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FOOD SCIENCE AND NUTRITION

Determination of the quality of cheese "Chihuahua" type: Sensory and physicochemical approaches

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Abstract

The physicochemical and sensory characteristics of eight formulations from Chihuahua cheese with differences in the number of maturation days were studied. There were significant differences in their (pH, internal temperature and color). Sensory characterizations were carried out by sorting task technique with a panel of 12 assessors. The sensory maps were analyzed by using Multidimensional Scaling (MDS) and Correspondence Analysis (CA) while a spatial comparison between configurations of physiochemical and sensory data were validated by the Multiple Factor Analysis (MFA) and *Rv coefficient*. A lexicon of 22 sensory attributes was generated by the assessors which further allowed them to discriminate cheese samples. The cheese eventually developed different attributes with respect to their maturation, especially in aspects of texture and aroma. A comparison of spatial configurations using Rv coefficients showed a moderate correlation between the methods of analysis. However, updates may be necessary for a complete sensory characterization by techniques such as QDA® for further study of Chihuahua cheese quality evaluation by consumers.

Key words: Chihuahua cheese, Physicochemical analysis, *Rv coefficient* and Sorting task

Introduction

Hispanic-style cheeses are cheeses developed in México, Latin America, and the Caribbean and are usually based on European cheese-making traditions but modified to accommodate local cheese-making conditions and consumer preferences (Van Hekken and Farkye, 2003). Although Chihuahua cheese is one of the major varieties of cheese consumed in northern Mexico and is usually consumed within one month after being manufactured, because after this time, the sensory characteristics perceived with great intensity in Chihuahua cheese, only a few papers have been published on its chemical and sensory properties (Van Hekken et al., 2008). In the Mexican state of Oaxaca, milk production is destined for the manufacture of different cheeses (López et al., 1997; Morales et al., 2003); the sensory attributes play an important role in the selection of a product by consumers (Issanchou et al., 1997) and at the same time, they are used for the sensory characterization (Ramón et al., 2011). The chemical and physical changes that occur during ripening cause the body of the freshly made cheese to lose its firmness and tough curd texture to become soft. Cheese ripening is a complex process involving many physicochemical changes such as pH changes and protein breakdown (Karoui and Dufour, 2003). Although research on sensory evaluation developed by Bárcenas et al., 2004; Drake et al., 2005; Ramón et al., 2011) have characterized different kinds of cheeses, there are currently few studies on the sensory quality of Chihuahua style cheese (Van Hekken et al., 2006; Van Hekken et al., 2008). However, this type of method, called sensory profile, is quite efficient but also very expensive and time consuming so most industries cannot routinely use this technique (Chollet et al., 2011). For this reason, it is necessary to develop other sensory methods in order to obtain sensory information about these products. As examples of new techniques for

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sensory description we have free sorting task or categorization (Abdi et al., 2007). However, during a task of categorization, products groups are formed based on the dissimilarities/similarities between among them to give us information about the criteria used by each judge (Cadoret et al., 2011). According to Bécue and Le (2011) and Leliévre et al. (2008) the sorting task is a cognitive process consisting of asking assessors to group items depending on their resemblances. This long-term practice task is gaining importance in sensory analysis to evaluate products from a global point of view (holistic approaches) and to compare different types of assessors (such as trained or naïve). In the sensory domain, sorting tasks were used in description of products such as beers (Chollet and Valentin, 2001; Leliévre et al., 2008; Chollet et al., 2011), wines (Bécue and Le, 2011), olive oil (Santosa et al., 2010) and cheese (Tang and Heymann, 2002; Nestrud and Lawless, 2009). Multidimensional scaling (MDS) is a useful statistical technique for generating perceptual maps that can systematically represent the similarity/dissimilarity of products in a spatial map (Tang and Heymann, 2002; Nestrud and Lawless, 2009). Other statistical techniques used are DISTATIS, Multiple Correspondence Analysis (MCA) (Chollet and Valentin, 2001, Abdi et al., 2007; Leliévre et al., 2008; Cadoret et al., 2009; Cadoret et al., 2011) and Correspondence Analysis (CA) (Chollet and Valentin, 2001;Bécue and Le, 2011). The information on the two different types of tools, physicochemical and sensory data, is very important to establish the quality of cheeses. Due the above, the use of multi-varied techniques such as Multiple Factor Analysis (MFA) and *Rv* coefficient allow for the determination of the degree of correlation between data on physiochemical and sensorial nature (Pagès and Husson, 2001; Husson et al., 2001).

Consequently, for the purpose of this study it was decided to investigate the changes in the sensory properties that occur during maturation and correlation with physicochemical data.

Materials and Methods

Samples of cheeses

Eight Chihuahua type cheeses were manufactured with lactic cultures (*Lactococcus lactys* + *cremoris*) and matured over different periods of days (0, 8, 10, 18, 24, 31, 34 and 41 days) in ripening chamber model Sobrinox rus 230 at a controlled temperature of 10°C. For the production of Chihuahua type cheese, the lactic cultures *Lactococcus lactys* + *cremoris* were used. They were shown at a temperature between 32-37° C for 45 minutes. At 32-35°C the rennet was added and left to set for 45 minutes. The cheese were prepared with whole milk and came from a single cow is why it was not necessary standardization fat protein. On obtaining a solid consistency, it was cut. The cut was made in a space of 2 cm and was carefully stirred to avoid mixing. Later, the curds were heated between 35 and 37°C and were left to set for 20 minutes, 2.5% salt is added and then it is pressed. The cheeses were cut into cubes of 3.5 x 3.5 cm and were sensorially and physicochemical evaluated at a temperature of 25°C (Bárcenas et al., 2004; Drake et al., 2009a; Drake et al., 2009b). The cheeses were transported in Styrofoam coolers on ice. Upon receipt, the cheeses were stored at 5ºC in the dark until analysis.

Physicochemical analysis

pH

The pH was measured on cheese slurry prepared from 10 g of cheese and 10 g of distilled water using a potentiometer Hanna model HI 99163 (Hernández et al., 2010).

Internal cheese temperature

A Hanna model HI 99163 potentiometer/ thermometer with an insertion electrode was used according to Villegas de Gante (2004). The temperature and pH are two of the factors that can influence microorganism growth in the lactic cultures being those temperatures in the range of 15 and 20°C where the maturation of semi hard doughy cheeses such as the Chihuahua cheese occurs.

Color measurements

In order to determine the color of the Chihuahua cheese samples**,** cross sections were analyzed by using Hunter X-rite model HI SP60L colorimeter, registering the value of L* [brightness, from 0 (dark) to 100 (light)] and *b**[from -60 (blue) to +60 (yellow)]. Before taking the measurements, the instrument was calibrated with its white reference tile. The color was measured in the grated cheeses (15 g) within the test tubes (27 mm diameter, 250 mm length). Eight to ten readings were taken from each test tube and the average value was used. The L* (whiteness) and b* (yellow to blue) values were used to evaluate color changes for the samples (Hernández et al., 2010). The pH, internal temperature and color analyses were performed four times.

Sensory analysis

Sorting task procedure

The panel dedicated to the sorting was composed of 12 assessors (6 men and 6 women) aged from 20 to 28 were recruited students and

personnel in the laboratory analysis and Food technology from the University of the Sea, Campus Puerto Angel, Oaxaca, Mexico. These assessors had previous experience in the evaluation of dairy products. The evaluation of cheese consisted of one single session. The sorting task procedure was realized according to Chollet and Valentin (2001), Leliévre et al. (2008) and Chollet et al. (2011). The assessors were presented with the entire set of cheese. The order of presentation of the samples was randomized prior to presentation and so it was different for each assessor. The assessors started by tasting all the cheeses, one at time. The assessors were free to make as many groups of cheeses between 2 and 7 (acoording to similar properties sensory) as they wanted and to put as many cheeses as they wanted into each group. Subjects are told that they must sort the samples into no fewer than two groups and into no more than 7, to ensure a group structure (Faye et al., 2004). After they had finished their sorting task, the assessors were asked to describe each group of cheese.

Data coding

Encoding free sorting data

For each assessor, the results are encoded in an individual similarity matrix (cheese x cheese), in which 1 stands for two samples set in the same group and 0 for two samples put in different groups. These individual matrices are added for all subjects or for sub-groups of subjects. The result co-occurrence matrix represents the global similarity matrix in which larger numbers indicate higher similarity between samples. The assumption underlying this method is that samples grouped together are more similar than samples sorted into different groups (Popper and Heymann, 1996).

Encoding terms

Each subject describes each group of products with words and expressions. For each subject, the terms given for a group of cheese are associated with each product from the group. The underlying assumption is that all the cheeses of the group are described by the terms in the same way. An occurrence matrix (Cheese x Attributes) is provided by the assessors. The global terms matrix is calculated by adding individual matrices. The resulting contingency table was then reduced and simplified (1) Attributes having similar meanings were grouped by the sensory analyst; (2) Only attributes having quotations for an attribute by the total possible number of quotations at a frequency higher or equal to 3 % are kept for the comparison of cheeses. This percentage was calculated by dividing the number of quotations for an attribute by the total possible number of quotations. Finally only the words whose frequency is at least 3 were left (Faye et al., 2004, 2006).

Statistical analysis

Sensory map of product and correlation physicochemical- sensory analysis

The resulting co-occurrence matrix was submitted to an MDS analysis in order to derive a spatial representation of cheese. The quality of fit was measured by the stress value, where the stress varies from 0 (perfect fit) to 1 (worst possible fit). A value below 0.2 is generally considered as a good agreement between the initial and final configuration (Chollet and Valentin, 2001; Leliévre et al., 2008; Chollet et al., 2011). The sensory map analyzed through CA offers a visualization of cheese, attributes and the associations between products and attributes (Perrin and Pagès, 2009; Bécue and Le, 2011). Sensory and physicochemical data were analyzed jointly using Multiple Factor Analysis (MFA) where the first step consists of performing a PCA per group of variables. Then, each variable is multiplied by the inverse of the first eigenvalue issued from the corresponding factor analysis. The internal configuration of each group is not modified. The second step consists of performing a PCA on the resulting data, yielding a configuration which takes into account all the weighed variables and measurements of the similarities between the date sensory and physicochemical given by the *Rv* coefficient (Lassoued et al., 2008). According to Cartier et al. (2006); Nestrud and Lawless (2008), an excellent agreement between configurations corresponds to a coefficient value close to 1 while Faye et al. (2004) concluded that there is a good agreement between two configurations at *Rv* values close to 0.67. MDS, CA, MFA and *Rv* coefficient were performed with the software XLSTAT, version 2009 (Addinsoft, New York, NY, USA).

Results and Discussion

In order to describe the cheese by sorting task, more than 20 attributes were generated by the assessors; however, other attributes such as fat odor, aroma of yoghurt, aged flavor, melts in the mouth were eliminated because of low frequency values. However, they were not statistically usable and they were not introduced in the CA. Figure 1 presents the 21 attributes which are used: acid, creamy, hard in the mouth, soft in the mouth, dry and yellow are included within the first more frequent words used by the assessors.

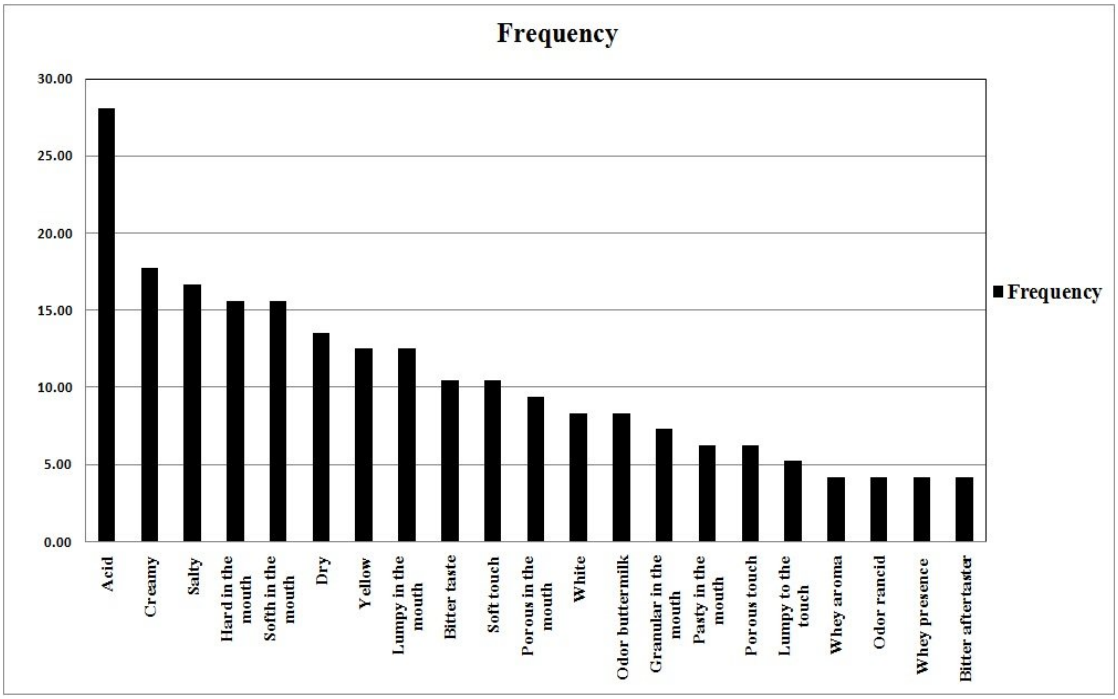

Figure 1. Glossaries of the most frequent word used.

Figure 2 shows the two-dimensional solution for multidimensional sorting data (Kruskal Stress= 0.14). This value confirmed the results obtained by Nestrud and Lawless (2010) who demonstrated the relative consistency of sorting data set of cheddar cheese (Kruskal Stress = 0.081). Tang and Heymann (2002) reported a Kruskal Stress value = as 0.12. The cheeses within the same general number of ripening days were plotted close together, especially in the cheeses with similar days of maturation. For example, the sensory map showed: cheeses H, A and D (group 1), cheese G and F (group 2), cheese E, B (group 3) and Cheese C (group 4). The CA was performed on the contingency table. Attributes having a quotation frequency higher or greater than 3 were introduced into the CA. The first factor of CA (see Figure 3) accounted for 82.60% of the total inertia and reveals that the cheese with 10 (A) and 18 (H) days maturation had a more acid intensity, bitter taste, soft to touch and mouthfeel. According to Hort et al. (1997) initially, that is within the first 7 to 14 days, residual coagulant enzymes are responsible for the relative rapid hydrolysis of the α_{s1}- casein to α_{s1}-l casein which reduces the rubbery texture of the cheese. This may be due to pH changes which are directly related to changes in the casein network. The cheese with 24 (F) and 31 (G) days respectively were classified as yellow, and salty. Aspects of texture were creamy and pasty mouthfeel. On the other hand, the cheeses B and E (34 and 41 days respectively) were described as hard and lumpy in the mouth. Hort et al. (1997) observed that the creamy character of the maturing cheddar cheese changes with time and shows a significant logarithmic relationship. However, the changes in the texture of the cheeses described above may be due to the result in the extent and type of protein–protein interactions in the protein network due to ripening (Karoui and Dufour, 2003). Where the increment in firmness and hardness could be attributed to the crystallization of the fat which will occur over time. Additionally, as proteolysis continues, less water is available which results in a harder, not as easily deformable cheese (Hort et al., 1997). Lawrence et al. (1987) observed that, during the second stage of maturation, the rate of proteolysis is considerably slower, with consequently less remarkable changes in the springiness and crumbliness attributes. Cheese texture may also change with the physical state of the fats depending on the storage temperature and time (Karoui and Dufour, 2003). The salt content of samples increased significantly until 30 days and then did not change statistically during the maturation period (Cambaztepe et al., 2009) as was

occurred in the case of cheese G. On the other hand, the changes associated with aromas incremented with maturation time developing the attributes of bitter, acidic, buttermilk smell, rancid odor, and aroma of whey. Muir et al. (1996) observed the attributes of cheddar aroma, cheddar flavor and acid flavor that were generated during the course of cheddar cheese maturation.

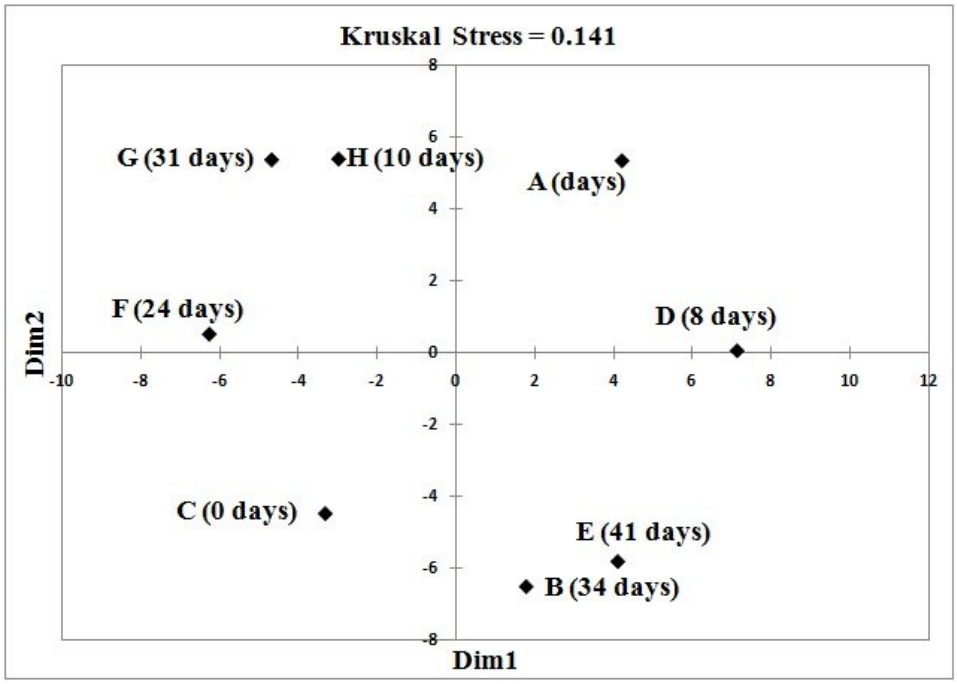

Figure 2. Perceptual space for cheese derived from multidimensional scaling for sorting data.

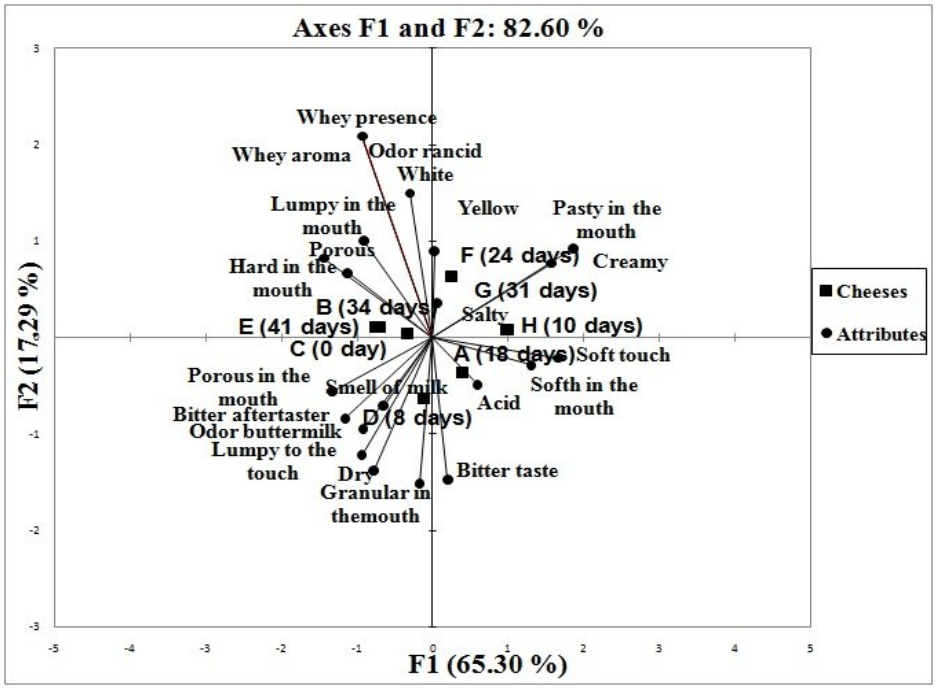

Figure 3. Cheeses and attributes projections on the first two dimensions of Correspondence Analysis.

Correlation of physicochemical and sensory analysis by MFA

The result of the first two principal axes of the MFA was 69.76% (see Figure 4). This value was higher than what was reported by Lassoued et al. (2008) at 51.42%, which noted similarities between the configurations of sensory description and instrumental data for the evaluation of bakery products using the flash profile. Figure 4 shows correlations between white and variable L* in the cheese C (0 days). On the contrary, variable b* is correlated with yellow (sensory), which is for the cheeses with more than 24 days of maturation (cheese F and G). According to Hernández et al. (2010), these differences in color were observed with increases in the yellowness at a greater ripening time. In other words, the L* value decreased significantly during ripening and increased the values b*, Pinho et al. (2004) observed this same effect during ripening (0 to 60 days) of "Terrincho" ewe cheese. The pH is correlated with acid and bitter taste which is attributed to acidification due to microbial growth (Hernández et al., 2010). According to Pinho et al. (2004) acidity increased slightly during ripening between 30 and 60 days. Internal temperature is correlated with attributes of dry, smell of milk and buttermilk odor.

The *Rv* coefficient provides a measure of similarity between the results of the methods of data collection and analysis. A high value of the coefficient would suggest that the methods were measuring the same underlying characteristics of the samples (King et al., 1998). The *Rv* coefficient for the physicochemical and sensory data were 0.51 which only reflected the differences between the two analysis methods for characterization of cheese A, B and G in which major differences between analysis for the evaluation of these cheeses, while in the cheeses C, D, E, F and H the distances between analysis were equidistant (see Figure 5).

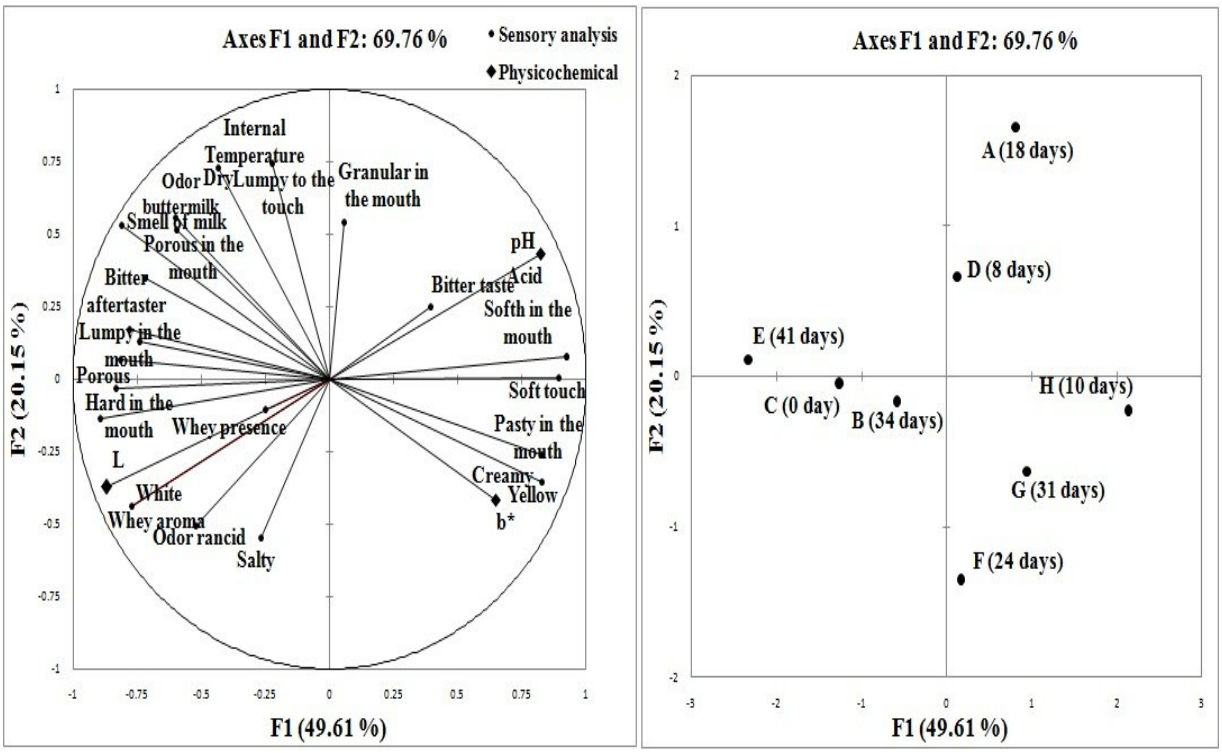

Figure 4. Correlation circle of Multiple Factor Analysis for physicochemical and sensory data.

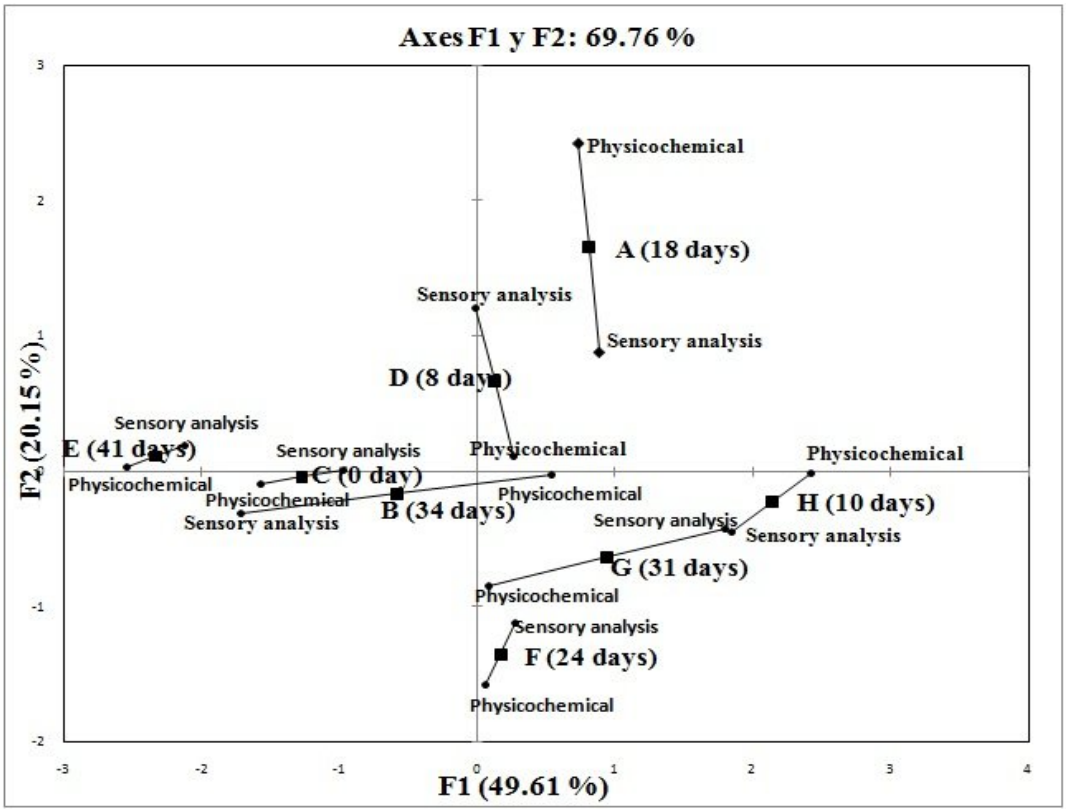

Figure 5. Multiple factor analysis on the table of the averages by the performed analysis.

Conclusion

It is clear from the preceding results that the main changes in physicochemical and sensory characters are associated with maturation. The combination procedures of sorting with multidimensional scaling analysis, correspondence analysis and multiple factor analysis showed in the perceptual map that the physicochemical and sensory properties are affected by biochemical processes such as proteolysis which caused changes in texture and aromas. However, updates may be necessary for a complete sensory characterization by techniques such as QDA® for further study of Chihuahua cheese quality evaluation by consumers.

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Impact of drying temperatures and air mass flow rates on the drying performance of a Parabolic Trough Solar Collector (PTSC) used for dehydration of apricots

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Abstract

An efficient parabolic trough solar collector (PTSC) was developed from locally available materials and used for dehydration of apricots. The apricots were cut into two halves and dried after pre-treatment of 1% with sodium benzoate solution. Apricots were dried at temperatures of 40, 50 and 60°C under three different air mass flow rates of 1.57, 2.29 3.56 kg.min^{-1} at less than 30% humidity Shortest apricots drying time was 16 hours at 60°C temperature and air flow rate of 3.56 Kg.min^{-1} with an average drying rate of 0.035 g.hr^{-1}.cm^{-2}.. It was followed by drying time of 19 hours at 55°C. The air mass flow rate was 2.29 Kg.min^{-1}with an average drying rate of 0.29 g.hr^{-1}.cm^{-2}. Initial moisture content of the apricots was 86% which reduced to less than 10% after drying. Results showed that both air mass flow rates and drying temperatures significantly ($P< 0.000$) affected efficiency, moisture lost, drying rate and drying time. The PTSCs must be operated at high temperatures and high air mass flow rates to achieve maximum performance. Apricots must be dried at high temperature and high air mass flow rates to get minimum time for getting valuable dried apricots.

Key words: Solar Energy, Parabolic trough, concentrating collector, Apricots, Moisture Loss, Drying Rate

Introduction

Apricot (*Prunus armeniaca*) is the sixth main fruit of Pakistan. Total area under apricot cultivation in Pakistan is about 28.8 thousand hectares with production of 210.9 thousand tons. The average production is 6.03 tons. ha^{-1} (MINFAL, 2011). Northern areas of Pakistan have the high production of apricots but almost 30-35% losses occur there as apricots cannot be stored for a longer period of time as they are perishable and spoil very quickly (Khalid et al., 2011). On-farm losses can greatly be reduced if apricots are dried with the help of solar dryers because they are one of the fruits best suited to drying (Henderson and Brenand, 2004). Dehydrators are the most efficient way to dehydrate apricots because they have a controlled heat source and a fan to circulate and remove the moist air. They are also able to dehydrate products more quickly and safely as pathogen attacks are minimized under control drying environment (Henderson and Charlotte, 2004; Istvan, 2011).

Hussain et al. (2008) studied dehydration of fruits by solar energy using thermo-convectional dehydrators of direct and indirect type made from indigenously available material. They concluded that agricultural products like apricots must be dried using solar thermal collectors as they dried the products under hygienic environment. The cost of dehydration was nominal, whilst the quality of the dried products was of marketable standards. The findings suggested the large scale use of such dehydrators as an economic means of drying fruits and vegetables. Yadollahinia et al. (2008) worked on experimental dryer and studied the effect of moisture and velocity of air on the product. They concluded that high velocities and low humidity accelerated the drying process. Rafiee et al. (2007) studied the effect of low humidity and high temperatures on drying. They concluded that there

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was significant effect of both temperature and humidity on the drying process. Drying occurs more quickly at high temperature and low humidity using a solar dehydrator. Hashemi and Shimizu, (2008) and Seiiedlo et al. (2010) studied the effect of temperature and air velocity on drying time of perishable fruits like apricots. Both of them concluded that increase in temperature and air velocity reduced drying time significantly. High temperatures effects the proteins present in the products and causes browning. They also concluded that temperatures higher than 60°C have a more negative effect on products to be dried.

Pakistan receives solar energy with intensity about 750 $w.m^{-2}$ throughout the year except for the monsoon. The prospect of utilizing solar energy is very bright (Ahmad, 2011). Like the rest of Pakistan, Peshawar and FATA receives about 1 $cal.cm^{-2}.min^{-1}$ which is almost 750 $w.m^{-2}$ (Khattak et al., 2010). The use of dehydrators in the form of solar collectors is increasing day by day in Pakistan. There is a good market of these technologies which is developed further in the future. The specific demand is of efficient solar drier that could dry more fruits in less time (ASF, 2011). The objective of this research was to develop a parabolic trough solar concentrating collector use to dry apricots in the Northern Areas of Pakistan. This research might enable us to develop processes and procedures to enhance performance of parabolic trough solar collector (PTSC) for valuable drying of apricots.

Materials and Methods

Experimental Site

The experiment was conducted at village Gundi, Khyber Agency (34° 0' 29" N / 71° 34' 22" E) of Federally Administrated Tribal Area (FATA), Pakistan. The collector was oriented facing south having a tilt angle of 34° equal to the latitude of the experimental site to receive maximum solar radiation.

