Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Effect of planting time and vermicompost on the proteomic pattern of fenugreek (*Trigonella foenum-graecum*)

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Received December 26, 2017; **Accepted** June 26, 2018; **Published** June 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.9.7

Abstract: The fenugreek is one of the most important medicinal plants belongs to Fabaceae, originated in West Asia, Iran and Mediterranean regions. This research included a qualitative study of fenugreek proteins using SDS-PAGE electrophoresis on polyacrylamide gel and the separation of protein bands of fenugreek leaves in different treatments of vermicompost fertilizer and cultivating dates. Results showed that a band (about 80 kDa) on the first planting date (May 31) is observed in all samples except for sample a1 (10 t/ha vermicompost on May 31). Another significant difference was the band contained in the third planting date (31 September) and in the molecular weight of about 15 kDa, which was not seen in other dates. This difference can be due to the synthesis of this protein with the mentioned weight under the conditions of reducing the temperature in the early fall. It also showed more differences in two-dimensional electrophoresis, for example, in 14 kDa and PI in the range of 4.5-4.7 in treatment without fertilizer, no protein expression was observed, which was consistent with the results of the SDS-PAGE test.

Key words: Planting times; Vermicompost; Proteomic pattern; Fenugreek (*Trigonella foenum-graecum*).

Introduction

Sustainable agriculture and the cultivation of organic crops is one of the contemporary agricultural debate. Also in recent years, people's tendency to use in medicinal plants has increased (1-9).

In recent years, public awareness of the process of vermicompost has increased and its use for conversion of organic wastes to vermicompost has expanded considerably (10, 11).

Vermicompost is a kind of compost produced by the earthworm, which is the result of the transformation and digestion of organic residues along the digestive tract of these animals. Earthworms convert organic fertilizers into vermicompost with organic fertilizers. This fertilizer, with its high microbial diversity, regulators and plant growth hormones such as humic acids, auxins and gibberellins, and high amounts of macro and microelements, can stimulate plant growth. Vermicompost contains enzymes such as protease, lipase, amylase, cellulase, ligninase and chitinase, which is effective in biological degradation of soil organic matter. This fertilizer is also rich in vitamins, antibiotics and growth hormones (4, 12-14).

Protein markers, which are considered a type of molecular marker, have two types of storage proteins and enzymes. The first type is highly stable and has a high polymorphism. Although environmental factors affect their amount and they have little effect on their presence in the seed. SDS-PAGE protein electrophoresis was initially performed by Lamelli (1970) and later modified by Fulington et al. (1983). Electrophoresis of proteins has been identified as one of the methods of identification, genetic diversity and classification of plant varieties. Plants are able to activate their compatible mechanisms in response to environmental factors and react to environmental factors by changing their expressions, so the proteomics technique is used to identify the proteins that respond to these factors (5, 8).

The aim of this study was to investigate protein changes in response to changes in planting dates and the use of different levels of vermicompost during growth of fennel plants.

Materials and Methods

Location and treatments

This experiment was carried out at the Research Farm of Agricultural and Natural Resources Campus of Razi University, Kermanshah, Iran and laboratories of

the Medical Biology Research Center of Kermanshah University of Medical Sciences. Experiments were carried out in two phases of field and laboratory. In the first stage, the treatments were applied and sampled from the leaves of the plant. Three sowing dates May 31 (A), July 31 (B) and September 31 (C)) and four different vermicomposts (0, 5, 10, and 15 t/ha) and a chemical fertilizer treatment (NPK) based on fertilizer requirement were evaluated.

Layout characterization

Preparation of plots with intervals of 75 cm. Each experimental unit was designed with a total area of 16 m^2. The time was the main plot and fertilizer treatments as subplots. After preparation of the seedbed, vermicompost fertilizer was carefully distributed in plots and mixed with soil. The treatments were applied to Fenugreek. Irrigation was carried out on the basis of field capacity and soil moisture was maintained until the application of the treatments at the field capacity level. Protein test specimens were placed in liquid nitrogen flask after isolation from the plant and then transported to a freezer temperature of 80 °C until the onset of protein experiments.

