Vol. 17, No. 2 (2018) 547-560
Revista Mexicana de Ingeniería Química

EFFECT OF OPTIMAL GERMINATION CONDITIONS ON ANTIOXIDANT ACTIVITY, PHENOLIC CONTENT AND FATTY ACIDS AND AMINO ACIDS PROFILES OF *Moringa oleifera* SEEDS

EFECTO DEL PROCESO DE GERMINACIÓN EN CONDICIONES ÓPTIMAS SOBRE ACTIVIDAD ANTIOXIDANTE, CONTENIDO FENÓLICO Y PERFILES DE ÁCIDOS GRASOS Y AMINOACIDOS DE LAS SEMILLAS DE *Moringa oleifera*

G. Servín de la Mora-López[1], J. López-Cervantes[2], R. Gutiérrez-Dorado[1,3], E.O. Cuevas-Rodríguez[1,3], J. Milán-Carrillo[1,3], D.I. Sánchez-Machado[2], C. Reyes-Moreno[1,3]*

[1]*Programa Regional de Posgrado en Biotecnología, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa. Ciudad Universitaria, AP 1354, CP 80000, Culiacán de Rosales, Sinaloa, México.*

[2]*Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora. 5 de Febrero 818 Sur, CP 85000, Ciudad Obregón, Sonora, México.*

[3]*Pograma de Posgrado en Ciencia y Tecnología de Alimentos, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa. Ciudad Universitaria, AP 1354, CP 80000, Culiacán de Rosales, Sinaloa, México.*

Received November 4, 2017; Aceptado January 22, 2018

Abstract

The object of the present research was to study the effect of the optimal germination conditions on antioxidant activity, phenolic content, and fatty acids and amino acids profiles of *Moringa oleifera* seeds. Response surface methodology was applied to identify the optimal germination conditions of moringa seeds that would result in a functional flour with maximum values of protein content (PC), antioxidant activity (AoxA) and total phenolic content (TPC), and a minimum lipid content (LC) of 20%. A central composite rotatable (CCR) experimental design with two factors [Germination temperature (GT, 25-40°C)/Germination time (Gt, 25-360 h)] in 5 levels (13 treatments) was used. The prediction models developed for each response variable showed high coefficients of determination, demonstrating their adequacy to explain the variations in experimental data. The germination bioprocess at optimal conditions increased PC, AoxA, and TPC, polyunsaturated fatty acids, and amino acids content, and decreased LC concerning the entire seed. Therefore, the germination under optimized conditions is an effective strategy to improve the nutritional, antioxidant, and functional value of *Moringa oleifera* seeds.

Keywords: moringa seeds, antioxidant activity, germination, optimization.

Resumen

El objetivo de la presente investigación fue estudiar el efecto de las condiciones óptimas de germinación sobre la actividad antioxidante, el contenido fenólico y los perfiles de ácidos grasos y aminoácidos de las semillas de *Moringa oleifera*. La metodología de superficie de respuesta se aplicó para identificar las condiciones óptimas de germinación de las semillas de moringa que dieran como resultado una harina funcional con valores máximos de contenido proteico (CP), actividad antioxidante (AAox) y contenido de compuestos fenólicos totales (CFT) y un contenido mínimo de lípidos (CL) de 20%. Se utilizó un diseño experimental compuesto central rotable (CCR) con dos factores [temperatura de germinación (TG, 25-40 °C) / tiempo de germinación (tG, 25-360 h)] y 5 niveles (13 tratamientos). Los modelos de predicción desarrollados para cada variable de respuesta mostraron altos coeficientes de determinación, demostrando su adecuación para explicar las variaciones en los datos experimentales. El bioproceso de germinación en condiciones óptimas aumentó CP, AAox y CFT, ácidos grasos poliinsaturados, y contenido de aminoácidos, y disminuyó CL en relación con la semilla completa. Por lo tanto, la germinación bajo condiciones optimizadas es una estrategia eficaz para mejorar el valor nutricional, antioxidante y funcional de semillas de *Moringa oleifera*.

Palabras clave: semillas de moringa, actividad antioxidante, germinación, optimización.

* *Corresponding author. E-mail*: creyes@uas.edu.mx

doi: 10.24275/10.24275/uam/izt/dcbi/revmexingquim/2018v17n2/Servin
issn-e: 2395-8472

1 Introduction

Moringa oleifera (Moringaceae family) is a small tree used in folk medicine in tropical Africa, Asia, and America. It is an important food commodity, which all plant parts (seeds, leaves, roots, flowers, and immature pods) possessing relevant nutritive and nutraceutical features that make them suitable for various therapeutic applications against gastrointestinal infectious, inflammatory, and cardiovascular diseases (Sánchez-Machado *et al*., 2010). The seeds of *M. oleifera* have valuable nutrients for the human diet. They contain about 42% of oil, which is rich in highly unsaturated fatty acid (75.8-82.9%), with oleic acid as the major component (71.2-79.5%) (Ogunsina *et al*., 2014). The second highest nutriment of the seeds are the proteins (about 30-40%), which has a right balance of essential amino acids, so that it can supplement cereal and tuber flours, which has low levels of protein and a deficient profile of essential amino acids (Ogunsina *et al*., 2010). Additionally, the seeds contain vitamins, such as provitamin A, B-complex, and C, and essential minerals as macro-elements such as sodium, potassium, calcium, phosphorus and magnesium, and micro-elements such as iron, copper, manganese, and zinc (Barakat and Ghazal, 2016). Many of the biological activities, as well as folk medicinal uses of *Moringa oleifera* are attributed to the presence of phenolic compounds (Saini *et al*., 2016). Phenolics are beneficial due to their ability to reduce the low-density lipoprotein (LDL) aggregation and for protecting cell membranes against the damage induced by reactive free radicals; phenolics act as antioxidant, anti-cancer, and antidiabetic agents (Fernandez-Orozco *et al*., 2009).

Due to the high demand for functional foods, moringa seeds have gained full recognition due to their health benefits. Germination is considered a simple and economic bioprocess to improve the nutritive value of grains by causing desirable changes in the nutrient availability, nutritive value, sensorial characteristics, texture, antioxidant, and nutraceutical properties (Perales-Sanchez *et al*., 2014).

During the germination bioprocess, a considerable breakdown of seed-storage compounds, and synthesis of cell wall components, structural proteins, vitamins, and secondary compounds take place; many of these compounds are considered beneficial as antioxidants and may change dramatically during the germination process (Kuo *et al*., 2004). This physiological process improves nutritional quality by increasing the levels of free amino acids, available carbohydrates, dietary fiber, and other components. The process has the added benefit of enhancing the functionality of the seeds due to the subsequent increase in the bioactive compounds and related antioxidant activity (Paucar-Menacho *et al*., 2017; Gómez-Favela *et al*., 2017).

