African Journal of Biotechnology

Volume 15 Number 15, 13 April 2016

ISSN 1684-5315

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Vol. 15(15), pp. 565- 570, 13 April, 2016
DOI: 10.5897/AJB2015.15171
Article Number: A210D9558047
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Spectrophotometric method for quantification of soil microbial biomass carbon

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Received 14 December, 2015; Accepted 2 March, 2016

The search for more suitable alternatives in analytical processes is a strategy to reduce environmental damages. The present study aimed to quantify the soil microbial biomass carbon (SMB-C), establishing a comparison between the methods involving quantification of SMB-C by titration of the samples with ferrous sulfate ammonia and molecular absorption spectrophotometry methods. This comparison was performed by two soil sample tests: (i) areas of grain crops with conventional management versus no-till farming; and (ii) areas with distinct phytophysiognomies in the region southern Mato Grosso do Sul state (Cerrado and Semideciduous Forest). It was found that molecular absorption spectrophotometry was an efficient tool for the determination of soil microbial biomass carbon, allowing replacement of the titrimetric method. There were significant differences in the levels of SMB-C determined spectrophotometrically in relation to those determined by titration. However, for the levels of SMB-C determined by spectrophotometry to be compared with those determined by titration, the values must be corrected by the linear regression equation $Y_{spectrophotometry} = - 151.38 + 0.92532 * X_{titration}$.

Key words: Potassium dichromate, soil quality, titration.

INTRODUCTION

The microbial biomass is the living and most active soil organic matter, being formed primarily of fungi, bacteria and actinomycetes (Jenkinson and Ladd, 1981; Roscoe et al., 2006). The determination of soil microbial biomass carbon (SMB-C) has been used to assess the size of the most active and dynamic reservoir of soil organic matter (Oliveira et al., 2001). Adequate levels of soil microbial biomass (BMS) are essential to the maintenance and productivity of agroecosystems, which depend in large part on processes mediated by microorganisms (Tótola and Chaer, 2002; Mendes et al., 2011).

In this context, the importance of quantifying BMS has been highlighted in gauging the quality of soil, being considered the most sensitive indicator to detect changes in agroecosystems (Mercante et al., 2008; Hungary et al., 2009). Several indicators of soil quality, including

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chemical, physical and biological attributes, have been evaluated for this purpose (Kaschuk et al., 2010). The search for the standardization of an efficient method for determination of BMS is aimed at greater practicality, minimization of the time needed analysis and adequate repeatability, thereby allowing the construction of a database for subsequent identification of soil quality indexes (Roscoe et al., 2006; Mendes et al., 2011). According to Faleiro et al. (2011), finding new standard and reproducible methods to analyze soil microbial biomass, able to avoid or reduce the use of toxic chemicals, is a challenge.

Currently, different methods can be employed to determine BMS. Among the most used are chloroform fumigation-incubation (CFI) and chloroform fumigation-extraction (CFE), described by Jenkinson and Powlson (1976) and Vance et al. (1987), respectively. Both are based on the partial sterilization of soil samples through fumigation with chloroform. The efficiency of these two methods was compared in studies conducted in acidic soils in Australia and New Zealand. According to Brandão-Junior et al. (2008), the CFI was the pioneer and is most widely used as the standard for the calibration of other methods to quantify SMB-C. However, certain analytical methods employed in the quantification of SMB-C, although efficient, require a long time for implementation, in addition to need to handle acids and carcinogenic substances, generating toxic compounds that cannot be discarded without treatment into the environment, such as potassium dichromate. This substance is soluble in water and highly toxic, because it freely diffuses through cell membranes and is a strong oxidizing agent (Knupp e Ferreira, 2011). According to Kotás and Stasicka (2000), chromium (Cr VI), in high concentrations, can cause serious cell damage, due to its high ability to interact with various organic compounds such as important functional enzymes, inhibiting them.

To reduce the pollution of soil and water, development of new methods and/or validation of methods are fundamental to obtain the most appropriate alternatives to reduce the toxic waste generated by the analytical processes. In this sense, spectrophotometry is gaining prominence for the determination of soil microbial biomass carbon (SMB-C) due to its speed, low cost and wide availability in laboratories (Marques et al., 2012). The aim of this study was to compare the methods of titration and molecular absorption spectrophotometry for measurement of soil microbial biomass.

MATERIALS AND METHODS

Characterizations of the experimental area and management systems

Considering that the microbial biomass controls the decomposition and accumulation of organic matter in the environment and is affected by environmental conditions, soil samples were collected in two experimental areas, as follows.

Table 1. Geographical location, altitude and soil type of the experimental areas with native vegetation (Semideciduous Forest and Cerrado) where soil samples were collected in the southern region of Mato Grosso do Sul [1].

[1]Soil	Latitude	Longitude	Altitude (m)
Semideciduous forest			
LVdf	20° 17 ’’05 ’S	54° 48 ' 37 ’’W	385
LV	22° 04 45 ’S ’’	55° 22 33 ’’W ’	428
Cerrado			
LVdf	22° 17 ’’’32 S	54° 48 ’’’26 W	381
LV	22° ’45 ’S ’05	55° 15 ’’’22 W	451

[1]LVdf and LV: typical Hapludox and Hapludox, respectively.

Test 1 – Comparison of conventional handling systems and tillage

The soil samples were collected as part of a long-term experiment (established in 1995) in an native forest fragment area (semideciduous forest), used as a reference for the original condition of the soil, and in two comparative management systems established in the experimental field of the Embrapa Western Agricultural Research Unit (Embrapa Agropecuária Oeste), in Dourados-MS (22° 16' S and 54° 49' W), in a typical Dystrophic Red Latosol with highly clayey texture. There were two soil management systems. The first was conventional (CS), consisting of planting soybeans (*Glycine max* (l.) Merrill) in summer and of oats (*Avena strigosa* Schreb) in autumn/winter, where the soil was prepared before each cultivation with disk harrows to 0.20 m depth, with pre-emergence herbicide application, in a 2.0 ha area. The second was a no-till system (NT), planted with soybeans and corn in the summer, rotated with wheat, oats and forage turnip in winter and millet in the spring. The soil sampling was carried out in July, 2012, at a depth of 0 to 0.10 m, with a Dutch auger, to obtain five composite samples from each system.

Test 2 – Evaluation of two phytophysiognomies (Cerrado and Semideciduous Forest) in the south of Mato Grosso do Sul

The soil sampling was carried out in April, 2013, in two distinct natural phytophysiognomies, in the southern region of Mato Grosso do Sul state, in a Red Latosol, with flat relief. The systems of native vegetation were selected on the basis of preliminary floristic surveys (Arruda and Daniel, 2007; Gomes et al., 2007), taking into consideration the two phytophysiognomies. The collections were made in four separate fragments (Table 1), at a depth of 0 to 0.10 m, to collect five samples in each system.

Determination of soil microbial biomass

Two methods were used to quantify the soil microbial biomass carbon (SMB-C): titration and molecular absorption spectrophotometry. The process of preparation and extraction of carbon was identical in both methods. Initially, soil samples were collected, homogenized and packed in plastic bags duly identified and stored

Table 2. Soil microbial biomass carbon (SMB-C), evaluated by spectrophotometry and titration methods, in soil samples collected in an experimental area under conventional system (CS), no-till system (NT) and native vegetation (NV), in typical Hapludox soil.

Systems	Spectrophotometry (μ g C g $^{-1}$ dry soil)	Titration (μ g C g $^{-1}$ dry soil)
NT	252^{abB}	426^{bA}
CS	158^{bB}	361^{bA}
NV	370^{aB}	637^{aA}

Values followed by lowercase letters in columns indicate average contrast between soil management systems and capital letters in rows indicate comparison between the spectrophotometry and titration, by the Tukey test at 5% probability.

in a cold chamber (±4°C), until analysis. The soil samples were ground, homogenized and sieved (< 2 mm), sprayed with distilled and deionized water to maximum capacity, and kept in closed containers for 12 h. After this step, the samples were weighed in cylindrical glass bottles with snap-on lids, using six aliquots of 20 g, repeated three times, for the determination of SMB-C, of which three subsamples were fumigated (packed in the desiccator with 10 mL of chloroform ($CHCl_3$), for 12 h), and three were not fumigated. For extraction of SMB-C, the sub-samples received 50 ml of potassium sulfate (K_2SO_4) 0.5 mol L^{-1}, being agitated horizontally at 30 rpm for 220 min.

Then, they were filtered through quantitative filter paper (125 mm) for separation of the extract. Soil moisture was determined by placing 50 g of moist samples in an oven at 105°C for 12 h and then weighed again.

For titration, an aliquot of 8.0 ml of each extract was placed in an Erlenmeyer flask and 2.0 ml of potassium dichromate ($K_2Cr_2O_7$), 0.066 mol L^{-1}, 5 ml of concentrated phosphoric acid (H_3PO_4) and 10 ml of concentrated sulfuric acid (H_2SO_4) were added. Then, the extract was heated over a hot plate (± 300°C) for 5 min and then cooled, after which 80 ml of distilled and deionized water was added. The aliquots were titrated with ferrous ammonium sulfate [(NH_4Fe $(SO_4)_{2.2}$ 6:00 0.033 mol L]$^{-1}$, using 1% diphenylamine ($(C_6H_5)_2NH$) as indicator (turning point of violet to green).

In the spectrophotometric method, a 2 ml aliquot of the same extract used for titration was placed in a test tube. Then, 3.0 ml of distilled and deionized water, 2.5 ml of working solution (300 ml of sodium pyrophosphate ($Na_2P_2O_7$) 0.1 mol L^{-1}, 46 ml of sulfuric acid (H_2SO_4) 0.5 mol L^{-1}, 20 ml of potassium permanganate ($Na_2P_2O_7$) 0.1 mol L^{-1}, 80 ml of manganese sulfate monohydrate ($MnSO_4H_2O$) 0.1 mol L^{-1}) and 2.5 ml of sulfuric acid (H_2SO_4) were added to each aliquot. The samples were then vortexed and left at rest for 2 h, and then submitted to molecular absorption spectrophotometry at a wavelength of 495 nm (Bartlett and Ross, 1988).

The results were submitted to analysis of variance and means were compared by Tukey test, with 5% probability. The statistical analyses were carried out with the Assistat program (Silva and Azevedo, 2009). The average values of SMB-C obtained in the two experiments were subjected to regression analysis, through the use of SAEG software (v. 9.1-2007), to establish the model best fitted to the levels determined by spectrophotometry versus those obtained by titration.

Among the regression models tested (linear, quadratic, and exponential square root), the linear model had the highest coefficient of determination, with significance of at least 5%.

RESULTS AND DISCUSSION

In the first test, we compared the levels of SMB-C obtained in native forest in relation to the two management systems (no-till and conventional system), in a typical Hapludox with highly clayey texture (Table 2). The values ranged between 159 (CS) and 637 μg C g^{-1} dry soil (native forest). In both the analytical methods evaluated, the no-till system provided higher SMB-C values compared to management under conventional tillage, which was expected, since in the no-till system the soil micro-aggregates are maintained, preserving the main niche for activity of microorganisms (Mendes et al., 2003). A similar result was observed by Balota et al. (1998), when evaluating SMB-C in soils submitted to successions of wheat/wheat/corn and soybean, under conventional and zero tillage.

The values of SMB-C determined in soil samples collected in the two locations, with two distinct natural physiognomies (Semideciduous Forest and Cerrado) (Table 3), ranged from 218 to 761 μg C g^{-1} dry soil. The samples collected in the forest area showed significantly higher values ($p < 0.05$) than those found in the Cerrado area. According to a study conducted in different native biomes in Brazil (Roscoe et al., 2006), the soil from the forest area had the highest figures. According to Borges et al. (2009), these values can be explained by the denser formation and the presence of a higher stratum in native vegetation found in forest systems. The absence of tillage, greater floristic diversity, maintenance of fungal hyphae and accumulation of litter on the soil surface contribute to more favorable conditions in forest areas than in cropped areas (Mercante et al., 2008). According to Roscoe et al. (2006), the main cause of low SMB-C values in soils with natural vegetation in Cerrado areas is due to the low concentrations of total organic carbon in this ecosystem. This result shows a significant correlation for Brazilian soils, since the measurements were performed in soils with varied textures, vegetation and management systems. When comparing the analytical methods evaluated (Tables 2 and 3), the levels of SMB-C determined by titration were significantly higher ($p < 0.05$) than the values obtained by spectrophotometry, regardless of the type of soil management adopted.

The joint analysis of the means obtained in two runs allowed fitting a linear regression model, with a high

Table 3. Soil microbial biomass carbon (SMB-C), evaluated by spectrophotometry and titration, in samples collected in two experimental areas with two soil types and systems of native vegetation (NV) and Cerrado, in the southern region of Mato Grosso do Sul state.

[1]Soil	System	Spectrophotometry (μ g C g $^{-1}$ dry soil)	Titration (μ g C g $^{-1}$ dry soil)
LVdf	Mata	593[aA]	761[aA]
LV	Mata	495[bA]	660[aA]
LVdf	Cerrado	314[cA]	548[bA]
LV	Cerrado	218[abA]	347[bB]

[1]LVdf and LV: typical Hapludox and Hapludox, respectively. Values followed by lowercase letters in columns indicate average contrast between soil management systems and capital letters in rows indicate comparison between the spectrophotometry and titration, by the Tukey test at 5% probability.

correlation coefficient (r = 0.957). This indicates a close relationship between the two analytical methods evaluated. However, according to Oliveira and Leite (2002), the simple observation of a high correlation coefficient between two analytical methods does not mean the results obtained are statistically identical.

Thus, these authors proposed a statistical procedure that combines the F-test with testing the average error and the linear correlation coefficient to check whether the results obtained through an alternate analytical method are equivalent to those obtained by a standard method. From the average SMB-C obtained in the two tests, we estimated the value of F (F_{calc} = 52.23), which was significantly higher ($p < 0.01$) to the F-value in the standard table for 2 degrees of freedom for the treatment and 5 degrees of freedom for the error ($F_{(2.5)}$ = 13.27). According to Leite and Oliveira (2002), in this situation the null hypothesis is rejected and it is assumed that the intercept (b_0) and angular coefficient (b_1) of the adjusted linear regression equation are zero and one, respectively, at 1% significance. These authors point out, however, that in conditions where a high correlation coefficient is obtained, the calculated F-value is greater, increasing the probability of rejecting the null hypothesis mistakenly. For this reason, they recommend applying the t-test in complement to average value $\left\{\bar{e} = \left[\sum \frac{(Y-X)}{X}\right] / n\right\}$ and comparing the correlation coefficients and the average errors [r_{yiyj} in relation to $(1-|\bar{e}|)$].

In this work, the means obtained resulted in error of -0.380, calculated t-value (t_{calc}) of -8.733 and tabled t-value (t_{tab}, in a bilateral test with 6 degrees of freedom for the residuals, at 1% probability) of 3.710. In this way, the value of t_{calc} in absolute value was higher than the value of t_{tab}, so it can be concluded that the differences between the levels determined by two analytical methods evaluated are caused randomly. Finally, since the correlation coefficient is 0.957 (Figure 1) and the expression $(1-|\bar{e}|)$ presents a value of 0.620, then $r \geq (1-|\bar{e}|)$. According to the criteria recommended by Leite and Oliveira (2002), the rejection of null hypothesis for $b_0 = 0$ and $b_1 = 1$, coupled with the rejection of the null hypothesis for $b_0 = 0$ and $b_1 = 1$ and the condition that $r \geq (1-|\bar{e}|)$ means that the two methods of determining SMB-C are statistically different.

In other words, there is a systematic discrepancy of 151.38 μg g^{-1} in levels of SMB-C obtained by means of spectrophotometry in relation to those determined by titration. Note also that there is a difference in sensitivity between the analytical methods evaluated, that is the amplitude of variation in levels of SMB-C determined through spectrophotometry is greater than that determined by the standard method. In this context, the results obtained by both analytical method can be compared, so one can correct the SMB-C value obtained by spectrophotometry using the linear regression equation $y_{spectrophotometry} = -151.38 + 0.92532 * x_{titration}$.

The feasibility of assessing SMB-C by spectrophotometry is important since it allows direct determination of carbon in soil extract, which avoids problems related to the variability of results by perception of the turning point in the titration process (Duda et al., 2005). Another advantage of spectrophotometry is the greater agility during the carbon quantification process, due to the possibility of prior preparation of the working solution in significant volumes (2 liters). On average, for a period of eight hours of work, the use of the titration method allows quantification of SMB-C in 10 samples, repeated three times, totaling 30 subsamples. On the other hand, by spectrophotometry in five hours, it is possible to analyze 20 samples with three repetitions, for a total of 60 subsamples.

Finally, spectrophotometry allows the use of potassium permanganate instead of potassium dichromate, which is potentially toxic and carcinogenic (Stout et al., 2008), as well as the reduction of approximately 75% of the need for sulfuric acid, resulting in less toxic waste generation.

In addition to this substantial reduction in environmental impact, the adoption of the spectrophotometric method results in a considerable reduction in the cost of

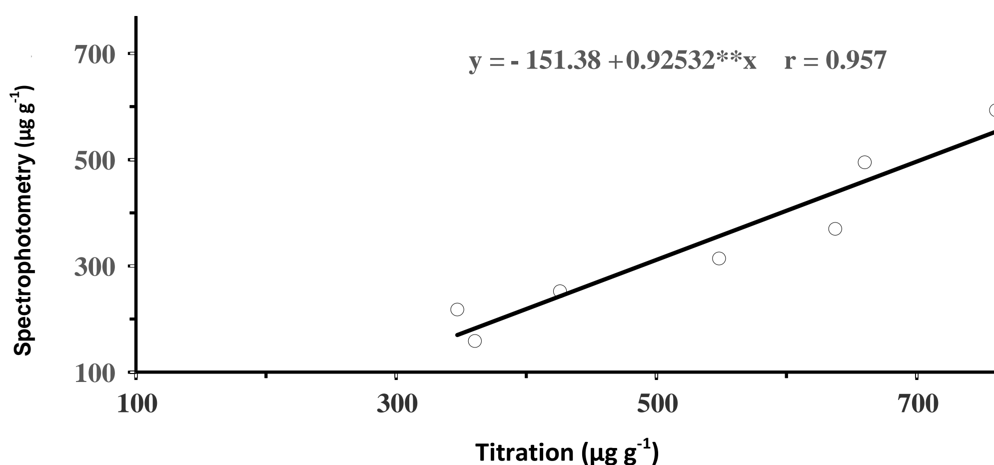

Figure 1. Linear regression between the levels of soil microbial biomass carbon (SMB-C), determined by molecular absorption spectrophotometry and titration in soil samples collected in areas under three management systems (conventional, no-till and native vegetation), two Cerrado systems and two Semidecidual forest in the experimental field of the Embrapa Western Agricultural Research Unit (Embrapa Agropecuária Oeste), Dourados-MS.

laboratory tests, since the phosphoric acid used to digest the solution in the titrimetric method has an average cost of more than five times that of the potassium permanganate used in spectrophotometry. Spectrophotometry is also better for human health, since the technician will not be exposed to potassium dichromate. Therefore, molecular absorption spectrophotometry can be an efficient tool for the evaluation of SMB-C, with the potential to replace the titration method (Ka and Ferreira, 2011).

Conclusions

1. Spectrophotometry is an efficient tool for determination of soil microbial biomass carbon, allowing it to replace the titrimetric method.
2. There are significant differences in the levels of SMB-C determined by spectrophotometry in relation to those determined by titration.
3. The SMB-C values obtained by spectrophotometry can be compared with those determined by titration by applying the linear regression equation $y_{spectrophotometry} = -151.38 + 0.92532 * x_{titration}$.

Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 15(15), pp. 571-579, 13 April, 2016
DOI: 10.5897/AJB2015.15098
Article Number: 91FD99458061
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Prospects of genetic modified maize crop in Africa

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Received 11 November, 2015; Accepted 11 March, 2016

Genetic modified maize crop increases annually as a result of food insecurity and limited land caused by rapid population increase of over seven billion in the world. Scientists have been playing their role to address this food insecurity problem. The use of genetically modified (GM) maize crop to feed people is one of the proposed ways, because it yields more compared to the conventional varieties. However, there are several contradictions which hinder the adoption of this new technology. Some studies have shown that GM maize is risky to human health, animals and not friendly to environmental conservation, which may lead to the death of other bio-diversities. Generally, other studies have supported the consumption of GM maize. However, after being approved by the scientist in the countries concerned, the GM maize varieties which seem to be hazardous to human health must be prohibited in research centres so as to avoid transportation to other countries. Regarding the new technology of GM maize, the conventional method of breeding is still important to keep maize seeds available in the gene bank. Therefore, researchers should consider this for further research issues on maize improvement.

Key words: *Bacillus thuringensis*-maize, *Bacillus thuringensis* protein, conventional breeding, environment, food crisis, genetic modified maize, genetic modified organism, health risk, landraces.

INTRODUCTION

Genetically modified (GM) maize ranked the second most important transgenic crop globally. It is planted in an area that accounts for 24% of global biotech crop area and about 14% of total maize grown globally (James, 2005). Farmers have rapidly adopted genetically modified organism (GMO) technology including GM maize crops (Lawson et al., 2009). GMO technology involves the incorporation of genetic engineering to improve crop productivity since over one billion people in the world face starvation and two billion people suffer from one or more micronutrient deficiencies, especially vitamin A, iodine and iron, often lumped as hidden hunger (Alnwick, 1996). The technology has been adopted by most of the developed countries which previously were not aware of it. GM crops are also used for food security purpose (Chondie and Kebede, 2015). GM crops were first grown commercially worldwide in 1996 (James, 2007). Due to the advancement of biotechnology, a number of GM or transgenic crops carrying novel traits have been developed and released for commercial purpose (Arthur, 2011). In GM maize crops technology, the desired traits are inserted into plant, unlike the conventional breeding methods, where traits from two crops are combined; for instance, maize crops (James, 2013; Chondie and

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Table 1. Genetically modified maize crop development in sub-Saharan Africa (Namuddu and Grumet, 2013).

Country	Crop/Trait	Status
Kenya	Maize/Stem borer resistance	Confined field trials
	Maize drought tolerance	Confined field trials
Mozambique	Maize/Drought tolerance	Stalled: awaiting regulatory framework
South Africa	Maize streak virus resistance	Greenhouse containment
	Maize/Drought tolerance	Greenhouse containment
Tanzania	Maize/Drought tolerance	Stalled: awaiting regulatory framework
Uganda	Maize/Drought tolerance	Confined field trials

Kebede, 2015). GM maize is useful for livestock and human consumption (Clive, 2008).

