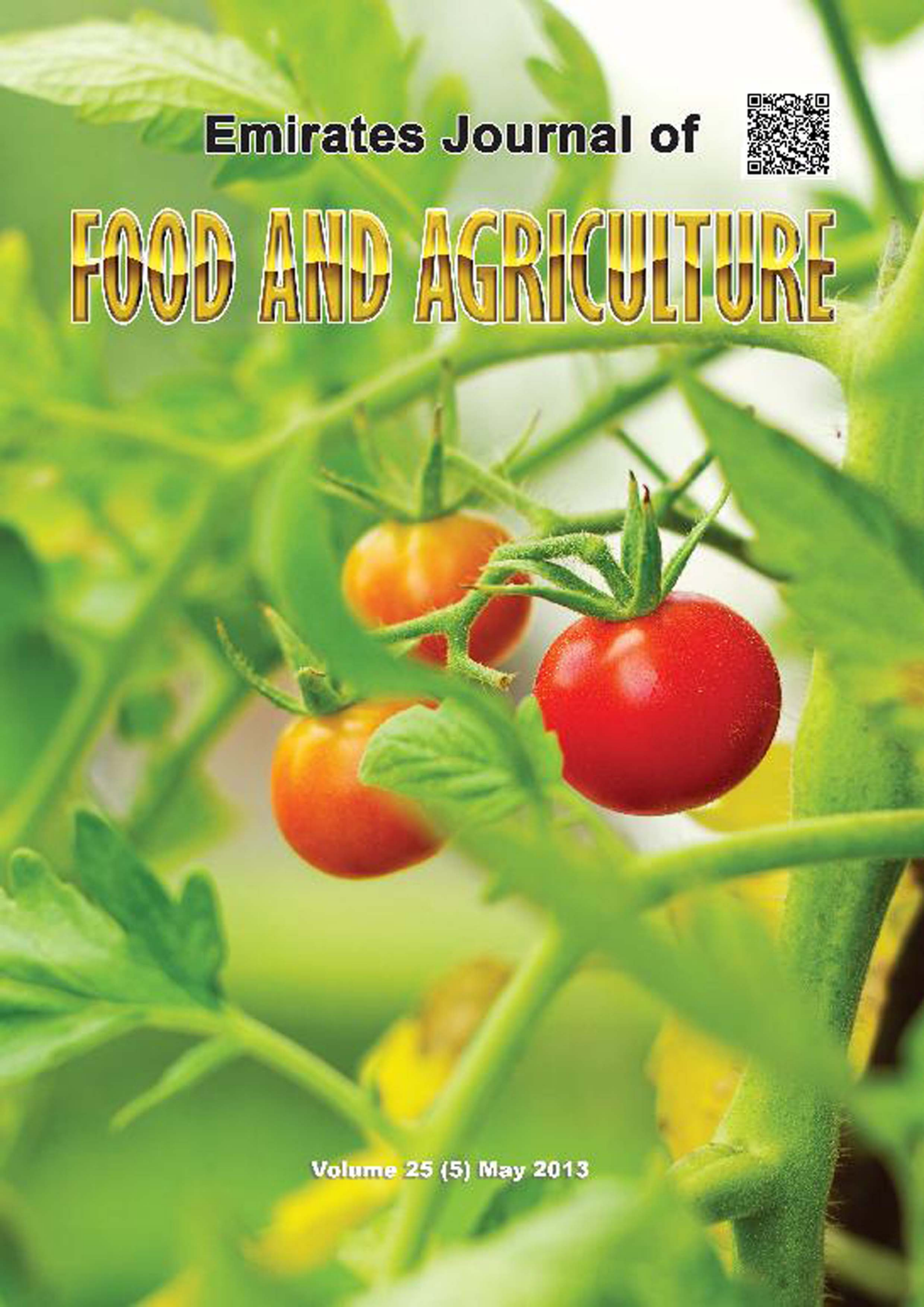
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Utilization of some vegetable oil blends rich in omega-3 fatty acids: Biological evaluation

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Abstract

The effect of feeding rats diet containing 9% of some prepared vegetable oil blends instead of corn oil for 6 months on body weight gain, weight of some selected internal organs, blood picture, lipid profile and liver histopathological changes were studied. Also, the oil blends were used for preparing microcapsules and omega fatty food products. The results indicated that the body weight gain of the rats was differed according to the type of oil and feeding period. Except liver, the weight of the other determined internal organs of the different rat groups were not significantly different. Plasma triglycerides level was ranged from 88.67 to 90.66 mg/dl. The value of LDL-cholesterol was relatively less in the plasma of the rats fed on diet containing canola oil with flaxseed oil (CF-2) blend rich in linolenic acid and less in oxidation stability, followed by olive oil with canola oil (OC-1), olive oil with flaxseed oil (OF) and corn oil, respectively. The less histological changes in the liver tissues were observed in the rats group fed on diet containing CF-2 oil blend followed by those containing OC-1 and OF then corn oil. Oxidation of prepared microcapsules oil caused a darkening in red color an increase in FFAs, PV, TBA, P-anisidine value and specific UV-absorbance. The results showed that the sensory properties of the fatty omega food products were acceptable by panelists.

Key words: Biological evaluation, Blends, Microcapsules, Omega-3, Utilization

Introduction

The increasing public awareness with personal health and fitness turned the attention of the nutritionists to investigate the role of various dietary fats in the maintenance of health and reduction of risk of chronic diseases such as heart diseases, cancer, hypertension, obesity and diabetes (Simopoulos, 1991).

Health and Welfare-Canada's Scientific Review Committee suggested that n-6 fatty acids (FAs) and n-3 FAs should provide at least 3% and 0.5% of energy, respectively with an n-6 to n-3 FAs ratio in the range of 4:1 to 10:1. The same ratio of n-6 to n-3 FAs was also recommended by World Health Organization (Richter, 2001). The factors affect this ratio are energy intake, total polyunsaturated fatty acids, absolute amount of n-6 and n-3 FAs in the diet (Maki et al., 2003). A lower ratio of n-6/ n-3 FAs is more desirable in reducing the risk of the chronic diseases of high prevalence in Western societies, as well as in the developing countries (Simopoulos, 1991; Gomez Candela et al., 2011). Consumption of recommended amounts of n-3 FAs can contribute to improvement of general health and welfare of entire population, especially young people (Sretenovic et al., 2009).

Both n-6 linoleic and n-3 linolenic acids interact with liver enzymes and convert to long chain fatty acids through a process of desaturation and elongation, respectively. The new produced fatty acids build hormone like eicosanoids that regulate immune and inflammatory responses. Such hormones form from 20 carbon atoms long and include prostaglandins, leukotrienes and thromboxanes (Fan and Chapkin, 1998). The parent fatty acids for eicosanoids are arachidonic acid (AA) from n-6 FAs family and eicosapentaenoic acid (EPA) from n-3 FAs family. The eicosanoids from n-6 FAs cause high risk of asthma allergic, arthritis, psoriasis, colitis and other inflammatory diseases. The functions of the eicosanoids of n-3 FAs have the opposite effects (Simopoulos, 1991). Therefore eating oils containing the proper ratio of n-6 to n-3 FAs will supply the body with essential fatty acids ideally for metabolism and will reduce the risk of host diseases including cardiovascular cancer, diabetes, obesity, arthritis, and asthma (Fan and Chapkin, 1998).

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In Egypt, the major edible vegetable oil sources are palm, soybean, cottonseed, sunflower and corn oils. Palm oil rich in saturated fatty acids and very low in n-3 FAs represents more than 70% of the total oil consumption. This was behind the increase of the cardiovascular diseases (CVD) and blood cholesterol between male and female in Egypt (Miladi, 1999). Increased n-3 FAs consumption can come from changes in diet. An alternative for increasing consumption would be to supplement with n-3 daily foodstuffs. Dietary supplements are a clear option for contributing to meet the established recommendation (Gomez Candela et al., 2011).

The aim of this study was to formulate vegetable oil blends rich in n-3 FAs and to evaluate their health claims in addition to estimate their palatability when use to prepare cold and warmed food products, as well as their storage stabilities when prepared in microcapsules form.

Materials and Methods

The following materials were obtained from Alexandria markets of Egypt, Netherland extra refined canola oil, cold press extra virgin olive oil (Wadifood Company, Egypt), fresh cold press crude flaxseed oil, refined corn oil (Arma Food Industries, Co., Egypt), Lurpak Danish Cow's butter (Dairy Aria Food Amba, Denmark), skimmed dry milk (Nestle Co., Egypt), minerals and vitamins mixtures (Altromin, D. 4937, Germany), baking powder and corn starch (Tag El-Melouk Co., Egypt), vinegar and mustard paste (Heinz Co., Egypt), 72% wheat flour extraction rate (Flour land Co., Egypt), sucrose powder (El-Doha, Co., Egypt), refined fine iodized common salt, fresh whole hen egg, with white shell, red cabbage, onion, yellow carrots, and lemon fruits.

Technological methods

Flaxseed oil pre-refining

The fresh crude pressed flaxseed oil was first degummed using 85% phosphoric acid, then neutralized with 15% sodium hydroxide, and bleached under vacuum at 90°C for 30 minutes using Tonsil ACCFF activated bleaching earth as described by Lillard (1982). The cooled bleached oil was packed in brown glass bottles and stored at 4°C until used.

Blending of oils

Olive oil (O) was individually mixed with each of canola (C) and flaxseed (F) oils and also canola (C) oil with flaxseed (F) oil at the following suggested ratios: 5.7 olive oil:1 canola oil (OC-1), 19 olive oil: 1 flaxseed oil (OF) and 9 canola oil : 1 flaxseed oil (CF-2). The blending was carried out at room temperature (22±2°C), then packed in brown glass bottles (100g) under stream of nitrogen gas. The oil bottles were kept at room temperature (22±2°C).

Encapsulation of oil blends

The CF-2 and OF oil blends, were embedded in gelatin capsules using centrifugal an extrusion encapsulation technique. This technique is based on utilizing nozzles consisting of concentric orifices located on the outer circumference of rotating cylinder. The encapsulating cylinder or head consists of a concentric food tube through which coating and core materials are pumped separately to the many nozzles mounted on the outer surface of the device. While the oil passes through the center tube, coating material flows through the outer tube (Schlameus, 1995).

Table 1. Recipes of the different omega food products prepared.

Ingredient in grams	Omega food products			
	Oil blend /butter	Mayonnaise	Fresh sauerkraut salad	Carrot cake
1-Butter	50	-	-	-
2-Oil blend*	50	125	9	75
3-Hen egg	-	10	-	20
4-Common salt	-	1.25	1.25	1.25
5-Mustard paste	-	4	2.5	-
6-Sucrose powdered	-	1	30	100
7-Lemon juice	-	20	-	-
8-Red cabbage	-	-	150	-
9-Red onion	-	-	10	-
10-Vinegar	-	-	50	-
11-Wheat flour	-	-	-	130
12-Baking powder	-	-	-	2.5
13-Grated carrots	-	-	-	150

*Both CF-2 and OF oil blends were used in preparing these products.

Preparation of omega food products

Table 1 shows the recipes of the different omega food products prepared in this study.

The preparation of the omega food products was done as described by Simopoulos and Robinson (1999). A- Oil blend/ Butter: Oil blend and butter were blended in food processor until combined and had a thick cream consistency then packed into a small polystyrene bowl, covered with aluminum foil and placed at 4°C to firm.

B- Mayonnaise: Twenty five grams of the oil blend plus the whole liquid egg, were placed into the bowl of the food processor and then salt, mustard paste and sucrose were blended until completely combined. To this mixture, the 50% of the remained oil blend was added in a very thin stream during the running of food processor then lemon juice was added with blending until combined. The final amount of oil blend was added and blended in a very thin stream as mentioned before. The obtained mayonnaise was packed in glass jar, covered with plastic cap and stored at 4°C until used.

C- Fresh sauerkraut salad: The cabbage and onion were sliced into thin slices. These slices were layered in the bottom of a bowl. The other ingredients (sucrose, vinegar, salt, mustard paste and oil blend) were mixed in a sauce pan, boiled and poured over the cabbage and onion layers while still hot. The resulted salad was kept at 4°C for 12 hours or more then occasionally stirred before presenting for taste panel testing.

D- Carrot cake: Oil blend, eggs and sugar were beaten in a mixer until obtaining a creamy texture. The dry ingredients were mixed in a separate bowl then added to the egg mixture and stirred until combined. The mixture was packed into a lightly greased baking pan and baked for about 35 minute at 350°C in a baking oven. After cooling at room temperature, it was packed in aluminum foil.

Analytical methods

Color of oil was assessed using Lovibond Tintometer, Model-E, 100000G, USA, using 0.5 inch cell (Mackinnery and Little, 1962). Free fatty acids (FFAs) as oleic acid, peroxide value (PV) as meq O_2/kg oil were determined as mentioned in AOCS (1985). Thiobarbituric acid (TBA) was colormetrically estimated at 538 nm as absorbance per kg oil (Pokorny et al., 1985) and P-anisidine value (P-An) was assayed at 350 nm as the absorbance resulting from the reaction of one gram oil with P-anisidine (Absorbance per g oil) (Egan et al., 1987). The values of specific absorbance of oil at K_{232} and K_{270} were determined as described by Kiritsakis (1991).

Biological methods

In this study, the experiments on animals were performed in accordance with the ethical guidelines and regulations set forth by experimental animal's laboratory, Home Economic Department, Faculty of Agriculture, Alexandria University. The experiments were done in accordance with the internationally accepted principles for laboratory animal use and care.

Sixty weanling male albino rats with a mean initial weight of 60±10 grams were divided into four groups of about equal weight. The rats in each group were hosted individually in metal cages. The rats were fed for a week as adaptation period on the control diet consisted of 37.5% skimmed milk, 30% corn starch, 13.5% powdered sucrose, 9% corn oil, 5% wood dust, 4% minerals and 1% vitamins (Abou-Arab and Hassan, 2006). After this period, the animals were switched to the experimental diets which contained instead of 9% corn oil, the same level from OC-1, OF and CF-2 oil blends. Each of control and experimental diets contained the following vitamins and minerals mixtures, 5.1g V.A, 0.0375g V. D2, 24g V. E., 1.2g V.K., 0.9g V. B_1, 1.5g V.B_2, 1.2g V. B_6, 6g V.C, 0.12g folic acid, 2.76g pantothenic acid, 6.0g nicotinic acid and 0.006g biotin per each one kilogram vitamin mixtures in lactose, the mineral mixture consisted of 33.4g Mn, 26.7g Zn, 20g Fe, 6.7g Cu, 0.83g I, 0.167g Co and 0.067g Sn per each one kilogram mineral mixtures in C_aCO_3. The feeding period on the experimental diet was continued for 12 weeks. Food and water were available to the animals during the experiment. Rat's body weight gain was determined through the feeding period (Abo-Arab and Hassan, 2006). At the end of the experiment, the animals were sacrificed after 14 hours of fasting. Weight of liver, kidney, spleen, heart, lung and testes were recorded and their ratios to body weight were calculated. Fresh liver kept in 10% formalin until histological assay performed.

Blood analysis: The blood sample was collected from each sacrificed animals and mixed with heparin as a blood anticoagulant in glass centrifugal tubes, left for 10 min then centrifuged at 8000 rpm for 10 min to separate serum from plasma. The obtained serum was kept at -20°C until analysis. Five samples of bloods of each group from five sacrificed animals were analyzed for: (**a**) Hemoglobin (Hb), according to Wintrobe (1965), using the following equation:

$$\text{Hb (g/dl)} = \frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times 36.77$$

(**b**) Red blood cell count (RBCs), using a light microscope at 40X magnification (Heplar, 1966); (**c**) White blood cell count (WBCs),using a light microscope at 25X magnification according to England and Bain (1976) method; (**d**) Hematocrit, according to Dacie and Lewis (1984), using the micro-method; (**e**) Calculation of Red cell values *Mean cell volume* (M.C.V.), *Mean corpuscular hemoglobin* (M.C.H.), *Mean cell hemoglobin concentration* (M.C.H.C.) according to Dacie and Lewis (1984); **(f**) Triglycerides (TG), according to the method of Fossati and Principle (1982), (**g**) total cholesterol (TCh) (Watson, 1960) as mg/dl using the following equation:

$$\text{Total cholesterol (mg/dl)} = \frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times 50$$

(**h**) High density lipoprotein (HDL) (Warnick et al., 1983), as mg/dl using the following equation:
HDL (mg/dl) = Optical density of sample × 220.

The methods of Warnick et al. (1983) were used to calculate LDL after measuring very low density lipoprotein (VLDL) using the following equations respectively,

VLDL = Triglycerides ÷ 5

LDL = Total cholesterol – (HDL + VLDL)

Histopathological examination: Three liver organ samples for each group were subscribed to histological measurement using haematoxylin and eosin (H & E) technique according to Drury and Willington (1983). Liver thin slices were stained by haematoxylin and eosin dyes. The slices viewed and photographed using Carlzeis light microscope.

Organoleptic methods

The omega food products were organoleptically evaluated using a set of 10 panelists from Food Science and Technology Department, Faculty of Agriculture, Alexandria Univ., Alexandria, Egypt. Each panelist was asked to evaluate the different sensory properties in each product according to the following methods:

a- Descriptive tests for oil blend / butter and carrot cake (Youssef, 1986; Abu-Foul, 1990).

b- Nine point hedonic scale ranging from 1 (Extremely dislike) to 9 (Extremely like) according to Gerczyca and Zabik (1979) for both mayonnaise and fresh sauerkraut salad.

Statistical methods

The data of the biological evaluation of oils were analyzed as a completely randomized design (Steel and Torrie, 1980) using the General Linear Model procedure of SAS (1986). Means were compared by Least Significant Difference (LSD) test (Steel and Torrie, 1980).

Results and Discussion

Biological effects of vegetable oil blends

In this part of the study the effect of feeding Albino male rats diet containing 9% of the prepared oil blends instead of corn oil for 3 months on their body weight gain, weight of some selected internal organs, blend picture, lipid profile and liver histopathological changes were studied.

Body weight gain

According to the data in Table 2, the body weight gain of the rats differed markedly due to type of oil in the rat feed and feeding period. Generally a significance gradual less in weight gain of the rats was noticed with extending feeding period. Such lower is mostly due to the less of rat appetite resulting from the oxidation of these types of vegetable oils.

The reduction in the body weight gain of the rats fed on OC-1 oil blend was noticed after the sixth weeks of feeding. The same observation was observed after 8 weeks in both group of rats which their diets contained OF oil blend and corn oil, the control group. These differences reflect the oxidation stability of these oils during feeding period and also the reaction of amino acids with the oxidized products of oils which caused a decrease of net utilization and digestibility of the protein, fat and fatty acids of feeds (Abou-Arab and Hassan, 2006).

Table 2. Changes in body weight gain in grams of rats feeding on oil blends*.

Type of oils	Feeding period (weeks)						
	1	2	4	6	8	10	12
CF-2	4.79^{c}±0.20	4.73^{c}±0.70	4.71^{d}±0.20	3.83^{d}±0.11	2.66^{c}±0.12	2.59^{d}±0.13	1.05^{d}±0.08
OC-1	9.94^{b}±0.09	9.89^{b}±0.17	8.26^{c}±0.36	5.29^{c}±0.19	4.03^{b}±0.09	3.01^{c}±0.08	1.63^{c}±0.11
OF	11.56^{a}±0.98	11.46^{a}±0.57	10.86^{a}±0.42	9.54^{a}±0.54	5.26^{a}±0.35	4.67^{a}±0.29	2.25^{b}±0.11
Corn oil	11.54^{a}±0.36	10.88^{a}±0.24	9.79^{b}±0.62	8.10^{b}±0.15	5.36^{a}±0.42	3.94^{b}±0.19	3.53^{a}±0.29

*Values are means ± standard deviation

The same letters in the same column were not significance.

Relative weight of some internal organs of rats

In the end of feeding experiment, the rats were slaughtered for measuring their organs weight. Results in Table 3 showed that, except liver, the weight of the others determined internal organs of the different rats groups feeding on different diets containing various types of oils were much closed. On the other hand, the rat liver weight was differed according to the type of oil in its diet. Rats group fed on diet containing corn oil (control diet) had the highest liver weight followed by those fed having CF-2, OC-1 and OF oil blends, respectively. According to Harman (1980) and Yetiv (1988), the negative approach of the intake of fat rich in polyunsaturated fatty acids (PUFA), is the risk of lipid peroxidation and free radical formation. The disorders of free radical formation include liver diseases, inflammation, malignancies, aging acceleration and atherosclerosis.

Blood picture

Table 3 showed that, the level of hemoglobin in the blood of the rats was very closed and ranged from 13.50 to 14.0 (g/dl). It lies in the normal hemoglobin range (13.5-18 g/dl) for male as reported by Tilkian et al. (1983). Generally, hemoglobin is an iron porphyrins attached to the globin protein. This conjugated protein able to combine reversibly with oxygen. The hem proteins function in oxygen binding, oxygen transport and electron transport. As seen from data in Table 3, the red blood cells (RBCs) or erythrocytes varied from 4.63 to 4.70×10^6 per cubic millimeter in the blood of the rats feeding diets having various types of oils. According to Tilkian et al. (1983) the normal count of RBCs of men ranges from 4.8 to 6.2×10^6/cmm. These cells are formed in bone marrow. The primary function of these cells is to transport oxygen to the tissues and to carry CO_2 to lungs. The white blood cells (WBCs) or leukocytes ranged from 8.17 to 8.30×10^3 per cubic millimeter in the blood of four groups of rats which feeding on diets having different oil types as stated in Table 3. This range is within the normal count of this type of cells ($4.5\text{-}11X10^3$/cmm) as stated by Tilkian et al. (1983).Also, the values reported in Table 3 for haematocrit, mean corpuscular hemoglobin (M.C.H), mean corpuscular hemoglobin concentration (M.C.H.C) and mean corpuscular volume (M.C.V) in the bloods of the four groups of rats feeding on diet having different types of oil were closed. But the mean corpuscular volume differed significantly in the bloods of the group of rats feeding on diet having OF oil blend and there were no significant differences within other groups. The normal value of M.C.H., M.C.H.C., M.C.V. and haematocrit in blood ranged from 26-32 pg, 32-36%, 78-94 FL and 34.0-40.0%, respectively (Heplar, 1966). The above results indicated that non-significant changes were observed among the blood pictures of the rats feeding on diets either having vegetable oil nearly free from n-3 fatty acids (corn oil) and/or containing various ratios of n-3 to n-6 fatty acids such as CF-2, OC-1 and OF oil blends.

Table 3. Changes in the relative weight of some organs, blood picture and lipid profile of rats feeding on oil blends*.

Property	Vegetable oil blends			Corn oil (Control)
	CF-2	OC-1	OF	
Weight of internal organs (grams)				
1- Liver	$3.61^b \pm 0.02$	$3.39^c \pm 0.1$	$3.27^c \pm 0.02$	$3.83^a \pm 0.03$
2- Kidney	$0.71^a \pm 0.03$	$0.76^a \pm 0.05$	$0.67^a \pm 0.08$	$0.70^a \pm 0.08$
3- Spleen	$0.40^a \pm 0.04$	$0.39^a \pm 0.06$	$0.39^a \pm 0.07$	$0.39^a \pm 0.02$
4- Lung	$0.90^a \pm 0.07$	$0.87^a \pm 0.02$	$0.95^a \pm 0.02$	$0.89^a \pm 0.07$
5- Heart	$0.34^a \pm 0.02$	$0.36^a \pm 0.03$	$0.35^a \pm 0.01$	$0.37^a \pm 0.02$
Blood picture				
Hb. (g/dl)	$13.9^a \pm 0.20$	$14.0^a \pm 0.70$	$13.9^a \pm 0.52$	$14.0^a \pm 0.60$
RBCs $10^6/mm^3$	$4.63^a \pm 2.06$	$4.63^a \pm 0.25$	$4.67^a \pm 0.25$	$4.70^a \pm 0.10$
WBCs $10^6/mm^3$	$8.30^a \pm 0.42$	$8.17^a \pm 0.21$	$8.20^a \pm 2.40$	$8.30^a \pm 0.10$
Haematocrit (%)	$41.56^a \pm 2.60$	$41.53^a \pm 2.54$	$41.40^a \pm 2.70$	$41.9^a \pm 2.04$
M.C.V (FL)	$89.76^a \pm 0.01$	$89.69^a \pm 0.15$	$88.65^b \pm 0.52$	$89.44^a \pm 0.35$
M.C.H (Pg)	$29.16^a \pm 0.20$	$30.24^a \pm 0.61$	$29.76^a \pm 0.20$	$24.78^a \pm 0.10$
M.C.H.C. (%)	$32.48^b \pm 0.20$	$33.71^a \pm 0.25$	$33.57^a \pm 0.30$	$33.11^a \pm 0.30$
Lipid profile (mg/dl)				
Total triglyceride	$88.67^a \pm 0.58$	$90.0^a \pm 3.00$	$89.66^a \pm 3.06$	$90.66^a \pm 1.16$
Total cholesterol	$178^a \pm 3.00$	$174^a \pm 2.60$	$178^a \pm 2.65$	$144^b \pm 4.00$
LDL-Ch.	$144.59^a \pm 1.53$	$147.0^a \pm 2.08$	$148.41^a \pm 1.93$	$110.87^b \pm 2.08$
HDL-Ch.	$15.67^a \pm 0.58$	$14.0^a \pm 1.00$	$14.66^a \pm 0.56$	$15.0^a \pm 2.65$

*Values are means ± standard deviation; The same letters in the same line were not significance

Table 4. Change of oil blends quality in the microcapsules during storage* (22±2°C).

Constitutes	Oils blend in microcapsules					
	CF-2			OF		
	Zero time	3 months	6 months	Zero time	3 months	6 months
Red color value	2.28	2.33	2.15	1.12	1.34	1.84
Free fatty acids (FFAs) as % oleic acid	0.34±0.04	0.51±0.01	0.73±0.05	0.24±0.04	0.49±0.06	0.74±0.05
Peroxide value (PV) as meq O_2/kg	2.17±0.08	5.88±0.02	9.58±0.04	2.07±0.06	4.86±0.15	7.14±0.07
Thiobarbituric acid (TBA) as absorbance /kg	0.031±0.00	0.115±0.00	0.498±0.01	0.016±0.01	0.040±0.00	0.374±0.03
P-anisidine value (P-Av) as absorbance / g	1.3±0.22	3.73±0.24	5.95±0.10	0.826±0.00	1.45±0.02	2.41±0.12
Absorbance values at K_{232}	0.52±0.03	1.21±0.09	1.89±0.14	0.51±0.02	0.95±0.04	1.59±0.04
Absorbance values at K_{270}	0.028±0.01	0.060±0.00	0.092±0.01	0.026±0.00	0.048±0.01	0.060±0.00

*Values are means ± standard deviation.

Lipid profile

The lipid profile comprises triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol. This group of tests is used in combination with factors such as age, blood pressures, obesity, smoking, diabetes and family history to assess coronary heart disease (CHD) risk and to select and monitor treatment (Dietscky et al., 1993). Data in Table 3 showed that the plasma triglycerides level ranged from 88.67 to 90.66 (mg/dl). It was slightly higher in plasma of the rats feeding on diet containing the free n-3 fatty acids oil, corn oil, than those having vegetable oil blends containing n-3 and n-6 fatty acids. The variation in this test did not vary significantly among the rats feeding on different diets. Generally, the values of the triglycerides in this table are within the normal range (35-165 mg/dl) of this component in plasma (Ismael et al., 2007). It is also cleared from the results in this Table that using rat diet containing oil blends instead of corn oil, caused a significant rise of the total cholesterol in the rat plasma. On the other side, this increase did not go as far as the upper limit of this component in human (200-250 mg/dl) (Taneja and Rakha, 2005). The value of LDL-cholesterol was more noticeable in the plasma of the rats feeding on diet containing OF vegetable oil blend, followed by OC-1, CF-2 and corn oil respectively. On the contrary, the HDL-cholesterol levels in plasma of the rats feeding on the diet containing different types of oils were much closed. The above results showed that the levels of total, LDL and HDL-cholesterol in the plasma profile of the rats did not only depend on n-3 to n-6 fatty acid ratio but also on the oxidation stability of the oil in their diets. According to Taneja and Rakha (2005) the presence of vitamin E with omega-3 fatty acids prevent the changes in blood lipid profile and impose no serious risk to the consumer. Ramirez-Tartosa et al. (1999) found that the consumption of olive oil plus fish oil caused a significant reduction in the plasma triglycerides compared with olive oil. Vegetable oil rich in the n-6 FAs have been promoted as lowering blood cholesterol concentration of the people in USA (Hass, 1999). Harris (1997) showed that the plant n-3 FA caused rise of LDL-cholesterol by 5-10% and HDL-cholesterol by 1-3%. Fickova et al. (1998) noticed that rats fed on the diet containing n-3 FA contained significantly lower level serum triglycerides compared with those fed on diets having n-6 FAs. The study of Mc-Donald et al. (1989) revealed that the diet containing canola oil or sunflower oil produced similar decrease in plasma total and low-density lipoprotein cholesterol and nearly no changes in both HDL-cholesterol and triglycerides. Pang et al. (1998) concluded from their research on replacement of linoleic acid with α-linolenic acid that both acids offer similar cardioprotective benefits with respect to lipid metabolism.

Histopathological of rat liver

Histology helps in the interpretation of the microscopic changes that occur under different pathological conditions. In this study, the liver tissues of the four groups of rats fed on diet containing 9% fat from different oil sources were microscopically examined. The results of such examination were illustrated in Figures 1 and 2. It is known that liver is one of the most important and large organs in the body. Generally it consists of hepatocytes, Von kupffer cells and blood sinusoids which transfer blood from the portal vein and hepatic artery to the central vein. The hepatocytes arrange in an anastomosing plates or laminates between the portal triad and the central vein or venule. It has polyhedral surface and contains mono or binucleus. The portal triad consists of three

branches, portal vein branch, hepatic artery branch and the bile duct branch. The three branches are surrounded with an indented collagen layer in the portal canal which has also the lymphatic vessels. Figure 1 show the liver tissues of the rats groups fed three months on diet containing 9% corn oil free from n-3 fatty acids. The following alterations were noticed in their liver tissues, (1) Enlargement in some hepatocytes, (2) Formation of some vacuolation in the intracytoplasm, (3) Dilation in portal tract and central vein, (4) Infiltration in portal tract within blood sinusoid, (5) Marked inflammation around portal tract and (6) Congestion in blood portal tract and central vein.

The influence of the diet containing CF-2 oil blend on the liver tissues of the rats was illustrated in Figure 1B. The examination of these figure showed only slight dilation in the blood sinusoid and mild inflammation with slight infiltration in portal tract. Nearly the liver tissues were normal.

The alteration in the liver tissues of the rats group fed on the diet having OC-1 oil blend included the following as shown from Figure 2A, (1) Mild dilation in the blood sinusoid, (2) Mild dilation in the tributary veins, (3) Market inflammation around portal tract and (4) Slight congestion in central vein.

In case of feed having OF oil blend, the main changes in the liver tissues of the rats were: (1) Enlargement in the hepatocytes, (2) Formation of few numbers of vacuolation in the cytoplasmic of the liver cells, (3) Mild dilation in the blood sinusoid as illustrated in Figure 2B.

The above results showed the important of the presence of n-3 fatty acids in the diet to reduce from the changes in the liver tissues. The less histological changes in the liver tissues was observed in the rats group fed on diet containing CF-2 oil blend followed by those containing OC-1 and OF then corn oil. These types of oils are easily metabolized in liver. The liver does not normally store fat, but it plays an essential role in the continual and rapid turnover of phospholipids, the saturation and desaturation of fatty acids, the oxidation and also the synthesis of fatty acids (Alwayn et al., 2005). It can be concluded from the data of the biological effect of oils on rats that both source and composition of oil in the diet had an effect on the weight gain, liver weight, triglycerides, total cholesterol, LDL-cholesterol of the plasma and histopathology of the liver tissues of the rats.

Utilization of oil blends

In this part of the study, the CF-2 and OF oil blends were used in preparing, microcapsules to use as a food supplement, omega fatty food products such as, oil blend/ cow butter and mayonnaise, fresh sauerkraut salad and carrot cake.

The following points were undertaken during preparing such products: 1-the products should be relatively low in saturated fat and omega-6-fatty acids; 2-the overall ratio of n-6 to n-3 fatty acids in these products is kept between 4-10: 1 as recommended by World Health Organization (Kris-Etherton et al., 2000), and n-3 fatty acids are found in all products; 3-the other prepared products than both fatty foods and the microcapsules are relatively low in calories; 4-such products should be simply, easily and rapid prepared.

Microcapsules

The encapsulation technique was used to prepare gelatin microcapsules containing CF-2 or OF oil blend. Each capsule contained 0.2 mg of oil blend. The prepared capsules were packed in a brown glass jar and kept at room temperature (22±2°C) for 6 months. The changes in FFAs, PV, TBA, P-AV and specific UV-absorption at 232 and 270 nm were determined to determine the oil stability in these capsules during storage period. The results were present in Table 4 and revealed that both composition of oil blend and storage period affected the quality of oil in the microcapsules. The rate of the changes in the determined parameters was more noticeable in CF-2 oil microcapsules than the OF ones. Because both blends containing flaxseed oil, the differences changes in the rate of the oil stability inside the capsules could be attributed to the other oil type in such blends. Canola oil had more unsaturated fatty acids especially linolenic acid than olive oil rich in oleic acid and nearly free from linolenic one (Sharara, 1998). Therefore, the rate of the oxidation was more expected in CF-2 blend than OF one. Also, extending the storage period to 6 months was associated with an increase in the rate of oil oxidation in microcapsules. The results of oil oxidation caused a darken in red oil color, an increase in FFAs, PV, TBA, P-AV, specific UV-absorbance at 232 and 270 nm particularly in microcapsules containing CF-2 oil blend. Generally, the rise in FFAs and PV values were less than the limits at which the oil should be rejected, (2% FFAs as oleic acid, and 10 meq O_2/kg oil PV value), respectively.