Description of the PTSC

A Parabolic Trough Solar Collector assembly (Figure. 1) consists of two units.

i. A Parabolic Trough used as Reflecting Unit

This composed of a parabolic trough solar reflector. The reflector was basically a steel sheet that concentrated the incoming solar radiation on the centrally located black painted steel pipe having a diameter of 5cm.. The pipe diameter was arbitrarily selected with the assumption that it will allow sufficient air flow under natural conditions as well. The pipe received ambient air that was heated inside it and then delivered to the drying unit for drying of apricots. Before constructing the parabolic structure, the desired focal length was by using the following equation (Price et al., 2002):

$$f = X^2 / 4Y$$

Where, f is focal length of the trough, X is radius and Y is the aperture of the trough.

ii. Drying Unit (Hanif et al., 2012)

This unit consisted of a steel box fully insulated on the inside with the help of polystyrene foam. The box was 1 m high, 0.55 m wide and 0.57 m long. There were three exhaust fans each having a diameter of 0.05 meters. The total volume of the drying box was 0.3135 m^3. The fans were used to suck hot air inside of the drying box through the absorber pipe. The flow rate of each fan was controlled by the help of dimmer fixed on the drying unit. There was an outlet fixed on the top of the drying unit having a diameter of 0.02 meters that removed humid air from the unit.

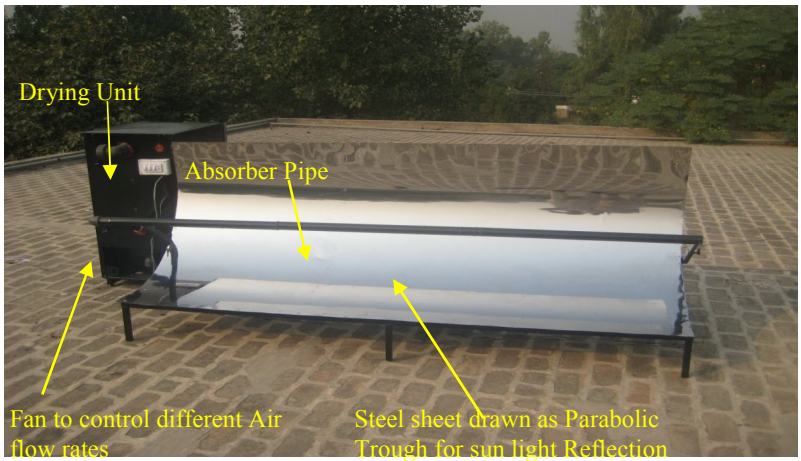

Figure 1. The Parabolic Trough Solar Collector showing its main parts.

Experimental design and treatment applications

The experiment was laid out as a randomized complete block design with a factorial arrangement of the treatments consisting of 3 levels of temperature and 3 levels of air mass flow rate, replicated 3 times and resulting in a total of 27 treatments. Factors and their treatment levels are given as below:

Factor #1 (Temperature)

T1 = 40°C, T2 = 50°C, T3 = 60°C

Factor#2 (Air mass Flow Rate)

F1 = 1.57 Kg.min^{-1}, F2 = 2.29 Kg.min^{-1}, F3 = 3.56 Kg.min^{-1}

Solar energy and its conversion to heat

Solar energy (kJ.m^{-2}.Sec^{-1}) available per unit time per unit area was measured with the help of mechanical pyranometer. The solar intensity was calculated using the equation (Hanif et al., 2012):

$$Ea = Pc \times Cv \qquad (1)$$

Where, Ea is the solar irradiance (kJ.Sec^{-1}), Pc is the pyranometer constant (367.78) for the one used in the experiment) and Cv is the chart value of pyranometer. This solar energy was converted into heat energy by the absorber pipe and was in the form of heat energy consumed (kJ.Sec^{-1}) by the drier in unit time. This energy is the product of the the total energy consumed by the air (medium) inside the dryer is given by equation (Karim and Hawlader, 2003):

$$E_h = Fr \times C_p (\Delta T) \qquad (2)$$

Difference between inlet and outlet temperatures of the collector (ΔT) was determined by the help of thermometers.

Air Mass Flow Rate

Air mass flow rate (kg.s^{-1}) is the mass of air flowing from the collector to the drying box in unit time. It is the product of velocity V (m.sec^{-1}) of air at the outlet area A (m^2) of the outlet and density (kg.m^{-3}) of air. Air mass flow rate determined using equation (Ahmed, 2011). The air mass flow rate was changed by changing the speed of the fan fixed at the outlet.

$$Fr = V \times A \qquad (3)$$

Efficiency of PTSC

Efficiency η (%) is the ratio of heat consumed by the drier unit Eh to heat available to the collector Ea in the form of solar radiation. Efficiency was calculated using the equation (Ahmed, 2011).

$$\eta = \frac{Eh}{Ea} \times 100 \qquad (4)$$

Moisture Loss and Drying Rate

Moisture loss (%) by apricots after each hour of drying was determined. The products were dried to less than 10% moisture content to minimize mold, insects and bacterial attack. The initial moisture content was determined by taking 3 samples in an oven and dried for 48 hours at a temperature of 105°C to completely remove the moisture present in them (Ehiem, 2009). After determining the total moisture, the moisture loss after each hour in drying was determined by using equation (Mohanraj and Chandrasekar, 2009).

$$MC = \frac{W_l}{Wi} \times 100 \qquad (5)$$

Also drying rate D_r (g_w.gdm^{-1}.cm^{-2}.hr^{-1}) was calculated using the equation (Henderson and Perry, 1976).

$$D_r = \frac{(W_l)}{(D_m \times A_p \times Dt)} \qquad (6)$$

where W_l is the moisture loss during time interval D_t from product with surface of A_p. The apricots were dried till the moisture content becomes less than 10%. The drying time is noted from the beginning till the moisture becomes less than 10%.

Experimental procedure

Only good quality apricots were used in these experiments. About six (6) kg of apricots were cut into halves and blanched for 5 minutes in hot water at 80°C. After that pretreatment of 1% sodium benzoate solution was given for two minutes. Then the initial moisture content was determined by oven-drying method. The apricots were put on trays of drying unit having 80% perforation and dried until the consecutive weights of samples remained constant i.e. the moisture content of 10% wet basis was achieved (Santos et al., 2005). The fans were switched on and the air mass flow rate through the outlet was adjusted according to the design of the experiment (1.57, 2.29, 3.56 kg.min^{-1}). The velocity of air at the inlet of the tray was measured with the help of digital anemometer. Solar intensity was measured with the help of mechanical pyranometer. Temperature and humidity at the inlet and outlet of the PTSC and drying unit were recorded every hour with the help of a hygrometer (Mahmood et al., 2005). All the experimental observations were made after transient state when the drier attains the steady state condition (Wilcke, 1980). The experiments were repeated three times. The characteristics of the PTSC like drier thermal consumption, available solar irradiance and inlet and outlet temperatures were studied and also drying characteristics of apricots

such as moisture content and drying rate were determined by using equations (1-6) respectively.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate the effect of temperatures and air mass flow rate on PTSC efficiency, drying time, drying rate and moisture loss by apricots. A two factorial Randomized Complete Block Design was used to find the main factor and the interaction effect of drying temperature and air mass flow rate on the performance of PTSC and drying mechanism of apricots. A software named SPSS version 17 was used for ANOVA. Comparison of means was performed with Duncan's Multiple Range Test.

Results

Solar energy and its conversion to heat

The solar energy available to the collector during the drying period is given in Figure 2. The solar intensity graph recorded by pyranometer shows that a total of 12 hours (6:00 am to 6:00 pm) of solar energy in the form of sunlight was available. The intensity of solar energy was higher at noon (12:00) which is about 367 KJ. At this time the PTSC collected the maximum heat (Figure 2) because of high efficiency of the PTSC. The Chi-squared goodness of fit test shows that the distribution of solar energy available (Ea) is normal with chi square value of 0.998 and P value = 0.009. Similarly the distribution of solar energy consumed (Eh) is also normal with chi square value of 0.967 and P value = 0.007. The site receives a brilliant amount of solar radiation. The intensity was 260 $KJ.min^{-1}$ which was far better for the purpose of solar drying. The intensity of sunlight was high at noon. The PTSC began to convert incident solar irradiance to heat from 9:00 am till 6:00 pm. Therefore, a total of 9 hours were available for apricots for drying under a temperature range of 40-60°C and humidity of less than 30%.

Efficiency of PTSC

The data regarding the efficiency of PTSC with different drying temperatures and air mass flow rates is shown in Figure 3. The variance analysis was done for finding the effect of temperature and the air mass flow rate of the efficiency of the PTSC. The significant difference ($P < 0.000$) was found by drying temperatures and mass flow rates as well as their interaction on the efficiency of PTSC (Table 1) while the replication effect was not significant. The mean values (Table 2) Shows that at T1 and T2 the efficiency was 44% which becomes higher at T3 with 49%. Efficiency on F1 was 45% which increase to 47% at F2. Similarly an increase in efficiency was recorded when air mass flow was increased to F3. Efficiency increased from 47% to 48%.

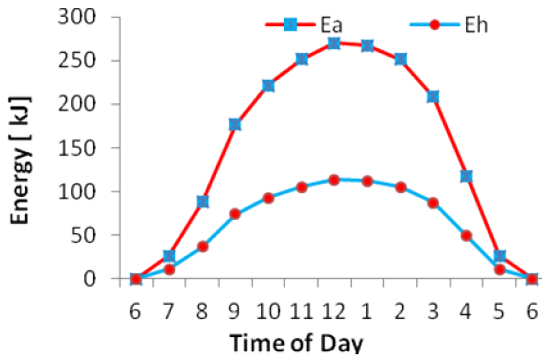

Figure 2. Energy available and consumed by the PTSC and drier.

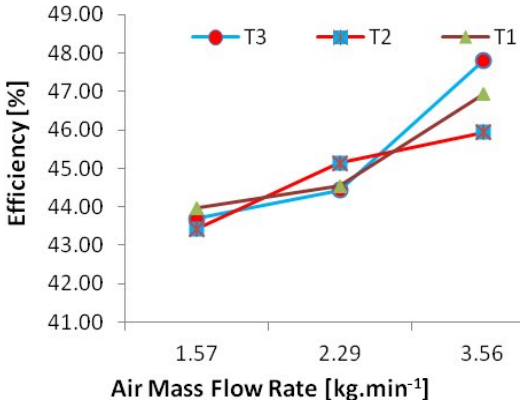

Figure 3. Efficiency of PTSC with different air mass flow rates under different temperatures.

Table 1. Analysis of variance for Efficiency.

Source	Degree of Freedom	Sum of Square	Mean Square	F- value	Prob
Replication	2	4.018	2.009	4.2799	0.0324^{Ns}
T	2	63.333	31.667	67.4556	0.0000 *
F	2	312.900	156.450	333.2659	0.0000 *
TxF	4	25.579	6.395	13.6219	0.0001*
Error	16	7.511	0.469		
Total	26	413.342			

The star (*) Indicates significance difference at 1% confidence level; Ns indicates non significant Effect

Table 2. Means Comparison of Efficiency @ 1% confidence interval by Duncan's Multiple Range Test.

Factor		Mean
Replication	R1	15.984a
	R2	16.231a
	R3	16.898a
Drying Temperature	T1	19.206a
	T2	17.498b
	T3	17.490b
Air Mass Flow Rate	F1	20.726a
	F2	15.972b
	F3	12.416c
LSD = 1.389		

Means followed by different alphabets are significantly ($\alpha = 0.01$) different from each other

Moisture Loss and Drying Rate

The initial moisture content of apricots was 86% and apricots were dried until the final moisture content reached to less than 10%. It was due the fact that pathogen attack was minimized when dried fruits having a moisture content less than 10%. The statistical analysis (Table. 3) Showed that drying temperature, air mass flow rate and their interaction have significant ($P < 0.000$) effect on moisture loss (%) from apricots. The means comparison (Table 4) shows that moisture loss from apricots was more at T3. It was about 4.7% in each hour. T3 is followed by T2 with 4.5% followed by T1 with 4.4%. Also the data of air mass flow rates were affecting the moisture loss. At F3 moisture loss was 4.9% followed by F2 and F1 with 4.6 and 4.2%.

The statistical analysis (Table. 5) showed that drying rate was significant ($P < 0.000$) affected by drying temperature, air mass flow rate and their interaction. The means comparison (Table. 6) Shows that the drying rate of apricots was more at T3. It was about 0.139 $g_w.g_{dm}^{-1}.cm^{-2}.hr^{-1}$ in each hour. T3 is followed by T2 with 0.123 $g_w.g_{dm}^{-1}.cm^{-2}.hr^{-1}$ followed by T1 with 0.111 $g_w.g_{dm}^{-1}.cm^{-2}.hr^{-1}$. Also the data of air mass flow rates were affecting the moisture loss. At F3 drying rate was 0.149 $g_w.g_{dm}^{-1}.cm^{-2}.hr^{-1}$ followed by F2 and F1 with 0.127 and 0.104 $g_w.g_{dm}^{-1}.cm^{-2}.hr^{-1}$. Moisture loss from apricots as a function of time is given in Figure. 4. At F1T1 the apricots took 24 hours to be dried. At F2T2 the apricots took 20 hours to be dried and at F3T3 the apricots took 16 hours to be dried. So drying time decrease as we increase the temperature and Mass flow rate.

Table 3. Analysis of variance for Moisture loss by Apricots.

Source	Degree of Freedom	Sum of Square	Mean Square	F- value	Prob
Replication	2	0.059	0.029	0.8240	0.0624^{Ns}
T	2	0.387	0.194	5.4550	0.0156^{Ns}
F	2	1.476	0.738	20.7875	0.0000 *
TxF	4	0.086	0.021	0.6050	0.0051*
Error	16	0.568	0.036		
Total	26	2.576			

*Indicates significance at the 1% confidence level

Ns indicates non significant Factor

Table 4. Means Comparison of Moisture loss by Apricots @ 1% confidence interval by Duncan's Multiple Range Test.

Factor		Mean
Replication	R1	4.544a
	R2	4.467a
	R3	4.578 a
Drying Temperature	T1	4.400b
	T2	4.500ab
	T3	4.689 a
Air Mass Flow Rate	F1	4.222b
	F2	4.789a
	F3	4.578a
LSD = 0.318		

Means followed by different alphabets are significantly ($\alpha = 0.01$) different from each other

Table 5. Analysis of variance for Drying rate of Apricots.

Source	Degree of freedom	Sum of Square	Mean Square	F- value	Prob
Replication	2	0.001	0	2.3846	0.0012*
T	2	0.003	0.002	12.0769	0.0000 *
F	2	0.007	0.003	23.6154	0.0000 *
TxF	4	0.001	0	2.3462	0.0091*
Error	16	0.002	0		
Total	26	0.015			

*Indicates significance at the 1% confidence level

Ns indicates non significant Factor

Table 6. Means Comparison of Drying rate of Apricots @ 1% confidence interval by Duncan's Multiple Range Test.

Factor		Mean
Replication	R1	0.123b
	R2	0.119c
	R3	0.131a
Drying Temperature	T1	0.111c
	T2	0.123b
	T3	0.139a
Air Mass Flow Rate	F1	0.149a
	F2	0.127b
	F3	0.104c
LSD = 0.006		

Means followed by different alphabets are significantly ($\alpha = 0.01$) different from each other

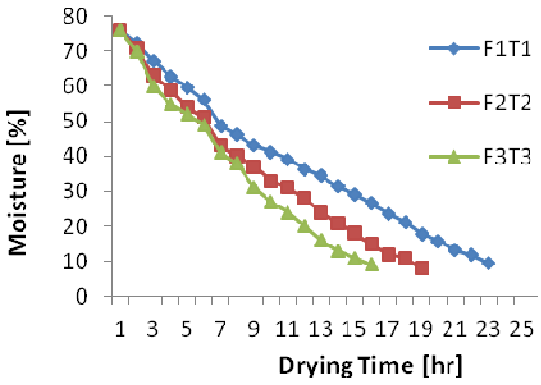

Figure 4. Interaction of Drying Temperature and Air mass flow ratee of Moisture loss from Apricots.

Discussions

The site received 260 KJ.min^{-1}, a brilliant amount of solar radiation, suitable for the purpose of solar drying. A total of 9 hours were available for apricots for drying under a temperature range of 40- 60^{o}C and humidity of less than 30%. The PTSC performance results showed that efficiency was significantly affected by drying temperature, air mass flow rate and their interactions. The greater the difference between the collector and drying unit's temperature and the ambient temperature the greater was the heat loss. At high temperatures of 60^{o}C, the collector has a high average efficiency at different flow rates as compared to 50^{o}C and 40^{o}C. The effect of drying temperature over increase in efficiency is significant having P value 0.000. The reason for this was that at low ambient temperature, difference between the outside environment and the collector temperature was more and thus the collector lost more heat to the surroundings. At a low ambient temperature the enthalpy of air was less and needed more heat to be warmed up. At high ambient temperatures the air had high enthalphy and needed less amount of heat to be warmed up. As a result the average efficiency of the collector at 60^{o}C was greater than that at 50^{o}C and 40^{o}C. Also collector shows high efficiency at high air mass flow rate. This is because at a high mass flow rate, more air was pumped into the collector that resulted in more heat delivery to the drying unit. This ultimately increased efficiency of the collector. These results are in the agreement with the findings of Karim et al. (2003), Ehiem et al. (2009), Khattak et al. (2010) and Hashemi et al. (2008) who recorded a significant increase in efficiency with an increase in drying temperature and air mass flow rate. The performance of PTSC to dry apricots was significantly affected by drying temperature, air mass flow rates and their interactions. Both moisture loss and drying rate of apricots were higher at high temperature and high air mass flow rates. As moisture loss and drying rate both are directly related with temperature and air mass flow rate, high temperature of 60^{o}C and high air mass flow rate of 3.56 kg. min^{-1} the moisture loss and drying rate were hgher. These results are also in the agreement with the findings of Ahmad (2011), Mahmood et al. (2005), Mohanraj and Chandrasekar (2009), Santos et al. (2005) and Wilcke (1980) who recorded a significant increase in moisture loss and drying rate with an increase in drying temperature and air mass flow rate. Acosta-Esquijarosa et al. (2011) also recorded that drying kinetics are affected by both drying temperature and air mass flow rates. They

concluded that drying is faster when temperature and mass flow rate in increased.

Conclusion

Based on the findings obtained in this study, it is concluded that:

The Efficiency of the PTSC increased significantly with the increase in the air mass flow rate as well as drying temperature. It is recommended to operate PTSC with air as medium of heat transfer at a higher air mass flow rate and within a high range of drying temperature to get maximum performance.

PTSC gives maximum performance as a drier and work efficiently for 9 hours in a day from 9:00 am to 6:00 pm in summer. It is recommended to dry apricots by the PTSC's drying chamber from 9:00 am to 6:00 pm.

Moisture loss from apricots and drying rate was more rapid at high temperature and high air mass flow rate. It is therefore, recommended to dry apricots at high air mass flow rate and within a high range of drying temperature. They will be dried quickly and will be of good quality and taste.

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FOOD SCIENCE AND NUTRITION

Formulation and characterization of vegetable oil blends rich in omega –3 fatty acids

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Abstract

Seven blends of vegetable oils were formulated from flaxseed, olive and canola oils differing in their content of n–6 to n–3 and n–9 to n–6 to n–3 fatty acids (FAs) ratio. FA composition, oil classes, triacylglycerol fractions and identity characteristics of the original three oils and their seven blends were determined. Results showed marked differences in the FAs composition between the three oils used for preparing the seven oil blends. The prepared blends had n–3 to n– 6 FAs ratio which varied from 1.2: 1 to 1: 9.1 with the highest ratio of n–3 to n– 6 FAs in flaxseed – canola oil blends. Increasing the level of canola oil in canola - olive oil blends increased from the linolenic acid and lowered from linoleic acid. Meanwhile increasing the proportion of olive oil in the vegetable oil blends was associated with a decrease in iodine value, linolenic acid, n–3 to n–6 and saturated to unsaturated FAs ratio. The opposite trend was noticed with increasing flaxseed and/or canola oil in such blend. Slight differences were observed in the specific gravity, refractive index and saponification values. Among the formulated blends, triacylglycerols were the major fractions of three oils and their seven blends. Also, the triacylglycerols of flaxseed and canola oil blends were fractionated into 9 groups rich in unsaturated triacylglycerols.

Key words: Fatty acids, Lipid classes, Oil blends, Omega-3, Triacylglycerols

Introduction

Nearly 85% of the Egypt's needs for oils and fats are obtained from imports. More than 70% of such amount is palm oil followed by sunflower, animal fats, soybean, cottonseed and corn oils in addition to limited quantity of canola and olive oils (FAO, 2005). Except soybean oil, the other's major imported oils are rich either in saturated fatty acids (Palm oil) and/or n–6 fatty acids, and very poor in n–3 fatty acids. Also, the rise in the consumption rate of Western diets in Egypt in the last few years has caused a significant increase (nearly 3 fold) of fat intake. This is accompanied by a significant rise in death due to cardiovascular disease (CVD) and the blood cholesterol (Miladi, 1999). Usually, PUFA and olive oil have been considered as healthy lipids because they reduce the incidence of CVD (Barzi et al., 2003; Mozaffarian et al., 2007).

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Nutritionists recommend 30% level or lower of calories as fat with the intake of saturated fat not to exceed 10% of total energy. The remaining can be divided equally between monounsaturated and polyunsaturated fats (Simopoulos, 1996). World Health Organization (WHO) recommends 4–10:1 ratio of n–6 to n–3 fatty acids (FAs) (Richter, 2001). Eating the proper ratio of n–6 to n–3 FAs from the right edible oil sources will help in enhancing thermogenesis, building muscles, and hormones like eicosanoids, reducing risk factors of CVD, high levels of blood pressure, triglycerides, lipoprotein–a, fibrinogen, clot formation and inflammation, muscle breakdown, improving brain functions, mood, intelligence, behavior and vision (Fan and Chapkin, 1998; Rodriguez– Leyva et al., 2010).

The main sources of n–3 FAs are fish and vegetable oils which are low in both saturated and n–6 FAs such as flaxseed oil (Consisting of 60% α–linolenic acid), canola oil, walnut oil and olive oil. Both olive and canola oils contain generous amount of monounsaturated FAs. Virgin olive oil is also rich in antioxidants and a substance called squalene that has antiflammatory properties, slows blood clot formation, lowers cholesterol and increases the amount of n–3 FAs taken up by body cells (Trichopoulou et al., 1995). Munoz et al. (2009)

found that the adherence to a Mediterranean dietary pattern rich in fruits and vegetables, fish and olive oil, was associated with high scoring for self–perceived health.

The aim of this study was to formulate vegetable oil blends differing in their content of n–6 to n–3 and n–9 to n–6 to n–3 FAs ratio using pre–refined flaxseed, virgin olive and refined canola oils. The characteristics and structure of these blends compared to their original oil sources was also determined.

Materials and Methods

Materials

Vegetable oils

Thirty kilograms of each of the following three vegetable oils were used in this study, (i) an imported Netherland extra refined canola oil from a big supermarket at Alexandria city Egypt, (ii) cold press extra virgin olive oil from Wadifood company, Egypt, and (iii) fresh cold press crude flaxseed oil from a private commercial flaxseed press mill at Alexandria city, Egypt. The crude oil was pre-refined in the same day of it's pressing in lab by degumming with 85% phosphoric acid, neutralizing with 15% sodium hydroxide and bleaching under vacuum at 90°C for 30 min. using Tonsil ACCFT activated bleaching earth as described by Lillard (1982). The cooled bleached oil was packed in brown glass bottles and stored at 4°C.

Vegetable oil blends

Seven vegetable oil blends were formulated as shown in Table 1 by mixing olive oil (O) with each of canola (C) and flaxseed (F) oils and also canola oil (C) with flaxseed oil (F).

Table 1. Vegetable oil blends.

Vegetable oil blends	Blending ratio (w:w)
OC–1	5.7 olive oil : 1.0 canola oil
OC–2	4.0 olive oil : 1.0 canola oil
OC–3	3.0 olive oil : 1.0 canola oil
OF	19.0 olive oil : 1.0 flaxseed oil
CF–1	19.0 canola oil : 1.0 flaxseed oil
CF–2	9.0 canola oil : 1.0 flaxseed oil
FC	1.0 canola oil : 1.3 flaxseed oil

The blending was carried out at room temperature (22 ± 2°C) in brown glass bottles under a stream of nitrogen gas. The bottles with oil blends were kept at 4°C until analysis.

Methods

Color of oils was assessed using Lovibond Tintometer, Model E, 100000 G, USA, using 0.5 inch cell as described by Mackinery and Little (1962). Specific gravity at 25°C (28.003 and 28.004), refractive index at 25°C (28.007), iodine number (28.019 and 28.020) were determined according to AOCS (1985) and saponification value was estimated as mentioned by Egan et al. (1987). Oils were fractionated into different classes as described by Mangold and Malins, (1960) using (20 × 20 cm) TLC plates coated with 0.25 mm thickness silica gel (Merk G, type 60), developing solvent system consisted of petroleum ether (40–60°C): diethyl ether: glacial acetic acid (70: 30: 2, v/v/v) and exposing to iodine vapors in a closed jar for visualization. Triacylglycerols were separated on chromatoplates coated with a slurry of silica gel (Merk G, type 60) in an aqueous silver nitrate 20% solution according to Gegiou and Georouli (1983) method using developing solvent consisting of toluene : diethyl ether (96: 4 v/v). Visualization was carried out by charring at 180°C for 10 min. Fatty acid methyl esters of oils were prepared as described by Radwan (1978) in a screw cap vial using 1% H_2SO_4 in methanol under a stream of nitrogen gas. The closed vials' were heated in an oven at 90°C for 90 min. Analysis of fatty acids was carried out by Shimadzu Gas Chromatography (GC–4 CM, PYE) using, SP–216 packing material, 130–190°C (2°C/min rise) as a column temperature, flame ionization detector at 250°C,5 mm/min. sheet speed, 0.5 ml/min air flow rate, 1 ml/min.H_2 flow rate and 30 ml/min nitrogen flow rate (Radwan, 1978). The n-6: n-3 and n-3: n-6: N-9 fatty acid ratios of flaxseed, canola and olive oils and their blends were calculated according to the determined values of linolenic, linoleic and oleic acids.

Statistical analysis

The standard deviation was calculated using the method described by Steel and Torrie (1980).

Results and Discussion

Fatty acids composition, classes, triacylglycerols and identity characteristics in addition to the calculation of n–3 to n–6 and n–3: n–6: n–9 fatty acids of the original three oils and their seven blends were determined.

Fatty acids composition

Fatty acid composition is depicted in Table 2:

1– Marked difference in the fatty acids composition between the three oils used for preparing the seven oil blends. Flaxseed oil was the highest source of linolenic acid (n–3 FA) while olive oil was the richest in oleic acid (n–9 FA), palmitic acid and nearly free from linolenic acid

among the three oil sources. Linoleic acid (n–6 FA) was found in the highest value in canola oil followed by flaxseed and olive oils, respectively. Generally, the unsaturated FAs represented 90.63, 90.46 and 81.87% of the total fatty acids of flaxseed, canola and olive oils, respectively. Therefore, the ratio between saturated to unsaturated FAs was nearly similar in flaxseed and canola oil, (1:9.5) and (1:9.67), respectively, in spite of the clear variation in their FAs composition, and lower in olive oil (1:4.5).

2– The FAs composition of the prepared seven oil blends was differed according to the type and proportion of the used oil in their formulation. Generally, they had lower linolenic and stearic, higher linoleic, oleic and palmitic acids than flaxseed oil. Also, they contained less amount of oleic and palmitic acids, higher value of linolenic, linoleic and stearic acids than olive oil.

3– Among the prepared seven oil blends, FC–blend was the highest in linolenic acid (n–3 FA), the lowest in oleic acid (n–9 FA), and medium in linoleic acid (n–6 FA). The ratio of saturated to unsaturated FAs of this blend was similar (1:9.56) with those of flaxseed and canola oils in addition to both CF–1 and CF–2 oil blends in spite of the obvious variation in their FAs composition.

4– Slight differences in FAs composition were observed between CF–1 and CF–2 oil blends. Both contained medium amount of linolenic and oleic acids and highest amount of linoleic acid among the prepared seven oil blends. Comparing with canola oil, they had more linolenic acid.

5– Except linolenic acid content, slight differences in FAs composition among OC–1; OC–2; OC–3 and OF oil blends were noticed. Comparing with olive oil, the four blends had higher levels of linolenic acid. The ratio of saturated to unsaturated FAs of these blends was ranged from 1:4.6 to 1:5.3.

The results of the fatty acids analysis of flaxseed, canola and olive oils in this study were in agreement with those stated in literatures. Linseed oil composed of 10.5% total saturated fatty acids, 20.5% oleic, 17.5% linoleic and 51% linolenic acids. Meanwhile, the oil of low–linolenic version of linseed consisted of 11% total saturated fatty acids, 16.2% oleic, 70% linoleic and 2.2% linolenic acids (Tomkrawczky, 1999).

Canola oil is characterized by a relatively high concentration of oleic acid (55.8%), a low concentration of saturated fatty acids (13%) and a moderate level of PUFAs (20%) of which linolenic acid contributes between 25 and 30% (Mc–Donald et al., 1989; Lawson, 1995). Petukhov et al. (1999) found that the 18:1, 18:2 and 18:3 FA levels were 56.5, 22.3 and 10.8% in canola oil, 58.2, 27.9 and 3.74% in low linolenic canola oil, 75.2, 8.0 and 5.5% in high oleic canola oil, respectively.

Table 2. Fatty acids composition of flaxseed; canola and olive oils and their blends.

Type of oils**	Fatty acids* (%)								Saturated : Unsaturated fatty acids ratio
	Saturated				Unsaturated				
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	
F	0.21	0.50	4.06	4.60	1.07	28.18	20.37	41.01	1 : 9.67
C	0.23	0.39	5.54	3.38	0.92	54.76	24.92	9.86	1 : 9.50
O	0.39	0.39	14.21	3.14	0.28	63.73	17.23	0.63	1 : 4.50
FC	0.26	0.44	4.73	4.04	0.99	40.13	22.42	26.99	1 : 9.56
CF–1	0.23	0.40	5.39	3.50	0.94	52.10	24.77	12.97	1 : 9.50
CF–2	0.23	0.41	5.46	3.44	0.92	53.43	24.69	11.42	1 : 9.50
OC–1	0.36	0.39	12.47	3.19	0.41	61.95	18.76	2.47	1 : 5.09
OC–2	0.34	0.38	12.05	3.21	0.44	61.49	19.15	2.94	1 : 5.30
OF	0.38	0.40	13.70	3.21	0.32	61.95	17.39	2.65	1 : 4.60
OC–3	0.36	0.39	12.92	3.18	0.38	62.38	18.38	2.01	1 : 4.93

*Values are means of duplicates.

**F= flaxseed oil, C = canola oil, O = olive oil, FC = 1 canola oil: 1.3 flaxseed oil, CF–1 = 19 canola oil: flaxseed oil, CF–2 = 9 canola oil: 1 Flaxseed oil, OC – 1= 5.7:1 canola oil, OC–2 = 4 olive oil: 1 canola oil, OC – 3 = 3 olive oil: 1 canola oil, OF = 19 olive oil: 1 flaxseed oil

Table 3. Calculated ratio of n–6: n–3 and n–3: n–6: n–9 fatty acids of flaxseed, canola and olive oils and their blends.

Type of oils	n–6 : n–3 ratio	n–3 : n–6 : n–9
F	1.00: 2.01	2.01:1.0:1.38
C	2.50: 1.00	1.00:2.50 :5.55
O	27.3 : 1.00	1.00: 27.30 :101.16
FC	1.00: 1.20	1.20:1.00: 1.79
CF–1	1.88 : 1.00	1.00:1.88 :4.02
CF–2	2.16 : 1.00	1.00:2.16 :4.68
OC–1	7.60: 1.00	1.00:7.60:25.08
OC–2	6.50 :1.00	1.00:6.50:54.93
OF	6.56: 1.00	1.00:6.56 :23.38
OC–3	9.10:1.00	1.00:9.10:31.04

Olive oil is composed of oleic acid (55.0–83.0%), palmitic acid (7.5–20.0%), linoleic acid (3.521.0%), stearic acid (0.5–5.0%) and linolenic acid (0.0–1.5%) (Wessels and Walking, 1981; Cuellar, 1990). Also, Teresa–Sature et al. (1995); Sharara (1998) indicated that the major fatty acids in olive oil were oleic (65–79%), palmitic (9–15%), linoleic (5–14%) and stearic (2–4%).

