

Emirates Journal of



FOOD AND AGRICULTURE



Volume 24 (3) June 2012

NUTRITION AND FOOD SCIENCE

Use of ozone and chlorine dioxide to improve the microbiological quality of turnip greens

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Abstract

The biocidal effect of ozone, chlorine dioxide (oxine), commercial sanitizer, and the combination of ozone and oxine on natural microbial flora found on turnip greens was investigated. Turnip greens were treated with tap water, 5 ppm ozone, 40 ppm oxine, 2% commercial sanitizer, and the combination of ozone and chlorine dioxide for 0, 1, and 5 min. Exposure times and types of sanitizers were evaluated. There were three trials per treatment. Tap water failed to achieve 1 log CFU/g reduction (0.44-0.55 log CFU/g). The combined ozone-oxine treatment was more effective in reducing population levels of these bacterial populations on turnip greens than individual treatments. The total bacterial count was reduced by 2.17 log CFU/g and the *E. coli* population was not detected when samples were treated with combination of 5 ppm ozone and 40 ppm oxine.

Key words: Pathogenic bacteria, Ozone, Turnip greens, Sanitizer

Introduction

Consumers nowadays are increasingly aware of the benefits of eating fresh fruit and vegetables, so the demand for produce has increased accordingly. Scharff (2010) reported that the health related costs associated with foodborne illnesses are estimated to be \$152 billion annually. The Center for Science in the Public Interest has ranked fresh produce as one of the leading causes of foodborne illnesses since 1990 (CSPI, 2002). In December 2002, McCall Farms of South Carolina recalled about 15,000 cases of Glory Foods brand kale in 27 ounce cans and about 9,000 cases of Glory Foods brand turnip greens in 27 ounce cans because the products were potentially contaminated with *Salmonella* and *Staphylococcus aureus* (FDA, 2003). The FDA issued a high alert in October 2005 on Dole pre-packaged salads. These salad products were linked to an outbreak of *E. coli* O157:H7 in Minnesota. The salad products involved were Classic Romaine, American Blend, and Greener Selection. Eleven people became ill and two were hospitalized (FDA,

2005). Again, in September 2006, the FDA issued a health alert advising people not to eat fresh-bagged spinach because of *E. coli* O157:H7 contamination that left 1 person dead and several people hospitalized across 21 states (FDA, 2006) in the U.S.

Some studies have been conducted on the use of different natural ingredients including organic acids as antimicrobials (Tajkarimi et al., 2010; Tajkarimi and Ibrahim, 2011). In the last few years the combined use of several disinfectant agents including lactic acid, chlorinated water, thyme essential oil solution, sodium lactate, citric acid, hydrogen peroxide, and ozone has also been reported (Allende et al., 2006). Copper in a combination with lactic acid have been used to decontaminate produce surfaces inoculated with *E. coli* O157:H7 (Gyawali et al., 2011; Gyawali and Ibrahim, 2012). Use of ozone and chlorine dioxide could be another effective sanitizing solution in reducing produce microbial population.

Ozone has been used in the food industry as an antimicrobial since its approval as a direct food additive for the treatment, storage, and processing of foods in gas and aqueous phases from the U.S. Food and Drug Administration (Federal Register 2001). Treating fresh fruits and vegetables with ozone has been found to increase shelf-life (Guzel-Seydin et al., 2004). Sharma et al. (2002) reported 0.40 – 1.75 log reduction of *E. coli* O157:H7 on

Received 30 January 2012; Revised 19 February 2012;
Accepted 21 February 2011

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inoculated alfalfa seeds using 4 ppm to 21 ppm of ozonated water.

Chlorine dioxide is a strong oxidizing agent with 2.5 times the oxidizing capacity of chlorine. It does not react with phenolic compounds to produce chlorophenols that taste and smell bad (Singh et al., 2002). Chlorine based sanitizers produce chlorinated organic compounds, such as trihalomethanes, which are potential carcinogens (Fawell, 200). Unlike chlorine, chlorine dioxide does not form carcinogenic products. In 1998, the FDA allowed the use of aqueous chlorine dioxide in sanitizing fruits and vegetables. Research has proven that chlorine dioxide is effective against bacteria on carrots, mushrooms, lettuce, cabbage, and strawberries. Singh et al. (2002) have reported that 5 mg/L of chlorine dioxide was as effective as 34 mg/L of chlorine.

According to the manufacturer, the commercial sanitizer used in this research was made of all natural ingredients that are effective against a wide range of pathogenic microorganisms and molds. It can extend the shelf life of produce up to three times. It can break down biofilm that could hinder the effectiveness of the sanitizer. It is considered safer than chlorine because it does not produce a residual effect and it is odorless. This research was conducted to find the most effective way to sanitize turnip greens. The objective of this study was to investigate the effect of ozone and chlorine dioxide, alone or in combination, on microbial reduction on turnip greens.

Materials and Methods

Turnip greens and sample preparation

Fresh turnip greens (20 lbs) were purchased from a farmer's market in Greensboro, North Carolina and stored in their original packaging at 4°C until the following day. Defective leaves and unusable stems were removed.

Preparation of treatments

Five treatments were applied to determine their effectiveness in improving the microbiological quality of turnip greens. These treatments were: (1) tap water (2) 5 ppm ozone (3) 40 ppm oxine (4) 40 ppm oxine with 5 ppm ozone, and (5) 2% commercial sanitizer. Samples without treatment served as a control.

5 ppm ozone

Pure oxygen was used as the feed gas for the ozone produced by the OZAT CFS-3A ozone generator (Ozonix Ltd., Switzerland). Ozone gas was bubbled into the water. The ozonated water was recycled between the washing sink and the

mixing tank. Ozone dissolves more effectively at low temperature; therefore, ice was placed in the water storage tank to maintain the water temperature at 0-2°C.

40 ppm oxine

Stabilized active oxine solution with 2% chlorine dioxide was purchased from Bio-Cide International, Inc. (Norman, OK). The concentration of oxine used was 40 ppm. The oxine solution was activated by adding 34 g of food grade citric acid to 106 mL of oxine. The resulting solution was placed on a stirring plate to stir the solution until the crystals of citric acid completely dissolved. The pH of the solution was adjusted to 2.2 ± 0.3 using a pH meter (Accumet Model AR 50 Fisher Scientific, Pittsburg, PA). The activated oxine was poured into 300 mL of distilled water and placed on a stirring plate. After mixing well, the solution was poured into 56 L of tap water in the vegetable wash sink.

2% Commercial sanitizer

A 2% commercial sanitizer (Bioremediation, Inc. Knox, IA) made of natural ingredients and described by the manufacturer as having strong antimicrobial activity was tested in this study. A volume of 1000 mL of the solution was mixed with 56 L of tap water in a wash sink.

Turnip greens washing

For each treatment, 1000 g of turnip greens were weighed and submerged in a wash sink for each treatment and washed for the designated amount of time. Three 10-g samples of treated turnip greens were withdrawn at different time intervals (0, 1, and 5 min) and placed in stomacher bags containing 100 mL of 0.1% sterile peptone water. Samples were homogenized (Stomacher 400 Circulator, Seward Ltd., UK) for 1.5 min. After homogenization, 1mL of liquid sample was withdrawn for microbial analysis.

Microbial analysis

Appropriate dilutions from each homogenized sample were plated onto Tryptic Soy Agar (TSA) for the enumeration of total counts, Eosin Methylene Blue (EMB) and MacConkey Sorbital Agar (MAC) for the enumeration of *E. coli*. The plates were incubated at 37°C for 24 hr. Log reductions in microbial load were calculated by subtracting microbial count after treatment from the initial microbial count of untreated turnip greens.

Statistical analysis

Data obtained from microbiological counts (CFU/g) of samples was first transformed to log

values and then analyzed by Analysis of Variance (ANOVA) by SAS (SAS Institute Inc., Cary, NC). Differences of means were separated by least significant difference at $P < 0.05$.

Results and discussion

Effect of tap water

Table 1 shows the effect of tap water on microbial quality of turnip greens as defined by total microbial count and *E. coli*. After 1 min of washing turnip greens in tap water, the total bacterial count was 5.03 log CFU/g and 4.78 log CFU/g for 5 min washing. The results show a 0.4 log CFU/g reduction after 5 min of exposure from the initial population of untreated sample (5.18 log CFU/g). When turnip greens were treated with tap

water, the *E. coli* population was reduced by 0.23 and 0.59 log CFU/g after 1 and 5 min respectively when compared to untreated samples (4.06 log CFU/g) in EMB agar. *E. coli* population was also determined using MAC agar. Turnip greens exposed to treatment for 1 and 5 min showed 0.33 and 0.54 log reduction respectively when compared to untreated samples (4.02 log CFU/g). These results indicated that using tap water alone as a rinsing agent was not an effective method for improving the microbiological quality of turnip greens. Tap water failed to achieve 1 log reduction. Kim et al. (1999) have also reported less than 1 log reduction (0.74 log CFU/g) when lettuce leaves were washed with tap water.

Table 1. Effect of tap water on survival of total bacterial count (TSA) and *E. coli* (EMB, MAC) on turnip greens

Treatment	Media	LogCFU/g		
		0 min	1 min	5 min
Tap water	TSA	5.18 ^a ± 0.34	5.03 ^a ± 0.11	4.78 ^a ± 0.21
Tap water	EMB	4.06 ^a ± 0.41	3.83 ^a ± 0.24	3.47 ^a ± 0.39
Tap water	MAC	4.02 ^a ± 0.22	3.69 ^a ± 0.05	3.48 ^a ± 0.28

Results are presented as mean ± standard deviation from the experiments in triplicate. Means with the same letters are not significantly different ($P > 0.05$).

Effect of ozone

Table 2 shows the effect of ozone 5 ppm on the microbial population on turnip greens. For total microbial count, 0.77 log CFU/g reduction was observed after 1 min of washing. After 5 min, the population was reduced by 1.43 log CFU/g. *E. coli* population was reduced by 0.76 and 1.81 log in EMB after 1 and 5 min respectively. Similarly, 1.17

and 3.13 log reduction was observed when samples were treated for 1 and 5 min respectively as observed in MAC agar. Garcia et al. (2003) conducted similar research on minimally processed lettuce using 5 ppm ozone as a sanitizer and observed a 1.1 log CFU/g reduction of microbial count.

Table 2. Effect of 5 ppm ozone on survival of total bacterial count (TSA) and *E. coli* (EMB, MAC) on turnip greens

Treatment	Media	LogCFU/g		
		0 min	1 min	5 min
5 ppm ozone	TSA	4.51 ^a ± 0.34	3.74 ^a ± 0.23	3.08 ^a ± 0.58
5 ppm ozone	EMB	4.10 ^a ± 0.41	3.34 ^a ± 0.38	2.29 ^b ± 0.47
5 ppm ozone	MAC	3.70 ^a ± 0.22	2.53 ^a ± 0.05	0.57 ^b ± 0.98

Results are presented as mean ± standard deviation from the experiments in triplicate. Means with the same letters are not significantly different ($P > 0.05$).

Effect of chlorine dioxide (oxine)

Table 3 shows the effect of chlorine dioxide (oxine) at 40 ppm on total microbial count and *E. coli*. The total microbial count after 1 min exposure to oxine showed a 1.2 and 1.43 log CFU/g reduction was achieved after 5 min. Exposure to 40 ppm oxine for 1 min and 5 min reduced *E. coli* on EMB by 0.95 and 1.03 log CFU/g respectively. Enumeration of *E. coli* using MAC showed that after 1 min of exposure, 0.82 log CFU/g reduction was achieved. After 5 min, *E. coli* was reduced by 2.42 log CFU/g when compared to the untreated sample (3.71 log CFU/g). Singh et al. (2002)

conducted research using aqueous chlorine dioxide on lettuce and baby carrots and achieved 1.48-1.97 log CFU/g reduction. In our study, we achieved 0.82-2.42 log CFU/g reduction of microbial population using TSA, EMB, and MAC media.

Effect of commercial sanitizer

Table 4 shows the effect of commercial sanitizer (2%) on total microbial population and *E. coli* found on turnip greens. When samples were washed for 1 and 5 min, total microbial population was reduced by 2.0 log compared to untreated 4.4 log CFU/g. There was no significant difference

($P > 0.05$) between treatments after 1 and 5 min. When compared to untreated samples (3.61 log CFU/g), the population of *E. coli* was reduced by 1.79 and 1.93 log after 1 and 5 min of wash respectively. We noticed a significant difference between untreated sample and 1 min treated sample. However, we did not observe any significant difference ($P > 0.05$) after 1 and 5 min of washing. MAC agar, which was also used for the enumeration of *E. coli*, showed 2.19 log reduction

compared to the untreated samples (3.80 log CFU/g). When samples were treated for 5 min, bacterial population was not detected using classical microbiological plate count method. Other commercial sanitizers have also been tested on tomatoes and apples, showing a nearly 3 log CFU/g reduction of microbial load (Kilonzo-Nthenge et al., 2006).

Table 3. Effect of 40 ppm oxine on survival of total bacterial count (TSA) and *E. coli* (EMB, MAC) on turnip greens.

Treatment	Media	LogCFU/g		
		0 min	1 min	5 min
40 ppm oxine	TSA	5.31 ^a ± 0.23	4.11 ^a ± 0.12	3.88 ^a ± 0.45
40 ppm oxine	EMB	4.23 ^a ± 0.51	3.28 ^a ± 0.90	3.20 ^a ± 1.29
40 ppm oxine	MAC	3.71 ^a ± 0.32	2.89 ^a ± 0.79	1.29 ^b ± 0.91

Results are presented as mean ± standard deviation from the experiments in triplicate. Means with the same letters are not significantly different ($P > 0.05$).

Table 4. Effect of commercial sanitizer on survival of total bacterial count (TSA) and *E. coli* (EMB, MAC) on turnip greens.

Treatment	Media	LogCFU/g		
		0 min	1 min	5 min
Sanitizer	TSA	4.4 ^a ± 0.33	2.40 ^b ± 0.19	2.37 ^b ± 0.22
Sanitizer	EMB	3.61 ^a ± 0.51	1.82 ^b ± 1.14	1.68 ^b ± 0.28
Sanitizer	MAC	3.80 ^a ± 0.53	1.61 ^b ± 0.93	ND

Results are presented as mean ± standard deviation from the experiments in triplicate. Means with the same letters are not significantly different ($P > 0.05$).

ND: below detectable level < 10 CFU/g.

Effect of combination of ozone and oxine

Table 5 shows the effect of a combination of ozone 5 ppm and 40 ppm oxine after samples were treated for 5 min. When samples were treated with 40 ppm oxine alone, or in combination with 5 ppm ozone, the reduction in total microbial population was similar (slightly > 2.0 log CFU/g). When samples were treated with 40 ppm oxine alone,

E. coli in EMB showed 2.37 log reduction compared to the untreated sample (3.88 log CFU/g). With the combination effect of oxine and ozone, *E. coli* population was below the detectable level. The bacterial count for MAC also showed the population below the detectable level. These results showed the synergistic effect of 5 ppm ozone and 40 ppm oxine against *E. coli*.

Table 5. Effect of the combination of ozone 5 ppm and oxine 40 ppm on survival of total bacterial count (TSA) and *E. coli* (EMB, MAC) on turnip greens.

Treatment	Media	LogCFU/g		
		Untreated	40 ppm oxine	5 ppm ozone + 40 ppm oxine
Ozone/Oxine	TSA	4.73 ^a ± 0.50	2.60 ^b ± 0.19	2.56 ^b ± 1.06
Ozone/Oxine	EMB	3.88 ^a ± 0.41	1.51 ^b ± 1.33	ND
Ozone/Oxine	MAC	3.80 ^a ± 0.33	ND	ND

Results are presented as mean ± standard deviation from the experiments in triplicate. Means with the same letters are not significantly different ($P > 0.05$).

ND: below detectable level < 10 CFU/g.

Garcia et al. (2003) observed that ozone alone had a 1.1 log reduction while the combination with chlorine had 2.5 log bacterial reductions on lettuce. A similar effect was also shown by Singh et al. (2002) with 3-4 log reduction on shredded lettuce

and baby carrots when samples were treated with a combination of ozone, chlorine dioxide, and thyme. The combination of ozone and citric acid showed 2.31 log CFU/g reduction of *E. coli* O157:H7 compared to 5 ppm ozone alone, which reduced the

population by only 1.09 log CFU/g on stainless steel squares (Moore et al., 2000). Yuk et al. (2006) have also reported that a combined treatment of 3 ppm ozone and 1% citric acid was the most effective in reducing populations of *E. coli* O157:H7 and *L. monocytogenes* on lettuce. Beltran et al. (2005) conducted research on potato strips that showed that ozone alone was not effective in lowering total microbial population. However, the combination of ozone and tsunami was the most effective sanitizer against total microbial populations.

Conclusion

Our results demonstrated that tap water showed the least microbial reduction on turnip greens. The average reduction was 0.23-0.4 log for total microbial count and 0.23-0.59 log CFU/g for *E. coli* population. This method of treatment failed to achieve at least 1 log reduction. The most effective treatment was the combination of 5 ppm ozone and 40 ppm oxine showing 2.17 log reduction in total count and below the detectable level for *E. coli* population after 5 min of exposure. This result indicates the synergistic effect of ozone and oxine. The combination of 5 ppm ozone and 40 ppm oxine could thus be a potentially effective treatment reducing the presence of bacterial cells on produce. Our treatment was able to reduce the microbial load in the treated food to the level that we could not detect using classical microbiological approach, plating method. Therefore, the use of other non-traditional approaches, specifically DNA/RNA-based molecular techniques, could be applied for the detection of the presence of viable bacterial cells in various food samples.

Acknowledgement

This work was supported by the USDA National Institute of Food and Agriculture, Hatch project number NC.X-234-5-09-170-1 in the Agricultural Research Program at North Carolina Agricultural and Technical State University.

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Evaluation of mayonnaise-like food emulsions with extracts of herbs and spices

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Abstract

This paper investigates mayonnaise-like food emulsions with natural spices and herbs and new type of extracts. The microbiological quality, oxidative stability and sensory perception of the food emulsions were analyzed. Mayonnaise and salad dressing were prepared with extra virgin olive oil at 50% and 25% oil-phase volume, respectively. Results demonstrate that the microbiological contamination of spice extracts is significantly lower than that of the corresponding natural spices. Food emulsions with spice and herbs extracts were not microbial contaminated. The differences in the oxidation processes were evaluated by measuring the formation of primary oxidation products. Mayonnaise with olive oil and natural spices proved to be most susceptible to oxidation. When comparing samples, these with extracts were more resistant to oxidation, and this was expressed most strongly in salad dressing with basil. Sensory evaluation indicated that there were little differences in the degree of perception in investigated sensory attributes of the emulsions with natural spices and herbs and their extracts. Consumers scored the samples with a higher fat content higher than the low-fat ones.

Key words: Food emulsions, Herbs and Spices extracts, Oxidative stability, Sensory evaluation

Introduction

Different mayonnaise-like food products like mayonnaise, mayonnaise with addition of different ingredients and dressing sauces are used in the food and catering industries. All such products are in the form of oil-in-water emulsions. One current trend in food technology is to incorporate plant oils as an oil phase in emulsions, with an emphasis on oils perceived as healthy, such as walnut, olive oil, canola oil, etc. (O'Donnell, 1995; Brandt, 1999; Paraskevopoulou et al., 2006). However, because of their unsaturated character, such oils are susceptible to oxidation (Chaiyasit et al., 2007). There are many articles concerning quality changes of bulk oils. In contrast, the oxidative and hydrolytic degradation that occurs in oil-in-water emulsions are less studied.

The addition of spices into food emulsions improves their consumer acceptance and flavour characteristics. Besides sensory profile, spices also improve oxidative stability of vegetable oils. They may serve as natural food preservatives, too.

Different authors have considered antioxidative activity spices and herbs added to pure oils (Özcan, 2003; Abdala and Roozen, 1999), but not many references have been found concerning the effect of spices on the oxidative stability of oil-in-water emulsions.

In present days several new techniques for extraction of spices and herbs are industrially involved – supercritical extraction, sub-critical liquefied gas extraction, etc. (McHugh and Krukonis, 1994). In this paper is considered the influence of new type of extracts (www.buy-extracts.com) derived through food grade sub-critical liquefied gas on some foods. There are no attempts for substitution of traditional researched spices with their type of extracts in mayonnaise-like food products.

As regards microbiological contamination, mayonnaise-like food products are relatively stable, because of their low values of pH (Smittle, 1977; Radford and Board, 1993). Moreover, high fat content is inappropriate environment for microbial grow. Nevertheless, extracts of natural herbs and spices can be used as additional ingredients for improving microbiological quality. On the other hand, if they are not microbial clean, some herbs and spices may contaminate the end product.

The objective of this research was to evaluate and compare the replacement of natural spices and

Received 30 July 2011; Revised 22 February 2012; Accepted 27 February 2012

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herbs with their new type of extracts in mayonnaise-like food emulsions from the point of their microbiological, antioxidative and sensory characteristics.

Materials and Methods

Materials

Table 1 shows the recipes for mayonnaise and salad dressing with natural spices and herbs.

Skimmed milk powder containing 4% moisture, 1.25% fat and 38% proteins was obtained from the local market. Cold-pressed extra-virgin olive oil, produced in Greece, had the followed characteristics: acidity (% oleic acid) – 0.27; peroxide value (meq O₂/kg of oil) – 1.2; K₂₃₂ [where K=absorbance/C (g/100 ml oil)] - 1.6. Modified corn starch, Instant Clearjel SD” obtained from

National Starch and gum “Lygomme KCT 45” obtained from Cargill Texturizing Solutions was used as thickening agents.

The other ingredients for the recipes were supplied from local market: dried basil herb (*Ocimum basilicum*), country of origin Bulgaria, crop 2009; Fresh leaves of parsley (*Petroselinum crispum*), country of origin Bulgaria; Ground black pepper fruits (*Piper nigrum* L.), country of origin Vietnam, crop 2009; Hot Paprika (*Capsicum annum* L.) country of origin China, crop 2009; salt, sugar and vinegar, country of origin Bulgaria.

Antimicrobial agents, sodium benzoate and potassium sorbate, were obtained from Qingdao Rich Trading Co. Ltd (China).

Table 1. Recipes for mayonnaise and salad dressing with olive oil and natural spices and herbs.

Products (Ingredients) g/100g	Mayonnaise (MO)	Salad dressing (SD)
Olive oil	50	25
Merigel 314	1.2	2
Lygomme KCT 45	0.1	0.2
Skimmed milk powder	3	3
Salt	1	1
Sugar	1	1
Sodium benzoate + potassium sorbate	0.15	0.15
Citric acid	0.4	0.4
Vinegar	2.1	2.1
Parsley	0.32	-
Ground black pepper	0.2	-
Basil	-	0.08
Hot Paprika	-	0.12
Water	40.53	64.95

The samples of mayonnaise with extracts of parsley and ground black pepper (MOE) and salad dressing with extracts of basil and hot paprika (SDE), were with the same composition, and the content of added extracts were aliquot of the natural ingredients.

Obtaining of herbs and spices extracts

The extracts were derived on a semi-industrial scale extraction unit using sub-critical liquefied gas 1,1,1,2-tetrafluoroethane (CAS-No. 811-97-2, Solkane 134a), as food grade solvent according to EU flavoring regulations. The extraction raw materials were the same as used for food emulsions flavoring.

The standardized extract Botfex™ Black pepper has the following technical data: INCI-Name (CTFA) Piper Nigrum Extract, CAS-No. 84929-41-9, EINECS-No. 284-524-7, appearance - brown clear oily liquid with yellow crystals, pungent taste and strong characteristic smell and

taste of raw material, volatile oil content – min. 50% (v/w), piperin content – min. 40% (w/w), yield 25-32 kg raw material per kg extract. The extraction parameters were: extraction time 60 min, temperature 20-23°C.

The standardized extract Botfex™ Basil has the following technical data: INCI-Name (CTFA) Ocimum Basilicum Extract, CAS-No. 84775-71-3, EINECS-No. 283-900-8, appearance - dark green brown oily liquid with strong characteristic smell of raw material, volatile oil content – min.50% (v/w), yield 350-400kg dried raw material per kg extract. The extraction parameters were: extraction time 90 min, temperature 20-23°C.

The standardized extract Botfex™ Parsley has the following technical data: INCI-Name (CTFA) Apium Graveolens Extract, CAS-No. 89997-35-3, EINECS-No. 289-668-4, appearance - dark green oily liquid with strong characteristic smell of raw material, volatile oil content – min. 40% (v/w), yield 250-300 kg fresh raw material per kg extract.

The extraction parameters were: extraction time 60 min, temperature 20-23°C.

The standardized extract Botfex™ Hot Paprika has the following technical data: INCI-Name (CTFA) Capsicum Annuum Extract, CAS-No. 84625-29-6, EINECS-No. 283-403-6, appearance - red brown clear oily liquid with strong characteristic smell and taste of raw material, volatile oil content – min. 15% (v/w), yield 40-60 kg dried raw material per kg extract. The extraction parameters were: extraction time 180 min, temperature 30-35°C.

Preparation of the mayonnaise-like food emulsions

The control samples were prepared in a homogenizer “Disho-Labor V 60/10” (“Koruma”), operated at 3900 min⁻¹.

Milk powder, salt, and sugar were blended in water and homogenized for 60 sec. The starch and gum were blended with part of the oil in a ratio of 1:3. The mixture was slowly added to the water phase under continuous mixing. After the entire quantity was added, the rest of the oil phase was slowly emulsified into the water phase. The duration of the process of emulsification was 150 sec for mayonnaise and 90 sec for salad dressing. Finally, the citric acid, dissolved in vinegar, was added to the emulsion and additional homogenization for 60 sec was done. The natural spices and herbs were added into the continuous phase; their extracts were blended with the oil phase.

Four samples were prepared: two with natural spices and herbs - Mayonnaise (MO) and Salad dressing (SD), and two with their extracts - Mayonnaise with extracts (MOE) and Salad dressing with extracts (SDE).

After preparation, emulsions were kept in refrigerator at 0°C to 4°C until their analysis.

Microbiological analysis

The following microbiological analyses of spices and spice extracts were performed: aerobic colony count according to ISO 4833:2004, yeasts and moulds according to ISO 7954:2002, *Escherichia coli* according ISO 16649-2:2002, *Salmonella* spp. according ISO 6579:2002, coliforms according ISO 16649-2:2002, coagulase-positive staphylococci according ISO 6888-1:2005+A1:2005, anaerobic sulfite-reducing bacteria according ISO 15213:2003.

Food emulsion samples were analyzed for aerobic colony count according to ISO 6610:2002, yeasts and moulds according to ISO 6611:2002,

coagulase-positive staphylococci according ISO 6888-1:2005+A1:2005 and anaerobic sulfite-reducing bacteria according to ISO 15213:2003 at the first day of the cold storage.

Oxidative stability evaluation

Experimental design

The olive-oil samples, mayonnaise-like food products, and sauces were stored in screw-capped glass containers (200 ml) at 4°C prior to analysis. Oil was separated from the O/W emulsions by repeated freeze-thaw cycles, followed by centrifugation (Jacobsen et al., 1998). Containers were sampled every five days. Two containers of each sample were independently analyzed in each sampling, and each parameter was measured twice. The results below are expressed as mean values.

Analytical determinations

The quality of olive oil and progress of lipid oxidation was assessed by measuring free acidity, peroxide value (PV), and specific extinction coefficient K_{232} . These quality indicators were determined according to European Union standard methods (Annexes II and IX in European Community Regulation EEC/2568/91).

Free acidity, given as % oleic acid, was determined by titration of an oil solution dissolved in ethanol/ether mixture with ethanolic solution of potassium hydroxide.

Peroxide value, given in milliequivalents of active oxygen per kilogram of oil (mequiv/kg), was determined as follows: a mixture of oil and chloroform/acetic acid 3:2 (v/v) was left to react in darkness with saturated potassium iodine solution; the free iodine was then titrated with a sodium thiosulfate solution.

The determination of the absorbance in the UV spectrum of the samples was measured at 232 nm as conjugated dienes according to standard methods (IUPAC, 1987), with minor modifications. Mayonnaise emulsions (0.15g) were dissolved in methanol (20 ml). The samples were centrifuged at 12 000 rpm for 5 min, and the absorbance of the supernants was measured. Two measurements were performed on duplicate samples, and the results were computed according to the following formula: $CD=A_{232}/c \times d$, where A is the absorbance reading at 232 nm, c denotes the concentration of the solution in gram per 100 ml and d is the length of the cell, in centimeters.

Sensory analysis

The sensory analysis was carried out in a sensory laboratory. Sensory evaluation was performed by a trained sensory panel consisting of

15 trained assessors. Panellists were trained in 2 h sessions prior to evaluation to be familiar with attributes and scaling procedures of food samples. Sensory evaluation was conducted on the samples after one day cold storage at 0°C to 4°C. The samples were tempered at 10°C±1°C before tasting. Each assessor was served representative mayonnaise samples of 5g placed on white plastic glass and labelled with a three-digit code at a temperature of 10°C±1°C. The performance conditions were standard, during day time, and under regular room temperature (20°C).

The following list of specific attributes and sensory descriptors was defined:

- **Appearance:** color (intensity of yellow color), brightness (mat to bright), emulsion stability (separate oil- stability);
- **Texture:** consistency (thin to thick), oiliness (watery to oily), homogeneity (homogenous to heterogeneous);
- **Smell** (weak to strong): olive, basil (for salad dressings) and parsley (for mayonnaise), rancid;
- **Taste** (weak to strong): salty, sour, bitter, chili, basil (for salad dressings) and parsley (for mayonnaise), rancid;
- **Aftertaste** (weak to strong): bitter, basil (for salad dressings) and parsley (for mayonnaise);
- **Overall acceptability:** bad to very good.

The analysis was performed by scoring attributes on a structural scale from 0 to 9 points, where higher score means more expressed attribute.

Each attribute had its own individual scale. After a statistical evaluation, the results were graphically presented, and sensory profiles were demonstrated.

Sensory characteristics: appearance, texture, smells, and taste were evaluated on a nine-point hedonic scale, with 1 being “dislike extremely” and 9 being “like extremely”.

Statistical analysis

All experiments were performed on duplicate samples. Statistical analysis was conducted with a software package implementing the one-way ANOVA method. Significant differences between means (at the level of $p < 0.01$) were determined using the Student's *t*-test.

Results and Discussion

Microbiological analysis

Results from microbiological analysis of natural spices and herbs and their extracts (Table 2) used for food emulsions production showed higher contamination with *Escherichia coli* counts of basil in comparison with ground hot paprika and black pepper. This herb had higher contamination with moulds, coliforms, other *Enterobacteria*, anaerobic sulfite-reducing bacteria counts, and other anaerobic bacteria. Small differences were found in yeasts, coagulase-positive and anaerobic sulfite-reducing bacteria counts of natural spices and herbs. The obtained results showed higher contamination with microorganisms in comparison with their natural extracts.

Table 2. Microbiological quality of spices and herbs and their extracts used for making of mayonnaise-like food products.

Microorganisms groups	Sample					
	Hot Paprika		Ground black pepper fruits		Dried basil herb	
	Row material	Extract	Row material	Extract	Row material	Extract
	CFU/g	CFU/cm ³	CFU/g	CFU/cm ³	CFU/g	CFU/cm ³
Aerobic colony count	6x10 ⁴	1x10 ²	1.7x10 ⁶	2.5x10 ²	5x10 ⁵	<10 ¹
Moulds	<10 ¹	<10 ¹	<10 ¹	<10 ¹	2.8x10 ²	<10 ¹
Yeasts	1x10 ²	<10 ¹	2x10 ³	<10 ¹	<10 ¹	<10 ¹
<i>Escherichia coli</i>	<10 ²	<10 ²	<10 ²	<10 ²	5.4x10 ³	<10 ²
<i>Salmonella</i>	ND	ND	ND	ND	ND	ND
Coliforms	<10 ²	<10 ²	<10 ²	<10 ²	1x10 ⁵	<10 ²
Other						
<i>Enterobacteria</i>	<10 ²	<10 ²	<10 ²	<10 ²	2x10 ⁵	<10 ²
Coagulase-positive staphylococci	10 ²	<10 ²	1.3x10 ³	<10 ²	2x10 ⁴	<10 ²
Anaerobic sulfite-reducing bacteria	7x10 ¹	<10 ²	8x10 ¹	<10 ¹	6x10 ²	<10 ¹
Anaerobic sulfite-reducing bacteria / vegetative cells /	<10 ¹	<10 ¹	<10 ¹	<10 ¹	1x10 ³	<10 ¹
Other anaerobic bacteria						
/vegetative cells/	<10 ¹	<10 ¹	<10 ¹	<10 ¹	1x10 ⁵	<10 ¹

ND – not detectable

Table 3. Microbiological quality of mayonnaise-like food products.

Microorganisms Groups	Sample			
	MO	MOE	SD	SDE
	CFU/g	CFU/g	CFU/g	CFU/g
Aerobic colony count	1×10^4	$< 1 \times 10^2$	1.7×10^3	$< 1 \times 10^2$
Moulds, Yeasts	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$
<i>Escherichia coli</i>	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
<i>Salmonella</i>	ND	ND	ND	ND
Coliforms	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
Coagulase-positive staphylococci	$< 10^2$	$< 10^2$	8×10^2	$< 10^2$
Anaerobic sulfite-reducing bacteria / vegetative cells/	$< 10^1$	$< 10^1$	1×10^1	$< 10^1$

ND = not detectable

The most significant difference between spices and their natural extracts was established for their aerobic colony counts. Aerobic colony counts of ground black paper and dried basil herb were 4 lg higher in comparison with their extracts. These results suggest the conclusion that microbiological contamination of spice extracts is significantly lower than that of the corresponding natural spices. As is evident from the results for aerobic colony counts, spices could be a significant source of microbial contamination for food emulsions. In contrast, spice extracts had significantly lower microbial contamination.

Evaluation of the effect of replacing spices and herbs with their natural extracts on the microbiological quality of food emulsions was performed by analysis of the microbial contamination of control samples (Table 3). According to many authors mayonnaise-like food products are considered safe in terms of microbiology stability. In this regard, a number of studies (Perales and Garsia, 1990; Hathcox et al., 1995) demonstrated microbiology safety of these foods. The observed bactericidal effect was due to the presence of preservatives and organic acids (Radford and Board, 1993). The investigated emulsion products were characterized by pH of 3.9, for all products.

Salad dressing was found to have high contamination in terms of aerobic colony counts, as well as of coagulase-positive staphylococci and anaerobic sulfite-reducing bacteria counts. This was due to the addition of dried basil and ground black pepper in salad dressing, which was responsible for an incoming contamination.

The obtained results showed that the same products, but with spice and herbs extracts, were significantly less contaminated microbially.

The results displayed in Table 2 and Table 3 show that replacing hot paprika, ground black

pepper fruits, and dried basil herb with their extracts improved significantly the microbiological quality of food emulsions.

Oxidative stability

The oxidative stability of olive oil and mayonnaise-like food products with olive oil and natural spices and herbs and their extracts were evaluated by means of measuring their acidity, free acidity, peroxide value (PV), and specific extinction coefficient K_{232} (K_{232}) during a storage period of twenty days.

Figure 1 shows increasing values of acidity in olive oil, as well as in other mayonnaise-like food products. Acidity is the most fundamental quality measurement of olive oil. It is the primary indicator of olive oil purity and freshness. The highest values of acidity were determined in mayonnaise with olive oil (MO), whereas the bulk olive oil had the lowest acidity. The acidity of mayonnaise with olive oil exceeded the acceptable value for the quality of olive oil (0.9). Regarding PV (Table 4), the same sample (MO) showed the highest values at the end of the storage period. When comparing samples with natural spices and herbs with those that contained extracts, the samples with extracts were more resistant to oxidation.

Numerous studies have considered the antioxidant effects of spices and herbs added to pure oil (Özcan, 2003; Yanishlieva et al., 2006), and have shown their efficiency. However, Frankel et al. (1994, 1996) studied the antioxidant activity in oils and food emulsions, and described difficulties in evaluating the effectiveness of antioxidants in different lipid systems and oxidation condition. The observed differences in antioxidative stability may be explained by the presence of a droplet membrane and interaction between the ingredients.

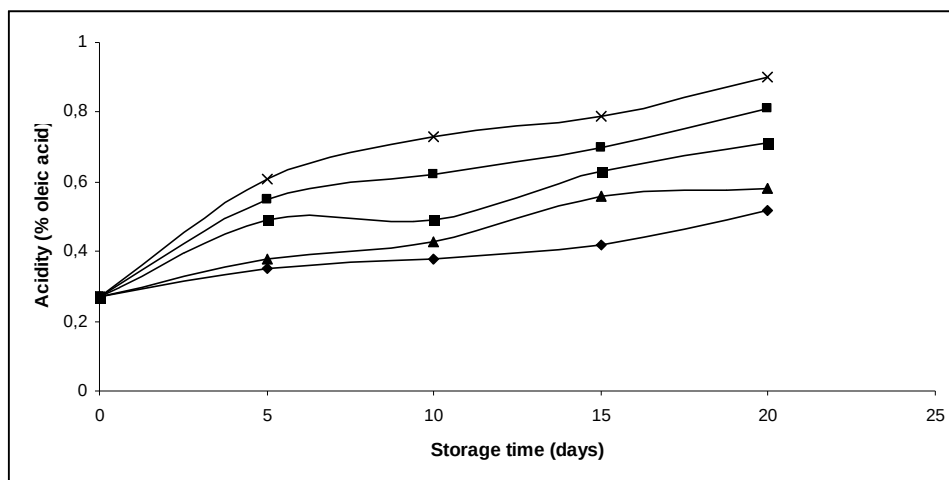


Figure 1. Changes in acidity over the storage period at 20°C in food emulsions with olive oil: olive oil (◆); salad dressing (■); salad dressing with extracts (▲); mayonnaise with olive oil (x); mayonnaise with olive oil and extracts (■).