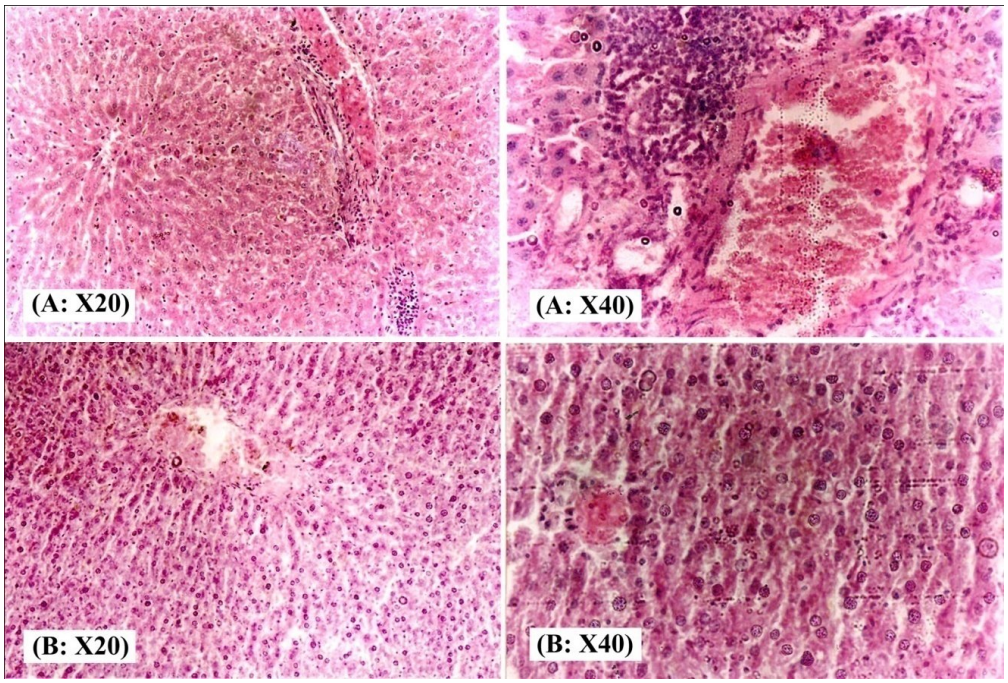

Figure 1. Light microscopic photograph of rat liver tissues.
A. Feeding on control; B. Feeding on CF-2 oil blend diet

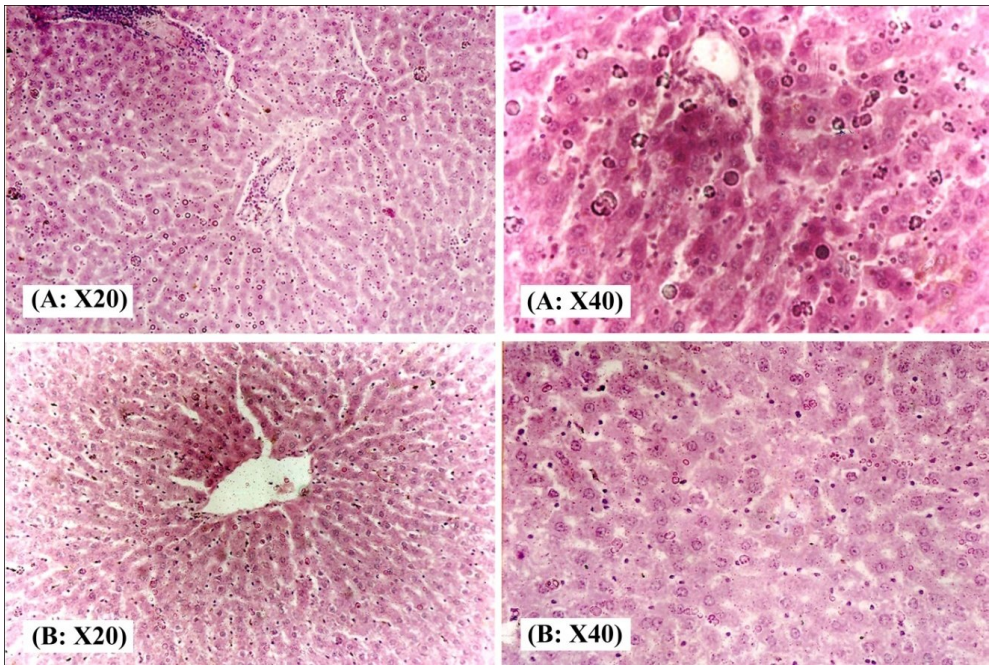

Figure 2. Light microscopic photograph of rat liver tissues.
A. Feeding on OC-1 oil blend diet; B. Feeding on OF oil blend diet

According to these results, the shelf life of such food supplement product can be recommended to be less than 6 months. The importance of such capsules is due to its high content of n-3 fatty acids which are an essential for brain function, hormone production and the other body functions. Also, the capsules provide a convenient way to cover the personal need from the right fat and its optimum level of the required EFAs. The daily need from oil blend capsules will depend on the total energy

intake, total polyunsaturated fatty acids intake and the absolute amount of n-6 to n- 3 fatty acids in the diet. According to Nielsen (1992), Denmark was the first country in the world which using microcapsules containing fish oil. Such capsules are used in preparing omega bread.

Omega fatty food products

(1) Oil blend / cow butter product: This product has about 50% of the saturated fat in cow butter and unlike margarine; it has a negligible amount of hydrogenated fat. Also it has a perfect consistency for spreading on toast or bread when uses after cooling and removing from refrigerator.

The color of this product was yellow and its flavor was an incipiently rancid. The acceptability of the color and flavor of such product were described by panelists as an excellent and good, respectively. No variations were noticed by panelists in the previous two characteristics between the products containing either CF-2 and/or OF oil blends. In contrast, the consistency of the product containing CF-2 oil blend was moderately spreading than that having the OF oil blend, slightly spreading. This can be attributed to the differences in the level and type of unsaturated fatty acids in the two oil blends. Therefore, the panelists described the consistency of the product containing CF-2 oil blend as good and the other product as fair. Generally leaving this product at room temperature for an hour or more caused a conversion of its consistency from plastic to nearly liquid. This problem can be solved by adding oil stabilizer to prevent the oil and butter from separating. Also, the flavor of such product can improve by adding some herbs or other different flavors.

(2) Mayonnaise: The color of this product was pale yellow and yellow in the products made from CF-2 and OF oil blends, respectively. The panelists preferred the color of the second product than the first one. They also noticed that the flavor of the last product was slightly rancid and its consistency was viscous and not semisolid. Therefore, the overall acceptability of this product was slightly like. On the other side, the second product prepared from OF oil blend was with unrancid flavor, semisolid consistency and a moderate like acceptance.

The above results showed that the sensory properties of the fatty omega products were depended on the fatty acids composition and the oxidation stability of the oil blend. Mayonnaise product made from oil with high level of unsaturated fatty acids was more susceptible for oxidation and had a thin consistency.

(3) Fresh sauerkraut salad: The results of the evaluation of the sensory properties of this product showed that the panelists did not detect differences in the sensory properties between the products containing CF-2 and OF oil blends. Both products had a red-purple color, crisp texture, and unrancid flavor. Therefore the overall acceptability of this product was very like as described by panelists. This product considers low calories, rich in dietary fibers and phytosterols (Simopoulos and Robinson, 1999).

(4) Carrot cake: This product contained carrot plus n-3 fatty acids from both CF-2 and/or OF oil blend. The results of the sensory evaluation indicated that the crust color of the carrot cakes prepared either by using CF-2 and OF oil blends was dark brown comparing with that made with butter and free from carrot, golden brown in color. Also the crumb color, porous distribution, porous structure, crumb texture and chewiness of the products containing the two oil blends were descried by panelists as yellow, fairly uniform, moderate, slightly soft and good, respectively. Such properties were less than those recorded for the control cake free from carrot and made of butter. The control cake had a white crumb color, uniform porous distribution; fine or thin cells porous structure, soft moist crumb texture and an excellent chewiness. The taste of the three cakes was accepted by panelists, the degree of the taste acceptability was excellent in both control cake and that made by CF-2 oil blend and good for the other product.

Conclusion

The biological effect of some vegetable oil blends rich in omega-3 fatty acids on rats showed that both source and composition of oil in the diet had an effect on the weight gain, liver weight, triglycerides, total cholesterol, LDL-cholesterol of the plasma and histopathology of the liver tissues of the rats. The results showed that the presence of n-3 fatty acids in the diets reduced the changes in the liver tissues. The shelf life of microcapsules containing oil blends to use as food supplement product can be recommended to be less than 6 months. Addition of antioxidants to such oil blends can extend their shelf life. The influence of such additives on biological effect of oil blends needs to examine. The capsules provide a convenient way to cover the personal need from the right fat and its optimum level of the required essential fatty acids.

The sensory properties of the prepared fatty omega products were acceptable by panelists.

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FOOD SCIENCE AND NUTRITION

Physicochemical characterization and sensory profile of 7 principal Tunisian date cultivars

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Abstract

The physicochemical characterization and the sensory evaluation of six Tunisian dates' cultivars, preselected on the base of the D-optimal design, have been made to compare them with the principal Tunisian dates cultivar, Deglet Nour. The morphological (fresh fruit weight and pulp content) and physicochemical (quality index) studies showed a great diversity among tested cultivars. In fact, the percentage of pulp indicated the existence of cultivars as interesting as Deglet Nour (89.3 ± 0.0), such as Horra (91.9 ± 0.1) and Alig (92.3 ± 0.1). Chemical analysis showed that Mnekher had high levels of total sugars (59.2 ± 0.0 of FM) and that Angou presented the highest ash content (3.6 ± 0.0%). Also, the sensory profiling revealed that each cultivar has its own distinctive characteristics (colour, texture and taste) and that Deglet Nour, Mnekher and Alig presented a tender and soft texture unlike the others, especially the cultivar Kintichi. In addition, the results relating to the hedonic study showed that Deglet Nour,, known as "finger of light", was the most appreciated (the best preference score) followed by Alig and Mnekher, whereas, the other studied cultivars were rather rejected by the consumers, especially Horra, Kintichi, Angou and Hamra. These two sensory evaluations revealed that the Tunisian consumer is more attracted by sweet and soft cultivars.

Key words: Correlation, Cultivar, Date, Quality, Sensory

Introduction

In Tunisia, date palm (*Phoenix dactylifera* L.) has a great nutritional and economical importance (Besbes et al., 2009; El Arem et al., 2011). In fact, date fruits are an integral part of Tunisian diet. The information accrued in the past four decades suggest that dates possess diverse medicinal uses including anti-hyperlipidemic, anticancer, gastro-protective, hepatoprotective and nephroprotective activities and thereby serving as an important healthy food in the human diet (Baliga et al., 2011). The observed pharmacological properties may be attributed to the presence of a high concentration of minerals (e.g. selenium, copper, potassium, and magnesium and moderate of manganese, iron, phosphorus and calcium) and various other phytochemicals of complex chemical structure (e.g. phenolics, vit A, vit B1, vit B2, vit B3, vit B6, vit B9 and vit C; and insoluble fibers) (Al Farsi and Lee, 2008).

There are more than 600 varieties of dates worldwide differing in shape and organoleptic properties of the fruits (Ahmed et al., 1995). Tunisian palm is represented by more than 250 cultivars (Rhouma, 1994), which are threatened primarily by the expansion of Deglet Nour (Ferry et al., 1998). The preservation of this heritage requires better knowledge about these cultivars including morphological, chemical, biochemical and especially sensory characterization. This would help improve characteristic, particularly in terms of taste. Sensory analysis is an indispensable pre-requisite to chemical analysis, in the definition of the characteristics and value of food products (Gerbi et al., 1997). However, sensory analysis for dates is particularly arduous because of the sweet taste of the product, that's why, the relationships between Tunisian consumer preference and the sensory properties of fresh dates have not been established in the scientific literature (Ismail et al.,

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2008). Tunisian researches affecting this sector are few. Some studies have focused on morphological characterization of dates or dates products (Rhouma, 1994) and others on the physicochemical and biochemical one (Reynes et al., 1994; Bouabidi et al., 1996), but few have considered the sensory evaluation of dates (Al Hooti et al. 1997; Ismail et al., 2001). And since no sensory study was performed on Tunisian cultivars, this work develops a comprehensive physicochemical and sensory characterization of seven Tunisian dates varieties, the majority of which do not benefit so far a commercial or industrial interest compared to Deglet Nour.

Materials and Methods

Plant material

Seventeen different cultivars of date palm fruits were harvested during the 2010 harvest season at Tmar maturation stage, from the southern Tunisia (Dgueche and Gabes cities) and stored 24 hours maximum at -20°C prior to analysis. The analyzed cultivars were: Angoua, Alig, Bent Essagni, Besser Helou, Boufaggous, Deglet Hassen, Deglet Nour, Mnekher, Hamra, Horra, Kintichi, Khlat Emmwachim, Khlat Saad, Khouet Alig, Tezerzit Safra, Trongea and Zehdi. All morphological and physicochemical analyses were repeated three times and were carried out on the 17 varieties.

Morphological characterization

Twenty-five fruits were selected randomly and were used for all morphological and physicochemical analyses. Each individual fruit, representing one replicate, was subjected to determination of length and diameter using a micrometer caliper (± 0.01 mm) (Ismail et al., 2008). Pulp and pit weights were determined through a precision balance (model EG-220-3NM, ± 0.002 g).

Physicochemical analysis

The amount of total and reducing sugars in fruit was evaluated by the method of Dinitro Salicylic Acid (DNS) after defection of the sample (Miller, 1959). To 1 mL of the date solution, 4 mL of the DNS reagent was added. Tubes were placed in boiling water bath for 5 min, transferred to ice to rapidly cool down and then brought to room temperature. The absorbance was measured at 540 nm.

The water content was measured by drying 2 grams of pulp in a drying oven at 80°C until constant weight was reached. Results are expressed as percent of fresh weight (El Arem et al., 2011).

The pH was measured using a pHmeter. Four grams of date pulp were dispersed in a flask with 200 ml of boiling water. After being cooled, this solution was used for the determination of the pH (El Arem et al., 2011).

The volatile acid was determined by titration (NT 52.28, 1983).

The ash content was determined as a result of mineralization of samples by incineration of 1 g (powdered flesh) in a porcelain container at 450 ° C for 5 h (El Arem et al., 2011). Ash contents were expressed as percent of dry weight.

To determine the colour, 25 fruits were selected randomly, and each individual fruit, representing one replication, was subjected to a chromameter (Model. CR 300, Minolta Camera, Ltd., Osaka, Japan) (CIE, 1978; van Eck and Franken, 1994).

Finally, the texture was evaluated by a firmness tester (model FT 327, EFFEGI Corp., Torino, Italy) according to Valero et al. (2007).

Sensory profiling

Eleven trained panellists were selected according to ISO 8586-2 (1994) to describe and rate sensory properties of dates. The selection of descriptors for establishing a sensory profile was made according to the method described by Stone et al. (1974). Ten attributes defining the sensory profile focused on the external aspect, colour, tactile texture, mouth texture, stickiness, astringency, sweetness, bitterness, sourness and sandiness. The evaluation of these attributes has been structured on a numerical scale from 1 to 10. Due to the large number of cultivars a reduction using MATLAB® (1999) software was necessary to realize sensory analyses. The selection method was based on the D-optimal design to reduce the number of cultivars (17) used in hedonic and sensory analysis because it is difficult to a judge to compare simultaneously more than 7 products (Claustriaux, 2001). The final number of selected cultivars was 6 (Alig, Hamra, Horra, Angou, Mnekher and Kintichi) to which the cultivar Deglet Nour was added.

Consumer test

Hedonic evaluation was conducted by the participation of 100 untrained Tunisian volunteers (Watts et al., 1991; Lespinasse et al., 2002).The group was formed by 50.1% female and 49.9% male (INS, 2012), 50% of them were between 20 and 40 years old, and 50% were between 40 and 60 years old. Evaluations took place in individual sensory booths, and daylight lighting was used (Lespinasse et al., 2002). The product was

introduced anonymously represented by 3 digits codes and arranged on table at random (Lespinasse et al., 2002). Consumers asked to rate each sample on a 9-point descriptive scale where 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely (Ahenkora et al., 1998).

Statistical analysis

Data analysis was performed using descriptive univariate analyses based on ANOVA for each descriptor. The statistical analysis was conducted using MATLAB ® (version 5.3, 1999) and nonrandom associations between categorical variables (at the level of $p<0.01$) were determined using the Fisher (Foucart et al., 1984). The multivariate analysis is done using the technique of principal component analysis (PCA) (Husson and Pagès, 2007). The analysis of consumer data was generated by MATLAB ® which allowed the classification of cultivars by their order of preferences and identified, accepted and rejected one.

Results and Discussion

Morphological characterization

The length of date palm fruits varies from 46.3 ± 0.5 mm to 27.7 ± 0.1 mm (Table 1). As for dates width, they are ranging from the simple (13.3 ± 0.1 mm for Khalt Emmwachim) to the twice (26.3 ± 0.1 mm for Tezerzit Safra).These results are similar to those obtained by Rhouma (1994). The average weight of the fruit varies from one to three, also depending on cultivar. It is between 5.5 g ± 0.2for Khlat Saad and 17.4 ± 0.4 g for Mnekher. Comparing to Deglet Nour (11.5 g ± 0.1), the weight of fresh fruit highlights other cultivars which have more interesting yield, such as Mnekher, Trongea and Boufaggous (respectively 17.4± 0.4 g, 15.2 ± 0.2 g and 14.7 ± 0.2 g). The percentage of pulp (weight of the pulp / total weight of the fruit) indicates the existence of cultivars as interesting as Deglet Nour (89.3 ± 0.0), such as Zehdi (89.0 ± 0.1), Bent Essagni (89.3 ± 0.6), Tezerzit Safra (89.6 ± 0.0), Mnekher (89.8 ± 0.1), Horra (91.9 ± 0.1) and Alig (92.3 ± 0.1). These results coincide with those developed by Reynes et al. (1994) and Bouabid et al. (1996) who worked on the South Tunisia dates characterization.

Chemical characterization

The total sugar expressed as a percentage of fresh material (FM) varies between 44.0 ± 0.0 and 62.7 ± 0.1 of FM (Table 2). It is interesting to note that cultivars Zehdi and Mnekher which have high levels of total sugars (respectively 62.7 ± 0.1 and 59.2 ± 0.0 of FM) are subject to rapid fermentation and can't be kept longer. However they can be used to obtain fermented products such as dates vinegar or wine.

The pH value extends over an interval between 5.6 ± 0.1 (Deglet Nour) and 6.7 ± 0.0 (Besser Helou). These values are close to those found by Reynes et al. (1994) (between 5.0 and 6.3). The levels of malate didn't vary a lot depending on cultivar. Allowing to Centeno et al. (2001), and despite the fact that the organic acid content of a fruit is regarded as one of its most commercially important quality traits when assessed by the consumer, relatively little is known concerning the physiological importance of organic acid metabolism for the fruit itself.

Dates' ash content has varied from 0.9 ± 0.1 to 3.6 ± 0.0% of dry matter. The highest ash content was noted for Angou. It is interesting to note that Deglet Nour (2.4 ± 0.1%) was within the range of the average, and several other cultivars can be found most interesting concerning the ash content.

Colour is defined by three parameters (L, a and b) that vary widely among cultivars. The parameter L is comprised between 24.5 ± 0.1 (Tezerzit Safra) and 73.8 ± 0.7 (Deglet Hassen) (Table 3). The cultivar Deglet Nour presented a good brightness (36.6 ± 0.4) but less than Deglet Hassen, Kintichi, and Angoua. These results indicated that the colour of these 3 cultivars is as good as Deglet Nour, which is known as "fingers of light". Concerning the parameter a, positive values indicate setting reddish colours, while negative ones indicate the greenish colours. The results showed values between 17.6 ± 0.1 for Angou and 2.4 ± 0.1 for Tezerzit Safra.

Finally, the parameter b indicates a range of colour between the yellow (for positive values) and blue (for negative values). Relative results showed a large difference between cultivars from 36.6 ± 0.2 for Kintichi and 1.7± 0.6 for Tezerzit Safra. The medium value for Deglet Nour cultivar was about 17.4± 0.4.

The study of these three colour parameter settings, allowed classifying cultivars into two groups:

• Dark cultivars (a> b): Alig, Tezerzit Safra, Boufaggous, Khouet Alig, Trongea, Bent Essagni and Hamra.

• Clear cultivar (a <b): Mnekher, Khlat Saad, Khlat Emmwachim, Besser Helou, Zehdi, Kintichi, Angou, Deglet Nour, Deglet Hassen and Horra.

Colour is an important distinctive characteristic in plant breeding and therefore often used in official Plant Breeders' Rights (PBR) tests (van Eck and Franken, 1994). The chromameter is generally used to objectively describe a colour and communicate about colour. It demonstrated a high potential to improve the objectivity of the description of any coloured varieties (van Eck and Franken, 1994). However, when assessment of colour is done visually, it may be subjective and depends on both individual and personal interpretation. The lack of suitable charts makes accurate description of samples hardly possible (van Eck and Franken, 1994). In addition, it is difficult to express the differences between the observed colour and a comparable colour chart.

Many substances are responsible for this fruit colour such as carotenoids (a class of natural fat-soluble pigments) and anthocyanins. Studies (Boudries et al., 2007) have also shown that dates contain the carotenoids; lutein, β-carotene and neoxanthin. A significant decline in the carotenoid level occurs during the transition from the Khalal through Tamar stage and that during the ripening process (Boudries et al., 2007). Anthocyanins are water-soluble vacuolar pigments and may appear in red, purple, or blue. They are widely distributed in many fruits as dates, and they have potential health benefits (Wang et al., 1997; Al Farsi et al., 2005).

The colour is an important parameter in the date consumer acceptance because it represents the first visual attraction with the product. The difference of texture (Table 3), intra and inter specific between cultivars (with and without pit), showed that Kintichi (dry dates) can carry a load of 5.9 ± 0.2 kg with pit and 5.2 ± 0.4 kg without pit; while Safra Tezerzit, which is a soft date, can't carry more than 1.3 ± 0.1 kg with pit and 1.1 ± 0.0 kg without pit. Moisture content and texture are often used to classify date palm varieties into 3 main types: soft, semi-dry, and dry cultivars. Local date palm varieties are numerous and well adapted to agroecological conditions. Their denominations are strictly local and originating, most often, from the place of cultivation, the colour or the shape of the fruits (Mohamed Vall et al., 2011).

Sensory Evaluation

Characterization sensory

Sensory analysis is considered an important technique to determine product quality. It comprises a set of techniques for accurate measurements of human responses to foods (Pérez Elortondo et al., 2007). It has been applied to determine appearance, odour, flavour and texture properties, which are important characteristics of food products quality. Sensory evaluation requires panels of human assessors, on whom the products are tested, and the responses made were recorded. This is the case of our study, in which different dates' cultivars were characterized.

Table 1. Morphological characteristics of 17 Tunisian date cultivars based on fruit measurements.

Cultivar	Weight (g)	Pulp (%)	Length (mm)	Width (mm)
Mnekher	17.4 ± 0.4	89.8±0.1	46.3 ± 0.5	24.1 ± 0.1
Alig	12.1 ± 0.1	92.3±0.1	44.5 ± 0.1	25.0 ± 0.1
Tezerzit Safra	9.9 ± 0.3	89.6±0.0	34.6 ± 0.0	26.3 ± 0.1
Boufaggous	14.7 ± 0.2	87.12±0.0	42.8 ± 0.0	24.7 ± 0.1
Zehdi	8.9 ± 0.1	89.0±0.1	37.9 ± 0.2	22.9 ± 0.1
Kintichi	6.2 ± 0.2	80.7±0.2	34.2 ± 0.1	21.7 ± 0.1
Khouet Alig	6.9 ± 0.2	92.0±0.1	43.2± 0.4	24.0± 0.2
Angou	5.7± 0.1	83.4±0.1	27.7± 0.1	23.0± 0.0
Deglet Nour	11.5± 0.1	89.3±0.0	41.2± 0.0	24.1± 0.2
Besser Helou	5.7± 0.2	74.9±0.3	29.3± 0.0	13.8± 0.2
Trongea	15.2± 0.2	77.6±0.5	31.5± 0.1	23.4± 0.2
Hamra	11.6± 0.4	83.5±0.0	44.2± 0.0	24.3± 0.1
Horra	11.9± 0.1	91.9±0.1	44.0± 0.2	21.8± 0.0
Khlat Saad	5.5± 0.2	86.1±0.0	46.2± 0.0	15.9± 0.2
Deglet Hassen	10.8± 0.2	85.4±0.2	39.7± 0.1	16.3± 0.2
Khlat Emwachim	9.6± 0.1	86.2±0.2	36.5± 0.1	13.3± 0.1
Bent Essagni	12.1± 0.2	89.3±0.6	36.9± 0.2	17.5± 0.2

Table 2. Chemical characteristics of 17 Tunisian date cultivars based on fruit measurements.

Cultivar	Total sugar (%FM)	Sucrose (%FM)	Dry Matter (%)	pH	Ash (% DM)	Malate (meq/100g FM)
Mnekher	59.2±0.0	4.0±0.0	81.7±0.0	6.2±0.1	2.5±0.1	0.1±0.0
Alig	52.9±0.6	8.0±0.0	75.0±0.1	6.5±0.1	3.2±0.0	0.1±0.0
Tezerzit Safra	44.0±0.0	0.0±0.1	68.6±0.2	6.3±0.0	2.9±0.0	0.2±0.1
Boufagous	52.0±0.0	13.3±0.2	81.7±0.0	5.7±0.0	2.1±0.1	0.2±0.0
Zehdi	62.7±0.1	10.6±0.8	91.1±0.0	6.2±0.1	0.9±0.1	0.3±0.1
Kintichi	52.0±1.0	45.3±0.5	84.0±0.1	6.0±0.1	2.7±0.6	0.3±0.1
Khouat Alig	51.7±0.0	9.6±0.3	80.2±0.1	6.3±0.1	1.3±0.0	0.2±0.0
Angou	52.7±0.6	5.3±0.1	80.7±0.1	6.2±0.1	3.6±0.0	0.3±0.1
Deglet Nour	54.1±0.0	41.4±0.1	87.7±0.1	5.6±0.1	2.4±0.1	0.2±0.0
Besser Helou	54.7±0.0	1.6±0.1	70.5±0.0	6.7±0.0	2.1±0.1	0.3±0.0
Trongea	54.1±0.1	2.7±0.0	48.7±0.0	6.1±0.1	3.1±0.1	0.2±0.1
Hamra	57.1±0.1	15.3±0.1	98.2±0.0	6.5±0.1	3.0±0.1	0.3±0.1
Horra	58.0±0.0	47.4±0.4	96.8±0.3	6.3±0.1	2.3±0.1	0.3±.0.0
Khlat Saad	52.3±0.1	8.8±0.4	75.4±0.1	6.3±0.1	2.7±0.1	0.2±0.1
Deglet Hassen	50.0±0.1	32.3±0.1	72.3±.0.4	5.8±0.0	2.4±0.1	0.2±0.0
Khlat Emwachim	52.3±0.9	32.7±0.1	70.0±0.0	6.0±0.1	3.3±0.0	0.3±0.0
Bent Essagni	45.9±0.2	11.3±0.2	76.4±0.5	5.6±0.1	2.9±0.1	0.2±.0.1

MF: Fresh matter, MS: Dry matter.

Table 3. Textural characteristics and colour of 17 Tunisian date cultivars based on fruit measurements.

Cultivar	TexN (Kg)	Tex (Kg)	L	a	b
Mnekher	3.2±0.0	2.6±0.2	27.4±0.0	7.9±0.0	9.0±1.0
Alig	1.7±0.2	1.6±0.2	26.7±0.1	6.4±1.2	5.4±0.7
Tezerzit Safra	1.3±0.1	1.1±0.0	24.5±0.1	2.4±0.1	1.7±0.6
Boufagous	2.3±0.1	1.5±0.2	27.6±0.0	9.3±1.0	7.6±0.7
Zehdi	2.2±0.2	2.0±0.2	36.3±0.8	13.8±0.3	20.4±0.2
Kintichi	5.9±0.2	5.2±0.4	51.1±0.1	14.3±0.5	36.6±0.2
KhouatAlig	2.2±0.1	2.0±0.0	27.3±0.6	11.9±0.8	8.8±1.0
Angou	3.8±0.1	3.3±0.2	39.9±0.4	17.6±0.1	24.1±0.7
DegletNour	1.4±0.0	1.3±0.1	36.6±0.4	7.3±0.7	17.4±0.4
BesserHelou	1.9±0.4	1.8±0.2	35.1±0.5	14.5±0.1	16.9±0.0
Trongea	2.7±0.0	2.5±0.2	32.2±0.3	7.07±0.8	6.7±0.1
Hamra	4.7±0.1	4.6±0.4	36.3±0.9	11.1±0.2	7.7±0.7
Horra	3.4±0.0	3.2±0.3	31.1±0.1	10.4±0.4	21.5±0.2
KhlatSaad	2.5±0.2	2.2±0.1	29.4±1.0	11.1±0.1	11.9±0.2
DegletHassen	1.9±0.1	1.4±0.1	73.8±0.7	9.4±0.3	11.9±0.7
KhlatEmwachim	3.8±0.1	3.4±0.2	32.2±0.9	9.9±0.1	11.6±0.3
BentEssagni	1.6±0.1	1.5±0.2	27.0±0.0	7.9±0.5	6.7±0.7

TexN: Charge applied to date with pit, Tex: Charge applied to date without pit, L* corresponds to levels of darkness/lightness between black and white, a* signifies the balance between red/green, and b* between yellow/blue.

The results obtained by sensory characterization, showed a statistically significant difference between the cultivars' descriptors studied at a level of confidence of 95%. Indeed, within the following criteria: colour, appearance, mouth texture (soft, sticky and astringent), taste (sweet and bitter), the existence of significant differences between studied cultivars was recorded (Table 4), whereas it wasn't the case of the acid flavor and sandy criterion (presence of sugar crystals). A graphic presentation of individuals (subjects) and variables (cultivars) was resumed in Figure 1. Indeed, the first two lines showed 90% of the total information. The first axis (FD1) is generally an axis of tactile texture or mouth texture, while the second axis (FD2) is an axis of the colour. It is clear that Deglet Nour, placed at the top right, stands out from the total group. It is also interested to note that the group Deglet Nour, Mnekher and Alig are rather in the right part of the plan, in contrast to assessments for

cultivars Kintichi, Hamra, Horra and Angoua. In fact, Deglet Nour, Mnekher and Alig present a tender and soft texture unlike the others, especially the cultivar Kintichi, which are qualified generally as dry and hard. Also, cultivars with a dark colour like Mnekher, Alig are located in the upper part of the plan, in contrast to Kintichi which is clearer. This graph is used to confirm the clear distinction between cultivars and the typicity of each of them, and it highlights the effect subject. Indeed, we can see clearly the discriminating power and degree of agreement of the tasting panel concerning Deglet Nour, Alig, Mnekher, Hamra and Kintichi. However, we noticed a great dispersal of spots concerning Horra and Angoua. Moreover, bitterness, astringency and sandiness were negatively correlated to axis 1 and opposed to appearance, mouth texture and feel, sweetness, stickiness and colour. The tender in the mouth is negatively correlated with the astringency and the sandiness that are highly correlated (correlation coefficient close to 1). The results showed generally division into three groups of cultivars (the first group contains Angoua, Kintichi, Hamra and Horra, the second Deglet Nour and Alig, and the latter contains only Mnekher) primarily determined by bitterness, astringency and sandiness which are opposed to appearance, texture and mouth touch, colour and stickiness. Deglet Nour and Alig presented the best results concerning appearance, texture, mouth and tactile stickiness.

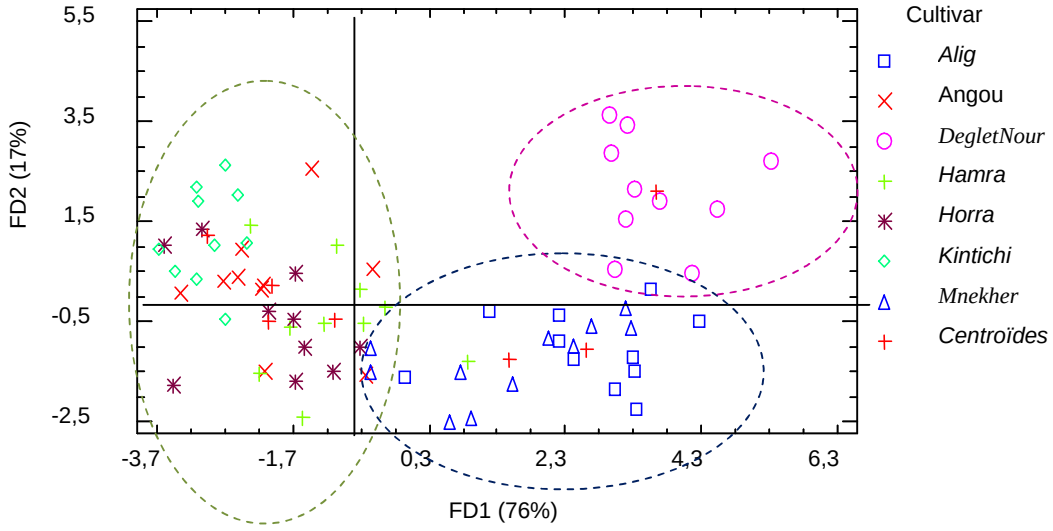

Figure 1. Score plot of Tunisian dates.

Table 4. Descriptor effect analysis calculated by Fisher ratio.

Descriptor	F	Signification level
Colour	18.32	0.0000***
Tactile texture	25.51	0.0000***
Mouth Texture	6.62	0.0000***
Criterion sticky	24.55	0.0000***
Criterion Astringent	6.09	0.0000***
Criterion sweet	2.55	0.0149**
Criterion bitter	8.09	0.0000***
Appearance	2.57	0.0145**

F : Calculated Fisher number, ** : P < 0.01, *** : P < 0.001

Hedonic evaluation

Results of panel evaluation (100 subjects) showed significant variation between the different assessed cultivars (Figure 3). Deglet Nour and Alig spots were significantly higher than those of the other cultivars. In fact, they attracted respectively 90% and 70% of the consumers (left part of the presentation) (Figure 3). Segmentation of Tunisian consumers appeared to be according to whether a sweet date, with a good mouth texture and a better appearance is preferred, whereas, bitter, astringent

and sandy dates are rejected. In addition, the histogram reflecting the average note of preference based on cultivars between men and women (Figure 4) showed that there is no significant difference, in order of preference, and that both of them prefer rather Deglet Nour and Alig. The study of the effect of age on consumer preferences showed that consumers aged between 20 and 30 years and those above 30 years, preferred successively, cultivars Deglet Nour, Alig, Mnekher, Horra, Kintichi, Hamra and Angou (Figure 5). However, it appears that subjects aged between 8 and 20 years advanced a different order of preference. They preferred successively cultivars Deglet Nour, Alig, Horra, Mnekher, Hamra, Kintichi and Angoua. However, Alig and Deglet Nour remained the two most appreciated cultivars by all subjects. The study of the difference in preference by region for the 7 cultivars studied (Figure 6), revealed that subjects from southern and northern Tunisia have nearly the same order of preference for the 7 dates cultivars. They prefer successively Deglet Nour, Alig, Mnekher, Horra, Hamra, Kintichi and Angoua. However, subjects from the center of Tunisia prefer successively cultivars Deglet Nour, Alig, Horra, Mnekher, Kintichi, Hamra and Angoua. At the end of this segmentation by region we can say that Deglet Nour, Alig and Angoua remained in the same position in terms of preference for individual consumers however there was a permutation between Horra-Mnekher and Hamra-Kintichi for subjects from the center of the country compared to those from southern and northern Tunisia.

Comparison between sensory evaluation and consumer ranking

In both panel evaluation and consumer ranking, Deglet nour was prescribed to have the best quality, by far, among the tested varieties. Also, Alig and Mnekher did not significantly differ in both panel evaluation and consumer ranking, they were in the second order of preference. However, as stated by Hollingsworth (1996) it is not easy to translate consumer language. For example, when they say that a product is too sweet, they might not want it to be less sweet, but probably they wanted something to balance the sweetness. On the other hand, within each variety, there are, sometimes, qualitative differences, thus, results from the taste panel made on samples from a known background might be different from other samples of the same variety coming from a different source (Ismail et al., 2001). Thus, this work should be pursued using a bigger number of varieties coming from different sources.

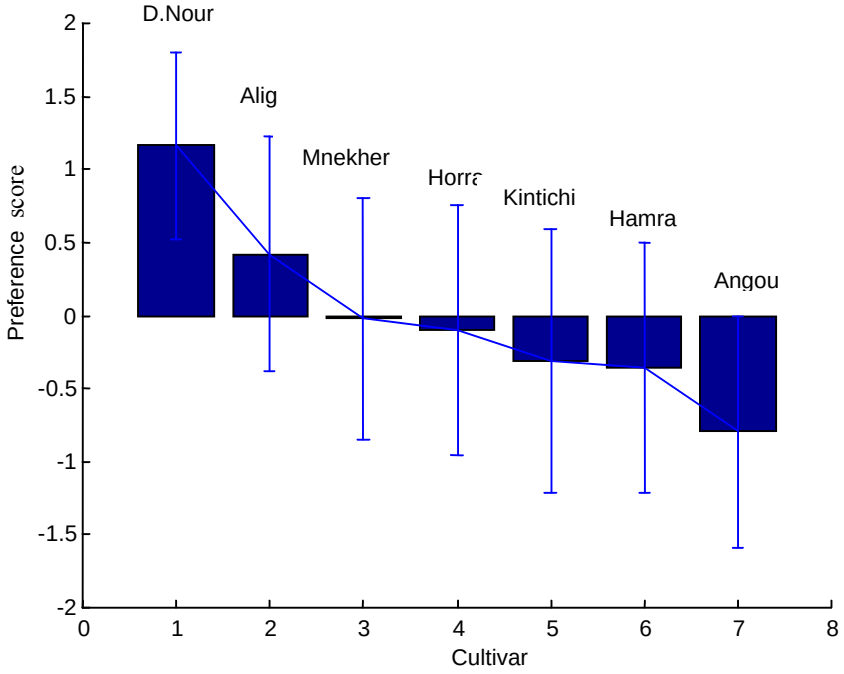

Figure 3. Preferences study (100 consumers – 7 Tunisian date cultivars).

According to Colomb and Stocker (2007), the brain collects two types of information from taste stimuli: the hedonic aspect (Is it good or bad?) and the sensory aspect (What kind of molecule is it?). While the hedonic information commands ingestion or rejection of food, molecular information is thought to be essential for modifying responses to food through learning. In mammals, the hedonic aspects (good versus bad) and sensory aspects (i.e., the molecular quality) of taste are associated with different brain regions.