Resistance to herbicide as well as insect is the most common trait that has been incorporated into GM maize. GM crops contain proteins that make them herbicide tolerant (Ht) and insect resistant (USDA, 2011). Herbicide tolerant crops are engineered to produce one or more proteins which allow them to survive even if sprayed with herbicides (Carman et al., 2013). A number of studies conducted have shown a significant number of GM crops which are approved for human and animal use. Crops containing several GM genes 'stacked' into one plant are among the recommended for consumption (Carman et al., 2013). GM maize varieties are common in some countries like United States of America (USA) (USDA, 2011). The GM maize from USA contains Ht or *Bacillus thuringiensis* traits, or a 'stacked' combination of them (Pioneer Hi-Bred, 2012). Of this, 2004 GMO technology accounted for 23% of all GM crops produced (James, 2004). It has been projected that in the coming decades, human population will reach over nine billion (Pinstrup-Andersen, 2010a, b). Therefore, the production of GM maize should not be stopped so as to make the new approved beneficial traits available to people (James, 2007). The common producers of GM maize globally are dominated by USA having 59% of land for production, followed by Argentina with 20%, Canada and Brazil with 6% each, and China with 5% of land. GM maize is in the initial stage in Africa (Moola and Munnik, 2007). Of all countries in African, only South Africa and Egypt have introduced new commercialized GM maize. However, Zimbabwe, Kenya, Nigeria, Mali, Egypt, and Uganda have also adopted GM maize in Africa. Other countries are still engaging in GM research, whereas others are in trials including Benin, Burkina Faso, Cameroon, Ghana, Malawi, Mauritius, Morocco, Namibia, Niger, Senegal, Tanzania, Tunisia, and Zambia (Moola and Munnik, 2007). Table 1 shows some of the GM crops under research in sub Saharan Africa.

Several studies on GM maize have been done to either introduce herbicide-tolerance (for instance Roundup (R)) or to produce a modified *B. thuringiensis* toxin insecticide or both (Seralini et al., 2014). Results have shown that these GM crops contain new pesticide residues for which new maximum residue levels (MRL) have been established in some countries. However, herbicide (glyphosate) tolerance and *B. thuringiensis* toxins make crop plants inedible to some insects (GineBordonaba, 2011; Hammond et al., 2006). It has been reported that no major physiological changes are attributable to the consumption of GM maize in sub-chronic toxicity studies (GineBordonaba, 2011; Hammond et al., 2006). GM maize crop has offered some solutions to future food production. According to Tirado and Johnston (2010), although solutions to food have been provided by GM maize, there has been increase in farmers' dependence on agro-industries compared to non-GM maize seed which farmers believe to be safe with less cost. An example of GM maize crop which has been approved for cultivation in Europe is MON 810 maize (Monsanto). It contains a Cry gene from the bacterium *B. thuringiensis*, which expresses *B. thuringiensis* protein, a toxin of insecticidal properties (Tirado and Johnston, 2010). Most European countries have banned the cultivation of GM maize, because biotechnological industries are not interested in introducing GM varieties resistant to drought, soil salinity, and cold due to their low marketing potential (Lisowska, 2011). Therefore, this review paper aims to explore the constraints, benefits, risk to human and animals' health and environment as well as the controversy of GM maize.

CONSTRAINTS OF GM MAIZE CROP

Studies about GM maize were reported for the first time in Africa through food aid shipments which were given in Southern Africa during the food crisis that occurred in 2002 (Zerbe, 2004). New GM technology is introduced due to critical food shortages caused by a number of

factors including climate change, limited arable land, water shortage, pests and diseases, debt, collapsing public services, and poor governance (ISF, 2011). However, Africans were not happy with GM maize. They debated against agricultural biotechnology, refusing to use GM products (Zerbe, 2004). Although GM maize prevents hunger, its safety has not been proved in any documents. Therefore, the countries which oppose GM maize could be correct to maintain health status (Konig et al., 2004; Seralini et al., 2009). Rats fed with the genetic modified maize (NK 603, MON 810, and MON 863), revealed 3 new GMOs side effects, which were sex and often dose-dependent (Spiroux de Vendômois et al., 2009). More side effects were observed in kidney and liver, the dietary detoxifying organs (Spiroux de Vendomois et al., 2009). It has been estimated that GM maize crop is taken up quickly than any other agricultural technology which is currently used by 16 million farmers (James, 2007). Maize imports from United States and Mexico seem to be worse in terms of subsistence farmers' trade. Hence, the production has been reported to decrease due to the loss of local varieties of maize and this would lose their resilience to environmental stress through contamination with genetically modified maize (Zietz and Seals, 2006).

European Corn Borer (ECB) is one of the major pests that have been affecting maize (corn) crops in North America for over 60 years (Park et al., 2011). The pest was controlled by dichlorodiphenyltrichloroethane (DDT) which later had negative effects; therefore, it was rejected. Organophosphate and pyrethroids were subsequently used (Park et al., 2011). The strains with high resistance to European corn borer were developed by plant breeders. The mixture showed success with transgenic maize expressing insecticidal protein derived from *B. thuringiensis* (Kaster and Gray, 2005). Avidin, produced commercially in GM maize for use in research and diagnostics, is toxic to certain insects (Park et al., 2011). There are also environmental issues relating to gene flow from GM crops to non-GM crops which may disturb genotypes and lower production. Further studies are needed to confirm the usefulness of GM maize to avoid risk to human and animals (Park et al., 2011). Left-over grains from GM maize modified to express biopharmaceutical compounds have been reported to germinate with soybeans grown on the same field in the season following the trial. This may cause contamination. GM maize technology has affected seed markets. An agrochemical company, Monsanto which has not been registered as traditional breeder, took advantage of seed production during the establishment of genetic engineering. The company made patents and use an opportunity to access the market and implement new strategies to obtain maximum profit (Vandana et al., 2011). Prices for seeds are increasing and the number of farmers using seeds from their own harvest is being endangered steeply and may likely disappear completely (Jacobsen et al., 2013).

BENEFITS OF GM MAIZE CROP

Genetic modified crops may provide better quality food, higher nutritional yields, inexpensive and nutritious food, crops and produce that require less chemical application, such as herbicide resistant maize. Previously, GM maize was grown for commercial purpose in 11 countries, including United States, Brazil, Argentina, South Africa, Canada, Philippines, and Spain (Singh et al., 2014). From the African perspective, GM technology so far has been deployed only in South Africa, Zimbabwe, Egypt, Kenya, Burkina Faso, Uganda, Malawi, and Mauritius. Of these, few countries, South Africa, Egypt, and Burkina Faso have commercialized their crops (Arthur, 2011; ASSAF, 2010). Since its introduction, GM technology has been found to reduce losses of maize incurred through damage by stem borers (Wanyama et al., 2004) and reduce herbicides application by introducing *B. thuringiensis* maize through transgenesis. *B. thuringiensis* maize used is of a better grain quality, and increases farmers' competitiveness; a healthier product, like mycotoxin is consistently well below mandatory regulations (Barros et al., 2009). Enrichment of transgenic maize in specific alimentary products makes such maize foods to frequently have higher utility value than traditional food products. The group of nutraceutics contains, first of all, vitamins A, C, E, plant pigments, alimentary cellulose, and pre- and probiotics (Kosicka-Gębska et al., 2009). The leading examples of cultivable edible vaccines are exemplified by varieties of rice, maize, soybean or potato, capable of producing antigens against various infections, including the effects of *Escherichia coli* toxins, rabies, infections of *Helicobacter pyloribacteria*, and viral type B hepatitis (Kramkowska et al., 2013). Several studies have reported that the reduction of pest damage after the introduction of *B. thuringienssis* maize instead of conventional maize results in enhanced yield (Huesing and English, 2004). At commercial level, after the approval of the glyphosate-tolerant soybeans, other twenty events have been approved for planting, food and feed consumption and commercialization, including 15 maize varieties. Gouse et al. (2005) found that large-scale commercial maize farmers benefit economically from the use of insect resistant yellow maize. Despite paying more for seeds, farmers who adopted *B. thuringiensis* yellow maize unlike conventional maize had increased income through savings from pesticides and increased yield due to better pest control (Huesing and English, 2004). Corn has been deliberately genetically modified to establish agronomically desirable traits. Traits that have been engineered into corn include resistance to herbicides and resistance to insect pests, the latter being achieved by incorporation of a gene that codes for the *B. thuringiensis* toxin (Singh

et al., 2014). GM maize varieties with high resistance to glyphosate herbicides have been produced. Pioneer Hi-Bred marketed corn hybrids with tolerance to imidazoline herbicides under the trademark "Clearfield" beng in Canada markets. Though in these hybrids, the herbicide-tolerance trait was bred without the use of genetic engineering. Therefore, these corn hybrids do not apply imidazoline-tolerant corn (Singh et al., 2014). The utilization of pesticides and herbicides globally has decreased. The decrease is due to the emergence of GM maize technology. However, there may be some variations of response from one variety to another (Benbrook, 2012). This has contributed to increase of agricultural production (Phipps and Park, 2002). However, Phipps and Park (2002) have established that the use of *B. thuringiensis* maize has the added advantage of reducing mycotoxin contamination, thus producing safer grain for both human and animals. In addition, *B. thuringiensis* -maize seeds have been distributed as new improved varieties which give high yields, thus consumers critically address the issue of food insecurity (Oliva et al., 2006).

CONTROVERSY OF GM MAIZE CROP

Regarding the high emphasis placed on innovation of technology in plant breeding especially GM maize crop which has expanded recently, there has been a debate about global food production focusing on GM crops, safety and regulatory approval process of GM crops and foods (Konig et al., 2004; Seralini et al., 2009). Many studies have been documented that, the countries which emphasize use of GM maize crop are often times more concerned about making profit first than health care (Seralini et al., 2014). Some scientists are against GM maize consumption; they have supporting evidence which shows that GM maize affects biodiversity leading to reduction of living organisms in the environment. Scientists are advised to carry out more research works so that they come up with effective conclusion about GM maize (Buiatti et al., 2013). This perspective is similar with Phipps and Park (2002) who stated that the sequencing of genomes provides capacity for selective breeding of crops suited to diverse ecologies including GM maize crop. Whilst scientists continue to debate risks, such as the effects of genetically engineered maize pollen on butterfly populations, drastic reductions in pesticide use achieved through the introduction of GM crops need to be researched (Ortiz et al., 2014). On the other hand, its adverse effects on the environment and human health have not been known for a decade. There are on-going researches which may come up with proper conclusion of whether to use GM maize or not (Buiatti et al., 2013). Spain, one of the countries which have adopted GM maize production technology, has been experiencing European corn borer pest which affects yield (James, 2008). Transgenic imidazolinone resistant maize is resistant to pest leading to the improvement of yield. However, the pest affected areas continue to spread northwards (Park et al., 2011). In other countries, for instance, Poland has allowed GM maize crop where *B. thuringiensis* maize has been commercially grown since 2006. However, other countries are in the first trial; for instance, Tanzania, Malawi, Ghana, and Uganda (Ortiz et al., 2014). The GM maize feed in Tanzania is in restriction awaiting approval from the government (Ortiz et al., 2014). Use of GMO feeds in Poland was permitted occasionally until the year 2012 (Maciejczak and Wąs, 2008). There is on-going debate at different levels regarding introduction of GMO crops in Poland. However, most public opinion shows that half of Polish society does not allow the introduction of GMO cultivations (Maciejczak and Wąs, 2008). *B. thuringiensis* gene, isolated from *B. thuringiensis* bacteria is considered resistance for the transgenesis of maize (Ronald, 2011).

It is assumed that a toxin coded by a bacterial chromosome, after transferring to plant tissues, allows the development of resistance to noxious insects which reduce crops, but have no negative influence on the health of humans and animals consuming the plants (Twardowski, 2010). This procedure proves that, the GM maize is resistant to corn borer (*Pyrausta mirilabilis*), while its commercial variety (MON810) is used for cultivation worldwide, including Poland and other countries of the European Union (Kramkowska et al., 2013). A similar report was documented by Stephenson (2010) that MON810 Monsanto's line of maize was developed through genetic modification to resist corn borer, an insect pest in Europe. Other countries like Spain have planted MON810 without experiencing negative effects (Stephenson, 2010). European Food Safety Authority (EFSA)'s findings indicate that MON810 (Monsanto pesticide produce GM maize) is environmentally safe (Stephenson, 2010). One of the diseases which forced scientists to adopt GM maize crop is the maize streak virus (MSV) of *Zea mays* L. This has made the production of maize in some parts of Africa virtually impossible, leading to critical food insecurity (Bosque-Perez, 2000). Arthur (2011)'s argument is against GM maize crop, because it affects value of maize genetic resources of landraces in the centre of crop diversity. Germany's law allows the banning of a GMO product if it potentially poses harm to the environment. Other countries should adopt the Germany's law to keep the environment useful for other living organisms (Stephenson, 2010). Southern African countries in 2001 rejected GM food aid from the U.S during a severe drought partly due to environmental concern. Scientists generally agreed that the possibility of actual potential environmental risk due to pollen disbursal is extremely remote (Stephenson, 2010).

For GM maize technology to be adopted in a number of countries, unfavourable GMO effects involved the case of

Aventis, the American producer of maize given the utility name of Star Link maize (Kramkowska et al., 2013). The modified plant contained an additional gene, conditioning natural resistance to pesticides (Benbrook, 2012). The transfer of genetic information from *B. thuringiensis* bacteria to the cell nuclei of maize yielded the expression product of Cry9c protein, which produced strong allergic properties (Kramkowska et al., 2013). Due to its specificity, Star Link maize gained the acceptance of the Environmental Protection Agency (EPA) and was permitted to enter the trade market exclusively as an animal fodder (Ramjoue, 2008). However, after commercialization of the transgenic plant, Star Link maize was detected in food products generally accessible in consumer markets (Ramjoue, 2008). In other study with high widespread press coverage, GM Star link maize which was approved for animal feed but not for human consumption was found unsafe for human consumption (Oliva et al., 2006). Spread of the information through mass media was followed by numerous consumers' reports related to symptoms of food allergy in the form of headache, diarrhoea, nausea and vomiting, which were supposed to develop following consumption of products containing the GM maize (Domingo, 2007; Dona and Arvanitoyannis, 2009; Batista and Oliveira, 2009). Before the release of GM maize crop, sufficient research should be conducted such that the recommendation on the use of new GM maize variety should not result in health risk to human, animals and environment in order to prevent biodiversity depletion (Ortiz et al., 2014). Countries which are in trial with GM maize crop should make laws which govern the use of GM maize. This will help to checkmate companies which aim to make profit by selling GM maize without considering the health risk involved (Ortiz et al., 2014).

Other reports are against the findings which emphasize MON810 maize to be safe for human and animal consumption. For instance, Kramkowska et al. (2013) reported harmful influences of MON810 maize (resistant to corn borer) on cells of the pancreas, intestines, liver and kidneys in rodents. Based on several European Union Member States' suggestion of provisional restriction or prohibition of marketing maize MON 810 in their territory, the EFSA GMO Panel concluded that, there is no scientific proof of health risk to human, animal and environment (EFSA, 2012) in using MON 810.

Rossi et al. (2011) noted positive evidence that piglets given maize MON 810 performed better than piglets given the control maize. They suggested that this difference was due to the lower level of fumonisin B1 in the diet. These additional feeding studies are good confirmations that maize MON 810 has no health risk to animals as other recommended maize for animal feed (EFSA, 2012). Results of other studies have shown the effects of different varieties of transgenic maize (MON810 and MON863) on living bodies. They showed that maize producing *B. thuringiensis* toxin is resistant to insects, NK603 and roundup herbicide, which has the potential to induce histopathological lesions first of all in liver and kidneys, and thus, in the principal detoxifying organs (Spiroux de Vendomois et al., 2010). This report has been confirmed in experiments involving two groups of rats: one group fed for 90 days with 11 or 33% components of transgenic maize and the control group which was given non-GM maize. However, Spiroux de Vendomois et al. (2009), in their previous study, suggested that, the chronic toxic effects of GM maize effect should finalized after long term evaluation for at least two years instead of three months.

Studies on similar crop by other authors agreed with previous findings: in South Africa markets, there is a significant reduction in pesticide use due to the availability of GM maize with better resistance to stem borer insect (Chondie and Kebede, 2015). Such varieties include *B. thuringiensis* maize that are already helping farmers in other African countries such as South Africa, Egypt and Burkina Faso (Chondie and Kebede, 2015). The common GM crops of research and commercial interest in Africa are maize, cotton, soybean, pigeon pea, banana, sweet potato and tobacco (Zerbe, 2004). Of these, trials have been conducted in various countries. Though, in Africa, only four countries (Burkina Faso, Egypt, South Africa and Sudan) are growing transgenic crops out of a total of 29 worldwide (Chondie and Kebede, 2015). The policies and legislation in place do not seem to cater for transgenic livestock research and deployment of livestock transgenic animals (Kiome, 2015). The studies on crops like livestock transgenic research in Africa are very weak only in African countries which have practiced transgenic research on livestock. However, no clear information has been displayed (Kiome, 2015). Chondie and Kebede (2015) also suggested that before any interventions be done in the GM crops in African countries, farms must be tested for GM crops by researchers to avoid potential risks. Furthermore, policy makers and researchers should evaluate environmental and socioeconomic risks, for instance, risks to biodiversity, the prospects of insufficient out-crossing distances, the relative absence of clear labelling and other threats to seed purity from adjacent traditional food production. This should be done before farmers change their conventional farming methods to GM (Azadi and Ho, 2010). Research centres must preserve the local landrace strains of maize for food security through seed saving. They should be confined in the gene bank in order to prevent genetic loss and transgenic contamination of the local landraces (Chondie and Kebede, 2015). John and Beringer (2000) believed that GM maize does not present a measurable risk to humans. In addition, the authors do not agree with statement that Novartis should persevere with a crop that is so widely perceived to be a threat to human health (John and Beringer, 2000) and whose construction is so

obviously awed from a public point of view. Moreover, there is clear evidence that GM maize is not easily contaminated by mycotoxins such as fumonisin and aflatoxin, toxins produced by fungi that infest maize cobs and which cause serious illnesses in man and animals (DeVilliers and Hoisington, 2011). With the exception of GM cotton, soybean and maize, only a limited number of commercially available GM crops are currently suitable for conditions in developing countries. Even though GM maize crop is promoted for high yield worldwide, Gurian-Sherman (2009) indicated that there is a strong market to grow non-GMO corn for the premiums in Kentucky compared to GM maize product. GM maize crop has contributed to lower its production in the United States (Gurian-Sherman, 2009). In contrast, Lee and Halich (2008) argued that farmers are concerned that they may be losing yield without using GMO hybrids because their finding showed that GMO hybrid yield was higher than the non-GMO sister at a range of 2.5 to 25.5 ratios. More similar studies need to be conducted to compare non-GM maize/GM maize pairs in the future to determine if these differences are consistent across a larger number of hybrids (Lee and Halich, 2008).

For subsistence farmers, for instance in parts of Africa, toxins also cause grave health problems, particularly for children. Mycotoxin contamination of GM maize may be reduced by preventing injuries to maize cobs (DeVilliers and Hoisington, 2011). One among the disadvantages of GMO is the inability of the farmers to save GM maize seed since harvested grains cannot be used as farmer-saved seed. This has been one of the critics of biotechnology which is often given as a reason why in African countries *B. thuringiensis* seeds are not suitable for smallholder farmers who mostly use farmer-saved seeds due to low income (DeVilliers and Hoisington, 2011). Previously, 70% of maize donated to Southern Africa in mid-2002 from United States was GM (Cooke and Downie, 2010). Zambia, along with Zimbabwe, Mozambique, and Malawi refused the food donated and in Zambia, about 18,000 tons of donated maize already in the country was put under security and ultimately transported back after knowing GM maize (Zerbe, 2004). They wanted GM maize which was milled to prevent contamination with domestic maize crop varieties (Cooke and Downie, 2010). On the other hand, Zambia wanted to mill maize to refugee camps that housed some Angolan and Congolese refugees. However, Zambian people were not allowed to consume it (Zerbe, 2004). The analysis of maize varieties collected from farms, household and research centres conducted in Cameroon proved the presence of genetic modified DNA, and probably the source could be from France, United States and South Africa which are the major sources of donated food (Roger and Gone, 2014). A case in point is the new *B. thuringiensis*-maize event called ‘Smartstax’ that was recently registered for environmental release in the USA and Canada (Hilbeck et al., 2011). This GM maize combines six insecticidal *B. thuringiensis*-toxins and resistance genes for two broad-spectrum herbicides. It entered the market with close to no testing for toxic or environmental impacts; it relied entirely on ‘the environmental risk assessment of the individual events’, except for one additional study with an unspecified non-target organism (Hilbeck et al., 2011; DeVilliers and Hoisington, 2011).

