0.71 to 169.98 ± 6.98 mg CE/100 g (dw) ($p < 0.05$). Pushparaj and Urooj (2011) observed similar behaviour during sorghum germination as explained by the hydrolysis of condensed tannins such as proanthocyanidin.

The saponin content increased by 82% in optimised germinated moringa seeds when compared to unprocessed moringa seeds. Saponins are triterpenes stored in the hypocotyl of leguminous seeds as glycoside conjugates. During germination, the saponin complex is hydrolysed, allowing the increment of free saponins. These changes have also been reported in sprouted black beans, in which sprouting increased the saponin content by 41% as compared to raw black beans (Mendoza-Sánchez *et al*., 2016).

Table 3. Antinutritional factors of unprocessed and optimised germinated moringa seeds.

Component	Sample	
	UM[1]	OGM[2]
Tannins[3]	62.58 ± 0.71[b]	169.98 ± 6.98[a]
Phytic acid[4]	1,062.06 ± 5.81[a]	847.25 ± 9.80[b]
Saponins[5]	374.98 ± 30.27[b]	681.50 ± 10.89[a]
Trypsin inhibitors[6]	2.87 ± 0.03[a]	0.77 ± 0.01[b]

[1]Unprocessed moringa seeds; [2]optimised germinated moringa seeds; [3]mg catechin equivalents (CE)/100 g (dw); [4]mg phytic acid equivalents (PAE)/100 g (dw); [5]mg diosgenin equivalents (DE)/100 g (dw); [6]TIU/mg (dw). Data are means of triplicate (n = 3) ± standard deviation. Means with different superscripts in the same row indicate significant differences ($p < 0.05$).

Effect of optimal germination conditions on TPC and AoxA of moringa seeds

Moringa seed germination under optimal conditions resulted in a decrease in bound phenolics and an increase in free and total phenolic compounds (Table 4). Increments in free and total phenolics could be explained by the increase in free forms of phenolics due to the dismantling of the cell wall during sprouting (Pramai *et al*., 2017). However, these results differ from those obtained by De La Mora-López *et al*. (2018), who applied optimised germination conditions (temperature and time) under a light/dark regime in moringa seeds because in that study, not only was the increase in the free phenolics content observed, but also the germination process allowed the increment of bound phenolics.

When comparing the effect of dark germination on the AoxA of moringa seeds, a significant increase was observed ($p < 0.05$) (Table 4). The improvement in AoxA could be attributed to the higher accumulation of compounds with peroxyl-scavenging activity, such as TPC (Andriantsitohaina *et al*., 2012). However, in the present work, AoxA

evaluated by the ORAC assay did not show a strong linear correlation with TPC in germinated moringa seeds obtained under different conditions (r^2 = 0.399), suggesting the presence of other compounds with antioxidant activities in the extracts assayed for AxoA, such as saponins (positively affected by the germination process) and small molecular weight peptides generated during germination by proteolysis of storage proteins.

Table 4. Phenolic content and antioxidant capacity of unprocessed and optimised germinated moringa seeds.

Properties	Sample	
	UM[1]	OGM[2]
Phenolics[3]		
Free	38.74 ± 2.78[b]	158.22 ± 10.98[a]
Bound	170.90 ± 2.96[a]	113.45 ± 10.90[b]
Total	209.64[b]	271.67[a]
AoxA[4]		
Free	2,695.88 ± 53.16[b]	5,397.81 ± 149.22[a]
Bound	6,063.76 ± 309.86[a]	5,574.38 ± 514.27[a]
Total	8,759.64[b]	10,972.19[a]

[1]Unprocessed moringa seeds; [2]optimised germinated moringa seeds; [3]mg gallic acid equivalents (GAE)/100 g (dw); [4]µmol Trolox equivalents (TE)/100 g (dw). Data are means of triplicate (n = 3) ± standard deviation. Means with different superscripts in the same row indicate significant differences ($p < 0.05$).

Conclusions

The optimum combination of process variables for the production of germinated moringa seeds with higher values of CPC, TPC, and AoxA were 336 h of germination at 30°C. The germination process under optimal conditions also significantly reduced trypsin inhibitor activity and phytic acid levels but increased tannin and saponin content in moringa seeds. The optimised dark germination process of moringa resulted in an effective strategy to maintain CPC, increase TPC and AoxA, and decrease some antinutritional components. The optimised germinated moringa seeds could be used as a source of natural antioxidants and protein in the formulation of functional foods.

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