The object of the present research was to identify the optimal germination conditions of *Moringa oleifera* seeds that would result in a functional flour with maximum values of protein content (PC), antioxidant activity (AoxA) and total phenolic content (TPC), and a minimum lipid content (CL) of 20%, as well as, study the effect of the optimal germination conditions on antioxidant activity, phenolic content, and fatty acids and amino acids profiles of the moringa seeds.

2 Materials and methods

2.1 Materials

The moringa seeds were provided by a regional supplier from Cd. Obregón, Sonora, México. The seeds were cleaned and stored in tightly sealed containers at 4°C until use.

2.2 Production of germinated moringa seed flour (GMSF)

A portion of 70 g of moringa seeds was disinfected in 1,000 mL of 0.1 % sodium hypochlorite for 10 min. Then, these seeds were washed with distilled water. During the germination process the soaking of the seeds was not carried out. Preliminary studies were made and it was observed that seeds soaked and not soaked had a similar growth at the end of germination. The hydrated seeds were placed in germination trays on wet laboratory paper. The trays were introduced to the germination chamber with temperature control. A relative humidity of 80 to 90 % was maintained within the chamber using trays of water. The bioprocess was achieved by applying combinations of germination temperature/time (GT/Gt) in the intervals of 25 to 40ºC and 24 to 360 h, respectively (Table 1) (13 treatments). In all cases, the seeds were germinated under light/darkness in periods of 50/50% of the germination time daily [light source: fluorescent tubes (light white, 16 W/2,700 K, Tecno Lite, China)]. The resulting bioprocessed moringa seeds were dried

(50°C/8h), tempered (25°C), and ground (80-US mesh=0.180 mm) to obtain germinated moringa seeds flour (GMSF). GMSF was packed and kept at 4ºC in tightly sealed containers until further analysis.

2.3 Antioxidant activity (AoxA)

Antioxidant activity for hydrophilic antioxidant capacities of free and bound phenolic compounds extracts was evaluated applying the ABTS radical cation decolorization assay, and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay using a Microplate Reader (SynergyTM HT Multi-Detection, BioTek, Inc., Winooski, VT, USA). For the ABTS assay, the extracts were evaluated against a standard of Trolox (Re *et al*., 1999). For the DPPH assay, the extracts were also evaluated against a standard of Trolox (Kim., 2002). The results of both assays were expressed as μmol of Trolox equivalents (TE)/100 g of dry weight (dw) sample. All measurements were made in triplicate.

2.3.1 Extraction of free and bound phenolic compounds

Free and bound phenolic compounds were extracted according Perales-Sánchez *et al*. (2014), employing as solvents 80 % chilled ethanol and ethyl acetate, respectively. All extractions were made in triplicate.

2.4 Total phenolic content (TPC)

The phenolic content present in free and bound extracts from ground samples was evaluated using the colorimetric method of Singleton *et al*. (1999). The absorbance was measured using a Microplate Reader (SynergyTM HT Multi-Detection, BioTek Inc., and Winooski, VT, USA). The TPC was expressed as milligrams of gallic acid equivalents (mg GAE)/100 g dw sample. All measurements were made in triplicate.

2.5 Proximate composition

The following methods of the Association of Official Analytical Chemists (AOAC, 2012) to evaluate proximate composition were used: Moisture (method 925.09B): drying at 130°C; lipids (method 920.39 C): defatting in a Soxhlet apparatus with petroleum ether; protein (method 960.52): micro-Kjeldahl (Nx6.25).

2.6 Soluble and insoluble dietary fiber (SDF/IDF)

The soluble and insoluble dietary fiber (SDF, IDF) were measured according to the enzymatic-gravimetric method (official method 985.29) for total dietary fiber (AOAC, 2012), using the total dietary fiber assay kit from Sigma-Aldrich (TDF 100 A).

2.7 Amino acid content (AAC)

The amino acid content of moringa seed flour samples was determinate using the method reported by Sánchez-Machado *et al*. (2003) with some modifications. In this work, the employed quantities of sample, the HPLC equipment, the column, and the gradient of the mobile phase were different to the originally used by Sánchez-Machado *et al*. (2003). The quantity of sample used to raw seed and germinated seeds were 500 and 300 mg, respectively. Amino acid analysis was done by Equipment HPLC (GBC, Dandenong, Australia), equipped with an auto-injector 1650 LC GBC, a thermostat for column LC 1150, a sensor with an LC 5100 diode array, and WinChrom software. Zorbax ODS C18 column (250 mm x 4.6 mm i.d.) with a particle size of 5 μm was used. The analysis was carried out at 29 °C, and the amount of sample injected was 20 μL. The mobile phase was a gradient prepared from solutions A and B. Solution A was sodium acetate buffer 0.14 M containing 0.05% (v/v) TEA (pH-adjusted to 6.4 with glacial acetic acid). Solution B was 60:40 (v/v) acetonitrile-water. The elution gradient (min: A%) was: 0:90, 12:70, 16:70, 22:54, 37:0, 38:0, 40:90, and 46:90. The flow rate was 0.9 mL min-1, and the detection wavelength was 254 nm.

2.8 Fatty acid content (FAC)

The fatty acid profile was evaluated by gas chromatography according Sánchez-Machado *et al*. (2015) with some modifications. In this work, the quantities of sample and solvent employed, and the operating conditions of the chromatograph were different to the originally used by Sánchez-Machado *et al*. (2015). The quantities of sample used were 0.6 and 0.8 g to raw seeds and germinated seeds, respectively. Also, 3 mL of toluene, 4 mL 5 vol% methanolic/HCl, and 5 mL of 6% K2CO3 solution were employed. The gas chromatograph (VARIAN 3800; Melbourne, Victoria, Austria) was equipped with a capillary column and flame ionization detector

CP-Sil 88 (60 m × 0.25 mm, VARIAN) was used. The operating conditions were: injector temperature: 270°C; carrier gas: helium; detector temperature: 300°C. The gradient of temperature in the oven of the column will start at 50°C/min increased by increments of 8°C/min up to 220°C, with subsequent increases of 4°C/min to 240°C and a dwell time of 5 minutes. Peak identifications were based on comparing the retention times with those of known standards obtained from Sigma (St. Louis, MI, USA). The areas of the peaks were quantified using the software Galaxie Workstation (Varian Inc., Palo Alto, CA, USA). The relative amount of each fatty acid (% of a fatty acid of the total fatty acids) was evaluated by integrating the area under the peak and dividing the result by the total area for all fatty acids.

