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Cover Page, Introduction, Contents, Call for Papers, All in one file

Contents

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Oxidative and biochemical alterations induced by profenofos insecticide in rats

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ABSTRACT: Profenofos is a persistent and toxic organophosphorous insecticide. Animal's exposure to profenofos occurs via food and water. It is largely known to cause toxicity in various organs, such as the liver and brain. The present study was designed to explore the effect of oral administration of profenofos (26.53 and 53.07 mg/kg body weight /day for 28 days) on lipid peroxidation, endogenous antioxidants (GSH, and catalase), of the liver and brain and serum biochemical changes of male rats. Lipid peroxidation, as measured by thiobarbituric acid reactive substances, (TBARS) was increased, with a decrease in GSH level. A highly increase in catalase activity was observed in the 53.07mg/kg/day dose of profenofos. Furthermore, profenofos exposure were associated with depletion of serum levels of vitamin C, E, A and β- carotene. As compared to the results obtained in control groups the study showed that a lower concentration of serum proteins and albumin were accompanied by decreased globulin alpha 1 and beta along with an increased gamma 2 globulin; and the activity of serum GGT, LDH and concentrations of cholesterol, triglyceride, LDL and VLDL were higher, whereas level of HDL was lower. . [Nature and Science. 2009;7(2):1-15]. (ISSN: 1545-0740).

Keywords: Oxidative; biochemical alterations; insecticide; rat

This study suggests that although profenofos in low concentrations had oxidative stress and induced serum biochemical alteration in male rats.

Introduction

Organophosphorous compounds (OPs) have been widely used for a few decades in agriculture for crop protection and pest control, thousands of these compounds have been screened and over one hundred of them have been marketed for these purposes (Hassall, 1990; Chirions and Geraud-Pouey, 1996 and Geraud-Pouey et al., 1997).

The common use of insecticides in public health and agricultural schedules has caused severe environmental pollution and potential health hazards including severe acute and chronic cases of human and animal poisonings. **(Moghadamnia and Abdollahi 2002 and Abdullahi et al., 2004)**

Toxicities of OP insecticides cause adverse effects on many organs **(Gupta 2006)**. Systems that could be affected by OPs are the immune system, **(Neishabouri et al.,2004),** liver **(Akhgari et al.,2003)**, muscles **(Pournourmohammadi et al., 2005)** urinary system **(Rodrigo et al.,2001),** reproductive system **(Joshi et al., 2003)**, pancreas **(Hagar and Fahmy 2002)** and hematological system **(de Blaquiere et al., 2000)**.

Certain OP`s are also associated with carcinogenesis. Since DNA damage has been correlated with cancer development **(Hagmar et al., 1994),** genotoxicity studies have been carried out on OP`s **(Rupa et al., 1991 and Dolora et al., 1994).** It has been reported that OPs can induce oxidative stress by generating free radicals and altering antioxidant levels of the free radical scavenging enzyme activity **Sharma et al., (2005)**.

Profenofos [0-4-bromo-2-chlorophenyl-0-ethyl S-propyi phosphorothioate] is a broad spectrum organophosphate insecticide and acaricide. Its main physiological effect is the inhibition of cholinesterase (ChE) activity **(Anderson et al., 1977).**

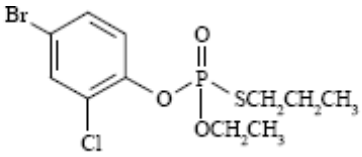
Biochemical Signs of hepatocellular injury and disturbed amino acid metabolism may be of value as markers of exposure to Profenofos, **Gomes et al., (1999).** Moreover, high doses of the profenofos induced tissue vacuolization, haemorrhage and hyperplasia of Kupffer cells in the liver. In addition, swelling of Bowman's capsules and tubular degeneration in the kidney were reported by **Fawzy et al., (2007).**Profenofos can induced oxidant stress which may be earlier diagnostic index in profenofos poisoning **(Lin et al.,2003).**

Therefore, the aim of this study was performed on the oxidative stress and biochemical effects of profenofos as an organophosphorous insecticide.

Materials and Methods

materials

Profenofos: is a pale yellow liquid, was provided by Central Agricultural Insecticides Laboratory (CAPL) Egypt.



Profenofos [O-(4-bromo-2-chlorophenyl)-O-ethyl-S-propyl phosphorothioate]

Laboratory animals:

Sixty adult male albino rats weighting (150 ± 10 g) were obtained from the farm of General Organization of Serum and Vaccine (Helwan Farm). The animals were housed in plastic cages in an air conditioned room where regular alternate cycles of 12 hr light and darkness were maintained and supplied with pelleted diet and tap water ad libitum. Animals were observed and signs of intoxication were recorded**.**

Experimental design:

The rats were divided into three groups of 20 rats each. Group (1) served as control and was given tap water only. Group II and III were given profenofos (72 EC, trade name: "Ictacrone") at a dose of 26.53 and 53.07 mg/kg body weight in 0.4 ml tap water through oral intubation. Dosages represent 1/8 and 1/4 LD, 50 respectively. LD, 50 value of profenofos (217.15 mg/kg b.W.) was determined orally (per os) according to **Weil, (1952).** The treatment was carried out for 28 days, the dose schedule being four days a week. Body weight was monitored twice a week and the dose was adjusted accordingly.

Sampling:

Individual blood samples were obtained after 28days from rats of each group, left to clot, sera were separated and kept at - 40°C for biochemical analysis Then animals were sacrificed and autopsy performed immediately; Brain, liver and kidney tissues were removed and washed with saline solution, then minced and homogenized (10% w/v) in ice-cold normal saline. The homogenate was centrifuged at 10,000xg for 20 min at 4C° and the resultant supernatant was used for antioxidant assay **(Chitra et al., 1999)**.

Biochemical Analysis

The biochemical assays of serum gamma glutamyle transferase (GGT) and lactic dehydrogenase (LDH) activities were determined according to methods of **(Szase et al., 1976)**. Triglyceride **(Wahlefeld, 1974),** cholesterol **(Watson, 1960)**, high density lipoprotein (HDL), low

density lipoprotein (LDL), **(Peace and Kaplan, 1987)**. Vitamin E, A, C and β carotene were performed according to **Henry et al., (1974).** Estimation of serum total protein and electrophoretic pattern were carried out after **SonnenWirth and Jaret (1980) and Davis (1964) ,**respectively.

Catalase activity; lipid peroxidation (TBARS) and reduced glutathione **(**GSH**)** in tissue were determined according to **Aebi, (1974); Ohkawa et al., (1979)** and **Ellman, (1959)**, respectively. The activity of catalase was expressed as IU per mg protein which estimated by **Bradford, (1976)**.

The obtained data were statistically analyzed using t`test after **Petrie and Watson (1999).**

Results and discussion

The present study has indicated the manner of organophosphorus poisoning in the experimental animals. Profenofos caused different symptoms of toxicity and revealed some biochemical changes especially in the enzymes activity of the liver and brain following two sublethal doses of profenofos in mice **(Saeed et al., 1995).** Animals dosed with 26.53 and 53.07 mg/kg body weight /day for 28 days showed significant signs of poisoning with in high dose.

It is reported by **Malkovics, (1995**) that OP, besides their inhibitory effect on AChE, also induce changes characteristic of oxidative stress (induced free radical)**.** Insecticides have been reported to induce production of reactive oxygen species and oxidative tissue damage **(Bagchi et al., and 1995**). All the major biomolecules like lipids, proteins, and nucleic acids may be attacked by free radicals, but lipids are probably the most susceptible **(Cheeseman and Slater1992)**. In this study, (table1) have shown that profenofos treatment result in a significant increase in MDA concentrations but a significant decrease was obtained in glutathione (GSH**)** levels of brain and liver tissues. The activity of CAT also increased in the brain, and liver (table 1), its increase was also remarkable in the brain of the rats as compared with control rats. These results were in agreement with **(Fortunato et al.,2006**and **Güney et al.,2007).**

Levels of MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, have been considered as an important indicator of lipid peroxidation **(Kalender et al., 2004**).

Table 1: Effect of profenofos insecticides on some anti-oxidant parameter of liver and brain tissues of male rats.

	Malondiahyde (mM/100g)		GHS (µmol/mg protein)		Catalase (IU/mg protein)	
	Liver	brain	Liver	brain	liver	brain
G1	0.79± 0.27	1.18± 0.22	408.22± 29.9	589.3± 18.16	5.19 ± 0.41	5.14 ± 0.73
G2	1.63± 0. 26*	2.10± 0.35*	315.31± 24.2*	501.88± 15.22**	6.26 ± 0.19*	8.45 ± 1.01
G3	3.21± 0.42***	4.64± 0.63***	240.5± 29.3***	394.4± 17.57***	8.65± 0.93**	10.14± 1.02***

Results are expressed as means ± SEM (n =5), student 't' test

* P < 0.05 ** P < 0.01 *** P < 0.001

Glutathione is the cell's natural antioxidant, which destroys free radicals formed in cells. Significant dose-dependent depletion of GSH levels confirmed the potential of the profenofos to induce oxidative stress in brain and hepatic tissue**.** (**Rajeswary et al., 2007). Lin et al., (2003)** reported that profenofos was increased the antioxidase activities (SOD, CAT and GSH-Px,) earlier than the decease of ChE activity. They suggested that profenofos can result in the increases of the antioxidant enzyme activities which may be earlier diagnostic index in profenofos poisoning.

Vitamin E and A (Vit E and A) is the primary liposoluble antioxidant, which may have an important role in scavenging free oxygen radicals and in stabilizing the cell membranes, thus maintaining its permeability **(Bjørneboe *et al.*, 1990, Navarro *et al.*, 1999).** Vitamin E and A may also affect oxidative changes which occur in other cell organelles **(Ibrahim et al., 2000).** Vitamin C is a potent scavenger of free oxygen radicals and it has been shown that marginal Vit C deficiency results in intracellular oxidative damage in the animal **(Hudécová and Ginter 1992, Nagyová *et al.*,1994, Tatara and Ginter 1994).** Our results showed that profenfose intoxication decreased the concentration of Vit C, E, A and b-carotene as compared to control animals (table .2).

Table 2: Effect of profenofos insecticides on serum vitamin C, E, A and β- carotene of male rats.

	Vit. c (µg/dl)	Vit. E (µg/dl)	Vit. A (µg/dl)	β- carotene (µg/dl)
G1	0.77±0.09	501.47±18.12	43.41±3.04	22.17±2.63
G2	0.56± 0.11	420.61± 14.77**	40.01±3.13	19.74 ± 1.22
G3	0.38± 0.08**	376.93± 17.22***	33.15 ±3.81*	12.97± 2.62*

Results are expressed as means ± SEM (n =5), student 't' test

* P < 0.05 ** P < 0.01 *** P < 0.001

The observed depletion of serum levels of vitamin C, E and A can be explained by impairment of liver function and peroxidative processes caused by profeofose **(Rajeswary et al., 2007).**

Prolonged exposure of rats to profenofos was also shown by this study to cause a significant increase in γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH) Activities, compared with the control group (table 3). This finding agreed with those of **Irfan et al., 2002**. The significant elevations in enzymes activities of GGT and LDH indicate damage to any or all organs producing these enzymes such as liver or kidneys injuries **(Amacher, 2002 and Ncibi et al., 2008).**

At the same table (3) profenofos significantly increased the levels of serum cholesterol, triglyceride (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL), while the level of high density lipoprotein (HDL) decreased. These results were in agreement with **Yousef et al., (2006)** and disagree with **Young and Koplovitz (1995).**

Table 3: Effect of profenofos insecticides on some serum enzymes and lipid profile of male rats

	G1	G2	G3
GGT u/l	4.48 ±0.42	6.51 ±0.45**	8.50 ±0.49***
LDH u/l	559.7±14.95	604.3±13.22*	711.5±16.45***
Cholesterol mg/dl	94.52± 6.47	118.54±5.21*	130.57±5.43**
Triglyceride mg/dl	130.36± 7.81	157.33±6.95*	175.91±8.11**
HDL mg/dl	60.05± 6.01	44.12± 2.98*	37.27±3.11**
LDL mg/dl	60.62± 8.11	105.86± 8.92***	127.37±8.47***
VLDL mg/dl	26.13±2.12	31.44± 1.98	35.07± 3.01*

Results are expressed as means ± SEM (n =5), student 't' test

* P < 0.05 ** P < 0.01 *** P < 0.001

Paraoxonase (PON) has been found to hydrolyzes various organophosphorus compounds **(Yamada et al., 2001)** and protect LDL and HDL from oxidation **(Mackness et al., 1998b; and Cao et al., 1999).** Organophosphorous inhibits PON activity in serum (**Ellenhorn, et al., 1997**). the activity of PON1 have been correlated with HDL-C and apoA-I levels, **Durrington, et al., (2001),** and therefore this may decrease the protective ability of PON to secure against free radicals **(Mackness et al., 2000)** Supporting the present data, the relationship between Op and the alteration of serum lipoprotein.

The electrophoretic pattern of serum protein (table 4) pointed out that profenofos provoked a significant lower serum protein concentration with higher of gamma-globulins and lower albumins and therefore A/G decreased.

Table 4: Effect of profenofos insecticides on serum total protein and electrophoretic pattern of male rats

	G1	G2	G3
T.protein	7.6 ±0.11	7.26 ±0.09*	7.02 ±0.1***
Albumin	2.46±0.22	1.98±0.12	1.81±0.14*
α. globulin	2.28 ±0.11	2.42±0.09	2.35±0.07
α_1	0.33 ±0.07	0.6±0.07*	0.71±0.08**
α_2	1.95 ±0.1	1.82±0.21	1.64±0.12*
β. globulin	1.56± 0.12	1.47± 0.1	1.46± 0.13
B1	0.98±0.09	0.77±0.06	0.68±0.04**
B2	0.58 ±0.05	0.7 ±0.04	0.78 ±0.06*
γ .globuli	2.28 ±0.11	2.42±0.09	2.35±0.07
γ_1	1.95 ±0.1	1.82±0.21	1.64±0.12*
γ_2	0.33 ±0.07	0.6±0.07*	0.71±0.08**
T.globulin	5.14 ±0.21	5.28 ±0.15	5.21 ±0.12
A:G ratio	0.48 ±0.03	0.37 ±0.02**	0.35 ±0.02***

Results are expressed as means ± SEM (n =5), student 't' test

* P < 0.05 ** P < 0.01 *** P < 0.001

Such changes in t. protein and albumin reflect hepatocellular injury and disturbed amino acid metabolism induced by profenofos **(Gomes et al.,1999 and Yousef et al.,2006).** As a matter of fact, free radicals can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to DNA resulting in strand breaks and cross-linking. Reactive oxygen species can also induce oxidation of

critical Sulfhydryl (SH) groups in proteins and DNA, which will alter cellular integrity and function (**Fatemeh-Teimouri**, **2006**). Exposure to organophosphorus insecticides has been shown to inhibit all the cytoplasmic proteases and some of the lysosomal proteases in the liver tissue, the major site for insecticide metabolism. **(Mantle, 1997)**.

The previous study added that α2, β1, γ1 content were decreased while α1, β2, γ2 globulins were increased **(Kossmann and Magner-Krezel 1992 and Gupta et al., 1994).** These findings may be related to impact of profenofos towered the hepatic cells and immune system **(Yousef et al., 2006).** Organophosphate-induced immunosuppression was associated with severe cholinergic stimulation **(Pruett et al., 1992).**

The immunosuppression may result from direct action of acetylcholine upon the immune system or it may be secondary to the toxic chemical stress associated with cholinergic poisoning **(Zahran et al., 2005).** The increase in α1-globulin (alpha-1antitirypsin) might be attributed to tissue destruction and inflammatory reaction as mentioned by **Peasce and Kaplan, (1987).**

From this study we can conclude that treatment with profenofos induces oxidative stress, alteration in some biochemical parameters and the changes in anti-oxidant enzymes indicate a situation of enhanced oxy-radicals generation. The brain was the most sensitive organ to the oxidative stress induced by porfenofose.

References:

Abdollahi, M.; Ranjbar, A.; Shadnia S.; Nikfar, S. and Reizaic, OF. (2004): Insecticides and oxidative stress: a review. Med Sci Monit, 10(6): RA141–RA147

Aebi, H. (1974): Catalase. In: Bergmeyer, HU (ed.), Methods of Enzymatic Analysis, Chemic Academic Press Inc, Verlag, NY,; 2: 673–85

Akhgari, M.; Abdollahi, M.; Kebryaeezadeh, A.; Hosseini, R. and Sabzevari, O. (2003): Biochemical evidence for free radical induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats. Hum Exp Toxicol. 22: 205_11.

Amacher, DE. (2002): A toxicologist's guide to biomarkers of hepatic responseHuman & Experimental Toxicology 21, 253 ±262

Andreson, R.A.; Aaraas, I.; Gaare, G. and Fonnum, F. (1977):"Inhibition of acetylcholinesterase from different species by organophosphorus compounds, carbamates and methylsulphonylflouride." Genetic Pharmac., 8: 331-334.

Bagchi, D.; Bagchi, M.; Hassoum, EA. and Stohs, SJ. (1995): In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage of selected insecticides. Toxicol, 104: 129

Bjørneboe, A.; Bjørneboe, G. and Drevon, C. (1990): Absorption, transport and distribution of vitamin E. *J Nutr* 120**:** 233-242.

Bradford, M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 7: 248–54

Cao, H.; Girard-Globa, A.; Berthezene, F. and Moulin, P. (1999): Paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and is unaffected by the Q-R genetic polymorphism. J Lipid Res 40:133-9.

Cheeseman, KH.; and Slater, JF. (1992): An introduction to free radical biochemistry. In: Cheeseman, KH. and Slater, TS.(1992):. Free radicals in medicine. New York: Churchill Livingstone;. p. 481-93.

Chirions, D. and Geraud-Pouey, F. (1996): "Effectors de algunos insecticides sobre rentomofauna." Interciencia, 21: 31-36.

Chitra, KC.; Latchoumycandane, C. and Mathur, PP. (1999): Chronic-effect of endosulfan on the testicular functions of rat. Asian J Androl; 1: 203-6.

Davis, B. (1964): Disk electrophoresis. II Method and application to human serum protein. Ann. N.Y. Acad. Sci., 121: 404-427.

de Blaquiere, GE.; Waters, L.; Blain, PG. and Williams, FM. (2000): Electrophysiological and biochemical effects of single and multiple doses of the organophosphate diazinon in the mouse. Toxicol Appl Pharmacol; 166: 81_ 91

Dolora,P., Torricelli,F. and Antonelli,N. (1994): Cytogenetic effects on human lymphocytes of a mixture of 15 insecticides commonly used in Italy. *Mutat. Res.*, 325, 47–51.

Durrington, P. N., Mackness, B. and Mackness. M. I. (2001): Review - Paraoxonase and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **21:** 473–480.

Ellenhorn, MJ., Schonwald, S.; Ordog, G.; Wasserberger, J.and Ellenhorn, S. (1997): medical toxicology: diagnosis and treatment of human poisoning. Williams & Wilkins Co, 1614_63.

Ellman, G. (1959): Tissue sulphydryl groups. Arch Biochem Biophys,; 82: 70–73

Fatemeh Teimouri; Amirkabirian, N.; Esmaily, H.; Mohammadirad, A.; Aliahmadi, A. and Abdollahi ,M (2006): Alteration of hepatic cells glucose metabolism as a non-cholinergic detoxication mechanism in counteracting diazinon-induced oxidative stressHuman & Experimental Toxicology 25: 697 _ 703

Fawzy I.; Iman Zakaria, Hamza A. and Ihab A: (2007): The Effect Of An Organophosphorous Insecticideon The Hepatic, Renal And Pulmonary Tissues Of Mice Fetuses Egypt J. Med. Lab. Sci., 16(2): 99 -113.

Fortunato, JJ.; Agostinho, FR.; Réus, GZ.; Petronilho, FC.; Dal-Pizzol, F. and Quevedo, J. (2006): Lipid peroxidative damage on malathion exposure in rats. Neurotox Res. 9(1):23-8.

Geraud-Pouey, F.; Chirinos, D. and Miranda, Tejera, A. (1997): "Side effects of insecticide treatments on melon, cucumis mel L. entofauma." Rev. Fac. Agrono. (LUZ), 14: 225-232.

Gomes, J.; Dawodu, AH.; Lloyd, O.; Revitt, DM. and Anilal, SV. (1999): Hepatic injury and disturbed amino acid metabolism in mice following prolonged exposure to organophosphorus insecticides. Hum Exp Toxicol.. 18(1):33-7.

Güney, M.; Demirin, H.; Oral, B.; Ozgüner, M.; Bayhan, G. and Altuntas, I. (2007): Ovarian toxicity in rats caused by methidathion and ameliorating effect of vitamins E and C. Hum Exp Toxicol. 26(6):491-8

Gupta, RC.; Goad, JT. and Kadel, WL. (1994): In vivo acute effects of carbofuran on protein, lipid, and lipoproteins in rat liver and serum. J Toxicol Environ Health. 42(4):451-62

Gupta, RC. (2006):Toxicology of organophosphates and carbamate compounds. Elsevier Academic Press,

Hagar, HH. and Fahmy, AH. (2002): A biochemical, histochemical, and ultrastructural evaluation of the effect of dimethoate intoxication on rat pancreas. Toxicol Lett; 133: 161_70.

Hagmar,L.; Brogger,A. and Hansteen,I.L. (1994): Cancer risk in human predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res., 54, 2919–2922

Hassall, K.A. (1990): "The biochemistry and uses of insecticides." 2nd Ed., MacMillan Press Ltd., Houndmills, Bacingestoke, Taiwan, P. 81.

Henry, R.J.; Cannon, D.C. and Winkeluran, J.W. (1974): Clinical chemistry principle and technics, 2nd ed. Harper and Row publisher, Hagers Tow

Hudecova, A. and Ginter, E. (1992): The influence of ascorbic acid on lipid peroxidation in guinea pigs intoxicated with cadmium. *Food Chem Toxicol* 30: 1011-1013.

Ibrahim, W.; Blagavan, H.; Chopra, R. and Chow, C. (2000): Dietary coenzyme Q10 and vitamin E alter the status of these compounds in rat tissues and mitochondria. *J Nutr* 130: 2343-2349

Irfan, A. ; Namik, D. ; Mustafa, D.; Ibrahim, K. and Numan, T. (2002): the effects of methidathion on lipid peroxidation and some liver enzymes: role of vitamins e and c. Archives of Toxicology, vol. 76, n°8, pp. 470-473.

Joshi, SC.; Mathur, R.; Gajraj, A. and Sharma, T. (2003): Influence of methyl parathion on reproductive parameters in male rats. Environ Toxicol Pharmacol; 14: 91_98.

Kalender, S.; Kalender, Y.; Ogutcu, A.; Uzunhisarcikli, M.; Durak, D. and Aŋikgoz, F. (2004): Endosulfan-induced cardiotoxicity and free radical metabolism in rats:the protective effect of vitamin E. Toxicology.202:227-35.

Kossmann, S. and Magner-Krezel, Z. (1992): Biochemical indices of liver function in workers from repair brigades in chemical plants in Jaworz. Medycyna Pracy 43: 505 ± 508.

Lin, L.; Liu, J.; Zhang, K. and Chen, Y. (2003): [An experimental study of the effects of profenofos on antioxidase in rabbits] Wei Sheng Yan Jiu. 32(5):434-5.

Mackness, B.; Mackness, MI.; Arrol, S.; Turkie, W. and Durrington, PN. (1998*b*): Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by high density lipoprotein against low density lipoprotein oxidative modification. FEBS Lett 423:57–60.

Mackness, MI.; Durrington, PN. and Mackness, B. (2000): How highdensity lipoprotein protects against the effects of lipid peroxidation. Curr Opin Lipidol; 11: 383_88.

Malkovics, B. (1995): Effect of organophosphates on the antioxidant systems of fish tissue. Acta Biol Hung 9:11.

Mantle, D. (1997): Effects of pirimiphos methyl on proteolytic enzyme activities in rat heart, kidney, brain and liver tissues in vivo. Clin Chim Acta 262: 89 ± 97.

Moghadamnia, AA. and Abdollahi, M. (2002): An epidemiological study of poisoning in northern Islamic Republic of Iran. East Mediterr Health J; 8: 88_94.

Nagyova, A.; Galbavy, S. and Ginter, E. (1994): Histopathological evidence of Vitamin C protection against Cdnephrotoxicity in guinea pigs. *Exp Toxicol Pathol* 46: 11-14.

Navarro, F.; Arroyo, A.; Martin, Sf.; Bello, R.; De Cabo, R.; Burgess, Jr.; Navas, P. and Villalba, Jm. (1999): Protective role of ubiquinone in vitamin E and selenium-deficient plasma membranes. *BioFactors* 9: 163-170.

Ncibi, S.; Ben Othman, M.; Akacha, A.; Krifi, MN. and Zourgui, L. (2008): Opuntia ficus indica extract protects against chlorpyrifos-induced damage on mice liver. Food Chem Toxicol. 46(2):797-802.

Neishabouri, EZ.; Hassan, ZM.; Azizi, E. and Ostad, SN. (2004): Evaluation of immunotoxicity induced by diazinon in C57bl/6 mice. Toxicology 196: 173-79

Okhawa, H.; Ohishi, N. and Yagi, N. (1979): Assay for Lipid peroxides in animal tissues by thiobarbituric acid reaction. Ann Biochem,; 5: 351–58

Peace, AJ. and Kaplan, LA. (1987): Clinical chemistery. Mosby st. Louis. USA.

Pournourmohammadi, S.; Farzami, B.; Ostad, SN.; Azizi, E, and Abdollahi, M. (2005): Effects of malathion subchronic exposure on rat skeletal muscle glucose metabolism. Environ. Toxicol Pharmacol; 19: 191_96.

Petrie, A. and Watson, A. (1999): Statistics for Veterinary and Animal Science. 1st Ed., pp. 90-99, the Blackwell Science Ltd, United Kingdom

Pruett, SB.; Han, Y.; Munson, AE .and Fuchs, BA. (1992): Assessment of cholinergic influences on a primary humoral immune response. Immunology. 1992 November; 77(3): 428–435.

Rajeswary, S.; Kumaran, B.; Ilangovan, R.; Yuvaraj, S.; Sridhar, M.; Venkataraman, P.; Srinivasan, N. and Aruldhas, MM. (2007): Modulation of antioxidant defense system by the environmental fungicide carbendazim in Leydig cells of rats. Reprod Toxicol. 24(3-4):371-80.

Rodrigo, L.; Hernandez, AF.; Lopez-Caballero, JJ.; Gil, F. and Pla, A. (2001): Immunohistochemical evidence for the expression and induction of paraoxonase in rat

Rupa, D.S., Reddi, P.P. and Reddi, O.S. (1991) Clastogenic effect of insecticides in peripheral lymphocytes of cotton-field workers. Mutat. Res., 261, 177–180

Saeed, R. M., Al-Koly, M. A., and Ali, M. A., (1995): Hepatic, renal and pulmonary responses in pregnant mice and their fetuses induced by profenofos. Journal of Union of Arab Biologists: Zoology 3(A):359-386.

Sharma, Y.; Bashir, S.; Irshad, M.; Gupta, SD.; and Dogra, TD.(2005): Effects of acute dimethoate administration on antioxidant status of liver and brain of experimental rats. Toxicology 206:49-57

Sonnenwirth, A. and Jareet, L. (1980): " Garduals Clinical Laboratory Methods and Diagnosis." Vol.1,8th Ed., Mosby.

Szase, G.; Gruber, W. and Bente, E. (1976): Clin. Chem.., 22:650-656.

Tatara, M, and Ginter, E. (1994): Erythrocyte membrane fluidity and tissue lipid peroxides in female guinea-pigs on graded vitamin C intake. *Physiol Res* **43:** 101-105.

Wahlefeld, AW. (1974): Cloremetric determination of enzymatic triglycol in serum. In Methodes of enzymatic Analysis, vol. 5 M.M. Bermeyer Ed., Academic Press, New York,PP. 1831-35.

Watson, D. (1960): Determination of serum cholesterol. Clin. Chem. Acta, 5: 637.

Yamada, A.; **Shoji, T**.; **Tahara, H**.; **Emoto, M**. **and Nishizawa, Y**. **(2001):** Effect of insulin resistance on serum paraoxonase activity in a nondiabetic population. Metabolism.50(7):805-11.

Weil, C.S. (1952):"Tables for convenient calculation of medium effective dose (LD_{50}) or ED_{50}) and instruction in their use" Biometrics, 8:263 -294.

Young, GD. and Koplovitz, I. (1995): Acute toxicity of cyclohexylmethylphosphonofluoridate (CMPF) in rhesus monkeys: serum biochemical and hematologic changes. Arch Toxicol. 69(6):379-83.

