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Short Communications

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Effect of Different Levels of Foliar Application of Potassium on Hysun-33 and Ausigold-4 Sunflower (*Helianthus annuus* L.) Cultivars under Salt Stress

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(received March 20, 2012; revised May 8, 2013; accepted May 27, 2013)

Abstract. A hydroponic study was conducted to see the growth response of two cultivars of sunflower (Hysun-33 and Ausigold-4) to K^+ nutrition under salt stress during the growing season 2011, at National Agriculture Research Centre, Islamabad, Pakistan. Nursery of *Helianthus annuus* was raised in sand and ten-day old seedlings per hole were transplanted in each pot having four holes per pot lid. Half strength Hoagland's nutrient solution was filled in each pot. After the establishment of seedlings, salt stress (6 dS/m) was developed artificially. The treatments were, control, 2 and 4 % K^+ as K_2SO_4 foliar applications. Salt present in the growing medium caused a significant (P<0.00l), reduction in fresh and dry weights of sunflower. Salt stress suppresses the K uptake from pot. Application of varying levels of K_2SO_4 improved the fresh and dry weights of sunflower under both control and saline conditions. However, the highest increase in fresh and dry weight of control and stressed plants was observed when 2% K was applied. Further increase in the level of K application did not improve fresh and dry weights of salt stress and unstressed plants. The growth medium salts reduced sunflower growth.

Keywords: *Helianthus annuus*; salinity; K foliar application; biomass

Introduction

The demand for oil seeds has increased several times for the last few years but the acreage cannot be increased due to the increasing competition with major cereal crops. Sunflower is one of the four major oilseed crops (soybean, peanut, rapeseed and sunflower) grown for edible oil in the world, which is cultivated on about 23.31 million hectares (mha) all over the world. In Pakistan, it is grown on about 0.363 mha (MINFA, 2010). Oil seed crops are mostly grown on marginal lands and the productivity of oilseed crops is much less on saline soils because salinity exerts a number of adverse effects on plants including osmotic effects, ion toxicity and nutritional imbalance resulting in reduced growth and yield (Riaz *et al*., 2008; Munns, 2005; 2002; Ashraf, 2004). High external Na^+ inhibits the uptake of other nutrients particularly K^+, by interfering with the transport mechanism at the root plasma membrane such as K^+ selective ion channels (Tester and Davenport, 2003). Potassium ions constitute the most important macronutrients taken up by plants in salt-affected soils. Na^+ competes with K^+ and reduces its uptake and causes potassium deficiency (Carden *et al.*, 2003). The capacity of plants to counter balance salt stress depends largely on the status of their K nutrition because it plays a vital role in many cell processes such as enzyme activity, cell turgor, regulation of stomatal movement and maintenance of osmotic pressure (Shabala *et al.*, 2005; Shabala, 2003). Potassium increases the protein content, improves the efficiency of water use and produces resistance to diseases and insects. However, salinity stress greatly reduces the uptake and translocation of nutrient ions like K^+ and Ca^{2+} (Nawaz *et al*., 2002; Rangel, 1992). These problems can be tackled by growing the tolerant crops i.e., the crops able to produce high yields on such marginal lands (Sandhu and Qureshi, 1986). Salt-affected soils comparatively demand more nutrients for plant growth and optimum yield. The major fraction of potash fertiliser directly applied to soil gets fixed with clay fraction and becomes unavailable to crop plants (Ali *et al*., 2005). Further, the price of K fertilisers is increasing and is becoming unaffordable to farmers (NFDC, 2005). Keeping in view the economic importance of sunflower as oil seed crop, hydroponic experiment was conducted to test foliar application of K_2SO_4 using two sunflower cultivars (Hysun-33 and Ausigold-4) under salt stress during the growing season 2011, at Soil Salinity Laboratory, Land Resources Research Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan.

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Materials and Methods

Nursery of both cultivars was raised in sand at NARC and 10-day old one seedling per hole was transplanted in each pot having four holes per pot lid. Half strength Hoagland's nutrient solution was filled in each pot and after the seedling establishment; a salt stress of 60 mM (6 dS/m) was developed artificially. The treatments were 0, 2 and 4 % K^+ as K_2SO_4 foliar application. Crop was grown up to 4 weeks and data on fresh biomass per plant was recorded at the time of harvest. Plant samples were dried in oven at 60 ºC to a constant weight and the dry matter yield was recorded. Ground plant samples were digested in diacid (perchloric-nitric acid 2:1 ratio) mixture (Rhoades, 1982) to estimate Na and K by atomic absorption spectrophotometer (Perkin-Elmer, 4000). The experiment was set up in completely randomised design (factorial) with four replicates. The data obtained were subjected to statistical analysis using MSTAT-C and the treatment means were compared using Duncan's Multiple Range (DMR) test (Gomez and Gomez, 1984).

Results and Discussion

The fresh and dry weights of two sunflower cultivars are given in Table 1-2. Application of varying levels of K_2SO_4 improved the fresh and dry weights of sunflower under both normal and saline conditions. However, the highest increase in fresh and dry weight of control and stressed plants was observed with 2% foliar application of K as K_2SO_4. Sultana *et al*. (2001) concluded that foliar application of nutrient solutions partially alleviates the adverse effects of salinity and yield related components through mitigating the nutrient demands of salt-stressed plants. Further increase in the level of K application did not improve fresh and dry weights of salt stressed and unstressed plants. The concentrations of Na^+ and K^+ in sunflower tissue are presented in Table 3-4. Salt stress suppresses the uptake of K from soil but foliar application of K_2SO_4 improved the growth of sunflower cultivars under both control and saline conditions. However, the highest increase of control and stressed plants was observed when 2% K applied as a foliar spray. These findings are supported with the results of Kaya *et al*. (2001a). They suggested that supplementary P and K can reduce the adverse effects of high salinity on plant growth and physiological development. Further increase in the level of K application did not further improve the growth of salt stressed and unstressed plants.

Table 1. Fresh biomass (g/plant) of sunflower as affected by foliar application of K

Treatments	Salinity levels (dS/m)					
	0			6.0		
	Hysun-33	Ausigold-4	Mean	Hysun-33	Ausigold-4	Mean
Control	1.26c	1.28c	1.27C	1.17c	1.19b	1.18C
2% K	1.62a	1.55a	1.59A	1.55a	1.76a	1.67A
4% K	1.58ab	1.37b	1.48AB	1.33b	1.77a	1.57AB
Mean	1.49	1.4		1.35B	1.57A	

Means followed by different letter (s) within the columns differ significantly at 1% level of significance.

Table 2. Dry weight of sunflower (g/plant) as affected by foliar application of K

Treatments	Salinity levels (dS/m)					
	0			6.0		
	Hysun-33	Ausigold-4	Mean	Hysun-33	Ausigold-4	Mean
Control	0.04	0.05	0.045B	0.05	0.06	0.045C
2% K	0.07	0.06	0.065A	0.07	0.08	0.075A
4% K	0.05	0.07	0.060A	0.06	0.07	0.065B
Mean	0.05	0.06		0.06	0.07	

Means followed by different letter (s) within the columns differ significantly at 1% level of significance.

Table 3. Na Concentration (%) in sunflower tissue at harvesting

Treatments	Salinity levels (dS/m)					
	0			6.0		
	Hysun-33	Ausigold-4	Mean	Hysun-33	Ausigold-4	Mean
Control	0.30bc	0.43b	0.35AB	0.43bc	0.58bc	0.50BC
2% K	0.43b	0.43b	0.43A	0.53b	0.65b	0.59B
4% K	0.80a	0.53a	0.44A	0.73a	0.83a	0.78A
Mean	0.51		0.45		0.56B	0.68A

Means followed by different letter (s) within the columns differ significantly at 1% level of significance.

Table 4. K Concentration (%) in sunflower tissue at harvesting

Treatments	Salinity levels (dS/m)					
	0			6.0		
	Hysun-33	Ausigold-4	Mean	Hysun-33	Ausigold-4	Mean
Control	2.88c	2.40bc	2.64C	3.73bc	2.70c	3.21C
2% K	4.18b	2.95b	3.56B	3.98b	3.43b	3.70B
4% K	6.50a	3.43a	4.96A	5.53a	4.68a	5.10A
Mean	4.53A	2.92B		4.43A	3.60B	

Means followed by different letter (s) within the columns differ significantly at 1% level of significance.

The growth medium salts caused a marked reduction in growth of sunflower. However, application of potassium sulphate improved the growth of sunflower plants under both non-saline and saline conditions. Kaya *et al*. (2001b) suggested that sodium concentration in plant tissues increased in both cultivars of strawberry in the high NaCl treatment. Concentrations of P and K were in the deficient range in plants grown at high NaCl and these deficiencies were corrected by foliar application of these nutrients under salt stressed conditions. These results can be correlated to the findings that exogenous application of potassium offset the adverse effects of salinity and improved the growth attributes in different crop plants eg., strawberry (Kaya *et al*., 2001c), cucumber and pepper (Kaya *et al*., 2003). Similarly, in a study on *Lagenaria siceraria*, Ahmad and Jabeen (2005) reported that foliar application of potassium nitrate counteracted the salt induced growth inhibition in rice plants. However, in the present study 2% K_2SO_4 was found to be effective in minimizing the adverse effects of salt stress on sunflower plants.

Conclusion

Application of varying levels of K improved the fresh and dry mass of sunflower under both control and saline conditions. However, the highest increase in fresh and dry weight of controlled and stressed plants were observed when 2% K was applied as foliar spray. Further, 2% K foliar application increased the K^+ concentration of plant tissues grown under normal as well as salt stress conditions resulting in more biomass production. Nevertheless, the foliar application has no significant effect on both genotypes.

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Growth and Yield Response of Sunflower (*Helianthus annuus* L.) to Sulphur and Boron Application

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Abstract. An experiment was conducted to study the growth and yield response of sunflower to sulphur and boron application. Sulphur (control, 15, 30 kg/ha) and foliar sprays of 1% boron solution (control, spray at 4 week after emergence, 20 days after first spray, at 4 weeks after germination + 20 days after first spray) was applied in soil. The results showed significant increase of all growth and yield parameters by varying levels of sulphur and boron application. Application of 30 kg sulphur/ha enhanced stem diameter (1.99 cm), number of achene per head (765.75) and oil content (36.42%). In case of combined use of sulphur and boron application, maximum plant height (171 cm), head diameter (20.71 cm), 1000-achene weight (54.56 g), biological yield (16.49 t/ha) and achene yield (3.99 t/ha) was recorded by the application of 2 sprays of boron solution (1%) at 4 weeks after germination and reproductive stage.

Keywords: sunflower, sulphur, boron, growth, yield

Introduction

The economy of Pakistan is agriculture based, accounting for over 21% of GDP, hence, it remains by far the largest employer. Among the oil seed crops, sunflower (*Helianthus annuus* L.) belonging to the family (Compositae) plays a major role in vegetable oil industry in the whole world. The protein content of sunflower cake ranges from 20-40% (Gandhi *et al*., 2008). In Pakistan, sunflower is a newly introduced oil seed crop. The area under sunflower crop in 2011-12 was 877 thousand acres with 473 thousand tonne achene yield (MINFAL, 2011).

Micronutrient deficiency can greatly disturb crop production which ultimately affects human and animal health (Malakouti, 2007). Among them sulphur is essential for growth and development of all crops. Plants absorb sulphur through roots in the form of sulphate. Sulphur is involved in the formation of vitamins, grains, oils, fruits and vegetables. Regarding chemical composition of seed, sulphur is very important for increasing the oil percentage in seeds (Welch, 2003). Boron is an essential element required in micro amount but producing a macro impact on plant growth and development. Boron is involved in numerous important processes, including protein synthesis, transport of sugar, respiration, RNA, plant hormones and carbohydrate metabolism, flowering and fruiting (Tariq and Mott, 2007; Havlin *et al.,* 2005) Moreover, functions of boron are related to cell wall synthesis, lignification and cell wall structure by cross-linking of cell wall polysaccharides as well as the structural integrity of bio membranes (Tanaka and Fujiwara, 2008). The requirement for boron fertilisation is rising because of higher crop yield and reduced quantity of organic matter and severe boron removal by crops. Boron is critical for the process of cell differentiation at all growing tips of plants (meristems), where cell division is active (Adiloglu and Adiloglu, 2006). Deformed flowering is a common symptom of boron deficiency. Many plants may respond by reduced flowering and improper pollination as well as thickened, curled, wilted and chlorotic new growth. On the basis of current knowledge about sulphur and boron evaluations, a field experiment was arranged to study the combined effect of these nutrients to assess the response of various growth and yield attributes of hybrid sunflower under Faisalabad, Pakistan conditions.

Materials and Methods

An experiment was conducted at the research area, Department of Agronomy, University of Agriculture, Faisalabad, to determine the effect of sulphur and boron on growth, yield and quality of hybrid sunflower. The experiment was laid out in randomised complete block design with factorial arrangement having three replica-

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tions with a net plot size of 4.5 m × 7.0 m. Before sowing of crop a portion of the prepared soil samples was used to analyse its various physicochemical properties. Soil was sandy clay loam (65% sand 16% silt and 19% clay). Soil pH (7.8) and electrical conductivity (1.3 dS/m) was measured in saturation extract using digital EC meter. Organic matter content (0.79 %), total N (0.047 ppm), available phosphorus (8.75 ppm), and available potassium (165 ppm) was recorded. Boron (0.39 ppm) and no sulphur were found.

The experiment comprised of soil application of sulphur (control, 15, 30 kg/ha) and foliar sprays of 1% boron solution (control, spray at 4 week after emergence, 20 days after first spray, at 4 weeks after germination + 20 days after first spray). The seedbed was prepared by cultivating the field for 2 times with tractor-mounted cultivator each followed by planking. Sunflower hybrid, Huysun-33 was sown on ridges in August 6, 2009 with the help of dibbler keeping R×R and P×P distances of 75 cm and 25 cm, respectively. Seed rate of 6 kg/ha was used. Sulphur and boron were applied as per treatment. Recommended doses of nitrogen and phosphorus were applied to all plots. DAP and urea was applied as a source of phosphorus and nitrogen. All recommended phosphorus (100 kg/ha) was applied at sowing time as a basal dose, while, nitrogen was applied in three splits i.e., 1^{st} at sowing time, 2^{nd} with first irrigation and 3^{rd} with second irrigation. Sulphur was applied at sowing stage and boron was applied as foliar spray at vegetative and reproductive stages of the crop using available resources of elemental sulphur and boric acid. All other agronomic practices, except treatments under study, were kept normal and uniform for all treatments. All the plant protection measures (weeds and insects pests' control) were taken according to the recommendation for sunflower crop. Crop was harvested manually at physiological maturity. Separate threshing was done for each experimental unit to obtain data regarding achene yield for each treatment. Plant protection measures were adopted to keep crop free of weeds, insect pests and diseases.

Ten plants were selected at random from each experimental unit. Height of those selected plants was measured and then averaged. Stem girth was measured of all ten plants with the help of Vernier caliper from top, mid and bottom of the plants and then averaged to calculate the diameter. The head diameter of ten randomly particular plants was measured and finally averaged. Ten heads were selected randomly from each treatment dried and threshed. Achene were counted and averaged to calculate the number of achenes per head. Ten samples of 1000 seeds were taken randomly from achene lot of each experimental unit. They were weighed and averaged to measure the 1000 achenes weight. After harvest at physiological maturity, the heads were separated from the plants, sun dried, threshed and the seed yield per plot was recorded with weighing balance. The achenes yield was converted into tonnes per hectare. Weight of sun dried plants (including heads) was taken on per plot basis and then transformed into tonnes per hectare to find out the biological yield. Seed samples were taken randomly from each subplot and oil contents were determined by AOAC (1990). Protein content of sunflower seed samples collected from each subplot were determined by the micro Kjeldahl method. One gram of each sample was transferred to Kjeldahl flask; a digestion tablet was added to 5 mL of concentrated H_2SO_4 and contents mixed thoroughly. The flask was placed on the digestion assembly; heater and exhaust fan were turned on. The digestion was continued with occasional shaking of flask. When the solution became clear and all the organic matter had been oxidised, the digestion was continued for another 30 min.

Results and Discussion

Plant height at maturity (cm). The data presented in Table 1 revealed that interaction between boron and sulphur application, being highly significant, proved a combination of 30 kg/ha sulphur and a single spray at 4 weeks after germination of 1% boron solution, to give the highest plant height (171 cm) at maturity. The effect of boron application was non-significant when no sulphur was applied. This might be attributed to the positive interaction between sulphur and boron. Sulphur is needed for chlorophyll formation and boron for increased cell division, which enhanced the intermodal distance and finally plant height. Results obtained in this study regarding plant height at maturity, are in line with Reddy and Chaturvedi (2009) and Sarker *et al.* (2002), who also reported the increase in plant height due to boron × sulphur application.

Stem diameter (cm). The potential growth of sunflower is determined from its vigorously growing stem. Significant differences in stem diameter of sunflower were observed in boron and sulphur level, however, their

Table 1. Growth and yield response of sunflower (*Helianthus annuus* L.) to sulphur and boron application

Treatments	Plant height (cm)	Stem diameter (cm)	Head diameter (cm)	Number of achene per head	1000-achene weight (g)	Achene yield (t/ha)	Biological yield (t/ha)	Oil contents (%)	Protein contents (%)
S_0 (0 kg/ha)	133.92 C	1.47 C	13.92 C	664.58 C	41.76 C	3.07 C	13.37 C	32.13 C	15.70
S_1 (15 kg/ha)	147.92 B	1.64 B	18.65 B	726.58 B	44.35 B	3.25 B	14.65 B	34.64 B	16.00
S_2 (30 kg/ha)	167.50 A	1.99 A	19.95 A	765.75 A	53.82 A	3.96 A	16.46 A	36.42 A	14.76
LSD value (for S at 5%)	6.39	0.14	1.46	4.99	3.88	0.28	1.23	2.86	NS
B_0 (No spray)	147.44	1.63	17.44	700.67 D	46.28 B	3.39	14.79	33.43 C	15.17
B_1 (1 spray at four weeks after germination)	150.78	1.71	17.75	712.78 BC	47.12	3.41	14.81	34.65AB	15.53
B_2 (1 spray at reproductive stage)	152.11	1.76	17.18	724.11 B	46.49 B	3.43	14.83	34.21BC	15.42
B_3 (B_1+B_2)	148.78	1.69	17.66	738.33 A	46.69AB	3.47	14.87	35.30 A	15.83
LSD value (for B at 5% probability level)	NS	NS	NS	6.65	0.38	NS	NS	3.82	NS
S_0B_0	133.33 e	1.41	13.77 e	638.33	40.66 b	3.05 f	13.35 f	30.67	13.57 h
S_1B_0	148.33 c	1.60	18.92 c	715.00	44.31 b	3.19 e	14.59 e	33.63	16.44 b
S_2B_2	160.67 b	1.90	19.62 b	748.67	53.87 a	3.92 b	16.42 b	36.00	15.51 e
S_0B_1	133.33 e	1.46	13.91 e	657.67	42.47 b	3.06 f	13.35 f	32.17	15.49 e
S_1B_1	148.00 c	1.65	18.74 cd	721.00	44.70 b	3.21 de	14.59 e	34.70	16.18 bc
S_2B_1	171.00 a	2.01	20.61 a	759.67	54.20 a	3.95 ab	16.42 b	37.08	14.93 f
S_0B_2	135.33 e	1.49	14.00 e	669.00	42.60 b	3.07 f	13.37 f	32.70	16.06 bcd
S_1B_2	153.00 c	1.69	18.67 cd	732.00	44.20 b	3.25 d	14.65 d	34.77	15.78 cde
S_2B_2	168.00 a	2.09	18.85 c	771.33	52.67 a	3.97 a	16.47 a	35.15	14.43 g
S_0B_3	133.67 e	1.51	14.00 e	693.33	41.29 b	3.08 f	13.38 f	32.97	17.70 a
S_1B_3	142.33 d	1.62	18.26 d	738.33	44.20 b	3.33 c	14.73 c	35.47	15.62 de
S_2B_3	170.33 a	1.95	20.71 a	783.33	54.56 a	3.99 a	16.49 a	37.46	14.18 g
LSD value (for S x B 5% probability level)	4.16	NS	5.83	NS	7.13	1.03	2.2	NS	0.56

Source of variation — Analysis of variance

	D.F	P.H	S.D	H.D	NAH	TAW	AY	BY	OC	PC
Replication	2	95.361	0.00610	0.2317	66.0	0.629	0.001	0.0011	2.904	0.002
Sulphur (S)	2	3414.694**	0.840**	120.811**	31225.4**	484.105**	2.673**	28.985**	55.861**	5.044**
Boron (B)	3	NS	NS	NS	2324.2**	1.162*	NS	NS	5.521**	0.677**
Sulphur x Boron	6	44.250**	NS	1.004**	NS	1.833**	0.002*	0.002*	NS	4.746**
Error	22	9.755	0.003	0.086	71.1	0.302	0.000	0.0006	0.932	0.080
Total	35									

* = significance at $P \leq 0.05$ level of probability; abbreviations: D.f = degree of Freedom, PH = plant height, SD = stem diameter, HD = head diameter, NAH = number of achenes per head, TAW = 1000-achene weight, AY = achene yield, BY = biological yield, OC = oil contents and PC = protein contents.

interactions were non-significant for the parameter under discussion. Maximum stem diameter (1.99 cm) of sunflower was obtained from plots, where, sulphur was applied at 30 kg/ha and minimum (1.47 cm) stem diameter was produced, where, no sulphur was applied. Among boron levels, single spray at reproductive stage produced maximum stem diameter of 1.76 cm, which was statistically at par with single sprays at 4 weeks after germination. Minimum (1.63 cm) stem diameter was produced, where, no spray of boron was applied, which, however, remained statistically similar to two sprays of boron at vegetative and reproductive stages (B_3). The improvement in stem diameter might be due to application of boron and sulphur, which enhanced the stem diameter of sunflower. These results are in line with the results of Tomar *et al.* (1997), who also reported increase in stem diameter with application of boron.

