INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0244/2019/21–4–786–794
DOI: 10.17957/IJAB/15.0957
http://www.fspublishers.org

***Full Length Article***

# Metabolomic and Proteomic Analysis of *Moringa oleifera* Cultivated with Vermicompost and Phosphate Rock under Water Stress Conditions

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## Abstract

Interest in the plant gender *Moringa oleifera* has grown in recent years as it is a plant that can potentially provide a good amount of nutrients necessary for human consumption. Thus, humans could benefit from nutrient enrichment in this plant and produce foods with high protein content. Protein and metabolite content is influenced by soil type, organic input and frequency of irrigation. For this reason we evaluated the effect of different culture conditions on the metabolomic profile and protein concentration leaf of *M. oleifera*. The cultivation of *M. oleifera* with organic inputs was conducted in a greenhouse. Nutrient sources were vermicompost nitrogen at a fertilization rate of 150 kg/ha, phosphate rock as a source of phosphorus at a rate of fertilization of 96 kg P/ha and urea (control) at a rate of 150 kg N/ha. The experimental design was a split plot with randomized complete block. The frequency of irrigation was monitored for each of the levels of water holding capacity (WHC), low (25% WHC), medium (40% WHC) and high (55% WHC). The application of soil-rock phosphate-vermicompost increases morphometric parameters significantly at 45 and 90 days. UPLC-ESI-MS/MS analysis of leaf extracts revealed that the most abundant metabolites were flavonoids, alkaloids and terpenes. Thus, water stress induced changes in the metabolomic profile and the morphometric variables of *M. oleifera*. These results are important for *Moringa* industrialization for metabolite production culture in different crop conditions. 

**Keywords:** Protein extraction; Vermicompost; Phosphate rock; Mass spectrometry; Secondary metabolite

## Introduction

*Moringa oleifera* Lam. is commonly known as horseradish tree and belongs to the family Moringaceae with around 33 species. Out of these, 4 are accepted, 4 are synonym and 25 have not been assessed whose importance lies mainly by its fruits, leaves, flowers, roots and its oil obtained from the seeds (Reyes *et al*., 2006; Arora and Onsare, 2014). This tree has great potential for cultivation in Mexico and other places of America, due to its combination of properties alone. Leaves are edible and rich in protein with a balanced amino acid profile, including essential amino acids. Also, they contain vitamins, especially A, B, C and E and mineral elements and comprise a rich and rare combination of bioactive secondary metabolites such as glucosinolates, flavonoids and phenolic acids (Anwar *et al*., 2007).

These secondary metabolites have the potential to reduce the risk of cardiovascular diseases and cancer (Makkar and Becker, 1996; Anwar *et al*., 2007; Ferreira *et al*., 2008; Pandey *et al*., 2011; Nouman *et al*., 2014; Zheng *et al.,* 2016). It is reported that different parts and preparations of *M. oleifera* have been used in traditional medicine and in the treatment of various diseases, such as nervous disorders, hypertension and diabetes (Fahey, 2005).

Although the leaves of *M. oleifera* have been extensively researched for its nutritional and pharmacological potential benefits for human uptake, they can also provide benefits to livestock feed. The nutritional profile of dry leaves has shown high levels of lipids and important amino acids used as feed for poultry, which increases productivity (Makkar and Becker, 1997). Recent research has found that the synergistic properties between individual bioactive compounds in the leaves influence different aspects of physiology, such as absorption and processing of nutrients. Also, antioxidant action has potential therapeutic effects (Anwar *et al.*, 2007; Wallace *et al.*, 2010; Mbikay, 2012). Previous studies on *Moringa* have focused mostly on its medicinal use and on the nutritional

**To cite this paper:** Guzmán-Albores, J.M., M.L. Ramirez-Merchant, E.C. Interiano-Santos, L.A. Manzano-Gómez, J.H. Castañón-González, R. Winkler, M. Abud-Archila, J.A. Montes-Molina, F.A. Gutiérrez-Miceli and V.M. Ruíz-Valdiviezo, 201x. Metabolomic and proteomic analysis of *Moringa oleifera* cultivated with vermicompost and phosphate rock under water stress conditions. *Intl. J. Agric. Biol*., 21: 786–794

uses of the tree parts (Vázquez-León *et al*., 2017) as well as on the use of the seed in clarification during waste-water treatment (Folkard *et al*., 1993; Fakayode and Ajav, 2016).