Yousef, MI.; Awad, TI.; and Mohamed, EH. (2006): Deltamethrin-induced oxidative damage and biochemical alterations in rat and its attenuation by Vitamin E. Toxicology. 29; 227(3):240-7.

Zahran, M.M.; Abdel-Aziz, K.B.; Abdel-Raof, A. and Nahas E.M. (2005): The Effect of Subacute Doses of Organophosphorous Insecticide , Nuvacron, on the Biochemical and Cytogenetic Parameters of Mice and Their Embryos Research Journal of Agriculture and Biological Sciences 1(3): 277-283, 2005

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التغيرات التاكسديةو البيوكيميائية المصاحبة لاستخدام مبيد للبيروفنيوفوس في الجرذان

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**المعمل المركزي للمبيدات- الدقي

الملخص العربي

يعتبر البروفينوفوس احد المركبات الفسفورية العضوية السامة، تتعرض الحيوانات لمثل هذه المركبات عن طريق المآكل والمشرب وهى تحدث سميتها في كثير من الأعضاء مثل الكبد والمخ.

وقد أجريت هذه الدراسة لاستبيان تأثير تعاطي البروفينوفوس عن طريق الفم (26.53 &53.07 ملجم/كجم من وزن الجسم/يوم لمدة 28 يوما) على المالونالدهيد و مضادات الأكسدة (الكتاليز و الجلوتاثيون في خلايا الكبد والمخ وبالإضافة إلى التغيرات البيوكيميائية في مصل ذكور الجرذان.

وقد حدث ارتفاع حاد في تركيز الدهون المؤكسدة مع نقص في معدل الجلوتاثيون و أظهرت النتائج زيادة معنوية ملحوظة في مستوى إنزيم الكتاليز بالنسبة إلى الجرعة 53.07 ملجم/كجم من البروفينوفوس.

التعرض للبروفينوفوس يصاحبه نقصا في معدل الفيتامينات ا, هـ, سى, البيتا كاروتين فى مصل الدم. وبالمقارنة مع المجموعة الضابطة وجد نقص معنوي في معدل البروتين الكلي والالبيومين مصاحبة لنقص فى جلوبيولنات الالفا-1 والبيتا مع ارتفاع في معدل جاما-2 جلوبيولن ونشاط كل من GGT و LDH , الكوستيرول ,التراى جلسريد,VLDL, LDL بينما وجد نقص في HDL.

وقد بينت هذه الدراسة ان البوفينوفوس في الجرعات القليلة له أثر مؤكسد ويتسبب في التغيرات البيوكيمائية في ذكور الجرذان.

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Influence of Subchronic Exposure of Profenofos on Biochemical Markers and Microelements in Testicular Tissue of Rats

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Abstract: ***Aim:*** To investigate the effect following subchronic exposure to the organophosphorous insecticide of common name profenofos, which extensively used in agriculture, on the key enzymes of fertility and the concentration of microelements in testicular tissues in male albino rats. ***Methods:*** Adult male albino rats were orally administered with profenofos at a does of 23.14 mg/kg body weight per day for 60 days, emulsifying in 0.4 ml tap water. The control group received equal volume of tap water. Twenty-four hours after the last treatment the rats were sacrificed using anesthetic ether. Epididymus and testes were collected, cleaned and weight. Then epididymus prepared in buffer saline and spermatozoa were examined with light microscopy for concentration and motility. Testes were fractionated and supernatant of testicular homogenate was obtained by centrifugation, activities of alkaline and acid phosphatases, lactate dehydrogenase and total protein as well as concentration of microelements; Copper, Iron, Zinc and Selenium were measured. Moreover, the testes were histologically examined. ***Results:*** The epididymus and testes weights were significantly decreased. Reduction in sperm count was recorded in cauda epididymus in profenofos treated group, associated with decreased motility. Total protein (TP) level exhibited an elevation in testicular tissue in comparison with the control group. There was significant decrease in the activities of alkaline and acid phosphatase (ALP and ACP) and lactate dehydrogenase (LDH). A totally different trend was observed for the level of microelements; Copper (Cu), Zinc (Zn), Iron (Fe) and selenium (Se) where a sharp augmentation in the element levels was noticed in profenofos-treated rats compared with the control group. Treatment-dependent histopathological changes were seen in testes. ***Conclusion:*** Profenofos alters testicular functions possible by inhibition the activities of marker enzymes and inducing alteration in microelements levels, thereby disrupting male reproduction. [Nature and Science. 2009;7(2):16-29]. (ISSN: 1545-0740).

Keywords: Profenofos; rats; microelements, Zinc, Copper, Iron, Selemnium; Enzymes; Acid and Alkaline phosphatase, Lactate dehydrogenase, Total Protein, Testes.

1- *Introduction*

Organophosphorous insecticides (OPIs) have been considered as genuine alternatives to chlorinated (O'Ch) insecticides due to their broad-spectrum pesticidal properties and relatively shorter persistence after applications **(Sharma et al., 2005).** OPIs in addition to their intended effects like control of insects or other pests are sometimes found even to effect non-target organisms including human beings **(Chantelli-Forti et al., 1993; Chaudhuri et al., 1999).**

Exposure to low level OPIs is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in humans and experimental animals **(Sutatos, 1994).** There is growing concern that environmental chemicals both natural and man-made, having estrogenic property may be causing a variety of reproductive disorders in wildlife and human population **(Chitra et al., 1999).**

The testes of humans and other mammals are highly susceptible to damage produced by genetic disorders, environment or occupational exposure to chemical or other means. Specific causes of testicular damage have been catalogued **(Jadaramkunti and Kaliwal, 2002).**

In man, much data are available about biochemical analysis of seminal plasma. However, not many studies have been conducted in animals yet **(Pesch et al., 2006).**

Analysis of enzyme activities and concentrations of microelements can estimate integrity and function of testes, in man; analysis of seminal plasma enzymes and microelements has been performed accurately and much is known about the importance of the "right contents" of seminal plasma **(Pandy et al., 1983; Chia et al., 2000; Huang et al., 2000 and Stanwell-Smith et al., 1983).**

It has been reported that, pesticides with such properties have been shown to cause overproduction of reactive oxygen species (ROS) in both intra and extra cellular spaces, resulting in a decline of sperm count and infertility in wildlife and human **(Gangadharan et al., 2001).**

Trace elements, such as Copper (Cu), Zinc (Zn), and Selenium (Se) have a pivotal role in the spematogensis **(Homma-Taked et al., 2003)** Ionic environment has a high influence on sperm function **(Hamameh and Gatti, 1998),** profenofos belongs to the phosphorothioate class of OPIs. It widely used for a variety of agricultural and public health applications, previous studies suggest that profenofos considered as one of the male reproductive toxicant **(Moustafa et al., 2007).**

In spite of the extensive use of profenofos in crop protection and in the household, information related to its effects on health with particular reference to reproductive toxicity are scarcely. Therefore, the objective of this study was to clarify the effect following subchronic exposure to profenofos on testicular functions by measuring the fertility indices (sperm count and motility), the activity of specific enzymes that responsible of spermatogenesis (alkaline and acid phosphatases and lactate dehydrogenase) and total protein level as well as concentrations of the essential microelements; Copper (Cu), Iron (Fe), Zinc (Zn) and Selenium (Se) in testicular tissue of male rats.

2-1 ***Materials***

The active substance profenofos produced by Syngenta multi national comp. under trade name: Selecron 72% EC was used.

Tap water was used for preparing emulsion of profenofos immediately before use and orally administered into animals by osophageal intupation (per OS.). The median lethal dose (LD_{50}) of profenofos (per OS.) was determined according to **Weil (1952)** and its value was 185.13 mg/kg body weight.

2-2 *Animals*

In this investigation, thirty male Wistar albino rats, rattus norvegicus were obtained from the breeding unit of the Egyptian organization for the Biology and vaccine production, Egypt. Male rats initially weighing 150±10g were used. Animals were allowed to be acclimatized to laboratory conditions; of temperature at 25±2^{0}C, humidity (30-70%) and light (12-h dark: 12-h light) and kept on balanced diet and water *ad libitum* for 2 weeks prior to the experiment. Animals were housed throughout the experiment in polypropylene cages (with each cage housing five animals) containing paddy husk as bedding.

2-3 *Experimental Design*

Rats were randomly divided into two comparable groups as follows, First group: (n = 10) served as normal control and animals were received the vehicle (tap water). Second group: (n = 20) animals were orally dosed for 60 days with profenofos at 23.14 mg/kg body weight (4 doses/week).

Clinical signs were monitored daily and animals were weighed twice weekly throughout the experiment and the dose was adjusted accordingly.

2-4 *Sampling*

After completion of treatment period (60 days), animals were anaesthetized with ether and sacrified. The testes and epididymus were removed immediately, cleaned of the adhering tissues and weighted. Fertility-related parameters (sperm count and motility) were performed by dissecting out the Cauda epididymus and teasing it in a known volume of normal saline at 37^{0}C. Sperm counting was done using a haemocytometer according to the method of **Feustan et al. (1989).**

The right testes were kept in a deep freezer (-40^{0}C) for biochemical estimations and microelements detection. Left testes were removed and fixed in 10 % formalin for routine histopathology.

2-5 *Biochemical Estimations:*

Frozen testes were washed with saline solution, then minced and homogenized (10% W/V) in ice-cold saline, using a chilled glass-teflon porter-Elvehjem tissue grinder tube. The homogenate was centrifuged at 10,000 xg for 20 min. at 4 ^{0}C and the resultant supernatant used for determination of protein contents, Tp (Bradford, 1976); alkaline phosphatase, ALP (Babson, 1965) and acid phosphatase ACP (Babson and Read, 1959). Also, a 10% homogenate of testes was prepared in ice-cold 0.1M phosphate buffer, the homogenate was centrifuged at 12,000 xg for 30 min. at 4^{0}C. the supernatant used for determination of lactate dehydrogenase, LDH **(Moss and Henderson, 1994).**

2-6 ***Histopathological Studies***

For the histopathological observations at light microscopic level, fresh testes were immersion fixed in 10% formalin saline.

Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5um thick sections were double stained with hematoxylin and eosin and observed under microscope **(Banchraft et al., 1996).**

2-7 Determination of microelements concentrations in testicular tissues:

The concentrations of the microelements Copper (Cu), Iron (Fe), Zinc (Zn) and Selenium (Se) in testicular tissues were measured according to the procedure which reported in **AOAC (2004),** by using atomic absorption spectrophotometer (Thermo Jarel Ash-AA-ScanI).

2-8 Statistical Analysis

Data analysis and evaluation of statistical significance among different values determined was done using the student's t-test. Statistical differences with a value of $p<0.05$ were considered significant **(Snedecor and Cochran, 1980).**

3- ***Results***

3-1 ***Testes and epididymus weights***

The variations in the testes and epididymus weights of animals subjected to profenofos treatment are shown in Table (1). There was significant decrease ($p<0.05$) and ($P<0.001$) in weights of the testes and epididymus, respectively, as compared to control group.

Table (1): Effect of oral administration of profenofos on testes and epididymus weights of rats after sub-chronic exposure (60 days)

Parameter	Control group	Profenfos-treated group 23.14 mg/kg body weight
Testes weight (g)	1.52 ± 0.040	1.40 ± 0.004*
Epididymus weight (g)	0.37 ± 0.014	0.02 ± 0.008***

Data represent mean ± SE, n = 5, * P< 0.05, *** P< 0.001 (Student's t-test)

3-2 Semen Parameters

The effect of oral administration of profenofos for 60 days on sperm count and motility in cauda epididymus is shown in Table (2). The spermatozoal density (count) increased significantly ($p<0.05$) in profenofos-treated group in comparison with the control group.

Similarly, spermatozoal motility was also found to be significantly decreased ($p<0.001$).

Table (2): Effect of oral administration of profenofos on semen parameters in cauda epididymus of rats after sub-chronic exposure (60 days):

Parameter	Control group	Profenofos-treated group 23.14 mg/kg body weight
Total sperm count (10^6/ml)	100 ± 3.536	80 ± 4.082*
Motility (%)	90 ± 1.58	65 ± 3.227***

Data represent mean ± SE, n = 5, * P<0.05 , *** P<0.001 (student's t-test)

3-3 *Biochemical assays*

Results of testicular biochemistry have been depicted in Table (3). Alkaline (ALP), acid (ACP) phosphatese and lactate dehydrogenas (LDH) activities were recorded to have decreased ($p<0.001$, $p<0.05$ and $p<0.01$, respectively) in profenofos-treated group as compared to control group.

In addition, total protein level was found to be significantly raised ($p<0.05$) in treated group in comparison with the control group.

Table (3): Effect of oral administration of profenofos on some testicular biochemical parameters in rats after sub-chronic exposure (60 days)

Parameters	Control group	Profenofos-treated group 23.14 mg/kg body weight
alkaline phosphatase(U/mg protein)	0.127 ± 0.002	0.067 ± 0.009***
acid phosphatase (U/mg protein)	0.108 ± 0.002	0.084 ± 0.008*
lactate phosphatase (U/mg protein)	1.60 ± 0.073	1.25 ± 0.042**
total protein (mg/g tissue)	17.28 ± 0.774	20.27 ± 0.348*

Data represent mean ± SE, n = 4, * P<0.05, P<0.01, *** P<0.001 (student's test)**

3-4 *Testicular histoarchitecture*

In addition to the findings listed above, we have observed the presence of microscopic changes in the testes of male albino rats.

Histological findings of testes from control and treated groups are presented in figs. 1, 2, respectively.

1 Normal control animals, revealed normal mature seminiferous tubules with complete series of spermatogenesis and high spermatozoal concentration in the lumen (fig.1) Profenofos-

intoxicated animals indicated that there were few numbers of sperm cells in the lumen of the seminiferous tubules (fig. 2), in correlation with the control one.

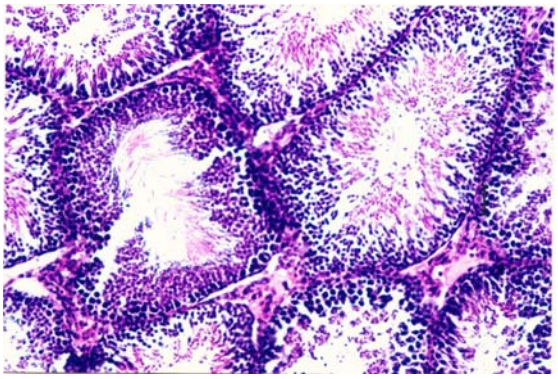

Fig. (1): Testes of rat in control gp. Showing the normal histological structure of the seminiferous tubules in nature active condition.

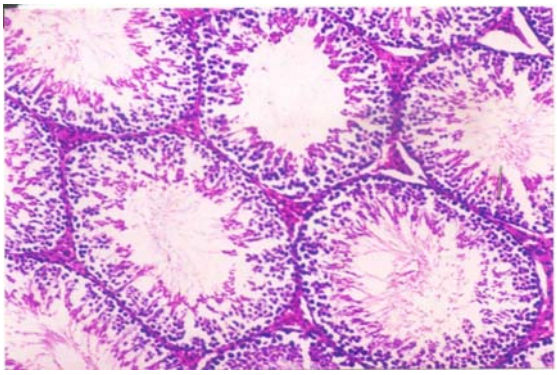

Fig. (2): Testes of rat treated by profenofos showing low amount of sperms in the lumen of the semineferous tubules.

3-5 *Microelements concentrations*

The effect of oral administration of profenofos for the 60 days on testicular tissue contents of microelements is depicted in table (4).profenofos treatment produced significant increase ($p<0.001$) in iron (Fe), copper (Cu), zinc (Zn) as well as in selenium (Se) levels.

Table (4):The Testicular tissue contents of microelements in profenofos-treated rats after sub-chronic exposure (60 days).

Element (ppm)	Control group	Profenofos-treated group 23.14 mg/kg body weight
Copper (mg/kg.tissue)	960.24 ± 3.136	1747.22 ± 3.747***
Ferric (mg/kg.tissue)	370.36 ± 1.659	700.19 ± 4.827***
Zinc (mg/kg.tissue)	9.93 ± 0.143	16.74 ± 0.158***
Selenium (mg/kg.tissue)	100.52 ± 0.808	162.37 ± 0.458***

Data presented mean ± SE of five individual values.

4- ***Discussion***

Organophosphates (OPIs) are among the most widely used synthetic insect pesticides. The wide spread use of OPIs has stimulated research into the possible extence of effects related with their reproductive toxic activity **(Joshi et al., 2007).**

The present study results demonstrated that 60 day's exposure of male rats to profenofos at the dose 23.14 mg/kg body weight (4 doses/week) resulted in decreased the testes and epididymus weights, male fertility indices (sperm count and motility), and activities of ALP, ACP and LDH but increased levels of total protein and microelements (Cu, Fe, Zn and Se) in testicular tissues.

Our results showed that the weights of testes and epididymus were significantly lower in the profenofos-treated rats than in the controls. The decrease in testicular weight in treated rats may be due to reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of leydig cells, a site of steroid biosynthesis **(Sujatha et al. ,2001 and Kaur and mangat, 1980).** The decrease in testicular weight in profenofos-treated rats may indicate impairment at testicular, pituitary, or hypothalamic level **(Chitra et al. ,1991).**

Similar results were recorded by Ref Joshi et al. (2007), who mentioned that chlorpyrifos (OPIs) at dose levels of 7.5, 12.5 and 17.5 mg/kg b.wt./day, for 30 days, decreased significantly the weight of testes.

The epididymus is androgen-dependant organ, relying on testosterone for its growth and function **(Klinefelter and Hess, 1998).**

On discussing the results with previous reports, it is proposed that profenofos probably imped the activity of testes and epididymus by inhibition of androgen production or its direct action on these organs **(Kaur and mangat, 1980),** thus, the reduction in the weights of testes and epididymus in our study may be due to lower bioavailability of androgen **(Sujatha et al. ,2001).**

Moreover, the deleterious effects of profenofos on reproductive organ weights might be due to a decrease in the testosterone (T) and thyroid hormone levels after 60 days from the onset of the treatment **(Takizawa and Horii, 2002).**

The present results confirm the previous reports of **(El-kashoury and El-far, 2004)** who mentioned that administration of rats with profenofos at 23.14 and 46.30 mg/kg body weight for 28 days and 60 days, respectively, induced significant decrease in thyroid hormone levels, there is ample evidence that thyroid hormone is essential to the normal development of testes in the neonate **(Cook et al. ,1994 and Hardy et al. ,1996),** as well as an elevation in cholesterol level, a precursor of steroid hormone had occurred. Authors also, mentioned that inhibition of hepatic microsomal
7-hydroxylation of cholesterol by profenofos leads to reduction of cholesterol break down and its accumulation.

Sperm count is one of the most sensitive tests for spermatogenesis and it is highly correlated with fertility. Our results revealed that, treatment of rats with profenofos significantly reduced the sperm count and motility.

The decreased sperm motility and density (count) after oral administration of profenofos is may be due to androgen insufficiency **(Chaudhary and Joshi, 2003)** which caused impairment in testicular functions by altering the activities of the enzymes responsible for spermatogenesis **(Sinha et al. ,1995 and Reuber, 1981).**

Histological structure of the testes confirmed the aforementioned results, where it is revealed degeneration in some of seminiferous tubules associated with low luminal spermatozoal concentration.

It is tempting to speculate that the decreased sperm motility in the present study may have been related to our earlier studies on profenofos **(El-kashoury and El-far, 2004)** which pointed that subclinical hypothyroid state in rats administered with profenofos for 60 days had occurred.

Also, men with hypothyroid have been reported to have lower sperm motility than euthyroid controls **(Corrales – Hernandez et al. ,1990)** and thyroxine (T4) replacement in men with hypothyroidism is reported to improve sperm motility **(Kumar et al. 1990).**

Moreover, it had been reported that chlorpyrifos brought about marked reduction in epididymal and testicular sperm counts in exposed males **(Joshi et al., 2007)**. Also, testicular atrophy and degenerative changes in the seminiferous tubules had been reported in experimental animals administered with various O'Ch and OPIs pesticides **(Dutta and Dikshith, 1973).**

Based on the data obtained in this study, administration of profenofos into male albino rats reduced the activities of acid and alkaline phosphatase and lactate dehydrogenase which reflect suppression in testicular function **(Johnson et al. ,1970).** Activities of markers enzymes viz ALP, ACP and LDH are considered to be functional indicators of spermatogenesis.

Our results confirm the findings of **(Salem et al. ,1989)** who investigated the influence of methamidophos (O'ps) on mammals. Results showed that treatment of male rats with methamidophos, at 100 ppm in drinking water for 9 and 45 days, reduced significantly acid and alkaline phosphatase and lactate dehyrogenase in testicular tissue.

Also, **(Mustafa et al. ,2007)** reported that profenofos considered as one of the male reproductive toxicants.

ALP is primary of testicular and epididymual origin and, therefore, suitable for differentiation of oligo-and azoospermia **(Turner and Sertich, 2001; Turner and McDonell, 2003).** Decline in ALP activity indicated that profenofos treatment produced a state of decreased steroidogenesis where the inter and intercellular transport was reduced as the metabolic reactions to channelize the necessary inputs for steroidogenesis slowed down **(Latchoumycandane et al. ,1997).** Acid phosphatases are enzymes capable of hydrolyzing orthophosphoric acid esters in an acid medium. The testicular acid phosphatase gene is up-regulated by androgens and is down-regulated by estrogens **(Yousef et al. ,2001).**

Activities of phosphatases enzymes have been shown to rise when testicular steroidogenesis is increased **(Mathur and Chattopandhyay, 1982).**

Also, **(Latchoumycandane et al. ,1997)** mentioned that a decrease in ACP activity in free state would thus reflect decreased testicular steroidogenesis in rats and this may be correlated with the reduced secretion of gonadotrophins. LDH is associated with the maturation of germinal epithelial layer of seminiferous tubules and associated with post meiotic spermatogenic cells **(Sinha et al., 1997).** An inhibition in the activity of LDH in testes of profenofos-treated rats points toward the interference of profenofos with the energy metabolism in testicular tissues **(Mollenhauer et al., 1990).**

The correlation between LDH and motility and living sperm could be a sign that extracellular LDH ensures metabolism of spermatozoa, perhaps even in anaerobic conditions **(Pesch et al., 2006).**

As regards the testicular protein, results of the present study exhibit an increase in its level in profefos-treated rats. The testicular fluid contains both stimulatory factors as well as inhibitory factors that selectivity alter the protein secretions **(Brooks, 1983).** Thus, the changes in protein suggested that there is a reduction in the synthetic activity in testes.

An elevation in testicular protein in the present study confirms the previous results by **(Joshi et al., 2007)** who mentioned that the protein content was raised at significant levels in chlorphrifos-treated rats.

Gupta et al. (1981) and Singh and Pandey (1989) illustrated that an elevation in the testicular protein may be due to the hepatic detoxification activities caused by endosulfan (O'ch) which results in the inhibitory effect on the activities of enzyme involved in the androgen biotransformation **(Dikshith and Dutta, 1972).**

Similar results showed the same trend in the protein content caused by several pesticides, at different periods and / or different concentrations, had been also reported **(Shivanandappa and Krishnakumari (1981), Bhatnagar and Malviya, 1986; Chitra et al., 1999; Choudhary and Joshi, 2003).**

In accordance with the findings of the present study, **Rao and Chinoy (1983)**, suggested that the accumulation of protein occurred in testes and epididymus due to androgen deprivation to target organs.

This deprivation effect also led to a reduction in testicular and cauda epididymus sperm population, loss of motility in the latter and an increase in number of abnormal spermatozoa, thereby manifesting 100% failure in treated animals.

Results of the present investigation showed that administration of profenofos into male rats increased the concentration of trace elements; Cu, Fe, Zn and Se in testicular tissue, which have a pivotal role in spermatogenesis **(Homma-Takeda et al., 2007).**

These findings are not in accordance with those of **Salem et al. (1989),** who stated that treatment of rats with methamidophos (OPIs), for 45 days, decreased the concentrations of Zn and Se in the testicular tissues.

On the other hand, similar results were recorded by **Al-Bayati et al. (1988),** who mentioned that 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), O'ch, produced atrophy, morphological changes and impaired spermatogenesis in testes of experimental animals. In addition, testicular tissue contents of Fe, Cu, and Zn were significantly increased in the treated rats.

Zinc (Zn) markedly increased the ALP and ACP activities and this occurred concomitantly with the appearance of spermatids and mature sperm cells **(Guha and Vanha-Perttula, 1983).**

Selenium is an essential trace nutrient for humans and animals. It is an essential at lower concentrations and toxic at higher concentrations. Se is required for normal testicular development and spermatogenesis in rats **(Behne et al., 1996).**

The selinodeiodinase enzymes (types I, II and III iodothyronine deiodinase) control the metabolism of thyroid hormone, which is essential for the normal development **(Defrance et al., 1995)** and function **(Latchoumycandane et al., 1997)** of testes in rats.

The above explanation supports our findings where elevated testicular tissue content of Se associated with decrease in testicular weight, sperm count and motility in profenofos-treated rats. In support of these findings, earlier results **(El-Kashoury and El-Far, 2004)** revealed that treatment of rats with profenofos at the same dose and time interval decreased markedly (T_3) level in plasma in comparison with the control group.

Cupper is necessary for many enzymes like the Cu-Zn-Superoxide dismutase (SOD), which is involved in cell protection against free (Oxygen) radicals. Copper is also needed for the cytochrome C oxidase that is responsible for energy supply and for cellular and humoral immunity **(Leonhard-Marek, 2001).**

As regards Cu concentrations, an administration of rats with profenofos increased testicular tissue contents of Cu by 2-fold, respectively. Elevated Cu concentrations reduced oxidative processes and glucolysis that may cause immotility and reduced viability **(Leonhard-Marek, 2001).**

A proposed mechanism could explain elevated iron concentrations in testicular tissues in profenfos-treated rats, is that iron is known to be essential and mostly bound to transferrin (produced by sertoli cells), haptoglobin (sertoli, leydig and germ cells) and lactoferrin (spermatozoa and vascular gland). These proteins contain catalytic inactive iron which avoids extensive oxidation **(Leonhard-Marek, 2001).**

Results of the present investigation suggested that profenofos may impede the utilization of micro-elements in the testes, consequently stagnation of Cu, Fe, Zn and Se in the testes occurred.

It is concluded that profenofos induced adverse effects on testicular function by altering biomarker enzymes activities as well as disrupting micro-elements levels, thus care should be taken and more studies should be done to increase the validity of those information.

Abbreviation used:

OPIs, organophosphorous insecticides; O'Ch, organochlorine, TP, total protein, ALP, alkaline phosphatase; ACP, acid phosphatase; LDH, lactate dehydrogenase; Cu, Copper; Zn, Zinc; Fe, Iron; Se, Selenium; Ec, Emulsifiable concentrate; T_4, Thyroxine; T_3, Triiodothyronine; T, Testosterone; Ros, Reactive oxygen species.

References

1. A.O.A.C. (2004). Association of official analytical chemistry.
2. Al-Bayati, Z.A.; Wahab, Z.Z. and Stohs, S.J. (1988). 2, 3, 7, 8 Tetrachlorodibenzo-p-dioxin (TCDD)-induced alterations in lipid peroxidation, enzymes and divalent cations in rat testis. Xenobiotica. 18: 1281-1289.
3. Babson, A.L. and Ready, A.P. (1959). Colourimetric method for the determination of total and prostatic acid phosphatase. Am. J. Clin. Path., 32 : 89-91.
4. Babson, L.A. (1965). Phenolphalein monophosphate methods for the determination of alkaline phosphatase. Clin. Chem., 11 : 789.
5. Banchraft, J.D.; Stevens, A. and Turner, D.R. (1996). Theory and practice of histological techniques. 4th Ed., Churchill Livingstone, New York, London, San Francisco, Tokyo.
6. Behne, D.; Weiler, H. and Kyriakopoulos A. (1996). Effects of selenium deficiency on testicular morphology and function in rats. J. Repord Fertile. 106: 291-297.
7. Bhatnagar, V.K. and Malviya, A.N. (1986). Changes in some biochemical indices in rat upon pesticide toxicity. Indian J. Biochem. Biophys., 15 : 78-81.
8. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem., 72 : 248-254.
9. Brooks, D.E. (1983). Effect of androgen on protein synthesis and secretion in various regions of the rat epididymis, as analysed by two dimensional gel electrophoresis. Mol. Cell Endocrinol., 29 : 255-270.
10. Chantcli-Forti, G.; Paolini, M. and Hrelia,P. (1993). Multiple end point procedure to evaluate risk from pesticides. Environ. Health perspect. 101, 15-20.
11. Chaudhuri,K.; Selvaraj, S. and Pal, A. K. (1999). Studies on the genotoxicity of endosulfan in bacterial systems. Mutat. Res. 439, 63-67.
12. Chia, S.E.; Ong, C.N.; Chua, L.H.; Ho, L.M. and Tay, S.K. (2000). Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men, J Androl. 21: 53-57.
13. Chitra, K.C.; Latchoumycandane, C. and Mathur, P.P. (1999). Chronic effect of endosulfan on the testicular functions of rat. Asian J. Andrology, 1 : 203-206.
14. Choudhary, N. and Joshi, S.C. (2003). Reproductive toxicity of endosulfan in male albino rats. Bull. Environ. Contam. Toxicol., 70 : 285-289.
15. Cooke, P.S.; Zhao, Y.D. and Bunick, D. (1994). Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal sertoli cell: Possible mechanism for increase adult testis weight and sperm production induced by neonatal goitrogen treatment. Biol. Reprod. 51: 1000-1005.
16. Corrales-Hernandez, J.J.; Miralles-Gareia, J.M. and Garcia-Diez, L.C. (1990). Primary hypothyroidism and human spermatogenesis. Arch. Androl. 25: 21-27.
17. Defrance, L.R.; Hess, R.A.; Cooke, P.S. and Russell, L.D. (1995). Neonatal hypothyroidism causes delayed sertoli cell maturation in rats treated with propylthiouracin: Evidence that sertoli cell controls testis growth. Anat. Rec. 242: 57-69.