Head diameter (cm). The production potential of sunflower crop is determined by the size of its head, which is an important yield contributing factor. The data shows that head diameter was significantly, affected by sulphur and boron seperately and their interaction.The head diameter of sunflower was highest (19.95 cm), when 30 kg/ha of sulphur was applied and it was lowest (13.92 cm), when no sulphur fertiliser was applied. Whereas, in case of boron, single spray at 4 weeks after germination proved to give the maximum head diameter (20.61 cm), which was statistically similar with two sprays at vegetative and reproductive stages. The minimum head diameter was recorded from single spray at reproductive stage and was statistically at par with control, where, no boron was applied (B_0). The interaction between boron and sulphur application, proved a combination of 30 kg/ha sulphur and a single spray of 1% boron solution at 4 weeks after germination to give the highest head diameter (20.71 cm) and the lowest head diameter was noted (13.77 cm), where, neither sulphur nor boron treatment was applied. This might be due to application of B, which enhanced pollen viability flower, fruit set, hormone formation and increased the stem diameter. The results of present study regarding the head diameter were at par with the results reported by Oyinlola (2007), who found that the head diameter of sunflower increases with boron application.

Number of achenes per head. Number of achenes per head contributes significantly, towards the final yield of the crop and it also determines the yield potential of the sunflower crop. Significant differences were observed in number of achenes per head among boron levels and sulphur levels, while, non-significant differences were found in boron and sulphur interactions. The minimum number of achenes was recorded from control, where, no sulphur was applied. The number of achenes increased significantly, with each increase in sulphur level. The maximum number of achenes (765.75) was recorded with sulphur application of 30 kg/ha. As far as boron levels are concerned, two sprays, one at vegetative and other at reproductive stage, were found best, which produced maximum number of achenes (738.33) as compared to minimum in control (700.67). The improvement in achenes per head might be due to more head diameter and proper and timely application of sulphur and boron. These results are in line with those of Sarkar *et al.* (1999), who reported increase in number of achenes per head with the application of boron and sulphur.

1000-achene weight (g). Stout and sturdy achenes are a sign of high yield and quality produce, which, contributes significantly, towards its final yield. 1000-achene weight was greatly influenced by sulphur and boron nutrition. Significant differences were observed among sulphur levels, boron levels and sulphur × boron interactions. In case of sulphur doses, 30 kg/ha elemental sulphur produced maximum thousand achene weight (53.82 g) and on the contrary, control produced minimum (41.76 g). Single spray of 1% boron solution at 4 weeks after germination produced maximum thousand achene weight (47.12 g). The interaction between sulphur and boron was also significant. Application of sulphur at the rate of 30 kg/ha with two sprays of boron on both vegetative and reproductive stages, produced maximum thousand achene weight (54.56 g), which was statistically at par with single spray of boron at 4 weeks after germination and application of sulphur at 0 kg/ha with no spray of boron produced the minimum thousand achene weight (40.66 g). Improvement in achene weight may be due to maximum diameter and number of achenes per head. Combined application of sulphur and boron also increased the 1000-achene weight. These results are also in line with those of Sarkar *et al.* (2002), who reported an improvement in 1000-achene weight with application of boron and sulphur.

Achene yield (t/ha). Achene yield is a function of integrated effects of the various yield components and any variation in them is liable to bring about variation in yield. Significant, differences in achene yield were observed in sulphur levels, boron levels and sulphur × boron interaction. The data indicated that sulphur at

the rate of 30 kg/ha produced highest (3.96 t/ha) achene yield as compared to control, which, produced lowest (3.07 t/ha) achene yield. In case of boron, two sprays (at both, vegetative and reproductive stages) produced maximum achene yield (3.47 t/ha) and control produced the minimum (3.39 t/ha). The interaction between sulphur and boron application for achene yield was significant. The maximum yield (3.99 t/ha) was obtained when sulphur was applied at 30 kg/ha with two sprays of boron on both, vegetative and reproductive stages and minimum (3.05 t/ha) was obtained without boron and sulphur application. Improvement in achene yield is due to maximum head diameter, number of achene per head and 1000-achenes weight. Similar results were also reported by Sarker *et al.* (2002) and Salim *et al.* (1997), who reported that achene yield can also be increased with application of boron and sulphur in combination.

Biological yield (t/ha). Biological yield is the total biomass produced by a crop by utilising the resources available at the time. Significant differences in biological yield were observed in sulphur levels, boron levels and sulphur × boron interaction. Maximum biological yield (16.46 t/ha) was obtained with sulphur at the rate of 30 kg/ha, as compared to control, which produced lowest (13.37 t/ha) biological yield. In case of boron, two sprays (at both, vegetative and reproductive stages) produced maximum biological yield of 14.87 t/ha and control produced 14.79 t/ha. In case of sulphur and boron interaction, significantly, maximum yield (16.49 t/ha) was recorded, where, sulphur at the rate of 30 kg/ha with two sprays of boron at both, vegetative and reproductive stages were applied. Minimum (13.35 t/ha) was obtained at 0 kg sulphur with no spray of boron. Increase in biological yield is attributed to proper utilisation of boron. Results of this study are also in line with those of Sarker *et al.* (2002), who also reported increase in biological yield due to sulphur and boron application.

Achene oil content (%). A crop rich in oil contents is the ultimate goal of the growers. Significant differences were observed in oil content of achenes among boron and sulphur levels, while, non-significant differences were found in boron and sulphur interaction. Maximum achene oil content (36.42%) was obtained from sulphur applied at the rate of 30 kg/ha and minimum achene oil content (32.13%) was found, where, no sulphur was applied. As far as boron levels are concerned, two sprays, one at vegetative and another at reproductive stage, were found best, which produced maximum achene oil content (35.30%) as compared to minimum in control (33.43%). Boron along with sulphur application increased the oil contents because sulphur is constituent of several amino acids, which are essential for protein production. These results are in line with those of Diggs *et al.* (1992), who reported an increase in oil content of seed with the increase in sulphur dose.

Achene protein content (%). Protein content in seeds indicates the quality and dietary value of the seeds. The data shows that significant differences were observed in protein content among sulphur levels, boron levels and their interaction. The data regarding the role of sulphur in promoting the protein content reflected that most efficient sulphur dose was 15 kg/ha, which produced highest (16%) protein content and on the other hand a high dose (30 kg/ha) of sulphur found to be producing lowest (14.76%) protein content. In case of boron, two sprays, one at vegetative and another at reproductive stage, were found best, which produced maximum (15.83%) protein content and minimum (15.17%) protein contents were noted in control. Whereas, in case of interaction of sulphur and boron, the best treatment combination was S_0 kg/ha with two sprays of boron; one at vegetative and another at reproductive stage and minimum protein content was recorded, where, S_0 kg/ha and no boron were sprayed.

Conclusion

From results of experiments it can be concluded that two sprays of boron solution (1%) first spray at 4 weeks after emergence and second 20 days after first spray in combination with S (30 kg/ha), significantly, increased head diameter (20.71), 1000-achene weight (54.56), achene yield (3.99 t/ha) and biological yield (16.49 t/ha) as compared to control.

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In vitro Antifungal Activities of Extracts of Fruits and other Morphological Parts of *Xanthium strumarium* Against the Plant Pathogen, *Rhizoctonia solani*

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Abstract. *In vitro* antifungal activity of different plant parts of *Xanthium strumarium* (Compositae) was investigated against *Rhizoctonia solani* to seek safe natural alternatives to the harmful synthetic fungicides. The most active plant parts of *X. strumarium* were seeds, extracted with *n*-hexane and the leaves, extracted with absolute ethanol. The two treatments resulted in growth inhibition diameters of 45 mm and 47 mm, respectively. The value of MIC lied between 350.0 and 175.0 µg of *Xanthium* oil/mL. Gas liquid chromatography of the seed oil of *X. strumarium* revealed the presence of the usual fatty acids, palmitoleic (7.6%), oleic (21.6%) and linoleic (70.4%). The oil was separated into free fatty acids fraction and unsaponifiable matter fraction. The unsaponifiable matter fraction was separated on TLC, out of six separated compounds, two were active against *R. solani.* The infrared spectra (FTIR) of these two purified compounds pointed to a long chain hydrocarbon back-bone for both, one of them possessing in addition, an alcoholic moiety.

Keywords: *Xanthium strumarium*, antifungal activity, *Rhizoctonia solani*, seed-oil, unsaponifiable matter fraction

Introduction

Rhizoctonia solani is a serious soil and seed borne pathogen that causes damping off disease resulting in severe losses all around the world, to various crop plants including important food crops (Kataria and Verma, 1992; Anderson, 1982) as well as forest trees (Camporota and Perrin, 1998). The fungus was first reported on wheat in Sudan (Elnur and Chester, 1967). We have also observed the fungus to infect other crops, e.g., cotton, sorghum, tomato and onions.

R. solani is very persistent in soil and only very few chemical fungicides are effective against it (Errampali and Johnson, 2001). Other, non-chemical methods are attempted for control of the fungus and most notably used is biological control agents (Fiddaman and Rossall, 1995).

Natural chemicals of plant origin represent a diversified, renewable and a safe source of potential pesticides. Natural antifungal products of plant origin were reviewed by Arif *et al*. (2009). *Xanthium strumarium*, commonly known as cocklebur or bur weed, belongs to the family Compositae and is an annual gregarious weed widely distributed in many parts of the world (Favier *et al*., 2005). Cocklebur enjoys a reputation in the folk medicine of several nations of the world as a remedy for a number of ailments including malaria, rheumatism, leprosy, etc. (Kamboj and Saluja, 2010). In addition to being toxic to man and animals, cocklebur was reported to possess interesting biological or pharmacological activities including antitrypanosomal (Talakal *et al*., 1995), anti-cancer (Ramirez-Erasa *et al*., 2007), anti-inflammatory (Han *et al*., 2007) and other activities (Akarte *et al*., 2009; Yoon *et al*., 2008).

X. strumarium is an invasive widely spreading weed in Sudan particularly in the irrigated agricultural schemes in central Sudan. The spiny fruits easily cling to the hair of grazing animals, aiding seed dissemination as well as causing some animal health problems. The rainy season, in addition, supports considerable growth of the plant outside these schemes, as part of the spontaneous autumn (Kharif) flora. Thus the plant's biomass is available in quantity. Crude extracts of *X. strumarium* have been shown to possess antifungal activity, although against fungi other than *R. solani* (Park *et al*., 2005).

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The objectives of this work was to test solvent extracts prepared from different morphological parts of *X. strumarium* for their activity against the plant disease causing fungus *R. solani* and to chemically characterise the active antifungal ingredients.

Meterials and Methods

Collection and preparation of plant material. Samples of wild-growing *X. strumarium* plants were collected from the Blue Nile river banks near the Gezira University area at Wad Medani city, Sudan (Herbarium No. NOPRI. NEOUprjt.005). The collected plants were dissected into morphological parts (leaves, roots, etc.) and allowed to dry at ambient temperature in the shade before grinding and subsequent solvent extraction.

Solvents and chemicals. All solvents, chemicals and media used in bioassays were obtained from E. Merck (Germany) or Sigma (USA).

Preparation of solvent extracts from *Xanthium* plant parts. Air-dried, powdered *Xanthium* plant material was extracted using either of three methods. The first was a cold method that involved maceration of powdered tissue with 20 volumes of solvent such as ethanol (95%) or *n*-hexane using conical flasks maintained at room temperature for 72 h with mild magnetic stirring. In addition two hot extraction methods were (a) involving Soxhlet extraction and (b) refluxing in an appropriate solvent. All extracts and their filtrates were concentrated in a rotatory evaporator and kept for further chemical analyses and bioassay.

***Xanthium* seed-oil fractionation.** *Xanthium* seed-oil was partially saponified using KOH, according to AOCS method (1993), and fractionated into free fatty acids and the unsaponifiable matter fractions. After saponification the unsaponifiable matter was recovered in chloroform. The remaining saponified fraction was acidified and free fatty acids were extracted with hexane.

Preparative thin layer chromatographic separations. The unsaponifiable matter fraction prepared as above from 1g of *Xanthium* seed oil was dissolved in a final volume of 10 mL of chloroform. Volumes of 100 µL of this solution were each applied to a TLC plate (20× 20 cm glass plates pre-coated with silica gel 60, without fluorescent indicator, layer thickness 0.5 mm; Merck, Germany). The plates were developed in a tank containing the solvent mixture, *n*-hexane: acetone (4:1). Bands were detected using the Antimony trichloride reagent. Provisions were made to detect separated components only at the edge of the plate, allowing recovery of others (to the inside of the plate), uncontaminated by detection reagent. These bands were scraped individually, transferred to sterilised sample bottles, eluted with solvent and taken for further studies.

Transmethylation of *Xanthium* oil fatty acids. Fatty acid methyl esters were derivatised using 0.5 N sodium methoxide. About 0.5 g of the oil was weighed in a stoppered flask and 0.5 N sodium methoxide was added (7-8 mL). The contents were heated for 3-5 min in a water-bath at 80 °C with shaking and then transferred to a separatory funnel. A few drops of glacial acetic acid were added followed by 15 mL of distilled water and 10 mL of *n*-hexane. After equilibration, the *n*-hexane layer was collected and kept dry over anhydrous sodium sulphate before GLC analysis.

Gas liquid chromatography. The fatty acid composition was determined by GLC using a Varian 3400 gas liquid chromatograph equipped with a hydrogen flame ionisation detector and a computing integrator. A fused silica gel column (25 m × 0.32 mm inner diameter) packed with polyethylene glycol-2-nitroterephthalic ester (film thickness 0.25 µm) was used. Analysis of fatty acid methyl esters was carried out isothermally at column oven temperature of 170 °C, column inlet and detector oven temperature of 180 °C and the carrier gas flow was 50 mL/min. The identification of fatty acids was carried out according to the retention time of esters of authentic samples of fatty acid methyl esters.

Bioassay of antifungal activity. Isolated and identified *Rhizoctonia solani* fungus was grown on PDA medium. Micro fibre glass discs (5 mm in diameter) saturated with plant extracts, as well as control solutions, were placed in the middle of the plate for 24 h before culturing (using the disc diffusion method essentially described by Bauer *et al*., 1966). Two discs (5 mm in dia) of the test fungus culture, were prepared using a cork borer, placed from each side of the petri-plates. Micro fibre discs saturated with the respective solvent, alone, were used as control treatments. The growth diameter (three replicas for each test) was periodically measured for up to three days and the inhibition zone was expressed in millimetre (± Standard Deviation).

Results and Discussion

Table 1 shows fungal inhibitory effects on the growth of *R. solani* Kuhn of crude extracts prepared from different morphological parts (seed, stem, leaf and root)

Table 1. Fungal growth inhibitory activities against *R. solani*, of crude extracts prepared from different parts of *X. strumarium* using different extraction methods and solvents. The disc diffusion method was used for antifungal assays

Treatment no.	Extraction		Zones of fungal growth inhibition (mm)			
	Solvent	Method	Seed	Stem	Leaf	Root
1.	Ethanol, absolute	Soxhlet	0.0	12.1 ± 0.2	37.2 ± 0.3	40.3 ± 0.1
2.*	Ethanol, absolute	Soxhlet	10.4 ± 0.2	-	-	-
3.	Ethanol, absolute	Maceration	-	-	38.9 ± 0.3	38.0 ± 0.4
4.*	Ethanol, absolute	Maceration	-	-	47.5 ± 0.4	45.1 ± 0.4
5.	Ethanol, absolute	Reflux	-	-	34.5 ± 0.3	35.0 ± 0.3
6.	Ethanol, aqu. 70%	Maceration	-	-	2.5 ± 0.3	30.3 ± 0.4
7.	*n*-Hexane	Soxhlet	32.0 ± 1.0	-	0.0	0.0
8.	Dichloromethane	Maceration	-	-	40.2 ± 0.2	40.6 ± 0.3

Notes: All pure solvent controls gave a fungal inhibition zone of 0.0 mm; (*) in treatments 2 and 4, plant material was first defatted by hexane extraction (Soxhlet) before preparing the absolute ethanol extract; (-) = not determined; aqu. = aqueous.

of cocklebur (*X. strumarium* L.). The dry powdered plant material was directly extracted with solvent, however, in two occasions (indicated in the Table) the plant material was first defatted by Soxhlet-extraction with hexane followed by re-extraction of the marc (Soxhlet) with absolute ethanol; the latter extract assessed for antifungal activity. Solvents used were absolute ethanol, 70% aqueous ethanol, *n*-hexane and dichloromethane.

The most active absolute ethanol extracts (Soxhlet method) were those of the plant roots and leaves, resulting in fungal growth inhibition diameters of 40 and 37 mm, respectively (Table 1, Treatment no.1). The ethanol extract of the stems was less active, while, that of the directly extracted seeds was completely inactive against *R. solani*. However, pre-extraction of the seeds with hexane (defatting) followed by subsequent extraction of the marc with absolute ethanol, resulted in a considerably active ethanol extract (Table 1, Treatment no. 2). On the other hand, hexane extracts of the seeds prepared by the Soxhlet method (consisting of the seed fixed oil) were very active, resulting in a fungal growth inhibition zone of 32.0 mm (Fig. 1), while, hexane extracts of the leaves and roots were completely inactive towards growth of *R. solani*.

A comparison of the efficiency of the three extraction methods (Soxhlet, maceration and reflux methods) in recovering the antifungal constituent(s) from *Xanthium* tissue can be made by considering Table 1 (Treatments no. 1, 3 and 5) in which absolute ethanol was used to extract leaf and root morphological parts. The three methods gave similar results of recovery of antifungal activity, although the reflux method yielded somewhat lower activity.

Using the maceration method, dichloromethane, as extraction solvent was as good as or slightly better than absolute ethanol in the cases of both leaf and root plant material (Table 1, Treatments no. 3 and 8). However, aqueous 70% ethanol gave an extract with less inhibitory activity towards the growth of *R. solani* especially when used with the leaf material (Table 1, Treatments no. 3 and 6). Again, as observed for seed material, prior defatting (extraction with hexane) followed by extracting the marc with absolute ethanol resulted in increased activity of absolute ethanol extracts of both leaves and roots (Table 1, Treatments no. 3 and 4).

Bahraminejad *et al*. (2011) screened 63 Iranian plant species, including *X. strumarium*, against three phytopathogenic fungi, including *R. solani*. These authors

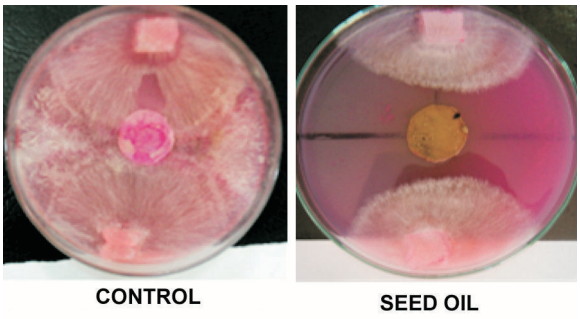

Fig. 1. Typical photos of inhibition of the growth of *R. solani* by the seed oil (hexane extract) of *Xanthium*.

reported weak activity for aqueous extracts of the shoots of *X. strumarium*, but considerable activity for the plant's shoot methanol extracts. So far, this is the only report available on the antifungal effect of extracts of *X. strumarium* on *R. solani*. Crude extracts of cocklebur (*X. strumarium*) have been reported to possess considerable antifungal activity against fungal species of *Phytophthora* (Bahraminejad, 2012; Kim *et al*., 2002), *Sclerotinia* and *Aspergillus* (Park *et al*., 2005). On the other hand, Murillo-Alvarez *et al*. (2001) reported a low activity for ethanol extracts of *X. strumarium* tested among extracts of 24 other plants, against *Candida albicans*. Gupta and Banerjee (1972) also tested crude extracts of 170 different West Bengal plants against the two fungi *Aspergillus niger* and *Trichophyton rubrum* and reported no antifungal activity for aqueous or ethanolic extracts of leaf, stem, root or fruit of *X. strumarium*. Extracts of other species of *Xanthium* also showed antifungal activity against fungi not including *R. solani*, e.g., *X. spinsum* (Ginesta-Peris *et al*., 1994) and *X. macrocarpum* (Lavault *et al*., 2005). Present report is the first to deal with growth inhibitory effects against *R. solani*, of extracts of any species of *Xanthium* prepared by different methods from morphological parts of the plant.

There are, however, reports of preparations made from plant species not belonging to the genus *Xanthium* that showed growth inhibition of *R. solani.* These included the volatile oil of *Chenopodium ambrosioides* (Dubey *et al*., 1983), dichloromethane extracts of *Desmos chinensis* (Plodpai *et al*., 2013) and a preparation obtained by mechanical expression of *Aloe vera* (Rodriguez *et al*., 2005).

It is important to choose the right extraction solvent properly for different morphological parts of the plant. The fact that antifungal activity of the seeds of *X. strumarium* was only when soluble in hexane (and not in absolute ethanol) and the reverse solubility property for compound(s) responsible for antifungal activity of the leaf and root (Table 1, Treatments no. 1 and 7) suggests the presence of at least two different types of compounds active against *R. solani*.

Improved recovery of antifungal constituents by absolute ethanol extraction, following removal of hydrophobic constituents (defatting) of *Xanthium* seed, leaf and root material (Table 1, Treatment nos. 1, 2 and 3, 4) suggests that some antifungal *Xanthium* constituent(s) has a distinct hydrophobic moiety.

Stability of antifungal activity of crude cocklebur extracts. Three extracts of *X. strumarium* were prepared from the seeds (using the Soxhlet method and hexane as extraction solvent) as well as from the leaves and roots of the plant (using the maceration method and absolute ethanol as solvent). Aliquots from each of the three extracts were applied to 5 mm diameter discs in amounts corresponding to equivalent plant powder weight of each plant part. The discs were then placed on the growth media used for the usual antifungal bioassay protocol and allowed to stand for time periods of 0, 24, 48, 72 and 96 h (at room temperature), before the antifungal bioassay was started by adding cultures of *R. solani*. Thus these time periods represent different times of storage of *Xanthium* extracts at room temperature. The results are shown in Table 2. With all three *Xanthium* extracts, fungal growth inhibitory activity somewhat increased initially with storage and was maximal in extract-loaded discs stored for 48 h before the fungus was introduced (Table 2). This increase could be due to increased diffusion of the antifungal components of the extracts with incubation time. Thereafter, antifungal activity only slightly decreased. After 96 h (4 days) of storage of the discs at room temperature, 100, 86 and 75% of the original antifungal activities of all three extracts still remained. Thus, the three extracts, particularly those of the seed were quite stable under the conditions of the test.