Plants are subjected to both biotic and abiotic stress during growth and development. Stress leads to upregulation of genes associated with the production of metabolites such as proteinase inhibitors, toxins, among others (Rani and Prasannalaxmi, 2014). Abiotic stress is mainly due to factors such as drought, extreme temperatures, cold weather, heavy metals or high salinity, which endanger plant growth and productivity. Abiotic stress has a direct impact on the photosynthetic apparatus and is characterized by stomatal closure, loss of turgor, reduction of relative water content. This seriously harms the growth and development of plants. Severe cases cause photosynthesis to slow down, alter metabolism, finally leading to plant death (Allen and Ort, 2001; Jaleel *et al*., 2008).

Tropical regions face a shortage of fertilizer supply, mainly nitrogen, which is essential nutrient for maximum performance of most crops (Pal and Shehu, 2001). Much attention has been paid in recent years to various organic wastes that can provide those nutrients. Sustainable agriculture has become a major topic of global concern, and farmers in Mexico are starting to cultivate their land without the use of chemicals, pesticides and herbicides (Prasad *et al.*, 1999).

It is difficult to increase or keep soil organic matter in the subtropics and tropics. Although replacement of organic matter is high, crop residues are removed from the field and are used as cattle fodder or burned (Prasad *et al.*, 1999). In Mexico, an alternative that could be explored is the use of vermicompost (obtained by composting of organic materials using worms). The country produces large quantities of organic waste, such as biosolids derived from wastewater of processing plants, excreta of animals in areas of production and domestic and agricultural waste (Ndegwa and Thompson, 2001). When used as compost or vermicompost, these organic wastes have application in horticulture, vegetable production and agriculture or in restoration of degraded soils (Krogman *et al*., 1997; Hernández *et al*., 2003). The aim of this study was to evaluate the effect of different culture conditions on the metabolomic profile and protein concentration leaf of *M. oleifera*.

## Materials and Methods

### Site Description and Soil Sampling

Soil samples were collected from "La Escondida" ranch, located in the irrigation area of district municipality 101 of La Concordia, Chiapas (México), with coordinates 16° 06'58 "N 92° 41'20" N, and at an altitude of 550 meters. The experimental site, located in the Fraylesca region, is characterized by a sub-hot and sub-humid-climate (ACw) with rains in summer. This type of climate is common in tropical regions of Mexico.

### Characterization of Vermicompost and Soil

The characterization of soil samples and vermicompost was carried out as described by López-Valdez *et al*. (2010). The parameters evaluated were moisture, water holding capacity (WHC), texture, pH, electrical conductivity, organic carbon and total nitrogen.

Establishment of the experiment: The seeds were collected in the Ranch "La Escondida" in Concordia (16° 07'' N, 92° 41'' W), Chiapas (México). The climate is characterized by a sub-hot and sub-humid-climate (ACw) with rains in summer. The vegetation is of forest of oak-pine and of high forest, where are a variety of species among which include cedar, oak, cypress, pine, rosemary, sabino, chamomile, oak, casoirol, ram, among others. These were grown in a greenhouse in polyethylene bags with 9 kg of soil. Seedlings were watered with tap water every two days. For the realization of the experiment, a design of divided plots with three completely randomized blocks was used. Treatments were as follows: soil+plant (control -), soil+plant+urea (control +), soil+vermicompost+plant, soil+phosphate rock+plant and soil+phosphate rock+vermicompost. Also, three levels of water retention capacity (CRA) were used, which were 25 (water deficit), 40% (optimal conditions) and 55% (excess water). All the plants were harvested at 45 and 90 days after the emergency to make each of the determinations.

### Preparation of the Biological Material

The preparation of the samples was performed by weighing 0.5 g of leaves of fresh *M. oleifera* for each treatment on a PA214 Ohaus scale. Samples were then macerated with liquid nitrogen to a fine powder (100 mesh), which were placed in 1.5 mL tubes and stored at 4°C until further use Chen *et al*. (2012).

### Determination of Morphometric Variables

The morphometric parameters were length and diameter of the plant, these were evaluated at 45 and 90 days after the emergency (45 dde). Plant length was measured with a Pretul flexometer, and plant diameter was measured with a Surtex Vernier.

### Determination of Chlorophyll Content

The chlorophyll content was determined in the apical leaves using a Konica Minolta Spad 502 computer at 45 and 90 days after the emergency, and units were reported in SPAD (Coste *et al*., 2010). Six replicas of different leaves were determined by experimental unit.

### Protein Extraction and Quantification

Protein extraction from *M. oleifera* leaves was performed by

using the modified extraction protocol used by Chen *et al*. (2012). The extraction buffer contained 0.1 *M* Tris-HCl+0.1% ascorbic acid+10% glycerine+1% polyvinylpolypyrrolidone+5% β-mercaptoethanol at a pH of 8.1 mL of buffer was added to each of the samples and incubated at 4°C for 4 h. Subsequently, the samples were centrifuged at 15,000 rpm for 15 min at 4°C in a Hermle Z326K centrifuge (Labortechnik Gmbh, Stuttgart, Germany). Subsequently, 1 mL of supernatant was taken for protein quantification by the Bradford method and 10 $\mu$L for electrophoresis.