The results of n–3 to n–6 and n–3 to n–6 to n–9 FAs ratios of the flaxseed, canola and olive oils and their blends were reported in Table 3. Generally, the n–3 to n–6 FAs ratio reveals the presence of the essential polyunsaturated fatty acids and their proportions in oil source. The height ratio of n–3 to n–6 fatty acids helps in establish and maintain the human health (Fan and Chapkin, 1998; Krauss et al., 2000). The highest value of n–3 to n–6 fatty acids ratio was found in flaxseed oil (2.01:1) rich in linolenic acid and the lowest one was in olive oil (1:27.3) rich in oleic and poor in linolenic acids. On the other hand, canola oil had 1:2.5 n–3 to n–6 fatty acids ratio. According to World Health Organization, the ratio of n–3 to n–6 fatty acid should be ranged from 1:4–10 (Kris–Etherton et al., 2000). Therefore, both flaxseed and canola oils are considered from the main sources of n–3 fatty acids among vegetable oils. But both oils, especially flaxseed oil, are subjected for high rate of autoxidation and thermal polymerization (Malcolmson et al., 2001). The prepared seven oil blends had n–3 to n–6 fatty acids ratio varied from 1.2:1 to 1:9.1. The highest ratio of n–3 to n–6 fatty acids was in FC oil blend (1.2:1) followed by CF–1, CF–2, OC–2, OF, OC–1 and OC–3, respectively. This ratio was much closed in both CF–1 (1:1.9) and CF–2 (1:2.1) and also in both OC–2 (1:6.5) and OF (1:6.56) oil blends. Generally, the prepared seven oil blends had n–3 to n–6 fatty acids ratio lies within the ratio recommended by World Health Organization, Health and Welfare–Canada's Scientific Review Committee (1:4–10 n–3 to n–6 fatty acid ratio) (Kris–Etherton et al., 2000). Calculation of n–3: n–6: n–9 fatty acids ratio reveals the variation in the unsaturated fatty acids and their proportion in oil source. It is known that the rate of oil oxidation increases with the increase in the number of double bonds in the fatty acids of the oil. Therefore, it is expected that the increasing of monounsaturated fatty acids (n–9 fatty acids) in oil with keeping the proper ratio of n–3 to n–6 fatty acids will lower from its exposure to oxidation. As seen from Table 2 both olive and canola oils contain high content of monounsaturated fatty acid, oleic acid. Therefore, both oils carry a lower risk of peroxidation than flaxseed oil. According to Viola and Audisio (1987) virgin olive oil contains α–tocopherol, as a natural antioxidant, squalene and rich in monounsaturated fatty acid. Squalene increases the amount of n–3 fatty acid taken up by the cells of body. The highest ratio of n–3: n–6: n–9 was in flaxseed oil followed by FC and CF–1 blends, canola oil, CF–2, OC–2, OF, OC–1, OC–3 oil blends and olive oil, respectively (Table 3). This sequence may reveal the degree of the peroxidation stability of these oil sources during preparation, storage and utilization.

Identity characteristics

Table 4 shows some identity characteristics of flaxseed, canola and olive oils in addition to their oil blends. Generally, no much difference in specific gravity, refractive index, and saponification value were observed between flaxseed, canola and olive oils and their blends. Specific gravity, refractive index and saponification value ranged from 0.911–0.915, 1.467–1.481 and 186.25–192.87 among the three oils and their blends respectively. Meanwhile, marked variation was noticed in iodine value between the three oils and their oil blends. Iodine value increased

with the increase of unsaturation degree or the numbers of double bonds in oils and fats. Therefore, the highest value of iodine value was in flaxseed oil rich in linolenic acid and the lowest one was in olive oil rich in oleic acid. The other oils can be arranged into three classes according to their iodine value. The class one includes FC oil blend with 153.7 iodine value and 1:9.56 saturated to unsaturated fatty acid ratio. The second class contains canola oil, CF–1 and CF–2 oil blends with 119.52 to 125.94 iodine value and 1:9.5 saturated to unsaturated fatty acid ratio. The third class includes the other vegetable oil blends, OC–1, OC–2, OF and OC–3, with 89.81 to 92.89 iodine value and 1:4.6–5.3 saturated to unsaturated fatty acids ratio. The variations in iodine value among these oil sources may be affected their peroxidation stability.

According to Schoene et al. (1998) flaxseed oil had 0.931–0.936 specific gravity at 15.5°C, 1.477–1.482 refractive index at 25°C, 189–195 saponification numbers and 165–204 iodine value (Wijs).

Canadian standards stated that canola oil should has a relative density at 20°C not less than 0.914 and not more than 0.920, a refractive index at 40°C not less than 1.465 and not more than 1.467, a saponification value not less than 182 and not more than 193, an iodine value (Wijs) not less than 110 and not more than 126 and an unsaponifiable matter content not more than 20 grams per kilogram (Genser and Eskin, 1982).

The data in literatures showed that virgin and refined olive oils had 1.4689–1.4700 and 1.4700–1.4702 refractive index at 20°C (Sonntag, 1979), 0.9090–0.9131 and 0.910–0.916 specific gravity at 20°C (Firestone et al., 1985), 184–196 and 184–196 saponification value, mg KOH/g (Cuellar, 1990) and 75–94 and 75–94 (Wijs) iodine value, respectively (Rahmani and Saari–Csallany, 1991).

Table 4. Some identity characteristics of flaxseed, canola, olive oils and their blends*.

Type of oils	Specific gravity at 25°C	Refractive index at 25°C	Iodine value (Wijs)	Saponification value (mg KOH/ g oil)
F	0.913±0.001	1.481±0.001	182.58±1.12	192.87±0.97
C	0.914±0.001	1.467±0.000	119.52±0.98	189.21±0.81
O	0.911±0.001	1.462±0.001	84.63±0.87	191.09±0.92
FC	0.913±0.002	1.474±0.001	153.71±0.96	191.22±0.84
CF–1	0.914±0.001	1.468±0.001	125.94±0.85	189.58±0.90
CF–2	0.915±0.001	1.468±0.000	123.06±0.80	188.99±1.10
OC–1	0.912±0.002	1.464±0.001	91.45±0.76	190.86±0.86
OC–2	0.912±0.002	1.464±0.001	92.89±0.84	190.79±0.70
OF	0.911±0.001	1.463±0.000	89.85±0.79	190.81±0.82
OC–3	0.911±0.001	1.463±0.001	89.81±0.83	186.25±0.93

*Values are means of triplicates ± standard deviation

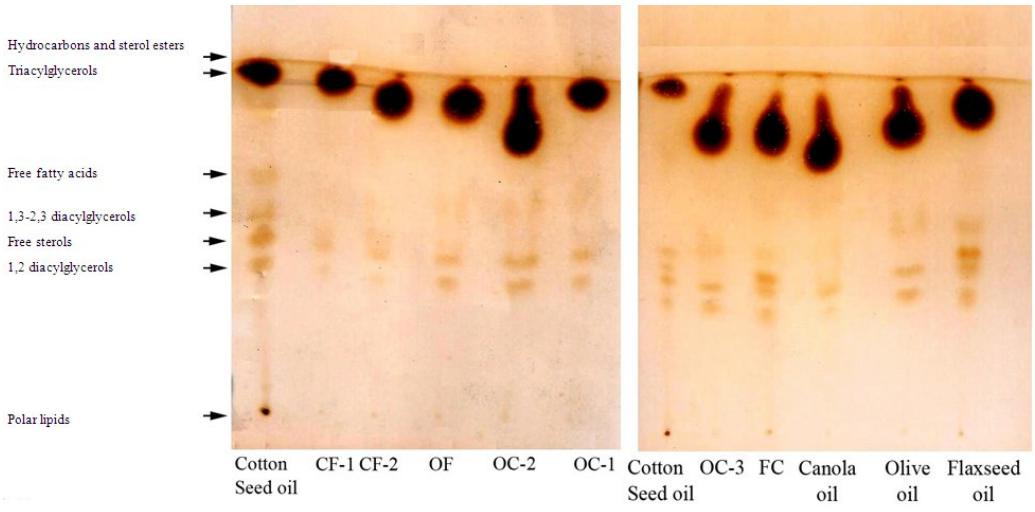

Figure 1. TLC separation of classes of flaxseed, canola, olive oils and their blends.

Lipid classes

Figure 1 illustrates the fractionation of the classes of three oil sources and their blends. Because refined flaxseed and canola oils in addition to the fresh virgin olive oil were used in this study, the main class of these oils and their blends was the triacylglycerols. The other classes which detected in small quantity were polar lipids, 1, 2–diacylglycerols, free sterols, hydrocarbons, esters and traces from 2, 3–diacylglycerols and free fatty acids. The 2, 3-diacylglycerols were clearly appeared in FC, olive oil and flaxseed oil. The free fatty acids band was more clearly appeared in flaxseed oil.

According to Gamel (1995) most of the fatty acids of olive oil are present as triacylglycerols and the non–glycerides fraction of olive oil contain non–glyceride fatty acids esters, hydrocarbons, sterols, phospholipids, chlorophylls and flavor compounds

Triacylglycerols

Figure 2 shows the triacylglycerols fractions of the three oil sources and their blends. Triacylglycerols of flaxseed oil was fractionated into 7 groups with 1, 4, 5, 6, 7, 8 and 9 double bonds. These groups can arranged according to their colour intensity which reveal their concentration in this oil as following, triacylglycerols with 8 double bonds then with 7, 4, 5, 6, 1 and 9 double bonds, respectively. Hui (1996) isolated the following triacylglycerols of linseed oil using counter–current distribution technique, trilinolenin (18.2%), linoleodilinolenin (12.3%), oleodilinolenin (19.5%) and dilinoleolinolenin (4.17%). Triacylglycerols of canola oil were also fractionated into 7 groups with 1, 2, 3, 4, 5, 6 and 7 double bonds. The sequence of these groups according to their colour intensity in TLC plate was the group with 3 double bonds followed by those with 2, 1, 4, 5, 6, and 7 double bonds, respectively. According to Prevot et al. (1990) canola oil had the following triacylglycerols LLnL, LLLn, OLnLn, LLL, OLLn, PLLn, OLL, OOLn, PLL, POLn, OOL, POL, OLGn, OOO, StOL, POO, PPO and StOO. Olive oil was fractionated into 7 triacylglycerol groups with 1, 2, 3, 4, 5, 6 and 7 double bonds. These groups can be arranged according to their color intensity in the following decreasing order, triacylglycerols with 3 double bonds followed by those with 2, 4, 5, 1, 6, and 7 double bonds, respectively. Sharara (1998) fractionated triacylglycerols into 8 groups. These groups included, 5.1% LLO, 0.2% LLP, 10.7% LOO, 5.2% LOP, 48.2% OOO, 22.8% OOP, 2.8% POO and 3.9% OOS. The oil blends which formulated from both flaxseed and canola oils, OF, CF–1 and CF–2, were fractionated into 9 triacylglycerols with 1, 2, 3, 4, 5, 6, 7, 8 and 9 double bonds. The color intensity of the fractionated groups was more obvious in triacylglycerol with 3 double bonds followed by those with 4, 5, 6, 7, 8, 1, 2 and 9 double bonds, respectively.

In case of oil blends which made of olive and flaxseed oils, OF blend, the triacylglycerol were separated into 9 groups with 1, 2, 3, 4, 5, 6, 7, 8 and 9 double bonds. Among these groups, the color intensity of triacylglycerol with 3 double bonds was deeper than other groups. It was followed by those with 4, 2, 5, 1, 6, 7, 8 and 9 double bonds, respectively. The triacylglycerol of canola–olive oil blends were fractionated into 7 groups with 1, 2, 3, 4, 5, 6 and 7 double bonds. The main group was that of 3 double bonds followed by those with 4, 2, 5, 1, 6 and 7 double bonds,

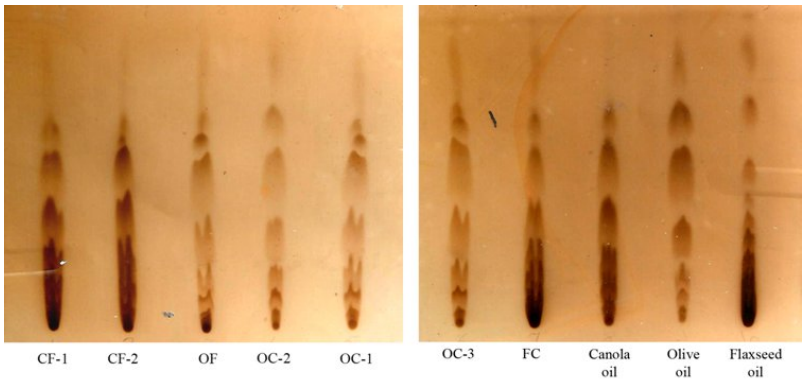

Figure 2. Ag+–TLC separation of triacylglycerols of flaxseed, canola and olive oils and their blends.

Conclusion

The identity characteristics, fatty acids composition and triacylglycerol fractions of the vegetable oil blends were based on type and level of their oils raw materials. The n–6 to n–3 FAs ration in the 7 prepared oil blends conforms to that recommended by WHO, 4–10:1. Therefore these blends are important for both nutrition specialists and oil manufacturers. Nutritionists will be interested to be sure from the availability and presence of n–3 FAs in proper concentration and their beneficial effects on human health. Moreover, the development of such blends and their successful utilization will be of great interest to food oil companies.

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PLANT SCIENCE

Development of transgenic cotton lines harboring a pesticidal gene (cry1Ab)

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Abstract

Each transgenic plant has its unique genetic architecture and level of expression. The present study was conducted to study each transgenic plant separately in order to develop pure transgenic lines to be further used in efficient cotton breeding programme. Earlier MNH-93 cultivar was transformed with the Bacillus thuringiensis (Bt) gene cry1Ab through Agrobacterium-mediated transformation method using mature cotton embryos as explants. Transformed plants obtained out of this transformation event were used to develop pure lines for five consecutive generations. The selection criteria contained confirmation of transgene through PCR and western dot blot, boll damage percentage, insect mortality percentage in lab bioassay with heliothis larvae, seed cotton yield (g), plant height (cm), number of monopodial branches per plant and number of sympodial branches per plant. All the characters were stably inherited in further generations. The expression of introduced genes was found to be variable; however it conferred protection against targeted insect pests. It was concluded that the transgenic pure lines are an excellent source of germplasm to be used in conventional breeding programme.

Key words: molecular evaluation; insect resistance; agronomic features; breeding programme

Introduction

Cotton is an important economic and fibre crop worldwide and likewise it is considered as backbone in Pakistan economy because of its major share in GDP (Economic Survey of Pakistan 20011-12). Genetically modified (GM) crops were cultivated on 148 million hectares globally in 2010. In Pakistan, insect Resistant cotton was grown on 2.4 million hectares out of 2.8 million hectares allocated land (James, 2011). Among them, transgenic cotton expressing insecticidal proteins from B. thuriengenesis (Bt) has been one of the most rapidly adopted GM crops in the world (James, 2002; Barwale et al., 2004; Dong et al., 2005) containing cry gene(s) such as cry1Ac, cry1Ac + cry2Ab or cry1Ac + cry1F.

Many laboratories have obtained insect-resistant (Zhang and Feng 1998; Hussain, 2002; Rashid et al., 2008; Bakhsh, 2010) or herbicide-resistant transgenic cotton plants via Agrobacterium-mediated transformation (Umbeck et al., 1987; Rajasekaran et al., 1996), particle bombardment (Finer and McMullen 1990; McCabe and Martinell, 1993), or pollen tube pathway (Zhang et al., 2000).

Stable inheritance and expression of transgenes in transgenic plants is of paramount importance in the successful application of genetic engineering in crop improvement. Some research revealed that once foreign genes were integrated into host cells, they could be faithfully transmitted to progenies through sexual generations and retained high meiotic stability and expression stability (Duan et al., 1996; Fearing et al., 1997; Scott et al., 1998). However, there are also many instances where foreign genes were lost through meiosis or inactivated or silenced in the progenies of transgenic plants (Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Srivastava et al., 1996; Zhang et al., 1996).

Whether transgenes could be stably inherited and expressed in progenies of transgenic plants has remained a prime question during the course of the successful employment of transgenic plants into traditional breeding programs. The present study

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was conducted to develop pure transgenic cotton lines out of a transformation event (Khan et al., 2011) for consecutive five years. The various molecular (gene integration and expression assays) as well as agronomic approaches were utilized to screen and further develop these pure lines which can ultimately be used in an efficient transgenic cotton breeding programme.

Materials and Methods

MNH-93 (a good yielding local cotton variety but susceptible to lepidopterans insect pests) was transformed with *Bacillus thuringiensis* gene (cry1Ab) using Agrobacterium tumefaciens strain C58C1 (Khan et al., 2011) at National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. The sterilized seeds of variety were soaked, placed in dark at 30°C overnight for the germination. The germinated embryos were used for the transformation. Bombardment with tungsten particles was done to create small wounds on the surface of the embryos which would facilitate DNA transfer from Agrobacterium. The prepared tungsten particles were coated on a filter assembly and allowed to dry for 1-2 minutes in a laminar hood. The filter assembly was fixed in leur-lock of Particle Bombardment Gun. The mature embryos were placed at a pre-optimized distance of 22 cm and bombardment was done under vacuum using helium gas at a pressure of 4.13 bar. The bombarded embryos were co-cultivated with *A. tumefaciens* strain C58C1 harboring pKMAB plasmid. The embryos were cultured on MS medium (Murashige and Skoog, 1962). Twenty five non-transformed embryos were cultured on MS medium as control. The plates were kept at 28 ± 2°C for 3 days. Thus, plantlets were sub-cultured on selection medium, i.e. MS containing 50 mg L^{-1} kanamycin. Cefataxime (250 mg L^{-1}) was also added to inhibit bacterial overgrowth. Subculturing was done after every 10 days. After 2 months selection, these seedlings were shifted to kanamycin free MS medium until fully developed plantlets were obtained which were further shifted to pots containing soil of equal proportion of clay, sand and peat moss (1:1:1). Finally the plants were shifted to greenhouse and were subjected to different molecular analysis. The transgenic cotton plants obtained were further used to develop pure lines in field as well as green house conditions in five consecutive generations.

First Generation

The plants of cotton variety MNH-93, transformed with the gene cry1Ab were grown under field conditions in 2003. International biosafety guidelines were followed while designing field experiments. Experimental fields were surrounded with 5 rows of untransformed MNH-93 and CIM-482 (another locally approved cotton cultivar) to serve as refugia to prolong insect resistance. Sorghum bicolor was grown around the field to isolate field from surroundings (NBC, 1999). The total number of bolls was counted at the end of season. Since cotton is an indeterminate crop and new bolls keep on forming throughout the year, both mature and immature bolls were included in counting. In addition to it, the transgenes were meant to control lepidopteran insects; therefore no insecticidal spray against the lepidopteran insects was applied. The plants were monitored for degree of damage due to lepidopteran insects during the entire season. The boll damage was calculated in percentage by dividing the number of damaged bolls with the total number of bolls on the plant, multiplied by 100. Laboratory Bioassay assay were also performed against targeted insect pests.

To check the efficacy of endotoxins against targeted insect pests, laboratory biotoxicity assays of cotton leaves with *Heliothis* larvae (2nd instar) were conducted. Five leaves from upper, middle and lower portion of each lines were detached in perti plate ,placed on moist filter papers, taken to laboratory and 2 instar larvae of *Heliothis* was fed to them. After 2-3 days mortality rates of larvae were recorded.

Second Generation

The progenies of the 1st generation plants were grown in 2004. The field layout was planned according to the international guidelines for *Bt*-crop growing. Each plant progeny consisted of two rows (each row of 10m length) accommodating 66 plants. The plant to plant and row to row distances were kept at 30 and 75cm, respectively. The transgenic plants were surrounded by 4.5m wide belt of non-*Bt* cotton as refugia. The whole cotton field was further surrounded by 4.5m wide sorghum belt, as an isolation boundary.

Boll damage percentage due to natural infestation of bollworms, insect mortality percentage in lab bioassay with *Heliothis* larvae, seed cotton yield (g), plant height (cm), number of monopodial branches per plant, number of sympodial branches per plant, confirmation of transgene through PCR and western dot blot were recorded on individual plant basis. Single plant selection was made on the basis of the following criteria: - The plant must be positive in molecular screening tests viz. PCR and western dot blot. The

plant must be higher yielding in terms of seed cotton. The plant must bear less than 5% boll damage due to natural infestation of bollworms. The plant must have shown at least 40% insect mortality in the lab bioassays. The plant should give Ginning Outturn Percentage equal or more than the control plants and the plant should be equal or short in stature than control.

Third Generation

The progenies of the selected plants from 2nd generation were raised in the green house during winter 2004-05. The plants were grown in tumbler-shaped earthen pots of 60cm height and 45cm upper diameter. Each pot accommodated a single plant. Each progeny consisted of ten plants. The light, temperature and humidity conditions of the green house were maintained by using high-powered lights, heaters and humidifiers. During the entire plant growing period, the temperature and humidity ranged between 25-40ºC and 30-70%, respectively.

The plants were again subjected to individual studies and molecular analysis. Plant selections were made on the basis of the criteria stated above. The plants in this generation showed 50% to 100% homozygosity; therefore the seed of selected plants from each progeny row was bulked to have four pure lines.

Fourth and Fifth Generation

The four pure lines mentioned above were further analyzed at molecular level, for two successive generations to eliminate false positive plants and to check for errors in selection. A sample of 30 plants, at random, was taken from each pure line during every year and analyzed at molecular level through PCR and western dot blot.

Results and Discussion

The primary objective of developing transgenic pure lines was to develop an insect resistance source. The plant selections were made in each generation to choose plants with high resistance also accompanied by other characteristics as are desirable for an efficient plant breeding programme. The methods adopted during selections were according to the standard procedures laid down in the books by Poehlman (1978); Khan et al. (2001) and Singh (2005).

First Generation

The transformation event as described by Khan et al., 2011) resulted in 26 positive plants. The plants were further grown under field conditions at CEMB in 2004. The data were recorded on various plant characteristics; the most important at this stage were boll damage (%age) due to natural infestation of bollworms (Figure 1). A boll which was completely or partially damaged (at least 10%) by any bollworm was counted as the damaged one. The bolls which were totally undamaged were counted as healthy bolls. The boll damage ranged from 8% to 100% in the plants (Table 1). The plants were also subjected to insect bioassays with 2nd instar *Heliothis* larvae under laboratory conditions (Figure 2). The insect mortality in lab bioassay ranged from 20% to 100% (Table 1).

Figure 1. Comparative view of damaged and healthy cotton bolls.
Among various plant characteristics; the most important one was Boll Damage (%age) due to natural infestation of bollworms. A boll which was damaged at least 10% by any bollworm was counted as the damaged one (A&C). The totally undamaged bolls were counted as healthy bolls (B&D). The boll damage in the 1st generation plants ranged from 8% to 100% in the plants.

Table 1. Insect Resistance and No. of Bolls of 1st Generation Plants.

S.No.	NAME	Insect mortality % shown in lab bioassay with Heliothis	No. of Total Bolls (Mature + Immature)	Average Boll Damage %age During the Peak Infestation Period (August-October)
1	CEMB-1	90	72	24
2	CEMB-2	50	63	14
3	CEMB-3	60	71	24
4	CEMB-4	80	79	8
5	CEMB-5	80	51	37
6	CEMB-6	20	19	26
7	CEMB-7	50	11	64
8	CEMB-8	90	2	100
9	CEMB-9	50	10	40
10	CEMB-10	70	22	86
11	CEMB-11	60	60	18
12	CEMB-12	80	52	12
13	CEMB-13	90	65	34
14	CEMB-14	70	22	50
15	CEMB-15	60	13	92
16	CEMB-16	70	7	29
17	CEMB-17	100	46	17
18	CEMB-18	100	40	15
19	CEMB-19	80	39	46
20	CEMB-20	100	29	24
21	CEMB-21	80	24	21
22	CEMB-22	100	37	19
23	CEMB-23	70	41	27
24	CEMB-24	80	57	18
25	CEMB-25	70	30	37
26	CEMB-26	100	36	25
27	Control	20	8	63

The boll damage due to naturally occurring bollworms in the field ranged from 8% to 100% in the plants. The insect mortality in lab bioassay ranged from 20% to 100%

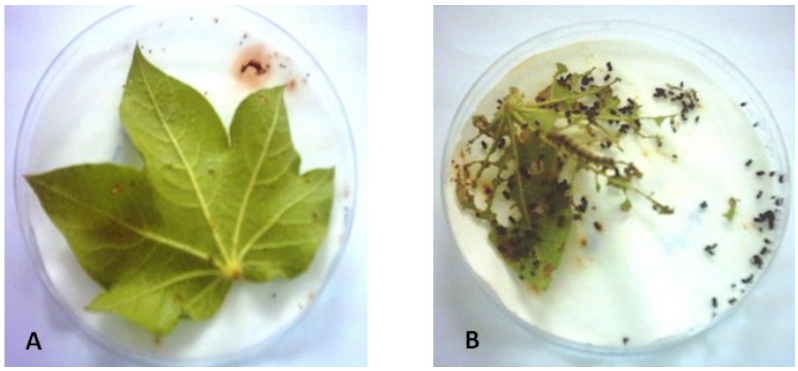

Figure 2. Laboratory bioassay with Heliothis larvae. The transgenic cotton plants were subjected to lab bioassays with American Bollworm (*Heliothis armigera*). Five fresh leaves from each plant were taken and placed on wet filter paper in petri plates accommodating one leaf per plate. One 1st/2nd instar larva, pre-fasted for 4-6 hours, was released in the each plate and allowed to feed on the leaf. After 48-72 hours feeding, the transgenic plants (A) showed insect mortality whereas the insect survived on control plant B.

The progeny rows of all 26 plants were desired to be grown but the seed setting in most of the plants did not take place under field conditions. The unfavorable weather conditions during the season besides late sowing hindered the bolls to mature. Moreover, most of the bolls which matured late season produced lighter seeds, which were discarded during seed selection for next sowing. At the end, sufficient seed of nine plants only could be obtained for further studies in progeny rows.

Second Generation

The progeny plants grown during in 2004 showed a considerable heterozygosity in plant morphology and insect resistance. The Boll Damage (%age) ranged from 0.0% to 100%. The *Heliothis* larval mortality %age in the lab bioassay ranged from 10% to 100%. The yield per plant ranged from 0.0 g to 165.1g. The plant height ranged from 47 to 230cm. The number of monopodial branches per plant ranged from 1 to 6 per plant. Similarly, the number of sympodial branches ranged from 4 to 40 per plant (Figure 3).

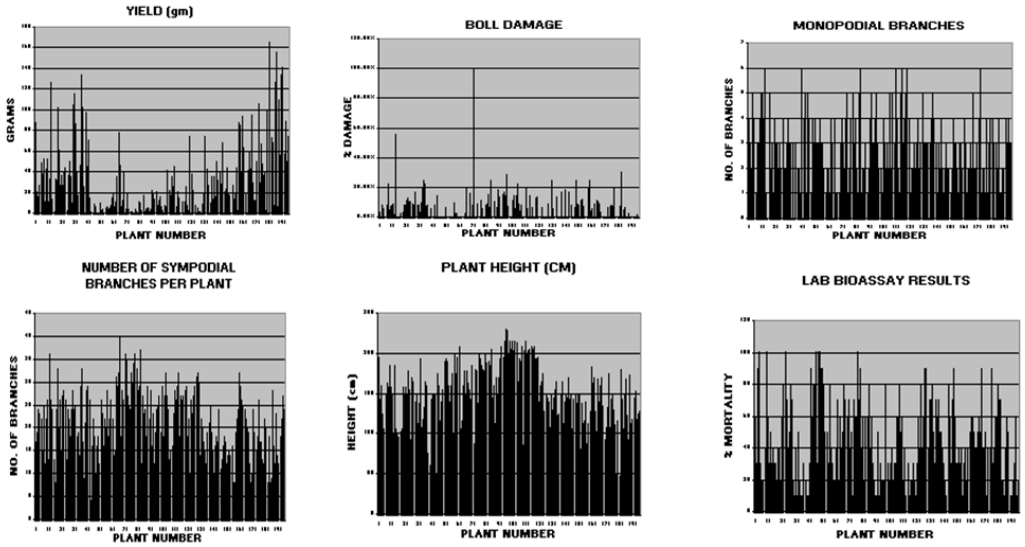

Figure 3. Data on different characters of all plants of 2nd generation, kharif, 2004The data on different characteristics of 2nd generation plants were recorded on individual plant basis.

Table 2. All plants of 2nd generation were subjected to screening on the basis of the data recorded regarding the above-mentioned traits. The data pertaining to finally selected five plants are shown here.

S. No.	Plant No.	PCR	Western Dot Blot	Insect Mortality - Lab Bioassay (%)	Boll Damage due to Natural Infestation (%age)	No. of Monopodial Branches	No. of Sympodial Branches	Plant Height (cm)	Yield (g)	Ginning Outturn (%age)
1	CEMB 3-2	+	+	60	3.33	4	17	195	87.8	43.96
2	CEMB 11-2	+	+	40	2.86	3	33	105	102.3	39.6
3	CEMB 16-10	+	+	50	2.63	1	12	172	109.3	41.0
4	CEMB 16-15	+	+	60	0.00	4	22	117	88.6	42.0
5	CEMB 17-25	+	+	50	3.45	5	19	170	95.3	38.5
6	Control	-	-	20	20	2	18	190	46.8	38.0

On the basis of the criteria stated above, selection of the best five plants was done. The data pertaining to the best five plants is given in the Table-2. The 1st plant CEMB 3-2 was positive in PCR and Western Dot Blot; it gave 87.8g seed cotton yield and 43.96% GOT; it had 4 monopodial branches, 17 sympodial branches, 195cm height; it showed 3.33% boll damage under natural conditions and killed 60% *Heliothis* larvae in the laboratory bioassay. The 2nd plant CEMB 11-2 was positive in PCR and Western Dot Blot; it gave 102.3g seed cotton yield and 39.6% GOT; it had 3 monopodial branches, 33 sympodial branches, 105cm height; it showed 2.86% boll damage under natural conditions and killed 40% *Heliothis* larvae in the laboratory bioassay. The 3rd plant CEMB 16-10 was positive in PCR and Western Dot Blot; it gave 109.3g seed cotton yield and 41.0% GOT; it had 1 monopodial branch, 12 sympodial branches, 172cm height; it showed 2.63% boll damage under natural conditions and killed 50% *Heliothis* larvae in the laboratory bioassay. The 4th plant CEMB 16-15 was positive in PCR and Western Dot Blot; it gave 88.6g seed cotton yield and 42.0% GOT; it had 4 monopodial branches, 22 sympodial branches, 117cm height; it showed 0.0% boll damage under natural conditions and killed 60% *Heliothis* larvae in the laboratory bioassay. Similarly, the 5th plant CEMB 17-25 was positive in PCR and Western Dot Blot; it gave 95.3g seed cotton yield and 38.5% GOT; it had 5 monopodial branches, 19 sympodial branches, 170cm height; it showed 3.45% boll damage under natural conditions and killed 50% *Heliothis* larvae in the laboratory bioassay.

Third Generation

The progenies of the selected five plants were raised in the green house during winter 2004-05. The plants were again subjected to individual studies and molecular analysis. The progeny plants were analyzed through PCR and Western Blotting. The plants of two progenies viz. CEMB 3-2 and CEMB 16-10 showed 100% positive results in PCR and Western Dot Blot. The plants of three progenies viz. CEMB 16-15, CEMB 11-2 and CEMB 17-25 showed 90% positive results in PCR and Western Dot Blot.

The *Bt* contents were quantified using Image Quant TL software of the Amersham BioSciences (Pvt). The *Bt* content ranged from 0.09% to 0.88% in the CEMB 3-2 plants, from 0.00% to 1.18% in the CEMB 11-2 plants, from 0.26% to 1.35%. in the CEMB 16-10 plants, from 0.00% to 0.83% in the CEMB 16-15 plants and from 0.00% to 0.76% in the CEMB 17-25 plants (Table 3).