HEALTH RISK OF GM MAIZE TO HUMANS AND ANIMALS

Regarding the safety of GM crops and foods for humans’ and animals’ health, a comprehensive review of animal feeding studies of GM crops was found. An equilibrium in the number of research groups suggests that a number of varieties of GM products are mainly maize (Hilbeck et al., 2015). The foreign protein synthesized from the transgene maize has an expected change and interaction in the new environment of a plant cell which may lead to alergenicity (Kosicka-Gębska et al., 2009). This is similar to Star Link maize which has been reported to cause food allergy (Taylor and Tick, 2001). In 2000, there were traces of Star Link maize, a GM maize variety approved for animals feed, but not for human consumption, because most of the consumers reported allergic reactions caused by eating taco shells. These studies were conducted by taking and analysing blood samples from the consumers. It was suggested that further studies be conducted to know if it affects animals (DeVilliers and Hoisington, 2011). According to the studies conducted in 2001 by the Centers for Disease Control and Prevention, there is no evidence that exaggerated people experienced related allergic reaction after consuming Star Link maize (Ramjoue, 2008). From the reported information, there is enough evidence that Star Link maize is associated with risk to human and animals’ health (Taylor and Tick, 2001). The Star Link producer incurred cost to compensate for those farmers who got losses due to the use of Star Link maize. This led to the rapid death of the company (Taylor and Tick, 2001). In order to prevent future problems of this nature, regulation needs to be established to reject GM maize which has not been authorized for animal and human consumption (Kiome, 2015). Other studies have reported long term-effects in rats that consumed two Monsanto products, a GM maize and its associated pesticide, Roundup, together and separately (Spiroux de Vendômois et al., 2009). The target parts of rat affected by GM maize containing Monsanto were liver and kidney (Spiroux de Vendômois et al., 2009). A study showed that rats fed for 2 years with GM glyphosate-tolerant NK603 maize developed cancerous tumors (Seralini et al., 2014). Further studies have reported health risk of some GM maize to animals; for instance, a laboratory experiment was conducted in United States to check for negativity of

pollen. It showed that pollen in maize rendered insect resistant through incorporation of a gene coding for a toxin from *B. thuringiensis*. This was toxic to larvae of the monarch butterfly (Losey et al., 1999). Several studies based on one type of toxin have been documented, for instance, Cry1Ab is present in GM maize varieties *B. thuringiensis*11 and MON810. A little is known about the toxicity of other types of *B. thuringiensis* toxin; for example Cry1F, present in the GM maize 1507 (Lang and Vojtech, 2006). Cry1F is highly likely to be toxic to non-target organisms; however, there is need for further studies to confirm it (EFSA, 2011). Most *B. thuringiensis* maize produces toxin from their roots into the soil which is threat to other living organisms in the soil (Flores et al., 2005). The long-term, cumulative effects of growing *B. thuringiensis* maize is toxic to aquatic organisms especially frog larvae and it affect plants which are useful for birds' survival (Zobiole et al., 2011a). Cry1Ab toxin is positive in human as it helps in rapid degradation in the human digestive system (Guimaraes et al., 2010). However, it contradicts recent studies which reported that, there is lack of degradation in the human gut. The toxin seems to have a greater potential to cause allergenic reactions (Guimaraes et al., 2010). This is in agreement with reported information of another recent study which found Cry1Ab *B. thuringiensis* toxin in the blood of pregnant women, and their foetuses showed the possibility of crossing the placental boundary (Aris and Leblanc, 2011).

CONCLUSION

In conclusion, possible costs, benefits and risks associated with particular GM maize crops should be assessed only on a case by case basis. Any of such assessment needs to take into consideration a variety of factors, including the gene or combination of genes being inserted and the nature of the target maize crop. Considering whether GM maize crops should be used or not, it is important to focus on the specific situation in a particular country. All possible health risk to human, animals and environment should be taken into account before releasing new GM maize varieties. The cost-effective, nutrition and the ability to afford an adequate diet should also be considered. Furthermore, research on the use of GM maize crop in developing countries should be sustained and governed by a reasonable application of the precautionary approach. There must be positive scientific results which address the current and future use of GM maize crops. The farmers' perspective and other stakeholders should be taken into consideration on recommendation of GM maize varieties before being publicized.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The authors acknowledge Nichrous Mlalila for his constructive comments during the writing of this review

Abbreviations

Bt, *Bacillus thuringensis*; **DDT,** dichlorodiphenyl-trichloroethane; **EFSA,** European Food Safety Authority; **EPA,** Environmental Protection Agency; **EU,** European Union; **GMO,** genetic modified organism; **Ht,** herbicide tolerant; **MRL,** maximum residue levels; **R,** roundup; **USDA,** United States Department of Agriculture

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Vol. 15(15), pp. 580-592, 13 April, 2016
DOI: 10.5897/AJB2015.15061
Article Number: D4716DB58067
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Genome-wide analysis of mechanosensitive channel of small conductance (MscS)-like gene family in common bean

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Received 22 October, 2015; Accepted 18 March, 2016.

Mechanosensitive (MS) ion channels are transmembrane proteins that open and close in response to mechanical forces produced by osmotic pressure, sound, touch and gravity. In plants, MS have an important role in different biological processes like gravity detection, maintenance of plastid shape and size, lateral root emergence, growth of pollen tube, and plant-pathogen interactions. In this study, homologous mechanosensitive channel of small conductance (MscS)-like gene family in common bean was identified. Nine *Phaseolus vulgaris* MscS-like (*PvMSL*) genes were found to be distributed on five chromosomes. A complete overview of *PvMSL* genes in common bean is presented, including gene structures, chromosome locations, phylogeny, protein motifs and expression pattern. Subcellular localization predictions of *PvMSL* family revealed their location to plasma and chloroplast membrane. Phylogenetic analysis of nine *PvMSL* proteins resulted in two main classes. The predicted gene structure, conserved motif, domain and presence of transmembrane regions in each *PvMSL* strongly supported their identity as members of MscS-like gene family. Four duplicate events of *PvMSL* genes were discovered in *P. vulgaris* chromosomes, and tandem and segmental duplication may cause the expansion of *PvMSL* genes. Furthermore, *PvMSL* genes displayed differential expression patterns in tissues and organs. This is the first step towards genome-wide analyses of MSL genes in common bean. Thus, the data obtained in this study provide resources to select candidate genes for future functional analyses that will help understand plant growth, development, and function of MSL gene family in *P. vulgaris*.

Key words: Mechanosensitive, phylogenetic analysis, gene duplication, plant, *in silico.*

INTRODUCTION

Plants perceive and respond to a lot of mechanical stimulli, including touch, gravity, osmotic pressure and stress (Ding and Pickard, 1993; Blancaflor and Masson, 2003; Braam, 2005; Furuichi et al., 2008a; Hamilton et

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al., 2015). Moreover, they respond to signals associated with plant development processes like emergence of lateral roots, growth of pollen tube, damages to the cell wall, and plant-pathogen interactions (Lucas et al., 2013; Appel and Cocroft, 2014; Jayaraman et al., 2014). In many of these cases, the application of a mechanical stimulus results in a quick explosion of the ion flow which can be attributed to mechanosensitive (MS) ions due to the response speed (Fasano et al., 2002; Jaffe at al., 2002; Monshausen and Haswell, 2013).

Some responses are rapid, such as regulation of volume and turgor pressure of guard cells and closing of Venus's flytrap leaf on its prey (Cosgrove and Hedrich, 1991; Haswell and Meyerowitz, 2006; Furuichi et al., 2008b). Other responses to mechanical perturbation are growth-related and more slowly like repeated tactile stimulation of *Arabidopsis* seedlings which causes late flowering and short inflorescences (Braam and Davis, 1990) and *Arabidopsis* roots that alter their course through the soil to avoid a barrier (Massa and Gilroy, 2003). It has recently been shown that at least some mechanical stimuli trigger rapid molecular events that are then transduced into a necessarily slower growth response. In *Arabidopsis*, cytoplasmic alkalinization of root gravi-responsive cells occurs within 2 min of gravity stimulation and touch can elicit immediate Ca^{2+} transients in root cells (Legue et al., 1997; Fasano et al., 2001).

The MS channels, formed by transmembrane proteins, work as mechano-electric transductors where mechanical stimuli are converted into electrical or chemical signals in live cells that perform numerous cellular processes associated with mechanosensory transduction (Kloda and Martinac, 2002). The first MSs were identified during the electrophysiological characterization of the plasmatic membrane of *Escherichia coli* (Martinac et al., 1987; Sukharev et al., 1993; Berrier et al., 1996). The MS channels were classified according to their ion conductance and the main ones are: Mechanosensitive Channels of Large Conductance (MscL) and Mechanosensitive Channels of Small Conductance (MscS) (Edwards et al., 2012; Cox et al., 2015). The MscL present conductance (larger than 2.4 nS), open undermembrane high tension and present rapid kinectic (Perozo, 2006). Each MscL subunit is composed of a polypeptide of 136 amino acids with two transmembrane helices, TM1 and TM2 (Sukharev et al., 1994, 1999). A 3.5 Å crystalline structure in *TbMsLC* ortholog (151-amino acids) in *Mycobacterium tuberculosis* revealed a homo pentamer in an apparently closed state (Chang et al., 1998). On the other hand, MS of small conductance is a 0.8 to 1-nS channel opened by moderate pressure (Perozo, 2006). *EcoMsSC* structure of the channel from *E. coli* (286 amino acids) was originally resolved at 3.9 Å (Bass et al., 2002) and recently at 3.45 Å in an open configuration (Wang et al., 2008).In bacterial systems and animals, MS ion channels mediate the perception of pressure, touch and sound.

Although plants respond to a wide variety of mechanical stimulli, the molecular nature of transmembrane protein mechanical perception in vegetal system has been little studied (Hedrich, 2012). MS ion channels were characterized in *Arabidopsis thaliana* and *Oriza sativa* model plants (Haswell and Meyerowitz, 2006; Saddhe and Kumar, 2015). In *Arabidopsis* and rice, ten and five MscS homologs, respectively, were identified and classified into two main classes, Class I and Class II. There has been no study done on *Phaseolus vulgaris* to identify MscS homologs.

Common bean (*P. vulgaris*) is a main socially important crop and is a major source of protein and essential nutrients. Common bean is the most consumed legume in the worldwide (Schmutz et al., 2014). Brazil is the largest producer with an average annual production of 3.5 million tons (MAPA, 2015). However, the grain yield in Brazil is considered low and several factors are related to this, as the adverse effects of climate conditions, as well as the occurrence of pests and diseases (Beebe et al., 2013).

Therefore, knowing the importance of MS ion channels of small conductance in plant development and the important role of MscS genes to overcome different stresses, and considering lack information of MscS in *P. vulgaris,* this study aimed to identify homologous MscS-like (MSL) genes in common bean and analyze their structure, subcellular location, phylogenetic relationship, expression pattern and chromosomal distribution. Our analysis may contribute to select candidate genes for future functional analyses that will aid researchers in understanding plant growth, development and function of MSL gene family in *P. vulgaris.*

MATERIALS AND METHODS

Identification of MSL genes in *P. vulgaris* genome

Protein sequences of MSL genes identified in *A. thaliana* and *O. sativa* model plants from databases of TAIR (http://www.arabidopsis.org/) and TIGR (http://rice.plantbiology.msu.edu/), respectively, were utilized for studies in Phytozome Database of *P. vulgaris* version 11.0 (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvulgaris), using BlastP algorithm (Altschul et al., 1997). The sequences were compared with other sequences deposited in GenBank database, utilizing BlastP and BlastX programs (National Center for Biotechnology Information, NCBI; http://www.ncbi.nlm.nih.gov) to confirm their identity. The initially collected sequences, whose ORFs were incomplete, were excluded from the analyses. The protein sequences were aligned utilizing Clustal Omega algorithm version 2.0.3 (Sievers et al., 2011) and the redundant inputs were removed.

Characteristics of predicted proteins, transmembrane regions and subcellular location of MSL genes in *P. vulgaris*

The physical and chemical characteristics of MSL proteins in bean were calculated by ProtParam tool (http://web.expasy.org/protparam), including the number of amino

Table 1. Comparison of number of members of each MSL class of genes between species *Phaseolus vulgaris, Arabidopsis thaliana, Glycine max, Solanum lycopersicum, Oryza sativa* and *Zea mays*, respectively.

Species	*Phaseolus vulgaris*	*Arabidopsis thaliana* [a]	*Glycine max* [b]	*Solanum lycopersicum* [b]	*Oryza sativa* [b]	*Zea mays* [b]
Class	n	n	n	n	n	n
Class I	4	3	8	4	3	2
Class II	5	7	6	4	3	3
Total	9	10	14	8	6	5
Size Genome (Mb)	580	115	1,115	950	420	2,500

[a]Haswell and Meyerowitz (2006). [b]Saddhe and Kumar (2015).

acids, molecular weight (KDa) and theoretical isoelectric point (PI). The transmembrane (TM) regions with drawing diagrams were predicted utilizing Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2/). All sequences of predicted MSL proteins were analyzed *in silico* regarding their subcellular location, using LOCTREE3 (https://www.rostlab.org/services/loctree3) and WoLF PSORT algorithms (http://wolfpsort.org/).

Phylogenetic analysis

Phylogenetic analysis was done by aligning the protein sequences utilizing Clustal Omega algorithm, version 2.0.3 (Sievers et al., 2011). The phylogenetic tree was built by Maximum-Likelihood (ML) method using pair-wise deletion with the help of MEGA program, version 6.06 (Tamura et al., 2013). 1,000 bootstrap replicates were utilized to test analysis confidence.

Analysis of conserved transmembrane motifs

The identification of transmembrane (TM) motif of predicted proteins were examined utilizing MEME (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi) with the following paramteres: sequence repetition: 1, maximum number of found motifs: 1 and ideal size: 70 to 80 amino acids. The resulting motifs were verified in the databases of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and PROSITE (http: //www.expasy.org) to verify their significance.

Genome structure

Complete sequences of genomic DNA and coding sequences CDS, corresponding to each gene using Gene Structure Display Server 2.0 (GSDS) (http://gsds.cbi.pku.edu.cn/), were used to identify the position of introns and the organization of exon/intron in genes.

Annotation, duplication and synteny of MSL genes in *P. vulgaris* genome

P. vulgaris MSL genes (*PvMSL*) were mapped in bean chromosome according to their genome coordinates. The genes were plotted in chromosomes using MapChart software and data on their physical location are avaialble in Phytozome. Duplications of *PvMSL* genes considered as parameters 50% identity and 80% coverage, adapted from Lopes-Caitar et al. (2013). Plant Genome Duplication Database (PGDD; http://chibba.agtec.uga.edu/duplication/) was used to search for orthologous genes in *P. vulgaris* and *Arabidopsis*. The synteny map was displayed using Circos software (http://circos.ca/) (Krzywinski et al., 2009).

Analysis of gene expression

Illumina RNA-seq datasets were downloaded from Phytozome Database (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvulgaris). The expression profile *in silico* of common bean *PvMSL* genes were calculated by Cufflinks in FPKM units (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced) (Mortazavi et al., 2008). FPKM values were log2 transformed and the heatmap was generated with the algorithm CIMMiner (http://discover.nci.nih.gov/cimminer).

RESULTS AND DISCUSSION

Identification and classification of MSL genes in the common bean (*P. vulgaris)* genome

In order to identify *MSL* gene family in common bean genome, the amino acid sequence PF00925 and a Hidden Markov Model profile of MSL protein were used to perform a BLASTP search against the Common Bean databases v1.1 (Phytozome v10.3: http://www.phytozome.net). In this study, a total of nine genes encoding putative proteins from a MS ion channel of small conductance were identified from the whole genome and were named *PvMSL1* to *PvMSL9.* Nine genes encoding MSL family were identified in *P. vulgaris*, while different numbers of genes were identified in other plant species (Table 1). The number of genes in MSL family identified in common bean was similar when compared with tomato (8), smaller than in *Arabidopsis* (10) and soybeans (14), and larger than in rice (6) and maize (5). Generally, the number of MSL genes distribution in dicotyledons is nearly double than in monocotyledons, possibly due to the higher rate of genome duplication events in the former. In *Arabidopsis*, Saddhe and Kumar (2015) attributed this to four different major genome duplication events over 100 to 200 million years ago. Moreover, the number of MSL genes in *P. vulgaris* is not associated with the size of the genome itself. Although *P. vulgaris* has a much larger genome

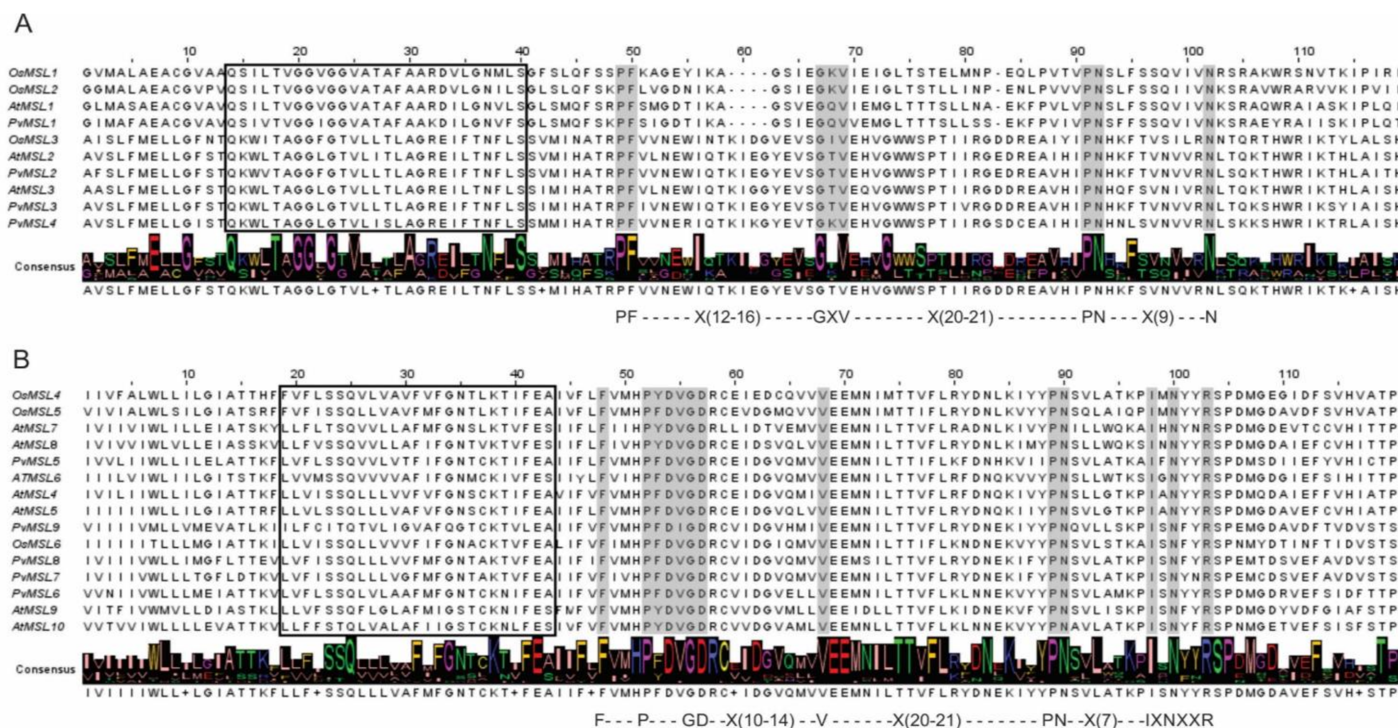

Figure 1. Alignment of MSL family conserved domains. Amino acid sequence of MSL family members from *A. thaliana* (*AtMSL1* to *AtMSL10*), *O. sativa* (*OsMSL1 to OsMSL6*) and *P. vulgaris* (*PvMSL1* to *PvMSL9).* Consensus sequences derived from this analysis is presented at the bottom of each alignment in dark type; the consensus sequence derived by Pivetti et al. (2003) is below and of each alignment in gray type. (A) Class I proteins. The box indicate the experimentally derived MscS TM3 domain. (B) Class II proteins. The box indicate the location of a consensus TM3 domain.

(580 Mb) (Schmutz et al., 2014) than *Arabidopsis* (145 Mb) (Huala et al., 2001), the structure and phylogenic arrangements are quite similar. Indeed, the number of MSL genes in monocotyledons as well as in dicotyledonous seemed to be quite similar, despite the different sizes of the genomes (Table 1). In fact, any irregular distribution of genes in MSL family among different plant species may have evolved from genomic losses or duplication events of a common ancestor, contributing to the expansion of or restriction on the number of these genes. All of the putative MSL proteins were divided into two classes (that is, I and II). The members of Class I resembled prokaryotic MsSC channels, and the ones of Class II form independent plant/fungus lineages (Pivetti et al., 2003; Haswell, 2007). Table 1 summarizes MSL protein classification of common bean genome and other plant species. Classes I and II had four and five members, respectively. According to Pivetti et al. (2003), the eukaryotic members of MscS family can be organized into two main classes based on sequence similarity. Proteins in Class I have transmembrane (TM) domains at the C-terminal region that resemble TM3 MscS which are rich in glycine and alanine residues, though the pattern is not conserved (Figure 1A). Conserved motif PF(X12–16)GXV(X20–21)PN(X9)N was identified at C-terminal for the TM domain in Class I MSL in common bean (*PvMSL1*, *PvMSL2*, *PvMSL*3 and *PvMSL*4). Class I proteins align relatively closely to *E. coli* MsSC and their C-terminal TM domains

Table 2. Physicochemical characteristics of *PvMSL* genes.

PvMSL	ID Phytozome	Nucleotide CDS bp	AA	PI	MW	Subcellular location
PvMSL1	Phvul.010G102400	1623	540	9.03	58.79	Plasmamembrane
PvMSL2	Phvul.002G147200	2094	697	9.34	76.8	Chloroplastmembrane
PvMSL3	Phvul.009G036900	2052	683	9.34	75.59	Chloroplastmembrane
PvMSL4	Phvul.009G037000	1896	631	9.41	70.34	Chloroplastmembrane
PvMSL5	Phvul.007G016600	2754	917	8.32	105.91	Plasmamembrane
PvMSL6	Phvul.009G081300	2295	764	8.95	86.86	Plasmamembrane
PvMSL7	Phvul.009G228800	2250	749	9.32	85.52	Plasmamembrane
PvMSL8	Phvul.009G228700	2232	743	7.59	85.19	Plasmamembrane
PvMSL9	Phvul.003G279800	2103	700	8.87	81.53	Plasmamembrane

PvMSL, Phaseolus vulgaris MscS like, ID Phytozome; CDS, coding sequences; bp, base pair; AA, amino acid; MW, molecular weight (KDa); pI, isoelectric point and subcellular localization.

resemble TM3 of MsSC. Similar motifs have been reported in bacterial MscS channel systems (Pivetti et al., 2003; Saddhe and Kumar, 2015). Proteins in Class II showed the presence of consensus sequence F(X3)P(X3)GD(X10–14)V(X20–21)PN(X7)IXNXXR at the C-terminal TM domain in *P. vulgaris* (*PvMSL5*, *PvMSL6*, *PvMSL7, PvMSL8* and *PvMSL*9) (Figure 1B). In Class II, C-terminal TM domain proteins are not glycine- or alanine rich; however, they contain amino acids with larger hydrophobic side chains. Large hydrophobic amino acids are conserved at certain positions within the domain. In agreement with a previous analysis by Pivetti et al. (2003), the conserved motif includes most C-terminal TM domain and surrounding sequence in *E. coli.* Generally, members of MscS family have good conservation throughout the TM3 helix (Pivetti et al., 2003; Miller et al., 2003; Saddhe and Kumar, 2015).