**Cuantification of Protein and SDS-PAGE**

A 0.5 g of leaves were homogenized in 1 mL of extraction buffer [0.1 *M* Tris–HCl (pH 8.0), 0.1% (w/v) antiscorbic acid, 10% (v/v) glycerin, 1% (w/v) polyvinyl pyrrolidone and 5% (w/v) b-mercaptoethanol], respectively. Samples were later transferred to 1.5 mL tubes, incubated at 4°C for 4 h, and centrifuged for 15 min at 15,000 rpm and 4°C. The supernatant was used for SDS-PAGE electrophoresis (Chen *et al*., 2012). The protein content analysis protocol developed by Bradford (1976) was used to measure protein contents.

SDS-PAGE was carried out on a 0.75 mm thick SDS-polyacrylamide slab gel with 10% separating gel and 4% stacking gel. The run conditions were: 80 volts for 20 min for the stacking gel and 120 volts for 40 min for gel separating. The gels were stained with 0.1% Coomassie Brillant Blue R 250 in a mixture of methanol, glacial acetic acid and water [50:7:43 (v/v)], and destained with a mixture of methanol and acetic acid (45.4:7.5%) described by Chen *et al*. (2012).

**Analysis of Metabolites by UPLC-ESI-MS/MS**

In order to improve metabolite extraction, 20 mg of lyophilized residue were reconstituted in 500 $\mu$L methanol for 10 min in a sonicator at 20ºC, and then centrifuged (11,356 × g, 10 min). Supernatants were collected and filtered through a 0.22 $\mu$m polypropylene membrane filter (ANOTOP10 plus; Whatman, Maidstone, UK). Each sample (10 $\mu$L) was analyzed in a UPLC-ESI-MS/MS system (LCQ Fleet, Thermo Finnigan, San Jose, CA, USA). This equipment used a C18 Hypersil gold column (50 x 2.1 x 1.9 mm) (Rhea *et al*., 2012). Operating conditions were as follows: oven temperature of 38°C, flow rate of 350 $\mu$L/min, where water in 0.1% formic acid was the gradient mobile phase A and methanol in 0.1% formic acid was mobile phase B. The gradients were 35% B (0–1.5 min), 35–86% of phase B (1.5–3 min), 86–100% of phase B (3–25 min); 100% of phase B (25–27 min) and 35% of phase B (27–27.5). Individual sample data was downloaded into "mzXML" digital files and analyzed with programming software and bioinformatics. The data were analyzed with Mzmine3 (Pluskal *et al*., 2010) and R Software (Ernest *et al*., 2012; Xia *et al*., 2015).

**Peroxidase Activity**

The peroxidase activity was determined at 45 and 90 days by measuring the appearance of tetraguaiacol at 470 nm. The reaction mixture contained 3 mL of extraction buffer, 0.25% (v/v) in 10 m*M* guaiacol sodium phosphate (pH 6.0) with 10 m*M* hydrogen peroxide and 100 µL of enzyme extract. The enzyme activity was calculated by measuring the ratio at 470 nm/min = 0.01, which is defined as 1 unit of enzymatic activity. The specific activity was expressed as IU /mg protein (Hanaa *et al*., 2011).

## Results

**Characterization of Vermicompost and Soil**

The soil of the "La Escondida" ranch has a near neutral pH and sandy clay loam as shown in Table 1. The phosphorus content was 4 mg $kg^{-1}$ and total nitrogen content was 1.7 g $kg^{-1}$ with a WHC of 857 and EC <1 dS $m^{-1}$.

**Protein Extraction and Quantification**

The content of protein was evaluated at 45and 90 days after emergence. At 45 days there was no statistically significant difference ($p$ <0.05) between treatments in protein concentration. However, protein content decreases significantly at 90 days (Table 2).

**Morphometric Parameters of *M. oleifera* in Greenhouse**

The growth parameters were evaluated at days 45 and 90. Plants amended with treatment S + R + V (soil+phosphate rock+vermicompost) have 44.8 cm and 6.71 mm of height and diameter at 45 days, respectively. On the other hand, after 90 days the diameter and height were 11.40 mm and 77.33 cm respectively, when plants were treated with phosphate rock + vermicompost and the diameter and plant height presented a statistically significant difference with respect to other treatments is shown in Table 3.

**Evaluation of Chlorophyll**

The content of chlorophyll from leaves in the different treatments is shown in Table 4. There was a significant statistical difference between the S + P + U treatment compared to the other treatments, finding values of 42.75 and 33.70 SPAD units at 45 days and 90 days, respectively. In this study, the treatment that allowed to find higher values was soil + plant + urea.