The seeds of the positive plants were picked in each descent and bulked at this stage. Since the progenies of the plant nos. 16-10 and 16-15 were identical and also share a common ancestor, the seed of these two lines was bulked jointly. The plants in the 3rd generation were phenotypically similar and were screened at molecular level; there was no need for further selfing. The bulked seed was thus declared to be pure lines and named as CEMB-3, CEMB-11, CEMB-16 and CEMB-17.

Table 3. Bt Protein %age in 3rd Generation Plants 2004-2005. The Bt contents were quantified using Image Quant TL software of the Amersham BioSciences (Pvt). The seeds of the positive plants were picked in each descent and bulked at this stage. Since the progenies of the plant nos. 16-10 and 16-15 were identical and also share a common ancestor, the seed of these two lines was bulked jointly.

	CEMB 3-2	CEMB 11-2	CEMB 16-10	CEMB 16-15	CEMB 17-25
Plant No.1	0.56	1.07	0.53	0.40	0.76
Plant No.2	0.88	1.02	0.32	0.44	0.54
Plant No.3	0.55	0.16	1.35	0.40	0.56
Plant No.4	0.40	1.18	0.26	0.39	0.58
Plant No.5	0.70	0.94	0.27	0.62	0.00
Plant No.6	0.09	0.85	0.32	0.62	0.39
Plant No.7	0.11	0.61	0.49	0.26	0.63
Plant No.8	0.20	0.20	0.39	0.00	0.40
Plant No.9	0.60	1.06	0.33	0.83	0.52
Plant No.10	0.46	0.00	0.30	0.68	0.55
Standard Deviation	0.26	0.44	0.33	0.24	0.20

Fourth and Fifth Generation

Besides included in the field trials, the pure lines mentioned above were further analyzed at molecular level, for two successive generations to eliminate false positive plants and to check for errors in selection. A sample of 30 plants, at random, was taken from each line during every year and analyzed at molecular level through PCR and western dot blot. All plants in both generations were found to be positive in molecular screening.

Most of the transgenic lines were superior as compared to untransformed control variety MNH-93 with respect to average number of bolls, boll damage %age, early in maturing and yield of plant. It is already reported that transgenic lines may be early or late in maturity (Jiang et al., 2000). Possible reason for this morphological variation may be somaclonal variation (Larkin et al., 1981), breakdown of plant genes caused by transgene insertion or insertion mutagenesis (Lijsebettens et al., 1991), pleiotropy or transgene induced endogenous silencing (Matzke et al., 2000).

In the present studies, Bt contents were quantified and expressed as percent of total protein. The *Bt* contents showed variation in successive generations. In the 3rd generation, the *Bt* contents ranged from 0.09% to 1.35% of the total protein whereas in the successive two generations, the *Bt* contents ranged from 0.21% to 0.29% of the total protein. Our findings were in confirmation of the findings of Sachs et al., (1998) who found that *Cry1A* gene expression was variable and strongly influenced by environmental factors. The expression of transgenes in our lines varied in different generations. These results are in agreement with previous studies conducted by Wu et al. (2002), Mahon et al. (2002), Xia et al. (2005), Adamczyk et al. (2009), Bakhsh et al. (2010) and Bakhsh et al. (2011, 2012) who have reported inconsistency in insecticidal gene expression over the crop growth period and in various generations. However, investigation at molecular, genetic, as well as physiological levels should help in understanding the differential expression of transgenes and the quantitative changes in insecticidal proteins in insect resistant cotton plants. Similarly, a varying behaviour of transgenic progeny plants in respect of resistance was recorded laboratory bioassays against *Heliothis* larvae. This has been in confirmation of many research workers. Chunlin et al. (1999) and Zeng et al. (2002) reported that some of the homozygous *Cry1Ac* transgenic rice plants of T_2 progeny showed high level resistance against striped stem borer (*Chilo suppressalis*) at field trial.

The transgenic pure lines expressing insecticidal gene *cry1Ab* were selected and further propagated to attain purity. These lines provided protection against lepidopterans insects throughout the growth period till the harvesting and were desirable for agronomic and morphological characteristics. Based on the molecular and agronomic data of five consecutive years, these transgenic lines proved to be an excellent source of germplasm for an efficient cotton breeding programme.

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PLANT SCIENCE

Phytoremediation potential of *Eichhornia crassipes* for the treatment of cadmium in relation with biochemical and water parameters

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Abstract

The present investigation was aimed to assess the effect of cadmium chloride on some biochemical parameters and the potential of cadmium uptake of *Eichhornia crassipes*. The effects of increasing concentrations of cadmium chloride on growth characteristics of *E. crassipes* including root length, leaf area and biomass production was studied. The root growth of *E. crassipes* was severely affected at all concentrations of cadmium in the trays where plants were grown and in general it was serially increased. Similar trend was observed in average leaf area with 11.28 cm2 on 12th day in control while with only 7.05 cm2 at 75 ppm concentration. Chlorophyll contents were also observed to be decreased serially with the increasing concentrations of CdCl2. The lowest total chlorophylls were 118.56 mg/100g in comparison with 239.09 mg/100g in control set at the end of treatment. Polyphenol and proline contents were increased indicating the stress conditions due to toxicity of cadmium. Highest polyphenol and proline were 303.27 mg/100g and 8.14 mg/100g respectively at 50 ppm set of the treatment. Moreover, Electrical conductivity and total dissolved solids content of the various dilutions were decreased remarkably after 12 days of treatment. pH of the solution tend to become neutral while increase in turbidity might be related with root exudates and dead organics by the plants growing in the tray whereas decrease in hardness, acidity, sodium and potassium content was observed with increase in concentration. Values for BOD and COD were slightly increased at the end of treatment. Estimation of bioconcentration factor is very much important. It indicates that the species is more favourable to tolerate higher concentrations of heavy metals and also helps a lot in decontamination of the land, water etc. In this study increasing concentration of cadmium showed higher accumulation capacities and may be better treatment option for cadmium by means of phytoremediation

Key words: Phytoremediation, Bioconcentration factor, Biochemical parameters, Physico-chemical parameters, Heavy metals, Wastewater

Introduction

In nature land and water are precious natural resources for the sustainability of agriculture and the civilization of mankind. However, rapid industrialization and urbanization shows maximum exploitation and severe pollution in these resources. Land and water pollution by heavy metals is a worldwide issue (Gade, 2000). Worldwide all countries have been affected, though the area and severity of heavy metal pollution vary enormously so there has been an increasing concern with regard to accumulation of heavy metals in environment as they pose big threat to both human health and natural environment (McKeehan, 2000). In Central and Eastern European countries about 1.7 million sites were affected by heavy metals moreover in USA, there are 600 000 brown fields which are contaminated with heavy metals and need reclamation due to which 1,00,000 ha of cropland, 55,000 ha of pasture and 50,000 ha of forest have been lost (McKeehan, 2000; McGrath et al., 2001). In developing countries particularly in India, China, Pakistan and Bangladesh soil and water pollution is also severe where small industrial units are pouring their untreated effluents in the surface drains, which spread over near agricultural fields. In these countries, raw sewage is often used for producing

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vegetables near big cities (Ragnarsdottir and Hawkins, 2005). Cadmium salts are considered to be significant water pollutants not only because of their direct toxicity in water but also due to their ability to concentrate and incorporate into the food chain by aquatic plants and organisms with the natural process of bioaccumulation and biomagnification (Cearley et al., 1973). The annual worldwide release of cadmium is about 9,39,000 tones (Singh et al. 2003). Mobilization of heavy metals in environment due to industrial activities is of serious concern due to toxicity of these metals in human and other forms of life (Vieira and Volesky, 2000). Removal of toxic heavy metals from industrial wastewater is essential from the standpoint of environmental pollution control (Yuan et al., 2001).

In order to maintain good quality of soil and water there are continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. Physicochemical approaches have been widely used for remedying polluted soil and water. However, they experienced more difficulties for a large scale of remediation because of high initial capital cost, larger volume of chemicals, continuous maintenance, skilled technicians and even generate large amount of sludge which adds into the secondary waste generation, posing threats to aquatic life and minimizes the acceptability of the treatment technique (Rai, 2008).

The use of plant species for cleaning polluted soils and waters named as phytoremediation has gained increasing attention since last decade, as an emerging cheaper technology. Rhizofiltration is the removal of pollutants from the contaminated waters by accumulation into plant biomass. Literature survey suggests that several aquatic species have been identified and tested for the phytoremediation of heavy metals from the polluted water such as, sharp dock (*Polygonum amphibium* L.), duck weed (*Lemna minor* L.), water hyacinth (*Eichhornia crassipes*), water lettuce (*P. stratiotes*), water dropwort [*Oenathe javanica* (BL) DC], calamus (*Lepironia articulate*), pennywort (*Hydrocotyle umbellate* L.) (Prasad and Freitas, 2003). Recently the roots of Indian mustard and sunflower are found to be effective in the removal of Cd, Cr, Cu, Ni, Pb, Zn, and Pb, U, Cs-137, Sr-90 from hydroponic solutions, respectively (Zaranyika and Ndapwadza, 1995; Wang et al., 2002; Prasad and Freitas, 2003).

Aquatic macrophytes play an important role in structural and functional aspects of aquatic ecosystems by numerous ways. The ability of aquatic macrophytes to take up heavy metals make them acceptable research applicants especially for the treatment of effluents having medium concentration level pollutants and city sewage waters (Sood et al., 2012). Water hyacinth possesses a well-developed fibrous root system and large biomass and has been successfully used in wastewater treatment systems to improve water quality by reducing the levels of organic and inorganic nutrients. This plant can also reduce the concentrations of heavy metals in acid mine water while exhibiting few signs of toxicity. Dos Santos and Lenzi (2000) tested *Eichhornia crassipes* in the elimination of Pb from industrial effluents in a green house study and found it useful for Pb removal. Water hyacinth accumulates trace elements such as Ag, Pb, Cd, etc. and is efficient for phytoremediation of wastewater polluted with Cd, Cr, Cu and Se (Zhu et al., 1999). Tolerance to metals in plants can be achieved by purposeful accumulation into the tissues which favors uptake of metals or in such tissues which use them in non-functional parts as an indicator of tolerance or remain sturdy under adverse natural conditions like wind, rainfall, temperature fluctuations etc. showing greater ability of accumulation (Mane et al., 2010).

The present investigation demonstrates phytoremediation potential of *Eichhornia crassipes* exposed to increasing concentration of cadmium chloride. The effect and accumulation was studied with reference to selected biochemical parameters and physico-chemical parameters of the water. Growth characteristics, chlorophyll, carotenoid, polyphenol and proline were studied so as to know the physiological changes associated with experimental species while water quality analysis was carried out to judge the natural ability to treat cadmium containing water. This was specially assessed with reference to electrical conductivity (EC), pH, total dissolved solids (TDS), turbidity, free CO_2, chemical oxygen demand (COD), biological oxygen demand (BOD), acidity, hardness, sodium and potassium content. Finally aaccumulation of cadmium (Cd^{++}) in *E. crassipes* was estimated to find out the tolerance capacity and toxicity from liquid medium.

Materials and Methods

Materials used in the study

Eicchornia crassipes **(**Mart.) Solms plants were collected from Mula-Mutha river of Pune city, and acclimatized in laboratory condition. A stock solution of analytical grade cadmium chloride $CdCl_2$. $2H_2O$ (1000 mg/l) was prepared in tap water and was later diluted as required. The plants were subjected to various concentrations of cadmium

chloride i.e. 25, 50 and 75 ppm along with a control set in four plastic tubs of ten-liter of capacity each. Uniform sized plants with similar shoot area and root length were selected for the study. They were washed thoroughly under a running tap water, propagated hydroponically for twelve days and subjected to heavy metal treatments. Whole experiment was run with the initial concentrations of cadmium chloride in hydroponics and all the parameters were estimated at 0 (two hours), 4, 8 and 12 days. The chemicals used in this study were of the highest purity available and of analytical grade.

Study of the growth characteristics:

Ten plants from each treatment tray were carefully removed and washed thoroughly with water to remove any dirt and dust particles on the surface of the plant parts and blotted to surface dry. This plant material was analyzed for their growth characteristics. Various growth parameters namely root length, average leaf area, fresh weight biomass, dry weight biomass and moisture content of the randomly sampled leaves were recorded.

Estimation of photosynthetic pigments

Chlorophyll content

The chlorophylls of the mature leaves were estimated by following the method of (Mane et al., 2011). Fresh plant material (1g) was roughly homogenized in mortar by keeping the temperature at 2^{o}C in dark condition and extraction was carried out using 90% acetone, with the addition of pinch of magnesium carbonate, to protect and stabilize the chlorophylls. This extract was filtered through Whatman No.1 filter paper under suction using Buchner's funnel. The residue was washed thoroughly 2-3 times with 90% acetone, collecting all the washings in the same filtrate and final volume of the filtrate was made to 100 ml with 90% acetone. Absorbance of chlorophyll 'a' and 'b' was recorded using double beam UV-Visible spectrophotometer (Elico SL-159, India), at 663 and 645 nm using 90% acetone as blank. Following formulae were used to determine the chlorophyll content.

Chlorophyll 'a' = X = 12.7 x A_{663} - 2.69 x A_{645}

Chlorophyll 'b' = Y= 22.9 x A_{645} - 4.68 x A_{663}

Total chlorophyll (a + b) = Z = 8.02 x A_{663} + 20.20 x A_{645}

$$\text{Chlorophyll a / b/ total (mg/100g fresh leaves)} = \frac{\text{X/Y/ Z x volume of extract x 100}}{\text{1000x weight of plant material (g)}}$$

Carotenoid content

The carotenoid content of the leaves was determined from the same extract used for chlorophyll estimation, by recording the absorbance at 480 nm using spectrophotometer by putting 90% acetone as the control. The carotenoids content was calculated by using the following formula (Borkar et al., 2011).

$$\text{Carotenoids content} = \frac{\text{A480 x volume of extract x 10 x 100}}{\text{2500 x weight of plant material (g)}}$$

Polyphenol content

The polyphenol content of the leaves was estimated following the method suggested by (Vesely et al., 2012). 2 ml of acetone extract used for chlorophyll was mixed with 10 ml, 20% sodium carbonate and adjust the volume to 35 ml with distilled water to this mixture 2 ml of Folin-Denis reagent was added, mixed thoroughly and final volume was adjusted to 50 ml with distilled water. The standard tannic acid solution (0.1 mg ml^{-1}) was used for the preparation of standard polyphenol curve by measuring the absorbance at 660 nm using double beam UV-Visible spectrophotometer.

Zinc (Zn++) concentration:

Zn^{++} concentration was estimated from the dried plant material (shoot and root combined). 0.5 g oven dried plant material was acid digested as per the standard method suggested by (Rivelli et al., 2012). Plant material was taken in a 150 ml clean beaker and to that 10 ml concentrated nitric acid was added. It was covered with a watch glass and kept for an hour till the primary reactions subsided. It was then heated on hot plate until all the material was completely dissolved. It was allowed to cool to room temperature and then 10 ml of perchloric acid (60%) were added to it and mixed thoroughly. It was then heated strongly on the hot plate until the solution became colorless and reduced to about 2-3 ml. While heating, the solution was not allowed to dry. After cooling, it was transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day it was filtered through Whatman No. 44 filter paper. The filtrate was stored properly and used for analysis of Zn^{++} by using Atomic Absorption Spectrophotometer (Perkin-Elmer, 3030A).

Bioconcentration Factor (BCF)

The bioconcentration factor provides an index of the ability of plant to accumulate the metal with respect to metal concentration in substrate. The bioconcentration factor was calculated as the ratio of the trace element concentration in the plant tissues at harvest to the concentration of the element in the external environment (Vesely et al., 2012; Rivelli et al., 2012). The BCF was calculated by using formula: BCF = *P/E*,

Where, *P* represents the trace element concentration in plant tissues (ppm), *E* represents the residual concentration in water (ppm) or in sediment (ppm dry wt).

Water quality analysis

Following parameters were determined from the liquid solution of the plastic trays after twelve days of period. The methods of analysis were in consistent with the standard methods mentioned in 'Handbook of Water Analysis' (Borkar et al., 2011; Maiti, 2004).

Electrical conductivity and total dissolved solids

Electrical conductivity (EC) and Total dissolved solids (TDS) of the water containing zinc chloride was determined after every 4 days by using ELICO EC-TDS meter (CM 183, Make-India) where the electrode was directly dipped into the well diluted and filtered solutions to get direct digital display of the result.

Turbidity and pH

Turbidity of the sample was measured by Nephalometric method using CL 52D model of ELICO. Sample was poured into the cell and turbidity was read directly from digital display of the instrument. pH of the water samples was determined by using ELICO LI 127 pH meter after every 4 days of incubation.

Hardness

The total hardness of the water samples was determined by EDTA titration methods. In which 50 ml of well mixed sample was taken in a conical flask and pH of solution was adjusted up to 10. A pinch of Eriochrome black-T was added and titrated with 0.01M EDTA till wine red solution changes to blue. The volume of EDTA consumed was noted and similarly a reagent blank with distilled water was run to calculate the hardness of the water samples.

$$\text{Hardness (mg/L)} = \frac{\text{C x D x 1000}}{\text{ml of Sample}}$$

Alkalinity

Alkalinity of the samples was determined of 100 ml sample with the addition of 2-3 drops of phenolphthalein indicator. Pink solution developed was titrated with 0.02N H_2SO_4 till it disappears. Again the 2-3 drops of methyl orange were added to the same flask and titration was continued till pH is down to 4.5 or orange color changes to pink. The volume of H_2SO_4 added was noted as for each time and the total alkalinity was calculated. The values obtained were expressed in mg l^{-1}.

Sodium and potassium

Sodium and potassium of the water samples were estimated with the use of Flame photometer (Systronics-128) by preparing standard concentrations of sodium and potassium. The air pressure was kept at 0.5 kg/cm² and the gas feeder knob was adjusted so as to obtain a blue sharp flame.

Chemical Oxygen Demand (COD)

COD determination was carried out with dichromate reflux method (Saratale et al., 2010) with the addition of 10 ml of 0.25N potassium dichromate ($K_2Cr_2O_7$) and 30 ml H_2SO_4+Ag_2SO_4 reagent in 20 ml diluted sample. The mixture was refluxed for 2 h and was cooled to room temperature. The solution was then diluted to 150 ml by using distilled water and excess $K_2Cr_2O_7$ remained was titrated with ferrous ammonium sulphate (FAS) using ferroin indicator.

$$\text{COD (mg/L)} = \frac{\text{(A - B) x N x 1000 x 8}}{\text{Volume of Sample}}$$

Where, A is the ml of FAS used for blank; B is the ml of FAS used for sample, N is the normality of FAS and 8 is milliequivalent weight of oxygen.

Biological Oxygen Demand (BOD)

The dilution method was followed to determine the BOD after three days. For the same dilution water was prepared with the addition of nutrients namely phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride. The diluted sample was transferred to BOD bottles. After determining initial dissolved oxygen (DO), final DO was estimated from the bottles kept for incubation period of three days.

Statistical analysis

Statistical analysis of the data was carried out by using Graph Pad software. Mean and standard deviation (SD) was calculated.

Results and Discussion

Growth analysis

Growth changes are often first and most obvious reactions of plants under heavy metal stress (Hagemeyer, 1999). In present study plant growth was measured in terms of root length, leaf area and biomass production in terms of fresh and dry weight. The causes of growth reduction differ (Munns et al., 1995), but it is not clear which mechanisms plants employ to maintain residual growth for short and long term responses.

Root length

From the results, it can be seen that the root length of *Eichhornia crassipes* responded differentially with respect to Cadmium chloride

treatment. Maximum root length was observed in control (12.5 cm) on 12th day while minimum root length was observed as 5.2 cm at 75 ppm concentration on the 12th day (Table 1). On the same day root length was observed to be decreased with an increase in concentrations showing perfect negative correlation. Literature survey showed that the heavy metals accumulations in water hyacinth increased linearly with the solution concentration in the order of leaves<stems<roots (Mane et al., 2011; Stratford et al. 1984). Munns (2003) concluded that the reduction might be attributed to the inhibition of hydrolysis of reserved foods and their translocation to the growing shoots. Growth changes are often the first and most obvious reactions of plants under heavy metal stress (Hagemeyer, 1999). Stratford et al. (1984) reported that Cd was toxic and caused substantial reduction in water hyacinth mainly by suppressing development of newly developing roots. It is known that Cd is non-essential heavy metal and has inhibitory effects on plant growth (Greger et al., 1991). Greger et al. (1991) reported that the uptake of Cd, both by roots and shoots, increased with the increasing metal concentration in the external medium. Stratford et al. (1984) found that the metal accumulation in water hyacinth increased linearly with the solution concentration in the order of leaves<stems<roots in water hyacinth. In general, most studies reported the higher concentrations of metals in roots than in shoots. Cd concentrations were reported to be higher in roots in most studies (Borkar et al., 2011; Rivelli et al., 2012). Some metals are accumulated in roots, probably due to some physiological barriers against metal transport to the aerial parts, while others are easily transported in plants. Translocation of trace elements from roots to shoots could be a limiting factor for the bioconcentration of elements in shoots (Zhu, et al., 1999). From the present investigation, it is clear that the root growth of *Eichhornia crassipes* was more sensitive and severely affected at higher concentrations of Cadmium in the water medium at the end of experiment. It might be due to higher uptake of Cd^{++} (Figure 1).

Leaf area

Maximum average leaf area was 13.06 cm^2 at 25 ppm on the 0 day and the minimum average leaf area was 7.05 cm^2 on the 12th day observed at 75 ppm concentration (Table 1). On the 12th day the leaf area of *Eichhornia* crassipes decreased with the increasing concentrations of $ZnCl_2$ and $CdCl_2$ (Table 1). From the present results, it is clear that leaf area per plant decreased when exposed to 25, 50 and 75 ppm concentration of Cadmium chloride. The mechanism of plants behind for growth reduction is not clear to maintain residual growth for short and long term responses (Munns, 2003). From the present results, it is clear that leaf area in *E. crassipes* affected at higher concentration of cadmium and might be associated with the toxic nature of the plant (Figure 1).

Table 1. Effect of Cadmium chloride (ppm) on root length, leaf area, fresh weight and dry weight of leaves of *Eichhornia crassipes*.

Duration	Root Length				Leaf area			
	Control	25	50	75	Control	25	50	75
0 Day	7.6 (±1.93)	6.34 (±2.26)	6.62 (±1.86)	6.7 (±1.63)	12.71 (±4.18)	13.06 (±3.01)	12.56 (±5.91)	12.98 (±2.13)
4th Day	9.7 (±2.48)	7.76 (±1.63)	5.78 (±0.50)	5.92 (±0.73)	11.49 (±3.56)	9.32 (±3.64)	7.88 (±0.67)	9.62 (±2.37)
8th Day	12.1 (±1.04)	7.54 (±0.55)	6.1 (±0.39)	5.16 (±0.61)	11.54 (±1.78)	8.87 (±4.90)	8.66 (±3.10)	8.01 (±1.43)
12th Day	12.5 (±0.84)	6.74 (±0.42)	5.9 (±0.35)	5.2 (±0.52)	11.28 (±2.38)	8.21 (±1.51)	7.23 (±2.35)	7.05 (±3.01)
	Fresh Weight				Dry weight			
Duration	Control	25	50	75	Control	25	50	75
0 Day	0.302 (±0.05)	0.305 (±0.09)	0.314 (±0.16)	0.325 (±0.12)	0.044 (±0.01)	0.042 (±0.02)	0.047 (±0.03)	0.047 (±0.02)
4th Day	0.336 (±0.13)	0.309 (±0.12)	0.297 (±0.02)	0.293 (±0.07)	0.058 (±0.03)	0.036 (±0.02)	0.035 (±0.00)	0.037 (±0.01)
8th Day	0.344 (±0.09)	0.320 (±0.17)	0.321 (±0.08)	0.305 (±0.08)	0.049 (±0.02)	0.051 (±0.23)	0.032 (±0.01)	0.031 (±0.01)
12th Day	0.355 (±0.06)	0.303 (±0.06)	0.295 (±0.12)	0.285 (±0.08)	0.055 (±0.01)	0.052 (±0.00)	0.030 (±0.02)	0.029 (±0.02)

Each value is a mean of ten observations and values in parenthesis indicate standard deviation

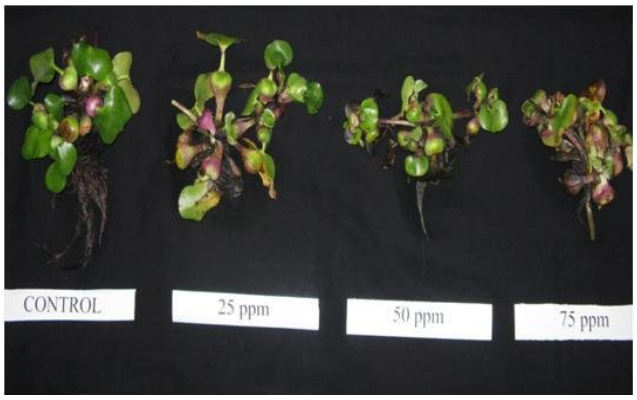

Figure 1. Effect of increasing cadmium chloride concentration on the growth parameters of *Eicchornia crassipes*.

Biomass

Fresh weight

It is evident from the results that the fresh weight of *E. crassipes* is sensitive parameter at various concentrations of Cadmium chloride. Fresh weight of the leaves was maximum with 0.355 g in control set on the 12th day and the minimum fresh weight was 0.285 g on 12th day at 75 ppm concentration (Table 1). On the 12th day the fresh weight decreased serially with the increase in concentration of cadmium. Though the plants showed slight increase in fresh weight on 4th and 8th day at 25, 50 ppm concentration over the control, it was adversely affected at higher dose at the end of treatment (Table 1). It is well known that aquatic biomass irrespective living or dead, exhibits capacity to remove heavy metals from wastewater. The reduction in shoot biomass production by the plant may be due to the chlorosis and necrosis of the leaves that reduce the photosynthetically active area (De Herralde et al., 1998). The decrease in fresh weight of the leaves of *E. crassipes* might be due toxic nature of $CdCl_2$ and the suppression of growth under such stress during the early developmental stages.

Dry weight

It is evident from the results that the dry weight of the plants is also another sensitive parameter like fresh weight. Dry weight of the leaves was maximum with 0.058 g on the 4th day in control set and minimum dry weight was 0.029 g on 12th day at 75 ppm concentration (Table 1). On the 12th day the dry weight decreased serially with increase in concentration showing a perfect negative correlation. The dry weight decreased with high concentration of $CdCl_2$ (Table 1). Munns (2003) concluded that the reduction might be attributed to the inhibition of hydrolysis of reserved foods and their translocation to the growing shoots. A decrease in dry weight of the leaves at the highest concentrations of $CdCl_2$ might be due to the inhibition in hydrolysis of reserved foods and their translocation to the growing shoots.

Moisture content

It is clear from the results that moisture percentage of plants slightly increased due to $CdCl_2$ in water medium but overall moisture content was not much affected. During Cadmium chloride treatment maximum moisture content was observed as 90.03% at 50 ppm concentration on 8th day and the minimum moisture content was observed as 82.73% in control set on 4th day (data not shown). On 12th day however there was increase in moisture content with the increase in the concentrations of cadmium. The decrease in moisture content in the leaves of *E. crassipes* at higher levels of $CdCl_2$ might be due to osmotic stress, which could not be maintained by plant while slight increase at higher levels might be the adaptation of plants to osmotic adjustment, which maintains water uptake and turgor with the accumulation of organic solutes (Li et al., 2005).

Photosynthetic pigments

Chlorophylls

It is evident from the results that chlorophyll content of *E. crassipes* is severely affected by the toxicity up to 75 ppm salt concentration showing drastic changes in the chlorophyll content of the leaves with Cadmium chloride treatment. Higher salt concentration in the liquid medium is inhibitory for chlorophyll in the species studied. It is also evident that chl.'a':chl.'b' ratio was decreased at 75 ppm salt concentration and it is quite clear that highest salt concentration (75 ppm) is certainly negatively influential on chl. 'a': 'b' ratio. *E. crassipes* appears to be rather sensitive in this respect and chl. 'a' appears to be more sensitive to toxicity than chl. 'b' (Table 2).

Table 2. Effect of Cadmium chloride (ppm) on chlorophyll content, photosynthetic pigments and their metal accumulation and antioxidant enzymes of *Eichhornia crassipes.*

	Cadmium Chloride (ppm)					Cadmium Chloride (ppm)			
Chlorophyll content	Control	25	50	75	Metal Accumulation	Control	25	50	75
Chl. 'a'	110.43 (±1.06)	88.01 (±0.36)	2.47 ±2.66)	2.04 (±1.36)	Residual concentration (mg/l)	0.084 (±0.004) 0.0[a]	0.36** (±0.15) +328	1.30* (±0.11)+ 1447	3.32 (±0.21) +3852
Chl. 'b'	128.73 (±3.83)	111.82 (±3.29)	73.35 (±3.27)	6.56 (±2.62)	*E. crassipes* (mg/kg)	11.42 (± 0.06) 0.0[a]	141.88* (±2.34) +1142	334.5* (±3.14 +2829	402.56 (±4.51 +3425
Total Chl.	239.09 (±3.63)	192.37 (±3.69)	145.77 (±3.04)	118.56 (±2.70)	Bioconcentration Factor	135.95 (± 0.09) 0.0[a]	394.11 (± 0.12) +189.9	257.3 (± 0.15) +89.26	121.25 (± 0.11) -10.81
Chl. 'a' : Chl. 'b' ratio	0.86 (±0.03)	0.79 (±0.02)	0.99 (±0.07)	0.78 (±0.04)					
Photosynthetic pigments	Control	25	50	75	Antioxidant enzymes	Control	25	50	75
Carotenoid	53.68 (±1.65)	52.72 (±1.89)	46.08 (±0.78)	31.52 (±1.01)	Catalase[b]	0.218 (±0.02)	0.225 (±0.02)	0.548 (±0.06)	0.184 (±0.01)
Proline	7.02 (±0.09)	7.33 (±0.1)	8.14 (±0.15)	4.39 ±0.21)	Peroxidase[b]	0.195 (±0.012)	0.245 (±0.02)	0.284 (±0.03)	0.175 (±0.01)
Polyphenol	151.63 (±2.31)	244.95 (±2.3)	303.27 (±4.26)	291.60 (±3.78)					

[a] The values indicates percentage variation relative to control values.
[b] Each value is expressed as Δ O.D. $min.^{-1}$ mg^{-1} protein
[c]Each value is expressed as mg 100^{-1} g fresh tissue
Each value is a mean of three determinations
Values in parenthesis indicate standard deviation

Chlorophyll content in plants correlates directly to the healthiness of plant (Li et al., 2005). Several cases of decreased chlorophyll content owing to metal toxicity have been reported in the plant kingdom growing in wetland ecosystems (Valavanidis et al., 2005). Chlorophyll degradation is the routinely observed response to stress or chiefly in elevated concentrations of various heavy metals (Chen and Djuric, 2001). Many deleterious environmental influences that inhibit plant growth, ranging from nutrient deficiencies to anthropogenic pollution, can result in decreased leaf chlorophyll and carotenoid contents (Chen and Djuric, 2001). Thus, changes in chlorophyll and carotenoid content and pigment ratios are important indicators of environmental stress and describes about the tolerance status of the species (Carter and Spiering, 2002). A decrease in chlorophyll content may be either due to inhibition of chlorophyll synthesis or its destruction or replacement of Mg ions (Chandra et al., 2009).

Carotenoids

The carotenoid content of the leaves of *E. crassipes* with Cadmium chloride treatment decreased with increasing concentrations of Cadmium chloride. Maximum carotenoid content was observed as 53.68 mg 100^{-1} in control set and a minimum of 31.52 mg 100^{-1} at 75 ppm on 12^{th} day of treatment (Table 2). Carotenoid content of the leaves decreased with increasing concentrations showing a negative correlation. Significant decrease in the carotenoid content implies decrease in protection action of carotenoid (Arora et al., 2006). According to (Vesely et al., 2012) carotenoids have two major functions in photosynthesis, they protect chloroplast from photo-oxidative damage and they also act as accessory light harvesting pigments because they absorb the light energy in the range of 400-500 nm (blue). Chen and Djuric (2001) were of the opinion that decrease in carotenoid content is a regular response to stress condition due to heavy metals. From the present investigation, it appears that toxic nature of cadmium might have caused a photo-oxidative damage in the leaves of *E. crasssipes*.