Table 2. Stability of fungal inhibitory activity (against R. solani) of Xanthium seed, leaf and root extracts stored at room temperature for different time periods

Storage time	Fungal inhibition zone (mm)		
(h)	Seed	Leaf	Root
0	30.0	40.0	37.0
24	34.0	35.0	40.0
48	45.0	45.0	47.0
72	43.0	42.0	45.0
96	30.0	30.0	30.0

The values represent means of three separate bioassay determinations.

Minimum inhibitory concentration (MIC) of the seed oil of cocklebur towards *R. solani*. Figure 2 shows antifungal activity against *R. solani* of different concentrations of *X. strumarium* seed oil obtained by Soxhlet extraction with *n*-hexane. The inhibition zone of fungal growth decreased with decreasing *Xanthium* seed oil concentration used to saturate the assay discs. The value of MIC lied between 350.0 and 175.0 μg of *Xanthium* seed oil/mL.

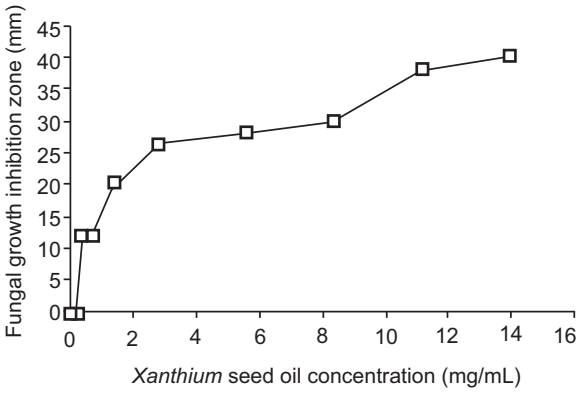

Fig. 2. Inhibition of *R.solani* growth by different concentrations of *Xanthium* seed oil.

Partial characterization of the active antifungal ingredients of cocklebur seed oil. The seed oil was chosen for further studies since it was quite active against *R. solani* and represented a somewhat more homogenous (lipid) fraction.

It is known that many of the so-called 'unusual' fatty acids, such as short-chain, cyclopropanoid or acetylenic fatty acids have antifungal activity (Carballeira, 2008). However, GLC analysis (Table 3) revealed that the oil contained no unusual fatty acids. Linoleic and oleic acids constituted over 90% of cocklebur seed oil fatty acids. Palmitoleic acid, a usual fatty acid was present in a relatively larger amount. Cocklebur seed oil was further separated into two fractions, the free fatty acid (FFA) and the unsaponifiable matter (UM) fractions. Both fractions were assayed for their inhibition of the growth of *R. solani*. No antifungal activity was detected for the FFA fraction and it was associated solely with the UM fraction (Table 4, Fig. 3). Subsequently the UM fraction was subjected to preparative TLC analysis yielding six major components. These were numbered (I) to (VI), according to increasing R_f value in the TLC solvent system *n*-hexane: acetone (4:1). Bands representing each of these components were scraped, eluted in a solvent and assayed for growth inhibition of *R. solani*. Two of the six TLC spots, designated as II and IV were active against the growth of *R. solani* (Table 5). The two compounds were subjected to infrared spectroscopic analysis. Thus, it is concluded that compound II is a long chain alcohol (absorption at 3453 cm^{-1} due to O-H; at 1165 cm^{-1} due to C-O; relatively intense absorption at 2960-2850 cm^{-1} and long chain methyl rocking at 725 cm^{-1}). The presence of a hydrophobic moiety (the long hydrocarbon chain) is consistent with the results observed for increased recovery of antifungal activity from seed, leaf and root tissues of *Xanthium* on defatting. This feature may assume significance in future studies on the mode of action of this antifungal compound.

Table 3. Fatty acid composition of *Xanthium* seed oil as determined by GLC

Fatty acid	%
Palmitic acid	0.5
Palmitoleic acid	7.2
Stearic acid	0.7
Oleic acid	21.6
Linoleic acid	70.0

Table 4. Antifungal activity of the two oil fractions of *X. strumarium* on growth of *R. solani*

Oil fraction	Fungal growth inhibition (mm)
Free fatty acids	0.0
Unsaponifiable matter	28.0

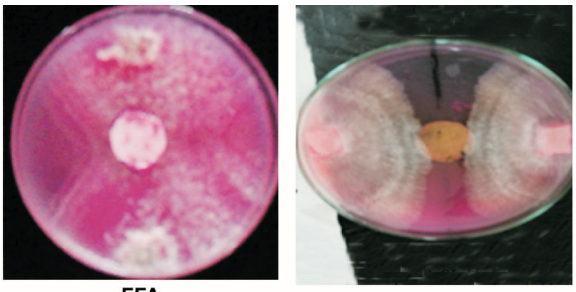

Fig. 3. Inhibition of the growth of *R. solani* by the free fatty acid (FFA) and unsaponifiable matter (UM) fractions of *Xanthium* seed oil.

Table 5. Antifungal activity of TLC separated components of *Xanthium* seed oil unsaponifiable fraction

Components	Fungal growth inhibition zone (mm)
I	0.0
II	30.0
III	0.0
IV	20.0
V	0.0
VI	0.0
Control*	0.0

* = eluate of blank (spot-free) silica gel.

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Effect of Plant Age on Cotton Leaf Curl Disease (CLCuD) in Relation to Environmental Conditions

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Abstract. The effect of cotton leaf curl disease (CLCuD) and weather variables were studied using same genotypes of cotton, planted at 15 days interval during 15th, 23rd standard weeks of 2010-2011. On an average basis, the 15th standard week planting showed, significantly, less disease incidence than all other sowing dates. The incidence increased as the sowing was delayed up to 23rd standard weeks. Among the sowing dates, regardless of genotypes, disease incidence differed, significantly. The CLCuD boost up during 25th to 29th standard (2010) and 27th to 31st standard (2011) weeks of the year, regardless of sowing date and genotypes. Disease incidence was low during 2011 as compared to 2010. The disease increased sharply during 2010 and gradually during 2011. Average maximum (34.8~39.8 °C), minimum (27.7~28.9 °C) temperature and relative humidity (62~79%) favoured CLCuD progression.

Keywords: cotton leaf curl disease, cotton cultivars, relative humidity, temperature

Introduction

Upland cotton (*Gossypium hirsutum* L.) is one of the important fibre and cash crops of Pakistan. Cotton occupies a unique position in textile world with millions of people engaged in its cultivation, processing and marketing etc.

The main reasons for low productivity of cotton is heavy attack by a number of insect-pests that results in various diseases starting from germination up to the harvest of crop. Among these diseases, cotton leaf curl virus disease (CLCuD) is the major reason for the decline in cotton production and productivity in Pakistan, especially in the Punjab. This disease is caused by cotton leaf curl virus (CLCuV) which belongs to begomovirus (Family Germiniviridae) transmitted in persistent manner by the vector, whitefly (*Bemisia tabaci* Gennadius). Characteristic symptom of the disease is upward or downward curling of leaves with thickened veins which is more pronounced on under side. The disease results in stunted plant growth with loss in yield (Hameed *et al.*, 1994).

CLCuD was reported for the first time in Pakistan during 1967 near Multan. At that time disease was of minor importance and did not get much attention. After 1988, the disease appeared in an epidemic form and damaged the crop on about sixty thousand hectares with a loss of 0.3 billions bales in production (Mahmood *et al.,* 1999). The geographic spread of CLCuD has increased tremendously and more than 7.7 million bales of cotton have been lost due to CLCuD from 1986 to 2002 (Akhtar *et al.,* 2004).

Losses due to this disease depend upon the variety and sowing time of cotton crop (Tahir *et al.*, 2004). Weather factors (individual and collectively) particularly temperature, relative humidity and rain fall influence the disease and vector (whitefly) population to great extent in host pathogen system. The hot and humid climate in north India during crop season is conducive for growth of host and the vector (Sharma *et al.*, 2006). However, meager information is available on the role of climatic factors affecting vector population and disease development. The present studies were therefore, carried out to understand the role of environmental factors on the development of CLCuD.

Materials and Methods

The present investigations were designed to work out the effect of temperature and relative humidity and different planting dates on cotton varieties at research area of Central Cotton Research Institute (CCRI), Multan, Pakistan.

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Delinted seed of three genotypes *viz.*, CIM-573, CIM-496 and CIM-608 (2010) and CIM-554, CIM591 and CIM-608 (2011) were planted on 15th, 17th, 19th, 21st, and 23rd standard weeks of each year by dibbling method. The plants were spaced 75 cm row to row and 30 cm plant to plant distance. The experiment was conducted in split plot (main plot: planting time, subplot: genotypes) design with four replication. All other cultural practices were performed in standard fashions to optimise the seed cotton yield.

Observations on the incidence of CLCuD were recorded at 15 days interval starting from 30 days after planting and continued up to 35th std., week.

Total numbers of plants showing leaf curl virus disease symptoms (upward curling with thickened veins on under side of leaf) were counted every time during observations. Plants with even a single leaf showing the symptoms of disease were counted as infected. The percentage disease incidence was counted by using following formula

$$\text{Disease incidence (\%)} = \frac{\text{total plants} - \text{healthy plants}}{\text{total plants}} \times 100$$

At the end of the season (35th std., week) each and every plant was examined in the field and different grades/ scales were allotted to them according to the level of infection in disease plants as described by Akhtar and Khan (2002). The rating scales are given in Table 1.

The percentage of disease index was calculated by using the following formula

$$\text{Disease index (\%)} = \frac{\text{summation of all disease ratings}}{\text{total plants}} \times \frac{100}{\text{maximum grade}}$$

The data on environmental variables were obtained from Meteorological Department, Central Cotton Research Institute, Multan, Pakistan. Data for fortnightly progression of disease incidence were calculated and compared with environmental parameters (maximum, minimum temperature & relative humidity) of that period of each year separately. The pooled data for both years were first analysed by simple regression. The disease index were subjected to standard statistical analysis (Steel *et al*., 1996) and the means were compared using DMR/LSD test (P=005).

Results and Discussion

The results of incidence of CLCuD monitored (fortnightly interval) right from 30 days after planting for all planting dates in each year are given in Table 2-3.

Progression of disease. Results reveal that expression of CLCuD and its progression during the crop seasons of (2010 and 2011) differed greatly with planting dates. Averaged across varieties, minimum incidence of disease (1.7%) was recorded at day 45 after planting and increased upto 11.9% at day 60 after planting. The disease boosted upto 82% at day 120 after planting on that crop planted on 15th std., week of the year.

The infection level was 9.5% at day 45 and attained its maximum (98.2%) at day 105 after planting on crop planted on 17th std., week of the year. However, incidence

Table 1. Disease rating scales on the basis of intensity of CLCuD for its index

Symptoms	Rating scale	Symptoms	Rating scale
Complete absence of symptoms	0	Large groups of veins involved and curling or top of the plant affected	4
Few small scattered vein thickening	1	All veins involved and severe curling or half of the plant affected	5
Small scattered vein thickening	2	All veins involved and severe curling and stunted plant or whole of the plant affected and stunting	6
Vein thickening involving small groups of veins	3	Enations	E

Table 2. Incidence of CLCuD as influenced by planting dates and strain during 2010

Planting time		Incidence of CLCuD (%)								
(Std., weeks)	Cultivar	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	CIM-608	0	0.52	7.17	16.54	28.41	33.09	61.48	87.41	96.05
	CIM-573	0	1.10	9.33	59.12	73.06	88.21	93.05	97.81	100
	CIM-496	0	3.42	19.21	66.59	78.66	88.92	91.44	100	100
	Average	**0**	**1.7**	**11.9**	**47.4**	**60.0**	**70.1**	**82.0**	**95.1**	**98.7**
17	CIM-608	0	7.8	25.8	51.59	56.39	95.78	97.65	100	-
	CIM-573	0.42	10.11	68.54	81.21	89.54	98.74	99.12	100	-
	CIM-496	0.42	10.72	60.87	84.07	100	100	100	100	-
	Average	**0.3**	**9.5**	**51.7**	**72.3**	**82.0**	**98.2**	**98.9**	**100**	**-**
19	CIM-608	0.92	30.48	74.92	99.06	100	100	100	-	-
	CIM-573	0.88	38.29	68.37	100	100	100	100	-	-
	CIM-496	2.26	49.66	96.35	99.53	100	100	100	-	-
	Average	**1.4**	**39.5**	**79.9**	**99.5**	**100**	**100**	**100**	**-**	**-**
21	CIM-608	33.03	97.05	100	100	100	100	-	-	-
	CIM-573	46.56	100	100	100	100	100	-	-	-
	CIM-496	44.45	100	100	100	100	100	-	-	-
	Average	**41.3**	**99.0**	**100**	**100**	**100**	**100**	**-**	**-**	**-**
23	CIM-608	88.21	100	100	100	100	-	-	-	-
	CIM-573	89.10	98.36	100	100	100	-	-	-	-
	CIM-496	94.81	100	100	100	100	-	-	-	-
	Average	**90.7**	**99.5**	**100**	**100**	**100**	**-**	**-**	**-**	**-**

* = days after planting.

Table 3. Incidence of CLCuD as influenced by planting dates and strain during 2011

Planting time		Incidence of CLCuD (%)								
(Std., weeks)	Cultivar	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	CIM-608	0	0.46	3.16	8.6	14.49	16.17	19.54	20.87	21.79
	CIM-591	0	0.40	5.41	16.37	37.43	47.47	49.75	51.65	52.56
	CIM-554	0	0.39	9.22	14.22	31.72	43.73	50.76	50.77	50.97
	Average	**0**	**0.41**	**5.91**	**13.06**	**27.08**	**35.79**	**40.01**	**41.09**	**41.77**
17	CIM-608	0	0.55	6.75	13.91	17.69	19.08	19.18	19.55	-
	CIM-591	0	1.02	11.55	28.97	48.62	59.99	63.34	64.3	-
	CIM-554	0	3.09	26.21	49.06	58.02	66.42	66.76	67.48	-
	Average	**01.55**	**14.83**	**30.64**	**41.44**	**48.49**	**49.76**	**50.44**	**-**	**-**
19	CIM-608	0.51	6.0	17.87	30.12	37.96	39.92	41.13	-	-
	CIM-591	0.88	8.34	27.29	69.44	89.71	100	100	-	-
	CIM-554	1.48	10.5	37.20	57.03	93.57	93.96	94.12	-	-
	Average	**0.95**	**8.28**	**27.45**	**52.19**	**73.75**	**77.96**	**78.41**	**-**	**-**
21	CIM-608	0.6	15.05	47.56	83.51	89.56	93.3	-	-	-
	CIM-591	2.71	24.84	61.07	93.79	98.04	99.3	-	-	-
	CIM-554	2.16	24.80	67.75	-	99.21	99.80	-	-	-
	Average	**1.82**	**21.56**	**58.79**	**98.63**	**95.60**	**97.46**	**-**	**-**	**-**
23	CIM-608	5.42	28.45	94.56	95.82	96.89	-	-	-	-
	CIM-591	7.07	32.43	98.69	99.57	100	-	-	-	-
	CIM-554	9.06	46.31	99.06	99.53	100	-	-	-	-
	Average	**7.18**	**35.73**	**97.43**	**98.30**	98.96	-	-	-	-

* = days after planting.

of CLCuD (39.5%) of day 45 and reached to its maximum level (99.5%) at day 75 after planting on crop planted on 19th std., week of the year. The incidence of the disease was 41.3% at day 30 and reached to maximum (99%) at day 45 after planting on crop planted on 21 std., week of the year. Further more crops planted on 23rd std., week of the year fell prey to CLCuD to 90.7% within 30 days after planting and to maximal

(99.5%) with in next 15 days (Table 2). Data revealed that the incidence of disease increased and period decreased (days after planting) as the planting time was increased (Table 2).

Averaged across varieties, disease started at low level (0.41%) at day 45 after planting and reached upto 5.91% at day 60 after planting. With the advancement of age, the incidence progressed gradually to 40.01% within next 60 days, on that crop planted on 15th std., week of the year. The infection level was 14.83 at day 60 and attained it maximum (48.4%) at day 105 after planting on crop planted on 17th std., week of the year. However, incidence of CLCuD was 8.28% at day 45 and reached to its maximum level 78.4% within rest days as crop planted 19 std., week of the year. The incidence of disease start from 1.82% at day 30 and reached upto (91.9%) at day 75 after planting on crop planted 21st std., week of the year. Furthermore, the crop planted 23rd std., week of the year fell prey to CLCuD to 97.4% with in 60 days after planting, which is 50% period less and incidence double than those crop planted on 17th std., week of the year (Table 3). Similar findings were made by Tahir *et al*. (2004) and Khan *et al*. (1988). They concluded that maximum incidence was recorded in June planting (21st std., week) and increased rapidly in the first week of August (29th std., week) in all planting dates.

Effect of planting date. One of the most important agronomic considerations for growers to optimise yield and quality is to select an appropriate planting time for cotton crop. Choosing the best time for planting in a particular region can often be difficult as it is a decision that must strike a balance between planting too early and too late and enduring problems of different pest and diseases. Planting too early and too late makes the crop susceptible to different diseases, like CLCuD. Data on the effect on planting on the disease index of CLCuD (Table 2-3) revealed that among sowing dates regardless of genotypes, disease incidence differed significantly. The CLCuD boost up during 25th to 29th std., (2010) and 27th to 31st std., (2011) weeks of the year regardless of sowing dates and genotypes.

Averaged across cultivars, minimum disease index of CLCuD were recorded on 35th std., week of the year (150 days after planting) on crop planted on 15th std., week of the year (2010). Whereas, in other planting, data showed that no difference in disease index was observed. (Fig. 1). Averaged across cultivations, minimum disease index of CLCuD was recorded on

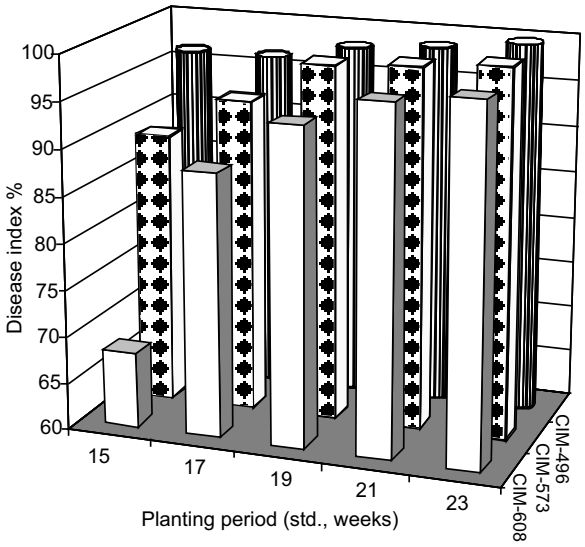

Fig. 1. Effect of CLCuD index as affected by different planting dates during 2010.

crop planted on 15th std. and 17th std., week compared with crop planted on 19th std., week of the year (2011). A little difference of disease index was recorded on crop planted during 21st and 23rd std., week of the year (Fig. 2). According to Sharma *et al*. (2006), the disease incidence increases rapidly between the mid of June to the last week of July (25th-31st std., week). Ghazanfar *et al*. (2007) also stated that sowing even earlier to 15th May (19th std., week) may have more effect on reduction in disease incidence which needs to be tested.

Varieties effects. The responses of varieties of cotton crop species toward the attack of its different pathogens are different. Some varieties display tolerant and others exhibit susceptible response. The maximum disease index of CLCuD was recorded on CIM-496 (97%), followed by CIM-573 (95%) and CIM-608 (89%) respectively, irrespective of the planting dates for the year 2010 (Fig. 1). On the basis of disease incidence (Table 2) and CLCuD index (Fig. 1) the strain CIM-608 showed some tolerance against the disease as compared to other varities/strains when planted on 15th std., week of the year. Whereas, in other planting, data showed that no difference in disease index was observed in all cultivars.

Averaged across planting dates, minimum disease index (33%) was recorded on cultivar CIM-608 followed by CIM-591 (59%) and CIM-554 (60%), respectively, during the year 2011 (Fig. 2). However, there was little difference of incidence and disease index when planted

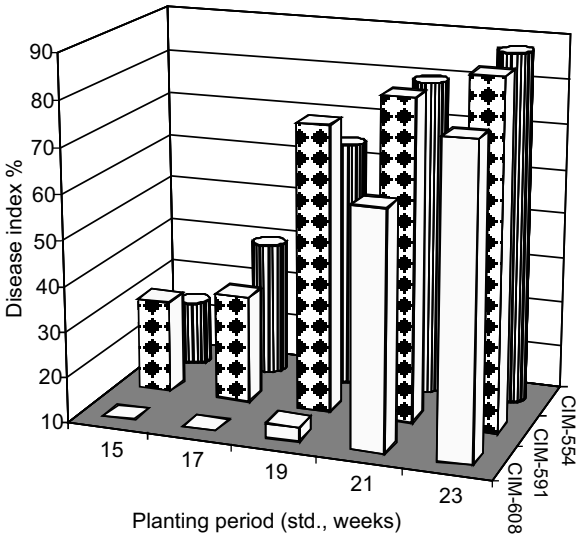

Fig. 2. Effect of CLCuD index as affected by different planting dates during 2011.

on 21st to 23rd std., week of the year as well as with the comparison of other strains/varieties. The cultivars CIM-554 and CIM-591 showed no effect on the reduction of disease index on any planting dates.

It is concluded from the two years studies on sowing dates in relation to CLCuD, the CIM-608 showed comparatively less incidence of disease when planted between 15th to 17th std., weeks of the year. Whereas, planting during 19th std., week of the year or late, it was heavily attacked by CLCuD (Table 4). It may be suggested that to plant the cultivar (CIM-608) before 19th std., week of the year to get maximum yield due to less disease incidence.

These findings are similar to that of Tahir *et al*. (2004) who found that in cotton cultivars under trial, the incidence was more on CIM-496 as compared to others. Akhtar *et al*. (2004) found that the age related to susceptibility to CLCuD was more apparent in late planting. Maximum increase in disease incidence occurred at 6 weeks after sowing. The incidence of different viral and fungal diseases is also influenced by altering the date of sowing as reported by Mirza (1992) and Singh *et al*. (1989).

Effect of weather parameters. The fortnightly increase in the disease of both years along with the environmental parameters of that period is given in Table. 5-6.