2.9 Response surface methodology (RSM), experimental design, statistical analysis, and optimization

A central composite rotatable experimental design was chosen for RSM, with two factors [Germination temperature (GT), germination time (Gt)] and five variation levels (Table 1). The stepwise regression procedure was applied. The terms that were not significant ($p > 0.1$) were deleted from a second order polynomial, and a new polynomial was used to obtain a predictive model for each response variable. The conventional graphical method was applied to obtain maximum values of protein content (PC), antioxidant activity (AoxA) and total phenolic content (TPC), and a minimum lipid content (CL) of 20%. Predictive models were used to graphically represent the system. Contour plots of each of the response variables were superimposed to obtain a contour plot for observation and selection of the best combination GT/Gt for producing optimized germinated moringa seed flour (OGMSF). The statistical software Design Expert version 7.0.0 (Stat-Ease, Minneapolis, MN, USA) was used for the RSM analyses. Results of the chemical composition, antioxidant activity, and total phenolics content of OGMSF were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with a 5% significance level. Unprocessed moringa seed flour (UMSF) was used as a reference.

Table 1. Experimental design[a] used to obtain different combinations of germination temperature/germination time for producing germinated *Moringa oleifera* seed flour, and experimental results for response variables.

Process variables			**Response variables**			
Assay[b]	Germination temperature (°C)	Germination time (h)	Protein content[c]	Antioxidant activity[d]	Total phenolic content[e]	Lipid content[f]
1	27.2	73.2	25.99	7,608	523	30.64
2	37.8	73.2	33.26	10,388	339	26.55
3	27.2	310.8	41.23	18,963	688	25.7
4	37.8	310.8	41.29	15,727	685	18.16
5	25	192	32.93	11,573	516	30.98
6	40	192	40.99	14,969	581	22.73
7	32.5	24	23.8	9,103	392	32.72
8	32.5	360	41.54	21,114	742	14.89
9	32.5	192	28.87	16,196	623	19.49
10	32.5	192	35.86	16,723	552	19.5
11	32.5	192	35.71	16,006	572	18.13
12	32.5	192	30.37	16,930	532	22.5
13	32.5	192	33.26	15,600	500	21.15

[a]Central composite rotatable design with 2 factors and 5 levels; 13 assays
[b]Does not correspond to order of processing, [c]% dw, [d] μmol Trolox equivalents (TE)/100 g (dw), [e] mg gallic acid equivalents (GAE)/100 g sample (dw), [f] % dw

Table 2. Germinated moringa seeds flours. Analyses of variance and regression coefficients of the second-order polynomial models showing the relationships between response variables (Y_k) and coded process variables (X)

Coefficient	Protein content (Y_{PC})	Antioxidant activity (Y_{AoxA})	Total phenolic content (Y_{TPC})	Lipid content (Y_{LC})
Intercept				
β_0	32.87	16291.05	557.38	20.16
		Lineal		
β_1	2.34**	543.35 NS	-11.77NS	-2.91***
β_2	6.04***	4210.16***	125.69***	-4.82***
Quadratic				
β_{11}	2.22**	-1764.62***	NS	3.33***
β_{22}	NS	-845.77*	NS	1.81**
Interactions				
β_{12}	-1.80NS	-1503.89**	45.14*	NS
P	0.0005	<0.0001	0.0002	0.0002
R^2	0.8998	0.9636	0.8794	0.9147

* Significant at P≤0.10 level, ** Significant at P≤0.05 level
*** Significant at P≤0.01 level; ^{NS} Not significant (P > 0.10 level)

3 Results and discussion

3.1 Optimal conditions for moringa seeds germination

3.1.1 Prediction models for protein content (PC), antioxidant activity (AoxA), and total phenolic and lipids contents (TPC, LC) of germinated moringa seed flours (GMSF)

The PC, AoxA, TPC, and LC experimental values of the GMSF varied from 25.99 to 41.54% (dw), 7,608 to 21,114 μmol TE/100 g sample (dw), 339 to 742 mg GAE/100 g sample (dw), and 14.89 to 32.72 % (dw), respectively (Table 1). Analyses of variance and regression coefficients of the prediction second order polynomial models showing the relationships among responses (PC, AoxA, TPC, and LC) and process variables are shown in Table 2. Predictive models using coded variables for the response variables were:

$$PC = 32.87 + 2.34(GT) + 6.04(Gt) - 1.80(GT)(tG) + 2.22(GT)^2$$

$$AoxA = 16291.05 + 543.35(GT) + 4210.16(Gt) - 1503.89(GT)(Gt) - 1764.62(GT)^2 - 845.77(Gt)^2$$

$$TPC = 557.38 - 11.77(GT) + 125.69(Gt) + 45.14(GT)(Gt)$$

$$LC = 20.16 - 2.91(GT) - 4.82(Gt) + 3.33(GT)^2 + 1.81(Gt)^2$$

The regression models explained 89.98, 96.36, 87.94, and 91.47% of the total variability in PC (p= 0.0005), AoxA (p< 0.0001), TPC (p = 0.0002) and LC (p = 0.0002), respectively (Table 2). The lack of fit was not significant (p>0.05), and the relative dispersion of the experimental points from the predictions of the models (CV) was found to be < 10%. These values indicated that the experimental models were adequate and reproducible. In general, PC, AoxA, and TPC of GMSF increased with Gt until reaching maximum values at 360 h. While LC decreased with GT and Gt, with a maximum value observed at 25°C and 24 h (Fig. 1).