Proline

Proline content of leaves of *E. crassipes* subjected to Cadmium chloride increased maximally as 14.39 mg 100^{-1} at 75 ppm concentration and minimum as 7.02 mg 100^{-1} in control on 12^{th} day

(Table 2). Proline occurs widely in higher plants and accumulates in larger amounts than other amino acids, regulates the accumulation of usable nitrogen (Abraham et al., 2003). Number of authors as an adaptive trait concerned has considered proline accumulation in response to environmental stresses with stress tolerance and it is generally assumed that proline is acting as a compatible solute in osmotic adjustment (Lutts et al., 1999). It is evident that in general proline accumulates in plants exposed to various environmental stresses and is not an exception to toxicity stress of cadmium chloride in water medium. The increased levels of proline in the leaves showed a perfect positive correlation with increasing levels metal salt and might be to maintain osmoregulation, stabilization of proteins and conservation of nitrogen and energy for a post-stress period alongwith some other organic solutes in the species (Mane et al., 2011; Borkar et al., 2011).

Polyphenols

Very little attention has been paid towards the influence of metals on the polyphenol metabolism in plants. Parida et al. (2002) were of the opinion that accumulation of polyphenols played a key role in plants towards stress. In the present study, polyphenol content of the leaves increased with the increasing concentrations of $CdCl_2$ especially from 25 ppm to 50 ppm however, it was slightly decreased at 75 ppm concentration on 12^{th} day. The maximum increase in polyphenol was observed as 303.27 mg 100^{-1} at 50 ppm concentration and minimum polyphenol content was observed as 151.63 mg 100^{-1} in control observed at the end of experiment (Table 2). It is clear that $CdCl_2$ induce accumulation of secondary metabolites in the experimental species in order to tolerate higher levels of toxicity stress and adverse conditions aroused.

Enzymes as antioxidants

In plant cell, antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) have been considered to act as a defensive team, whose combined purpose is to scavenge reactive oxygen species (ROS) and protect cells from oxidative damage (Odjegba et al., 2007; Mittler, 2002; Mittova et al., 2003). In plants, the links between ROS production and photosynthetic metabolism are particularly important (Rossel et al., 2002). However, an elaborate and highly redundant plant ROS network, composed of antioxidant enzymes and antioxidants, is responsible for maintaining the levels of ROS under tight control (Mittler, 2002).

Catalase (E.C. 1.11.1.6)

Catalase is a common enzyme found in nearly all living organisms, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. It is the most effective antioxidant enzyme preventing oxidative damage (Mittler, 2002). Hydrogen peroxide is a harmful byproduct of many normal metabolic processes and to prevent damage, it must be quickly converted into other less toxic substances. To end this, catalase is frequently used by cells to rapidly catalyse the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Mittler, 2002; Mane et al., 2011; Rivelli et al., 2012). The activity of this enzyme was observed to be decreased with Cadmium chloride treatment. Maximal activity of enzyme catalase was observed as 0.548 at 50 ppm concentration on the 12^{th} day, and the minimum activity of the enzyme catalase was observed as 0.184 Δ O.D. $min.^{-1}$ mg^{-1} protein at 75 ppm concentration on the same day (Table 2). Initial increase at lower levels of $CdCl_2$ toxicity and then decrease at higher level indicates the strong adjustment developed by the leaves to tolerate higher toxic environment.

Peroxidase (E.C. 1.11.1.7)

It is well documented that in plants subjected to cadmium stress the activity of peroxidase increases (Saratale et al., 2011) an in the present study maximal activity of enzyme peroxidase (POD) was observed as 0.284 Δ O.D. $min.^{-1}$ mg^{-1} protein at 50 ppm concentration on 12^{th} day and minimal activity was observed as 0.175 at 75 ppm concentration on the same day (Table 2). POD is widely distributed in higher plants where it is involved in various processes, including lignification, auxin metabolism, salt tolerance and heavy metal tolerance (Odjegba et al., 2007). The increase in the activity of peroxidase under $CdCl_2$ stress may be regarded as an inhibition of stimulated secondary metabolism. It may also be involved in scavenging the reactive oxygen species in plants grown under stress condition.

Inorganic constituents

Cadmium accumulation and bio-concentration factor (BCF)

The toxic effect of metals on physiological functioning within plants is connected to their accumulation in different plant tissues (Liu et al., 2005). Several cases of accumulation of heavy metals such as Zn, Cu, Pb, Cd, Ni and Cr, have been thoroughly studied in several wetland plant species, such as *E. crassipes*, *Typha latifolia, Spartina alterniflora* and *Phragmites australis* (Liu

et al., 2005). Cadmium has no biological function and is extremely toxic, even at low concentrations and is easily assimilated by plants (Milone et al., 2003). Cadmium enters into the aquatic environment through the sources like electroplating industries, batteries, chemicals and various other applications. The accumulation of Cd^{++} by aquatic plants and their subsequent toxic effect has been reported by several investigators (Liu et al., 2005; Vesely et al., 2012). Cadmium affects various steps in plant metabolism particularly stimulation in activities of several enzymes has been also demonstrated (Rivelli et al., 2012; Borkar et al., 2011).

Bioconcentration factor (BCF) is a useful parameter to evaluate potential of plants in accumulating metals and this value is calculated on a dry weight basis. The appropriateness of a plant for phytoremediation potential is often judged by its BCF. The change in BCF of *E. crassipes* was studied to know capacity of *E. crassipes* to concentrate cadmium from varied effluent concentrations. An increase in cadmium (Cd^{++}) accumulation in *E. crassipes* leaves with increasing concentrations of cadmium in the hydroponics was observed (Table 2). The maximum accumulation of Cd^{++} by *E. crassipes* was 402.56 mg/kg at 75 ppm concentration of cadmium chloride after 12 days while it was minimum at control set with 11.42 mg/kg. In the present study, increase in concentration of cadmium in *E. crassipes* at elevated levels might be due to increasing levels already added in the liquid medium, which could also be related with Cd^{++} tolerance. The residual concentration was minimum by 0.084 mg/l in control set while the highest 3.32 mg/l was observed at 75 ppm of cadmium chloride (Table 2). Vesely et al. (2012) found that the metals accumulations in water hyacinth increased linearly with the solution concentration in the order of leaves<stems<roots in water hyacinth.

As a fact, larger BCF implies better phytoaccumulation capability and tissues with BCF greater than 1,000 are considered high, and less than 250 low, with those between classified as moderate (Rivelli et al., 2012; Borkar et al., 2011). The BCF values were increased at 25 (394.11) and 50 (257.30) ppm concentration of cadmium chloride and was lesser than control set at 75 (-10.81) ppm concentration. Only 25 ppm concentration showed highest BCF values which in equivalent with the previous study carried out by Xiaomei et al. (2004). This clearly shows that the 25 ppm concentration is the limiting concentration and later concentrations are harmful for the growth, development and contributes only as toxicity to the plant though higher accumulation is observed in dry tissues. BCF value at 25 ppm shows that *E. crassipes* has capacity to accumulate Cd^{++} through liquid medium after twelve days exposure period, which is also in accordance with other studies (Table 3) and it can be concluded that at 25 ppm concentration *E. crassipes* has moderate accumulating capacity. The sequestration of heavy metals in plants is achieved mainly by absorption and accumulation mechanisms (Dhir, 2010). The toxic effect of metals on physiological functioning within plants is connected to their accumulation in different plant tissues (Liu et al., 2005).

Analytical parameters

Electrical conductivity (EC) and Total Dissolved Solids (TDS)

Electrical conductivity is the most common measure of water and is indicative of the ability of an aqueous solution to carry an electric current. It is a fact that excess salts in water detrimentally affect plants, both physically and chemically. It is obvious that EC of the water increases with the increasing levels of cadmium chloride in the solution. It was maximum by 693.67 µS on the 0 day at 75 ppm concentration and a minimum by 85.26 µS on the 12^{th} day in control set. On 12^{th} day the EC increased with increasing concentration and showed a perfect positive correlation (Table 4).

Table 3. Bioconcentration factors (BCF) for zinc in various plants used for phytoremediation.

Sr. No.	Plant species	Bioconcentration Factor	Reference
1)	*Eichhornia crassipes(Leaf)*	394.11	Present study
2)	*Eichhornia crassipes*	622.3	Xiaomei et al. (2004)
3)	*Azolla pinnata (root)*	24,000	Sela et al. (1989)
5)	*Elodea nuttalli*	1700	Nakada et al. (1979)
6)	*Lemna polyrrhiza*	650	Jain et al.(1990)

The varying levels of EC in water containing different concentrations of $CdCl_2$ might be due the external additions of salts and also might be due to different binding capacities of the roots, root excretions and competition by the roots to get nutrients from the liquid medium. This also clearly shows the capacity of *E. crassipes* to treat cadmium containing water. Similar increase in electrical conductivity in rooting medium has been reported earlier (Gokhale et al., 2008; Mane et al., 2011). Mahmood et al. (2005) also observed the reduction in conductivity due to absorption of pollutants by plants.

TDS is simply the sum of the cations and anions concentration expressed in mg/l. It is obvious that TDS in the water increases with the increasing levels of cadmium chloride. The maximum TDS content was observed as 349.33 mg/l on the 0 day at 75 ppm concentration and the minimum value was recorded on 12th day by a value of 43.0 mg/l in control set (Table 4). On the 12th day, the TDS content increased with the increase in concentration, showing a perfect positive correlation. Khosravi et al. (2005) reported the importance of TDS uptake by *Azolla filiculoides* for their growth in wetlands. Groudev et al. (2011) observed reduction of total dissolved solids from 2620 ppm to 1230 ppm in treatment of acid mine drainage from an uranium deposit by means of a natural wetland. A good reduction (90 %) of total suspended solids by constructed wetland plants with a retention time of 7 days was reported (Amelia, 2001). The varying levels of TDS in the cadmium containing water were obviously due to the external additions of $CdCl_2$ and it's interactions with root and organic-inorganic ions already present in water.

pH and turbidity

pH of the water is the measure of H^+ ion activity of the water system. It indicates whether the water is acidic, neutral or alkaline in nature. The maximum pH was recorded with 8.85 on 12th day in control on 0 day while it was minimum by a value of 5.48 on 12th day in control set (Table 4). In general, pH was observed to be slightly acidic with the addition of cadmium chloride in the hydroponic medium. The change in pH may be due to release of some root exudates in response to the stress to adapt itself with the existing environment. Wagner (1997) found that at water temperature of 25 ^{o}C, both *A. pinnata* and *A. filiculoides* showed maximum growth at pH values from 5-7. pH seems to be the most important parameter in the biosorptive process: it affects the solution chemistry of the metals, the activity of functional groups in biomass and competition of metallic ions (Li et al., 2005).

Table 4. Effect of Cadmium Chloride (ppm) on electrical conductivity, total dissolved solids, pH and turbidity of water after 12 days of incubation with *Eichhornia crassipes.*

Duration	EC				TDS			
	Control	25	50	75	Control	25	50	75
0 Day	144.22 (±1.51)	345.67 (±5.77)	518.33 (±6.35)	693.67 (±3.21)	73.13 (±0.06)	175.1 (±0.10)	260.00 (±0.14)	349.33 (±0.58)
4th Day	134.6 (±1.01)	308.33 (±0.58)	313.0 (±1.0)	378.67 (±0.58)	63.14 (±0.21)	155.24 (±0.25)	185.65 (±0.34)	223.61 (±0.21)
8th Day	102.37 (±0.21)	167.80 (±0.26)	202.0 (±1.00)	223.33 (±2.08)	51.40 (±0.10)	103.63 (±1.06)	164.92 (±0.92)	131.93 (±0.58)
12th Day	85.26 (±0.40)	129.45 (±0.62)	181.24 (±0.23)	189.25 (±0.28)	43.0 (±0.61)	96.67 (±1.89)	121.95 (±1.31)	124.10 (±1.02)
Duration	pH				Turbidity			
	Control	25	50	75	Control	25	50	75
0 Day	8.85 (±0.05)	6.57 (±0.02)	6.68 (±0.01)	6.74 (±0.01)	0.75 (±0.04)	2.11 (±0.06)	2.59 (±0.08)	2.85 (±0.07)
4th Day	7.75 (0.04)	6.25 (0.01)	6.35 (0.02)	6.51 (0.01)	3.98 (±0.07)	7.52 (±0.12)	8.42 (±0.15)	9.89 (±0.16)
8th Day	6.89 (0.01)	6.02 (0.00)	6.41 0.01)	6.42 (0.01)	4.47 (±0.06)	5.30 (±0.14)	5.27 (±0.12)	5.70 (±0.18)
12th Day	5.48 (±0.00)	6.25 (±0.01)	6.34 (±0.00)	6.28 (±0.01)	4.60 (±0.09)	7.10 (±0.11)	7.98 (±0.08)	10.60 (±0.19)

Each value is a mean of ten observations and values in parenthesis indicate standard deviation

Maximum turbidity was observed as 10.60 on the 12th day at 75 ppm concentration and a minimum turbidity was observed as 0.75 on the 0 day in control set. On the 12th day the turbidity increased with the increase in the concentration. It is definite that the increase in turbidity might be due to the external addition of salt and also due to the root exudates released to face the stress condition (Table 4).

Hardness and alkalinity

Maximum hardness was observed as 101.33 mg l^{-1} on 0 day at 75 ppm concentration and the minimum hardness was observed as 333.33 mg l^{-1} on 12th day in control (Table 11). On 12th day hardness of water containing Cadmium chloride increased with increase in concentration showing a perfect positive correlation and such an increase is definitely due to added salt to the water medium. Maximum alkalinity was observed as 268.0 mg l^{-1} on 12th day at 75 ppm concentration and the minimum was 87.33 mg l^{-1} on 0th day in control (Table 5). At the end of experiment alkalinity also increased similar to that of hardness of water with the increase in the cadmium chloride concentration.

Sodium and potassium

Maximum sodium content was observed as 17.19 mg l^{-1} on 0 day at 50 ppm concentration and minimum sodium content observed by a value of 12.34 mg l^{-1} on 12th day of the experiment at 25 ppm concentration of cadmium chloride (Table 5). On the same day 12th day sodium content slightly decreased with increase in concentration which might be used by plant for development and to minimize metal stress. It decreased from 50 to 75 ppm concentrations of Cadmium chloride treatments. Potassium is essential in nearly all the processes needed to sustain plant growth and reproduction. It plays a vital role in photosynthesis, translocation of photosynthates, protein synthesis, activation of plant enzymes, control of ionic balance and regulation of plant stomata. Potassium is essential in nearly all the processes needed to sustain plant growth and reproduction. Maximum potassium content observed with a value of 26.92 mg l^{-1} on 0 day in control while minimum potassium content observed was 11.12 mgl^{-1} on 12th day of experiment at 75 ppm (Table 5). It was also observed that potassium content of water also decreased with increase in concentration. Such a decrease in potassium content at the end of the experiment with the increase in concentration of cadmium chloride might be due to uptake by *Eichhornia* for development and to minimize the stress condition aroused.

COD and BOD

Chemical oxygen Demand determines the oxygen required for the chemical oxidation of most organic matter and oxidisable inorganic substances with the help of a strong chemical oxidant. Highest value for COD was observed 28.65 mg l^{-1} on 12th day at 75 ppm concentration while the minimum COD content was observed as 15.24 mg l^{-1} on 0 day in control. At the end of experiment COD content of water increased in all the sets with increase in the concentration of cadmium chloride.

Table 5. Effect of Cadmium Chloride (ppm) on selected water quality parameters.

Sr. No.	Duration	Hardness				Alkalinity			
		Control	25	50	75	Control	25	50	75
1	0 Day	42.67 (±1.15)	46.67 (±1.15)	95.33 (±1.15)	101.33 (±2.31)	87.33 (±1.41)	97.33 (±3.06)	126.67 (±1.15)	151.00 (±2.31)
2	12th Day	33.33 (±1.15)	41.67 (±2.31)	80.67 (±1.15)	84.67 (±1.15)	154.00 (±3.46)	242.67 (±1.15)	260.0 (±3.46)	268.0 (±0.00)
		Sodium				Potassium			
3	0 Day	15.16 (±0.26)	16.25 (±0.15)	17.19 (±0.18)	16.54 (±0.19)	26.92 (±0.45)	25.13 (±0.36)	22.82 (±0.32)	15.13 (±0.26)
4	12th Day	14.23 (±0.22)	12.34 (±0.12)	13.21 (±0.14)	13.01 (±0.11)	24.56 (±0.54)	22.31 (±0.31)	18.62 (±0.28)	11.12 (±0.25)
		COD				BOD			
5	0 Day	15.24 (±1.57)	16.25 (±1.68)	19.24 (±1.71)	26.21 (±2.01)	4.95 (±0.19)	5.64 (±0.13)	5.49 (±0.18)	6.54 (±0.19)
6	12th Day	16.25 (±1.51)	18.95 (±1.89)	23.54 (±0.95)	28.65 (±1.01)	5.46 (±0.11)	5.78 (±0.25)	7.65 (±0.24)	7.45 (±0.89)

Each value is a mean of ten observations and values in parenthesis indicate standard deviation

The increase in the COD content is definitely due to the external addition of salt and root secretions over time. Dissolved oxygen (DO) measures are vital for maintaining aerobic conditions in natural waters that receive polluted matter. Maximum BOD content was observed as 7.65 mg l^{-1} on the 12th day at 50 ppm concentration while a minimum BOD content was observed as 4.95 mgl^{-1} on 0 day in control (Table 5). It is definite that the degrading plant parts and root exudates are the main reasons behind increase in BOD content at the end of the experiment.

Conclusions

The water hyacinth is available in Indian water bodies and where it is possible to use it in wastewater treatment by setting wastewater treatment ponds with the application of this ecofriendly technique. From the present study, the floating hygrophyte *E. crassipes* based treatment has proved as a promising tool for the treatment of cadmium containing effluents especially below 25 ppm. It is clear that the growth characteristics of Eichhornia *crassipes* were more sensitive and severely affected at higher concentrations of Cadmium in the water medium at the end of experiment and might be due to higher uptake of Cd^{++}. Decreased levels of chlorophyll and carotenoid might be related with the cadmium as a metal stress indicator and describes about the tolerance status of the species. The increased levels of proline in the leaves might be to maintain osmoregulation and face the metal stress. Cadmium chloride might induce accumulation of secondary metabolites in the experimental species in order to tolerate higher levels of toxicity stress and adverse conditions aroused. The increase in the activity of peroxidase under $CdCl_2$ stress may be regarded as an inhibition of stimulated secondary metabolism at higher levels. We found that the 25 ppm concentration is the limiting concentration and later concentrations are harmful for the growth, development and contributes only to toxicity in the plant though higher accumulation is observed in dry tissues. The varying levels of TDS in the cadmium containing water were obviously due to the external additions of $CdCl_2$. It is definite that the increase in turbidity might be due to the external addition of salt and also due to the root exudates released to face the stress condition. The degrading plant parts and root exudates could also be the main reasons behind increase in BOD. Bioconcentration factor showed a clear conclusion and supportive proof about the species as a tool for phytoremediation of cadmium containing wastewaters. We also suggest the need to understand processes that affect metal availability, metal uptake and translocation in E. crassipes. Detail investigations and understandings of various aquatic species, accumulation of different metals under influence of various effluents should be studied with respect to physiological changes associated with the plants and their ability to treat the industrial and domestic wastewaters.

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PLANT SCIENCE

Azorella trifurcata and *Mulinum echegarayii* obtained from central region of Argentina: antibacterial activity of their organic extracts

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Abstract

The natural products derived from medicinal plants have proven to be an abundant source of compounds with antibacterial activity. The antibacterial activity of extracts of *Azorella trifurcata* and *M. echegarayii* was evaluated against strains of Staphylococcus aureus ATCC 43300, Pseudomonas aeruginosa ATCC 27853, *Listeria monocytogenes* CLIP 74902 and *Escherichia coli* ATCC 35218. Organic extracts were prepared using n-hexane, mixtures of n-hexane and ethyl acetate of increasing polarity and a mixture of ethyl acetate and methanol on flash chromatography. All the extracts of *A. trifurcata* showed antibacterial effects against gram-positive bacteria (CIM between 0.5 and 2 mg/ml). Four extracts (100% n-hexane, 40:60/50:50 acetate: n-hexane, 70:30 ethyl acetate: n-hexane and 2:98 methanol: ethyl acetate) of *A. trifurcata* showed antibacterial activity against gram- negative bacteria. *M. echegarayii* 2:98 methanol: ethyl acetate was active against all gram-negative and gram-positive bacteria (CIM between 1 and 2 mg/ml). The values of MBC of the extracts assayed were one or two times higher than corresponding MIC values. The discovery of organics extracts with antibacterial properties could contribute to the treatment of bacterial infections.

Key words: Antibacterial activity, *A. trifurcata*, *M. echegarayii*, Organic extracts

Introduction

In recent decades has increased significantly the development of microbial resistance to conventional antimicrobials and the study of antibacterial properties of plant extracts and natural phytochemicals continues offering a large number of therapeutic options. Latin American countries have a rich tradition in the use of medicinal plants in folk medicine. The use of these plants is very important in primary health care in rural and semi-rural areas. They are used in many circumstances in which the disease is considered as a loss of physical, emotional and spiritual conditions (Estomba et al., 2006; Ladio et al., 2008; Lozada et al., 2006; Eyssartier et al., 2009). *Azorella trifurcata* (Gaertn.) Pers (Apiaceae) is a plant known by the vernacular names "Yareta". This species is used in folk medicine as antitussive and expectorant, to treat asthma, colds and bronchitis, as well as antiseptic, antiparasitic, antirheumatic and hypoglycaemic (Padín,1999; Delporte Vergara et al., 2003; Fuentes et al., 2005).

Mulinum echegarayii (Hieron) is endemic specie to the high Andes of Mendoza and San Juan, western Argentina, where he lived between 2,500 and 3,000 m above sea and has no trivial names. There are no previous data in the literature on this species and are not known references in the literature on its use in folk medicine. Both species belongs to the family Apiaceae or also Umbeliferae called. Species of this family synthesize secondary metabolites as natural diterpenes, which have carbon skeletons and azorellane, mulinane, madreporane and yaretane types (Martinez, 1999; Loyola et al., 2002; Chiaramello et al., 2003a). The purpose of the study presented here, was to evaluate *in vitro* the antibacterial activity of organic extracts of *Azorella trifurcata* and *Mulinum echegarayii.*

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Materials and Methods

Plant material

Azorella trifurcata and *Mulinum echegarayii* Hieron were collected in Malargüe, Department, Mendoza, Argentina. A specimen of both species was available in the Herbarium of the National University of San Luis, Voucher N° 9247 (Figure 1) and N° 494 (Figure 2) respectively.

Figure 1. Aerial parts of Azorella *trifurcata* specimen collected in Mendoza, Argentina.

Figure 2. Aerial parts of *Mulinum echegarayii* specimen collected in Mendoza, Argentina.

Preparation of extracts

A. trifurcata (6.950 kg) and *M. echegarayii* (2.100 kg) were processed separately. The aerial parts of plant previously dried at ambient temperature and finely ground in mill blades, were macerated with cold acetone for 48 hours. Acetone extract was separated by filtration. This procedure was repeated three times. The combined extraction liquids were concentrated under reduced pressure yielding, 86g and 270g respectively, of syrupy material. Those acetone extracts was dissolved in the same solvent and adsorbed on 400g of silica gel 60 G. after evaporation of the solvent proceeded to prepare chromatography "flash" column using n-hexane (HEX) as eluents and mixtures of ethyl acetate (EtOAc) - HEX increasing polarity until reaching 100% EtOAc. The progress of the separation was monitored by thin layer chromatography (TLC), using as mobile phase benzene: dioxane: acetic acid (AcOH) (120:20:4) as developer and a mixture of H_2SO4: AcOH: H_2O (4:20:1) or anisaldehyde: H_2SO_4: ethanol: H_2O (1:20:90:90) followed by heating to 120°C.

Microorganism

A total of four bacteria were selected for this study, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74902 (Collection *Listeria* Institute Pasteur) and *Escherichia coli* ATCC 35218.

Antibacterial activity

Determination of Minimal Inhibitory Concentration (MIC)

The antibacterial activity was assayed in vitro using microplate method (microwell dilution) according to the CLSI method in tripticase soya broth (Britania, Argentina) pH 7.2 supplemented with 0.01% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) used as visual indicator of bacterial growth (CLSI, 2011). The inoculum of each strain was prepared from 24h broth culture and adjusted to concentration of 10^6CFU/ml. Organic extracts were dissolved in dimethylsulfoxide and tested in a concentration ranging from 8 to 0.25 mg/ml. The 96-well plates were prepared by dispensing into each well 95µl of nutrient broth and 5 µl of the inoculum (final concentration of 10^4 CFU/ml). One hundred microlitres aliquot from the serial dilutions of extracts was transferred into four consecutive wells. The final volume in each well was 200 µl. Controls of nutrient broth, strains and extracts were included. After 24 h incubation at 37ºC, the antibacterial activity of the extracts (MIC) was defined as the lowest concentration of the extract in the medium in which there no visible grown. The experiments were replicated at least twice.

Determination of minimal bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bactericidal effect. The presence or absence of bacterial growth was determined by visual inspection. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

Results and Discussion

In the present study, all the extracts of *A. trifurcata* showed antibacterial effects against Gram-positive pathogenic bacteria tested. Extracts

of *A. trifurcata* 100% HEX, 10:90 AcOEt:HEX, 20:80/30:70 AcOEt:HEX, 70:30 AcOEt:HEX and 100% AcOEt showed a MIC of 2 mg/ml against methicillin-resistant *S. aureus.* *A. trifurcata* 40:60/50:50 AcOEt:HEX and 2:98 MeOH:AcOEt showed activities against this bacterium at doses of 0.5 mg/ml and 1 mg/ml respectively. *L. monocytogenes,* whose isolates have been increasing in number and severity over the last years, was inhibited by all these extracts showing MIC values between 1 and 2 mg/ml. Four extracts of *A. trifurcata* tested (100% HEX, 40:60/50:50 AcOEt:HEX, 70:30 AcOEt:HEX and 2:98 MeOH:AcOEt) showed antibacterial activity against Gram- negative bacteria (CIM between 2 and 4 mg/ml) (Table 1). *A. trifurcata* 10:90 AcOEt:HEX, 20:80/30:70 AcOEt:HEX and 100% AcOEt extracts, were no active against *P. aeruginosa* and *E. coli* (Table 1).

Table 1. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of *Azorella trifurcata* extracts.

Azorella trifurcata extracts MIC/MBC (mg/ml)							
	HEX	AcOEt: HEX				AcOEt	MeOH:AcOEt
Bacterial strains	100%	10:90	20:80/30:70	40:60/50:50	70:30	100%	2:98
S. aureus ATCC 43300	2/4	2/4	2/4	0.5/1	2/4	2/4	1/2
P. aeruginosa ATCC 27853	4/ND	NA	NA	2/4	2/4	NA	2/4
L. monocytogenes CLIP 74902	1/2	1/2	1/4	1/2	2/4	2/4	1/4
E. coli ATCC 35218	4/ND	NA	NA	2/4	2/4	NA	2/4

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; HEX: n-hexano; AcOEt: ethyl acetate; MeOH: methanol; ND: no detected; NA: no activity.

Table 2. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of Mullinum echegarayii extracts.

	Mullinum echegarayii extracts MIC/MBC (mg/ml)				
	HEX	AcOEt: HEX		AcOEt	MeOH:AcOEt
Bacterial strains	100%	10:90	30:70	100%	2:98
S. aureus ATCC 43300	1/4	1/2	0.5/1	4/ND	1/2
P. aeruginosa ATCC 27853	NA	NA	NA	NA	2/4
L. monocytogenes CLIP 74902	NA	NA	4/ND	4/ND	1/4
E. coli ATCC 35218	NA	NA	NA	NA	2/4

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; HEX: n-hexano; AcOEt: ethyl acetate; MeOH: methanol; ND: no detected; NA: no activity.

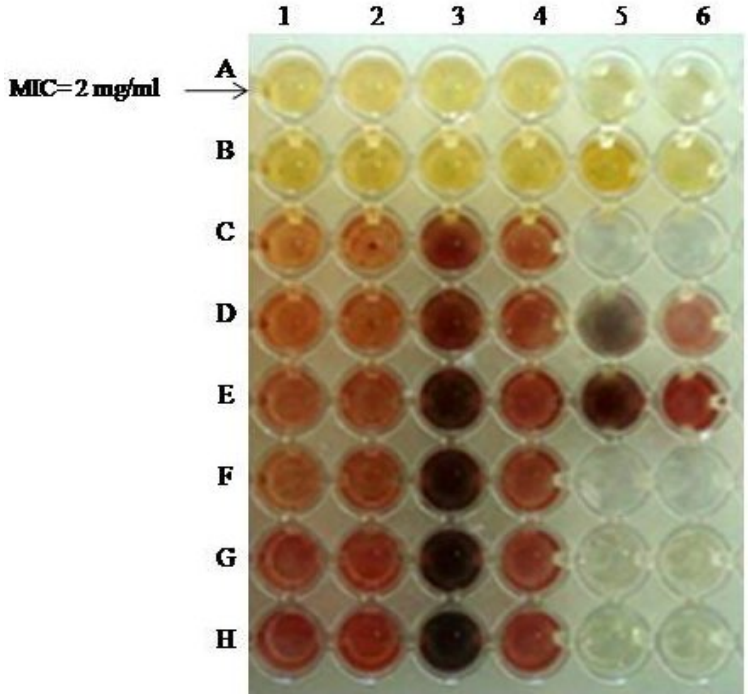

Figure 3. Microdilution plate used for broth microdilution method with *A. trifurcata* 70:30 Acetato de etilo: n-hexane extract against *S. aureus* ATCC 43300 (file 1); *L. monocytogenes* CLIP 74902 (file 2), *P. aeruginosa* ATCC 27853 (file 3); *E. coli* ATCC 35218 (file 4). File 5-6 (A-B): extracts controls; File 5-6 (D-E): controls strains.

An interesting finding was that both extracts of *A. trifurcata* and *M. echegarayii* 2:98 MeOH:AcOEt were active against all Gram-negative and Gram-positive bacteria tested. On the other hand, *S. aureus* was inhibited by all extracts of *M. echegarayii* with MICs between 0.5 and 4 mg/ml (Table 2). Methicillin-resistant *S. aureus* was sensitive to all *A. trifurcata* and *M. echegarayii* extracts. It is important to remember that infections caused by these bacteria have reached epidemic proportions, and therapeutic options are limited because these strains are often multidrug resistant (Chua et al., 2011). All extracts studied showed better antibacterial activity against Gram-positive bacteria, in comparison with Gram-negative bacteria. Several authors have shown that Gram-positive bacteria are more susceptible to the plant extracts that Gram- negative bacteria (Bele A.A. et al., 2009). Therefore it is theorized that Gram-positive bacteria are more susceptible than Gram-negative bacteria due to the differences in their cell wall structure. Gram-negative organisms are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances, including antibiotics (Lewis, K., 2001).

The Figure 3 shows the MIC obtained from *A. trifurcata* 70:30 AcOEt/HEX extract against the tested bacteria. The values of MBC of the extracts assayed were one or two times higher than corresponding MIC values. The MBC was not active at the highest concentration tested for extracts of both species whose MIC was 4 mg/ml. Table 1 and 2. An interesting group of bioactive metabolites, such as triterpenoids and diterpenoids with azorellane and mulinane skeletons have been obtained from *Azorella*, and *Mulinum* genera (Borquez et al.,2011; Chiaramello et al., 2003a; Colloca et al.,2004; Molina-Salinas et al.,2010; Morales et al., 2003; Areche et al., 2009). So, some authors demonstrated that these metabolites display a wide variety of biological activities, including antimycobacterial activity (Molina-Salinas et al., 2010), trichomonicidal activity (Loyola et al., 2001), trypanocidal activity (Araya et al., 2003) and antiplasmodial activity (Loyola et al., 2004). To our knowledge, there are few reports available in the literature on activity of organic extracts of *M. echegarayii* and *Azorella trifurcata* against pathogenic bacteria tested in this study. Chiaramello et al. (2003b) isolated from both plant species, secondary metabolites mulinane and azorellane-type such as azorellolide and mulinénico acid from *A. trifurcata* and 17-acetoximulinic acid, 14-α-hidroximulinolic acid, azorellolide and sphatulenol from *M. echegarayii* (Chiaramelo et al., 2003b; Chiaramello et al., 2007a; Chiaramello et al., 2007b). Some authors reported that diterpenoid acids isolated from *Azorella compact* showed inhibitory activity against methicillin-resistant *S.aureus,* methicillin-susceptible *S.aureus*, *Enterococcus faecium,* and *E.coli* (Wächer et al., 1999). Therefore, it could be possible to attribute the antibacterial activity of *A. trifurcata* and *M. echegarayii* organic extracts, partly or completely to the presence of diterpenoid acids as previously demonstrated in both plant species used in this study (Chiaramelo et al., 2003b; Chiaramello et al., 2007a).