On an average basis of planting dates the fortnightly increase of the maximum disease started from 25th- 29th std., week of the year (2010) during which period the range of maximum temperature was 35.5 to

Table 4. Incidence of CLCuD as influenced by planting dates on cultivar CIM-608 during 2010-11

Planting time	Incidence of CLCuD (%)								
(Std., weeks)	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	0	0.49	5.16	12.5	21.4	24.6	40.5	54.1	58.92
17	0	4.1	16.2	41.7	37.0	57.0	57.4	59.7	Ê
19	0.7	18.2	46.3	64.5	68.9	69.6	70.5	Ê	Ê
21	16.8	56.0	73.7	91.7	94.7	96.6	Ê	Ê	Ê
23	46.8	64.2	97.2	98.4	-	-	-	-	-

* = days after planting.

Table 5. Relationship between fortnightly increase in CLCuD with weather parameters during 2010

Planting time	Fortnightly increase on std., weeks								
(Std., weeks)	19	21	23	25	27	29	31	33	35
15	0	1.7	10.2	35.5	12.6	10.0	11.9	13.1	0.6
17	-	0.3	9.3	42.2	20.6	9.7	16.2	0.8	0.4
19	-	-	1.4	38.1	40.4	19.7	0.5	0.0	0.5
21	-	-	-	41.3	57.7	1.0	0.0	0.0	0.0
23	-	-	-	-	90.7	8.7	0.5	0.0	0.0
Average	-	-	**6.9**	**39.2**	**44.0**	**9.8**	**5.8**	**2.8**	**0.3**
Max. °C	39.9	42.2	38.1	39.8	37.0	35.5	33.5	34.4	37.9
Min. °C	24.5	26.5	27.7	28.9	28.8	28.9	27.6	27.1	26.7
Difference	15.4	15.7	10.4	10.9	8.2	6.6	5.9	7.3	8.2
RH%	49.0	40.5	52.0	62.5	68.7	79.6	89.6	82.7	80.9

Table 6. Relationship between fortnightly increases in CLCuD with weather parameters during 2011

Planting time	Fortnightly increase on std., weeks								
(Std., weeks)	19	21	23	25	27	29	31	33	35
15	0	0.4	5.5	7.1	15.0	7.9	4.2	1.1	0.6
17	-	0.0	1.6	10.3	15.8	10.8	7.1	1.3	0.4
19	-	-	1.0	7.3	22.2	24.7	21.6	3.9	0.5
21	-	-	-	1.8	19.7	37.2	33.2	3.5	2.0
23	-	-	-	-	7.2	28.5	61.7	1.1	0.6
Average	-	-	-	**6.6**	**16.0**	**21.8**	**25.5**	**2.2**	**0.8**
Max. °C	39.8	41	40	38.6	36.6	36.5	34.8	34.3	32.3
Min. °C	25.3	28.4	28.4	30.2	28.5	28.8	28.8	27.4	26.7
Difference	14.5	12.6	11.6	8.4	8.1	7.7	6.0	6.9	5.6
RH%	52.6	53.4	54.6	67.8	72	76.1	75.4	82.5	56.8

39.8 °C with R.H from 62.5 to 79.6%. The fortnightly increase of the disease remains negligible or low before 25th std., week because during those days the temperature remained high with low R.H and after 29th std., week due to low temperature with high relative humidity, irrespective of planting dates; whereas the incidence remained low (during 25th -29th std., weeks of the year) on that crop planted on 15th - 17th std., week of the year as compared to other planting dates (Table 5).

On an averaged basis of planting dates the maximum fortnightly increase of the disease starts from 27th - 31th std., week of the year (2011). Among environmental parameters the maximum temperature range was 34.8-38.6 °C, minimum temperature, 28.5-28.8 °C with relative humidity 72.0-76.1% during the above mentioned period. The fortnightly increase of the disease remained low up to 25th std., week of the year and same conditions were found after 31st std., week of the year (Table 6).

It is clear that CLCuD increased rapidly during the period 25thto 27th std., week of the year 2010, whereas, the disease gradually increased during the 29th to 31th std., week of the year 2011. If it is compared with the weather parameters it indicates that the maximum temperature was high, minimum temperature remained constant with relative humidity 62-79% in the year 2010 as compared to 2011 during 25th to 31th std., week of the year.

It is concluded that the disease does not express its symptoms if the temperature is greater than 40 °C and less than 50% of relative humidity during the early season of the crop. During the end of the season the disease also does not exhibit its symptoms if temperature is less than 34 °C with greater than 80% relative humidity. The results are in accordance with the findings of Sherma *et al.* (2006). In this study it is concluded that the disease was highly influenced by mean temperature and morning humidity. The morning relative humidity and mean temperature explained the variability in disease incidence.

Conclusion

Planting time plays an important role to get maximum cotton yield. The age of cotton plant was directly related of CLCuD susceptibility in late planting. Among cultivars the strain CIM-608 showed comparatively less incidence of CLCuD when planted between 15th to 17th std., weeks of each year. It is suggested that CIM-608 may be cultivated before 19th std., week of the year to get less disease incidence. The CLCuD increased rapidly, when the weather parameters remain average i.e., maximum temperature 34.8 to 39.8 °C, minimum temperature 27.7 to 28.9 °C, and relative humidity 62 to 79%. In case of early planting, plants had attained strong vigour before prevalence of these environmental conditions so plants were less affected by CLCuD as compared to late planting. In late planting, plants were week and tender at these weather phases and caused more CLCuD infection.

One of the tools used in reducing environmental risks and increasing the possibilities of profitable yield is cultivar development through breeding and genetics.

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Effect of Plant Age on Cotton Leaf Curl Disease (CLCuD) in Relation to Environmental Conditions

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Abstract. The effect of cotton leaf curl disease (CLCuD) and weather variables were studied using same genotypes of cotton, planted at 15 days interval during 15th, 23rd standard weeks of 2010-2011. On an average basis, the 15th standard week planting showed, significantly, less disease incidence than all other sowing dates. The incidence increased as the sowing was delayed up to 23rd standard weeks. Among the sowing dates, regardless of genotypes, disease incidence differed, significantly. The CLCuD boost up during 25th to 29th standard (2010) and 27th to 31st standard (2011) weeks of the year, regardless of sowing date and genotypes. Disease incidence was low during 2011 as compared to 2010. The disease increased sharply during 2010 and gradually during 2011. Average maximum (34.8~39.8 °C), minimum (27.7~28.9 °C) temperature and relative humidity (62~79%) favoured CLCuD progression.

Keywords: cotton leaf curl disease, cotton cultivars, relative humidity, temperature

Introduction

Upland cotton (*Gossypium hirsutum* L.) is one of the important fibre and cash crops of Pakistan. Cotton occupies a unique position in textile world with millions of people engaged in its cultivation, processing and marketing etc.

The main reasons for low productivity of cotton is heavy attack by a number of insect-pests that results in various diseases starting from germination up to the harvest of crop. Among these diseases, cotton leaf curl virus disease (CLCuD) is the major reason for the decline in cotton production and productivity in Pakistan, especially in the Punjab. This disease is caused by cotton leaf curl virus (CLCuV) which belongs to begomovirus (Family Germiniviridae) transmitted in persistent manner by the vector, whitefly (*Bemisia tabaci* Gennadius). Characteristic symptom of the disease is upward or downward curling of leaves with thickened veins which is more pronounced on under side. The disease results in stunted plant growth with loss in yield (Hameed *et al.*, 1994).

CLCuD was reported for the first time in Pakistan during 1967 near Multan. At that time disease was of minor importance and did not get much attention. After 1988, the disease appeared in an epidemic form and damaged the crop on about sixty thousand hectares with a loss of 0.3 billions bales in production (Mahmood *et al.,* 1999). The geographic spread of CLCuD has increased tremendously and more than 7.7 million bales of cotton have been lost due to CLCuD from 1986 to 2002 (Akhtar *et al.,* 2004).

Losses due to this disease depend upon the variety and sowing time of cotton crop (Tahir *et al.*, 2004). Weather factors (individual and collectively) particularly temperature, relative humidity and rain fall influence the disease and vector (whitefly) population to great extent in host pathogen system. The hot and humid climate in north India during crop season is conducive for growth of host and the vector (Sharma *et al.*, 2006). However, meager information is available on the role of climatic factors affecting vector population and disease development. The present studies were therefore, carried out to understand the role of environmental factors on the development of CLCuD.

Materials and Methods

The present investigations were designed to work out the effect of temperature and relative humidity and different planting dates on cotton varieties at research area of Central Cotton Research Institute (CCRI), Multan, Pakistan.

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Delinted seed of three genotypes *viz.*, CIM-573, CIM-496 and CIM-608 (2010) and CIM-554, CIM591 and CIM-608 (2011) were planted on 15th, 17th, 19th, 21st, and 23rd standard weeks of each year by dibbling method. The plants were spaced 75 cm row to row and 30 cm plant to plant distance. The experiment was conducted in split plot (main plot: planting time, subplot: genotypes) design with four replication. All other cultural practices were performed in standard fashions to optimise the seed cotton yield.

Observations on the incidence of CLCuD were recorded at 15 days interval starting from 30 days after planting and continued up to 35th std., week.

Total numbers of plants showing leaf curl virus disease symptoms (upward curling with thickened veins on under side of leaf) were counted every time during observations. Plants with even a single leaf showing the symptoms of disease were counted as infected. The percentage disease incidence was counted by using following formula

$$\text{Disease incidence (\%)} = \frac{\text{total plants} - \text{healthy plants}}{\text{total plants}} \times 100$$

At the end of the season (35th std., week) each and every plant was examined in the field and different grades/ scales were allotted to them according to the level of infection in disease plants as described by Akhtar and Khan (2002). The rating scales are given in Table 1.

The percentage of disease index was calculated by using the following formula

$$\text{Disease index (\%)} = \frac{\text{summation of all disease ratings}}{\text{total plants}} \times \frac{100}{\text{maximum grade}}$$

The data on environmental variables were obtained from Meteorological Department, Central Cotton Research Institute, Multan, Pakistan. Data for fortnightly progression of disease incidence were calculated and compared with environmental parameters (maximum, minimum temperature & relative humidity) of that period of each year separately. The pooled data for both years were first analysed by simple regression. The disease index were subjected to standard statistical analysis (Steel *et al*., 1996) and the means were compared using DMR/LSD test (P=005).

Results and Discussion

The results of incidence of CLCuD monitored (fortnightly interval) right from 30 days after planting for all planting dates in each year are given in Table 2-3.

Progression of disease. Results reveal that expression of CLCuD and its progression during the crop seasons of (2010 and 2011) differed greatly with planting dates. Averaged across varieties, minimum incidence of disease (1.7%) was recorded at day 45 after planting and increased upto 11.9% at day 60 after planting. The disease boosted upto 82% at day 120 after planting on that crop planted on 15th std., week of the year.

The infection level was 9.5% at day 45 and attained its maximum (98.2%) at day 105 after planting on crop planted on 17th std., week of the year. However, incidence

Table 1. Disease rating scales on the basis of intensity of CLCuD for its index

Symptoms	Rating scale	Symptoms	Rating scale
Complete absence of symptoms	0	Large groups of veins involved and curling or top of the plant affected	4
Few small scattered vein thickening	1	All veins involved and severe curling or half of the plant affected	5
Small scattered vein thickening	2	All veins involved and severe curling and stunted plant or whole of the plant affected and stunting	6
Vein thickening involving small groups of veins	3	Enations	E

Table 2. Incidence of CLCuD as influenced by planting dates and strain during 2010

Planting time		Incidence of CLCuD (%)								
(Std., weeks)	Cultivar	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	CIM-608	0	0.52	7.17	16.54	28.41	33.09	61.48	87.41	96.05
	CIM-573	0	1.10	9.33	59.12	73.06	88.21	93.05	97.81	100
	CIM-496	0	3.42	19.21	66.59	78.66	88.92	91.44	100	100
	Average	**0**	**1.7**	**11.9**	**47.4**	**60.0**	**70.1**	**82.0**	**95.1**	**98.7**
17	CIM-608	0	7.8	25.8	51.59	56.39	95.78	97.65	100	-
	CIM-573	0.42	10.11	68.54	81.21	89.54	98.74	99.12	100	-
	CIM-496	0.42	10.72	60.87	84.07	100	100	100	100	-
	Average	**0.3**	**9.5**	**51.7**	**72.3**	**82.0**	**98.2**	**98.9**	**100**	**-**
19	CIM-608	0.92	30.48	74.92	99.06	100	100	100	-	-
	CIM-573	0.88	38.29	68.37	100	100	100	100	-	-
	CIM-496	2.26	49.66	96.35	99.53	100	100	100	-	-
	Average	**1.4**	**39.5**	**79.9**	**99.5**	**100**	**100**	**100**	**-**	**-**
21	CIM-608	33.03	97.05	100	100	100	100	-	-	-
	CIM-573	46.56	100	100	100	100	100	-	-	-
	CIM-496	44.45	100	100	100	100	100	-	-	-
	Average	**41.3**	**99.0**	**100**	**100**	**100**	**100**	**-**	**-**	**-**
23	CIM-608	88.21	100	100	100	100	-	-	-	-
	CIM-573	89.10	98.36	100	100	100	-	-	-	-
	CIM-496	94.81	100	100	100	100	-	-	-	-
	Average	**90.7**	**99.5**	**100**	**100**	**100**	**-**	**-**	**-**	**-**

* = days after planting.

Table 3. Incidence of CLCuD as influenced by planting dates and strain during 2011

Planting time		Incidence of CLCuD (%)								
(Std., weeks)	Cultivar	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	CIM-608	0	0.46	3.16	8.6	14.49	16.17	19.54	20.87	21.79
	CIM-591	0	0.40	5.41	16.37	37.43	47.47	49.75	51.65	52.56
	CIM-554	0	0.39	9.22	14.22	31.72	43.73	50.76	50.77	50.97
	Average	**0**	**0.41**	**5.91**	**13.06**	**27.08**	**35.79**	**40.01**	**41.09**	**41.77**
17	CIM-608	0	0.55	6.75	13.91	17.69	19.08	19.18	19.55	-
	CIM-591	0	1.02	11.55	28.97	48.62	59.99	63.34	64.3	-
	CIM-554	0	3.09	26.21	49.06	58.02	66.42	66.76	67.48	-
	Average	**01.55**	**14.83**	**30.64**	**41.44**	**48.49**	**49.76**	**50.44**	**-**	**-**
19	CIM-608	0.51	6.0	17.87	30.12	37.96	39.92	41.13	-	-
	CIM-591	0.88	8.34	27.29	69.44	89.71	100	100	-	-
	CIM-554	1.48	10.5	37.20	57.03	93.57	93.96	94.12	-	-
	Average	**0.95**	**8.28**	**27.45**	**52.19**	**73.75**	**77.96**	**78.41**	**-**	**-**
21	CIM-608	0.6	15.05	47.56	83.51	89.56	93.3	-	-	-
	CIM-591	2.71	24.84	61.07	93.79	98.04	99.3	-	-	-
	CIM-554	2.16	24.80	67.75	-	99.21	99.80	-	-	-
	Average	**1.82**	**21.56**	**58.79**	**98.63**	**95.60**	**97.46**	**-**	**-**	**-**
23	CIM-608	5.42	28.45	94.56	95.82	96.89	-	-	-	-
	CIM-591	7.07	32.43	98.69	99.57	100	-	-	-	-
	CIM-554	9.06	46.31	99.06	99.53	100	-	-	-	-
	Average	**7.18**	**35.73**	**97.43**	**98.30**	98.96	-	-	-	-

* = days after planting.

of CLCuD (39.5%) of day 45 and reached to its maximum level (99.5%) at day 75 after planting on crop planted on 19th std., week of the year. The incidence of the disease was 41.3% at day 30 and reached to maximum (99%) at day 45 after planting on crop planted on 21 std., week of the year. Further more crops planted on 23rd std., week of the year fell prey to CLCuD to 90.7% within 30 days after planting and to maximal

(99.5%) with in next 15 days (Table 2). Data revealed that the incidence of disease increased and period decreased (days after planting) as the planting time was increased (Table 2).

Averaged across varieties, disease started at low level (0.41%) at day 45 after planting and reached upto 5.91% at day 60 after planting. With the advancement of age, the incidence progressed gradually to 40.01% within next 60 days, on that crop planted on 15th std., week of the year. The infection level was 14.83 at day 60 and attained it maximum (48.4%) at day 105 after planting on crop planted on 17th std., week of the year. However, incidence of CLCuD was 8.28% at day 45 and reached to its maximum level 78.4% within rest days as crop planted 19 std., week of the year. The incidence of disease start from 1.82% at day 30 and reached upto (91.9%) at day 75 after planting on crop planted 21st std., week of the year. Furthermore, the crop planted 23rd std., week of the year fell prey to CLCuD to 97.4% with in 60 days after planting, which is 50% period less and incidence double than those crop planted on 17th std., week of the year (Table 3). Similar findings were made by Tahir *et al*. (2004) and Khan *et al*. (1988). They concluded that maximum incidence was recorded in June planting (21st std., week) and increased rapidly in the first week of August (29th std., week) in all planting dates.

Effect of planting date. One of the most important agronomic considerations for growers to optimise yield and quality is to select an appropriate planting time for cotton crop. Choosing the best time for planting in a particular region can often be difficult as it is a decision that must strike a balance between planting too early and too late and enduring problems of different pest and diseases. Planting too early and too late makes the crop susceptible to different diseases, like CLCuD. Data on the effect on planting on the disease index of CLCuD (Table 2-3) revealed that among sowing dates regardless of genotypes, disease incidence differed significantly. The CLCuD boost up during 25th to 29th std., (2010) and 27th to 31st std., (2011) weeks of the year regardless of sowing dates and genotypes.

Averaged across cultivars, minimum disease index of CLCuD were recorded on 35th std., week of the year (150 days after planting) on crop planted on 15th std., week of the year (2010). Whereas, in other planting, data showed that no difference in disease index was observed. (Fig. 1). Averaged across cultivations, minimum disease index of CLCuD was recorded on

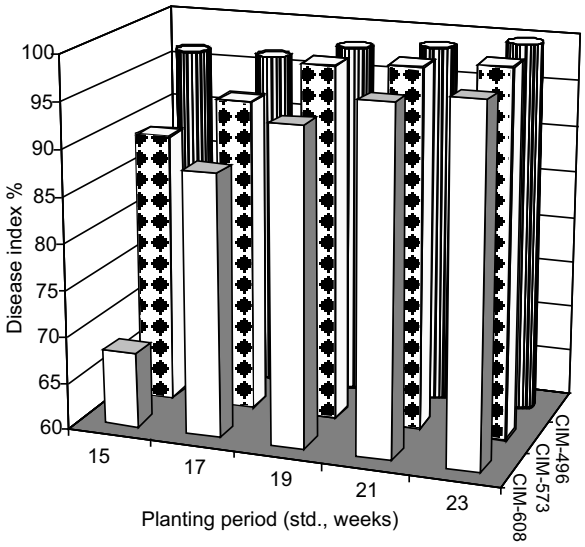

Fig. 1. Effect of CLCuD index as affected by different planting dates during 2010.

crop planted on 15th std. and 17th std., week compared with crop planted on 19th std., week of the year (2011). A little difference of disease index was recorded on crop planted during 21st and 23rd std., week of the year (Fig. 2). According to Sharma *et al*. (2006), the disease incidence increases rapidly between the mid of June to the last week of July (25th-31st std., week). Ghazanfar *et al*. (2007) also stated that sowing even earlier to 15th May (19th std., week) may have more effect on reduction in disease incidence which needs to be tested.

Varieties effects. The responses of varieties of cotton crop species toward the attack of its different pathogens are different. Some varieties display tolerant and others exhibit susceptible response. The maximum disease index of CLCuD was recorded on CIM-496 (97%), followed by CIM-573 (95%) and CIM-608 (89%) respectively, irrespective of the planting dates for the year 2010 (Fig. 1). On the basis of disease incidence (Table 2) and CLCuD index (Fig. 1) the strain CIM-608 showed some tolerance against the disease as compared to other varities/strains when planted on 15th std., week of the year. Whereas, in other planting, data showed that no difference in disease index was observed in all cultivars.

Averaged across planting dates, minimum disease index (33%) was recorded on cultivar CIM-608 followed by CIM-591 (59%) and CIM-554 (60%), respectively, during the year 2011 (Fig. 2). However, there was little difference of incidence and disease index when planted

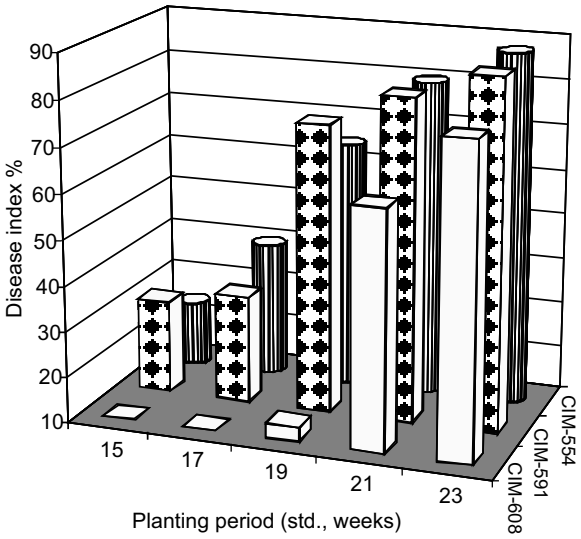

Fig. 2. Effect of CLCuD index as affected by different planting dates during 2011.

on 21st to 23rd std., week of the year as well as with the comparison of other strains/varieties. The cultivars CIM-554 and CIM-591 showed no effect on the reduction of disease index on any planting dates.

It is concluded from the two years studies on sowing dates in relation to CLCuD, the CIM-608 showed comparatively less incidence of disease when planted between 15th to 17th std., weeks of the year. Whereas, planting during 19th std., week of the year or late, it was heavily attacked by CLCuD (Table 4). It may be suggested that to plant the cultivar (CIM-608) before 19th std., week of the year to get maximum yield due to less disease incidence.

These findings are similar to that of Tahir *et al*. (2004) who found that in cotton cultivars under trial, the incidence was more on CIM-496 as compared to others. Akhtar *et al*. (2004) found that the age related to susceptibility to CLCuD was more apparent in late planting. Maximum increase in disease incidence occurred at 6 weeks after sowing. The incidence of different viral and fungal diseases is also influenced by altering the date of sowing as reported by Mirza (1992) and Singh *et al*. (1989).

Effect of weather parameters. The fortnightly increase in the disease of both years along with the environmental parameters of that period is given in Table. 5-6.