**Electrophoretic Pattern of Protein at 45 and 90 days**

SDS-PAGE using 12% Bis/Tris the protein band patterns of *M. oleifera*. The molecular size was uniformly and homogeneously separated for each of the treatments with molecular weight of bands ranging from 20 to 150 kDa with a predominant band of 45 kDa under optimum conditions of

**Table 1:** Characterization of soil sample (SN) and vermicompost (V)

| | pH | WHC ($g\ g^{-1}$ soil) | Humidity (%) | EC ($dS\ m^{-1}$) | Phosphor (mg/kg) | Organic C ($g\ kg^{-1}$ soil) | Total N ($g\ kg^{-1}$ soil) | C/N | Textural classification |
|---|---|---|---|---|---|---|---|---|---|
| SN | 6.77 | 0.41 | 5.99 | 4.31 | 4 | 9.65 | 1.77 | 5.5 | Sandy Clay |
| *V | 7.41 | 0.92 | 49.25 | 8.00 | 357.34 | 233 | 11.8 | 19.7 | - |

**Table 2:** Protein concentration of leaves of *Moringa* at 45 and 90 days in the greenhouse

| Treatment | 45 dde Protein (%) | 90 days Protein (%) |
|---|---|---|
| S+R | 23.38 a | 4.21 ab |
| S+P+U | 22.91 a | 4.14 ab |
| S+P | 23.03 a | 4 b |
| S+V | 24.09 a | 4.17 ab |
| S+R+V | 23.69 a | 4.27 a |
| DMS | 2.80 | 0.0239 |

Statistical analysis was performed using the Statgraphics Centurion XV program with a multifactorial ANOVA analysis and $P$ <0.05.Los values represent the average of three replicates, different letters indicate significant statistical difference between treatments, DMS is the least significant difference. The treatment abbreviations were: S = Soil, R = Rock phosphate, P = plant, V = vermicompost.

**Table 3:** Analysis of morphometric variables in leaves of *Moringa oleifera* at 45 and 90 days in the greenhouse

| | 45 days | | 90 days | |
|---|---|---|---|---|
| Treatment | Height (cm) | Diameter (mm) | Height (cm) | Diameter (mm) |
| S+R | 28.61 b | 4.70778 b | 58.22 b | 8.80 b |
| S+P+U | 30.11 b | 4.17 b | 62.66 b | 9.07 b |
| S+P | 31 b | 4.7 b | 57.22 b | 8.94 b |
| S+V | 39.33 ab | 5.43 ab | 79.66 a | 10.60 a |
| S+R+V | 44.88 a | 6.71 a | 77.33 a | 11.40 a |
| DMS | 11.5758 | 1.3065 | 7.3151 | 0.2723 |

Statistical analysis was performed using the Statgraphics Centurion XV program with a multivariate analysis of ANOVA and $P$ <0.05. Values represent the mean of three replicates, different letters indicate statistically significant difference between treatments, DMS is the least significant difference. The treatment abbreviations were: S = Soil, R = Rock phosphate, P = plant, V = vermicompost.

**Table 4:** Content of chlorophyll in leaves of *Moringa oleífera*

| | 45 days | 90 days |
|---|---|---|
| Treatment | Clorophyll (units SPAD) | Clorophyll (units SPAD) |
| S+R | 40.13 b | 30.12 c |
| S+P+U | 42.75 a | 33.70 a |
| S+P | 41.11 b | 33.54 ab |
| S+V | 40.88 b | 32.02 b |
| S+R+V | 39.57 b | 33.12 ab |
| DMS | 1.6357 | 1.568 |

Statistical analysis was performed using the Statgraphics Centurion XV program with a multivariate analysis of ANOVA and $P$ <0.05. Values represent the mean of three replicates, different letters indicate statistically significant difference between treatments, DMS is the least significant difference. The treatment abbreviations were: S = Soil, R = Rock phosphate, P = plant, V = vermicompost.

culture (40% CRA) (Fig. 1 and 2). However, when there was water stress due to water deficiency (25% CRA) and excess water (55% CRA) the molecular weight varied between 20 and 120 kDa.

## Evaluation of the Effect of Water Stress on Plant Growth and chlorophyll

The effect of the WHC levels with each of the treatments, with respect to the response variables at 45 days after the emergency was evaluated (Table 5). For the variables of height and diameter of the plant, there was a significant statistical difference ($p$ <0.05) with a water deficiency level (25% WHC) and optimum culture conditions (40% WHC), with respect to an excess of water (55% WHC). Protein content showed a significant statistical difference between different levels of WHC. Moreover, for the enzymatic activity there was a statistically significant difference between 25 and 55% of WHC. The chlorophyll content had significant statistical difference between 55%, 40% and 25% of WHC. In this study, we found that height and diameter of the plant decreased significantly when there is an excess of water.