Protein properties and subcellular localizations of *PvMSL* proteins

The parameters used to describe *PvMSL* proteins are listed in Table 2 and included gene locus number, deduced protein length, molecular weight, isoelectric point and subcellular localization. The deduced length of *PvMSL* proteins ranged from a minimum of 540 amino acids (*PvMSL1*) to a maximum of 913 residues (*PvMSL5*), whereas PI ranged from 7.59 (*PvMSL8*) to 9.41 (*PvMSL1*). The molecular weight ranges from 58.79 to 105.91 KDa for *PvMSL1* and *PvMSL5,* respectively. In general, members of Class I showed smaller size amino acids and lower molecular weight, while Class II presented higher isoelectric point than Class I. Most *PvMSL* proteins share similar physical and chemical characteristics within the same class.

All putative *PvMSL* proteins were found in the membrane. Class I *PvMSL* genes (*PvMSL1-PvMSL4*) were localized either in the plasma membrane or chloroplast membrane, whereas Class II *PvMSL* genes (*PvMSL5 - PvMSL9*) were present only in the plasma membrane (Table 2). Consistently with studies in *A. thaliana*, *AtMSL2* and *AtMSL3* genes were localized in the plastid envelope, most likely in the inner membrane, and are observed in foci at the plastid poles (Haswell and Meyerowitz, 2006; Wilson et al., 2011). MSL genes of Class I were reported to be located either in the plastid envelop or mitochondria (Haswell, 2007; Saddhe and Kumer, 2015). Class II genes were found in the plasma membrane. Saddhe and Kumer (2015) showed that all Class II members of *O. sativa* (*OsMSL4* - *OsMSL7*) are located in the plasma membrane. *AtMSL9* and *AtMSL10* genes in *A. thaliana* were found in the plasma membrane of root cells (Haswell et al., 2008). Immunofluorescence of algal *Chlamydomonas reinhardtii* similarly revealed a complex localization pattern for *MSC1* genes located in the chloroplast and cytoplasm (Nakayama et al., 2007).

The predicted topology of *PvMSL* genes were illustrated in Figure 2. In Class I, *PvMSL1* and *PvMSL3* proteins demonstrated the presence of five transmembrane (TM) regions, while *PvMSL2* and *PvMSL4* exhibited the presence of six and seven TM regions, respectively. In Class II, the number of TM regions ranged from six or seven. *PvMSL6, PvMSL7* and *PvMSL9* proteins exhibited the presence of six TM, while PvMSL5 and PvMSL8 showed the presence of seven TM regions. In *E. coli*, MscS channel topologies varied from 3 to 11 putative transmembrane segments (TMS) and adopted an N-terminal out and C-terminal in configuration (Miller et al., 2003; Pivetti et al., 2003). Indeed, the results identified in this study were similar to the ones in *Arabidopsis* and Rice Class I members which showed the presence of five TM domains while Class II members exhibited the presence of six TM domains (Haswell, 2007; Saddhe and Kumer, 2015).

Phylogenetic analyses and identification of conserved domains of *PvMSL* family

To clarify the phylogenetic relationships among MSL

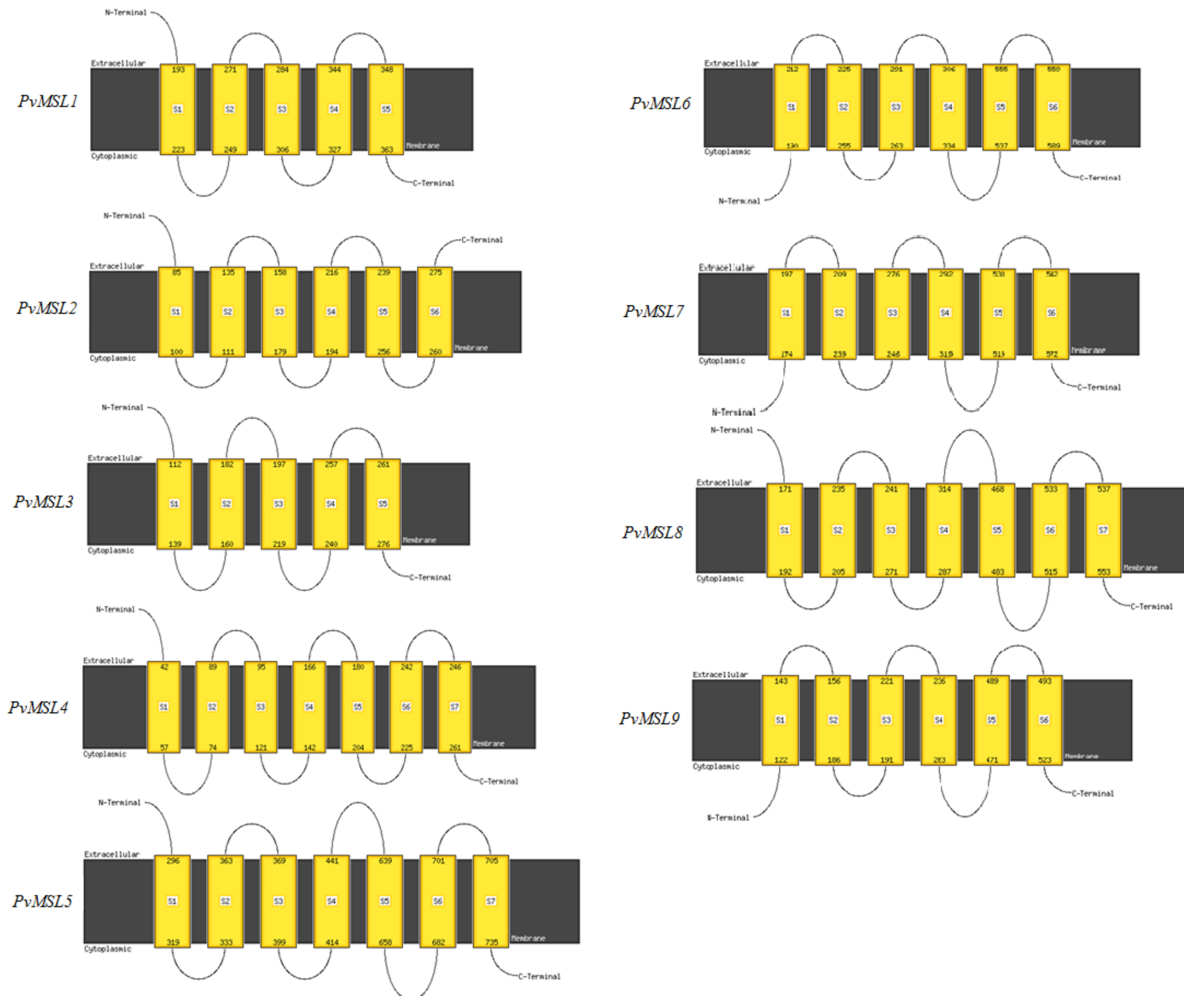

Figure 2. A cartoon of the transmembrane (TM) helix topology summarizing the linear coordinates for the helices and indicating the protein's extra- and intercellular regions. Transmembrane helix region represented by the yellow boxes labeled S1, S2, S3, S4, S5, S6 and S7. Membrane regions were represented by the black boxes. N and C terminal regions were labeled and represented as a single line.

gene family, a phylogenetic tree was constructed based on the alignment of *Arabidopsis*, rice and common bean of MSL proteins sequences (Figure 3). The Maximum-Likelihood (ML) phylogenetic tree divided MSL genes into two classes (Figures 3 and 4), supported by bootstrap values and the occurrence of conserved protein motifs (Figure 4A and B). In *Arabidopsis* and rice, two classes, I and II, have been classified (Haswell and Meyerowitz, 2006; Saddhe and Kumar, 2015) and the corresponding groups in common bean are labeled in Figures 3 and 4A. The Class I and II common bean contains four and five *PvMSL* genes, respectively, according to the classification in *Arabidopsis*. The phylogenetic analysis revealed that common bean MSL genes showed similar phylogenetic relationships with *Arabidopsis* and rice. Among these, Class II constituted the largest clade, containing 15 members and accounting for 60% of total MSL genes, while Class I contained 10 members and accounted for 40% of total MSL genes. Additionally, in rice, a monocotyledon, the number of members within the two classes was the same, while in dicotyledon, *Arabidopsis* and common bean, the number of members was larger, indicating that these genes could have originated from a common ancestor by frequent duplication gene after the split between mono and dicotyledon.

To further reveal the diversification of MSL genes in common bean, putative motifs were predicted using the program MEME, and 10 distinct motifs were identified (Figure 4B). The schematic distribution of 10 motifs among the two gene classes is shown in Figure 4B; these motifs are represented in their relative location within the protein. The identified multilevel consensus sequence for the motifs is shown in Table 3. The 10 motifs identified by MEME were annotated by InterProScan. The superfamily database of structural and functional protein annotations

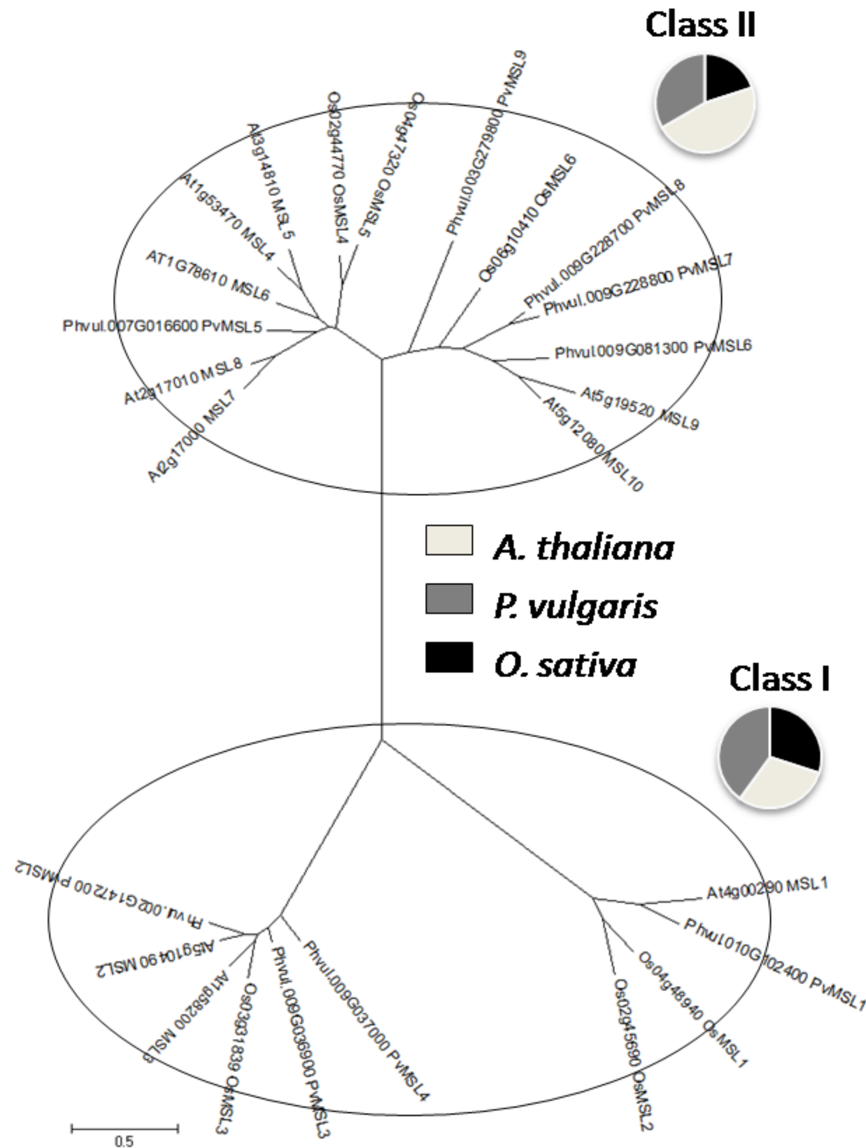

Figure 3. Phylogenetic tree of *A. thaliana, O. sativa,* and *P. vulgaris* MSL genes. The tree was based on 25 MSL sequences (including both N-terminal and C-terminal domains) from three species using the Maximum-Likelihood method.

showed that MS channel and like-Sm (LSM) domains were present in all common bean *PvMSL* protein sequences. The Pfam analysis showed that the MS channel domains were present in all *PvMSL* proteins. Protein information resource (PIR) database showed that common bean MSL, *PvMSL5* to *PvMSL9* have a unique MscS-like plant/fungus domain which is restricted to plant and fungus lineages. Motif 2 is a frequently occurring sequence and is present in most *PvMSL* genes, consisting of MS channel domain. As expected, most of the closely related members in the phylogenetic tree had common motif compositions, suggesting functional similarities among MSL proteins within the same class (Figure 4B). For example, motifs 2, 7 and 9 were specific to Class I, while motifs 3, 4, 5, 7, 8 and 10 were specific to Class II. Motifs3, 4 and 5 showed a casein kinase C phosphorylation site. All members of Class II(*PvMSL5* to *PvMSL9*) were located in plasma membrane, which might be regulated by phosphorylation during protein kinase C. These similarities in motif patterns might be related to similar functions of MSL proteins within the same class.

Structural analyses, genome distribution, duplications and synteny of *PvMSL* genes

It is well known that gene structural diversity is a possible mechanism for the evolution of multigene

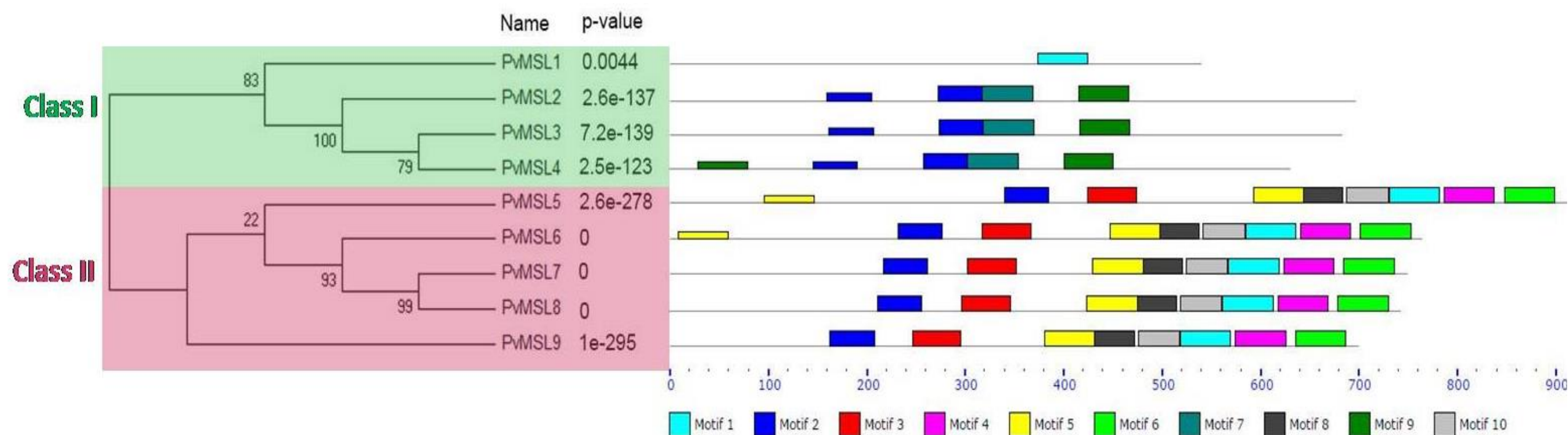

Figure 4. Relationships and schematic representation of motif compositions of 9 members of bean MSL family. Unrooted ML phylogenetic tree was based on nine *PvMSL* protein sequences using MEGA6.06. The motifs identified by MEME software are represented by colored boxes and their consensus sequences are shown in Table 3.

Table 3. Conserved motif compositions of *PvMSL* proteins.

ID[a]	Consensus sequences	E-values	Sites	Width
1	FEAIIFVFVMHPFDVGDRCVIDGVQMVVEEMNILTTVFLRYDNEKIYYPN	5.5e-118	5	50
2	VLVTLCGRLVTTWFINVIMFHIERNFLFNEWVQYFIYGYKKSGQ	9.4e-083	8	44
3	GAAIWLVKTLLIKMLASKFHVTTYFDRIQESIFHQYILQTLSGPPLME	8.1e-067	5	48
4	PISNYYRSPEMCDSVEFAVDVSTPIESIGALKHKIKWYCESKPQHWHPNH	1.8e-065	5	50
5	INSEWEAKAAAYRIFGNVAKPGCKYIEEDDLMRFMKNEEVHNVYPLFEGA	8.6e-069	5	50
6	KMKMAIYVTHTMNFQNYGEKNRRRSELVLELKKIFEELNIKYHLLPQEIH	4.9e-058	5	50
7	EHVGWWSPTIIRGDDREAVHIPNHKFTVNVVRNLSQKSHWRIKTHLAISH	4.6e-046	3	50
8	ETGRITRKSLKNWLIKVYYERRALAHSLNDTKTAVDQLN	2.7e-042	5	39
9	SCFVKTSHFEEYLCVKEAILLDLLRVISHHRARLATPIRTVQKIYSDTD	5.3e-042	3	49
10	IVIIVIIIVWLLIMEFATTKVLVFCSSQLVLVGFMFGNTCK	2.6e-036	5	41

[a]Means motif ID.

families. In order to gain further insight into the structural diversity of MSL genes, the exon/intron structure of each member of *PvMSL* family in common bean was analyzed. A detailed illustration of exon/intron structures is shown in Figure 5. According to their predicted structures, all *PvMSL* genes have introns in their structure and the number of exons varied from 4 to 12. These exon/intron

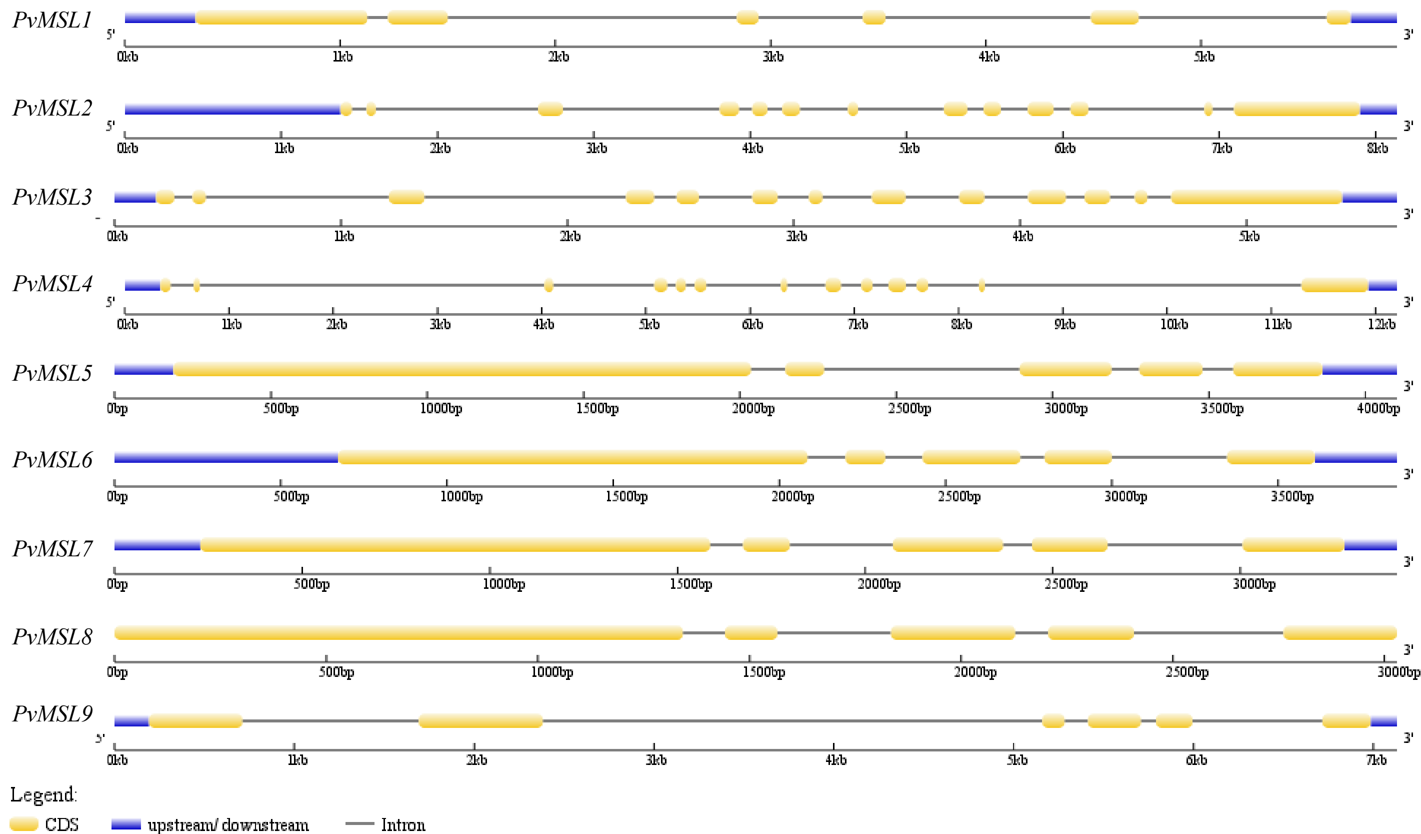

Figure 5. Gene structure schematic diagram for *PvMSL* genes. Exons were demonstrated by filled yellow boxes and introns were demonstrated by black lines. Untranslated region (UTR) was displayed by blue boxes at both ends.

structures are similar to rice (Saddhe and Kumer, 2015). The most closely-related members in the same class generally showed the same exon/intron pattern in which the number, position and length of the intron almost completely conserved within classes (Figure 5). For instance, the gene structure appeared to be more variable in *PvMSL* genes of Class I, where the number of introns ranged from 5 to 12, while the members of Class II contained only four or five introns. Most genes containing introns were clustered into the same class suggesting evolutionary conservation in *P. vulgaris.*

The chromosomal locations of *PvMSLs* were determined based on the information from Phytozome Database of *P. vulgaris.* Genome chromosomal location analyses revealed that *PvMSL* were randomly distributed in 5 out of 11 chromosomes (Figure 6). Among these chromosomes, chromosome 9 contained the largest number of MSL genes, while the other chromosomes contain only one MSL gene. *PvMSL2, PvMSL9*, *PvMSL5* and *PvMSL1* genes were localized in chromosomes 2, 3, 7 and 10, respectively, as shown in Figure 6. Five genes, *PvMSL3, PvMSL4*, *PvMSL6*, *PvMSL7* and *PvMSL8,* are located in chromosome 9. Substantial clustering of *PvMSL* genes was evident in chromosome 9, with high densities of the genes.