On an average basis of planting dates the fortnightly increase of the maximum disease started from 25th- 29th std., week of the year (2010) during which period the range of maximum temperature was 35.5 to

Table 4. Incidence of CLCuD as influenced by planting dates on cultivar CIM-608 during 2010-11

Planting time	Incidence of CLCuD (%)								
(Std., weeks)	30*	45*	60*	75*	90*	105*	120*	135*	150*
15	0	0.49	5.16	12.5	21.4	24.6	40.5	54.1	58.92
17	0	4.1	16.2	41.7	37.0	57.0	57.4	59.7	Ê
19	0.7	18.2	46.3	64.5	68.9	69.6	70.5	Ê	Ê
21	16.8	56.0	73.7	91.7	94.7	96.6	Ê	Ê	Ê
23	46.8	64.2	97.2	98.4	-	-	-	-	-

* = days after planting.

Table 5. Relationship between fortnightly increase in CLCuD with weather parameters during 2010

Planting time	Fortnightly increase on std., weeks								
(Std., weeks)	19	21	23	25	27	29	31	33	35
15	0	1.7	10.2	35.5	12.6	10.0	11.9	13.1	0.6
17	-	0.3	9.3	42.2	20.6	9.7	16.2	0.8	0.4
19	-	-	1.4	38.1	40.4	19.7	0.5	0.0	0.5
21	-	-	-	41.3	57.7	1.0	0.0	0.0	0.0
23	-	-	-	-	90.7	8.7	0.5	0.0	0.0
Average	-	-	**6.9**	**39.2**	**44.0**	**9.8**	**5.8**	**2.8**	**0.3**
Max. °C	39.9	42.2	38.1	39.8	37.0	35.5	33.5	34.4	37.9
Min. °C	24.5	26.5	27.7	28.9	28.8	28.9	27.6	27.1	26.7
Difference	15.4	15.7	10.4	10.9	8.2	6.6	5.9	7.3	8.2
RH%	49.0	40.5	52.0	62.5	68.7	79.6	89.6	82.7	80.9

Table 6. Relationship between fortnightly increases in CLCuD with weather parameters during 2011

Planting time	Fortnightly increase on std., weeks								
(Std., weeks)	19	21	23	25	27	29	31	33	35
15	0	0.4	5.5	7.1	15.0	7.9	4.2	1.1	0.6
17	-	0.0	1.6	10.3	15.8	10.8	7.1	1.3	0.4
19	-	-	1.0	7.3	22.2	24.7	21.6	3.9	0.5
21	-	-	-	1.8	19.7	37.2	33.2	3.5	2.0
23	-	-	-	-	7.2	28.5	61.7	1.1	0.6
Average	-	-	-	**6.6**	**16.0**	**21.8**	**25.5**	**2.2**	**0.8**
Max. °C	39.8	41	40	38.6	36.6	36.5	34.8	34.3	32.3
Min. °C	25.3	28.4	28.4	30.2	28.5	28.8	28.8	27.4	26.7
Difference	14.5	12.6	11.6	8.4	8.1	7.7	6.0	6.9	5.6
RH%	52.6	53.4	54.6	67.8	72	76.1	75.4	82.5	56.8

39.8 °C with R.H from 62.5 to 79.6%. The fortnightly increase of the disease remains negligible or low before 25th std., week because during those days the temperature remained high with low R.H and after 29th std., week due to low temperature with high relative humidity, irrespective of planting dates; whereas the incidence remained low (during 25th -29th std., weeks of the year) on that crop planted on 15th - 17th std., week of the year as compared to other planting dates (Table 5).

On an averaged basis of planting dates the maximum fortnightly increase of the disease starts from 27th - 31th std., week of the year (2011). Among environmental parameters the maximum temperature range was 34.8-38.6 °C, minimum temperature, 28.5-28.8 °C with relative humidity 72.0-76.1% during the above mentioned period. The fortnightly increase of the disease remained low up to 25th std., week of the year and same conditions were found after 31st std., week of the year (Table 6).

It is clear that CLCuD increased rapidly during the period 25thto 27th std., week of the year 2010, whereas, the disease gradually increased during the 29th to 31th std., week of the year 2011. If it is compared with the weather parameters it indicates that the maximum temperature was high, minimum temperature remained constant with relative humidity 62-79% in the year 2010 as compared to 2011 during 25th to 31th std., week of the year.

It is concluded that the disease does not express its symptoms if the temperature is greater than 40 °C and less than 50% of relative humidity during the early season of the crop. During the end of the season the disease also does not exhibit its symptoms if temperature is less than 34 °C with greater than 80% relative humidity. The results are in accordance with the findings of Sherma *et al.* (2006). In this study it is concluded that the disease was highly influenced by mean temperature and morning humidity. The morning relative humidity and mean temperature explained the variability in disease incidence.

Conclusion

Planting time plays an important role to get maximum cotton yield. The age of cotton plant was directly related of CLCuD susceptibility in late planting. Among cultivars the strain CIM-608 showed comparatively less incidence of CLCuD when planted between 15th to 17th std., weeks of each year. It is suggested that CIM-608 may be cultivated before 19th std., week of the year to get less disease incidence. The CLCuD increased rapidly, when the weather parameters remain average i.e., maximum temperature 34.8 to 39.8 °C, minimum temperature 27.7 to 28.9 °C, and relative humidity 62 to 79%. In case of early planting, plants had attained strong vigour before prevalence of these environmental conditions so plants were less affected by CLCuD as compared to late planting. In late planting, plants were week and tender at these weather phases and caused more CLCuD infection.

One of the tools used in reducing environmental risks and increasing the possibilities of profitable yield is cultivar development through breeding and genetics.

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Stability of Microbial and Chemical Indicators of the Minced Beef Meat under Freezing and Refrigerated Temperature

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Abstract. The microbial spoilage and chemical changes in minced beef meat were monitored during storage at freezing and refrigerating temperatures. Total viable count of *Pseudomonas*, *Streptococcus* faecal count, faecal coliform and *Staphylococcus aureus* in minced beef meat collected from supermarkets at day 0 were 4.3, 3.2, 2.5, 2.2 and 3.9 $\log_{10}$ CFU/g, respectively. These counts increased after 5 days of storage at 4±1 °C to 7.3, 7.1, 3.8, 5.0 and 3.3 $\log_{10}$ CFU/g, respectively. These counts decreased after 6 months at -10±1 °C to 3.2, 2.6, 2.0, 1.2 and 1.0 $\log_{10}$ CFU/g, respectively. The results also indicated that the total viable count of *Pseudomonas*, *Streptococcus* faecal count, faecal coliform and *Staphylococcus aureus* were higher in small butcher shop as compared to supermarket at day 0. On day 0 the thiobarbituric acid reactive in minced beef meat samples collected from supermarket and small butcher shop were 0.89 and 1.15 mg malonaldehyde/kg, respectively. After 5 days of storage at 4 °C, the thiobarbituric acid reactive in minced meat beef collected from supermarket and small shop increased and reached upto 2.95 and 3.74 mg malonaldehyde/kg, respectively. It increased to 3.02 in minced beef from supermarket, and 3.85 mg from small shop after 6 months at -10±1 °C. Lightness, redness and yellowness of minced beef meat decreased, when meat was kept under cooling and freezing temperature, however, lightness, redness and yellowness of minced beef meat were higher in density in supermarket samples than those of meat obtained from small butcher shops.

Keywords: cooling, freezing temperature, minced beef meat, TBARS, colour

Introduction

The minced beef meat is of high value in terms of nutrition and economy. It contains most of the nutrients specially essential amino acids. The minced beef meat is used in many types of meals. This type of meat is highly perishable due to its suitablity for the growth of microorganisms as the large exposed surface area facilitates spoilage. Fresh minced beef meats are commonly marketed at refrigerated temperature (4±1 °C). Total count and coliform bacteria are good indicators of the hygienic quality of minced meat (Skrökki, 1997). *Enterobacter*, Lactobacilli, Pseudomonads, *Brochothrix thermosphacta* and *Shewanella putrefaciens* are responsible for spoilage of fresh meat and meat products (Huffman, 2002; Garbutt, 1997). The chilled temperatures with high moisture will favour the *Pseudomonas alcaligenes*. Extended refrigeration may have the growth of *Pseudomonas*, *Acinetobacter* and *Moraxella* and causes spoilage of fresh meat. Yeasts may grow under aerobic conditions on meat and causes sliminess, lipolysis, discolourations, off odours and taste. Coliform is often used as hygiene indicator of foods of animal origin. The presence of coliforms in meat indicates that inadequate treatment or post-process contamination occur during handling or manufacturing stages (Crowley *et al*., 2005). Meat is contaminated with many types of microorganisms during slaughtering, evisceration, chilling, handling, and grinding (Jay, 2002). *Pseudomonas* spp. are ubiquitous and able to grow aerobically at low temperatures and are generally recognized to dominate in meat during storage at refrigeration temperature. Many *Pseudomonas* are responsible for spoilage of meat and meat products by degradation of glucose and amino acid, even at refrigeration temperatures (Koutsoumanis *et al*., 2006; Skandamis and Nychas, 2005; Ellis and Goodacre, 2001; Labadie, 1999). Also *Serratia*, *Enterobacter*, *Pantoea*, *Proteus*, and *Hafnia*, often contribute to meat spoilage (Jay *et al*., 2003; Gram *et al*., 2002; Labadie, 1999; Nychas and Drosinos, 1999; Borch *et al*., 1996). Podpeèan *et al*. (2007), isolated *S. aureus* in 62.5% (10/16) of specimens from ground meat. *S. aureus* grows, when meat is stored in an inadequate environment for longer period of time. During refrigeration, the hygienic quality of meat declines

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rapidly due to microbial growth through contamination from different sources and lipid oxidation, which eventually leads to meat spoilage. Reduction of contamination, cross contamination, delaying or inhibiting spoilage and growth of pathogenic bacteria improves meat quality, enhance safety and increase its shelf life. There are some factors, which can be effective for keeping meat quality that includes temperature, time of ageing and packaging (Beriain and Lizaso, 1997). Minced beef meat is susceptible to microbial contamination during processing and handling stages (Nam and Ahn, 2003). According to Irkin *et al*. (2011), minced beef meat is acceptable, when the total count is in the range from 5×10^6 to 1×10^7 CFU/g.

Oxidation and rancid off-flavour can occur during refrigerated and frozen storage (Pearson and Young, 1989). Meat colour is the main factor and discolouration and oxidative rancidity and off-flavour in red meat are related to the oxidation of myoglobin and the autoxidation of fat, which affects the consumer buying decisions (Brewer *et al*., 2002; Faustman and Cassens, 1990). Aerobic bacterial growth during logarithmic phase increases the rate of beef oxidation due to its high oxygen demand for the oxidation of myoglobin to metmyoglobin (Seideman *et al*., 1984). The sensory deterioration of minced beef meat is due to the growth of microorganisms and consumption of meats nutrients such as sugars and free amino acids that liberate undesirable volatile metabolites. Off-odours such as "cheesy" or "buttery" odours usually happens when the microbial load in minced beef meat over 10^7 CFU cm^2. Off-odours "fruit" evolves, when microbial loads increase and become moldy due to free amino acids consumed by microorganisms and numbering over 10^9 CFU cm^2 (Ercolini *et al*., 2006; Jay, 2000; Dainty *et al*., 1985).

Meat processors usually need meat of high quality with low microbial load. Therefore, it is very important for meat manufacturers to collect data that can be used by meat industry to validate or change their current practices of processing in order to increase the ground beef quality, safety and shelf life.

The main objective of this study was to evaluate the effects of cooling and freezing temperature (4±1 °C and -10±1 °C) on the microbial and chemical quality of minced beef meat.

Materials and Methods

Sample preparation. Minced beef meat samples were collected from four supermarkets butcheries and four butcher shops in Riyadh city area (capital of Saudi Arabia). About 250±10 g of each of the ground meat samples were collected, packed in sterile polyethylene bags. The samples were kept at low temperature (0/ -1 °C) and transported to the lab within 1 h, each replica consisted of 32 samples under aseptic conditions. On arrival at the laboratory, the samples were analysed for microbial count, colour and lipid oxidation. The rest of the samples were stored at temperatures appropriate to the study. Each batch consisted of 16 samples of minced beef collected from supermarkets butcheries and 16 samples collected from small shops. Eight samples from supermarkets and 8 samples from the small shops were stored at 4±1 °C, while, 8 samples each from supermarkets and small shops were stored at -10±1 °C.

Storage. Lipid oxidation, colour and microbial analysis were carried out at 0, 1, 3 and 6 months of frozen storage (-10±1 °C) and at 0, 2, 4 and 5 days of refrigerated storage (4±1 °C). Samples for lipid oxidation, colour and microbial analysis were stored without vacuuming in sterile bags.

Microbiological analyses. The microbial load in minced beef was determined at the beginning (before storage) using FDA procedures (Koch *et al*., 2001). Five methods were used for identification and enumeration of microorganisms in ground beef samples: total viable cell count of *Pseudomonas*, *Streptococcus*, faecal coliform and *S. aureus.* Aseptically, approximately 25 g of minced beef meat were 10-fold diluted in 225 mL buffered peptone water and homogenised in a stomacher bag for 1 min. Serial decimal dilutions were made and the following analyses were carried out on agar plates in duplicate: (1) total viable count on aerobic plate count incubated at 30 °C for 48 h (2) *Pseudomonas* count on aerobic *Pseudomonas* media incubated at 30 °C for 24 h (3) *Streptococcus* count on tryptic soy agar incubated at 35 °C for 24 h (4) faecal coliform at aerobic Violet Red Bile Agar incubated at 30 °C for 24 h (5) *S. aureus* using aerobic *Staphylococcus* medium 110 incubated at 35 °C for 48 h.

Lipid oxidation measurement. Lipid oxidation was evaluated by the determination of 2-thiobarbituric acid

reactive substances (TBARS), which is a good indicator for measuring the lipid peroxidation in minced meat, due to its speed and simplicity (Raharjo and Sofos, 1993; Shahidi and Hong, 1991). TBARS is formed in meat as a byproduct of lipid peroxidation that are generated under conditions of oxidative stress. It is component, which results from the decomposition of polyunsaturated fatty acid lipid peroxides. TBARS determination were performed since this parameter is one of the most widely used tests for evaluating the extent of secondary oxidation, owing to its sensitivity and relatively simple procedure (Fernindez *et al*., 1997). TBARS values were calculated from a standard curve of malonaldehyde (MA) and expressed as mg MA/kg minced beef meat sample.

Meat colour measurement. A Hunter-Lab Digital Colour Difference Meter was used to measure *L* (luminosity), *a* (redness), and *b* (yellowness) values. Three measurements were taken uniformly spaced over the surface and the colourimetric values were averaged.

Statistical analysis. The factorial experiment in the completely randomised design was done with three replicates. Three replica was done for each independent. ANOVA for the factorial experiment in the completely randomised design was carried out according to Gomez and Gomez (1984). The means were compared using the least significant difference (LSD) at the 5% level according to Waller and Duncan (1969). SAS software package was used (SAS, 2001).

Results and Discussion

Microbial load, lipid oxidation and colour changes are the most important quality criteria for storage of minced meat (Anthoula *et al*., 2012; Ozlem *et al*., 2011; Brooks *et al*., 2008). The microbiological quality of frozen and cooling packed minced meat are shown in Table 1. The initial total viable count on minced meat samples collected from supermarkets and small shops were 4.3 and 4.8 $\log_{10}$ CFU/g, respectively. A similar level of initial contamination was reported by Anthoula *et al*. (2012); Coleen *et al*. (2012); Ozlem *et al*. (2011) and Brooks *et al*. (2008). On day 0, 2, 4 and 5 there was a significant decrease ($P<0.05$), in the number of total viable bacteria between cooled minced meat samples collected from supermarkets and samples collected from small butcher shops, respectively. The total viable count increased rapidly on samples packed in air and stored in refrigerator, which could be due to the grounding process, initial contamination and cold storage (Papadopoulou *et al*., 2012; Ozlem *et al*., 2011). A similar trend was observed for *Pseudomonas*. In all minced meat samples packed in air and stored chilled, *Pseudomonas* were found to be the dominatinant flora. Similar results were also reported by Abderrahmane *et al*. (2011) and Brooks *et al*. (2008). Meat is a highly perishable food product which, unless correctly stored, processed, packaged and distributed, spoils quickly and becomes hazardous due to the microbial growth (Odekerken *et al*., 2012; Papadopoulou *et al*., 2012; Zhou *et al*., 2010; Nychas *et al*., 2008). High counts in meat indicates contaminated raw materials or unsatisfactory processing or cross contamination after processing from a sanitary point of view (ICMSF, 1988).

Staphylococcus aureus is a major human pathogen associated with a wide spectrum of diseases from relatively benign skin infections to life-threatening endocarditis, toxic shock syndrome and necrotising pneumonia (Luning *et al*., 2011; Diane *et al*., 2010). This bacterium can contaminate several foods, including processed meat products and minimally processed ready-to-eat vegetables (El-Hadedy and Abu El-Nour, 2012; Diane *et al*., 2010). The initial contamination levels of *S. aureus* on minced meat samples collected from supermarkets and small shops were 2.6 and 3.3 $\log_{10}$ CFU/g, respectively. The presence of pathogens in the food supply in low numbers is considered a major cause of world-wide gastrointestinal disease (Luning *et al*., 2011). The number of *S. aureus* on minced meat stored at 4 °C increased, significantly ($P<0.05$), during 5 days of storage. In contrast the number of *S. aureus* on minced meat stored frozen (at -10 °C), significantly decreased ($P<0.05$), on all samples during 180 days of storage. It could be concluded that frozen storage reduces the risk of *S. aureus* pathogen on minced meat.

High counts of coliforms indicated poor hygiene in the food supply chain (George *et al*., 2011; Luning *et al*., 2011; Norrung and Buncic, 2008). The initial contamination level of fecal coliform on minced meat samples collected from supermarkets and small butcher shops were 2.2 and 4 $\log_{10}$ CFU/g, respectively. The $\log_{10}$ CFU/g of faecal coliform on minced meat stored in refrigerator at 4 °C, significantly increased ($P<0.05$), during 5 days of storage. In contrast all samples stored frozen at -10 °C, the $\log_{10}$ CFU/g of faecal coliform, significantly decreased ($P<0.05$), during 180 days of storage. Similar trend was observed for *Streptococcus.*

Table 1. The growth of microbial indicators in minced meat stored at 4 °C and -10 °C collected from supermarkets and small shops at Riyadh

Location	Chilled minced beef (4 °C)				Frozen minced beef (-10 °C)			
	Day 0	Day 2	Day 4	Day 5	Day 0	Day 30	Day 90	Day 180
	Total viable count (log_{10} CFU/g)				Total viable count (log_{10} CFU/g)			
Supermarkets	4.3^{Aa}	5.2^{Ba}	6.1^{Ca}	7.3^{Da}	4.3^{Aa}	4.8^{Ba}	3.7^{Ca}	3.2^{Da}
SD	0.3	0.4	0.2	0.5	0.25	0.6	0.4	0.4
Small butcher shops	4.8^{Ab}	5.7^{Bb}	6.6^{Cb}	7.8^{Db}	4.8^{Ab}	5.3^{Bb}	4.1^{Cb}	3.5^{Da}
SD	0.6	0.15	0.7	0.8	0.5	0.3	0.3	0.2
	Pseudomonas (log_{10} CFU/g)				*Pseudomonas* (log_{10} CFU/g)			
Supermarkets	3.2^{Aa}	4.2^{Ba}	4.9^{Ca}	7.1^{Da}	3.2^{Aa}	4.1^{Ba}	3.1^{Ca}	2.6^{Da}
SD	0.4	0.2	0.5	0.4	0.2	0.4	0.1	0.3
Small butcher shops	3.8^{Ab}	4.9^{Bb}	5.5^{Cb}	7.4^{Db}	3.8^{Ab}	4.4^{Bb}	3.6^{Cb}	3.0^{Da}
SD	0.5	0.2	0.7	0.7	0.25	0.3	0.2	0.4
	Streptococcus faecal (log_{10} CFU/g)				*Streptococcus* faecal (log_{10} CFU/g)			
Supermarkets	2.5^{Aa}	3.0^{Ba}	3.3^{Ba}	3.8^{Ca}	2.5^{Aa}	2.0^{Ba}	2.5^{ACa}	2.0^{BDa}
SD	0.2	0.2	0.15	0.4	0.2	0.2	0.1	0.2
Small butcher shops	3.9^{Ab}	4.6^{Bb}	4.8^{Bb}	5.3^{Cb}	3.9^{Ab}	3.0^{Bb}	3.1^{Ab}	2.6^{Da}
SD	0.3	0.3	0.4	0.5	0.35	0.4	0.2	0.25
	Faecal coliform (log_{10} CFU/g)				Faecal coliform l (log_{10} CFU/g)			
Supermarkets	2.2^{Aa}	3.7^{Ba}	4.5^{Ca}	5.0^{Da}	2.2^{Aa}	2.1^{Aa}	1.6^{Ba}	1.2^{Ca}
SD	0.3	0.3	0.5	0.4	0.1	0.15	0.3	0.4
Small butcher shops	4.0^{Ab}	4.3^{Ab}	4.9^{Bb}	5.6^{Cb}	4.0^{Ab}	3.5^{Bb}	2.3^{Cb}	1.5^{Da}
SD	0.1	0.2	0.4	0.6	0.3	0.4	0.2	0.2
	Staphylococcus aureus (log_{10} CFU/g)				*Staphylococcus aureus* (log_{10} CFU/g)			
Supermarkets	2.6^{Ab}	4.0^{Bb}	3.8^{Ba}	3.3^{Ca}	2.6^{Ab}	2.4^{Ab}	1.4^{Bb}	1.0^{Da}
SD	0.3	0.3	0.2	0.2	0.1	0.3	0.2	0.1
Small butcher shops	3.3^{Aa}	4.5^{Ba}	3.9^{Ca}	3.4^{Da}	3.3^{Aa}	2.3^{Ba}	1.5^{Ca}	1.2^{Da}
SD	0.2	0.4	0.3	0.2	0.4	0.2	0.1	0.1

Log_{10} CFU/g values stated refer to three samples; values with the same superscripts in the same horizontal row (A-D) or vertical column (a-b) are not significantly different at (P=0.05); SD: standard deviation.