## Impact of Water Stress on Metabolomic Profile of *Moringa* leaves

In order to obtain a first metabolic profile on the analysis of metabolites in leaves of *M. olefiera* subjected to water stress, a heatmap was constructed using the software Mzmine with language R (Fig. 3). Informatics analysis and

**Table 5:** Effect of water retention capacity on height, diameter, proteins and enzymatic activity

| WHC | Height (cm) | Diameter (mm) | Proteins (mg/g) | Enzymatic Activity (UAE/mg of protein) | Chlorophyll (units SPAD) |
|---|---|---|---|---|---|
| 25% | 39.4 a | 5.658 a | 24.32 b | 179.06 a | 39.78 b |
| 40% | 38.6 a | 5.733 a | 28.44 a | 127.2 ab | 40.42 b |
| 55% | 26.36 b | 4.04 b | 17.50 c | 86.4 b | 42.48 a |
| DMS | 9.1551 | 1.0148 | 0.75 | 59.041 | 1.267 |

Values represent the mean of three replicates, different letters indicate statistically significant difference between treatments, DMS is the least significant difference.

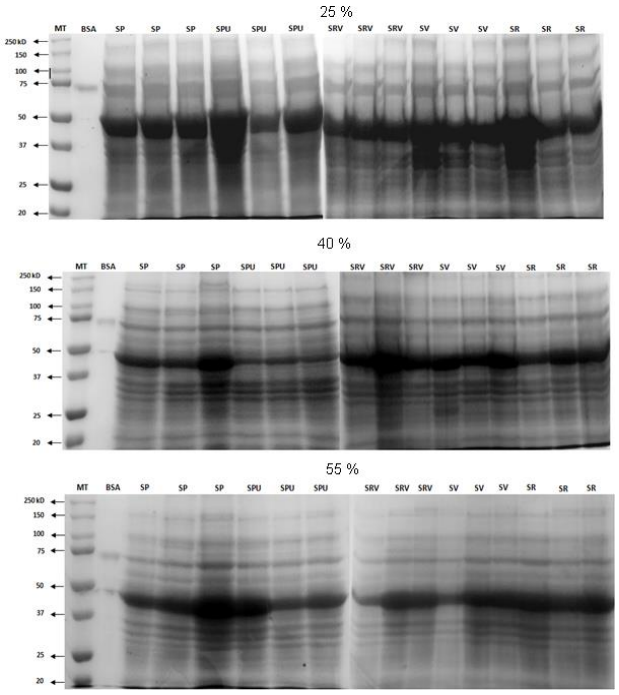


**Fig. 1:** Electrophoretic patterns of *Moringa oleifera* grown with organic inputs. Lane 1: molecular weight marker (MT); Lane 2: bovine serum albumin (BSA); Lane 3,4,5: soil + plant (SP); Lane 6,7,8: Soil + plant + urea (SPU), lane 9,10,11: soil + phosphoric rock + vermicompost (SRV), lane 12,13,14: soil + vermicompost (SV), lane 15,16,17: soil + rock Phosphoric (SR) at different levels of water stress (25, 40 and 55% WHC)

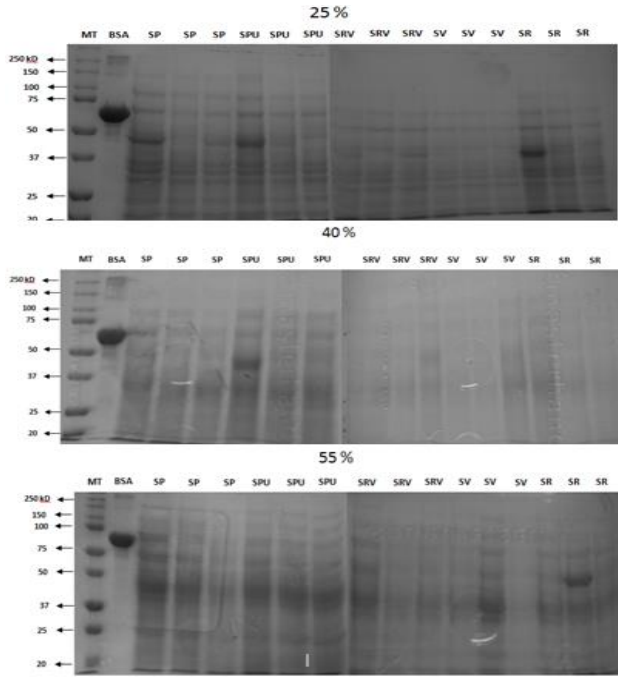


**Fig. 2:** SDS-PAGE electrophoresis of *Moringa oleifera* culture at 90 after emergence. Lane 1: molecular weight marker (MT); Lane 2: bovine serum albumin (BSA); Lane 3,4,5: soil + plant (SP); Lane 6,7,8: soil + plant + urea (SPU); Lane 9,10,11: soil + phosphoric rock + vermicompost (SRV); Lane 12,13,14: soil + vermicompost (SV); Lane 15,16,17: soil + phosphoric rock (SR) at different levels of water stress (25, 40 and 55% of WHC)

statistical visualization were performed with MetaboAnalyst software (Xia *et al.*, 2015).