18. Dikshith, T.S.S. and Dutta, K.K. (1972). Pathological changes induced by pesticides in the testes and liver of rats. Exp. Pathol., 7 : 309-316.
19. Dutta, K.K. and Dikshith, T.S.S. (1973). Histopathologic changes in the testes and liver of rats repeatedly exposed to pesticides. Exp. Pathol., 8 : 363-370.
20. El-Kashoury, A.A. and El-Far, F.A. (2004). Effect of two products of profenofos on thyroid gland, lipid profile and plasma APO-1/FAS in adult male albino rats, Egypt. J. Basic and Appl. Physiol., 3: 213-226.
21. Feustan, M.H.; Bodnai, K.R. and Kerstetter, S.L. (1989). Reproductive toxicity of 2-methoxy ethanol applied dermally to occluded and non-occluded sides in male rats. Toxicol. Appl. Pharmacol., 100 : 145-165.
22. Gangadharan, B.; Murugan, M.A. and Mathur, P.P. (2001). Effect of methoxychlor on antioxidant system of goat epididymal sperm in vitro. Asian J. Androl., 3 : 285-288.
23. Guha, K. and Vanha-Perttula, T. (1983). Acid phosphatases in the mouse testis: activity changes during development. Arch-Anorl. 10 (1): 7-16.
24. Gupta, P.K.; Shrivastava, S.C. and Ansari, R.A. (1981). Toxic effects of endosulfan on male reproductive organs in rats. Indian J. Biochem. Biophys., 18 : 159-163.
25. Hamameh, S. and Gatti, J.L. (1992). Role of the ionic environment and internal pH on sperm activity, Hum Repord Suppl. 4: 20-30.
26. Hardy, M.P.; Sharma, R.S.; Arambepola, N.K.; Sottas, C.M.; Russell, L.D.; Bunick, D.; Hess, R.A. and Cooke, P.S. (1996). Increased proliferation of leydig cells induced by neonatal hypothyroidism in the rat. J. Androl. 17: 231-238.
27. Homma-Takeda, S.; Nishimura, Y.; Watanabe, Y. and Yukawa, M. (2007). Site-specific changes in zinc levels in the epididymus of rats exposed to ionizing radiation. Nuclear instruments and methods in physics research section B. Beam interactions with materials and atoms. 260: 236-239.
28. Huang, Y.L.; Tsen, W.C.; Cheng, S.Y. and Lin, T.H. (2000). Trace elements and lipid peroxydation in human seminal plasma, Biol. Trace element. 76: 207-215.
29. Jadaramkunti, U.C. and Kaliwal, B.B. (2002). Dicofol formulation induced toxicity on tests and accessory reproductive organs in albino rats. Bull. Environ. Contam. Toxicol., 69 : 741-748.
30. Johnson, A.D.; Gomes, M. and Vandemark, N.L. (1970). The testis. 1st Ed., Academic Press, New York and London.
31. Joshi. S.C.; Mathur, R.; Gilati and Tags, N. (2007). Testicular toxcity of chlorpyrifos (an organophosphate pesticide) in albino rat. Toxicol Ind Health. 23: 439-444.
32. Kaur, C. and Mangat, H.K. (1980). Effects of estradiol dipropionate on the biochemical composition of testes and accessory sex organs of adult rats. Andrologia, 12 (4) : 373-378.
33. Klinefelter, G.R. and Hess, R.A. (1998). Toxicology of the male excurrent ducts and accessory sex glands. In : Reprod. and Dev. Toxicol., pp. 553-591, New York, Basel, Hong Kong; Marcel Dekker, Inc.
34. Kumar, B.J.; Khurana, M.L.; Ammini, A.C.; Karmarkar, M.G. and Ahuja, M.M.S. (1990). Reproductive endocrine functions in men with primary hypothyioidism: Effect of thyroxine replacement. Horm. Res. 34: 215-218.
35. Latchoumycandane, C.; Gupta, S.K. and Mathur, P.P. (1997). Inhibitory effects of hypothyrodism on the testicular functions of postnatal rats. Biomed. Lett., 56 : 171-177.
36. Leonhard-Marek, S. (2001). Influence of drugs, pollution and trace elements on male fertility. In: Busch, W. and Holzmann, A. Editors, Andrology in veterinary medicine, Stuttgart, Schattauer, pp. 474-481.

37. Mathur, P.P. and Chattopadhyay, S. (1982). Involvement of lysosomal enzymes in flutamide-induced stimulation of rat testis. Andrologia, 14 : 171-176.
38. Mollenhauer, H.H.; Morre, D.J. and Rowe, L.D. (1990). Alteration of intracellular traffic by monensin : mechanism, specificity and relationship to toxicity. Biochem. Biophys. Acta, 1031 : 225-246.
39. Moss, D. W. and Handerson, A.R. (1994). Lactate dehydrogenase 2nd Ed., Textbook of clinical chemistry, chapter 20, Enzymes, pp. 812-818
40. Moustafa, G.G.; Ibrahim, Z.S.; Hashimoto Y; Alkelch A.M.; Sakamoto K.Q.; Ishizuka M. and Fujita S. (2007). Testicular toxicity of profenofos in matured male rats. Arch Toxicol.
41. Pandy, V.K.; Parmeshwaran, M. and Soman, S.D. (1983). Concentrations of morphologically normal, motile spermatozoa: Mg, Ca and Zn in the semen of infertile men, Sci Total Environ 27: 49-52.
42. Pesch, S.; Bergmann, M. and Bostedt, H. (2006). Determination of some enzymes and macro-and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology. 66: 307-313.
43. Rao, M.V. and Chinoy, N.J. (1983). Effect of estradiol benzoate on reproductive organs and fertility in the male rat. Eur. J. Obstet. Gynecol. Reprod. Biol., 15 (3) : 189-198.
44. Reuber, M.D. (1981). The role of toxicity in the carcinogenicity of endosulfan. Sci. Total. Environ., 20 : 23-47.
45. Salem, H.A.; Younis, M.; El-Tohamy, M.; El-Herrawie, M.; Soliman, M. and Khalaf, A. (1989). Thyrotrophic and thyroid hormones concentration and some testicular biochemical parameters as affected by oral administration of methamidophs (Organophosphorus) into adult male rats. J. Egypt. Vet. Med. Ass. 49: 667-675.
46. Sharma, Y.; Bashir, S.; Irshad, M.; Nag, T.C. and Dogra, T.D. (2005). Dimethoate-induced effects on antioxidant status of liver and brain of rats following subchronic exposure. Toxicology, 215 : 173-181.
47. Shivanandappa, T. and Krishnakumari, M.K. (1981). Histochemical and biochemical changes in rats fed dietary Benzene hexachloride. Indian J. Exp., 19 : 1163-1168.
48. Singh, S.K. and Pandey, R.S. (1989). Differential effects of chronic endosulfan exposure to male rats in relation to hepatic drug metabolism and androgen biotransformation. Indian J. Biochem. Biophys., 26 : 262-267.
49. Sinha, N.; Narayan, R. and Saxena, D.K. (1997). Effect of endosulfan on the testes of growing rats. Bull. Environ. Contam. Toxicol., 58 : 79-86.
50. Sinha, N.; Narayan, R.; Shanker, R. and Saxena, D.K. (1995). Endosulfan-induced biochemical changes in the testis of rats. Vet. Hum. Toxicol., 37 (6) : 547-549.
51. Snedecor, G.W. and Cochran, W.G. (1980). Statistical methods (7th ed). Lowa State University Press. Ames.
52. Stanwell-Smith, R.; Thompson, S.G.; Haines, A.P.; Ward, R.J. Cashmore, G. and Stedronska, J. (1983). A comparative study of zinc, copper, cadmium and lead levels in fertile and infertile men, Fertile Steril. 40: 670-677.
53. Sujatha, R.; Chitra, K.C.; Latchoumycandane, C. and Mathur, P.P. (2001). Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. Asian J. Androl., 3 : 135-138.
54. Sultatos, L.G.(1994). Mammalian toxicity of organophosphorus pestiside. J. Toxicol. Environ. Health 43, 271-289.
55. Takizawa, S. and Horii, I. (2002). Endocrinological assessment of toxic effects on the male reproductive system in rats treated with 5-fluorouracil for 2 or 4 weeks. J. Toxicol. Sci., 27 (1) : 49-56.

56. Turner, R.M. and McDonnell, S.M. (2003). Alkaline phosphatase in stallion semen : characterization and clinical applications. Theriogenology, 60 : 1-10.
57. Turner, R.M. and Sertich, P.L. (2001). Use of alkaline phosphatase activity as a diagnostic tool in stallions with a zoospermia and oligospermia. Anim. Reprod. Sci., 68 : 315-316.
58. Weil, C.S. (1952). Tables for convenient calculation of medium effective (LD50 or EC50) and instruction in their use. Biometrics, 8: 249-263.
59. Yousef, G.M.; Diamandis, M.; Jung, K. and Eleftherios, P. (2001). Molecular cloning of a novel human acid phoshatase gene that is highly expressed in the testes. Genomics, 74 (3) : 385-395.

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Hydrochemical Analysis of Drinking Water Quality of Alwar District, Rajasthan

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ABSTRACT: Hydrochemical study of 13 ground water samples of Alwar district was done. Alwar districts lies in the eastern plains of Rajasthan State, covers an area 8,380 km^2 and lies between 27°-57' N latitude and 76°-6'E longitude. Different sampling stations were selected for the study purpose in the district. Sample collected from the Bore-wells and hand pumps were analyzed for the various physicochemical parameters like pH, electrical conductivity (EC), sodium(Na), potassium (K), Calcium (Ca^{+2}), magnesium (Mg^{+2}), fluoride (F^-), chloride (Cl^-), sulphate (SO_4^{-2}), nitrate (NO_3^-), total dissolved solids (TDS) and total hardness (TH). The results revealed that most of the water samples were out of limit according to the WHO standards (1996). The potability of ground water is going to be deteriorate. There is a rigorous fluoride problem in various parts of Alwar region. The results revealed that the values of pH were found to be ranging between 7.18-7.86, EC ranged from 592 to 1810 μs/cm and chloride content differed from 69.98 to 299.91 mg/l and total hardness varied from 295.23 to 865.70 msg/l. The most important parameter fluoride was found to be 0.45 to 3.6 ppm, which is more than the permissible limit in most of the samples studied. The results revealed that the quality of drinking water of Alwar region is very poor, which can be used for drinking and cooking only after prior treatment. [Nature and Science. 2009;7(2):30-39]. (ISSN: 1545-0740).

Keywords: Ground water, Alwar district, Fluoride, Total dissolved solids, WHO

INTRODUCTION

One of the most important crises of the twenty-first century is the scarcity of drinking water. Most freshwater bodies the world over are becoming increasingly polluted, thus decreasing the potability of water (Dixit et al.,2005). Increasing urbanization is taking place along coastlines and estuaries and causing increased use of groundwater that will have a large impact on the quality and quantity of aquifer water (Campbell et al, 1992). Water is also one of the most convenient of the natural resources as it is capable of diversion, transport, storage, and recycling. All these properties have great utility for human beings(Kumar et al.,2005). According to National Water Policy (2002) in the planning and operation of systems, water allocation priorities should be broadly as: (i) drinking water, (ii) irrigation, (iii) hydropower, (iv) ecology, (v) agro-industries and non-agricultural industries, and (vi) navigation.

Water pollution is a serious problem in India. The contamination of groundwater due to the human activities can lead to adverse effects on human health and ecosystem(Al-Khashman, 2007). Two types of water pollutants exist; point source and non point source. Point sources of pollution occur when harmful substances are emitted directly into a body of water. A nonpoint source delivers pollutants indirectly through environmental changes. Nearly 70% of surface water resources and a large number of groundwater reserves of India are already contaminated by biological, organic and inorganic pollutants. The difference of dissolved ions concentration in groundwater are generally governed by lithology, velocity and quantity of groundwater flow, nature of geochemical reactions, solubility of salts and human activities (Bhatt et al. 1996; Karnath, 1997). Water is often is contaminated by pollutants like excess amount of fertilizers, pesticides, effluents, discharged from industries, sewage and so on. Near about 70% rivers and stream of world contain polluted water because of disposal of sewage, industrial waste, radioactive waste etc. It is reported that 15 out of 1000 children born in the developing countries die before the age of five from diarrhoea caused by drinking polluted water (UNESCAP, 2000). The evaluation of water quality in developing countries has become a critical issue in recent years, especially due to concerned that freshwater will be a scare resource in the future. Various workers in our country have carried out extensive studies on water quality. Groundwater and wastewater quality for irrigation purpose and various studies on wastewater and groundwater of Jaipur City have also been study in our laboratory(Sharma et al., 1988,1989).

At present there is no major industry in and around the study area, yet household waste water and garbage (municipal sewage) are directly discharged into water bodies. The water supply for human consumption is often directly sourced from ground water without biochemical treatment and the level of pollution has become a cause for major concern. The water used for drinking purpose should be free from toxic elements, living and non-living organisms and excessive amount of minerals that may be harmful to health. Suitable quality of groundwater become a more crucial alternative resource to meet the drastic increase in social, agricultural, and industrial development and to avoid the expected deterioration of groundwater quality due to heavy abstraction for miscellaneous uses. Hence, hydrochemical investigations are the main objectives for the groundwater quality.

The purpose of this study is to monitor the hydrochemical characteristics of groundwater in Alwar. The data will be used to characterize the groundwater. This will help water resource planning in the area and will provide a baseline for future studies of water quality and trends. Therefore, in the present study an attempt is made to evaluate the the suitability of the groundwater of Alwar city, Rajasthan for the purposes of drinking and irrigation, with reference to recommended limits set by WHO.

MATERIAL AND METHODS

Alwar district (27°-57' N latitude and 76°-6'E longitude), which is located in the eastern part of Rajasthan State, covers an area 8,380 km^2, is undergoing rapid urbanization and industrialization. Ground water sample collected from the Bore-wells and hand pumps of fifteen sampling stations were analysed during pre monsoon session (Table 1). Samples were collected in clean Teflon bottles of 1 L capacity. Analysis was done during pre monsoon session (March 2008 to June 2008). High pure quality chemicals and double distilled water was used for preparing solutions for analysis.

Physical parameters like pH, TDS and EC were measured using digital meters immediately after sampling. The concentration of major cations and anions were analysed in the laboratory using the standard methods (Grasshoff et al., 1983; APHA, 1985).

- Sodium and potassium in groundwater samples were analysed using Flame photometer.
- Calcium and magnesium were estimated by EDTA titrimetric method.

- chloride was determined by Mohr's argentometric titration using standard silver nitrate as reagent.
- Carbonate and bicarbonate concentrations of the groundwater were determined titrimetrically.
- Sulphate concentration was carried out following turbidity method using double beam UV-Visible spectrophotometer.
- Nitrate and Fluoride concentration was determined by spectrophotometer.

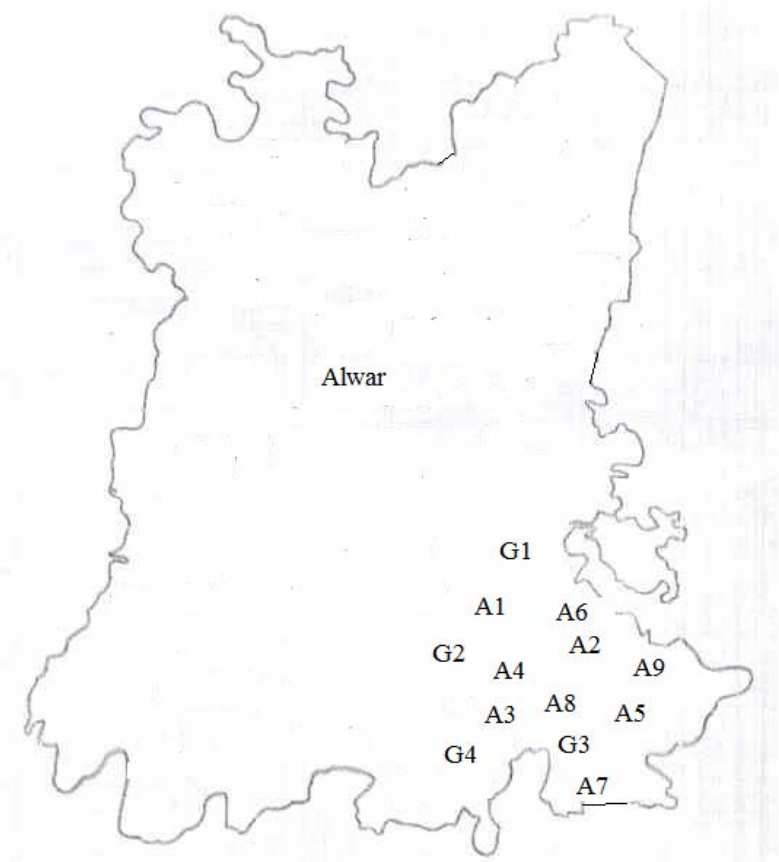

Figure 1 : A map view of study site

RESULT AND DISSCUSSION

Table 1 presents an overview Ionic Variation of groundwater in study area during pre monsoon season. The Values of TDS, TA, TH, RSC, SAR and Na% of groundwater are presented in Table 2. Permissible limit of parameters are described in Table 3. Table 4 illustrate Correlation Matrix among 13 water quality parameters of groundwater of Alwar City.

Chemistry of groundwater

In the studied localities groundwater samples were free from color and odor. The pH values of groundwater were varied from 7.18 to7.86 indicating slightly alkaline nature. The slight alkaline nature of groundwater may be due to the presence of fine aquifer sediments mixed with clay and mud. In general the pH was within the limits of the standard values (APHA 1985). For drinking water, a pH range of 6.0‑8.5 is recommended (De 2002). EC and TDS show the inorganic load in water. EC is a numerical expression of ability of an aqueous solution to carry electric current. WHO recommended permissible limit for electrical conductivity (EC) is 1400 µmhos/cm. The values of EC ranged from 592 to 1810 µmhos/cm. Minimum and maximum EC was reported from A7 and G3 water samples respectively.

Some of the water samples showed EC higher than permissible limit (Table 2). EC signifies the amount of TDS in water. The total dissolved solids (TDS) in drinking water reveal the saline behaviour of water. According to classification by (Rabinove et al., 1958), only one sample was slightly saline category (TDS value range between 1,000 and 3,000 mg l−1). TDS ranged from 360.02 to 1085.63 mg/l. Minimum (360.02 mg/l) and maximum (1085.63 mg/l) concentration of TDS was observed in A7 and G3 samples respectively (Table 1). According to WHO (2000), TDS should be 600 mg/l.

CO_3^{-2} was absent in most of the water samples. The concentration of HCO_3^- ion in groundwater of study area varied from 110.2 (A7) to 597.8 mg l^{-1} (G3) which is quite high. CO_3^{2-} and HCO_3^- together make total alkalinity. The value of Total Alkanility (as $CaCO_3$) of water samples ranges from 90.33 (A7) to 535 (G3) mg l^{-1}(Table 2). Alkalinity was higher than permissible limit i.e. (200 mg/l) in more than 50% samples (Table 2).

Calcium hardness (Ca-H) ranged from 40.08 to 194.39 mg/l. Minimum Ca-H (40.08 mg/l) was observed in A7 sample whereas maximum Ca-H (194.39 mg/l) was reported in G3 sample. Magnesium hardness (Mg-H) ranged from 34.05 to 92.42 mg/ l. Minimum (34.05 mg/l) and maximum (92.42 mg/l) values were reported A9 and G3 samples respectively. High concentration may cause laxative effect, while deficiency may cause structural and functional changes. Ca-H and Mg-H combined to form total hardness. TH varied from 295.23 to 865.70 mg/l. Minimum (295.23 mg/l) and maximum (865.70 mg/l) was reported from A9 and G3 water samples respectively (Table 2). WHO recommended safe permissible limit for hardness i.e. 500 mg/l (Table 3). Water hardness in most groundwater is naturally occurring from weathering of limestone, sedimentary rock and calcium bearing minerals. Hardness can also occur locally in groundwater from excessive application of lime to the soil in agricultural areas. Very hard groundwater results in urinary concretions, diseases of kidney or bladder or stomach disorder.

Chloride (Cl^-) varied from 69.98 to 299.91 mg/l. Minimum (69.98 mg/l) was reported in A7 and maximum (299.91 mg/l) was observed in A4 water samples (Table 1). The chloride values in the water samples due to dissolution of rocks surrounded the aquifer and probably due to leakage of sewage and anthropogenic pollution (agricultural activities). High concentration of chloride gives salty taste to water and may result in hypertension, osteoporosis, renal stones and asthma (McCarthy 2004). Sulphate concentration is varying from 22 to 137 mg/l and these values are within permissible limits prescribed by ISI, ICMR and WHO. Minimum (22 mg/l) and maximum (137 mg/l) sulphate (SO_4^{2-}) content was observed from A1 and G3 samples respectively.

Minimum (24 mg/l) and maximum (158 mg/l) Sodium (Na^+) content was observed from A8 and A9 samples respectively (Table 1). More than 50% samples contained higher

concentration of Na^+. The higher concentration of Na^+ may pose a risk to the persons suffering from cardiac, renal and circulatory diseases.The acceptable limit for Na^+ is 50 mg/l according to WHO (Table 3). Potassium (K^+) content of water samples varied from 0 to 5 mg/l. Minimum (0 mg/l) and maximum (5.0 mg/l) K^+ content were observed from A3 and G4 samples respectively (Table 1). All the water samples (100%) contained K^+ content lower than permissible limit i.e. 20 mg/l (Table 2).

Minimum (12 mg/l) and maximum (87 mg/l) nitrate (NO_3^-) content was observed from G3 and G2 samples respectively. Due to its solubility and anionic form, nitrate is very mobile and can easily leach into the water table (Fetter, 1988). The most common sources of nitrate in groundwater are atmospheric fallout, sanitation facilities, irrigational activities and domestic effluents (Ritzi *et al*., 1993). WHO recommended safe permissible limit for nitrate i.e. 50 mg/l (Table 3). Almost 50% water samples had NO_3^- concentration higher than permissible limit. Higher concentration of NO_3^- in water causes a disease called "Methaemoglobinaemia" or known as "Blue-baby Syndrome". It is particularly infant disease upto 6 months of child. The concentration of F^- in the studied water samples ranged from 0.4 to 3.6 mg/l . Minimum (0.4 mg/l) and maximum (3.6 mg/l) fluoride (F^-) content was observed from A8 and A2 samples respectively (Table 1). F− at low concentration (1 mg/l) in drinking water has been considered beneficial but high concentration may causes dental fluorosis (tooth mottling) and more seriously skeletal fluorosis (Ravindra et al., 2006).

Correlation:

Correlation is a method used to evaluate the degree of interrelation and association between two variables (Nair et al, 2005). A correlation of +1 indicates a perfect positive relationship between two variables. A correlation of –1 indicates that one variable changes inversely with relation to the other. A correlation of zero indicates that there is no relationship between the two variable. Table 4 represents the Correlation Matrix among thirteen Water Quality Parameters of Groundwater of Study Area. EC and TDS showed good positive correlation with major water quality parameters. The correlation(r = 0.9848) between these two parameters for the analyzed samples in this study showed a linear correlation. Some of the other highly significant correlation at p = < 0.001 were found between EC and Ca^{2+} (r = 0.8867) and between TDS and Ca^{2+} (r = 0.8881)also showed linear correlation. Some of the negative correlations were found between calcium and pH (r = −0.1060), between chloride and pH (r = −0.2015), between nitrate and pH (r = −0.2158), between calcium and nitrate (r = −0.1378), between carbonate and nitrate (r = −0.4872), between potassium and carbonate (r = −0.0511), between fluoride and nitrate (r = −0.0032). E.C. is solely a function of the major ion concentrations (Ca^{2+}, Mg^{2+}, Na^+, K^+, HCO_3^-, Cl^-, SO_4^{2-} and NO_3^-), the multiple regression model can be applied between EC and the ion concentrations expressed (in mg/L), as follows in equation:

$EC = -8.03 + 5.05\ Ca + 9.0\ Mg + 4.33\ Na + 2.37\ K + 0.05\ HCO_3 - 0.09\ CO_3 + 0.23\ Cl - 0.43\ SO_4 - 0.48\ NO_3$.

Table 1: Ionic Variation of Groundwater in Study Area during Pre Monsoon Season:

CODE	pH	EC	TDS	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	CO_3^{2-}	HCO_3^{-}	Cl^{-}	SO_4^{2-}	NO_3^{-}	F^{-}
A1	7.60	948	535.79	80.16	43.78	75	1.2	0	231.8	164.95	22	33	1.2
A2	7.30	975	548.98	84.17	49.86	36	2.4	0	189.10	172.45	86	24	3.6
A3	7.76	963	544.93	42.08	85.12	48	0	0	146.6	232.43	25	39	2.1
A4	7.18	1478	829.09	146.29	71.74	60.7	2.1	0	164.7	299.91	111	54	1.6
A5	7.62	1324	772.42	102.20	62.02	92	3.1	0	280.6	214.9	98	60	3.2
A6	7.40	1104	703.10	124.25	68.1	36	1	0	213.5	222.43	122	22	0.6
A7	7.74	592	360.02	40.08	49.86	36	2	0	110.2	69.98	60	47	1.1
A8	7.45	925	513.53	52.10	72.96	24	1.8	0	268.4	112.47	42	74	0.4
A9	7.53	1175	729.87	62.12	34.05	158	4.1	0	195.2	204.94	104	66	2.7
G1	7.69	1368	797.43	116.23	83.9	53	1.1	0	244.0	269.20	112	40	1.2
G2	7.58	1518	838.71	160.32	60.8	45	3.2	0	420.9	199.94	72	87	1.4
G3	7.86	1810	1085.63	194.39	92.42	81	2	18	597.8	249.92	137	12	0.8
G4	7.24	1198	734.65	136.27	42.56	63	5	0	292.80	247.42	35	59	0.45

All values are in mg/L. except pH and EC

Table (2). The Values of TDS, TA, TH, RSC, SAR and Na% in the Study Area

CODE	TDS	TA	TH	RSC	SAR	Na%
A1	535.79	190	380.31	-	1.67	30.20
A2	548.98	155	415.35	-	0.77	16.39
A3	544.93	120.16	455.34	-	0.98	18.66
A4	829.09	135	660.50	-	2.1	16.95
A5	772.42	230	510.41	-	1.77	28.56
A6	703.10	175	590.48	-	0.64	11.88
A7	360.02	90.33	305.25	-	0.9	20.95
A8	513.53	220	430.32	-	0.50	11.25
A9	729.87	160	295.23	-	4.0	54.18
G1	797.43	200	635.47	-	0.91	15.52
G2	838.71	345	650.51	-	0.77	13.56
G3	1085.63	535	865.70	-	1.20	17.12
G4	734.65	240	515.40	-	1.21	21.78

Table 3 : Standards for Drinking Water Quality

S. No.	*Parameters*	*BIS: 1999*	*ICMR: 1975*	*WHO: 2000*
1.	pH	6.5–8.5	7.0–8.5	6.5–9.5
2.	EC (μseimens/cm)	–	–	1400
3.	TDS	2000	500	600
4.	Na^+	–	–	–
5.	K^+	–	–	20
6.	Ca^{2+}	200	200	100
7.	Mg^{2+}	100	200	150
8.	Cl^-	1000	200	250
9	CO_3^{2-}	–	–	–
10	HCO_3^-	–	–	–
11	SO_4^{2-}	400	200	250
12	NO_3^-	100	50	50
13	TH	600	600	500

Note: All values except pH and EC are expressed in mg/l.