Protein studies

In order to protein extraction, four g of each leaf sample powdered in liquid nitrogen. 100 mg of powder was obtained in 1 ml of cold acetone containing 10% (w/v), Trichloroacetic acid (TCA) and 0.07% (w/v) of Dithiothreitol (DTT) in a microtip and after the vortex, the microtips incubated at a temperature of 20 °C for one hour to precipitate the proteins. Samples were centrifuged at 20 °C for 4 min at 20 °C for 20 min after leaving the freezer. After centrifugation, the samples were washed three times with cold acetone solution containing 0.07% diothirotryl and at the end after removing the supernatant, the precipitate was dried at room temperature. For removal of mucilages and purification of proteins, 50 ml of the sample was added from 450 ml of chloroform.

Dissolving of protein

The following method was used to dissolve proteins. To each microtube containing the precipitated stage, 350 µl of lysis buffer and 3.5 µl of PMSF were added. The solution was continuously vortexed for one hour at 40 °C. Then microtips were centrifuged at 13500 g and 10 °C for 15 min. From the supernatant, a small amount (about 5 µl) was taken to determine the protein concentration and, according to the results of the concentration determination, the rest of the sample was prepared for loading to determine the protein concentration desired by the Bradford method as the following: First, to the standard curve, the fifteen test tubes were prepared and 2.5 ml Bradford solution was poured into each one. Then, different concentrations of albumin (BSA) and lysis buffer were combined. Then the tubes were vortexed and incubated at room temperature for 10 min and then read their absorption at 595 nm. Finally, the standard curve was drawn.

First dimension: Isoelectric focusing in the IPG strip (IPG-I)

The rehydration of IPG strips (pH 3-10, 18 cm, GE Healthcare) was performed. The IPG strips were removed from the freezer and allowed to reach room temperature. According to the colourimetric method (Coomassie Blue R-250) and IPG strip (18 cm in length), about 250 µl of protein solution with a total concentration of 12 µg/µl was taken. The protein solution was loaded along the tray slots. The full rehydration was carried out during the overnight.

The isoelectric focusing of proteins: IPG-IEF

First, the ceramic tray was clean and dry. The strips were placed face down on a ceramic tray so that the positive pole of the strip was placed on the positive side of the tray and the negative pole on its negative side. Two paper electrode wicks were removed and soaked with 500 µl of distilled water and gently placed on the two ends of the strip. The electrodes were placed on two strips of gel and the strips were covered with mineral oil. The desired program was given to the device and after completing the program, the gels were immediately put into the balancing solution.

Second dimension: SDS-PAGE

Preparation of acrylamide gel

To prepare the acrylamide gel, the first running gel buffer (pH 8.8) and acrylamide stock were prepared.

Then, the materials were mixed. After the addition the tetramethylethylenediamine (TEMED), the solution was quickly poured in the distance between the two glasses, so that the upper surface of the gel was about one centimetre away from the edge of the glass. The dish was kept at room temperature for at least one hour until the acrylamide was completely polymerized.

Balancing of IPG strips and performance of the second dimension

For each strip, 10 ml of the balancing solution removed from the freezer to reach room temperature. The first balancing solution should contain 1% DTT. The solution was poured onto IPG strips and placed on a shaker for 20 min. Then the first solution was thoroughly discarded and the second solution, containing 2% iodoacetamide was added to the IPG strips and again placed on a shaker for 20 min.

After the completion of the balancing steps, the IPG strip was placed gently on the upper edge of the acrylamide gel, and the 0.5% melted agarose was poured into it. After filling the electrophoresis tank to the required volume from the electrode buffer, the electrophoresis was adjusted to 175 volts. The proteins were set up to run until the electrophoresis on the second dimension. To determine the molecular weight of the dots, a protein marker including proteins of a specific molecular weight containing ovotransferrin (78 kDa), albino bovine (66 kDa), ovalbumin (45 kDa), actinidine (29 kDa), beta-lactoglobulin (18 kDa) and lysozyme (14 kDa) were used as molecular weight standards in the gel.

Colouring and decolouring of the gel for the appearance of protein

The gel was placed in a container and added 200

ml of the colouring solution and placed on a shaker for about 12 h. After colouring, the colouring solution was removed, the gel washed with distilled water, then added a decolouring solution to the container and placed on a shaker for at least one hour. After dyeing, the decolouring solution was removed and replaced with fresh solution. This continued until the gel was clear and the protein dots were clearly visible.