## Discussion

Total nitrogen content of the soil was 1.77 g $kg^{-1}$ soil similar to those reported for different soils of the state of Chiapas Gómez-González *et al.* (2018). These results indicate that these soils are conducive to the establishment of different types of crops. The C/N ratio is an indicator of fertility and soil productivity. This ratio was higher for soils that have not been cultivated or plowed. Changes in the C/N ratio are attributed to climate change (Ostrowska and Porebska, 2015) due to the interaction with microorganisms. However, the C/N ratio values lower than 10 for arable soils are associated with a high amount of nitrogen, mainly due to the excessive use of fertilizers (Ostrowska and Porebska, 2015). This result indicates that biomass in soil organic matter (SOM) is not sufficient for nitrogen mineralization, which generates leachates that contaminate the soil (Nave *et al.*, 2009). Therefore the addition of vermicompost favored the C/N ratio, this ratio was higher (19) compared to the native soil (5.5). The C/N ratio has been considered one of the most important factors because it is directly related to the essential nutrients for microbial activites (Bernal *et al.*, 2009; Sánchez-Monedero *et al.*, 2010). A C/N ratio of less than 20 indicates an advanced degree of stabilization of organic matter and a satisfactory degree of maturity of vermicompost. This is suitable for soil amendment and is necessary becausse plants can not assimilate nitrogen unless the C/N ratio is less than 20 (Khwairakpam and Bhargava, 2009). Moreover, there are substantial evidences in the literature stating that the use of organic amendment

**Table 6:** Secondary metabolites in *M. oleifera* leaves

| Metabolites | m/z | RT | Activity | Reference |
|---|---|---|---|---|
| Daidzin | 416.077 | 13.74 | Suppression of inflammation and oxidative stress in the cornea | Xiao *et al.*, 2018 |
| Kaempferol-3-O-alpha-L-rhamnoside | 432.3779 | 13.73 | Cardioprotective, anti-inflammatory and anti-oxidant | Mahobiya *et al.*, 2018 |
| Isoschaftoside | 564.4161 | 20.66 | Antioxidant activity | Leong *et al.*, 2010 |
| 7-aminoflunitrazepam | 285.2276 | 21.54 | Tranquilizers | Kiss *et al.*, 2012 |
| Adiphenine | 311.2594 | 22.01 | Atropinic properties | Jordan *et al.*, 1977 |
| Isocoridine | 341.1541 | 8.52 | Inhibitorof topoisomerase I | Guterres da-Rosa *et al.*, 2013 |
| Flupentixol | 434.1851 | 9.79 | Iinflammatory and antioxidant properties | Kim and Song, 2016 |
| Neriifolin | 534.3398 | 17.21 | Anti-carcinogenic | Jui-Cheng *et al.*, 2018 |
| Lovastatin | 404.6343 | 11.83 | Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase) | Mouafi *et al.*,2016 |