TDS = Total Dissolved Solids

EC = Electrical Conductance

TH = Total Hardness

Table 4: Correlation Matrix among thirteen Water Quality Parameters of Groundwater of Study Area :

	pH	EC	TDS	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	CO_3^{2-}	HCO_3^{-}	Cl^{-}	SO_4^{2-}	NO_3^{-}
EC	0.0572											
TDS	0.0680	0.9848										
Ca^{2+}	-0.1060	0.8867	0.8881									
Mg^{2+}	0.3986	0.4479	0.4111	0.3306								
Na^{+}	0.1300	0.3016	0.3648	0.0361	-0.3692							
K^{+}	-0.3999	0.2171	0.2542	0.2485	-0.6241	0.4669						
CO_3^{2-}	0.4654	0.5919	0.6288	0.5671	0.4883	0.1620	-0.0511					
HCO_3^{-}	0.3237	0.7634	0.7626	0.7558	0.3698	0.1154	0.2173	0.7938				
Cl^{-}	-0.2015	0.7517	0.7577	0.6462	0.3910	0.2681	0.0486	0.2158	0.2557			
SO_4^{2-}	0.0275	0.6217	0.6759	0.5602	0.3240	0.2702	0.0434	0.4475	0.3322	0.4558		
NO_3^{-}	-0.2158	-0.0128	-0.0780	-0.1378	-0.3089	0.1183	0.5103	-0.4872	-0.0645	-0.1673	-0.3057	
F^{-}	-0.0420	-0.0480	-0.0821	-0.2681	-0.2615	0.3767	0.1301	-0.2208	-0.2793	0.0406	0.1456	-0.0032

Conclusion:

Total hardness of the groundwater of the most of the study area fall in the hard category. Higher concentration of EC, TDS, Cl^{-}, F^{-} and NO_3^{-} in the study area indicates sign of deterioration, which calls for at least primary treatment of groundwater before being used for drinking. The groundwater quality improves with the increase in depth and distance of the well from the pollution source. Although, the concentrations of few contaminants do not exceed drinking water standard even then the ground water quality represent a significant threat to public health.

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References:

1. Dixit S., Gupta S. K. Tiwari S., Nutrient overloading of a freshwater lake in Bhopal, India. Electronic Green Journal 2005; 21: 2–6
2. Campbell E. and Bate, G. Groundwater in the Alexandria dune field and its potential influence on the adjacent surf-zone. Water SA 1992; 17: 155-160.
3. Kumar Rakesh, Singh R. D. and Sharma K. D. Water resources of India. CURRENT SCIENCE 2005; 89(5).
4. National Water Policy, Ministry of Water Resources, New Delhi, 2002.
5. Al-Khashman Omar Ali. Study of water quality of springs in Petra region, Jordan: A three-year follow-up. Water Resour Manage 2007; 21:1145–1163.
6. Bhatt K.B. and Salakani S. Hydrogeochemistry of the upper Ganges River. India. J. Geol Soc. India 1996; 48: 171-182.
7. Karanth K.B. Groundwater assessment, development and management. Tata McGraw-Hill Publishers, New Delhi 1997.
8. UNESCAP, "State of the Environment in Asia and the Pacific, 2000". United Nations, New York 2000.
9. Grasshoff K., Ehrhardt M. and Kremling K. Method of sea water analysis. 2nd ed. Weinheim, Deerfeild Beach, Florida Basel, Vorlagchemie 1983.
10. APHA : Standard methods for the analysis of water and waste water. 16th edn. American Public Health Association, Washington D.C. 1985.
11. De, A. K. Environmental chemistry (4th edn.). New Delhi, India: New Age International Publishers 2002 (232).
12. Rabinove C. J., Long Ford R. H., and BrookHart J. W. Saline water resource of North Dakota. U.S. Geological. Survey Water-Supply Paper 1958; 1428: 72.
13. Nair G.A., Mohamed A.I., Premkumar K. Physico - Chemical Parameters and Correlation Coefficents of Ground Waters of North–East Libya. Pollution Research 2005; 24(1):1-6.
14. Ritzi R.W., Wright S.L., Mann B. and Chen M. Analysis of temporal variability in hydro geochemical data used for multivariate analysis. Groundwater 1993; 31: 221–229
15. Ravindra K. and Garg V. K. Distribution of fluoride in groundwater and its suitability assessment for drinking purpose. Int. J. Environ. Health Res. 2006; 16: 1–4.
16. Fetter C.W. Applied Hydrogeology, 2nd edn. Merrill publishing Company, London 1988 p 592.
17. McCarthy M. F. Should we restrict chloride rather than sodium? Medical Hypotheses 2004; 63:138–148.
18. Sharma D.K., Jangir J.P., Chandel C.P.S. and Gupta C.M. Studies in quality of water in and around Jaipur: Part-1. J. Ind. Water Works Asso. 1988; 20(3): 257.
19. Sharma D.K., Sharma Alka, Jangir J.P. and Gupta C.M. Studies of trace elements in water in and around Jaipur, Ind. J. Environ. Protection 1989; 9(4): 294.

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Changes in epipedal development in soils of a gravelly hilly terrain

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Abstract: This study investigated variations in properties of surface soils of 4 physiographic positions on a hilly landscape in 2007. A transect was used to align sampling sites on the identified topographic land units of summit, midslope footslope and valley bottom. Ten soil samples (replicates) were collected from each physiographic position (treatment) and the experiment was arranged in a randomized complete block design. Soil data were subjected to analysis of variance using the PROC mix-model of SAS computer software. Results showed significant differences ($P < 0.05$) in soil properties except bulk density. There were also variations in soil morphological properties. Based on these, topsoils from summit, midslope and valley bottom were classified as ochric epipedons while those of valley bottom were categorized as plaggen epipedons. There is need to study similar landscapes in the study area for the purpose of using data for delineating soil in terms of suitability for different land uses. [Nature and Science. 2009;7(2):40-45]. (ISSN: 1545-0740).

Keywords: Edaphology, epipedon, pedogenesis, topography, tropical soils.

Introduction

Soil is a product of interactions between climate, parent material, relief and organisms over a period of time. Its formation involves complex pedogenic processes (Buol *et al*., 1997) such as additions, losses, translocations and transformations. While climate (Johnson. Maynard *et al*., 2004) and organisms (Quideau *et al*., 2001) actively influence soil formation, topography indirectly affects rate of pedogenesis and distribution of soil nutrients (Wang *et al*., 2001).

Differences in soil formation along a hillslope result in differences in soil properties (Brubaker *et al.,* 1993) which affect pattem of plant production, litter production and decomposition (Wang *et al.,* 2001). Whereas organic matter varied with landscape position (Bhatti *et al*.,1991), C and N processes were found to be influenced by the same factor (Hobbie,1996). In addition to the above edaphic properties, landscape influences soil texture, penetration resistance (Bruand *et al.,* 2004) root development (Busscher *et al*., 2001) exchangeable basic and acidic cations (Stutten *et al.,* 2004), soil exchange chemistry (Clien *et al*., 1997), and nutrient budget (Mallarino 1996) hence important in fertilizer management (Paz-gonzalez *et al*., 2000).

These changes in soil physicochemical properties affect greatly epipedal or surface horizons of soils being an interface between the earth's crest and atmosphere with direct influence of climo-and bio-sequence on it. It is in this regard that an index was developed to characterize and monitor changes in near surface soils in soil survey and land evaluation studies (Grossman *et al*., 2001; Seybold *et al.,* 2004). Field morphological evaluations of epipedons of near surface soils have not be actively applied in soil quality assessments (Grossman *et al.,* 2001). Absence of such near surface evaluations could be responsible for the spate of land degradation in the hilly landscape of the southeastern Nigeria. Most pedological studies in the study area dwell on profile pit studies including subsurface horizons (Akamigbo and Igwe, 1990; Igwe *et al.,* 2005; Onweremadu, 2008) and in lowland area of southeastern Nigeria. It becomes necessary to characterize epipedal horizons of soils of a hilly landscape for the purpose of sustained use of soil and for environmental friendliness. Based on the above, the major objective of this study was to investigate the selected physico chemical characteristics of gravelly topsoils overlying a hilly slope in southeastern Nigeria for the purpose of classification and use in soil management

Materials and Methods

Study Area

The study was carried out before the on-set of wet season in 2007 on a gravelly hilly uncultivated landscape at Okigwe, southeastern Nigeria. It is located on latitude $5^0$48'46. 970''N longitude $7^0$35'54.810"E and with an altitude of 300 m (Handheld Global Positioning System Receiver readings- Garmin Ltd Kansas, USA). Soils of the study are derived from falsebedded sandstones (Ajalli formation) of the maestrichtian geologic era and proximal to the upper coal measures (Nsukka formation) of the Danian geologic era. Okigwe has a humid tropical climate, having a mean annual rainfall of 2250 mm and a mean annual temperature range of 27-28 ^{0}C (FDALR, 1985). Orographic rainfall is common in the area occupying over 25 km^2 land area, and the windward side of hills receive more rainfall than the leeward landscape. it has a sparsely vegetated shrubby rainforest with windward portions of hills having taller and varied plant species occurring in distinct tiers. Hillside farming, stone mining, hunting, quarrying, gathering of fruits especially cashew (*Anarcadium occidentale*), nomadism and several agro-based cottage industrial ventures constitute major socio-economic activities.

Field Sampling

A windward side of the hilly landscape was used for the study. The method of Brubaker *et al*. (1993) guided field sampling of soils. In this method, categories of landscape positions were identified as upper interfluves, lower interfluves, shoulder, upper linear, lower linear and footslope. However, the study was divided into 4 landscape positions namely summit, midslope, footslope and valley bottom and these physiographic positions were connected by a transect. Soil sampling (topsoil) was done along the transect. Abney level was used in measuring slope percent while Munsell colour chart was used to determine colour of peds. Ten soil samples were collected from each sampling point giving a total of 40 soil samples from the 4 landscape positions .The 4 landscape positions constituted treatments while 10 samples were replicates and the experiment was laid out in a Randomized Complete Block Design (RCBD) in order to accommodate other sources of variation being a field study. Soil samples were air-dried, sieved using a 2-mm sieve and stored in polyethylene bags in readiness for laboratory analyses. Gravel content was estimated by weight of the total soil (50 g for each soil sample).

Laboratory Analyses

Particle size distribution dispersed in sodium hexametaphosphate, was determined by hydrometer method according to the procedure of Gee and Or (2002). Bulk density was measured by core procedure (Grossman and Reinsch, 2002). Water holding capacity was determined on undisturbed samples as the difference of water contents at – 0.03 MPa, determined by pressure plate and –1.5 MPa, determined by pressure membrane (Dane and Hopmans, 2002). Total soil carbon was estimated by combustion at 1140 ^{o}C using Leco (R-12 analyzer (Leco Corp, St. Joseph, M1). Soil pH was measured potentiometrically on a 1:2 soil/water solution (Henderson *et al.*, 1993). Cation exchange capacity was estimated by ammonium acetate at pH 7 (Soil Survey Staff, 2003). Calcium carbonate equivalent (CCE) was measured by treating soil sample (<2 mm) with HCl and evolved $C0_2$ estimated manometrically (Soil Survey Staff, 2003).

Data Analyses

Soil data were subjected to analysis of variance (ANOVA) using PROC Mix –model of SAS (Little *et al.*, 1996) and means were separated using a standard error of the difference (SED) at 5% level of probability.

Results and Discussion

Soil morphology: Morphological features of studied soils are shown in Table I, indicating thin epipedons (0 –8 cm) for soil of the summit, midslope and footslope while epipedons of the valley bottom were thick (0 – 58 cm) with few artifacts. Except in valley bottom, soils of other physiographic positions were well drained and redder. Soils of the summit and footslope were weak fine granular–structured with soils of the midslope exhibiting massive structure. Soil rupture-résistance was dominated by very friable status at all the epipedons except in those originating from valley bottom. Soils were predominantly A- (Summit and Midslope) and Ap-horizons (Footslope and Valley bottom). Variability in depth of soils could be as a result of colluviation, although in a similar landscape in Sweden, Allen (2002) attributed it to vertical schistosity. Colour changes in the study site could be due to drainage differences since soils might have originated from similar parent material (Ajalli and Nsukka formations). Mechanization difficulties may constrain the use of soil of the summit and midslope due to slope (>16%) coupled with high gravel content. Indeed, intensive cultivation of soils of the higher physiographic positions may lead to land degradation. However, adoption of conservation measures such as terraces and vegetative strips may sustain arable agriculture (11RR and ACT, 2005) .

Soil physical properties: Soil physical properties are presented in Table 2, with soils exhibiting significant ($P< 0.0001$) variations in particle size distribution and moisture content while bulk density showed non-significant differences among physiographic land units. Sandiness decreased downslope while the other particle sizes increased in the same direction. This could be due to larger size of sand and its decreased transportability while silt and clay sizes are smaller and lighter hence easily moved in suspension towards the valley bottom. Silt – clay ratio, which is an index of age of soil, decreased downlope, indicating that soils of the summit are younger due to instability caused by erosion and colluviation unlike epipedons of lower physiographic positions. Despite non-significant variability in bulk density, the attrribule was found to be higher in valley bottom possibly due to seasonal flooding of soils. Continued wetting and drying of soils decreases aggregate stability (Caron *et al.,* 1992) , leading to collapse of soil pores and production of finer particles and macro-aggregates (Levy and Miller, 1997), implying increased bulk density and decreased macro-porosity .In a similar study on a fragipan, Scalenghe *et al.(*2004) reported higher density on wetted soil when compared with dry soil.

Water holding capacity increased towards the valley bottom physiographic position, and this could be attributed to higher values of clay downslope .Similar findings were reported by Ezeaku and Anikwe (2006)in soils of southeastern Nigeria. In addition to clay content, organic matter distribution contributed significantly ($P\leq 0.05$) to differences in soil moisture content (Table 3.) in line with the findings of Dekker *et al.* (1999) in topsoils in Netherlands, France, Sweden and Germany. However, soil organic matter interacts with other soil properties to influence water behaviour in soils (Ellerbrock *et al*., 2005; Eynard *et al.,* 2006).

Other soil chemical properties of epipedons in the study site are shown in Table 3 and they varied significantly ($P\leq 0.05$) along physiographic positions. Soil pH decreased towards the summit, suggesting possible loss of basic cations which finally accumulate at the valley bottom. Differences in soil pH were very significant among physiographic positions, and this could be used in delineating the hilly landscape into different arable land use types since soil pH governs the distribution crop nutrients in soil. Although soils were generally acidic, topography may have contributed to local differences in its distribution in the studied soils. Results of soil pH were higher (pH water = 5.27-7.6) and contrasted with findings of Onweremadu (2007) in a similar study (pH water =4.0-4.7 in the same agroecology. This variation could be due to land use and topography. Calcium carbonate equivalent (CCE) values were low in all physiographic units particularly in epipedons from the summit. This could be as a result of combined effect of leaching and runoff losses. The study site is within the northernmost part of the rainforest agroecology of southeastern Nigeria while higher values of CCE are expected in drier ecological zones of Nigeria.

Presley *et al.* (2004) reported higher values of CCE in semi-arid soils of Kansas in USA, and these values increased with depth of soils. However, this study did not investigate sub-horizons of studied soils.

Classification of epipedons

The soils of summit, midslope and footslope were thin (0-3,0-5, and 0-8 cm respectively) with Munsll colour values greater than 4 (moist) and chroma of 4. In addition to these, soils of summit, midslope and footshop had CCE of 10, 12 and 14 g kg, respectively (Table 3), suggesting the classification of these topsoils as ochric epipedones. Soils of the valley bottom contained artifacts with Ap horizon of 0- 58 cm thick possibly due to cultivation and prominent marks of farm tools unlike the other soils , hence classified as plaggen epipedons.

Table 1. Selected Soil Morphological Properties

Physiography	Horizon	% slope	Depth (cm)	Colour (moist)	Structure	Artifacts	Consistency (moist)	Gravel (32-2 mm)	Drainage
Summit	A	21	0-3	LRB 5YRS 6/4	1fgr	Nil	Vfr	46	WD
Midslope	A	16	0-5	LRB 5YR 6/4	0 ma	Nil	Vfr	43	WD
Footslope	Ap	8	0-8	RB 5YR5/4	2 fgr	Nil	Vfr	32	WD
Valley bottom	Ap	0-2	0-58	DG 5YR 4/1	2 m abk	Few	fi	26	PD

LRB = light reddish brown, RB = Reddish brown, DG = Dark gray
O = Structure less, 1 = weak, 2 = moderate, f = fine m = medium Ma = massive base = angular blocky, Vfr = very friable, WD= well drained, PD = poorly drained, fi = friable

Table 2. Soil Physical Properties

Physiographic horizon Depth	Sand g kg^{-1}	Silt g kg^{-1}	Clay g kg^{-1}	SCR	BD M gm^{-3}	WHC g kg^{-1}
Summit	850	90	60	1.5	1.41	24
Midslope	845	70	85	0.7	1.37	28
Footslope	800	90	110	0.8	1.32	36
Valley bottom	780	95	126	0.7	1.43	44
SEDp=0.05	1.38	1.95	5.06	0.05	0.02	1.22
P = values	<0.0001	<0.0001	<0.0001	0.0001	NS	<0.0001

SCR = silt – clay ratio, TC = textural class WHC = water holding capacity.

Table 3. Selected soil chemical properties

Physiography	Horizon	Depth cm	pH water	CEC cmo1kg^{-1}	CCE g kg^{-1}	OC g kg^{-1}
Summit	A	0-3	5.2	5.6	10	11.6
Midslope	A	0-5	5.4	7.8	12	14.2
Footslope	Ap	0-8	5.5	7.9	14	34.4
Valley Bottom	Ap	0-58	6.1	9.8	24	39.8
SEDp= 0.05			0.09	1.56	0.08	0.07
P-values			<0.001	<0.001	<0.001	<0.0001

CEC = cation exchange capacity, CCE = calcium carbonate equivalent, OC=organic carbon.

Table 4. Classification of studies epipedons

Physiography	Horizon	Depth (cm)	Epipedon
Summit	A	0-3	Ochric
Midslope	Ap	0-5	Ochric
Footslope	Ap	0-8	Ochric
Valley Bottom	Ap	0-58	Plaggen

References

Akamigbo F.O.R and C.A Igwe. 1990 Morphology, geography, genesis and taxonomy of three soils series in eastern Nigeria, *Samaru J. Agric Res*., 7:33-48.

Allen, C.E. 2002. The influence of schistocity on soil weathering on large boulder tops, Karkevageje, Sweden.*Catena*, 49:157-169.

Bhatti, A.U., D.J. Mulla and B.E. Frasier .1991. Estimation of soil properties and wheat yields in a complex eroded hills using geostatistics and thematic mapper images. *Remote Sens Environ.*, 37:181-191.

Bruand, A., C. Hartmann, S. Ratana-Anupap, P. Sindtusen, R, Poss and M hardy, 2004 .Composition, fabric and porosity of an Arenic Haplustalf of Northeast Thailand: Relation to penetration resistance. *Soil Sci. Soc. Am. J.*, 185-193.

Brubaker. S.C., A. J Jones, D.T. Lewis and K Frank 1993. Soil properties associated with landscape position. *Soil Sci Soc. Am. J*. 57:235-239.

Buol S.W., F.D. Hole, R.J. McCracken and R.J. Southard. 1997. *Soil genesis and classification*. 4th edition lowa State University Press, Ames.

Busscher, W.J. J.R. Federick and P.J. Bauer. 2001. Effect of penetration resistance and timing of rain on grain yield of a narrow–row corn on a coastal plain loamy sand. *Soil Till. Res*., 63:15-24.

Caron, J. B.O. Kay and J.A. Stone. 1992. Improvement of structural stability of a clay loam with drying .*Soil Sci. Soc Am. J*., 56:1583-1590.

Chien, Y.J., D.Y. Lee H.Y. Guo and K.H. Honng. 1997. Geoststisical analysis of soil properties of mid –west Taiwan soils. *Soil Sci.*, 162:291-298.

Dane, J.H. and J.W. Hopmans 2002. Water retention and storage: Laboratory methods. In: J.H. Dane and G.C. Topp (eds.) .*Methods of soil analysis.* Part 4 Physical methods. Soil Sci. Soc. Am. Book series No 5, ASA and SSSA, Madison WI. Pp. 675-720.

Dekker, L. W., C.J. Ritsema, O. Wendroth, N. Jarvis, K. Ostindie W. Pohi, M. Larsson and J.P. Gaudet 1999. Mositure distributions and wetting rates of soils os experimental fields in Netherlands, France, Sweden and Germany. *J. Hydrol.*, 215:4-22

Ellerbrock, R.H., H.H. Gerke, J. Bachimann and M.O. Goebel. 2005. Compsition of organic matter fractions for explaining wettability of three forest soil. Soil Sci: Soc Am. J., 64: 57-66.

Eynard, A., T.E. Schumacher, R.A. Kohl and D.D. Malo 2006, Soil wettability relationships with soil organic C and aggregate stability. 12th World congress of soil Science, Philadephia, Pennyslavia, USA 9-15 July 2006.

Gee, G.W, and D. Or. 2002. Particle size analysis. In: J.H. Dane and G.C. Topp (eds.) *Methods of soil analysis* Part 4, Physical methods. Soil Sci. Soc. Am. Book Series No. 5, ASA and SSSA, Madison WI pp. 255-293.

Grossman, R.B. and T.G. Reinsch. 2002 Bulk density and linear extensibility. In: J.H. Dane and G.C. Topp (eds) *Methods of soil analysis*. Part 4, Physical methods. Soil Sci. Soc Am. Book series No5, ASA and SSSA Madison WI. Pp. 201-228

Grossman, R.B., D.S. Harms, C.A. Seybold and J.E. Herrick. 2001. Coupling use-dependent and use-invariant data for soil quality evaluation in the United States. *J. Soil Water Conserve.*, 56:63-68.

Hendershot, W.H., H. Lalande and M. Duquette. 1993. Soils reaction and exchangeable acidity. In: M.R. (carter (ed). *Soil sampling and methods of analysis* Can Soc. Soil Sci, Lewis Publishers, London. pp 141-145

Hobbie, S.E. 1996 Temperature and plant species control over litter decomposition in Alaskan tundra. *Eco. Monogr*., 66:503-522.

Igwe, C.A., M. Zare, and K. Stahr, 2005. Mineral and elemental distribution in soils formed on the River Nigeria floodplain, eastern Nigeria. *Austr. J. Soil Res*., 43:147-158.

IIRR and AC (International Institute of Rural Reconstruction) and (African Conservation Tillage). 2005. *Conservation agriculture: A manual for farmer and extension workers in Africa.* Majestic printing Work Ltd, Narrobi Kenya pp 251.

Johnson-Maynard J.L., P.J. Shouse, R.C Graham, P. Castiloglione and S.A. Quideau. 2004. Microchimate and pedogenic implications in a 50-year old chaparral and Pine biosequence. *Soil Sci. Soc. Am. J.* 68: 876-884.

Lamotte, M., A Bruand, F.X. Humbel, A.J. Herbillon and M. Revi. 1997. A hard sandy-loam soil from Northern Cameroon. I. Fabric of the groundmass. Eur J. Soil Sci., 48:2130225.

Levy, G.I. and W.P. Miller. 1997. Aggregate stability of some southern Us soils. *Soil Sci Soc Am. J.*, 61: 1176-1182.

Mallarino, A.P. 1996. Sparial patterns of phosphorus and potassium in non-tilled soils for two sampling scales. Soil Sci Soc. Am. J., 60:473-1481.

Onweremadu, E.U. 2007. Soil carbon distribution in a hilly landscape. *Int J. Agric. Res*, 2 (4): 377-384.

Onweremadu, E.U. 2008. Physico-chemical characterization of a farmland affected by wastewater in relation to heavy metals. *J. Zhejiang University Sci.*, 9:366-372..

Paz-Gonzalez, A. S.R. Vierira and M.T Taboada-Castro. 2000. The effect of cultivation on the spatial variability of selected properties of an umbric horizon. *Geoderma*, 97:273-292.

Presley, D.R., M.D. Ransom, G.J. Kluitenberg and P. R. Finnell 2004. Effects of thirty years of irrigation on the genies and morphology of two semi-and soils of Kansas. *Soil Sci Soc. Am J*., 68:1916-1926.

Quideau, S.A., O.A. Chadwick, R.C. Graham, S.E. Trumbore, J.L. Johnson-Maynard R.C. Graham and M.A. Anderson. 2001 Vegetation control on soil organic matter dynamics.*Org, Geochem*., 32:247-252.

Scalenge, R. G. Certini, G. Corti, E. Zanini and F.C. Ugolini, 2004 Segregated ice and liquefaction effects on compaction of fragipans. *Soil Sci. Soc Am. J.*, 68:204-214.

Seybold C.A., R.B. Grossman, H. Hoper, G. Muckel and D.L. Karlen 2004 Soil quality morphological index measured in the 1996 NRi pilot study. *Soil Surv. Hor.*, 45(3):86-95.

Soil Survey Staff 2003. *Keys to soil taxonomy*, Ninth Edition USDA/NRCS, Was hington D.C. pp. 332

Stutter, M.I. L.K. Deeks and M.F. Billett, 2004. Spatial variability in soil ion exchange chemistry in agranitic upland catchment. *Soil Sci Soc. Am J.*, 68:1304-1314.

Wang, J. B.Fu, Qi and L. Chen. 2001. Soil nutrients in relation to land use and landscape position in the semi-and small catchment on a loess plateau China.

Tilapia Heat Shock Protein: Molecular Cloning and Characterization

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Abstract

A new member of heat-shock protein family (HSP 9) identified in Tilapia *Oreochromis niloticus* immunized by killed *Flavobacterium columnarae*. Suppressive subtractive hybridization (SSH) was utilized to construct a cDNA library and a semi-quantitative RT-PCR analysis used to examine HSP9 immune gene regulation. *O. niloticus* heat shock protein (ONHSP9) cDNA composed of 1228 bps with a 1167 bps open reading frame, the predicted gene product is 389 amino acid with molecular weight of 42.1 kDa, it shares 92% similarity with that of *Danio rerio*. Compared to ß-actin, the semi-quantitative RT-PCR revealed that ONHSP9 expressed in tissues of stimulated fish as up-regulated gene suggesting that this member of heat-shock genes is probably involved in the general immune response against the pathogenic bacteria. [Nature and Science. 2009;7(2):46-57]. (ISSN: 1545-0740).

Keywords: Tilapia, cDNA library, Immune genes, Heat-shock protein.

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Introduction

Heat shock proteins (HSPs) are widely distributed in nature, and are highly conserved proteins among prokaryotes and eukaryotes (Lindquist, *et al*., 1986). They are known to be involved in immune responses to many bacterial and parasitic pathogens (Kaufmann *et al*., 1990, 1994), and in the pathogenesis of some types of autoimmune diseases (Mo¨ller, 1991). HSPs have long been suspected to act as danger signals (Young *et al*., 1990 and Van Eden *et al*., 1988). Initially described as a family of intracellular proteins essential for various vital cell functions (Raulet, 1989 and Ohga *et al*, 1990), HSPs have later been demonstrated to stimulate potently immune responses. HSPs are attractive as danger signals because they are evolutionarily highly conserved, constitutively present in virtually all body cells and upregulated and released in response to cellular stress. In infections, this release could support induction of immunity. HSPs release, however, is not specific to infections but also occurs in response to physical stimuli, such as heat or irradiation, and in noninfectious inflammatory diseases (Ohga *et al*, 1990); so, members of the HSPs family are candidate molecules that potentially signal tissue damage or cellular stress to the immune system. The expression of HSPs is up-regulated rapidly during several forms of cellular stress and HSPs can be released from damaged tissue. Thus, in recent years, substantial interest has focused on the interplay of HSPs with the immune system (Wallin, *et al*., 2002).

Bacterial infection induces changes in the expression of host cell genes. A global knowledge of these modifications should help to better understand the bacteria / host cell interactions. The host response to bacterial stimulation represents a complex coordination of gene products, which are precisely turned to activate or inactivate specific pathways and finally counteract the effects of the bacterial antigen. *Flavobacterium columnare* has been recognized as a worldwide pathogen of freshwater fish, it is the etiological agent of columnaris disease, characterized by gill necrosis, greyish white spots on the body, skin erosion, and fin rot (Annemie ***et al.***, 1997).