In fact, food quality is a multivariate notion ('it tastes good – it looks traditional, safe, healthy, etc.'). Traditional foods, like dates, are sometimes used to carry an image of foods tasting good, but in the same time could be perceived either good for health (as related to natural products, no chemical modification, no additives). These aspects, taste and health, have to be improved in parallel and clarified for the consumers (Cayot, 2007).

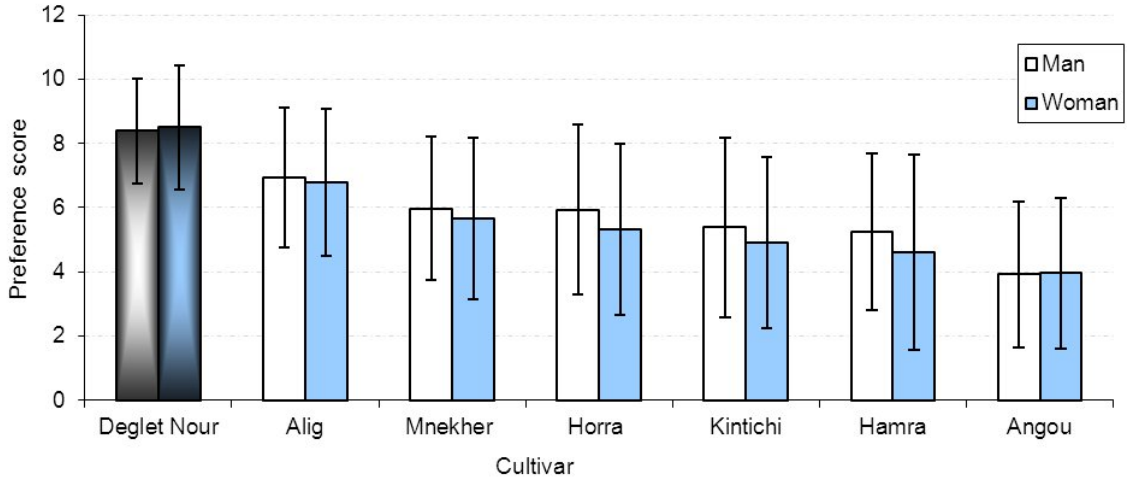

Figure 4. Average notes of the preferences expressed by men and women according to Tunisian.

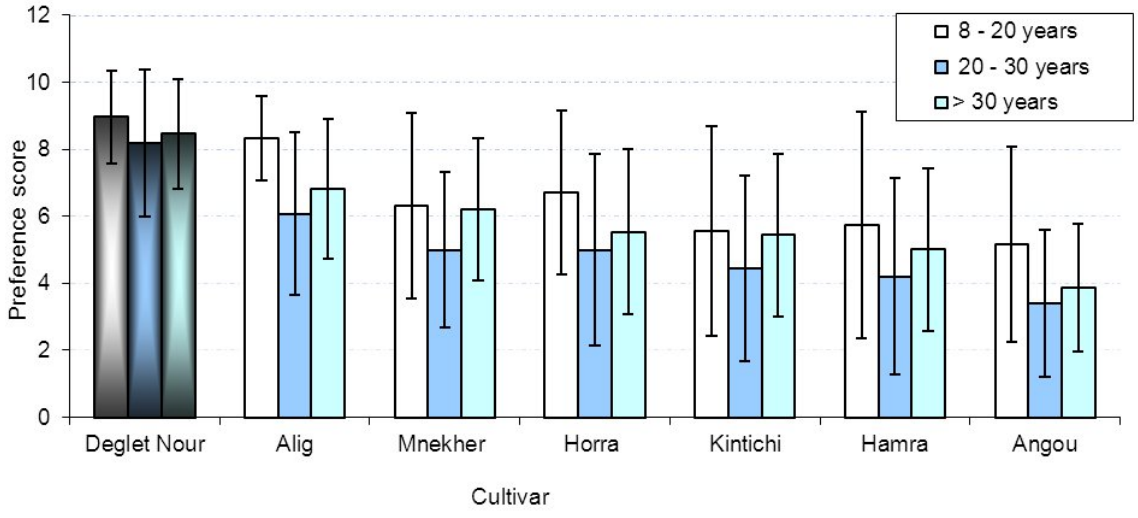

Figure 5. Average notes of the preferences expressed by age according to Tunisian cultivars.

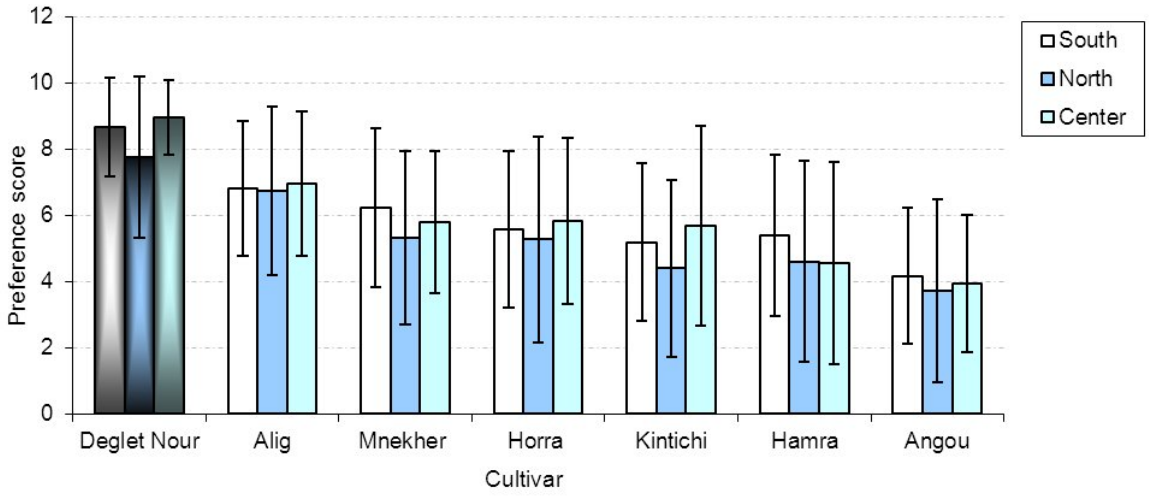

Figure 6. Average notes of the preferences expressed by area according to Tunisian cultivars.

Conclusion

Sensory characterization of 7 Tunisian dates cultivars, which, unfortunately, the majority does not benefit so far of a commercial or industrial fit, proved that each cultivar is distinguished by several criteria (appearance, mouth texture and feel, sweetness, bitterness, acidity, astringency, stickiness and sandiness). Significant differences among cultivars have been observed through the cultivars analysis results. For example, each cultivar presents its own colour: Kintichi is qualified as the clearest when Alig as the darkest. Also, no significant difference was seen for acidity and sandiness for all cultivars. This sensory characterization has already allowed the chain dates to validate the organoleptic interest of new cultivars as Alig and Mnekher which have almost similar characteristics of Deglet Nour. Hedonic characterization of these cultivars showed that the cultivar Deglet Nour was held the top spot for preference followed successively by Alig and Mnekher and that other cultivars have not been appreciated by consumers. Indeed, the cultivar Angoua was the most depreciated monitoring Hamra, Kintichi and Horra. On the other hand, this sensory analysis, on its two aspects analytical and hedonic, revealed that the Tunisian consumer is attracted by soft and sweet cultivars, with the best mouth texture and best appearance. This study allowed concluding that the cultivar Mnekher, which is a rare and decommissioned date, featured prominently on the preferences' map because it covered 50% of potential preferences. Such result suggests that local extension of the work should help farmers to grow these cultivars and that more awareness companions should help the development of its consumption or valorization (Al-Farsi, 2007; Besbes et al., 2009). Due to its nutritional importance, abundance and low cost, date fruit remains a species with tremendous potential and countless possibilities for further investigation.

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FOOD SCIENCE AND NUTRITION

Phytase production by *Candida melibiosica* 2491 alkalophylic strain

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Abstract

It has been proved that *Candida melibiosica* 2491 yeast strain, an intracellular phytase producer, can be well adapted to a nutritious medium with a pH range from 3.0 to 9.0. The best development of the strain was achieved at pH 8.0, which refers to the rarely encountered alkalophylic yeast strains. The maximum biosynthesis was obtained in a medium with pH of 8.5, 24th–hour aged inoculum added at concentration of 6.6% (v/v) with optical density OD600 of 0.68, aeration degree 500 dm^3/dm^3.h, stirring speed 300 rpm, and 24h duration of the cultivation in bioreactor. The optimal phytase activity was reached at 60°C and pH 4.5. However, over 80% of the maximum enzymatic activity was observed at the pH interval ranging from 4.0 to 8.5, which provides great potential for practical applications.

Key words: Alkalophylic strain, *Candida melibiosica*, Phytase activity

Introduction

Phytases (myo–inositolhexakisphosphate phosphohydrolases) are enzymes, which catalyze the stepwise decomposition of phytic acid (IP6), resp. phytates, to inositol phosphate esters (IP5–IP1) and inorganic phosphate. Different classifications of phytases have been proposed: i) depending on the C–atom (dephosphorilation of myo–inositol ring is initiated from it) – 3–phytases (E.C. 3.1.3.8), 6–phytases (E.C. 3.1.3.26) and 5–phytases (E.C. 3.1.3.72); ii) according to their pH optima – acid and alkaline phytases iii) based on the catalytic mechanism - histidine acid phytases, β–propeller phytases, cysteine phytases and purple acid phytases (Mullaney and Ullah, 2003, Greiner and Konietzny, 2006).

There is a great potential for the use of phytases in the processing and manufacturing of forage or foods for human consumption (Haefner et al., 2005) as well as for environmental protection (Lei and Stahl, 2001). The pH stability of *Candida melibiosica* 2491also determines the strains possible application in bioremediation and biofuel cells (Hubenova and Mitov, 2011).

In both plant seeds and microorganisms, constitutive as well as inducible phytases have been identified. In non–limiting media, the majority of the bacterial phytases formation is turned off in exponentially growing cells and starts as soon as the cultures enter the stationary phase. In molds, the phytase formation was shown to be growth associated (Dvořáková, 1998; Konietzny and Greiner, 2002).

Phytase yeast producers receive more attention due to their easy handling and improved cultivation conditions, as well as due to their non-pathogenic features which allow their application in direct food supplement production. Data regarding phytases produced by yeast from genera *Schwanniomyces* (Lambrechts et al., 1992), *Saccharomyces* (Žyla, 1994), *Rhodotorula* (Bindu et al., 1998), *Arxula* (Sano et al., 1998), *Candida* (Quan et al., 2001), *Pichia* (Vohra and Satyanarayana, 2001), *Kodamaea* (Li et al., 2007), *Criptococcus* (Pavlova et al., 2008) were reported. Each microorganism's required specific cultivation however necessitates studies aimed at improving the phytase expression and activity.

In our previous paper, we reported that *Candida melibiosica* 2491 yeast strain possesses the highest phytase activity among the one hundred and eighteen screened microorganisms (Georgiev

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and Gargova, 2006). The aim of this study is to optimize *Candida melibiosica* 2491 cultivation conditions as well as to establish enzyme optima of the phytase.

Materials and Methods

Yeast strain

The yeast strain *Candida melibiosica* 2491 is maintained on YPfru agar nutrition medium containing (in g/L): fructose – 30.0, yeast extract – 5.0, meat peptone – 8.0, and 0.2 mM KH2PO4. YPfru broth was used for the preparation of the inoculum.

Flask cultivation

A 30 mL mixture of yeast cell inoculum and liquid medium with the above mentioned composition was placed in 300 mL Erlenmeyer flask and put on a shaker (3.7 Hz) at 28°C (Georgiev and Gargova, 2007).

Optimization of cultivation conditions

In order to optimize the cultivation conditions, the experiments were carried out in broth media with different initial pH, and inoculum of different age and quantity was used. The nutritious media had initial pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Inocula were taken at the 18th, 24th, 36th and 48th hours of cultivation, and their optical density was standardized to OD600=0.68. From each sample, various concentrations between 1.65 and 10% (v/v) were used for further cultivation. At the 30th hour of the yeast development, the dry biomass (DW) and the phytase activity (PhA) were determined.

Bioreactor cultivation

In a 2L bioreactor MBR Mini, (AG, Switzerland) a periodic cultivation process was carried out, using 1L yeast cells/medium at initial pH 8.5. The pH, dry biomass, trace phosphorous (Pi) quantity, quantity of the reducing sugars and phytase activity were monitored during the process. The influence of the cultivation conditions, such as aeration degree (50 – 1000 dm3/dm3.h) and stirring speed (300 to 600 min-1), was also studied.

Temperature and pH profiles of phytase activity

The influence of temperature on the hydrolysis of sodium phytate was examined at 30, 37, 45, 50, 55, 60, 65, 70 and 80 oC. The pH of the substrate solutions was adjusted accordinglyby using the following buffers: 0.2 M glycine – HCl (pH 2.0, 2.5, 3.0, 3.5); 0.2 M sodium acetate – acetic acid (pH 4.0, 4.5, 5.0, 5.5, 6.0); 0.05 M Tris – HCl (pH 6.5, 7.0, 7.5, 8.0, 8.5), 0.05 M glycine – NaOH (pH 9.0, 9.5, 10.0).

Analytical methods

Yeast cells, centrifuged at 10000 rpm, were washed twice with 0.2 M sodium acetate buffer, pH 5.5, and then re-suspended in 5 mL of the same buffer (Žyla, 1994). The growth rate and the biomass accumulation were determined by means of optical density measurement (OD600). The reducing sugars were quantitatively determined by means of 3.5 - DNS method (Miller, 1959).

The phytase activity was estimated from the quantity of the formed inorganic phosphate, determined by the Engelen's method (Engelen et al., 1994). The optical density of the color solution containing ammonium heptamolybdate (Scharlau, AM0349) and ammonium monovanadate (Scharlau, AM0467) was measured at 415 nm. One unit phytase activity is equivalent to the production of 1 μmol inorganic orthophosphate and is denoted as U/g dry biomass (Segueilha et al., 1992).

Statistical processing

The results are presented as their mean values and standard deviation of three independent experiments "Sigma Plot 11" software was used for presentation of the results and statistical analysis.

Results and Discussion

Flask cultivation

A good yeast cell development was observed in cultivation media with a wide range of the initial pH (Figure 1). The maximum growth, however, was achieved at pH 8.0, which determines *Candida melibiosica* 2491 as an alkalophylic strain. It should be noted that the strain also develops well at acidic pH from 3.0 to 5.5, which is an indication of the rich physiological potential of this microorganism. The enzymatic assays, however, show that phytase expression is observed at or above pH 5.0, and the maximum enzyme productivity was at pH 8.5. At this pH value, the highest yield factor (Ybiomass/substrate) of 42.6% was also obtained. In a similar study, Quan et al. (2001) established almost the same development of *Candida krusei* in the pH range from 4.0 to 9.0. However, the highest phytase activity was obtained at pH 7.0, and it was close to the maximum at pH 6.0 and pH 8.0.

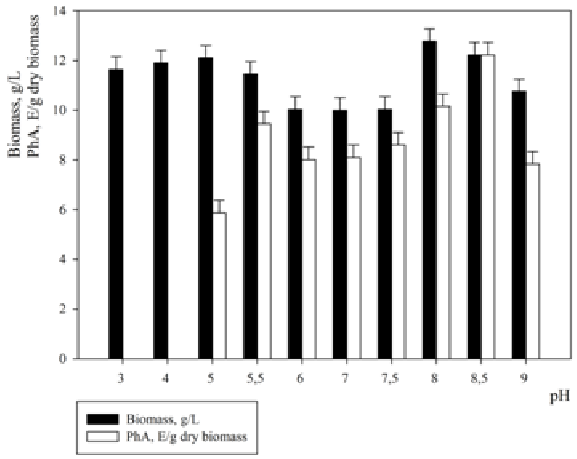

Figure 1. Influence of the medium's initial pH on the development of *Candida melibiosica* 2491 and phytase biosynthesis.

The inoculum age only slightly affects the culture development, but significantly influences the phytase productivity. Maximum enzyme activity was achieved when 24 h-aged inoculum was used (Figure 2a). The increase of inoculum quantity enhances the yeast biomass production. However, maximum phytase activity was observed when 6.6% inoculum was used (Figure 2b). During the optimization of the chemical composition of the nutrient medium was determined that 0.2 mM KH2PO4 stimulates the production of phytase. At higher Pi levels the phytase activity is inhibited, while lower Pi levels decrease but do not inhibit the enzyme production. The introduction of larger inoculum quantities probably leads to a decrease in the biomass/Pi proportion. Based on the obtained results, 24h-aged, 6.6% inoculum/media was chosen as optimal for *Candida melibiosica* 2491 cultivation and phytase production.

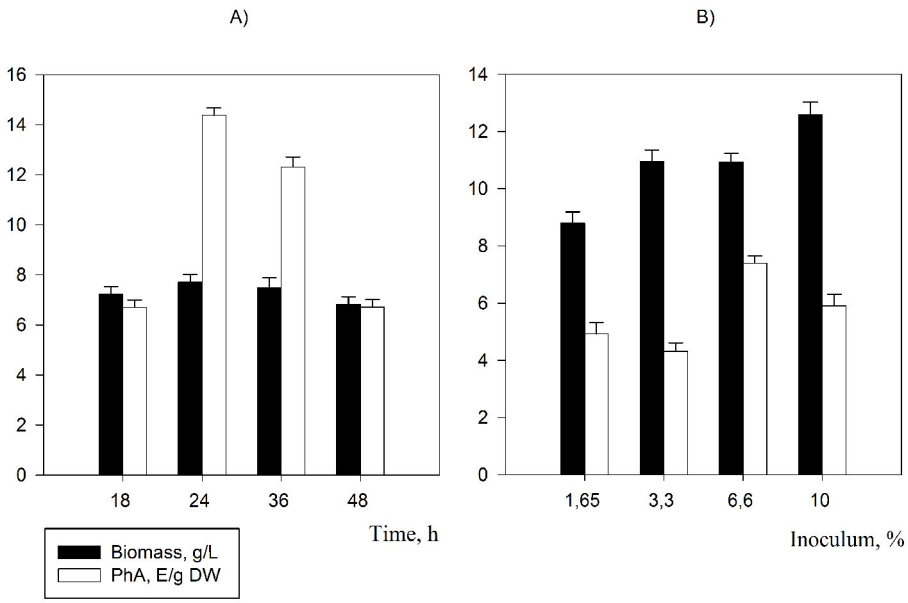

Figure 2. Influence of: a) the inoculum age and b) the inoculum quantity on the Candida melibiosica 2491 development and phytase productivity.

Bioreactor cultivation

The transition from flask cultivation to bioreactor cultivation is an important scale-up stage in the development of the biotechnological process for producing intracellular phytase from the yeast strain *Candida melibiosica* 2491.

After determining the optimal conditions in flask (pH of media, age and quantity of inoculum), cultivation of *Candida melibiosica* 2491 in a bioreactor was performed. The influence of the aeration degree and stirring speed on the yeast growth and phytase activity was determined. The aeration degree α (dm3/dm3.h) was estimated by the equation α = Q/V, where, Q is the air debit (dm3/h), V is the working volume (dm3).

The yeast biomass formation was enhanced by increasing the aeration degree. At low aeration degree (α = 50 dm^3/dm^3.h), both the accumulated

biomass and enzyme activity were approximately at one half of the maximum values (Figure 3). The best development of the culture was achieved at α = 1000 dm^3/dm^3.h (19.4 g/L biomass), even though at α = 500 dm^3/dm^3.h the obtained biomass quantity was also very close to the maximum. The highest phytase activity of 13.2 E/g dry biomass was measured at α = 500 dm^3/dm^3.h, which determined this aeration degree as optimal for further experiments.

The variation of stirring speed from 300 to 600 min^{-1} did not affect significantly the cell development. However, it was an important factor for the phytase biosyntheses (Figure 4). The enzyme activity determined at 600 min^{-1} was almost two times lower than the maximum achieved at 300 min^{-1} (13.0 E/g dry biomass).

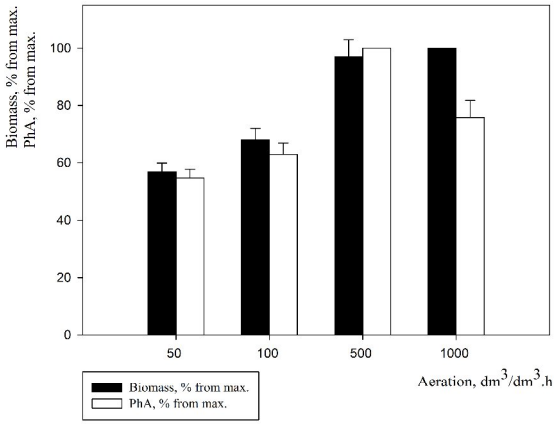

Figure 3. Influence of the aeration on *Candida melibiosica* 2491 development and enzyme activity in 2L bioreactor: pH 8.5; stirring rate 300 min–1; temperature 28°C; duration 24 h.

In the progress of the biosynthetic process, the main carbohydrate source fructose was intensively assimilated, and at the 12th hour residual sugars were not been measured (Figure 5). The total exhaustion of carbohydrates corresponds to the end of the exponential growth phase of *Candida melibiosica* 2491 and the maximal accumulation of biomass. The inorganic phosphate (Pi) in the medium rapidly decreased during the first 8 hours, and its complete depletion was achieved at the 18th hour of cultivation. The phytase formation continuously grew with the highest rate between the 8th and 18th hour of cultivation, corresponding to the exponential and early stationary phase of the culture development. pH of the cultural medium did not vary in a wide range – from the initial value of 8.5, it reached minimum of 7.5 at the 12th hour and after that increased to 8.0 at the end of the process. In their study Vohora et al. (2011) also investigated the process dynamics but in Pichia anomala (Vohora et al., 2011). For this train the carbon source quantity is 4%, the inoculum is 5% and the process duration is 24 hours.

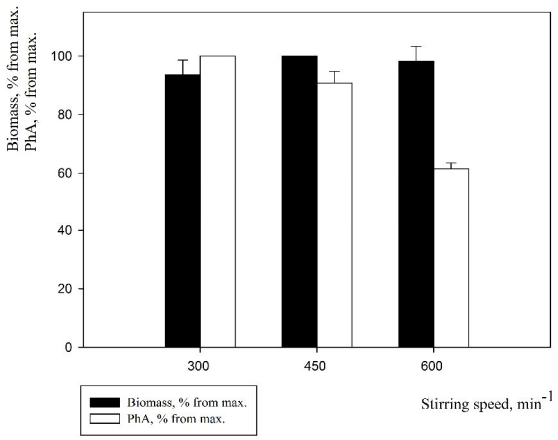

Figure 4. Influence of the stirring speed on Candida melibiosica 2491 development and enzyme activity in 2L bioreactor: pH 8.5; aeration degree 500 dm^3/dm^3.h; temperature 28°C; duration 24 h.

Enzyme optima

The temperature profile of the intercellular phytase activity showed a typical behavior – the activity gradually grew up to 60oC, then it sharply decreased and was totally lost at 80oC (Figure 6a). The observed profile is in agreement with other established data. However, the temperature optima for different phytase producers varies greatly: *Candida krusei* WZ – 001 – 40oC (Quan et al., 2001); *Pichia anomala* – 60oC (Vohra et al., 2001); *Schwanniomyces castellii* – 77oC (Segueilha et al., 1992); *Arxula adeninivorans* – between 75oC and 82oC (Sano et al., 1998).

It was determined that the phytase produced by *Candida melibiosica* 2491 is active in a very wide pH range from 4.0 to 8.5 with pH optimum at 4.5 (Figure 6b). The activity at pH 4.0 is 88% and at pH 8.5 – 80% of the maximum. The most often reported yeast phytases also exhibit pH optima in the 4.0 – 5.0 pH interval: *P. anomala* – pH 4.0 (Vohra et al., 2001); *A. adeninivorans* - pH 4.0 (Sano et al., 1998). An exception is the phytase from *P. pastoris*, which has a broad pH optimum at pH values from 2.0 – 5.5 (Yin et al., 2005).

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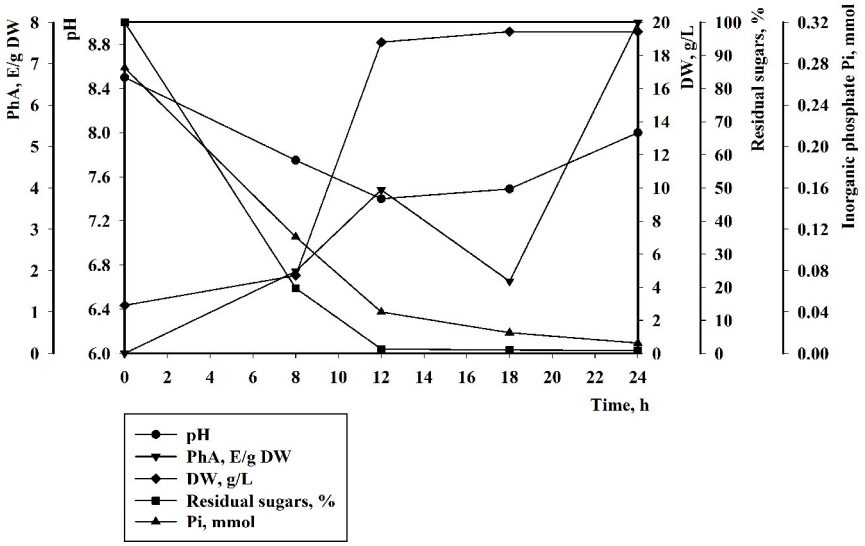

Figure 5. Dynamics of phytase biosynthesis by *Candida melibiosica* 2491, cultivated in 2L bioreactor: YPfru medium initial; pH 8.5; 6.6% inoculum; stirring rate 300 min^{-1}; aeration degree 500 $dm^3/dm^3.h$; temperature 28°C; duration 24 h.

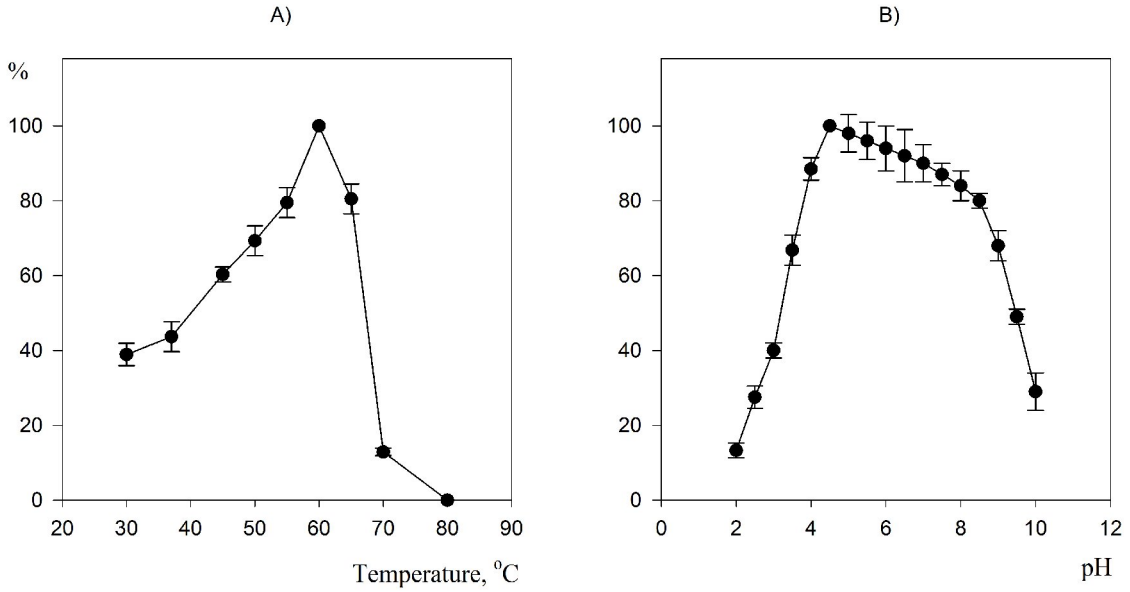

Figure 6. Influence of: a) the temperature and b) pH on the phytase activity.

The high phytase activity at different pH values - from acidic to weak alkaline range - reveals potential for applications of the investigated strain as a supplement for animal and human diets (Fu et al., 2008). As an enzyme active at acidic pH, the phytase is suitable for supplementing monogastric animals' dietaries. Acidic phytases might induce a more effective hydrolysis of phytate in both the stomach and small intestine of animals in terms of the pH of the animal gastrointestinal tract (Park et al., 1999). They show broad substrate specificity and hydrolyze metal-free phytate at the acidic pH range, producing myo–inositol monophosphate as the final product. In contrast, the alkaline phytases exhibit strict substrate specificity for the calcium–phytate complex, existing in the animals` digestive tract, and produce myo-inositol trisphosphate as the final product (Oh et al., 2004).

Further studies regarding the biotechnological development of *Candida melibiosica* 2491 for animal nutrition and human health are currently being investigated.

Conclusions

It is proved that *Candida melibiosica* 2491 has an optimum activity for phytase production at pH 8.5. Based on the literature survey, this is the only strain with an alkaline pH optimum. Under flask cultivation, higher enzyme activity was achieved, which is due to the better control over the cultivation parameters, especially pH and aeration control. In addition, better heat and mass transfers were also observed. In the laboratory bioreactor, a higher amount of biomass was reached, and because of this the total amount of enzyme is higher. Although *Candida melibiosica* 2491 is an alcaliphilic strain, it exhibits great physiological potential regarding the initial pH values. The high protein content of the strain combined with its phytase activity will improve the quality of feed by its introduction in industrial production.

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PLANT SCIENCE

Management of rice blast disease (*Pyricularia oryzae*) using formulated bacterial consortium

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Abstract

Rice blast caused by *Pyricularia oryzae* is a major disease affecting rice production grown in upland and wet-land rice. Application of beneficial bacteria as seedling root dip and spraying method to protect against the disease may be an alternative strategy to chemical control. This research was aimed to explore the bacterial consortium that may control blast disease on rice plants. In this study, the following bacterial cultures and their consortiums were used: *Bacillus firmus* E65, *Serratia marcescens* E31, *Pseudomonas aeruginosa* C32b, *Bacillus cereus* II.14, and its combination for their suppression ability against *P. oryzae* under in-vitro conditions. The results showed that A2 (*Bacillus firmus* E65) and A6 consortium (*Bacillus firmus* E65, *Bacillus cereus* II.14, and *Pseudomonas aeruginosa* C32b) significantly reduced the mycelial growth of *P. oryzae* with the percentage inhibition of 73-85% and 66-83%, respectively. Further greenhouse testing conducted with use of formulative preparation of the two selected best treatments using talc, bentonite, palm oil, and suspension-based carriers showed that spraying with suspension formulation had good effect in suppressing blast disease compared with that of other carriers evaluated.

Key words: *Bacillus firmus*, Blast disease, Consortium of bacteria, *Pseudomonas aeruginosa*, *Pyricularia oryzae*, Rice, *Serratia marcescens*

Introduction

Blast disease caused by *Pyricularia oryzae* is a major disease affecting rice cultivation that affects about 12% of the total area of rice fields in Indonesia (BPS, 2008). Rice blast was reported to infect rice causing a lowered yield of about 30-50% in Southeast Asia and South America (Shimamoto, 2001). Disease severity has increased recently due to the use of intensive agronomic practices that favor disease development. Blast disease severity was triggered by the excess of N fertilization (Faria et al., 1982; Huber, 1990) as well as rainfall and high humidity conditions.

The current control strategy of the disease is through fungicides application, however; their adverse effects on environment and beneficial soil micro-organisms are quite evident. Most studies have shown that various types of microbial are a potential substitute for inorganic chemical compounds (fertilizer and pesticides) that can be applied in the field on a wide scale.

A number of microbial agents have been reported to be effective as biological control of plant diseases i.e. *Bacillus, Bdellovibrio, Dactylella, Gliocladium, Penicillium, Pseudomonas*, and *Trichoderma* (Fravel, 1988). Biocontrol approach for managing blast disease is considered to be a practical and economical alternative. Production of secondary metabolites such as antibiotics, Fe-chelating siderophores, and cyanide are most often associated with fungal suppression by fluorescent Pseudomonads in the rhizosphere of several crops (Howell and Stipanovic, 1980). Most antagonistic studies of *Bacillus* were conducted on *B. subtilis* and *B. cereus,* and occasionally on *B. firmus*.

The success of biocontrol may depend on suitable formulations as well as survival of the microbial agents. Bacteria as biological control agents have advantages over fungal biological control agent i.e. the bacterial cells mass can be

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produced more easily and faster than the fungus. In addition, they are generally effective when applied as a preventive application to suppress the disease. As in line with this study, we have previously evaluated several strains of bacteria isolated from rice plant for their antagonistic ability against *M. grisea* and *R. solani* (Suryadi et al., 2011).

This study was aimed to test the candidate microbial consortium (*B. cereus* II.14, *B. firmus* E65, *P. aeruginosa* C32b, and *Serratia marcescens* E31) using various formulations as biological agents to control blast disease caused by *P. oryzae*.

Materials and Methods

Isolation and in-vitro screening of bacterial consortium against rice blast

The indigenous Indonesian bacterial isolates *viz. B. firmus* (E 65), *P. aeruginosa* (C 32b), *S. marcescens* (E 31), and *B. cereus* (II.14) were obtained from the microbial gene banks culture collection of Microbiology Laboratory (BiogenCC), and Department of Biology, Faculty of Mathematics and Natural Sciences (IPB). Stock cultures of bacteria were prepared using Nutrient Broth (NB) medium. Colonies were selected, purified and used for further studies. The list of bacterial isolates and their origin is presented in Table 1.

Table 1. List of isolates used in this study.

Bacterial codes	Host	Source
Bacillus cereus II.14	chili	IPBCC
Bacillus firmus E65	rice	BiogenCC
Pseudomonas aeruginosa C32b	soil	BiogenCC
Serratia marcescens E31	rice	BiogenCC

Table 2. Treatment code and bacterial isolates used in the study.

Treatment code	Isolates/mix cultures
A1	*B. cereus* II.14
A2	*B. firmus* E65
A3	*P. aeruginosa* C32b
A4	*S. marcescens* E31
A5	*B. firmus* E65 + *P. aeruginosa* C32b
A6	*B. firmus* E65 + *P. aeruginosa* C32b + *B. cereus* II.14
A7	*B. cereus* II.14 + *P. aeruginosa* C 32b + *S. marcescens* E31
A8	*B. firmus* E 65 + *P. aeruginosa* C32b + *B. cereus* II.14 + *S. marcescens* E31
A9	Copper sulphate 56% (chemical control)
A10	Untreated Control (water)

Dual culture test formulative bacteria against *Pyricularia oryzae*

Bacterial isolates were grown on NB medium in the Erlenmeyer flask until the population reaches 10^6-10^7 CFU/ ml; incubated at room temperature for 24-48 hours, then stored in a refrigerator at 4°C. Mixing the bacterial suspension was then performed as indicated in Table 2.

P. oryzae fungus inoculums was isolated from blast-infected rice cv. Inpari 13 obtained in Kuningan district, West-Java. Rice seeds cv. Inpari 13 used in this study were obtained from Muara experiment station, Bogor, West Java. Pure cultures of *P. oryzae* were grown on petri dishes containing potato dextrose agar (PDA) medium and incubated for one week at room temperature. Production of *P. oryzae* spores was carried out by growing the fungus on oat meal agar (OMA) medium, incubated for 8-10 days at room temperature. Fungal colonies were added with 2 µg/50 ml streptomycin to eliminate aerial hyphae; Isolates were then irradiated with fluorescent lamps 300 volts to induce the growth of spores for 5-6 days; then washed with 1 liter of sterile distilled water containing 0.2% (v/v) Tween 20. Spores were harvested by rubbing using a brush to the surface of the fungus colony that has been sterilized by immersion in absolute alcohol. Spore suspension was then filtered and collected in sterile Erlenmeyer flasks, and the spore density was observed using Neubauer-haemocytometer.