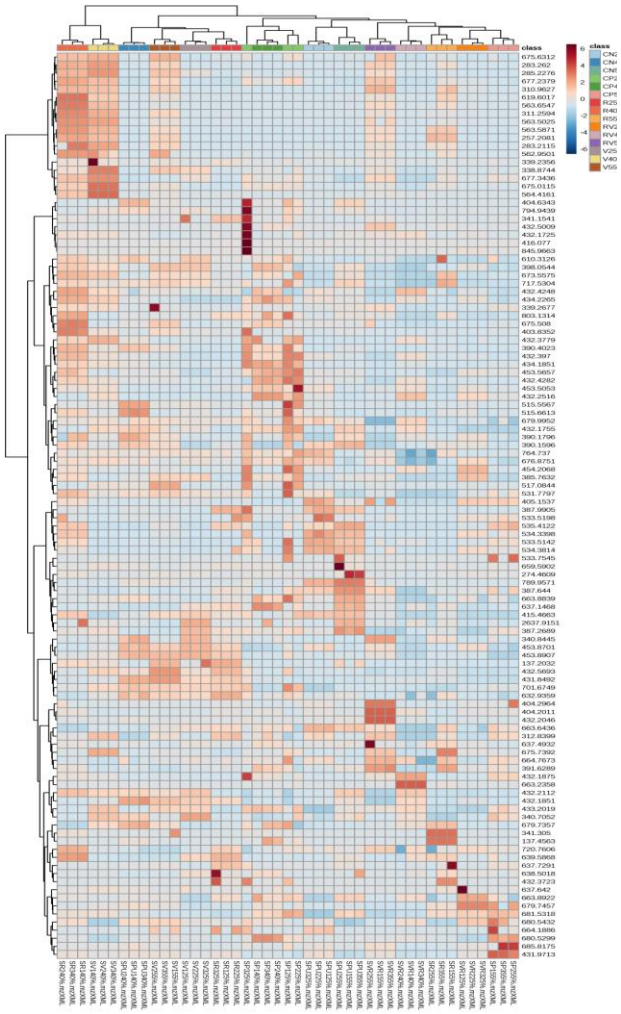

**Fig. 3:** Heatmap of leaves of *M. oleifera* illustrating the metabolites that differ among cultivated with organic inputs subjected to water stress. Colors indicate relative quantity of each metabolite

increases SOM (Zerzghi *et al*., 2010; Annabi *et al*., 2011). In turn, this increase in SOM improves soil quality by increasing soil aggregate stability and other physical properties such as WHC, soil porosity, water infiltration, and percolation (Celik *et al*., 2004; Leroy *et al*., 2008).

At 45 days, the protein content did not show a significant statistical difference between the fertilization treatments, however, at 90 days there was a statistical difference between the S + R +V interaction with respect to the S + P control (Table 2). This result may be due to the physiological stage of the plant. Zhang *et al*. (2015) reported a decrease in protein concentration in *Platycladus orientalis* leaves as it continued to grow. Also, this decrease may be due to an increase in the fresh biomass of the leaves of *M. oleifera* as was reported by Abid *et al*. (2018), who evaluated the protein content in leaves of *Triticum aestivum* L. and found a similar behavior under water stress conditions. Also, at 90 days, this result indicates that the application of vermicompost increases of protein content in the leaves of *M. oleifera* with respect to control (soil without vermicompost) using a fertilization rate of 150 kg N $ha^{-1}$. Similar results were reported by Mendieta-Araica *et al*. (2012), who found that the protein content increased when they used a fertilization rate of 521 kg N $ha^{-1}$ in clay soils. However, Adebayo *et al*. (2011) reported that the addition of organic amendment (cow dung) increased both vegetative and dry matter yield of *Moringa*. In order to create a suitable scenario with the necessary conditions for the cultivation of the plant, it primary starch and lipids). In this way, the decrease in protein concentration as the plant continues to develop can be explained (De-la-Cruz *et al*., 2013).

The increase of height and diameter could be due to vermicompost containing plant growth regulating materials, such as humic acids (Atiyeh *et al*., 2002) and plant growth regulators such as auxins, gibberellins and cytokinins (Singh *et al*., 2008) which are responsible for increasing plant growth and yield of many crops produced by increasing the activity of microorganisms such as (Arancon *et al*., 2005) solubilizing bacteria and mycorrhizal fungi, which degrade different components of soil organic matter. Further, phosphate rock supplies phosphorus to the soil, which is fundamental to the development of plant nutrients. Souza *et al*. (2013) reported that the addition of phosphate rock increases plant height. Pramanik *et al*. (2009) described how the use of phosphate rock with vermicompost increases the available phosphorus in the soil through the stimulation of microbial activity. This effect could also be due to the presence of humic acids and phosphate solubilising bacteria vermicompost (Hameeda *et al*., 2006).

The increase of urea could be due to the presence of nitrogen, a fertilizer widely used in agriculture, as it is easy to use, is chemically stable, of low cost and has high nitrogen content (46%). It is also conveniently soluble in water, which allows rapid decomposition into ammonium ($NH_4^+$) (Wang *et al*., 2008). Nitrogen is known to increase the cation exchange capacity of the roots to the plant, which makes the absorption of other nutrients more efficient. It is also a necessary element for cell multiplication and development of plant organs, and has an important partaking in the synthesis of chlorophyll and in the process of photosynthesis (Raven *et al*., 2007) assimilation and synthesis of organic products.

Studies on the proteomic profile of *M. oleifera* leaves have reported a molecular weight of 45 kDa (Interiano-Santos, 2015). However, no research reporting the proteomic profile of *M. oleifera* leaves cultivated with organic amendments such as vermicompost and phosphoric rock and subjected to water stress was found. In addition, it is very important to know the effect of the amendments on the synthesis of metabolites in the leaves of *M. oleifera*, as these metabolites (flavonoids) are of pharmaceutical interest.