Table 4. Changes in peroxide values over a storage period at 20°C in olive oil and mayonnaise-like food emulsions.

	PV (meq O ₂ /kg)				
	Day				
	0	5	10	15	20
Olive oil	1.2±0.02a	1.35±0.11a	7.23±0.23a	11.61±0.22a	13.64±0.21a
SD	1.2±0.02a	1.48±0.02b	8.95±0.22b	13.44±0.16b	15.21±0.07b
SDE	1.2±0.02a	1.45±0.07b	8.01±0.31c	12.50±0.29c	14.53±0.15c
MO	1.2±0.02a	2.62±0.16c	10.20±0.56d	14.87±0.10d	17.25±0.22d
MOE	1.2±0.02a	1.95±0.05d	10.03±0.16e	13.32±0.11b	16.26±0.13e

Mean ± SD Values in the same vertical column with different superscripts are significantly different (P < 0.01).

The measurement of the K_{232} coefficient was used to determine the level of conjugated dienes present in olive oil. The oxidation products of oils display characteristic spectra in the ultraviolet region and at 232 nm, and the determination of the K_{232} specific extinction coefficient is an indication of the state of oil oxidation. No significant differences ($p < 0.05$) were measured between the samples of olive oil, salad dressing and mayonnaise

with extracts during the storage period (Table 5). The K_{232} coefficient was slightly higher for MO in comparison with SD. However, by the 20th day, the values of this coefficient did not exceed the quality limit of 2.5. Clearly, in this relatively short period of study, it was not possible to measure significant processes of decomposition reactions in lipids, and therefore changes in the sensory properties in mayonnaise-like food products were not detected.

Table 5. Changes in specific extinction coefficient values (K_{232}) over the storage time at 20°C in olive oil and mayonnaise-like food emulsions.

	K_{232}				
	Day				
	0	5	10	15	20
Olive oil	1.5±0.14a	1.63±0.11a	1.81±0.07a	2.02±0.09a	2.24±0.07a
SD	1.5±0.14a	1.84±0.06b	1.96±0.07b	2.17±0.26b	2.36±0.29b
SDE	1.5±0.14a	1.65±0.13a	1.80±0.07a	2.08±0.05a	2.25±0.02a
MO	1.5±0.14a	1.87±0.11b	2.11±0.22c	2.33±0.13c	2.47±0.12c
MOE	1.5±0.14a	1.70±0.07a	1.92±0.13b	2.03±0.07a	2.22±0.12a

Mean ± SD Values in the same vertical column with different superscripts are significantly different (P < 0.05).

Sensory analysis

Figure 2 shows a graphic representation of the intensity of appearance and texture for the salad

dressing samples. Differences between the attributes of color and homogeneity were established. Although the recipe did not contain

eggs, the color of salad dressings was evaluated as yellowish. This color was perceived well by consumers, as it is typical for this type of food products. The actual reason is the typical yellow-green color of olive oil. The salad dressing with extracts was evaluated to have a more expressive, typical yellow color. Regarding the homogeneity of

the sample, the salad dressing with natural spices and herbs (SD) was perceived by the panelists as a product with more homogenous consistency. The panelists did not find differences between other attributes (oiliness, consistency, stability and brightness) of the evaluated samples.

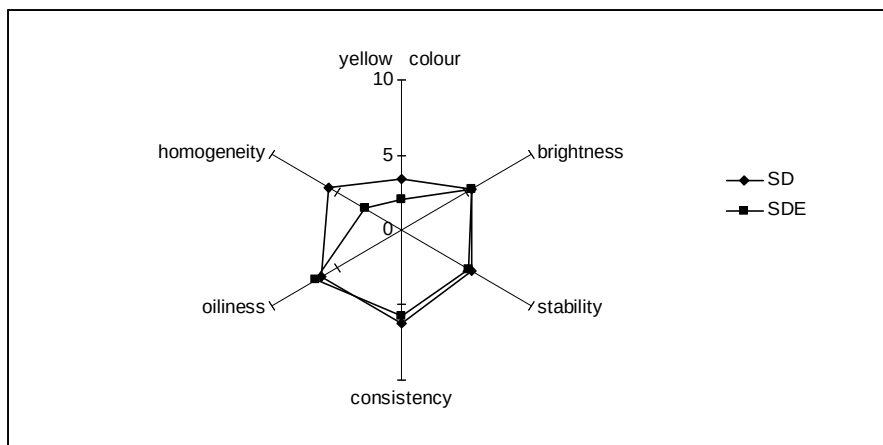


Figure 2. Sensory profiles of appearance and texture of salad dressing (SD) and salad dressing with extracts (SDE).

There was no significant difference among taste, smell, aftertaste profiles, and overall acceptability of the investigated food products

(Figure 3). The most noticeable difference was perceived in the attribute of basil aftertaste in the samples of salad dressing with extracts.

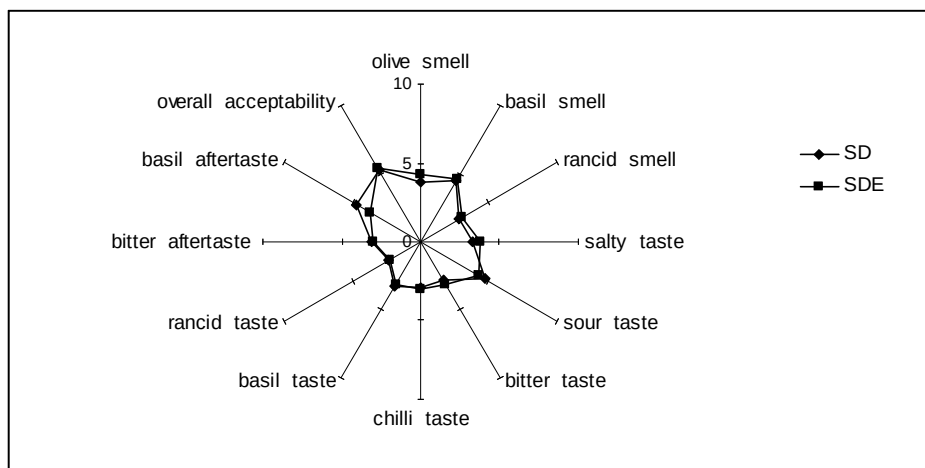


Figure 3. Sensory profiles of odor and taste of salad dressing (SD) and salad dressing with extracts (SDE).

The products with higher fat content mayonnaise with natural spices and herbs (MO) and their extracts (MOE) were evaluated as different

with respect to the attributes of homogeneity, color and brightness. The panelists determined MO to have high homogeneity and yellow color.

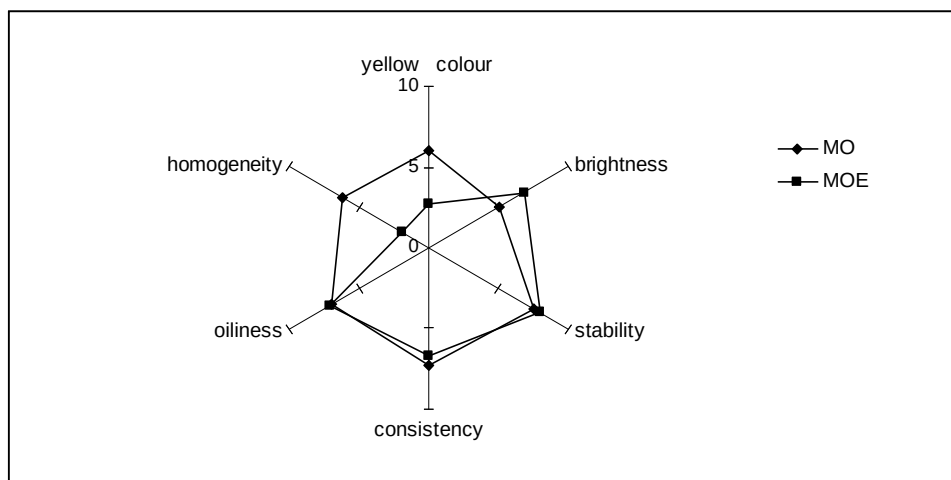


Figure 4. Sensory profiles of appearance and texture of mayonnaise (MO) and mayonnaise with extracts (MOE).

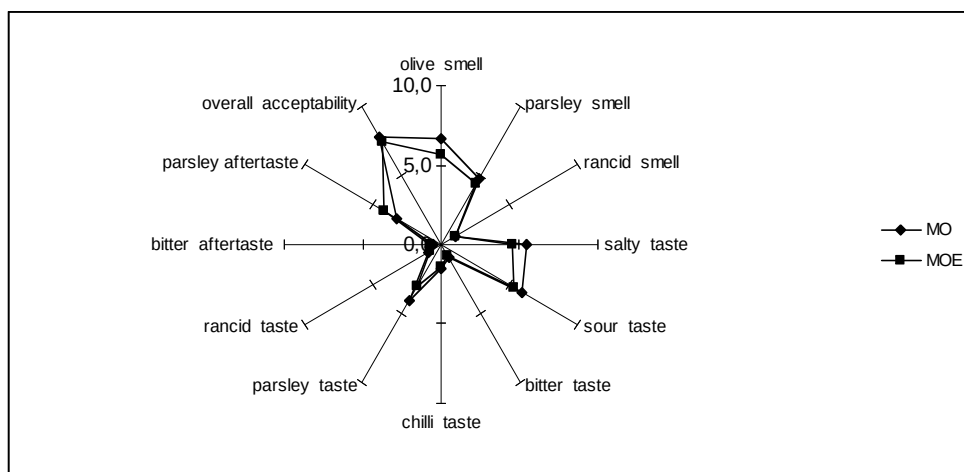


Figure 5. Sensory profiles of odor and taste of mayonnaise (MO) and mayonnaise with extracts (MOE).

The panelists did not perceive either rancid or bitter taste or bitter aftertaste, in spite of the high content of olive oil (50%) in the investigated

samples. The extract of parsley influenced the higher score of parsley aftertaste of MOE.

Results of sensory analysis by nine-point hedonic scale are shown in Table 6.

Table 6. Sensory scores for the degree of liking of mayonnaise-like food products.

Samples	Appearance	Texture	Smell	Taste
SD	5.8±0.35 ^a	6.1±0.27 ^a	6.1±0.51 ^a	5.4±0.37 ^a
SDE	5.8±0.44 ^a	6.2±0.35 ^a	6.0±0.64 ^a	5.8±0.44 ^b
MO	6.7±0.51 ^b	6.2±0.52 ^a	6.1±0.87 ^a	6.4±0.55 ^c
MOE	7.2±0.48 ^c	6.8±0.43 ^b	7.0±0.68 ^b	7.4±0.71 ^d

Mean ± SD Values in the same vertical column with different superscripts are significantly different ($P < 0.01$).

The lowest score for the degree of liking was reported for the appearance and taste of the salad dressings. With regard to the degree of liking of texture and smell, it was the same for both salad dressings and mayonnaise with natural spices. The mayonnaise-like product with spice extracts

showed the highest consumer acceptance and degree of liking. Izidoro et al. (2007) and Karas et al. (2002) found that standard mayonnaise as opposed to low-fat mayonnaise gained higher grades for most sensory attributes.

Conclusions

The focus of this study was to investigate mayonnaise-like food emulsions with extracts of herbs and spices, with emphasis on microbiological quality, oxidative stability, and sensory perception. The obtained results showed that replacing spices and herbs with their extracts improved significantly the microbiological quality of food emulsions. When spices were replaced by their extracts, pure olive oil, salad dressing and mayonnaise were less susceptible to oxidation, due to their high antioxidant activity. Food emulsions were found not to impact significantly various sensory descriptors. Mayonnaise with extracts had the highest score of the degree of liking for its sensory attributes.

The data show that new type of extracts of different spices and herbs could be used to improve the consumer acceptance and nutritional quality of food emulsions compared to traditional spicing. These extracts could be a viable technical and economical alternative to natural spices.

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NUTRITION AND FOOD SCIENCE

Textural and rheological properties of hydrolyzed Konjac Glucomannan and Kappa-Carrageenan: Effect of molecular weight, total content, pH and temperature on the mixed system gels

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Abstract

Rheological and textural properties of mixed gels of hydrolyzed Konjac glucomannan (KGM) and kappa-carrageenan (kC) were studied. The objective of the work was to investigate the effect of ratio, total content, molecular weight, pH and temperature of KGM on the mixed system gels. At 40% KGM the gel showed better hardness; whereas, the total content (1.0%-2.0% (w/w)) demonstrated that when it's higher, the hardness increased significantly ($P < 0.05$) and lowers the springiness. In addition, hardness values were relatively higher than would be expected for hydrolyzed KGM in comparison with native KGM and kC mixed system gels. It was found out that the hydrolyzed KGM (LM-2) exhibited better texture properties and suitable for the mixed system gels KGM/kC. The influence of pH shift on the hardness was stabilized after the pH5. The rheological properties, showed the significant temperature effect difference between kC alone and kC-LM-2 with $34 \pm 0.2^\circ\text{C}$ and $42 \pm 0.2^\circ\text{C}$, respectively, where the gels' moduli G' and G'' meet at frequency 10 rad/s and 1% strain. Although, KGM and kC mixed system gels behaviors are said to be influenced by the choice β -mannanase in the hydrolysis and preparation mechanisms.

Key words: Hydrolyzed KGM, kC, Mixed system gel, Texture, Rheological properties

Introduction

Konjac gum is a hydrocolloid gum derived from the tubers of *Araceae amorphophallus*, which comprises 60–70% konjac glucomannan. It has been recognized as GRAS (generally recognized as safe) by a consensus of scientific opinion since 1994 (Khanna and Tester, 2006; Case et al., 1992). It is widely utilised in areas of the food industry, chemical engineering and petroleum drilling (Al-Ghazzewi et al., 2007). KGM has been utilized as a raw material in the food and polymer industries in China since ancient times (He et al., 2011). KGM is regarded as a non-caloric food and in its traditional form is a source of indigestible high quality dietary fiber (Pan et al., 2011). However, health Canada advisory released a counter-indication stating that natural health products containing the ingredient glucomannan in tablet, capsule or powder form, which are currently on the Canadian market, have a potential for harm if taken without at least 8 ounces

of water or other fluid (Health Canada Advisory, 2010). As a result of its high viscosity, konjac gum is generally added to other hydrocolloids to increase the viscosity of blended systems and improve food quality (Yaseen et al., 2005). KGM can also be used for modification of carbohydrate in diabetes metabolism and also used in the preparation of composite materials, edible film, coating/packaging film, biodegradable film and controlled release matrix (Gao and Nishinari, 2004; Seleiman et al., 2010).

Polysaccharide, such as carrageenans are extracted from *Gigartina*, *Chondrus*, *Iridaea* and *Eucheuma* species. carrageenans can exist in forms of κ -, λ - and ι -; among which κ -carrageenan is characterised by its repeating disaccharide units of 3-linked- β -D-galactose-4-sulfate and 4-linked-3,6 anhydro- α -D-galactose. κ -carrageenan have the ability to form a gel in the presence of specific cations such as potassium (Morris and Robinson, 1980).

Food systems can contain complex mixtures of ingredients, some of which are added to control viscosity, form a gel or act as water binding agents such as proteins and polysaccharides (Abhyankar and Auty, 2010). Texture and rheological properties

Received 03 December 2011; Revised 16 February 2012;
Accepted 21 February 2012

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of starch-based products are normally improved by hydrocolloids (Shi and BeMiller, 2002; Salehifar and Shahedi, 2007). Generally, rheological properties of gels depend on the molecular structure of the gelling agent. However, this can be achieved through the use of small quantities of hydrocolloids, products modification to earn higher viscosity. Usually, κ -carrageenan is deficient in hardness, springiness, water holding capacity and it is also fragile (Nishinari and Watase, 1983; Powell et al., 1982) markedly affects gel properties.

Natural polymers are known to have better biocompatibility, biodegradability, non-toxicity and easily modified ability than various synthetic materials; thus, more researches have focused on natural polymer-based hydrogels (Figuly et al., 1997). Kohyama et al. (1996) reported that KGM being a neutral polysaccharide with few acetyl groups, ionic effects on various KGM molecules was minimal, though, addition of hydrolyzed KGM to kC have been an improvement to weakness of kC alone gel properties. Previous researches in this domain have shown the effect of higher molecular weight distribution of KGM on kC gel (Nishinari and Watase, 1983; Abhyankar and Auty, 2010). Therefore, the objective of this study was to evaluate the textural and rheological properties of β -mannanase hydrolyzed KGM and kC mixed system gels as influence by system ratio, total content, molecular weight, pH and temperature.

Materials and Methods

Materials

Powdered KGM from Konjac tuber and three fractions of KGM with different molecular weights prepared by an enzymatic degradation, were kindly supplied by Jiecheng Food Additive co. Ltd (Shantou, China). The native KGM (ND) was treated with an enzyme (β -mannanase, Xiasheng Co. Ltd. Ningxia, China) for different reaction times at optimum temperature. And LM-1, LM-2, LM-3 fractions with low molecular weights were obtained. All other chemicals were obtained from the chemical reagent Co. (China) and were of food grade quality.

Methods

The kC used in this study contained substantial amount of Potassium 79.2mg/g and Sodium 43.6mg/g. the contents of minerals in both ND and hydrolysed KGM were far behind to that of kC (Table 1 (supplementary information)), this clearly indicates that their minerals content have no or little influence on the kC gel properties, even though minerals mainly affect the elastic modulus of gels. Konjac glucomannan (ND and LM-2) and kC gels

were prepared at pH 3.0-9.0 using Na_2HPO_4 -Citric acid buffer with 40% KGM and total content (1.5% (w/w)).

Table 1. Minerals analysis for native konjac glucomannan (ND) Hydrolyzed KGM (LM-2) and kappa-carrageenan (kC).

	LM-2	ND	KC
Sample			
Fe ($\mu\text{g/g}$)	18.7	25.4	<1.0
Na (mg/g)	1.64	1.94	43.6
K (mg/g)	1.97	2.33	79.2
Mg (mg/g)	4.29	5.43	7.45
Ca (mg/g)	3.69	3.82	6.57

Molecular weight determination

Molecular weight of each KGM fraction was determined by High Performance Gel Filtration Chromatography (HPGFC) under the chromatographic condition of Column: Ultrahydrogel™ Linear 300mm×7.8mm mid×2 Mobile phase: 1mol/L NaNO_3 Flow: 0.9 ml/min Column temperature: 45°C. Sample preparation: Samples were dissolved in mobile phase, filtered with a microfiltration membrane for injection and the standards used in molecule weight calibration curve were: MW2000000, MW133800, MW21400, and MW250.

Texture and springiness measurement

The KGM solution was prepared by stirring mixed KGM powder with water continuously at the room temperature. Considering the ratio and the total content, κ -carrageenan was added into the solution and the mixture was heated in a water bath at $75\pm1^\circ\text{C}$ for a certain time (according to experimental design) with continuous stirring. Then the gel stands at room temperature for twelve hours.

The texture was determined using the Texture Profile Analysis method (three replicates per treatment) using TA.XTplus Texture Analyzer (Lotun Science Co. Ltd, Beijing, China). Samples were compressed with a P/50 probe at pre-test speed: 2.0 mm/s test speed: 1.0 mm/s post-test speed: 5.0 mm/s. The deformation level was 40% of the original sample height and the gels were compressed twice.

Hardness was expressed as the maximum force exerted during the first compression cycle. Springiness was determined by adaptation of the AACC (1986) method into a 'Hold Until Time' test by using the Texture Analyzer.

Rheological measurement

The sample was prepared like in the texture measurement. KGM and kC were mixed and the

total content was adjusted to 1.0% (w/w). Rheological measurements were performed using an AR-G2 Rotary Rheometer from TA Company of USA equipped with cone plate geometry of 40 mm diameter (cone angle 4°, gap 108 micro m), at various temperatures and frequencies in the linear viscoelastic regime. The surface of the sample was covered with silicone oil in order to prevent drying.

Strain sweep tests were performed from 0.1% to 100% at standard frequency of 10 rad/s at

25±1°C (Figure 1) and used for subsequent analysis in this linear regime. The frequency sweeps tests were performed from 0.1 to 100 rad/s of angular frequency, at a strain of 1% in the 25±1°C. Lastly, the temperature sweep tests was carried out by heated the gel from 20 to 75±1°C with 1.0°C/min increment using 1% strain and the 10 rad/s frequency. Experimental data of G' and G'' were recorded.

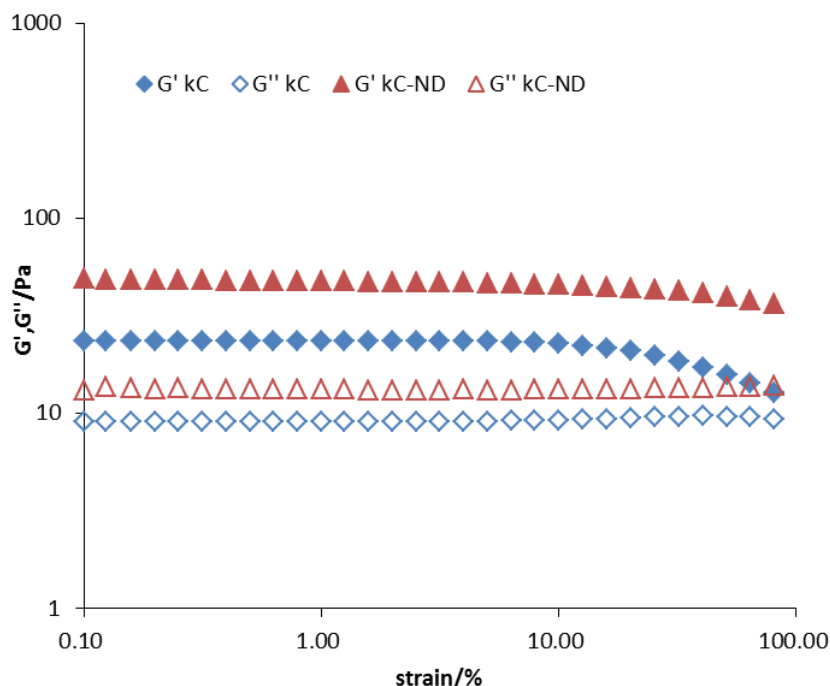


Figure 1. Strain dependence of storage modulus G' and loss modulus G'' for 10 rad/s frequency at 25±1°C.

Statistical analysis

The experimental results were evaluated by ANOVA and Tukey's multiple tests using SPSS statistical software, Version 18.0, USA. The statistical analyses were carried out within the 95% confidence interval.

Results and Discussion

Textural studies revealed that hydrolyzed KGM with different molecular weights exhibited different gelation characteristics. Our study showed that hydrolyzed KGM (LM-2) has significant textural properties against its counterpart molecular weight distribution LM-1, LM-3. Figure 1 shows that the moduli G' of kC alone and kC-ND remain larger than their G'' , therefore, the mixture system constituents can produce good gel with 40% KGM and 1.0% (w/w) total content.

Effect of Konjac glucomannan concentration and molecular weight on the mixed system gel

Table 2 compares the hardness and springiness of different parameters obtained by variation of KGM concentration using ND and LM-2 both selected for comparing the best molecular weight of hydrolyzed KGM (LM-2) to its native form (ND). At 40% the hardness of kC-ND and kC-LM-2 was the highest, moreover, the springiness was the lowest (Table 2). Although, the variation of KGM in gel making decreased rather than increased the texture and viscoelasticity after 40% due to the kC-KGM ratio molecular interaction, it made a major contribution to the textural properties of the gel. The springiness was inversely proportional to hardness. The kC-LM-2 exhibited higher textural and appearance than its counterpart kC-ND.

Kohyama et al. (1996) reported that the junction zone in the mixed gels was formed only by kC molecules. In other words, KGM creates weak junction zones which contribute to different rheological characteristics. Therefore, the higher

the molecular weight, the longer the KGM chain. The increase in molecular weight of KGM increases the number of weak junction zones and makes the contour length of flexible chains which connect junction zones longer.

Table 2. Effect of Konjac glucomannan (KGM) concentration on gel properties.

Concentration	0%	10%	20%	30%	40%	50%	60%	70%	80%
Hardness (g)									
ND	410.6±32.4 ^g	1934.0±107.8 ^c	2134.6±82.5 ^{bc}	2336.5±115.7 ^b	2762.8±04.4 ^a	1539.0±7.7 ^d	1057.6±84.2 ^e	769.6±84.9 ^f	393.1±40.7 ^g
LM-2	410.6±32.4 ^g	2163.1±139.0 ^d	2325.8±7.3 ^{dc}	2634.6±131.6 ^c	3156.6±71.2 ^b	2090.9±42.0 ^d	1429.5±45.5 ^e	1052.7±12.4 ^f	428.5±27.2 ^g
Springiness									
ND	0.92±0.003 ^d	0.890±0.004 ^e	0.887±0.002 ^e	0.871±0.002 ^{fg}	0.865±0.003 ^g	0.874±0.002 ^f	0.919±0.003 ^d	0.949±0.003 ^c	0.960±0.002 ^b
LM-2	0.92±0.003 ^c	0.882±0.003 ^e	0.884±0.001 ^e	0.864±0.004 ^f	0.857±0.002 ^g	0.867±0.002 ^f	0.906±0.002 ^d	0.950±0.002 ^c	0.957±0.001 ^b

ND: non-degraded Konjac glucomannan, LM-2: hydrolyzed Konjac glucomannan, total content: 1.5% (w/w), heating temperature: 75±1°C for 20 minutes.

Data is expressed as mean ± standard deviation of three determinations. Mean with different letters within the row are significantly different ($P < 0.05$).

Thus, the hardness of the gel with bigger molecular weight KGM will be higher than the smaller molecular weight KGM. Figure 2 (supplementary information) shows that LM-2 has the highest hardness and closely the slowest springiness which lays our choice when compares to others fractions of hydrolyzed KGM. Indeed, the KGM chain were been shortened through this β -mannanase hydrolysis. This is probably due to the enzyme and pH factors that change the structure of the mixed gels (Chen et al., 2006; Li et al., 2004).

The experimental data of frequency dependence (G') and (G'') moduli for with 1% strain at 25±1°C of kC alone, kC-ND and kC-LM-2 KGM at frequency consistency obtained by Mixolab is shown in Figure 3. The values of G' remained higher than G'' throughout the frequency range indicating a better gel structure. G' and G'' values increased with increasing angular frequency from 1 to 100 rad/s. The slight increase noticed for kC can be attributed to the absence of binding agents; however, kC-ND and kC-LM-2 KGM are

more stable. Hence, the modulus G' was larger than G'' throughout the experimentally-accessible frequency range and both moduli were little dependent on frequency showing a linear regime (Kohyama et al., 1996; Penroj et al., 2005).

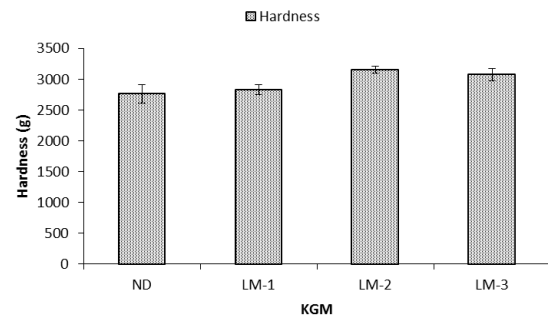


Figure 2. Effect of Konjac glucomannan (KGM) molecular weight on the gel hardness properties.

Table 3. Effect of total content on the final gel properties.

Total Content	1.0%	1.2%	1.4%	1.6%	1.8%	2.0%
Hardness (g)						
ND	739.7±77.1 ^f	1568.4±157.2 ^e	2341.7±89.3 ^d	2957.4±98.0 ^c	3510.6±59.1 ^b	4020.5±146.4 ^a
LM-2	1120.8±77.5 ^f	1855.8±101.2 ^e	2513.8±71.5 ^d	3311.6±55.4 ^c	3827.5±111.5 ^b	4397.8±67.3 ^a
Springiness						
ND	0.913±0.002 ^a	0.897±0.003 ^b	0.889±0.002 ^c	0.876±0.003 ^d	0.864±0.002 ^e	0.849±0.003 ^f
LM-2	0.902±0.002 ^a	0.898±0.001 ^b	0.884±0.002 ^c	0.869±0.002 ^d	0.858±0.003 ^e	0.843±0.004 ^f

ND: non-degraded Konjac glucomannan, LM-2: hydrolysed Konjac glucomannan, KGM concentration: 40%, heating temperature: 75±1°C for 20 minutes.

Data is expressed as mean ± standard deviation of three determinations. Mean with different letters within the row are significantly different ($P < 0.05$).

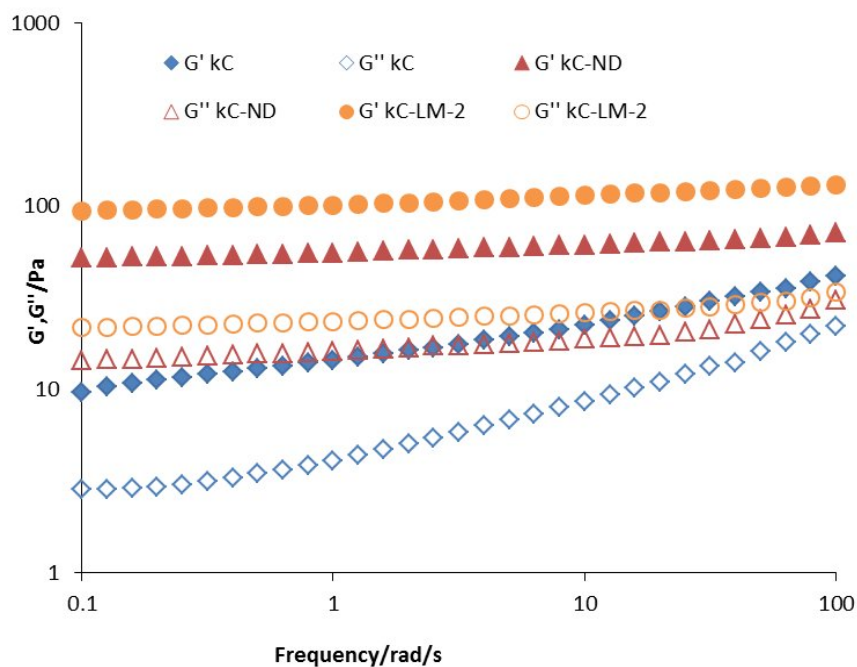


Figure 3. Frequency dependence of storage modulus G' and lost modulus G'' at 1% strain at $25\pm 1^\circ\text{C}$.

Effect of total content on the gel properties

Textural determination of KGM mixed with kC revealed significant ($P<0.05$) increase in hardness with increase of total content and conversely, showed significant ($P<0.05$) decrease in springiness (Table 3).

Table 3 shows that both hydrolyzed KGM and native KGM observed the same behavior with addition of total content in the mixed gel preparation. Native KGM is known as a gum with very high viscosity (Nishinari et al., 1992; Jacon et al., 1993). The enzyme treatment increased the ratio of lower molecular weight polymers, however, the hydrolyzed fraction LM-2 showed higher hardness than the native KGM and slightly lower springiness than the ND (Table 3). Previous studies such as x-ray diffraction have demonstrated that there is weak interaction between kappa carrageenan and KGM mixture molecules (Cheetham and Mashimba, 1988). Furthermore, it was concluded that KGM was not incorporated into kappa carrageenan crystalline junction zones. This phenomenon could be caused by the gelation rate of native KGM due to the high viscosity, which seems to inhibit the aggregation of the molecules and further delay the formation of junction zones.

Effect of temperature difference on the gel moduli

The weak gel behavior is the intermediate rheological behavior between that of the concentrated polymer solution and that of the elastic or true gel. True gels are formed by a sufficient number of junction zones in kC solutions in the presence of sufficient salts (Nishinari et al., 1996; Penroj et al., 2005). Throughout, the temperature heating, the behavior was typical of a concentrated polymer solution and those curves (G' and G'') were less dependent on temperature after $50\pm 0.2^\circ\text{C}$ as it is shown in Figure 4. The kC-LM-2 gel exhibited significant G' at early temperature rising but at higher temperature (Figure 4) the G' drop drastically leaving the G'' larger than G' , having a melting point of $42\pm 2^\circ\text{C}$. Therefore, this implies the temperature dependence on the gel properties. Moreover, similar behaviors were observed in the mixture systems kC-ND and kC gel alone where the kC modulus G' dropped sharply at earlier temperature of $28\pm 0.2^\circ\text{C}$ and $G'=G''$ at $34\pm 0.2^\circ\text{C}$ which implies the kC melting point. This data corroborate with previous findings reported by Nishinari et al. (1996).

Effect of pH on the gel hardness

The samples hardness increases up to pH 5 and then began to decrease slightly and stabilized until pH 9.0 (Figure 5). The variation of Konjac glucomannan (ND and LM-2) and kC gels properties at pH 3.0-9.0 indicate the influence of pH on the gel preparation. Meng et al. (2003) reported that kappa-carrageenan is stable in the alkalinity and neutral environment, and start to hydrolyze below pH 4, thus, the hardness of the gel

become lower. The pH shift is a significant factor in the stability of mixed gels. Our results corroborate with the work of Li et al. (2004) in their study on the kinetics for enzymatic degradation of KGM. Pan et al. (2011) concluded that the alkalinity of modifiers is a dominant factor for deacetylation of KGM, which led to improvement of gel characteristics, such as swelling property, viscous stability and thermal stability.

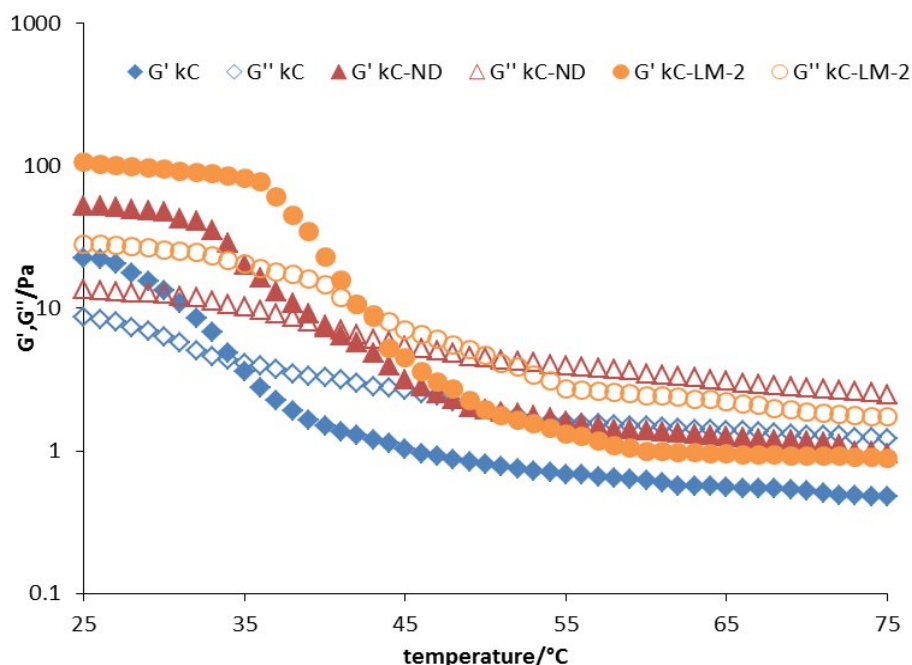


Figure 4. Temperature dependence of storage modulus G' and loss G'' during the heating process for 10 rad/s frequency and 1% strain.

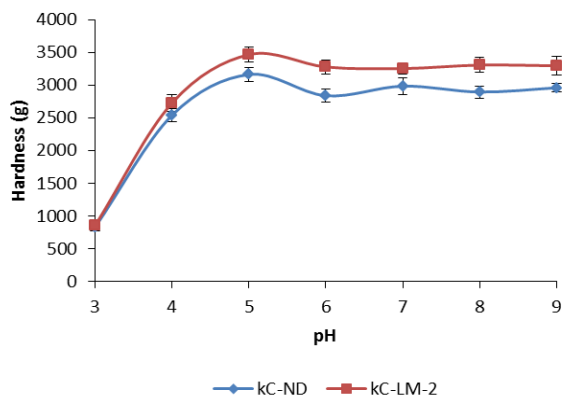


Figure 5. Effect of pH on the gel mixture system (kC-ND and kC-LM-2) hardness properties.

Effect of time at different pH on the gel hardness

The treatments were carried out with 1.5% (w/w) total content, 40% KGM concentration at $75 \pm 1^\circ\text{C}$, as shown in the Table 4, the hardness was the lowest at the pH 4 and highest at the pH 5 with the same time. Both kC-ND and kC-LM-2 exhibited the highest hardness at the time 20 minutes, when time increases the hardness reduces especially at pH 4, because the kC will be more hydrolyzed. This reflects the ability of kappa carrageenan and hydrolyzed KGM gel formation in respect with the time attributed to a segregate interaction between the two biopolymers. The possibility that the difference in hardness of the gel could be explained by the fact that difference in time scales of measurement (Penroj, 2005).

Table 4. Effect of time at different pH on the gel hardness (g) properties.

Time (min)	10	20	30	40	50
kC-ND					
pH=4.0	1995.8±187.4 ^d	1837.4±103.2 ^{dc}	1662.5±73.2 ^c	1449.9±51.8 ^b	1134.2±53.1 ^a
pH=5.0	2269.3±132.0 ^a	2962.6±65.7 ^{bc}	3189.4±87.7 ^{cb}	3059.8±107.2 ^{cb}	2944.9±67.5 ^{bc}
pH=6.0	2374.7±96.6 ^a	2937.8±84.0 ^b	3185.7±78.7 ^c	3105.4±68.8 ^{bc}	3073.8±73.2 ^b
LM-2					
pH=4.0	2186.5±163.0 ^{ba}	2726.9±91.9 ^{dc}	2511.9±52.6 ^d	2277.3±108.2 ^d	2027.2±90.8 ^c
pH=5.0	2539.7±142.3 ^a	3465.9±41.8 ^a	3321.4±57.2 ^a	3295.7±121.2 ^a	3184.5±176.8 ^a
pH=6.0	2452.4±115.9 ^a	3277.3±55.2 ^{ba}	3225.6±80.9 ^{ba}	3194.0±71.0 ^{ba}	3152.9±93.6 ^a

ND: non-degraded Konjac glucomannan, LM-2: hydrolyzed Konjac glucomannan total content: 1.5% (w/w), KGM concentration: 40%, heating temperature: 75±1°C.