The isolates and mix cultures were screened for their suppression ability against rice blast pathogen, *P. oryzae* by dual culture technique following the method of Rabindran and Vidyasekaran (1996). Bacterial isolate used as bacterial whole cell cultures (BWC) was streaked at one side of petri dish (3 cm away from the edge) containing PDA. Five mm mycelial plug from seven-day-old PDA cultures of *P. oryzae* were placed at the opposite side of petri dishes (diameter 9 cm) perpendicular to the bacterial streaks. Petri dishes inoculated with fungal discs alone served as control. The treatments were arranged in completely randomized design with three replications. The plates were incubated at 28ºC until fungal mycelia completely covered the agar surface in control plate. Observations were done by measuring inhibition zone and mycelial growth of the pathogen, and percent inhibition of pathogen growth was calculated using formula I %=C-T/C x 100; where I = inhibition of mycelial growth, C= growth of pathogen in the control plate (cm) and T= growth of pathogen in dual cultures (cm).

Dual culture test using culture filtrates of the selected best formulation

Two effective consortiums resulting from the above dual culture studies were selected and tested for their suppressing ability using bacterial culture filtrate (BCF). An agar plug (9 mm diameter) taken from actively growing fungal culture was placed on the surface of the plate-enhancing medium. Bacteria initially were grown on NB medium and incubated for 24 hours. One ml of culture suspension was centrifuged at 8944*g* for 5 minutes. A total of 100 mL supernatant of BCF was transferred into a petri dish and then poured to the homogenized PDA medium. Plates inoculated with fungal agar plugs alone were used as control. A piece of *P. oryzae* isolate was placed by using a cork bore diameter of 0.5 cm into the PDA medium in petri dishes. Each treatment was performed using 8 replications. The entire petri dish containing treatment was incubated for one week at room temperature, and then the radial growth of *P. oryzae* was measured and percent inhibition of pathogen growth was calculated (Kumar et al., 2000).

Efficacy of biocontrol agents against *P. oryzae* (green house test)

The carrier materials used in this study were talc, bentonite, oil, and suspension with the composition and mixing process of the materials as follows: (a) talc: 300 ml suspension of bacterial isolates, 1 kg of talc, 10 g of carboxymethyl cellulose (CMC), 15 g of $CaCO_3$, (b) bentonite: 300 ml of suspension bacterial isolates, 1 kg of bentonite, 10 g of CMC, 15 g of $CaCO_3$, (c) oil: 300 ml suspension of bacterial isolates, 6 ml of palm oil, 0.15 ml of Triton X-100, and (d) suspension: 300 ml of bacterial suspension (10^9 CFU/ml).

Rice seeds cv Inpari 13 was sown for 18 days in a plastic bag (15 x 30 cm^3) containing field soil. Prior to transplanting the seedlings were immersed in the following treatment regimens: immersion in talc and bentonite for one night; whilst immersion in oil and suspension was given for 3 hours; respectively.

Fungal inoculation was done by spraying using *P. oryzae* spores with a density of 5.5 x 10^7 spores/ml. Inoculated plants were placed under humid conditions with 90% relative humidity. Rice plants treated without *P. oryzae* infection and formulations served as healthy control; whilst plants inoculated with *P. oryzae* alone without formulation served as untreated control. 100 ml of bacterial formulations was applied using three times spraying at 3 days, 7 days, and 9 days after inoculation (d.a.i). Data was taken from eight plants (4-5 leaves per plant) for each treatment. Observations was done at 14 d.a.i based on blast disease assessment given by the standard evaluation system of IRRI (1996), using 9 scale basis i.e.; 0= no lesions; 1= small, brown, specks of pinhead size; 3= small, roundish to slightly elongated, necrotic, gray spots about 1-2mm in diameter; 5= typical blast lesions infecting < 10% of the leaf area; 7= typical blast lesions infecting 26-50% of the leaf area; 9= typical blast lesions infecting >51% leaf area and many dead leaves. Then the severity of blast is calculated using the formula:

$$DS = \frac{\sum n \times v}{N \times V} \times 100\%$$

DS = disease severity
n = number of leaves infected by blast
v = value score of each category attack
N = number of leaves observed
V = value the highest score

Results

Isolates of bacteria can grow well after 48 hours and fresh pure culture was obtained on NA media, while the *P. oryzae* fungus require around 7 days to grow up on PDA media. Hyphae in the early growth of *P. oryzae* were white in color and became dark after one week. Observation of spores using light microscopy showed the spores morphology was an avocado like shape with hyaline brownish color (Figure 1).

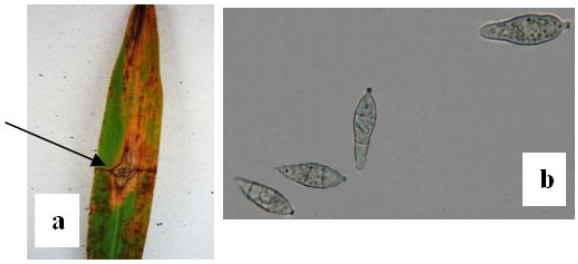

Figure. 1. Typical blast symptom on rice leaves 14 days after inoculation (a) an arrow indicate leaf spot; *P. oryzae* spores at magnification 400x (b).

Dual culture test of formulative bacteria against *Pyricularia oryzae*

Dual culture studies of these bacteria against the pathogen revealed that the inhibition of blast *P. oryzae* ranged from 10-53%. Among the isolates, E65 was found to be highly effective in controlling the pathogen with inhibition of 53.32%. The other effective strains were E31, and C32b that showed

inhibition of test pathogen in the range of 26.2 to 33.65% (Table 3).

Results from dual culture tests indicated the presence of inhibitory zones. This is evident of the formation of inhibitory activity against *P. oryzae* fungi by bacteria. The result revealed that isolates E65 (A2), C32b (A3) and mix cultures of E65, C32b and E31 (A6) showed high degree of inhibition with the mean average of the inhibition area of 53.32 cm^2, 33.65 cm^2 and 30.22 cm^2, respectively. The untreated control showed no inhibitory activity. In contrast to the standard chemical control (Copper sulphate 56%), *P oryzae* did not grow at all, which indicates the maximum inhibition.

Dual culture test using culture filtrates of the selected best formulation

Prior to the formulation of biocontrol agents, the inhibition test of further selected bacterial isolates using E65 (A2) and E65, C32b and E31 (A6) on NA medium containing supernatant of both treatments showed the average of *P. oryzae* radial growth of 2.80 cm^2 and 5.76 cm^2 with the inhibition of 95.59% and 91.00%, respectively (Figure 2); while on the control treatment, *P. oryzae* can grow up to 63.59 cm^2 across the surface of petri dish. This confirms inhibitory activity of bacterial isolates against *P. oryzae*.

Efficacy of biocontrol agents against *P. oryzae* (green house test)

Observation of the effect of carrier formulations application agents was performed at 14 days after inoculation. Rice blast fungus (*P. oryzae)* showed spots with brown edges; white or grayish center patches. The shape and color of spots varied depending on environmental conditions, and the degree of rice resistance. Following the assessment of blast disease based on SES, IRRI (1996); A2 and A6 treatments in the form of a suspension carrier provides slower blast disease progression than other formulas with the percentage of blast severity was 28.68% and 35.67%, respectively; whilst on untreated control, the blast severity was 57.85% (Table 4).

Table 3. Effect of bacterial consortium used as BWC on the pathogen growth inhibition.

Treatment Codes	Radial growth of *P. oryzae* (cm^2) ± SD	Mean inhibition over control (%) ± SD
A1	63.59 ± 3.1a	0 ± 0 e
A2	10.27 ± 6.3 d	53.32 ± 6.2 b
A3	29.94 ± 12.8 c	33.65 ± 12.9 c
A4	37.27 ± 4.05 bc	26.32 ± 4.06 cd
A5	44.32 ± 6.64 b	19.27 ± 6.6 d
A6	33.36 ± 10.46bc	30.22 ± 10.4 c
A7	44.26 ± 5.29 b	19.32 ± 5.3 d
A8	42.55 ± 6.11 b	21.04 ± 6.1 d
Copper sulphate 56% (chemical control)	0 ± 0 e	63.59 ± 0 a
water (untreated control)	63.59 ± 3.1 a	0 ± 0 e
CV(%)	20.36	

Note: numbers in each column followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at P=0.05.

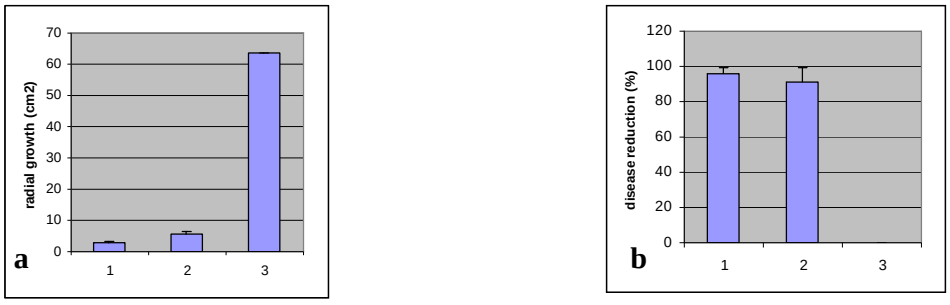

Figure 2. (a) Mean radial growth (±SD) and (b) percentage inhibition (±SD) of *P. oryzae* using bacterial culture filtrate (BCF) after 7 days incubation. 1=A2, 2= A6, 3= water (untreated control).

Table 4. Effect of different formulation to the severity of blast disease in Green House test.

Treatment codes	Mean average of blast severity (%) ± SD	inhibition over control (%) ± SD
Healthy plants control	0 ± 0 f	100 ± 0
Untreated control	57.85 ± 8,54 a	0 ± 0
A2 talc	45.83 ± 9,79 abcd	20.78 ± 16.27
A2 bentonite	39.72 ± 8,92 cde	31.34 ± 20.39
A2 palm oil	53.89 ± 9,33 ab	6.85 ± 3.11
A2 suspension	28.68 ± 12,23 e	50.42 ± 24.61
A6 talc	44.17 ± 7,27 abcd	23.65 ± 12.58
A6 bentonite	40.49 ± 11,13 bcde	30.01 ± 17.60
A6 palm oil	50.76 ± 13,91 abc	12.26 ± 9.9
A6 suspension	35.76 ± 7,77 de	38.18 ± 18.99
CV (%)	24.09	

Note: numbers in column followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at P=0.05.

Table 5. Cell viability of consortium bacteria during period of storage.

Bacterial consortium codes	Formulated carrier	Month- 1 Cells number (CFU/ml)	Month -3 Cells number (CFU/ml)
A2	Talc	8.1×10^8	4.5×10^8
	Bentonite	6.2×10^8	9.2×10^8
	Palm oil	3.3×10^8	6.6×10^8
	Suspension	4.4×10^8	9.6×10^8
A6	Talc	1.1×10^9	4.4×10^7
	Bentonite	1.56×10^9	1.6×10^8
	Palm oil	7.15×10^8	1.6×10^8
	Suspension	1.75×10^9	1.9×10^8

The effective formulation of A2 and A6 using talc and bentonite were also reduced blast severity over 20% inhibitions; whereas the inhibition of oil formula ranged from 6.85 to 12.26%. At the initial formulations process, the bacterial isolates had an average of 4.0×10^9 CFU /ml; whereas single cultures of *B. cereus* II. 14, *B. firmus* E65, *P. aeruginosa* C32b and *S. marcescens* E31 had viability of 2.5×10^9, 2.6×10^9, 5.1×10^9, and 6.0×10^9, respectively. After the bacterial isolates were mixed in the form of formulations, at month-1 and month-3 the quantity of E65 isolate (A2) decreased in average of 5.5×10^8 CFU/ml. The average of bacterial cells viability of A6 consortium was approximately 3.01×10^8 CFU/ml. The overall cells viability was slightly decreased during period of storage (Table 5).

Discussion

Rice cultivar Inpari 13 used in this study was derived from backcross between OM606 and IR18348-36-3-3 that was introduced in late 2009 by the ICRR Sukamandi, although little was known among farmers about its popularity. This variety has the advantage of high potential productivity of 8.0 tones/ha with an average of 6.59 tones/ha and has 103 days maturity (Darajat and Rojakurniati, 2011). This variety showed resistance to brown plant hopper and bacterial leaf blight, however Inpari 13 are still susceptible to blast disease particularly in Kuningan district, West Java.

P. oryzae the causal agent of blast disease is a type of fungi that is morphologically similar with *P. grisea* known to attack many types of grasses and weeds (Rush, 1992). *P. oryzae* has long conidiophores and rarely branched. At the terminal end of the conidiophores, the conidia had oval-shaped with pointed tip, with the average size of 20-22 x 10-12 µm. Under light microscope observation, conidia of *P. oryzae* consist of 2-3 cells, with hyaline brownish color. The result of greenhouse experiment under moist condition demonstrated that blast disease symptoms begin to appear at 4 days after inoculation which was characterized by the emergence of small spots. The latent period of blast disease in the tropics occur at 4-5 days after inoculation (Ou, 1985).

In recent years the use of bioformulations as biocontrol product of bacterial origin is gaining great interest in crop protection, and the product may be supplemented as an alternative to chemical control (Fravel, 1988). Dual culture test using bacterial isolates showed the formulation containing *B. firmus* E65 (A2) *B. cereus* II.14 (A3)

and consortium of *B. firmus* E65, *B. cereus* II.14, and *P. aeruginosa* C32b (A6) had the highest inhibitory effect compared to other bacterial isolates. Control treatment using chemical fungicide (Nordox 56WP with 56% copper-sulfate active ingredient) showed maximum inhibition of *P. oryzae*. Further inhibition test using bacterial cells cultures filtrate showed that A2 and A6 consortium could effectively suppress *P. oryzae* with the percentage inhibition of 79.05% and 69.92%, respectively.

Several studies have reported potential microbial biocontrol agents against *P. oryzae* fungus. It was reported that the fungus *Exserohilum monoceras* had the ability to inhibit the growth of the *P. oryzae* fungus by 61.8% - 71%. The use of *Pseudomonas fluorescence* strains 4-15 and 7-14 also showed growth inhibitory activity against *P. oryzae* with a percentage of 59% and 47% (Tsukamoto et al., 1999; Gnanamanickam and Mew, 1992)

The result of bacterial isolate selection through dual culture test has been reported previously using single isolate of *B. firmus* E65, *B. cereus* II.14, and *P. aeruginosa* C32b (Suryadi et al., 2011). Those isolates have activity against *P. oryzae* fungi causing blast disease on rice cv IR 64. *Bacillus* is a genus of Gram-positive bacteria that produce endospores, and can be a potential biological agent due to its resistance to heat and drought conditions, thus suitable for applications in the field (Wayne et al., 2000); whilst *Pseudomonas* is a genus of Gram-negative bacteria that can live in a simple nutrient conditions, and usually colonize the roots of rice.

Similar to the *in vitro* test, the outcome of the greenhouse trial was highly encouraging because the bacterial formulations significantly reduced blast disease. Mechanism of action of biological control agents are generally classified as activities of substances competition, parasitism, and antibiosis (Fravel, 1988; Weller, 1988). In the previous study, it was reported that several bacterial isolates could suppress the growth of pathogenic fungi P. oryzae (Suryadi et al., 2011). The mechanisms of bacterial isolates particularly isolates E65, E31, C32b and II.14 produced cell wall degrading enzymes such as chitinase and glucanase that can degrade cell walls of fungi with varying effect. For example the glucanolytic index produced by those bacteria was 2.21; 0.99; 0.50 and 0.63, respectively. In this study, the mechanism of inhibition of bacterial isolates against *P. oryzae* presumably caused by the activity of substance produced by bacteria. *Bacillus* produces a variety of antibiotics that are effective against bacteria and fungi such as zwittermicin-A (He et al., 1994), kanamisin and lipopeptida of iturin, surfaktin, and fengycin (Stabb et al., 1994). Huang et al. (1993) reported that antibiotic iturin and surfaktin can inhibit the growth of pathogenic fungi. Fengycin produces by *Bacillus subtilis* had inhibitory activity against fungi *P. oryzae* (Joshi and Gardener, 2006). In addition, several species of *Bacillus*, such as *B. cereus* II.14 showed chitinolytic activity (Mubarik et al., 2010). Meanwhile, Hassanein et al. (2009) reported *Pseudomonas* sp. had the ability to produce secondary metabolites such as antibiotics, ammonia, and cyanide. Examples of antibiotics produced by *Pseudomonas* sp. namely pyrolnitrin effective in suppressing the growth of *Rhizoctonia solani* and pyolutcorin that can suppress the growth of pathogenic fungi *Pythium ultimum* (Howell and Stipanovic, 1980). Siderophore chelating irons (Fe) have the ability to bind iron elements of the environment. Pathogenic fungi do not have the ability to produce the siderophore as *Pseudomonas* sp; hence pathogenic fungi- iron element deficit can lead to stunted growth of pathogens (Neilands and Leong, 1986). The ability of bacteria to produce antibiotic compounds was considered as most appropriate biological control agents compared with other means such as competition and parasitism.

Single bacterial and consortium treatment in this study showed variability of activity. For example, the dual culture test showed that A1 treatment (*B. cereus* II.14) as single bacterium had no inhibitory activity against *P. oryzae*. However, the effect of *B. cereus* was seen on A5 and A6 consortium (mix cultures). Consortium A6 containing *B. firmus* E65, *P. aeruginosa* C32b, and *B. cereus* II.14 had inhibitory activity against *P. oryzae* much better than the A5 consortium containing *B. firmus* E65, and *P. aeruginosa* C32b alone. These results may indicate *B. cereus* II.14 plays synergistic role in the inhibitory activity of *P. oryzae*.

This phenomenon that occurs in bacterial consortium was allegedly a process of quorum sensing (QS). This process is a form of inter-bacterial communication mechanism with the use of chemical signaling molecules called auto inducer (AI). AI is a secreted signaling molecule, accumulated, reabsorbed, and recognized by the bacteria during the process of QS (Rukayadi and Hwang, 2009). QS does not occur by an individual bacterium, but it would be done simultaneously by a large number of bacterial cells and this communication can occur in bacteria or interspecies-intraspecies (Waters and Bassler,

2005). The phenomenon of QS is involved in the regulation of important biological functions such as, production of antibiotics, plasmid transfer, motility, virulence, and expression of genes in the *Pseudomonas aeruginosa* and *Bacillus* sp pathogen (Dong et al., 2002; Zhang and Dong, 2004).

The effect of formulation application of bacteria to the severity of blast disease of rice was carried out during 14 days after inoculation. Treatment combinations of bacterial immersion and spraying formulation of the selected bacteria showed the suppressing activity against blast disease. Suspension-formulations of A2 and A6 showed potential suppressing effect that causing low blast severity than other formulations. Suspension based formulation is a formula containing only NB medium and bacterial isolates. NB is a common medium for bacterial growth that contains complete nutrition elements needed by bacteria. In this study, the most effective suspension might affect the growth and activity of bacteria. Bentonite and talc based formulations also showed the potential suppression of blast disease, although not as good as suspension formulations. CMC serves as an adhesive agent so that the formulation can be attached to the surface of plant organs, whereas $CaCO_3$ as a nutritional source of calcium for the growth of bacteria and pH medium becomes neutral (Ardakani et al., 2010). Bentonite and talc based carriers have similar physical appearance in terms of fine powder form, lightweight, and good ability to absorb liquids. A difference between the physical colors of bentonite is rather dark gray, whilst talc has pure white color. During the mixing process of the materials used, visible liquid absorption of bentonite was better than talc. Bentonite absorbs liquid containing bacteria more quickly and evenly; while the talc media had a little longer to absorb the NB, hence the structure eventually form large clumps. At this stage of formulation; bentonite applications seems to be more efficient; easily dissolved in sterile water compared with that of talc.

Research on the carrier material bioformulation using talc and bentonite had also been done by Ardakani et al. (2010) that showed biocontrol with carrier formulation of bentonite, CMC, and the bacterium *P. fluorescence* effectively suppresses the growth of pathogenic fungi *Rhizoctonia solani* on cotton plants. Formulation with oil mixture showed less potential for suppression of blast, causing less percentage of blast inhibition. Use of colloids Triton X-100 in this study may serve as oil emulsifier that can be mixed perfectly with NB medium containing bacteria. The use of oil based formulation should pay more attention due to disturbing effect on the physiological growth of plants. The rice plants-oil applied formulation has shorter than other formulations application.

Observation of bacterial cell viability was performed three times namely at the beginning of mixing process of formulations, one month and three months after the formulation stored at room temperature (solid formulation) and refrigerator (± 4ºC) for liquid formulations, respectively. At the initial formulations process, the bacterial isolates had an average of 4.0×10^9 CFU /ml). After the bacterial isolates were mixed in the formulations at month-1, the quantity of E65 bacterial isolate decreased in average of 5.5×10^8 CFU/ml. The decrease of viable bacteria at month-1 and month-3 may occur due storage adaptation that allows bacteria to survive or died. A phase of adaptation can occur because bacterial culture was transferred from nutrient-rich media (NB) to the limited nutrient content media.

Development of biocontrol formulation can be successful if microbial agents could survive during storage, as well as competitive and aggressiveness after inoculation process (Beatty and Jensen, 2002; Selim et al., 2005). In this study, although the potential suspension-based formulation showed good effect in blast suppression, but for large scale this formulation is less efficient in terms of packaging and use. Talc and bentonite based formulations should be further developed because of their efficiency and further research needs to be done to increase the suppression of blast disease.

It has been reported that several diseases are caused by exposure to harmful chemicals during spraying (Rola and Pingali, 1993). The most direct benefit from the use of bacterial consortium is the reduction in disease pressure (blast disease) may imply some savings in the use of traditional pesticides and labor for spraying pesticides. The reduced number of sprays was 5 for traditional pesticides compare with only 3 times for bacterial formulations would have a positive effect on health. Because of reduced severity of blast disease farmers may spent only 5$ US/kg/ha for bacterial formulations compared with farmers expenditure of using pesticides (15$ US). There is also substantial savings in the amount of labor used for spraying bacterial formulations.

The results obtained here pointed out the possibility use of bacterial consortium such as A6 (*B. firmus* E65, *B. cereus* II.14, and *P. aeruginosa* C32b) in rice fields for blast disease suppression.

However, further research is needed to elucidate in detail the mechanism of action of these strains and their compatibility with other components in integrated management of rice diseases.

Conclusion

Based on *in vitro* test, A2 (*Bacillus firmus* E65) and A6 consortium (*Bacillus firmus* E65*, Bacillus cereus* II.14, and *Pseudomonas aeruginosa* C32b) could suppressed the radial growth of *P. oryzae* fungus with the inhibition of 19.27% and 53.32%, respectively. It was shown that A2 (*Bacillus firmus* E65) suspension revealed good inhibitory producing the smallest of blast severity (28.68%) under greenhouse condition.

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Development and evaluation of Modified Atmosphere (MA) packages employing lamination technique for Royal Delicious apple

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Abstract

Modified atmosphere (MA) packages were prepared for Royal Delicious apple with an aim to achieve the optimum storage atmosphere within the packages in minimum possible time. Apples were harvested at their commercial maturity and respirtion rate of the fruits was measured at temperatures between 0 – 25°C. On the basis of preliminary investigations, air composition with 3% O_2 and 3% CO_2 was found appropriate for MA packaging of apple. The MA package was finalized with a size of 24 cm x 19 cm for 1 kg fill-weight of apples. As the gas transmission rates of individual polymeric films could not match the gas transmission requirements of MAP, judicious combinations of the films were made to bring gas transmission characteristics close to the required values. The equilibrium concentrations of O_2 and CO_2 were established within 36-72 h under different storage temperatures. The mathematical model, developed applying enzymatic kinetics based respiration equation coupled with the Arrhenius model, effectively predicted the equilibrium time and the levels of O_2 and CO_2 at equilibrium. MA packages were evaluated through assessment of different quality parameters of apple during storage and were found to increase its shelf life by 125-210% that of unpacked fruits at different storage temperatures.

Key words: Apple, Respiration rate, Gas transmission modelling, MA packaging, Quality

Introduction

The Delicious group of cultivars predominates the apple market and the Royal Delicious apples account for 50-60% of the total apple production in India. Post-harvest constraints like limited shelf life, susceptibility to many diseases and pest, faster fruit ripening at warmer temperatures etc. limit apples storage period (Kaul and Gupta, 1987; Kaushal and Sharma, 1995). It is important to prolong the storage period and improve its keeping quality during transport and marketing. Application of improved packaging systems is one such possibility.

Modified Atmosphere Packaging (MAP) is simple, economical, and effective way of extending shelf life of fresh commodities (Sandhya, 2010; Nicolais et al., 2011b). The modification of the storage atmosphere within the package is achieved by the interplay of two processes, the respiration rate of the packaged products and the gas permeation rates through the packaging materials (Smith et al., 1987; Fonseca et al., 2002; Mahajan et al., 2007; Meetoo, 2011). It is generally desirable to achieve an atmosphere low in O_2 and high in CO_2 to regulate the metabolism of the packaged produce, and the activity of decay-causing organisms to increase the storage life (Mangaraj et al., 2009a).

Rocha et al. (2004) stored apple for 6.5 months in MAP with good marketable quality using 100 µm polypropylene at 4°C and 85% R.H. The incidence of bitter pit that developed during controlled and modified atmospheres storage of 'Cox's Orange pippin apple for 5 weeks at 2°C was reduced from 50% to less than 5% (Hewett, 1984). Guan et al. (2004) packed 'Fuji apples' in optimal MAP that had good quality after 7 months storage at 0°C and 10°C with reduction of scald rate. Rocculi et al. (2006) reported that active modified atmosphere decreased the rate of oxygen consumption compared with passive MA, in particular at the beginning of storage.

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The objective of MAP design is to achieve the equilibrium concentrations of O_2 and CO_2 within the package as quickly as possible on account of the interactions of the produce with the package and the external atmosphere. These concentrations need to lie within the desired levels required for extending the storage life of the commodity (Oliveira et al., 2010; Costa et al., 2011). The desired levels of gas concentrations could be achieved by matching the film permeation rates for O_2 and CO_2 with the respiration rate of the packaged produce. As different products vary in their respiration rates and as MA-packages are exposed to a dynamic environment, each package needs to be optimized for specific productss (Tano et al., 2007; Montanez et al., 2010). A MAP system, which is not properly designed, may be ineffective or even may shorten the storage life of a commodity. If the desired atmosphere is not established rapidly the package would have no benefit, and the product might experience serious damages and its storage life might be shortened (Mahajan et al., 2007; Mangaraj and Goswami, 2009b). Therefore, it is important to carry out a systematic design of modified atmosphere packaging for different commodities by considering all factors affecting respiration and permeation. The present study was under taken to design MAP system for Royal Delicious apple and to evaluate its quality attributes under various storage temperatures.

Materials and Methods

Measurement of respiration rate of apple

Royal Delicious apples were harvested from an orchard at Shimla, Himachal Pradesh at their commercial maturity i.e. 125-130 days after full bloom. The respiration rate data for the fruits were experimentally determined for different temperatures using the closed system method (Mangaraj and Goswami, 2008). A mathematical model applying Michaelis-Menten type equation coupled with the Arrhenius model was developed for predicting the respiration rate of apple (Lee et al., 1991, 1996; Mangaraj and Goswami, 2008, 2009c; 2011a,b).

Measurement of GTR of selected films

With the objective of meeting MAP requirements for apple, BOPP and PVC films were selected considering gas permeability, water vapor transmission rate, sealing reliability, clarity, strength, and durability (Kader et al., 1989; Del Nobile et al., 2009). The gas transmission rates (GTR) of the films were determined employing equal pressure method (Mangaraj et al., 2009). Arrhenius-equation was fitted to the experimental data to depict the relationship of GTR with temperature (Exama et al., 1993).

Target level of MA package air composition for apple

Preliminary investigation with various designs of optimum combinations of O_2 and CO_2 for MA packaging of apple was carried out. Also, the MA packages were evaluated for sub-optimal air compositions with a view to provide a factor of safety against the development of deletarious levels of O_2 and CO_2 in the packages at any stage, throughout the distribution chain. On the basis of preliminary investigations and the sub-optimal package air composition, the MA packages for apple were designed with target air composition of 3% O_2 and 3% CO_2.

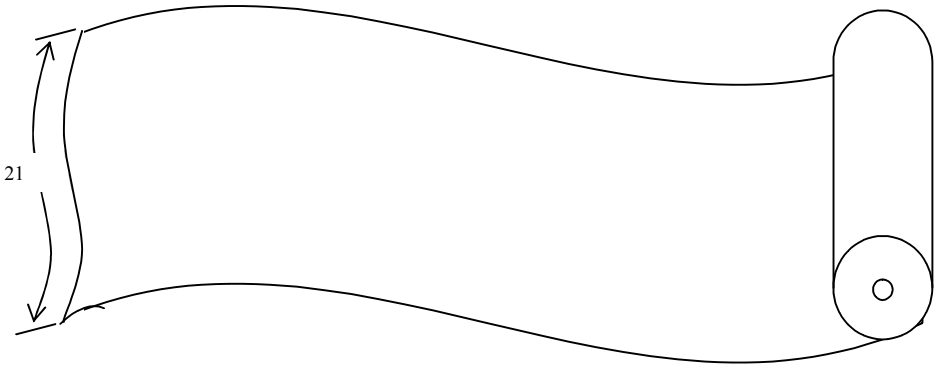
Modeling of gaseous exchange in MAP system

Once the fruit is sealed in the package, the O_2 and CO_2 concentration gradients develop due to respiration and the polymeric film serves as the regulator of O_2 inflow and CO_2 outflow. Considering that there is no gas stratification within the packages and that the total pressure is constant, the basic mass balance relationships describing the O_2 and CO_2 concentration changes in a package are:

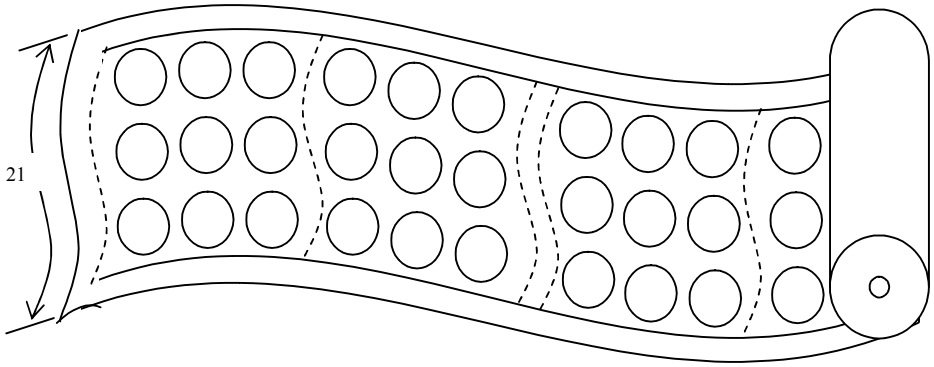
Rate of O_2 inflow – Rate of O_2 consumed by product = Rate of O_2 accumulation in package space, and;
Rate of CO_2 generated by the fruits – Rate of CO_2 outflow = Rate of accumulation CO_2 in package space

Preparation of film laminates for MA Packaging of apple

The mass balance relationships and the results of preliminary investigation were used for the optimization of package parameters. The MA packaging system was restricted to medium size apples with each package of 24 cm x 19 cm weighing 1 kg. The GTR requirement of the MA package was calculated using respiration data (Mangaraj et al., 2012a,b). The GTR of the selected films were compared with the gas transmission requirement (GTR_{req}) of MAP for apple. Neither BOPP nor PVC film alone could meet the GTR_{req} of package satisfactorily. Thus, the two films were laminated (Figure 1) to bring the GTR of the laminates close to the required values (Ahvenainen, 2003; Mangaraj et al., 2011). The areas of the two individual films were optimized and two types of film laminates i.e. LFR-1 (BOPP-30µ + PVC-50µ) and LFR-2 (BOPP-45µ + PVC-35µ) were developed employing lamination technique (Prasad, 1995; Mangaraj et al., 2011).

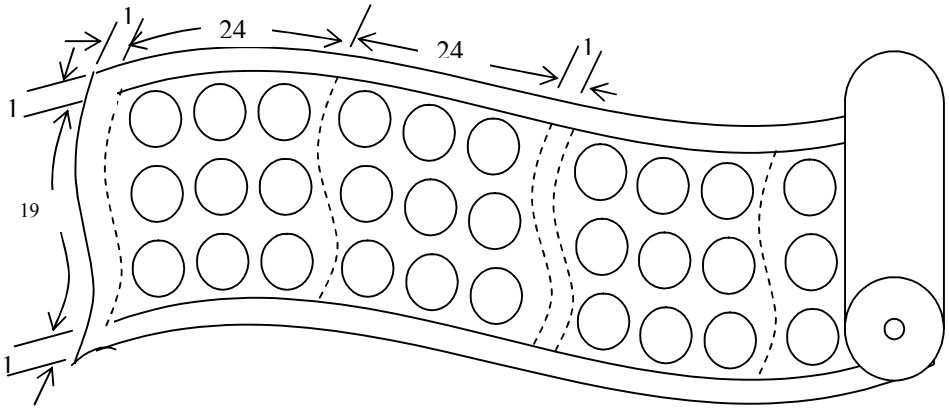


(a) Un-perforated film roll



(b) Perforated film Roll

A*ll dimensi*ons are in cm



(c) Laminated film rolls

Figure 1. Laminated film rolls for MA packaging of fruits.

Optimization of the area of films for OTRreq of MAP

The areas of the laminated films and un-perforated film were optimized to match the oxygen transmission rate requirement of MAP by employing the following equations (Mangaraj et al., 2011; Mangaraj et al., 2012a).

$$OTR^{req} = OTR^{la}\ a_1 + OTR^{upf}\ a_2 \quad (1)$$

$$a_1 = \frac{A_1}{A}\ ;\ a_2 = \frac{A_2}{A} \quad (2)$$

$$a_1 + a_2 = 1 \quad (3)$$

Where, OTR^{req} is the oxygen transmission requirements of film laminate for MAP; OTR^{la} is the oxygen transmission rate of the combined film; OTR^{upf} is the oxygen transmission rates of un-perforated single film; a_1 is the fractional area of laminated portion; a_2 is the effective fractional area of un-perforated film; A_1 is the area of laminated portion of the film laminate; and A_2 is the effective area of un-perforated film of the laminate.

Calculation of OTRla

The oxygen transmission rate of the combined film was measured experimentally. However, the values of OTR^{la} were also calculated by employing the following equation.

$$\frac{1}{OTR^{la}} = \frac{x_1}{\left(x\ OTR^{1}\right)} + \frac{x_2}{\left(x\ OTR^{2}\right)} \quad (4)$$

Where, OTR^1 and OTR^2 are the oxygen transmission rates of individual films having thickness of x_1 and x_2, respectively and x is the thickness of the film laminate.

Determination of a1 and a2

The values of a_1 and a_2 were determined by incorporating the values of OTR^{req}, OTR^{la} and OTR^{upf} in equations (1) and solving this with equation (3).

Calculation of A1 and A2

The area of laminated portion (A_1) and effective area of un-perforated film (A_2) was calculated using equations (2) as shown in equations (5) and (6).

$$A_1 = a_1\ A \quad (5)$$

$$A_2 = a_2\ A \quad (6)$$

Calculation of size and nos. of circular disc for removal from film

For the development of film laminates, an area equal to A_2 was removed in the form of circular disc from the perforated film. The sizes and numbers of discs required to be removed from film was calculated by employing the following equation (7).

$$A_2 = \pi/4\left(d_1^2\ N_1 + d_2^2\ N_2 + \ldots\ldots\ldots + d_n^2\ N_n\right) \quad (7)$$

Where, d_1, d_2, $d_3 \ldots d_n$ are the diameter of the circular disc to be removed, and N_1, N_2, $N_3 \ldots N_n$ are the numbers of discs to be removed from the film.

Calculation of OTR of film laminate (OTRfl)

The oxygen transmission rate of the film laminates was calculated by putting the values of a_1, a_2, OTR^{la} and OTR^{upf} in equation (1) and given in equation (8).

$$OTR^{fl} = OTR^{la}\ a_1 + OTR^{upf}\ a_2 \quad (8)$$

If, $OTR^{fl} = OTR^{req}$ then, the MA package design can be considered to be successful.

Calculation of CTR of film laminate (CTRfl)

Similarly, the CTR value of the film laminate (CTR^{fl}) was calculated by putting the values of a_1, a_2, CTR^{la} and CTR^{upf} as given in equation (9).

$$CTR^{fl} = CTR^{la}\ a_1 + CTR^{upf}\ a_2 \quad (9)$$

Where, CTR^{la} is the carbon dioxide transmission rates of the combined films and CTR^{upf} is the CTR of un-perforated film.