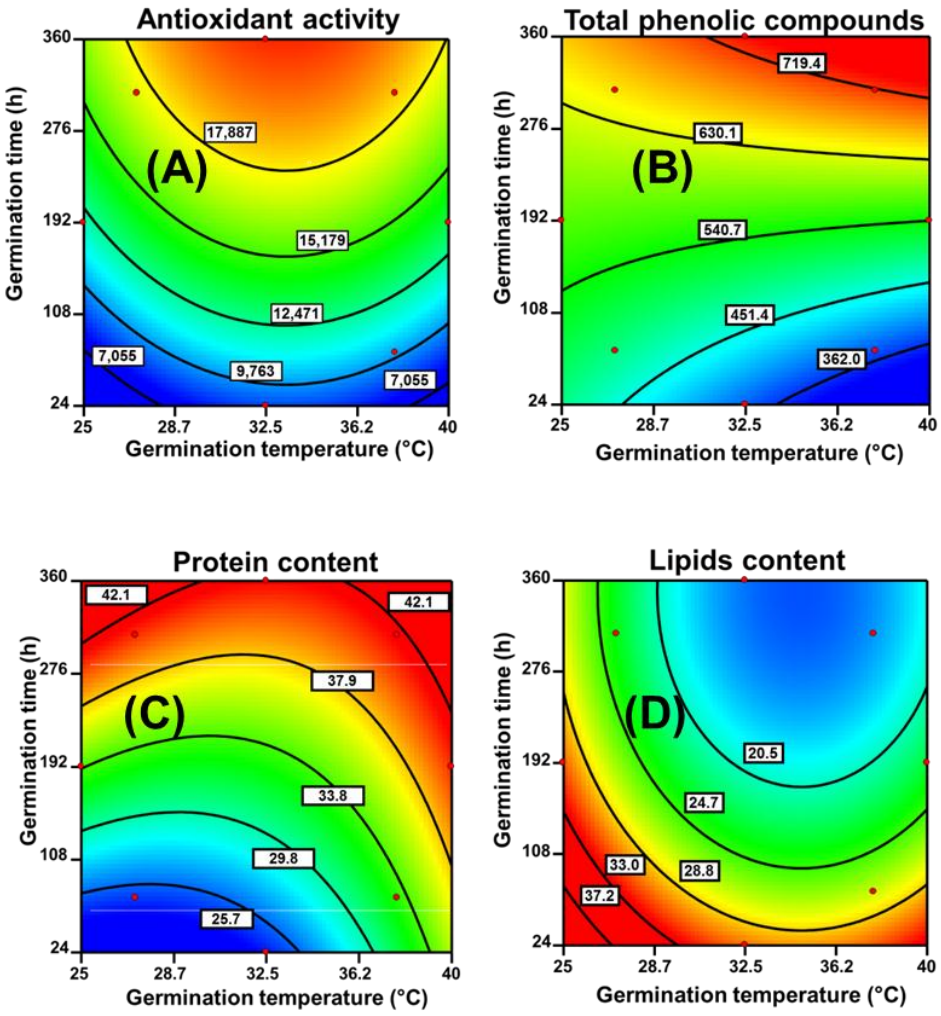

Fig. 1 Contour plots showing the effect of germination temperature and time on (A) antioxidant activity, (B) total phenolic content, (C) protein content, and (D) lipids content.

3.1.2 Optimization of the moringa seeds germination bioprocess

Fig. 2, corresponding to the superimposition contour plot, was used to determine the best combination of process variables for producing OGMSF. The central point of the optimization region in Fig. 2 corresponds to the optimum combination of bioprocess variables (GT= 32.92°C, Gt= 182 h) for OGMSF production with the highest PC, AoxA, and TPC values and the lowest LC value but not less than 20%. The predicted values of PC, AoxA, TPC, and LC, using the predictive models of each response variable and the optimal conditions of bioprocessing, were 33% (dw), 15,982 μmol TE/100 sample (dw), 546 mg GAE/100 sample (dw), and 20%, respectively. OGMSF was produced by applying the best combination of germination bioprocess variables; the experiment using optimal conditions was replicated three times. The experimental values of PC, AoxA, TPC, and LC of OGMSF (Tables 3 and 4) were similar to the predicted values mentioned above, indicating that the optimal conditions of germination bioprocess are appropriate and reproducible.

Table 3. Proximate composition and fatty acid profile in moringa seed flour

Property	Unprocessed moringa seed flour (UMSF)	Optimized germinated moringa seed flour (OGMSF)
Chemical composition (%, dw)		
Proteins	15.78±1.03^b	25.89±0.51^a
Lipids	39.46±2.19^a	21.38±1.43^b
Dietary fiber		
Soluble fiber	0.65±0.07^a	0.26±0.08^b
Insoluble fiber	3.79±0.02^b	4.50±0.5^a
Total fiber	4.44±0.22^b	4.76±0.30^a
Minerals	3.38±0.01^a	3.55±0.11^a
Carbohydrates	37.18±2.64^b	44.69±1.02^a
Fatty acids (% total fatty acids)		
Saturated		
Tetradecanoic acid (C14:0) - Myristic	0.22±0.22^a	0.17±0.08^a
Hexadecanoic acid (C16:0)-Palmitic	8.77±0.64^a	7.52±0.12^b
Octadecanoic acid (C18:0)-Stearic	5.28±0.08^a	4.69±0.04^b
Eicosanoic acid (C20:0)-Arachidonic	0.09±0.04^b	0.30±0.01^a
Docosanoic acid (C22:0)-Behenic	2.56±0.21^b	2.95±0.12^a
Total	16.94±0.25^a	15.66±0.06^a
Monounsaturated		
cis-9-Hexadecenoic acid (C16:1ω7) - Palmitoleic	2.24±0.16^a	1.73±0.04^b
cis-9-Octadecenoic acid (C18:1ω9) - Oleic	76.79±1.29^a	76.62±0.08^a
cis-11-Eicosenoic acid (C20:1ω9) - Eicosenoic	2.28±0.12^a	2.27±0.02^a
Total	81.39±0.41^a	80.63±0.03^a
Polyunsaturated		
9,12-Octadecadienoic acid (C18:2ω6) - Linoleic	0.73±0.04^b	2.08±0.24^a
9,12,15-Octadecatrienoic acid (C18:3ω3) - α Linolenic	1.39±0.03^b	1.60±0.07^a
Total	2.13±0.01^b	3.69±0.07^a

Data are expressed as means ± standard deviation

Means with different superscripts (a-b) in the same row are different (Duncan, p ≤0.05)

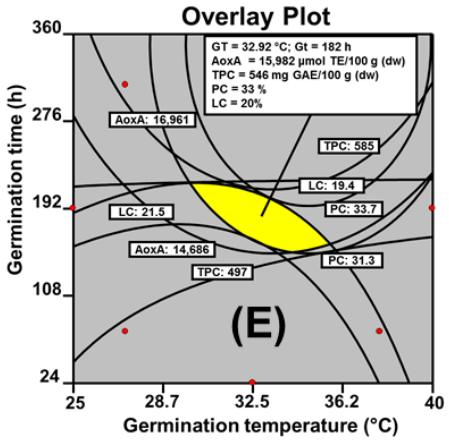

Fig. 2 Region of the best combination of process variables (GT=Germination temperature, Gt = Germination time) for production of optimized germinated moringa seeds flour (OGMSF).

3.2 Effect of optimal germination conditions on chemical composition and fatty acid profile of moringa seeds

Raw moringa seeds have 16% (dw) of protein. The protein content of germinated moringa seeds after 182 h at 32.92 °C trended toward a significant increase (64%) compared to that in the raw seeds (Table 3). The increase in the protein content of optimized germinated moringa seeds flour (OGMSF) compared to the protein content in the unprocessed moringa seeds flour (UMSF) can be explained mainly by the loss of lipids during the germination process of moringa seeds. The lipid content decreased from 39.46 in UMSF to 21.38% in OGMSF (46% decrease) (Table 3), which means a loss of approximately 18 grams of this chemical component per each 100 g of moringa seeds on a dry basis. The protein content during germination increased from 15.78%