Data is expressed as mean ± standard deviation of three determinations. Mean with different letters within the row are significantly different (P < 0.05).

Conclusion

The effect the ratio of konjac glucomannan (KGM) and kappa carrageenan (kC), total gel content, molecular weight, pH and temperature on the gelation are responsible for system kC and KGM texture and rheological properties. The increase in hardness of LM-2 molecular weight KGM compare to Non-Degraded KGM was likely due to enzyme used in the hydrolysis of native KGM, and the springiness were found to be inversely proportional to the hardness. The kC-LM-2 was found to be the prominent gel for our texture properties. Further studies are needed on specific effect of β -mannanase on this mixed system gel and its application is now being undertaken in our laboratory.

Acknowledgement

Thanks to the financial support by Guangdong provincial ministry of Science & Technology with the project collaboration which involve production, teaching & research (No. 2010B090400293), and thanks to Guangdong Shantou Jiecheng Food Additives Co. Ltd for providing free raw material.

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PLANT SCIENCE

Phytoremediation potential of aquatic macrophyte *Azolla caroliniana* with references to zinc plating effluent

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Abstract

The effect of zinc plating industrial effluent on some biochemical parameters and the phytoremediation capacity of floating aquatic macrophyte *Azolla caroliniana* was assessed. The effects of increasing zinc concentrations on growth characteristics of *A. caroliniana* namely root length, leaf area and biomass production was studied. The root growth of *A. caroliniana* was severely affected at higher concentration of zinc effluent (especially at 3 and 4%). Overall slight fluctuations in the moisture content represent the favorable nature of this essential micronutrient especially at 1% and 2% concentration. Maximum chlorophyll contents were observed at 2% concentration of the effluent with an increase by 41.29% over the control. The least amount of carotenoid was observed at 4% concentration. Polyphenol content of the fronds of *A. caroliniana* was lowest at 4% while higher accumulation was observed at 2% effluent concentration. The level of proline in the leaves of *A. caroliniana* was considerably increased under the influence of zinc especially at 1, 2 and 3% of the effluent concentration. It was also seen that electrical conductivity (EC) and total dissolved solids (TDS), content of the various dilution were decreased remarkably after 10 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 plant growing in the tray. The decrease in hardness, acidity, sodium and potassium content of the effluent were observed with increase in concentration of the effluent. Values for biological oxygen demand (BOD) were decreased in the effluent concentration of 2% while the decrease in chemical oxygen demand (COD) was maximum in the same effluent concentration after 10 days treatment period. Zinc was accumulated in increasing order in the plants treated with increasing concentration of zinc. The bio concentration factor (BCF) values were increased serially as per increasing concentration of zinc plating effluent and all the plants growing in these concentrations showed higher accumulation capacities. 4% of the effluent recorded with higher BCF while minimum for control treated biomass was observed. In the present study increasing BCF shows that the *A. caroliniana* has capacity to accumulate the Zn^{++} through liquid medium after ten days of exposure period.

Key words: *Azolla caroliniana*, Bioaccumulation, Biochemical and effluent parameters, Bioconcentration factor, Heavy metals, Phytoremediation

Introduction

Rapid industrialization and urbanization have resulted in elevated levels of heavy metals in the biosphere (Lu et al., 2004). Toxic levels of some heavy metals appear as a result of environmental pollution due to removal from mining, automobile traffic, smelting, manufacturing and agricultural wastes (Oncel et al., 2000). Over recent decades,

the annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al., 2003). Chemical methods, to effectively decrease heavy metals to acceptable levels require a large excess of chemicals, which increase the costs because of generating the voluminous sludge (Spearot and Peck, 1984). Aquatic macrophytes play an important role in structural and functional aspects of aquatic ecosystems by various ways. The ability to take up heavy metals makes them interesting research candidates especially for the treatment of industrial effluents and sewage waters (Andra et al., 2010). Zinc compounds are widely used in industry to make paint, rubber, dyes, wood preservatives and

Received 28 November 2011; Revised 04 February 2012;
Accepted 21 February 2012

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ointments. Some amount of zinc is also released into the environment by natural processes, but most comes from human activities like mining, steel production, coal burning and waste burning (Andra et al., 2010). Zinc plays an important role in many biochemical reactions within the plants (Lu et al., 2004). Plants under stressful conditions adapt very differently from one another, even from a plant living in the same area (Mane et al., 2010). Phytotechnologies involving use of plants for pollutant removal is relatively a new approach and has gained importance during the last two decades (Dhir, 2010). The use of plants to degrade, assimilate, metabolize or detoxify contaminants is cost-effective and ecologically sound. Phytoremediation is rapidly gaining interest and promises in effective and inexpensive cleanup of hazardous waste sites contaminated with metals, hydrocarbons, pesticides and chlorinated solvents (Kumar and Chandra, 2004; Liao and Chang, 2004; Saratale et al., 2011). Several plants species, such as water lettuce (*Pistia stratiotes*) (Mishra et al., 2008), water lilies (*Nymphaea spontanea*) (Choo et al., 2006), parrot feather (*Myriophyllum aquaticum*), creeping primrose (*Ludwigia palustris*), and watermint (*Mentha aquatic*) (Kamal et al., 2004) have been studied to determine their potential in accumulating heavy metals.

Azolla caroliniana is a small aquatic fern which freely floats on the water surface but do not have roots embedded in sediment; it freely move with wind and water currents. It contains a symbiotic cyanobacterium-anabaena azolae within its leaf cavities. The *Azolla* macrophyte called frond ranges from 1.0-2.5 cm length in species *Azolla pinnata* and to 15 cm or largest, in *Azolla nitolica*. *Azolla* is available in Indian water bodies, where it is possible to use it in wastewater treatment by setting wastewater treatment ponds and by the way of ecofriendly technique. The N-fixing capacity of *Anabaena azolae* enables *Azolla* to thrive on nitrogen-free waters. It has been used as a fertilizer in botanical gardens because of nitrogen-fixing capability (Peters and Meeks, 1989). For several decades *Azolla* has been used as a green manure in rice fields. On the other hand, it has negative effects on the aquatic ecology due to its capable of colonizing rapidly to form dense mats over water surfaces (Ashton and Walmsley, 1976). *Azolla* has been shown to effectively adsorb hexavalent and trivalent chromium, zinc and nickel from solutions and electroplating effluent (Zhao et al., 1999) and gold from aqueous solution (Antunes et al., 2001). *Azolla* utilization in wastewater treatment to remove contaminants, particularly phosphorus, was

also suggested by some authors (Fisher, 1988). *Azolla* is unique among floating macrophytes, because it can grow in waters devoid of combined nitrogen, due to the symbiosis with a N_2 fixing cyanobacterium, *Anabaena azollae* that lives in the dorsal lobe cavity of its leaf. So, this plant can grow even after the exhaustion of combined nitrogen in secondary effluents, improving an adequate phosphorus removal (Kitoh et al., 1993).

The present investigation demonstrates phytoremediation potential of *Azolla* exposed to zinc electroplating effluent. The effect was studied with reference to selected biochemical parameters and physico-chemical parameters of effluent. Growth characteristics, chlorophyll, carotenoid, polyphenol and proline content were studied so as to know the changes associated with the experimental species. Effluent quality analysis was carried out to assess the natural ability to treat the effluent. This was assessed with reference to electrical conductivity (EC), pH, total dissolved solids (TDS), total solids (TS), total suspended solids (TSS), turbidity, free CO_2 , chemical oxygen demand (COD), biological oxygen demand (BOD), acidity, hardness, sodium and potassium content. Accumulation of zinc (Zn^{++}) was estimated to find out the extent of accumulation by *Azolla* from the effluent. Bioconcentration factor was also estimated to make out a clear conclusion about the phytoremediation potential of *Azolla* for the electroplating effluent at various concentrations.

Materials and methods

Materials

Zinc plating effluent was collected from MIDC Chikhli, Pune ($20^{\circ}19'17''N$ and $76^{\circ}15'36''E$). Fresh *Azolla* was collected from the surface of a water body near Damodar Nagar, Pune ($18^{\circ}28'28''N$ and $73^{\circ}49'53''E$) and cultured in a nutrient medium. Healthy mature plants (ten days old) were used for further study and estimation of biochemical and physico-chemical tests. Experiment was performed on a known weight basis in 500 ml plastic containers. As the effluent was much toxic and concentrated, lower dilutions were used for the experimental purpose. *Azolla* plants were exposed to tap water (control) and under elevated concentration of zinc plating effluent (1%, 2%, 3% and 4%). Plants were harvested after ten days of exposure for growth characteristics namely root length, leaf area, wet and dry biomass, moisture content, chlorophyll, carotenoid, polyphenol and proline content. The chemicals used in this study were of the highest purity available and of the analytical grade.

Estimation of photosynthetic pigments

Chlorophyll content

The chlorophylls of the mature fronds were estimated following the method suggested by Arnon et al. (1949) with slight modification. Fresh plant material (1g) was homogenized in mortar by keeping the temperature at 2°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. Final volume of the filtrate was made to 100 ml with 90% acetone. Absorbance of the extract 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.

$$\text{Chlorophyll 'a'} = X = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{Chlorophyll 'b'} = Y = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{Total chlorophyll (a + b)} = Z = 8.02 \times A_{663} + 20.20 \times A_{645}$$

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

Carotenoid content

The carotenoid content of the *A. caroliniana* fronds was determined from the same extract used for chlorophyll estimation, by recording the absorbance at 480 nm. The carotenoids content were calculated by using the following formula (Kirk and Allen, 1965).

$$\text{Carotenoids content (mg/100g)} = \frac{A_{480} \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}}$$

Polyphenol content

The polyphenol content of the treated *A. caroliniana* fronds was estimated following the method suggested by Folin and Denis (1915). 2 ml of acetone extract used for chlorophyll was mixed with 10ml of 20% sodium carbonate and final volume was adjusted to 35 ml with distilled water, to this mixture 2 ml of Folin-Denis reagent was added, mixed thoroughly and final volume was made 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.

Proline content

Proline was estimated from the oven dried *A. caroliniana* fronds, following the method described by Bates et al. (1973). The plant material was homogenized in 10 ml, 3% sulfosalicylic acid and

the extract was filtered through Whatman No.1 filter paper. For assay, 0.5 ml of the filtrate was mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin reagent. The contents were boiled for 1 h. After cooling, 4 ml of toluene was added to test tube with vigorous shaking and the absorbance of toluene chromophore was recorded at 520 nm against toluene as blank. Standard curve of proline (0.1 mg ml⁻¹) was prepared by taking different concentrations of L-proline. From this standard curve, the proline content of different parts was calculated.

Growth characteristics

Ten fronds of *A. caroliniana* from each treatment tray were carefully removed, washed thoroughly with water to remove any dirt and dust particles and blotted to surface dry for the measurement of leaf area. Same material was used for the measurement of root length while completely grown material of each tray was removed carefully, blotted and used to estimate wet, dry biomass and moisture content.

Water quality analysis

Following parameters were determined from the diluted solutions of effluent taken out from plastic trays after 10 days. The methods used for analysis were in consistent with the standard methods mentioned in 'Handbook of Water Analysis' (Maiti, 2004).

Electrical conductivity (EC), total solids, total suspended solids and total dissolved solids (TS, TSS and TDS)

Electrical conductivity and total dissolved solids of the diluted effluent concentrations namely 1%, 2%, 3% and 4% were determined by using ELICO EC-TDS meter (CM 183, Make-India) where electrode was directly dipped into the respective solutions for the direct display of result on a digital scale. Total solids were determined by gravimetric method and then suspended solids were calculated by using equation $TS = TDS + TSS$

Turbidity and pH

Turbidity of the sample was determined by using CL 52D ELICO Nephelometer while pH of the samples was recorded by using ELICO LI 127 pH meter.

Sodium (Na) and potassium (K) content

The sodium and potassium content of treated and untreated samples was determined with the help of Systronics-128 flame photometer. 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. Each sample was aspirated into a flame in the form of a fine spray under carefully controlled excitation. After recording readings a standard graph of absorbance vs concentration was plotted to estimate sodium and potassium contents.

Acidity

For determining acidity of the samples, 50 ml of content was mixed with 2-3 drops of methyl orange. In all the samples, methyl orange acidity was absent then the same content was titrated against 0.02N NaOH with the addition of 2-3 drops of phenolphthalein to obtain pink end point.

$$\text{Acidity (mg/L)} = \frac{\text{ml of 0.02 N NaOH} \times 1000}{\text{ml of sample}}$$

Total hardness

The total hardness of the water samples was determined by EDTA titration method where 50 ml of well mixed sample was mixed with 1-2 ml buffer of pH 10 and a pinch of Eriochrome black-T indicator. The contents were then titrated with 0.01M EDTA till wine red solution changes to blue.

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

Where C=ml of EDTA for titration, D= mg of CaCO₃ equivalent to 1ml of EDTA

Chemical oxygen demand (COD)

COD determination was carried out with dichromate reflux method with the addition of 10 ml of 0.25N potassium dichromate (K₂Cr₂O₇) and 30 ml H₂SO₄+Ag₂SO₄ 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 K₂Cr₂O₇ remained was titrated with ferrous ammonium sulphate (FAS) using ferroin indicator.

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

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 at 27°C. 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 of 300 ml capacity. After determining initial dissolved oxygen (DO) final DO was estimated from the bottles kept for incubation period for three days.

Zinc (Zn⁺⁺) concentration

Zn⁺⁺ concentration from the dried *A. caroliniana* plant material (frond and root

combined). 1g oven dried plant material was acid digested as per the standard method suggested by Toth et al. (1948). Plant material was mixed with 10 ml concentrated nitric acid and then heated until all the material was completely dissolved. After cooling, 10 ml of perchloric acid was added and again heated until the solution became colorless and reduced to about 10%. After cooling, it was diluted to 100 ml with distilled water. Filtrate was used for analysis of Zn⁺⁺ by using atomic absorption spectrophotometer (Perkin-Elmer, 3030A). Water samples from treated and untreated trays were directly used after filtration for the metal analysis.

Bioconcentration factor (BCF)

The bioconcentration factor provides an index of the ability of plant to accumulate metal with respect to metal concentration in substrate. The bioconcentration factor was calculated as the ratio of trace element concentration in plant tissues at harvest to the concentration of element in the external environment (Zayed et al., 1998). The BCF was calculated by using formula

$$\text{BCF} = P / E$$

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).

Percent removal efficiency

It was calculated by using following formula

$$\text{Percent removal efficiency} = \frac{\text{inlet pollutants} - \text{outlet pollutants}}{\text{inlet pollutants}} \times 100$$

Statistical analysis

Statistical analysis of the data was carried out by using Graph Pad software. Mean standard deviation (SD) and percent variation was calculated. 'One Way Analysis of Variance' (ANOVA) was tested in order to check the statistical difference among the means. For the same Tukey-Kramer multiple comparison test of significance was carried out which suggested the variation among the column means is significant or not at different levels of significance. The data was analyzed for three different levels of significance based on the 'p' values as,

- * Significant (p = 0.01 to 0.05),
- ** Very Significant (p = 0.001 to 0.01) and
- *** Extremely Significant (p < 0.001)

Results and discussion

Physico-chemical analysis of zinc plating effluent was carried out with respect to selected parameters (Table 1). The concentration of zinc (Zn⁺⁺) was observed to be at higher levels as expected and was 751.65 mg/l. The effluent was

acidic with higher electrical conductivity, in addition with increased levels of TDS, TSS and in turn total solids. Sodium and potassium content of the samples were also much higher and might be due to the salts used in the various processing units for the electroplating. In general, the chemical constituents and dissolved contents were higher and are directly responsible for increase in COD and BOD values of the untreated effluent.

Table 1. Characteristics of untreated zinc plating industrial effluent.

Sr. No.	Parameter	Value
1)	pH	3.11 (± 0.11)
2)	EC	7740.12 (± 12.73)
3)	TDS	4330.34 (± 10.64)
4)	TSS	320.45 (± 8.25)
5)	TS	4650.79 (± 14.41)
6)	Turbidity	9.9 (± 0.31)
7)	Hardness	1250.45 (± 5.61)
8)	Acidity	1680.71 (± 6.38)
9)	Na	550.51 (± 6.13)
10)	K	240.5.56 (± 11.42)
11)	Zn	751.65 (± 4.82)
12)	Fe	22.32 (± 1.42)
13)	COD	1164.23 (± 16.57)
14)	BOD	232.41 (± 13.45)

Values are mean of three observations with $\pm$ SD in parenthesis, expressed in mg/l except pH unitless, EC in μ S and turbidity in NTU.

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, frond area and biomass production in terms of fresh and dry weight. The analysis of growth characteristics indicates that *Azolla* can grow quite well in treated urban wastewater, having growth rates and

productivities great enough to encourage its utilization as biofilter for nutrient removal and biomass production (Zhao et al., 1999). Alicia et al. (1994) reported that roots of some aquatic plants could retain both coarse and fine particulate organic materials present in water bodies supporting their growth.

Root length

Maximum root length was observed the biomass grown at 2% of the effluent with 4.5 cm while minimum was observed as 1.9 cm in the tray supplied with 4% zinc plating effluent (Table 2). The root length was also observed to be decreased with the increase in concentration of zinc in the sets provided with 2, 3 and 4% of the effluent which directly indicates the negative effect of such higher concentration of zinc present in hydroponics. Schat et al. (1996) had reported that Zn toxicity in plants was associated with reduced root growth of fronds. It has been reported that the non-lethal concentration of Zn^{++} shows an inhibitory effect on cell division in root tips of *Nigella sativa* and *Triticum aestivum* (El-Ghamery et al. 2003). Literature survey showed that the heavy metals accumulations in water hyacinth increased linearly with the solution concentration in the order of leaves<stems<roots (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. From the present investigation, it is clear that the root growth of *A. caroliniana* was sensitive and severely affected at higher concentration of zinc (especially at 3 and 4%) which might be due to higher uptake of Zn^{++} and harmful nature of effluent.

Table 2. Effect of zinc plating effluent on selected growth parameters of *A. caroliniana* (after 10 days treatment).

Parameter	Zinc plating effluent concentration %				
	Control	1	2	3	4
Root length (cm)	4.3 (0.19) 0.0 ^a	4.5* (± 0.25) 4.65	4.1*** (± 0.29) -4.65	2.4** (± 0.15) -44.19	1.9(± 0.34) -55.81
Frond area (cm ²)	4.5(0.11) 0.0 ^a	4.7(± 0.45) 4.44	4.4*(± 0.34) -2.22	3.7***(± 0.24) -17.78	3.2*** (± 0.09) -28.89
Fresh weigh (g)	4.040.0 ^a	4.99**23.51	5.65*39.85	4.09*** 1.24	3.85*** -4.70
Dry weigh (g)	1.320.0 ^a	1.35*2.27	1.5114.39	1.54** 16.67	1.64* 24.24
Moisture content (%)	67.40.0 ^a	68.57***1.74	73.2**8.61	62.32* -7.54	59.90*** -11.13

^a The values indicates percentage variation relative to initial values. Values are mean with $\pm$ SD. Significantly different from the control at *P<0.05, **P<0.01 ***P<0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Frond area

It is evident from the results that the frond area was 4.7 cm² in 1% zinc effluent and was maximum while minimum was observed at 4% effluent by a value of 3.2 cm² 10th day of experiment. It was slightly decreased at 2% of effluent in the potting medium. Average frond area also showed decreasing trend with the increasing concentrations of zinc in the growing medium similar to root length. The mechanism of plants behind for growth reduction is not clear to maintain residual growth for short and long term responses (Munns, 2003). The growing grass leaf represents an inherent combination of growth, developmental and micro-environmental zones and this combination can be used to identify signaling events, which are involved in the regulation of growth and growth responses to stress (Fricke et al., 2006). From the present results it is clear that frond area affects at higher percent of effluent and might be associated with higher levels of zinc in the potting medium.

Biomass

Fresh weight

It is well known that aquatic biomass irrespective living or dead, exhibits capacity to remove heavy metals from wastewater. In the present study, maximum fresh weight as 5.65g was recorded in 2% effluent concentration while minimum fresh weight as 4.11g in the tray supplied with 4% concentration of zinc plating effluent (Table 2). The increase in the fresh weight of the plants at lower concentrations i.e. at 1% and 2% of Zn plating industrial effluent might be due to favorable nature of this essential element in plant growth. The reduction in biomass production is associated with chlorosis and necrosis of the leaves

that reduce the photosynthetically active area (DeHerralde et al., 1998).

Dry weight

Maximum dry weight was observed at 4% concentration when whole biomass was weighed after drying. It was 1.64g while minimum dry weight was 1.32g in control tray. Increase in dry weight of the experimental species showed perfect positive correlation with the increase in the concentration of the effluent. Jarunee et al. (2003) observed the inhibition in dry weight of shoots and roots in *Sesbania rostrata* while Jain et al. (1990) found that the addition of Zn⁺⁺ at low concentration had a favorable effect on growth of water hyacinth, which may be attributed to the favorable nature of Zn⁺⁺ as micronutrient for the growth. In the present study an increase in dry weight of *A. caroliniana* might be due to accumulation of zinc and its favour by the azolla biomass as a micronutrient.

Moisture content

It is seen from the Table 2 that the moisture content was not much affected and increased at 1 and 2% concentration of effluent. There was slight decrease with the increased concentration later on. Maximum moisture content was observed by 73.20% in 2% of the effluent at the end of the treatment whereas minimum moisture content was 59.90% at 4% effluent in the tray. The decrease in the moisture content at 4% concentration shows that prolonged and concentrated exposure had a negative effect on the biomass growth. Overall slight fluctuations in the moisture content represent the favorable nature of this essential micronutrient especially at 1% and 2% concentration.

Table 3. Effect of zinc on chlorophyll content of *A. caroliniana*.

Chlorophyll Content	Zinc plating effluent concentration %				
	Control	1	2	3	4
Chl. 'a'	34.56 (±1.02) 0.0 ^a	40.14** (±2.21) 16.15	57.72*** (±1.98) 67.01	34.50*** (±2.15) -0.17	33.27* (±0.95) -3.73
Chl. 'b'	25.98 (±2.15) 0.0 ^a	28.8* (±3.01) 10.85	27.87** (±2.57) 7.07	25.5*** (±3.29) -1.85	23.52*** (±3.51) -9.47
Total Chl.	60.54 (±3.24) 0.0 ^a	68.94*** (±3.42) 13.88	85.54*** (±2.58) 41.29	60.0*** (±2.49) -0.89	56.79*** (±3.81) -6.19
Chl. 'a' : Chl 'b'	1.33** (±0.029) 0.0 ^a	1.39** (±0.024) 4.77	2.08* (±0.21) 55.99	1.35*** (±0.032) 1.71	1.41*** (±0.035) 6.34

^a The values indicates percentage variation relative to initial values. Values are mean of three experiments and are expressed in mg 100-1g of fresh tissue with ±SD. Significantly different from the control at *P<0.05, **P<0.01 ***P<0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Biochemical parameters

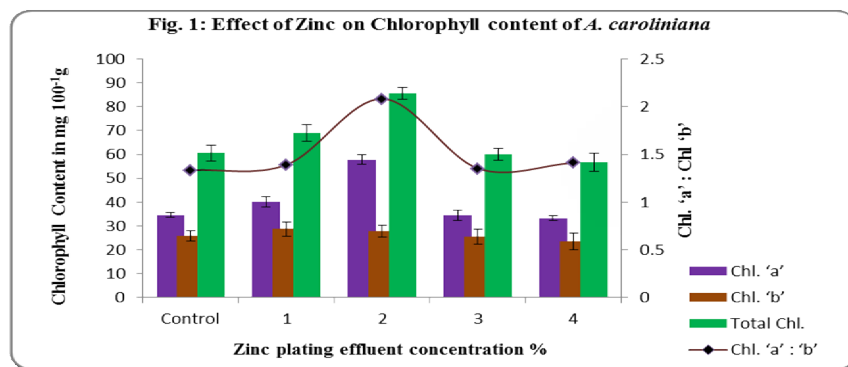
Effect of zinc on chlorophyll content of *A. caroliniana*

The effect of zinc samples on chlorophyll content of mature fronds of *A. caroliniana* is shown

in Table 3 and Figure 1. It is evident from the results that the total chlorophyll content of the mature fronds was increased considerably due to increasing concentrations upto 2% of zinc plating effluent but later on decreased with further increase

in concentrations. The decrease in chlorophyll contents at 3 and 4% was not remarkable; it was slight as compared to the control set. Maximum chlorophyll content was 85.54 mg 100⁻¹g was recorded at 2% effluent concentration while minimum chlorophyll content was observed at 4% concentration by a value of 56.79 mg 100⁻¹g. In general chlorophyll 'a' appears to be dominant over chlorophyll 'b', this fact was observed to be true even in case of elevated levels of zinc plating effluent. The ratio was not much affected and was increased markedly by a value of 2.08 at 2% concentration of the effluent which shows the favorable nature of the zinc present in the hydroponics. Thus, it is quite clear that the higher salt concentration (4%) is not much negatively influential on 'a': 'b' ratio. 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). Our results on chlorophyll pigments are in accordance with the findings of Mishra et al. (2007). Similarly, concentration dependent reduction in chlorophyll content over control was also observed in the leaves of *Lycopersicon esculentum* (Gaubha et al., 2007). The slight decrease in chlorophyll content at 3 and 4% effluent in *A. caroliniana* might be due to accumulation of Zn⁺⁺ and other toxic effluent constituents. The increase in total chlorophyll content in fronds of *A. caroliniana* might be due to osmotic adjustment mechanism developed by experimental species. The total chlorophyll content decreased as the concentration further increased from 2% to 4%.



Effect of zinc on carotenoid content of *A. caroliniana*

According to Armstrong (1996) 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), which is not accessible by the chlorophylls and pass this excitation energy to chlorophyll molecules. They are also one of the non-enzymatic antioxidants along with vitamin C, vitamin E and lipoic acid and play an important role in the protection against oxidative stress (Kojo, 2004). Kenneth et al. (2000) reported that an increase in carotenoid content was observed in Cr treated plants of *E. crassipes* and such an increase for the protection from free radical formation is a common response to xenobiotics. From the results it is clear that the carotenoid content of the fronds of *A. caroliniana* showed a decreasing trend but it was not much remarkable

and was directly proportional to the increase in the concentration of effluent in the liquid medium (Table 4 and Fig. 2). Chen and Djuric (2001) were of the opinion that decrease in carotenoid content is a regular response to stress condition due to heavy metals. Maximum amount of carotenoid content was observed with a value of 25.2 mg 100⁻¹g in the fronds grown in control pot while least carotenoid content was observed at 4% concentration with 21.28 mg 100⁻¹g. It is clear that total carotenoid content of the fronds of *A. caroliniana* was reduced due to the increasing zinc concentration in the pots and proved to be a negative photo-oxidative impact while a slight increase at 4% but below the control, might resist the toxic nature of effluent.

Effect of zinc stress on levels of polyphenol content of *A. caroliniana*

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, increased levels of polyphenols especially at 1 and 2% of effluent might have induced accumulation of secondary metabolites in the experimental species to tolerate the stress condition while decrease at 4% level might be due to damage in polyphenol production process. Polyphenol content of the fronds of *A. caroliniana* was lowest by 144.27 mg 100⁻¹ g at 4% while higher accumulation was observed at 2% effluent concentration by a value of 334.89 mg 100⁻¹g (Table 4 and Figure 2). Increase in polyphenols of the fronds of *A. caroliniana* at all concentrations of the zinc plating effluent might be related to the modified tolerance mechanism adopted by the plants for overall growth and development.

Effect of zinc stress on levels of proline content of *A. caroliniana*

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). Proline accumulation is considered to be involved in stress resistance mechanisms (Lutts et al., 1999). It is clear from the results (Table 4 and Fig. 2) that the level of proline in the leaves of *A. caroliniana* was considerably increased under the influence of zinc especially at 1, 2 and 3% of the electroplating effluent. Maximum increase (79.06%) in proline content was observed by 14.54 mg 100⁻¹ g at 1% of the effluent concentration and the minimum (-12.32%) proline content was observed by a value of 7.12 mg 100⁻¹ g at 4% of effluent concentration. It is evident that in general proline accumulates in plants exposed to various environmental stresses and is not an exception to toxicity stress of zinc plating effluent at various concentrations. The increased levels of proline in the leaves of *A. caroliniana* might be able to maintain osmoregulation and conservation of energy for a stress developed due to zinc containing effluent while decrease at 4% concentration indicates the toxicity of the effluent.

Table 4. Effect of zinc plating effluent on carotenoid, polyphenol and proline content of *A. caroliniana*.

Parameter	Zinc plating effluent (%)				
	Control	1%	2%	3%	4%
Carotenoid	25.2(±2.58) 0.0 ^a	24.08*(±3.04) -4.44	23.44***(±3.61) -6.98	22.0*** (±3.46) -12.70	21.28** (±1.59) -15.56
Polyphenols	156.24(±6.32) 0.0 ^a	334.89** (±11.45) 114.34	305.24* (±8.78) 95.37	164.78*** (±4.92) 5.47	144.27* (±3.78) -7.366
Proline	8.12(±1.48) 0.0 ^a	14.54** (±1.35) 79.06	9.64*** (±0.98) 18.72	8.58* (±1.05) 5.67	7.12*** (±0.86) -12.32

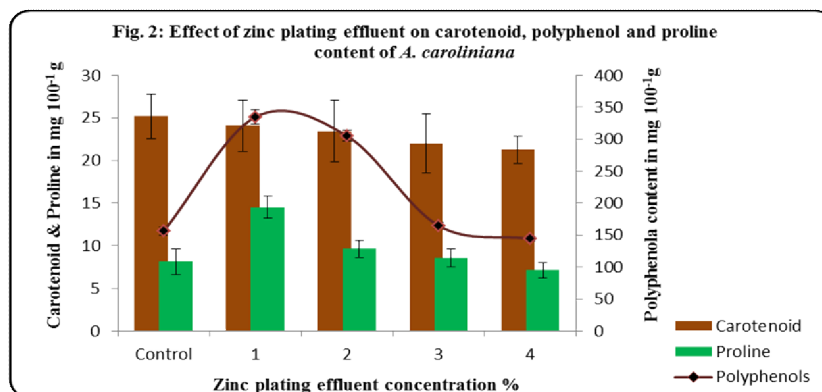
^a The values indicate percentage variation relative to initial values. Values are mean of three experiments and are expressed in mg 100⁻¹g of fresh tissue with ±SD. Significantly different from the control at *P<0.05, **P<0.01 ***P<0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Effect on water quality parameters

Electrical conductivity (EC) and total dissolved solids (TDS)

It is evident from the Table 5 that the EC of the samples was decreased after 10 days of treatment in all trays. Maximum reduction was observed by in control set (-18.34) followed by 2% of effluent concentration (-16.65). This clearly shows the capacity of *A. caroliniana* to treat the effluent. Mahmood et al. (2005) also observed the reduction conductivity due to absorption of pollutants by plants. TDS content of the samples was also decreased after 10 days treatment period where it was maximally reduced in control set followed by 2% and 3% of the effluent. Even in 4% of the

effluent the TDS content was reduced by 29.68%. 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. The reduction of TS was due to the retaining coarse and fine particulate organic materials present in water bodies supporting their growth by the root system while TSS content was reduced in all the cases after the retention time of 15 days (Ghaly, 2004). A good reduction (90%) of total suspended solids by constructed wetland plants with a retention time of 7 days was reported (Amelia, 2001).



pH and turbidity

It is evident from the Table 5 that the pH of samples decreased with the increase concentration of effluent initially on day zero but when compared to final values with respective percentage of the solutions the values tend towards higher pH. The minimum pH was observed as 4.45 at 4% concentration on zero day while maximum pH as 5.91 was observed in the same tray after 10 days of treatment with *Azolla*. It clearly indicates that the experimental species try to grow and maintain the pH towards neutral which might be due to the root secretions produced to avoid negative effect of toxins. Optimum growth of *Azolla* in culture solution is in pH range of 4.5-7, but *Azolla* can

survive in pH 3.5-10 if all essential elements are available (Ashton and Walmsley, 1974). Wagner (1997) found that at water temperature of 25 °C, both *A. pinnata* and *A. filiculoides* showed maximum growth at pH values from 5-7. Watanabe (1984) reported that *Azolla* growth was decreased by increasing pH level at low iron concentration. 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 (Galun, 1987). The pH was reduced from alkaline to nearly neutral by treatment with aquatic macrophytes (Mahmood et al., 2005).

Table 5. Reduction in electrical conductivity (EC), total dissolved solids (TDS), pH and turbidity of zinc plating effluent grown with *A. caroliniana* after 10 days.

Zinc plating effluent(%)	EC (μS)		TDS (mg/l)		pH		Turbidity (NTU)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control	299.21 (±2.62) 0.0 ^a	244.34 (±2.61) -18.34	149.59 (±1.62) 0.0 ^a	89.47 (±0.56) -40.19	5.25 (±0.21) 0.0 ^a	5.29 (±0.15) 0.76	0.18 (±0.08) 0.0 ^a	1.23 (±0.02) 583.33
1	356.52* (±3.25) 0.0 ^a	312.64*** (±3.12) -12.31	179.58** (±1.48) 0.0 ^a	119.61* (±0.48) -33.39	5.17** (±0.15) 0.0 ^a	5.37*** (±0.19) 3.87	1.3* (±0.07) 0.0 ^a	6.26 (±0.03) 381.54
2	416.86** (±2.65) 0.0 ^a	347.45* (±3.32) -16.65	207.61 (±2.12) 0.0 ^a	137.48** (±0.98) -33.78	5.08** (±0.24) 0.0 ^a	5.36* (±0.25) 5.51	1.2** (±0.08) 0.0 ^a	6.17** (±0.03) 414.17
3	480.45** (±3.14) 0.0 ^a	411.19*** (±2.65) -14.42	239.78** (±2.17) 0.0 ^a	174.95*** (±0.75) -27.04	4.57*** (±0.24) 0.0 ^a	5.73** (±0.29) 25.38	1.8** (±0.05) 0.0 ^a	7.19*** (±0.04) 299.44
4	622.94*** (±2.85) 0.0 ^a	570.94*** (±2.46) -8.39	313.92*** (±1.04) 0.0 ^a	220.75* (±0.71) -29.68	4.45*** (±0.11) 0.0 ^a	5.91*** (±0.31) 32.81	1.9* (±0.04) 0.0 ^a	7.73** (±0.05) 306.84

^a The values indicate percentage variation relative to initial values. Values are mean of three experiments and with ±SD. Significantly different from the control at *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Similarly it is evident from the Table 5 that the turbidity of samples increased with increase in

concentration of zinc in samples. The turbidity was observed to be increased on 10th day as compared to

its values on 0th day. The minimum turbidity observed was 0.18 NTU for control sample on 0 day while the maximum turbidity observed was 7.37 NTU at 4% sample on 10th day. The turbidity increased considerably on 10th day as compared to the turbidity on 0th day and it continued following the increasing trend with increase in zinc concentration in samples (Figure 2). The increase in turbidity of the samples after 10 days of treatment with *Azolla* might be due to the secretions and fall of dead biomass in solution of pots. On the other hand Gudekar and Trivedi (1989) reported 59.54% reduction of turbidity in treatment of engineering industry waste with water hyacinth.

Hardness and acidity

Table 6. Reduction in hardness, acidity, sodium and potassium of zinc plating effluent grown with *A. caroliniana* (after 10 days).

Zinc Concentration (%)	Hardness		Acidity		Na		K	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control	47.95	30.15	55.16	40.49	11.71	11.19	7.21	4.56
	(2.89)	(1.23)	(1.56)	(2.56)	(0.56)	(0.82)	(0.48)	(0.76)
	0.0 ^a	-37.12	0.0 ^a	-26.60	0.0 ^a	-4.44	0.0 ^a	-36.75
1%	69.46**	45.65**	64.24*	50.65**	13.25	11.98*	9.75*	7.93
	(3.65)	(2.56)	(2.89)	(1.64)	(1.02)	(1.05)	(0.98)	(0.48)
	0.0 ^a	-34.28	0.0 ^a	-21.16	0.0 ^a	-9.58	0.0 ^a	-18.67
2%	84.64**	70.36*	73.48***	60.84*	14.73**	12.43**	11.54***	8.74***
	(4.56)	(3.15)	(1.57)	(3.45)	(0.99)	(0.84)	(0.49)	(0.47)
	0.0 ^a	-16.87	0.0 ^a	-17.20	0.0 ^a	-15.61	0.0 ^a	-24.26
3%	107.78*	90.26***	105.19***	80.41***	16.27***	13.75*	17.88**	11.26**
	(3.48)	(2.48)	(3.42)	(3.56)	(1.15)	(0.21)	(0.69)	(0.43)
	0.0 ^a	-16.26	0.0 ^a	-23.56	0.0 ^a	-15.49	0.0 ^a	-37.02
4%	134.49***	113.91**	117.75**	100.56***	23.16*	18.76***	36.42*	17.34***
	(4.64)	(3.48)	(4.15)	(3.46)	(1.43)	(0.53)	(1.49)	(1.14)
	0.0 ^a	-15.30	0.0 ^a	-14.60	0.0 ^a	-19.0	0.0 ^a	-25.39

^a The values indicates percentage variation relative to initial values. Values are mean of three experiments and expressed in mg/l with $\pm$ SD. Significantly different from the control at

*P<0.05, **P<0.01 ***P< 0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Sodium (Na⁺) and potassium (K⁺) content

Sodium content of the samples also decreased after 10 days of treatment with *Azolla*. It was obvious that Na⁺ content was in increasing trend from 1 to 4% on zero day and 10th day as there was external addition of the effluent (Table 6). On the other hand it is also clear that the experimental species has ability to treat Na⁺ and K⁺ from electroplating effluent. Maximum reduction for Na⁺ content was observed at 4% of the sample and was 19% while for the K⁺, the same concentration was treated efficiently by *Azolla* biomass with 25.39% reduction as compared to zero day concentration. Overall decrease in initial concentration of Na⁺ and K⁺ indicate that *Azolla* has better ability of survival under toxic effluent. In short, floating macrophyte *Azolla* is one of the efficient candidates for sodium

and potassium removal. A reduction of 56% of sodium and 99.39 per cent potassium respectively with 100 per cent and 25 per cent concentration of textile industry waste treated with *Eichhornia crassipes* after four days of treatment had been recorded by Trivedi and Gudekar (1987). Water hyacinth (*Eichhornia crassipes*), is a floating macrophytes whose appetite for nutrients and explosive growth rate has been put to use in cleaning up municipal and agriculture wastewater (Gupta, 1980). In the present study, sodium and potassium content of effluent treated with *Azolla* is normal and as per earlier studies (Lumpkin and Plucknett, 1980). Moreover, it was determined that more growth of biomass was nearly led to more removal of metals (Khosravi, 2005).