Conclusions

There is considerable evidence that the organic extracts of the plants tested could be used in therapies preventative or treatment of bacterial infections.

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PLANT SCIENCE

Nutritional composition of jujube fruit

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Abstract

The jujube, also known as ziziphus, is grown and cultivated in India, Russia southern Europe, China and the Middle East. The fruit has been used in Chinese medicine for over 4,000 years. In India, the jujube is grown for just a few niche markets. Jujube contains potassium, phosphorus, manganese and calcium as the major minerals. There are also high amounts of sodium, zinc, iron and copper. Jujube also contains vitamin C, riboflavin and thiamine. The vitamin and mineral content of the fruit helps to support cardiovascular health and enhance metabolism. This brief review provides information on nutritional composition, changes in nutritional components with maturity and its maturity indices of Indian (*Ziziphus mauritiana* Lamk.) and Chinese (*Zizyphus jujuba* Mill.) jujube.

Key words: Chinese jujube, Indian jujube, Ber, Nutritional composition, Maturity indices

Introduction

Indian jujube (*Ziziphus mauritiana* Lamk.) is also known as ber, desert apple or Indian plum. It belongs to family Rhamnaceae. It is a tropical/subtropical fruit native to the northern hemisphere (Lyrene, 1979). The genus *Ziziphus* has 135 to 170 species (Islam and Simmons, 2006), of which 17 are native of India (Watt, 1883; Bailey, 1947; Singh et al., 2000). *Z. mauritiana* is cultivable ber in drier parts of the Indian subcontinent (Sebastian and Bhandari, 1990). Ber is also cultivated on marginal lands in some African countries (Johnston, 1972). The *Ziziphus* species are distributed throughout the tropical, subtropical and temperate regions of both the hemispheres (Rendle, 1959). Small or large plantations of ber exist in Afghanistan, Iran, Syria, Burma, Australia (Nijjar, 1975), France (Evreinoff, 1949; Munier, 1973), the United States of America (Thomas, 1924; Lanham, 1926; Riley, 1970) and the Russia (Mukherjee, 1967).

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Figure 1. External and internal of Indian jujube fruit.

Ber fruit is a drupe, globose to ovoid in shape, up to 6 x 4 cm in size; skin smooth or rough, glossy, thin but tough, yellowish to reddish or blackish; flesh white, crisp, and juicy, sub acid to sweet, becoming mealy in fully ripe fruit (Figure 1, 2). Irregular furrowed stones are found in tuberculate seed which contains 6 mm long brown kernels of elliptic shape. Ber fruit is generally eaten fresh and is a rich source of ascorbic acid, essential minerals and carbohydrates (Pareek, 1983; Abbas et al., 1988; Pareek et al., 2002). Colour of fruit is changed from green to yellow to chocolate brown with the maturity and ripening (Figure 3).

Jujube (*Zizyphus jujuba* Mill.) is cultivated from ancient time in China and reported that

cultivated for last 5000 years. Chinese jujube is found in subtropics of Asia and largely in China. Sixty lakh quintals of fruits were produced from 30,000 ha area in 2009 in China (Cui, 2009). China alone contributed 90% of world production of jujube (Li et al., 2005; Su and Liu, 2005). 'Chinese Winter Jujube', a new jujube cultivar, is known as "the king of jujube in China". This cultivar characterized with thin peel, crisp flesh and rich in nutritional components (Sun et al., 2007). Jujube fruit contains flavonoids, vitamins, amino acids, organic acids, polysaccharides, and microelements (Li et al., 2007) and found useful in spleen diseases and nourishment of blood in Chinese system of medicine (Shen et al., 2009).

Nutritional composition

Ber fruit have a high nutritive value, being a rich source of vitamin C, A, and B complex, and also of Ca, K, Br, Rb, and La (Tiwari and Banafar, 1995). In general, the fruit contain 81-83% moisture**,** 17.0% carbohydrates, 0.8% protein, 0.07% fats, 0.76-1.8% iron, 0.03% each of calcium and phosphorus, 0.02 mg/100g carotene and thiamine, 0.020-0.038 mg/100g riboflavin, 0.7-0.9 mg/100g niacin, 0.2-1.1 mg/100g citric acid, 65-76 mg/100g ascorbic acid, about 22 g/100g sugar, about 1.3 g/100g fiber, about 0.2 g/100g fat with a calorific value of 104/100g (Morton, 1987). Galactose, fructose and glucose are the major sugars found in ber fruit (Muchuweti et al., 2005). p-Hydroxybenzoic, caffeic, ferulic and p-coumaric acids are the most abundant phenolic compounds in ber with concentrations of about 366, 31, 20 and 19 mg/kg dry mass, respectively, whereas vanillic acid is the least abundant with a concentration of about 2.5 mg/kg. Guil-Guerrero et al. (2004) analyzed several ber varieties from Spain for fatty acid and carotene contents. Tryglycerides having medium chain fatty acids were most abubdant in all samples. The main fatty acids were 12:0, 10:0, 18.2n6, 16:1n7, 16:0, and 18:1n9 in total saponifiable oil. On an average 1.3 g/100g saponifiable oil was found in fruit on a dry weight basis. In comparison to other fruits, carotenes were found in appreciable amount (4 to 6 mg/100g on a dry weight basis). Citric, malonic and malic acids were identified as major organic acids in ber (Muchuweti et al., 2005). Pareek (1983) recorded 81-97% pulp in fresh, mature fruit and Jawanda et al. (1980a, b) reported a range of 91-93%. General nutritive composition of ber fruit is given in Table 1 (Morton, 1987; Pareek and Dhaka, 2008; Pareek et al., 2009).

Table 1. Nutritional composition of Indian jujube fruit (on fresh weight basis).

Constituents	Amount (per 100 g)
Moisture (g)	81.6-83.0
Protein (g)	0.8
Fat (g)	0.07
Fiber (g)	0.60
Carbohydrates (g)	17.0
Total Sugars (g)	5.4-10.5
Reducing Sugars (g)	1.4-6.2
Non-Reducing Sugars (g)	3.2-8.0
Ash (g)	0.3-0.59
Calcium (mg)	25.6
Phosphorus (mg)	26.8
Iron (mg)	0.76-1.8
Carotene (mg)	0.021
Thiamine (mg)	0.02-0.024
Riboflavin (mg)	0.02-0.038
Niacin (mg)	0.7-0.873
Citric Acid (mg)	0.2-1.1
Ascorbic acid (mg)	65.8-76.0
Fluoride (ppm)	0.1-0.2
Pectin (% dry basis)	2.2-3.4

Source: (Morton, 1987; Pareek and Dhaka, 2008; Pareek et al., 2009)

Li et al. (2007) provided the proximate composition of five cultivars of Chinese jujube. Total phenols, minerals and vitamins were also determined for fruits of 'Jinsixiaozao', 'Yazao', 'Jianzao', 'Junzao', 'Sanbianhong' cultivars. Significant variations were recorded for moisture (17.38-22.52%), carbohydrates (80.86–85.63%), proteins (4.75–6.86%), lipids (0.37–1.02%), soluble (0.57–2.79%) and insoluble (5.24–7.18%) fibres, reducing sugar (57.61–77.93%) and ash (2.26–3.01%). Fructose and glucose were identified as major soluble sugar in all the five cultivars, while rhamnose, sorbitol and sucrose contribute in lesser amount. Among the minerals K, P, Ca and Mn contributed major portion, while Fe, Na, Zn and Cu were also found in good amount. Chinese jujube is rich in ascorbic acid and it ranged from 192 – 359 mg/100g among the cultivars studied. Thiamine and riboflavin contents were 0.04–0.08 and 0.05–0.09 mg/100g, respectively. Total phenolic contents ranged from 5.18 to 8.53 mg/g (Table 2). In some cultivars of Spain triglycerides of medium chain fatty acids were present in abundant quantity. On total saponifiable oil basis 12:0, 10:0, 18:2n6, 16:1n7, 16:0, and 18:1n9 were identified as major fatty acids. On dry weight basis fruits contain 1.33 +/- 0.17 g/100 g saponifiable oil. Carotenes in these Spanish cultivars vary from 4.12 to 5.98 mg/100g on a dry weight basis. Vitamin A value was found in medium range and it was 38 µg RE/100g on a fresh weight basis (Guil-Guerrero et al., 2004).

Correlation studies suggest that there were no correlation found between antioxidants and phenolics or with ascorbic acid in Chinese jujube (Li et al., 2007).

Pareek (2001) gave details of the composition of Chinese jujube pulp. They include 9.6 – 33% sugar, 0.3-2.5% acids (Ahmedov and Halmatov, 1969), 2.9% protein and 136-363 mg/100g of vitamin C (Tasmatov, 1963; Baratov et al., 1975; Ristevski et al., 1982; Ciressa et al., 1984). The average dried *Z. jujuba* sugar content is 50.3 – 86.9 g/kg, while the protein content is 3.3 – 4.0 g/kg, and fat content is 0.2 – 0.4 g/kg. Chinese jujube also contains 18 kinds of amino acids, including eight essential amino acids, and is rich in vitamins and minerals (Annon., 1989). *Z. jujube* fruits are very rich in vitamin C, thiamin and riboflavin (Troyan and Kruglyakov, 1972; Kuliev and Guseinova, 1974). Consumption of one ber fruit in a day would meet the diet requirements for vitamin C and vitamin B complex of an adult man recommended by WHO. It is also known to have high vitamin P (bioflavonoid) content.

Fruit growth and development

Jujube fruit showed double sigmoid growth (Bal and Singh, 1978; Abbas et al., 1994; Abbas and Fandi, 2002). On the basis of diameter and weight, it has been confirmed that jujube fruit having double sigmoid growth from 40 to 88 days after petal fall (Lu et al., 2012). Cultivar differences were found for days taken to maturity e.g., 'Changhong' Jujube fruit matures in 80 days (Lu et al., 2011), whereas 'Zaytoni' took 124 days (Abbas and Fandi, 2002). Due to the rapid cell elongation and cell division (Bollard, 1970) as well as high levels of growth hormons particularly auxins, gibberellins, and cytokinins (Fandi, 1999) the first rapid growth was observed between 40 to 48 days after petal fall (Lu et al., 2012). A lag phase was observed after first rapid growth. The lag phase period was also different for different cultivars and it was more than two weeks in Indian jujube (Bal and Singh, 1978; Abbas et al., 1994) and less than two weeks in 'Zaytoni' (Abbas and Fandi, 2002) and 'Changhong' (Lu et al., 2012) jujube fruit. The occurrence of a short lag phase of growth resulting in early maturity. The fruit then entered a second period of rapid growth. This stage of fruit growth is mainly due to cell enlargement and is associated with high levels of gibberellins, whereas cytokinins are no longer detectable (Fandi, 1999).

Figure 2. Fruits of Indian jujube cultivars.

Table 2. Nutritional composition of five cultivars of Chinese jujube (on dry weight basis).

Component	Cultivars				
	Jinsixiaozao	Yazao	Jianzao	Junzao	Sanbianhong
Carbohydrate (%)	81.62	80.86	84.85	82.17	85.63
Reducing sugar (%)	57.61	60.24	77.93	58.73	67.32
Fructose (%)	19.1	18.6	42.9	25.1	22.8
Glucose (%)	22.5	26.3	19.2	19.6	27.2
Rhamnose (%)	12.2	12.5	14.1	10.5	14.7
Sorbitol (%)	2.1	0.3	1.9	2.6	3.5
Sucrose (%)	14.1	11.5	0.21	17.4	11.3
Soluble fiber (%)	2.79	1.46	1.51	1.07	0.57
Insoluble fiber (%)	6.11	7.18	5.24	5.83	5.56
Lipid (%)	0.37	1.02	0.39	0.71	0.65
Protein (%)	5.01	6.86	4.75	6.43	6.60
Moisture (%)	18.99	20.98	17.38	21.09	22.52
Ash (%)	2.26	2.78	2.41	3.01	2.56
Potassium (mg/100g)	79.2	458	375	201	344
Phosphorus (mg/100g)	110	59.3	72.3	105	79.7
Calcium (mg/100g)	65.2	91.0	45.6	118	76.9
Manganese (mg/100g)	39.7	36.5	51.2	24.6	42.1
Iron (mg/100g)	4.68	6.93	6.42	7.90	6.01
Sodium (mg/100g)	6.34	7.61	6.21	5.96	3.22
Zinc (mg/100g)	0.55	0.63	0.47	0.42	0.35
Copper (mg/100g)	0.26	0.27	0.42	0.31	0.19
Thiamine (mg/100g)	0.05	0.04	0.09	0.06	0.05
Riboflavin (mg/100g)	0.07	0.07	0.05	0.09	0.05
Vitamin C (mg/100g)	359	192	203	296	315
Phenols (GAE mg/g)	7.42	8.53	8.36	7.01	5.18
Antioxidant capacity (FRAP µmol/g)	1173	1025	794	563	342

Source: (Li et al., 2007)

Figure 3. Changes in color with maturity and ripening of Indian jujube fruits.

Changes in nutritional composition during growth and maturation

Ascorbic acid and phenolics

The ascorbic acid content of ber fruits was initially low, and continued to increase till the fruit reached physiological maturity (Abbas, 1997). The increase in ascorbic acid with the advancement of ripening was noticed in ber fruit and reached peak value i.e., 559 mg/100g on 15th day of storage (Kader et al., 1984). Bal et al. (1995) also noted the increase in vitamin C content as the maturity advanced in 'Umran' ber fruits. Highest content of ascorbic acid was observed at 56 days after petal fall and after this period it continuously decreased up to maturity (Lu et al., 2012). A significant difference in ascorbic acid was reported by various workers. Comparatively lesser amount (250-600 mg/g fresh weight) was reported by Wu et al. (2010) whereas it was as high as 721 mg/g fresh weight at 88 days after petal fall (Lu et al., 2012). Total phenols increased from 40 to 48 days after petal fall, decreased from 48 to 56 days after petal fall, again increased between 56 and 64 days after petal fall and decreased steadily after 64 days to maturity (Lu et al., 2012).

Sugars

Reducing sugars were increased from 40 days to 72 days after petal fall and then decreased until maturity and ripening (Lu et al., 2012). However, in some Indian jujube cultivars ('Zaytoni', 'Umran', 'Sanaur', and 'Kaithli') reverse trend was reported (Bal and Singh, 1978; Bal et al., 1979; Jawanda and Bal, 1980; Abbas and Fandi, 2002). Reducing sugars tended to accumulate over most of the growth period in jujube fruit cvs. 'Bambawi' and 'Mallacy' (Abbas et al., 1994). Soluble sugars content continuously increased throughout growth and development of fruit and highest increase was noticed between 40 and 48 days and 80 to 88 days after petal fall. The soluble sugars content in ripe jujube fruit was 121.58 mg/g (Lu et al., 2012) and Indian jujube was in the range 85–145 mg/g (Teotia et al., 1974). It was found as low as 58-79 mg/g in 'Mallacy' and 'Bambawi' (*Zizyphus spina-christi* L. Willd) jujube fruit (Abbas et al., 1994) and as high as 179 mg/g was found in 'Zaytoni' jujube fruits (Abbas and Fandi, 2002). Reducing and non-reducing sugars increased up to maturity (Bal and Man, 1978; Bal et al., 1979; Jawanda and Bal, 1980). Total sugars increased gradually up to certain period of growth and then increased rapidly (Bal and Singh, 1978; Bal and Man, 1978; Jawanda and Bal, 1980; Gupta et al., 1984; Bhatia and Gupta, 1985; Pandey et al., 1990; Kadam et al., 1993). In 'Umran' ber, sucrose and fructose continued to increase whereas glucose decreased slightly with advancement of ripening. The stages of harvest had a significant effect on total sugars (Kudachikar et al., 2000). Delaying the picking of fruits to later maturity stages resulted in higher sugars after ripening (Bal and Chauhan, 1981; Bal, 1986). While working on four Indian jujube cultivars ('Gaolangyihao', 'Xinshiji', 'Mizao', 'Miandianchangguo') in China, Ling et al. (2008) reported that the soluble sugar mainly consisted of sucrose, glucose and fructose. The rate of increase in sucrose accumulation was highest during mid-late growth period to ripening in all cultivars studied, however the rate of sucrose accumulation in 'Gaolangyihao' was faster than that of the other three cultivars. The cultivar difference was found in glucose and fructose content of total sugars and fructose was significantly higher than that of the glucose in 'Mizao' and 'Miandianchangguo' fruit while fructose and glucose was almost equal in'Gaolangyihao' and 'Xinshiji' (Ling et al., 2008).

Carotenoids, pH and phenols

In 'Changhong' jujube fruit, carotenoid content increased from 40 to 56 days after petal fall and staying more or less unchanged until 72 days after petal fall. At the later fruit development stage, the carotenoid content rose again (Lu et al., 2012). The pH increased at early stage and decreased at middle stage of jujube fruit development, and at maturity stage, the pH increased again (Lu et al., 2012).

The total phenols decreased with the advancement of maturity (Bal and Singh, 1978; Bal, 1981; Bal et al., 1995; Al-Niami et al., 1992). This reduction in phenolics during the ripening could be due to its hydrolysis into sugars, acids or any other compounds or owing to their transformation from a soluble into an insoluble form (Singh et al., 1981). Both on tree as well as stored 'Umran' ber fruits showed decrease in total phenols with the advancement of ripening (Sharma, 1996) and tannins also decreased (Kadam et al., 1993). Total phenolics were decreased in Chinese ber after 3 days at 20ºC and again increased on 15 days of storage period (Kader et al., 1982).

Conclusions and direction for future research

Chinese jujube is grown in temperate regions while Indian jujube is cultivated in hot arid regions of India. Both the fruits are rich in nutritive value. Vitamin C content is very high in Chinese jujube

and it is fairly high in Indian jujube fruits. More than 300 varieties are available in Indian jujube but very few are in cultivation. The complete nutritional profile is not known for both these fruits. Proximate composition, fraction of sugars, vitamins, carotenoids, minerals, amino acids, volatiles etc. should be studied in both jujube fruits for particular cultivars. Nutritional changes with the advancement of growth, maturation and ripening should be measured.

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PLANT SCIENCE

Effects of electromagnetic field on the nitrogen, protein and chlorophyll content and peroxidase enzyme activity in oil palm (*Elaeis guineensis* Jacq.) leaves

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Abstract

The effects of electromagnetic fields (EMF) from 275 kV high voltage transmission line on physiological, biochemical and antioxidant system changes in oil palm leaves were investigated under field conditions. Oil palm leaves were exposed to EMF from power lines at distances of 0, 8.8, 17.6, 26.4, 35.2, 44, 52.8 and 61.6 m away from the transmission lines for six months and seven years. The results showed that EMF exposure increased the content of chlorophyll a, b and the total chlorophyll in oil palm leaves planted nearer to the power lines (at 8.8 m) and decreased it with increasing distance from the lines. The protein banding profile of the oil palm exposed to electromagnetic field of different strength for seven years were insignificantly different. Oil palm exposed to the EMF for six months had three different protein banding patterns, which were different in the banding intensities of certain protein bands. The peroxidase enzyme activity (POX) of oil palm directly below the 275 kV power line was significantly ($p \leq 0.05$) higher. However, decreasing EMF strength signifying that exposure on oil palm for duration of both seven years and six months resulted in a stress related response. The POX activity bands from the oil palm exposed to EMF for both seven years and six months respectively showed that more banding intensity and number of bands were found in those planted nearer to the power lines with higher electromagnetic strength exposure.

Key words: *Elaeis guineensis* Jacq, Electromagnetic field, Oil palm, Protein marker

Introduction

In many countries including Malaysia, thousands and hundreds of kilometres of electrical power line runs across highways and oil palm plantation lands. Electric distribution and transmission lines is the largest sources of electromagnetic field in the environment (Moon and Chung, 2000; Martinez et al., 2002). Therefore, it is inevitable for a large area like agricultural plantation to be free from electromagnetic field emitted from the power lines.

Although a number of studies have been done on the effects of electromagnetic filed on plants growth, very little is known about the specific physiological, biochemical and molecular changes that take place (Nucitelli, 1988; Karcz and Burdach, 1995; Liang et al., 2009). Oil palm *(Elaeis guineensis* Jacq.) is one of the major plantation crop grown extensively in Malaysia. With no visual differences observed in the oil palms, the biochemical analysis can provide an insight of how the plant is responding to the external stress, namely electromagnetic field.

Generally on occasions of environmental stress to plants, the present of visual symptoms signifies that the apparent damage to the plant is either irreversible or a valuable period of the vegetative growth has been lost which will be reflected in the final yield (Heimer et al., 1990; de Souza 2006; Liang et al., 2009). Therefore, biochemical studies are crucial as it provides information on the biochemical indicators of stress and plant physiology. The objective of the work is to

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determine the effects of EMF exposure from 275 KV high transmission line on physiological, biochemical and antioxidant system changes in oil palm leaves under field conditions. In an attempt to increase the understanding of the exposure of electromagnetic field on plants, this study was conducted to determine whether the exposure over a short-term period of several months and a long-term period of several years caused detrimental or encouraging biochemical changes in oil palm grown within the electrical environment of a 50 Hertz 275kV high voltage transmission line.

Materials and Methods

Plant materials

The oil palm (Elaeis guineensis var. tenera) grown in the vicinity of high voltage transmission lines were obtained from Guthrie Plantations, Sua Betong in Port Dickson, Malaysia. Six months old and seven years old oil palm grown at different distances from the power lines were exposed to different electromagnetic field strength for their duration of growth. Fully expanded leaf from the first frond of the oil palm plants were taken at 0, 8.8, 17.6, 26.4, 35.2, 44, 52.8, 61.6 m at ground level from the 275 kV high voltage transmission line for the biochemical studies. Ten leaf samples were randomly taken from each marked location at every 8.8 m from the 275 kV power line until 61.6 m away where the EMF were at ambient levels. Plants at 0 m referred to those planted directly below the line. The same leaf position was maintained for all the plants sampled to avoid variation during analysis.

Electromagnetic exposure conditions

Magnetic field (mT) reading was taken at the marked distances using the extremely low frequency field strength meter. Electromagnetic field readings were taken three times at each marked positions to ensure consistent readings and the mean readings were used to compute the final data.

Soluble protein and enzyme extraction

The harvested leaves were weighed and pulverized with liquid nitrogen before homogenizing with protein buffer using a chilled pestle and mortar at a ratio of 1:3 (g sample: mL extraction buffer). The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The pellet was discarded and crude soluble fraction was subjected to ammonium sulphate precipitation.

Assay for soluble protein and soluble nitrogen content

Soluble protein content of the leaves obtained was determined according to the Coomassie Blue dye-binding method of Bradford (1976). Soluble nitrogen content of the leaves obtained was determined according to the Cadavid and Paladini (1964).

Assay for Peroxidase enzyme activity (POX) enzyme activity

The enzyme extract used for the determination of peroxidase enzyme activity was prepared following the protein extraction method. Peroxidase activity (POX) (EC 1.11.1.7) was measured by monitoring the formation of tetraguaiacol (extinction coefficient 26.6 mM-1cm-1) from guaiacol using the method of Chance and Maehly (1955).

Total chlorophyll and carotenoid content

The total chlorophyll and carotenoid of the sampled leaves were determined according to the method of Harborne (1984).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The protein banding pattern of both six months and seven years old oil palm leaves planted at different distances from the 275 kV power lines were compared using SDS PAGE.

Enzyme activity banding pattern

Enzyme activity banding pattern were done using native polyacrylamide gel electrophoresis (PAGE). Enzyme extracts was prepared according to the soluble protein content method and 10 µg proteins were loaded onto the gel wells. The native PAGE was run at 4ºC for approximately 4 h.

Peroxidase enzyme activity bands

After the non-denaturing polyacrylamide gel electrophoresis was completed, the gels were stained for POX enzyme activity bands.

Glutamate oxaloacetate transaminase enzyme activity bands

Similarity, native electrophoresis gels were stained for glutamate oxaloacetate transaminase.

Statistical analysis

All biochemical measurements were made on triplicate samples and each experiment was repeated thrice (n=3) with independent extraction to ensure reproducibility of results. The data obtained were subjected to analysis of variance (ANOVA) of one-way and means between treatments were compared using Duncan Multiple Range Test (DMRT) at the significance level of 0.05.

Results and Discussion

Electromagnetic field (275 kV) from the seven years old oil palm plot

The electromagnetic field measured from the 275 kV transmission line in Port Dickson, Malaysia reduces with increasing distance as shown in Table

1. Electric and magnetic field range measured was from 0.0014 to 0.609 kV m^{-1} and 0.3 to 1.39 mT and this indicates that different location of the power lines emits different electromagnetic field range as many factors may affect the readings such as the electricity load or demand at the time of measurement and the location of the power line in either an urban or rural area (Kuehn, 2001).

Table 1. Electromagnetic field at different distances from the 275 kV Power Line overlooking the seven years old oil palm in Port Dickson, Malaysia.

Distance from Power Line (m)	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)
0	60.90a	1.39a
8.8	0.87b	1.15b
17.6	0.17c	0.99c
26.4	0.16c	0.81d
35.2	0.14c	0.62e
44.0	0.15c	0.41f
52.8	0.17c	0.28g
61.6	0.14c	0.30g

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Electromagnetic field from the six months old oil palm plot

At a separate site where six months old oil palm were planted at different distances from the 275 kV power line, the electromagnetic field reading obtained were different from the first plot. Higher electric field range from 0.11 to 4.54 kVm^{-1} and magnetic field range from 0.54 to 17.3 mT were obtained instead (Table 2). Although electromagnetic field from 275 kV transmission line were measured in a similar way previously, the readings obtained supported the fact that different location of the transmission line emits different range of electromagnetic field depending on the electricity load or demand. However, the inverse relationship between electromagnetic field and the distance from the source prevails where the electromagnetic field reading was found to reduce with increasing distance from the power line (Moulder, 1996).

Power line EMFs negatively and significantly influenced the soluble protein content in oil palm leaves. The palm planted nearer to power lines (0, 8.8, and 7.6 m) showed lower protein content than did those further away (26.4, 44.0, 52.8 and 61.6 m) (Table 3). The least protein content was reached for the palms grown beneath the 275 KV power line for seven years. Nemec and Meredith (1981) added that a general characteristic of plants subjected to stress is the increased level of free amino acids and reduced rates of protein synthesis which leads to decreased protein levels (Table 3).

Table 2. Electromagnetic field at different distances from the 275 kV Power Line overlooking the six months old oil palm in Port Dickson, Malaysia.

Distance from Power Line (m)	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)
0	4.54a	17.3a
8.8	0.82b	3.12b
17.6	0.52c	1.44bc
26.4	0.35cd	1.24bc
35.2	0.30cd	0.87c
44.0	0.22d	0.63c
52.8	0.14d	0.57c
61.6	0.11d	0.54c

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

The low protein content of oil palm leaves grown directly below the line may be due to the seven years exposure to strong electromagnetic field. The long-term exposure to the high electromagnetic field may have broken the peptide bonds between peptides and polypeptides, thus reducing the formation of globular or functional protein and releasing more free amino acid or nitrogen containing compounds. Similarly, Shalaby and Al-Wakeel (1995) suggest that amino acids accumulation found in stress treated plants are possibly due to the increase in protein breakdown. Although the soluble nitrogen content in the oil palm leaves grown directly below the power line were the lowest, high soluble nitrogen content in an increasing order was found at 8.8, 26.4, 35.2 and 17.6 m distance. The soluble nitrogen content was significantly decreased at 44.0, 52.8 and 61.6 m distances without statistical differences in comparison with 0 m.

Hamada and Khulaef (1995) added that at these conditions, the increase in soluble proteins was at the expense of other amino acids and vice versa. The lack of anabolic processes such as protein synthesis as seen in Table 3 leads to ammonia accumulation, which are later sequestered into nitrogen containing compounds. Rabe (1990) reported that the accumulation of free amino acids might serve as a nitrogen source compound during periods of inhibited growth, thus suggesting that the high soluble nitrogen content observed in the oil palm grown between 8.8 m to 35.2 m may be undergoing some growth disruption.

Table 3. Effects of seven years exposure to electromagnetic field at different distances from the 275 kV Power Line on the soluble protein and nitrogen content of oil palm.

Distance from Power Line	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Soluble protein (mg/g fresh weight)	Soluble nitrogen x 10^{-2} (mmole/g fresh weight)
0	60.90a	1.39a	5.5g	1.67d
8.8	0.87b	1.15b	12.2f	2.60c
17.6	0.17c	0.99c	35.9d	3.03a
26.4	0.16c	0.81d	45.2ab	2.83b
35.2	0.14c	0.62e	23.7e	2.87ab
44.0	0.15c	0.41f	40.7bc	1.77d
52.8	0.17c	0.28g	38.1cd	1.70d
61.6	0.14c	0.30g	46.1a	1.73d

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Barnett and Naylor (1966) explained that the increase in the total free amino acids and decrease in the soluble protein were due to the free amino acids which were preferentially synthesized and not readily incorporated into protein during stress period. Furthermore, the increment in soluble nitrogen content may be due to the presence of non-protein amino acids, which often increase under stress conditions. Rabe (1990) reported that the lack of feedback inhibition on nitrogen uptake and reduction during periods of reduced growth or protein synthesis causes ammonia-ammonium accumulation and preferential synthesis of specific amino-containing compounds. The preferential synthesis of amino acids normally incorporated into proteins resulted in reduced protein level and higher nitrogen-containing compounds in the cells.

Six months electromagnetic field exposure

The highest soluble protein content was obtained from the oil palm leaves planted at 17.6 m away from the line, with EMF strength of 0.52 kV m^{-1} and 1.44 mT, and 26.4 m away from the line, with EMF strength of 0.35 kV m^{-1} and 1.24 mT (Table 4). Similarity, the soluble nitrogen content was also higher at the two distances mentioned. Kordas et al. (1998) reported that intermediate strength magnetic field treatments resulted in a more pronounced effect when compared with high electromagnetic field strength treatment, which yields negligible difference.

The difference between the effects of EMF on the soluble protein and nitrogen content of the seven years and six months old oil palm may be due to the variation in the EMF strength and the duration of exposure on the crop. According to Lamattina et al. (1987), protein biosynthesis is a dynamic process that is vulnerable to changes in the environment. Protein breakdown in plant cells is important in determining developmental and adaptive phenomena where variations in degradation rates are a quick and primary strategy of cells under stress to regulate their protein mass.

Peroxidase enzyme activity (POX): Seven years electromagnetic field exposure

POX activity was significantly decreased with increasing distance from power lines, reaching the lowest values (99.61) at 61.6 m (Table 5). Oil palm grown nearer to the power line had significantly ($p \leq 0.05$) higher POX specific enzyme activity with the highest being directly under the line with 364.87×10^2 U mg^{-1} protein compared to 0.51×10^2 U mg^{-1} protein at 52.8 m away from the line. This result is correlated with those obtained for seven years in which higher protein content is reached. Thus, lower POX enzyme activities are correlated with higher protein content.

Table 4. Effects of six months exposure to electromagnetic field at different distances from the 275 kV Power Line on the soluble protein and nitrogen content of oil palm.

Distance from Power Line	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Soluble protein (mg/g fresh weight)	Soluble nitrogen x 10^{-2} (mmole/g fresh weight)
0	4.54a	17.3a	26.5d	1.10d
8.8	0.82b	3.12b	35.3c	1.57c
17.6	0.52c	1.44bc	68.0a	2.37a
26.4	0.35cd	1.24bc	65.2a	2.47a
35.2	0.30cd	0.87c	16.6e	1.77b
44.0	0.22d	0.63c	30.7d	1.67bc
52.8	0.14d	0.57c	46.5b	1.57c
61.6	0.11d	0.54c	18.0e	1.70bc

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Table 5. Effects of seven years exposure to electromagnetic field at different distances from the 275 kV Power Line on peroxidase enzyme activity of oil palm.