in UMSF to 25.89% in OGMSF (Table 3) which represents an increase of approximately 10 grams of protein per each 100 g of moringa seeds on a dry basis. In literature there are several reports about important losses of lipids during germination of seeds, mainly those seeds that have a high content of lipids (Herchi *et al*., 2015; Gómez-Favela *et al*., 2017). The resting amount of lipids lost during germination (approximately 8%), which was not occasioned by the increase in the protein content, can be explained mainly by the increase in the carbohydrate content (approximately 7.5 grams per each 100 grams of moringa seeds on a dry basis) during germination of moringa seeds. The metabolic activity that occur during the germination of seeds demand energy production to the synthesis of biological molecules such as DNA, RNA, enzymes, structural proteins, among other, and which can be supply by the consumption of lipids and/or carbohydrates (Perales-Sánchez *et al*., 2014; Gómez-Favela *et al*. 2017). In the present research work, based on the results obtained, it seems that the energy requirements during the germination of moringa seeds could have been provided mainly by lipids instead of carbohydrates, similar to what happened in other research works, where were used seeds with high lipid content (oilseeds) for the germination process, as was mentioned above. The utilization of lipids as energy sources in this type of seeds is basically for start the germination process or synthesis of certain structural constituents in the young seedling. Storage lipids breakdown plays an important role in the life cycle of many plants by providing the carbon skeleton that supports growth immediately followed germination. This metabolic process is initiated by lipases which catalyze the hydrolysis of triacylglycerols to release glycerols and free fatty acids, some of which are oxidized into acetyl-CoA, and then transformed into simple carbohydrates that are transferred to the embryo as saccharose. Also, a part of fatty acids and glycerol produced by lipases, are metabolized in glyoxysomes and other organelles. It is well known that germinating seedlings of many oilseeds are a rich source of lipases (triacylglycerol acyl hydrolases) (Dawood *et al*., 2013).

The soluble dietary fiber decreased by 69% during germination by 32.92 h at 182°C in the present study, while insoluble and total dietary fiber contents increased by 25% and 12%, respectively (Table 3). Martín-Cabrejas *et al*. (2003) found that the total dietary fiber increased by nearly 100% during the germination of peas. In other studies, a significantly increases in soluble, insoluble and total dietary fiber concentration has been reported in several germinated seeds (kidney, mung, soybeans, peanuts) (Megarat *et al*., 2016). This increase in dietary fiber has been reported to be mostly due to an increase in the cellular structure of the plant during germination, which occur by production of hemicellulose, cellulose, and pectic polysaccharides during the development of sprouts and seedlings. Also, the structure of cell wall polysaccharides of the seeds is modified during germination process affecting the fiber content and the soluble/insoluble fiber ratio; this probably occur because the intactness of tissue histology is affected and the protein carbohydrate interaction is disrupted by the germination process (Martín-Cabrejas *et al*., 2003, Gómez-Favela *et al*., 2017). Dietary fiber is an important component of a healthy diet because it greatly influences gut physiology. There is evidence that dietary fiber contributes to lowering serum LDL cholesterol and blood pressure, and protects against diseases, such as atherosclerosis or cardiovascular disease. Additionally, many studies have demonstrated a beneficial effect of dietary fiber in the prevention of chronic diseases and in reducing the incidence of several types of cancer, such as colorectal, prostate, and breast cancer (Zhu *et al*., 2015).

The composition of fatty acids presents in UMSF and OGMSF are shown in Table 3. Ten types of fatty acids were found in both raw and germinated moringa seeds, inside of which 5 are saturated fatty acids: C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C20:0 (arachidonic acid), and C22:0 (behenic acid); 3 are monounsaturated fatty acids: C16:1ω7 (palmitoleic acid), C18:1ω9 (oleic acid) and C20:1ω9 (eicosenoic acid), and 2 corresponded to the group of polyunsaturated acids: C18:2ω6 (linoleic acid) and C18:3ω3 (α-linolenic acid). Fatty acids are the integral constituents of every fat or oil. The degree of complexity of the glycerides depends on the number of fatty acids and their amounts. Fatty acids play a major role in the seed because they provide a source of energy to the germinating seedling, especially in early development (Dawood *et al*., 2013). The monounsaturated fatty acids fraction represented the highest proportion (80.63 - 81.39%) of the fatty acids total content in both flour types (UMSF and OGMSF), followed by the saturated fatty acids fraction (15.66 - 16.94%); polyunsaturated fatty acids content presented the lowest proportion (2.13% - 3.69%) in these samples. Only, the polyunsaturated fatty acids total content of moringa seeds was affected ($p < 0.05$) by the germination process, which increased

about 14.2%. The other fatty acids fractions were not affected significantly by this bioprocess (Table 3). The palmitic and stearic acids were the most abundant in the saturated fatty acids fraction of both samples (7.52 - 8.77% palmitic acid and 4.69 - 5.28% stearic acid for OGMSF and UMSF, respectively), which decreased 14.2 and 11.2%, respectively, after the germination process. The oleic acid was the most abundant fatty acid (about 77% of the total fatty acids) in the two flour types, and its content was not significantly affected by germination. The content of the polyunsaturated fatty acid ω6 (linoleic acid) represented 0.73 - 2.08% in OGMSF and UMSF, respectively, while the content of ω3 (α-linolenic acid) represented 1.39 - 1.60% in these samples; both polyunsaturated fatty acids increase (184.9% ω6 and 15.1% ω3) after germination, but the content of ω6 was the most affected by this process. Fatty acid metabolism during germination varies among different plant species and even changes for the same species. The changes in fatty acids composition of moringa seeds after germination could be attributed to the oxidation of fatty acids (Dawood *et al*., 2013). The corresponding result of oleic acid (C18:1ω9) content found in this study coincides with the results reported by some authors (Ogunsina *et al*., 2014; Sánchez-Machado *et al*., 2015). However, this does not coincide with the study realized by Ijarotimi *et al*. (2013), who reported that the most abundant fatty acid found in raw and germinated moringa seeds was linoleic acid (58.69% for raw seeds and 60.70% for germinated seeds), whereas oleic acid was the one with less abundance in both samples (13.18% and 10.25% for raw seeds and germinated seeds, respectively). These variations may be due to the type of climate and geological conditions of the respective regions (Anwar *et al*., 2003). Based on these results, the content of fatty acids present in *M. oleifera* seeds seems to be very important for human nutrition, since the consumption of polyunsaturated fatty acids (PUFA) n-3 are considered essential because the enzymes present in the human body and animals cannot introduce double ligatures in positions prior to carbon nine, counting from the terminal methyl group, therefore only you can get them through diet. The biological importance of PUFAs n-3 is that they are part of the cell membranes and are involved in the processes of brain and retinal formation in fetuses; also in the production of eicosanoids, which are regulators of inflammation, fever, thrombosis and dilation. In addition, PUFA n-3 consumption has been linked to the prevention of coronary, neuromuscular, immunological, allergic and cancer diseases, as well as can be adjusted to accommodate low-density cholesterol and total cholesterol (Aly *et al*., 2016; Correa-Leyva *et al*., 2017). A diet rich in monounsaturated fatty acids helps in the prevention of atherosclerosis and has a beneficial effect on the plasma concentrations of low-density-cholesterol (LDL) and high-density-cholesterol (HDL) (Jansen *et al*., 2000).