Table 7. Reduction in BOD and COD of zinc plating effluent grown with *A. caroliniana* (after 10 days).

Electroplating effluent %	BOD		COD	
	Initial	Final	Initial	Final
Control	1.6	1.4	4.5	4.2
	(±0.15)	(±0.16)	(±0.29)	(±0.86)
	0.0 ^a	-12.50	0.0 ^a	-6.67
1	3.4*	2.9*	7.8	6.2**
	(±0.18)	(±0.18)	(±0.37)	(±0.67)
	0.0 ^a	-14.71	0.0 ^a	-20.51
2	4.8***	3.9***	11.6**	7.8***
	(±0.2)	(±0.19)	(±0.54)	(±1.01)
	0.0 ^a	-18.75	0.0 ^a	-32.76
3	5.2**	4.6***	18.9***	15.6
	(±0.18)	(±0.28)	(±1.24)	(±1.04)
	0.0 ^a	-11.54	0.0 ^a	-17.46
4	6.7***	5.9**	22.6***	16.8***
	(±0.24)	(±0.24)	(±1.28)	(±1.32)
	0.0 ^a	-11.94	0.0 ^a	-25.66

^a The values indicates percentage variation relative to initial values. Values are mean of three experiments and expressed in mg/l with ±SD. Significantly different from the control at *P<0.05, **P<0.01 ***P< 0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Biological oxygen demand and chemical oxygen demand

Marked reduction in BOD and COD of all final treated samples was observed when compared with zero day values (Table 7). Value for BOD was decreased maximally by 18.75% in the effluent concentration of 2% while the decrease in COD was by 32.76% in the same effluent concentration after 10 days treatment period. Greater reduction in values of COD was observed as compared to BOD values and might be due to the more amounts of dissolved minerals present in the samples than organic matter. Significance decrease in concentration of BOD was observed by various scientists when the effluents from various industries were treated with constructed wetlands. Different effluent samples from *Eichhornia* sp., *Salvinia* sp., *Pistia* sp. and *Typha* sp. showed marked reduction in BOD where maximum reduction of BOD in undiluted effluent was with *Eichhornia* sp. based CWs (490.9%) followed by *Typha* sp. based CWs (400%) (Santos et al., 1987). An evaluation of ten systems utilizing surface flow wetlands by Conley (1991) showed BOD removal rates ranged from 64 to 96%. Kirzhner et al. (2008) working on phytoremediation reported that BOD of industrial

effluents was removed by 65 to 70% after four days of retention period by floating macrophytes.

Zimmels et al. (2006) noticed 360% reduction in COD in a pilot study with *Eichhornia crassipes* and *Pistia stratiotes* for treatment of urban sewage in Israel. Zhang et al. (2007) reported that the efficiency of COD removal varied a lot for various species to different contaminants. In case of undiluted effluent, *Typha* sp. based CWs removed maximum amount of COD. Treatment of textile dye using anaerobic baffled reactor by wetland plants removed 70-90% COD (Bell and Buckley, 2003). The reduction in COD and BOD can result in an increase in dissolved oxygen concentration of wastewater (Brix, 1998) and is an important outcome in relation with the species used to treat the effluent.

Zinc accumulation and bio-concentration factor (BCF) in the *A. caroliniana*

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). An increase in zinc (Zn^{++}) accumulation in *A. caroliniana* with increasing concentrations of zinc in the hydroponics was observed (Table 8). The maximum accumulation of Zn^{++} by *A. caroliniana* was 5610 mg/kg at 2% of the effluent concentration while it was minimum at 4% of effluent concentration by a value of 1420 mg/kg. In the present study increase in concentration of zinc in *A. caroliniana* at elevated levels might be due to their response to favor metal tolerance and involvement in maintenance of plant growth for overall development. Kumar et al. (1989) have also investigated elemental composition of certain aquatic plants by EDAX and found high level of heavy metals such as Al, Si, Mn and Fe accumulated in *Vallisneria spiralis*, *Hydrilla verticillata* and *Azolla pinnata*. Accumulation of 110, 841 and 1260 mg Zn^{++} /kg (dry mass) was recorded by Khosravi et al. (2005) in *Azolla filiculoides*.

Table 8. Accumulation of zinc in *A. caroliniana* at various treatments and their effect on bio-concentration factor.

Sr. No.	Zn ⁺⁺ content	Zinc concentrations (%)				
		Control	1%	2%	3%	4%
1	Initial concentration (mg/l)	3.7(±0.23) 0.0 ^a	7.68*(±0.24) 107.57	11.58**(±0.86) 212.97	17.42***(±0.48) 370.81	20.18*(±1.01) 445.41
2	Residual Water 'x' (mg/l)	2.61(±0.03) 0.0 ^a	3.06(±0.05) 17.24	4.11***(±0.05) 57.47	1.18***(±0.02) -54.47	0.52***(±0.01) -80.08
3	<i>A. caroliniana</i> 'y' (mg/kg)	1816 (±7.09) 0.0 ^a	3616*** (±12.66) 99.12	5610***(±5.0) 208.92	3108**(±7.37) 71.15	1420**(±0.58) -21.81
4	Bio-concentration factor (x/y)	696.85(±7.83) 0.0 ^a	1183.35*** (±23.45) 69.81	1365.13** (±18.84) 95.90	2641.97*** (±38.51) 279.13	2732.08*** (±52.54) 292.06

a The values indicates percentage variation relative to initial values. Values are mean of three experiments and expressed in mg/l with ±SD. Significantly different from the control at P<0.05, **P<0.01 ***P<0.001 by one-way ANOVA with Tukey-Kramer multiple comparisons test.

Bioconcentration factor (BCF) is a useful parameter to evaluate potential of the plants in accumulating metals and this value is calculated on a dry weight basis. The change in BCF of *A. caroliniana* was also studied in present investigation to know capacity of *A. caroliniana* to concentrate zinc from varied effluent concentrations at the end of treatment. 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 (Zayed et al., 1998). The BCF values were increased serially as per increasing concentration of zinc effluent. All effluent concentrations showed higher accumulation capacities as the BCF values were more than 1000. 4% of the effluent recorded with higher BCF as 2732.08 while minimum for control treated biomass was observed by a value of 696.85. In the present study increasing BCF shows that the *A. caroliniana* has capacity to accumulate Zn⁺⁺ through liquid medium after ten days exposure period which is also in accordance with other studies (Table 9). The sequestration of heavy metals in plants is achieved mainly by absorption and accumulation mechanisms (Dhir, 2010). Arora (2006) studied chromium BCF range between 243 and 4617 for the three species of azolla in which *A. microphylla* showed highest bioconcentration potential. They further concluded that the azolla species can be exploited for treatment of tannery and chromium contaminated wastewaters. Moreover, it was determined that the more growth of biomass was nearly led to more removal of metals (Khosravi, 2005). While Jain et al. (1990) reported the BCF value for Zinc in *Azolla pinnata* as only 44.

Table 9. Bioconcentration factors (BCF) for zinc in various plants used for phytoremediation.

Sr. No.	Plant Species	Bioconcentration Factor	Reference
1	<i>Azolla caroliniana</i>	2732.08	This study
2	<i>Eichhornia crassipes</i>	788.9	Xiaomei et al. (2004)
3	<i>Azolla pinnata</i> (root)	12,000	Sela et al. (1989)
4	<i>Myriophyllum exalbescent</i>	1,640	Franzin and McFarlane (1980)
5	<i>Ricciocarpus natans</i>	3,700-8,800	Caines et al.(1985)
6	<i>Elodea nuttalli</i>	3,000	Nakada et al. (1979)
7	<i>Lemna polyrrhiza</i>	44	Jain et al.(1990)

Conclusions

Azolla caroliniana can be used to treat zinc from the polluted waters which accumulate in its biomass. *A. caroliniana* showed maximum efficiency of accumulation of Zn⁺⁺ up to 4% of concentration. Photosynthetic pigments of *Azolla* were also observed to increase under the influence of zinc and other contents of effluent. The reduced level of electrical conductivity and pH after treatment period also shows the ability of experimental species to treat the effluent. The TDS, COD and BOD were also reduced after treatment period. The decrease in sodium and potassium content along with other selected water parameters mainly hardness, acidity were definitely associated with the use of effluent contents for the normal growth and development under the influence of toxic effluent. *Azolla* with its BCF values can be

considered as a high accumulator for Zn. Effluents containing zinc at slightly higher concentration can be treated with the use and growth of *Azolla* biomass. From the present study, the floating hygrophyte *A. caroliniana* based treatment has proved as a promising tool for the treatment of zinc electroplating effluent at lower dilutions. Its favorable growth and growing biomass has favored rhizosphere activity in the liquid medium thereby enhancing dissolved substances and metal uptake. It may also be possible to use the biomass as a biofertilizer after harvesting. We also suggest the need to understand processes that affect metal availability, metal uptake and translocation in *azolla*. Detailed investigations and understanding 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.

Acknowledgements

Authors are thankful to Principal Dr. R. G. Pardeshi, Fergusson College, Pune for constant support. We are also grateful to Head, Ms. Rupali Gaikwad, Department of Environmental Sciences, Fergusson College, Pune for providing necessary facilities.

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PLANT SCIENCE

The effects of cultural management on the lesser date moth (*Batrachedra amydraula* Myer) infestation

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Abstract

This research has been carried out in Khuzestan province of Iran, to evaluate the effects of cultural practices on the lesser date moth infestation in date palm. Incidentally cluster-sampling and survey research method were applied. Sampling society divided to 30 plots then one sample was selected randomly in each plot. Fifteen trees were selected randomly in each plot and their infestations were evaluated. Different cultural management factors including tillage, fertilization, irrigation, pruning, chemical control, bunch covering and bunch arrangement were evaluated using questionnaire data. Correlation analyses were performed, and then management factors that had significant correlation with pest infestation were used for further multiple regression analysis. Based on correlation analysis, it is found that, bunch remnant pruning ($r = -0.61$), bunch covering ($r = -0.55$) and chemical control ($r = -0.51$) had suitable effects on decreasing the lesser date moth infestation. Multiple regression analysis showed that the effect of chemical control (cc), bunch remnant pruning (bp) and bunch covering (bc) increased respectively and sum of effects decreased the pest infestation. The ultimate formula of the model is % infestation = $1.664 - 0.464cc - 0.427bp - 0.234bc$ (and Durbin-watson statistic = 1.73). Integrated effects of all three factors together are better than the effects of each alone.

Key words: Date palm, Lesser moth, Cultural management

Introduction

The lesser date moth (*Batrachedra amydraula* Myer) is a pest that damages fruits in both fields and stores (Dowson, 1982). The pest distribution is in Egypt, Iraq, Kuwait, Libya, Qatar, Oman, UAE, Yemen, Bahrain and Saudi Arabia. It is one of the major pests of date palm in Iran and its injury increased so that in some areas such as Bam region it's damage reached 50-70 percent. Larvae may attack and damage large number of young fruits from April to June or July (Zaid, 1999). Injured fruits usually fall, although the stout webbing spun by the larvae attaches some of fruits to the stands or to other fruits before perforation. The larva enters the fruit by making a small hole near the calyx and then feeds on the pulp and immature seed. A larva seldom eats more than a third of the fruit before it seeks another one and may damage three or four fruits during its life time. The larvae that attack

fruit just before ripening leave and debris, so that affected fruit must be culled (Dowson, 1982). The adult moth has a wingspread of about 11 - 14 mm, brownish color, marked by a grey longitudinal central stripe on the narrow forewings. The eggs are small, about 0.7 mm in diameter and yellow with a pearly sheen. The mature larvae are about 12 mm long. Some of them develop directly into adult moths, so that two or three generations occur in the summer. The larvae of last generation overwinter in the cocoon before pupating (Dowson, 1982).

The life table of *B. amydraula* Meyer (Lep.: Batrachedridae) was studied at $30 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and 24 hrs darkness, in the laboratory. The raw data was analyzed based on the age-stage two-sex life table, in order to take both sexes and the variable developmental rate among individuals and between sexes into consideration. Mean development times of immature stages were 51.91 ± 1.61 and 53.3 ± 2.3 for males and females, respectively. The intrinsic rate of increase (r_m), the finite rate of increase (λ), the net reproduction rate (R_0), the growth reproduction rate (GRR) and the mean generation time (T) of *B. amydraula* were 0.039 d⁻¹, 1.0398 d⁻¹, 9.39 offspring, 59.68 offspring and 57.37 day respectively (Rahmani et al., 2008).

Different methods were recommended for controlling the pest (Buxton, 1921; Dowson, 1982;

Received 18 January 2011; Revised 30 May 2011; Accepted 01 June 2011

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Garib, 1990; Zaid et al., 1999), but the usage of cultural control method is reduced by pesticide application, which is the most ancient control method. Nowadays, pronounced understanding of biological relationships, social condition and restriction of pesticide application in date orchards attracted the attention to the cultural control strategies again. The cultural control is covered under the following headings and subheadings: organization of crops (multiple cropping or intercropping; monoculture); soil management (preparation; amendments and fertilizers); water management; crop management (place and date of sowing; sowing density; transplantation; earthing up, staking and pruning; cover crops; living barriers; weed management); and postharvest management (Brar et al., 1996).

Date palm orchards tillage is common in Iran that it performed once / twice per year. Chemical and manure fertilizers were used for date palm fertilization. Chemical fertilizers were used based on soil experiment and manure disseminated and mixed with soil around date palm trees. Date palm orchard irrigation performed as ebb and flow, superficial. Pruning of date orchards was done for annihilating dead and injured tissues. Five kinds of pruning are common in Iran date palm orchards including: Leaf pruning, midrib pruning, prickly pruning, bunch remained pruning after harvesting and offshoot pruning (Dowson, 1982). Significant changes could be achieved in the date palm industry through the implementation of advanced cultural practices and dependable means of pest control. Improvement of date palm requires full understanding of the role of all cultural practices

and their effects on date pests. This article focuses on the effects of cultural practices on reducing the lesser date moth infestation. These cultural practices include pollination, pruning, irrigation, fertilization, tillage, bunch covering, bunch arrangement and chemical control.

Materials and Methods

Incidentally cluster-sampling method was applied in this experiment (Dowson, 1982). At first, sampling society divided into 30 plots then one sample was selected randomly in each plot in Khuzestan province for two years. Khūzestān is one of the 30 provinces of Iran. It is in the southwest of the country, bordering Iraq's Basra Province and the Persian Gulf. Its capital is Ahwaz and covers an area of 63,238 km². Other major cities include Behbahan, Abadan, Andimeshk, Khorram Shahr, Bandar Imam, Dezful, Shushtar, Omidiyeh, Izeh, Baq-e-Malek, Mah Shahr, Dasht-i Mishan/ Dasht-e-Azadegan, Ramhormoz, Shadegan, Susa, Masjed Soleiman, Minoo Island and Hoveizeh.

Cities of Khuzestan province were selected in terms of geographical location and classification under cultivation in each city by random sample method. Experiment method was survey research (Dangerfield et al., 1999). Data were collected by two methods that included:

Estimation of the lesser date moth infestation

Macheak method (Machacek, 1949) was used for estimating the pest infestation. Fifteen trees were selected randomly in each orchard then percentages of infestations were evaluated based on Table 1.

Table 1. Estimation of the lesser date moth infestations.

Infestation quality	Selective code	Infestation amount
No infestation	0	0 percentage
Little infestation	1	0-25 percentage
Mean infestation	2	25-50 percentage
Much infestation	3	50-75 percentage
Too much infestation	4	Up to 75 percentage

Infestation percentage of each tree was evaluated as:

$$\% \text{ Infestation} = (a + 2b + 3c + 4d) / n$$

a = Number of bunch with little infestation

b = Number of bunch with mean infestation

c = Number of bunch with much infestation

d = Number of bunch with too much infestation

n = Total number of bunch in each trees

Infestation percentage of each orchard was evaluated based on the 15 trees. The pest injury is a

biological index that indicated by its infestation percentage (Southwood, 1975).

Estimation of cultural management factors: Different cultural management factors were evaluated based on questionnaire by orchard survey, interview with date gardener and agriculture service centers. Cultural management factors including tillage, fertilization, irrigation, pruning, chemical control, bunch covering and bunch arrangement. Questionnaires were containing

various options for assessing the implementation of appropriate options based on the five-choice Likert spectrum. Credit test (reliability assessment) was used by Cronbach's α coefficient reliability test and was 0.86. It is commonly used as a measure of the internal consistency or reliability of a psychometric test score for a sample of examinees (Zinbarg et al., 2006).

Analysis

Data had been analyzed by multiple regressions. As a matter of fact, the lesser date moth infestation was evaluated as dependent factor and cultural management factors that sponsored on research theories as independent factors. Correlation analysis had been done before multiple regression then management factors that had significant correlation with pest infestation used for

regression analysis. The effects of cultural management factors graded as:

$R < 0.5$ -weak effect; $0.5 < R < 0.7$ - suitable effect;

$0.7 < R < 1$ -powerful effect

In addition, the negative and positive signs of the correlation coefficients pointed to decreasing and increasing effects of cultural management factors on the pest infestation respectively. Based on that bunch remains pruning, bunch covering and chemical control had suitable effects on decreasing of the lesser date moth infestation

Results

Correlation relationship between the infestation and management factors: The effects of performance/nonperformance of cultural management on pest infestation were studied by correlation relationships, the results shown in Figure 1.

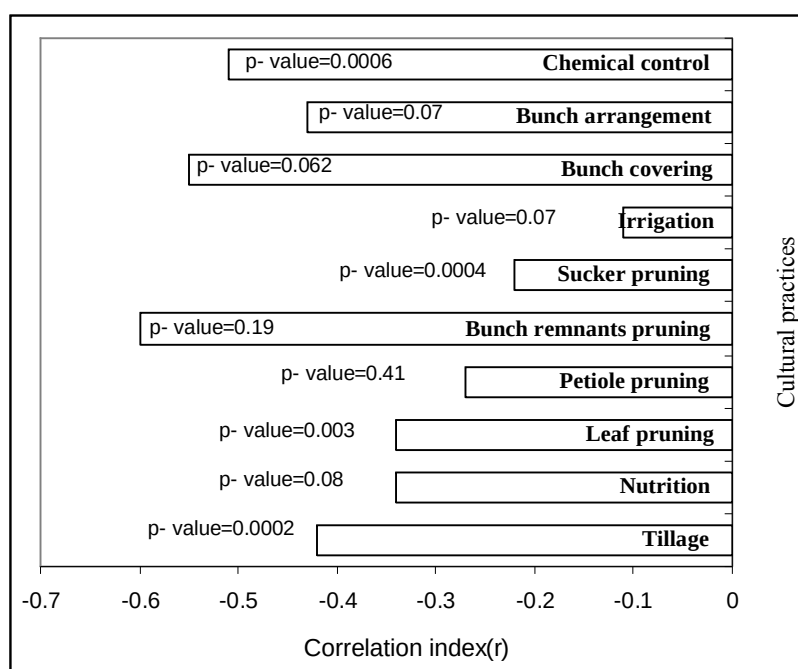


Figure 1. Correlation coefficients for describing the relationship between the lesser date moth infestation and cultural management factors.

Integrated effects of cultural management factors: Integrated effects of the significantly correlated factors on pest infestation were studied by multiple regression analysis, the results shown in Tables 2 and 3.

According to Table 2, the effect of chemical control, bunch- remained pruning and bunch covering increased respectively.

According to Table 3, the model is significant at 1% and sum of the processes have decreased effects on pest infestation. The ultimate formula of the model is (Figure 2):

$$\% \text{ infestation} = 1.664 - 0.464cc - 0.427bp - 0.234bc$$

Table 2. Estimation of cultural management factors coefficient indices of multiple regression model.

Factors	B	Standard Errors	t	P-value
Constant	1.664	0.197	8.457	0.00000
Chemical control(cc)	-0.464	0.187	-0.013	0.00016
Bunch- remain pruning(bp)	-0.427	0.139	-3.076	0.00033
Bunch covering(bc)	-0.234	0.154	-1.348	0.00980

Table 3. Analysis of variance of multiple regression models for describing integrated effects of the correlated cultural management factors on pest infestation.

Factors	Df	Ss	Ms	F	P-value
Model	4	7.403	1.851	7.84	0.0001
Residue	55	12.988	0.236		
Total	59	20.390			

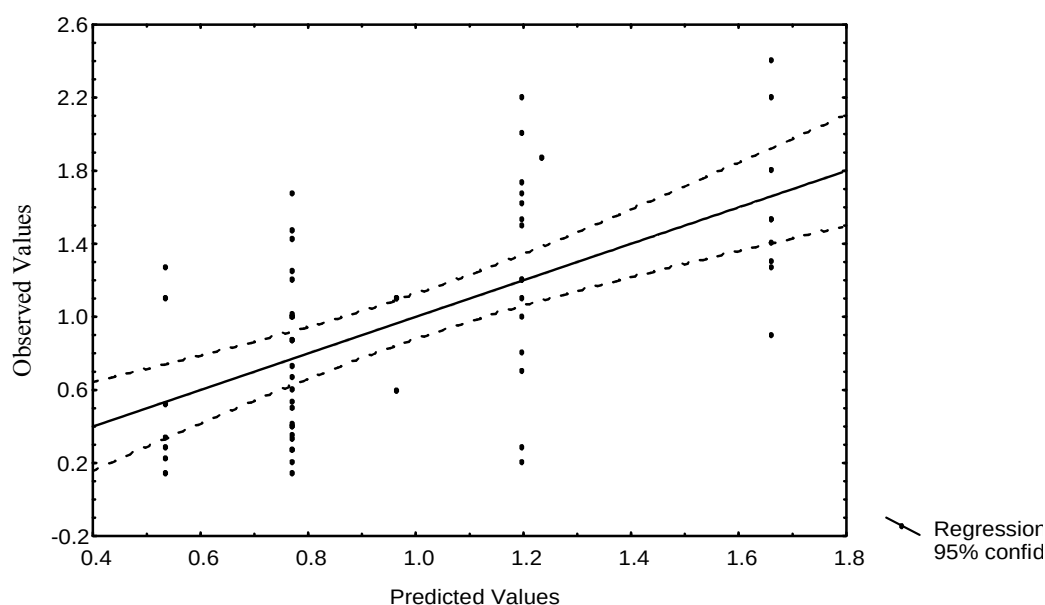
R²=66.3; Durbin-Watson statistic=1.73

Figure 2- Multiple regression analysis curve(predicted vs. observed value)

Discussion

Pest chemical control

Pest chemical control was done by twice spraying that appreciated 7-10 day after pollination and 4-5 weeks after that respectively. The efficiency of the factor is low ($r=-0.51$) because resistance to insecticides and low efficiency of sprayers. Other studies showed that the application of malathion insecticide to the bunches, protected the date fruits from the increase infestation of lesser date moth. After ten days of application the infestation decreases (Ba Angood, 1978). Larvae of the lesser date moth cause damage to dates in Iraq. A formulation based on a mixture of equal parts of wheat flour and pollen grains and containing either chlorpyrifos, fenitrothion or pirimiphos-methyl (all at 5 mg kg⁻¹) was dusted (5 g formulation bunch⁻¹) onto female clusters of dates at the time of

pollination. Numbers of larvae yield of dates and insecticide residues were monitored over a three-month period. Results showed that, by this technique, all three insecticides were effective in controlling the pest but that fenitrothion and chlorpyrifos were better than pirimiphos-methyl (Al-Samarraie et al., 2006).

Bunch remain pruning

Some of the lesser date moth hibernated population overwinter on bunch remains and transfers to warehouse by infested fruits. So bunch- remains pruning decreased overwintering locations and therefore, pest population will be reduced on future generations.

Bunch covering

The lesser date moth laying on bunch is prevented by bunch covering and reduced the pest

damage. Also, the factor reduces the wasps and birds damages. Bagging the bunches just after pollination minimized the damage of the fruits and reduce the infestation of lesser date moth, and increase the size of the fruits at the early stage of the fruits growth, but not at later stages of the fruits growth, the increase in size of the fruits buy the enlargement of the cells of fruits when they are covered with paper sacks. After the removal of the sacks, the cells did not enlarge that much in the un thinned bunches because of the high number of the fruits in the strands and the bunch. The lower infestation by lesser date moth in the bagging and unthinned treatment lead to increase the number of the fruits in the strands and therefore increase the yield of the trees- Furthermore the lower infestation by lesser date moth in the thinned treatment lead to increase the size, diameter and length of the fruits, because of accumulation of the food in a limited number of fruits in the strands and bunches, but it reduce the total yield of the tree. This agreed with the results obtained by Abbas (1990). Among the bagging treatments there is a significant difference (50%) in the yield. The higher yield was obtained by the unthinned treatment.

Integrated effects

Integrated effects of three factors are better than application each of them alone and reduce the pest damage below of the economic injury level.

The lesser date moth, *B. amydracula*, is one of the most important pests on date palm in the UAE that may cause more than 50% loss of the crop. Management programs for this pest on date palm have not been developed or implemented at the farm level. The study was conducted, from February 2004 to May 2005 in four date palm plantations in Abu-Dhabi Emirate. Such as the result of this study, control of the moth was achieved by the removal and destruction of the dormant larvae in an operation of cleaning from the fibrous strands around the bases of the terminal fronds. Pheromone lures and insecticide sprays gave excellent control of this pest in the field experiments. The development of management strategies for the inclusion of these lures and/or insecticides in the date moth integrated management programs would be worth considering (Kaakeh, 2006).

Other study was conducted during the two subsequent seasons of 2001 and 2002 in El-Beheira Governorate, Egypt, to evaluate the efficiency of some agricultural practices on the incidence of the lesser date-moth, *B. amydracula* on some date-palm varieties, i.e. Sammany, Hayany and Halawy. The

studied agricultural practices were: covering clusters of flowers with paper craft, covering date bunches with porous plastic cloth, thinning of bunches and/or diverging strands of bunches using special metal rings (Harhash et al., 2003).

Studies about other pest showed similar results. Cultural practices have great potential for developing integrated management schemes to control *Bemisia tabaci*. Some of the most promising practices are crop-free periods, planting dates, crop rotations, crop residue disposal, weed destruction, seedbeds covered with fine netting, floating covers, high planting densities, living barriers, ground covers, companion crops and sprinkler irrigation (Hilje, 2000). The effect of different combinations of three cultural practices (shredding, burning and conventional tillage) on the carryover population of hibernating larvae of *Scirpophaga incertulas* in a rice-wheat system was observed in Ludhiana, India, during 1992-93 and 1993-94. The mean number of larvae and percentage of tiller infestation were significantly less in the combinations of cultural practices having conventional tillage as a component than those having no tillage. Furthermore, the combination of conventional tillage with shredding proved superior in reducing the carryover of hibernating larvae compared with all the other treatments (Brar et al., 1996).

Field studies were carried out during 1995-96 in Egypt to investigate the influence of sowing date, water regime and spacing after transplanting on *Hydrellia prosteralis* incidence in rice (Sherif et al., 1997). Farmers' insect management practices on tomato in Dalat, Vietnam, were evaluated by a formal survey of farmers, agricultural officers and extension officers between January to March, 1997. The results showed that farmers insect control practices were primarily chemical, followed by cultural practices and physical removal. Microbial control (Bt) was not widely used (Huyen et al., 1998).

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PLANT SCIENCE

Prospects of biotechnology for crop cultivation in low-lying coastal areas

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Abstract

During severe weather events rice and other crops in nations inhabiting low-lying coastal areas can be submerged under salty sea water long enough to eventually kill them. In fact, many farmers already suffer from these events in the coastal areas of Bangladesh, Mauritius, and Grenada. To survive such natural calamities, the development of salt and submergence resistant-crops could be an effective option. Recent progress in the field of molecular biotechnology has shown that rice plants with tolerance to salt and submergence can be developed in near future.

Key words: Transgenic rice plant, *Oryza sativa*, Salt-tolerance, Submergence tolerance, Climate change and sea level rise, Pokkali rice, *Tecticornia pergranulata*

Agriculture began about 10,000 years ago after the last ice age (Hamilton, 2009); during that period people collected seeds and plants with useful traits and started using breeding practices for improving livestock. In contrast, modern biotechnology started just about 60 years ago when the double helical structure of DNA was reported by Watson and Crick; the research at the molecular level has expanded greatly since then, creating a revolution in the field of agriculture, pharmaceuticals, diagnostics, etc. Genome sequencing of many viruses, bacteria, fungi, plants, drosophila, mouse, insects, human and many more have been completed (<http://genomics.energy.gov/>). Exploitation of the sequences can help our ability to confront emerging crop diseases and changing environment of the world. Agricultural field is now challenged to develop crops with enhanced yields, stress-tolerance, disease-resistance, improved nutritional content of foods, etc. In agriculture, biotechnology has huge applications as have been seen already in generating some pest-resistant crops, frost-resistant crops, and delaying ripening

of fruits like tomato, etc. (Oeller et al., 1991). In the future, biotechnology also has the potential to produce food crops which have elevated essential amino acids or contain vitamins as in 'golden rice' (Zimmermann and Hurrell, 2002; Tang et al., 2009).

A popular mechanism of generating transgenic plants employs the bacteria *Agrobacterium tumefaciens*. This type of bacteria contains the Ti plasmid that helps them integrate into the plant genome. A genetically modified Ti plasmid has the capacity to introduce a 'gene of interest' to many types of plant cells. Successful transformation of rice has been reported (Rashid et al., 1996). The most challenging part of this process is identification of the gene(s) responsible for conferring the trait. The usual protocol for this kind of work is to (a) clone gene / cDNA, (b) transform into target plant, (c) obtain optimum level of expression, (d) modify location of expression, and (e) proper function of the gene product. An *Arabidopsis thaliana* plant gene *SOS1* (*Salt Overly Sensitive 1*), which is up-regulated in response to NaCl stress, has been cloned and characterized to be Na⁺/H⁺ antiporter (Shi et al., 2000). They have reported expression of *SOS1* complemented the salt-hypersensitive phenotype of *sos1-1* mutant plants. Identification of R genes from plants showed to provide resistance to a relatively wide range of pathogens (McDowell and Woffenden, 2003). Similarly, disease-resistance genes like *LR17* and *LR26* were identified that provides resistance to leaf rust (Abdul, 2011). Constitutive

Received 06 July 2011; Revised 26 February 2012; Accepted 27 February 2012

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expression of *GASA4* in *Arabidopsis* led to elevated heat tolerance in transgenic lines (Ko et al., 2007; Dias and Lidon, 2010). Transgenic rice plants expressing *cry1Ia5* gene (*Bacillus thuringiensis* toxin) has been reported to be resistant to stem borer (Moghaieb, 2010). Identification of drought response genes and their expression is currently under way in plants (Iskandar et al., 2011).

Over 600 million people live in coastal areas that are less than 10 meters above sea level, and two-thirds of the world's cities that have populations over five million are located in these at-risk areas (<http://www.npr.org/templates/story/story.php?storyId=9162438>). The countries with the largest share of their populations living in low-elevation areas are Bahamas, Suriname, the Netherlands, Vietnam, Guyana, Bangladesh, Djibouti, Belize, Egypt, and Gambia. About 1/3 of world's irrigated land has been estimated to be contaminated with high salt levels every year (Shereen et al., 2011). In November 2007, Bangladesh faced one of the most devastating cyclones in its history, causing enormous damage in terms of human loss, crop loss, animal loss, etc. In the foreseeable future, the country is likely to be affected by the larger, long lasting and global scale human induced disaster - climate change and sea level rise (CCSLR). Bangladesh is thought to be one of the most vulnerable countries of the world to CCSLR. For low lying coastal areas like Bangladesh, biotechnology can provide the best possible solution for better crop yields, as it faces floods and cyclones in the coastal areas every year.

Some plants synthesize "osmo-protectants", low molecular weight non-toxic compounds that protect and stabilize cellular macromolecules from damage by high salt levels, but these may not be enough to sustain extreme conditions (Apse and Blumwald, 2002). The demand for rice is constantly rising in Bangladesh and an increase in rice production must be achieved at a faster rate than in most other countries (<http://www.worldbank.org/html/cgiar/newsletter/june97/9bang.html>). Recently, the identification of the *Oryza sativa* ssp. *indica* cultivar FR13A has given hope among the crop cultivators. This rice species can survive up to 2 weeks of complete submergence under water. The gene that has been implicated in the resistance is named the *Submergence 1* (*Sub1*) locus in chromosome 9 of *Oryza sativa* (Xu and Mackill, 1996; Perata and Voesenek, 2007). Structurally this is a cluster of 3 genes (encoding putative ethylene response factors), and recently it has been reported that overexpression of *Sub1A-1*

conferred enhanced tolerance in other rice species (Xu et al., 2006).

In addition, salt stress is a major problem in the coastal areas, because the water submerging crops here is saline. *Oryza sativa* is not salt-resistant and genetic improvement for salt tolerance is needed. Studies of naturally salt-tolerant rice have given great hope in this regard. The *Pokkali* system of rice cultivation in the acid saline soils of Kerala, India is a unique method of rice production (Shylaraj and Sasidharan, 2005). The VTL5 rice variety of the Pokkali in land of Kerala is a high yielding salt-tolerant rice strain (Figure 1). The *Saltol*-loci has been identified in chromosome 1 in Pokkali strain (Xu et al., 2006; Walia et al., 2005; Kim et al., 2009; Kumari et al., 2009).



Figure 1. Pokkali Rice in natural habitat.
Source: USDA-Agricultural Research Service

A recent article from Islam's laboratory in the University of Dhaka, Bangladesh, has reported another *Oryza sativa* rice strain, Horkuch, obtained from the southwest coast of Bangladesh, also expressing salt-sensitive genes (Lisa et al., 2011). This could be a potential alternative to Pokkali rice for the future. However, salt-resistance by these plants was tested at up to 15 dS/m NaCl which is well below salinity level (50 to 60 dS/m NaCl) in sea water. Therefore, a rice strain that is tolerant to salt-stress similar to sea water as well as tolerant to

submergence is needed; this could be the best solution to maintain or improve rice crop yields in low lying coastal areas. Theoretically, this may be possible to achieve; we just need to identify the gene(s) from naturally more salt tolerant plants. A recent report on the halophytic stem-succulent *Tecticornia pergranulata* (Blackseed Samphire), naturally found in salt marshes of Western Australia (Figure 2), is greatly encouraging (Colmer et al., 2009). These plants were reported to have tolerance to both submergence and high salt exposure. These plants can maintain underwater photosynthesis at up to 40 dS/m NaCl and, most promising; retain 70% of activity at 80 dS/m NaCl which is substantially above sea water salinity.



Figure 2. *Tecticornia pergranulata* (Blackseed Samphire) in marsh lands.
 Source: Sikder B. Ullah

Moreover, *T. pergranulata* displays conservation of substrates under submergence for 28-56 days helping plants quickly recover after floodwaters recede; this phenomenon is called 'quiescence response' (Bailey-Serres and Voesenek, 2008). This could provide the breakthrough we are waiting for. This work, however, would need the identification of the gene(s) giving those particular traits shown by *T. pergranulata*. Then the genes could be engineered into the rice plants like *Oryza sativa* that would also have the overexpressed sub1 gene. The engineered *Oryza sativa* rice expressing higher Sub1A-1 and

Saltol-like gene obtained from *T. pergranulata* may offer the possibility of resistance to both submergence and high salt levels.

So, farmers from the coastal areas can hope for a useful solution in near future to save their rice crops during adverse climatic conditions.

Acknowledgments

The authors wish to thank Prof. Cindy Howard and Prof. Ron Mills at the University of Houston-Clear Lake, for critical reading of the article.

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PLANT SCIENCE

Effect of autoexhaust emission on germination and seedling growth of an important arid tree *Cassia siamea* Lamk.

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Abstract

Automobile exhaust emission showed toxic effects on seed germination and seedling growth of *Cassia siamea*. Germination and growth of *C. siamea* seeds was significantly ($p < 0.05$) affected in seeds collected from M. A. Jinnah Road, Sharah-e-Faisal, Nazimabad, and Gulshan-e-Iqbal as compared to University campus. The seeds of *C. siamea* collected from the Karachi University campus, which is considered as control site showed better percentage of seed germination and seedling growth as compared to the seeds of the same species collected from the city area. Higher percentage of decrease in seed germination was found for seeds collected from M. A. Jinnah road, followed by Shahrah-e-Faisal, Nazimabad, and Gulshan-e-Iqbal as compared to control. Seedling length and root length was also highly decreased for seeds of same species collected from M. A. Jinnah road as compared to control. High percentage of decrease in seedling dry weight was found for seeds of same species collected from M.A. Jinnah road, followed by Shahrah-e-Faisal, Nazimabad, Gulshan-e-Iqbal as compared to control. According to tolerance test it was observed that seedling growth of *C. siamea* showed lowest percentage of tolerance in samples collected from M. A. Jinnah Road followed by Sharah-e-Faisal, Nazimabad and Gulshan-e-Iqbal as compared to Campus.