Generally, all microbial groups showed viable counts higher for samples stored chilled than those frozen. Meanwhile, samples collected from small butcher shops showed higher viable counts as compared to samples collected from supermarkets. The low number of viable counts minced beef meat in supermarkets may be due to practicing good sanitation and food handling techniques. After 180 days of storage at -10 °C, there were significant decreases ($P<0.05$), in the number of total viable bacteria, *Pseudomonas*, faecal coliform, *Streptococcus* and *S. aureus*, on minced meat samples, collected from supermarkets and small shops as compared to the initial number (day zero). The frozen storage for minced beef meat enhanced the microbiological quality and reduced the risk of pathogens. Although the hygienic quality of minced meat was still low, due to the high initial contamination level of pathogenic bacteria.

Lipid oxidation is one of the most important causes of minced meat deterioration during refrigerated or frozen storage, which affects fatty acids, particularly polyunsaturated fatty acids. This lipid auto oxidative degradation gives products that changes the meat quality, e.g., the

colour, aroma, flavour, texture and even the nutritive value (Descalzo and Sancho, 2008; Duong *et al*., 2008; Balentine *et al*., 2006; Montgomery *et al*., 2003). The method of measuring oxidative stress by TBARS method is the best way for screening and monitoring the lipid peroxidation, a major indicator of oxidative stress (Armstrong,1998). TBARS content of minced beef collected from supermarkets and small butcher shops stored in the refrigerator at 4 °C and frozen at -10 °C is shown in Table 2. The initial TBARS number was 0.89 and 1.15 mg MA/kg of minced beef collected from supermarkets and small shops, respectively. Values detected in fresh minced meat were in agreement with the published data (Ozlem *et al*., 2011; Brooks *et al*., 2008). After 4 days of storage at 4 °C the number of TBARS increased rapidly and reached to 1.88 and 2.37 mg MA/kg in samples collected from supermarkets and small butcher shops, respectively. Lipid oxidation was particularly pronounced in ground meats, where, the disruption of muscle cell structure exposed lipid components to oxygen (Ozlem *et al*., 2011; Duong *et al*., 2008; Balentine *et al*., 2006). Meanwhile, the number of TBARS, of samples stored frozen increased at a slower rate as compared with samples stored chilled. Vieira *et al*. (2009), stated that TBARS of fresh meat was, significantly, lower than meat stored for 90 days at -20 °C. Such observations indicate that frozen storage is not necessarily sufficient to prevent oxidation from occurring. A similar trend was obtained in this study. According to Coleen *et al*. (2012) and Estévez (2011) the optimum temperature for the frozen storage of meat has been reported to be -40 °C. This fraction of water is believed to be bound to other food constituents and thus is chemically inactive (Coleen *et al*., 2012; Singh and Heldman, 2001; Nesvadba, 2008). The chemical reactions can occur during stored ground beef at 4 °C that initiate primary lipid oxidation (peroxidation) in the meat (Lynch *et al*., 2001).

Meat purchasing decisions are influenced more by colour consideration than any other quality factor because consumers use discolouration as an indicator of freshness and wholesomeness (Mancini and Hunt, 2005). The colour of muscle depends on the amount and oxidation/ reduction state of myoglobin (Ozlem *et al*., 2011; Mohamed *et al*., 2008; Mancini and Hunt, 2005). The effect of cold and frozen storage on colour properties (*L**, *a** and *b** values) of minced beef meat collected from supermarkets and small shops is shown in Table 3.

The data indicate that lightness, redness and yellowness in minced beef meat collected from supermarkets were higher in density than those of collected from small butcher shops. Lightness, redness and yellowness in minced beef meat decreased during storage in cooling and freezing temperatures. The results of colour confirmed those obtained for microbiological quality (Table 1) and lipids oxidation, TBARS (Table 2).

The shelf life of minced meat is limited because the large exposed surface area facilitates spoilage. The rate of deteriorative changes depends on meat composition, hygienic practices during cutting, grinding and preparation and, finally, storage conditions (Anthoula *et al*., 2012; Limbo *et al*., 2010). The results of microbiology, lipid oxidation and colour indicate that the shelf life of samples, stored chilled at 4 °C and frozen at -10 °C were 4 and >90 days, respectively. Microbial quality, lipid oxidation and discolouration are the causative spoilage of minced meat stored chilled. Meanwhile, lipid oxidation and discolouration are mainly the causative spoilage of samples stored frozen.

Table 2. Malonaldehyde value of minced meat collected from supermarkets and small butcher shops at Riyadh

Location	Refrigerated minced beef (4 °C) Malonaldehyde mg/kg				Frozen minced beef (-10 °C) Malonaldehyde mg/kg			
	Day 0	Day 2	Day 4	Day 5	Day 0	Day 30	Day 90	Day 180
Supermarkets	0.89^{Aa}	1.48^{Ba}	1.88^{Ca}	2.95^{Da}	0.89^{Aa}	1.65^{Ba}	1.69^{Ca}	3.02^{Da}
SD	0.10	0.15	0.20	0.40	0.20	0.20	0.10	0.20
Small butcher shops	1.15^{Ab}	1.74^{Bb}	2.37^{Cb}	3.74^{Db}	1.15^{Ab}	1.87^{Bb}	2.10^{Cb}	3.85^{Db}
SD	0.30	0.20	0.10	0.50	0.20	0.15	0.30	0.40

Each number is mean of three replicates; values with the same superscripts in the same horizontal row (A-D) or vertical column (a-b) are not significantly different at (P=0.05); SD = standard deviation.

Table 3. Colour of minced meat collected from supermarkets and small butcher shops at Riyadh

Location	Chilled minced beef (4 °C)				Frozen minced beef (-10 °C)			
	*L**				*L**			
	Day 0	Day 2	Day 4	Day 5	Day 0	Day 30	Day 90	Day 180
Supermarkets	41.4^{Aa}	40.9^{Ba}	39.6^{Ca}	37.9^{Da}	41.4^{Aa}	39.1^{Ba}	38.7^{Ca}	37.63^{Da}
SD	5.0	3.0	6.0	4.0	5.0	4.0	5.0	4.0
Small butcher shops	40.3^{Ab}	40.4^{Aa}	39.1^{Ca}	38.0^{Ca}	40.3^{Ab}	38.8^{Bb}	38.1^{Cb}	37.42^{Db}
SD	3.0	5.0	4.5	3.0	6.0	3.0	5.0	4.3
	*a**				*a**			
Supermarkets	16.4^{Aa}	15.34^{Ba}	12.2^{Ca}	9.13^{Da}	16.4^{Aa}	15.1^{Ba}	12.7^{Ca}	$8.85.6^{Da}$
SD	2.0	1.7	2.1	0.95	1.20	1.7	1.3	1.1
Small butcher shops	15.1^{Ab}	13.4^{Bb}	10.8^{Cb}	8.42^{Db}	15.1^{Ab}	13.2^{Bb}	11.4^{Cb}	7.92^{Db}
SD	1.3	1.6	1.2	0.65	1.30	1.5	1.3	0.95
	*b**				*b**			
Supermarkets	16.2^{Aa}	$15..1^{Ba}$	13.9^{Ca}	12.2^{Da}	16.2^{Aa}	15.5^{Aa}	14.1^{Ba}	$11..7^{Ca}$
SD	2.0	1.7	1.4	1.2	2.1	1.8	1.1	1.3
Small butcher shops	15.7^{Ab}	14.8^{Bb}	13.3^{Cb}	11.7^{Db}	15.7^{Ab}	13.7^{Bb}	13.5^{Cb}	10.4^{Db}
SD	1.5	1.5	1.	1.2	1.6	1.4	1.9	1.1

Each number is mean of three replicates; values with the same superscripts in the same horizontal row (A-D) or vertical column (a-b) are not significantly different at (P =0.05); SD = standard deviation; *L** = lightness; *a** = redness; *b** = yellowness.

Conclusion

From the results, it is clear that the refrigerator temperature was not sufficient to inhibit the growth of microorganisms and enzymes work and freezer temperature was sufficient to inhibit the growth of microorganisms, but did not stop the enzymes activity. It was also noted that the minced beef meat from supermarkets were lower in the microbial load and the percentage of rancidity. The spoilage of minced meat stored at 4 °C is mainly due to microbial growth, lipids oxidation and discolouration. Meanwhile, the deterioration of minced meat stored at -10 °C is mainly due to lipids oxidation and discolouration. Although freezing storage improved the microbial quality of minced meat, the hygienic quality of frozen meat was still low due to the high initial contamination level from pathogen and spoilage bacteria. Further studies are needed to evaluate the roots of contamination in minced meat industry to reduce the contamination level, improve safety, quality and increase its shelf life.

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Enrichment of Soymeal Medium to Increase the Rapamycin Production by *Streptomyces hygroscopicus*

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Abstract. Research was carried out to study the improved and increased production of rapamycin by *Streptomyces hygroscopicus* with soymeal enriched media. Media containing soymeal produced rapamycin upto 82.89 mg/L. The medium was enriched with different additives that can interfere with biosynthesis process. L-tyrosine supplementations led to noticeable increase in the rapamycin production to 112 mg/L. However, the progress was achieved upon addition of the shikimic acid (precursor rapamycin moiety), where, it reached 160 mg/L. The greatest increase was recorded after addition of calcium superphosphate (CaP) and the production achieved 176 mg/L. Other substances like vitamins and trace elements had either no or negative effects on the biosynthesis of rapamycin. The study also showed the ability of low concentrations of calcium phosphate to replace the expensive large amount of shikimic acid.

Keywords: *Streptomyces hygroscopicus,* rapamycin, immunosuppressants, soymeal

Introduction

Rapamycin is a potent immunosuppressant, anticancer and antifungal medicine. Rapamycin was early discovered in 1975 as an antifungal agent produced by *Streptomyces hygroscopicus* and has a great potency against *Candida albicans* (Vezina *et al.,* 1975). Up to date, rapamycin was approved twice from American FDA, the first one in 1999 for its ability to prevent host rejection in kidney transplanting and the second was in 2003 for its use in drug eluting stent to prevent restenosis of coronary arteries following angioplasty (Vezina *et al.,*1975).

In the recent years extensive research aiming at exploring more rapamycin activities ascertained that it represents an unreal tapped resource of clinical activities and can afford a lot of in drug realm as multifunction medication. It is anti-inflammatory and reduces the expression of several genes related to inflammation. In addition, it has antiangionenic, antiplorefiration and antifibrotic (Nuhrenberg *et al.,* 2005; Morris, 1992). Rapamycin protects against hypoxic damage in primary heart culture *via* Na/Ca exchange activation (El-Ani *et al.,* 2011). Also rapamycin found to play role in regulation of gastric hormones (Xu *et al.,* 2010). Furthermore, rapamycin does not affect post-absorptive protein metabolism in human skeletal muscle (Dickinson *et al.,* 2013).

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There are some trials to use rapamycin in treatment of acute myeloid leukemia, retinal and choriodal vascular diseases (Recher *et al.,* 2005). With the tremendous nature of rapamycin activities the demands on this drug would be increased in future. Therefore, research work is focussed to improve its productivity and to reduce the production costs. The cuurent study aims increament of the rapamycin production by *S. hygroscopicus* through enrichment of natural media containing soybean meal. The media was selected after comparison with other synthetic and natural media. Additives that could interfere with the rapamycin biosynthesis supplied and tested for their effects.

This is the first time for such results to be recorded and present findings may be helpful for economic production of Rapamycin.

Materials and Methods

Microorganisms. The strain *Streptomyces hygroscopicus* ATCC 29253, was the experimental organism throughout the current study. *Candida albicans* ATCC 10231 was used in bioassay.

The Rap producer, *S. hygroscopicus* ATCC 29253, was grown on slants of oat meal medium (contained oat meal, 20 g/L; agar, 20 g/L and pH 7) for 10 days at 28 ºC after which spores were collected by addition of 4 mL of 10% (v/v) glycerol to each slant. Spore

suspensions were then pooled together to get a suspension of 25.8 × 10^6 c.f.u./mL that was then dispensed in cryopreservation vials, each contained 1 mL, and stored at -20 ºC.

Culture media. A medium composed of soybean meal, 30 g; glucose, 20 g; $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 5 g (Sehgal *et al.,* 1975), was selected after comparison with other seven synthetic and natural media of different compositions. The components dissolved in tap water of pH 6 was used.

Fermentation. The culture was initiated by propagating 1 mL of thawed spore suspension containing 25.8×10^6 c.f.u. in 50 mL medium contained in 250 mL Erlenmeyer flask. Then each Erlenmeyer flasks (250 mL) containing 50 mL fermentation medium inoculated with 3 mL of inoculum. The inoculated flasks were incubated at 25 ºC ±2 and 150 rpm for 7 days. The fifth day was the optimal, where, after all fermentations were allowed to run for only 5 days.

Growth estimation. The dry cell weight was determined by placing a 10 mL sample from the whole fermentation medium into a pre-weighed 15 mL tube and centrifuged at 3500 rpm for 5 min. The microbial residue in the tube was dried at 80 ºC for 2 days and then weighed. The growth yield expressed as gram dry weight per liter fermentation medium. Packed cell volume percentage was determined by placing a 3 mL sample of whole fermentation medium into 10 mL tube and centrifuged at 3500 rpm for 5 min. The percentage of packed cell volume to the total volume of the whole fermentation medium sample was the desired estimate of growth.

Analysis. Aliquots of 3 mL were taken and the microbial growth was separated by centrifuging at 3500 rpm for 5 min and extracted twice by shaking with 3 mL methanol for 30 min. The two extracts were pooled to be assayed for Rap concentration. Bioassay determination of Rap was achieved by paper-disc agar diffusion method as described by Sallam *et al.* (2010) and Kojima and Demain (1998). A sample of 20 µL was analysed with HPLC (SYKAM apparatus equipped with Injector, S5111; Pumps (two pumps), S1122; Column Thermo controller, S4011; Degaser, S7505; Column, Phenomenex Gemini C18 (250x4.6 mm, 5 µ); Detector, Jasco UV-2070Plus; Software, Autochro-3000). The sample injected and eluant of acetonitrile: water 9:1 was pumped at the rate of 1 mL/min in column controlled at 55 ºC. Rap was assessed by absorbance at λ 277 nm.

Results and Discussion

Amino acids addition. Different amino acids were added individually in the concentration of 5 g/L. The results presented in Fig. 1 show that, addition of L-tyrosine accelerated the production to be 48% greater than in control sample, whereas, L-lysine neither enhanced nor suppressed the production. All the other added amino acids suppressed Rap biosynthesis by different degrees and the complete cessation was in case of L-cysteine. Similar results were obtained by L-histidine, DL-aspartic acid, L-arginine, L-glutamic acid, glycin, L-asparagine and L-cystine, while, sharp deleterious effect was observed in case of L-tryptophan, L-alanine and DL-methionine. Also, with addition of L-valine and L-proline, Rap titre was dropped down to be around the third of that in control sample.

Growth measurement revealed some significant features. There was some degree of correlation between growth of the producing strain and Rap biosynthesis. In most cases, amino acids negatively affected the growth as well as Rap biosynthesis. In addition, L-tyrosine, that has potentiated Rap biosynthesis, has also favoured the growth. With addition of L-proline, fermentation process showed the same profile like in control sample, the same growth and final pH of fermentation medium, yet it had remarkable decrease in Rap titre.

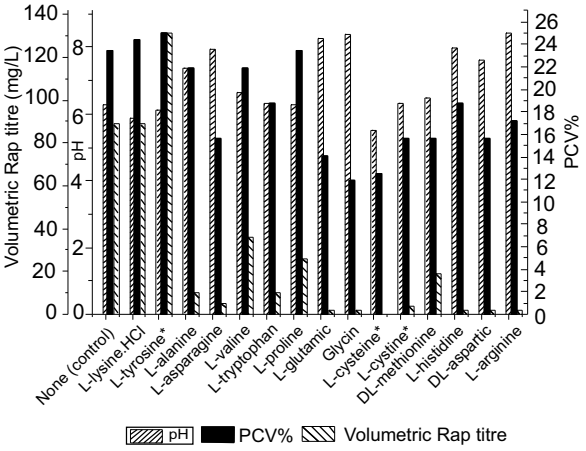

Fig. 1. Role of amino acids in biosynthesis of Rap by *S. hygroscopicus* ATCC 29253.

Biosynthesis of Rap in different augments of L-tyrosine. L-tyrosine was added to fermentation medium in different supplements namely; 1, 2, 5, 7.5, 10, 15, 20 and 30 g/L. The results that are represented in Fig. 2, show that decreasing L-tyrosine quantities to 2 g/L can successfully afford the same promotion in

Rap biosynthesis that was achieved at 5 g/L, and it yielded the best growth. More increase in L-tyrosine (more than 5 g/L) caused gradual depletion in Rap without effect on the microbial growth. The pH values remained constant at 6.

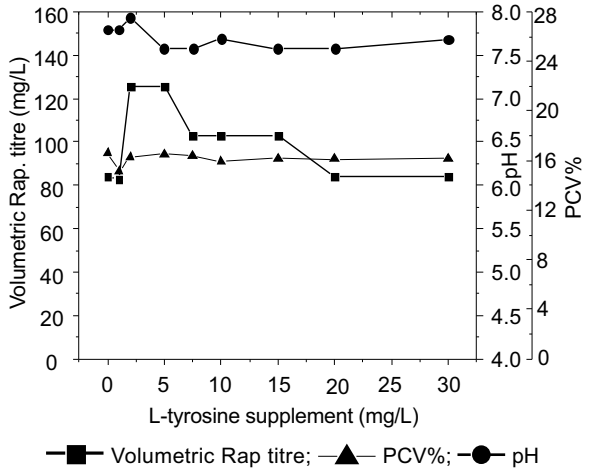

Fig. 2. Biosynthesis of Rap by *S. hygroscopicus* ATCC 29253 in different augments of L-tyrosine. PCV = packed cell volume.

Effect of time of L-tyrosine addition on Rap biosynthesis. According to the preceding investigations, L-tyrosine may play an obvious role in promoting Rap biosynthesis. In the current experiment, L-tyrosine (2 g/L) was added at different times during fermentation. Time of L-tyrosine addition has a critical role in its promoting action (Table 1). The results revealed that L-tyrosine should be added at the start of fermentation and not after more than 1 day of fermentation. The effect of L-tyrosine could be inversed to remarkable suppression to the Rap biosynthesis as well as the growth, if it was added at the late stages of fermentation or at least after two days.

Effect of trace elements on Rap biosynthesis. With a production medium containing L-tyrosine (2 g/L), trace elements were added, individually and in mixture, in concentrations like that were used by Lee *et al*. (1997). The data in Fig. 3 revealed that Rap biosynthesis was not favoured by addition of any of the studied trace elements. All of them have retarded Rap biosynthesis with only one exception in case of zinc salt, where, the results remained the same as that of control treatment. Copper ions (in very low concentration i.e., 1.3 mg/L) exerted reduction in Rap to less than half of that in control sample. However, copper ion increased the

Table 1. Effect of time of L-tyrosine addition on Rap biosynthesis by *Streptomyces hygroscopicus* ATCC 29253

Time of L-tyrosine addition (days)	Final pH of fermentation medium	Packed cell volume (PCV%)	Volumetric Rap titre (mg/L)
Control*	6.30	25.94	82.87
0	6.21	28.13	120.53
1	6.23	28.13	120.53
2	6.23	21.88	51.88
3	6.23	22.18	37.97
4	6.34	21.56	27.79

* = no L-tyrosine addition.

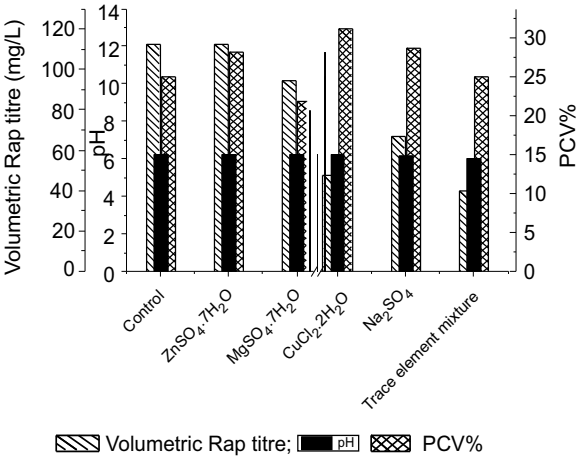

Fig. 3. Effect of trace elements on Rap biosynthesis by *S. hygroscopicus* ATCC 29253. PCV = packed cell volume.

growth from 25 to 31% PCV. The same effect was also observed in case of boron, molybdenum and sodium formulated as $Na_2B_4O_7$, $(NH_4)_6MoO_{24}.4H_2O$ and anhydrous Na_2SO_4, respectively. Mixture of trace elements resulted in negative effect on Rap production higher than that with all individual trace elements. With all studied elements, limited variation in pH of fermentation medium was observed.

Effect of addition of some vitamins on Rap biosynthesis. Different vitamins and vitamin precursors were added individually to the fermentation medium. Five milligrams of each vitamin were dissolved in 5 mL distilled water and the solution was then filtered and sterilised using syringe filter (of 0.45 µm pore diameter) to be added under aseptic conditions to 250 mL Erlenmeyer flask containing 45 mL sterile medium. From the results in Fig. 4, it was obviously

noted that Rap biosynthesis needs no medium enrichment with any of studied vitamins or vitamin precursors because none of them could increase Rap biosynthesis. On the contrary, only riboflavin had not hindered Rap biosynthesis, while, all the others exhibited inhibitory effect in different degrees. *p*-amino benzoic acid favoured the microbial growth. Remarkable suppression in Rap biosynthesis was observed with addition of biotin, pyridoxine and cyanocobalamine. The highest inhibition was in case of thiamine-HCl, nicotinic acid and L (+) ascorbic acid.

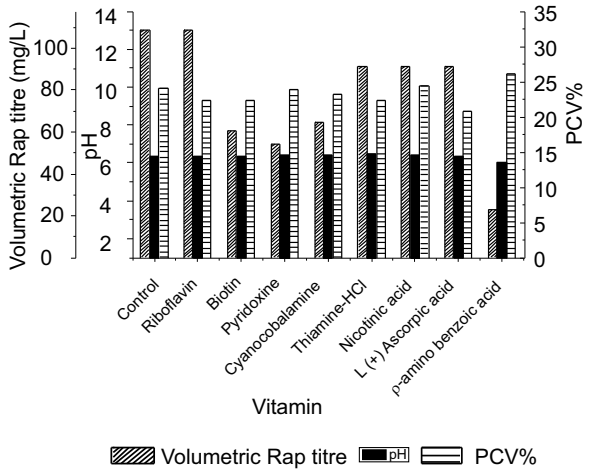

Fig. 4. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media supplemented with different vitamins. PCV = packed cell volume.