3.3 Effect of optimal germination conditions on amino acid profile, antioxidant activity, and phenolic content of moringa seeds

Table 4 shows the amino acid profile for unprocessed and optimized germinated moringa seed flour (UMSF, OGMSF). These results indicate that during germination, certain amino acids, such as aspartic acid, glycine, lysine, phenylalanine, and tyrosine increased significantly (+ 120-166%), while arginine, alanine, and methionine had an increase between 92 and 99%. The most abundant amino acid was arginine, with 9.35 g/100 g proteins (dw) in UMSF and 16.71 g/100 g proteins (dw) in OGMSF, while aspartic acid was found to a lesser extent, with 0.45 and 1.10 g/100 g proteins (dw) in UMSF and OGMSF, respectively (Table 4). The germination process increased the content of amino acids with respect to the entire seed. These results are consistent with those reported by Ijarotimi *et al*. (2013), in which germinated moringa seeds showed increased amino acids during this process, and glutamic acid was found to be the most prevalent amino acid. Ochanda *et al*. (2010), also reported that the germination process is a technique that increases the protein content and amino acid profile. This increase can be attributed to the fact that during the process of germination, lipases increase, causing hydrolysis of lipids, which are used as a source of energy for protein synthesis during the development of the plant, increasing the content of free amino acids (Botero *et al*., 2012). Also, this changes in the amino acids profile after germination of moringa seeds can be due to that storage proteins were broken down by proteases (endo- and exo-proteases) which are present in determinate regions of dry seeds or were synthetized in the seeds during germination process. Proteolytic enzymes play central role in the biochemical mechanism of germination.

Table 4. Amino acid profile, antioxidant activity, and phenolic content in moringa seed flour

Property	Unprocessed moringa seed flour (UMSF)	Optimized germinated moringa seed flour (OGMSF)
Amino acid *(g/100 g proteins)*		
Alanine	2.64 ± 0.52^b	5.27 ± 0.83^a
Arginine	9.35 ± 1.22^b	16.71 ± 2.45^a
Aspartic acid	0.45 ± 0.16^b	1.10 ± 0.39^a
Glutamic acid	4.46 ± 1.08^b	7.74 ± 1.41^a
Glycine	2.49 ± 0.72^b	6.62 ± 1.01^a
Histidine	2.75 ± 0.23^b	3.94 ± 0.46^a
Isoleucine	1.72 ± 0.26^b	2.88 ± 0.62^a
Leucine	1.72 ± 0.21^b	2.73 ± 0.54^a
Lysine	0.83 ± 0.21^b	1.89 ± 0.40^a
Methionine	1.11 ± 0.44^b	2.13 ± 0.29^a
Phenylalanine	1.98 ± 0.56^b	4.55 ± 0.63^a
Proline	6.55 ± 0.69^b	9.83 ± 0.19^a
Serine	1.96 ± 0.52^b	3.61 ± 0.72^a
Threonine	3.26 ± 1.11^b	5.28 ± 1.09^a
Tyrosine	1.22 ± 0.43^b	2.68 ± 0.47^a
Valine	1.66 ± 0.32^b	2.88 ± 0.45^a
Total	44.15 ± 1.56^b	79.84 ± 1.26^a
Antioxidant activity DPPH		
Free phytochemicals	$1{,}920 \pm 164^b$	$5{,}886 \pm 56^a$
Bound phytochemicals	$2{,}949 \pm 49^b$	$3{,}986 \pm 42^a$
Total phytochemicals	$4{,}869 \pm 116^b$	$9{,}872 \pm 92^a$
Antioxidant activity ABTS		
Free phytochemicals	$6{,}146 \pm 519^b$	$9{,}854 \pm 423^a$
Bound phytochemicals	$5{,}509 \pm 265^b$	$7{,}142 \pm 436^a$
Total phytochemicals	$11{,}655 \pm 782^b$	$16{,}996 \pm 611^a$
Phenolic content		
Free phenolics	232 ± 10^b	260 ± 12^a
Bound phenolics	263 ± 15^b	299 ± 13^a
Total phenolics	495 ± 12^b	559 ± 15^a

Means with different superscripts in the same row are significantly different (Duncan, $p \leq 0.05$)
Antioxidant activity: μmol Trolox equivalents (TE)/100 g (dw)
Phenolic content: mg gallic acid equivalents (GAE)/100 g sample (dw)

During germination period, the storage proteins are degraded by a variety of proteases which convert the insoluble storage proteins in to soluble peptides and these peptides are further hydrolyzed to free amino acids. These free amino acids are mobilized to the embryonic axis to support its growth and also to provide energy (Ramakrishna and Ramakrishna, 2006). The proteases have been classified into four major groups based on their active site catalytic mechanisms: serine proteases, sulfyhydryl proteases, metalloproteases and add proteases. Within this broad generalized classification, the enzymes can be more specifically classified with regard to their substrate specificities, i.e. endopeptidases, carboxypeptidases, or aminopeptidases, depending upon whether they attack internal peptide bonds of polypeptides (endo-proteases) or cleave single amino acid residues from the terminal ends (exo-proteases). In germinating seeds the endopeptidases and carboxypeptidases are apparently involved primarily with breaking down reserve proteins. However, the variations in levels of proteolytic activities in seeds from different

genera during germination suggest that there may be more than one way for the seeds to regulate proteolysis during the breakdown of storage proteins, although the overall proteolytic processes may be quite similar. Some seeds respond to the initiation of germination by producing large amounts of protease or protease activity, whereas other seeds apparently have considerable protease activity already present at the onset of germination (Ryan, 1973).

In general, AoxA of moringa seeds increased after bioprocessing at optimal germination conditions (32.92°C/182 h) (Table 4). The AoxA, evaluated by ABTS assay, for free (+60%), bound (+30%), and total (+46%) phytochemicals increased after germination (Table 4). Both DPPH and ABTS methods showed similar tendencies. Singh *et al.* (2014), reported that germination increased the antioxidant activity of legumes. The increase in AoxA with the germination bioprocess is one of the many metabolic changes that take place upon germination of seeds, mainly due to an increase in the content of phenolic compounds by the action of the endogenous hydrolytic enzymes. The increase in phenolic content and antioxidant activity in legume seeds shows a potentially important role of phenolic during seed germination, as well as the potential enhancement of the nutraceutical value of seeds by the germination process (Cevallos-Casals *et al.*, 2010).