Acquisition of images from SDS-PAGE and two-dimensional gels

The complex protein mixture that has been separated by two-dimensional electrophoresis and isolated as separate proteins, was extracted to acquire two-dimensional gel images after staining using a Bio-Rad GS-800 scanner. Similar operations were performed on gels from the SDS-PAGE test.

Results

SDS-PAGE

After electrophoresis and staining of the gel, as shown in Fig. 1, the protein bands of the leaves of the Fenugreek plant appeared well. Most of the bands were the same among all the samples, but there were some interesting differences between the samples, which could be due to the difference in sampling date and the effect of different vermicompost values, that have been shown as an arrow in the image. The polymorphism may be as down or up gene regulation or seen in switch on or off gene expression. One of the cases found in this study was a polymorphism, a band at a molecular weight of about 80 kDa which appeared on the first planting date (May 31) in all samples except A1 (10 t/ha vermicompost on April 31). Another significant difference was the band in the samples from the third planting date (September 31) and in the molecular weight of about 15 kDa, which cannot be seen on other planting dates. This difference can be due to the synthesis of this protein under the conditions of lowering the temperature and shortening the day length of the early fall.

Amini and Ehsanpour (2009) reported the protein changes of Isfahani tomato plants under salinity stress as SDS-PAGE and two-dimensional electrophoresis qualitatively and ophthalmologically. They observed some bands with different thicknesses in salinity stress and normal conditions (15).

Selection of samples with the highest difference in the SDS-PAGE gel for performing 2D electrophoresis

Based on comparing the protein pattern of different treatments in the SDS-PAGE experiment, considering that in the sowing date of September 31, a more distinct polymorphism was observed among different fertilizer treatments. Two samples C1 (application of 15 t/ha of vermicompost on September 31) and C5 (planting on September 31 without fertilizer application) were selected as two samples with a significant difference of

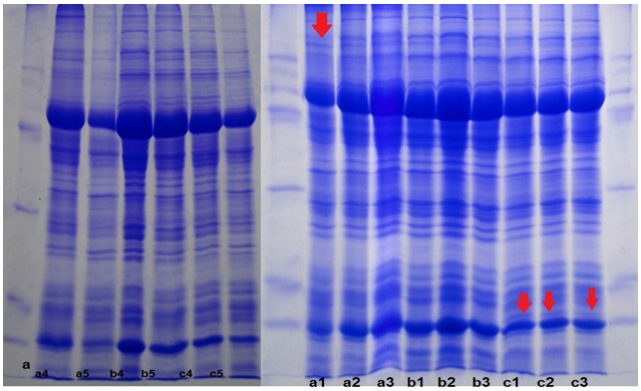

Figure 1. Protein pattern of fenugreek leaf in polyacrylamide gel. a, b and c: planting date (on May 31, July 31 and September 31, respectively) and 1- 5: different fertilizer treatments (15, 10, 5 t/ha vermicompost, NPK and control respectively). Protein size markers: ovotransferrin (78 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), ctinidi (29 kDa), lactoglobulin (18 kDa) and lysozyme (14 kDa)

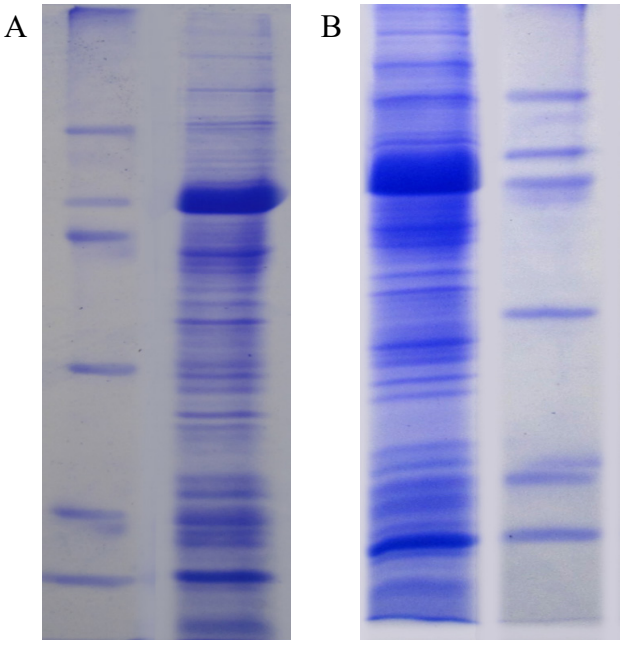

Figure 2. Selected samples from SDS-PAGE for two-dimensional electrophoresis in fenugreek. A. C5 sample (right) with the marker (left), B) sample C1 (left with the marker (right).

about 15 kDa in SDS-PAGE for two-dimensional electrophoresis. The results of the SDS-PAGE of the above two samples separately and with the size marker is shown in Fig. 2.