With the use of large-scale screening of mRNA changes, it is possible to define changes in gene expression that underlie the host response to bacterial pathogens and to gain specific insights into the molecular nature of the host pathways that govern bacterial pathogenesis. Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Tsoi *et al*., 2004, Chang *et al*., 2005). Numerous cDNA subtraction methods have been reported in general, they involve hybridization of cDNA from one population (tester) to excess of cDNA from other population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences. SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non target DNA amplification. In this regard, to obtain a more comprehensive view of the tilapia response and to identify the immune genes and their expression in response to *Flavobacterium columnarae*, a model of stimulation, we used the subtractive suppressive hybridization and the semiquantitative RT-PCR methods.

Materials and methods

Tilapia *Oreochromis niloticus* were obtained in September 2007 in Wuhan, Hubei province, China. The fish were acclimatized in an aerated freshwater tank at room temperature under natural photoperiod for a week. They were stimulated for four days each with intraperitoneal injection of 10^7 *Flavobacterium columnari*, killed using 0.5% formaldehyde in PBS. A healthy fish was injected with PBS and used as a control. Two stimulated tilapia, 24.5 cm and 27 cm in fork length were selected with the gills, liver; spleen, head kidney and intestine were dissected out for the construction of the infected group subtracted library. One healthy fish 23.7 cm in fork length was used also for the subtracted library with the mentioned organs dissected out. All samples were stored in -70°C for further use

Total RNA and poly A^+ RNA isolation

Total RNA was prepared for RT-PCR detection using Trizol Reagent (Invetrogen, USA). About 1.5 g of mixed tissues containing approximately equal amount of different formally mentioned tissues from both infected and uninfected fish were used as tester and driver samples, respectively. The mRNA was then isolated from total RNA using the poly Atract Isolation System (Promega). Concentration of mRNA was determined using a spectrophotometer.

Driver and tester Preparation (SMART cDNA synthesis)

Driver cDNA was synthesized from 2 μg. each of different tilapia poly (A) RNA using the Great Lengths manufacture's instructions. First- and second-strand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the manufacturer's protocol. The resulting cDNA pellet was dissolved in 50 μl of deionized water and digested by *Rsa*I in a 50 μl reaction mixture containing 15 units of the enzyme for 6 h. at 37°C. The cDNAs were then extracted, precipitated, by column using the DNA binding and washing buffers, and resuspended in 5.5 μl of deionized water.

Tester was Prepared as described above for the driver. Digested tester cDNA (1 μl) was diluted in 5 μl of H2O. The diluted tester cDNA (2 μl) was then ligated to 2 ml of adapter 1 and adapter 2 (10 mM) and 6ml ligation mixture (2μl 5x ligation buffer, 1μl T4 enzyme ligase, 3μl H2o) in separate ligation reactions in a total volume of 10 μl at 16°C overnight, using 0.5 units of T4 DNA ligase (Life Technologies) in the buffer supplied from the manufacturer. After ligation, 1 μl of 0.2 M EDTA was added and the samples were heated at 70°C for 5 min to inactivate the ligase and stored at 20°C.

Analysis of ligation:

Ligation efficiency was examined using beta-actin forward primer (1μl) and reverse (1μl) for tester 1 (ligated adaptor 1) and tester 2 (ligated adaptor 2) in a PCR mix. Of 22 μl. to verify that a proper quantity of the cDNAs has adaptors on the both ends.

Suppression Subtractive Hybridization and PCR Amplification

1.5 microliters of Rsa 1 digested driver dscDNA was added to each of two tubes containing 2μl of adaptor 1- and adapter 2-ligated tester cDNA. The samples were mixed and resuspended in 1.0 μl of 4X hybridization buffer (CLONTECH). The solution was overlaid with mineral oil, the DNAs were denatured (1.5 min, 98°C), and then allowed to anneal for 10 h at 68°C. After this first hybridization, the two samples were combined in a second hybridization and a fresh portion of heat-denatured driver (1μl) in 1.0 ml of hybridization buffer in a total volume of 4 μl was added. The sample was allowed to hybridize overnight at 68°C. The final hybridization was then diluted in 200 ml of dilution buffer (CLONTECH).

For each subtraction, we performed two PCR amplifications. The primary PCR was conducted in 25 ml. It contained 1 μl of diluted, subtracted cDNA, 1 μl of PCR primer P1 (10 μ M), and 24 ml of PCR master mixture prepared using the PCR-select ^{TM} cDNA subtraction kit (CLONTECH). PCR was performed with the following parameters: 94°C for 5min; 30 cycles

at (94°C for 30 sec; 66°C for 30 sec; 72°C for 1.5 min); and a final extension at 72°C for 10min. The amplified products were diluted 9-fold in deionized water. Some of the product (1 μl) was then used as a template in secondary PCR using the nested PCR primer PN1 and PN2 and performed with the following conditions, 94°C for 5min; 13 cycles at (94°C for 30 sec; 68°C for 30 sec; 72°C for 1.5 min); and a final extension at 72°C .

PCR analysis of subtraction efficiency

Beta-actin was used as a positive control to confirm the reduced relative abundance of SSH libraries following the PCR selection procedure. The beta-actin gene primers were designed according to the conseved domains in Tilapia nilotica, forward: CGAGGGTTATGCCTTGCC and reverse: TGTAGGTGGTTTCGTGGATT. PCR condition was 94°C for 5min; 35 cycles at (94°C for 30 sec; 56°C for 30 sec; 72°C for 40 sec); and a final extension at 72°C . 5 μl PCR product from each reaction for 15, 20, 25, 30, and 35 cycles were represented on 2% agarose/EtBr gel.

Cloning and dot blot hybridization:

Products from the secondary PCRs were inserted directly into pGEM-T using a pGEM-T Easy Vector System (Promega), which was then transformed into Escherichia coli and screened by the the colonieswhich were selected and amplified with nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested PCR primer 2 (5'- AGGGTGGTCGCGGCCGAGGT-3'). 3 μl of PCR product were denatured in 3 ml of 0.5 M NaOH. Two identical H-bond N^+ nylon membranes were prepared by loading 1 μl of denatured PCR product of each clone on the same location, after 5 min. neutralization in 0.5 M Tris-Hcl (pH7.5), the membranes were baked for 30 min at 120 °C to cross link the cDNAs.

Forward-subtracted cDNAs were digested with Rsa1 and labeled as probes with digoxigenin using a DIG High Prime system (Boehringer Mannheim) by following the manufacture's instruction. Positive clones were sequenced using the dedoxy chain sequencer (ABI Applied Biosystems Model 337).

Sequence homology, alignment and phylogenetic analysis

The sequences were compared with the sequences in the database using the BLASTX program at the web server of the National biotechnology information. Protein prediction was performed using software at the ExPASY Molecular Biology Sever (http://expasy.pku.edu.cn) and

SAPS program (Statistical Analysis of Protein Sequences). The open reading frame of *Oreochromis niloticus* HSP9 was searched using the NCBI server ORF finder. Sequences were aligned, employing the distance matrix; a neighbor-joining tree was constructed using Clustal W (version 1.83).

Tissue specific expression of HSP9 by RT-PCR

Total RNA from different mixed tissues was treated with DNase, 2 μg RNA was reverse transcribed with M-MLV reverse transcriptase using hexanucleotides (Promega) to prim the reaction**.** The first strand cDNA was used as templates for RT-PCR with a pair of HSP specific primers designed forward 5'- ACAGTGCGGCGGATAAGG-3' and
reverse: 5'ACGGCTGAACAAGACCAGAATA3' .
The PCR cycling parameters were one cycle of 94 ° C for 5 min, 35 cycles of 94° C for 30 s, 58° C for 30 s and 72 ° C for 1 min, with a final extenssion step of 72° C for 10 min. The RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel with PCR products derived from beta actin of the infected and non infected tilapia as controls.

Results

The electrophoresis analysis of ligation efficiency showed that tester and β-actin are about the same intensity (Fig. 1)

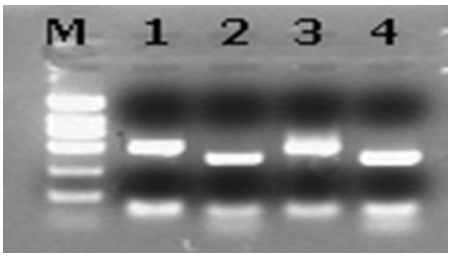

Fig.(1): ligation analysis efficiency (Lane 1, 3: Tester; lane 2, 4 :β-actin and M: marker)

Subtracted cDNA library

Subtracted cDNA library specific for immunized tilapia fish were evaluated by PCR analysis using β actin gene after the subtractive hybridization was performed (Fig.2). The subtraction efficiency was noticed as shown in fig. 2. The β actin product could be observed at 15 cycles for the unsubtracted cDNAs, while the amplified product was seen at 30 cycles in the subtracted cDNAs.

The abundance of β actin was theoretically calculated as 2^{15} between the unsubtracted and subtracted cDNAs, this indicates the enrichment of cdNAs specific for tilapia immunized by *F. columnarae* about 2^{15} times by SSH.

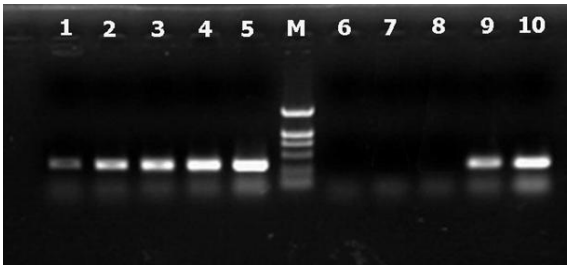

Fig.(2): Reduction of β actin abundance by SSH, PCR analysis was performed on secondary PCR product, 15 cycles(Lane 1,6); 20 cycles (lane 2,7); 25 cycles (Lane 3,8); 30 cycles(Lane 4,9); 35 cycles (Lane 5,10); M=DNA marker.

Isolation of differentially expressed clones

The comparison of pooled RNA samples from *Oreochromis niloticus* immunized by *Flavobacterium columnarae* against unimmunized healthy *Oreochromis niloticus* yielded multiple differentially expressed clones as revealed by dot blot analysis (Fig.3).

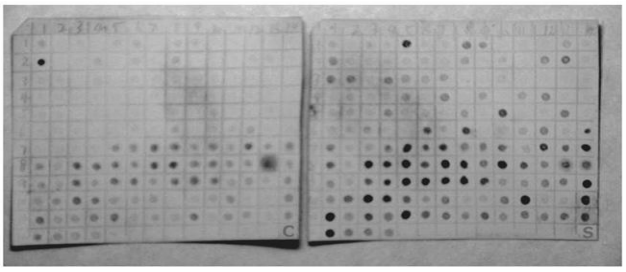

Fig.3: Screening of colonies with differential cDNA fragments from subtractive cDNA library by dot blot analysis. Two identical nylon membranes in which each colony was dotted in the same place were hybridized with subtractive cDNA library probd(s) and control cDNA library probs(c), respectively.

Identification of cDNA sequence of ONHSP9

Blast queries of the sequenced nucleotides showed that some of the sequenced genes were immune- related genes, HSP gene was identified as acute- phase reactant. The deduced amino acid sequence of ONHSP9 (Fig.4) with the genebank accession no. GH159106 contains 1228 nucleotide bps with 1020 bps open reading frame encoding a protein of 389 aa and molecular weight 41.2 kda. A signal peptide was predicted using Signal P 3.0, the signal peptide probability = 0.026, signal anchor probability = 0.126 and the Max cleavage site probability: 0.017 between residues 44 and 45.

```
  1 SVVAAEVLLS ILDHVLNVIF TEASLDHNLP LAACAFVLGR HMDDAVGVDV ECDLDLRDSA
 61 RGWRDSYQSK LTQQLVVCCH LSLTLAHFDL HLSLSISCCG EHLALLGGNC GVPVDELGKG
121 TTQSLDTQRQ WSHIQKQHIG YIASQNTTLD GCSNSDSFIR VHRLAGGSAK QILDCLLNLG
181 HACHASHQHL SDVSLGHFSI LHGLLARSHS AADKVSHNTF KLSTGLHVKM FGTGGVHSQV
241 GEVDVSLQRG QLTLCLLSSL SDSLKSHVVL HHVYTRLSLE LLDNVSERCS KSSPPGSVTI
301 GGLHLKTPFC ISRMRCQTYL HLDHIQHYLI LVLFSRRPAT TVSLSTQSSN STAGGARNSR
361 IPVRLESIMA YGSVKLRQSN QGAVNLGSL
```

Fig.4: Deduced amino acid sequences of ONHSP9.

Alignment analysis and phylogenetic tree construction

A Basic Genebee ClustalW 1.83 was performed on a variety of HSP genes from *Homo sapiens, Pongo abilii, Bos Taurus,* Equus caballus and *Danio rerio*, GeneBank accession nos. AAH00478, NP_001126860, NP_001029696, XP_001502580, AAH44175 and GH159106 (Fig. 5). A phylogenetic tree was constructed based on six aligned amino acids sequences using Clustal W program (Fig. 6).

```
Homo            MISASRAAAAR---LVGAAASRGPTAARHQDSWNGLSHEAFRLVSRRDYASEAIKGAVVG
Pongo           MISASRAVAAR---LVGAAASRGPTAARYQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Bos             MISASRAAVSR---FVGTAASRGPTAARHQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Equus           MISVSRAAAAR---LVGAAASRGPTAARHKDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Danio           MLSVSRTARLVRNVSCSQKTSSGVSDLIKKACLNGWTQKTLQTAARRHYASEAIRGAVIG
Oreochromis     ------------------------------------------------------------
Homo            IDLGTTNSCVAVMEGKRAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Pongoab         IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Bos             IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Equus           IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Danio           IDLGTTNSCVAVMDGKNAKVLENAEGARTTPSVVAFTSDGERLVGMPAKRQAVTNPNNTL
Oreochromis     -----SVVAAEVLLSILDHVLN----------VIFT------------EASLDHNLPL
                     :  .. *: .   :**:          * **             :*  : * .:
Homo            YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Pongo           YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Bos             YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Equus           YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
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Danio                YATKRLIGRRFDDAEVQKDLKNVPYKIVRASNGDAWLEVHGKMYSPSQAGAFILIKMKET
Oreochromis          AACAFVLGRHMDDAVG----VDVECDLDLRDSARGWRDSY--------------------
                      *   ::**: **.       :*  .:   ... .* : :

Homo                 AENYLGRTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Pongo                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Bos                  AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Equus                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Danio                AESYLGQSVKNAVVTVPAYFNDSQRQATKDAGQIAGLNVLRVINEPTAAALAYGLDKTQD
Oreochromis          -----------------------------QSKLTQQLVVCCHLSLTLAHFDLHLSLSIS
                                                   .:::   *:    . * * :   *. : .

Homo                 KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Pongo                KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Bos                  KIIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Equus                KIIAVYDLGG-TFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Danio                KIIAVYDLGGGTFDISVLEIQKGVFEVKSTNGDTFLGGEGFDQHLLRHIVKEFKKESGVD
Oreochromis          CCGEHLALLGGNCGVPVDELGKGTTQSLDT-------QRQWSHIQKQHIGYIASQNTTLD
                           * * . .:.: *: **. :  .*         . :.:   :**    .::: :*

Homo                 LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLTRAQFEGIVTD
Pongo                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLSRAQFEGIVTD
Bos                  LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVTD
Equus                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVAD
Danio                LMKDNMALQRVREAAEKAKCELSSSLQTDINLPYLTMDASGPKHLNMKLTRSQFEGIVAD
Oreochromis          GCSNSDSFIRVHRLAGGSAKQILDCLLN--------------------------------
                       .:. :: **:. *  :  :: ..: .

Homo                 LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Pongo                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Bos                  LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Equus                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Danio                LIRRTVAPCQKAMQDAEVSKSDIGEVLLVGGMTRMPKVQQTVQDLFGRAPSKSVNPDEAV
Oreochromis          -LGHACHASHQHLSDVSLGHFSILHGLLARSHSAADKVSHNTFKLSTGLHVKMFGTGGVH
                      : ::  ..:: :.*..:.: .* . :*. . :   **.:.. .*      * .... .

Homo                 AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Pongo                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Bos                  AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Equus                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Danio                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Oreochromis          SQVGEVDVSLQRGQLTLCLLSSLSDSLKSHVVLHHVYTRLS--------------------
                           *  *  **           *  * * *
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Homo                 MISASRAAAAR---LVGAAASRGPTAARHQDSWNGLSHEAFRLVSRRDYASEAIKGAVVG
Pongo                MISASRAVAAR---LVGAAASRGPTAARYQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Bos                  MISASRAAVSR---FVGTAASRGPTAARHQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Equus                MISVSRAAAAR---LVGAAASRGPTAARHKDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Danio                MLSVSRTARLVRNVSCSQKTSSGVSDLIKKACLNGWTQKTLQTAARRHYASEAIRGAVIG
Oreochromis          ------------------------------------------------------------
Homo                 IDLGTTNSCVAVMEGKRAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Pongo                IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Bos                  IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Equus                IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Danio                IDLGTTNSCVAVMDGKNAKVLENAEGARTTPSVVAFTSDGERLVGMPAKRQAVTNPNNTL
Oreochromis          -----SVVAAEVLLSILDHVLN----------VIFT------------EASLDHNLPL
                          :  .. *: .   :**:           * **                :*  : * .:

Homosapiens          YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Pongoabelii          YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Bostaurus            YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Equuscaballus        YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Daniorerio           YATKRLIGRRFDDAEVQKDLKNVPYKIVRASNGDAWLEVHGKMYSPSQAGAFILIKMKET
Oreochromisniloticus AACAFVLGRHMDDAVG----VDVECDLDLRDSARGWRDSY--------------------
                       *   ::**: **.       :*  .:   ... .* : :

Homo                 AENYLGRTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Pongo                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Bos                  AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Equus                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Danio                AESYLGQSVKNAVVTVPAYFNDSQRQATKDAGQIAGLNVLRVINEPTAAALAYGLDKTQD
Oreochromis          -----------------------------QSKLTQQLVVCCHLSLTLAHFDLHLSLSIS
                                                   .:::   *:    . * * :   *. : .

Homo                 KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Pongo                KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Bos                  KIIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Equus                KIIAVYDLGG-TFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Danio                KIIAVYDLGGGTFDISVLEIQKGVFEVKSTNGDTFLGGEGFDQHLLRHIVKEFKKESGVD
Oreochromis          CCGEHLALLGGNCGVPVDELGKGTTQSLDT-------QRQWSHIQKQHIGYIASQNTTLD
                           * * . .:.: *: **. :  .*         . :.:   :**    .::: :*

Homo                 LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLTRAQFEGIVTD
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Pongo                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLSRAQFEGIVTD
Bostaurus            LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVTD
Equus                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVAD
Danio                LMKDNMALQRVREAAEKAKCELSSSLQTDINLPYLTMDASGPKHLNMKLTRSQFEGIVAD
Oreochromis          GCSNSDSFIRVHRLAGGSAKQILDCLLN--------------------------------
                        .:. :: **:. *  :  :: ..: .
Homo                 LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Pongo                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Bos                  LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Equus                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Danio                LIRRTVAPCQKAMQDAEVSKSDIGEVLLVGGMTRMPKVQQTVQDLFGRAPSKSVNPDEAV
Oreochromis          -LGHACHASHQHLSDVSLGHFSILHGLLARSHSAADKVSHNTFKLSTGLHVKMFGTGGVH
                      : ::  ..:: :.*..:.: .* . :*. . :   **.:.. .*      * .... .
Homo                 AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Pongo                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Bos                  AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Equus                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Danio                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Oreochromis          SQVGEVDVSLQRGQLTLCLLSLSDSLKSHVVSLHHVYTRLS----------------------
                     :  . :: .:  *::*  ** .::    .  .*  *:*:*
Homo                 GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Pongo                GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Bos                  GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Equus                GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Danio                GQTQVEIKVCQGEREMATDNKVLGQFTLVGIPP-APRGV-QIEVTFDIDANGIVHVSAKD
Oreochromis          ------LELLDNVSERCSKSSPPGSVTIGGLHLKTPFCISRMRCQTYLHLDHIQHY------
                           ::: :.  * . ...  *..*: *:   :*  : ::.    :. : * *
Homo                 KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Pongo                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Bos                  KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Equus                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Danio                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRRKDRVEAVNMAEGIVHDTESKM
Oreochromis          ------LILVLFSRRPATTVSLSTQSSNSTAGGARNSRIPVRLESI--------------
                           :*: *    :. .:..  .*:   . .: *   *:*::
Homo                 EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Pongo                EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Bos                  EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Equus                EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Danio                EEFKDQLPADECNKLKEEISKVRELLSRKDTETGENIKQAATSLQQASLKLFEMAYKKMA
Oreochromis          ------------------------------------------------------MAYGSVK

                                                                          *** .:
Homo                 SEREGSGSSGTGEQKEDQKEEKQ
Pongo                SEREGSGSSGTGEQKEDQKEEKQ
Bos                  SEREGSGSSGTGEQKDNQKEEKQ
Equus                SEREGSGSSSTGEQKEDQKEEK-
Daniorerio           SEREGSSGSSSSGEAGEKKEGQQ
Oreochromis          LRQSNQGAVNLGSL---------
                      .:..... . .
```

Fig. 5. The alignment of deduced amino acid sequences of HSP gene from human, Pongo abilii, Bos Taurus and Danio rerio, GeneBank (accession nos. AAH00478, NP_001126860, NP_001029696, XP_001502580 , AAH44175 and GH159106), identical amino acids are indicated with an asterisk, similar amino acids are shaded. Dashes are gaps generated by alignment.

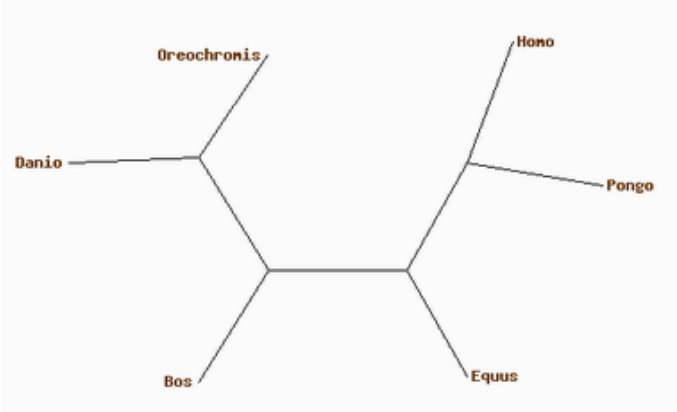

Fig.6: phylogenetic tree showing the relationship among the HSPs from different vertebrates

Expression of HSP9 in *Oreochromis niloticus*

ONHSP9 expression studied by RT-PCR in mixed tissues from infected and uninfected control fish was detected only in the infected tilapia (Fig.7).

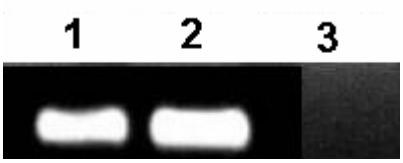

Fig.7: Tissue specific expression revealed by RT-PCR in *Oreochromis niloticus*; 1=βactin, 2=immunized fish, 3= unimmunized fish.

Discussion

Suppression subtractive hybridization (SSH) has led to the enrichment of specific expression genes as the non-specific expression of beta actin was reduced to about 2^{15} time lower after subtraction, suggesting that non-specific expressed genes have been avoided and immunization- specific genes have been enriched efficiently in this study. The identification of new genes involved in the bacterial immunization provides the foundation for further research on the immunoilogical interaction between host and the bacteria, In this study, cDNA library construction in case of *O. niloticus* immunized by *F. columnarae* introduced some new immune genes, one of them was Heat shock protein 9. HSP9 is a member of HSPs family which are intracellular proteins that can be released in various forms of cellular stress (Andreas *et al*., 2005)**.** HSPs, which are expressed constitutively in all cells, are also essential for several, important cellular processes, such as protein folding, protection of proteins from denaturation or aggregation, and facilitation of protein transport through membrane channels (Hartl *et al*., 1996). A wide variety of stressful stimuli, such as heat shock, ultraviolet radiation, and viral or bacterial infections, induce an increase in the intracellular synthesis of HSPs, clearly, HSPs play an important role in antimicrobial, as well as autoimmune, responses and have potent effects in inducing antigen-specific immunity to bound material upon immunization of animals (Wallin**,** *et al*., 2002), thus it is important to identify and characterize these genes which may help best understanding the process of host-pathogen relationship. ONHSP9

identified and characterized as it is composed of 1228 bps with a 73bps 5'UTR, a 135 3'UTR and 1020 bps open reading frame, the predicted gene product is 389 amino acid with molecular weight of 42.1 kDa. A signal peptide was predicted and suggested that HSP9 is a non secreatory protein. ClustalW alignment performed on variety of HSPs, ONHSP shares overall identity of 62.6% - 92% with other known vertebrates. Using Clustal W, a phylogenetic tree was constructed to demonstrate the distance similarity. Tissue expression of ONHSP9 was examined by RT-PCR and demonstrated that it is constitutively expressed in the immunized fish in contrast to unimmunized fish, thus it is suggested to be included in the immune response against *F. columnare*.

It could be concluded that killed *F. columnarae* did stimulate the immune response in the host fish, tilapia, some immune genes could be identified and characterized, one of these genes is HSP9, which may have a role and included in the immune response to bacterial infection, thus the present study provides some information for further research on the structure of HSP9 gene as an immune gene in relation to bacterial infection.

Acknowledgments

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References

Andreas L., Dirk B., Frank E., Daniel E., Svenja E., Minka B., Emma Hamilton-W., Sabine S., Achim H., Jürgen F., Arne von Bonin and Christian K.(2005).Heat Shock Protein 60 Is Released in Immune-Mediated Glomerulonephritis and Aggravates Disease: *In Vivo* Evidence for an Immunologic Danger Signal, J Am Soc Nephrol. 16: 383-391.

Annemie, D., Freddy, H., Andluca., D.(1997).Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibactercolumnaris*) from diseased fish. J. clinical microbiology, 322–324.

Chang M. X. , Nie P. , Liu G. Y. , Song Y. and Gao Q. (2005). Identification of immune genes in grass carp *Ctenopharyngodon idella* in response to infection of the parasitic copepod *Sinergasilus major*.J. Paracitol. Res. 96: 224-229.

Hartl, F.U. *(1996)* Molecular chaperones in cellular protein folding. Nature. 381, 571–579,

Kaufmann, S. H. E., Schoel, B.,Wand-Wu¨ rttenberger, A., Steinhoff, U., Munk, M E. and Koga, T. (1990) T-cells, stress proteins, and pathogenesis of mycobacterial infections. Curr. Top. Microbiol. Immunol.155:125.

Kaufmann, S. H. E. and Schoel, B. (1994). Heat shock proteins as antigen immunity against infection and self. In Morimoto, R. I., Tissieres, A. and Georgopoulos, C., eds, The Biology of Heat Shock Proteins and Molecular Chaperons, p. 495. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Lindquist, S. 1986. The heat-shock response. Annu. Rev. Biochem.55:1151.

Mo¨ller, G. 1991. Heat-shock proteins and the immune system. Immunol. Rev. 121:1.

Ohga, S., Yoshikai, Y., Takeda, Y., Hiromatsu, K. and Nomoto, K.(1990). Sequential appearance of γ/δ-and α/β- bearing T cells in the peritoneal cavity during an i.p. infection with Listeria monocytogenes. Eur. J. Immunol.20:533

Raulet, D. H. 1989. Antigens for g/d T cells. J.Nature, 339:342.

Robert P.A.Wallin, Andreas Lundqvist,Solveig H. Moré, Arne von Bonin, Rolf Kiessling and Hans-Gustaf Ljunggren(2002). Heat-shock proteins as activators of the innate immune system. J. TRENDS in Immunology, 23 (3):130-135.

Tsoi S. C. M, Ewart K. V., Penny S. , Melville K. , Liebscher R. S. , Brown L . and Douglas S. E. (2004). Identification of Immune-Relevant Genes from Atlantic Salmon Using Suppression Subtractive Hybridization. J. Mar. Biotecnol.6, 199-214

Van Eden, W., Thole, J. E. R., Van der Zee, R., Noordzij, A., Van Embden, J. D. A., Hensen, E. J. and Cohen, I. R. 1988.Cloning of the mycobacterial epitope recognized

Young, R. A. (1990). Stress proteins and immunology. Annu. Rev. Immunol.8:401.

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naturesciencej@gmail.com

Conservation through *in vitro* method: A case of plant regeneration through somatic embryogenesis in *Quercus semecarpifolia* Sm.