The free, bound, and total phenolic contents in UMSF were 232, 263, and 495 mg GAE/100 g sample (dw), respectively (Table 4). The amount of TPC is lower than those reported by Ilyas *et al.* (2015) in moringa seed powder. The variations in the results could be due to the procedures applied for polyphenolic compounds extraction, the degree of polarity of the solvents, and geographical locations of the plants. As shown in Table 4, the germination bioprocess increased ($p < 0.05$) free (+28%), bound (+36%), and total (+64%) phenolic contents. Khang *et al.* (2016), investigated the phenolic profiles and antioxidant activity of germinated legumes. These researchers reported that the phenolic concentration dramatically increased ($p < 0.05$) in all legumes (black beans, mung beans, peanuts, adzuki beans, soybeans, and white cowpeas) after five days of germination at 30°C. The germination is an effective bioprocess for increasing the concentration of phenolic compounds in seeds. This phenomenon may be due to the release and biosynthesis of phenolic compounds. The phenolic compounds, such as hydroxycinnamates (e.g., p-coumaric and ferulic acids), are bound to nonstarch polysaccharides in grain cell walls through associations, such as ester and ether bonds. The action of cell wall-degrading enzymes (mainly esterase) on these bonds contributes to the release of bound phenolic compounds (Gómez-Favela *et al.*, 2017). On the other hand, during germination of seeds the metabolic activity of the plant cell is restarted showing biosynthesis of secondary metabolites as a response to oxidative stress. The seeds in germination produce reactive oxygen species (ROS), which are neutralized by phenolic compounds, that also are synthetized during the germination process, because ROS need to be maintained below toxic concentration for the plant. The activation of key enzymes in phenolic biosynthesis (e.g. phenylalanine ammonia lyase) during germination of seeds has been amply reported (Díaz-Sánchez *et al.*, 2018). The polyphenolic compounds present in raw or germinated seeds make a good preventative tool against different diseases. Consequently, the presence of these compounds in raw or germinated seeds of Moringa can modulate the lipid peroxidation involved in atherogenesis, carcinogenesis, and thrombosis in humans (Siddhuraju *et al.*, 2003). Also, evidence indicates that phenolic compounds have potent antioxidant properties and free radical scavenging capabilities (Singh *et al.*, 2012).

The present study indicated that AoxA had a stronger correlation (R^2 = 0.8794, P= 0.0002) with TPC respectively. Xue *et al.* (2016), carried out a study on bioactive compounds and antioxidant activity of some germinated legumes (mung bean, soybean, and black bean). They observed that during the germination process, after an analysis of relative contribution, that TPC made the highest (41.74-82.94%) contribution to total antioxidant activity.

Conclusions

The optimal germination conditions of *Moringa oleifera* seeds produced a functional flour with high values of protein content, antioxidant activity and total phenolic content, and a lipid content of 20%. The optimized germination process is an effective strategy to increase the content and quality of proteins, antioxidant activity, total phenolic content, and polyunsaturated fatty acids, as well as decrease lipid content of moringa seeds. Therefore, the optimized germinated moringa seeds flour (OGMSF) could be used as a source of natural antioxidants, good quality proteins, dietary fiber, and unsaturated fatty acids as oleic (ω9), linoleic (ω6) and α-linolenic (ω3) acids in

the formulation of functional foods.

Acknowledgements

The first author is grateful to CONACYT (364511) for the scholarship granted for doctoral studies, also the authors wish to acknowledge the financial support provided by Programa de Fomento y Apoyo a Proyectos de Investigación (PROFAPI) - 2015 Universidad Autónoma de Sinaloa, Sinaloa, México.

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ANOVA	analysis of variance
AoxA	antioxidant activity
DPPH	2, 2-diphenyl-1-picrylhydrazyl
GAE	gallic acid equivalents
GMSF	germinated moringa seed flour
GT	germination temperature
Gt	germination time
IDF	insoluble dietary fiber
LC	lipid content
OGMSF	optimized germinated moringa seed flour
PITC	phenylisothiocyanate
PC	protein content
RSM	response surface methodology)
SDF	soluble dietary fiber
TEA	triethylamine
TPC	total phenolic content
UMSF	unprocessed moringa seed flour

References

Aly, A., Maraei, R., Ali, H. (2016). Fatty acids profile and chemical composition of Egyptian *Moringa oleifera* seed oils. *Journal American Oil Chemistry Society 93*, 397-404.

Anwar, F., Bhanger, M. (2003). Analytical characterization of *Moringa oleifera* seed oil grown in temperate regions of Pakistan. *Journal Agricultural Food Chemistry 51*, 6558-6563.

AOAC. (2012). Official Methods of Analysis of the Association of Official Analytical Chemists. Methods: 925.09B, 920.39 C, 960.52, and 985.29. Washington, USA: AOAC.

Barakat, H. and Ghazal, G.A. (2016) Physicochemical properties of *Moringa oleifera* seeds and their edible oil cultivated at different regions in Egypt. *Food and Nutrition Sciences 7*, 472-484.

Botero, M., Fong, C., Rothschild, J., Patrick, F. (2012) Effects of germination on the nutritional profile of gluten-free cereals and pseudocereals: a review. *Cereal Chemistry 89*, 1-14.

Cevallos-Casals, B.A., Cisneros-Zevallos, L. (2010) Impact of germination on phenolic content and antioxidant activity of 13 edible seed species. *Food Chemistry 119*, 1485-1490.

Correa-Leyva, C., Tejeda-Mansir, A., Martin-García, A.R., García, H.S., Noriega-Rodríguez, J.A. (2017) Cinética de esterificación enzimática de ácidos grasos poliinsaturados n-3 a glicerol: mecanismo ping-pong multi-sustrato multi-producto. *Revista Mexicana de Ingeniería Química 3*, 805-812.

Dawood, M., Sadak, M., Rayad, B.Y., El-Sayed, A.S.M., El-Gayar, S.H. (2013) Changes in chemical composition during germination of some canola varieties Changes in oil content and fatty acid composition. *Scientia Agriculturae 2*, 77-82.

Díaz-Sánchez, E.K., Guajardo-Flores, D., Serna-Guerrero, D., Gutiérrez-Uribe, J.A., Jacobo-Velázquez, D.A. (2018) The application of chemical elicitors improves the flavonoid and saponin profiles of black beans after soaking. *Revista Mexicana de Ingeniería Química 17*, 123-130.

Fernandez-Orozco, F.J., Zielinski, H., Muñoz, M., Piskula, M.K., Kozlowska, H., Vidal-Valverde, C. (2009). Evaluation of bioprocess to improve the antioxidant properties of chickpeas. *Lwt-Food Science and Technology 42*, 885-892.

Gómez-Favela, M.A., Gutiérrez-Dorado, R., Cuevas-Rodríguez, E.O., Canizalez-Román, V.A., León-Sicairos, C.R., Milán-Carrillo, J., Reyes-Moreno, C. (2017) Improvement of chia seeds with antioxidant activity, GABA, essential amino acids, and dietary fiber by controlled germination bioprocess. *Plant Foods for Human Nutrition 72*, 345-352.