Rap biosynthesis in media enriched with precursors and inducers. Knowing the building units and precursors involved in Rap biosynthesis justified the need to clarify the ability of these compounds to accelerate its production. The current experiment was developed to study the effect of adding acetate (building unit), shikimic acid (precursor of cyclohexane moiety in Rap) and calcium superphosphate (CaP) on biosynthetic rate of Rap. Graphically reflected results demonstrated strong promotion in Rap production under the effect of shikimic acid and CaP addition (Fig. 5). More than 42% achievement in Rap productivity was attained by addition of shikimic acid, which raised the titre from 112 to 160 mg/L. More close to that achievement was the increase from 112 to nearly 150 mg/L that had been attained with CaP. Beside the strong promoting action of shikimic acid and CaP in Rap biosynthesis, they showed slight decrease in growth yield comparing with control sample. On the other side, sodium acetate afforded complete suppression in Rap biosynthesis associated with very low PCV%.

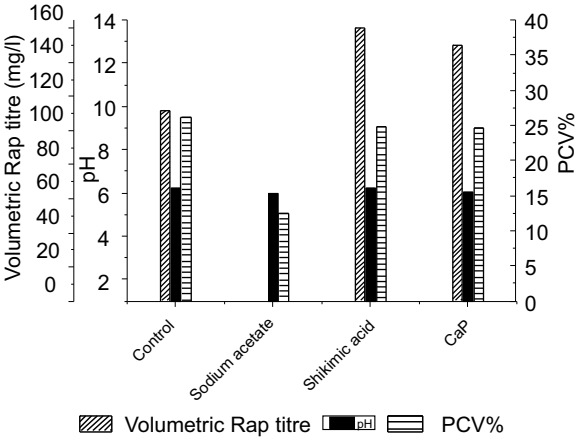

Fig. 5. Effect of medium enrichment with precursors and inducers on Rap biosynthesis by *S. hygroscopicus* ATCC 29253. CaP = calcium superphosphate; PCV = packed cell volume.

Rap biosynthesis in media enriched with different concentrations of shikimic acid and CaP. This experiment was directed to follow up the Rap biosynthesis in presence of different concentrations of shikimic acid and CaP which applied in supplements ranging from 0.1 to 5 g/L. As indicated in Fig. 6, promotion in Rap biosynthesis attained by 5 g/L shikimic acid could be achieved with lower concentration of 2 g/L. Shikimic acid was able to accelerate Rap production even at low concentration as 0.1 g/L. However, increasing its concentration to 0.7 g/L was associated with non-significant increase in Rap titre. With little increase in its concentration from 0.7 to 1 g/L, shikimic acid exhibited considerable advance in Rap productivity. On the other side, the results in Fig. 7 revealed that CaP was also strong Rap stimulator when supplied in special amount. Interestingly, it was found that promoting action of 0.7 g/L CaP that was recorded in the former experiment could be intensified by lowering its concentration to 0.4 g/L. Comparing with control culture, Rap titre achieved more than 53% increase in cultures amended with 0.4 g/L CaP. Concentration of 0.4 g/L was the best for maximum acceleration in Rap biosynthesis and minor change upward or downward caused a drop in Rap production. When CaP concentration exceeded 1 g/L, final pH of fermentation began to move

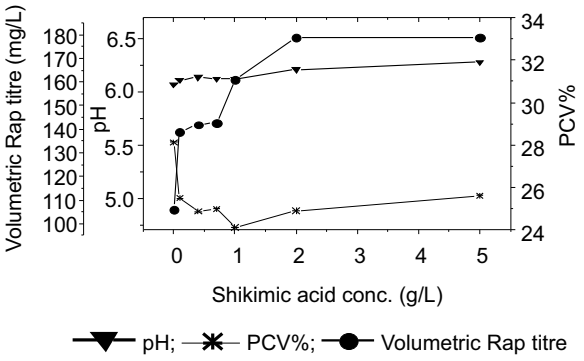

Fig. 6. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media of different shikimic acid concentrations.

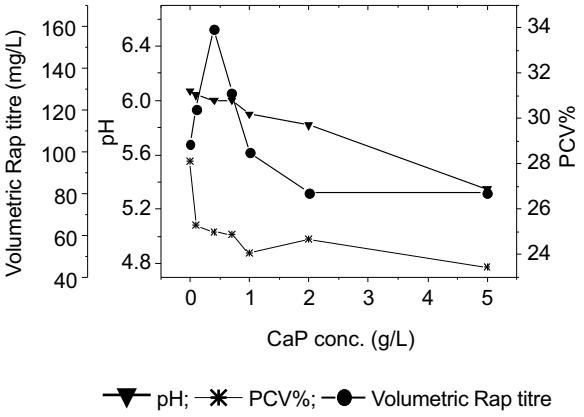

Fig. 7. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media of different CaP concentrations.

belw 6 and Rap biosynthesis considerably decreased (Fig.7). The critical role of CaP in Rap biosynthesis, if well modulated, could be used to replace the promoting action mediated by high priced shikimic acid. In addition to its very low price comparing to shikimic acid, CaP works effectively in minor concentrations, which affords an extra advantage. The results indicated low growth yield in cultures amended with shikimic acid or CaP and this provides an industrial economic input.

Applying L-tyrosine at different amounts showed that each of 2 or 5 g/L supplements could afford the same promoting action in Rap biosynthesis though 2 g/L was the optimal for growth. Promoting action of L-tyrosine was attained only, when it was added at start of fermentation or at most after one day lapse. At the second day of fermentation, addition of L-tyrosine gave rise to severe drop in Rap titre (51.88 mg/L), which was lower than that in tyrosine free culture (82.87 mg/L). It was proposed that incorporating tyrosine after time lapse of fermentation stimulates the metabolism to direct towards L-tyrosine, which may interrupt already working metabolic activities, and cells would spend some lagging period before being adapted to metabolise tyrosine. The lost time is reflected as a reduction in growth and Rap titer as it has shown in the current results.

Various metals affect the production of antibiotics (Iwai and Omura, 1982). The results showed that Rap biosynthesis was not favoured by any of studied trace elements. Copper ions (in very low concentration of 1.3 mg/L) exerted reduction in Rap titre to less than half of that in control sample together with increasing growth from 25 to 31% PCV. With all studied trace elements, limited variation in pH of fermentation medium was shown indicating that variation in outputs of Rap and growth was not attributed to shift in medium pH.

Enriching fermentation medium with different vitamins and vitamin precursors elucidated the retarding effect. ρ-amino benzoic acid caused the greatest drop in Rap titre and in the same time it had favoured the microbial growth which reflected the difference between requirements needed for growth and Rap biosynthesis. The role of vitamins in Rap biosynthesis may better be investigated in chemically defined medium. It is important to note that soy meal, a medium component, contains self constituents of vitamins (USDA National Nutrient database). The negative action in the obtained results may be attributed to rise in vitamin concentration after exogenous vitamin supplementations.

Shikimic acid and acetate are known precursors in Rap biosynthesis (Reynolds and Demain, 1997). The results of the current work showed strong promotion in Rap biosynthesis under the effect of shikimic acid addition. Also, calcium superphosphate (CaP) has potentiated comparable promotion. Beside the strong promoting action of shikimic acid and CaP in Rap biosynthesis, they showed slight decrease in growth yield comparing with control sample. These findings may point to a specific positive interaction of shikimic acid and CaP with metabolic activities incorporated in Rap biosynthesis. Decrease in growth yield may be result of

stimulatory effect of these precursors that initiate Rap biosynthesis earlier after short time of growth proliferation. Low growth yield may provide an advantage due to conserved downstream processing and extracting Rap from less biomass yield. Although Fang and Demain (1995) have elucidated the stimulatory action of shikimic acid on Rap production in chemically defined medium, they failed to record its promotion in complex medium. As such, stimulatory action of shikimic acid in a complex medium was reported here for the first time. Relatively low concentration (0.7 g/L) could markedly induce Rap biosynthesis greater than 5 g/L shikimic acid. Calcium superphosphate may need extensive future studies to resolve such role. Omura *et al.* (1980) reported the tremendous promotion, 2-15 times, in production of spiramycin, a macrolide antibiotic, by *Streptomyces ainbofaciens* ATCC 23877 after CaP addition. Thus, the inductive action of CaP in biosynthesis of Rap, as one of the most valuable recent drugs, was recorded here for the first time. Complete suppression of Rap biosynthesis, very low PCV% value and non changed medium pH, in case of fermentation amended with sodium acetate makes the suggestion that sodium acetate has strong toxic effect on the experimental organism. It was found that shikimic acid concentration of 2 g/L has the same effect of 5 g/L, where about 176 mg/L of Rap was obtained and the effect of CaP was found to be intensified by 53% by lowering its concentration from 0.7 to 0.4 g/L. Another feature in CaP stimulatory function; when CaP concentration exceeded 1 g/L, the final pH was decreased and Rap biosynthesis was delayed. It may be assumed that relatively higher CaP concentrations (nearly more than 1 g/L) alter the fermentation medium pH, which decreases the Rap production. Care should be taken to select the suitable buffer of non-interference with metabolic activities of the experimental organism. Thus, replacement of 2 g/L shikimic acids which is expensive substance, with 0.4 g/L of cheap calcium superphosphate will be of economic value.

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Utilization of Poultry Excreta for High Density Production of *Daphnia carinata* (King 1853): Cost Effective and Environmental Friendly Technique

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Abstract. *Daphnia carinata* was cultured for 21 days using poultry excreta to fertilise the medium at the rate of 500 ppt and maximum density of 5633.32 ± 88 Ind./L was recorded on 11th day of culture in the tanks, where, the feed was administered with 25% dosage followed by 50% dosage (1894.44 ± 9.68 Ind./L) and 75% dosage (1103.55 ± 17.80 Ind./L) of the initial dosage (500 ppt). A 50% renewal of the medium thrice a week proved optimal for the population development. The water analysis showed that the temperature range of 28 ± 2 °C and pH of 6-7 was conducive for optimal growth of *D. carinata.*

Keywords. *Daphnia carinata,* chicken manure, live feed, fish farming, aquaculture

Introduction

Mass culture of zooplankton of required quality and quantity within a short time period has been the subject of many investigations during recent years and is one of the important basic requirements for scientific management in the fish farms. An important factor in the success of aquaculture is the continuous availability of suitable food, at a reasonable cost. It is well known that the mass culture of zooplankton in high density is a basic necessity for fish production. Most fish and prawn species rely on zooplankton at some stage of their life span, while some others are exclusively zooplankton feeders throughout their life (Kumar *et al.,* 2005). The extended studies have further revealed that production of zooplankton is also desirable for consumption so as to obtain optimum growth of developmental stages until harvesting under fish culture in addition to controlled and coordinated supplementary feedings. Several studies document, why natural food is indispensable in the early life history of fish (Kumar *et al.,* 2005; Adeyemo *et. al.,* 1994; Sorgeloos *et al*., 1980). Zooplankton constitutes an important food source in both nursery ponds and outdoor enclosure systems (Jana and Chakrabarti, 1997). There is a growing need to mass culture of indigenous live food organisms for use in fish and shrimp hatcheries. A wide range of live and inert feed can successfully be used in culturing live feed organisms. Poultry manure; a cheap food, available worldwide, will be helpful in reducing the cost of expenditures on live feed production.

The cladoceran *Daphnia carinata* (Class: Crustacea, Order: Cladocera) popularly known as water flea is a preferable food item for many freshwater larval fishes. This group is one of the dominant groups of freshwater zooplankton and contributes significantly, to the productivity and energy flow in aquatic ecosystem. They are autotrophic producers as well as feed on detritus (SureshKumar, 2000). The ability of cladocerans to ingest food of wider range and their higher filtering rates give them a better competitive edge over the rotifers. *Daphnia* species with the help of specialised combs of setae on the thoracic appendages can utilise algae, bacteria, fungi, protozoans and organic debris and even small food particles of 1-60 µm range (Srivastava *et al.,* 2006). They contain many essential amino acids required for fish and shrimp larvae. These nutrients occur in them far above the level recommended for larvae (Kibria *et al*., 1997). Being natural food of fish and prawn larvae, cladocerans are collected from natural resources for use as diet for the larvae of ornamental fish in many hatcheries. Altaff *et al.* (2002) and Kahan (1982) opined that this is an unreliable source for commercial production in quality and quantity due to uncontrolled fluctuation and drawbacks of collecting method. Further, they may introduce harmful organisms in the hatchery. Waste water cultured *Daphnia magna*

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supports survival and growth of rainbow trout larvae and are mainly used alive or preserved as food for fish in aquaculture (Kumar *et al.,* 2005). Daphnids play an important role in the dynamics of inorganic turbidity in large tropical reservoir and produce lose faeces that disaggregate easily after release (Filella *et al*., 2008).

One of the major problems associated with the culture of zooplankton is its unstable nature, often exhibiting a rapid population decrease immediately after attaining a peak density (Srivastava *et al.,* 2006; Jana and Chakrabarti, 1997). The unstable nature of zooplankton populations is a major setback for mass culture because sustained production throughout year cannot be achieved. Use of manure provided at different dosages and frequencies may significantly, influence the water quality and assist in defining the optimal conditions required for continuous and high density culture of cladocerans. In this context experiments were carried out on mass culture of *D. carinata* using poultry excreta which will make it a cheap and easily available food source to the fish culturists. Hence, the main focus of this study was to check the feasibility of using chicken manure and the effect of different feeding rates for fertilising the medium to culture *D. carinata,* which is a cost effective and eco-friendly approach.

Materials and Methods

Chicken manure was collected from a local broiler chicken shop and was dried for two days to remove the moisture and stored in plastic jars for further use. Chicken manure was micronised by grinding and required quantity was dissolved in distilled water to get suspensions of 500 ppt for fertilisation of culture medium. Micronisation of chicken manure is necessary for an efficient filtration of the suspended particles by daphnids. Zooplankton sample was collected from fish rearing pond at Centre for Aquaculture Research and Extension (CARE), St. Xaviers College Palayamkottai, Tamilnadu, India and was brought to the laboratory with least disturbance. The adult *Daphnia carinata* were separated using binocular dissection microscope based on the key characters outlined by Altaff (2004). The experimental aquarium tanks of 50 L capacity were filled with 40 L of filtered water and were fertilised with chicken manure at the rate of 500 ppt (0.5 g in 1 litre of water). The culture water used in all experiments was tap water, previously aerated for 24 h to dechlorinate the water (Altaff and Mehrajuddin, 2010). The tanks were arranged in triplicate. *D. carinata* were inoculated in each experimental tank after 4 days at the rate of 50 ± 5 Ind./L containing both adults and neonates. The culture experiment was conducted for 21 days. To avoid anaerobic conditions in the medium, the sediment (unconsumed food, faeces, and pseudofaeces) was siphoned from the bottom three times a week. Excessive fouling was also removed from the walls of the tanks. Water change was carried out at every 3 days interval by removing 50% of the water. Food was administered at the rate of 25%, 50% and 75% of the initial dosage (500 ppt) after every two days.

Wet weight of the animals was determined after draining 10 L of the culture medium over a nylon gauze of 200 µm mesh size and washed several times to remove other debris. The remaining water was absorbed using tissue paper and the animals were weighed on a digital balance with 1µg sensitivity. Population density was estimated by counting samples on 3rd, 7th, 9th, 11th, 14th, 17th and 21st day of culture taken at random with one litre beaker, after mixing the culture volume. Sub samples of 100 mL and then 10 mL were drawn from these samples. Samples were immobilised using alcohol and counting was carried out using Sedgwick Rafter cell under a binocular dissection microscope. Results were expressed as number of individuals per litre (Ind./L).

Results and Discussion

In general, density of *D. carinata* was significantly, higher (5633.32 ± 88 Ind./L) in tanks, where, 25% dosage was given/supplied, while, the least (1310 ± 15.27 Ind./L) was found in 75% dosage. The values of most of the physical and chemical parameters recorded in the present study were within the desirable range. Atmospheric and water temperature during culture period was 28 ± 2 °C, and 26 ± 2 °C, respectively, while, pH ranged between 6-7 in the present experimental conditions. Protozoans reached to a peak density on 7th day of culture (40.33 ± 1.83 Ind./mL) and represented mostly by *Paramecium* sp., *Euglena* sp., and *Cryptodifflugia* sp. The population of *D. carinata* ranged between 50 ± 5 and 5633.32 ± 88 Ind./L during the culture period of 21 days (Fig. 1). Maximum density was observed on the 11th day of the culture (5633.32 ± 88 Ind./L). The wet weight (mg) obtained during different days of culture is summarised in Fig. 2. The highest wet weight (1156.7 mg/10L) was obtained on the 11th day of the culture period. A 50% renewal of the medium thrice a week proved optimal for the population development. Frequent application of low doses of

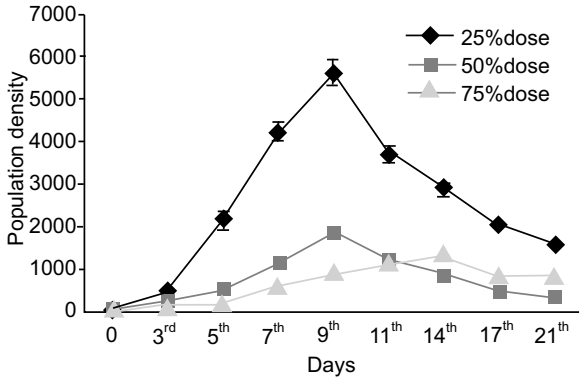

Fig. 1. Population density of *Daphnia carinata* during different days of culture at different feeding rates (Mean ± SE).

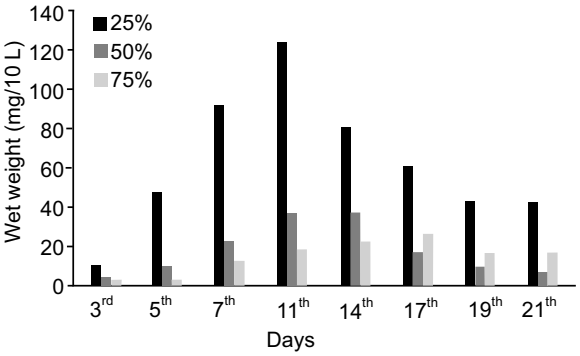

Fig. 2. Wet weight obtained during different days of culture period (Mean ± SE).

poultry excreta resulted in high density production of *D. carinata* and good water quality. The density of *D. carinata* increased steadily and reached to its peak (5633.32 ± 88.19 Ind./L) on the 11th day of culture, and thereafter, the production declined, such population dynamic was also reported by Pangano *et al.* (2000). Who observed maximium density on 7th day. It is interesting to note that the population of protozoans increased during the course of the culture and peaked (40.33 ± 1.83 Ind./mL) on the 7th day of culture and declined (28.33 ± 0.57) on the 14th day (Fig. 3). The advantage of culturing *D. carinata* by frequently applying low doses of poultry waste might provide sufficient food to the animals as well as maintaining the favourable water quality, leading to fast growth, early maturation and relatively long life span. Ammonia and nitrite are the main nitrogen compounds that are considered risk factors in the growth of crustaceans, when the manure decays and its nitrification produces acidity. Water in which high densities of *Daphnia* occurred exerts allelopathic effects on the growth and reproduction of smaller

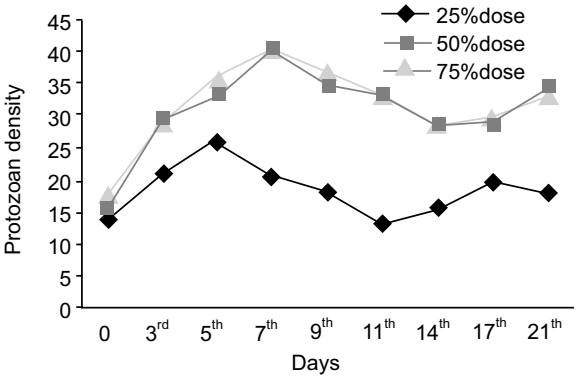

Fig. 3. Protozoan density (Ind./L) during different days in the culture medium fertilised with different concentrations of poultry excreta (Mean ± SE).

daphnids, probably through chemical compounds released into the medium that are not the products of food metabolism (Martínez-jerónimo *et al.,* 2007). Hence, higher concentration of the manure used to fertilise the water will lead to more acidic conditions rendering unfavourable conditions. Therefore, it suggests that frequent application of low doses might be best for achieving high density and continuous culture of *D. carinata*.

The physicochemical parameters appear to play an important role in the successful culture of cladocera. Tay *et al*. (1991) observed that there is no report to suggest the relationship between the physicochemical parameters and physiological process of zooplankton. Shrigur and Indulkar (1987) proposed the range of water temperature between 27-31 ºC for optimum growth of *Moina micrura*. In the present study, temperature range of 28 ± 2 ºC appeared to produce optimum density of cladocerans. It has been reported by Tay *et al*. (1991) that the upper lethal limit, where 50% mortalities of cladocerans occur is close to 40 ºC.

Dissolved oxygen content of the culture medium is another important factor for the growth of population. In the present study, in all culture media dissolved oxygen level of 4-5 mg/L was maintained through aeration (debris was removed on alternate days and by using aerator) and fertilisation of the medium at regular intervals. DePauw *et al*. (1981) also reported similar results, where dissolved oxygen level of culture medium for *D. magna* was above 5 mg/L with aeration. Tay *et al*. (1991) have stressed that aeration is an important culturing parameter and correlation studies showed that dissolved oxygen decrease with organic loading of the media (Sivakumar, 2005).