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Abstract: An efficient and reproducible protocol for *in vitro* propagation via somatic embryogenesis (direct as well as indirect) induced on cotyledon halves (with embryo) taken from seeds of *Quercus semecarpifolia* (Sm.) has been developed. Direct as well as indirect somatic embryogenesis was induced from the cotyledons on Woody plant (WP) medium supplemented with 6-Benzyladenine (BA) + Indole-3-butyric acid (IBA), and, BA + 2,4-Dichlorophenoxyacetic acid (2,4-D), respectively. Somatic embryos thus obtained were multiplied profusely on Schenk and Hildebrandt (SH) + Murashige and Skoog (MS) basal as well as BA supplemented media. Germination and conversion of somatic embryos into plantlets was achieved on SH+MS medium supplemented with BA (0.44- 8.88 μM). Rooting of *in vitro* produced shoots was achieved on WP (1/2 macro + full concentration of rest of the constituents) medium supplemented with IBA (14.76 μM). The plants were hardened *ex-vitro* and transferred to earthen pots containing garden soil. [Nature and Science. 2009;7(2):58-67]. (ISSN: 1545-0740).

Key words: *Quercus semecarpifolia*, brown oak, somatic embryogenesis, micropropagation.

INTRODUCTION

The genus *Quercus* has a wide distribution range; mostly trees, either deciduous or evergreen and is of enormous ecological and economical value. One of the species of *Quercus*, i.e., *Quercus semecarpifolia* Sm. (family-Fagaceae); common name-brown or kharsu oak; is the main forest forming evergreen tree species around 2400 m amsl in parts of Indian Himalaya (Singh and Singh, 1987). In view of the general importance of this species and problems associated with its regeneration (Tamta et al. 2008), in the present study attempt has been made for the first time to develop an efficient *in vitro* micropropagation method through somatic embryogenesis.

Micropropagation through somatic embryogenesis offers considerable advantages over other methods of clonal propagation; this route has a high proliferation potential. It has been

considered as a very promising method of oak micropropagation (Chalupa, 1995, Wilhelm 2000, Purohit et al.2002), and was found to be highly reproducible in this study on *Q. semecarpifolia*. Efficient protocols on SE induction and plant regeneration have recently become available for many plant species, including *Arabidopsis thaliana*, a model plant in genetics and embryogenesis (Gaj, 2004).

MATERIALS AND METHODS

Plant material and surface sterilization

Seeds of *Quercus semecarpifolia* Sm. were collected from well grown adult tree in the natural forests at Kilbury, Nainital (2100-2400 m amsl; 29° 24′ 30″ N- 29° 27′ N lat. and 79° 25′ E- 79° 29′ 40″ E long.), Uttarakhand, India. Following surface disinfection (Tamta et al. 2008), the seed coat was removed and seeds were divided into two halves; one half containing only one cotyledon while the other half contained the other cotyledon along with the embryo. These seed halves were used as explants for inoculation.

Media and culture establishment

Three basal media, namely MS (Murashige and Skoog, 1962), WP (Lloyd and McCown, 1980) and SH+MS, i.e., a combination of macronutrients of SH (Schenk and Hilderbrandt, 1972) and the remaining constituents of MS, were used. The basal media were supplemented with various concentrations of auxins, cytokinins and gibberellins. The sucrose concentration was 3.0% (w/v) and the media were solidified with 0.8% agar (w/v). The experiments were done using glass petridishes (10 cm dia, 25 ml medium per petirdish) or conical flasks (250 ml volume, 100 ml medium per flask). Incubation of cultures was carried out at 25 ±1 °C in a 16 h light and 8 h dark cycle, with 42.0 µmol $m^{-2}s^{-1}$ and 60.0 µmol $m^{-2}s^{-1}$ irradiance inside and outside the culture flasks, respectively by cool fluorescent tubes (Philips TI 40 W/54).

Production of somatic embryos

Seed halves turned green when inoculated on WP basal medium. After seven days, cotyledons with or without the zygotic embryo, were transferred on to WP or MS medium supplemented with either BA (0.44 µM) alone or in combination with 2,4-D (4.53 µM) or IBA (4.92 µM) or GA_3 (2.89 µM). Direct as well as indirect somatic embryogenesis with the intervening callus phase was induced within 13 weeks and 18 (10 weeks for callus establishment and proliferation + 8 weeks for induction of somatic embryos) weeks of culture, respectively. In both the cases, the presence of zygotic embryo seems to have some role in the production of somatic embryos. The callus raised

from cotyledons without the zygotic embryos did not survive on further subculture and degenerated. For germination of somatic embryos, formed both from the direct as well as indirect pathways, SH+MS medium supplemented with BA (0.44-8.88 μM) was used. The somatic embryos germinated to form well developed shoots, leaves and tap root system.

Adventitious rooting of microshoots

The survival rate of plantlets thus obtained; after transfer to *ex vitro* conditions was very poor (data not shown). Therefore, the main tap root was excised and the shoots were transferred to the rooting medium, i.e., WP (1/2 macro + full concentration of rest of the constituents) or SH + MS (macro of SH + rest of the constituents of MS) media supplemented with different auxins (Table 4), containing sucrose (3.0%; w/v) and phytagel (0.25%; w/v). Well developed adventitious roots were found to form within 4 weeks.

Transfer of plantlets to soil

After 5 weeks, the shoots with well developed roots were taken out from the culture flasks, the roots gently washed with water to remove traces of phytagel and the plantlets were then transferred to small plastic cups (8.0 cm ht; 7.0 cm dia) containing garden soil and the cups were covered with a transparent polythene sheet. Plants were kept inside a polyhouse for acclimatization for 1 month. After that the plants were transferred to the earthen pots (18 cm high; 20 cm dia) containing the same soil.

Statistical analyses

Experiments were conducted using a randomized block design to determine the effect of treatments and were repeated as described in individual experiments. For all the experiments explants were used in triplicates.

RESULTS AND DISCUSSION

Direct somatic embryogenesis

Globular structures were found to develop directly on the periphery of cotyledons with attached zygotic embryo, after 13 weeks on WP medium supplemented with BA and IBA (Table 1). These structures were loosely attached to the surface of cotyledons (Fig. 1A). On subculture these globular structures were converted into bipolar somatic embryos (Fig. 1B). This has been reported in some other species of *Quercus* (Chalupa, 1995; Gingas and Lineberger, 1989). Bipolar somatic embryos were also observed in *Q. robur* (Cuenca et al., 1999) and in *Q. suber* (Puigderrajols et al., 1996), which were reported to be translucent or opaque-white in appearance. These somatic

embryos were multiplied by secondary embryogenesis (Fig. 1C), and the frequency of secondary embryo formation was found to increase when subcultured on SH+MS medium, without any growth regulators. In *Q. suber* also secondary embryogenic lines were mainted on medium lacking PGRs (Fernandez-Guijarro et al., 1995). Proliferation of secondary embryos was most prolific from the root pole of the somatic embryos. Secondary embryos were produced mostly from the root pole end of the primary embryos as also observed by El Maataouti et al. (1990) and Gingas (1991). Cotyledons without the embryonic axes failed to give rise to direct embryos.

Indirect somatic embryogenesis

Callus was induced from the surface of cotyledons inoculated on both MS or WP media supplemented with BA and 2,4-D or IBA (Table 1). The creamy yellow callus developed on MS medium was slow to proliferate and degenerated on further subcultures. On the other hand friable callus was formed on WP medium after 10 weeks on cotyledonary halves with embryo (Fig. 1D); subsequently this callus was subcultured on MS basal medium (half or full strength) supplemented with CH (0.02%, w/v) and activated charcoal (1.0%, w/v) (Table 2). The friable callus developed on WP medium supplemented with BA (0.44 μM) and 2,4-D (4.53 μM) (Table 1) was found to turn embryogenic after 8 weeks (two months) of subculture (Table 2; Fig. 1E) when transferred to the above medium, i.e., MS basal (half or full strength) medium supplemented with CH (0.02%; w/v) + AC (0.1%; w/v). Somatic embryos could be multiplied through secondary embryogenesis on SH + MS medium supplemented with BA (0.44-8.88 μM) (Table 3). BA, a potent cytokinin, alone or in combination with auxins, particularly IBA or 2,4-D, has been known to induce somatic embryogenesis from the zygotic embryos (Chalupa, 1995; Gingas and Lineberger, 1989; Sasamoto and Hosoi, 1992; Kim et al. 2006). Somatic embryos of all stages (globular, heart and torpedo shaped) could be observed on the same medium.

The rate of multiplication of somatic embryos through secondary embryogenesis varied from 1.66 to 3.14 secondary embryos per somatic embryo, over a period of 5-6 weeks, depending upon the PGR supplements (Table 3). It is often reported in case of *Quercus* that calli turn embryogenic when transferred to the basal medium (Gingas and Lineberger, 1989; Guijarro et al., 1995; Kim et al., 1994).

Germination of somatic embryos

Somatic embryos (produced from the direct as well as indirect pathways) were transferred to BA (0.44-8.88 μM) supplemented SH+MS medium for germination. Some of the somatic embryos germinated and produced root and shoot in a well coordinated manner (Fig. 1F). In a number of

somatic embryos only the root primordia elongated (Fig. 1G); its frequency varied from 4.0-27.0 per cent depending upon the concentration of BA in the medium. The overall conversion frequency of somatic embryos was only around 10 per cent. BA at 2.22 μM was found to be optimum for germination and conversion of somatic embryos into plantlets (Table 3). The frequency of conversion of somatic embryos into full plants in oaks is usually quite low (Chalupa, 1995); this is a matter of future investigations. Fig. 1H shows the germination of somatic embryo.

Adventitious rooting of microshoots excised from germinating somatic embryos

Out of various media tried (MS, WP, SH+MS) supplemented with various auxins (IAA, NAA, IBA) in different concentrations (4.92 μM - 28.55 μM), WP medium supplemented with IBA (14.76 μM) was found to be most effective (100.0%) in inducing rooting without any callus formation at the basal end (Table 4). The root initials were observed within 10 days and well developed roots were formed in four weeks (Fig. 1I). The average number of roots was 12.46 with maximum length of 6.97 cm (Fig. 1J). WP medium supplemented with NAA or IAA also induced rooting (16.6% and 50.0%, respectively). However, the average number of roots was 3.0 and 3.02 and the length of the longest roots were 0.2 and 2.2 cm, respectively. When IBA was added to SH+MS medium, this combination also induced rooting (100.0%) but the formation of callus was invariably seen at the base of the explant, and the average number of roots (4.3) and length of the longest root (0.21 cm) were also considerably less. The addition of NAA to SH+MS medium totally failed in inducing rooting, whereas IAA induced rooting in 40.0% shoots with the average number roots being 4.0. However, the roots did not elongate and the length of the longest root never exceeded beyond 0.2 cm. Secondary roots were found to develop only on WP medium supplemented with IBA with profuse adventitious rooting. Addition of IBA to the rooting medium gave better results in comparison to another auxin, NAA, in *Q. suber* (Manzanera and Pardos, 1990) also.

Hardening: Well rooted plants were taken out of the culture vessels and the adhering phytagel was carefully removed; the delicate roots were then gently and thoroughly washed before transferring to plastic cups containing garden soil (Fig. 1K). The survival of these plants was only 20.0 per cent. After one month, these plants were transferred to earthen pots containing same soil and maintained inside the polyhouse until new leaves were found to emerge (Fig. 1L).

In conclusion, the present study describes, for the first time, the effective multiplication protocol for *in vitro* propagation of *Q. semecarpifolia*.

Table 1: Effect of treatments on seed halves of *Q. semecarpifolia* in different media

S. No.	Treatments	MS medium	WP medium
1	Control	-	-
2	BA (0.44 μM)	-	-
3	BA+2,4-D (0.44 μM+4.53 μM)	Callus	Callus*
4	BA+IBA (0.44 μM+4.92 μM)	Callus	Direct SE
5	BA+ GA_3 (0.44 μM+2.89 μM)	-	-

*embryogenic callus, - nil, SE: somatic embryogenesis, data recorded after 10 weeks of culture for callus formation and after 13 weeks for direct somatic embryo formation

Table 2: Callus proliferation and somatic embryogenesis in *Q. semecarpifolia*

Medium constituents	Callus Proliferation	Embryogenesis	No. of embryos/ petri dish
MS	++	***	125
MS+CH (0.02%)	+++	**	96
MS+CH (0.02%) +AC (0.1%)	+	-	NA
1/2 MS + CH (0.02%)	+	-	NA
1/2MS+CH (0.02%) + AC (0.1%)	++	***	110

The callus was initiated on WP medium supplemented with BA and 2,4-D;
MS: Murashige and Skoog medium; CH: Casein hydrolysate, AC: activated charcoal, all concentrations are w/v basis; + poor, ++ medium, +++ prolific; * poor, ** moderate, *** abundant, - nil, NA: not applicable; data recorded after 8 weeks (2 months) of culture; 6 petridishes were used per treatment with 4 callus pieces per petridish; the experiment was repeated twice with similar results

Table 3: Response of somatic embryos of *Q. semecarpifolia* on SH+MS medium supplemented with various concentrations of BA

BA (μM)	No. of somatic embryos transferred	Germination of somatic embryos (%)	Secondary embryogenesis*	Frequency of root formation (%)
0.44	30	0	1.66	26.60
0.88	97	4.50	2.28	18.40
1.78	44	5.20	3.14	18.18
2.22	49	6.90	3.00	14.00
4.44	68	2.90	1.85	4.40
8.87	90	0.89	1.76	4.10

* No. of total somatic embryos after six weeks/no. of somatic embryos initially inoculated per flask; each treatment consisted of 12 flasks, data was recorded 6 weeks after transfer of somatic embryos to the medium. The experiment was repeated twice with similar results.

Table 4: Effect of auxins and media on *in vitro* rooting of SE derived microshoots of *Q. semecarpifolia*

Medium	PGRs (conc.in μM)	Shoot ht (cm) ±SD	% callusing	% rooting	No. of roots/ shoot ±SD	Length of longest root (cm)±SD	Sec. roots
WP	IBA (14.76)	2.20 ±1.04	0.00	100.00	12.46 ±4.87	6.97 ±1.47	+
	NAA(16.11)	1.33 ±0.68	100.00	16.60	3.00 ±1.22	0.20 ±0.03	-
	IAA (17.13)	2.17 ±0.69	0.00	50.00	3.02 ±4.24	2.20± 0.57	-
SH+MS	IBA (14.76)	2.56 ±0.42	100.00	100.00	4.30 ±2.07	0.21 ±0.13	-
	NAA (16.11)	1.93 ±0.89	48.00	0.00	NA	NA	NA
	IAA (17.13)	2.00 ±1.31	0.00	40.00	4.00 ±2.3	0.20 ±0.11	-

WP: 1/2 macro + full concentrations of rest of the constituents; SH+MS : macro (SH) + rest of the constituents of MS; SE: somatic embryo, +: occurred; -: did not occur; NA: not applicable; SD: standard deviation, data recorded 5 weeks after transfer to rooting medium, treatments were carried out in triplicate and each flask contain 9 microshoots

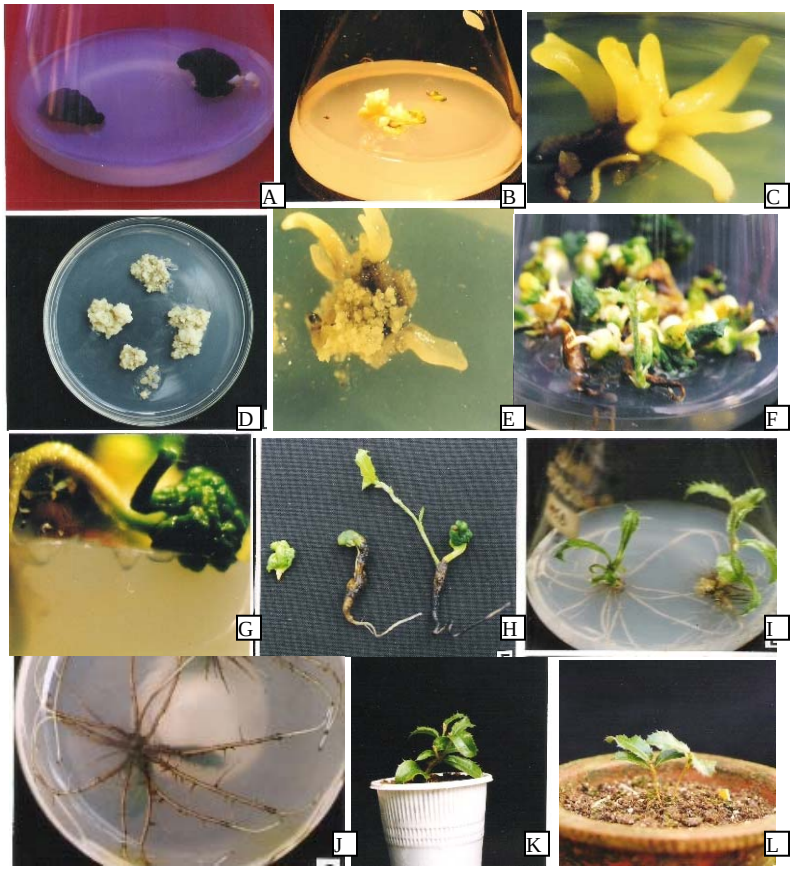

Fig. 1

Legends: Fig. 1. *In vitro* propagation of *Q. semecarpifolia*
(A) Globular structures loosely attached to the surface of the cotyledon.
(B) Bipolar somatic embryos
(C) Secondary embryogenesis
(D) Friable embryogenic callus on WP medium
(E) Indirect somatic embryogenesis
(F) Germination of somatic embryo
(G) Elongation of root primordial from the somatic embryo
(H) Different stages of somatic embryo germination
(I) Well rooted plantlets after 4 weeks of culture on WP medium supplemented with IBA
(J) Rooting from basal view
(K) Well rooted plant 1 month after transfer to plastic cup containing garden soil
(L) Two –months-old in vitro propagated plant in earthen pot

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REFERANCES

Chalupa V (1995) Somatic embryogenesis in oak (*Quercus* spp.). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in Woody Plants. Angiosperms. Kluwer Academic Publishers, Dordrecht, The Netherlands, vol. 2, pp 67 – 87

Cuenca B, San-Jose MC, Martinez MT (1999) Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Rep. 18: 538-543

El Maataoui M, Espagnac H, Michaux-Ferriere N (1990) Histology of callogenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) cultured *in vitro*. Ann. Bot. 66: 183-190

Fernandez-Guijarro B, Celestino C, Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell Tiss. Org. Cult. 41: 99-106

Gaj DM (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulation. 43(1): 27-47

Gingas VM (1991) Asexual embryogenesis and plant regeneration from male catkins of *Quercus*. Hort. Sci. 26: 1217-1218

Gingas VM, Lineberger RD (1989) Asexual embryogenesis and plant regeneration in *Quercus*. Plant Cell Tiss. Org. Cult. 17: 191-203

Guijarro FB, Clestino C, Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell Tiss.Org. Cult. 41: 99-106

Kim YW, Lee BC, Lee SK, Jang SS (1994) Somatic embryogenesis and plant regeneration in *Quercus acutissima*. Plant Cell Rep. 13: 315-318

Kim Y, Moon H, Son S (2006) Repetitive somatic embryogenesis and plant regeneration in Zizyphus jujube Mill. InVitro Cell Dev Bio Plant. 42(3): 247-251

Lloyd G. and McCown B. (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by shoot tip culture. Comb. Proc. Intl. Plant Prop. Soc. 30: 421-427.

Manzanera J.A. and Pardos J.A. (1990) Micropropagation of juvenile and adult *Quercus suber* L. Plant Cell Tiss. Org. Cult. 21: 1 – 8.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473 – 497.

Puigderrajols P, Fernandez-Guijarro B, Toribio M, Molinas M (1996) Origin and early development of secondary embryos in *Q. suber* L. Int. J. Plant Sci. 157: 674-684

Purohit V.K., Palni L.M.S., Nandi S.K. Vyas P. and Tamta S. (2002) Somatic embryogenesis in *Quercus floribunda*, an important Central Himalayan oak. In: Role of plant tissue culture in biodiversity conservation and economic development. Eds. Nandi S.K., Palni L.M.S. and Kumar A. Gyanodaya Prakashan, Nainital, pp. 41-52.

Sasamoto H, Hosoi Y (1992) Callus proliferation from the protoplasts of embryogenic cells of *Quercus serrata*. Plant Cell Tiss. Org. Cult. 29: 241-245

Schenk R.U. and Hildebrandt A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204

Singh JS, Singh SP (1987) Forest vegetation of the Himalaya. Bot. Rev. 53 (1): 80-192

Tamta S., Palni L.M.S., Purohit V.K, and Nandi S.K. (2008) *In vitro* propagation of brown oak (*Quercus semecarpifolia* Sm.) from seedling explants (accepted: 21 March 2008 for publication in In Vitro Cell.Dev.Biol.-Plant; 44(2):136-141.

Wilhelm E (2000) Somatic embryogenesis in oak (*Quercus* spp.). InVitro Cell Dev Bio Plant. 36: 349-357

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The Effect of Chemical Modification on the Thermal Stability of Protease from Local Isolate Bacteria, *Bacillus subtilis* ITBCCB148

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Abstract: This research aims to study the effect of chemical modification on the thermal stability of protease enzyme from local bacteria isolate *Bacillus subtilis* ITBCCB148 which was modified using dimethyladipimidate (DMA). To approach this aims, the production, isolation and purification of the enzyme were done. The purified enzyme was then modified with DMA. The number of modified lysine residue was shown by the modification degree determined using trinitrobenzenesulfonic acid. The success in the chemical modification was done by comparing the thermal stability of the enzyme before and after modification. The results showed that the modified enzymes with DMA produced modified enzyme with modification degree of 69, 75 and 76%. The enzyme thermal stability of the modified enzyme with modification degree of 69, 75 and 76% at 60°C were shown with the following data: $t_{1/2}$ = 96.25 min, k_i = 0.0072 min^{-1}, ΔG_i 106.868 kJ $mole^{-1}$; $t_{1/2}$ = 119.48 min, k_i = 0.0058 min^{-1}, ΔG_i 107.466 kJ mol^{-1}; and $t_{1/2}$ = 210 min; k_i = 0.0033 min^{-1}; ΔG_i 109,028 kJ $mole^{-1}$, respectively, whereas the thermal stability of the purified enzyme has data of: $t_{1/2}$ = 52.1 min, k_i = 0.0133 min^{-1}, 105.168 kJ $mole^{-1}$. The chemical modification on the purified protease enzyme has been able to increase the thermal stability up to 2 – 4 times compare to that of the purified one based on the decrease of K_i value. [Nature and Science. 2009;7(2):68-75]. (ISSN: 1545-0740).

Keywords: Protease, chemical modification, dimehyiladipimidate, *Bacillus subtilis* ITBCCB148

Introduction

Protease is enzyme that breaks the peptide bond into oligopeptide and amino acids. This enzyme is widely used in industrial sector such as food, skin, detergent and pharmaceutical industries (Rao *et al*., 1998). The use of this enzyme as biocatalyst in industry must fill some criteria, which are it must have high thermal stability and is able to work in wide pH range (Vieille and Zeikus, 1996). This condition is not own by most of the enzymes, as generally an enzyme works at physiological condition and cannot stand against extreme condition especially temperature and pH. In industrial processes which use the enzyme in enzymatic reaction, the use of en enzyme in an extreme condition from its optimum condition, can cause the enzyme lose its catalytic activity. Therefore in order to find an enzyme which can be used in industrial process, we must be able to get enzyme which is able to work in extreme conditions. This can be achieved in a few ways such as direct isolation from organism which lives in extreme condition (extremophilic) or by chemical modification to the enzyme from organism which does not live in extreme condition (mesophilic) (Wagen, 1984). The stabilization of enzyme obtained from mesophilic microbia is preferred way to get stable enzyme (Mozhaev and Martinek, 1984). According to Mozhaev *et al.* (1984), three ways to increase stability of an enzyme are amobilization, chemical modification, and directed mutagenesis. Chemical modification is a method to increase the stability of an enzyme which is soluble in water. The use of enzyme amobilization has a weakness, i.e. the decrease of binding capacity or enzyme reactivity due to the mass transfer inhibition by amobil matrix. In directed mutagenesis needs complete information about the primer structure and the picture of three dimension structure. According to Janecek (1993) in amobil process, the work mechanism of the enzyme in clinical sector, during interaction with receptor or other components of cellular membrane might be altered due to the presence of long matrix. While chemical modification, the interaction of enzyme with substrate is not hindered by the presence of insoluble

matrix, so the decrease of enzyme activity can be minimized. Based on this fact, the chemical modification is suggested way to increase the stability of enzyme.

In order to obtain the enzyme by chemical modification with stable covalent bond can be done by utilizing bifunctional reagent, modification with nonpolar reagent, addition of charge group or new polar group and hydrophilization the surface of protein (Mozhaev *et al*., 1990).

Modification with bifunctional reagents (cross-bond reagents) has been used many times to increase the enzyme stability. An example of bifunctional cross-bonded reagent is dimethyladimipidate. Kazan *et al.* (1996) reported that penicillin G acylase has increased its pH working range after reaction with DMA. Erarslan and Ertan (1995) have done thermostablization of penicillin G acylase from *E. coli* mutant with dimethyladipimidate (DMA), dimethylsuberimidate (DMS) and dimethyl-3,3'-dithiobispropionimidate (DTBP). The higher thermostabilization increased was observed after cross bond with DMA at temperature above 50^{o}C.

Based on the results of the above researches, the chemical modification using dimethyladipimidate to increase the thermal stability of protease enzyme from local bacteria isolate *Bacillus subtilis* ITBCCB148 is chosen.

Materials and Methods

Materials: Dimethyladipimidate (DMA) and other chemicals used were purchased from Sigma Aldrich and used without further purification. Local bacteria isolate *Bacillus subtilis* ITBCCB148 was obtained from Microbiolgy Laboratory, Chemical Engineering Department, Bandung Institute of Technology, Bandung, Indonesia. Buffer pH was measured at the temperature of use, and the pH reported is that at the temperature of the incubation.

Production of protease enzyme: the production of enzyme is done using fermentation media containing peptone 0.5%, yeast extract 0.15%, glucose 0.036%, and NaCl 0.25% (Yandri *et al.* 2007).

Purification of protease enzyme: The purification of protease enzyme is done in few steps: the separation of enzyme liquid from the cell with cold centrifugation to get the raw enzyme extract, precipitation with ammonium sulphate, ion exchange column chromatography and molecule filtration column chromatography (Yandri *et al*., 2007, 2008).

The protease enzyme activity test and protein content determination: The protease enzyme activity test was done based on the modified of Kunitz method (Yamaguchi *et al.,* 1982). Protein content determination was done based on the Lowry *et al.* (1951) method.

The modification of purified enzyme with DMA: Into 10 mL (0.082 mg/mL) of purified protease enzyme in 20 mM of phosphate buffer pH 7 was added with solid DMA until the concentration of DMA were 0.5%, 1%, and 1,5% (^{W}/v) to reach inactivation of kinetic work at different pH and temperature and then leave them for 1 h at room temperature (Kazan *et al.*, 1996)..

Determination of modification degree: Determination of modification degree was done based on the method used by Synder and Sobocinski (1975) and as following 0.1 mL of modified enzyme is dissolved into 0.9 mL borate buffer (pH 9.0) and then added with 25 µl 0.3 M 2,4,6-trinitrobenzenesulfonic acid. The mixture is then shaken and left it at room temperature for 30 min. The standard solution is made with the same composition but using the purified enzyme, while the blank contains 1 mL borat buffer 0.1 M pH 9 and 25 µl 0.3 M 2,4,6-trinitrobenzenesulfonic acid. The absorbance is measured at the λ_{max} 420 nm.

Determination of optimum pH and temperature before and after the modification: To know the optimum pH of the enzyme before and after the modification, the phosphate buffer 0.1 M was used with pH variations of 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; and 9.0. The temperature was kept constant at the determined optimum pH. To find the optimum temperature, the variations of temperature used were 50; 55; 60; 65 and 70°C, and then followed by the measurement of enzyme activity with Kunitz method.

Thermal stability test and stability of the enzyme pH before and after the modification: the enzyme thermal stability before and after the modification was done by measuring the residual activity of the enzyme after being incubated for 0, 60, 120, 180, 240, and 300 min optimum pH and temperature based on the method applied by Yang *et al*. (1996)

Determination of half life (t½), ki and ΔGi: determination of ki value (rate constant of termal inactivation) of purified enzyme and the modified enzyme was done using the first order of inactivation kinetics equation (Equation 1) (Kazan *et al.* 1997

$$\ln(Ei / E0) = -ki\ t \quad (1)$$

The denaturation energy change (Δ Gi) of the purified and modified enzymes was done using the Equation (2) (Yandri, 2007):

$$\Delta Gi = -RT \ln (kih/kBT)$$

Results and Discussion

Determination of modification degree with DMA: Modification of the purified enzyme with DMA was done in three concentration variations of DMA which was mixed with 10 mL of purified enzyme in phosphate buffer pH 7.0. The concentration variations of DMA used were 0.5%, 1%, and 1.5%. The result of determination of modification degree with 2,4,6-trinitrobenzenesulfonic acid were 69, 75, and 76%. Determination of modification degree is based on the ratio of number of lysine residues after and before modification.