Ijarotimi, O.S., Adeoti, O.A., Ariyo, O. (2013). Comparative study on nutrient composition, phytochemical, and functional characteristics of raw, germinated, and fermented *Moringa oleifera* seed flour. *Food Science Nutrition 1*, 452-463.

Ilyas, M., Arshad, M.U., Saeed, F., Iqal, M. (2015) Antioxidant potential and nutritional comparison of moringa leaf and seed powders and their tea infusions. *Journal Animal and Plant Science 25*, 226-233.

Jansen, S., López-Miranda, J., Castro, P., López-Segura, F., Marín, C., Ordovás, J.M., Paz, E., Jiménez-Pérez, J., Fuentes, F., Pérez-Jiménez, F. (2000). Low-fat and high-monounsaturated fatty acid diets decrease plasma cholesterol ester transfer protein concentrations in young, healthy, normolipidemic men. *American Journal Clinical Nutrition 72*, 36-41.

Khang, D.T., Dung, T.N., Elzaawely, A.A., Xuan, T.D. (2016) Phenolic profiles and antioxidant activity of germinated legumes. *Foods 5*, 1-10.

Kim, H. (2002). Modulation of redox-sensitive transcription factors by calorie restriction during aging. Mechanism of Ageing Development 123:1589-1595.

Kuo, Y.H., Rozan, P., Lambein, F., Frias, J., Vidal-Valverde, C. (2004). Effects of different germination conditions on the contents of free protein and non-protein amino acids of commercial legumes. *Food Chemistry 86*, 537-545.

Mamilla, R.K., and Mishra, V.K. (2017). Effect of germination on antioxidant and ACE inhibitory activities of legumes. *LWT-Food Science and Technology 75*, 51-58.

Martín-Cabrejas, M.A., Ariza, N., Esteban, R., Molla, E., Waldron, K., López-Andreu, F.J. (2003) Effect of germination on the carbohydrate composition of the dietary fiber of peas (*Pisum sativ um* L). *Journal Agricultural Food Chemistry 51*, 1254-1259.

Megat, R.M.R., Azrina, A., Norhaizan, M.E. (2016). Effect of germination on total dietary fiber and total sugar in selected legumes. *International Food Research Journal 23*, 257-261.

Ochanda, S., Onyango, C., Mwasaru, A., Ochieng, J., Mathooko, F. (2010) Effects of malting and fermentation treatments on group B-vitamins of red sorghum, white sorghum and pearl millets in Kenya. *Journal Applied Biosciences 34*, 2128-2134.

Ogunsina, B.S., Radha, C., Govardhan-Singh, R.S. (2010). Physicochemical and functional properties of full-fat and defatted moringa (*Moringa oleifera*) kernel flour. *International Journal Food Science Technology 45*, 2433-2439.

Ogunsina, B.S., Indira, T.N., Bhatnagar, A.S., Radha, C., Debnath, S., Gopala-Krishna, A.G. (2014) Quality characteristics and stability of *Moringa oleifera* seed oil of Indian origin. *Journal of Food Science and Technology 51*, 503-510.

Paucar-Menacho, L.M., Martínez-Villaluenta, C., Dueñas, M., Peñas, E. (2017). Optimization of germination time and temperature to maximize the content of bioactive compounds and the antioxidant activity of purple corn (*Zea mays* L.) by response surface methodology. *LWT-Food Science and Technology 76*, 236-244.

Perales-Sánchez, J.X.K., Reyes-Moreno, C., Gómez-Favela, M.A., Milán-Carrillo, J., Cuevas-Rodríguez, E.O., Valdez-Ortiz, A., Gutiérrez-Dorado, R. (2014). Increasing the antioxidant activity and total phenolic and flavonoid contents by optimizing the germination conditions of amaranth seeds. *Plant Foods for Human Nutrition 69*, 196-202.

Ramakrishna, V. and Ramakrishna Rao, P. (2006). Storage protein degradation in germinating Indian bean (*Dolichos lablab* L. var. *lignosus*) seeds. *Seed Science and Technology 34*, 161-168.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine 26*, 1231-1237.

Ryan, C.A. (1973). Proteolytic enzymes and their inhibitors in plants. *Annual Review of Plant Physiology 24*, 173-96.

Saini, R.K., Sivanesan, I., Keum, Y.S. (2016) Phytochemicals of *Moringa oleifera*: a review

of their nutritional, therapeutic and industrial significance. *3 Biotech 6*, 203.

Sánchez-Machado, D.I, López-Cervantes, J., Núñez-Gastélum, J.A, Servín de la Mora-López, G., López- Hernández, J., Paseiro-Losada, P. (2015). Effect of the refining process on *Moringa oleifera* seed oil quality. *Food Chemistry 187*, 53-57.

Sánchez-Machado, D.I., López-Cervantes, J., López-Hernández, J., Paseiro-Losada, P., Simal-Lozano. (2003). High-performance liquid chromatographic analysis of amino acids in edible seaweeds after derivatization with phenyl isothiocyanate. *Chromatographia 58*, 159-163.

Sánchez-Machado, D.I., Núñez-Gastélum, J.A., Reyes-Moreno, C., Ramírez-Wong, B., López-Cervantes, J. (2010). Nutritional quality of edible parts of *Moringa oleifera*. *Food Analytical Methods 3*, 175-180.

Siddhuraju, P., Becker, K. (2003) Antioxidant properties of various solvent extracts of total phenolic constituents from 3 different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam). *Journal Agricultural Food Chemistry 15*, 2144-2155.

Singh, G., Sharma, P., Bajaj, R., Solah, V. (2012) Effects of incorporating germinated brown rice on the antioxidant properties of wheat flour chapatti. *Food Science Technology International 18*, 47-54.

Singh, P.K., Gautam, A.K., Panwar, H., Sing, D.K., Srivastava, N., Bhagyawant, S.S., Updhayay, H. (2014). Effects of germination on antioxidant and antinutritional factors of commonly used pulses. *International Journal of Research in Chemistry and Environment 4*, 100-104.

Singleton, V.L, Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymology 299*, 152-178.

Xue, Z., Wang, C., Zhai, L., Yu, W., Chang, H., Kou, X., Zhou, F. (2016) Bioactive compounds and antioxidant activity of mung bean (*Vigna radiate* L.), soybean (*Glicine max* L.) and black bean (*Phaseolus vulgaris* L.) during the germination process. *Czech Journal Food Science 34*, 68-78.

Zhu, F., Du, B., Zheng, L., Li, J. (2015) Advance on the bioactivity and potential applications of dietary fiber from grape pomace. *Food Chemistry 186*, 207-212.