Image acquisition and analysis of two-dimensional gels

The scan of two-dimensional gel images of the proteins of C1 and C5 samples have been shown in Figures 3 and 4, respectively. Two-dimensional electrophoresis experiments were performed in this experiment by comparing the protein points of the C1 and C5 samples.

In the two-dimensional electrophoresis experiment, significant differences were observed in the severity of the protein dots, or the removal and addition of dots between the plants for the application of vermicompost and control. Among the observed points of the gels, about 16 dots or areas were identified by eye analysis, which had the greatest difference between the two gels (Fig. 5).

These modified dots can be classified into three different groups. Group 1: dots that their expression increased in plants that received vermicompost. Group 2: Protein dots that originated in plants that received NPK fertilizer. Group 3: Protein dots that declined or disappeared in plants that did not receive fertilizer (control).

As shown in Figure 5, in the range of 14 kDa and PI is about 4.5-4.7 (circle 16), the lack of protein expression with the above characteristics is observed, which is consistent with the results of the SDS-PAGE test (Fig. 2). Another dot was observed with the same molecular weight, but PI is about 3.5% in the treatment of vermicompost fertilizer. However, this dot was not found in the treatment without fertilizer (circle number 14). This result is also consistent with the results of the SDS-PAGE test. The expression of this protein range can be due to the use of vermicompost fertilizer.

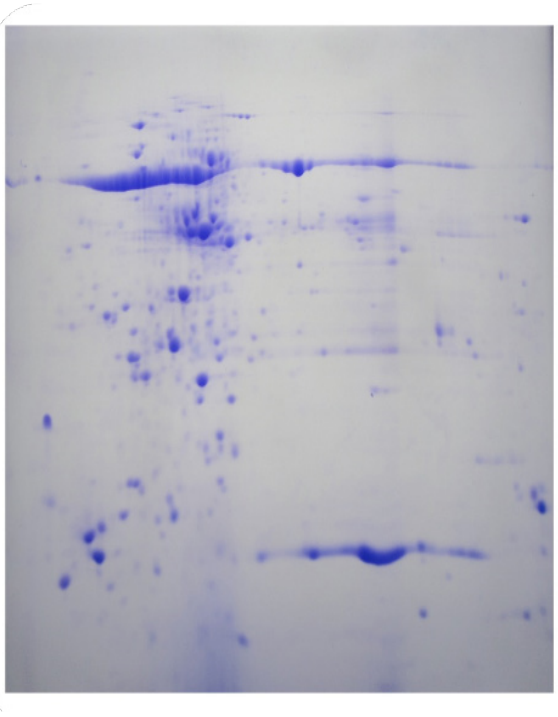

Figure 3. 2D gel sample of C1 for the planting of fenugreek on September 31 and application 15 t/ha of vermicompost.

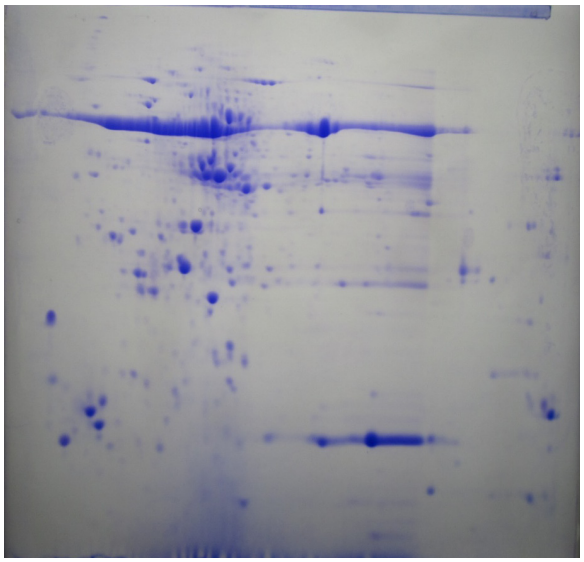

Figure 4. 2D gel sample of C5 for the planting of fenugreek on September 31 and without fertilizer application.