MA packages for apple

Using different coded film laminates i.e. LFR-1 and LFR-2, two types of MA packages (PCG-LFR1, PCG-LFR2) of size 24 cm x 19 cm were prepared. Six apples were inserted in each package and the packages were heat-sealed. Silicon rubber septums were glued to the packages to facilitate gas sampling. The MA packages were labeled and kept for subsequent storage study at 10, 15, 20 and 25^{o}C temperature. The representative samples were taken from the fruit lot and the physico-chemical attributes were determined employing standard techniques and procedures.

Performance evaluation of MA packages

The performance of various packages was evaluated for their ability to establish equilibrium at target levels, and ability to extend the shelf life of the packaged fruit while maintaining quality. The experimental variation of in-pack O_2 and CO_2 concentrations (Y_{O2}^{in} and Z_{CO2}^{in}) from the time of sealing the fruits in the package to the time of establishing equilibrium were recorded at 6 h interval. The samples of package air were analyzed for the variation of O_2 and CO_2 concentration in the package with time using GC. The equilibrium concentration of O_2 (Y_{O2}^{eq}), CO_2 (Z_{CO2}^{eq}) and

equilibrium time (t_{eq}) were subsequently determined. The different quality attributes such as PLW, volume reduction, firmness, TSS, titratable acidity, color (l*, a*, b*, hue angle, chroma, total color difference) of MA packaged as well as unpacked apples, with storage time were determined by standard methods (Ranganna, 1995). Various sensory attributes such as color, texture, taste and mouth feel of the fruit samples were evaluated using Fuzzy logic models (Das, 2005).

Statistical methodology

Three-factor analysis of variance was carried out to find the direct two-factor and three-factor interaction effects of temperature, storage system and storage period on the quality parameters of apple (Das and Giri, 1986). Response surface methodology (RSM) was employed to evaluate the effects of temperature, storage periods and their interaction on the quality parameters of MA packed and unpacked fruits (Khuri and Cornel, 1987; Mangaraj and Singh, 2011).

Results and Discussion

Respiration rate at transient state of apple

The effect of changes in O_2 and CO_2 concentrations on respiration rates at various levels of storage temperature under simulated transient state of MAP were analyzed. The decrease in O_2 concentration from 20.5% to 4.2% reduced R_{O2} and R_{CO2} from 9.76 to 2.3 and 11.5 to 2.19 cc/kg-h, respectively, for apple at 2.0% CO_2 concentration level and 10ºC storage temperature. For similar reduction in the O_2 concentration at 5% CO_2 level, R_{O2} and R_{CO2} were found to have reduced from 7.24 to 1.84 and 9.85 to 1.78 cc/kg-h, respectively. At 3% O_2 and 3% CO_2, the respective values of R_{O2} and R_{CO2} were found to be 1.93 and 2.11 cc/kg-h, and those under normal air were 9.43 and 11.76 cc/kg-h, respectively. The results indicated 78 to 82% reduction in respiration rate. Similarly at 15ºC, the percent reduction of R_{O2} and R_{CO2} from normal air to modified atmosphere of 3% O_2 and 3% CO_2 was found to be 80-85% (Figure 2). The percentage reduction in R_{O2} and R_{CO2} were found to be higher at higher levels of temperature.

Similarly increase in CO_2 reduces respiration rates at all the level of O_2. The effect of variation in O2 on respiration rates was found to be much higher than that of the variation in CO_2. The effect of CO_2 level on R_{O2} and R_{CO2} was found more pronounced at higher level of O_2 concentration. These results are in close agreement with those obtained by Talasila et al. (1992). Overall, at all the reference temperatures, R_{O2} decreased by decreasing the O_2 and increasing the CO_2 levels and this effect was more significant for low O_2 concentration and higher temperatures. Similar trends were observed for CO_2 production rates (R_{CO2}). Therefore, low O_2 and high CO_2 atmospheres imposed a decreasing trend in the respiration rate of apple, which should increase its shelf life.

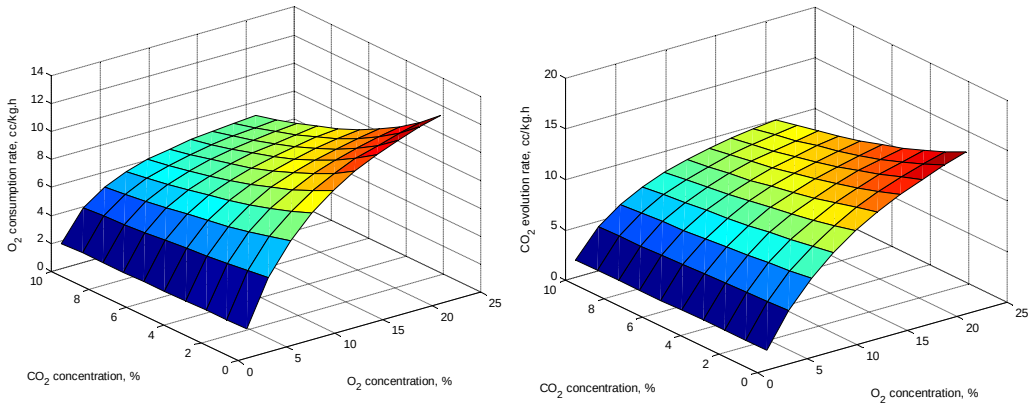


Figure 2. Respiration profile of apple under simulated transient state of MAP at 15ºC.

Gas Transmission Rates of Selected Polymeric Films

The O_2 transmission rate (OTR) and CO_2 transmission rates (CTR) of the selected films as well as the combined film laminates were determined at 10, 15, 20 and 25ºC and are given in Table 1. The gas transmission rates have been expressed for the total film thickness and not for unit film thickness. The GTR of the films increased with the increase in temperature. However, the magnitude of the increase varied with the film type and thickness. Among the selected films, the GTR and the TR of PVC film were found to be significantly higher as compared to BOPP film.

Equilibrium concentrations of O_2 and CO_2 in MA packages

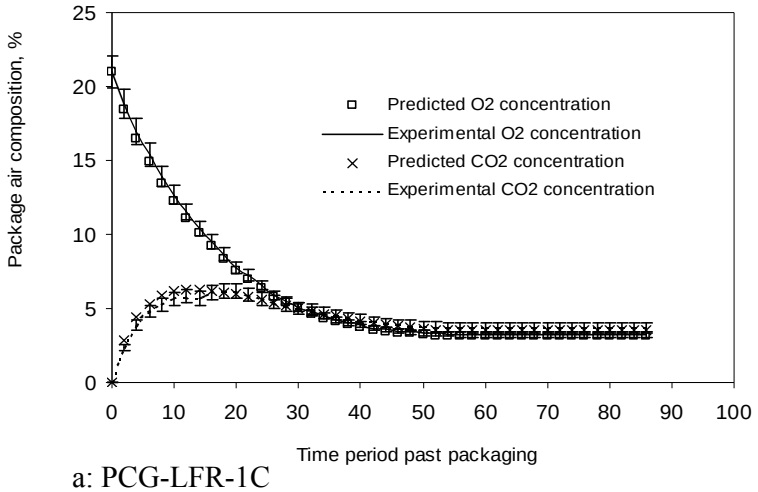
The predicted as well as experimental values of Y_{O2}^{eq}, Z_{CO2}^{eq} and t_{eq} for various MA packages at different storage temperatures have been presented in Table 2.

Table 1. GTR of Selected Polymeric films and laminates at various temperatures.

Polymeric films/ Laminates	Film code	Thickness (μ)	Gas transmission rates (Cm^3 (m^2.h. ΔC)$^{-1}$)							
			10ºC, 90% RH		15ºC, 80% RH		20ºC, 75% RH		25ºC, 70% RH	
			OTR	CTR	OTR	CTR	OTR	CTR	OTR	CTR
BOPP-I	PFR-1	30	43.15	190.72	61.72	278.13	88.59	408.62	125.86	596.57
BOPP-II	PFR-2	45	25.76	87.19	38.54	171.42	57.93	263.57	79.13	368.72
PVC-I	PFR-3	25	650.91	3968.81	943.37	5830.16	1320.84	8263.27	1894.30	11992.64
PVC-II	PFR-4	35	417.59	2527.64	614.38	3784.81	846.17	5264.08	1289.38	8148.89
PVC-III	PFR-5	50	290.26	1712.58	431.29	2609.83	585.52	3579.78	896.45	5566.95
BOPP-I + PVC-III	LFR-1	80	92.22	428.967	132.89	629.79	188.53	915.48	271.98	1349.77
BOPP-II + PVC-II	LFR-2	80	43.69	150.95	65.33	294.37	97.78	451.01	134.26	633.21

Table 2. Predicted and Experimental Equilibrium Concentration of O2 and CO2 for PCG-LFR-1 Package at Different Storage Temperatures.

Temperature (ºC)	Y_{O2}^{eq-pre} (%)	Y_{O2}^{eq-exp} (%)	Z_{CO2}^{eq-pre} (%)	Z_{CO2}^{eq-exp} (%)	t^{eq-pre} (h)	t^{eq-exp} (h)
Package fill weight (W_p): 1.0 kg, V_{fp} : 890 ml						
10	3.11	3.17	3.53	3.74	60.00	66.00
15	3.14	3.22	3.51	3.68	54.00	58.00
20	3.31	3.24	3.59	3.74	46.00	50.00
25	3.30	3.17	3.90	4.17	40.00	42.00
Package fill weight (W_p): 0.9 kg, V_{fp}: 1024 ml						
10	3.21	3.25	3.73	3.86	72.00	80.00
15	3.19	3.23	3.66	3.79	66.00	74.00
20	3.28	3.19	3.62	3.73	62.00	60.00
25	3.23	3.16	3.90	4.11	56.00	54.00
Package fill weight (W_p): 0.95 kg, V_{fp}: 967 ml						
10	3.12	3.10	3.57	3.74	68.00	74.00
15	3.14	3.17	3.54	3.55	62.00	64.00
20	3.26	3.31	3.55	3.68	56.00	52.00
25	3.27	3.20	3.89	3.82	48.00	44.00
Package fill weight (W_p): 1.05 kg, V_{fp}: 818 ml						
10	3.01	3.06	3.37	3.49	58.00	62.00
15	3.08	3.11	3.40	3.52	50.00	52.00
20	3.23	3.16	3.44	3.48	44.00	46.00
25	3.20	3.12	3.71	3.65	38.00	34.00
Package fill weigh (W_p): 1.10 kg, V_{fp}: 762 ml						
10	3.00	2.94	3.34	3.45	52.00	58.00
15	3.05	3.10	3.35	3.50	46.00	50.00
20	3.20	3.13	3.39	3.34	40.00	36.00
25	3.15	3.17	3.60	3.52	36.00	34.00



a: PCG-LFR-1C

Figure 3. Experimental and predicted variation in package air composition with time for pcg-lfr-1c at 15ºC storage temperatures.

The experimental as well as predicted variations of O_2 and CO_2 levels in MA packages PCG-LFR-1C, at 15ºC storage temperatures have been shown in Figure 3. Most of the packages have established equilibrium at such levels of O_2 and CO_2, which were fairly close to the target levels. The predicted and experimental values of Y_{O2}^{eq}, Z_{CO2}^{eq} were found to be higher than the target levels for all the MA packages. There was good agreement between predicted as well as experimental values of Y_{O2}^{eq} and Z_{CO2}^{eq}. The experimental values of Y_{O2}^{eq} varied between 3.10 – 3.31% whereas those of Z_{CO2}^{eq} varied between 3.34 – 4.17% for all types of MA packages for apple, at all the reference temperature levels. During steady state period, the experimental values of O_2 and CO_2 were found to be nearly constant for an extended period of storage. By and large, all types of MA packages have accommodated varying fill weight between 0.9–1.1 kg and have established dynamic equilibrium state without causing any unfavorable deviation from the target levels of O_2 and CO_2 at all the reference storage temperatures.

Equilibrium time

For various packages, the predicted values of equilibrium time were found to have varied between 36–72 h; whereas those of experimental values varied between 34–80 h; for all types of MA packages for apple, respectively at all the reference storage temperatures as given in Table 2. The experimental values somewhat deviated from the predicted ones.

The development of quasi-equilibrium conditions and the variations in the free volume in the package (V_{fp}), because of the varying fill weight, were probably the cause of such deviations in equilibrium time (t_{eq}) values. In fact, small variations in V_{fp} are always possible in a flexible package. Hence, it is unrealistic to expect a constant value of V_{fp} in the flexible packaging system.

It has been seen that the variation in O_2 affects both R_{O2} and R_{CO2} significantly. With the variation in R_{O2} and R_{CO2}, the O_2 consumption as well as the CO_2 evolution of the package varies which in turn affects O_2 and CO_2 level in the internal atmosphere of the package. Thus, as O_2 decreases, R_{CO2} reduces which in turn reduces CO_2 in the internal atmosphere of the package. Reduction in CO_2 level tends to retrieve R_{CO2} slightly. However, the amount of reduction in CO_2 due to decrease in O_2 is greater than the amount retrieved. As such, with the decrease in O_2 level, CO_2 level also decreases, though by small amounts. Thus, though the equilibrium condition for CO_2 level appears to be approaching earlier than that of O_2 level but in true sense, CO_2 level becomes stable only when O_2 level attains equilibrium as shown in Figure 3. It implies that the equilibrium for both, O_2 and CO_2 is attained simultaneously in MA packaging. Also, in view of the fact that O_2 level has more pronounced effect on respiration rates than CO_2 level, the equilibrium time (t_{eq}) for O_2 level

assume greater importance. The single equilibrium time (t_{eq}) approach, advocated in this study, is in agreement with the study of Rocculi et al. (2006), Torrieri et al. (2007), Rai and Paul (2007), González-Buesa et al. (2009), Tariq et al. (2009) and Mangaraj et al. (2011, 2012a,b).

Validation of the MAP model

The mathematical model using enzymatic kinetics based respiration equation coupled with the Arrhenius model was developed and solved numerically using MATLAB programme. The model was used to determine (i) the variation of O_2 and CO_2 with time within the MA packages; and (ii) the time to reach the equilibrium concentration within the MA package as well as the level of O_2 and CO_2 concentration at equilibrium state. The equilibrium O_2 and CO_2 concentration obtained from the model was verified against the experimental data for MA packaging of apple. The mean relative deviation moduli (E) between the equilibrium concentration of O_2 and CO_2 as predicted by the developed model and that obtained through experiment were found to be in the range of 5.92-8.60% and 7.14-9.35%, respectively. This indicates the developed model is in good agreement with the experimental data. The equilibrium time for both, O_2 and CO_2 has been observed to be attained simultaneously in MA packaging.

Quality assessment of MA packaged and unpackaged fruits

The variations in physico-chemical quality attributes of Royal Delicious apples under MA packaged and un-packaged condition with different storage temperature were evaluated and compared. The data of different quality attributes of apple at 15ºC for different storage periods is presented in Table 3. Variations of some important parameters with storage temperature are shown in Figure 4 to 6.

Table 3. Variations in Quality Attributes of MA Packed and Unpacked Apple at 15°C.

Quality parameters	Packaging system	Storage periods, days						
		4	17	31	45	59	73	87
PLW (%)	PCG-LFR-1	0.00	0.60	1.23	1.40	2.00	2.60	3.52
	PCG-LFR-2	0.00	0.68	1.31	1.72	2.40	3.10	3.87
	CS	0.00	2.62	4.37	9.38	14.39	20.34	28.56
Vr (%)	PCG-LFR-1	0.00	0.17	0.25	0.31	0.42	0.55	0.78
	PCG-LFR-2	0.00	0.18	0.24	0.38	0.45	0.68	0.91
	CS	0.00	1.71	3.72	4.82	5.72	6.34	7.40
Firmness (g)	PCG-LFR-1	7625.00	7560.00	7516.00	7240.00	6850.00	6510.00	6142.00
	PCG-LFR-2	7625.00	7540.00	7423.00	7180.00	6770.00	6420.00	6073.00
	CS	7625.00	7130.00	6730.00	6125.00	5740.00	4700.00	4238.00
Puncture strength	PCG-LFR-1	397.00	390.00	383.00	361.00	328.00	281.00	247.00
(g)	PCG-LFR-2	397.00	389.00	380.00	355.00	322.00	282.00	243.00
	CS	397.00	338.00	300.00	262.00	232.00	192.00	140.00
TSS (°Brix)	PCG-LFR-1	12.26	12.57	13.00	13.72	14.31	14.85	16.23
	PCG-LFR-2	12.26	12.60	13.12	13.68	14.45	15.00	16.45
	CS	12.26	13.14	14.40	15.52	15.92	16.68	14.52
TA (% malic acid)	PCG-LFR-1	0.25	0.242	0.232	0.221	0.21	0.196	0.182
	PCG-LFR-2	0.25	0.239	0.229	0.219	0.206	0.192	0.178
	CS	0.25	0.235	0.21	0.172	0.136	0.121	0.108
Ascorbic acid	PCG-LFR-1	7.87	7.52	7.10	6.73	6.14	5.53	4.63
(mg/100ml of juice)	PCG-LFR-2	7.87	7.54	7.00	6.82	5.92	5.38	4.55
	CS	7.87	6.82	5.12	4.27	3.24	2.52	1.69
Starch-Iodine Index	PCG-LFR-1	4.50	4.65	4.72	4.81	5.16	5.37	5.87
	PCG-LFR-2	4.50	4.69	4.83	4.75	5.22	5.32	5.79
	CS	4.50	4.92	5.65	6.19	6.82	7.52	8.29
L*	PCG-LFR-1	40.43	39.74	39.14	38.29	37.45	36.54	35.46
	PCG-LFR-2	40.43	39.60	38.90	38.14	37.23	36.32	35.39
	CS	40.43	39.36	38.24	37.00	35.26	33.75	32.12
a*	PCG-LFR-1	42.18	42.58	43.73	44.36	45.10	45.97	47.30
	PCG-LFR-2	42.18	42.73	43.90	44.50	45.22	46.11	47.42
	CS	42.18	43.96	45.61	47.38	47.92	47.56	46.85
b*	PCG-LFR-1	18.73	18.12	17.85	17.52	16.50	14.74	13.56
	PCG-LFR-2	18.73	17.99	17.63	17.35	16.33	14.72	13.48
	CS	18.73	17.46	15.84	13.78	12.54	12.40	12.26

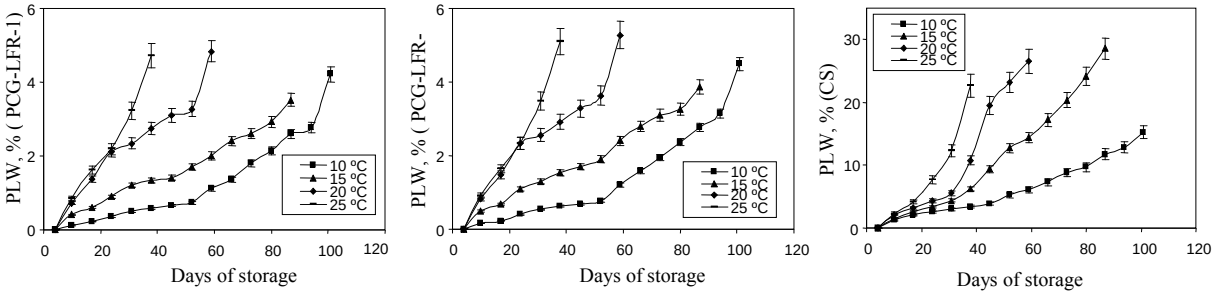


Figure 4. Variation in PLW (%) of MA packaged and control sample at various storage temperature.

During storage, an increase in PLW (%) was observed in all conditions. However, the fruits stored under MAP and at low temperature recorded slower changes. This might be due to the effect of high humidity atmosphere inside the package that reduced the rate of water loss from the fruits and also the films acting as a barrier to moisture loss from fruit surface to the environment. The activities of ripening enzymes (pectin esterase, polygalacturonase and cellulase) were reduced due to modified environment. Also the biochemical metabolic activity involved in different physiological processes was reduced and the carbohydrate utilized in physiological process was also low. The % PLW in unpackaged apples was 3.65-7.11 times higher than that of MA packaged apples at different storage temperatures (Figure 4). At elevated temperature, the reserve carbohydrate utilization increased, and hence the physiological loss in weight was more and so also the reduction in volume. The % reduction in volume of PCG-LFR-1 packed apple was found to be 10 - 14.6 times lower than that of the control at the given temperature ranges. In MAP, apart from the reduction in respiration and heat of respiration, the evaporation of water from the fruit surface is drastically reduced (Arvanitoyannis and Bosnea, 2004).

There was gradual decline in fruit firmness and puncture strength of apple in MA packed as well as control during storage. However, the application of MAP proved more effective in retention of higher fruit firmness of apple than normal (control) storage (Figure 5). The retention of relatively higher fruit firmness under MA condition at lower temperature could be attributed to slower metabolic activities leading to lower substrate utilization, minimum ripening changes, regulation of ethylene biosynthesis and cellular disintegration (Gorny and Kader, 1996; Hardenburg, 1971; Kader, 1986; Hind and Abu-Bakr, 2003; Goswami and Mangaraj, 2011; Mangaraj et al., 2012a). The loss of firmness in fruits is also attributed to the change in pectic substances (Mohsenin, 1980; Mangaraj et al., 2005). The increase of soluble pectins was retarded by lowering storage temperature and increasing CO_2 level (Awad and Jager, 2003). Also CO_2 level was reported to have pronounced effect on hydrolytic changes. In MAP, low O_2 and high CO_2 levels retard cellular disintegration of cellular walls, membrane permeability, senescence as well as hydrolytic changes which in turn help retaining flesh firmness for longer periods (Soliva-Fortuny, 2001; Nicolais et al., 2011a).

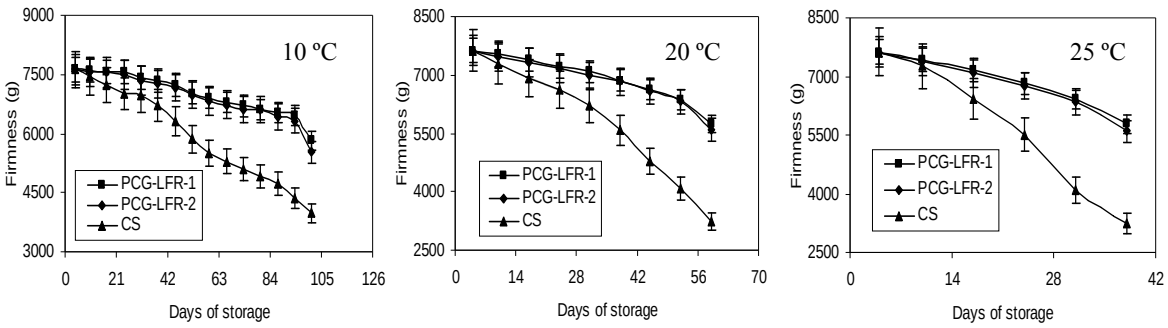


Figure 5. Variation in firmness (g) of MA packaged and unpackaged apple during storage.

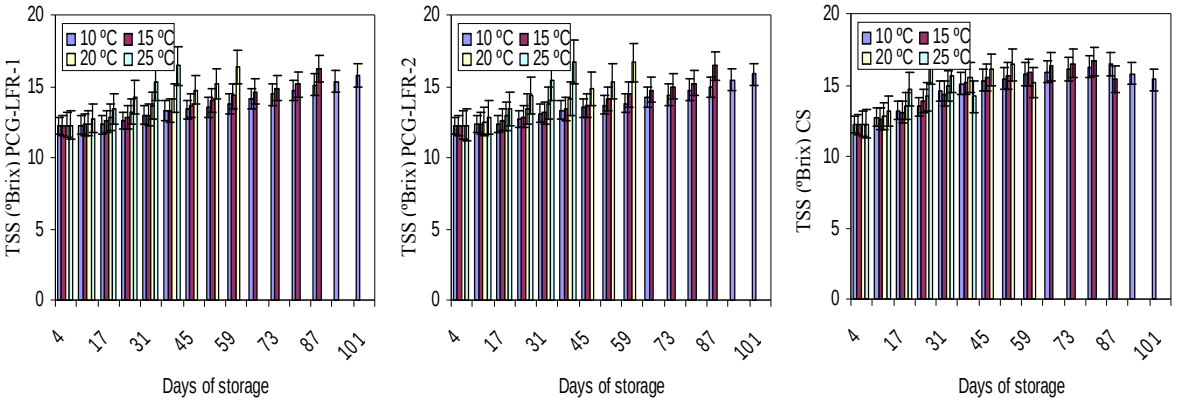


Figure 6. Variation in TSS (ºbrix) of MA packaged and control sample at different storage temperature.

The TSS content of apple was found to have increased with storage period. In case of MA packaged apples, the increase in TSS content was slow and gradual (Figure 6). The increase in TSS during storage is attributed to the numerous catabolic processes taking place in the fruit, preparing it for ripening and senescence. With time, starch gets hydrolyzed into mono and disaccharides (hydrolysis of insoluble polysaccharides in to simple sugar), which in turn may lead to an increase in TSS and sugars. On complete hydrolysis of starch, no further increase in TSS occured and subsequently TSS declined since they are the primary substrate for respiration (Podesedek et al., 2000; Johnston et al., 2001). These findings are further supported by the observation of Lelievre et al. (1997). Titratable acidity (TA) was found to have decreased with storage period. During storage, increased respiration is responsible for the declining of malic acid content, which is the principle metabolic substance together with sugar in apple. It was observed that the rate of decline in TA of MA packaged apple at low temperature was lower as compared to that of control. Similar experimental variations in TA values with storage period have also been reported by Lelievre et al. (1997) and Rocha et al. (2004).

The reduction in ascorbic acids in unpackaged apples was 2 - 2.5 times higher than that of MA packaged apples at different storage temperatures. It is reported that high oxygen level could be responsible for acceleration of ascorbic acid loss through increased oxidation by ascorbate oxidase during storage (Singh and Pal, 2008). The slow decrease of ascorbic acid content also influenced the shelf life of fruit. Ascorbic acid can scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen. The extent of ascorbic acid loss decreases as oxygen level decreased, and carbon dioxide level increased (Lee and Kader, 2000). MA packaging was found effective in preventing the ascorbic acid losses as evident from the findings.

For most apple cultivars, rather than having a constant level, there is an increase in ethylene during the ripening process (Johnston et al., 2001). This increase in ethylene concentration may allow the different ripening characters to be co-ordinated so that starch conversion always occurs before excessive fruit softening or the production of attractant volatiles. The rate of increase in starch Iodine Index value of apple was slow and steady at lower temperature and under MAP but it was faster in the control fruits at higher temperatures. This indicated that the atmospheric modification retarded the amount of starch formation in the apple flesh (Table 3). The scores between 4 and 6 using 1 to 9 scales for starch-Iodine index were considered as matured but not ripened.

The values of L* and b* decreased, while that of a* increased during storage (Table 3). The royal delicious apple was harvested when 75 - 80% of the yellow skin was covered with red strip. The ripening of apple is governed by the radiation, enzyme activity and pigmentation. During storage, the surface area as well as the intensity of redness increased which is indicated by the increase in a* value (Mangaraj et al., 2006). When the whole surface area was covered with red strips then the apple achieved maximum redness and the apple was

considered to have ripened completely. It was observed from the control stored fruits that after achieving total redness, the a* value decreased very slightly and remained constant throughout the storage periods, meaning thereby that the color did not change significantly after ripening. However, other physiological changes in the apple took place as indicated by the change in TSS, acidity, firmness (Table 3). Similar observation but reverse trend was observed for yellowness values. As the surface area for redness increased, the yellowness decreased. After certain period the b* value remained constant and decreased very slightly in control sample. It was observed that the fruits stored under MA packaging maintained better colour than unpacked apple. The retention of relatively higher colour values under MA condition may be due to the lower metabolic activities leading to retarded ethylene biosynthesis, lowering ripening changes and delaying senescence. As storage progressed, the redness increased, however, there was no shifting of colour spectrum during the entire period of study.

The ΔE of apple increased during storage. However, in control sample, it was high at the initial stage then decreased and remained constant (Figure 7). This was due to the facts that after complete redness, the overall colour did not change significantly. The ΔE value of MA packaged apple was lower than the air-stored. This indicates that the rate of total color change of MA packaged was lower than that of the control fruits. The effect of temperature on ΔE with different packaging system shows that rate of change of color is faster and higher at higher temperature during storage (Figure 7). The hue angle, which represents the pure / exact colour of the fruit, gradually decreased with storage time in MA packaged as well as unpackaged apple. The hue angle of fruits stored in air was decreased from 23.94 to 14º during storage at different temperatures reflecting the increase in redness. However, there was no shift of colour from one spectrum to other as the apple was harvested when the color was 75 - 80% red. During storage, only redness increased as is evident from the observations (Table 3). The hue angle of fruits under MAP was lower than that of the control for the same storage periods indicating the preservation of color pigment under modified atmosphere. The increase in chroma value during the storage period, reveals that the brightness increases till the development of full colour (Figure 7). However, in control sample the chroma values decreased after attainment of complete ripening. The color of the MA packaged fruits was comparable with the color at harvesting maturity (Mangaraj and Goswami, 2009d). The MA packaged apple was slightly lighter than that of the control during shelf life extended storage period as reflected in its higher L*, lower a*, higher hue angle, lower chroma and ΔE values. The increase in chroma value of apple indicates the increased in brightness or intensity of red colour during extended storage.

Altogether, Chlorophyllase is considered as the important enzyme in the pathway of chlorophyll degradation of apple. The MA packaging of fruits delayed the increases in the activities of cell wall hydrolytic enzymes and chlorophyllase activity that affects the fruit colour.

The results of the quality attributes of apple (cv. *Royal Delicious*) under MA packaging were in agreement with those found by Rocha et al. (2004), which stated apples (cv. Bravo de Esmolfe) packed in MA showed reduced weight loss, preserved better colour and firmness than fruits stored in air. Prasad (1995) reported that MA packed golden delicious apples retained orchard freshness for longer period of time with increased shelf life. The incidence of bitter pit that developed during storage of New Zealand 'Cox's Orange Pippin' apples were progressively reduced from 50% to less than 5% in MA Packaging (Hewett, 1984). Geeson et al. (1994) found that application of polymeric film lining systems for MA box packaging provided effective, reproducible atmospheres of 7-10% CO_2 and 5-7% O_2, which was beneficial in shelf-life extension of Bramley's Seedling and Cox's Orange Pippin apples, and hence was introduced into the UK fruit market. Guan et al. (2004) reported that Fuji apples in optimal MA package had good quality retention during storage. Dipping treatment and MA affected the respiratory activity of the packed product and preserved better quality of Golden Delicious minimally processed apples (Rocculi et al., 2006). Mondial Gala' apples stored under controlled atmospheres maintained quality parameters better, in addition to the highest titratable acidity and firmness values than the air stored fruits (Echeverria et al., 2008).

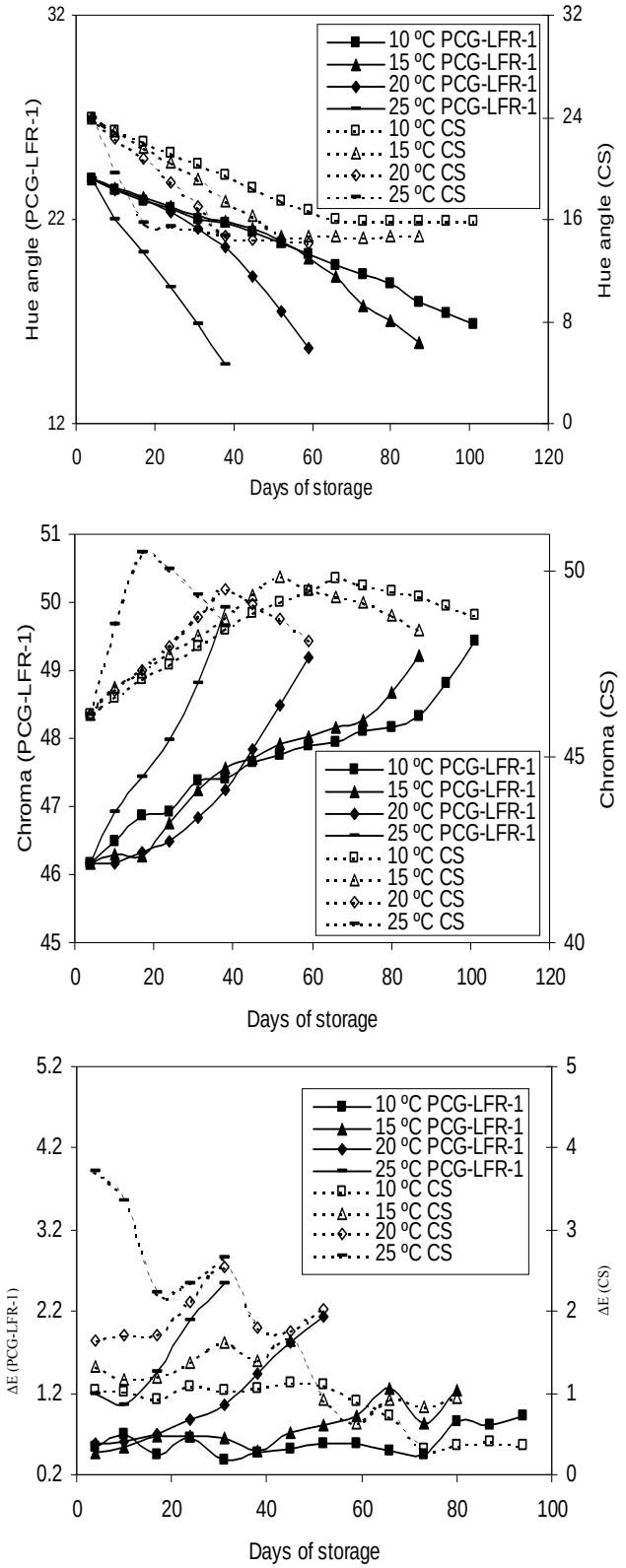


Figure 7. Colour values of MA packaged (PCG-LFR-1) and control stored apple at various temperatures.

ANOVA of quality parameters during storage

Three-factor ANOVA revealed the direct effect as well as two and three factor interaction effects of temperature, storage system and storage periods on quality parameters of apple at 1% level of significance. The results of the three factors ANOVA for some of the quality attributes of apple are given in Table 4.

Second order response surface regression was fitted to the experimental data on different quality parameters of apple. The regression coefficients of all the quality parameters obtained from response surface regression analysis are presented in Tables 5 to 7, which revealed the linear, quadratic and interaction regression coefficients of temperature, days of storage and their interaction are significant at 1% level of significance for all the variables except PLW and Reduction in Volume, for which significance is at 5% level of significance.

Table 4. ANOVA for Quality Parameters of MA Packed and unpacked Apple.

Sources of variation	DF	MSS	F-Value
PLW (%)			
Model	73	32.957783	322.81**
Replication	2	0.0621005	0.61
Days	5	120.3673185	1178.96**
Temp	3	108.4747963	1062.48**
Storage system	2	235.9413671	2310.97**
Days*Temp	15	20.8267859	203.99**
Days*Storage system	10	35.5647721	348.35**
Temp*Storage system	6	23.1515060	226.76**
Days*Temp*Storage system	30	6.6563895	65.20**
Error	142	0.102096	---
Firmness			
Model	73	1795609.2	6878.31**
Replication	2	49.85	0.19
Days	5	7777706.97	29793.5**
Temp	3	10331251.09	39575.2**
Storage system	2	9333356.51	35752.6**
Days*Temp	15	1545338.50	5919.62**
Days*Storage system	10	999983.55	3830.57**
Temp*Storage system	6	772586.87	2959.49**
Days*Temp*Storage system	30	157164.66	602.04**
Error	142	261.1	---
TSS			
Model	73	3.7890336	83.71**
Replication	2	0.0406000	0.01
Days	5	28.5697233	631.21**
Temp	3	17.7102179	391.29**
Storage system	2	11.5424014	255.02**
Days*Temp	15	1.3858149	30.62**
Days*Storage system	10	1.0272697	22.70**
Temp*Storage system	6	0.5719211	12.64**
Days*Temp*Storage system	30	0.7680910	16.97**
Error	142	0.0452615	---

Storage system: MAP and Control ; ** = Significant at 1%

Table 5. Parameters of Response Surface Analysis for PCG-LFR-1 of Apple.