Usually, when environmental conditions are favourable (adequate food quality and quantity, population density, temperature and photoperiod), the progeny constitutes parthenogenetic females, which are clones of mother. When the environmental conditions become unfavourable asexual reproduction changes to sexual, which leads to the production of males and females, eventually leading to formation of resting eggs that enter into diapause, until the conditions become favourable again (Martínez-jerónimo *et al.,* 2007). Population density can exert indirect effects, modifying feeding conditions by releasing and accumulating chemical substances, or through behavioural signs. *D. carinata* can be grown to a high density using chicken manure. However, an absolute prerequisite is the exact dosing of chicken manure at different densities during the culture period. Overfeeding can quickly cause problems in water quality, regardless of the type of the media used, therefore, it should be started with small amounts of feed or fertiliser and slowly increase the amount used as the density increases. Dried algae in some cases are also excellent food but they are too expensive to be used in large scale systems (DePauw *et al.,* 1980). It is evident from the results that it is possible to mass culture *D. carinata* using chicken manure which is cost effective, thereby, reducing the expenditures on the seed production in hatcheries.

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Short Communication

Heat Processing and Cold Storage Effects on Vitamins B_1 and B_2 of Buffalo Milk

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Abstract. Heat processing and cold storage effects on vitamins B_1 and B_2 contents of whole and skimmed buffalo milk were investigated. Whole and skimmed buffalo milk was heated at various temperatures (90-140 °C) for different time periods (2-90 min). Losses in vitamins B_1 and B_2 occurred to various extents depending upon the temperature and time period of heating and the storage conditions. Maximum losses in vitamins B_1 and B_2 were found on heating milk at 110 °C for 90 min and 140 °C for 8 min. Maximum losses in vitamins B_1 and B_2 were found to be 32.5 and 29.9% at 110 °C and 37.5 and 32.6% at 140 °C for whole buffalo milk, 30.4 and 26.4% at 110 °C and 34.8 and 29.6% at 140 °C for skimmed buffalo milk, respectively. Similarly, after 15 days cold storage, maximum amount of vitamins B_1 and B_2 was lost from heated whole and skimmed buffalo milk. Losses in these two water soluble vitamins were comparatively higher in case of whole buffalo milk than skimmed buffalo milk after heat treatment. However, losses in vitamin B_1 were higher than vitamin B_2 contents in all samples.

Keywords: buffalo milk, vitamins B_1 and B_2, heat processing, cold storage

Shelf life of milk is very short which is usually extended by heat treatment but heating causes considerable losses of B-vitamins (B_1, B_2, B_6 and folic acid) in buffalo and cow's milks depending upon the severity of heat treatments (Sharma and Lal, 1998; Burton, 1984; Mehriz and Ganguli, 1980). Informations are available in the literature about the heat treatment on vitamins of milk (Burton, 1984; Haddad and Loewenstein, 1983; McLaughlan *et al*., 1981). Severe heat treatment of skimmed cow milk (121 °C for 20 min) destroyed all the vitamins B_{12}, about 60% of the thiamin and vitamin B_6, 70% of the ascorbic acid and about 30% of the folate (Kilshaw *et al*., 1982). Losses in vitamins C, B_1 and B_2 from goat and cow milk were observed as a result of heat treatment. However, vitamins B_6, B_{12}, thiamin and riboflavin in milk were found relatively stable to heat treatment (Lavigne *et al*., 1989; Scott and Bishop, 1986). Storage stability of nutrients including lactose, lysine and water soluble vitamins have already been studied in processed milk (Rehman, 2002; Sierra and Vidal-Valverde, 2001; Lavigne *et al*., 1989). The present work was undertaken to study the effect of heat processing and cold storage on vitamins B_1 and B_2 contents of buffalo milk.

Whole and skimmed buffalo milk was directly collected from a local dairy industry, which was heated in sealed stainless steel tubes (3.5 mL capacity) in a thermostatically controlled oil bath in a temperature range of 90° to 140 °C, with holding times ranging from 2-90 min. After heating for a specified period, tubes were placed in an ice bath to stop the reaction before carrying out the analysis of vitamins B_1 and B_2 in heated milk.

Whole and skimmed buffalo milk heated at 90°-140 °C for different time periods (2-90 min) was distributed into 50 mL flask without leaving head space. These flasks were kept refrigerated at 4.5±0.5 °C for 15 days and analysed for vitamins B_1 and B_2 contents every 5 day of cold storage. Milk sample, 10.5 mL was mixed with 1g trichloroacetic acid (TCA) in a 50 mL centrifuge tube (30 mm diameter). The mixture was thoroughly shaken for 1 min over a magnetic stirring plate and then centrifuged for 10 min at 1250 g to separate the two phases. Liquid phase was collected in 10 mL volumetric flask and the volume was filled with 4% TCA. This acid extract was filtered through a 0.45 µm filter paper prior to HPLC analysis. The analysis of vitamins was carried out on HPLC (Albala-Hurtado *et al*., 1997).

Initially, vitamin B_1 contents in whole and skimmed buffalo milk were 40 and 46 µg/100 mL, respectively, which were lost to various extends depending upon temperature and time period of heating. After heating

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at 90, 100 and 110 °C for 90 min, losses in vitamin B_1 contents were 17.5, 25.0 and 32.5% for whole buffalo milk, 17.4, 23.9 and 30.4% for skimmed buffalo milk, respectively, (Fig. 1). However, vitamin B_1 contents in whole and skimmed buffalo milk were lost by 20.0 and 21.7% at 120 °C, 32.5 and 28.3% at 130 °C and 37.5 and 34.8% at 140 °C, respectively, after heating for 8 min.

On heating at 90, 100 and 110 °C for 90 min, vitamin B_2 contents were lost by 14.4, 18.1 and 29.0% from whole buffalo milk and by 12.0, 17.6 and 26.4% from skimmed buffalo milk. As a result of heating at 120, 130 and 140 °C for 8 min, losses in vitamin B_2 contents were 15.4, 22.7, 32.6% for whole buffalo milk and 12.8, 21.6, 29.6% for skimmed buffalo milk, respectively, (Fig. 2).

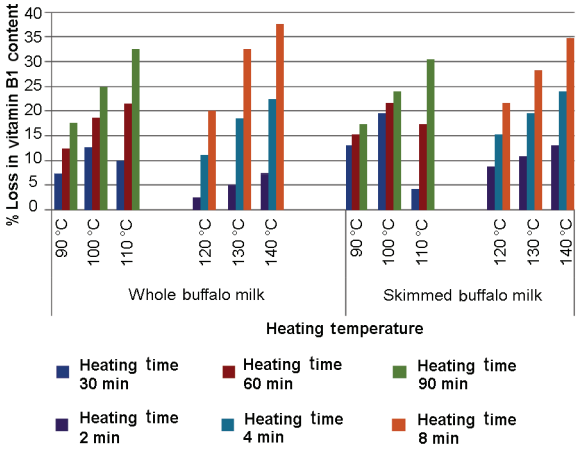

Fig. 1. Effect of heating on vitamin B_1 contents of whole and skimmed buffalo milk.

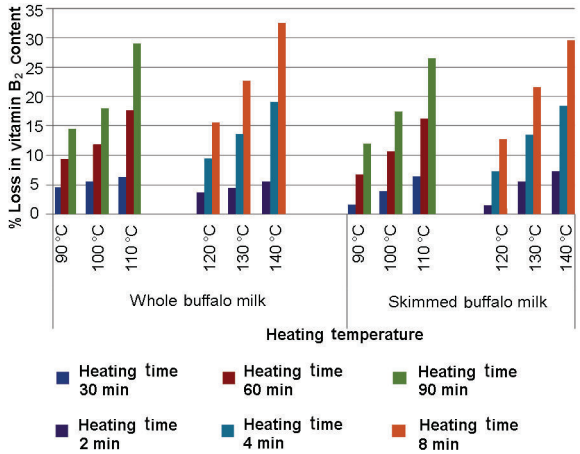

Fig. 2. Effect of heating on vitamin B_2 contents of whole and skimmed buffalo milk.

These results showed that losses in vitamin B_1 were higher than vitamin B_2 as a result of heat treatment. These results also show that loses in both, these water soluble vitamins (B_1 and B_2) were comparatively higher in case of whole buffalo milk than skimmed buffalo milk.

In fact, these results are in agreement with the observation of Lavigne *et al*. (1989). Contrary to these observations, Sierra and Vidal-Valverde (2000) found no change in vitamin B_1 contents in microwave and conventionally heated cow milk due to lower temperature and less time of heating. However, a slight decrease in vitamin B_1 by 3% was observed in heat treatment of milk at 90 °C for 30 sec. (Hartman and Dryden, 1974).

Figures 3-4 summarizes the effect of cold storage on the contents of vitamins B_1 and B_2 in heated whole and skimmed buffalo milk. During the first 5 days cold

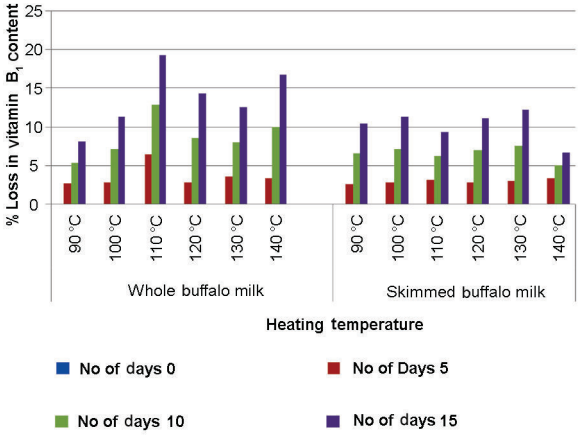

Fig. 3. Effect of cold storage on vitamin B_1 content of heated whole and skimmed buffalo Milk.

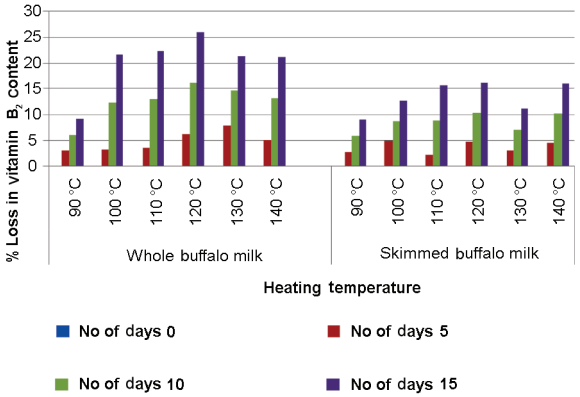

Fig. 4. Effect of cold storage on vitamin B_1 content of heated whole and skimmed buffalo milk.

storage losses in vitamins B_1 and B_2 were not significant in whole and skimmed buffalo milk but decreased thereafter, resulting losses in vitamin B_1 and vitamin B_2 by 8.1–19.23% and 9.18–26.04% for whole buffalo milk, 6.66–11.42% and 9.09–16.19% for skimmed buffalo milk, respectively, after 15 days cold storage.

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Short Communication

Biochemical Composition of Koi (*Anabas testudineus*) Collected from Paddy Field of Mymensingh, Bangladesh

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Abstract. An experiment was undertaken to analyse proximate composition of Koi (*Anabas testudineus*), collected from a rice field from Mymensingh, Bangladesh. Fishes were grown as a second crop along with rice. Moisture, protein, lipid and ash percentage (%) were evaluated as 70.07±1.10, 16.97±0.82, 13.01±0.47, 0.95±12, respectively, in wet basis. When compared to previous reports, a lot of variation was observed in the case of lipid.

Keywords: proximate composition, *Anabas testudineus*, paddy field, lipid

The gross content of important chemical parameters (moisture, protein, lipid and ash) represents the proximate composition of fish. The fish body contains moisture (66-81%), protein (16-21%), fat (0.2-25%), mineral (1.2-1.5%) and carbohydrate (0-0.5%) in a wide range (Minar *et al*., 2012) but they may be influenced by food, space, temperature, salinity, physical activity etc., (Begum *et al*., 2012). Waterlogged paddy field can be used as habitat for small indigenous species (Ahmed *et al*., 2012). Dugan *et al*. (2006); Gurung and Wagle (2005) and Halwart and Gupta (2000), have studied different aspects of rice cum fish farming, its diversification, intensification, productivity, profitability and sustainability.

Studies regarding proximate composition of fish collected from local water are available (Mazumder *et al*., 2008; Naser *et al*., 2007), but reports of investigation on nutritional composition of freshwater species that are cultured in paddy field is still fragmentary. Therefore, in view of these facts, present study was designed to conduct the proximate composition of Koi (*Anabas testudineus*) collected from the paddy field of Mymensingh area, Bangladesh to find, whether it will be helpful to fulfill the nutrient demand of human.

Samples were collected from various paddy fields of Trishal, Mymensingh (Fig. 1) during August to November, 2012. The fishes were taken to the Fish Technology Research Section, Institute of Food Science and Technology, Bangladesh Council of Scientific and Industrial Research, Dhaka, for conducting experiment. Total 30 fishes were used in this study. The size was ranged from 5.3-7.3 inch and weight was ranged from 50-90 g. Moisture and ash contents of the fish were determined by AOAC method (1990). The crude protein was conducted by Micro- Kjeldhal method (Pearson, 1999).

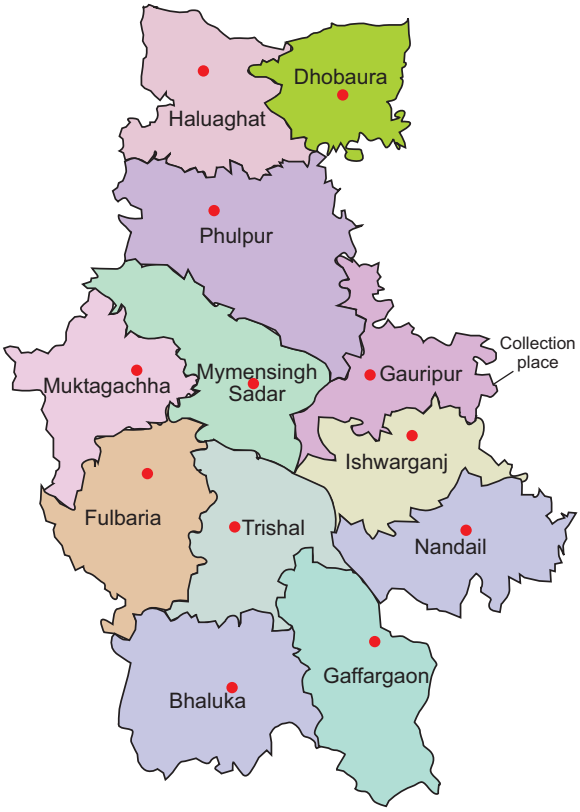

Fig. 1. Study area.

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Fat content was determined by Bligh and Dryer method (1959). The data were analysed by using SPSS 11.5 and Microsoft Office Excel 2007.

Calculation of moisture:

$$\text{Moisture (\%)} = \frac{\text{weight loss}}{\text{original weight of the sample taken}} \times 100$$

Calculation of protein:

N_2 (%) = (titration reading-blank reading) × strength of acid × 100/5 × 100/weight of the sample.

For most routine purpose the % of protein in the sample is the calculated by multiplying the % of N_2 with an empirical factor 6.25 for the fish:

Protein (%) = % of total N_2 × 6.25

Calculation of lipid:

$$\text{Fat (\%)} = \frac{\text{weight of the residue}}{\text{weight of the sample taken}} \times 100$$

Calculation of ash:

$$\text{Ash (\%)} = \frac{\text{weight of dry samples}}{\text{original weight of the sample taken}} \times 100$$

The result of the estimated parameters are given in Table 1. Variation of moisture, protein, lipid and ash content in the experimental fishes are shown in Fig. 2.

Moisture content. Moisture (%) levels (Table 1 and Fig. 2) in the analysed samples were ranged from 69.09 to 71.90 are similar to the findings of Kamal *et al.* (2007). Nargis (2006) found that the moisture content of Koi was 79.11±0.65% in males and 78.99% in females throughout the year. The dissimilarity of result may be due to time period i.e., previous study was conducted throughout the year but present study was conducted only for a short period. Another reason might be the collection place as the fish samples were collected from the paddy field directly. Besides the percentage may also vary according to size, sex and season of the year (Minar *et al*., 2012).

Protein content. The estimated protein content (Table 1 and Fig. 2) was 16.97±0.82 (%), which is similar to the findings of CSRI (1962). It showed variation from the findings of Kamal *et al.* (2007) collected from Mouri river, Khulna. Nargis (2006) found that protein content was 10.24 ± 0.79% for males and 11.07 ± 0.31% for females. In both sexes the maximum values were obtained in January where the fish sample was collected from Rajshahi. This difference may be due to availability of different food found in the paddy field. Besides the

Table 1. Estimated percentage of the tested parameter (wet basis)

S. No.	Moisture	Protein	Lipid	Ash
		(%)		
1	69.09	17.18	13.05	0.72
2	71.90	16.85	12.75	1.09
3	68.09	18.85	13.39	0.90
4	70.45	17.46	12.80	0.97
5	69.90	16.25	13.13	1.09
6	71.00	16.20	12.90	0.98
7	69.46	17.30	13.65	0.80
8	70.07	17.05	12.01	1.03
9	71.11	16.23	13.57	0.94
10	69.65	16.35	12.89	1.02
Mean±SD	70.07±1.10	16.97±0.82	13.01±0.47	0.95±12

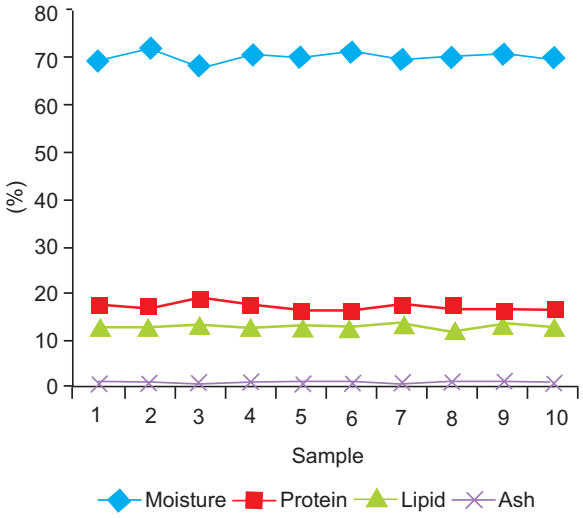

Fig. 2. Variation of moisture, protein, lipid and ash content in the experimental fishes.

value is close to the protein content (%) for *G. chapra, C. soborna, A. punctata, C. psendeutropius atherinoides, P. sarana,* (16.78, 17.31, 18.17, 16.69, and 16.73 respectively) (Begum and Minar, 2012).

Lipid content. The percentage of lipid obtained (Table 1 and Fig. 2) from the study is 13.01 ± 0.47 which was very much higher than the findings of Kamal *et al.* (2007), who found that the lipid contents of muscle of seven freshwater fishes from the River Mouri, Khulna, Bangladesh, was ranging between 3.45 and 7.90%. This result is very much different from present results. Besides, Hossain *et al.* (1999) reported the lipid contents

of some selected muscle of SIS (Kachki, mola etc.) fishes from Mymensingh District, Bangladesh ranging between 1.87 and 9.55%. This is an indication that, the fishes have probably been exposed to intense feeding with minimum activities in the paddy field (Ahmed *et al*., 2012). Another reason might be due to the larger size of the *A. testudineus* (Nargis, 2006).

Ash content. Normally ash may be defined as the residue that lacks water and volatile constituents containing carbon dioxide, oxides of nitrogen. In the present study, ash level (Table 1 and Fig. 2) in analysed samples was 0.95±12, whereas, Nargis (2006) found that ash content was found to vary from 1.32 to 2.15% with an average of 1.72±0.07% for males and 1.45 to 2.21% for females (1.78 ± 0.06%) *A. testudineus*. But Chowdhury (1981) found the values of ash very high, it may be due to habitat, season, sex and size. The main cause of change is due to amount and quality of food it eats along with its movement (Minar *et al*., 2012).

Result obtained from the present study may be useful in developing a nutritious, cost effective production of Koi fish alongwith the rice.

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Short Communication

First Record of *Cyphonotus testaceus* (Pallas, 1781) (Coleoptera: Scarabaeidae: Melolonthinae: Melolonthini) from Pakistan

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Abstract. The melolonthine species *Cyphonotus testaceus* (Pallas, 1781) is for the first time recorded from Pakistan. Geographic distribution of this rarely collected species is summarized. Habitus photo of the male collected in Pakistan is presented.

Keywords: *Cyphonotus testaceus*, new record, melolonthini, palaearctic region, oriental region, Pakistan

Pakistani fauna of chafers belonging to the tribe Melolonthini is still known only insufficiently. Since there is no any comprehensive taxonomic treatment, data about Melolonthini of Pakistan could be extracted from few faunistic papers and/or catalogues only (Bezdìk, 2006; Hashmi and Tashfeen, 1992; Chaudhry *et al.,* 1966). The striking exception is quite recent taxonomic contribution on *Melolontha* of Pakistan (Keith and Saltin, 2012).

The genus *Cyphonotus* Fischer von Waldheim, 1824 differs from all other Palaearctic and NW Oriental genera of Melolonthini by the following combination of characters: body large and stout (26-40 mm), outer margin of protibia with three strong teeth, subapical calcar of protibia missing in both sexes, clypeus with widely rounded anterior angles, antennal club 4-segmented and very short in both sexes (Baraud, 1992; Medvedev, 1951). Currently, the genus comprises only two valid species (Bezdek, 2006; Nikolaev, 1976; Petrovitz, 1962), insufficiently known *C. bicoloratus* Petrovitz, 1962 described according to a single female from Iraq, Assur and rather widely distributed *C. testaceus* (Pallas, 1781). In 2012, the senior author discovered one male specimen of *C. testaceus* in the vicinity of the village Jamaldini in SW Pakistan representing the first record of this chafer for Pakistan.

Material examined. Pakistan (Balochistan province): village Jamaldini (29 °33'N 65 °59'E, 950 m a.s.l.), district Nushki, 12. vi. 2012, leg. Shuja Jan, 1 male at light (Fig. 1), deposited in Zubair Ahmed's collection.

Fig. 1. Habitus photo of the male *Cyphonotus testaceus*.

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Geographic distribution. *C. testaceus* is a rather widely distributed species. It is known from eastern Ciscaucasia (lowland of river Terek), eastern Transcaucasia (Armenia and Nakhichivan Auto-nomous Republic) (Iablokoff-Khnzoryan, 1967), Turkmenistan, Uzbekistan, southern Kazakhstan and Iran (Medvedev, 1951). Moreover, Medvedev (1951) listed it also from Baluchistan, but without more precise data. Here, we report the first reliably confirmed record for Pakistan.

Acknowledgement

We are grateful to BRSP (Balochistan Rular Support Programme) for providing necessary facilities during the visit of Nushki.

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