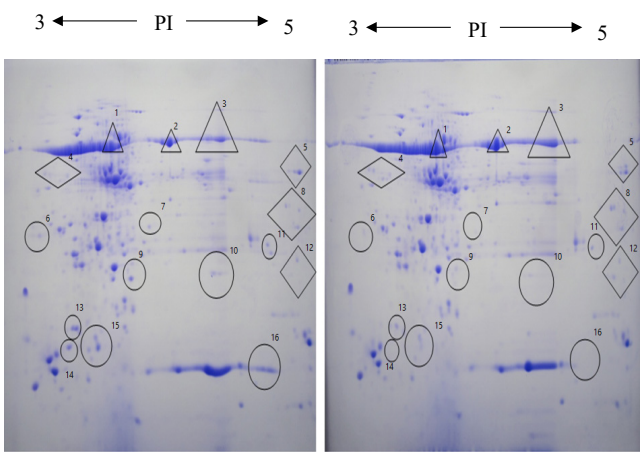

Figure 5. Two-dimensional of the Fenugreek protein leaves (right: sample C5 and left: sample C1). Triangle: Difference in protein expression (Up and down). Increasing protein expression in a plant that has not received fertilizer. Diamond: Increasing protein expression in a plant that has not received fertilizer rather than those plants that have received vermicompost. Circle: Increasing protein expression in a plant that has received vermicompost rather than those plants that have not received fertilizer.

One of the other significant differences observed in the range of 16 kDa and PI about 3.6 (circle number 15), which has increased significantly when fertilizer was used. Similar results in other areas of the gel include the range of 29 kDa and PI of about 4.8 (circle 11), the range of 25 kDa and PI is about 4.5 (circle 10), the range of 29 kDa and the PI is about 3.9 (circle number 9), the range of 32 kD and PI is about 3.2 (circle number 6) and the range of 35 kD and PI is about 4 (circle number 7), which was not visible in the SDS-PAGE experiment because in SDS-PAGE the proteins are separated only the basis of the molecular weight.

Increasing the expression protein in the molecular weight range of about 47 kDa in PI is about 4.5 (triangle number 3), PI is about 1.4 (triangle number 2) and PI is about 3.6 (triangle number 1) is clearly visible, which can indicate the effect vermicompost fertilizer in increasing of this category of proteins.

Discussion

For many proteomics experiments, one-dimensional electrophoresis method is used for selecting a protein among of mixture proteins. In a one-dimensional electrophoresis, proteins are separated based on their molecular weight. One-dimensional electrophoresis is used simply to isolate proteins of molecular weight of 10 to 300 kDa. The most common application of one-dimensional electrophoresis is the description of proteins after purification. If we encounter protein complexes, we should use two-dimensional electrophoresis. In two-dimensional electrophoresis, proteins are separated by two distinct properties, they are first separated in the first dimension based on pure charge, and then in the second dimension, based on molecular weight. The combination of these two techniques gives rise to higher resolution (detection power) than one-dimensional electrophoresis. One of the high abilities of two-dimensional electrophoresis is that it can isolate proteins influenced by some changes after translation (16).

In this experiment, more differences were observed in the analysis of the second dimension of the protein that they were not found in the SDS-PAGE that protein band patterns were similar in the most of the samples. However, a significant difference was observed in the samples of the third planting date (31 September) and in the 15 kDa molecular weight which was not seen in the other planting times. This difference can be due to the synthesis of this protein in the case of decreasing the temperature or shortening the day length in early autumn.

Exposure of plants at low temperatures or their growth in temperature-varying environments causes changes in their physiological characteristics and metabolism. For example, cold creates relatively similar changes to other environmental stresses in plant metabolism (17).

Nielsen et al. (2010) reported that especially high-temperature fluctuations due to global warming have a great impact on crop production. For example, high-temperature stress causes differences in protein pattern in stressed rice versus control, resulting in reduced yields. They also reported that under non-biological stress conditions, rice contains a protein of 15 kDa molecular weight, which was related to mannose-bound lectin protein (18).

A protein of similar molecular weight was also observed in this experiment, but it was necessary to test for confirmation such as sequencing to determine the type of protein.