Key words: Automobile exhaust emission, *Cassia siamea*, Pollution, Seeds, Tolerance

Introduction

Karachi is the largest industrial city of Pakistan. The transport system consists of buses and minibuses as a primary mode of conveyance. According to traffic engineering bureau (TEB) in Karachi, a total number 1355,972 all kind of vehicles were registered with the motor vehicle registration department in Sindh till June 2005 (Mohiuddin, 2005). Pakistan is plagued with environmental problems due to unlimited population growth and unchecked vehicular emissions (Wahid, 2006). These toxic materials such as carbon particles, unburned and partially burned hydrocarbons, fuels, tar materials, lead compounds and other elements which are the constituents of petrol and lubricating oils deposit on the surface of plants. These pollutants in combinations cause greater or synergistic effects to plants. Toxicity of mixed chemicals is not always

equal to the sum of those by the individual chemicals (Nagata et al., 2008).

Emissions from automobiles and trucks operating on public roads represent a major portion of the air pollutants included in emission inventories (Skiba and Davydova-Belitskaya, 2003). Trees in cities faces an exceptionally stressful growing environment such as air pollution, environmental degradation, pressure for land space, traffic congestion, destruction of trees and green areas to accommodate urban development which suppresses performance and shorten life span (Jehan and Iqbal, 1992; Sawidis et al., 1995; Jim, 1998). The pollutants emitted from the autoexhaust adversely affect the germinability of seeds (Türkan, 1988; Mehmood and Iqbal, 1989; Qadir and Iqbal, 1991). Automobile activities has increased the level of metals in the environment of the major cities of the world and produced ill effects on plant growth (Abdullah and Iqbal, 1991; Al-Saleh and Taylor, 1994; Alfani et al., 1996; Ara et al., 1996; Hussain et al., 1997; Alam and Ahmad, 1998; Aksoy and Sahn, 1999; Aksoy et al., 2000; Shafiq and Iqbal, 2007). Vehicular traffic is one of the major sources of heavy metal contamination, either in soils or roadside dusts in urban areas, and it has increased in the last years (Colville et al., 2001; Celik et al.,

Received 06 January 2011; Revised 10 August 2011; Accepted 26 August 2011

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2005: Ahmed and Ishiga, 2006: Ovadnevaitė et al., 2006). Plants not only have an ornamental function in urban areas, but they may also improve the quality of urban life (Akbari, 2002: Brack, 2002), having a role in carbon dioxide sequestration and oxygen releasing through photosynthesis. The seeds of *Dalbergia sissoo* Roxb., collected from different polluted areas of the city showed significant reduction in germination as compared to seeds collected from the less polluted areas (Mehmood and Iqbal, 1989). The seeds of *Cassia surattensis* Burm F., *Leucaena leucocephala* (Lam.) de-Wit, *Parkinsonia aculeata* L., *Sesbania sesban* (L.) Merrill collected from the polluted areas showed significant reduction in germination as compared to control (Siddiqui and Iqbal, 1994).

The exploitation of fossil fuels is integral to modern living and has been a key element of the rapid technological, social, and cultural changes of the past 250 years (Wilkinson et al., 2007).

The sensitivity of seeds to vehicular emission has raised question about the potential impact of pollution on plant growth. Therefore, the present research was carried out to investigate the effects of automobile pollutants on seed germination and seedling growth of *Cassia siamea* an important tree species growing in different areas of the city of Karachi.

Materials and Methods

Site description

Karachi is situated on the coast along the Arabian Sea. Karachi city is spread over 3,530 km² (1,360 sq mi) in area. Karachi is located in the south of Pakistan, on the coast of the Arabian Sea. Its geographic coordinates are 24°51' N 67°02' E. Most of the land comprised largely of flat or rolling plains, with hills on the western and Manora Island and the Oyster Rocks (Wikipedia, 2010). Chaudhry (1961) has characterized the climate of Karachi as subtropical maritime desert. Whereas, the bioclimate of Karachi, determined by Holdrige (1947) comes in the category of "Tropical desert bush formation". Average wind velocity is 12 M Sec⁻¹ during June and July and 3.5 M Sec⁻¹ from January to March. During the southwest monsoon season winds blow from the sea towards the coast, whereas during the northeast monsoon their direction is reversed. Therefore, pollutants are pushed inland during the southwest monsoon season and are blown out to sea during the northeast monsoons (UNEP, 1992). The hot and humid rainy season, which is variable, lasts from June to September. The winter season is very short lasting from middle of November to middle of

February. The rest of the months constitute the summer, autumn and spring seasons. Temperature is mild with no frost. Dew formation is quite common, the relative humidity is high and the differences in day and night temperatures are great. The climatic conditions at the control site (Karachi University Campus) are not different from other sites of the city.

Selection of sites

The site in urban area is disturbed mainly by autovehicular activities, includes all main traffic network (Gulshan-e-Iqbal, Nazimabad, Shahrah-e-Faisal and M.A. Jinnah Road) whereas, Karachi University is relatively a clean area (Figure 1). Brief description of the study area is given below:

A. Karachi University Campus: Karachi University Campus is situated at the outskirts of the city. This site is relatively free from the autovehicular activities as compared to other sites of the city. Karachi University Campus is clean and 20 km away from Quaid-e-Azam tomb.

B. Gulshan-e-Iqbal: Gulshan-e-Iqbal is located about 8 km North East of the Quaid-e-Azam tomb. This place is comparatively open with low traffic. However, traffic congestion in the morning hours is common due to trade activities at old vegetable and fruit market.

C. Nazimabad: Nazimabad handles a large traffic volume, moving from north to west of the city. This site is about 10 km away from the North of Quaid-e-Azam tomb.

D. Shahrah-e-Faisal: Shahrah-e-Faisal has many multistoryed buildings. This site is 15 km from the eastern site of the Quaid-e-Azam tomb and heavily influenced by traffic activities.

E. M.A. Jinnah Road: This site is the most congested site along the Quaid-e-Azam tomb. The density of traffic from Gulshan-e-Iqbal, Nazimabad, Liaquatabad and Shahrah-e-Faisal passes through this road. Multistoryed buildings are common at this site. The slow movement of traffic starts building up toxic pollutants in the area.

The seeds of *Cassia siamea* Lamk. in sufficient quantities were collected in summer season from the polluted sites of the city as well as from a comparatively clean area of the Karachi University Campus (Figure 2). Seeds of *C. siamea* were rubbed by sand paper to break the seed dormancy. All the seeds were soaked in 1% bleach solution for one minute and later on wash with double distilled water to avoid any type of fungal contamination during germination. Ten seeds of each species were kept in plastic pot (130-mm diameter and 30 mm in depth). The pots were filled with manure and soil in

ratio of 1:2 and watered daily. There were three replicates and the experiment was completely randomized. The seed germination was regularly checked. After six weeks of growth period in the natural environmental conditions during September-October (Atmospheric temperature day

32°C max. $\pm$ 25°C min.) all the seedlings were carefully removed from the pots and were washed thoroughly with tap water. The root and seedling lengths were measured and their oven dry weights were obtained.

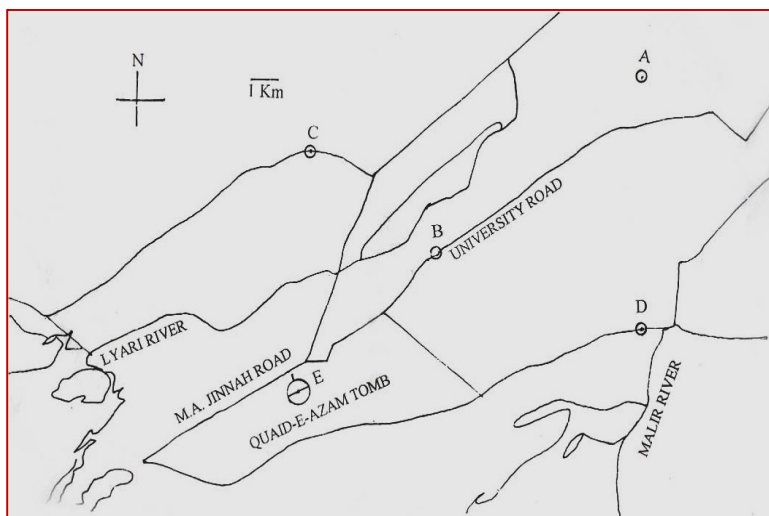


Figure 1. Map of the study area
 (A= Karachi University, B= Gulshan-e-Iqbal, C= Nazimabad, D= Shahrah-e-Faisal, E= M. A. Jinnah Road).



Figure 2. *Cassia siamea* in the campus.
 Courtesy Mr. Zia ur Rehman Farooqui

An index of tolerance was determined by the following formula:

$$\text{Index of tolerance} = \frac{\text{Mean root length of polluted area seeds}}{\text{Mean root length of campus seeds}} \times 100$$

The data collected for various growth parameters from different experiments was

statistically analyzed by analysis of variance techniques (ANOVA) Steel and Torrie (1961) and Duncan's Multiple Range Test (1955).

Results

Seeds of *C. siamea* collected from the polluted areas showed low percentage of germination,

seedling length, root length and seedling dry weight as compared to seeds from the clean area (Figure 3-5). Germination of *C. siamea* seeds showed better percentage of seedling growth in samples collected from University campus as compared to Gulshan-e-Iqbal, Nazimabad, Shahrah-e-Faisal and M.A. Jinnah Road (Figure 3).

Decrease in seed germination was found to be higher for seeds collected from M.A. Jinnah road (37%), followed by Shahrah-e-Faisal (30%), Nazimabad (34%), Gulshan-e-Iqbal (19%) as compared to control. Seedling length was also decreased for seeds of *C. siamea* collected from M. A. Jinnah road (45%), followed by Shahrah-e-Faisal (41%), Nazimabad (35%), Gulshan-e-Iqbal (25%) as compared to control. The root growth of *C. siamea* was also affected in seedlings which were raised from the seeds of polluted areas as compared to less polluted areas of the city.

The high percentage of decrease in root growth was observed at M.A. Jinnah road (44%), followed by Shahrah-e-Faisal (40%), Nazimabad (36%), Gulshan-e-Iqbal (16%) as compared to control. Seedling dry weight of *C. siamea* was greatly decreased in seeds collected from the city area as compared to control. The high percentage of seedling dry weight was found for seeds of same

species collected from M.A. Jinnah road (59%), followed by Shahrah-e-Faisal (48%), Nazimabad (34%), Gulshan-e-Iqbal (29%) as compared to control.

Automobile emission showed significantly ($p < 0.05$) toxic effects on seed germination and seedling growth of *C. siamea*. Germination of *C. siamea* seeds was significantly ($p < 0.05$) affected in seeds collected from M. A. Jinnah Road, Shahrah-e-Faisal, Nazimabad, Gulshan-e-Iqbal as compared to University campus (Figure 4). Seedling length of *C. siamea* was also greatly affected in sample collected from M. A. Jinnah Road (54 mm), Sharah-e-Faisal (58 mm), Nazimabad (64 mm), Gulshan-e-Iqbal (73 mm) as compared to campus (98 mm). Similarly root length of *C. siamea* was significantly ($p < 0.05$) reduced in sample collected from M.A. Jinnah Road (28 mm), Sharah-e-Faisal (30 mm), Nazimabad (32 mm), Gulshan-e-Iqbal (42 mm) as compared to Karachi University campus (50 mm), respectively. Seedling dry weight of *C. siamea* also showed decrease in samples collected from M. A. Jinnah Road (0.025 g), Sharah-e-Faisal (0.032 g), Nazimabad (0.040 g), and Gulshan-e-Iqbal (0.043 g) as compared to Campus (0.061 g).

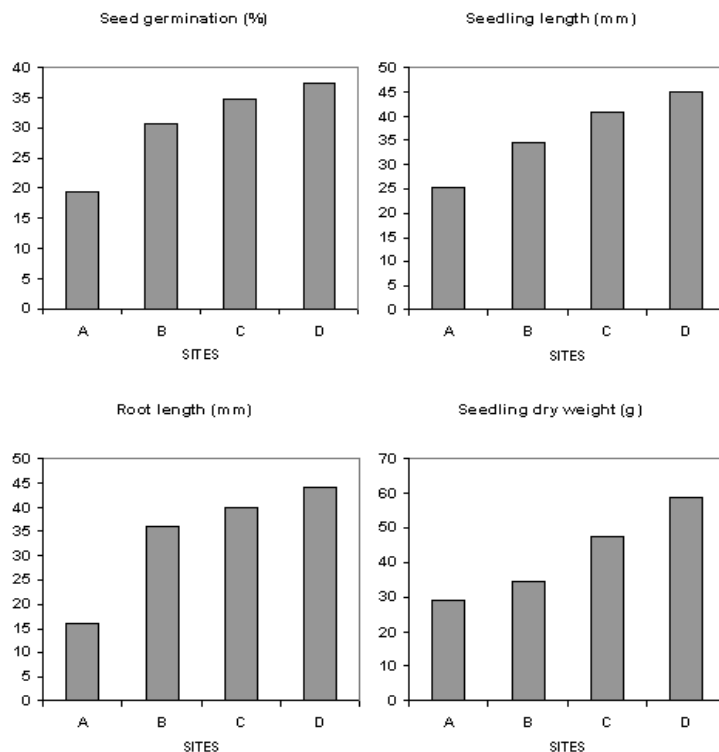


Figure 3. Percentage decrease in seed germination, seedling length, root length and seedling dry weight of *Cassia siamea* as compared to control. SITES: A= Gulshan-e-Iqbal, B= Nazimabad, C= Shahrah-e-Faisal, D= M.A. Jinnah Road.

According to tolerance test it was observed that seeds of *C. siamea* collected from the polluted area of the city showed low percentage of tolerance in seedling growth as compared to seeds of *C. siamea* collected from the less polluted areas (Figure 5). Seedling growth of *C. siamea* showed lowest

percentage of tolerance in samples collected from M.A. Jinnah Road (56%) and Sharah-e-Faisal (60%) as compared to Campus. Similarly, seedling growth of *C. siamea* showed better percentage of tolerance in samples collected from Nazimabad (64%) and Gulshan-e-Iqbal (84%) as compared to Campus.

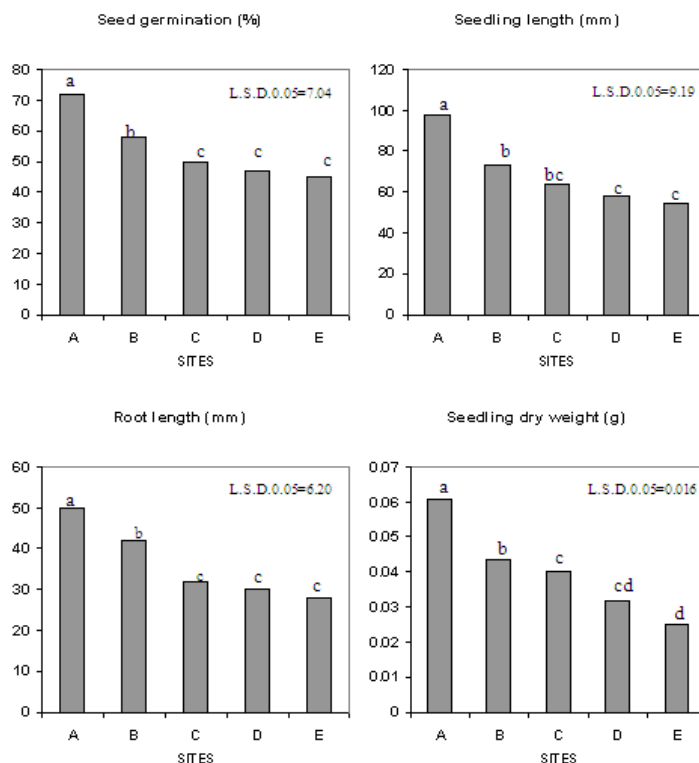


Figure 4. Effects of autoemission on seed germination, seedling length, root length and seedling dry weight of *Cassia siamea* as compared to control. Statistical significance determined by analysis of variance. Numbers followed by the same letters in the same bar not significantly different ($p < 0.05$) according to Duncan's multiple range tests.

SITES: A= Karachi University campus, B= Gulshan-e-Iqbal, C= Nazimabad, D= Shahr-e-Faisal, E= M.A. Jinnah Road

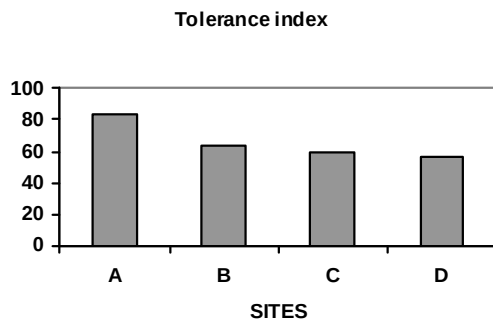


Figure 5. Toerance index of *Cassia siamea* as compared to control.

SITES: A= Gulshan-e-Iqbal, B= Nazimabad, C= Shahr-e-Faisal, D= M.A. Jinnah Road

Discussion

More recently, attention is being focused on the global environmental problems (Hasanuzzaman et al., 2010). Environmental conditions are becoming increasingly worse in Pakistan and its major cities are plagued with environmental problems due to unchecked vehicular emission (Wahid and Marshall, 2000). This paper examines the effects of autovehicular exhaust emission impact on seed germination and seedling growth of *Cassia siamea* an important arid trees growing along the main busy roads of the city of Karachi, Pakistan. Most of the studies have observed that in industrialized cities like Karachi, where the automobiles activities

increasing day-by-day, automobile exhaust emission is a major source of atmospheric pollution (Qadir and Iqbal, 1991) affecting plant growth. Trees in cities areas are subjected to high under pressure for proper growth performance due to emissions of pollutant from the automobile. Motor vehicle traffic produced considerable emission of air pollutant. Pollutant derived from the auto emission can directly affect the plant ability to produce and store food in the seeds. The source of Zn and Cu in the street dust has been ascribed to corrosion of the metallic parts of cars like engine wear, thrust bearing and brush wear (Divrikli et al., 2003; Al-Khashman and Shawabkeh, 2006). Pb is directly related to the emissions from motor vehicles (Koepe, 1981; Al-Khlaifat and Al-Khashman, 2007). Pollutant derived from the auto emission can directly affect the plant ability to produce and store food in the seeds. In recent years, the increasing contribution of the transport sector to greenhouse gas (GHG) emissions resulting from the use of fossil fuels, and their effects on global warming and climate change have been a major concern. The estimated emissions from motor vehicles in Chennai in 2005 were 431, 119, 46, 7, 4575, 29, and 0.41 tons/days respectively for CO, VOC, NO_x, PM, CO₂, CH₄ and N₂O reported (Nesamani, 2010).

Roadside trees in the city are coming under pressure and are lost due to vehicular-traffic infrastructure (Jim, 1998). The pollutants emitted from the exhaust detrimentally influence the germination ability of seeds. Information is available on the effect of air pollution on tomato seed quality (Guderian, 1977). MacLean and Schneider (1976) have reported a decrease in fruit production of beans and tobacco cultivars due to oxidants. The results of the present investigations are testifying this grave situation facing for plants growing at the polluted sites of the city. In our previous investigation it was observed that biomass production of some roadsides trees were more sensitive to air pollutant particularly at M.A. Jinnah road as compared to other less polluted areas of the city (Shafiq and Iqbal, 2005).

In present study, the seeds of *C. siamea* collected from different polluted and less polluted sites of the city behaved differently for germination and growth. The seed germination, seedling growth, root growth and seedling dry weight of *C. siamea* seed collected from different sites of the city was found low as compared to seeds collected from the campus areas. Significant ($p < 0.05$) reduction in seed germination was observed in seeds of *C. Siamea* collected from M. A. Jinnah Road as

compared to Sharah-e-Faisal, Nazimabad, Gulshan-e-Iqbal and University campus. The possible reason for reduction in seed germination of *C. siamea* could be due to the development of unhealthy seeds produced by trees growing along the main road of the city. The reduction in seed germination of *C. siamea* seeds collected from the polluted areas of M. A. Jinnah Road, Nazimabad, Sharah-e-Faisal and Gulshan-e-Iqbal as compared to seeds of *C. siamea* collected from campus agrees with the findings of Iqbal and Siddiqui (1996). They have found reduction in seed germination of *Pongamia pinnata* (L.) Merrill and *Sesbania sesban* (L.) Merrill collected from other parts of the polluted areas. Similarly, in another investigations seeds of *Dalbergia sissoo* Roxb. Collected from different areas of the city showed significant reduction in germination as compared to clean area (Mehmood and Iqbal, 1989; Qadir and Iqbal, 1991). The seed of *Albizia lebbbeck* (L.) Bth. collected from the polluted sites of the city showed significant reduction in germination as compared to less polluted area (Iqbal et al., 1997). The other reason of high percentage of reduction in seed germination of *C. siamea* collected from M. A. Jinnah Road and Shahr-e-Faisal, which is highly polluted sites of the city, could be due to structure of the pod. The pods of *C. siamea* have some rough surface and hair like structure. Pollutants released from the automobile might be able to enter into seeds through the pods therefore, leads to the development of unhealthy seeds. The accumulation of toxic products, excess fall of autodust, which contain hydrocarbon and metals, are likely to be additional important factor responsible for production of low viability seeds of *C. siamea*. The deposition of lead and soot particles on the surface of pods from automobile are additional cause for low production of healthy seeds. Krishnaya and Bedi (1986) had observed the toxic effects of automobile exhaust on germination of *Cassia tora* L. and *Cassia occidentalis* L. The percentage germination of seed viability was improved as the distance of 60 m from the road increased. The decrease in reduction in viability of seeds was directly proportional to the amount of lead accumulated in the plants. An inhibition and delay in germination of seeds of *Cucumis sativus*, cv. Beite alpha and *Triticum aestivum* cv., cumhuriyet 75 have been also recorded with the application of exhaust gas (Turkan, 1988). The tolerance of plants to pollutants enables them to produce a greater number of viable seeds, as supported by the work of Pell and Puente (1988). Furthermore, decrease in seedling growth of *C. siamea* can be attributed to the presence of toxic pollutants in the environment of M. A.

Jinnah Road, Nazimabad, Sharah-e-Faisal and Gulshan-e-Iqbal sites from where the seeds were collected. In autovehicular pollution, some hidden or physiological changes or metabolic disorders of plants take place which affect the reproductive capacity of plants and enforce plants to produce low viability of seeds. Low percentage of seedling dry weight of *C. siamea* has proved the loss of viability of the seeds. This is also demonstrated that the seeds of *C. siamea* collected from M. A. Jinnah road are less tolerant to auto exhaust emission than the seeds of same species collected from the Shahr-e-Faisal, Nazimabad, Gulshan-e-Iqbal and University Campus, respectively. The degree of different percentage of decrease in seedling weight is due to their adaptation to the different environment. This study further confirms that the seed viability of *C. siamea* was more affected in seed collected from plants growing close to the roadside. The root growth of *C. siamea* was highly decreased in seedlings raised from the polluted seeds as compared to control. Wong et al. (1984) have studied the inhibition in root growth of *Brassica chinensis* and *Brassica parachinensis* roadside plants. The pollutant emitted from the autoexhaust has greatly influenced the seedling dry weight capability of roadside plants. The seedling dry weight was reduced in the polluted seedlings as compared to control. The yield of seeds and fruits of *Guaiacum officinale* and *Azadirachta indica* was lessened at polluted sites (Bhatti and Iqbal, 1988). In the polluted atmosphere the chlorophyll is disintegrated and CO₂ uptake is reduced followed by a reduction in synthesized assimilates.

The growth of plants could be affected by several reasons and one of them is the presence of toxic pollutants derived from the auto vehicular exhaust emission. The deposition of lead and soot particles on the surface of leaves causes clogging of stomata and this clogging ultimately leads to reduction in photosynthetic rate which, in turn, causes a reduction in the contents of sugar, chlorophyll and protein as found by Constantinidou and Kozlowski (1979) on *Ulmus americana* seedlings and by Satoh et al. (1987) in *Phaseolus vulgaris*. The other reason of inhibitory factor in controlling the germination and seedling growth of *C. siamea* can be attributed to the physical constraints induced by dust and diesel fuel particles available in the nearby soil and absorbed by the seed due to automobile activities. Diesel fuel would cause a film of oil to form around the seed, which would act as a physical barrier, preventing or reducing both water and oxygen transfer to the

seed. This physical impedance was shown to delay seed emergence and therefore could be an additional factor in the overall inhibitory effect of diesel fuel contamination on germination and growth of plants (Adam and Duncan, 2002). Steady growth in vehicular populations has put environmental stress on urban centres in various forms particularly causing poor air quality. The severity of the problem arises when the traffic flow is interrupted and the delays and start-stops occur frequently (Pandian et al., 2009). *C. siamea* can be also used a pollutant indicator.

Conclusion

Environmental pollution can have a powerful negative effect on germination and growth of plants. It is important to understand the relationships between plant and the environment in which they are growing, if we want to conduct safe and clean environment on a long-term basis. In creating a sustainable clean environment, environment protection needs a major consideration. The present study confirms that the seeds of *C. siamea* collected from the polluted areas had some potential of adaptability to some extent. As we are aware that once contamination of pollutant established in environment, it is difficult to eradicate completely. Although this has been an intense area of research need further studies. The current finding will contribute some interesting perspective on this complex issue. In view of the destructive and hazardous role of the air pollution due to toxic pollutant discharged from the automobile, it becomes necessary, to grow resistant plant species and low leaded fuel to help in reducing the automobile pollution. Existing roadside plants must be saved otherwise would be lost due to constant increase in vehicular traffic density infrastructure. Strong dedication from all-civic agencies is required to preserve this economic important arid tree species from the extinction in future at least at M.A. Jinnah road site, which is extremely highly polluted area of the city.

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PLANT SCIENCE

Soil productivity improving attributes of Mexican sunflower (*Tithonia diversifolia*) and siam weed (*Chromolaena odorata*)

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Abstract

In the present investigation, soils fallowed to Mexican sunflower (*Tithonia diversifolia*), siam weed (*Chromolaena odorata*), spear grass (*Imperata cylindrica*) and soil cropped to cassava were chemically analysed. Soil physical properties such as bulk density, total porosity and moisture content were also determined. The soils were also used to grow maize in screen house. Soils fallowed to *Tithonia* and *Chromolaena* had higher mineral values like nitrogen, phosphorus, potassium, calcium, magnesium and also organic matter. The plant morphological parameters like maize height, girth and leaf area were also found higher than cropped land and soil fallowed to spear grass. Soils under *Tithonia* and *Chromolaena* had more favorable physical properties compared with soils under cassava and spear grass as indicated by lower bulk density and higher total porosity. This can be adduced to the ability of the *Tithonia* and *Chromolaena* weeds to protect the soil, proliferate surface soil with their roots, and attract fungi, increase biomass and organic matter.

Key words: Soil productivity, Mexican sunflower, Siam weed, Spear grass, Cassava

Introduction

High cost of chemical fertilizers in tropical agriculture necessitated dependence on biological means of maintaining soil fertility and productivity. Mulching with residues of weeds such as siam weed and Mexican sunflower was found to increase yield of crops such as maize (Ojeniyi and Adetoro, 1993; Falade and Ojeniyi, 1997; Awodun and Ojeniyi, 1999) and soil nutrients content. The mulches have fertilizer effect also (Taiwo and Makinde, 2005).

Tithonia (Mexican sunflower) originated in Mexico, but is now widely distributed throughout the humid and sub-humid tropics in Central and South America, Asia and Africa. *Tithonia* is an aggressive weed growing to a height of about 2.5m and adaptable to most soils. It had been observed to be widely spread in Nigeria where it is found growing on abandoned/waste lands, along major roads and waterways and on cultivated farmlands. Evidence from other parts of the world suggests

that *Tithonia* can be used for a wide variety of purposes. These include fodder, poultry feed, fuel, compost, land demarcation, soil erosion control, building materials and shelter for poultry (Nill and Nill, 1993; Liasu and Atayese, 1999). The stems and leaves of *Tithonia* had been reported to contain sesquiterpene lactones e.g. tagitinins (terpene) that prevents attack by termites (Adoyo et al., 1997) and possess antimicrobial properties. Hence, *Tithonia* with low lignin (6.5%), polyphenol (1.6%) and considerably high nitrogen (3.50%), phosphorus (0.37%) and potassium (4.10%) contents (Jama et al., 2000) has great potential for use as soil amendment. *Tithonia* has been found to produce high biomass and was reported as effective biomass for mulching and increasing yield of rice and tomato (Liasu and Achakzai, 2007) and is also effective as nutrient source for maize, beans and vegetables in Kenya, Malawi and Zimbabwe (Jama et al., 2000) and yam in Nigeria (Adeniyi et al., 2008). The abundance and adaptation of this weed species to various environment couples with its rapid growth rate and very high vegetative matter turn over makes it a candidate species for soil rejuvenation (Olubode et al., 2007).

Tithonia has received less research attention in Nigeria compared with siam weed as to its effect

Received 02 December 2010; Revised 12 March 2011; Accepted 15 March 2011

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on soil properties and productivity. In the guinea savanna zone of Nigeria, Atayese and Liasu (2001) found that soil under *Tithonia* and siam weed had higher pH, porosity, moisture content, nitrogen, phosphorus, potassium, sodium, calcium, mycorrhizal fungi spores and earthworm cast density and lower bulk density compared with bare soil. The aim of this work was to investigate soil depth to which *Tithonia* and siam weed influence soil chemical properties in humid zone of southwest Nigeria, and relative effect of their natural fallows on soil properties and growth of maize. Soils fallowed to *Tithonia*, siam weed and spear grass were compared to determine their relative effect as soil improvers.

Materials and Methods

Two studies were carried out in separate phases. The first study investigated effect of *Tithonia*, siam weed and spear grass on soil properties and maize. The second study investigated effect of *Tithonia* and siam weed at different soil depths on the soil properties.

[a] In the first study, soil samples were collected from plots that were under three year fallow of *Tithonia*, siam weed and spear grass (i.e. soils that were naturally grown of *Tithonia*, siam weed and spear grass) and plots cropped to cassava for three years in the same locality. Ten samples were randomly collected with auger from fallow and cassava plots and later bulked into three per plot. The twelve samples were air dried and sieved using 2 mm sieve. Portions of soil samples were kept for chemical analysis before they were weighed into 4-litre polythene pots. There were three pots per treatment given 36 pots on the whole. Pots were laid out in screen house using randomized complete block design. They were wetted to saturation and allowed to drain for 3 days. Maize seeds were planted and thinned to one seedling per pot. Wetting was done at two days interval using watering can. Plant height, stem girth, ear leaf length (L) and width (W) were determined at 6 weeks after planting. Leaf area was calculated as product of L x W x 0.75 (Sinoquet and Andrieu, 1993; Olufayo et al., 1994).

Steel core samples collected from plots in triplicate were used for determination of bulk density, gravimetric moisture content and total porosity. Total porosity was calculated from bulk density and particle density of 2.65 g/cm³.

[b] In the second study, soil samples were collected in triplicate from 0-5, 5-10 and 10-15 cm depths in a pit dug at each plot dominated by *Tithonia*, siam weed and cropped to cassava to

determine their chemical properties at different depths.

Soil chemical analysis

Air-dried 2 mm sieve soil samples collected from the two studies were subjected to routine chemical analysis. Total N was determined by micro Kjeldahl approach and available P was determined by molybdenum blue colorimetry. Exchangeable K, Ca and Mg were extracted using ammonium acetate, K was determined on flame photometer and Ca and Mg by atomic absorption spectrophotometer (Okalebo et al., 1993).

Results and Discussion

Compared with soil cropped to cassava and fallowed to spear grass, *Tithonia* and *Chromolaena* had significantly higher ($p=0.05$) values of soil N, P and K (Table 1). Cropped soil had least values of the chemical properties. Compared with soil cropped to cassava, *Tithonia* and *Chromolaena* increased soil N by 206 and 189%, respectively. The percentage increases for available P were 41 and 41%, respectively while the percentage increases for exchangeable K were 57 and 33%, respectively. Soil N and P between soils fallowed to *Tithonia* and *Chromolaena* were not statistically different from each other, but soil fallowed to *Tithonia* was significantly higher ($p=0.05$) than *Chromolaena* in K by 18%. The influence of *Tithonia* and *Chromolaena* fallow in increasing the soil nutrient contents had been widely reported (Obatolu and Agboola, 1993; Ademiluyi and Omotoso, 2008; Chukwuka and Omotayo, 2009). This was attributed to the conversion of biomass to organic matter, which increased the levels of these nutrient elements, indicating that fallow improved soil properties.

Table 1. Effect of weed fallow on soil chemical properties.

Fallow	N %	P mg/kg	K cmol/kg
<i>Chromolaena</i>	0.52	8.2	0.40
<i>Tithonia</i>	0.55	8.2	0.47
Spear grass	0.25	6.0	0.21
Cassava	0.18	5.8	0.30
LSD (0.05)	0.19	0.64	0.08

Table 2 shows data on values of soil chemical properties at 0-5, 5-10 and 10-15 cm depths. Soils fallowed to *Tithonia* and *Chromolaena* had higher values of N, P, K, Mg, Ca and organic matter than cropped soil at 0-5 and 5-10 cm depths. At 10-15 cm depth soils under weed fallows also had higher values of N, P, Ca and organic matter. The

parameters reduced in the order *Tithonia*, *Chromolaena* and cassava soils. However, when K and Mg are considered at 10-15 cm depth, cassava soil had highest K content and relatively high Mg content. This could be due to leaching of the cations in the more exposed cassava soil. The fallow plants (*Tithonia* and *Chromolaena*) increased soil chemical properties to 15 cm depth.

Generally soil nutrients reduced with depth from 0 to 15 cm depth. The findings that soil nutrients reduced with depth from 0 to 15 cm depth could be attributed to higher concentration of organic matter in the upper soil layer than in the sub soil layers since organic matter is known to be ultimate determinant of soil fertility in most tropical soils (Obatolu and Ibiremo, 1999).

Table 2. Effect of fallowing on soil chemical properties at different depths.

Fallow/depth (cm)	N %	P mg/kg	K cmol/kg	Ca cmol/kg	Mg cmol/kg	OM %
<i>Chromolaena</i> / 0-5	0.52	8.2	0.40	4.1	1.20	2.94
<i>Tithonia</i> / 0-5	0.55	8.3	0.47	3.8	0.93	2.96
Cassava / 0-5	0.25	6.1	0.31	2.2	0.39	1.59
LSD (0.05)	0.10	1.1	0.01	0.36	0.29	0.96
<i>Chromolaena</i> / 5-10	0.32	8.3	0.33	3.2	0.50	2.94
<i>Tithonia</i> / 5-10	0.41	8.4	0.38	3.2	0.86	2.96
Cassava / 5-10	0.20	6.1	0.22	2.0	0.26	1.59
LSD (0.05)	0.12	0.7	0.10	0.5	0.25	0.69
<i>Chromolaena</i> / 10-15	0.32	6.5	0.28	2.6	0.41	2.03
<i>Tithonia</i> / 10-15	0.29	5.9	0.33	2.9	0.65	2.37
Cassava / 10-15	0.16	5.5	0.53	1.7	0.61	1.20
LSD (0.05)	0.11	0.6	0.10	0.6	NS	0.52

Physical conditions of soils under *Tithonia* and *Chromolaena* respectively were better than that of soil under cassava and spear grass (Table 3). Soils under *Tithonia* and *Chromolaena* had significantly lower ($p=0.05$) soil bulk density and significantly higher ($p=0.05$) total porosity compared to soils fallowed to spear grass and cropped to cassava. Soil under spear grass had the highest moisture content which was significantly higher ($p=0.05$) compared to soils under *Chromolaena* and cassava, but not statistically different from soil under *Tithonia*. Cassava soil had least total porosity and highest soil bulk density, and it also had least moisture content. The quick growing weeds should have returned biomass and enhanced organic matter which is known to enhance soil physical and chemical properties. Hence soils under the weeds had relatively high values of nutrients content.

Table 3. Effect of weed fallow on soil physical properties.

Fallow	Bulk density g/cm ³	Moisture content %	Total porosity %
<i>Chromolaena</i>	1.1	28.2	58
<i>Tithonia</i>	1.0	35.7	62
Spear grass	1.3	37.5	51
Cassava	1.4	20.2	47
LSD (0.05)	0.08	7.2	5

Table 4 shows the effect of weed fallow on maize growth. Maize plant height, stem girth and leaf area were significantly affected by weed fallow. Soil under *Tithonia* had the highest maize plant height, stem girth and leaf area which were significantly higher ($p=0.05$) compared to soils fallowed to *Chromolaena*, spear grass and cropped to cassava. Soil under *Chromolaena* fallow produced maize plant height, stem girth and leaf area which were significantly higher ($p=0.05$) than soils fallowed to spear grass and cropped to cassava. Soil cropped to cassava had the least maize plant height, stem girth and leaf area which were significantly lower ($p=0.05$) when compared to soils dominated by *Tithonia*, *Chromolaena* and spear grass. In the overall performance, maize height, girth and leaf area increased in order of cassava, spear grass, *Chromolaena* and *Tithonia* soils. The more improved soil physical and chemical properties under *Tithonia* and *Chromolaena* fallows led to better growth of maize compared with maize grown to cassava and spear grass soils. Fallowing soil to weeds improved soil physical and chemical properties and maize performance as opposed to cropping. The better performance of maize growth under *Tithonia* fallow compared to other organic sources could be attributed to its higher nutrient concentrations and a faster rate of decomposition of nutrient release.