Tandem and segmental duplications have been suggested to be two of the main causes for gene family expansion in plants (Cannon et al., 2004). Two or more genes located in the same chromosome are confirmed as a tandem duplication event, while gene duplication between different chromosomes is designated as a segmental duplication event (Liu et al., 2011; Cai et al., 2013). Two pairs of MSL paralogues genes were identified in chromosomes 2, 3 and 9. Segmental duplications with high similarity (55%) were detected between *PvMSL2* in the terminal region of chromosome 2 and *PvMSL3* in the same region of chromosome 9.

PvMSL9 in chromosome 3 also showed a probable (50%) duplicated region shared with *PvMSL8* in chromosome 9, both of which are located in the lower arm of chromosomes. According to Holub (2001), tandem duplications are 200 kb regions in a chromosome that contains two or more genes with high similarity. Therefore, two MSL tandem duplication clusters were identified. Four genes (*PvMSL3, PvMSL4, PvMSL7*, and *PvMSL8*) were arranged in two clusters in 7.7 and 33.7 kb segments in chromosome 9, respectively (Figure 6). This strongly suggested that they might be generated by tandem duplications. The duplication prediction analysis indicated that the evolution of *PvMSL* gene family in

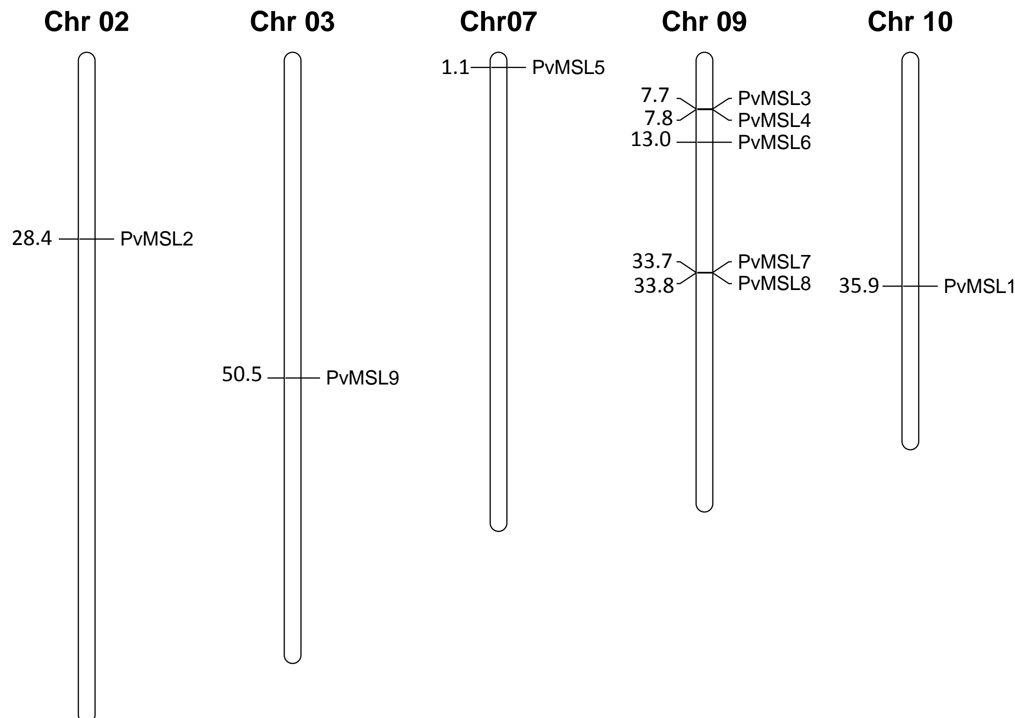

Figure 6. Chromosomal location of *PvMSL* genes. The chromosome number is indicated at the top of each linkage group. The chromosomal positions of putative *PvMSL* were mapped according to genome coordinates.

common bean genome resulted from a total of 4 gene duplications, 2 segmental and 2 tandem duplication events (Figure 6).

In our study, one comparative syntenic map of *P. vulgaris* associated with *A. thaliana* (Figure 7) was built. A total of three pairs of syntenic relations were identified, three *AtMSL* genes (*AtMSL1*, *AtMSL7* and *AtMSL10*) and three *PvMSL* genes (*PvMSL1*, *PvMSL5* and *PvMSL6*) were found to be associated with synteny events. These synteny events suggest that many MSL genes arose before the divergence of *Arabidopsis* and common bean lineages. Thus, this result may indicate that *PvMSL* genes in *P. vulgaris* share similar structure and function with *AtMSL* genes in *Arabidopsis*.

Expression profiles of *PvMSL* genes

It has been noted that MSL gene family exhibits differences among different organisms and different tissues to exert different physiological functions. Thus, gene expression patterns can provide important clues for gene function. To further analyze the tissue specificity of MSL gene family members, the expression profiles of the nine *PvMSL* genes were analyzed as shown in Figure 8. Most of MSL genes showed distinct tissue-specific expression patterns across the nine examined tissues. Some of them were constitutively expressed in almost all tissues and organs, and the expression levels were high, such as those of *PvMSL1*, *PvMSL2, PvMSL3* and *PvMSL6*. The expression levels of *PvMSL1*, *PvMSL2* and *PvMSL3* were relatively higher in leaves and roots, which indicates that they could play a role in the development of plant root and leaves. These expression patterns were similar to *AtMSL9* and *AtMSL10* in *Arabidopsis*, which are expressed at high levels in root and responded to mechanical and gravity stimuli (Haswell, 2007; Kimbrough et al., 2004; Haswell et al., 2008). Likewise, *AtMSL2* and *AtMSL3* were found in leaves and control plastid size, shape, and perhaps division during normal plant development by altering ion flux in response to changes in membrane tension (Haswell and Meyerowitz, 2006). In eukaryotic, MSL genes presented varied distribution and function, in addition to serving as a safety valve (Saddhe and Kumar, 2015). *PvMSL6* and *PvMSL9* were relatively higher expression in nodules and young trifoliates, respectively. *PvMSL4*, *PvMSL7* and *PvMSL8*showed very low expression in almost all tissues and organs of common bean. Detailed analysis of the expression patterns of *PvMSL* showed that some of the genes clustered in the same class of the phylogenetic tree (Figure 4) had similar expression patterns, also indicating the existence of redundancy among *MSL* genes in this class. For example, most *PvMSLs* in Class I were mainly expressed in leaves and roots while all genes in Class II were little expressed in different tissues

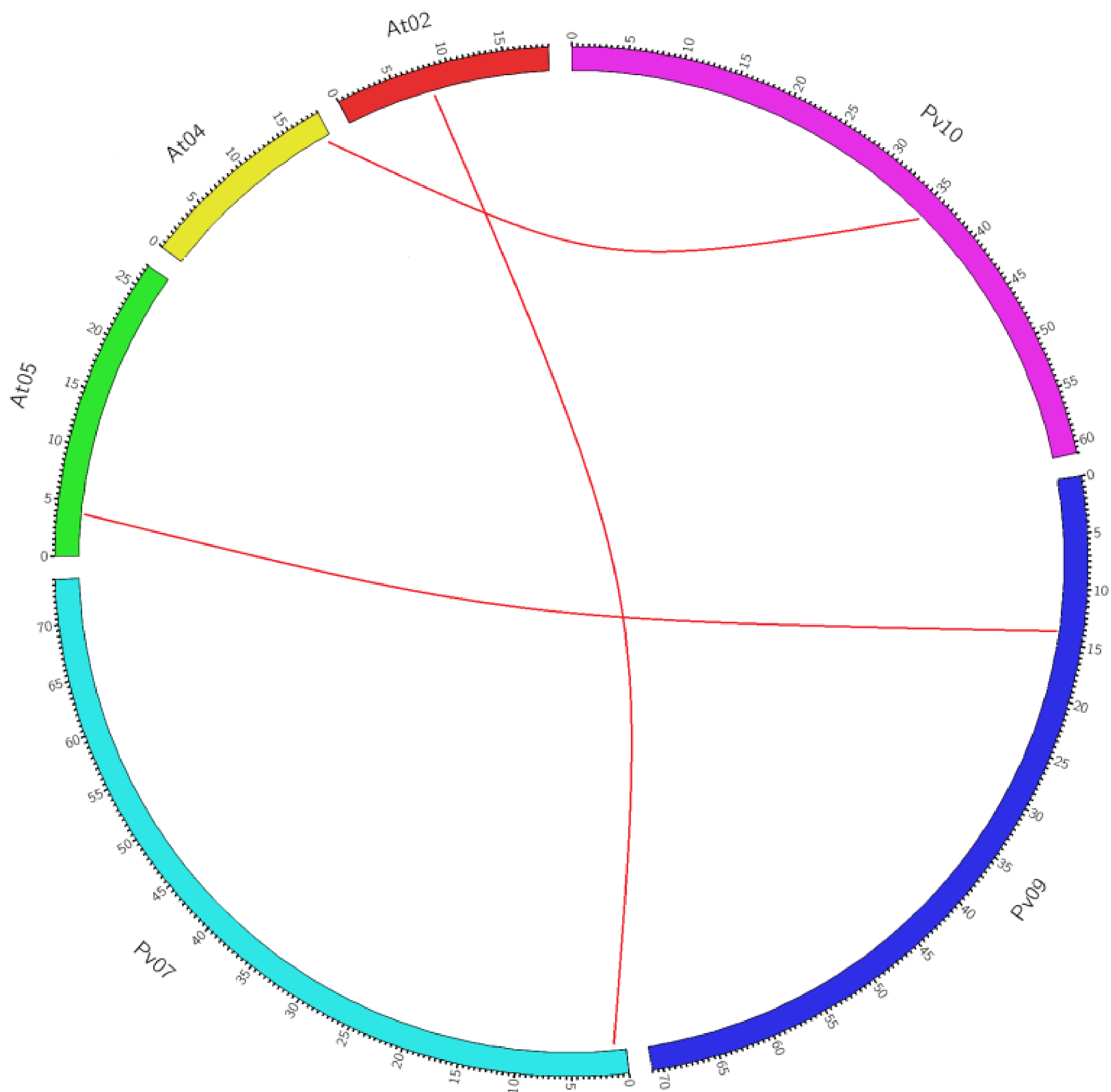

Figure 7. Synteny map showing the orthologous gene positions of MSL genes between *Phaseolus vulgaris* and *Arabidopsis*. *P. vulgaris* (Pv07, Pv09 and Pv10) and *Arabidopsis* chromosomes (At02, At04 and At05) maps were based on the orthologous and demonstrate highly conserved synteny. Each block represents individual chromosome and red lines connecting two chromosomal regions denote syntenic regions of genomes.

and organs of common bean. In addition, paralogous *PvMSL* genes, such as *PvMSL2-PvMSL3* and *PvMSL8-PvMSL9*, revealed distinct expression patterns. These expression profiles suggest a divergence in the biological functions of *PvMSL* genes during plant development.

Conclusion

Based on *in silico* approach, this is the first study that provides some information about MSL family in *P. vulgaris* and has a crucial role in the plant growth and development.

Understanding the genetic bases of MS ion channels provide resources to select candidate genes for future functional analyses of *PvMSL* family in common bean, increasing the possibility to genetically engineer new traits of importance for agriculture and food production.

Conflict of Interests

The authors have not declared any conflict of interests.

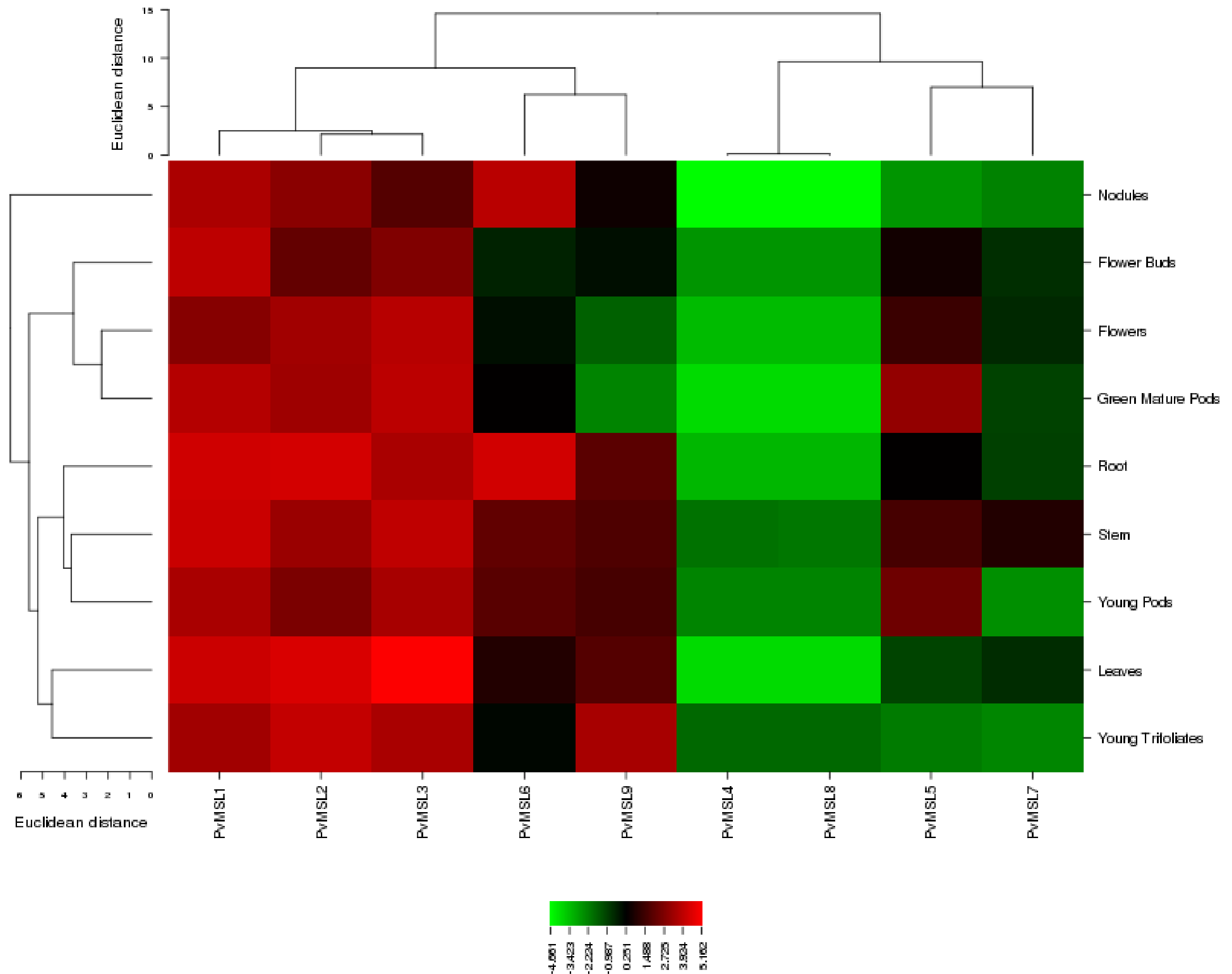

Figure 8. Differential expression patterns of common bean *PvMSL* genes. Heat map showing differential expression profile of common bean *PvMSL* genes inspecifictissues: leaves, roots, nodule, young trifoliates, flower buds, stem, flowers, green mature pods and young pods.

ACKNOWLEDGEMENTS

The authors thank UNIPAR for the financial support. TMI and TC thank CAPES for the fellowship.

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Vol. 15(15), pp. 593-600, 13 April, 2016
DOI: 10.5897/AJB2015.14841
Article Number: 28330C258072
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Biochemical effects of ethylene diamine tetra-acetic acid (EDTA) on cadmium treated maize (*Zea mays* L.) and cowpea (*Vigna unguiculata* L.)

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Received 7 July, 2015; Accepted 11 March, 2016

The effects of ethylenediaminetetra-acetic acid (EDTA) on germination, length of stem, area of leaf, fresh weight, level of lipid per oxidation, alkaline phosphatase, acid phosphatase, super oxide dismutase (SOD) and catalase in the roots of cadmium (Cd) treated maize (*Zea mays* L) and cowpea (*Vigna unguiculata* L) seedlings after 7 and 21 days of germination were determined in this study. The results obtained, indicate that at the end of 7 and 21 days of exposure to Cd, percentage germination of the seeds were not significantly different in both control and test soil ($p>0.05$). Morphological parameters (area of leaf, length of stem and fresh weight) were significantly reduced by Cd after 7 and 21 days. The supplementation of the soil sample with EDTA (0.5 mM or 1.0 mM) reversed the effect of Cd on these parameters as it significantly increased length of stems, area of leaf and plant fresh weight. There was a significant decrease in root acid phosphatase, root alkaline phosphatase, super-oxide dismutase (SOD) and catalase activity in both plant species. Both used doses of EDTA to ameliorate the above biochemical parameters. Increased level of root lipid peroxidation in Cd treated maize and cowpea seedlings was observed after 7 and 21 days of germination. Albeit, the level of lipid peroxidation in the root of Cd treated maize was significantly higher than that of cowpea, an indication that cowpea may be more tolerant than maize to Cd toxicity. The treatment of plant with, concentrations of EDTA (0.5 and 1.0 mM) failed to decrease the Cd induced, but increased the level of root lipid peroxidation. These results indicate that EDTA (0.5 mM and 1.0 mM) could be used for the treatment of Cd toxicity in plants; although, EDTA did not totally protect cowpea seedling from oxidative stress.

Key words: Heavy metal, phytoremediation.

INTRODUCTION

Cadmium is a heavy metal with, no known biological function. However, it is highly toxic to plants and animals (Chandler, 1996; Tran and Popova, 2013). Its effects on plants are manifested by inhibition of the normal uptake and utilization of mineral nutrients (Liu et al., 2003). Cadmium is thought to enter the environment mainly from industrial sources and during application of phosphate fertilizer; hence, it can be of high levels in agricultural

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soils. It is a major pollutant present in areas with heavy road traffic and near smelters and sewage sludge areas (Rascio et al., 1993). When taken up in excess, Cd can disturb almost all physiological processes in plants (Siedlecka and Baszyń-Aski, 1993). It is one of the most toxic metals in plants, active at concentration much lower than those of other heavy metals (Kudo et al., 2011). The metal can affect the overall metabolism through alterations in both the behavior of the key enzymes of important pathways (Shah et al., 2001), and membrane composition and function (Fodor et al., 1995; Madejón et al., 2006), including lowering the control of the cell redox state which ultimately causes oxidative stress (Gratao et al., 2005). Cadmium inhibits the photoactivation of photosystem 2 (PS2) by inhibiting electron transfer (Kudo et al., 2011). Hence, Cd can lead to the generation of reactive oxygen species (ROS) indirectly by production of disturbances in the chloroplasts. As a non-redox metal, Cd is unable to perform single electron transfer reactions, and hence does not directly produce ROS such as the superoxide anion (O_2•–), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), but thought to generate oxidative stress by interfering with the antioxidant defence system (Tran and Popova, 2013). In addition, some other reports suggested that Cd may stimulate the production of ROS in the mitochondrial electron transfer chain (Hassan and Aarts, 2011). Acid phosphatase and alkaline phosphatase activity can be used as marker enzymes for Cd toxicity in plant as the enzymes have been shown to be sensitive to Cd. Also the toxicity of Cd has been related with the increase of lipid peroxidation and alterations in antioxidant systems in plants (Fornazier et al., 2002; Tran and Popova, 2013).

Lipid peroxidation is the destructive oxidative process by ROS or nitrogen radicals of animals and plants fatty acids (Kudo et al., 2011). Thus, these polyunsaturated fatty acids are converted to lipid hydroperoxides (LOOH), rendering damage to biological membrane (Khan et al., 2013).

Alkaline phosphatase (ALP) is a hydrolase with sole function to removing phosphate groups from biological molecules including nucleotides, proteins and alkaloids. The process of removing the phosphate group is called dephosphorylation (Coleman, 1992). The presence of phosphate groups usually prevents organic molecules from passing through the membrane; therefore, dephosphorylating them may be important for plant uptake of organic compounds (Coleman, 1992; Chatchawal et al., 2008).

Acid phosphatases are widely distributed in plants and significantly differ in their susceptibility to inhibition by various compounds (Penheiter et al., 1997). Acid phosphatases specifically catalyze the hydrolysis of a variety of phosphate esters in acidic environment and these enzymes function in the maintenance of the phosphorus status of the plant, particularly with accessing phosphorus from the soil (Duff et al., 1994). Several factors have been shown to influence acid phosphatase activity; however, heavy metals effect on acid phosphatase is poorly understood.

Removal of excess metal ion from the soil is brought by chemical as well as biological means. Chemical remediation involves the use of chemicals to clean the environment, however, this cannot be considered universal as one chemical cannot be used for all metal ions (Shah et al., 2001). Examples of chemicals used for remediating Cd pollution in soils are, EDTA and pyrene.

Ethylenediaminetetraacetic acid with the formula $[CH_2N(CH_2CO_2H)_2]_2$, is a polyamino carboxylic acid. Its usefulness arises because of its ability to sequester metal ions such as Fe^{3+}, Cd^{2+}, Pb^{2+}, etc. After being bound by EDTA, metal ions remain in solution but exhibit diminished reactivity. Hence, it is important in biological system as a chelating agent. The complex of EDTA with Cd reduces its uptake by plants. However, very little work has been done on the effect of EDTA on Cd toxicity in plants. Hence, this study, specifically, investigated the effect of EDTA on germination, growth, fresh weight, root acid and alkaline phosphatase activity as well as root lipid peroxidation of Cd treated *Zea mays* L and *Vigna unguiculata* L. The results shows the co-treatment of Cd treated maize and cowpea seedlings with EDTA at 0.5 mM and 1.0 mM reversed the inhibition of growth rate, levels of SOD and catalase, acid and alkaline phosphatase activities in both seedlings.