In molecular weights of about 80 kDa on the first planting date (May 31) in all samples except a1 (10 t/ha vermicompost on May 31) was observed, which can be due to the use of vermicompost.

Amini and Ehsanpour (2009) reported the results of the SDS-PAGE and second dimension of a proteomic experiment in the study of protein changes of Isfahani tomato plants in Isfahan. They also showed up, down, on and off bands in two conditions of salinity and normal stress in the first and second dimensions (15).

Miraghaee (2012) compared the protein pattern of adapted Kiwi to the Kermanshah conditions and in the north of Iran using two-dimensional electrophoresis. The more differences observed between the two samples of kiwifruit in the two-dimensional electrophoresis than the SDS-PAGE (19).

There is no report on the effect of vermicompost on the protein pattern of the plant for comparison with the results of this study. However, the results of two-dimensional electrophoresis experiments showed more pronounced differences. Identification of these differences, especially dot No. 68 which seems to be mass spectrophotometry and then sequencing.

In this research, it can generally be concluded that factors such as planting date, which include factors such as day length or temperature, as well as different amounts of vermicompost, affect the protein pattern of the Fenugreek plant. These effects were more remarkable in some dots, and elsewhere, weakly increasing or decreasing expression.

References

1. Eftekharinasab N, Zarei D, Paidar S et al. Identification of wild medicinal plant in Dalahoo mountain and their used indigenous knowledge (Kermanshah, Iran). Ann Biol Res. 2012; 3(7): 3234-3239.

2. Kahrizi D, Molsaghi M, Faramarzi A et al. Medicinal plants in holy Quran. Am J Sci Res. 2012; 42: 62-71.

3. Kahrizi D, Rostami Ahmadvandi H, Akbarabadi A. Feasibility Cultivation of Camelina (Camelina sativa) as Medicinal-Oil Plant in Rainfed Conditions in Kermanshah-Iran's First Report. *J Asian Nat Prod Res.* 2015; 2: 215-217.

4. Kahrizi D, Soorni J. Study on shoot regeneration and somatic embryogenesis in cumin (Cuminum cyminum L.) landraces. Biharean Biologist. 2013; 7(1): 37-41.

5. Masoumi SM, Kahrizi D, Rostami-Ahmadvandi H et al. Genetic diversity study of some medicinal plant accessions belong to Apiaceae family based on seed storage proteins patterns. Mol Biol Rep. 2012; 39(12): 10361-10365.

6. Molsaghi M, Moieni A, Kahrizi D. Efficient protocol for rapid Aloe vera micropropagation. Pharmaceuti Biol. 2014; 52(6): 735-739.

7. Rostami-Ahmadvandi H, Cheghamirza K, Kahrizi D, Bahraminejad S. Comparison of morpho-agronomic traits versus RAPD and ISSR markers in order to evaluate genetic diversity among Cuminum cyminum L. accessions. Aust J Crop Sci. 2013; 7(3): 361.

8. Rostami-Ahmadvandi H, Kahrizi D, Zebarjadi A, Mostafaie A, Sohrabi-Babahadi F, Kiani S. Study of Peganum harmala genetic diversity based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. J Agric Sci Technol A. 2011; 1: 1300-1302.

9. Soorni J, Kahrizi D, Molsaghi M. The effects of photoperiod and 2, 4-D concentrations on callus induction of Cuminum cyminum's leaf explant: an important medicinal plant. Asian J Biol Sci. 2012; 5(7): 378-383.

10. Aalok A, Tripathi A, Soni P. Vermicomposting: a better option for organic solid waste management. J Hum Ecol. 2008; 24(1): 59-64.

11. Domfnguez J. 20 State-of-the-Art and New Perspectives on VermicompostingResearch. Earthworm ecology: CRC Press Boca Raton, FL, USA; 2004: 401-424.

12. Dominguez J, Edwards C, Subler S. A comparison of vermicomposting and composting. Biocycle. 1997; 38: 57-59.

13. Jeyabal A, Kuppuswamy G. Recycling of organic wastes for the production of vermicompost and its response in rice–legume cropping system and soil fertility. Eur J Agron. 2001; 15(3): 153-170.

14. Rupani PF, Singh RP, Ibrahim MH, Esa N. Review of current palm oil mill effluent (POME) treatment methods: vermicomposting as a sustainable practice. World Appl Sci J. 2010; 11(1): 70-81.