This agrees with the findings of Nziguheba et al. (1998) that *Tithonia* is a high quality organic source in terms of nutrient release and supplying capacity. Lower C/N ratio of *Tithonia* (8:1) compared to moderate C/N ratio of *Chromolaena* (12:1) (Olabode et al., 2007) and relatively high

C/N ratio of spear grass (20:1) indicates a faster decomposition. Palm and Rowland (1997) also listed high N and P contents and high soluble fraction and moderate lignin content resulting in high biodegradation as the strong points of *Tithonia* as source of organic matter.

Table 4. Effect of weed fallow on maize growth.

Fallow	Plant height (cm)	Leaf area (cm ²)	Stem girth (cm)
<i>Chromolaena</i>	76.6	158	3.2
<i>Tithonia</i>	106.0	267	4.4
Spear grass	69.4	106	2.8
Cassava	48.9	63	2.1
LSD (0.05)	18.8	27	0.7

Tithonia and *Chromolaena* serve as protective cover against erosion and are agents of nutrient cycling and supply. Atayese and Liasu (2001) found that soils under *Tithonia* and *Chromolaena* contained arbuscular mycorrhizal fungi spore which enhanced absorption of nutrients from soil to biomass. *Tithonia* is known to be rich in N, P and Ca (Taiwo and Makinde, 2005; Liasu and Achakzai, 2007). Because of the ability of the weeds to protect the soil, proliferate surface soil with their roots, attract fungi, increase biomass and organic matter; the fallows were able to improve soil structure and porosity, reduce bulk density, and increase soil fertility and maize growth.

Conclusion

Mexican sunflower and siam weed respectively reduced soil bulk density and increased total porosity and soil water content. They also increased the soil nutrient levels and subsequently produced high growth of maize. This study has shown that *Tithonia* and *Chromolaena* have high fertilizing and sound potentials for building soil organic matter to adequate levels that will meet nutritional needs of crops as well as improve the nutrient element status of arable fields when bush fallows are dominated by such organic resources than when such fields are taken over by grasses like spear grass. *Tithonia* and *Chromolaena* are potential soil improvers and are therefore recommended for use as fallow plants in rainforest zone of southwest Nigeria for enhanced productivity.

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PLANT SCIENCE

Biodiversity of cyanobacteria from Uppanar estuary, south-east coast of India

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Abstract

The spatial and seasonal patterns of distribution of cyanobacterial species and their abundance in relation to physicochemical parameters were studied during April, 2002 to March, 2003 from two stations of Uppanar estuary in the south-east coast of India (Lat. 11°42'N: Long. 79°49'E). Presently 15 species of cyanobacteria were recorded which belong to different families viz. Chroococcaceae (4), Oscillatoriaceae (7) and Nostocaceae (4). The concentration of nutrients was low during summer season and more number of cyanobacteria was recorded in monsoon season.

Key words: Physico-chemical, Cyanobacteria, Seasonal distribution, Uppanar estuary

Introduction

Estuaries act as natural laboratories for studying the dynamics of terrigenous chemical constituents borne by the river water on their way in to the sea, alongwith interactions and adaptations of organisms to wide range of environmental situations. Hydrographic features like temperature, salinity, dissolved oxygen, pH and transparency constitute the important environmental requisites which govern the distribution and abundance of flora and fauna in estuaries (Rajashree Gouda and Panigrahy, 1993). Cyanobacteria are oxygen evolving photosynthetic prokaryotes, which inhabit various types of marine environments. They occur in oceans, estuaries, saline backwaters, salt lakes, salt marshes and hypersaline salt pans (Fogg et al., 1973).

Cyanobacteria are photoautotrophic picoplankton distributed over vast tracts of the world's oceans where they occupy a key position at the base of the marine food web and contribute significantly to global primary productivity (Partensky et al., 1999). The cyanobacteria are found distributed from polar through temperate to

tropical waters and are generally more abundant in nutrients rich surface waters. The basic and fundamental requirement for initiating marine cyanobacterial biotechnology is first to enumerate the natural biodiversity and to understand their innate properties which could be useful for a variety of purposes. Hence, the present investigation of the Uppanar estuary was taken up to understand the seasonal variations of various physico-chemical characteristics and their influence on the cyanobacteria in uppanar estuary.

Materials and Methods

Uppanar estuary (Lat. 11° 42' N; Long 79° 49'E) (Figure 1) is formed by the confluence of Gadilam and Paravanar rivers, and opens in to Bay of Bengal near Cuddalore old town on the south east coast of India. The present study is focussed on the various hydro- biological and ecological parameters of the Uppanar estuary. Surface water samples were collected from Uppanar estuary covering two stations (Station 1 - mouth and station 2 - estuary) at monthly intervals for a period of one year from April 2002 to March 2003, for the estimation of various physicochemical parameters.

Rainfall data were obtained from the Statistical (Meteorological) Department at Cuddalore. Temperature (air and surface water) was measured using a standard centigrade thermometer; light penetration in the water column was measured with the help of a Secchidisc and the light extinction coefficient (LEC) was calculated using formula of Pool and Atkins (1929). Salinity was estimated

Received 01 February 2011; Revised 16 March 2011; Accepted 26 May 2011

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with the help of a Salinometer Model-E-2 and pH was measured using an Elico pH meter (Model L`-120).

Dissolved oxygen, nutrients like nitrate, nitrite, phosphate and reactive silicate were estimated adopting the standard procedure described by Strickland and Parsons (1972).

Planktonic cyanobacteria were collected using the plankton net made up of bolting silk cloth No.

25 (pore size 64 μ M) and the net was towed for 15 minutes in the surface water. The collected planktonic sample was transferred to the laboratory. A portion of this sample was preserved in 5% formalin and subsequently the sample was analyzed under the microscope. Identification of specimen was done using the taxonomic publications (Biswas, 1949; Geitler, 1932; Prescott, 1951; Desikachary, 1959; Anand et al., 1986).

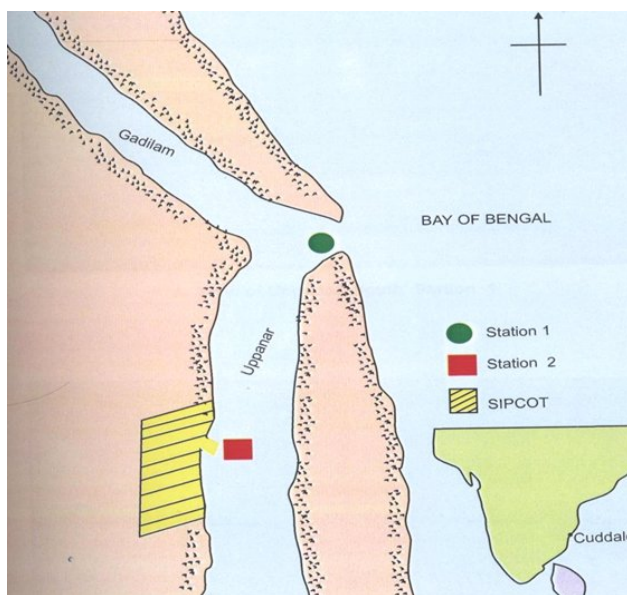


Figure 1. Map showing the study area.

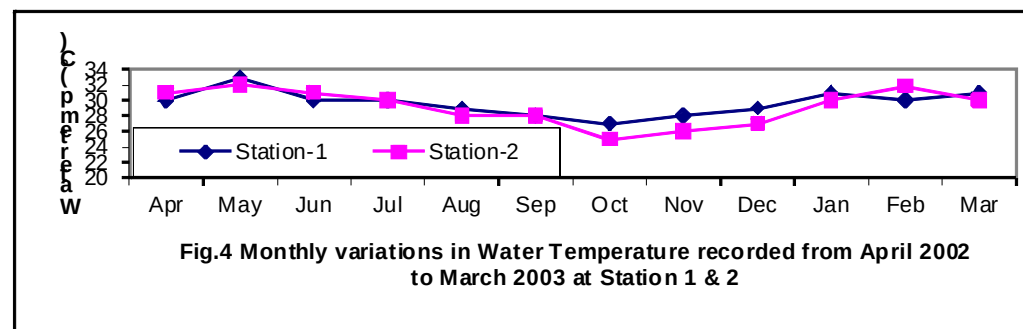
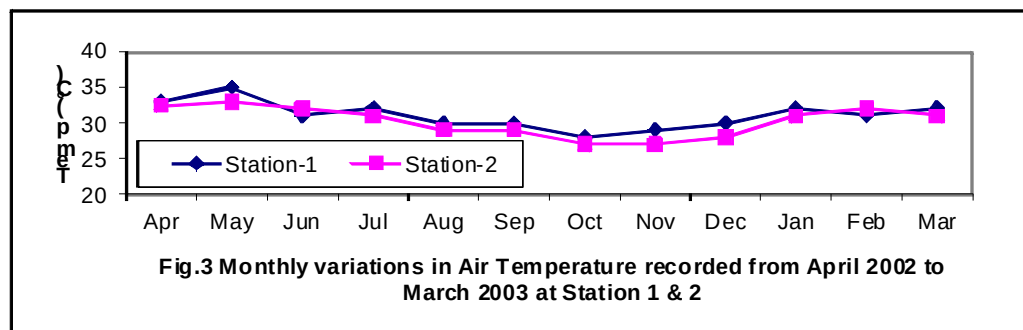
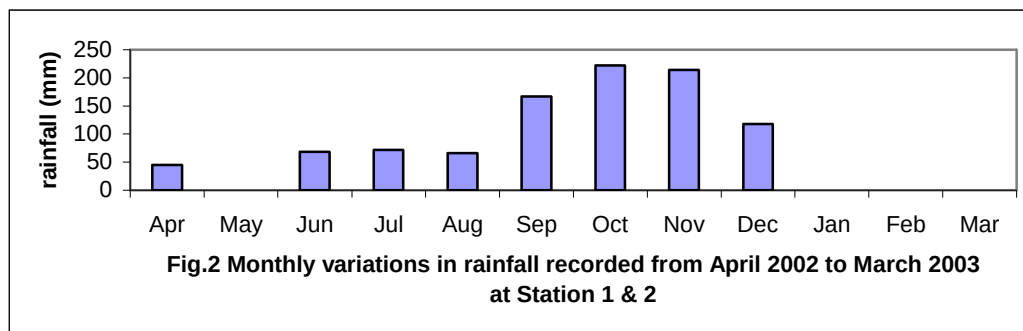
Results and Discussion

The physicochemical characteristic features of the estuarine system which closely follow the pattern of river runoff and tidal variations were mostly controlled by the seasonal regimes. Rain fall is the most important cyclic phenomenon and it brings about important meteorological changes in the physical and chemical characteristics of the coastal and estuarine environments. In the present study, peak values of rainfall were recorded during the monsoon season in October, 2002 (222. mm) (Figure 2). This is because, the study area received bulk rainfall during the northeast monsoon compared to the south west monsoon (May to July).

Temperature variations in the estuarine environment can influence the other physicochemical characteristics. High atmospheric temperature was recorded during the summer season (Figure 3) due to clear sky with more solar radiation less solar radiation with cloudy sky and more rainfall during the monsoon season greatly reduced the atmospheric temperature. The surface water temperature largely depends on the intensity of solar radiation, evaporation and fresh water

influx. The presently recorded summer peaks 33°C at Station – 1 and 32.1°C at station – 2 (Figure 4) are closely resemble the data generated earlier by several workers from the east and west coast of India (Vijayakumar et al., 2000; Srinivas Rao and Umamaheswara Rao, 2002).

Light extinction co-efficient (k) at both the stations was high during the monsoon season (Figure 8) due to low intensity of solar radiation, higher concentration of dissolved organic matter, suspended sediments, increased freshwater discharge, wave action and wind action. Similar observations have been made from Vellar estuary by Mani (1989) and Sujata Mishra et al. (1993) from Bahuda estuary. Low light extinction co-efficient was observed during summer season at both stations and it could be due to the high solar penetration, reduced freshwater inflow and land run-off. In general, these values were high during rainy months mostly due, to the biogenic turbidity of the water column. This was due to the monsoon floods which brought in lot of silt and terrigenous material to the biotope coupled with reduced of sunshine (Subramanian and Kannan, 1998).



Salinity at both stations showed high values during the summer season due to less rain fall, decreased freshwater inflow and rise in temperature of the estuary and low during the monsoon season due to high rainfall and land runoff. Salinity is one of the important factors which profoundly influence the abundance and distribution of the fauna and flora in the estuarine environment which in turn depends on the inflow of fresh water and the prevailing temperature. During the monsoon season low salinity recorded (Figure 5) due to heavy rainfall and large quantity of fresh water inflow. Thus, the variations in salinity were mainly influenced by the rainfall and entry of freshwater (Sasinayar et al., 2000).

The pH was higher during the summer season while it was low during the monsoon period (Figure 6) due to the uptake of CO₂ by the photosynthesizing organisms, especially phytoplankton and planktonic cyanobacteria from the seawater could have

increased the pH level during the summer season (Subramanian and Mahadevan, 1999). The low pH observed during the monsoon due to the influence of freshwater influx and dilution of seawater, reduction of salinity and temperature and decomposition of organic matter (Zingde et al., 1987; Subramanian and Kannan, 1998).

Dissolved oxygen concentration varied from 2.8 to 6 ml/l at station-1 and from 3.2 to 6.3ml/l at station-2 (Figure 7) with the maximum during the monsoon season in November and minimum during the post monsoon in March at both the stations. Distribution and behavior of nutrients in the coastal environments particularly in the nearshore waters and estuaries would exhibit considerable seasonal variations depending upon the local conditions of rainfall, fresh water inflow, tidal incursion and some biological activity (Choudhary and Panigrahy, 1991).

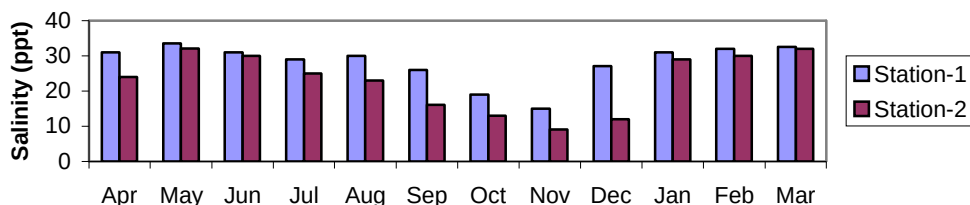


Fig.5 Monthly variations in Salinity recorded from April 2002 to March 2003 at Station 1 & 2

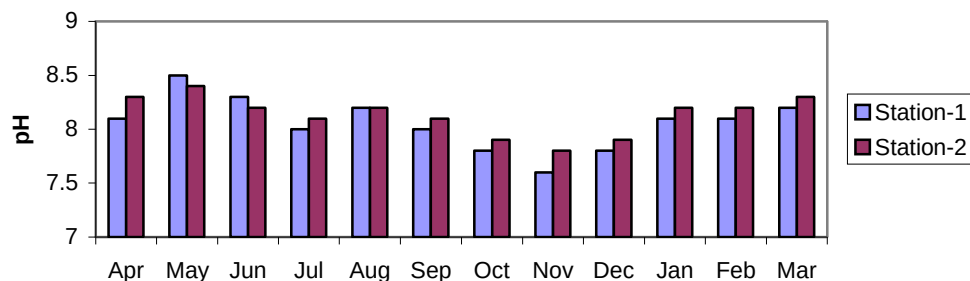


Fig.6 Monthly variations in pH recorded from April 2002 to March 2003 at Station 1 & 2

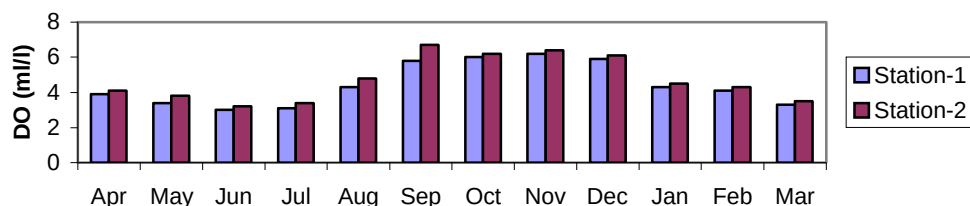


Fig.7 Monthly variations in Dissolved Oxygen recorded from April 2002 to March 2003 at Station 1 & 2

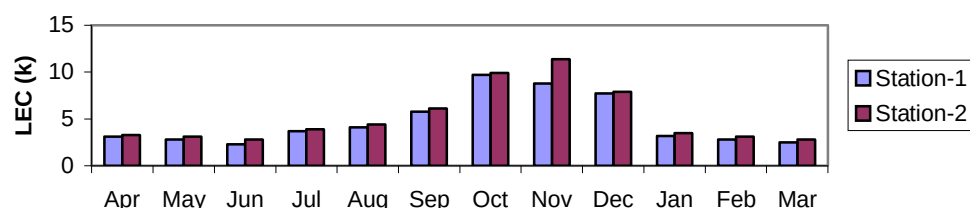


Fig.8 Monthly variations in Light extinction Co-efficient recorded from April 2002 to March 2003 at Station 1 & 2

The higher values of nitrate during the monsoon could be due to heavy rainfall, river and terrestrial run – off (Padmavathi and Sathyanarayana, 1999). The low nitrate concentration during the summer season: 9.04 μm at station-1 and 9.75 μm at station – 2 (Figure 10) were observed during summer months and at the end of the post monsoon which could be attributed to the rapid utilization of nitrate by phytoplankton

and Cyanobacteria, (Panigrahy et al., 1999). The higher values of nitrite concentration recorded (Figure 9) at both stations during the monsoon season could be related to the terrigenous input by river and the low values of nitrite observed during the summer season might be due to the lesser amount of freshwater inflow and higher salinity (Sasinayar and Gouda, 1999).

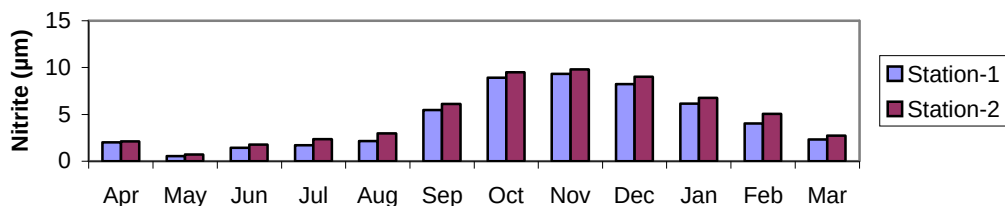


Fig.9 Monthly variations in Nitrite recorded from April 2002 to March 2003 at Station 1 & 2

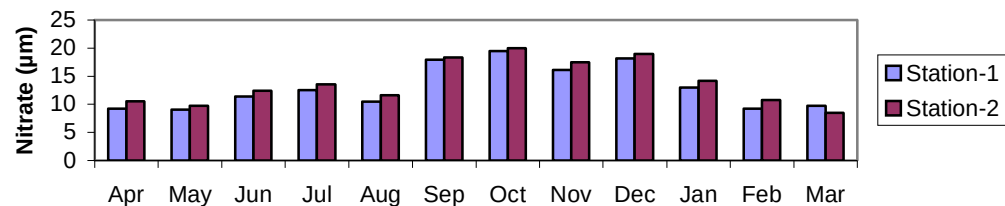


Fig.10 Monthly variations in Nitrate recorded from April 2002 to March 2003 at Station 1 & 2

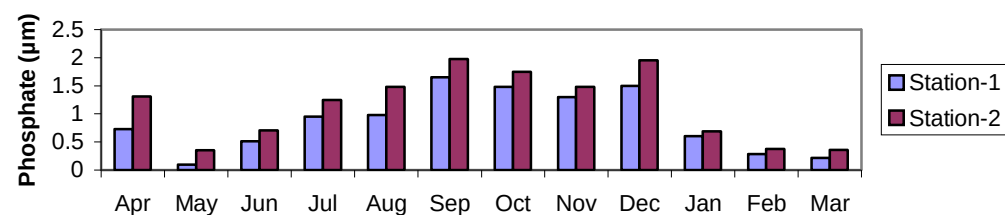


Fig.11 Monthly variations in Phosphate recorded from April 2002 to March 2003 at Station 1 & 2

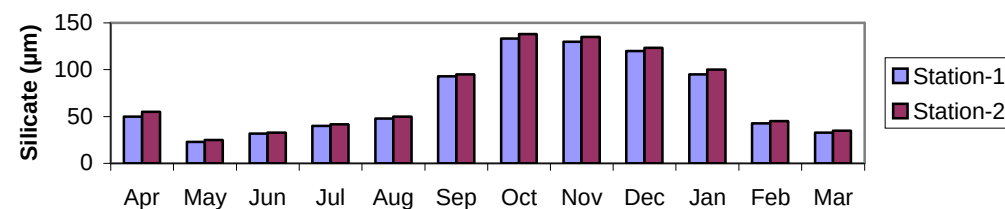


Fig.12 Monthly variations in reactive silicate recorded from April 2002 to March 2003 at Station 1 & 2

Inorganic phosphate registered its peak values during the monsoon season 0.10 to 1.65 μm (Figure 11) and there was a decrease in the concentration during the post monsoon season to summer. The high value in the estuary during the monsoon season was due to fertilizers from the agricultural land run-off and erosion of phosphate rock (Jailalram, 2001). Low concentration of phosphate observed during the summer season was due to the decreased land drainage, utilization by phytoplankton (Srinivasa Rao and Umamaheswara Rao 2002; Sevimpolat and

Mine PercinPiner, 2002). The reactive silicate concentration was comparatively higher than other nutrients. High concentration was recorded during monsoon season and low concentrations was recorded during summer and pre-monsoon seasons which were due to the considerable reduction in the freshwater input and greater utilization of this nutrient by the abundantly occurring phytoplankton and cyanobacteria for their biological activity. This is in agreement with the earlier observation in Mulki estuary by Vijayakumar et al. (2002).

Table 1. Cyanobacteria recorded in Uppanar estuary during April 2002 to March 2003.

S. No.	Family	Species	Station-1	Station-2
1.	Chroococcaceae	<i>Microcystis littoralis</i> [Hang.] Forti.	-	+
		<i>Chroococcus tenax</i> (Kirch) Hiron	+	+
		<i>Gloeocapsa aeruginosa</i> (Carm.) Kütz	+	-
		<i>Synechococcus elongatus</i> Näg	-	+
2	Oscillatoriaceae	<i>Spirulina subsalsa</i> Oerst. ex. Gomont.	+	+
		<i>Oscillatoria agardhii</i> Gomont	+	+
		<i>O. salina</i> Biswas	+	-
		<i>Phormidium tenue</i> (Menegh.) Gomont	+	+
		<i>P. fragile</i> (Menegh.) Gomont	-	+
		<i>Lyngbya aesturii</i> Liebon. ex. Gomont	+	+
		<i>Trichodesmium erythraeum</i> Ehrenberg ex. Gomont	+	-
		<i>Nostoc paludosum</i> Kütz. ex. Born. et Flah	-	+
3.	Nostocaceae	<i>Anabaena variabilis</i> Kütz	-	+
		<i>A. ambigua</i> Rao. C.B	+	-
		<i>Cylindrospermum majus</i> kuetz. ex Born. et Flah	+	+

+ = Present; - = Absent

Cyanobacteria are unique group of photosynthetic prokaryotic microorganisms. They are better known till recent times as blue green algae. Cyanobacteria constitute one of the commercially important marine resources having unique characters of fixing atmospheric carbon and nitrogen; In general, cyanobacteria seem to be more abundant in natural or slightly alkaline habitats. Among the planktonic species several forms blooms. The most common bloom-forming marine cyanobacterium in the tropical water is *Trichodesmium*.

In the present study, an attempt has been made to study the cyanobacteria occurring in estuarine environment. Totally 3 Families, 12 Genera and 15 cyanobacterial species were recorded from both stations (Table 1). The estuary showed a good planktonic cyanobacterial population during monsoon season. The species that occurred in the two stations during monsoon were two colonial form and 3 filamentous forms namely *Anabaena variabilis*, *Cylindrospermum majus*, *Chroococcus tenax*, *Microcystis littoralis* and *Oscillatoria agardhii* (Table 1). In general, higher number of species composition and distribution of cyanobacteria were recorded at both the stations during monsoon season. This could be attributed to the presence of fresh water species of cyanobacteria. They would enter in to this ecosystem during monsoon season through the irrigation channels. This was apparent as cyanobacteria occurred in abundance near station-2 of the fresh water connecting inputs in the estuarine ecosystem.

The simple correlation co-efficient analysis between physico-chemical characters and cyanobacterial diversity revealed no significant

relationship between them. Similar observation were reported earlier by Selvakumar and Sundararaman (2001) and Thajuddin and Subramanian (1992). Hence, it is necessary to document the biodiversity of Cyanobacterial resources in the estuarine ecosystems. A number of Cyanobacteria from estuarine habitat have potential and commercial value.

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ANIMAL SCIENCE

Inhibition of the growth of rats by extruded snacks from bean (*Phaseolus vulgaris*) and corn (*Zea mays*)

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Abstract

There is a need to develop new food products with high protein quality and a high caloric value, high acceptability and low costs for low income families. The aim of this investigation was to evaluate in vivo an extruded bean-corn product, supplemented with Ca and Zn, as a potential nutritional snack. Extruded and non-extruded bean-corn flours were fed to rats. Antinutritional factors, rat weight and length, femur weight and heart weight and volume were determined. Microscopy pictures of rat liver were taken. The antinutritional factors present in the studied bean variety did not affect rat growth or internal organ characteristics. Bean-corn diets affected ($p < 0.05$) rat weight and produced liver alterations, probably because of interference by bean protein with intestinal or systemic metabolism. Ca and Zn supplementation is not necessary in a bean-corn extruded snack for rats.

Key words: Bean flour, Extrusion, Maize flour, Mineral supplementation, Steatosis

Introduction

Malnutrition is common among neonates and infants in developing countries. There is a need to develop new food products with high protein quality and a high caloric value, high acceptability and low costs for low income families (Kannan et al., 2001; Hussain et al., 2010; Atienzo-Lazos, 2011; Rodríguez-Miranda et al., 2011, 2012).

Legumes play an important role in the diet of many people throughout the world. They are drought resistant and salt-tolerant (Messina, 1999; Sagarpa, 2004; Akhtar et al., 2010; Rao and Shahid, 2011). Beans are rich in lysine but deficient in sulfur amino acids. On the other hand, corn contains high amounts of sulfur amino acids, but is poor in lysine. A combination of legumes and cereals can result in a product with high protein quality, even comparable to casein (Gujska and

Khan, 1990; Stoecker et al., 2006; Dhingra and Jood, 2007; Hussain et al., 2010; Giwa and Ikujenlola-Abiodun, 2010; Atienzo-Lazos, 2011; Rodríguez-Miranda, 2012).

Beans and corn, as well as other plants, have antinutritional factors that affect growth, nitrogen balance, intestinal sugar and amino acid absorption, and the immune system, and reduce the bioavailability of cations (Mamiro et al., 2001; Manary et al., 2002; Devine 2002; Marzo et al., 2002; Nestares et al., 2003; Boccio and Monteiro, 2004; Dhingra and Jood, 2007; Martinez et al., 2012). Phytic and oxalic acid, which are present in cereals and legumes, can bind minerals such as Ca, Fe and Zn, reducing the availability of these minerals in cereal foods (Ockenden, 1997; Zhou and Erdman 1995; Adams et al., 2002; Lönnerdal et al., 2011). Phosphate can cause significant zinc loss and influence its bioavailability. Dehulling can reduce phytic acid content by 60 – 90% in cereals, since phytic acid is located principally in the germ and aluerone layer (Hatzack et al., 2000; Linares et al., 2007; Lönnerdal et al., 2011). Although phytate is the principal chelating agent, water-soluble components are responsible for 39% of the binding power of whole bran (Linares et al., 2007). Other

Received 20 January 2012; Revised 25 February 2012; Accepted 27 February 2012

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authors also found that the highest amount of phytate-P in cereals is found in the bran fraction (Alonso et al., 2000). Another antinutritional factor in beans are lectins. The presence of 0.5% lectins in the rat diet can be enough to introduce impairment of intestinal absorption and produce death (Martinez et al., 2012). The presence of both trypsin inhibitors and lectins in the diet increases fecal nitrogen excretion (Morrison et al., 2005; Tan et al., 2006; Macedo et al., 2007; Wati et al., 2009). Since lectins are located in the cytoplasm of the cotyledon, dehulling has no effect on its content. Detoxification of lectins can be obtained by traditional cooking, but some industrial processes, such as dry heat, do not lead to complete detoxification (Tan et al., 2006). Extrusion of bean-corn flour is more effective than soaking for inactivating antinutritional factors such as lectins (Dhurandhar and Chang, 1990; Zhou and Erdman, 1995; Ockenden et al., 1997; Hatzack et al., 2000; Mamiro et al., 2001; Adams et al., 2002; Martinez et al., 2012). To counter the loss of minerals caused by the phytic acid present in cereals and legumes, flours may be complemented with minerals such as Ca, Fe and Zn from natural sources (Hussein et al., 2010; Ramkumar et al., 2012). There is little information regarding the nutritional effect of extruded bean-corn flours. The purpose of the present study was to evaluate *in vivo* an extruded bean-corn product, supplemented with Ca and Zn.

Material and Methods

Bean and corn flour

The bean cultivar Pinto villa (*Phaseolus vulgaris*) and the corn (*Zea mays*) cultivar H-131 were used (INIFAP-Durango, Mexico). Beans and corn were conditioned at 16% humidity for six hours. The flour (<200µm diameter) was obtained with a laboratory mill (Brook Control Gear, West Yorkshire, England). Composite flour containing a mix of 30% bean and 70% corn flour was prepared, since higher bean flour concentrations reduces expansion index and quality of the extrudates.

Extrusion process

The bean-corn flour was extruded with a Brabender Extruder 19/25 D (Germany) with a compression ratio of 1:6, 25 rpm at 150°C and 20% humidity. The duration of the extrusion process was constant for all experiments.

Chemical composition

Fat, protein starch, ash and fiber were determined by AOCS (1983) methods. Results are presented as the mean of two replicates.

Antinutritionals

Trypsin inhibitors were determined as described by Alonso et al. (2000). Phytic acid was determined by the method described previously (AOCS, 1983). Lectins were determined by the method described by Haug and Lantzsch (1983). Results are presented as the mean of three replicates.

In vivo assays

Sprague-Dawley female rats (n = 54), 37 days old, with a mean weight of 100g at the beginning of the study, were used. They were kept in plastic metabolic cages and were divided into nine groups. Each group was formed by six individuals. The groups were fed and named as follows: (1) commercial rat feed, Teklad 2018S Rodent Diet, Harlan, USA (control), (2) bean-corn flour without addition of Ca and Zn (Bean-corn-flour), (3) bean-corn flour supplemented with Ca and Zn (Bean-corn-flour Ca-Zn), (4) bean-corn flour supplemented with Ca (Bean-corn-flour Ca), (5) bean-corn flour supplemented with Zn (Bean-corn-flour Zn), (6) extruded bean-corn flour without supplementation of Ca and Zn (Bean-corn-extrudates), (7) extruded bean-corn flour supplemented with Ca and Zn (Bean-corn-extrudates Ca-Zn), (8) extruded bean-corn flour supplemented with Ca (Bean-corn-extrudates Ca) and (9) extruded bean-corn flour supplemented with Zn (Bean-corn-extrudates Zn). During the 22 days of the experiment, the rats of the control group were fed only with the basal diet described by Tejerina et al. (1977). The diets of the eight other groups were supplemented with the recommended concentrations of minerals and with 27 UI of vitamin E as DL - α - tocopherol (Tejerina et al., 1977). The rats were kept at 22°C and 50% H. Water was given every day.

Body weight and weight gain/day were recorded twice a week and are presented in g as a mean of six replicates per day (Rodriguez et al., 1998). After 22 days the rats were sacrificed. Femur lengths (mm) were measured as described by Rodriguez et al. (1998), and is presented as the mean of six replicates.

Hearts were removed as described by Ceislar et al. (1998). Heart weight is presented in g, while heart volume is presented in ml as the mean of six replicates.

The amount of Ca and Zn present in the diets and femur was determined by atomic absorption (Alonso et al., 2000).

Histological studies

The liver was removed as described by Ceislar et al. (1998). Liver weight is presented in g as the mean of six replicates. Histological sections were made with Hematoxylin - Eosin at the Faculty of Medicine at the Juarez University, Durango, Durango.

Statistical analysis

Data were analyzed by ANOVA using SIGMASTAT (SPSS. Inc.) at $p < 0.05$.

Results and discussion

Chemical composition

The fat, protein, starch, ash and fiber content of corn, beans and bean-corn flour are summarized in Table 1. The fat and starch content in corn was higher ($p < 0.05$) than in bean, while protein and

fiber were higher in beans ($p < 0.05$) than in corn. The starch, protein, fat and ash contents of bean were comparable to results obtained by other authors (Gujska and Khan, 1991; Atienzo-Lazos, 2011; Rodriguez-Miranda et al., 2011, 2012). The protein and fiber content were higher ($p < 0.05$) in the bean-corn flour mixture than in corn. Our results are consistent with other studies, which showed a higher amount of protein and fiber in beans than in corn (Sagarpa, 2004; Rao and Shahid, 2011). Mixing bean and corn flour can significantly ($p < 0.05$) increase dietary protein and fiber as shown in this work and other findings, whereas composite flours can improve the nutritional value of food (Dhingra and Jood, 2007; Giwa and Ikujenlola-Abiodun, 2010; Hussain et al., 2010).

Table 1. Fat, protein, starch, ash and fiber content of milled bean, corn and of the bean-corn flour mixture (70/30).

Sample	Chemical characteristics [%]				
	Fat	Protein (N x 6.25)	Starch	Ash	Fiber
Control diet (Harlan)	6.0±0.08 ^a	18.8±0.15	41.2±0.22	5.9±0.03	3.8±0.05
Corn	4.8±0.09	12.0±0.06	65.5±0.32	3.1±0.06	4.3±0.09
Bean	1.8±0.13	18.6±0.18	51.9±0.50	3.7±0.09	11.0±0.20
Bean-corn flour	3.9±0.09	13.3±0.11	59.8±0.38	2.3±0.05	8.1±0.09

^a standard deviation of the mean.

The bean-corn flour had 37.8% less ($p < 0.05$) ash content than beans, due to dehulling of beans before extrusion.

The bean-corn flour used for rat diets and for extrusion contained 35, 29 and 61% less fat, protein and ash content than the control diet (Table 1).

Lectin activity and Trypsin inhibitors

Lectin activity is summarized in Table 2. Relatively high lectin activity was detected in the bean-corn flour mixture before extrusion. Other authors have also found high levels of lectin activity in legumes (Macedo et al., 2007; Wati et al., 2009). Extruded bean-corn flour showed no lectin activity, as found by other authors (Ockenden et al., 1997; Atienzo-Lazos, 2011). Extrusion is more effective at inactivating lectins than dry heat (Rodriguez-Miranda, 2012). Lectin activity was destroyed by the extrusion conditions used in this study. Cooking at 100°C also inactivates lectin (Dhurandhar and Chang, 1990).

Trypsin inhibitor activity was not present in the control diet and it was completely destroyed by extrusion at 150°C in 30 sec. (Table 2). Dhurandhar and Chang (1990) found a trypsin inhibitor activity after 30 min of cooking beans at 100°C. Other

researchers also found that traditional cooking shows a rest trypsin inhibitor activity. Generally, extrusion inactivation of trypsin inhibitors was more effective than in other studies (Dhurandhar and Chang, 1990), which reported 43 to 87% of activity after boiling for 1 hour. Other methods such as dry roasting have been reported to be ineffective in destroying trypsin inhibitor activity. Extrusion is a very effective process for inactivating protein-antinutrients such as lectins and trypsin inhibitors in beans. Bean products containing a high amount of protein-antinutrients do not support the normal growth of rats even when the diet is supplemented with essential amino acids (Martinez et al., 2012).

Table 2. Trypsin inhibitors and lectin activity of control, bean-corn flour mixture and extrudates.

Diets	Trypsin inhibitors [TIU/g]	Lectin activity [HU/mg protein]
Control (Harlan)	0	0
Bean-corn flour mixture	10856.9±1.56 ^a	640
extrudates	0	0

^a standard deviation of the mean.

Body weight of rats

Table 3 shows the data obtained in the growth experiments where the control diet and bean corn flour diets, either supplemented with Ca and/or Zn, raw or extruded, were fed to young rats. Both raw and extruded bean-corn flour caused a weight loss ($p < 0.05$) within 22 days, compared to the weight of rats (100g) at the beginning of the experiment. All rats fed with raw or extruded bean corn flour lost around 2.5 g/day. After 22 days the rats started to die. Other authors have shown that heat-treated bean protein incorporated into a basal diet produced a normal rate of growth in rats. These authors also showed that other legumes, with no heat treatment, led to weight gain, although growth was inferior to that when fed casein protein. There was no significant improvement in the nutritive value of extruded bean-corn flour compared to raw bean-corn flour, even though trypsin inhibitors and lectins were inactivated (Tables 2 and 3). Marked inhibition of growth was observed with diets consisting of 100% bean-corn flour, even after extrusion. The results show that growth inhibition is not necessarily due to the trypsin inhibitors and lectin activity present in the bean flour. Apparently, trypsin inhibitors stimulate pancreatic enzyme secretion, which causes pancreatic hypertrophy (Oates and Morgan, 1982). Other researchers have shown lower weight gains and food conversion efficiency in rats fed with legume seeds, due to lower protein and lipid intake (Erlwanger et al., 1999; Grant et al., 1995a; Grant et al., 1995a, 1995b; Nell et al., 1992; Olivera et al., 2003). Protein plays a major role in nutrition; it has been demonstrated that a diet based only on plant protein is not enough to obtain a comparable body weight to rats fed with milk protein. In a human diet, bean protein should be a complement in the diet but not a substitute for animal protein.