Effect of modification toward optimum pH: Based on the data obtained, the modified enzyme with modification degree of 75% get decrease its optimum pH , while the optimum pH of modified enzyme with modification degree of 69% and 76% are the same as the purified enzyme (Figure 1).

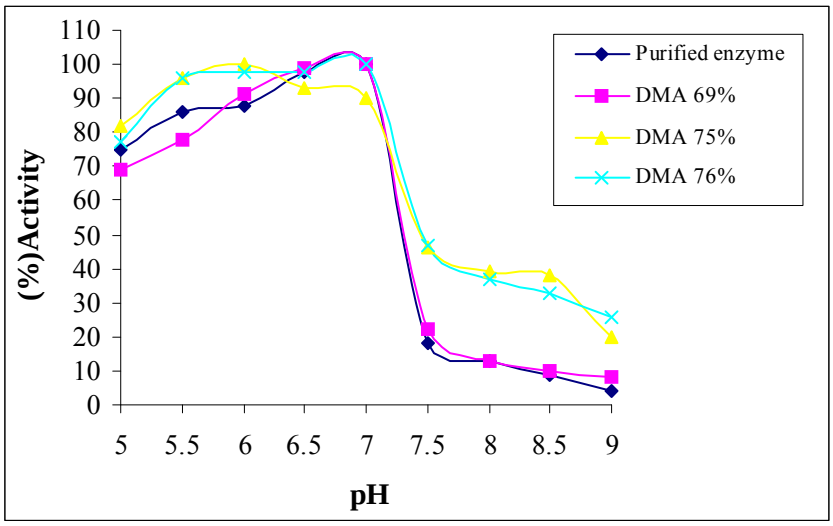

Figure 1. The Optimum pH of the Purified and Modified Enzyme (69%, 75%, 76%)

The decrease of pH optimum might be due to the change in enzyme conformation which causes the change on the active site of the enzyme, so the reaction ability of enzyme is also changed in its environment. Although there is decrease in optimum pH, but the (%) activity of the modified enzyme (DMA 75% and 76%) is higher at pH 7.5-9.0 when they are compared to modified enzyme with DMA 69% and the purified one. The high activity value (%) of the modified enzyme illustrates that the modified enzyme attain stability increase toward pH when compared to that of the purified one. The purified enzyme can retain its stability at pH 6.0-7.0, but there is a drastic decrease at pH 7.5-9.0. Whereas the modified enzymes with DMA 75% and 76% have wider working pH range, i.e. 5.0-7.0, although there is activity decrease at pH 7.5-9.0, but the decrease is not too high and the (%) activity is higher in that pH range compared to modified enzyme with DMA 69% and the purified enzyme. The activities (%) of purified and modified DMA 69% at pH 9.0 were 4% and 9 %, respectively, while those for modified DMA 75% and 76% were 20% and 26%, respectively. These results showed that the modified enzymes are more stable toward pH especially at basic condition than the purified enzyme. This phenomenon can be explained that at basic condition, the higher concentration of OH^- ion will bind to H^+ ion of carboxyl group on enzyme molecule to form H_2O. By modifying the enzyme, besides the binding of NH_2 groups of enzyme molecule by carbon group from DMA molecule, the hydrogen bond between H atom from carboxyl group with N of amine group in DMA. Due to the binding of H^+ of carboxyl group of enzyme by OH^- to form H_2O is not occurred. Therefore, the modified enzyme will be more stable at basic pH. The activity increase of modified enzyme was also occurred at acidic condition, but not too high and less significant. Based on this discussion it has been shown that DMA is able to increase enzyme stability toward pH.

The effect of modification toward optimum pH: The modified enzymes with DMA 69%, 75%, and 76% have optimum pH the same as the enzyme before it is modified (Figure 2), however their activity is higher than that of purified one.

Soemitro (2005) stated that the activity increase occur due to temperature increase did not change the accuracy of the tertier structure of enzyme before and after modification which bring close the side chain of some amino acids of stereospecific substrate side bond maker or amino acids of catalytic side support, so the optimum temperature of the enzyme did not change. This result agreed to those reported by Yandri *et al.,* (2008), Kazan *et al*. (1997) and Francis *et al*. (1992) that the chemical modification must not always cause the change of optimum temperature of the modified enzyme. Although the optimum temperature of the modified enzyme did not change, but the stability of the enzyme has increased at temperature range of 55 – 65^{o}C. At 55^{o}C the purified enzyme has activity of 75%, while at 65^{o}C was 24%. The modified enzymes with DMA 69%, 75%, and 76% at 55^{o}C have activity of 87%, 82%, and 96%, respectively, while at 65^{o}C has activity of 42%, 60% and 40%, respectively. This result illustrated that the modification caused the enzyme was more rigid, so the enzyme was more endure toward temperature.

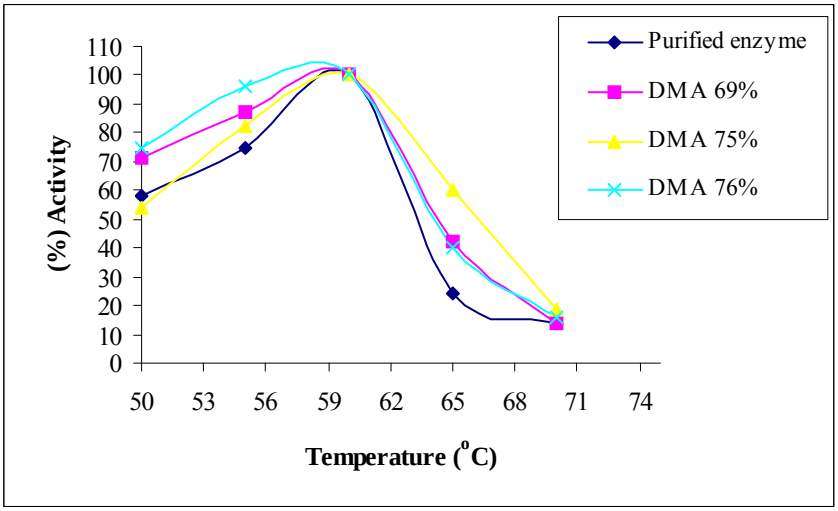

Figure 2. Optimum Temperature of the Purified and Modified Enzyme (DMA 69%, 75%, 76%)

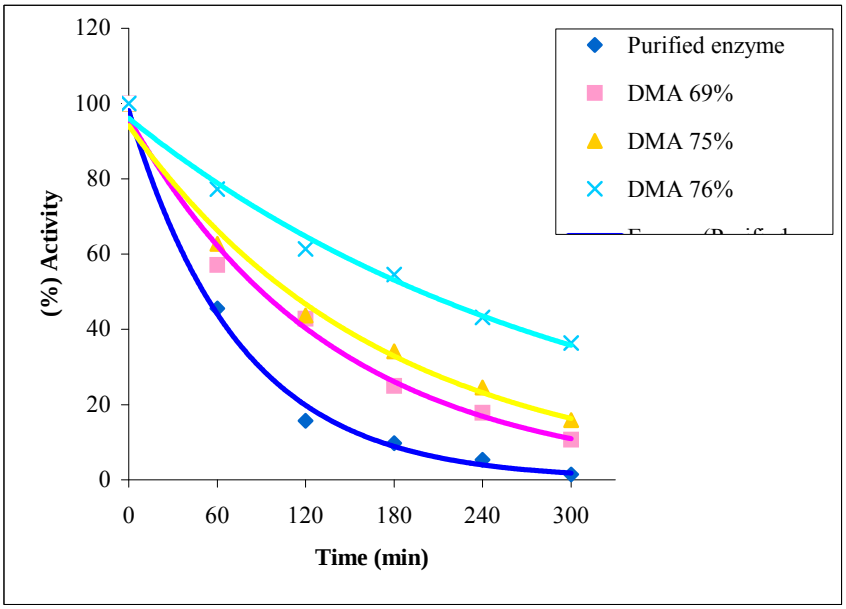

Figure 3. Thermal Stability Expressed as Graphic between Residual (%) activityPurified Enzyme and Modified Enzyme (DMA 69%, DMA 75%, DMA 76%) at 60°C *vs* Time

The effect of modification toward thermal stability: The test of thermal stability effect to the modified enzyme was done by calculating the percentage of residual activity of purified and modified enzymes. This was done by incubating each enzyme at 60°C for 300 minutes. The activity was measured every 60 minutes, and the result is presented in Figure 3.

Figure 3 illustrates the residual (%) activity of each enzyme. The purified enzyme has residual activity of 1.47% and then after modification with DMA 69%, DMA 75% and DMA 76% the residual activities were 10.71%, 15.87% and 36.36%, respectively. Based on graph in Figure 3, it is clear that the modified enzymes have much higher thermal stability than that of purified one.

In line with modification degree, the higher modification degree, the higher the thermal stability of the enzyme, and the use of DMA as the modifying agent has a good effect on the structure stability. The mechanism of enzyme structure stability by DMA is occurred through cross-bond of inter and intramolecular of DMA to form small circles on enzyme, to protect the unfolding of tertier structure of enzyme and protein oligomer dissociation into subunits. The cross bond to lysine residue in the surface will protect the hydrophobic site of the enzyme to contact with solvents. Besides that, there is addition of hydrogen bond between the nitrogen atom of amine group in DMA molecule and hydrogen atom of carboxyl groups of the enzyme. Since DMA is bifunctional reagent with two functional groups which can bind to two enzyme molecules, consequently, the hydrogen bond form will more. Kazan *et al*. (1996) reported that the change of ionization degree of organic groups on enzyme due to the formation of hydrogen bond causing the rigidity increase of penicillin G acylase structure modified with DMA. The more hydrogen bonding formed, the higher the modification degree used, so the enzyme rigidity is also increased. This result supported previous result obtained that the higher the modification degree, the more stable the enzyme will be (Kazan *et al*., 1996).

Table 1. The Values of Rate of Thermal Inactivation (k_i), Half-life ($t_{1/2}$) and Denaturation Energy Change (ΔGi) of Purified and Modified Enzymes (DMA 69%, DMA 75%, DMA 76%)

Enzyme	k_i (min^{-1})	$t_{1/2}$ (min)	ΔGi (kJ $mole^{-1}$)
Purified enzyme	0.0133	52.1	105.168
DMA 69%	0.0072	96.25	106.868
DMA 75%	0.0058	119.48	107.466
DMA 76%	0.0033	210	109.028

Half-life (t½) and rate of thermal inactivation (ki): The half lifes of the modified enzymes have increased 2-4 times compared to the purified enzyme. Stahl (1999) stated that half-life will determine the stability of the enzyme. The results obtained in this research, the half-lives of modified enzyme with DMA 69%, 75% and 76% have all increased from 52.1 minutes to 96.25, 119.48 and 210 minutes, respectively. These data showed us that the higher the modification degree used, the longer the half-life will be. The decrease of k_i value (Table 1) showed that the decrease of enzyme denaturation has occurred on the modified enzymes. The increases of enzyme stability based on the decrease of ki value are 2-4 times compared to that of purified enzyme. The lower of value k_i illustrates that the enzymes is less flexible in water due to the bond formation between DMA and NH_2 group of side chain lysine residue on the surface. This causes the decrease in protein unfolding so the structure of enzyme will be more rigid and more stable. The same result was also obtained by Kazan *et al*. (1996) where the rate of thermal inactivation of DMA cross-bonded with penicillin G acylase (PGA) was always lower than the pure PGA.

The energy change due to denaturation (ΔGi): Table 1 shows the ΔGi increase of modified enzyme compared to that of the purified enzyme. The higher ΔGi value of the modified enzyme demonstrates that the enzyme is more rigid and less flexible in water. The tertier structure of the denaturated enzyme has changed and the enzyme structure that is more rigid has stronger bond, so the enzyme conformation is not easily opened that the tertier enzyme structured can be maintained. To denature this sort of enzyme will require more energy, so the ΔGi value obtained will also be higher. Therefore the higher ΔGi value indicates that the enzyme is more rigid, less flexible and is hard to denature and *vice versa.* Kazan *et al.* (1996) reported that the higher ΔGi of

penicillin G Acylase which crossed-bond to DMA indicated the stabilization of PGA by cross bond at extreme pH. In this research, the ΔGi value of purified enzyme obtained was 105.168 kJ mole^{-1} while for the modified enzyme with DMA 69%, 75%, 76% were 106.868; 107.466; 109.028 kJ mole^{-1}, respectively. The increase of enzyme stability is higher with the increase of modification degree, although the ΔGi value is not too high, but the thermal stability is increased 2-4 times.

Conclusion

Based on the results obtained and the discussion above, it can be concluded that: the optimum temperature of the modified enzyme is the same as the purified enzyme, but the stability is increased at 55 – 65^{o}C. The thermal stability test at storage temperature of 60^{o}C for 300 min shows the modified enzyme with DMA (69%) has residual activity of 10.71%, $t_{1/2}$ = 96.25 min; k_i = 0.0072 min^{-1}; ΔGi = 106.868 kJ mole^{-1}.

DMA (75%) has residual activity of 15.87%; $t_{1/2}$ = 119.48 min; k_i = 0.0058 min^{-1}; ΔGi = 107.466 kJ mole^{-1}. DMA (76%) has residual activity of 36.36%; $t_{1/2}$ = 210 min; k_i = 0.0033 min^{-1}; ΔGi = 109.028 kJ mole^{-1}. While the purified enzymes has residual activity of 1.47%; $t_{1/2}$ = 52.1 int, k_i = 0.0133 min^{-1}, 105.168 kJ mole^{-1}. The modification of enzyme with DMA has been able to increase the thermal stability of the enzyme up to 2-4 time based on the decrease of k_i value.

Future works and suggestion

Based on the results obtained, we suggest to do further research to find out the three dimension structure of protease enzyme *B. subtilis* ITBCCB148 to explain the stability of the enzyme. The modification of this enzyme with other bifunctional reagent is interesting to be done to compare the stability might be obtained.

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References

1. Erarslan A, Ertan H. Thermobilization of Penicillin G Acylase Obtained from a Mutant of *Escherichia coli* ATCC 11105 by Bisimidoesters as Homobifunctional Cross-Linking Agents. Enzyme and Microbial Technology 1995;17(2):629-635.
2. Francis GE, Delgado C, Fisher D. PEG Modified Protein in Stability of Protein Pharmaceuticals Part B, Ahern, T. J and M. C. Manning Editor. Manning Editor, Plenum Press, New York. Pp. 1992: 246 – 247.
3. Janecek S. Strategies for Obtaining Stable Enzymes. Process Biochemistry 1993; 28:435-445
4. Kazan D, Ertan H, Erarslan A. Stabilization of Penicillin G Acylase Against pH by Chemical Cross-Linking. Process Biochemistry 1996;31(2): 135-140.

5. Kazan D, Ertan H, Erarslan A. Stabilization of *Escherichia coli* Penicilin G Acylase Against Thermal Inactivation by Cross-linking with Dextran Dialdehyde Polymers. Applied Microbiology and Biotechnology 1997;48: 191-197.
6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurment with the Folin phenol reagent, Journal of Biological Chemistry 1951;193:265 - 275.
7. Mozhaev VV, Martinek K. Structure Stability Relationship in Proteins: New Approaches to Stabilizing Enzymes. Enzyme and Microbial Technology 1984;6:50-59.
8. Mozhaev VV, Melik-Nubarov NS, Siksnis V, Martinek K. Strategy for Stabilizing Enzymes. Part two: Increasing Enzyme Stability by Selective Chemical Modification. Biocatalalysis 1990;3:189-196.
9. Rao MB, Tanksale AM, Gate MS, Desphande VV. Molecular anf Biotechnology aspects of Microbial Proteases. Microbiology and Molecular Biology Reviews 1998; 62:597-628.
10. Stahl S. Thermophilic Microorganisms: The Biological Background for Thermophily and Thermoresistance of Enzymes in *Thermostability of Enzymes* (Gupta, M.N. editor), Springer Verlag, New Delhi, 1999: 59-60.
11. Soemitro S. Pengaruh Modifikasi Kimiawi Selektif Terhadap Kestabilan α-Amilase dari *Saccharomycopsis fibuligera*. Bionatura 2005;7(3) :259-273 (Indonesian).
12. Synder SL, Sobocinski PZ. An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines, Analytical Biochemistry 1975;64: 284-288.
13. Wagen ES. Strategies for increasing the stability of enzymes, in *Enzyme Engineering* 7, The New York Academy of Sciences, New York. 1984:1-19.
14. Vieille C, Zeikus JG. Thermozymes: Identifying molecular determinant of protein structural and functional stability, Trends in Biotechnology 1996;14(6):183-189.
15. Yamaguchi T, Yamashita Y, Takeda I, Kiso H. Proteolytic enzymes in green asparagus, kiwi fruit and miut: occurence and partial characterization, Agricultural and Biological Chemistry 1982;46:1983-1986.
16. Yandri AS, Dian H, Tati S. Isolasi, pemurnian dan karakterisasi enzim protease termostabil dari bakteri isolat lokal *Bacillus subtilis* ITBCCB148, Jurnal Sains MIPA (*Special Edition*) 2007;13(2):100- 106. (Indonesian)
17. Yandri AS, Herasari D, Suhartati T, Hadi S. The Chemical Modification of Protease Enzyme Isolated from Locale Bacteria, *Bacillus subtilis* ITBCCB148, with Polyethylenglycol Cyanuric Chloride. European Journal of Scientific Research 2008;23(1):177-186.
18. Yang Z, Michael D, Robert A, Fang XY, Alan JR. Polyethylene glycol-induced stabilization of subtilisin Enzyme and Microbial Technology 1996;18:82-89.

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Movement and Ranging Behaviour of Asian elephants *Elephas maximus* in and around the Rajaji National Park, North-West India

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Short Title: Movement and Ranging Behaviour of Asian Elephant in North-West India.

Abstract: The seasonal and annual movements and home ranges of two different recognized elephant herds and bull elephant were assessed by ground based data and direct observations in Chilla forest of the Rajaji National Park and its adjoining protected habitats from May 2005 to June 2007. During summer elephants use the lower slopes of Chilla forest but during monsoon their movement was towards upper areas of Luni, Rawasan and Pulani forest (south-east axis) and towards Shyampur and Chiriapur forests (south axis) and during the winter elephants again return back to plains of Ganges, where they utilize the riparian corridors. Large migration was observed during onset of summer (February-March). Herd's movement was observed within an area of about 80 Km^2 moving on the average less than 8 kilometers per day whereas bull elephant's movement was observed to be about 390 Km^2 moving on the average less than 27 kilometers per day. Home range sizes of herds varied between 13 Km^2 (winter) and 24 Km^2 (monsoon) and of bulls between 63 Km^2 (winter) to 177 Km^2 (monsoon). Annual home ranges of herds were estimated 18 Km^2 (summer), 24 Km^2 (winter) and 21 Km^2 (monsoon) for Chilla herd and 20 Km^2 (summer), 13 Km^2 (winter) and 24 Km^2 (monsoon) for Garhwal herd, whereas bulls home ranges were estimated to be 83 Km^2 (summer), 63 Km^2 (winter) and 87 Km^2 (monsoon) for bull Ist (Ganesh) and 174 Km^2 (summer), 84 Km^2 (winter) and 177 Km^2 (monsoon) for bull IInd (Hitler) respectively. Data from ground based observations confirmed that elephants frequently moved across outside of the Rajaji National Park boundary towards Laldhang forest of Lansdowne forest division and Shyampur and Chiriapur forest of Hardwar forest division. All of these data support the importance of protecting the Rajaji National Park and its adjoining protected habitats as an important elephant range and corridor, linking different protected areas in north-west India. [Nature and Science. 2009;7(2):76-94]. (ISSN: 1545-0740).

Keywords: Asian elephant; movement; ranging behaviour; Rajaji National Park; protected habitats; north-west India.

Introduction

India has between 21,000 and 25,000 Asian elephants (*Elephas maximus*) in the wild and among them Uttarakhand state harbours 1346 elephants distributed within 14 protected areas. India currently has the largest surviving population of the Asian elephant, approximately 50 % of the total world population of the species (Daniel, 1996). A number of wildlife habitats have undergone or are being threatened with fragmentation due to various anthropogenic factors and this has adversely affected the large mammal populations residing in them (Johnsingh et al., 1990). Recently, developmental activities and habitat destruction have caused major decline in the abundance of the terrestrial megafauna. As most of the wild animals are presently categorized under threatened category therefore, there is increasing concern that the area-wise decline of the elephant will have unexpected and grave consequences for the long-term viability of the terrestrial ecosystems.

The Rajaji National Park was established to enhance the long-term survival of the Asian elephant in a sub tropical moist deciduous forest in India. But during the recent past natural continuous forest ranges of India has been broken up into many parts due to agriculture, urbanization, increasing road traffic and development related activities as well as other anthropogenic activities. This situation creates many problems for various organisms living in forests especially for large size mammals like elephant. Genetic isolation, limitation of dispersal, migration and the decline of populations of animals requiring large territories are the most common problems connected with fragmentation of forests and other components of the environment. Shivalik landscape (lesser Himalayan zone) is one of the last few places in the world where elephants exist and offers urgent need for conservation. From conservation point of view Rajaji National Park appears to be India's one of the most successful national park and its management has helped to boost the population of Asian elephant in their natural habitat. Before the Gujjar rehabilitation programme elephants must scarify the feeding grounds in order to feed on the short grasses due to domestic buffaloes being grazed (Joshi and Pande, 2007).

Human settlements in and around the park area have created the shrinking of elephant's natural paths. The human population around the Rajaji National Park alone has doubled during past one decade and rapid urbanization and industrialization has resulting in the loss of many forestlands to townships and to various development related activities. The factors that contribute to the killing of humans by elephants are the presence of people into elephant's habitat to collect firewood and fodder, conflict over water and cultivation of palatable crops near the forest boundary. In between years 1986 to 2004, elephants have killed 47 persons and injured 43 persons in and around the Rajaji National Park area. And in Hardwar forest range, elephants have killed 26 persons and injured 11 persons in between year 1985 to 2001. On the other hand from 1987 to 2004, more than 134 elephants succumbed in the wild to various reasons (train accident, poaching, electrocution, fallen through hillock, disease, bull fight and natural). Human settlements in and around the park area have created the shrinking of elephant's natural paths. The human population around the Rajaji National Park alone has doubled during past one decade and rapid urbanisation and industrialisation has resulting in the loss of many forestlands to townships and to various development related activities (Joshi and Singh, 2007).

Movements in large mammals are considered to be one of the most important ecological factor, which influence the distribution of other small herbivores. Elephants travel long distance as part of their migrational activities and at the same time they stay within different forest habitats those are enriched with water and fodder species. Once elephants of this track were known to perform extensive migration from river Yamuna to Sharda but during the last three decades, elephants are pocketed in small habitats due to escalated rate of developmental activities and fragmentation of large habitats into smaller ones. This has declined the high in-breeding rate among different population of elephants and constrained them to live within smaller habitats. Migration within large mammals also influenced due to water availability as during the dry period elephants required tremendous amount of water and at that time their local movements is quite frequent near to riparian corridors. Thus the home range studies are better options for wildlife management as these highlights the preferred habitats of any animal and the rate of fragmentation of habitat.

Methods

Study Area

Rajaji National Park [29^0 15' to 30^0 31' North Latitude, 77^0 52' to 78^0 22' East Longitude] is spread over an area of 820.42 Km^2 in and around the Shivalik foothills, which lies in the lesser Himalayas and the upper Gangetic plains (Figure 1). Rajaji National Park (RNP) was notified in 1983 by amalgamating three erstwhile wildlife sanctuaries namely, Rajaji, Chilla and Motichur. Spread across Hardwar, Dehradun and Pauri districts of Uttarakhand state, Rajaji National Park has been designated as a reserved area for the "Project Elephant" by the Ministry of Environment and Forests, Government of India with the sole aim of maintaining the viable population of Asian

elephants in their natural habitat. The Shivalik foothills offer the most prominent geomorphic features of this tract. The river Ganges has cut across these hills at Hardwar. The Chilla forest area of the RNP lies in the east of the river Ganges and is attached by the Garhwal Forest Division. The study was conducted in Chilla (District-Pauri) forest range of the RNP. Besides, Laldhang forest range (Lansdowne forest division, LFD), Shyampur and Chiriapur forest ranges (Hardwar forest division, HFD) were also included in this study. The altitude lies between 302-1000 m asl.

This protected area in India's lesser Himalayan region falls under sub tropical moist deciduous forest type with extensive stands of *Shorea robusta* (Sal), *Mallotus phillipinensis* (Rohini), *Acacia catechu* (Khair), *Adina cordifolia* (Haldu), *Terminalia bellirica* (Bahera), *Ficus bengalensis* (Bar) and *Dalbergia sissoo* (Shisham) in its premise besides many other important fodder plant species. This entire belt is natural home of Asian elephants (*Elephas maximus*) besides many other wild animals like *Panthera tigris* (tiger), *Panthera pardus* (leopard), *Melursus ursinus* (Sloth bear), *Hyaena hyaena* (Hyaena), *Muntiacus muntjak* (Barking deer), *Axis axis* (Spotted deer), *Cervous unicolor* (Sambhar), *Sus scrofa* (Wild boar) and *Ophiophagus hannah* (King cobra).

Data Collection

It is not easy to sight elephants in dense forest habitats due to thickness of the undergrowth and foggy climate especially during the monsoon and winter period. Also there are chances of any casualty. In few of the forest pockets it was difficult to observe directly the elephants in RNP because of dense sub-tropical vegetation and presence of undulating foothills with bushes taller than the animal. Thus the study incorporated both direct as well as indirect methods.

Direct Method

For assessing the movement behaviour of elephant's four forest ranges (Chilla, Shyampur, Chiriapur and Laldhang) of the RNP, HFD and LFD were selected and in-depthly surveyed. All the field observations were made during 2005 to 2007. It was not possible to observe the elephants during monsoon as most of the areas are dominated with tall grasses and dry period was the best time to observe the elephants especially near to water sources. The study area was visited at weekly intervals during which observations on elephants were made along the motorable forest track, present in between different forest habitats. Few other connected rough routes, which link the grassland habitat with motorable road were also used during the course of study. As few forest beats of the study area does not comprises of any road, therefore, study was made on foot. Although some animals were observed up to a maximum distance of 100 meter, most of the observations fell within 50 meter. Besides, all the potential habitats (water dominant areas, cool shaded areas, fodder enriched areas and rough forest routes) were also investigated on foot during early morning, mid-day and evening hours. Cool shaded trees like *Ficus bengalensis*, *Adina cordifolia* and *Ficus glomerata* and dense forest of *Mallotus phillipinensis* and other favourite fodder species were examined mostly during mid day (March-June) hours as elephants generally take rest under these cover. Whereas all the water sources (perennial/annual) were investigated alternatively during evening hours.

As the elephants in RNP have been known to emerge from the forest predominantly during evenings, all sightings of elephants were made between 1500 hours and 1900 hours. Besides, observations were also done during early morning hours (0600 hours to 10 hours). Different forest blocks of concerned forest ranges were selected one after another sequentially and searched for elephants for about 10 – 12 hours (depending upon weather conditions) in a single day search. The observations started at early hours in the morning being the best time to search and observe the elephant in open areas and four hours in the afternoon i.e. before the sunset. The data collected was as part of the animal monitoring activities and the daily record was based on direct sighting of animals, indirect evidences like feeding sign, footprints impression time and fresh dung piles. The direct sighting were noted in duly prepared proforma, recording the Herd composition, age and sex, if observed in Herds and also the place of sighting, time and vegetation type. Besides, villagers of

adjoining areas, Gujjars (where available), staff of forest department, the researchers from various scientific institutions and non-government organizations and other individuals working on this problem, were also interviewed. Field binocular was also used for observing their movement behaviour without disturbing the animal from an adequate and safe distance.