Distance from Power Line	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Total enzyme activity x 10 (Units/mL)	Specific enzyme activity x 10^2 (Units/mg protein)
0	60.90a	1.39a	99.61a	364.87a
8.8	0.87b	1.15b	10.52b	17.44b
17.6	0.17b	0.99c	2.99e	1.62e
26.4	0.16c	0.81d	5.24d	2.22e
35.2	0.14c	0.62e	7.84c	7.69d
44.0	0.15c	0.41f	2.96e	1.45e
52.8	0.17c	0.28g	0.98f	0.51e
61.6	0.14c	0.30g	2.96e	12.84c

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Table 6. Effects of six months exposure to electromagnetic field at different distances from the 275 kV Power Line on peroxidase enzyme activity of oil palm.

Distance from Power Line	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Total enzyme activity x 10 (Units/mL)	Specific enzyme activity x 10^2 (Units/mg protein)
0	4.54a	17.3a	208.3a	19.92a
8.8	0.82b	3.12b	58.7d	4.13e
17.6	0.52c	1.44bc	47.1e	1.72f
26.4	0.35cd	1.24bc	42.6f	16.08b
35.2	0.30cd	0.87c	70.4c	10.62c
44.0	0.22d	0.63c	49.1e	3.97e
52.8	0.14d	0.57c	77.1b	4.14e
61.6	0.11d	0.54c	35.0g	4.78d

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

The results obtained signifies that the oil palm that were grown nearest to the power line and exposed to the high electromagnetic field for a long period of seven years yielded some stress response by increasing their anti-oxidative capacity via the increase in the POX enzyme level. Although the POX enzyme activity of oil palm leaves planted at the furthest distance of 61.6 m were higher than to those at 52.8 m, it may not be due to the effects from the electromagnetic field as the readings at the furthest distance have been deemed minimal and insignificant. Table 1 showed that the difference in the EMF strength was only 0.03×10^{-2} kV m^{-1} and 0.02 mT between the two distances, 52.8 and 61.6 m.

Same trend was observed in oil palms, which were grown under the influence of electromagnetic field for six months. A clear trend is showed within 17.6 m from the line where the POX enzyme activity decreases with increasing distance from the line (Table 6). Significantly ($p\leq0.05$) higher POX enzyme activity was observed in the crop planted directly below the line at 0 m. However, no trend could be observed in the POX enzyme activity of oil palm at 26.4 and further from the line. Perhaps the effects of electromagnetic field were not significant at the said distances in the oil palm plot.

Therefore, it can be concluded from Table 7 and Figure 4 that the electromagnetic field from the 275 kV transmission line, regardless of their period of exposure on the oil palm, both six months and seven years of exposure resulted in a stress-induced proteins response from the plants. Although a decreasing trend of total peroxidase enzyme activity and specific enzyme activity alongside the decreasing electric and magnetic field couldn't be establish, drastic increase in the peroxidase enzyme level especially in those grown directly below the line was significantly ($p\leq0.05$) observed. The enzymatic response to the electromagnetic field, a non-ionizing radiation is similar to the case of ionizing radiation as was reported by Adrain (1999) who suggests that the primary damage induced by ionizing radiation is the modification of the enzymatic repair processes.

Table 7. Effects of seven years exposure to electromagnetic field at different distances from the 275 kV Power Line on the chlorophyll and carotenoid content of oil palm.

Treatments (kV/m)	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Chlorophyll a (ug/mL)	Chlorophyll b (ug/mL)	Chlorophyll a:b	Total Chlorophyll x 10^{-1} (mg/g fresh weight)	Carotenoid x 10^{-2} (mg/g fresh weight)
0	60.90a	1.39a	11.0g	8.2g	1.34c	2.89f	6.78c
8.8	0.87b	1.15b	22.0a	22.3a	0.99g	6.64a	4.97d
17.6	0.17c	0.99c	19.2c	15.0c	1.29d	5.13c	8.28ab
26.4	0.16c	0.81d	19.8b	17.8b	1.11f	5.93b	7.64bc
35.2	0.14c	0.62e	16.1f	10.6f	1.52b	3.99e	6.71c
44.0	0.15c	0.41f	10.5h	4.8h	2.17a	2.28g	7.92ab
52.8	0.17c	0.28g	17.8d	13.6e	1.31cd	4.70d	7.88ab
61.6	0.14c	0.30g	17.1e	14.2d	1.21e	4.70d	7.90a

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Total chlorophyll and carotenoid content: Seven years electromagnetic field exposure

Table 7 showed the effects of electromagnetic field from 275 kV power line on the total chlorophyll and carotenoid content of oil palm grown at different distances from the line for seven years. Although the low chlorophyll content was obtained from the oil palm leaves planted directly below the line, the total chlorophyll content was significantly ($p<0.05$) highest in those planted at 8.8 m away from the line and the chlorophyll content seemed to be decreasing with increasing distance from the line. Generally, the trend could be seen from 8.8 m up till 44 m.

The significant ($p\leq0.05$) difference in chlorophyll content at 0 and 8.8 m may be due to the drastic difference in the electromagnetic field at the two distances where 0.609 kV m^{-1} and 1.39 mT were measured at 0 m; and 0.087 kV m^{-1} and 1.15 mT were measured at 8.8 m away from the 275 kV power line. Therefore, the electromagnetic field strength at 0 m may be too high especially over a seven year exposure period for encouraging increased chlorophyll development in the oil palm leaves. On the other hand, the electromagnetic field at 8.8 m may be optimal for the increased production of chlorophyll, thus as the electromagnetic field sway away from the optimal at 8.8 m with further distance from the line, so does the chlorophyll content.

Therefore, the results from Table 7 suggest that small doses of EMF at further distance of 8.8, 17.6 and 26.4 m from the 275 kV power lines may stimulate plant growth by increasing the chlorophyll formation in leaves while higher electromagnetic field directly below the power lines may be detrimental as indicated by the high peroxidase enzyme activity reported in Table 5.

Table 8. Effects of six months exposure to electromagnetic field at different distances from the 275 kV Power Line on the chlorophyll and carotenoid content of oil palm.

Treatments (kV/m)	Electric Field x 10^{-2} (kV/m)	Magnetic Field (mT)	Chlorophyll a (ug/mL)	Chlorophyll b(ug/mL)	Chlorophyll a:b	Total Chlorophyll x 10^{-1} (mg/g fresh weight)	Carotenoid x 10^{-2} (mg/g fresh weight)
0	4.54a	17.3a	12.6b	11.4a	1.11g	3.61a	8.81d
8.8	0.82b	3.12b	10.4c	10.0b	1.05h	3.06ab	7.12e
17.6	0.52c	1.44bc	12.5b	9.0b	1.39e	3.22ab	10.94b
26.4	0.35cd	1.24bc	11.7b	10.1b	1.17f	2.95b	10.37bc
35.2	0.30cd	0.87c	8.6d	5.3f	1.64c	2.10c	7.02e
44.0	0.22d	0.63c	14.2a	7.5de	1.90a	2.06c	9.48cd
52.8	0.14d	0.57c	12.0b	7.9cd	1.52d	2.98b	13.01a
61.6	0.11d	0.54c	11.9b	6.4ef	1.87b	2.74b	10.38bc

Means with the same letter in columns do not show significant differences according to DMRT (p=0.05).

Similarly to the trend observed on increasing chlorophyll content with increasing electromagnetic field in Table 7 from 8.8 to 44 m. Table 8 showed that the highest chlorophyll content was obtained from the oil palm grown at 0 m from the power line and the total chlorophyll content decreases with increasing distance from the line up till 44 m. Perhaps the total chlorophyll content in oil palm leaves at 0 m were not reduced as in Table 7 because the exposure period in Table 8 were only six months compared to the former with seven years.

However, in comparison with the EMF strength of 0.609 kV m^{-1} and 1.39mT obtained from the 275 kV power line at the seven years old oil palm planting site, the EMF strength at the six months old oil palm planting site was much higher with 4.54 kV m^{-1} and 17.3mT. The significantly ($p \leq 0.05$) lower chlorophyll a to b ration of oil palm planted at 8.8 m away from the power line may indicate the senescing effect of electromagnetic field on the crop. Nedunchezhian and Kulandaivelu (1995) reported a decrease in the chlorophyll a to b ratio during leaf senescence due to the relatively faster decrease of chlorophyll a than chlorophyll b. Total carotenoid content was higher at further distances from the power line in which low electromagnetic field radiation is received (Table 8).

SDS PAGE protein banding pattern: Seven years electromagnetic field exposure

Figure 1 showed the effects of seven years electromagnetic field exposure at different distances from the 275 kV power line on the 12.5% SDS PAGE protein banding profile of oil palm. Generally, SDS PAGE protein banding profile from oil palm leaves yield low-resolution bands due to the high amount of fibre in the leaves, polyphenols, certain lipids and nucleic acids that interfered with polypeptide resolution as was reported by Yuffa et al. (1994) although different extraction and purification steps were tried in order to extract the most protein from the oil palm leaves.

All the protein banding profiles of the seven years old oil palm at different distances from the 275 kV power line were insignificantly different quantitatively except for the enhanced intensity in bands from 35.2 and 61.6 m. Table 3 showed that the highest protein content was obtained from the leaves of oil palm planted at 61.6 m away from the line. Therefore, the enhanced banding intensity in lane 8 may be related to the high protein content originally contained in the leaf samples since the same amount of protein were loaded into each lane of the polyacrylamide gel.

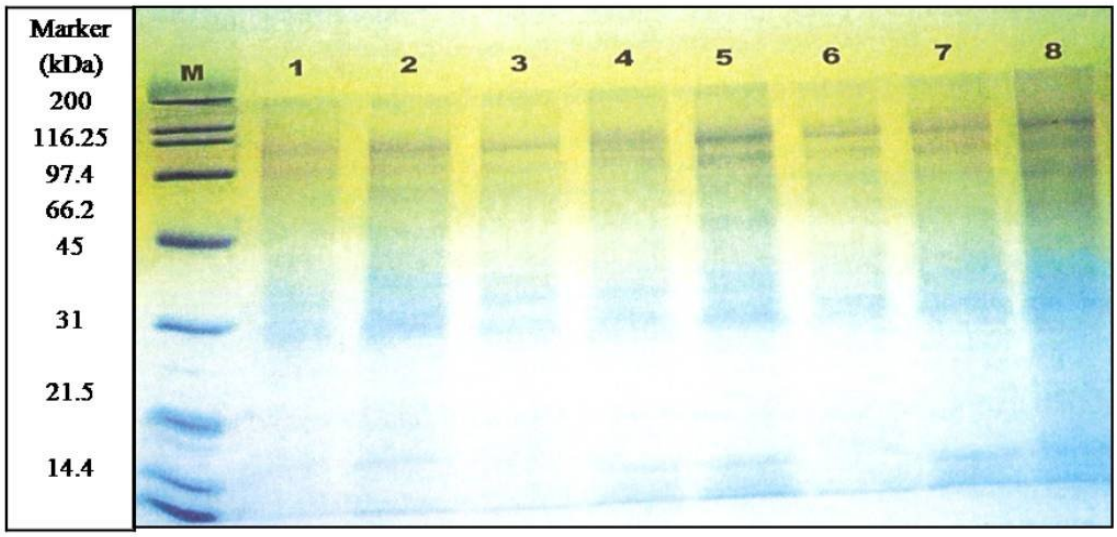


Figure 1. Effects of seven year electromagnetic field exposure at different distances from the 275 kV power line on the 12.5% SDS PAGE protein banding profile of oil palm. Lane M: Marker, Lane 1:0 m, Lane 2: 8.8 m, Lane 3: 17.6 m, Lane 4: 26.4 m, Lane 5: 35.2 m, Lane 6: 44m, Lane 7: 52.8 m, Lane 8: 61.6 m.

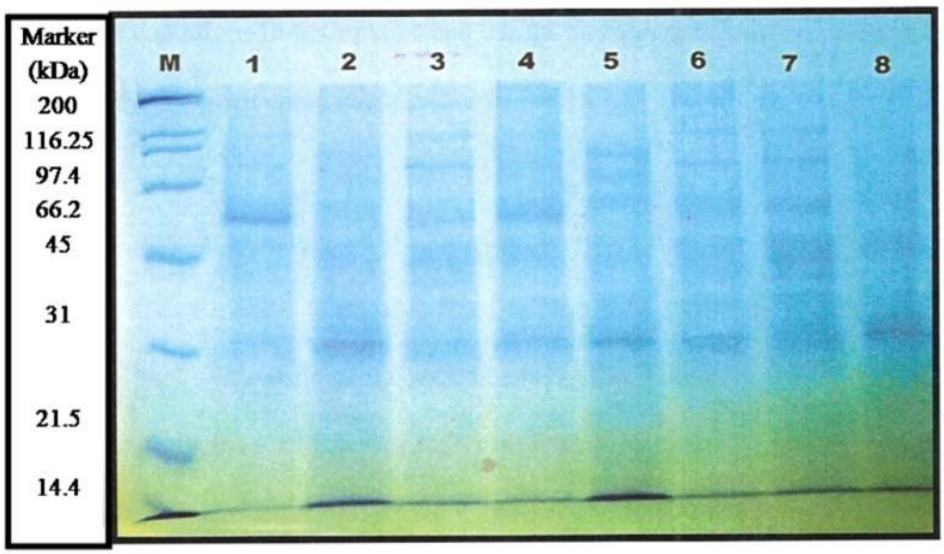


Figure 2. Effects of six months electromagnetic field exposure at different distances from the 275 kV power line on the 12.5% SDS PAGE protein banding profile of oil palm. Lane M: Marker, Lane 1:0 m, Lane 2: 8.8 m, Lane 3: 17.6 m, Lane 4: 26.4 m, Lane 5: 35.2 m, Lane 6: 44m, Lane 7: 52.8 m, Lane 8: 61.6 m.

SDS PAGE protein banding pattern: Six months electromagnetic field exposure

Figure 2 showed the effects of six months electromagnetic field exposure at different distances from the 275 kV power line on the 10% SDS PAGE protein banding profile of oil palm. Similarly, the overall resolution of bands was low due to the oxidation of high phenolic compounds in the oil palm grown at 0 m up till 61.6 m away from the 275 kV power line. At lane 1 (0 m), a significant protein band was observed at a molecular weight between 45 and 66.2 kDa. Similar protein banding pattern was also observed in lane 4 (26.4 m) except that the lane 4 had a less intense band at the molecular weight between 45 and 66.2 kDa but it had a more intense protein band at 31 kDa compared to lane 1 (0 m).

Oil palm planted 26.4 m away from the electromagnetic field source, which received and exposure of 0.35 kV m^{-1} and 1.24 mT had a prominent increase in the relative amount of at least one polypeptide species at 31 kDa. This may be due to a consequence of a highly preferential synthesis of the polypeptide in the cells. The increase in the relative abundance of a particular polypeptide band detected by SDS PAGE may be either from an increase in the rate of synthesis or from the rate of synthesis being relatively unaffected while synthesis of other polypeptides are reduced. Webster (1980) reported that the usual increase in the relative amount and synthesis of at least one specific polypeptide in stressed plants signifies a common mechanism of plants dealing with environmental stress. The preferentially synthesized polypeptides are common responses to stress conditions in both plants and animals as a gene regulation attempt in higher eukaryotic cells experiencing stress.

A different protein banding profile from the above were observed in lane 2 (8.8 m), lane 5 (35.2 m) and 8 (61.6 m). All three of the lane's protein profile had a significant protein band with a molecular weight of 31 kDa. However, clearer protein banding profile was obtained at lane 5 (35.2 m). The third type of protein banding profile was obtained at lane 3 (17.6 m), lane 6 (44 m) and lane 7 (52.8 m) with three clear bands at a molecular weight between 116.25 and 200 kDa, at 116.25 kDa and between 66.2 and 97.4 kDa. The magnitude of the increase in band intensity or number could not be related with the magnitude of the electromagnetic field strength. This indicates that the response of plant to electromagnetic field treated plants was exclusive to certain EMF strength.

Peroxidase enzyme activity bands: Seven years electromagnetic field exposure

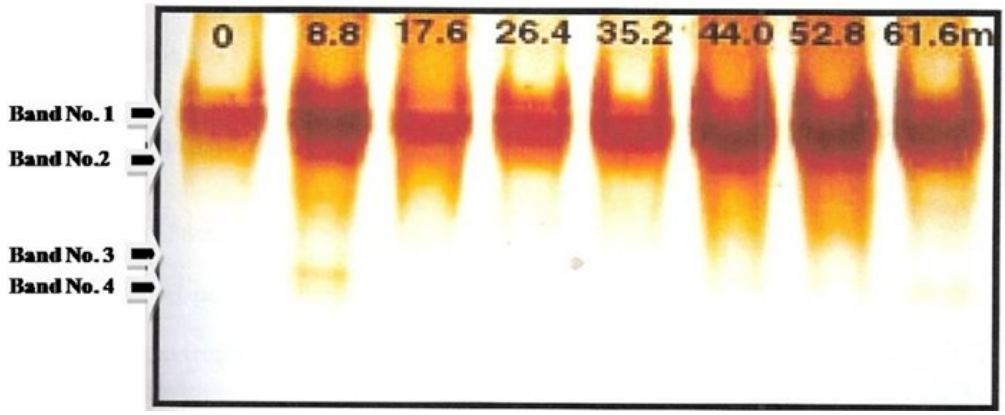
Figure 3 showed the effects of the seven years exposure to electromagnetic field on oil palm grown at different distances from the 275 kV power line. Most of the treatments had a total of two enzyme activity bands at R_f 0.08 and 0.13 except

for lane 7 (52.8 m) and lane 6 (44.0 m) which had a very faint extra enzyme activity band at R_f 0.40 while lane 8 (61.6 m) and lane 2 (8.8 m) and additional two enzyme activity bands at R_f 0.40 and 0.44 (Figure 3). The changes in peroxidase enzyme activity bands due to the exposure to EMF suggest the need for further genetic studies to determine the root cause of the changes.

The highest intensity of POX enzyme activity band at R_f 0.40 were from lane 2 (8.8 m) while the highest intensity of POX enzyme activity band at R_f 0.08 were from lane 7 (52.8 m), lane 6 (44.0 m), lane 2 (8.8 m) and lane 8 (61.6 m). The increase in the POX enzyme activity bands intensity may be related to the POX enzyme activity as Table 4 revealed that the highest POX specific enzyme activity was from the oil palm leaves grown at 0 m from the line followed by 8.8 m. Furthermore, Figure 3 showed that from 0 to 44 m away from the power line, the number of POX enzyme activity band decreases with increasing distance from the power line similarly to Table 5 which showed that the POX specific enzyme activity decreases with increasing distance from the power line, thus with decreasing electromagnetic field strength.

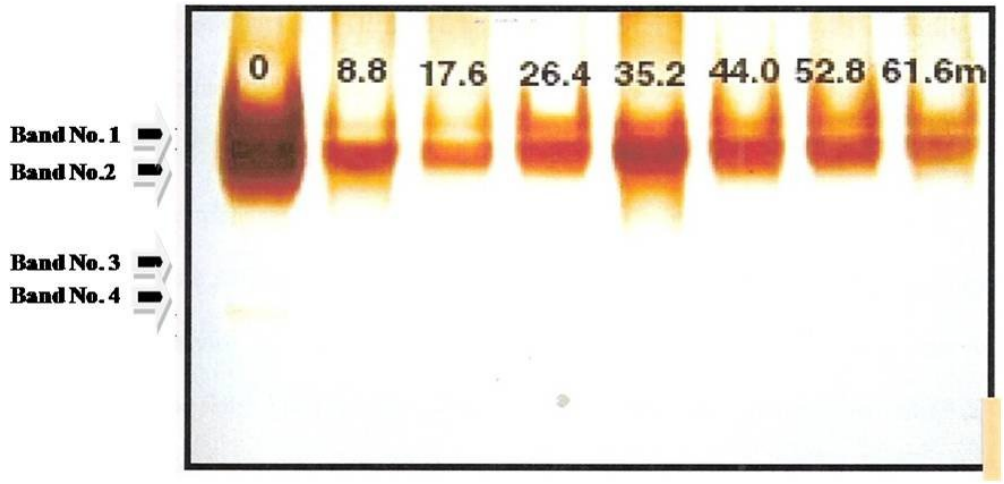
Peroxidase enzyme activity bands: Six months electromagnetic field exposure

Figure 4 showed the peroxidase enzyme activity bands and the corresponding R_f values of oil palm grown at different distances from the 275 kV power line for six months. All the treatments had a total of two enzyme activity bands at R_f 0.08 and 0.14 except for lane 1 (0 m) where the leaf samples were obtained from the oil palm planted directly below the 275 kV power line. Lane 1 (0 m) had two additional POX enzyme activity bands at R_f 0.40 and 0.44. According to Yurenkova et al. (1995), the formation of new isozymes can be due to their biosynthesis *de novo*, or by transformation of active or inactive precursors into new molecular forms. Similarly to Figure 3 on the seven years electromagnetic field exposure on oil palm directly below the line, the six years electromagnetic field exposure on oil palm directly below the line also yielded a total of four POX enzyme activity bands and the number of enzyme activity bands decreases with increasing distance from the
power line.



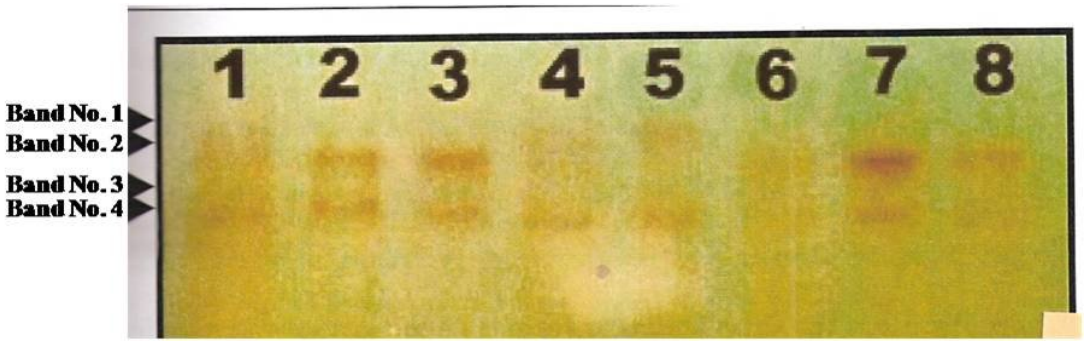
Lane	1	2	3	4	5	6	7	8
DFPL (m)	0	8.8	17.6	26.4	35.2	44.0	52.8	61.6
Band No. 1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Band No. 2	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Band No. 3	-	0.40	-	-	-	0.40	0.40	0.40
Band No. 4	-	0.44	-	-	-	-	-	0.44

Figure 3. Peroxidase (POX) enzyme activity bands on 7.5% polyacrylamide gel and the R_f values of oil palm planted at different distances from the 275 kV power line for seven years. DFPL : Distance from the power line.



Lane	1	2	3	4	5	6	7	8
DFPL (m)	0	8.8	17.6	26.4	35.2	44.0	52.8	61.6
Band No. 1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Band No. 2	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Band No. 3	0.40	-	-	-	-	-	-	-
Band No. 4	0.44	-	-	-	-	-	-	-

Figure 4. Peroxidase (POX) enzyme activity bands on 7.5% polyacrylamide gel and the R_f values of oil palm planted at different distances from the 275 kV power line for six months. DFPL: Distance from the power line.



Lane	1	2	3	4	5	6	7	8
DFPL (m)	0	8.8	17.6	26.4	35.2	44.0	52.8	61.6
Band No. 1	-	-	-	-	0.14	-	-	-
Band No. 2	-	-	-	0.16	-	0.16	-	0.16
Band No. 3	0.19	0.19	0.19	0.19	-	-	0.19	-
Band No. 4	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30

Figure 5. Glutamate oxaloacetate transminase enzyme activity bands on 10% polyacrylamide gel and the Rf values of oil palm planted at different distances from the 275 kV power line for six months. DFPL: Distance from the power line.

Therefore, the different form of the POX enzyme activity bands obtained from the oil palm planted directly under the high voltage transmission line suggests that further study is needed to determine if the changes is due to the addition in the number of polymorphic bands corresponding to

specific alleles in the peroxidase locus. Such genetic interpretation has been made several times in different plant species (Shields et al., 1983). Saker et al. (2000) reported that the reason behind the variation at the gene expression level obtained could be due to gene amplification, chromosomal irregularities, point mutation or alteration in DNA methylation. However, since only a small fraction of the bases in DNA have a coding or regulatory role, majority of mutations are silent and have no functional effect (King, 2000).

The peroxidase enzyme activity bands were most intense in lane 1 (0 m) followed by lane 5 (35.2 m). The increase in the abundance at R_f 0.14 indicates the high POX activity in the leaves of the oil palm grown under the exposure of electromagnetic field. The least intense POX enzyme activity bands were observed in a lane 3 (17.6 m). The intensity of the POX enzyme activity bands correlate with the POX specific enzyme activity in Table 5 where the highest activity was found in treatment 0 m and the least was in treatment 17.6 m.

Glutamate oxaloacetate transaminase enzyme activity bands: Six months electromagnetic field exposure

Successful glutamate oxaloacetate transaminase enzyme activity band staining was obtained in the oil palm exposed to electromagnetic field for six months. Figure 5 showed that all the treatments resulted in a total of two glutamate oxaloacetate transaminase enzyme activity bands inclusive of a common band at R_f 0.30. Lane 1 (0 m), 2 (8.8 m), 3 (17.6 m) and lane 7 (52.8 m) had two enzyme activity bands at R_f 0.19 and R_f 0.30. Lane 4 (26.4 m), 6 (44 m) and 8 (61.6 m) had a slightly smaller R_f band number one of 0.16 instead of 0.19, and lane 5 (35.2 m) had an even smaller R_f band number one with 0.14 (Figure 5). Higher intensity of the enzyme activity bands were observed in lane 7 (52.8 m), followed by lane 2 (8.8 m), lane 3 (17.6), and lane 1 (0 m). Lane 8 (61.6 m), lane 5 (35.2 m), lane 4 (26.4 m) and lane 6 (44 m) had less intense enzyme activity bands with lane 6 having the least. Although such observations could be made, no relationship could be established between the observations and EMF strength, thus, suggesting the need for a more in depth study in the future.

In the present study, the soluble protein content in oil palm planted beneath the 275 kV power line for both seven years and six months respectively were least directly below the line and increase with increasing distance when comparison were made up till 26.4 m away from the line. Similar trend was observe in both the oil palm exposed to EMF from 275 kV power line for seven years and six months, whereby the POX specific enzyme activity directly below the line was significantly ($p \leq 0.05$) higher and the enzyme activity decreases with decreasing EMF strength.

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ANIMAL SCIENCE

Performance and hematological parameters of broilers fed neem, turmeric, vitamin e and their combinations

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Abstract

An experiment was conducted to study the efficiency of inclusion of neem, turmeric, vitamin E and its combinations on performance and hematological parameters for a period of six weeks with 288 day old Raja II broiler chicks. Basal diet was supplemented with *Azadirachta indica* (Neem 8g/kg feed), Curcuma longa (Turmeric 2g/kg feed) and Vitamin E 0.2g/kg feed) individually and in combination to form eight test diets. Each test diet was fed ad libitum for 42 days. The result of the present stumdy identified no significant difference in body weight of chicks fed with supplemented diets. Whereas, addition of either neem alone or in combination with turmeric and vitamin E induced significant growth depression in birds compared to control birds. Supplementation of neem, turmeric, Vitamin E and their combinations in broiler diets did not exhibit any significant effect on feed intake and feed conversion ratio during all the weeks of age. The percentage livability of birds under different treatments was statistically non-significant. Hematological parameters in broilers revealed that the hemoglobin concentration in neem fed group was significantly ($P<0.05$) higher (10.5 g/dL) when compared to turmeric fed group (9.85 g/dL), otherwise diet containing turmeric alone recorded lowest value of hemoglobin. Feeding of neem alone and its combination with turmeric and vitamin E increases significantly PCV values as compared to control. The supplementation of turmeric or vitamin E alone to basal diet did not significantly change PCV values.

Key words: Neem, Turmeric, Humoral, Cell mediated Immunity, Broilers

Introduction

Poultry production in India has witnessed a phenomenal growth from backyard farming to scientific poultry farming involving commercial egg and meat production. In spite of numerous hurdles, there has been a perceptible rapid stride towards development and production, mainly contributed by indigenous scientific methods and advancement in the technology of poultry production (Gowda et al., 1998). Increase in the productive cost of meat is observed mainly due to the cost of broiler feed stuffs which constitute about 80 per cent of the total production cost. This has made the researchers to focus on natural feed resources which can be used as feed additives to increase feed efficiency and growth rate of broilers.

Antibiotics are mostly used at sub therapeutic level to improve the production performance of poultry birds. However, consistent use of antibiotic will not only lead to various health issues, could be a major contributors to higher feed cost. Thus, it's imperative to sort out alternatives that could effectively and economically substitute antibiotics (Toghyani et al., 2011). Emphasize on herbal use as growth promoter and preventive remedies for several infections in poultry birds could be the best possible sources under subtropical environment that could result in economic and healthy gains keeping in view their easy availability and effective use as medicinal plants in large animals and human beings. Several herbal sources have been effectively in poultry production for better gains and health chicken production (Guo et al., 2004).

Neem (*Azadirachta indica*) and turmeric (*Curcuma longa*) are herbal plants of India which

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have demonstrated to exhibit various beneficial pharmacological properties including performance and immunomodulatory effect (Ismail et al., 2004) in broilers. *Curcuma longa* is an extensively used spice, food preservative and coloring material that has biological actions and medicinal applications. The main component of turmeric is curcumin that is a a good antioxidant and an inducer of immune response in animals (Burt, 2004). Neem (*Azadirachta indica*) leaves contains (g/ kg) 938.9 ± 0.16 Dry matter, 35.3 ± 0.08 Ether Extract, 181.6 ± 1.46 Crude Protein, 113.3 ± 0.86 Crude Fiber and 130.7 ± 0.07 Ash (Esonu et al., 2005) and has been found to exert some beneficial effects in various animals (Chand, 1987).

In addition, vitamin E along with selenium has been found to be essential for the proper growth and development (Marsh et al., 1986) and for the overall immunocompetence of growing broilers. However, use of these medicinal plants or feed additives in diets of broiler chicken with the objectives of improving their performance and hematological parameters status has not been fully addressed. Hence, the present study has been proposed to evaluate the efficiency of neem, turmeric, vitamin E and their combinations on performance and hematological parameters in broilers.

Materials and Methods

Experimental birds

Two hundred and eighty eight indigenously developed Raja II colored broilers were procured from the Department of Poultry Science, Veterinary College, Bangalore. The birds were divided into 8 treatments with 3 replicates per treatment and 12 birds each replicate. Twenty four pens were made and the replicates were randomly allotted using lottery method under deep litter system and reared for 6 weeks.

Diets

Diets were formulated using maize and soybean meals. The broiler starter and finisher rations were formulated as per BIS (1992). Starter mash (12.2 MJ/kg ME, 22% CP) was fed from one to 21 days and finisher (12.2 MJ/kg ME, 20% CP) from 22 to 42 days. A practical type of broiler diet was compounded to serve itself as control (T1). Basal diet was fortified with *Azadirachta indica* (Neem) powder of 8 gm/kg feed (T2), *Curcuma longa* (turmeric) of 2 gm/kg feed (T3)and Vitamin-E of 0.2 gm/kg feed (T4) and likewise 4 experimental diets were prepared using their combination and were designated as T5, T6, T7 and T8 (Table 1).

Body weight

Individual body weights were recorded at the beginning of the experiment and further body weight were recorded at the end of each week to monitor the pattern of body weight changes. Group wise average weights under different treatments were arrived. The weighing of the birds was done in the early hours of the day before feeding.

Table 1. Experimental design.

Treatment	Experimental diets		
	Neem (%)	Turmeric (%)	Vitamin-E (%)
T1 (control)	-	-	-
T2	0.8	-	-
T3	-	0.2	-
T4	-	-	0.02
T5	0.8	0.2	-
T6	-	0.2	0.02
T7	0.8	-	0.02
T8	0.8	0.2	0.02

Feed consumption

The daily amount of the concerned diet was weighed and offered to the 12 bird replicate group. The feed consumption in each replicate was recorded weekly by subtracting the weight of residual fed from the total quantity of feed supplied during the respective week.

Feed conversion ratio

The feed conversion ratio (FCR) was determined through the relationship between amount of feed consumed (FC) to the body weight gain (BWG) under each group of birds (FCR = FC g/BWG g).

Livability

Mortality in respective group was recorded at occurrence in starter and finisher period.