Variables	b0	b1	b2	b11	b22	b12	R2
PLW	1.8326	-0.2468	-0.0486	0.0077	0.0002	0.0050	0.8942
Red.vol	1.7605	-0.2259	-0.0360	0.0061	-0.0002	0.0039	0.7414
Firmness	5923.72	227.8709	19.4673	-6.4944	-0.1266	-2.0641	0.9184
Puncture	246.07	20.7073	2.0234	-0.6187	-0.0129	-0.2047	0.9305
TSS	15.4818	-0.4395	-0.0318	0.0124	0.0002	0.0045	0.9552
Acidity	0.1875	0.0078	0.0007	-0.0002	-0.0001	-0.0001	0.9457
Ascacid	4.9529	0.3866	0.0316	-0.0108	-0.0002	-0.0039	0.9447
Starch II	6.0544	-0.1702	-0.0225	0.0044	0.0001	0.0015	0.8950
L*	43.7376	-0.4416	-0.0119	0.0120	0.0001	0.0042	0.9482
a*	35.1994	0.8604	0.0375	-0.0229	-0.0001	-0.0062	0.9119
b*	26.5583	-0.9739	-0.0947	0.0269	0.0005	0.0079	0.9335
Hue ang	36.8806	-1.6077	-0.1349	0.0440	0.0007	0.0127	0.9274
Chroma	44.0075	0.2666	-0.0150	-0.0067	0.0002	-0.0016	0.9044
ΔE	5.9119	-0.6822	-0.0760	0.0198	0.0003	0.0050	0.8499

Table 6. Parameters of Response Surface Analysis for PCG-LFR-2 of Apple.

Variables	b0	b1	b2	b11	b22	b12	R2
PLW	1.4150	-0.1623	-0.0575	0.0050	0.0003	0.0054	0.8700
Red.vol	1.8966	-0.2436	-0.0394	0.0067	-0.0001	0.0042	0.7567
Firmness	5680.05	254.5104	21.9174	-7.2367	-0.1477	-2.2184	0.9139
Puncture	235.13	22.5502	1.5645	-0.6793	-0.0086	-0.2000	0.9170
TSS	15.6269	-0.4520	-0.0336	0.0127	0.0001	0.0048	0.9521
Acidity	0.1813	0.0085	0.0008	-0.0002	-0.0001	-0.0002	0.9363
Ascacid	4.6152	0.4189	0.0345	-0.0117	-0.0002	-0.0041	0.9372
Starch II	5.8273	-0.1690	-0.0064	0.0049	0.0003	0.0009	0.7844
L*	43.7548	-0.4378	-0.0059	0.0119	-0.0001	0.0043	0.9355
a*	35.3345	0.8264	0.0405	-0.0218	-0.0002	-0.0067	0.9223
b*	27.2901	-1.0551	-0.0775	0.0290	0.0004	0.0078	0.8638
Hue ang	36.7602	-1.5751	-0.1331	0.0430	0.0007	0.0132	0.9252
Chroma	44.2698	0.2328	-0.0228	-0.0057	0.0002	-0.0015	0.8981
ΔE	5.4603	-0.6375	-0.0687	0.0191	0.0003	0.0047	0.8149

Table 7. Parameters of Response Surface Analysis for Control Stored Apple.

Variables	b0	b1	b2	b11	b22	b12	R2
PLW	11.4190	-1.0852	-0.4963	0.0175	0.0020	0.0427	0.9636
Red.vol	1.2372	-0.1095	-0.0987	-0.0005	-0.0002	0.0145	0.8704
Firmness	3736.36	527.9729	21.4651	-15.0395	-0.0801	-4.53420	0.9241
Puncture	196.35	27.4302	-0.9443	-0.7923	0.0083	-0.22830	0.9505
TSS	12.1702	-0.1180	0.1266	0.0050	-0.0008	-0.00020	0.8911
Acidity	0.2336	0.0033	-0.0003	-0.0001	-0.0001	-0.00010	0.9825
Ascacid	2.7140	0.6962	-0.0206	-0.0195	0.0002	-0.00600	0.9673
Starch II	6.8510	-0.2803	-0.0179	0.0069	0.0001	0.00370	0.9745
L*	51.2674	-1.4384	-0.0778	0.0402	0.0004	0.01240	0.8835
a*	31.6256	1.3970	0.0831	-0.0379	-0.0002	-0.01510	0.9425
b*	32.5417	-1.7606	-1.7606	0.0481	0.0010	0.01870	0.9549
Hue ang	46.2025	-2.8679	-0.2410	0.0779	0.0013	0.03110	0.9538
Chroma	47.2560	-0.0301	-0.1050	-0.0001	0.0008	0.00080	0.6689
ΔE	6.8790	-0.8480	-0.0634	0.0275	0.0001	0.00660	0.8801

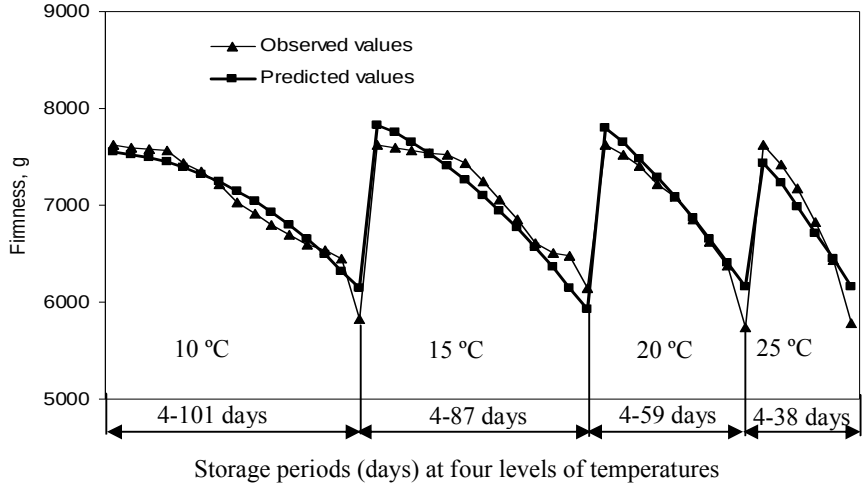


Figure 8. Experimentally observed and predicted values of firmness during different storage periods at various temperatures for apple.

From the regression coefficients (Table 5, 6, and 7) it is inferred that there are lower rates of changes in all the quality attributes of apple in MA packages as compared to the control samples during storage. It also indicates that the effect of temperature is more pronounced than the storage periods for the changes in quality parameters during storage.

The developed regression models were verified for all the quality parameters of fruits. The comparison of experimental and predicted quality parameters yielded the mean relative deviation modulus (E) value of less than 5, which indicated that the developed regression models have good agreements for predicting the quality attributes of MA packaged and unpackaged fruits during storage. The comparison of experimentally determined firmness of apple with predicted values under PCG-LFR-1 package is shown in Figure 8.

Conclusions

The laminated MA packages, developed in this study, were found to have met the GTR requirements of the MAP precisely. Most of the packages achieved equilibrium of O_2 and CO_2 levels, which were fairly close to the target levels. The experimental values of equilibrium levels of O_2 and CO_2 varied between 3.10 – 3.31% and 3.34 – 4.17%, respectively, for various MA packages of apple at different temperatures. Apples packed in MA packages exhibited reduced weight loss, reduced amount of starch formation, maintained good colour and preserved firmness as compared to the fruits stored in normal atmosphere. The shelf-life of fruits under MAP was extended up to 94, 80, 52 and 31 days at 10, 15, 20 and 25ºC storage temperatures, respectively. The MA packaging system increased the shelf life of apple by 125-210% as compared to the unpacked fruits at various storage temperatures with a quality comparable with the freshly harvested commodities. The mean relative deviation moduli between the equilibrium concentrations of O_2 and CO_2 predicted by the model and obtained through experiment were found to be 5.92-8.6% respectively, indicating good agreement with the experimental data. Three- factor ANOVA revealed that the direct as well as two and three factor interaction effects of temperature, storage system and storage periods on quality parameters of apple were significant at 1% level of significance.

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PLANT SCIENCE

Developing Vegetation Health Index from biophysical variables derived using MODIS satellite data in the Trans-Gangetic plains of India

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Abstract

Basic requirement for crop growth monitoring is the efficient tool for retrieving different plant biophysical variables and its accuracy or reliability. In the present study, statistical approach is used to retrieve the three biophysical parameters i.e. leaf area index (LAI), Chlorophyll content (Cab), and leaf equivalent water thickness (Cw). Normalized Difference Vegetation Index (NDVI), Red Index (RI) and Normalized Difference Water Index (NDWI) were used to retrieve LAI, Cab and Cw respectively. Study area was Trans-Gangetic plain comprising of whole Punjab, Haryana, Chandigarh, Delhi and some parts of Rajasthan. Field measurements were done at 190 locations. Spectral measurements (wheat crop and soil) with ground held spectroradiometer (FieldSpec3), LAI using Canopy Analyzer (LICOR-2000) and leaf samples were collected for further analysis in laboratory. Satellite data used was MODIS Surface Reflectance Product (MOD 09). Vegetation Health Index (VHI) was developed using the retrieved LAI, Cab and Cw and study area was categorized into four groups based on VHI.

Key words: Chlorophyll content, Equivalent water thickness, NDVI, Leaf area index, Vegetation health index

Introduction

Assessment of vegetation condition particularly caused by biotic or abiotic stress such as drought or pest infestation can be critical for countries where the economy is dependent on the crop harvest. Early assessment of yield reductions could avert a disastrous situation and help in strategic planning to meet demands. Basic requirement for crop growth monitoring is an efficient tool for retrieving different plant biophysical variables and its accuracy or reliability. Remote Sensing has been found to be potentially valuable in estimating these biophysical variables. The methods to estimate canopy biophysical variables from reflectance data are (1) Statistical approach and (2) Process based approach (using Radiative Transfer Models). Empirical or statistical approaches are based on observations of the spectral contrasts of reflectance and consist of fitting a relationship between reflectance and some biophysical variables, mainly by the use of vegetation indices (Tripathi et al., 2012).

Leaf Area Index (LAI) has been an important variable that is directly related to photosynthesis, evapotranspiration, and productivity of agro-ecosystems. Measurement of LAI in the field is very difficult, and requires a great amount of time and efforts. The mapping of LAI in large geographic area may be impossible when we rely on the field measurement. To solve this problem, there have been continuing efforts to develop methodologies to estimate LAI using remotely sensed data. Because of the obvious difference of the optical features between leaf and non-vegetative surface, it is possible to assess LAI by applying remotely sensed data.

The common approach has been used to correlate ground-measured LAI with the simple ratio or the normalized difference vegetation index (NDVI), and modified simple ratio (Leonard Brown and Chen, 1999). The normalized difference vegetation index was the most commonly used. Hui et al. (2003) indicated that there is a good linear correlation between LAI by ground measurement

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and NDVI calculated from Landsat ETM data. Increase in the amount of canopy chlorophyll, either through an increase in leaf chlorophyll content or leaf area index (LAI) will lead absorption well in vegetation reflectance spectra centred on 680 nm (Gitelson et al., 1996).

To estimate chlorophyll content of plant, the method of indices is generally used. Indices R_{NIR}/R_{700} and R_{NIR}/R_{550} for chlorophyll assessment were recently developed as a result of signature analysis of the reflectance spectra of two deciduous species (maple and chestnut) (Gitelson and Merzlyak, 1994a, 1994b; Gitelson et al., 1996) and of tobacco plant (Lichtenthaler et al., 1996). These indices allow for chlorophyll estimation in dark-green to yellow leaves within a wide range of pigment variation. It was reported also that the index *R760/R695* is a sensitive indicator of plant stress. Vegetation water content (VWC) is an important indicator in agricultural and forestry applications. The VWC could possibly provide information for agriculture that can be used to infer water stress for irrigation decisions, aid in yield estimation and in the assessment of drought conditions. At a whole-plant level, soil drought and leaf water deficit lead to a progressive suppression of photosynthtesis, and is associated with alterations in carbon and nitrogen assimilation (Zlatev and Lidon, 2012). The principle application to forestry is determining fire susceptibility (Pyne et al., 1996). The VWC is also used in retrieving soil moisture from microwave remote sensing observations. Ceccato et al. (2002 a, b) found that the short wave infra red (SWIR) channel was critical to estimating VWC and the near Infra red (NIR) channel was needed to account for variation of leaf internal structure and dry matter content variations.

Moderate Resolution Imaging Spectroradiometer (MODIS) is a key instrument aboard the Terra and Aqua earth observation system satellites. Terra's orbit around the Earth is timed so that it passes from north to south across the equator in the morning, while Aqua passes south to north over the equator in the afternoon. Terra MODIS and Aqua MODIS are viewing the entire Earth's surface every 1 to 2 days. MODIS has a viewing swath width of 2330 km and views the entire surface of the Earth every one to two days. MODIS is playing a vital role in the development of validated, global, interactive Earth system models able to predict global change accurately enough to assist policy makers in making sound decisions concerning the protection of our environment.

Keeping all these in background this study was conducted with the objective of retrieving Leaf Area Index, chlorophyll and moisture content of wheat crop at regional scale from TERRA MODIS products using statistical approaches and to develop a composite vegetation health index.

Materials and Methods

The study area lies between 72°38'54.44"E to 77°36'11.74"E and 27°39'19.38"N to 32°30'26.85"N, with altitude varies between 590 to 3600 ft (180 metres to 1200 metres) above sea level, which covers whole states of Punjab and Haryana, Delhi and two districts of Rajasthan in India which is commonly known as Trans-Gangetic Plain (Figure 1).

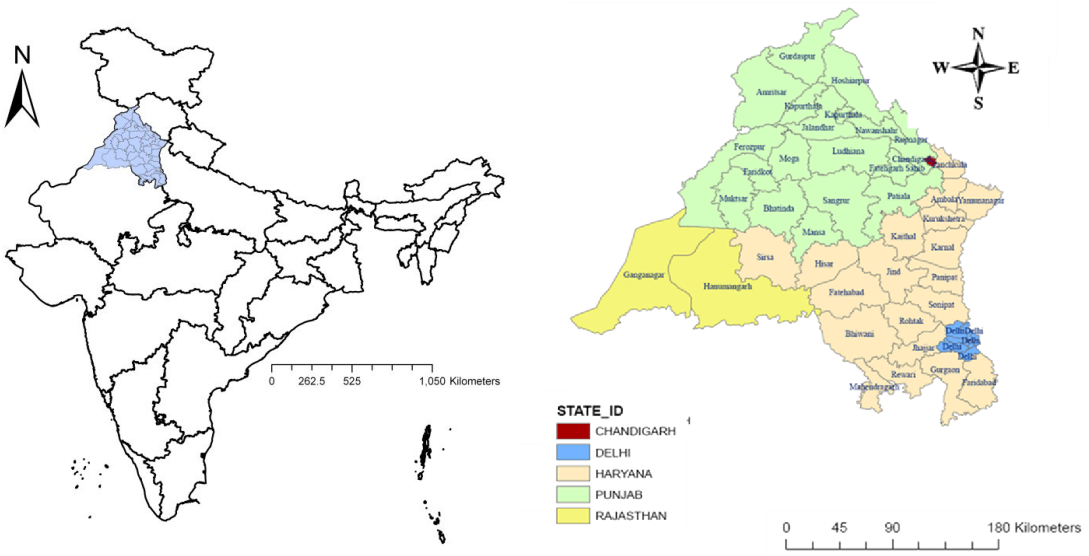

Figure 1. (a) Location of study area in the map of India. (b) Study area (Trans-Gangetic Plains) with district boundaries.

Satellite Data used

MODIS Surface reflectance data product (in the blue, green, red and near-infrared wavelength regions i.e. MOD09) were acquired from the EOS Data Gateway (https://wist.echo.nasa.gov/api/) Brief description of these products are given below

MODIS Surface Reflectance Product (MOD 09)

The MODIS Surface-Reflectance Product (MOD 09) is 8-day composite product, computed from the MODIS Level 1B land bands 1, 2, 3, 4, 5, 6, and 7 (centered at 648 nm, 858 nm, 470 nm, 555 nm, 1240 nm, 1640 nm, and 2130 nm, respectively). The MODIS Level 1B (L1B) algorithm performs radiometric calibration of data from the MODIS sensors on the EOS-Terra and EOS-Aqua satellites. Input to L1B is the Level 1A (L1A) and geolocation data. L1A includes the raw digital counts from the sensor and telemetry data for each Earth-view pixel. The Level 1B Code generates data products containing calibrated radiances for all MODIS bands. The product is an estimate of the surface spectral reflectance for each band as it would have been measured at ground level if there were no atmospheric scattering or absorption (Vermote et al., 1999).

Image Dimensions = 2400 x 2400 rows/columns
File Size = 50 – 500 MB
Resolution = 500 meters
Projection = Integerized Sinusoidal
Data Format = HDF-EOS
Composite MOD 09 product of the duration Feb 10 -20, 2008 was used for the present study. For satellite image processing ENVI 4.4 and ArcGIS 9.2 softwares have been used.

Ground Truth and and Spectral Data Collection

A field visit was conducted in early February, 2008 in the wheat growing regions of the study area with the purpose of in situ measurement of LAI using canopy analyzer (LICOR-2000), spectral measurement (wheat crop and soil) and other biophysical variables of the wheat crop. Wheat leaf samples were also collected for measurement of chlorophyll content and equivalent water thickness in the laboratory. The ground truth was conducted using handheld GPS (Garmin) and locations (latitude and longitude) of the sample collection points were recorded (Figure 2).

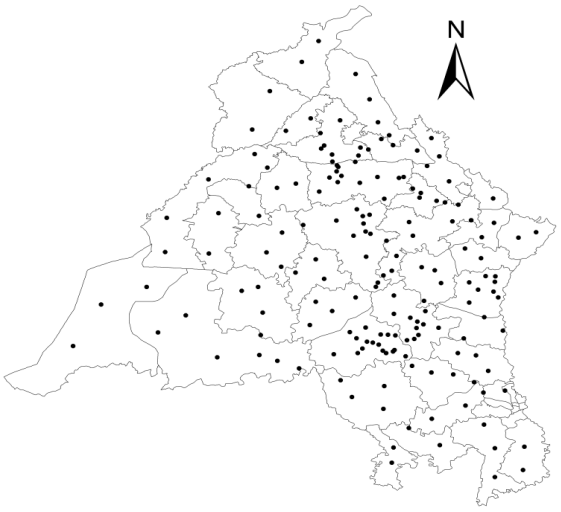

Figure 2. Ground truth locations acquired through hand held global positioning system in the Trans-Gangetic Plains of India.

Spectral Reflectance Data Collection

Spectral reflectance of wheat crop grown in different farmers' fields were collected using ASD Field Spec™ 3 ground held spectroradiometer having 25° Field of View (FOV) synchronizing with observation of other biophysical parameters. The spectral data has not been used for this particular study.

***In situ* - Leaf Area Index measurement**

In situ LAI measurement was done in various farmers' field of the study area using canopy analyzer (LICOR-2000). The instrument was set to take four below canopy measurements and one above canopy measurements to estimate the LAI. Canopy analyzer (LICOR-2000) measures the gap fraction P(θ) in five zenith angle (θ) ranges with midpoints of 7°, 23°, 38°, 53° and 67°. LAI is determined by inverting simple radiative transfer model foliage information. This indirect LAI estimate specifically represents an effective leaf area index for the agricultural crops and an effective plant area index, including branch components, for deciduous forests. The assumption of a random spatial distribution of the leaves as made in the model inversion is generally satisfied for these crops. Where a nonrandom spatial distribution of canopy foliage is observed, an accurate description of gap size is essential to avoid large errors in LAI estimation (Chen et al., 1997; Gower et al., 1999).

LAI is calculated according to Gower and Norman (1991) from the LAI-2000 gap fraction measurements:

$$LAI = 2 \int_0^{\pi/2} \ln[1/P(\theta)] \cos\theta \sin\theta \, d\theta$$

LAI measurement was done in 190 locations having two measurements in each field.

Leaf Moisture Thickness

Equivalent leaf moisture thickness was calculated by using the expression

$$= \frac{(\text{Fresh weight - Dry weight})}{\text{Leaf Area}}$$

and expressed in gm cm^{-2} or cm.

Leaf chlorophyll content

Fresh leaf samples from different treatments were cut and 100 mg was weighed and chlorophyll was extracted by a non-macerated method equilibrating it with 10 ml *D*i *M*ethyl *S*ulf*O*xide (DMSO) in a capped vial and keeping in an oven at 65°C for about 4hrs. The decanted solution was used to estimate the absorbance at 645 and 663nm wavelength using Spectrophotometer Spectronic-20. The total chlorophyll content was calculated using the formula given by Arnon (1949).

$$\text{Total Chlorophyll content (mg/g of fresh weight)} = \frac{[20.2 x A_{645} + 8.02 A_{663}]XV}{1000XW}$$

Where,

A_{645} = Absorbance at 645 nm

A_{663} = Absorbance at 663nm

V = Final volume of chlorophyll extract in DMSO

W= Weight of plant sample

Spectral indices

The following vegetation indices have been derived using MODIS bands (Table 1).

Table 1. MODIS bands and their corresponding wavelengths.

MODIS Bands	Wavelengths (nanometer)
Band 1	620-670
Band 2	841-876
Band 3	459-479
Band 4	545-565
Band 5	1230-1250
Band 6	1628-1652
Band 7	2105-2155

Normalized Difference Vegetation Index

The traditional approach for LAI estimation using Vegetation indices is based on the combination of a chlorophyll sensitive band (typically the red band) and a band located in the high reflectance plateau of vegetation canopies (NIR band). The high canopy penetration ability of the NIR band makes it highly useful for tracking variations in biomass and LAI. Normalized difference vegetation index has been widely used for the retrieval of LAI as it combines the NIR band (ρ_{nir}) and red band (ρ_{red}) reflectances.

$$NDVI = \frac{\rho_{nir} - \rho_{red}}{\rho_{nir} + \rho_{red}}$$

Red Index (RI)

It combines the NIR band (ρ_{nir}) and red band (ρ_{red}) reflectances.

$$RI = \frac{\rho_{nir}}{\rho_{red}} - 1$$

Red Index (RI) were evaluated for the estimation of total canopy chlorophyll concentration

The Normalized Difference Water Index (NDWI)

Gao (1996) NDWI was employed for the determination of vegetation water content. NDWI uses two bands centered at approximately 860 nm and 1240 nm and follows the simplicity of NDVI.

$$NDWI = \frac{\rho_{858} - \rho_{1240}}{\rho_{858} + \rho_{1240}}$$

where ρ_{858} is the MODIS NIR band centered at 858 nm and ρ_{1240} is the MODIS MIR band centered at 1240 nm.

Development of the composite vegetation health index (VHI)

The aim of developing vegetation health index (VHI) was to give a single value to the vegetation condition on the basis of three parameters (leaf area index, chlorophyll content and equivalent water thickness) which are most important for assessing the vegetation condition.

The following four steps were followed for the development of vegetation health index:

1. Selection of variables
2. Transformation of the variables of different units and dimensions to a common scale
3. Assignment of weight or score to the variables
4. Aggregation of three variables to produce a final index

Transformation of the parameters of different units and dimensions to a common scale

Different vegetation health index parameters are expressed in different units. For example Chlorophyll content is expressed in µg cm^{-2}, equivalent water thickness in centimeters, and leaf area index is unitless. In other words, different

parameters occurred in different ranges and were expressed in different units. To formulate the index, all the parameters were transformed into a single scale beginning with zero and ending at one. Linear stretching was performed on all the parameters, using the following formulae for rescaling.

$$DN'' = \left(\frac{DN - MIN}{MAX - MIN} \right)$$

Where,

DN'' = Digital number assigned to pixel in output image

DN = Original digital number of pixel in input image

MIN = Minimum value of input image, to be assigned a value of zero in the output image

MAX= Maximum value of input image, to be assigned a value of 1 in the output image

Assignment of weight to the biophysical variables

Since the three parameters i.e. LAI, Chlorophyll content and equivalent water thickness were equally important, equal weight of 1/3 was allotted to each of the three parameters for deriving the vegetation health index.

Aggregation of three parameters to produce a final index score. The index score was obtained with a linear sum aggregation function. The function consists of the weighted sum of three parameters i.e. leaf area index (LAI), chlorophyll content and equivalent water thickness divided by the sum of the weights.

$$VHI = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1} w_i}$$

Where, w_i = weightage given to the i^{th} parameter

x_i = i^{th} parameter

Results and Discussions

Regression model for the retrieval of leaf area index

Normalized difference vegetation index (NDVI) was used for the retrieval of LAI using regression analysis. NDVI map was generated using the equations described above. The empirical approach based on exponential relationships was used to relate NDVI to ground measured LAI in the present study (Table 2). A regression analysis between NDVI and measured LAI was performed. Regression analysis between NDVI and measured LAI resulted in a R^2 value of 0.667 (Figure 3). The exponential model of NDVI-LAI relationship was used to generate LAI map (Figure 4). It was observed that R^2 value and RMSE between the ground measured LAI and estimated LAI through regression were 0.6759 and 0.5833 respectively.

Our results are in accordance with other researchers. Houborg and Eva (2008) found that LAI–NDVI relationships resulted in good agreements between estimated and measured LAI for barley, wheat and maize sites with an overall root mean square deviation of 0.74. Some researchers have observed low correlation between NDVI and LAI in numerous studies (Chen and Cihlar, 1996, Cohen et al., 2003; Turner et al., 1999). NDVI has been a popular index with which to estimate LAI across diverse systems, and numerous studies have noted a strong contribution of short wave infra-red band to the strength of relationships between reflectance and LAI (Brown et al., 2000; Cohen and Goward, 2004).

Regression model for the retrieval of chlorophyll content (Cab)

Red index (RI) was used for the retrieval of Cab using regression analysis. Red index map was generated using the equations described above. The empirical approach based on linear relationships was used to relate RI to measured Cab values (Table 2). The overall R^2 observed was 0.96 for RI–Cab relationship (Figure 5). The linear model using RI-Cab regression analysis was used to generate chlorophyll map (Figure 6). It was observed that R^2 value and RMSE between the ground measured Cab and estimated Cab through regression were 0.8515 and 5.2043 respectively.

Houborg and Eva (2008) compared estimates from the composited Cab map (generated thorough regression approach) with field measurements at different wheat, barley and maize sites which recorded a root mean square deviation of only 4.9 $\mu g\ cm^{-2}$.

Regression model for the retrieval of Equivalent leaf moisture thickness (Cw)

Regression analysis was performed between normalized difference water index (NDWI) and Cw for the retrieval of Cw. Normalized difference water index map were generated using the equations described above. The empirical approach based on exponential relationships was used to relate NDWI to measured Cw in the present study. A regression analysis between NDWI and Cw (measured) showed a R^2 value of 0.726 (Figure 7). The exponential model using NDWI-Cw was used to generate Cw map (Figure 8). It was observed that R^2 value and RMSE between the ground measured Cw and estimated Cw through regression were 0.73 and 0.01 respectively.

Table 2. Regression models developed for retrieval of Leaf area index, Chlorophyll content and Equivalent water thickness through regression of corresponding indices' with measured values.

S. No.	Parameter	Method	Regression equation	R^2 Value with measured parameter
1	Leaf area index	Regression with NDVI	Y = 1.835 exp (1.51 x)	0.68
2	Chlorophyll content	Regression with RI	Y = 4.45 x + 9.45	0.85
3	Equivalent water thickness	Regression with NDWI	Y = 0.23 x + 0.02	0.73

NDVI: Normalized difference vegetation index; RI:Red Index; NDWI: Normalized difference water index

Absorption by vegetation liquid water near 0.86 µm is negligible. Weak liquid absorption at 1.24 µm is present. Canopy scattering enhances the water absorption. As a result, NDWI is sensitive to changes in liquid water content of vegetation canopies. Normalized difference water index (NDWI) employing the MODIS mid-infrared band centered at 1.24 µm was much better candidate than enhanced vegetation index and NDVI for the estimation of vegetation water content (Houborg et al 2007).

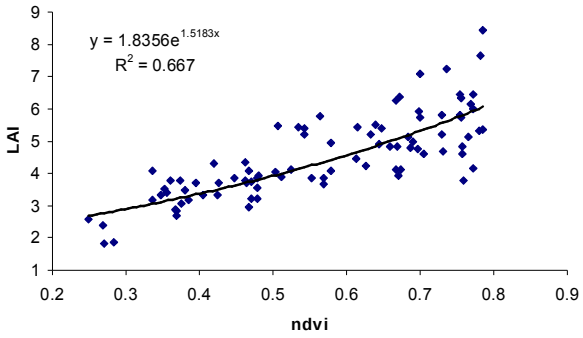

Figure 3. Regression relationship between the normalized difference vegetation index (NDVI) derived from MODIS data and the ground measured LAI.

Developing vegetation health index and zonation of the study area based on the VHI

The proposed VHI is a composite index was derived from most important three crop growth variables such as LAI, Cab and Cw. Leaf area index has direct relationship with biomass and yield of the crop. Chlorophyll content (Cab) is majorly governed by nutrient status (mainly nitrogen) of the plant which indicates indirectly N stress. Equivalent water thickness (Cw) reflects water status in the canopy and thereby water stress. The composite indicator considering all these three parameters may provide information about the vegetation health there by the index is named vegetation health index which can be used to prioritize zones at regional scale for site specific interventions for enhancing crop yield. Since LAI, Cab and Cw retrieved using vegetation indices approach were found to be more reliable and hence used for the development of the vegetation health index.

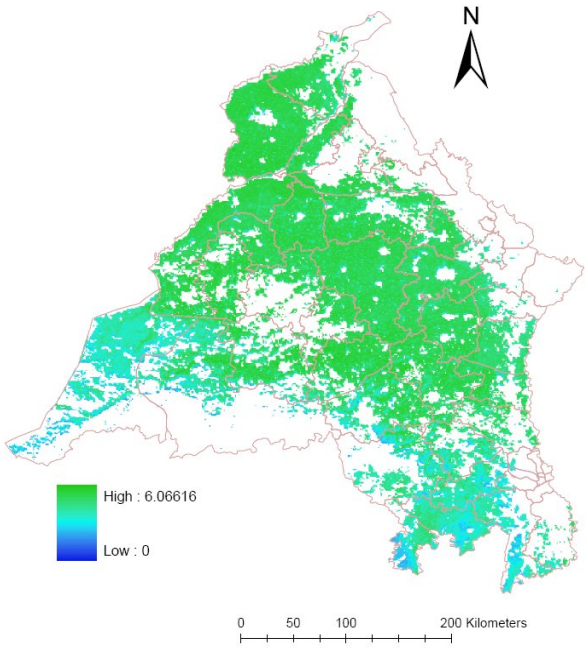

Figure 4. Leaf area index (LAI) map generated through exponential regression model between normalized difference vegetation index (NDVI) and ground measured LAI.

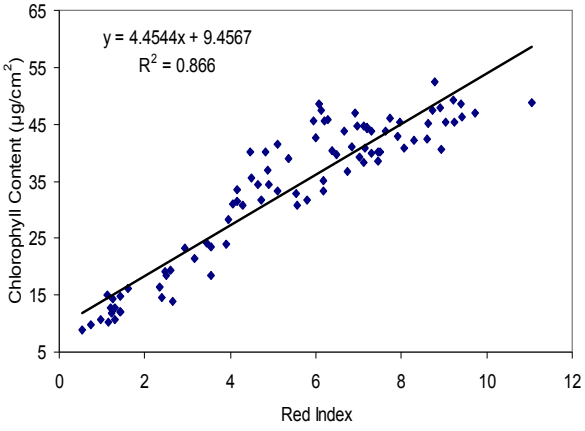

Figure 5. Regression analysis between chlorophyll content and MODIS Red Index.

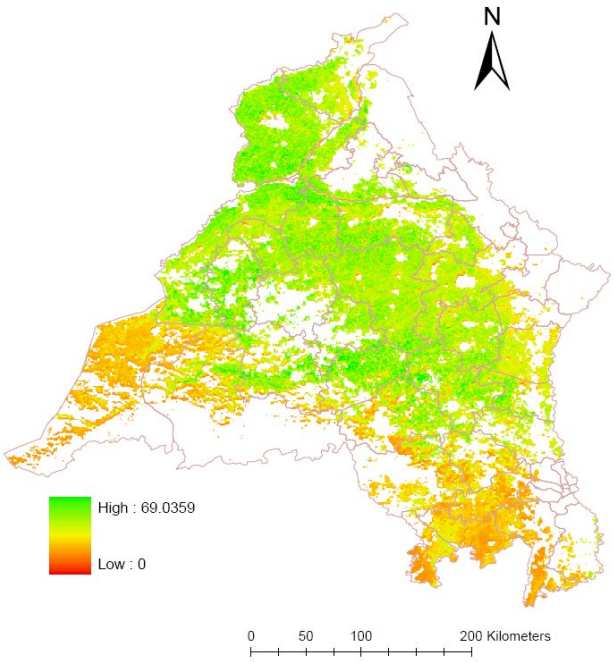

Figure 6 Chlorophyll Content (Cab) map (µg/cm2) retrieved through Linear regression model between measured Cab with Red Index.

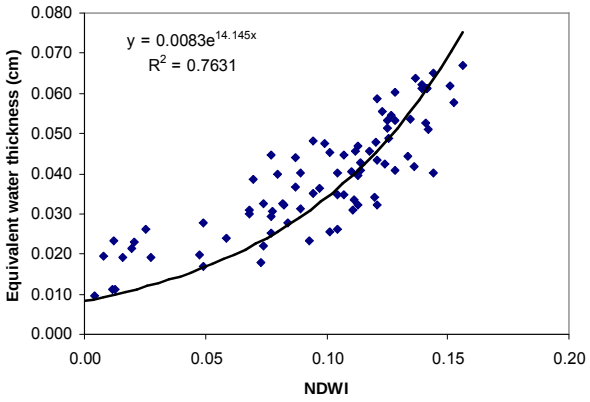

Figure 7. Regression analysis between normalized difference water index (NDWI) derived from MODIS data and measured equivalent water thickness (cm).

Vegetation health index was developed using the equation described (Figure 9). Vegetation health index is unit less ranging from 0 to 1. Based on the VHI, study region was divided into four zones corresponding to four growth conditions of wheat crop (Table 3). Wheat area with very poor growth conditions were having VHI ranging from 0 - 0.25 and 0.26 - 0.50 for poor growth, 0.51 to 0.75 to good and above 0.75 for very good conditions (Figure 10).

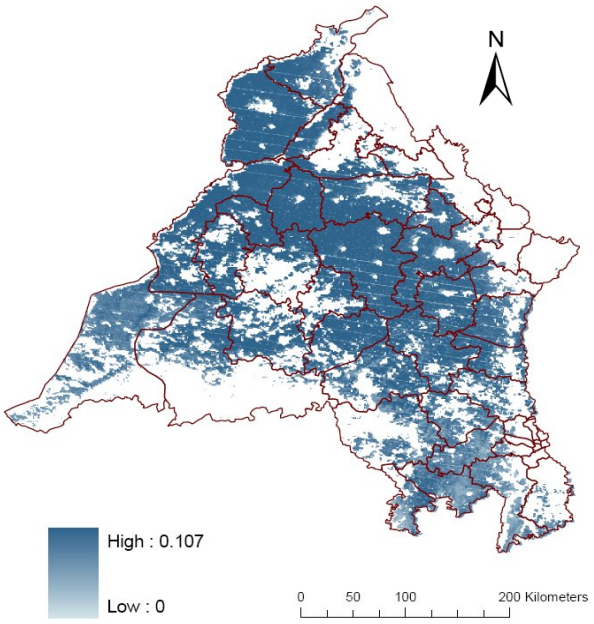

Figure 8 Leaf equivalent water thickness(Cw, in cm) map retrieved through regression model developed between normalized difference water index (NDWI) and measured Cw.