Contrary to findings by other authors (Olivera et al., 2003), the protein inhibitors present in the bean-corn flour did not affect rat growth. The poor growth of rats fed with bean-corn flour and bean-corn extrudates cannot be explained by the presence of trypsin inhibitors and lectins. Lower rat weight and weight loss may be explained by diet composition. The fat, protein and ash content in the bean-corn diets is significantly ($p < 0.05$) lower than in the control diet (Table 1). The quality of protein in the bean-corn flour is also lower than that of the casein protein present in the control diet. Both may explain the lower body weight of rats fed with bean-corn mixtures, compared to rats fed with the control diet (Table 3). Complementation of bean flour with corn flour was not enough to compensate for the lack of casein protein in the diet. Although the starch and

fiber content in the bean-corn flours is higher ($p < 0.05$) than in the control diet (Table 1), this does not compensate for the lower protein content. Legume proteins are less digestible than high quality protein such as lactalbumin (Dhingra and Jood, 2007; Martinez et al., 2012; Martinez et al., 2012; Olivera et al., 2003). Legume protein is rarely retained by rats (Nielsen 1991; Rubio, 2000), probably because it may interfere with intestinal or systemic metabolism (Rubio et al., 1995), but not due to the low digestibility of the dietary protein or amino acid deficiency. The bean-corn flour and extruded diets have high fiber concentrations (Table 1). Insoluble fiber can also be responsible for the low energy availability of legume diets (Olivera et al., 2003).

Ca and Zn supplementation had no effect ($p > 0.05$) on rat weight and weight gain compared to the un-supplemented bean-corn flour and extruded diets. In this case the amount of Ca and Zn used to supplement bean-corn flour was not sufficient to show a positive effect on body weight (Table 3). The chelating effect of phytic acid was not saturated with the amount of Ca and Zn applied. The control diet showed a much higher ($p < 0.05$) ash concentration than the bean-corn flour, due the high concentration of Ca present in casein (Belitz and Grosch, 1999) and to the fiber in the control diet. As well as protein, starch has an effect on rat weight (Olivera et al., 2003), depending on the concentration of resistant starch present in the starch samples (Table 3). Legume starch also affects food conversion efficiency and body lipid and protein increase in the body. The energy intake of rats fed with legume starch is less than in those fed cereal starch (Grant et al., 1995; Olivera et al., 2003).

Table 3. Body weight, weight gain and length of rats after 22 days of diet, fed with bean-corn flour mixture and extrudates, with or without Ca and/or Zn supplementation.

Diet	Rat weight [g]	weight gain/day [g]
Control (Harlan)	127.5 ± 4.61 ^a	2.0 ± 0.40 ^a
Bean-corn flour	78.0 ± 3.83 ^b	-2.4 ± 0.13 ^b
Bean-corn flour Ca-Zn	76.8 ± 4.55 ^b	-2.4 ± 0.28 ^b
Bean-corn flour Ca	73.6 ± 3.17 ^b	-2.9 ± 0.17 ^b
Bean-corn flour Zn	81.5 ± 1.69 ^b	-2.7 ± 0.25 ^b
Bean-corn extrudates	87.0 ± 2.15 ^b	-2.2 ± 0.22 ^b
Bean-corn extrudates Ca-Zn	77.1 ± 2.52 ^b	-2.3 ± 0.20 ^b
Bean-corn extrudates Ca	81.9 ± 1.65 ^b	-2.3 ± 0.25 ^b
Bean-corn extrudates Zn	78.8 ± 4.04 ^b	-2.9 ± 0.24 ^b

*Standard deviation of the mean; a-b Different letters indicate significant differences at $p < 0.05$.

Femur length, heart weight and volume

The femur lengths were comparable for all diets (Table 4). Those rats fed with Ca- and Zn-supplemented diets showed no differences, which indicates that supplementation was not needed to assure good femur growth.

Neither heart weight nor volume was affected by diets based on bean–corn flour or extruded bean–corn flour (Table 4). Other authors have also shown that diets based on legumes do not alter the weight

of internal organs such as small intestine, heart and pancreas (Grant et al., 1993, 1995b; Olivera et al., 2003). Legume starch does not affect heart size (Erlwanger et al., 1999; Nell et al., 1992; Olivera et al., 2003). These results demonstrate that trypsin inhibitors and lectins have no effect on heart weight, heart volume or femur length. The results also show that the chelating effect of phytic acid present in cereals and legumes did not affect femur length in rats.

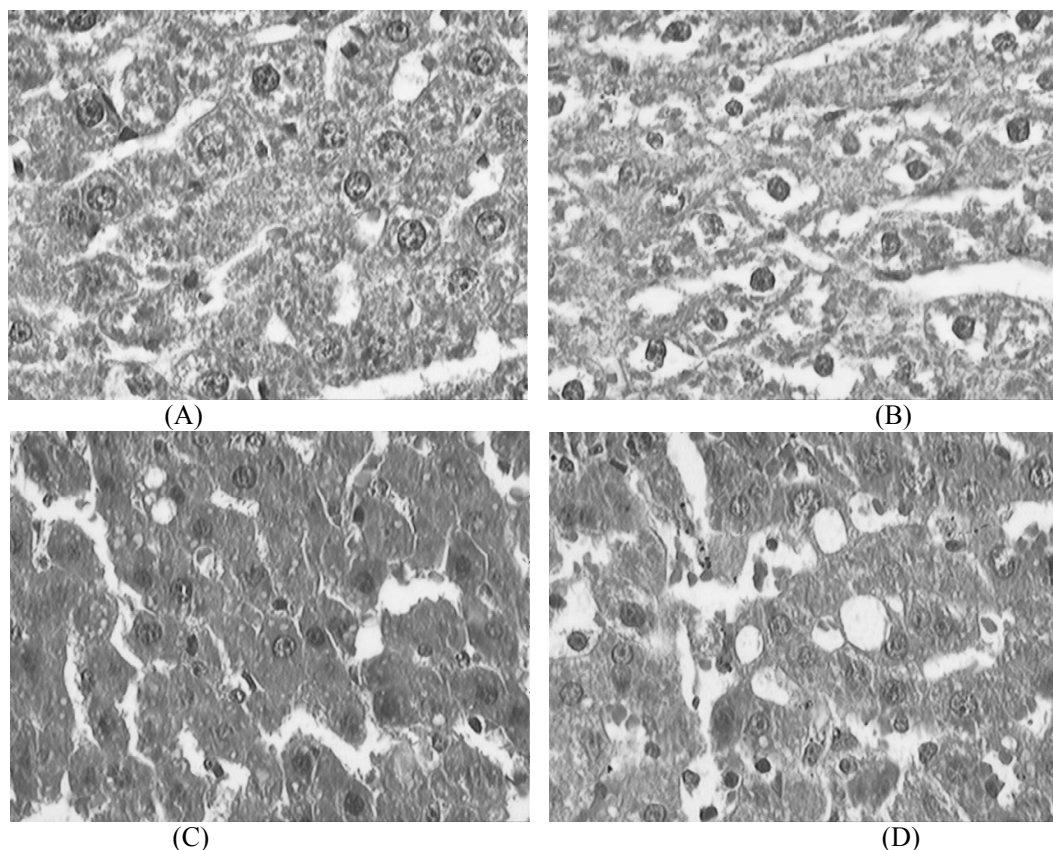


Figure 1. Microscopic pictures of liver with no signs of steatosis in rats fed with the control diet (A), liver with signs of vascular formation in rats fed with bean–corn flour supplemented with Ca (B), liver with signs of microvascular steatosis in rats fed with bean–corn flour supplemented with Zn (C), liver with signs of macrovesicular steatosis in rats fed with extruded bean–corn flour (D)(40X).

Liver

No signs of steatosis were observed in the liver of rats fed with the control diet. The addition of Zn to the extruded bean–corn flour produced a small amount of microvesicular steatosis or none at all (Figure 1). Bean–corn flour supplemented with Ca showed liver balonization. When extruded bean–corn flour was not supplemented with Ca or Zn, intense macrovesicular steatosis (as lipid vacuole accumulation) in the hepatocytes of the liver was

observed. Severe gastrointestinal malabsorption is a cause of steatosis. Lipids accumulate when lipoprotein transport is disrupted and/or when fatty acids accumulate. Since we know that legume proteins may cause intestine enlargement and malfunction (Nielsen, 1991; Rubio, 2000; Olivera et al., 2003; Martinez et al., 2012), this may be responsible for the gastrointestinal malabsorption leading to steatosis in the rats fed with bean–corn flour and extrudates. The concentration of trypsin

inhibitors and the lectin activity present in the bean–corn flour (Table 2) did not induce changes in the liver of the rats. Bowman-Birk-type inhibitors may have little or no effect on some rat species

(Rubio et al., 1995). Extruded diets caused the most severe liver alteration, while Zn supplementation seems to reduce liver alterations induced by bean–corn flour extruded diet.

Table 4. Heart weight, heart volume and femur length of rats fed with bean-corn flour mixture and extrudates, with or without Ca and/or Zn supplementation.

Diet	Heart weight [g]	Heart volume [ml]	Femur length [mm]
Control (Harlan)	0.80 ± 0.100 ^a	0.42±0.083	26.0±0.72
Bean corn flour			
no Ca nor Zn	0.70 ± 0.100	0.66±0.083	25.9±0.42
with Ca and Zn	0.70 ± 0.058	0.65±0.076	25.3±0.42
with Ca	0.77 ± 0.033	0.50±0.000	25.5±0.22
with Zn	0.80 ± 0.058	0.50±0.000	25.5±0.22
Extruded bean corn flour			
no Ca nor Zn	0.70 ± 0.058	0.60±0.058	26.8±0.31
with Ca and Zn	0.80 ± 0.000	0.70±0.050	25.5±0.24
with Ca	0.85 ± 0.050	0.50±0.250	26.3±0.20
with Zn	0.77 ± 0.088	0.78±0.117	25.8±0.60

^a a standard deviation of the mean.

Conclusions

The demonstration that a diet based only on bean-corn flour significantly affects rat weight and produces liver alterations, may have important implications in the field of human nutrition. The chelating effect of phytic acid present in cereals and legumes did not affect ($p < 0.05$) femur length in animals and probably also would not in humans. As shown in this study, trypsin inhibitors and lectins present in composite bean-corn flours had no negative effect on the growth and internal organs of rats. Trypsin inhibitors and lectin can be totally inactivated by extrusion. Care must be taken in translating the above findings to humans, until further investigation is done, since not all of the population is vegetarian, and diets contain more than just beans and corn. For this reason bean–corn flour extruded snacks can be used for human nutrition as a complement in the diet.

Acknowledgement

This work has been made possible by the financial support of the Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) and Consejo Nacional de Ciencia y Tecnología (CONACyT).

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ANIMAL SCIENCE

Influence of in-ovo administration with vitamin E and ascorbic acid on the performance of Muscovy ducks

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Abstract

Nutrient administration in-ovo could be considered as an alternative method to improve hatchability and duckling weights followed by better economic performance. On the 12th day of incubation, fertile duck eggs (n= 500) were distributed into 5 groups: uninjected control; 0.1 ml corn oil; 0.1 ml corn oil plus 10 mg vitamin E; 0.1 ml saline; and 0.1 ml saline plus 3 mg ascorbic acid. In-ovo injection of vitamin E but not ascorbic acid resulted in higher hatchability percentage compared to the uninjected control; however this was not statistically confirmed. In-ovo injection of either vitamin E or ascorbic acid resulted in significantly ($P < 0.05$) higher body weights at hatch, final body weights, and feed intakes than the uninjected control group. However, both male and female ducklings hatched from eggs injected with either vitamin E or ascorbic acid had better feed conversion during the starting period only. The carcass characteristics and relative lymphoid organ weights did not differ ($P < 0.05$) between the in ovo injected groups and the uninjected controls. In-ovo injection of either vitamin E or ascorbic acid resulted in significant increase ($P < 0.05$) in the geometric mean of the 1st antibodies titers of males and the 1st and 2nd antibodies titers of females compared to the uninjected control. It could be concluded that in-ovo injection of vitamin E and ascorbic acid improve the embryonic and post-hatch growth of Muscovy ducks.

Key words: In-ovo, Muscovy ducks, Growth, Vitamins

Introduction

The subsequent development of avian embryos and hatched chicks are influenced by the yolk nutrient status (Al-Murrani, 1982). Many nutrients have important structural, physiological, and immunological roles in avian embryogenesis and growth performance. In-ovo injection of nutrients may help overcome any constraint of inadequate egg nutrition. During early development, there is rapid oxidative metabolism that leads to the production of large quantities of free radicals in many tissues, making them more susceptible to oxidative damage. Antioxidants are a critical defense against these free radicals. The developing embryo may use antioxidants found in the yolk in order to reduce the impact of free radicals. Vitamin E has received the most attention over the past few

years relative to immunity and more recently meat quality. Increasing egg vitamin E content in order to maximize the vitamin E available to the embryo and newly hatched chick becomes of interest either through dietary supplementation of vitamin E to breeder hens or directly via injection into the egg yolk (Cherian et al., 1997; Surai et al., 1999). Exogenous vitamin E administered in-ovo at a time of increased fatty acid oxidation, which begins at day 14 of incubation (Cherian et al., 1997), could be beneficial in reducing the production of free radicals that cause serious damage to the highly poly un-saturated fatty acids of cellular membranes (Cherian and Sim, 1992, 1997).

On the other hand, there are several reports regarding the effect of ascorbic acid as anti-stress agent on the productive performance parameters of chickens such as growth and reproductive traits, including fertility and hatchability. Ascorbic acid is synthesized by the embryo (Surai et al., 1996; Wilson, 1990) and the accumulation of this water-soluble antioxidant by the developing tissues will be independent of the dietary intake by the breeding hen. On the other hand, there were clear differences between the species (turkey, goose, duck, and

Received 19 December 2011; Revised 13 February 2012;
Accepted 21 February 2012

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chicken) concerning the concentrations of ascorbic acid especially by the brain during the development (Surai et al., 1998). Additionally, incubated eggs and their embryos may be subjected to stress caused by excessive production of metabolic heat during the latter part of egg incubation (Tullett, 1990), producing some internal environmental stress for embryo, which may be not suitable for the embryo to hatch successfully. In-ovo injection of ascorbic acid as an anti-stress agent may be beneficial. Our objective is to examine the effect of in-ovo injection of vitamin E or ascorbic acid on the performance of Muscovy ducklings.

Materials and Methods

Vitamin E and ascorbic acid preparation

Vitamin E was prepared as a solution of 1 g of water dispersible vitamin E (E-Viton®; 100mg alpha tocopheryl acetate per one capsule; Kahira Pharmaceutical and Chemical Industries Company, Egypt) per 10ml of corn oil (vitamin E solvent according to Ameenuddin and Sunde, 1984) and 0.1 ml from this solution is equivalent to 10 mg of vitamin E (Schaal, 2008). Ascorbic acid was prepared as a solution of 3 g of ascorbic acid (99% ascorbic acid®; SD Fine-Chem-Limited, India) in 100ml 0.9% saline solution (ascorbic acid solvent) and 0.1ml from this solution is equivalent to 3mg of ascorbic acid (Zakaria and Al-Anezi, 1996).

Eggs incubation and injection

Incubated fertile duck eggs ($n = 500$) were obtained from Muscovy breeders ducks fed on adequate nutritional diet. Fertility was verified by candling with a hand ultraviolet lamp at day 12 of incubation, Eggs were distributed into five groups of 100 eggs each. Eggs were incubated at 37 to 37.5°C and 70% relative humidity during the first 32 days of incubation. Eggs were turned automatically every hour until the 32nd day. All eggs were transferred to the hatchery at the end of the 32nd day of incubation, and placed in hatching boxes at 37°C temperature and 70-75% relative humidity until hatching occurred between days 35 and 36. On the 12th day of incubation, the groups of fertile eggs were distributed into five groups: uninjected control; 0.1 ml corn oil; 0.1 ml corn oil plus 10 mg vitamin E; 0.1 ml saline; and 0.1 ml saline plus 3 mg ascorbic acid. The treatment solutions or sham control were injected into the yolk of the 12-day-old embryo which was identified by candling with a hand ultraviolet lamp, through a pinhole made at the broad end of the egg, using a 25 mm needle. Prior to in-ovo injection the injection site was disinfected with 70% ethanol and the solutions were warmed to 30°C. The pinhole site was sealed with sterile paraffin wax

immediately after injection. The injected eggs were returned to the incubator after injection.

Duckling management and feeding

All hatched ducklings from each treatment were weighed and sexed to males and females subgroups. The ducklings in each treatment group either males or females were randomly assigned to 4 replicates of 8 to 10 ducklings depending upon the hatch size, each replicate with a pen in each treatment. All ducklings were reared under similar managerial and hygienic conditions. The ducklings were raised in clean, well-ventilated, previously disinfected room. A continuous lighting program was maintained throughout the nine weeks experimental period. Temperature was adjusted at 32°C $\pm$ 2 in the first week and then reduced by 2°C each successive week then maintained at 22°C $\pm$ 2. The ducklings of all groups were vaccinated once against Avian Influenza with 0.50 ml single dose of Reassortant H5N1 Avian Influenza vaccine (Re-1 vaccine) subcutaneously in the lower back of the neck at the 14th day old (Middleton et al., 2007). Ducklings of different experimental groups fed on basal starter (0-3 weeks), grower (3-6 weeks) and finisher (6-9 weeks) rations as shown in Table (1). Basal rations were formulated to meet the nutrients requirements for Muscovy ducks as recommended by French Group Company, Sadat City, Egypt (Strain' origin). Nutrients compositions of the used rations were calculated according to the feed composition tables given by NRC (1994). The ducklings were fed ad libitum on dry mash ration and fresh clean water was constantly available.

Growth parameters

After hatching, the body weights (BW) of the ducklings in each group were recorded. The body weight of individual ducklings in each group and feed consumption of each pen were recorded at 3, 6 and 9 weeks of age. The feed conversion ratio (FCR) was calculated accordingly.

Sampling

Blood samples were taken from tibial vein of eight birds in each group (4 males and 4 females) at 5 and 9 week of age by needle under aseptic precaution. Separation of serum was carried out by centrifugation of coagulated blood at 3000 rpm for 15 minutes for determination of antibodies titer against Avian Influenza disease virus by Haemagglutination Inhibition test (Thayer and Beard, 1998). At the end of the experimental period (9 weeks of age) 8 birds (4 males and 4 females) were selected from each treatment group and were slaughtered after being fasted for 12 hours. After

slaughter and complete bleeding, the birds were dressed. The carcass and some other components (liver, gizzard, visible fat, heart, bursa of Fabricius, spleen, and thymus) were weighed.

Dressing percentage = $[(\text{Dressed carcass weight} / \text{Live body weight}) \times 100]$.

Relative organ weights were calculated as percentages of body weight = $[(\text{Organ weight} / \text{Body weight}) \times 100]$.

Total edible parts were calculated as percentage

of body weight = $[(\text{Weight of liver} + \text{gizzard} + \text{heart} + \text{visible fat}) / \text{Body weight}] \times 100]$.

Statistical Analysis

The obtained data were presented as means $\pm$ SE. Analysis of variance (ANOVA) was used to test the significance of the difference between different treatments and statistical differences were established using a Duncan's Multiple Range Test (Duncan, 1955) at the level of $P < 0.05$.

Table 1. Composition and nutritional levels of experimental diets.

Ingredients (%)	Ration		
	Starter	Grower	Finisher
Ground yellow corn	61.40	67.00	68.10
Soya bean meal (44 % CP)	30.00	23.40	20.00
Corn gluten meal (60 % CP)	4.30	5.00	5.00
Vegetable oil ¹	0.40	0.70	3.00
Mono calcium phosphate ²	1.36	1.42	1.36
Ground limestone	1.73	1.72	1.54
Salt (NaCl)	0.31	0.30	0.40
Mineral and Vitamin Premix ³	0.30	0.30	0.40
L-Lysine ⁴	0.10	0.10	0.15
L-Methionine ⁵	0.10	0.10	0.10
Calculated composition			
ME MJ/Kg diet	12.2	12.6	13.1
Crude protein (g/kg)	211.0	191.0	177.0
Calcium (g/kg)	9.7	9.6	8.7
Available phosphorus (g/kg)	4.2	4.2	4.0
Lysine (g/kg)	10.8	9.3	8.8
Methionine (g/kg)	4.6	4.4	4.2
Methionine + Cystine (g/kg)	8.1	7.7	7.3

¹Vegetable oil composed of a mixture of soybean, cotton seed and sunflower oils.

²Monocalcium phosphate; 21% phosphorus, and 15% calcium.

³Minerals and Vitamins mixture (Pharma Mix). Each 3 kg contain: 12,000,000 I.U. Vitamin A, 2,500,000 I.U. Vitamin D3, 10,000 mg Vitamin E, 2,000 mg Vitamin K3, 1,000 mg Vitamin B1, 5,000 mg Vitamin B2, 1,500 mg Vitamin B6, 10 mg Vitamin B12, 30,000 mg, Niacin 1,000 mg, Folic acid, 50 mg Biotin, 10,000 mg Pantothenic acid, 10,000 mg Copper, 10,000 mg Iodine, 100 mg Selenium, 30,000 mg Iron, 60,000 mg Manganese, 50,000 mg Zinc, 100 mg Cobalt, CaCo3 add to 3000 gm.

⁴L-lysine: 78% produced by Archer Daniels Medland Company De Caur I.L. made in U.S.A. (ADM).

⁵DL-methionine: 99% Canadian registration number 990137 Guaranteed analysis, DL-Methionine 99%.

Results and discussion

Hatchability and duckling weight

The actual process of hatching a duck is complicated and there are many factors influencing the normal procedure. Poor breeder hen nutrition can result in embryo mortality at all times during incubation. The hatchability and embryonic mortality of fertile eggs were influenced by the type of injected substance into the eggs. In-ovo injection of vitamin E (78%) into the yolk of fertilized Muscovy duck eggs at the 12th day of incubation increased hatchability percentage as compared to uninjected control and oil injected group (72%). However, in-ovo injection of saline solution with or without ascorbic acid decreased hatchability percentage (68%) in comparison with the

uninjected control (74%). Exogenous vitamin E administered around day 14 of incubation (the time of intense fatty acid oxidation) may be beneficial in reducing the production of free radicals that cause a serious damage to the cellular membranes (Cherian and Sim, 1992, 1997; Cherian et al., 1997) and increased lipid utilization for energy production in order to improve hatchability (Schaal, 2008). Therefore, this increase in hatchability percentage after in-ovo injection of vitamin E might be due enhancement of the antioxidant status of the eggs (Surai, 2000) and/or the vitamin E may protect the protein and lipids from oxidation (Puthongsiriporn et al., 2001). The reduced hatchability and increased embryonic mortality percentages with in-ovo injection of ascorbic acid might be due to the

relatively high dose of ascorbic acid during this injection time (12th day of incubation) or the site of injection (yolk), since Zakaria and Al-Latif (1998) demonstrated that either premature or excessively high doses of ascorbic acid injected in-ovo can cause worsening of chick hatchability. Moreover, greatest improvement in hatchability was obtained when Pekin ducks eggs were injected with 6 mg/egg of ascorbic acid into the air cell on the 20th day of incubation (Nowaczewski et al., 2012). This decrease in hatchability after in-ovo injection of saline with or without ascorbic acid may also probably due to the varied osmolality of the injection solution, since the osmolality of the injected solution was a critical factor affecting hatchability of turkey (Ferket et al., 2005). On the other hand, Zakaria and Al-Anezi (1996) found that injection of ascorbic acid at a dose 3 mg on day 15 of incubation in broilers' eggs improved hatchability, and therefore in-ovo injection of ascorbic acid may become effective and successful when it is carrying out before incubation by eggs dipping in ascorbic acid solution (Shafey, 2002) or by its injection during the latter period of incubation, when there is a risk of heat stress (Tullet, 1990).

In-ovo injection of vitamin E (53.23g) or ascorbic acid (52.20g) significantly increased ($P < 0.05$) body weights of ducklings at hatch compared to the uninjected control (45.95g). On the other hand, there was no significant difference between the uninjected control and sham groups (47.10g and 44.10g for oil and saline injected groups, respectively). This higher body weight might be due to the prevention of hydro peroxides impacts and more energy production by vitamin E injected eggs which enhances the embryonic growth (Schaal, 2008). However, Schaal (2008) reported different results when they injected the same dose into fertile chicken eggs at the 14th day of incubation. Similarly, this is contrary to the result of Bhanja et al. (2007) whose injected 0.5 IU of vitamin E into the yolk of fertile chicken's eggs. These conflicting results may be due to the late injection time in the former study and to the lower dose in the latter. However, in the case of ascorbic acid these results are in agreement with Bhanja et al. (2007) and Zakaria and Al-Anezi (1996) who found an improved hatchling weight by in-ovo injection of ascorbic acid into broiler's fertile eggs.

Growth parameters

It was documented that vitamin E has an important role in maintaining normal rate of growth of chicks (Marsh et al., 1981) and improving broiler

performance under commercial condition (Kennedy et al., 1992). In-ovo route of vitamin E supplementation enhances the post-hatch performance as compared with the traditional means of dietary feeding, which requires more time to build up the vitamin E levels necessary for optimal performance (Gore and Qureshi, 1997). The obtained results revealed that the ducklings from the eggs injected with vitamin E significantly exhibited heavier body weights for both males and females than those of the uninjected control and sham group during the experimental periods (Table 2). The heavier body weights of vitamin E injected group may be due to the vitamin E requirement for early post-hatch growth (Bhanja et al., 2007). These results agreed with those obtained by Hossain et al. (1998) who observed that higher final body weight at 42 days with broilers hatched from eggs injected with 2.5 or 5 mg of vitamin E, and Bhanja et al. (2006 and 2007) who reported that body weight of broiler chickens was more at 14 and 28 day of age in vitamin E injected group than the uninjected control. In-ovo injection of 3 mg ascorbic acid significantly increased body weight of males than those of the uninjected control and sham group. The same result was recorded with the females of the same treatment except during the finishing period; there was no significant difference (Table 2). The heavier body weights of ascorbic acid injected group may be due to the important role of ascorbic acid during incubation in the subsequent growth of broiler chickens to market weight (Zakaria et al., 1998) with relate to the limited capacity of ascorbic acid biosynthesis in male chicks (Hornig and Frigg, 1979). Furthermore, the rapid growth rates of male broiler considered as a stressful condition and consequently increases ascorbic acid requirement. The obtained results are in consistent with the results of Zakaria et al. (1998) and Zakaria (2001), they recorded that in-ovo injection of 3 mg ascorbic acid at the 15th day of incubation resulted in greater body weight of males and no significant differences were observed for female broiler chickens. In our study, there was significantly decrease in the body weight of males and females injected with either saline or corn oil during the starting period only.

In-ovo injection of vitamin E increased feed intake of males and females than the uninjected control (data not shown). While, it caused significant decrease in feed conversion of male and female ducks except during the starting period, which wasn't affected for males and improved for females as compared to the uninjected control and sham group (Table 3). These results were in agreement with the results of Bhanja et al. (2006)

who found that vitamin E injected birds had significantly better feed conversion than the un-injected control birds during 0-14 days of age, and Bhanja et al. (2007) who observed that no significant difference in feed conversion between vitamin E injected birds and un-injected control birds until 28 days of age. In-ovo injection of ascorbic acid increased feed intake of male and female ducks except during the finishing period for females as compared to uninjected control and sham group (data not shown). Moreover, in-ovo

injection of ascorbic acid caused a significant decrease in feed conversion of male ducks except during the starting period and no significant changes in females except during the finishing period; it significantly decreased as compared to the control group (Table 3). This higher feed intake of ducklings hatched from eggs injected with either vitamin E or ascorbic acid may attribute to greater body weight as feed intake is a function of live body weight.

Table 2. Body weight (g) of male and female Muscovy ducklings hatched from eggs injected with vitamin E and ascorbic acid in different growth periods (mean $\pm$ SE).

Group Period	Control	Vitamin E	Corn oil	Ascorbic acid	Saline
<i>Male</i>					
Starting	551.6 $\pm$ 12.8 ^c	845.5 $\pm$ 6.1 ^a	466.3 $\pm$ 5.8 ^d	784.1 $\pm$ 11.9 ^b	404.3 $\pm$ 15.4 ^e
Growing	2196.6 $\pm$ 41.2 ^b	2588.7 $\pm$ 17.1 ^a	2253.3 $\pm$ 16.8 ^b	2640.0 $\pm$ 29.5 ^a	2238.9 $\pm$ 21.4 ^b
Finishing	4109.2 $\pm$ 55.2 ^b	4458.0 $\pm$ 21.2 ^a	4078.3 $\pm$ 25.4 ^b	4429.2 $\pm$ 52.6 ^a	4090.0 $\pm$ 11.5 ^b
<i>Female</i>					
Starting	572.0 $\pm$ 8.1 ^c	715.0 $\pm$ 3.5 ^a	406.0 $\pm$ 2.9 ^e	605.7 $\pm$ 7.0 ^b	490.1 $\pm$ 5.3 ^d
Growing	1770.5 $\pm$ 27.7 ^c	2003.8 $\pm$ 10.5 ^a	1772.5 $\pm$ 10.3 ^c	1850.61 $\pm$ 15.8 ^b	1759.2 $\pm$ 11.0 ^c
Finishing	2801.9 $\pm$ 32.6 ^b	2937.5 $\pm$ 14.8 ^a	2772.5 $\pm$ 12.7 ^b	2747.5 $\pm$ 11.8 ^b	2640.0 $\pm$ 13.9 ^b

a-b Means within the same raw having different superscript are significantly different ($P < 0.05$). Treatments: Corn oil (0.1 ml); corn oil plus vitamin E (10 mg); saline (0.1 ml); and saline plus ascorbic acid (3 mg). Number of replicates is 4 of 8 to 10 ducklings depending upon hatch size.

Table 3. Feed conversion ratio (FCR) of male and female Muscovy ducklings hatched from eggs injected with vitamin E and ascorbic acid (mean $\pm$ SE).

Group Period	Control	Vitamin E	Corn oil	Ascorbic acid	Saline
<i>Male</i>					
Starting	1.47 $\pm$ 0.04 ^a	1.47 $\pm$ 0.01 ^a	1.51 $\pm$ 0.02 ^a	1.49 $\pm$ 0.02 ^a	1.42 $\pm$ 0.05 ^a
Growing	1.85 $\pm$ 0.04 ^c	2.19 $\pm$ 0.01 ^a	1.82 $\pm$ 0.01 ^c	1.98 $\pm$ 0.02 ^b	1.78 $\pm$ 0.07 ^c
Finishing	2.21 $\pm$ 0.04 ^c	2.51 $\pm$ 0.03 ^b	2.24 $\pm$ 0.01 ^c	2.60 $\pm$ 0.04 ^a	2.23 $\pm$ 0.01 ^c
<i>Female</i>					
Starting	1.40 $\pm$ 0.02 ^a	1.30 $\pm$ 0.01 ^b	1.38 $\pm$ 0.02 ^a	1.40 $\pm$ 0.01 ^a	1.41 $\pm$ 0.01 ^a
Growing	2.25 $\pm$ 0.04 ^b	2.35 $\pm$ 0.01 ^a	2.23 $\pm$ 0.04 ^c	2.25 $\pm$ 0.02 ^b	2.25 $\pm$ 0.01 ^b
Finishing	3.30 $\pm$ 0.06 ^b	3.75 $\pm$ 0.02 ^a	3.17 $\pm$ 0.01 ^c	3.66 $\pm$ 0.03 ^a	3.18 $\pm$ 0.01 ^b

a-b Means within the same raw having different superscript are significant different ($P < 0.05$).

Treatments: Corn oil (0.1 ml); corn oil plus vitamin E (10 mg); saline (0.1 ml); and saline plus ascorbic acid (3 mg). Number of replicates is 4 of 8 to 10 ducklings depending upon hatch size.

Carcass characteristics

There have been considerable studies looking at improving carcass traits. In-ovo injection of nutrients is interested possible method to meet this objective, but there is very little research looking at in-ovo supplementation with vitamins. In this study, in-ovo injection of vitamin E or ascorbic acid had no significant effect on dressing and total edible parts (as % of body weight) except the dressing percentage of females hatched from eggs injected with ascorbic acid, it significantly increased as compared to the uninjected control and saline injected group (Table 4). In male ducklings, in-ovo injection of either corn oil or

saline significantly decreased ($P < 0.05$) dressing percentage compared to the uninjected control. Moreover, in-ovo injection of oil solution with or without vitamin E significantly decreased ($P < 0.05$) relative weights of liver and heart as compared to uninjected control. On the other hand, in-ovo injection of saline solution with or without ascorbic acid significantly decreased ($P < 0.05$) relative weight of liver and increased ($P < 0.05$) relative weight of heart compared to uninjected control. Further investigations are needed to highlight the effect of in-ovo injection of vitamin E and ascorbic on the development of digestive organs in Muscovy ducks.

Table 4. Carcass characteristics of male and female Muscovy ducklings after in-ovo injection with vitamin E and ascorbic acid (mean $\pm$ SE).

Group Item (as % of BW)	Control	Vitamin E	Corn oil	Ascorbic acid	Saline
<i>Male</i>					
Dressing	82.50 $\pm$ 1.10 ^a	80.50 $\pm$ 0.80 ^a	71.60 $\pm$ 0.70 ^c	82.20 $\pm$ 0.90 ^a	75.60 $\pm$ 1.29 ^b
Liver	2.25 $\pm$ 0.12 ^a	1.90 $\pm$ 0.02 ^b	1.75 $\pm$ 0.03 ^b	2.07 $\pm$ 0.01 ^b	2.00 $\pm$ 0.03 ^b
Gizzard	2.52 $\pm$ 0.08 ^{ab}	2.66 $\pm$ 0.09 ^a	2.53 $\pm$ 0.04 ^{ab}	2.56 $\pm$ 0.03 ^{ab}	2.32 $\pm$ 0.11 ^b
Heart	0.67 $\pm$ 0.01 ^b	0.59 $\pm$ 0.03 ^c	0.60 $\pm$ 0.01 ^c	0.69 $\pm$ 0.01 ^{ab}	0.74 $\pm$ 0.03 ^a
Visible fat	2.30 $\pm$ 0.30 ^a	2.53 $\pm$ 0.30 ^a	1.95 $\pm$ 0.23 ^a	2.52 $\pm$ 0.50 ^a	2.30 $\pm$ 0.20 ^a
Total edible parts	7.85 $\pm$ 0.54 ^a	7.52 $\pm$ 0.28 ^a	7.34 $\pm$ 0.20 ^a	7.72 $\pm$ 0.10 ^a	7.32 $\pm$ 0.19 ^a
<i>Female</i>					
Dressing	75.70 $\pm$ 0.10 ^b	76.50 $\pm$ 0.80 ^b	73.60 $\pm$ 0.80 ^b	89.70 $\pm$ 0.80 ^a	74.40 $\pm$ 0.70 ^b
Liver	1.92 $\pm$ 0.02 ^{ab}	1.86 $\pm$ 0.01 ^{ab}	1.81 $\pm$ 0.02 ^b	1.93 $\pm$ 0.02 ^a	1.85 $\pm$ 0.04 ^{ab}
Gizzard	2.63 $\pm$ 0.02 ^a	2.53 $\pm$ 0.09 ^a	2.50 $\pm$ 0.08 ^a	2.56 $\pm$ 0.03 ^a	2.50 $\pm$ 0.11 ^a
Heart	0.77 $\pm$ 0.01 ^a	0.83 $\pm$ 0.01 ^a	0.76 $\pm$ 0.01 ^a	0.80 $\pm$ 0.01 ^a	0.79 $\pm$ 0.01 ^a
Visible fat	2.64 $\pm$ 0.30 ^a	3.13 $\pm$ 0.13 ^a	2.45 $\pm$ 0.30 ^a	2.26 $\pm$ 0.26 ^a	2.74 $\pm$ 0.30 ^a
Total edible parts	7.96 $\pm$ 0.12 ^a	8.35 $\pm$ 0.04 ^a	7.52 $\pm$ 0.01 ^a	7.55 $\pm$ 0.06 ^a	7.96 $\pm$ 0.26 ^a

a, b Means bearing different letters in the same row differ significantly ($P < 0.05$).

Treatments: Treatments: Corn oil (0.1 ml); corn oil plus vitamin E (10 mg); saline (0.1 ml); and saline plus ascorbic acid (3 mg). Number of replicates is 4.

Immune status

In-ovo injection of vitamin E caused no significant changes in the relative weights of thymus gland, bursa of fabricus, and spleen for males and females as compared to the uninjected control. The relative weights of lymphoid organs were comparatively higher in the vitamin E injected females group than the uninjected control (Table 5). These results are agree with the results obtained by Gore and Qureshi (1997) who found that turkey and chick embryos exposed to 10 IU vitamin E into the amnion 3 days prior to hatch had no differences in

the bursa and spleen weights compared to the control group. In-ovo injection of ascorbic acid caused no significant changes in the relative weights of lymphoid organs of males and females groups except that's of bursa of females significantly increased as compared to the uninjected control group. The relative weights of these lymphoid tissues were comparatively higher in ascorbic acid injected males and females group than the uninjected control group (Table 5). Comparative researches looking at in-ovo injection of ascorbic acid are unavailable.

Table 5. Relative weight of lymphoid organs (as % of body weight) of male and female Muscovy ducklings hatched from eggs injected with vitamin E and ascorbic acid (mean $\pm$ SE).