Hematological parameters

Hematological parameters including hemoglobin (Hb) concentration, packed cell volume (PCV), total leukocyte count (TLC) and differential leukocyte count (DLC) was evaluated as per the procedures outlined by Benjamin (1978).

Data pertaining to various parameters obtained during the experiment was analyzed as Completely Randomized Block Design according to the methods described by Snedecor and Cochran (1994). Significance level was set at $P \leq 0.05$.

Results and Discussion

Body weight

The results of the present study indicated that addition of either neem alone or in combination with turmeric and vitamin E to broiler diets induced significant growth depression in birds as compared to birds fed with control diet (Table 2). These results are in line with the findings of Kumar and D'Mello (1995) who observed that inclusion of neem leaf meal above 0.5% significantly ($P \leq 0.05$) reduced body weight in broilers. The depression in growth upon feeding neem was attributed to the fact that neem leaf meal has imparted a bitter and unpalatability thus inhibiting feed consumption in birds resulting in poor body weight gain. Contrary to findings of present study, several workers have reported that supplementation of neem leaf meal increases body weight gain in broilers (Verma et al., 1998, Chakravarty and Prasad, 1991, Onyimonyi et al., 2009).

The body weights obtained in birds fed with diets containing turmeric, vitamin E and their combination were unisignificatly lower compared to that of birds fed with control diet. These results are not line with Al-sultan et al. (2003) and Kumari et al. (2007) who observed a significant increase in body weight of broilers fed with turmeric powder. Further, it was noticed that combination of vitamin E and turmeric had no influence on the body weight of broilers when compared with birds fed with either vitamin E or turmeric alone.

Feed conversion ratio

Effect of various treatments on FCR revealed statistically a non-significant difference between the control and different treatment groups (Table 3). The findings were in agreement with Adebiyi et al. (2011) and Mehala and Moorthy (2008) who reported better FCR values in birds fed with Vitamin E and Turmeric had no significant effect on the FCR in broilers. The results of the study revealed that birds fed with neem and its combinations had negative effect on FCR as compared to control and other treatment groups. The findings were in line with findings of Kumar and D'Mello (1995), Esonu et al. (2006) who observed no or negative effect of neem on FCR in broilers.

Table 2. Effect of neem, turmeric, vitamin E and their combination on body weight (mean± SE) in broilers.

Treatments	Week (g)					
	I	II	III	IV	V	VI
Basal diet	115 ± 2.08^{a}	259 ± 6.08^{a}	452 ± 15.3^{ab}	802 ± 13.20^{a}	1143 ± 23.20^{a}	1350 ± 32.37^{a}
Neem (0.8%)	103 ± 2.17^{bc}	231 ± 5.20^{bc}	410 ± 9.22^{bc}	694 ± 19.20^{be}	991 ± 29.00^{be}	1159 ± 28.76^{bc}
Turmeric (0.2%)	113 ± 1.97^{ad}	258 ± 4.09^{ad}	457 ± 7.50^{ae}	794 ± 13.70^{ac}	1143 ± 30.00^{ad}	1279 ± 29.86^{ab}
Vitamin E (0.02%)	112 ± 2.04^{da}	258 ± 3.82^{da}	449 ± 8.68^{ca}	781 ± 20.30^{ca}	1074 ± 21.60^{abdf}	1328 ± 26.41^{da}
Neem+ Turmeric	105 ± 2.13^{dcb}	236 ± 4.93^{cb}	430 ± 7.28^{cb}	728 ± 21.00^{abc}	1003 ± 21.60^{bcg}	1196 ± 29.31^{cb}
Turmeric + VE	104 ± 3.47^{abc}	251 ± 4.09^{abc}	450 ± 8.29^{ba}	766 ± 17.70^{bca}	1080 ± 22.80^{abc}	1279 ± 30.01^{abc}
VE + Neem	105 ± 2.05^{bcd}	240 ± 4.46^{bca}	412 ± 10.60^{be}	718 ± 13.80^{bd}	977 ± 19.80^{dcbe}	1189 ± 27.98^{bce}
Neem +Turmeric+ VE	105 ± 2.18^{cdb}	240 ± 4.83^{cab}	427 ± 9.24^{abc}	707 ± 13.20^{bde}	996 ± 24.30^{efg}	1190 ± 25.08^{cbe}

Means bearing atleast one common superscript within the column does not differ significantly ($P \leq 0.05$)

Table 3. Effect of neem, turmeric, vitamin E and their combination on feed conversion ratio (mean± SE) in broilers.

Treatment	Week					
	1	II	III	IV	V	VI
Basal diet	0.99 ± 0.02	1.59 ± 0.06	1.83 ± 0.05	1.88 ± 0.10	1.95 ± 0.13	2.38 ± 0.15
Neem (0.8%)	1.08 ± 0.08	1.75 ± 0.07	1.93 ± 0.05	2.07 ± 0.09	2.14 ± 0.12	2.57 ± 0.11
Turmeric (0.2%)	0.93 ± 0.01	1.49 ± 0.07	1.77 ± 0.06	1.86 ± 0.05	1.90 ± 0.06	2.35 ± 0.07
Vitamin E (0.02%)	0.98 ± 0.01	1.57 ± 0.03	1.86 ± 0.02	1.98 ± 0.09	2.1 ± 0.12	2.37 ± 0.08
Neem+ Turmeric	0.97 ± 0.04	1.64 ± 0.08	1.88 ± 0.05	2.03 ± 0.05	2.19 ± 0.07	2.64 ± 0.04
Turmeric + VE	1.02 ± 0.06	1.55 ± 0.04	1.75 ± 0.04	1.95 ± 0.07	2.03 ± 0.06	2.42 ± 0.09
VE + Neem	1.03 ± 0.03	1.69 ± 0.02	1.97 ± 0.09	2.10 ± 0.09	2.26 ± 0.08	2.58 ± 0.03
Neem +Turmeric+ VE	1.03 ± 0.03	1.56 ± 0.13	1.88 ± 0.14	1.98 ± 0.09	2.1 ± 0.13	2.45 ± 0.04

Livability

The results of study revealed good livability of birds among different treatment groups and was not altered by any dietary treatments. The percentage livability of birds under different treatments was statistically non-significant ranging from as low as 94.4% in Control) and by Neem+ Turmeric to as high as 100% by Neem and byNeem + Vitamin E + Turmeric. This might be due to the anti-oxidant and antimicrobial activities of neem, turmeric and vitamin E which suppress the pathogenic bacteria and enhance immune status.

Hematological parameters

The results of various hematological parameters are presented in Table 4 and 5.

Hemoglobin

The dietary inclusion of neem, vitamin E and their combination revealed significantly (P≤0.05) higher hemoglobin concentration when compared to that of turmeric added and control group. Birds fed with combination of neem, turmeric and vitamin E recorded the highest hemoglobin value (10.90g/dl) followed by neem (10.50 g/dl) and neem + vitamin E diets (10.50 g/dl). Diets containing turmeric alone recorded lowest value of hemoglobin whereas in combination with neem and vitamin E the values were higher indicating the additive effect. These findings were in agreement with Nagalakshmi et al. (1996) who opined that neem possess strong influence on the hematological traits in broilers particularly hemoglobin. Contrary to our findings, Biu et al. (2009) and Akbari et al. (2008) opined that inclusion of neem and vitamin E at higher levels in the diets of broilers significantly decreases hemoglobin concentrations.

Packed cell volume

The results of study showed a significant (P≤0.05) difference in PCV values between treatment groups and control. Feeding of neem alone) and its combination with turmeric and vitamin E significantly (P≤0.05) increased PCV values as compared to control. In contrast,the supplementation of turmeric and vitamin E alone did not significantly change PCV values. This was similar to the findings of Akbari et al. (2008) who reported that inclusion of vitamin E in the diet of broilers has no effect on PCV values. Similar results have been reported by Gowda et al. (1998) who observed a significant increase in PCV values at 10% inclusion levels. On the contrary Biu et al. (2009) reported decreased PCV values on inclusion of neem in broiler diets. The observed relative polycythemia may be due to reduction in the fluid component of the blood from insufficient fluid intake or due to slight hepatomegaly (Esonu et al., 2006). Neem leaf meal has high fibre content which may have resulted in dehydration due to insufficient water intake (Onyimonyi et al., 2009).

Total leukocyte count

The total leukocyte count observed in the present study across the entire treatment groups were within the normal range. The dietary inclusion of neem alone in the diet of broilers significantly (P≤0.05) increased TLC value as compared to that of other treatments and control. The present findings were in contrary to the findings of Esonu et al. (2006) who found significant reduction in circulatory leukocytes on feeding 10 % neem leaf meal layer diet and to Nagalakshmi et al. (1996) and Akbari et al. (2008) who reported no significant effect of inclusion of neem on total leukocyte count in broiler diet. The result on the effect of turmeric obtained in the present study were in line with the findings of Kumari et al. (2007) who reported no negative effect on circulating leukocytes in broilers. Whereas, Al-Sultan (2003) reported an increase in circulating total leukocyte count in broiler chicks fed on diet incorporated with turmeric.

Table 4. Effect of neem, turmeric, vitamin E and their combination on Hb, PCV and TLC (mean± SE) in broilers.

Treatment	Hematological parameters		
	Hb (%)	PCV (%)	TLC (x 10^3)
Basal diet	09.75 ± 0.05^{a}	31.4 ± 0.40^{a}	16.9 ± 0.10^{a}
Neem (0.8%)	10.50 ± 0.05^{bc}	34.1 ± 0.05^{bc}	17.9 ± 0.10^{bc}
Turmeric (0.2%)	09.85 ± 0.05^{ad}	32.0 ± 0.05^{ad}	17.0 ± 0.15^{ac}
Vitamin E (0.02%)	10.20 ± 0.05^{bdf}	32.4 ± 0.40^{da}	17.3 ± 0.30^{ca}
Neem+ Turmeric	10.20 ± 0.05^{dbg}	32.9 ± 0.10^{bde}	17.2 ± 0.20^{bc}
Turmeric + VE	10.40 ± 0.05^{cb}	33.5 ± 0.30^{cbf}	17.3 ± 0.15^{cb}
VE + Neem	10.50 ± 0.05^{bde}	34.9 ± 0.10^{be}	17.7 ± 0.20^{ab}
Neem +Turmeric+ VE	10.90 ± 0.10^{fg}	35.5 ± 0.20^{ef}	17.8 ± 0.10^{ba}

Means bearing atleast one common superscript column wise does not differ significantly (P<0.05)

Table 5. Effect of neem, turmeric, vitamin E and their combination on differential leukocyte count (mean± SE) in broilers.

Treatment	Differential leukocyte count			
	Heterophills	Eosinophills	Lymphocytes	Monocytes
Basal diet	57.5 ± 0.40^{a}	2.00 ± 0.02	33.90 ± 0.10^{a}	6.60 ± 0.50^{a}
Neem (0.8%)	59.0 ± 0.10^{bcfh}	2.50 ± 0.50	35.10 ± 0.20^{bc}	3.40 ± 0.80^{bce}
Turmeric (0.2%)	58.8 ± 0.10^{cde}	2.50 ± 0.50	34.80 ± 0.25^{ca}	3.90 ± 0.35^{ca}
Vitamin E (0.02%)	58.8 ± 0.30^{cbd}	2.00 ± 0.08	34.90 ± 0.15^{ab}	4.40 ± 0.10^{ba}
Neem+ Turmeric	58.9 ± 0.15^{bcdg}	3.50 ± 0.50	35.10 ± 0.05^{ac}	2.60 ± 0.70^{bcdf}
Turmeric + VE	58.2 ± 0.15^{ab}	3.0 ± 0.06	35.00 ± 0.05^{cab}	3.90 ± 0.20^{abc}
VE + Neem	59.9 ± 0.20^{fg}	2.00 ± 0.0	35.60 ± 0.45^{cb}	2.60 ± 0.65^{dbcg}
Neem +Turmeric+ VE	60.0 ± 0.05^{hg}	3.40 ± 0.4	35.90 ± 0.15^{bcd}	0.80 ± 0.50^{efg}

Means bearing atleast one common superscript column wise does not differ

Differential leukocyte count

The WBC counts obtained in the present study revealed that feeding of neem significantly ($P \leq 0.05$) increased heterophill, lymphocytes and lowered monocytic values. Whereas, addition of turmeric and vitamin E to diets had no effect on lymphocyte and monocyte counts. The percentage of heterophills increased significantly ($P \leq 0.05$) among the birds fed ration containing neem and its combination of diets with turmeric and vitamin E. The lymphocyte count in the control group differed significantly from birds fed on ration containing neem alone or in combination.,Also there was reduction in circulating monocytes among birds placed on neem and other neem based combination of diets. Results of the study are in accordance with the findings of Akbari et al. (2008) who reported a non-significant effect of vitamin E on the percentage of lymphocytes, eoisinophills and heterophills. The increased heterophills and lymphocyte values observed in the present study due to feeding of neem confirm the finding of Esonu et al. (2006).

Conclusion:

Inclusion of neem, turmeric, Vitamin E and their combinations in diet of broilers has been found to be effective in terms of improving performance and hematological parameter. However, further studies are essential to assess the impact of these additives as immunomodulatory agents in broiler diets before drawing final conclusion.

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ANIMAL SCIENCE

Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (bekasam) and their antimicrobial activity against pathogenic bacteria

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Abstract

Bekasam is an Indonesian fermented fish product that tastes sour and mostly contains lactic acid bacteria (LAB). The aim of this study is to obtain and characterize LAB isolates from bekasam and to study their potency in inhibiting the growth of pathogenic bacteria, i.e. Escherichia coli, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes*. LAB were isolated from bekasam using media of MRSA supplemented with $CaCO_3$ 0.5%. Incubation was at 37°C for 48 hours. The pure cultures were verified as LAB based on morphological and biochemical characteristics. Eight bekasam samples showed that the total average of LAB were 1.4 x 10^8 to 9.0 x 10^8 CFU/mL. Seventy four isolates were successfully isolated. It was found that 62 isolates (84%) belonged to LAB, and 23 isolates of them could inhibit the growth of the five pathogenic bacteria. The highest inhibition zone was accounted for S. aureus. However, neutralized supernatant of the LAB culture did not inhibit the growth of the pathogenic bacteria. These results indicate that the LAB inhibition to the pathogenic bacteria was due to the organic acid, and that perhaps the main factor in the bekasam preservation by LAB.

Key words: Antimicrobial, Bekasam, Fermented fish, Inhibition index, Lactic acid bacteria

Introduction

Bekasam is an Indonesian fermented fish product with a sour taste and it is a popular food in Central Java, South Sumatra and South Kalimantan. Generally the production of bekasam involves a spontaneous, fermentation process of freshwater fish, supplemented by salt, and rice or fermented cassava (Murtini et al., 1997).

Lactic acid bacteria LAB is the dominant microorganism found in fermented fish. In som-fak, rapid growth of LAB cousing pH to decrease below 4.5 in two days is essential to prevent spoilage and to ensure safety of the product (Ostergaard et al., 1998). LAB has an important role in food fermentation that caused changes in taste, smell and texture with improved preservation of the product (Hugas, 1998). This preservation process inhibits the growth of spoiled and pathogenic bacteria. However the salt too had ability to inhibit the growth of spoiling and pathogenic bacteria, because of antibacterial activity. The LAB produces some metabolites i.e. organic acid (lactic acid and acetate), hydrogen peroxide, diacetil and bacteriosin (Ross et al., 2002; Diop et al., 2007; Galves, 2007).

LAB was reported in the fermented fish product from Thailand such as pla-ra, pla-chom, plaa-som and som-fak. The dominant bacteria on fermented product of som-fak, plaa-som, pla-ra and pla-chom were LAB i.e. *Lactococcus lactis* subsp. lactis, *Leuconostoc citreum*, *Lactobacillus paracasei* subsp. paracasei, *Weisella confuse*, *L. plantarum*, *L. pentosus, Pediococcus pentosaceus*, *Lactobacellus alimentarius/farciminis, Lactococcus garvia, Lactobacillus acidipicis* sp. nov and *Weissella thailandensis* sp nov (Paludan-Muller et al., 1999; Tanasupawat et al., 2000; Paludan-Muller et al., 2002). *Weissella cibaria* 110 (plaa-som) produced bacteriosin known as weissellicin 110 (Sriounnual

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et al., 2007), and plantaricin w produced by *Lactobacillus plantarum* PMU33 (som-fak) (Noonpakdee et al., 2009). However, there is limited information on the microbiological aspects of bekasam fermentation due to the fact that its production is on a small or house-hold scale. Rahayu (2010) stated that most research in Indonesia related to LAB is mostly in the area of exploration for the potency of these bacteria in fermented foods, biopreservative and probiotics. Therefore this study describes the characteristics of LAB isolated from bekasam and their inhibitory action on the growth of pathogenic bacteria.

Materials and Methods

Isolation and Selection of LAB

Bekasam was collected from local small scale processors in Panganjang, Indramayu residence (West Java), Indralaya, Ogan Ilir Residence (South Sumatera) Cengal and Kayu Agung, Ogan Komiring Ilir Residence (South Sumatera). Each sample was measured for its pH, NaCl and lactic acid contents using titration method. The number of cell was measured using plating method on Plate count Agar (PCA) for the total aerobic bacteria and de Man Rogosa Sharper Agar (MRSA) supplemented with $CaCO_3$ 0.5% for total of LAB (Veljovic et al., 2007). LAB were isolated from the plates of MRSA. As many as 10-20 of colonies were isolated using scratched plating method. Incubation was at 37°C for 48 hours (Tanasupawat et al., 1998). The pure culture isolate was verified as LAB using gram staining, spore staining, motility, catalase test, gas production from glucose (Palludan-Muller et al., 2002; Kopermsub et al., 2006; dan Veljovik et al., 2007).

Antimicrobial activity test

Antimicrobial activity of LAB was determined using double layer method against *E. coli, S. typhimurium* ATCC 14028, *B. cereus, S. aureus,* and *L. monocytogenes* (Nurhasanah, 2004). As much as 50 µL of the idicator bacteria (10^8 CFU/mL) was suspended in 50 mL Nutrient Agar (NA) consisted of 0.75% agar and then poured 10 mL into the surface of lactid acid isolate on MRSA media. All the culture was incubated at 37°C for 24 hours. The bacteria having an ability to produce antimicrobial compound showed clear zone (inhibition zone) around the colony. The inhibition index was calculated using the following formula:
Inhibition index=(inhibition zone diameters (mm)-colony diameters (mm))/(colony diameters (mm)).

Isolates of lactic acid bacteria showed antagonistic activity against indicator bacteria was examined for their antimicrobial compound using agar well diffusion method.

Culture supernatant was obtained from an overnight culture by centrifugation at 10.000 rpm for 10 min at 4°C.The supernatant was neutralized (at pH 7.0±0.2) with 1N NaOH, and sterilized by filtering with acrodisc (pore size 0.22 µm). The antimicrobial activity of neutral supernatant was carried out by using agar well diffusion methods. A volume of 20 µL culture of the indicator bacteria was inoculated to 20 mL of liquid Molten Muller Hinton agar, and after it solidified the cultures were incubated overnight. The culture was bored using cork bore (5 mm in diameter). As much as 70 µL of cell-free supernatant (CFS) was dispensed into the wells. All plates were incubated overnight at 37°C. Inhibition zone was determined by measuring clear area surrounding agar well with modifications Diop et al. (2007).

Results and Discussion

LAB from Bekasam

Eight samples of bekasam taken from 4 locations contained 2.34% - 7.28% and 1.13% - 2.50% of total salt and lactid acid, respectively (Table 1). Before fermentation, bekasam consisted of salt 15% - 25% and carbohydrate source from rice 30% - 50 %. LAB total in bekasam was $1.4x10^8$-$9.0x10^8$ CFU/mL. Lactic acid produced by LAB was decreased pH up to 3.60 – 5.30. Adding of salt could inhibit growth of spoiled bacteria. This indication was shown from the data of aerobic microbes that was smaller than the total of LAB (Table 1).

Seventy four isolates of acid producing bacteria were examined for their morphological and biochemical characteristics i.e. catalase production, motility and gas production from glucose. Lactic acid bacteria generally were defined as a group of lactic acid producer, which have characteristics of low G+C, non-spore forming, gram-positive rod and cocci, fermentative, negative catalase, anaerob facultative, non-motile and tolerant against acid (Hutkins, 2006). The result of morphological and biochemical characteristics of LAB from bekasam was shown on Table 2.

Based on the morphological characteristic with gram's staining, the percentage of rod shaped cells to sphere shape cells was determined. Among the 74 isolates, 54% were gram-positive with rod shaped cells (bacilli) and 46% gram-positive with sphere shape cells (cocci). And 1% was spore forming bacteria and 99% non-spore forming bacteria. All isolates were non-motile. Eleven isolates performed positive reaction for the catalase

test. Based on gas production from glucose fermentation test, 69 isolates had no gas formation from glucose fermentation. Based on morphological and chemical characteristics it was concluded that 62 isolates belonged to LAB group.

In a study that investigated milk fish bekasam, Chandra et al. (2007) stated that among the five acid producing isolates isolated from bekasam, four were Gram-positive cocci and one was gram-positive bacilli. All isolates were no spore and non motil. This research was also obtained that LAB isolates were isolated from milk fish bekasam (BI.8 sample) was dominated by gram-positive cocci, and all isolates were also no spore and non motil (Table 2).

Table 1. Bekasam characteristics and their chemical and microbiological parameters.

No	Sample	Location	Type of fish	Length of Fermentation (day)	pH	Salt concen tration (%)	Total of lactic acid (%)	Total of aerobic microbe (CFU/ml)	Total of LAB (CFU/ml)	Isolates obtained
1	BP.4	Indralaya, Ogan Ilir residence (South Sumatera)	Pearl gourami	4	4.60	2.34	2.41	$1.7x10^8$	$4.0x10^8$	14
2	BP.8		Pearl gourami	8	3.62	3.45	241	$1.2x10^8$	$3.2x10^8$	4
3	SI.7		Rasbora	7	3.71	7.28	2.41	$6.6x10^6$	$1.4x10^8$	10
4	SK.7	Kayu Agung, Ogan Komiring Ilir residence (South Sumatera)	Rasbora	7	3.60	4.62	1.43	$1.2x10^8$	$5.0x10^8$	7
5	NS.4	Desa Sungai Pasir, Distric of Cengal, Ogan Komiring Ilir residence (South Sumatera)	Nile tilapia	4	5.30	nd	1.13	$4.2x10^8$	$9.0x10^8$	7
6	SS.8		Pearl gourami	8	4.45	nd	2.50	$5.6x10^7$	$4.8x10^8$	12
7	PS.8		Cat fish	8	4.23	nd	1.67	$4.3x10^7$	$4.7x10^8$	5
8	BI.8	Panganjang, Indramayu residence (West Java)	Milk fish	8	4.09	4,01	2.20	$4.8x10^7$	$2.7x10^8$	15
Total										74

nd= not determined

Table 2. Number of lactic acid bacteria isolated from bekasam with their morphological and biochemical characteristics.

No	Sample code	Isolates code	Number isolates	Number of isolates with Cell morphology			Biochemical properties				Number of LAB isolates
				cocci	bacilli	Gram-positive	No spore	Negative catalase	Non motile	Homo fermen-tative	
1	BP.4	BP (1-20)	14	7	7	14	14	13	14	13	13
2	BP.8	BP(21-30)	4	0	4	4	3	4	4	4	3
3	SI.7	SI(1-15)	10	9	1	7	10	4	10	10	4
4	SK.7	SK(1-20)	7	3	4	7	7	6	7	7	6
5	NS.4	NS(1-17)	7	0	7	7	7	7	7	7	7
6	SS.8	SS(1-17)	12	2	10	12	12	12	12	9	12
7	PS.8	PS(1-17)	5	0	5	5	5	5	5	5	5
8	BI.8	BI(1-20)	15	13	2	15	15	12	15	14	12
		Total	74	34	40	74	73	63	74	69	62

In a study that investigated plaa-som, a similar fermented fish product likes bekasam. Kopermsub et al. (2006) stated that among the ninety acid producing bacterial isolates isolated from plaa-som, 79% were gram-positive rod shaped cells (bacilli) and 21% gram-positive sphere shape cells (cocci). From 90 isolates, eighty isolates were confirmed as belonging to the LAB group. Three genera were identified as *Lactobacillus* spp., *Pediococcus* spp., and *Aerococcus* spp. at 79%, 18% and 3%.

Yahya et al. (1997) reported that LAB isolated during bekasam fermentation from Nile tilapia (*Oreochromis mossambicus*) were *Leuconostoc mesenteroides* which was isolated from sample of 1 – 7 days fermentation. *Lactobacillus acidophilus* from day 5 to 7, *L. plantarum* and *L. fermentans* on the first day, *L. buchneri, L. reuteri* on the third day, *Pediococcus pentacaseus, L. bifermentans, L. tolerans, Pediococcus acidilactici, L. bulgaricus*, Leu. dextranicum are isolated on the seventh day. There are four isolates that have antimicrobial activity against *Staphylococcus aureus* FNCC 0047; they are *Lactobacillus plantarum*-IB2, L. fermentum-IB5, *L. acidophilus* IIIB5 and *Pediococcus acidilactici*-IVB2.

Gram-positive rod shaped cells (bacilli) were generally the predominant bacteria in all samples. While gram-positive sphere shaped cells (cocci) were more dominant in bekasam of rasbora (*Rasbora* sp.) from Indralaya and milk fish (*Chanos chanos*) bekasam from Panganjang. This difference might be caused by the type of raw material utilised. Bekasam processing still uses spontaneous fermentation process so the bacteria dominating in this fish fermentation process is indigenous bacteria that come from each of the raw material mainly the fish.

Some researchers reported that LAB is part of native microbiota of aquatic animals (Ringo, 2004). Itoi et al. (2008) reported that halotolerant strains of *Lactococcus lactis* subsp lactis isolated from the intestinal tract of the pufferfish *Takifugu niphobles* caught in Shimoda, Shizuoka, Japan. Nair and Surendran (2005) isolated lactic acid bacteria from various samples of fresh and frozen fish and prawn. Thirteen species of *Lactobacillus* were identified among the 64% isolates. Among them, *L. plantarum* was the dominant species. The remaining 36% isolates of *Lactobacillus* could not be assigned to any species with the available taxonomic schemes.

According to Kopermsub et al. (2006) fermented food characteristics varied with raw material and additional material used that caused the microbial diversity in the food. Palludan-Muller et al. (1999) reported that LAB bacteria isolated from fish, rice, garlic and banana leaf and during som-fak fermentation process. *Lactococcus lactis* subsp. lactis and *Leuconostoc citreum* were specifically associated with fish fillet and minced fish, *Lactobacillus paracasei* subsp. paracasei with boiled rice and *Weisella confusa* with garlic mix and banana leaves. In addition, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Pediococcus pentosaceus* were isolated from raw materials. A succession of aciduric, homofermentative lactobacillus species, dominated by *L. plantarum/pentosus*, was found during fermentation.

This study showed that 69 isolates consisted of homofermentative characteristics. It showed that these isolates had the ability to change more than 90% of sugar substrates to become lactic acid. This is different from heterofermentative bacteria that produce less than 50% of lactic acid and 50% acetate acid, ethanol and carbon dioxide. According to Rose et al. (2002) the group of homofermentative bacteria consists of *Lactococcus, Pediococcus, Enterococcus, Streptococcus* and some *Lactobacillus* use Embden-Meyerhof-Parnas pathway to change glucose to lactic acid.

Antimicrobial activity of LAB isolates

As many as 62 LAB isolates from bekasam were tested for their antimicrobial activity against five indicator bacteria associated with foodborne deseases. Figure 1 shows that five of LAB isolates had antimicrobial activity against five indicator bacteria.

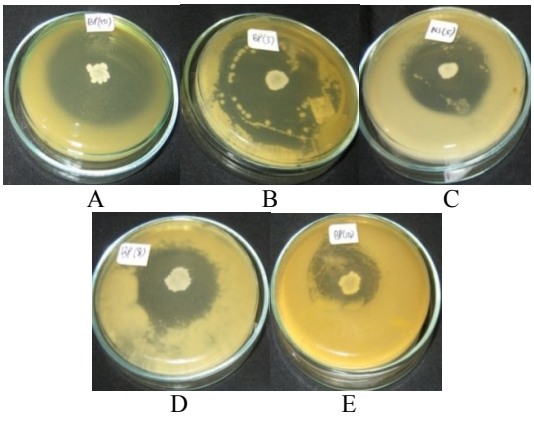

Figure 1. Antimicrobial activity of LAB isolates against indicator bacteria of *E. coli* (A), *S. typhimurium* (B), *L. monocytogenes* (C), *B. cereus* (D), dan *S. aureus* (E) with double layer method.

Table 3. The range of inhibition zone and Inhibition index on each indicator bacteria.

No.	Indicator bacteria	Number of isolates that inhibit	Inhibition zone (mm)	Inhibition index
1	*L. monocytogenes*	56 (90%)	2 -29	0.3 – 5.4
2	*S. typhimurium*	49 (79%)	3 -38	0.4 – 5.4
3	*E. coli*	45 (73%)	4 -32	0.3 – 4.0
4	*B. cereus*	44 (71%)	2 - 33	0.3 – 4.7
5	*S. aureus*	41 (66%)	2 - 44	0.2 – 6.1

There were 23 (38%) isolates that inhibited the five indicator bacteria. And 56 (90%) isolates of LAB produced inhibition zone against *L. monocytogenus*, while the LAB isolates that inhibit growth of *S. typhimurium, E.coli, B. cereus* and *S. aureus* were 79%; 73%; 71% and 66% respectively. The highest range of inhibition zone and inhibition index was on *S. aureus* (Table 3).

All isolates exhibited inhibition zones against the indicator bacteria which is indicative of their antimicrobial activity. Each isolate had different ability to inhibit growth of the five indicator bacteria. The inhibition activity of various antimicrobial substances against several species of pathogen bacteria was different. The antimicrobial activity of LAB was mainly caused by organic acid produced from glucose metabolism. At this study, the concentration of lactic acid for all samples was 1.13 – 2.50% with pH up to 3.60 – 5.30. LAB can produce some metabolites like organic acid (lactic and acetate acid), hydrogen peroxide, diacetil and bacteriosin (Ross et al., 2002; Diop et al., 2007; Galves, 2007).

All the neutralized cell-free supernatant of 53 LAB isolates did not show the inhibition zones against the five indicator bacteria. While non-neutralized cell-free supernatant showed inhibition zone about 1–10 mm. This indicates that inhibition activity of LAB isolates was caused by the lower pH of supernatant due to organic acid content. The supernatant pH after incubating for 24 hours from 53 LAB isolates was in range of 3.5 to 5.7. This indicated that the antimicrobial activity of LAB isolates from bekasam was caused by organic acid. Palludan-Muller et al. (2002) reported that the primary role of LAB is to ferment the available carbohydrates thereby decreasing in pH. It is important to inhibit pathogenic and spoilage bacteria and guaranty of product safety. The organic acids produced (mainly lactic acid) is the main preservative factor in bekasam fermented fish products.

The positive control of lactic acid solution with a pH of 4, 5 and 6 showed that at pH 5 and 6 did not show inhibitory zone against the five indicator bacteria, whereas at pH 4 showed inhibitory zones in range of 3-7 mm. According to Allokami et al. (2000), antimicrobial activity of lactic acid was shown at concentration of 5mM or at pH 4. This acid causes prominent permeabilization on the outer membrane of *Escherichia coli* O157: H7, *Pseudomonas aeruginosa*, and *Salmonella enterica serovar typhimurium.*

Conclusions

Sixty-two isolates obtained from bekasam were member of the LAB group. And 90% of LAB isolates inhibited growth of *L. monocytogenes*. And the percentage of LAB isolate that inhibited the growth of *S. typhimurium, E. coli, B. cereus* and *S. aureus* were 79%, 73%, 71% and 66%, respectively. The highest inhibition zone and inhibition index was on *S. aureus*. However, culture supernatant of the LAB isolates did not produce inhibition zone at neutral pH. This result indicates that inhibition activity of LAB from Indonesian bekasam was from organic acids, and they are probably the main preservative factor in the bekasam. The antibacterial compounds produced by LAB or its LAB obtained from bekasam may be used to combat the growth of pathogenic microorganisms in the bekasam fermentation processing. They could give a beneficial effect to fermented product, ie more stable product and metabolites which are produced during fermentation will improve flavor, aroma and texture of the product. Base on this results, improvement of bekasam fermentation could carry out, using LAB isolates as a starter culture. This is possible utilization for large scale production.

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