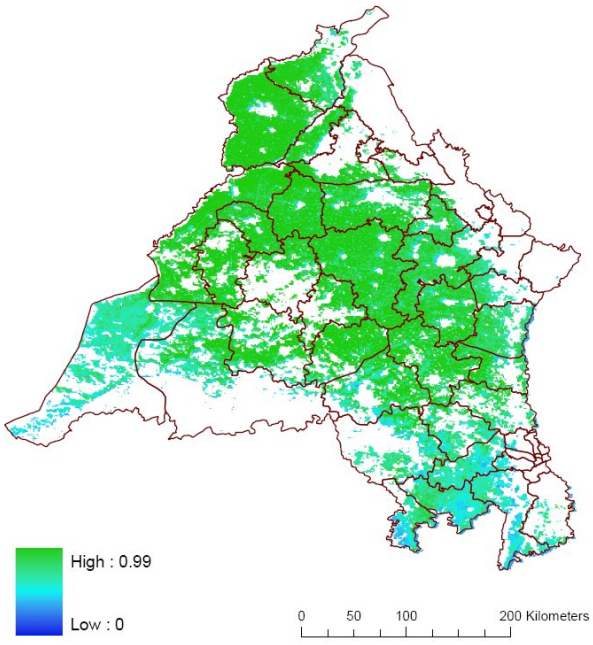

Figure 9. Vegetation Health Index map composited from leaf area index, chlorophyll content and equivalent moisture thickness of wheat.

Table 3. Four groups of vegetation growth condition based on vegetation health index.

Vegetation Health Index	Vegetation growth condition
0 – 0.25	Very poor
0.26 – 0.50	Poor
0.51 – 0.75	Good
0.76 – 1.0	Very good

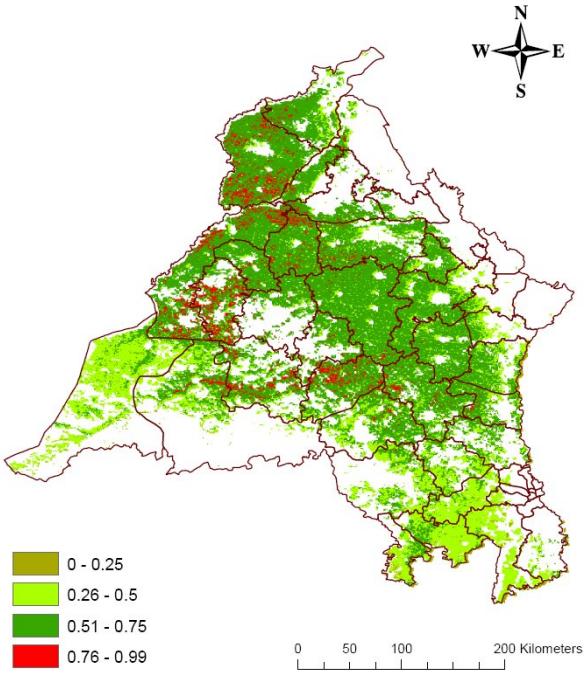

Figure 10. Zonation based on vegetation health index developed by combing chlorophyll content, Leaf area index and equivalent water thickness.

Conclusions

Crop growth monitoring at regional scale is very crucial for policy decisions. In the present study NDVI, RI and NDWI were proved to give reliable accuracy in retrieving these parameters. Vegetation health index thus developed can be used for site specific crop management for better utilization of available resources and maximizing the crop yield. This study has demonstrated the benefits of mapping the green leaf area index, total chlorophyll content and vegetation water content using Terra MODIS reflectance data. Using this technique other plant biophysical variables may also be retrieved for land cover classes characterized by contrasting canopy architectures, leaf inclination angles, and leaf biochemical constituents. The biophysical retrieval scheme used in this study is suitable for large-scale operations because the vegetation indices reflect differences in internal and external factors caused by plant species diversity, variable environmental and background conditions, and view and illumination geometry differences. The flexibility of this approach allows for easy implementation and evaluation for other environments and vegetation species compositions. Further, the use of directional satellite data, acquired from multi-angle sensors like the Multi-angle Imaging Spectro Radiometer (MISR) or from multi-temporal acquisitions from sensors with a wide range in viewing angles, in addition to extra wavebands located in the mid-infrared spectrum could probably enhance the retrieval accuracy of plant biophysical variables.

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PLANT SCIENCE

Vermicompost management: An alternative to meet the water and nutritive demands of tomato under greenhouse conditions

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Abstract

Different studies have suggested that the use of vermicompost as part of the plant growth media can provide nutrients and retain moisture while promoting the development of crops. To corroborate this assumption we tested the effects of vermicompost supplementation to tomato (saladette type) under greenhouse conditions. The evaluated treatments included four mixtures (T1, T2, T3, and T4) of vermicompost and river sand, with volume ratios 0:1, 1:1, 1:2 and 1:3, respectively. Physical and chemical tests were performed in each mixture to determine nutritional elements (N, P, K, Ca, Mg, Na, Fe, Cu, Zn, Mn, organic matter, pH, texture, cation-exchange capacity, electric conductivity, and apparent density) and water holding capacity. Treatment with 0:1 volume ratio (T1) was used as control, and it was fertilized with a nutrient solution [KNO_3, $Ca(NO_3)_2$, $Mg(NO_3)_2$, phosphoric acid concentrate, and multi Maxiquel (Bioagro ®)]. Seeds were sown in polystyrene trays with 200 cavities, padded with peat moss; seedlings were transplanted 37 days after sowing in 20 L black plastic bags. Harvest, including up to the fifth cluster, was performed manually, when the fruits reached a pink color. The treatment effects on tomato were evaluated considering the number of fruits, number of locules, equatorial and polar diameters, pulp thickness, soluble solids, fruit weight and fruit yield. The four treatments were repeated eight times in a completely randomized design. Data were statistically analyzed by analysis of variance and means were separated by the $LSD_{0.05}$ test. Five of the variables studied - number of fruits, number of locules, soluble solids, pulp thickness, and yield- showed highly significant difference ($P \leq 0.01$) among treatments; the polar diameter showed significant differences ($P \leq 0.05$), and both equatorial diameter and weight of fruit were not significantly different among the substrates tested. The maximum yield (50.29 $t \bullet ha^{-1}$) was obtained in treatment T2 with a water volume of 40 L•pot-1, followed by T1 (49.93 $t \bullet ha^{-1}$), applying a water volume of 95.72 $L \bullet pot^{-1}$. Derived from the results of the best treatment (T2), and under conditions described, the productivity was estimated in 30.66 $kg \bullet m^{-3}$. Since no synthetic fertilizers were used during the crop production, the results indicate that the vermicompost was able to satisfy the nutrient demand of tomato plants and reduces the volume of water required by this crop.

Key words: Earthworm, Humus, Organic manure, *Solanum lycopersicum*, Water holding capacity

Introduction

Today, one of the most viable alternatives to solve the problem of solid waste disposal is the process of composting (Valadares-Veras and Povinelli, 2004). The composting process is a suitable treatment used to produce a stable product, rich in humic-like substances that are environmentally safe and practicable at acceptable operational costs (Salas and Ramírez, 2001; Amir et al., 2003; Valadares-Veras and Povinelli, 2004), from a diverse range of organic waste to generate high-quality fertilizer for crops (Santamaría-Romero et al., 2001, Sharma et al., 2005). From time immemorial, humans -and mainly the farmers- have used the wastes generated by anthropogenic activities, such as organic amendments, to improve the physical, chemical and biological properties of soil (Leal and Madrid-de-Cañizalez, 1998; Romero-Lima et al., 2000).

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The use of these products is based on the fact that the presence of organic matter in the soils promotes, among other things: a) the conservation of moisture, b) increased permeability, c) the slow release and solubilization of nutrients for plants, d) improvement of soil structure, e) the soil buffering capacity, f) soil biological activity, and g) the natural control of pests and plant diseases (Cruz-Rodrigues et al., 2003). In addition, the incorporation of biodegradable materials to the soils promotes the synthesis of organic compounds that bind particles into aggregates, the increase of pore space, greater and more rapid infiltration, and water storage (Leal and Madrid de Cañizalez, 1998; Acevedo-Sandoval et al., 2001). Organic compounds are also reservoirs of macro and microelements, essential for plant development (Leal and Madrid de Cañizalez, 1998). In fact, the application of amendments to the soils, such as manure from different animals and sewage sludge is a very environmentally attractive practice, since it also provides a useful destination for these wastes (Dick et al., 2002).

In recent decades, the use of organic fertilizers has become increasingly important for several reasons, among which: a) from the ecological point of view, there is an increased concern for promoting ecofriendly agricultural practices (the use of organic fertilizers improves soil conditions that have been damaged by excessive use of agrochemicals and their over-exploitation), and b) from the economics point of view, the use of fertilizers and organic products has been promoted by organic agriculture, which represents an added value to the obtained products (their prices are higher than those of conventional agriculture products, so this practice becomes more attractive to the farmers) (Nieto-Garibay et al., 2002).

This suggests that the use of vermicompost (VC), as part of the growth media, allows meeting the nutrient demand, as well as retaining and meeting the water needs of crops developed under greenhouse conditions. Therefore, we evaluated the effect of different mixtures of VC with river sand on the availability of nutrients and moisture during the growth of tomato (*Solanum lycopersicum* L.) under protected conditions (greenhouse).

Materials and Methods

The study was carried out in a greenhouse of the Horticulture Department at the Universidad Autonoma Agraria Antonio Narro, Unidad Laguna (UAAAN-UL), in Torreon, Coahuila, México (101°40' and 104°45' W long and 25°05' and 26°54' N lat) (Schmidt, 1989). According to Aguirre (1981), the climate of this region is dry desert with rainfall in summer and cool winters. The annual precipitation is 241.9 mm and the average temperature is 21.5°C, ranging from 33.7°C maximum and 7.5°C minimum. The average annual evaporation is approximately 2,396 mm. The relative humidity in this region varies according to season, with 31, 47, 58 and 40% in spring, summer, fall, and winter, respectively (CNA, 2002).

The greenhouse used in this study is semicircular, has reinforced acrylic coating, damp wall, extractors, gravel floor, and measures 8 x 23 m, width and length, respectively. It has side windows of 1.20 m high, also covered with reinforced acrylic, which can be rolled up and are protected with anti-aphids mesh. The acrylic cover is protected with shade mesh during the warmer seasons.

The experiment was conducted in the period summer-fall-winter 2008, using the tomato variety Sun-7705 (Nunhems USA, Inc. ®) saladette type of indeterminate cycle, which were sown on 8 July 2008 in polystyrene trays of 200 cavities, using Peat moss (Canadian Sphagnum Peat Moss Association ®) (Atiyeh et al., 2000) as substrate, which were previously saturated with water, then filled the tray and deposited one seed per cavity. The trays were placed inside the greenhouse, covered with black plastic and watered with tap water (pH 7.57 and classified as C1S1) every three days until the time of transplant, which was performed at 44 days after sowing (DAS), when the plant had an approximate height of 15 cm, placing one seedling per pot. Black polyethylene 20 L capacity bags, 500 gauge, type nursery and 20 L of capacity were used as gavels. At the greenhouse, the gavels were placed in a line to double array and "*tresbolillo*" arrangement, and a distance of 30 cm between plants, with a population density of 4.1 gavels•m^{-2}.

The materials used for filling the pots were VC and river sand (RS) in four volume ratios VC:RS, 0:1, 1:1. 1:2 and 1:3, v:v, each of which corresponds to T1, T2, T3 and T4, respectively. The mixtures used in each treatment (T1 – T4) were analyzed physically and chemically (Table 1). The RS was previously washed with tap water and sun-dried for 48 h, while the VC was prepared from a mixture of three types of manure (goats, horses, rabbits, 1:1:1 ratio by volume) digested by *Eisenia fetida* Saving., for three months (Atiyeh et al., 2000; Bansal and Kapoor, 2000; Ndegwa et al., 2000).

In the control treatment T1 (VC:RS, 0:1, v:v) nutrient solution was applied on the basis recommended by Zaidan and Avidan (1997) matched for the synthetic products applied. This solution was prepared with highly soluble substances of technical grade, available in the regional market [KNO_3, $Ca(NO_3)_2$, $Mg(NO_3)_2$, phosphoric acid concentrate and multi Maxiquel (Bioagro ®)], and diluted in water. The concentrations of the macroelements of this solution, applied according to phenological stage of crop, are presented in Table 2.

Considering the objective of evaluating the effect of different mixtures VC:RS on the availability of nutrients and moisture during the development of tomato, the gavels with mixtures that included VC (T2 – T4) were watered with tap water without the application of the nutrient solution, seeking to meet the metabolic needs only with VC, which contains an increasing availability of nutrients (Mangrich et al., 2000; Atiyeh et al., 2001; Gunadi et al., 2002; Valadares-Veras and Povinelli, 2004).

The water volume and frequency of irrigation applied (Table 3) in treatments T2 - T4 was determined according to: a) the content of VC in each gavel, since the use of VC as growth media promotes moisture retention (Atiyeh et al., 2000, 2001 and 2002, Cruz-Rodrigues et al., 2003); b) curves of soil moisture retention of each treatment (Figure 1); and c) considering four critical stages during the physiological development of tomato [1st stage = transplant to flowering (12 days on average), 2nd stage = flowering to fruit set (10 days on average), 3rd stage = Fruit set at the beginning of ripening (14 days on average) 4th stage = Harvest (30 days on average until the fifth cluster)]. One more physiological stage was added to the stages suggested by González-Meza and Hernández-Leos (2000) [1st stage = transplant to onset of fruit formation, 2nd stage = training fruit first cut, and 3rd stage = period harvest, which demand the greatest amount of irrigation] in response to climatic conditions and past experience in the management of this crop under greenhouse conditions in the Comarca Lagunera.

Table 1. Physical and chemical characteristics of VC and mixtures (VC, RS) used as substrates for the development of tomato.

Components (concentration)§	VC	RS (T1)	T2	T3	T4
N (%)	1.55	0.008	0.17	0.18	0.09
P (ppm)	879.12	4.49	244.75	234.15	145.85
K (meq•100 g^{-1})	14.70	0.11	2.46	1.84	0.84
Ca (meq•L^{-1})	10.67	0.05	5.28	2.14	26.70
Mg (meq•L^{-1})	12.35	0.08	1.56	1.06	0.90
Na (meq•L^{-1})	4.30	3.04	3.00	1.65	40.54
Fe (ppm)	13.08	12.72	12.96	11.91	12.06
Cu (ppm)	8.64	5.31	5.19	5.37	5.31
Zn (ppm)	8.04	2.10	4.35	3.30	2.94
Mn (ppm)	10.86	3.90	6.72	6.21	5.82
EC (mS•cm^{-1})	31.90	0.55	2.42	1.89	1.44
OM (%)	24.65	0.20	2.80	1.77	1.32
CEC (meq•100 g^{-1})	20.00	4.50	4.00	8.00	6.00
pH	8.52	7.48	8.00	7.97	7.94
BD (g•cm^{-3})	0.69	1.47	1.32	1.39	1.45
Texture	SCL	SL	SL	SL	SL

VC = Vermicompost; RS = River Sand; SCL = Sandy clay loam; SL = Sandy loam; §OM = organic matter (Walkley Black); Nt = total nitrogen Kjeldahl; P (modified Olsen); Cu, Fe, Zn and Mn (extracted by DTPA and determination by atomic absorption, Perkin – Elmer 2380); Ca, Mg y Na (soil saturation extract and determination by atomic absorption, Perkin – Elmer 2380); CEC = Cation-exchange capacity, with ammonium acetate as saturated solution; pH and EC = Electric conductivity, saturation extract and determined with Orion model 162; Texture = Bouyoucos method; BD = Bulk Density

Table 2. Concentration of macro elements in the nutrient solution.

	N	P	K
Plant phenological stage	(mg•L^{-1})		
Transplanting to flowering (1st stage)	100 – 120	40 – 50	150 – 160
Flowering to fruit set (2nd stage)	150 – 180	40 – 50	200 – 220
Fruit set at the beginning of ripening (3rd Stage)	80 – 200	40 – 50	230 – 250
Harvest (4th stage)	80 – 200	40 – 50	230 – 250

Table 3. Irrigation frequency and amount of water applied during the stages of physiological development of tomato.

Treatments	Irrigation (h)	1st stage	2nd stage	3rd stage	4th stage	TVWAPGT (L)
		Water volume applied per pot (L¶)				
AR (T1)	24	0.560	0.80	1.5	2.0	95.72
T2	72	1.0	2.0	2.0	2.0	40
T3	48	1.0	1.0	2.0	2.0	55
T4	24	1.0	1.0	1.0	2.0	96

1st stage = transplant to flowering (12 days on average), 2nd stage = flowering to fruit set (10 days on average), 3rd stage = Fruit set at the beginning of ripening (14 days on average) 4th stage = Harvest (30 days on average until the fifth cluster); h = period of time in hours between watering, ¶In each treatment, the volume of water for irrigation was performed in two applications: half in the morning (between 9:00 and 10:00 h) and half in the afternoon (between 16:00 and 17:00 h); TVWAPGT = Total volume of water applied per gavel for tomato phenological cycle.

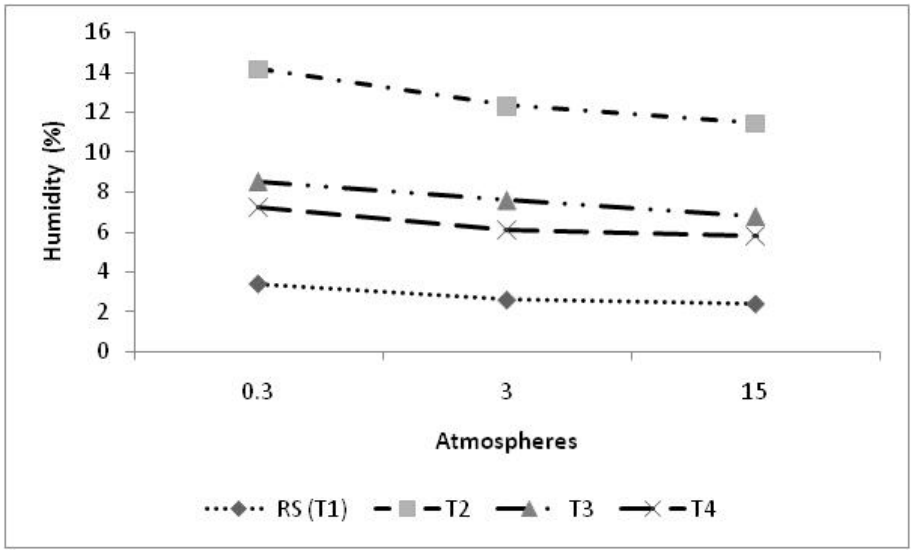

Figure 1. Water retention curves of the mixtures VC:RS used as substrates in the treatments evaluated.

Different agronomic practices were carried out during the development of the tomato crop inside the greenhouse, the most important of which were a) formation pruning (removing the side shoots, leaving one main stem per plant), b) removal of basal leaves after making the harvest of the uppermost cluster (this promotes air circulation, decreases the humidity level, and reduces the risk of disease), c) tutoring of plants (coiling the plant around a raffia fastened to the gavel and the upper structure of the greenhouse, to avoid contact of the plant with ground), d) pollination (carried out daily between 11:00 and 14:00 h, at early flowering of the crop by hand, using an electric toothbrush for placing on the stalk of the inflorescence for 3 s). The scissors used for pruning were disinfected with sodium hypochlorite solution 5% (Cloralex, AlEn ®) between sessions.

The control of pests and diseases was carried out as follows: for silver leaf whitefly (*Bemisia argentifolii*, Bellows and Perring), the use of FLY-NOT (Agrorgánicos Nacionales, SA de CV ®) (20 mL•8 L^{-1} water) and Vel Rosita detergent (Colgate-Palmolive Company ®) (1 mL•1 L^{-1} water) was implemented on a weekly basis and in periods of peak daily presence; for thrips (*Frankliniella occidentalis*), the use FLY-NOT in the same previous dose, and KALIL 95 (Sociedad de Producción Rural, Ganadera, Agrícola y Forestal, MAPIMI R.I.®) (50 mL•10 L^{-1} of water) mixed with 5 mL of oil to achieve adhesion of the product to the leaves; powdery mildew (*Leveillula taurica*), which appeared prior to the conclution of the harvest, was controlled with weekly application of Fungibac plus (Bilper Group ®) (15 mL•20 L^{-1} of water).

The fruit harvest was done weekly by hand, once they reached a pink color and until the genotype reached the fifth cluster. The treatments' effects on tomato were evaluated considering the number of fruits per plant (NFPP), number of locules (NL), equatorial diameter (ED), polar diameter (PD), pulp thickness (PT), soluble solids (SS), fruit weight (FW), and yield (R). The four treatments were repeated eight times in a completely randomized design. Data were statistically analyzed by analysis of variance and means were separated by the $LSD_{0.05}$ test (Olivares-Sáenz, 1993).

Results and Discussion

Quality variables

Five of the quality variables evaluated in the fruit -NFPP, NL, SS, PT, and R- showed highly significant difference ($P \leq 0.01$) among treatments; PD showed significant difference ($P \leq 0.05$), and both ED and FW were not significantly different among the substrates tested. Comparison of treatment means with the $LSD_{0.05}$ test showed that treatment T2 exceeded or was statistically similar to the control treatment for the variables PD, NFPP, NL, PT and R, but not for SS, where T1 was higher. Both treatments T1 and T2 surpassed the treatments T3 and T4 (Table 4). The amount of VC used in treatment T2 produced a similar effect on the results of Atiyeh et al. (2001), who reported that the growth of tomato seedlings was greater when the VC from pig manure replaced the commercial growth media Metro-Mix 360 with amounts ranging between 25 and 50% by volume.

Polar diameter

In the case of the polar diameter, the highest value was registered in treatment T2 (5.31 cm) (Table 4). Therefore, according to the standard NMX -FF-031-1997-SCFI, these tomatoes were considered medium-sized. This value was slightly lower than the range of PD (5.7 to 6.1 cm) reported by de la Cruz-Lazaro et al. (2009) during the development of hybrid-7705 Sun using compost and VC as substrates in different proportions. In the same way, the recorded diameter of 5.3 cm was also similar to those reported by Rodriguez-Dimas et al. (2008), who determined values of 6.1 and 5.4 cm in PD for Big Beef and Miramar genotypes, respectively, on treatment with the mixture VC:RS (which also exceeded the control treatment with sand and nutrient solution) highlighting that genotypes used by Rodriguez Dimas correspond to globe type tomatoes.

Equatorial diameter

In a similar behavior as the observed in a study by de la Cruz Lazaro et al. (2009), the ED of the Sun-7705 hybrid used in the present study showed no statistical differences for the evaluated sources of variation. The highest average value for ED was recorded in treatment T1 with 4.47 cm and the lowest averages corresponded to the treatments that included VC in different proportions. However, it is important to notice that in all treatments, the ED ranged between 4.03 and 4.47 cm, which is slightly below the range for this type of tomato, as reported by the same authors, whose values ranged between 4.24 and 5.10 cm when developed on growth media with different proportions of compost and VC.

The average ED of fruits grown with treatments that included VC was 4.14 cm, therefore these fruits can be classified as medium-sized. This results can be of great importance due to the desirable effect that could be achieved on fruit size of hybrid globe type tomatoes, with the use of VC in the production system; as noted by Ucan-Chan et al. (2005), the international market -specially USA- and selected national markets of Mexico prefer and pay more for fruits with ED larger than 6 cm.

Table 4. Mean values of quality variables assessed during the development of the tomato crop in mixtures of river sand and VC under greenhouse conditions.

Treatments	PD (cm)	ED cm)	NFPP	NL	SS (ºBrix)	PT (mm)	FW (g)	R ($t \cdot ha^{-1}$)
T1	5.11 ab	4.47 ns	19.87 a	2.41 ab	5.36 a	5.33 ab	57.61 ns	49.93 a
T2	5.31 a	4.28 ns	20.75 a	2.57 a	4.92 b	5.76 a	60.23 ns	50.29 a
T3	4.87 b	4.11 ns	16.37 b	2.51 a	4.37 c	4.86 b	54.89 ns	39.81 b
T4	4.88 b	4.03 ns	17.00 b	2.39 b	4.27 c	4.90 b	57.03 ns	41.04 b
Mean	5.04	4.22	18.50	2.47	4.73	5.21	57.44	45.27
SD	0.21	0.20	2.14	0.08	0.51	0.42	2.20	5.62
SEM	0.11	0.10	1.07	0.06	0.25	0.21	1.10	2.81
CV (%)	6.1	8.9	14.5	5.01	6.07	9.1	14.9	14.8

RS = River sand; PD = polar diameter; ED = equatorial diameter; NFPP = number of fruits per plant; NL = Number of locules; SS = soluble solids; PT = pulp thickness; FW= fruit weight; R = yield; SD = Standard Deviation; SEM = Standard Error of the Mean. The same letters by column correspond to non-significant differences at LSD ($P \leq 0.05$).

Number of fruits per plant

The analysis of variance of this variable, closely related to yield, showed a highly significant difference ($P \leq 0.01$) among treatments. The highest average value was obtained with treatment T2 (20.75 fruits•plant^{-1}), as shown in table 4. Considering whole digits, the descending order of treatment results for this variable was T2>T1>T4>T3, with values of 20, 19, 17 and 16 fruit•plant^{-1}, respectively.

The number of fruits per plant obtained in treatment T2 (VC without application of synthetic fertilizers) was lower only by two fruits, compared to 22.4 fruit•plant^{-1} reported by Ucan-Chan et al. (2005), who fertilized tomato crops with a nutrient solution (N 250, P 60, K 300, Ca 300, S 200, Mg 75, Fe 3, Mn 0.5, B 0.5, Cu 0.1 and Zn 0.1 mg•L^{-1}).

The range of NFPP obtained in this experiment (16 to 20 fruits•plant$^{-1)}$ was comparable to the range reported by Ramírez et al. (2005) in two experimental saladette tomato hybrids with determinate and indeterminate growth habit (18 to 25, and 16 to 23 fruits•plant^{-1}, respectively) after application of prohexadione-Ca, a growth retardant whose toxic effect on humans has not yet been fully clarified.

Number of locules

The analysis of variance showed highly significant difference ($P \leq 0.01$) for this variable among treatments with an average of 2.4 locules and a CV of 5.1%. The treatment T2 recorded the highest number of locules with 2.57, and the treatment showed the lowest number with 2.39 locules (Table 4).

Treatments T2 and T3, which included the largest concentrations of VC, statistically outperformed the control treatment T1 (artificially fertilized). As a result of application of VC to globe tomato, Moreno-Resendez et al. (2008) reported a similar behavior (5 and 4.9 locules on André and Adela genotypes, respectively) with the mixture VC:Sand; in this study, the treatment (12.5:87.5;%:% based on weight) also had a greater NL than the control group (100% sand with application of nutrient solution).

Soluble Solids

The analysis of variance for soluble solids demonstrated highly significant differences ($P \leq 0.01$) between treatments with an average of 4.73 °Brix and a CV of 6.07%. Comparison of means showed that control treatment (T1) recorded the highest value (5.36 ºBrix), followed by treatment T2 (4.92 °Brix), as shown in Table 4. Treatments T4 and T3 were statistically equal, and recorded the lowest values of SS.

Soluble solids concentration recorded in the present work, which ranged from 4.27 to 5.36 °Brix, was slightly higher than the range of 4.1 and 5.0 °Brix reported by de la Cruz Lazaro et al. (2009) when they evaluated tomato production using the hybrid SUN-7705, under greenhouse conditions by applying compost and VC mixed with sand, as growth media. Two tomato hybrids, Big Beef and Miramar, were studied by Rodríguez Dimas et al. (2008), and some results showed that in earthworm humus growth media, the concentration of SS ranged between 4.8 to 5.3 and 4.9 to 5.3 °Brix, respectively. The range of SS determined in this experiment greatly exceeded the 4.04 °Brix reported by Márquez-Hernández et al. (2008) when they used organic substrates during the production of tomato, with genotype Bosky, under greenhouse conditions.

Although the SS produced by treatments T2, T3, and T4 (which included the VC) were inferior to those of the control treatment (T1), their range of results (4.27 - 4.92 °Brix) can be fairly compared to the range that Diez (1995) established as optimal levels for tomato (4.4 to 5.5 °Brix). Therefore, it can be argued that VC application has a positive effect of on SS content.

It is possible to assume that the conductivity recorded in the VC (31.90 mS•cm^{-1}, Table 1), favored the SS content, which is consistent with the assumption reported by Goykovic-Cortez and Saavedra del Real (2007), that salinity on tomato plants causes positive effects because the fruit not only has higher acidity and carotenoid pigments, but also increases the SS contents. This benefit is of great importance, although the latter authors also have noted that the salts have adverse effects on germination and in different organs of tomato plants.

Pulp thickness

The treatments showed highly significant difference ($P \leq 0.01$) among each other when PT variable was tested. The highest value, 5.76 mm, was reached by treatment T2, followed by treatment T1, while treatments T3 and T4 showed decreasing values as VC content in the growth media was reduced (Table 4).

The results obtained in this experiment were surpassed by the values reported for globe tomatoes, genotypes Miramar and Big Beef, raised with earthworm humus by Rodríguez Dimas et al. (2008), who determined pericarp thickness of 8.4

and 7.0 mm, respectively; Márquez-Hernández et al. (2008) with genotype Bosky, using organic substrates as growth media, showed values between 7.0 and 8.9 mm. These differences are probably due to the type of tomato saladette evaluated in this experiment. However, the present study reveals that VC promotes the development of the outer covering of tomato, which could probably lead to a longer shelf life of the fruit, since the strength of the pericarp also increases fruit firmness (Mena-Violante et al., 2009).

Fruit weight

The fruit weight had no statistical difference among the different treatments evaluated. However, in Table 4 it can be seen that in treatment T2, the fruit weight was heavier than fruits from treatments T1, T3 and T4, with at least 2.6 g more of weight•fruit^{-1}. Even though there is no statistical difference, it can be noticed that as the amount of VC in the evaluated mixtures decreases, FW becomes lighter. This situation is complementary to the results reported by Atiyeh et al. (2000), who concluded that the application of small amounts of VC which were mixed with standard and high quality growth media, significantly enhanced the development of tomato plants.

The weight reported for fruits of saladette tomato from 54.89 to 60.23 g in this experiment was very similar to the ranges of 42.5 to 62.5 g and 61.3 to 63.4 g reported by Ramírez et al. (2005) for the same type, with indeterminate and determinate growth, respectively, after applying prohexadione-Ca, in order to slow plant growth, stimulate the formation of flowers and thereby increase the number of fruits. However, although in the case of this product is not clarified its mechanism of action, from the standpoint of endogenous hormone, there is evidence that other growth retardants, such as daminozide and chlormequat, have the disadvantage of a long period of persistence in plant tissue and toxicological effects to humans, which would restrict its application in organic farming systems.

Tomato yield

The analysis of variance for this variable, of great importance in the selection of an appropriate treatment to increase tomato production, recorded highly significant difference ($P \leq 0.01$) among treatments. As shown in Table 4, the maximum yield was obtained in treatment T2 (50.29 t•ha^{-1}), followed by control treatment T1 (49.93 t•ha^{-1}). Additionally, when comparing the best treatment (T2), treatmentsT3 and T4 were overpassed by 10.48 and 9.25 t•ha^{-1}, respectively.

The yields obtained in this study exceeded those reported by de la Cruz Lázaro et al. (2009), who assessed the same hybrid SUN-7705 with a mixture of vermicompost and sand 50%, and obtained 39.81 t•ha^{-1}; the difference in yield could be due to raw material and the time of preparation of VC used in each experimental work. As described above, the VC, whose chemical characteristics are presented in Table 1 (all the determinations were carried out in triplicate) used in this study was obtained after passing a mixture of three types of manure (goats, horses, rabbits, 1:1:1 ratio by volume), by a process of decomposition of 90 days, moisture was maintained to about 60–70% of water-holding capacity, watered with potable water (Mangrich et al., 2000; Ndegwa et al., 2000), while that de la Cruz Lazaro et al. (2009) used for the preparation of VC waste of cattle manure, Bahia grass (*Paspalum notatum* Flügge), and black soil (1:1:1, by volume) over a period of 60 days. Both procedures employed earthworms of the species *Eisenia fetida*.

It is necessary to comment that the maximum yield recorded in this experiment was obtained by harvesting only to the fifth cluster and was far below the 200 t•ha^{-1} reported by Flores et al. (2007), who used type saladette tomato, variety Tequila, with synthetic fertilizers, and harvested up to the eighth cluster. When averaged, the harvest per cluster obtained by Flores et al. (2007), can be estimated at 25 t•ha^{-1}, so that the yield on the fifth cluster would be 125 t•ha^{-1}; with this extrapolation, this value exceeds by 60% the maximum yield (50.29 t•ha^{-1}) obtained by treatment T2.

When talking about irrigation requirements, the above comparison becomes relevant when the productivity data of Flores et al. (2007) is extrapolated to obtain the number of kilograms of fruit per cubic meter of water used to the fifth cluster; the estimated productivity of these authors was 20.32 kg•m^{-3} (143 L•plant^{-1}), while the same variable found in the present study reached 30.66 kg•m^{-3}(40 L•plant^{-1}), as seen in Table 4. This situation corroborates that VC, as stated by Atiyeh et al. (2001) and Sharma et al. (2005), is a finely divided peat-like material with high porosity, aeration, drainage, water-holding capacity, and high microbial activity.

Conclusions

Since no synthetic fertilizers were used during the crop production, the results obtained in the present study suggest that VC, due to its physical,

chemical, and biological characteristics, is able to satisfy the nutrient demand and to reduce the volume of water required by saladette type tomato, variety Sun-7705. Therefore, VC has potential to support the growth of the vegetable species when they are used as part of the potting media.

Also, from the results obtained it is possible to assume that VC, besides it provides essential elements in adequate quantities to meet crop nutrient demand, and due to its ability to promote moisture retention, could significantly reduce water consumption during the crop cycle without adverse consequences for productivity, which is of vital importance to the economy of farmers and the development of agriculture in semi-desert and desert regions of countries like México.

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PLANT SCIENCE

Evaluation of oil-resin activity of *Copaifera* sp. on gastric emptying in *Rattus novergicus*

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Abstract

The Copaiba oil-resin (*Copaifera* sp.) is widely used in folk medicine, especially as a healing, anti-inflammatory, antiseptic, to treat ulcers and skin diseases. Increased gastric emptying was observed with oil-resin of *Copaifera langsdorfii*, highlighting those others species from the genus of *Copaifera* can act over the gastrointestinal tract. This study evaluated the activity of the *Copaifera* sp oil-resin on gastric emptying in *Rattus novergicus*. Eighteen male rats were fasted for 24h and orally received Tween-80 and Copaiba oil-resin 100 and 200 mg/kg; one hour later they all received phenol red (PR) 0.5 mg/mL. Then, the parts of gastrointestinal tract (stomach and intestine) were analyzed. The results showed that *Copaifera* sp oil-resin at 200 mg/kg increased the gastric emptying in Rattus novergicus.

Key words: *Copaifera* sp., Gastric emptying, *Rattus novergicus*

Introduction

According to Copaiba's biologic classification, this plants belongs to Leguminosae family, subfamily Caesalpinoideae, genus *Copaifera* (Brito, 2000; Cascon, 2004; Maciel, 2002; Veiga Junior, 2005; Oliveira, 2006), and there is 72 species identified (Index Kewensis, 1996). It is natural from tropical region of Latin American and Occidental Africa (Francisco, 2005). At Brazil is possible to find 16 species exclusived find there, the most are observed at the southwest, center-west and Amazonia (Francisco, 2005).

From copaiba tree is possible to obtain its oil-resin, a transparent liquid which color ranges from yellow gold to brow, depending on the specie. However, *Copaifera langsdorfii* oil-reins are red-colored (Francisco, 2005).

Oil-resin from copaibeiras (*Copaifera* sp.) has na historical importance owing to its medical uses (Pieri, 2009), being widely used at folk medicine mainly anti-inflammatory (Rigamonte Azevedo, 2004; Araújo Júnior, 2005; Brito, 2005; Freire, 2006; Pacheco, 2006; Ramos, 2006; Silva, 2006), antibacterial (Veiga Junior, 2002, 2005; Drumond, 2004; Gonçalves, 2005; Freire, 2006; Pieri, 2007), antitumor (Maciel, 2002; Veiga Junior, 2002, 2005; Rigamonte Azevedo, 2004; Araújo Júnior, 2005; Francisco, 2005; Freire, 2006; Oliveira, 2006; Pacheco, 2006; Silva, 2006;) and healing (Brito, 2000; Maciel, 2002; Veiga Junior, 2002, 2005; Araújo Júnior, 2005; Brito, 2005; Francisco, 2005; Ramos, 2006; Silva, 2006). Gastroprotection was observed with *Copaifera langsdorfii* (0.63 mL/kg) oil-resin, besides laxative effects (Brito, 2000). Those facts highlight that other species of *Copaifera*'s genus can act over the gastrointestinal tract. The plant studied by our group is popular known as white copaiba. It had already showed antiulcerogenic activity and is in identification process and chemical characterization. Preliminary data showed that the major constituents are sesquiterpenes (trans-α-bergamotene, β-bisabolene e β-selinene), chemical compouds with known gastroprotective activity.