MATERIALS AND METHODS

Plant materials and soil samples preparation

Maize (*Zea. mays)* and cowpea *(Vigna unguiculata*) seeds used for the study were sourced locally. Humus soil was sieved to remove debris and stones of the sieved soil (1.4 kg) was weighed and treated with 100 ml (100 mg /L) cadmium chloride ($CdCl_2$), and 100 ml (0.1 M) EDTA). The control soil was similarly treated with 100 ml of water before planting in perforated bags. Treatment of the soil was carried out by gradual mixing in a solution of $CdCl_2$ alone, and $CdCl_2$ containing 0.5 ml (1.0 mM EDTA) for the experimental samples. The control soil was treated with an equivalent volume of water.

Experimental design

The seeds of maize and cowpea, sown in various treated soils are described above. The polyethene bags containing the soil implanted with maize seedlings were kept in a greenhouse for germination. Each treatment including control consisted of a set of 3 seeds per bag and was replicated seven times. All treated soil was kept moist with water to aid seed germination. Record of seed germination was taken after first, second, third, and fourth day of germination, respectively, and the seeds which fails to grow or sprout after that time was regarded as not germinated. Plants from each treatment group were harvested after 7 and 21 days, respectively for morphological and biochemical studies.

Early seedling growth

Early seedling growth rate was measured using vine length of 7 and

Table 1. Effect of EDTA on length of stem, fresh weight and area of leaf of cadmium treated maize seedlings.

Parameter	Control	100 mg/L Cd^{2+}	100 mg/L Cd^{2+} + 0.5 mm EDTA	100 mg/L Cd^{2+} + 1.0 mm EDTA
7 days				
Length of stem (cm)	11.2±0.28[a]	9.6±0.53[b]	11.1±1.12[a]	11.1±0.66[a]
Fresh weight (g)	2.25±0.28[a]	1.73±0.10[b]	2.02±0.41[a]	1.86±0.42[b]
Area of leaf (cm^2)	39.9±10.29[a]	29.0±2.49[b]	29.3±3.24[b]	33.1±3.32[c]
21 days				
Length of stem (cm)	16.5±1.29[a]	14.3±0.45[b]	14.5±1.73[b]	15.9±1.14[a]
Fresh weight (g)	5.82±0.60[a]	3.72±0.98[b]	4.24±1.08[c]	3.66±0.52[b]
Area of leaf (cm^2)	64.8±18.02[a]	47.8±8.46[b]	63.1±0.45[a]	49.8±6.76[b]

21 day old plants. The vine length was measured from the soil level of the terminal bud.

Measurement of fresh weight

Fresh weight was determined from specimens of 7 and 21 days old maize and cowpea plants using the Setra BL-40s precision electronic balance. The uprooted specimen were carefully rinsed with portable water and dried before weighing.

Measurement of leaf length and weight

Leaf lengths were determined from specimen of 7 and 21 days old seedlings using a ruler and its value recorded in cm^2.

Collection of samples

The roots of maize plants were collected randomly from each of the treatment group as pooled samples. These roots, pooled together as a sample were used for biochemical analysis. They were stored at -10°C until required for homogenization, done within 24 h.

Preparation of samples

Weighed quantities of roots were homogenized in pre-chilled mortar using 5 ml of 1-X normal saline (0.9% NaCl) solution. The homogenate was centrifuged at a speed of 5000 X g and the supernatant transferred into a labeled test tube until required for biochemical analysis.

Assay for acid and alkaline phosphatase

The activity of acid and alkaline phosphatase was determined using the method of Giese (1971). The amount of p-nitrophenol produced was estimated from a standard curve. The activity of the enzyme is expressed as µmole p-nitrophenol produced/ min^1/ ml.

Assay for lipid peroxidation

Level of lipid peroxidation was determined using the method of Gutteridge and Wilkins (1982), and Halliwell and Gutteridge (1984).

Statistical data analysis

The data are presented as ±standard error of mean (SEM), [following similar analysis by Nwachukwu et al. (2014) and Nwose et al. (2015)] and are analysed statistically by one-way analysis of variance (ANOVA). This is followed by Duncan's multiple range test using SPSS 10.0 computer software package (SPSS Inc., Chicago, U.S.A). The correlation analysis was performed, quoting the Pearson correlation coefficients and test of significance, with significance accepted at P≤<0.05.

RESULTS

The present study investigated the effect of EDTA on Cd toxicity in plants using maize (*Z. mays*) and cowpea (*V. unguiculata*) as plant model. Moreover, the experiments was also designed to establish the effect of EDTA on germination, growth, fresh weight, root acid and alkaline phosphatase activity as well as root lipid peroxidation of Cd treated *Z. mays* L. and *V. unguiculata* L. as these were critically examined.

The effect of EDTA on length of stem, fresh weight and area of leaf of Cd treated maize seedlings is presented in Table 1. The length of stem of Cd (in soil exposed) treated plants was significantly decreased in relation to control after 7 days of germination. Conversely, the Cd and EDTA significantly increased the length of stem of the plants as compared to the plants treated with Cd only. The length of stem was not significantly different from the control after 7 days of germination.

A similar trend was observed in maize plants treated for 21 days, with exposure to Cd (Table 1). However, supplementation of the soil with 1.0 mM EDTA reversed the effects of Cd on the length of stem of maize plants (Table 1), while no significant changes was observed in this parameter in plants grown in soils treated with Cd and 0.5 mM EDTA as compared to those grown in soils treated with Cd alone. The fresh weight of Cd treated plants was significantly decreased relative to control after 7 days of germination. However, supplementation of the soil with 0.5 mM EDTA reversed the effects of Cd on

Table 2. Effect of EDTA on length of stem, fresh weight and area of leaves of cadmium treated cowpea plants.

Parameter	Control	100 mg/L Cd^{2+}	100 mg/L Cd^{2+} + 0.5 mm EDTA	100 mg/L Cd^{2+} + 1.0 mm EDTA
7 days				
Length of stem (cm)	20.1±0.42[a]	19.6±1.69[a]	20.51±0.54[a]	23.32±0.84[b]
Fresh weight (g)	1.96±0.06[a]	1.66±0.35[a]	1.85±0.11[a]	1.80±0.22[a]
Area of leaf (cm^2)	36.75±2.71[a]	35.05±4.24[a]	36.27±26.7[a]	27.01±2.13[b]
21 days				
Length of stem (cm)	27.8±2.21[a]	22.7±3.16[b]	24.87±2.71[c]	24.6±3.71[c]
Fresh weight (g)	5.16±1.143[a]	2.35±0.65[b]	4.05±1.14[a]	4.59±2.303[a]
Area of leaf (cm^2)	35.94±10.58[a]	31.73±4.73[b]	34.57±10.04[a]	25.34±5.21[c]

Results expressed as mean±standard deviation (SD). Values with the same letters are not significantly different (p>0.05).

fresh weight of the maize plant, while no significant changes was observed in this parameter in plants grown in soils treated with both Cd and 1.0 mM EDTA as compared to those treated with Cd alone. The fresh weight of the plants treated with both Cd and 0.5 mM EDTA were not significantly different from the control after 7 days of germination.

Also, the exposure of the plants to Cd decreased fresh weight relative to control after 21 days of germination. However, supplementation of the soil with 0.5 mM EDTA significantly increased fresh weight of maize plant, while no significant change was observed in fresh weight of plants grown in soils treated with both Cd and 1.0 mM EDTA as compared to those grown in soils treated with Cd alone (Table 1). The fresh weight of plants treated with both Cd and 0.5 mM EDTA was also significantly different from the control after 21 days of germination.

The area of leaf of Cd treated plants (grown in cadmium treated soil) was significantly decreased relative to control after 7 and 21 days of germination. Supplementation of the soil with 1.0 and 0.5 mM EDTA significantly increased leaf area of the maize plants relative to the plants treated with Cd after 7 and 21 days, respectively. Thus, the study shows that EDTA reversed the effect of Cd on morphological parameters of maize seedling, albeit, the influence of EDTA was not dose dependent.

Similarly, the effects of EDTA on length of stem, fresh weight and area of leaf of Cd treated cowpea plant were studied and the results presented in Table 2.

The length of stem of Cd treated cowpea plants showed no significant difference relative to control after 7 days of germination, but was significantly decreased relative to control after 21 days (Table 2). The effects of Cd on length of stem of the plants was reversed by addition of 0.5 mM concentrations of EDTA after 21 days of germination as the stem length of the plants was significantly increased relative to Cd treated plants.

Moreover, the fresh weight of Cd treated cowpea plants was not significantly different relative to control after 7 days of germination, neither did the administration of EDTA alter the fresh weight of the Cd treated plants as compared to plants treated with Cd after the same period of exposure (Table 2). As noticed, the fresh weight of Cd treated cowpea plants was significantly decreased after 21 days relative to the plants treated with Cd only. However, treatment of the Cd treated cowpea plants with both concentrations (0.5 to 1.0 mM) of EDTA restored the fresh weight of the plants to levels comparable with the control.

The area of leaf of Cd treated cowpea plants was significantly decreased relative to control after 21 days of germination however, no significant difference was observed after 7 days. The simultaneous treatment of plants with Cd and 0.5 mM EDTA significantly increased the area of leaf of the 21 days plants relative to plants treated with only Cd (Table 2), whilst the simultaneous treatment of the plant with Cd and 1.0 mM EDTA appear to be adverse as it decreased leaf area as compared to other experimental groups (Table 2). Thus, the study also established that morphological parameters of cowpea were significantly decreased after 21 days of germination and the co-treatment of Cd treated cowpea plants with EDTA tend to restore the levels of these parameters as they were significantly increased relative to plants treated with Cd only, albeit the effectiveness of EDTA was not specific to a concentration.

Also, the effect of EDTA on root acid phosphatase activity of Cd treated maize plants was studied (Table 3). The root acid phosphatase activity of Cd treated maize plants was significantly decreased relative to control after 7 and 21 days of germination. The co-treatment of maize plants with Cd using both concentrations of EDTA was observed to significantly increase root acid phosphatase activity at 7 and 21 days of germination as compared to plants treated with Cd only.

Tables 4 and 5 show the effect of EDTA on root alkaline phosphatase activity of Cd treated maize and cowpea plants, respectively. The result data shows no significant change in root alkaline phosphatase activity in both control and test soils after 7 days. However, the root alkaline phosphatase activity of Cd treated plants was

Table 3. Effect of EDTA on root alkaline acid phosphatase activity of cadmium treated maize plants.

Parameter	Control	100 mg/L Cd^{2+}	100 mg/L Cd^{2+} + 0.5 mm EDTA	100 mg/L Cd^{2+} + 1.0 mm EDTA
7 days				
Alkaline phosphatase	7.75±0.526[a]	7.50±0.346[b]	7.55±0.300[a]	7.70±0.740[a]
Umol/PNP/min/ml				
Acid phosphatase	39.9±10.29[a]	2.22±0.218[b]	2.39±0.679[b]	2.68±0.197[c]
21 days				
Alkaline phosphatase	12.07±0.946[a]	8.15±1.099[b]	9.67±0.582[c]	10.38±0.462[c]
Umol/PNP/min/ml				
Acid phosphatase	5.04±0.621[a]	3.47±0.315[b]	4.00±0.194[c]	4.16±1.275[c]

Results expressed as mean±standard deviation (SD). Mean with the same letter in the same root is not significantly different ($p>0.05$).

Table 4. Effect of EDTA on level of alkaline acid phosphatase in roots of cadmium treated cowpea plant.

Parameter	Control	100 mg/L Cd^{2+}	100 mg/L Cd^{2+} + 0.5 mm EDTA	100 mg/L Cd^{2+} + 1.0 mm EDTA
7 days				
Alkaline phosphatase	17.8±1.68[a]	16.7±1.79[a]	17.0±3.40[a]	17.0±1.20[a]
Acid phosphatase	3.3±0.37[a]	2.03±0.45[b]	2.3±0.62[b]	3.38±0.75[a]
21 days				
Alkaline phosphatase	21.8±1.804[a]	13.2±1.407[b]	18.2±2.250[c]	19.5±1.804[c]
Acid phosphatase	5.3±0.86[a]	4.6±0.41[b]	5.1±0.37[a]	5.41±1.13[c]

Results are expressed as mean±standard deviation (SD). Values with same letters are not significantly different ($p>0.05$).

Table 5. Effect of EDTA on levels of SOD, catalase and lipid peroxidation in roots of cadmium treated maize seedlings.

Parameter	Control	100 mg Cd/L	100 mg Cd/L + 0.05 mM EDTA	100 mgCd/L + 1.0 mM EDTA
7days				
SOD	74.5±4.2[a]	52.6±3.5[b]	70.0±7.5[a]	68.5±6.4[a]
CAT	2.56±0.4[a]	1.8±0.3[b]	2.4±0.3[a]	2.6±0.4[a]
LPO	413.0±16.77[a]	469.0±12.45[b]	445.4±9.64[b]	484.7±4.51[c]
21 days				
SOD	60.2±4.5[a]	34.5±3.0[b]	58.5±3.6[a]	45.0±4.0[c]
CAT	2.3±0.6[a]	1.2±0.3[b]	2.4±0.5[a]	1.8±0.6[c]
LPO	412.1±19.46[a]	657.1±17.4[b]	621.3±8.80[b]	652.1±17.47[b]

Results expressed as mean± standard deviation (SD). SOD, Super oxide dismutase; LPO, lipid peroxidation; CAT, catalase.

significantly decreased after 21 days of germination. Thus, the treatment of maize and cowpea plants with Cd at both concentrations of EDTA (0.5 and 1.0 mM EDTA) significantly increased root alkaline phosphatase after 21 days of germination as compared to plants treated with Cd only. These results are firm indication that both

Table 6. Effect of EDTA on levels of SOD, catalase and lipid peroxidation in roots of cadmium treated cowpea seedlings.

Parameter	Control	100 mg Cd/L	100 mg Cd/L + 0.05 mM EDTA	100 mgCd/L + 1.0 mM EDTA
7days				
SOD	85.0±5.2[a]	40.8±4.0[b]	63.5±4.5[c]	48.0±3.8[b]
CAT	2.8±0.6[a]	1.5±0.2[b]	2.7±0.4[a]	1.2±0.2[b]
LPO	333.3±10.8[a]	463.8±8.33[b]	441.0±9.5[c]	420.0±4.4[c]
21 days				
SOD	80.0±4.5[a]	54.2±4.0[b]	78.5±4.0[a]	75.4±5.0[a]
CAT	2.5±0.3[a]	2.3±0.2[a]	2.4±0.2[a]	2.5±0.3[a]
LPO	358.81±8.35[a]	635.5±4.53[b]	599.4±5.97[c]	573.0±10.5[c]

Results expressed as mean± standard deviation (SD). Values with same letters are not significantly different ($p>0.05$). SOD, Super oxide dismutase; LPO, lipid peroxidation; CAT, catalase.

concentrations of EDTA reversed the effects of Cd on root alkaline phosphatase activity after 21 days of germination.

The effect of EDTA on level of root SOD, catalase and lipid peroxidation of cadmium treated maize and cowpea seedlings are presented in Table 6. Conversely, the level of lipid peroxidation of both Cd treated maize and cowpea plants were significantly increased relative to control after 7 and 21 days of germination, whereas that of SOD and catalase were significantly decreased after 7 and 21 days except for catalase activity of cowpea after 21 days. The simultaneous treatment of maize seedlings with both concentrations of EDTA could not restore the level of lipid peroxidation induced by Cd relative to control although there was a significant decrease for cowpea. However, supplementation with 0.5 and 1.0 mM EDTA restored the activity of SOD and catalase compared to test for maize after 7 and 21 days. On administration of 0.5 mM EDTA to Cd-treated cowpea seedlings, a significant increase was observed in the levels of SOD and catalase compared to Cd-treated cowpea plants. No significant change was seen on treatment with 1.0 mM EDTA except for SOD level after 21 days.

DISCUSSION

Cadmium from various sources of chemical pollutants accumulates in the soil and is taken up by plants (Ernst, 1980). In the present study, a weighed quantity of soil (1.4 kg) was treated with 100 ml (100 mg Cd/L), two solutions of 100 mg Cd/L containing 0.5 mM EDTA, and 100 mg Cd/L containing 1.0 mM EDTA and an equivalent volume of water was used for control samples. Seeds of maize (*Z. mays* L.) and cowpea (*V. unguiculata* L.) were planted in these soils. The percentage germination, length of stem, area of leaves, fresh weight as well as root acid phosphatase and root alkaline phosphatase and levels of root lipid peroxidation, SOD and catalase were monitored on both plants and comparison made to show the level of cadmium toxicity and possible effects of EDTA on cadmium treated plants.

Generally, there are five basic applications of Cd. About three-fourth of Cd is used as electrode materials in nickel-cadmium batteries; most of the remaining one-fourth is used mainly as pigment in plastics and glasses, protective plating for steel, stabilizers for plastic (Poly Vinyl Chloride (PVC), and as a component of various alloys. Also, Cd has ability to absorb neutons, so it is used as a barrier to control nuclear fission (Scoullos et al., 2001). Recently, the role of Cd in biology was discovered, as carbon dependent carbonic anhydrase has been found in matrine diatoms. Cadmium does the same job as zinc in other anhydrases, but the diatoms live in environment with very low zinc concentrations, thus biology has taken Cd rather than zinc (Lane and Morel, 2000; Lane et al., 2005).

Results obtained from the study, show that germination of both maize and cowpea seeds were not influenced by treatment with Cd alone or the co-treatment of Cd and EDTA at 0.5 and 1.0 mM concentrations, respectively. This, result suggests that Cd has no effect on the germinating ability of the plants, which may not be surprising as they lack prominent roots at early stage of seed germination. Similar results have been reported previously (Dong et al., 2006). Thus, growth rate which was assessed by the length of stem, fresh weight and areas of leaf of Cd treated maize and cowpea seedlings was inhibited at 7 and 21 days of exposure, an indication of toxicity of the metal on these plants (Table 1). This retardation in growth rate could be due to interference with mineral station, which hampered the uptake and translocation of essential elements (Dong et al., 2006).

Previous study by Ci et al. (2009), reported that heavy metals such as Cd affects plants by inhibiting the normal uptake and utilization of mineral nutrients within the plant cells. Hence, high amounts of Cd therefore may lead to the displacement of micronutrients such as; Zn, Mn, Fe, etc. including other essential nutrient sharing similar valency from their sites of action. This may in turn lead to

physiological changes resulting in the stimulation of some enzymatic activities that limit cell growth and consequently accelerating tissue senescence (De-Pasquale et al., 1988; Dunwei et al., 2009). Therefore, the significant decrease in root acid phosphatase (Tables 4 and 5), and alkaline phosphatase activities of Cd treated maize and cowpea seedlings may be due to the displacement of metal ion cofactors for example Mg^{2+} by Cd at high concentration from the active site of the enzyme as both phosphatase are metallo-enzymes. Furthermore, other possible mechanisms for Cd toxicity in plants at high concentrations can and may be through its interference with PO_4^{3-} binding sites of the enzymes (Gonçalves et al., 2007).

The exposure of maize and cowpea seedling to Cd treated soil for 7 and 21 days caused an increase in level of root lipid peroxidation of the plants (Table 6). Similar trend has been reported in *Phaseolus vulgaris* (Sandalio et al., 2001; Chaoui et al., 1997), *Helliantus annuus* (Benavides et al., 2005), *Pisum sativum* plant (Lozano-Rodriguez et al., 1997) which caused irreversible damage to cell function thus affecting growth rate. However, both concentrations of EDTA (0.5 mM and 1.0 mM) reversed the Cd-induced increase in level of root lipid peroxidation in Cd treated cowpea seedling after 7 and 21 days of exposure.

Ethylenediaminetetraacetic acid is known to be a good chelating agent, hence, in biological systems, EDTA are used to bind metal ions in the form of chelation therapy, e.g. for mercury and lead poisoning. It is used in similar manner to remove excess iron from the body (Huldé and Harju, 1980). This therapy is used to treat the complication of repeated blood transfusions, as would be applied to patient with thalassemia phenotype. EDTA acts as a powerful antioxidant to prevent radicals from injuring blood vessel walls (Lamas et al., 2014).

Results obtained in the present study show that the toxicity conferred on maize and cowpea seedlings by Cd stress was reversed when test soils were supplemented with EDTA at 0.5 and 1.0 mM, respectively, albeit, effects was not dose dependent. This suggests that the supplementation of test soils with EDTA made Cd unavailable to the plants. Similarly, Wang et al. (2008) found that EDTA also affected Cd translocation from plant roots to plant shoots and that the complexation of EDTA with Cd reduced the uptake of Cd by plants. It has also been reported that the uptake of free Cd^{2+} in nutrient solution is diminished in the presence of EDTA, suggesting that the Cd-EDTA complex is unable to penetrate the membranes (Greger and Lindberg, 1986). Also, Debra et al. (2007) study, on the effect of chelating agents on Cd treated plants had shown EDTA to increase the aqueous solubility of Cd by 36 times over the soil matrix thereby increasing the possibility of leaching. Among all chelating agents used, EDTA has the most marked effect with a 45% reduction in total Cd (Debra et al., 2007).

The significantly higher levels of root lipid peroxidation of maize as compared to cowpea seedling after 7 and 21 days of germination is a likely indication that cowpea seedlings may be more tolerant than maize to Cd toxicity (Table 6).

It is however noteworthy that a higher concentration (1.0 mM EDTA) has an adverse effect on the growth parameters of Cd exposed cowpea (Table 2). Available studies had indicated in highly polluted environment, excessive uptake of EDTA, enhances uptake of metals, thus, increasing phytotoxicity. The fact is high internal concentration disturbs almost all physiological processes in plants. Despite achievements in sequestering Cd phytotoxicity, its physiological nature is not fully understood. Thus, it poses huge problems in regions of heavy metals pollution in which a sharp decrease in agricultural crop production has been observed.

Since Cd is readily taken up by root of many plant species with its toxicity considered to be far higher than that of other heavy metals; Cd pollution is of great interest in scientific research (Gussarson et al., 1996). Due mainly to modern industrial practices, more and more toxic ions are being added to the natural environment with adverse consequences to the ecosystem. Thus, metals like Cd and Pb, present in high concentration in soil showed potential toxicity on overall growth and plants metabolism. The bio-accumulation of such toxic metals in plants possesses a threat to human and animal health.

In plants, it was previously established that Cd inhibits the photosynthetic rate with toxic Cd effects depending on the applied concentrations, the species and cultivation characteristics, the age of leaves and the phonological development of plants (Lagriffoul et al., 1998; Lux et al., 2004; Khan et al., 2013). The rate of photosynthesis, chlorophyll content, activities of photosystem 1 (ps1) and ps11 declined progressively with increasing concentration of applied Cd (Lux et al., 2004; Dunwei et al., 2009; Kudo et al., 2011). Moreover, human exposure to Cd and Cd containing compounds are known to be carcinogenic, hence can induce kidney dysfunction, renal disease that are characterized by severe tubular combined with glomerular disorders (Friberg, 1983).