Group Item	Control	Vitamin E	Corn oil	Ascorbic acid	Saline
<i>Male</i>					
Thymus	0.545 $\pm$ 0.006 ^a	0.545 $\pm$ 0.006 ^a	0.540 $\pm$ 0.008 ^a	0.580 $\pm$ 0.012 ^a	0.541 $\pm$ 0.006 ^a
Bursa	0.131 $\pm$ 0.004 ^{ab}	0.136 $\pm$ 0.001 ^a	0.120 $\pm$ 0.008 ^b	0.137 $\pm$ 0.001 ^a	0.119 $\pm$ 0.007 ^b
Spleen	0.054 $\pm$ 0.001 ^a	0.055 $\pm$ 0.006 ^a	0.053 $\pm$ 0.004 ^a	0.054 $\pm$ 0.004 ^a	0.054 $\pm$ 0.003 ^a
<i>Female</i>					
Thymus	0.620 $\pm$ 0.016 ^a	0.665 $\pm$ 0.006 ^a	0.621 $\pm$ 0.014 ^a	0.660 $\pm$ 0.008 ^a	0.658 $\pm$ 0.006 ^a
Bursa	0.118 $\pm$ 0.007 ^b	0.127 $\pm$ 0.006 ^b	0.160 $\pm$ 0.016 ^b	0.225 $\pm$ 0.038 ^a	0.142 $\pm$ 0.017 ^b
Spleen	0.050 $\pm$ 0.005 ^a	0.052 $\pm$ 0.004 ^a	0.050 $\pm$ 0.004 ^a	0.055 $\pm$ 0.006 ^a	0.050 $\pm$ 0.001 ^a

a-b Means within the same raw having different superscript are significant different ($P < 0.05$).

Treatments: Corn oil (0.1 ml); corn oil plus vitamin E (10 mg); saline (0.1 ml); and saline plus ascorbic acid (3 mg). Number of replicates = 4

Table 6. Serum antibody titers (HI titer log₂) of male and female Muscovy ducks after in-ovo injection with vitamin E and ascorbic acid (mean ± SE).

Group Item	Control	Vitamin E	Corn oil	Ascorbic acid	Saline
<i>Male</i>					
1 st antibody titer	3.5 ± 0.2 ^b	4.5 ± 0.2 ^a	3.0 ± 0.4 ^b	4.5 ± 0.2 ^a	3.5 ± 0.2 ^b
2 nd antibody titer	3.0 ± 0.4 ^{ab}	4.0 ± 0.4 ^a	2.5 ± 0.2 ^b	3.5 ± 0.2 ^{ab}	2.5 ± 0.2 ^b
<i>Female</i>					
1 st antibody titer	3.5 ± 0.2 ^c	5.5 ± 0.2 ^a	3.5 ± 0.2 ^c	4.5 ± 0.2 ^b	3.5 ± 0.2 ^c
2 nd antibody titer	3.5 ± 0.2 ^b	4.5 ± 0.2 ^a	3.5 ± 0.2 ^b	4.5 ± 0.2 ^a	3.5 ± 0.2 ^b

Means bearing different letters in the same row differ significantly ($P < 0.05$).

Treatments: Corn oil (0.1 ml); corn oil plus vitamin E (10 mg); saline (0.1 ml); and saline plus ascorbic acid (3 mg). Number of replicates = 4

In-ovo injection of vitamin E or ascorbic acid resulted in significant increase in the geometric mean of the 1st estimate for antibodies titers of males and the 1st and 2nd estimate for antibodies titers of females as compared to the uninjected control and sham groups (Table 6). These results were in agreement with those obtained by Gore and Qureshi (1997) who reported that the hatched turkeys and chicks from the 10 IU vitamin E exposed embryos were high responders compared to the control group for antibody production, and Hossain et al. (1998) observed that the antibody titer to killed Newcastle disease vaccine were increased significantly by the level of vitamin E supplements in the diets or its direct injection into eggs.

Conclusion

It could be concluded that early supplementation of nutrients through in-ovo injection such as vitamin E can be regarded as a possible method to improve hatchability, body weight at hatch, marketing weights, and immune status of Muscovy ducks. Further investigations are needed to highlight the effect of in-ovo injection of Vitamin E and ascorbic acid on the humoral, cell mediated immunity, and development of digestive organs in Muscovy ducks.

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ANIMAL SCIENCE

Phenotypic classification of Saudi Arabian camel (*Camelus dromedarius*) by their body measurements

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Abstract

In order to identify homogeneous groups of camels according to their conformation, 212 camels (155 females and 57 males) from 9 regions of the Kingdom of Saudi Arabia, and belonging to 12 different camel breed or types were measured. The body measurements included the length of the head, of the neck, of the udder and of the teat, the height at the withers, and the circumference of the neck, of the thigh and at girth. The 12 breeds were compared according to their mean body measurements and the groups with similar conformation were identified by Automatic Hierarchical Classification on Ward distance. Finally, 4 types of female camel conformation were identified: small size breed from mountains and Red Sea coast, big size camel from desert areas, and 2 breeds, Zargeh with small size but with wide chest and neck, and Asail (racing camel) with very thin neck and leg and poorly developed udder. Six groups of males are identified also but with a different distribution. This classification is close to the typology based on the ecosystem distribution of camel breeds in Saudi Arabia.

Key words: Body measurements, Camel breed, Classification, Dromedary camel.

Introduction

The total population of dromedary is estimated to be around 1.6 million camels within the Arabian Peninsula, about 53% found in Saudi Arabia (source: FAO statistics, 2011). However, there is an important gap between the official number of camel heads according to FAO statistics (280,000 in 2009) and the national estimation published by the Ministry of Agriculture in Saudi Arabia (830,000 heads). Based on this last estimation, the camel population is 51% of the total tropical livestock unit (TLU) in the country. The camel population is increasing since the year 1961, passing from 80,000 to 280,000 in 2009; i.e. approximately a growth of 5.2%/year. The importance of camel in Saudi Arabia is clearly underlined by these data (Gaili et al., 2000).

However, during the same time the milk and meat productivity increased but in similar proportion: 5.4%/year for milk production and 6.4%/year for meat production (Faye and Bonnet,

2012). This increase of milk and meat productivity in camel was mainly linked to the population growth. Regarding the higher growth in meat production, it was rather due to the increase of slaughtering rate than to the growth of meat productivity. Indeed, the mean carcass weight was the same in 2009 compared to 1961 (224 kg) and the slaughtering rate increased by 6.62%/year. The dairy productivity did not change also for the last 48 years and the increase of dairy production was linked to the increase of proportion in dairy animals passing from 62 to 69%; i.e. a growth by 5.53% per year (Faye and Bonnet, 2012). Thus, the increase in available production was mainly mechanical and due to the population growth.

Elsewhere, the Arabian Peninsula is probably one of the main area where the dromedary camel was domesticated 5000 to 6000 years ago (Uerpman and Uerpman, 2002), and the place where the camel biodiversity is one of the most important in the world. Formerly, a dozen of "breeds" are described (Faye et al., 2011) mainly based on their coat color.

However, their differences on other parameters as their general conformation were not clearly described. There is no clear classification of Saudi Arabia camel populations with ecological, morphological and utilities criteria generally mixed (Almathen et al., 2012). Yet, it would be an

Received 03 December 2011; Revised 24 February 2012;
Accepted 27 February 2012

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important step to propose control of performances for further potential selection programs in order to contribute for the improvement of camel productivity. Thus, the present study aims to classify the described camel breed of Saudi Arabia on the base of their body measurements only in order to identify some groups with similar conformation.

Materials and Methods

The survey

As the whole, 152 camel owners were visited. They lived in 9 regions of the kingdom (Al-jouf, Arar, Tabuk, Tabarjal, Riyadh, Qassim, Hail, Jazan, Al-Bahah). They were selected on the basis of the variability in the breed composition of their camel farms. In each farm, a questionnaire was applied and body measurements were achieved: all female and male camels had between 5 and 10 years old (adults having finished their growth), and they were regarded by their owner as the more characteristic specimens for a given breed. By this participative approach, the total size of the sample could be limited. Finally, the data involving 212 camels (155 female and 57 males) belonging to 12 different camel breeds (or types) were collected. As the description of these different camels are not yet clear, the terms "breed" or "types" will be used indistinctly.

The measurements

The measurements were achieved on standing animals with a meter-ribbon and reported in cm. The following distances were collected: (i) the length of the head from nose to occipital (LH), (ii) the length of the neck (lower part) from base of head to the chest (LN), (iii) the circumference of the neck at the middle of the neck (CN), (iv) the height at the withers (HW), (v) the girth circumference in front of the hump (GC) and the thigh circumference at the middle of the thigh (TC), then in females (vi) the length of the left front teat (LT) and finally (vii) the length of the udder from the front to hind attach (LU).

The statistical analysis

The statistical analysis included three steps:

(i) Each breed was described by the mean ($\pm$ S.D) of the different reported measurements and their within variability. To compare the different breeds, a variance analysis was applied in order to identify the significant differences for each measurement between breeds.

(ii) The correlations between the different measurements were assessed by calculating the correlation coefficient of Pearson for a given sex.

(iii) In a third step, a table including the 12 identified breeds (in row) and the different mean values of body measurements (in column –see table 1) was analyzed by automatic clustering. The principle of the method of automatic classification (or clustering) was based on the identification of homogeneous groups of individuals (clusters) in the population (here, camel breed). Two camel breeds belonging to the same group were somehow close to each other (similar means of body measurements). At reverse, two camel breeds belonging to different groups are somehow far from each other (they have significant different body measurements). The classification consists to build a partition of the population into homogenous clusters (having low within variability), different one from other (having high between variability). Each retained cluster, identified by the convenient cutting of a dendrogram (graphic expressing the dissimilarity between clusters or classes) would represent a "type" or a "cluster". The convenience of the cutting was estimated when the gain in between-cluster variance is not significant. The retained clustering is expressed by the total between-cluster variance explained by the model. The interpretation of the types was achieved by analyzing the contribution of the different variables to the class. Only variables with significant contribution (assessed by Chi square test) at $P > 0.05$ were retained for the final interpretation (Jain et al., 1999).

The statistical analyses were applied separately on females and males and the software XLSTAT (Addinsoft ©) was used.

Results

Succinct individual description of the camel breeds (Plate 1)

Hadhana breed is a yellowish to red coat color camel with a fine head, flat forehead, thin neck, narrow feet and medium size legs. He has a round and symmetric hump and no long hair on its body. It is mainly encountered in the hills around Al-Baha town in a limited area.

Awadi breed is a camel from southern part of the Kingdom with red to white coat color. A small head with a slight forehead, short and thin neck, small udder, pilosity in ears, head and neck, narrow feet and round hump are the main characteristics of the breed.

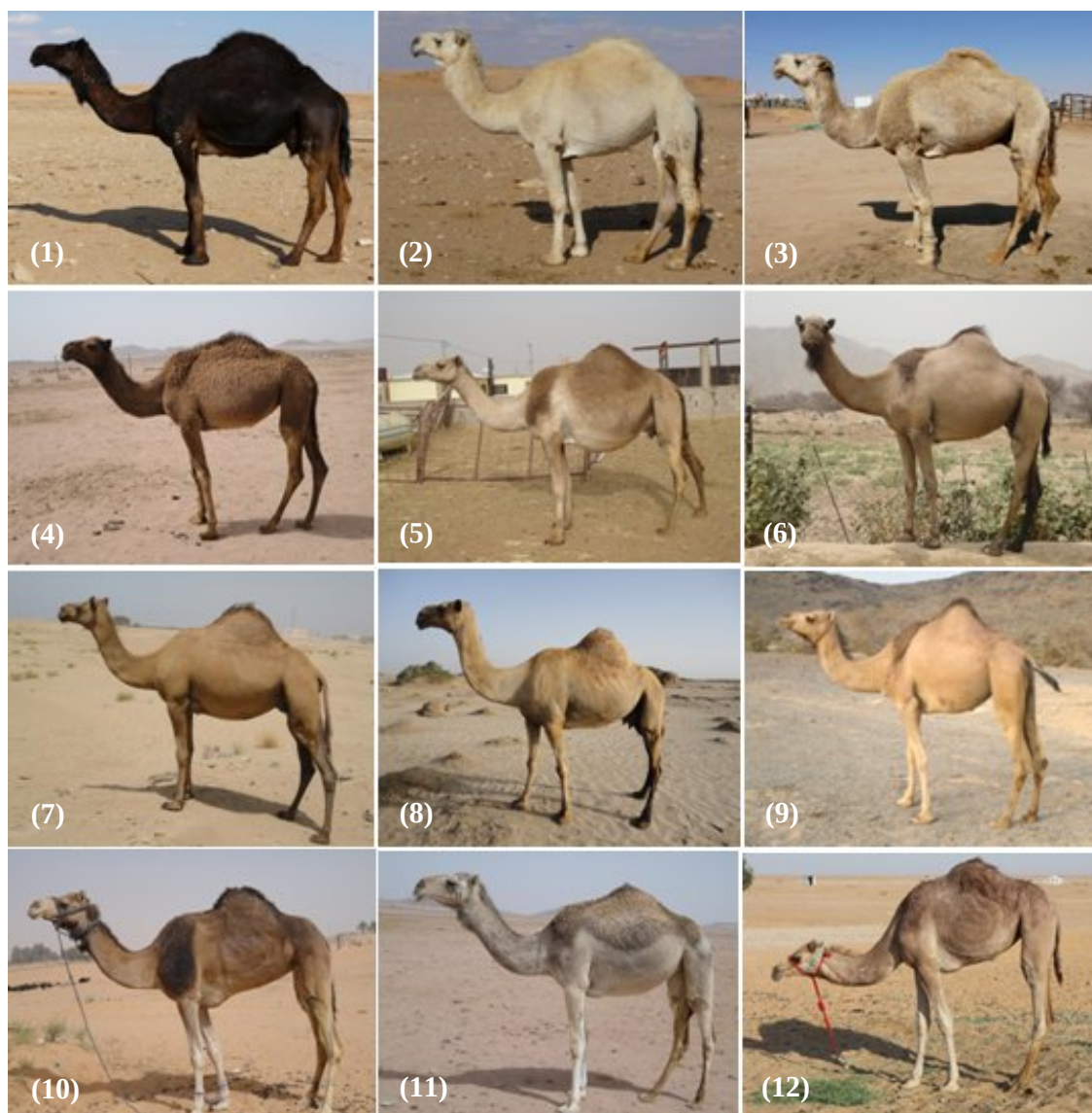


Plate 1. Camel breeds phenotypes photos from Saudi Arabia. (1) Majaheem (2) Waddah (3) Homor (4) Sofor (5) Shaele (6) Aouadi (7) Saheli (8) Awrk (9) Hadhana (10) Asail (11) Zargeh (12) Shageh.

Asail is a racing camel characterized by a fine head with flat forehead, pointed ears, very narrow feet and long legs, round hump in the hind of the back, small but balanced udder with very small teats. Its yellow to brown coat color has short hairs.

Awrk (Awarik) camel has a clear coat color, almost white, with short hair. It differs from Saheli breed by a more developed udder, medium neck circumference, pointed ears and its hump rather at the hind of the back. It is widely distributed in Jazan region. Its milk production is moderate.

Homor (or Hamrah) camel is a medium size dairy breed with a characteristic clear brown coat color. Its forehead is slightly marked in male and

the ears are pointed with low pilosity. It is distributed in small number all over the country, but more common in the northern part.

Majaheem (also named Malah) is a characteristic black coat camel originated from north-east part of the country. A pointed hump, placed in the middle of the back, long hair covering all the body, long legs, wide feet, well developed udder are its main characteristics. It is one of the best dairy producers of the Kingdom.

Saheli, a typical camel living all along the Red Sea coast, has a characteristic red coat color, a small head with a pronounced forehead (even in

female), a small and thin neck, a small udder, round ears without hairs, narrow feet and round hump.

Shaele breed is a medium size camel and has a grey coat color going sometimes to brown-red especially in male, pointed ears, narrow feet, short legs and well symmetric hump. It is a common type in South-eastern part of the Kingdom.

Shageh is a small-head camel with short and thick neck, narrow chest but developed udder. It is close to Zargeh breed but its coat is greyer. It is more common in the southern part of the Kingdom.

Sofor (or Safrah) has a dark brown coat color with medium size, long head and neck, well developed udder, wide chest and long legs. The ears are pointed and the feet are large. Its body is widely covered with long hair. It is distributed in same area than Homor camel. It well resists to the climatic constraints of the desert.

Waddah (or Maghatir) has a white coat color, sometimes almost yellow. The characteristics are

pointed ears with low pilosity, long legs and well developed hump. The male have a developed forehead and long penis case.

Zargeh, a southern camel, has a blue-grey coat color with long hair on the head, the neck, the shoulder and the hump. It has small head with short neck and flat forehead, pointed ears, small udder, unbalanced teats, a wide chest on short legs. The hump could be pointed and placed in hind part of the back. Sometimes, the coat color on shoulder and on the back is darker.

The mean values for females (Table 1)

The ranges for the individual measurements were 31-60 cm for the length of the head, 75-114 cm for the length of the neck, 57-111 cm for the neck circumference, 158-272 cm of the height, 102-274 cm for the girth circumference, and 60-110 cm for the thigh circumference. The udder length range was 10 to 50 cm and the teat length was 1 to 26 cm.

Table 1. Mean body measurements of 12 types or breeds of female camel in Saudi Arabia (in cm). ^{a,b,c,d} Means within column with different superscript differ ($P < 0.05$).

Breed	Lhead	Lneck	cNeck	Lteat	Ludder	Height	GirthC	ThighC
Hadhana	42.1 ^b	87.8 ^d	74.8 ^d	4.2 ^b	17.0 ^b	173.0 ^c	180.5 ^{a,b}	73.3 ^c
Aouadi	42.4 ^b	97.6 ^{c,d}	79.3 ^{b,c,d}	4.7 ^b	15.7 ^{b,c}	174.3 ^c	191.3 ^{a,b}	83.6 ^b
Asail	42.3 ^b	94.3 ^{c,d}	86.3 ^{a,b}	2.0 ^b	6.3 ^c	185.8 ^c	199.3 ^{a,b}	78.3 ^{b,c}
Awrc	41.5 ^b	92.0 ^{c,d}	88.8 ^{a,b}	4.6 ^b	18.5 ^{a,b}	199.3 ^a	201.0 ^{a,b}	88.8 ^{a,b}
Homor	46.5 ^a	107.1 ^{a,b}	83.9 ^{a,b}	4.7 ^b	25.6 ^a	186.7 ^c	217.3 ^a	93.1 ^a
Majaheem	46.9 ^a	110.7 ^a	89.4 ^a	6.8 ^a	25.0 ^a	192.2 ^{a,b}	219.2 ^a	94.9 ^a
Saheli	42.8 ^b	96.2 ^{c,d}	86.4 ^{a,b}	5.1 ^{a,b}	16.7 ^b	176.0 ^c	195.9 ^{a,b}	84.3 ^b
Shaele	46.9 ^a	104.5 ^{a,b}	83.0 ^{a,b,c}	4.1 ^b	24.8 ^a	187.0 ^{b,c}	213.5 ^a	86.9 ^b
Shageh	39.3 ^b	92.3 ^{c,d}	92.0 ^a	5.2 ^{a,b}	17.0 ^b	182.7 ^c	180.7 ^{a,b}	90.3 ^{a,b}
Sofor	48.1 ^a	98.7 ^{b,c}	81.0 ^{a,b,c}	4.3 ^b	22.7 ^{a,b}	185.3 ^c	220.9 ^a	85.3 ^b
Waddah	47.4 ^a	108.6 ^a	79.0 ^{c,d}	4.8 ^b	25.4 ^a	186.7 ^c	221.8 ^a	93.0 ^a
Zargah	40.5 ^b	87.0 ^d	91.0 ^a	4.5 ^b	22.0 ^{a,b}	185.0 ^c	222.0 ^a	86.5 ^b

The length of the head was significantly higher in Homor, Majaheem, Shaele, Sofor and Waddah breed. The length of the neck was higher in Majaheem, Homor and Waddah and the lower significant values were observed on Hadhana and Zargeh breed. Majaheem, Shageh and Zargah had a significant higher neck circumference than Hadhana, Awadi and Waddah. The higher breeds were Majaheem and Awrk. The shorter were Hadhana, Awadi and Saheli. Regarding girth circumference, there was a high significant difference between Zargah, Waddah, Sofor, Majaheem and Homor in one hand, and Hadhana in another hand. Waddah, Majaheem and Homor had a high thigh circumference reverse to Hadhana and Asail. The length of the udder was quite more important for the

breeds Shaele, Waddah, Majaheem and Homor and quite smaller in Asail. The length of the teat was quite higher in Majaheem attesting its dairy vocation. Asail had also very short teat.

The mean values for males (Table 2)

For the different body measurements, the values range were 41-59 cm (LH), 85-152 cm (LN), 55-119 cm (CN), 113-226 cm (HW), 134-249 cm (HG) and 73-123 cm (TC) showing a higher homogeneity than for females.

In male, there was no significant difference between breeds for the head length. The neck was significantly longer in Majaheem, Homor, Waddah and Shageh, reverse to Asail and Hadhana characterized by a shorter neck. The highest neck

circumferences were observed on Majaheem, Wadda, Saheli and Shageh while no significant difference was observed for height.

The girth circumference was also higher in Majaheem, Waddah, Homor and Sofor, but Shageh

and Saheli were in the same group. The lowest measurement was reported on Awadi. The thigh circumference was highest in the same breeds than female, but also in Shageh. Asail breed has the finest thigh.

Table 2. Mean body measurements of 10 types or breeds of male camel in Saudi Arabia (in cm). ^{a,b,c,d} Means within column with different superscript differ (P < 0.05).

Breed	Lhead	Lneck	Cneck	Height	GirthC	ThighC
Hadhana	49.5 ^a	96.5 ^b	73.0 ^c	183.0 ^a	209.0 ^{a,b}	95.5 ^{a,b}
Aouadi	46.0 ^a	107.0 ^{a,b}	83.0 ^{b,c}	178.0 ^a	176.0 ^b	100.0 ^{a,b}
Asail	46.0 ^a	98.8 ^b	82.0 ^c	184.8 ^a	199.6 ^{a,b}	84.8 ^b
Homor	49.3 ^a	114.6 ^a	96.7 ^{a,b}	198.6 ^a	228.0 ^a	103.6 ^a
Majaheem	51.5 ^a	119.7 ^a	100.8 ^a	203.3 ^a	230.8 ^a	106.7 ^a
Saheli	45.0 ^a	103.0 ^{a,b}	115.0 ^a	179.0 ^a	220.0 ^a	91.0 ^b
Shaele	48.8 ^a	117.2 ^b	88.6 ^{b,c}	193.2 ^a	200.7 ^{a,b}	94.4 ^b
Shageh	54.0 ^a	133.0 ^a	119.0 ^a	193.0 ^a	235.0 ^a	104.0 ^a
Sofor	51.5 ^a	104.5 ^{a,b}	93.5 ^{a,b}	191.5 ^a	219.0 ^a	98.0 ^{a,b}
Waddah	51.4 ^a	122.8 ^a	97.9 ^a	190.2 ^a	226.0 ^a	104.1 ^a

Sexual differences

On average, the male measurements were higher in male for all the parameters. The male head was 11% longer, the neck 12% longer, the neck circumference 13% higher, the thigh circumference 14%. The difference in height (4%) and girth circumference (6%) was less marked.

The sexual difference was more important in Shageh breed: considering all the parameters, the body measurements of the Shageh male was 27% higher than female. This difference was 13% for Hadhana, 11% for Saheli, 10% for Waddah, 9% for

Majaheem, 8% for Homor and Sofor, 6% for Aouadi, 5% for Shaele and 3% only for Asail.

Correlations between measurements

The correlation matrix was achieved for each group of camel (male or female) showing globally significant correlations between most of the quantitative parameters. There were no significant negative correlations. Among the parameters, in both sex, the thigh circumference appeared correlated to all the others parameters (Tables 3 and 4).

Table 3. Correlation matrix between the quantitative measurements of the female camel in Saudi Arabia. Significant coefficients at P < 0.05 were in bold.

parameter	L head	L neck	Neck C	L teat	L udder	Height W	Girth C	Thigh C
L head	1	0.540	-0.034	0.132	0.489	0.260	0.238	0.311
L neck	0.540	1	0.104	0.172	0.389	0.303	0.345	0.353
Neck C	-0.034	0.104	1	0.163	-0.132	0.348	0.088	0.233
L teat	0.132	0.172	0.163	1	0.294	0.119	0.111	0.201
L udder	0.489	0.389	-0.132	0.294	1	0.116	0.360	0.267
Height W	0.260	0.303	0.348	0.119	0.116	1	0.289	0.366
Girth C	0.238	0.345	0.088	0.111	0.360	0.289	1	0.410
Thigh C	0.311	0.353	0.233	0.201	0.267	0.366	0.410	1

Table 4. Correlation matrix between the quantitative measurements of the male camel in Saudi Arabia. Significant coefficients at $P < 0.05$ were in bold.

Variables	L head	L neck	Neck C	Height W	Girth C	Tigh C
L head	1	0.537	0.310	0.190	0.286	0.439
L neck	0.537	1	0.388	0.244	0.331	0.434
Neck C	0.310	0.388	1	0.249	0.574	0.621
Height W	0.190	0.244	0.249	1	0.294	0.303
Girth C	0.286	0.331	0.574	0.294	1	0.568
Tigh C	0.439	0.434	0.621	0.303	0.568	1

Classification of the females

The clustering of the female camel breeds was optimal with a partition within 4 groups of breeds which explained 59.6 % of the variance. A first group (type A) included Hadhana, Aouadi, Saheli, Shageh and Awrc breed. They were characterized by short dimensions and medium size udder (Figure 1 and Table 5). A second (type D) gathered Homor,

Majaheem, Shaele, Sofor and Waddah. This group had significantly higher measurements (LH, LN, HW, GC, TC) and well developed udder. Zargah and Asail appeared different and separated from the 2 other groups. Zargah (type B) was medium but with high girth circumference. Asail (type C) was also medium but with low development of the udder and narrow chest.

Table 5. Mean body measurements of the 4 clusters of female camel breed in Saudi Arabia (in cm). ^{a,b,c} Means within column with different superscript differ ($P < 0.05$).

Cluster	Lhead	Lneck	cNeck	Lteat	Ludder	HeightW	GirthC	ThighC
A	42.1 ^b	93.3 ^b	82.5 ^a	4.7 ^{b,c}	16.8 ^b	177.5 ^b	190.1 ^b	81.9 ^b
B	42.7 ^{a,b}	93.3 ^b	85.3 ^a	8.3 ^a	29.3 ^a	183.6 ^{a,b}	226.6 ^a	81.7 ^b
C	42.2 ^b	94.2 ^b	86.2 ^a	2.0 ^c	6.2 ^c	185.7 ^{a,b}	199.2 ^{a,b}	78.2 ^b
D	47.0 ^a	107.8 ^a	84.0 ^a	5.1 ^{a,b}	24.9 ^a	188.3 ^a	218.7 ^a	92.7 ^a

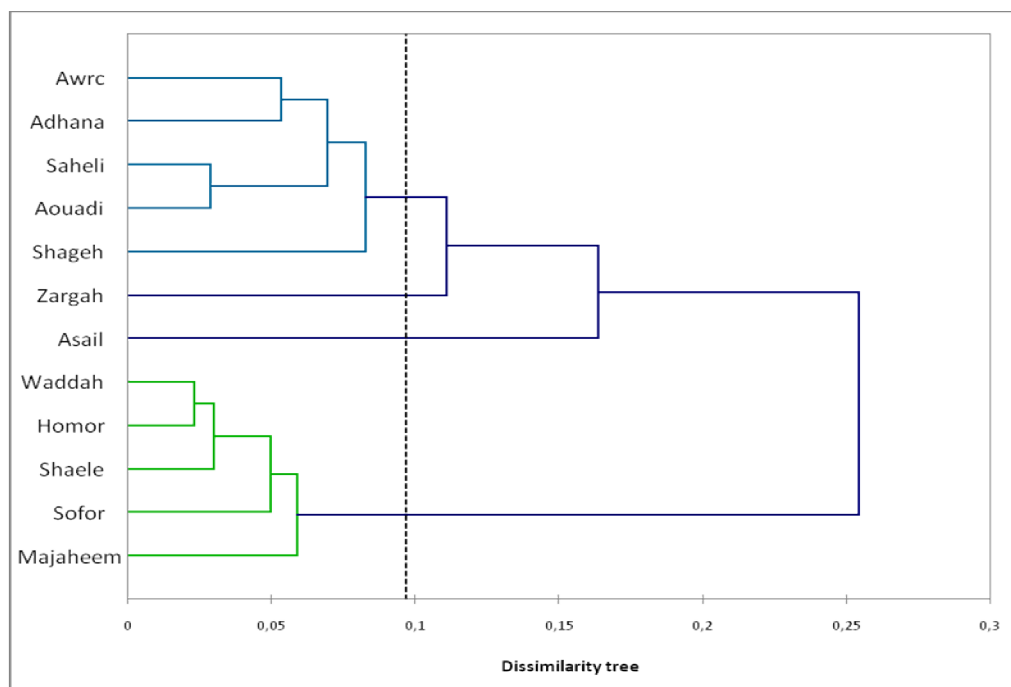


Figure 1. Classification tree of the 12 female camel breeds of Saudi Arabia according to their body measurements showing a partition within four types of camel breed.

Classification of the males

The optimal classification of male camel breeds gave a partition in 6 clusters explaining 77% of the variance (Figure 2). The type A (Asail and Shaele) had fine neck, thin leg and narrow chest, but was high. They are rangy camel useful for racing and riding. The type B (Homor, Majaheem, Sofor and

Waddah) included big size camels. The type C included only Hadhana characterized by a short and fine neck and medium other measurements. The type D (Aouadi) was a small head animal with very narrow chest. The type E (Shageh) was the biggest type. The type F (Saheli) had a thick neck and a thin thigh with a medium chest (Table 6).

Table 6. Mean body measurements of the 6 clusters of male camel breed in Saudi Arabia (in cm). ^{a,b,c} Means within column with different superscript differ ($P < 0.05$).

Cluster	Lhead	Lneck	Cneck	Height	GirthC	ThighC
A	47.8 ^a	110.6 ^{a,b}	86.2 ^b	190.2 ^a	200.3 ^b	91.0 ^b
B	50.9 ^a	119.1 ^a	97.8 ^a	194.7 ^a	226.9 ^a	104.0 ^a
C	49.5 ^a	96.5 ^b	73.0 ^b	183.0 ^a	209.0 ^{a,b}	95.5 ^{a,b}
D	46.0 ^a	107.0 ^{a,b}	83.0 ^b	178.0 ^a	176.0 ^b	100.0 ^{a,b}
E	54.0 ^a	133.0 ^a	119.0 ^a	193.0 ^a	235.0 ^a	104 ^{a,b} .0
F	45.0 ^a	103.0 ^{a,b}	115.0 ^a	179.0 ^a	220.0 ^{a,b}	91.0 ^b

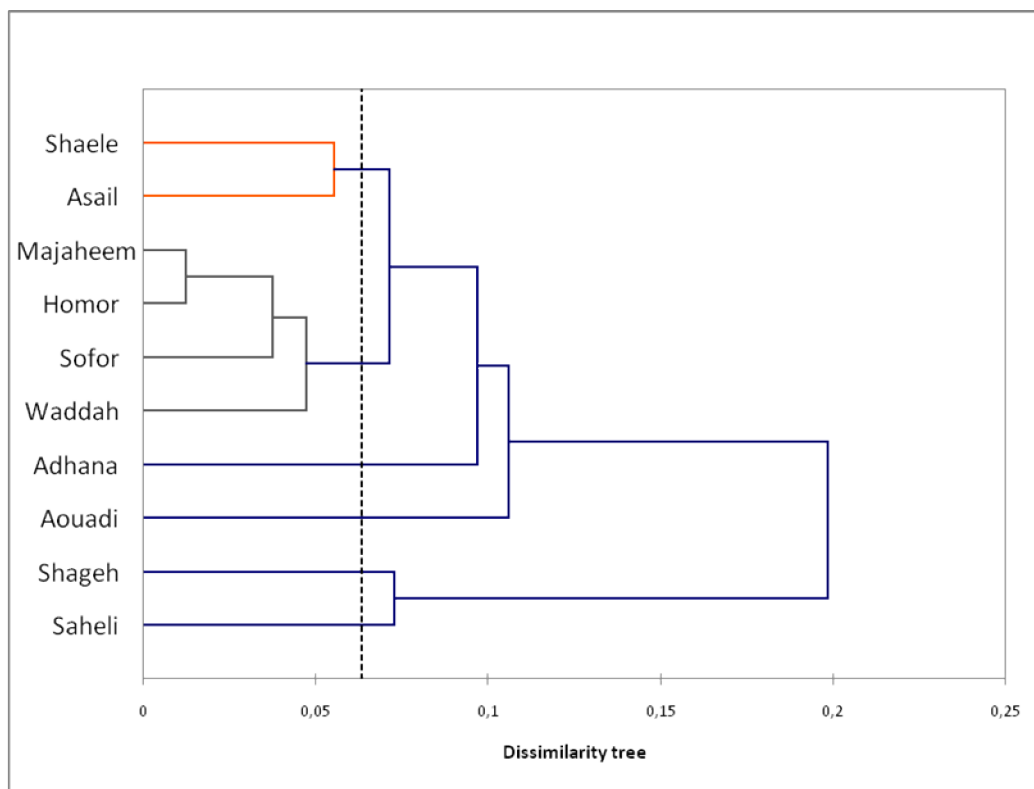


Figure 2. Classification tree of the 10 male camel breeds of Saudi Arabia according to their body measurements showing a partition within six types of camel breed.

Discussion

The body measurements were currently used for describing the camel phenotypes. Ishag et al. (2011) described the phenotypes of Sudanese camel breeds by measuring the heart girth, the barrel girth, the height at shoulder and the body weight. In our

study, the barrel girth was not taken in account, because this measurement is depending of the hump size which varies according to the body condition score of the animal (Kamili et al., 2006). Indeed, the hump is the main fat storage form in camel representing on average 85% of the adipose tissue

(Faye et al., 2001a). Except for the thigh circumference, the body measurements were not significantly correlated to all the other measurements. The thigh circumference seems to be a good indicator of the camel conformation, including the udder in female. Besides, the thigh circumference is commonly used by the butchers for predicting the weight of the carcass (Faye et al., 2001b).

The clustering of the different breeds was slightly different between male and female. The sexual difference in the general conformation of camel conducted naturally to a different distribution between the breeds. On average, the measurements were higher in male compared to female, but in different proportion according the breed.

By regarding, the only clustering of the females, a close relationship with the classification of Al-Eknah (unpublished results) based on the ecosystem (desert, hill, coast) or the use (riding, racing or production) was observed. Three main groups of camel could be described: (i) the big size camels from desert areas with good dairy potential (Homor, Majaheem, Waddah, Shaele, Sofor); (ii) the small size camels from hill or beach having multipurpose vocation (Aouadi, Awrk, Saheli, Hadhana); (iii) the medium size rangy camel breed for riding and racing (Asail, Zargeh, Shageh).

This classification is corresponding partially to the typology of Blanc and Hennesser (1989) which distinguish (i) the big size camel living in desert plain, (ii) the medium size camel used for riding, and (iii) the small size camel living in mountainous areas.

In Saudi Arabia, these phenotypes are linked partly to different farming system (Abbas et al., 2000; Al-Khamis and Young, 2006): (i) the big size camels are mainly used for dairy production, adapted to desert areas but able to undergo intensification process with settlement around the towns, (ii) the small size camels from coastal or hill environment are mainly associated to small scale camel farm in more favorable climatic environment characterized by a diversification of the livestock rearing activities and the use of camel as multipurpose animals, (iii) the medium size camels are linked to racing stables where the animals received enriched diet and undergo special exercise for competitions. For racing camels however, genetic exchanges with neighboring countries could lead to specific classification (Shorepy, 2011).

The described phenotypes are also mainly linked to geographical distribution as it has been observed in other countries (Ould Ahmed et al., 2010).

The big size camels are more common in the northern and eastern part of the Kingdom of Saudi Arabia, and the small size camel in the south-western part. The medium size camel having a riding and racing vocation, they are more widely distributed in the country. The preliminary results of camel genotyping in Saudi Arabia produce similar conclusions showing a geographical classification distinguishing the camel in the northern part from the camel breeds in the East, themselves different from the camel breed living in the western mountainous part of the Kingdom (Mahrous et al., 2011). These results were recently confirmed (Almathen et al., 2012) revealing three genetically separated groups of dromedary in Saudi Arabia with distinct, although likely overlapping, geographic distribution in the Southern west region (Awrk, Awadi, Hadhana and Saheli), East region (Majaheem and Shaele) and Northern part (Waddah, Sofor, and Homor) of the Kingdom of Saudi Arabia respectively. The results also highlighted that the Awrk and Awadi camel populations were genetically distinct from the other camel populations.

Conclusion

The fine phenotype description could be a first step for establishing a classification of the different breeds or types, and for valorizing the camel biodiversity in the Kingdom of Saudi Arabia. Elsewhere, the current changes in the camel farming systems (settlement, intensification, indoor feeding systems, implementation around the towns, etc.) would increase the demand for more productive and specialized animals. For this reason, a clear description of the potential is an important step for the future improvement of the camel breeds. The present phenotype study limited to the body measurements would be an opportunity to underline the richness of this emblematic animal for all the Saudi people whatever their social rank or the place where they are living.

Acknowledgements

This study has been achieved within FAO project UTF/SAU/021/SAU with the support of Camel and range research Center (CRRC). The authors thank Mr Sallal Issa Al-Mutairi, head of the CRRC for his encouragements and support. We thank also all the camel owners and shepherds who help us to manage their animals and gave us their agreement for measuring them. This work was done with the powerful help of all staff of Ministry of Agriculture in different regions of the Kingdom. Thanks also to FAO regional officer, Dr Mohammed Bengoumi who supported this project

from the beginning. And finally, thanks to all the camel who accepted to be measured.

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