This aim of this study was to evaluate the activity of the *Copaifera* sp oil-resin on gastric emptying in *Rattus novergicus*.

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Materials and Methods

The gastric emptying and small intestinal transit were assessed by the phenol red content assay, modified from the method described by Izbeki et al. (2001). Briefly, three groups of 6 male rats (242-292 g), were fasted for 24 h and orally received Tween-80 1% in water (5 mL/kg, vehicle control-VC) and copaiba oil-resin (100 and 200 mg/kg – experimental groups, Cop100 and Cop 200, respectively. One hour later, they all orally received phenol red 0.5 mg/mL in glucose 5 g% (1.5 mL/animal). After 20 min, the animals were euthanized with an overdose of sodium thiopental (100 mg/kg, i.p.) and the stomach and small intestine were removed. The small intestine was divided in the proximal (40%), medial (30%) and distal (30%) portions and each segment was homogenized in 100 mL of 0.1 N NaOH. Tissue proteins (in 5 mL homogenate) were precipitated with 0.5 mL of 20 g% trichloroacetic acid and centrifuged out (20 min, 3000 rpm). From the supernatant, an aliquot of 3 mL was added to 4 mL of 0.5 N NaOH and the concentration of phenol red was determined by absorbance at 560 nm (Biospectro SP-220 UV-VIS spectrophotometer, EQUIPAR Ltda., Curitiba, Brazil). The content of the dye in each segment was calculated and the retention of the marker was expressed as the percentage of the total amount of phenol red recovered in the four segments.

Statistical analysis

The data were analyzes by ANOVA and Tukey pos-test.

This study was approved by the Ethics Committee for Animal Research at the Federal University of Piauí (085/2010).

Results and Discussion

The rats from Cop200 group showed lower retention of phenol red ($p<0,05$) at stomach(G), proximal (P) and distal (D) portions of small intestine and higher($p<0,01$) at medial (M) portion of small intestine (G: 13,6 ± 2,8; P: 7,5 ± 1,2; M: 64,2 ± 10,0; D: 5,8 ± 0,8) (see Figure 1; Figure 2; Figure 3; Figure 4), when compared with the animals from VC group (G: 30,3 ± 3,1; P: 19,3 ± 2,1; M: 35,1 ± 3,5; D: 13,3 ± 1,9) (see Figure 1; Figure 2; Figure 3; Figure 4). The animals from Cop100 group did not presented significantly retention of phenol red (G: 37,7 ± 3,8; P: 25,5 ± 2,4; M: 26,0 ± 2,8; D: 11,3 ± 1,1) compared with VC group (see Figure 1; Figure 2; Figure 3; Figure 4).

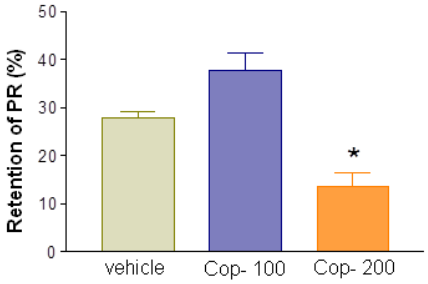

Figure 1. Shows the perceptual of phenol red at the stomach of the three groups evaluated.
*p<0,05 compared to vehicle (ANOVA and Tukey).

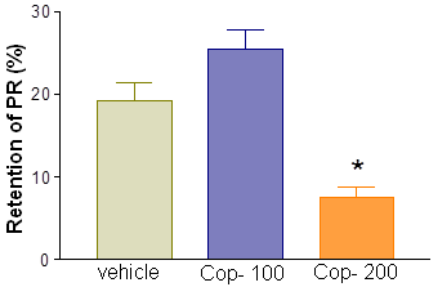

Figure 2. Shows the perceptual of phenol red at the proximal intestine of the three groups evaluated.
*p<0,05 compared to vehicle (ANOVA and Tukey post-test).

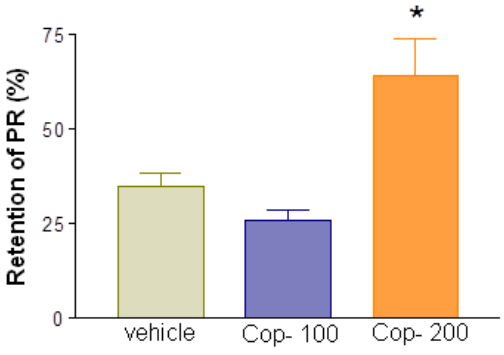

Figure 3. Shows the perceptual of phenol red at the medial intestine of the three groups evaluated.
*p<0,05 compared to vehicle (ANOVA and Tukey post-test).

The effect of a substance depends on the amount administered, but if the dose selected is below the subliminal dosing, the effect will be absent (Brunton, 2006). Depending on the nature of the effect to be measured, ascending doses may cause the effect to increase in intensity. That is why every drug has a graded dose response curve that

expresses a certain response to increasing doses of the chemical agent. (Lüllmann, 2000; Silva, 2002; Brunton, 2006; Gary, 2006; Katzung, 2006).

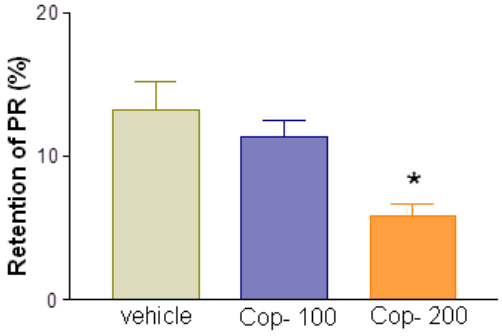

Figure 4. Shows the perceptual of phenol red at the distal intestine of the three groups evaluated.
*p<0,05 compared to vehicle (ANOVA and Tukey post-test).

In fact, the intensity of a pharmacologic response is proportional to the number of receptors with which a drug interacts (Silva, 2002; Katzung, 2006). The receptors denote the component of the organism with which the medicament is presumed to interact, modifying the function of the body component and thereby initiating biochemical and physiological changes that are characteristic of the response to the drug (Lüllmann, 2000; Brunton, 2006; Gary, 2006; Katzung, 2006). So, even if at a certain dose the drug produces no response, response can be achieved with higher doses (Lüllmann, 2000; Silva, 2002; Brunton, 2006; Gary, 2006; Katzung, 2006).

The elimination of the copaiba of the human body is made by the lungs, kidneys, sebaceous and sweat glands (Veiga Junior, 2005; Opção Fênix, 2011). In large quantities it can cause side effects like gastrointestinal irritation, vomiting, nausea, salivation, diarrhea, irritative effect in the peritoneum, causing adhesions and abscess formation and cavitary central nervous system depression. In normal doses it has anti-inflammatory effects without causing gastric damage. Thus, it becomes an agent clinically safe and potentially useful (Veiga Junior, 2007; Pinto, 2002; Opção Fênix, 2011).

As a drug, the oil-resin of Copaifera sp. is not different. Scientific studies showed that administration of 0.4 ml of the oil-resin via transdiaphragmatic caused diarrhea (Sousa, 2000). Researches with commercial copaiba oil in rats showed the occurrence of diarrhea, weight loss and irritating action on the behavior of rats at doses of 0.63 ml / kg. It is also known that gastrointestinal irritation, diarrhea, drooling and central nervous system depression are adverse effects of high doses of the oil (Veiga Junior, 2005).

Therefore, due to the proven link between the plasma concentration of the drug (which depends directly on the dose) and the proportion of the desired therapeutic effect (Brunton, 2006), the oil-resin of *Copaifera* sp., at 200 mg/kg, increased significantly the speed of gastric emptying in *Rattus novergicus*, however, at 100 mg/kg no kinetic activity was showed by *Copaifera* sp. oil-resin.

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ANIMAL SCIENCE

Effect of Cameline Nisin isolated from *Lactococcus lactis* sub sp. lactis on *Staphylococcus aureus* sp.

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Abstract

The camel milk differs from milk of other species by the presence of a powerful protector system, linked to relatively high levels of lysozyme, lactoperoxidase, lactoferrin, component-3 of proteose peptone (PP3), organic acid, hydrogen peroxide and bacteriocins produced by lactic acid bacteria. The study aims to estimate the part of a bacteriocin (type nisin) produced by a strain of *Lactococcus lactis* sub sp lactis isolated from camel milk, in this protective system and its antibacterial activity against a strain of *Staphylococcus aureus* isolated from a bovine milk sample. Optimize conditions for a nisin production, by the producing strain isolated and purified, was carried out, by glucose addition (1%) to the M17 medium. Antagonism tests by the disk method showed inhibition zones (IZ Ø) from 6 to 8mm in diameter depending on the incubation time of the producing strain. Thus, for incubation periods of 18h, the production of camel nisin appeared to be maximum (IZ= Ø 8mm) with a minimum growth of *Lactococcus lactis* sub sp lactis (pH 6, 37). Optimum growth of camel *Lactococcus lactis* sub sp lactis strain (pH 6.17) and an optimum production of nisin (IZ= Ø 8mm) were registered for a 24 hours incubation period.

Key words: Bacteriocins, Camel, *Lactococcus lactis* sub sp lactis, Milk, *Staphylococcus aureus*

Introduction

Milk represents about 22% of total food imports in our country. Algeria is thus the second importer of milk and derivatives, after Mexico. It has therefore need every resource in milk, namely that of the goat, sheep and camel particularly adapted to the harsh agro-climatic conditions of the Sahara.

The camel milk has a balanced composition in basic nutrients (proteins, carbohydrates and lipids). This composition is similar to that of bovine milk. It also provides adequate concentrations in minerals and vitamins including vitamin C and niacin, which makes it a nutrient medium favourable to the development of undesirable microbial species such as *Staphylococcus aureus* responsible for mastitis infections and food intoxication.

To defend itself against the development of these microorganisms, camel milk is characterized by a powerful protector system due firstly, to whey protein (lysozyme, lactoferrin, lactoperoxidase, immunoglobulins and component 3 of proteose peptones (PP 3) and secondly to antibacterial substances produced by these lactic acid bacteria (Klaenhammer et al., 1994). These substances include organic acids, oxygen peroxide and bacteriocins purpose of this study.

Materials and Methods

Four samples of camel milk of the Sahrawi population living in extensive in the region of Ouargla were used. They were transported to the microbiology laboratory in a cooler with an ice pack. Bovine milk samples provided by a farm near the laboratory were also used in this study.

The isolation of a lactococci strain was performed on M17 medium and a *Staphylococcus aureus* strain on Chapman's hypersaline medium.

Choice of the target strain

Staphylococcus aureus strain was chosen for:

- sensitivity to bacteriocins (Choi et al., 2000; Allouche et al., 2010) including nisin (Raimbiault, 1995, Sharma et al., 2010);
- belonging to the pathogenic contamination flora in food products (Trias et al., 2008). It is a

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species that can be found in mastitis milk, (Larpent et al., 1997);
- resistance to antibiotics (Rahal, 1984);
- its property "catalase +" to eliminate the inhibitory effect of H_2O_2 in antagonism tests and in mixed cultures;
- its pathogenicity for humans and animals
- its exigency of a selective medium (Chapman medium)

Culture and purification of nisin producing strain and the target strain

Producing strain grew on M17solid medium for 24 to 72 hours at 30°C, the target strain on Chapman medium for 24 h at 37°C.

After incubation, both strains were tested for identification and purified by 4 to 5 subculturing by streak of exhaustion

Propagation of the *Lactococcus lactis sub sp lactis* strain

To prepare a mother -culture, for the production of bacteriocin (nisin), it was preceded to the "propagation of the strain" by the realization of a preculture. This was done by using a protocol reported by Doumandji et al. (2010).

The propagation of the strain consisted in inoculate a colony previously purified from *Lactococcus lactis* (inoculum) in 1 ml of M17 broth and incubated at 30°C for 24 hours: the "intermediate culture 1" was obtained. After incubation, it was proceeded to a second subculture which was transplanted with 1 ml of the previous culture into a tube containing 9 ml of M17 medium: a second culture: "intermediate culture 2" was obtained.

Optimization culture conditions of *Lactococcus lactis subsp lactis*

Supplementation of culture medium with glucose, magnesium, peptone, and yeast extract enhances production of bacteriocins (Heng et al., 2007). Since the - M17 medium contained three types of peptone, yeast extract and magnesium sulfate, only Glucose was added (Heng et al., 2007; Sharma et al., 2010).

Another factor may influence the bacteriocins production. Indeed, Sharma et al. (2010), reported that a maximum growth of *Lactococcus lactis subsp lactis* was observed after 24 h incubation at 35 ° C. Because bacteriocin production is a growth-dependent physiological trait and hence follows primary metabolite kinetics (De Vuyst and Leroy, 2007), two incubation temperatures were used in the present study: 18 hours (usually used) and 24 hours. Two lots were obtained, each lot being divided into two sublots (Table 1).

Table 1. Constitution of the experimental lots of the nisin-producing strain culture.

Lot N°	Sublots	Glucose addition (1%)	Incubation period
1	a	-	18 h
	b	-	24 h
2	a	+	18 h
	b	+	24 h

a: incubation for 18 h **b**: incubation for 24 h

Nisin isolation

After incubation for 24 hours and 18 hours, experimental lots of culture strains were subjected to centrifugation at 8000 r.p.m. (Universal 16 R), for 10 minutes at 4°C.

The resulting supernatant was neutralized to pH 6.5 with NaOH (5N) to remove the antibacterial activity which could be exerted by organic acids (Nykanen et al., 2000).

Study of the antimicrobial activity of bacteriocin from the supernatant

The agar diffusion test by the disc method was performed to investigate the effect of nisin produced by *Lactococcus lactis sub sp lactis* contained in the supernatant on *Staphylococcus aureus* strain.

Results and Discussion

Identification of the strain isolated on M17 and those - isolated on hypersaline Chapman medium

Macroscopic and microscopic examinations and physico-chemical and biochemical tests and biochemical identified the strain of lactic acid producing nisin, as a strain of *Lactococcus lactis subsp lactis* (Plate 1). Other macroscopic and microscopic examination, allowed identifying the target strain, as a strain of *Staphylococcus aureus* (Plate 2).

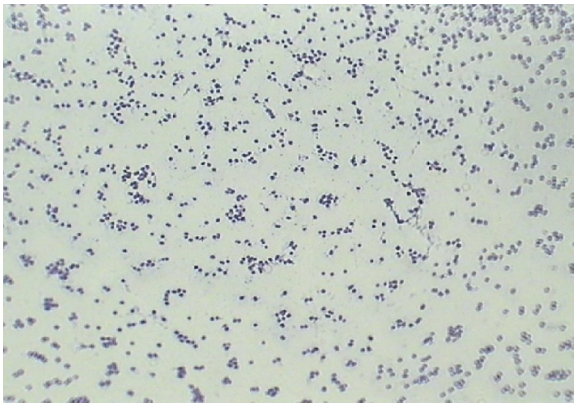


Plate 1. Lactococcus strain after Gram staining (camel milk) (G x 100).

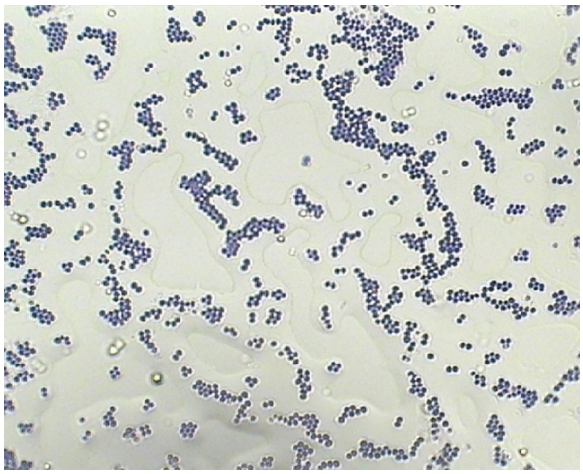


Plate 2. *Staphylococcus aureus* strain after Gram staining (G x 100).

After 4 to 5 successive subcultures of previously isolated strains, pure colonies were obtained.

Culture conditions and optimization of the producing strain

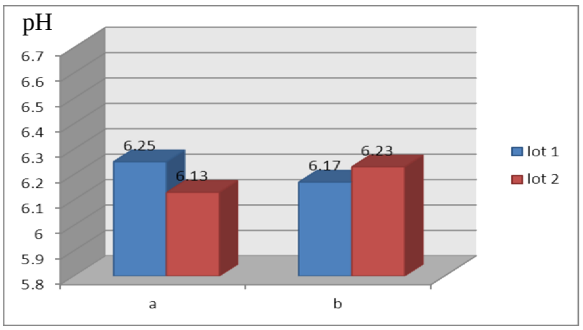
This step was performed with the primary aim to get optimal production of nisin. Bacteriocins production is physiologically dependent on the growth of the producer strain (De Vuyst and Leroy, 2007). This is related to the metabolic activity of the strain, itself linked to the production of lactic acid (Beal et al., 1994) and thus decreasing pH.

Biomass separation

After centrifugation, bacterial cultures and biomass separation, the two lots from supernatants of *Lactococcus lactis subsp lactis* were recovered and the pH measured (Figure 1).

The pH measurement of the supernatant indicated that:

- The glucose addition in a culture of *Lactococcus lactis subsp lactis* incubated for 18 hours increased its growth;
- The extension of the incubation period to 24 hours with the glucose addition had no effect on the growth of the strain;
- the chnage of the incubation period from18 hours to 24 hours of a pure culture of *Lactococcus lactis subsp lactis* enhanced its growth .



a: incubation for 18 hours
b:incubation for 24 hours

Figure 1. pH values of supernatant lots.

Antibacterial activity of supernatants

Tests by the disc method allowed revealing antibacterial activity (Plate 3). Indeed, in all Petri dishes, the IZ appearance was observed. The ZI, recorded in this study was relatively small compared to those reported in the literature. They were between 6 and 8 mm (Keramane, 2009; Doumandji et al., 2010). This result coud be caused by the possibility of partial adsorption of the peptide molecules of nisin on the *Lactococcus lactis subsp lactis* wall and thus its elimination with the pellet (Holo et al., 2002; Rajaram et al., 2010; Sharma et al., 2010). It may also be due to the low concentration of nisin in the supernatant (Holo et al., 2002). Moreover, the diameter of inhibition zones varies with the type of culture medium used and the species used as indicator strain or target strain (Trias et al., 2008).

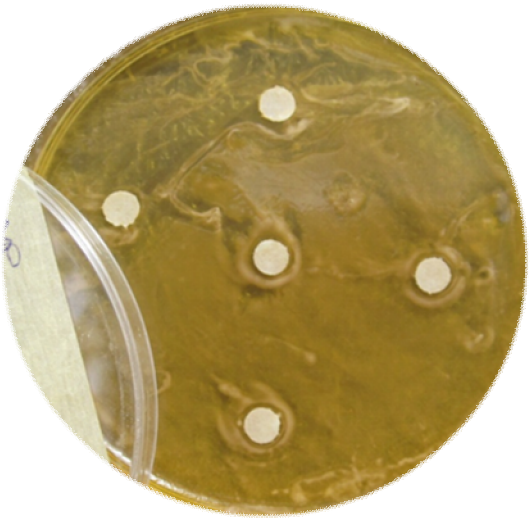


Plate 3. IZ appearance by the disks test.

Nevertheless, the IZ appearance indicated that there was an antibacterial effect of supernatant against *Staphylococcus aureus* strain. Remembered that the antibacterial effect of organic acids, diacetyl and that of H_2O_2, has been discardes by the neutralization of supernatants and by the property of the target strain (catalase +), diacetyl having inhibitory activity against the Gram negative rather than against Gram positive. This means that the appearance of IZ was only due to the production of bacteriocin (type nisin) by *Lactococcus lactis subsp lactis* isolated from camel milk.

For a 24 hours incubation, without glucose addition, better growth (pH 6.17) and increased production of nisin (IZ = Ø 8mm) were recorded.

This result agrees with those of Siboukeur (2007) and Siboukeur and Mati (2008) in which the evolution of the halotolerant contamination flora, during storage at room temperature (30°C on average) has allowed to highlight the appearance of self-purification aspect particularly effective in this milk. The microbiological study undertaken by the same authors showed that the rate of this halotolerant flora decreased during the first three days of storage, while that of lactic acid bacteria tended to increase. This result highlighted the part of bacteriocins in the particular protector system of camel milk.

Conclusion

This study showed that the natural system of camel milk was reinforced by considerable action of nisin produced by *Lactococcus lactis subsp lactis* species. This bacteriocin was particularly effective against one species may accidentally contaminate milk (*Staphylococcus aureus*) and which have developed a resistance to antibiotics according to many authors. It appeared that the synergistic effect of whey protein, organic acid, H_2O_2 and nisin, were responsible for the self-purification effect of camel milk stored, and who keeps it for many hours in relatively high temperatures (about 28°C).

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ANIMAL SCIENCE

Monitoring of monthly SCC in she-camel in relation to milking practice, udder status and microbiological contamination of milk

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Abstract

Somatic cell counts and bacteriological examinations were measured in 28 camel milk samples from 2 farms, one with milking machine (farm A) and the second with hand milking (farm B). The milk was analyzed for 6 months after the parturition, every month, the first one occurring one week approximately after delivery. The somatic cell count was higher at the first sampling in the two farms but significantly more in farm B. The microbiological contamination was also higher in farm B (37% samples were contaminated) than in farm A (12%). The somatic cell count decreased all along the lactation stage and increased with the parity but the trends were not significant due to the high variability of the values. On average, the normal level of somatic cell counts is low compared to cow.

Key words: Camel, Subclinical mastitis, Bacterial contamination, Somatic cell count

Introduction

The consumption of camel milk is a long-standing tradition in Saudi Arabian and the demand for camel milk and other products in the Kingdom and elsewhere is rising (Faye and Bonnet, 2012). Even if many factors affect milk production and quality, such as breed, age, management, nutrition, parity, and lactation stage, mastitis is the single most important factor affecting production in dairy animals, including camels. While clinical mastitis can be easily recognized, subclinical mastitis almost always passes unnoticed, which accounts for its high prevalence among lactating camel herds in many countries (Guliye et al., 2002; Mohammed et al., 2005; Abdel Gadir et al., 2006; Hawari and Hassawi, 2008; Abera et al., 2009).

Traditional hand milking, use of anti-suckling devices, presence of teat lesions, and failure to apply basic hygienic measures are important predisposing factors for mastitis in camels (Mohammed et al., 2005; Abdel Gadire et al., 2005).

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Somatic Cell Count (SCC) is widely used in cow for monitoring the milk quality in dairy industry. In camel, the use of SCC is not very common, but was applied as indirect diagnostic tool for detecting uninfected and infected quarters (Abdurahman, 1995; Abdurahman et al., 1995; Saleh and Faye, 2011). However, because the basal levels of cells and their physiological variations are not yet established in this species (Abdurahman et al., 1992), the interpretation could be problematic. Notably, the variation along the lactation was rarely documented. The aim of the present work was to monitor monthly SCC in camel milk for six month after calving and udder contamination in two farms with similar practices except the milking practice, hand milking *vs* machine milking.

Materials and Methods

Animals

Fourteen lactating camels (*Camelus dromedarius*) were kept at the farm of Camel and Range Research Center (farm A) at Al-Jouf region (Saudi Arabia) where animal was milked by machine and fourteen lactating camels from one surrounding herd milked manually (farm B). The lactating camels were of various parties and suckling their calves and they were housed together and fed with similar diet, i.e. alfalfa, concentrates and hay. All camels were free from clinical mastitis and any abnormalities in udder and teats. The mean parity was similar in the both farm (3.30) but the

distribution was different (Figure 1). The calving season was concentrated between October and April in the both farms and the distribution of parturitions was similar also (Figure 2).

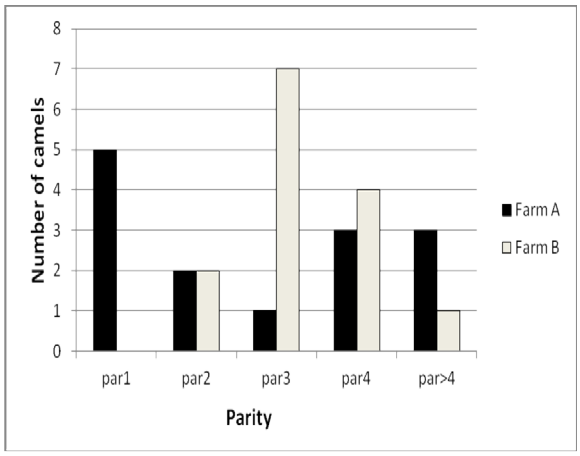

Figure 1. Distribution of the camel parities between farms A and B.

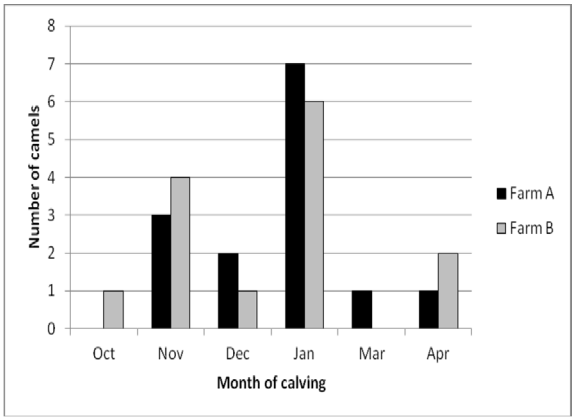

Figure 2. Seasonal distribution of the camel calving in farms A and B.

Sampling procedure

A total of 168 milk samples from the 28 lactating camels were collected at the morning milking through six months after calving to monitor the monthly somatic cell count (SCC) and bacterial contamination in milk. The camel calves were allowed to suckle in order to stimulate milking. The udder and teats were washed and cleaned with 70% alcohol. The first few strips of milk were discarded. About 15ml of milk (four quarters mixed) was then collected into sterile glass vials. The samples were kept on ice during transportation. The milk samples were subjected to bacteriological isolation and also tested for SCC.

Bacteriological examination

An aliquot of the milk samples (0.01 ml) from each animal were streaked on blood agar and Baird-Parker agar plates. Plates were incubated for 24-48 h at 37°C. The plates were then examined for growth colony morphology. Individual colonies will be picked for identification according to the Scandinavian recommendations on examination of bovine milk samples (Klastrup, 1975).

Somatic Cell Count (SCC)

The somatic cell counts (cell/ml) for the milk samples were determined using NucleoCounter SCC-100 (coulter electronic-Chemometec A/s, Denmark). As the SCC values are log-normal (Shook, 1982), means and S.D were calculated after logarithm transformation of the raw data (Neperian logarithm base e=2.718). However, the values were expressed in cells/ml to be more explicit. To get values in cells/ml, the inverse transformation according to the function e^x was applied.

Statistical analysis

To compare the monthly results between farms A and B, a variance analysis on repeated measures (ANOVA) was applied on the log-transformed data. When the number of cells/ml was below the detection limit of the SCC counter (10,000 cells/ml), the value of 10,000 was retained for calculation.

For all statistical analysis, the XLstat software (Addinsoft©) was used (repeated anova procedure)

Results

Bacteriological examination

Regarding the bacteriological findings, intramamary infections were presented in 19 (12%) of the 84 milk samples collected in farm A and in 31 (37%) of the 84 milk samples examined from camels in farm B (Table 1). Coagulase negative staphylococci (CNS) and micrococci represent 17.8% and 4.8% of the isolates recovered from collected milk samples in farm A respectively and 26.2% and 7.1% of the isolates recovered from collected milk samples in farm B respectively. While *Staphylococcus aureus* represent 3.6% of the isolates in farm B where milking procedure was done by workers hand, *S. aureus* was not isolated in farm A which used machine during milking process.

Table 1. Bacteriological finding of camel udder milk samples in two farms with different milking process.

Isolated bacteria	Farm A		Farm B	
	Milking process by machine		Milking process by hand	
	No.	%	No.	%
Coagulase-negative Staphylococci	15	17.8	22	26.2
Staphylococcus aureus	-	-	3	3.6
Micrococcus	4	4.8	6	7.1
No growth (non infected)	65	77.4	53	63.1
Total	84	100	84	100

Somatic cell count

SCC varied from 11,000 to 298,000 cells/ml. in farm A and from 14, 000 cell/ml to 643,000 in farm B after parturition. The values decreased highly after parturition in both farms (Table 2), but this decrease was more marked in farm B. The difference between the farm A and farm B was significant at month 1 ($P < 0.05$) and 3 ($P < 0.001$) (Figure 3). After 6 months, the values were similar in the 2 farms (approximately 15,000 cells/ml).

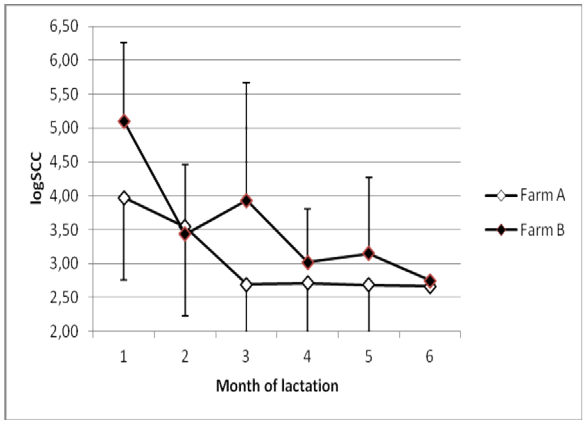

Figure 3. Somatic cell count (in log) in camel farms A (milking machine) and B (hand milking) according to the physiological stage.

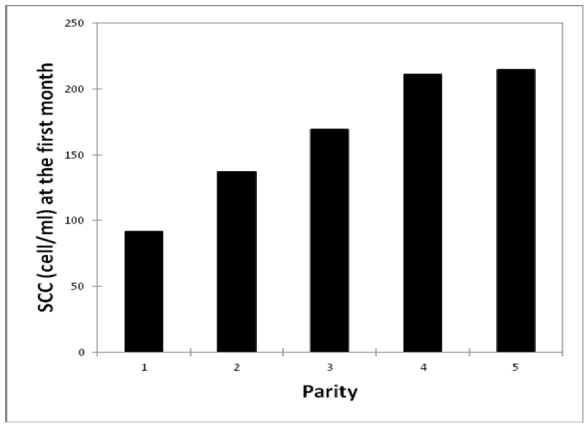

Figure 4. Somatic cell count (in cells/ml) in camel milk according to the parity of the camel.

At the month 1, the SCC increased with parity, passing on average from 91.000 cells/ml. in primiparous camel to 115.000 for parity more than 4, but the difference was not significant (Figure 4). By including all the data along the 6 first month of lactation, no significant change was observed.

Regarding the seasonal variations, there was a tendency of SCC decreasing all along the year (Figure 5) since October but it was no significant. The SCC appeared higher in camel with early calving (October and November) and in late calving (April), but this trends were not significant also (Figure 6).

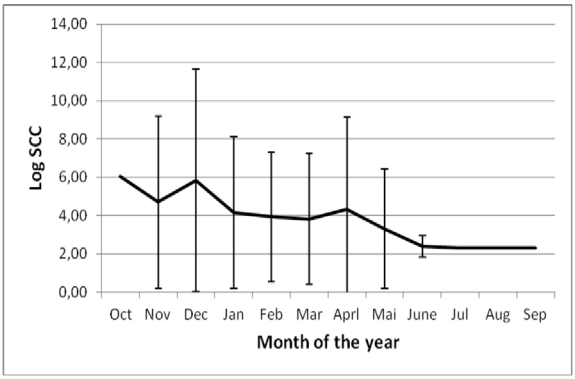

Figure 5. Seasonal variation of somatic cell count (in log) in camel milk.

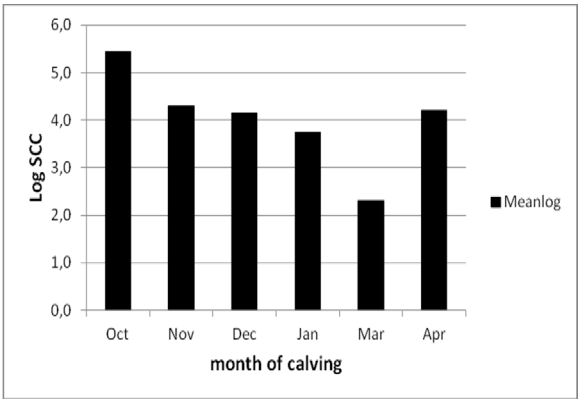

Figure 6. Somatic cell count (in log) in camel milk according to the month of calving.

Table 2. Compared values of mean SCC in camel milk samples in two farms with different milking process.

	Monthly mean SCC/ml.					
	M1	M2	M3	M4	M5	M6
Farm A	53,068	34,628	14,762	15,090	14,721	14,372
Farm B	164,110	30,929	50,843	20,461	23,292	15,571

Discussion

Udder health status of camel

The present results showed a clear difference between the udder health status in the two sampled farms, the farm with hand milking being more affected than the farm with milking machine, at least at the beginning of the lactation. However, the type of milking was not probably only in cause. Indeed, the general hygiene would be more probably responsible of the results revealing that herd B with poor hygiene of milking process had a higher prevalence of subclinical mastitis (Aljumaah et al., 2011) and intramammary infections. Indeed, poor hygiene during milking is identified as a risk factor for occurrence of mastitis as well in bovine (Abdurhman, 2006) as in camel (Tourette et al., 2002; Saber et al., 2010).

This might be due in farm B to absence of udder washing, milking of she camels with milkers which have cuts and chaps on their hands and using of common udder cloths, which could be vectors of spread especially for contagious mastitis. The bacteriological findings were comparable with the results obtained on SCC. CNS and *S. aureus* were the main bacteria recovered in farm B using milking by hand.

Bacterial contamination of camel milk

Kospakov (1976) isolated staphylococcal strain from udder tissue, bulk milk and udder skin of Bactrian camels. The microorganism found in the present study was regarded as important pathogens causing mastitis in dromedary camels (Bakhiet et al., 1992; Abdurahmann, 1996; Abera et al., 2010).

As described by Younan et al. (2001), the prevalence of Staphylococci varies according to different studies, but there is nearly no publication on bacteriological hygiene of milk where Staphylococci are not mentioned (Eberlein, 2007).

Also, Radostits et al. (2000) asserted that *S. aureus* was well adapted to survive in the udder and usually establishes a mild subclinical infection of long duration, from which it shed in milk, facilitating transmission to healthy animal mainly during milking process. This agrees with the data presented in this study.

Somatic cell count variation

SCC in milk is widely used as an indicator of the degree of inflammation of the udder and to predict udder infection since long time in cattle (Poutrel and Rainard, 1982). It is also the basis of most indirect tests for subclinical mastitis. The references in camel are more recent (Merin et al., 2004) and the variation factors, except in case of intramammary infections were not widely studied.

In our study, the first sampling occurring one week after parturition had on average the highest values. The first stage of lactation could be associated with decreased resistance of mammary gland to infection as result of immune depression following the stresses and hormonal changes that occur around the time of parturition and onset of lactation may leads to high prevalence of subclinical mastitis (Sordillo, 2005; Burvenich et al., 2007). At reverse, the change within lactation characterized by a significant decrease after the first month was not reported in cow where, on average, the pattern was reverse to lactation curve (Serieys, 1985).

An increase in the number of SCC in camel milk with infected quarter has been reported by Mostafa et al. (1987). The increase of SCC or mastitis with age of dairy animals or parity was widely observed in cow (Serieys, 1985; Faye et al., 1986) like in our study on camel in spite it was not significant due to the high variability within parity.

Conclusion

It appeared that camel SCC can be in less quantity than in normal cow milk as many of our samples are below the detection threshold of 10,000 cells/ml. Anyway, it is recommended that in order to reduce the prevalence of mastitis, improved milking hygiene, prevention of skin lesions, culling of chronic mastitis carriers and treating of clinically infected she camel should be practiced. Post calving milk SCC could be useful surveillance tool for monitoring mastitis, although the values require appropriate interpretation due to the lactation stage dependent physiological variation after calving.

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