Conclusion

Results of the present study show that the co-treatment of Cd treated maize and cowpea seedlings with EDTA at 0.5 and 1.0 mM, respectively reversed the inhibition of growth rate, levels of SOD and catalase, acid and alkaline phosphatase as well as activities in both seedlings. However, the effect of EDTA observed was not dose dependent.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Vol. 15(15), pp. 601-612, 13 April, 2016
DOI: 10.5897/AJB2015.15199
Article Number: AACECC858074
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Effect of monosodium glutamate and aspartame on behavioral and biochemical parameters of male albino mice

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Received 30 December, 2015; Accepted 29 March, 2016

The present study aimed to investigate the individual and combined effect of mono-sodium glutamate (MSG) and aspartame (ASM) on biochemical, blood parameters and neuro-behavioral aspects of mice. The results indicated that exposure induced many changes in fear and anxiety behavior. The non-social and social behavior of the exposed mice was significantly affected, showing an increase in the former and a decrease in the later stages, respectively. The elements of social behavior including attack, numbers of fights and bites, naso-nasal and naso-genital contacts were decreased significantly. The latencies to threat and attack and first bite were increased significantly. Locomotor activity and neuromuscular coordination (grip strength) were decreased in treated animals as compared to the control group. There was a significant decrease in the red blood cell count, packed cell volume, hemoglobin concentration, white blood cell count platelets count and testosterone hormone in the treated males. The activity of acetylcholinesterase enzyme decreased as compared to the control. In conclusion, the current study indicated that exposure to food additives MSA and ASM was dangerous to mice in relation to behavior and biochemical analysis. In addition, these food additives need more scientific researches to investigate their effect on other parameters.

Key words: Mono-sodium glutamate, mono-sodium aspartame, fear and anxiety, locomotory behavior, grip strength, acetylcholinesterase.

INTRODUCTION

Food additives that are intended for human use are generally approved after testing for their toxicity through animal toxicity tests (Kokoski et al., 1990). The overall goal of such tests is twofold: to assess the additive's potential to cause toxic effects in humans and to determine if safe conditions of use can be established. However, evaluation for the safe consumption of such food additives is usually based on their toxicity data obtained from animal studies since human data are scantily available (Lin et al., 1992).

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Monosodium glutamate (MSG) is one of the most popular flavoring agents of modern time and is widely used in many commercially packed food and restaurant and household cooking. It is reported that neonatal exposure to MSG (4 mg/g body weight) in rats and mice causes learning difficulty (Abu-Taweel et al., 2014), obesity (Nagasawa et al., 1974), and gonadal dysfunction (Pizzi et al., 1978). Brain damage induced by the neurotoxicity of MSG has also been established in experimental chicken (Robinzon et al., 1974). MSG injected i.p. at 2 and 4 mg neonatally in mice produced lesions in the arcuate nucleus region of the brain affecting the regulation of water drinking (Morley and Flood, 1980). Acute ingestion of MSG has been associated with adverse symptoms that include general weakness, muscle tightness or tenderness, flushing or sweating, headache, paresthesias, arrhythmias and tachycardia in healthy individuals (Shimada et al., 2015).

Aspartame (ASM) is a dipeptide (L-aspartyl-L-phenylalanine methyl ester) and is used as an artificial sweetener that is now in wide and frequent use. It was discovered by chance over 25 years ago and was first used as a food additive 13 years ago (Lajtha et al., 1994). ASM is used in a variety of food products; however, ASM-related neurological disturbances such as dizziness, headaches, gastrointestinal symptoms, mood alterations, allergic type reactions and alterations in menstrual patterns have also been reported (Nagasawa et al.,1974; Abu–Taweel et al., 2014). Aspartame is metabolized in the gastrointestinal tract into 50% of phenylalanine, 40% of aspartic acid and 10% of methanol. The degradation of aspartame may be due to both temperature changes and the time spent before usage. d-Phenylalanine, d-aspartic acid and methanol may be formed on exposure to heat, during shipping, baking, heated foods and beverages. Effect of aspartame consumption on behavior is the most important aspect to be considered because controversial reports do exist for aspartame (Ashok et al., 2014). The present study aimed to investigate the individual and combined effect of mono-sodium glutamate and mono-sodium aspartame on biochemical and neuro-behavioral aspects in mice.

MATERIALS AND METHODS

Experimental animals

Forty male Swiss-Webster strain mice (8–10 weeks old, bred and reared under controlled conditions) were housed in opaque plastic cages measuring 30 × 12 × 11 cm (5 animals per cage) under hygienic conditions in the animal facility of the Zoology Department, King Saud University, Riyadh, Saudi Arabia. All animals were maintained under reversed lighting conditions with white lights from 22.00 to 10.00 h local time. The ambient temperature was regulated between 20 and 22°C. Food (Pilsbury's Diet) and water were available *ad libitum*, unless otherwise indicated. All procedures were carried out in accordance with the ethical guidelines for care and use of laboratory animals, and all protocols were approved by the local Ethics and Care of Experimental Animals Committee.

MSG and ASM administration

All animals were randomly divided into four different groups with ten animals each. Group I consisted of untreated mice and served as naive controls since they were given only plain tap water. Group II was treated with monosodium glutamate (MSG) at a dose of 8 mg/kg body weight/day, dissolved in drinking water. Group III was treated with aspartame (ASM) at a dose of 32 mg/kg body weight/day, dissolved in drinking water. Group IV was treated with MSG and ASM together with the same doses as in groups II and III dissolved together in drinking water. The doses were selected on the basis of our pilot studies and from available literature. All exposures were through oral administration in their drinking water that formed the only source of drinking fluid for a period of one month. Pilot studies have shown that a normal adult mouse on average consumes 30 ml of water per day. Thus, all doses of MSG and ASM were prepared in such a manner that the required doses of MSG and ASM (individually and in combination) were consumed by the animals per day through their daily consumption of water. MSG and ASM of analytical grade, from Sigma Chemical Company, USA, were used in this study. After the exposure period of one month, the animals were subjected to cognitive behavioral tests in a shuttle box and a water maze. Subsequently, the animals were sacrificed and the neurotransmitters and oxidative stress parameters were estimated in their forebrain tissue.

Behavioral studies

Social behavior in all male animals was measured in the “standard opponent” test and the “tube restraint test”.

Anxiety behavior in the elevated plus-maze test

The elevated plus-maze (with 2 opened and 2 enclosed arms) is frequently used as a measure for evaluating the risk assessment and anxiety behavior of an ethologically derived animal model (Wall and Messier, 2001). The plus-maze was elevated to a height of 80 cm above the floor. The mice were individually placed onto the central platform facing one of the open arms and were observed for 5 min while freely exploring the maze. The animal was considered to have entered an arm when all four limbs were inside the arm. Duration of time spent in open and enclosed arms and number of entries in open and enclosed arms were measured during the test period. On completion of the test, the maze was cleaned with a 10% ethanol solution for control.

Standard opponent test

All males from each treatment category and control group were individually housed in new cages for 14 days. After this isolation period, these male animals were subjected to “standard opponent” tests under dim red lighting (ca. 8 lux). The docile and age-matched male “standard opponents” were rendered anosmic by applying 25 µl of 4% zinc sulphate solution to the nasal tract under ether anesthesia for three days prior to encounters. The anosmic ‘standard opponent’ intruders were introduced in the home cages of ‘test animals’ and the “standard opponent” test of each ‘test animal’ was observed visually for 500 s. The opponents were used to assess the selected “elements” of behavior as studied earlier by standard procedures (Brain et al., 1987; Ajarem and Ahmad, 1991; Abu-Taweel and Ajarem, 2008).

Tube restraint test

The males from treated and control group were used for the 'tube

restraint test'. The apparatus was based on the equipment described by standard procedure (Abu-Taweel et al., 2006) and consisted of a cylindrical transparent perspex tube 13 cm in length and with an internal diameter of 3.1 cm. One end of the tube was blocked by a perforated perspex wall through which a 2 cm long metal target was attached to a telegraph key/electronic counter arrangement. This enabled recording the number of bites directed by the restrained mouse towards the target. The test was conducted visually for 500 s under normal laboratory white lighting and temperature.

Motor activity test in automated activity meter

Spontaneous motor activity has been used extensively in rodents to study the pharmacological and toxicological effects of chemicals. Motor activity was measured using automated electronic activity meter (Ugo Basile, Comerio-Varese, Italy) (Kim et al., 2015). The horizontal and vertical motor activities were detected by arrays of infrared beams located above the floor of the testing arena. Each interruption of the beams on the x or y axis generated an electric impulse which was recorded on a digital counter. Each animal was tested separately and the motor activity was recorded for a period of 2 min in the activity meter (Abu-Taweel et al., 2013).

Neuromuscular coordination in grip strength meter

Neuromuscular coordination was measured using automated electronic grip strength meter (U.S. company products Biocompare). Device is consists of two parts; first a square base is composed of Perspex material, ending with rectangular column length of 15 cm, on the column cylinder fixed by anther column, and out of brass pieces length of about 5 cm (catching the mouse during the test). Test maintenance of mouse was started gently in the tail; it was placed in front of a copper piece. It remained in the front by raising the hind limbs when measuring the power base forelimbs. It was laid at the rear base to measure the tensile strength of the parties Quartet. Grip strength was measured by kg/m after two minutes (Ali et al., 2004).

Blood parameters

After completion of behavioral tests, the blood was collected from the retro-orbital plexus of the animals in heparinized tubes at the end of the experiments. Blood parameters namely, red blood count, packed cell volume, hemoglobin content, total white blood count and blood platelets were measured using the automated parameter hematology analyzer (T 450, USA).

Estimation of testosterone in plasma

The collected blood was centrifuged at 4000 rpm/min for 10 min and plasma was obtained and kept at -30°C until it was used for hormones estimation. Testosterone was estimated using hormone analysis instrument (Hitachi-Eleceys 2010-Roche Diagnostic, USA) by the method of electrochemiluminescence immunoassay.

Biochemical studies in brain tissue

The brain of animals were removed and gently rinsed in physiological saline (0.9% NaCl), and then blotted on Whatman filter paper. Their fresh weights were recorded, and organs were then frozen.

Brain homogenate preparation

A 10% (w/v) homogenate of each frozen brain was prepared in teflon-glass homogenizer at 4 ± 1°C, centrifuged at 1000 xg for 10 min to remove cell debris and the supernatant was used for enzyme assays. The brain homogenate was prepared in an ice-cold phosphate buffer (0.067 M, pH 7.2) solution.

Estimation of AChE

The AChE activity in the homogenised brain tissue was estimated by the method of Ellman et al. (1961), utilizing acetylthiocholine iodide (ATCI) as substrate. The rate of production of thiocholine was determined by the continuous reaction of the thiol with 5,5-dithiobis-2-nitrobenzoate (DTNB) ion to produce the yellow anion of 5-thio-2- nitro-benzoic acid. Spectrophotometric assay of enzyme activity was performed by adding 0.4 ml of the supernatant to a cuvette containing phosphate buffer (2.6 ml, pH 8) and 0.2 ml of 5,5%-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma Chem. Co., St. Louis, MO,USA). After adjusting the absorbance to zero, 0.02 ml of the substrate acetylthiocholine iodide (Sigma Chem. Co., St. Louis, MO, USA) was added and change in absorbance over 5 m was recorded. The specific activity of AChE was expressed as μ moles of acetylthiocholine iodide hydrolyzed/min/g of wet tissue.

Statistical analysis

The data of standard opponent and tube restraint tests were compared within the experimental groups by the analysis of variance (ANOVA) and were subsequently analyzed using Mann-Whitney U tests. Data of fear and anxiety in Plus-Maze, locomotry behavior, neuromuscular activities in grip strength meter, blood parameters and biochemical analysis were compared within the experimental groups by the analysis of variance (ANOVA) using minitab computer programme and were subsequently analyzed by Student's t-test (Yamane, 1973).

RESULTS

Anxiety behavior in the elevated plus-maze test

Exposure to MSG and ASM both (individually and in combination) caused many disorders in fear and anxiety behavior. The treated animals spent more time in the closed arm ($P < 0.001$) while the time spent in opened arm was shorter ($P < 0.001$) in the control animals (Figure 1). Figure 2 shows that the number of entries of open arm decreased ($P < 0.001$) while the number of entries of closed arm increased ($P < 0.001$) in the treated animals as compared to the controls.

Social behavior (standard opponent and tube restraint tests)

The results indicate that exposure to MSG and ASM led to changes in social behavior of mice. The data on standard opponent and tube restraint tests (Tables 1A, B and 2) showed that MSG and ASM increased significantly nonsocial investigation, number of wall rears and rears,

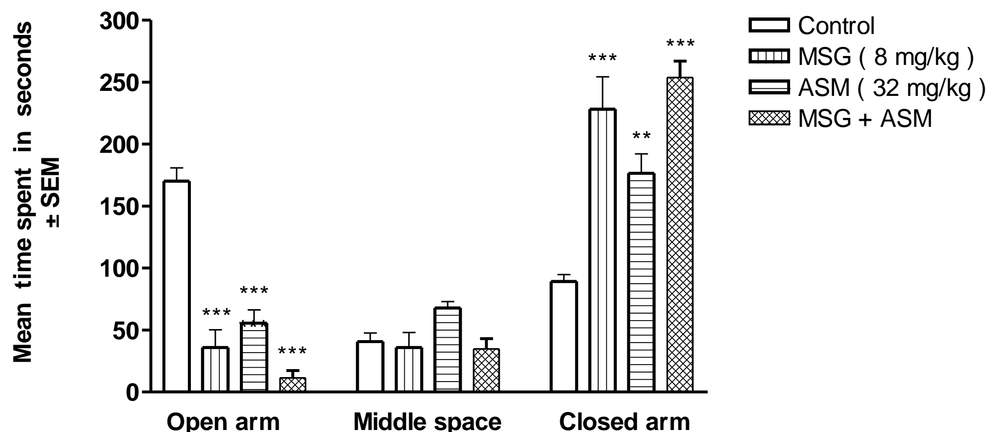

Figure 1. Effects of MSG and ASM on time spent in closed and open arms. ** and *** statistically significant at $p<0.01$ and $p<0.001$ respectively, as compared to the control group by ANOVA and student's t-test.

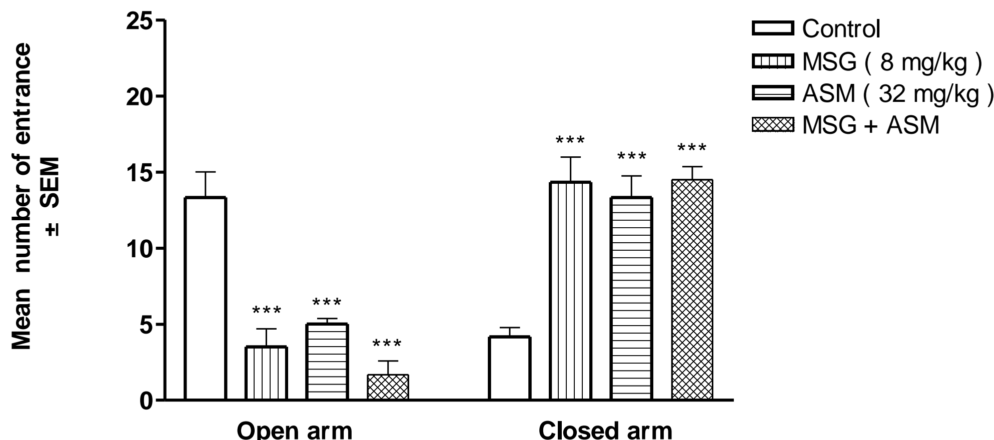

Figure 2. Effects of MSG and ASM on entries number of closed and open. *** statistically significant at $p<0.001$, as compared to the control group by ANOVA and student's t-test.

latency to threat ($p < 0.05$, $p < 0.001$), attack and latency to first bite ($p < 0.05$, $p < 0.01$, $p < 0.001$), while it decreased significantly social investigation, time of defense, attack and displacement ($p < 0.05$, $p < 0.01$, $p < 0.001$), threat ($p < 0.05$), number of fights, naso-nasal and naso-genital contacts, and number of bites ($p < 0.001$). Furthermore, the data in these tables show that exposure in combination has more effect than individual toxicity of MSG and ASM.

Motor activity test in automated activity meter

Figure 3 shows the decrease in motor activity induced by exposure (individually and in combination) to MSG and ASM. Horizontal and vertical activities were reduced significantly ($p<0.001$ and $p<0.05$, $p<0.001$ and $p<0.001$, respectively) as compared to the control group.

Neuromuscular coordination in grip strength meter

Exposure to MSG and ASM (individually and in combination) impact the neuromuscular coordination in mice. MSG and ASM reduced significantly neuromuscular coordination in treated animals ($p<0.05$, $p<0.01$ and $p<0.001$), respectively as compared to their control (Figure 4).

Blood parameters

MSG and ASM exposure individually or in combination led to significant depletion in some of the observed blood parameters like the red blood cell count, packed cell

Table 1A. Effect of monosodium glutamate (MSG) and aspartame (ASM) on social behavior of male mice.

Group	Median number (with ranges) of seconds allocated to behaviours like					
	Nonsocial investigation	Social investigation	Defense	Threat	Attack	Displacement
Co	150.00(94.30-170.60)	224.00(214.10-241.30)	18.00(15.20-20.70)	20.00(13.30-30.00)	64.00(50.40-100.20)	20.00(4.90-56.60)
MSG (8 mg/kg)	349.00***(323.80-350.30)	50.00***(4.50-53.30)	4.00***(3.20-18.80)	50.00**(37.40-70.80)	30.00**(23.90-55.00)	24.00(12.00-46.60)
ASM (32 mg/kg)	290.00***(250.90-335.10)	100.00***(88.20-147.20)	2.00***(0.70-8.80)	42.00(19.00-56.80)	30.00**(0.00-58.90)	14.00*(9.50-46.80)
MSG + ASM	362.00***(320.00-418.00)	41.00***(28.100-100.60)	2.00***(0.00-7.40)	14.00*(10.00-42.20)	16.00***(0.00-100.10)	9.00**(2.10-25.30)

*, ** and *** significantly different (*p<0.05*, *p<0.01*and *p<0.001* respectively*)* from the control groups by ANOVA and Mann-Whitney U test.

Table 1B. Effect of monosodium glutamate (MSG) and aspartame (ASM) on social behavior of male mice.

Group	Median number (with ranges) of acts and postures						
	Latency to threat (s)	Latency to Attack (s)	Number of fights	Number of Naso-Nasal contacts	Number of Naso-Genital contacts	Wall rears	Rears
Co	15.00(5.00-30.00)	62.00(20.00-70.00)	20.00(15.00-21.00)	27.00(23.00-29.00)	18.00(14.00-22.00)	13.00(10.00-16.00)	9.00(8.00-11.00)
MSG (8 mg/kg)	50.00(15.00-80.00)	70.00(50.00-100.00)	11.00(4.00-20.00)	15.00(0.00-18.00)	8.00***(3.00-12.00)	10.00(8.00-19.00)	7.00(5.00-8.00)
ASM (32 mg/kg)	120.00*(90.00-200.00)	200.00(120.00-220.00)	27.00(0.00-43.00)	43.00**(42.00-81.00)	7.00***(6.00-9.00)	30.00***(2.00-52.00)	7.00(4.00-20.00)
MSG + ASM	350.00***(40.00-70.00)	212.00(0.00-365.00)	5.00(0.00-55.00)	25.00(15.00-42.00)	3.00***(1.00-4.00)	10.00(9.00-20.00)	6.00(5.00-10.00)

*, ** and *** significantly different (p<0.05, p<0.01 and p<0.001 respectively) from the control groups by ANOVA and Mann-Whitney U test.

volume, hemoglobin content, white blood cell count and platelets count in the males (Figure 5A, B, C, D and E).

Testosterone

Testosterone in male (Figure 6) mice depleted significantly (p<0.01, p<0.001) due to MSG and ASM exposure, respectively. This depletion increased significantly in the combination (p<0.001) than individually as compared to the control.

Acetylcholinesterase enzyme

Figure 7 shows that the activity of acetyl-cholinesterase (AChE) enzyme decreased significantly (p<0.001) in MSG and ASM animals as compared to the control.

DISCUSSION

Food additives are substances that are part of a food product when added (intentionally or unintentionally) during the processing or production of that food. They include using salt to preserve meats, adding herbs or spices to foods, or pickling foods in vinegar solutions. However, concerns about food additives most often relate to artificial ingredients added to them (Rangan and Barceloux, 2009). The safety of the two food additives, L-glutamic acid or mono-sodium glutamate (MSG) and Aspartame, 1-methyl N-L-alpha-aspartyl-phenylalanine (ASM) were examined widely and fears of toxic effects were expressed (Rothman and Olney, 1995).

Monosodium-glutamates or MSG carries the number 621 in food additives. MSG occurs naturally in many plants such as tomatoes, spinach and grapes. This form of MSG is bound to proteins and is in low concentration. MSG is a flavor-enhancing food additive used in Asian cooking and also commonly found in fast foods as well as commercially packaged food products such as chips, crackers, soups and soup mixes, lunch meats, salad dressings and many others (Yu et al., 1997). Some people find that consuming MSG, especially in large quantities, can trigger various side effects and symptoms,

Table 2. Effect of monosodium glutamate (MSG) and aspartame (ASM) on social behavior of male mice.

Treatment group	Measures (median values with ranges)	
	Latency to first bite (s)	Number of bites
Control	15.0(10.0 - 40.0)	70.0(45.0 - 96.0)
MSG 8 mg/kg	85.00 **(0.00 - 150.00)	14.00 **(0.00 - 54.00)
ASM 32 m/kg	132.0 ***(0.0 - 180.0)	13.0 ***(0.0 - 54.0)
MSG + ASM	35.00* (0.00 - 150.00)	5.00 ***(0.00 - 15.00)

*, ** and *** significantly different at $p<0.05$, $p<0.01$ and $p<0.001$ respectively, from the control group by ANOVA and Mann-Whitney U test.