This could be due to the fact that under these conditions, oxygen rapidly decreases due to the slow diffusion of gases in the water (Jackson and Colmer, 2005), and due to the rapid consumption of $O_2$ by soil microorganisms, which are the main cause of damage to plants grown in flooded soils. The immersion implies low light stress, limited gas diffusion, soil nutrient spillage, mechanical damage, and increased susceptibility to pests and diseases (Greenway and Setter, 1996; Ram *et al*., 1999).

The growth and development of most plants is affected for waterlogged soils (Nishiuchi *et al*., 2012). During water stress, an excess of reactive oxygen species (ROS) can be produced that cause oxidation of proteins, lipids, nucleic acids in increasing amounts and even to induce mutations, thus causing a decrease in protein concentration (Liu *et al*., 2012). The response of plants to different types of stress generally includes altered expression of proteins. This coincides with findings in this experiment, where changes in the proteomic profile and molecular size of the *M. oleifera* leaves proteins under conditions of water stress were observed. These changes are generally related to increased or decreased gene-specific expression, and depend on the nature, duration and severity of stress (Liu *et al*., 2012). Thus, for the enzymatic activity the peroxidase activity was evaluated. Table 5 shows an increase in activity when plants are subjected to water deficit stress (25%) after 45 days after emergence compared to the control block (40%). This is due to closure of stomatal, which plays an important role in protecting the plant in response to water stress (Liu *et al*., 2012). However, in the block with an excess of water (55%), there was a decrease in peroxidase activity. This has also been reported by Hossain *et al*. (2009) in citrus crops.

Every experiment with an excess of water induced an increase in chlorophyll was observed, while water deficits showed a decrease with respect to the control, finding values of 42.48 and 39.78 SPAD units at 45 days and 90 days, respectively. The low content of chlorophyll may be due to the limitations of the assimilation of $CO_2$ imposed by the closure of the stomata, but it can also be due to deteriorated conductance of the mesophyll and/or biochemical and photochemical restrictions, as described by Pinheiro and Chaves (2011).

The first step of the chemometric analysis was a Data Integrity Check, with the objective of evaluating peaks via the m/z and tr relationship. All triplicates of the measurements were pooled correctly, which confirm the reproducibility of the analysis. The heat map generated with the mass of the most frequently metabolites found across the profiles showed that most of them were found in higher relative abundance in water stress conditions. It was found that when the plant undergoes water stress, the most abundant putative secondary metabolites identified were flavonoids such as Kaempferol-3-O-alpha-L-rhamnoside (m/z: 431.8492–432.3779), daidzin (m/z: 416.077) and isoschaftoside (m/z: 564.4161), followed by alkaloids such as: 7-aminoflunitrazepam (m/z: 83.262–285.2276), adiphenine (m/z: 311.2594), medifoxamine (m/z: 257.2081) Ergocryptine (m/z: 575.6613) and isocoridine (m/z: 341.1541), while in lower abundance, steroids such as flupentixol (m/z: 434.1851), neriifolin (m/z: 534.3398) and dexamethasone acetate Z: 434.1851) and finally lovastatin (m/z: 404.6343), a terpene. Regarding the level of these secondary compounds, this was the first putative metabolomic study of leaves of *M. oleifera*, where we could only detect the abundance of the metabolites present in the different treatments. To our knowledge, the latter groups have not been reported for *M. oleífera* leaves. However, there are reports on the activity of these metabolites as seen in Table 6.

These results indicate that biosynthesis of metabolites of the flavonoid group may be influenced by the effect of water stress, as was reported by Ritesh *et al.* (2013). In this study, they evaluated the effect of prolonged water stress on specialized secondary metabolites in *Artemisia annua* L. where they reported an even greater increase in phenolics content due to mild and moderate water stress (Ritesh *et al*., 2013).

## Conclusion

To our knowledge, there are no studies related with the protein content of *M. oleifera* leaves and the fertilization with organic fertilizers such as vermicompost and phosphoric rock and the effect of water stress on metabolome and morphometric characteristics. Thus, overall results obtained in the present study revealed that the interaction of phosphoric and vermicompost rock caused significant differences in morphometric variables as compared to control. However, when plants were under

water stress, abundant secondary metabolites and a decrease in protein content are found. For future studies, carrying out *in vitro* and *in vivo* testing could contribute more information to the medicinal and food industries.

## Acknowledgements

The project was funded by project "5663.15-P" and "6211.17-P" from 'Tecnológico Nacional de México' (TNM, México) and J.M. Guzmán-Albores and L.A Manzano-Gómez and received grant aided support from CONACyT. We thank the owner of the 'Rancho la Escondida' and NOVA LIFE SPR DE RL for providing us with the *Moringa* seed and soils.

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**(Received 14 February 2018; Accepted 03 November 2018)**