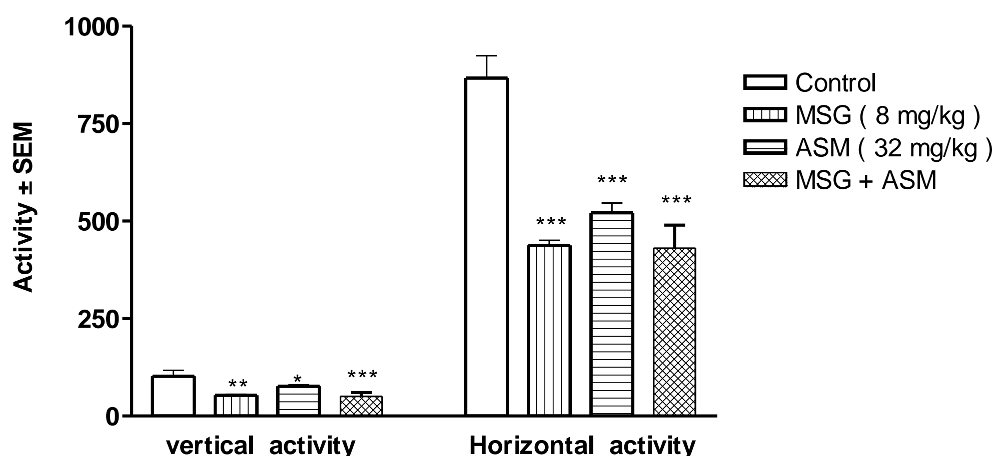

Figure 3. Effects of MSG and ASM on motor activity of mice. *, ** and *** statistically significant at p<0.05, p<0.01 and p<0.001 respectively, as compared to the control group by ANOVA and student's t-test.

Figure 4. Effects of MSG and ASM on neuromuscular coordination of mice in grip strength meter. *, ** and *** statistically significant at p<0.05, p<0.01 and p<0.001 respectively, as compared to the control group by ANOVA and student's t-test.

including headaches, nausea, dizziness, rapid or irregular heartbeat, flushing or excessive sweating, skin rash, numbness, intense thirst, lethargy or sleepiness, ringing ears and tingling in the mouth (MSG Standard, 2007).

The number of aspartame in food additives is 951 and it took 20 years of debate before it was approved by the US

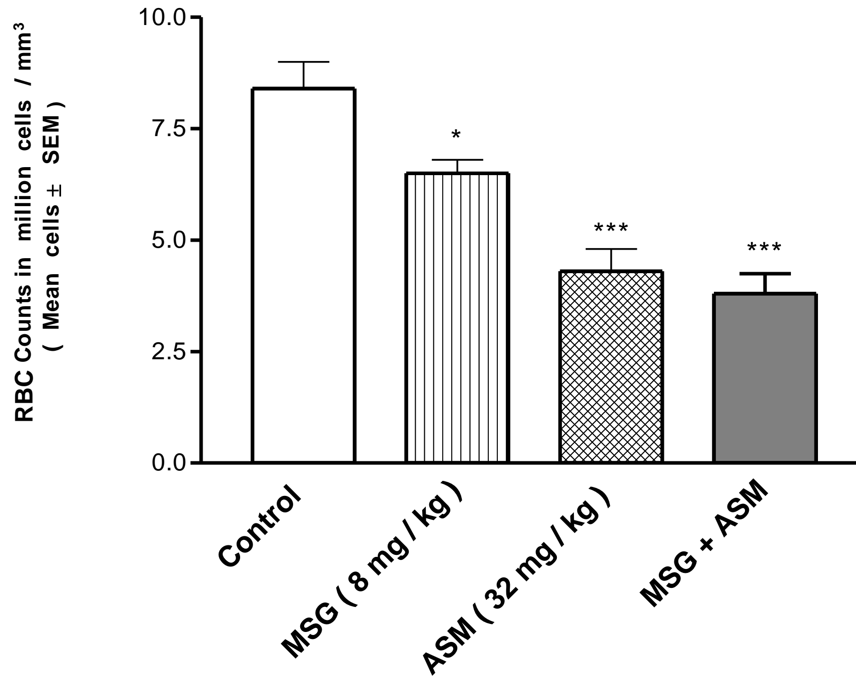

Figure 5A. Effects of MSG and ASM on RBC counts. * and *** statistically significant at $p<0.05$ and $p<0.001$ respectively, as compared to the control group by ANOVA and student's t-test.

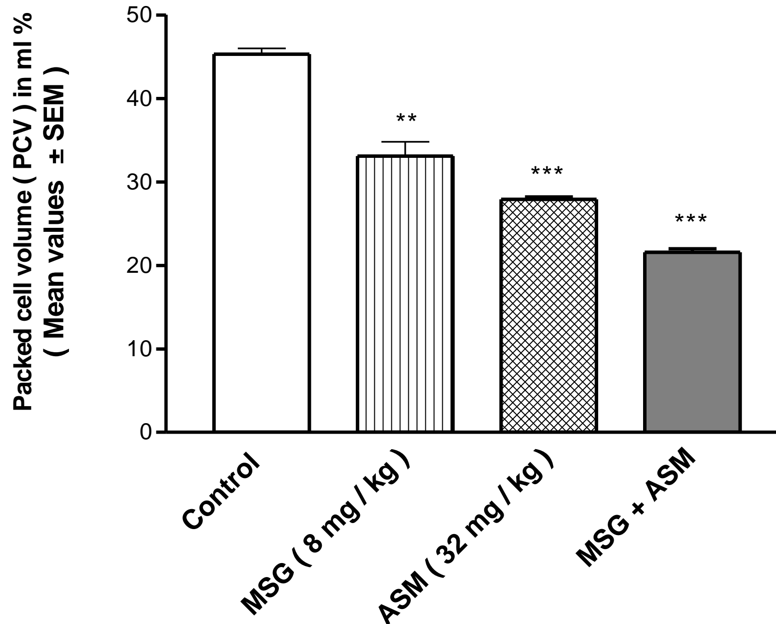

Figure 5B. Effects of MSG and ASM on packed cell volume. ** and *** statistically significant at $p<0.01$ and $p<0.001$ respectively, as compared to the control group by ANOVA and student's t-test.

Food and Drug Administration (FDA). Aspartame is made up of methanol (10%), phenylalanine (50%) and aspartic acid (40%). ASM is a widely used artificial sweetener in soft drinks and low calorie food. There have been reports of adverse neurological effects such as headache, insomnia and seizures after ingestion of aspartame

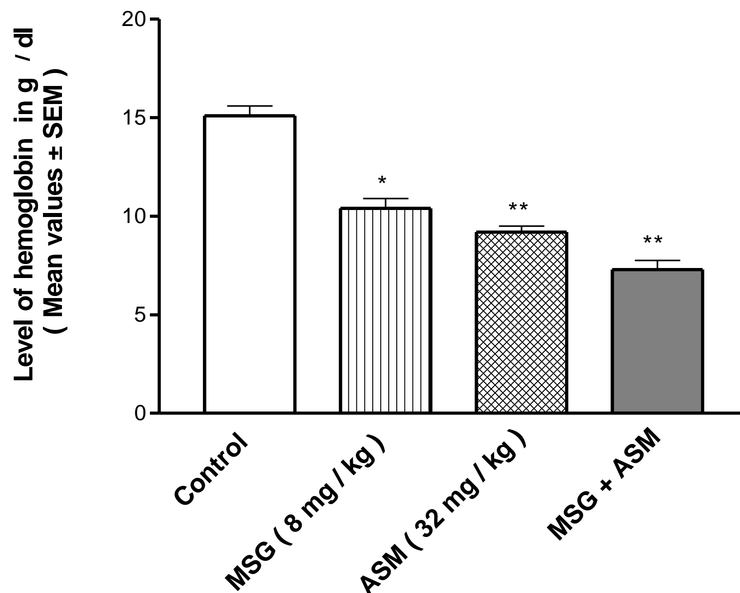

Figure 5C. Effects of MSG and ASM on hemoglobin concentration. * and ** statistically significant at p<0.05 and p<0.01, respectively, as compared to the control group by ANOVA and student's t-test.

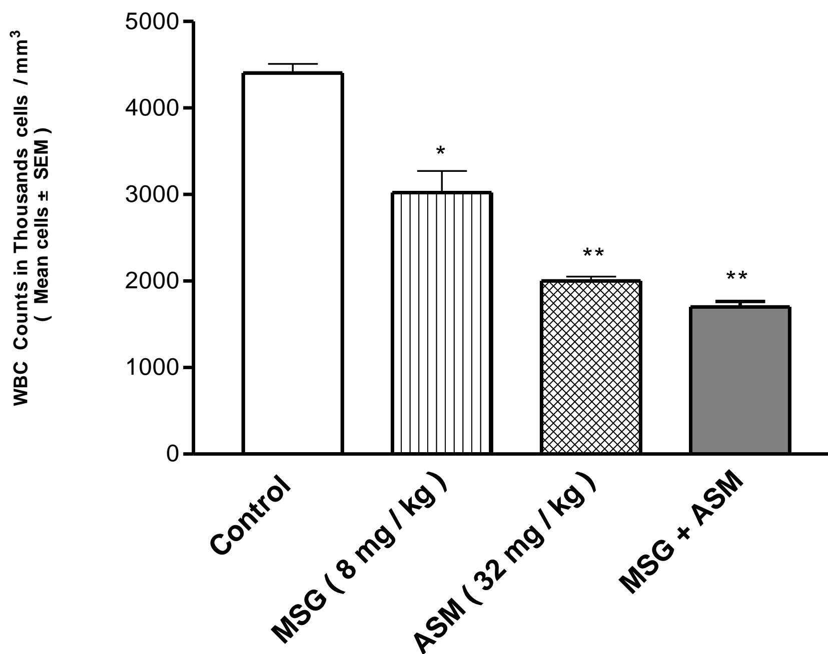

Figure 5D. Effects of MSG and ASM on white blood cell count. * and ** statistically significant at p<0.05 and p<0.01 respectively, as compared to the control group by ANOVA and student's t-test.

(Bergstrom et al., 2007). The present study and previous studies suggested that exposure to monosodium glutamate (MSG) or aspartame (ASM) is very dangers.

The term anxiety covers four aspects of experiences an individual may have: mental apprehension, physical tension, physical symptoms and dissociative anxiety

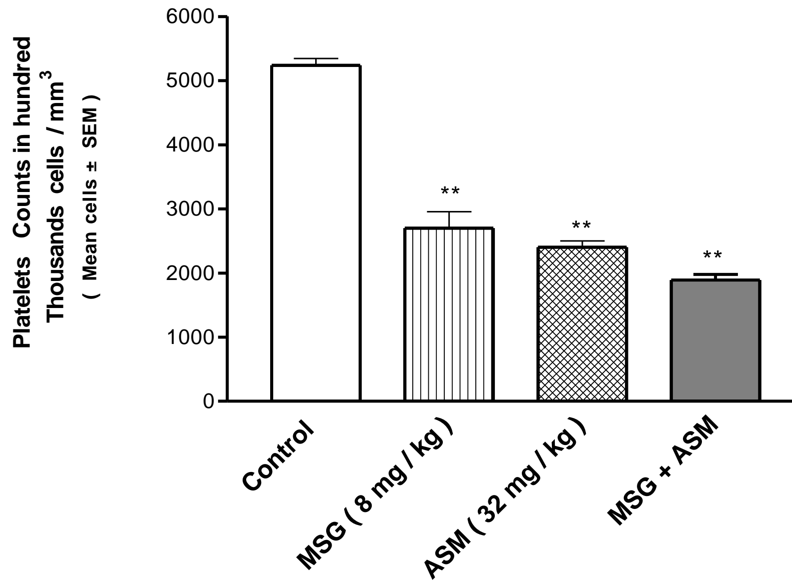

Figure 5E. Effects of MSG and ASM on platelets count. ** statistically significant at $p<0.01$ as compared to the control group by ANOVA and student's t-test.

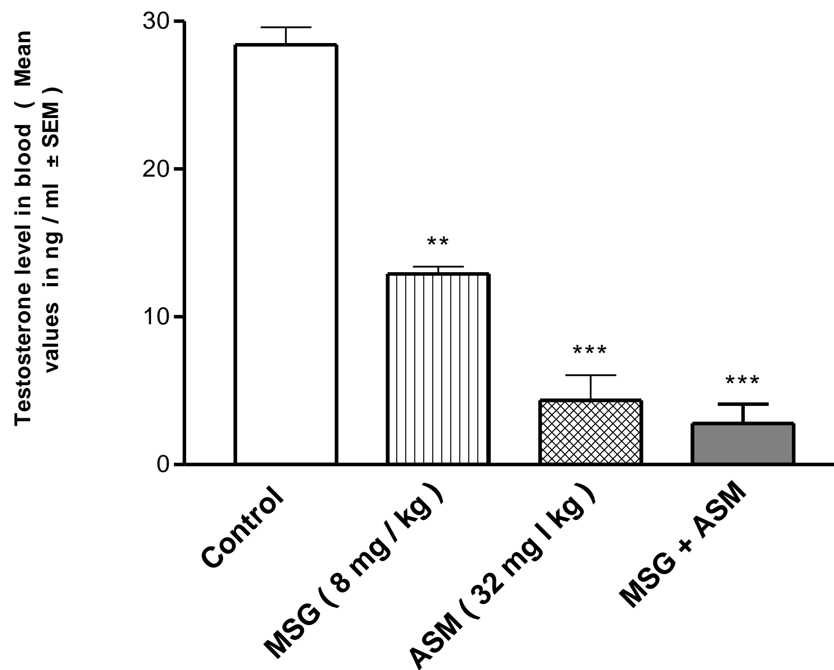

Figure 6. Effects of MSG and ASM on testosterone level in plasma. ** and *** statistically significant at $p<0.01$ and $p<0.001$ respectively, as compared to the control group by ANOVA and student's t-test.

(Pizzi and Barnhart, 1975). The single exposure to food additives (MSG, ASM) or synergistic led to many disturbances in the behavior of fear and anxiety. The residence time in the closed arm and the number of times it enter closed arm increased, while the residence time in the open arm and the number of times it entered the open arm decreased as compared to the control group. The present study results agreed with that of Caputo

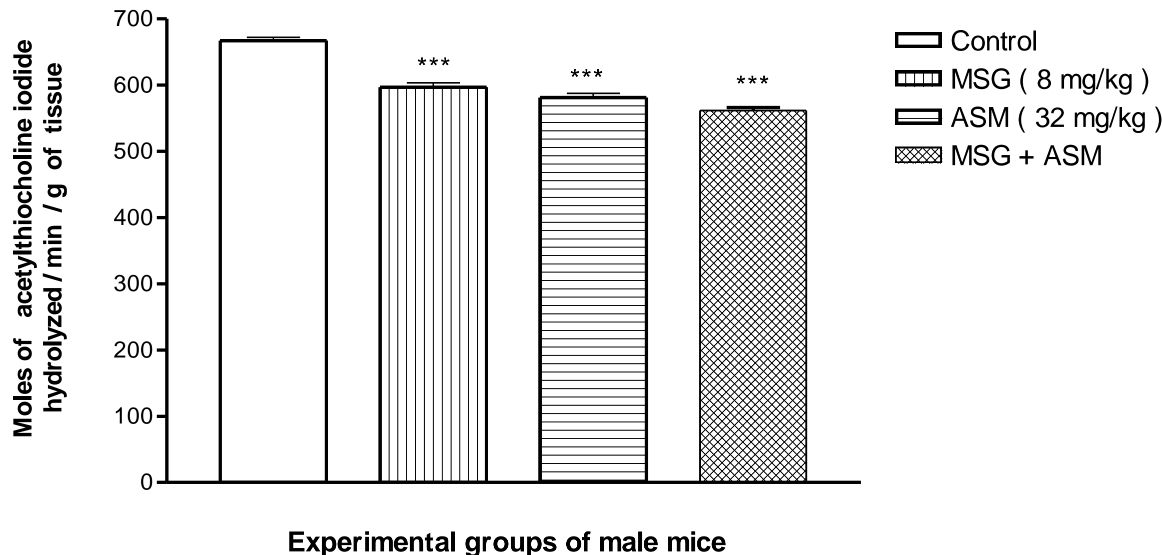

Figure 7. Effects of MSG and ASM on Acetylcholinesterase activity. *** statistically significant at $p<0.001$ respectively, as compared to the control group by ANOVA and student's t-test.

et al. (1996).

Rosen and Schulkin (1998) justified the changes in the behavior of concern to the imbalance in the hippocampus and amygdala regions which play an important role in the onset of symptoms as upset of their electroencephalography (EEG) and increases in their blood flow when diagnosing the disease in those infected. Studies carried out by researchers proved that aluminum affects the hippocampus area (Beas-Zaratea et al., 2002), and discovered that the area of mice brain die due to cellular apoptosis and necrosis when exposed to these food additives commonly available in the market (Gonzalez-Burgos et al., 2001). The changes in the anxiety behavior may be caused by alternations of essential elements for body growth like iron and zinc that result from exposure to MSG and ASM (Collison et al., 2010).

Similar mechanisms of these food additives and lead influence adrenocorticotropic hormone (ACTH). Lead increases this hormone that is produced by the pituitary gland and activates the adrenal cortex to increase the secretion of corticosterone hormone. These hormones increase pressure and anxiety (Nieto-Fernandez et al., 2006). This is done through overlapping element in the axis that reaches the hypothalamus with pituitary and adrenal glands called hypothalamic-pituitary-adrenal gland axis (Virgolini et al., 2006). Lack of weight of adrenal gland might lead to the secretion of orticosterone hormone. Some scientific reports indicated that exposure to food additives (MSG, ASM) reduced relative weight of adrenal, pituitary and thyroid glands (Zelena et al., 1998).

Some scientists showed the existence of a potential link between anxiety disorders and imbalance in the dynamic equilibrium of several neurotransmitters of the brain, such as serotonin, dopamine, noradrenaline and amino acids (Tsunoda, 1998). Cecci et al. (2002) pin-pointed the overlapping imbalance in neurotransmitters like mental disorders, cognitive and neurological disorderly attention, anxiety and aggression. Some research works show anxiety disorders change serotonin, which is linked to changes in mood and anxiety (Shinagawa, 1994). Studies have shown those changes in serotonin in cortex, hippocampus, striatum, hypothalamus, the olfactory lobe, cerebellum and brain stem in rats exposed to MSG and ASM (Kristova et al., 1998).

It could be that alternations in some brain peptides cause increased anxiety in mice treated with food additives in the current study, such as peptides of bombesin (BB). Merali et al. (1999) found that bombesin (BB), responsible for loss of appetite and increased sense of false satiety (Sidhu and Nehru, 2003), also encourages anxiety and stress by inducing secretion of the ACTH hormone. This hormone activates adrenal cortex hormone, which helps the emergence of fear, anxiety, loss of appetite and lack of physical activity (Koob, 1999). Among other neuropeptides causing anxiety peptides factor prompts adrenal cortex Corticotropin-releasing factor (CRF), which is composed of forty-one amino acids that spark at the initial excitation of HPA to counter pressures. It also represents the link between the nervous system of self-autonomic nervous system and some components of behavior to control and avoid damage. It also participates in the feedback process for hypothalamus response to external pressures (Spiga et al., 2006).

Social behavior reflects group of animals' relationship and the extent of their ability to interact and exchange

things within the group. Our results indicated that the single exposure to food additives (MSG, ASM) or synergistic interaction led to many disturbances in social behavior. The non-social and social behavior of the exposed mice was significantly affected; there was an increase in the former and a decrease in the later, respectively. The elements of social behavior including attack, number of fight, naso-nasal and naso-genital contacts were decreased significantly. The latencies to threat and attack were also increased significantly. Overall, the results indicate that social behavior is significantly decreased due to exposure to MSG and ASM. Conversely, anti-social behavior and its elements like fears, in treated animals were increased significantly. Our results agreed with studies of Ramanathan et al. (2007).

Changes in social behavior caused by MSG and ASM in the nervous system affect the olfactory nerve which controls the sense of smell in mice (Park et al., 2000). It has been shown also that exposure to food additives leads to the appearance of many behavioral changes, like aggressiveness and changes in activity as a result of damage caused by MSG and ASM in the hippocampus.

Food additives work to reduce androgens which indirectly affect the axis connecting the pituitary gland. This has negative effects on social behavior and the relative weight of members of the sex -producing hormone (Sun et al., 1991). Deficiencies of the testosterone hormone might cause social behavioral changes because of its importance in the regulation of aggression in mammals (Terry et al., 1981). Studies have indicated that exposure to MSG and ASM negatively affects the concentration of neurotransmitters. This suggests it causes changes in social behavior, increases isolation and lack of social movements in the current study. Current study indicated that treatment with food additives led to decreased locomotion activity and neuromuscular coordination (Grip strength) in treated animals compared to the control group.

Food additives affect the composite structure and function of neurons in the hands and feet, hurt the process of molecular adhesion between nerve cells, disrupt the process of cellular communication between neurons, astrocytes. These happen through the influence of the cellular communication channels with gaps or by changes in the function or installation of structural proteins (Sakr, 2004), resulting in imbalance in the transfer of nerve impulses, or reduction in the speed of nerve impulses and conduction velocity (NCV). This is because myelin membrane is removed (Demyelination) which covers axes neurons. The composite materials that lead to the breakdown of nerve cells in the nervous system reduce or delay access commands to the muscles, resulting in a slow response.

Aspartic acid, another ASP hydrolysis product, is a dicarboxylic amino acid that may exert toxic effects when administered at very high doses; although species susceptibility varies considerably (Simintzi et al., 2007). The observed lowered enzyme activities may be due to an increase in reactive oxygen species and intracellular $Ca2^{+}$ concentrations by the metabolite, as reported previously (Sureda et al., 1996). Free radical attacks on unsaturated bonds of membrane fatty acids result in an autocatalytic process called membrane lipid peroxidation, which can impair the function of membrane AChE. MeOH is totally absorbed and may interact with lipids of the mice forebrain cell membrane and/or protein parts of mice forebrain AChE, resulting in a reduction of its activity (Oyama et al., 2002).

Overall, the present study concluded that MSA and ASM were dangerous to behavioral and biochemical analysis in mice. In addition, these food additives need more scientific researches to investigate their harmful effects on other parameters.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

ACKNOWLEDGEMENT

The author extends sincere appreciation to the Deanship of Scientific Research at Dammam University for funding this research through the Research Project no: 2012062.

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