Movement behaviour

Two different Herds and two solitary adult male elephants were selected and alternatively followed in Chilla (RNP), Shyampur and Chiriapur (HFD) and Laldhang (LFD) forests during the early morning hours and evening hours. First Herd consists of 21 individuals and the other one 13 individuals (Table 1). The classification of individuals in the Herds was based on Eisenberg and Lockhart (1972) – namely adult, sub adult, juvenile and calf. Photographs of the herds and bulls were also taken for confirmation upon repeated sightings (Figure 2, 3, 4 & 5). Whenever any marked Herds or solitary males were encountered during early morning circuit, their location was noted along with ongoing activity. An attempt was again made later in the evening hours to relocate the animal that had been observed in the morning. Video camera was also used to cover small footages of Herd composition along with different behaviours of recognized Herds. Whenever herds were encountered, they were observed until they disappeared from sight or until darkness made further observations impossible. As elephant movement was restricted in between Chilla – Motichur and Rajaji – Corbett corridors, therefore, it has made us easy to follow and observe these recognized Herds and bulls those were performed their movements in eastern part of river Ganges.

The movement pattern and the present home range used by identified herds and bull elephants were calculated by plotting sighting locations on a map (1:50,000 scale) (McKay, 1973; Ali et al., 1986; Daniel, 1988; Datye and Bhagwat, 1995). Sometimes, movements of both the herds and bulls were overlapped simultaneously in the same area, and it has helped us to trace their movements sharply.

Table 1. Composition of elephants in Herd Ist (Chilla herd) and Herd IInd (Garhwal herd).

Herd No.	Adult females	Sub-adult females	Juvenile males	Juvenile females	Calves	Total
1	7	5	2	4	3	21
2	5	3	1	1	3	13

Identification of the elephants was important to verify their movement as in the same area there was a possibility that the same Herd was observed in the different forest beats. Therefore, distinctive features, with certain identification marks of individual elephants were noted like; shape of the ears, tusk size and shape, scars and tubercles on the body, tail length, total number of individuals (all ages separately), body mass and nature of Herd or solitary bull.

Indirect Method

In few of the places the indirect count method was followed for checking their number in study area (Dawson and Dekker, 1992; Ramakrishanan et al., 1991; Santiapillai and Suprahman, 1986). This involves path counts and frequency of elephant signs. For conducting the study on elephant's presence, all the observations were made from a vehicle and through adopting the road-strip count method (Hirst, 1969; Santiapillai et al., 2003) to monitor the fluctuations in elephant numbers.

Results

Movement is one of the most important ecological factors that represent the home range as well as habitat utilization of an animal. Both movement and migration depends upon the availability of natural food and water. Changes in season and scarcity of water and natural fodder species force

wild animals to leave a place for few months and reached to new feeding grounds for fulfilling their feeding and breeding requirements. There are seasonal variations in fodder species as RNP area falls under sub-tropical moist deciduous forest vegetation type. Elephants use whole of the park area as their natural habitat but mostly they leave some of the areas having less vegetation cover and water for few months and move towards other ranges richer in fodder species and natural water. Although at that time few of them (mostly solitary bulls) use the same feeding grounds or move frequently in all the forest beats of the park as a general rule of migration of any species.

Movement and Ranging Behaviour of Elephants

Elephant use Chilla forest round the year because of altitude wise variation of rich fodder species. Elephant used to live regularly in lower patches of Chilla forest (Chilla, Mundal, Jhabargarh and Khara forest) during dry period but on the arrival of rainy season elephants migrates towards upper areas (Luni, Pulani, Rawasan and Kasaan forest beats) and that was the time when elephants start their long term migration towards Lansdowne forest division and Hardwar forest division (south-east axis). Again on the onset of winter elephant's movement is towards lower areas (south-east to north-west direction) and at the same time elephants also utilize the adjoining forest of river Ganges, which is spread up to Rishikesh along the river. Besides, many of the Herds and solitary bulls perform their movements in all of the forest beats round the year for their local movements. During the summer elephant's movement was also frequent in the Gohri forest range, which is in the north of the Chilla forest.

Elephants also use the Ghasiram water stream for visiting to river Ganges especially when their movement was frequent in adjoining forest beats, which are major riparian corridors of different tributaries to river Ganges. One bridge over to Ganga canal (Soni shroath) is also utilized during dry period for inter-changing the forest. Some Herds were also observed to use the Shyampur and Chiriapur forest of HFD during rainy season because of availability of Ganga canal water. At the same forest elephants perform their movements towards river Ganges through crossing the Hardwar-Bijnor National Highway. Currently only bull elephants are utilizing this track whereas no Herds were reported during last three years. As per last 6-7 years data, Herds of the elephants were observed in the same area but rapid developmental activities has restricted the frequent movement of elephant's Herd towards river Ganges.

Few of the main reasons affecting local movement of the elephants in rainy season are:

a) During rainy season the elephants were seen moving towards upper areas of the park. This is because the low lying areas become swampy and unfit for free movement of the elephants.
b) Another major factor contributing to their upward movement is the abundance of a blood sucking fly locally called as 'daans' in low lying areas which irritates elephants by hovering around their ears and trunk. This fly is commonly found affecting the cattle stock of Gujjars and villagers.
c) Forest fire is also one a factor to force the elephant's movement to a separate area where fire had not been so extensive. This fire if spread extensively then the movement of such a large animal also restricts to the same area for some time.

The movements of the Rajaji's herds are entirely seasonal and similar seasonality was also observed in the appearance of herds at adjoining protected habitats. Figure 6 and 7 shows the movements of both the Herds (21 and 13 elephants), which constituted part of the population inhabiting the Chilla forest of the RNP from May 2005 to June 2007. During this two year period elephant's movement was observed within an area of about 80 Km^2 moving on the average less than 08 kilometers per day (Figure 6 & 7). Bull elephant's movement was observed to be more as

compared to herd movement and they ranges about 390 Km^2 moving on the average less than 27 kilometers per day (Figure 8 & 9).

Herd Movements

During the study period Chilla herd was located on 194 days (summer), 183 days (winter) and 127 days (monsoon) while Garhwal herd was located on 181 days (summer), 162 days (winter) and 114 days (monsoon). The annual home range of Ist herd was observed to be 18 Km^2 in summer, 24 Km^2 in winter and 21 Km^2 in monsoon. Similarly, the range utilization by IInd herd was 20 Km^2 in summer, 13 Km^2 in winter and 24 Km^2 in monsoon respectively. The home ranges of both the recognized herds were almost same throughout the year and they were observed to utilize the same seasonal feeding grounds in both the years. It indicates that both of these herds have had a fixed home range to some extent. Generally herds comprise of baby elephants and their seasonal ranging was also occurred in a fixed manner, therefore, their movements were restricted to particular areas. Longer movements were only observed during the course of migration or when environmental conditions are unfavourable for example forest fire and scarcity of water.

Figures 6 and 7 shows the movements and areas covered by two herds, the Chilla herd and the Garhwal herd over periods of two year each. As can be seen from both of these figures, each of the herds remained in a particular area for period of 2-3 months, although the Chilla herd did remain primarily in the Chilla region itself for the period extending between February and June. From July to September, these animals were moving fairly regularly and covered an area of approximately 24 Km^2 throughout this period. In November, they were observed making a long movement into an area where they remained between December to June. It was unfortunately not possible to follow the herd in this area during the monsoon season so; indirect evidences like presence of footprints, feeding signs and presence of dung piles (recognised herds) were followed to compare their movements during this time. The movements of the Garhwal herd (Figure 7) was somewhat similar in that the animals used only small areas of their total range for periods of 1-4 months.

The movement of both the herds in dry season was restricted around the Chilla forest of the RNP mainly due to presence of natural water in some of the forest pockets. Besides, river Ganges is flowing adjoining to this forest, which further ensures the long-term movement of elephants in the adjoining forest areas. At the onset of rains the herds extended their movements to Shyampur and Chiriapur forest (HFD) and Laldhang forests (LFD) area. For a long period of time (4-5 months) Garhwal herd movement was also occasionally observed in Bijnor forest division adjoining to Sigaddi forest beat of Lansdowne forest division. There was considerable difference in the seasonal and annual home range sizes of the herds (Table 2).

Table 2. Annual and Seasonal Home Range Size (km^2) of the Herd Ist (Chilla Herd), Herd IInd (Garhwal Herd), Bull Ist (Ganesh) and Bull IInd (Hitler) during 2005-2007.

S. No.	Group / Bull Elephant	Home Range Size (Km^2)				Number of Locations of Sightings			
		Summer	Winter	Monsoon	Annual	Summer	Winter	Monsoon	Annual
1.	Herd Ist (Chilla Group)	18	24	21	63	194	183	127	504
2.	Herd IInd (Garhwal Group)	20	13	24	57	181	162	114	457
3.	Bull Ist (Ganesh)	83	63	87	233	147	123	97	367
4.	Bull IInd (Hitler)	174	84	177	435	129	113	89	331

Bull Movements

The movement of the first bull elephant (Ganesh) in dry season was restricted around the Chilla forest of the RNP and its adjoining areas whereas the movement of second bull (Hitler) was not confined to ant particular area. As both the bulls are sharp crop raiders, therefore, the movement of both the bulls was regularly observed in Shyampur forest of the Hardwar forest division. First bull elephant was located on 147 days (summer), 123 days (winter) and 97 days (monsoon) and the second bull 129 days (summer), 113 days (winter) and 89 days (monsoon). The annual home range of second bull (Hitler) was approximately two times larger than the first bull (Ganesh). The summer home range of the first bull was 83 Km^2 while the second bull was 174 Km^2. Similarly, the winter and monsoon home range of first bull was 63 Km^2 and 87 Km^2 while that of second bull was observed to be 84 Km^2 (winter) and 177 Km^2 (monsoon) (Table 2).

Bulls always range over a longer area as part of their habit. In RNP and its adjoining areas bull were sharply observed to raid crops in out skirts of protected areas as compared to Herds. During the musth period the movements of bull elephants were enhanced and they traveled long distances. The first bull (Ganesh) was more than 45 years old whereas the second bull was aged between 35-40 years. Our long course of study on the behaviour of elephants in this region indicated that the second recognized bull (Hitler) was very aggressive and as per our earlier observations it has killed about eight persons in the same area during the last two years. The summer and monsoon home range size of the second bull (Hitler) was comparatively larger, whereas the home range size of the first bull (Ganesh) showed a definite movement pattern with a greater monsoon home range size. The home range of both the bulls was more overlapped with herd's movements during summer.

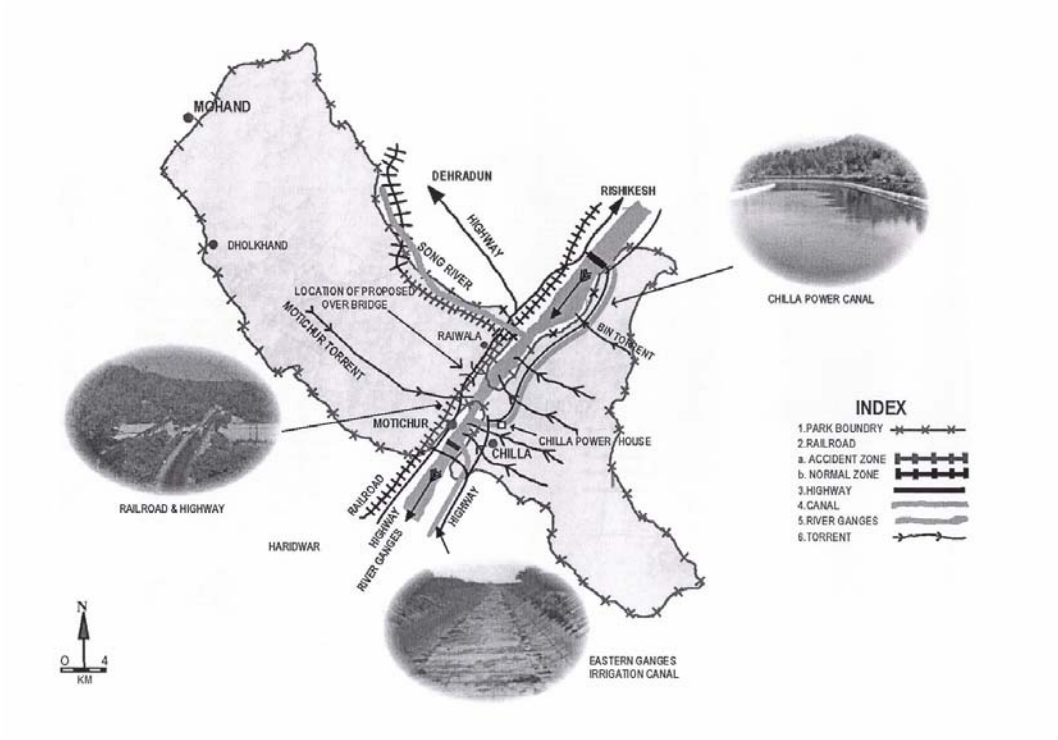

Figure 1. Map of the Study Area (RNP).

Figure 2. Chilla Herd (Herd Ist) at Luni river of the Rajaji National Park.

Figure 3. Garhwal Herd (Herd IInd) at Siggadi Forest of Lansdowne forest division.

Figure 4. Bull Ist (Ganesh) at Chilla Forest of the Rajaji National Park.

Figure 5. Bull IInd (Hitler) at Mundal Valley of the Rajaji National Park.

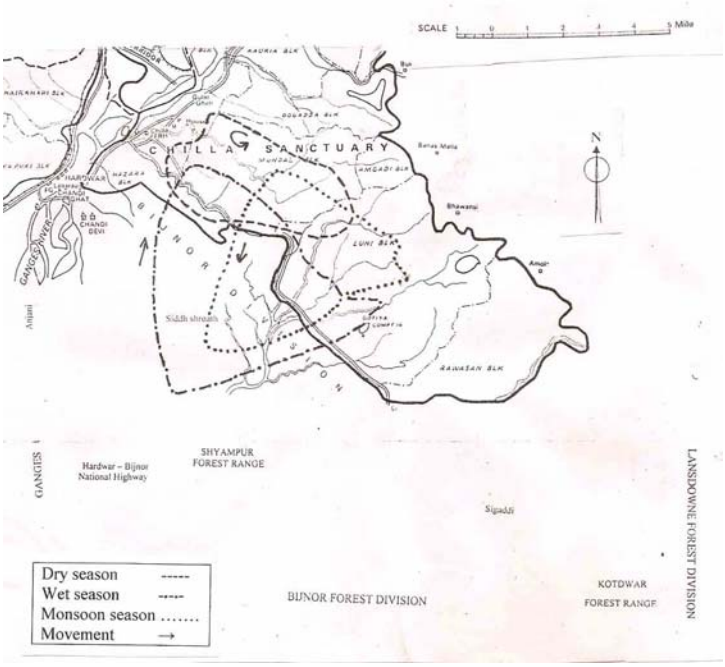

Figure 6. Seasonal Home Range of Ist Herd (Chilla Herd) in and adjoining to Chilla Forest of the Rajaji National Park.

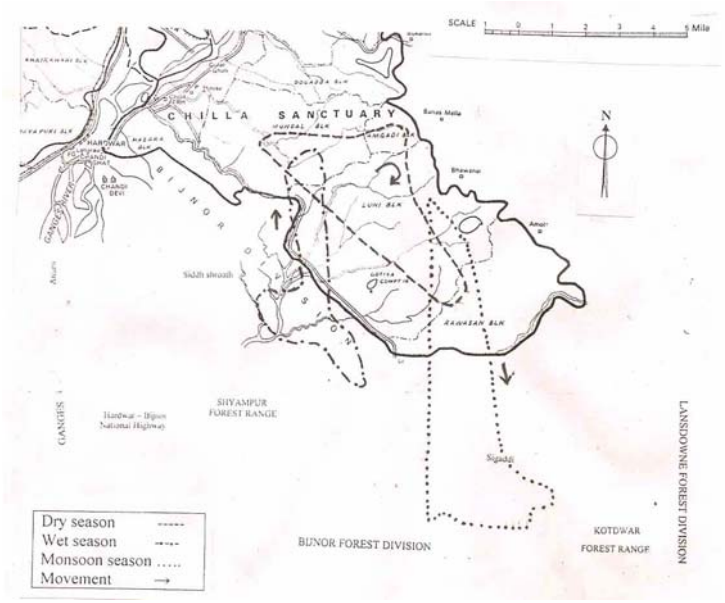

Figure 7. Seasonal Home Range of IInd Herd (Garhwal Herd) in and adjoining to Chilla Forest of the Rajaji National Park.

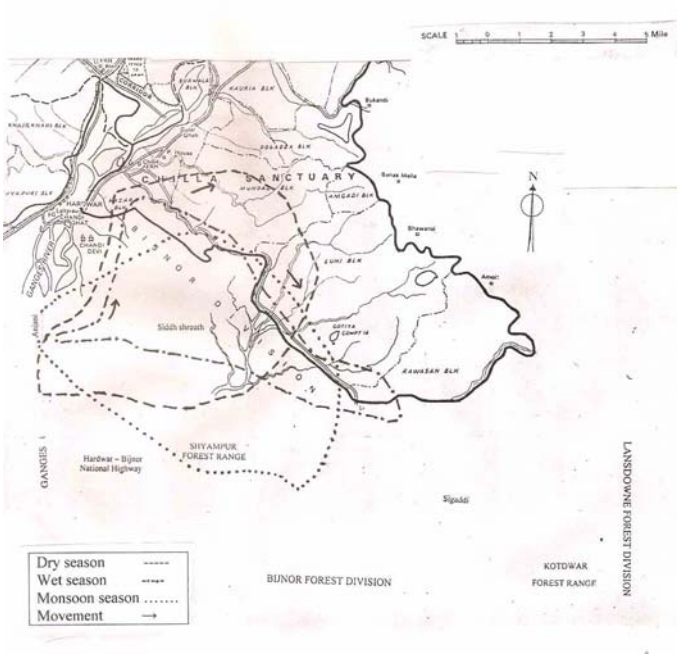

Figure 8. Seasonal Home Range of Ist Bull (Ganesh) in and adjoining to Chilla Forest of the Rajaji National Park.

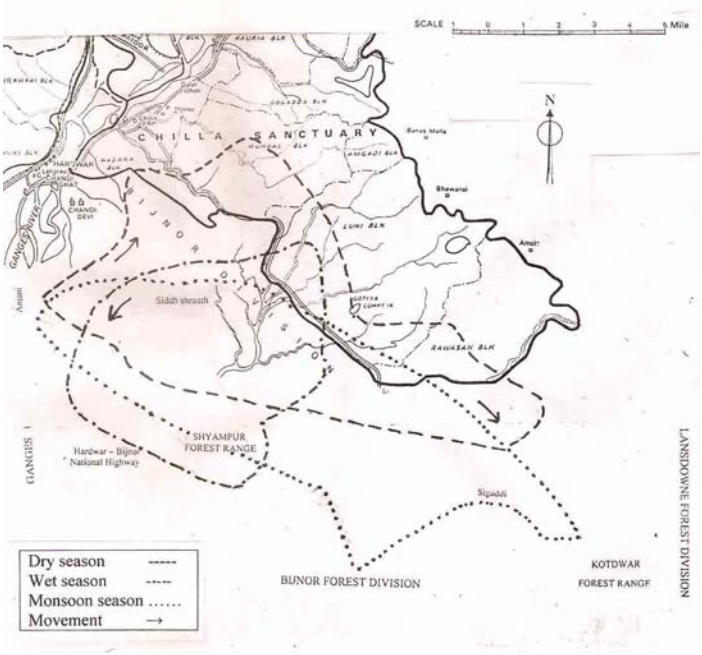

Figure 9. Seasonal Home Range of IInd Bull (Hitler) in and adjoining to Chilla Forest of the Rajaji National Park.

Figure 10. Recording the Geo-Positioning of Ganesh during his walk at Shyampur forest.

Figure 11. Observing the Associational Behaviour of Ganesh (facing behind).

Discussion

During the recent past extensive work has been carried out on the movement pattern and habitat utilization of Asian elephants (Singh, 1969; McKay, 1973; Ali et al., 1986; Santiapillai and Suprahman, 1986; Easa, 1988; Daniel, 1988; Sukumar, 1989; Datye and Bhagwat, 1995; Williams and Johnsingh, 1996; Williams, 2002; Joshi, 2002; Joshi and Singh, 2007) but enhancing rate of developmental and anthropogenic activities forced elephants to change their traditional routes and therefore, regular documentation of movement activities is highly required to know the exact population persistence of species. Besides, wildlife biologists have also explored new conservation tools regarding to long term survival of various endangered wild species but currently there is a need to obtain more and more biological information about wild species to enhance the status of their habitat and population in forest habitats.

Seasonality of Movements

Some herds, especially those in the eastern and western regions of Corbett Tiger Reserve, showed distinct seasonal movements. Others, such as the elephants in the Kalagarh region show little seasonal movement (Singh, 1969). Movement pattern of elephants in the study area indicated that elephants alternatively utilize the Chilla forest (RNP), Shyampur and Chiriapur forest (HFD) and Laldhang forest (LFD). During the summer, elephant's movement was more in the Chilla forest and at the onset of monsoon they migrate towards Laldhang and Shyampur forest. And at that time their movement was more common near the foothills dominant areas as all the small ponds were fulfilled with water during the said period. At the onset of winter they again migrated towards lower slopes and their movement was more observed in grassland dominated areas. During the winter elephant's movement activities generally enhance and they travel more distances up to the onset of summer. Few of the bull elephants and occasionally Herds use whole of the forest for their movements.

Overlapping of the home ranges of Herd Ist and IInd and bull Ist and IInd was more observed during summer season, which was also the time for mating in elephants. Mixing of the adult bulls, selection of prospective partner to mate, smelling of genital organ and discharge of urine were few of the major features of mating behaviour in elephants. Breeding season in Rajaji National Park was noted to be extending maximum from May to November, which through embraces the hot, rainy and beginning of cold seasons but largely the warm period (Joshi, 2008). Water and fodder are one of the important factors, which influence the distribution of elephants. Besides, forest fire also influences the range utilization in elephants. But, fodder species presence did not restrict the movement of elephants in study area as all the forest zones are enriched with huge amount of food plant species, which the elephants like.

Elephant's diet in Rajaji mainly consisted of 50 plant species, which are available to them alternately round the year. Alteration between a predominantly browse diet throughout the year with a grass diet during the early dry season was related to the seasonally changing mineral content of grasses. Consumption of tree species (74%) was highest as compared to grasses (14%) and shrubs (8%) but their diet was mainly dependent on availability of seasonal food round the year and on their migration (Joshi and Singh, 2008b). The seasonal movement of the Chilla herd (Herd Ist) and Garhwal herd (Herd IInd), which took place during 2006-2007 (summer, winter and monsoon) was almost similar to that of 2005-2006 but a slight change was observed in the movement of Herd Ist during their migration towards Chilla area in the beginning of winter. During 2007 heavy flood was observed in annual water streams of Chilla forest (Mundal, Ghasiram and Hazara) and due to this different grassland has got destructed. A large extent of the Chilla forest was affected from the impact of heavy floods during July, August and September, 2007. On one hand floods has affected the migrational pattern of elephants and on the other hand disrupts the natural regeneration potential of several grass species those grow especially in between dry river beds (Joshi and Singh, 2008a).

Riparian wildlife corridors (annual water stream beds) inside the RNP generally comprises of several fodder grass species like *Saccharum munja*, *Saccharum spontaneum*, *Desmostachya bipinnata* and *Cynodon dactylon*. All of these species are the favourite food item for elephants. Besides, few tree species those grow generally nearer to the Gangetic plains like *Acacia catechu, Dalbergia sissoo, Bauhinia variegata*, *Albizzia lebbek*, *Ehretia laevis* and *Lagerstroemia parviflora* were also damaged due to heavy floods. Mundal valley consists of larger feeding grounds (grasslands) but this environmental event has destroyed whole of the area. Water flow has also caused damage to some extent in higher slopes of the protected area. Grass species which grow in profusion in higher altitude area like *Neyraudia arundinacea* were severely affected due to floods (Joshi and Singh, 2008a).

The movement of the Chilla herd was nearer to the *Haplophragma adenophylla* (Kut Sagaun), *Tectona grandis* (Sagaun) and *Mallotus phillipinensis* (Rohini) species during 2006 whereas next year their movement was not observed nearer to *Haplophragma adenophylla* and *Tectona grandis* forests as these species were infected by termites during 2007 in some parts of Chilla forest beat. As bull elephants were observed to be sharp cop raiders, therefore, they perform extensive movements nearer to human habitation areas besides, their movements is also influenced by musth phenomenon and to search prospective group and female. Some time their movements were also observed in National Highways passing in between the protected habitats while moving towards adjoining village to feed on cultivated crops (Figure 10 & 11).

As per the observations of a previous study on radio-telemetry of elephants in Chilla forest, the annual home range of bull and the cow was observed to be 200 Km^2 and 34 Km^2 (Joshua and Johnsingh, 1995). Elephant's home ranges were estimated to range from 188 Km^2 to > 400 Km^2 but no differences were found between male and female home ranges (Williams, 2002).

In Kuppam, Palamaner, Gudiyattam, Panganur and Bangarupalayam (south India) elephant's home range was estimated to 409 Km^2. Similarly, a study in south India estimated the home range of elephant's herds to be 105 Km^2 and 115 Km^2 (Sukumar, 1989). Home range size of two different Herds was estimated to be 34.7 Km^2 (dry season) and 87.2 Km^2 (wet season) and 81.3 Km^2 (dry) and 46.1 Km^2 (wet) in Parambikulum Wildlife Sanctuary, south India (Easa, 1988). Movement of elephants in Dudhwa National Park has been also seasonal and erratic. Animals of the western Nepal population range south along the Karnail river from the reserve to a patch of riverine forest across the border along India (Javed, 1996). The home range estimated to be 258.6, 3343.1 and 4348.9 Km^2 for three different bulls in Dalma Wildlife Sanctuary and Chhotanagpur Plateau (central India). Study also highlighted that home ranges expanded to be maximum in winter and shrunk to minimum in summer (Datye and Bhagwat, 1995).

The same populations of elephants used to perform their movements in Gohri, Chilla, Laldhang, Kotdwar, Shyampur, Chiriapur and Sonanadi forest whereas Herd movement was almost restricted towards Corbett National Park area as both of the forest zones are disconnected mainly due to huge amount of anthropogenic and developmental activities. It was also observed during the study period that Chilla forest was the favourite place for elephants during summer and large number of elephants stayed in this forest and among them some herds represent their seasonal traditional movements adjoining to river Ganges. In few of the places, elephants utilize the same feeding grounds round the year (recognised Herds).

Elephants inter-change the forests of Rajaji and Corbett National Park as their part of traditional migration. But presently in few of the areas their traditional feeding grounds and corridors are denied to them, which have causes man–elephant conflict. The long-term effects will include genetic isolation, habitat fragmentation within the same forest and enhancement in the human-elephant conflict in adjoining areas. Genetic isolation of elephant populations may also increase the chances of replacement of interbreeding to intrabreeding, and thereby reduce the population persistence even for wide ranging wildlife species (Joshi and Singh, 2008a).

Same situation is with other corridors present adjacent to the RNP area. Kotdwar – Lansdowne road runs parallel to the river Kho and crosses the Rajaji-Corbett corridor, the major movement

track of northwestern elephant population between the Yamuna and river Sharda. This road serves as the major transport link between Pauri town and Kotdwar area. The presence of traffic on the road, construction of steep retaining walls by the side of road and the presence of human population along the entire corridor area have almost restricted the migration of elephants using this corridor (Johnsingh and Williams, 1999). The motor roads, which are adjacent to the forests like Hardwar-Dehradun National Highway and BHEL roads have heavy traffic pressure. As per a preliminary study, the average number of vehicles passing on Dehradun-Hardwar road per day is 7,929 and all the wild animals, including elephants, are not in a position to cross this track at any time due to the presence of heavy traffic (Singh and Sharma, 2001).

A large mammal like the elephant could be expected to move more considerable distances even with a short period and families of a clan seemed broadly coordinated in their seasonal movements (Sukumar, 1989). In the dry months (January to April), when no rainfalls occur, the Herds seek the neighbourhood of streams and shady forests. From the month of July, after the first shower, they start roaming and feed on the fresh grass. This grass in hill tracts become long and course by July and August, the elephants then shows their upward movements. The reason for the elephants and other animal's migration is the high lands, continuous and uninterrupted hilly terrain for grazing, assured food, ideal breeding ground and thick population (Sinha, 1981).

During the last 5-6 years, state Government has constructed about six flyovers in Hardwar – Bijnor National Highway. As a result of anthropogenic activities about 18 kilometers forest stretch existing on both the sides of highway has got disturbed. Besides, agricultural expansion near river Ganges has lead to the loss of forest wealth, which has also hindered the traditional movement of elephants. This forest stretch is one of the major corridor for elephant movement and presently has got disturbed mainly due to habitat loss around the national highway. Sometimes few of the male elephants associate to enter the forest near to river Ganges through this route. Elephants cross the national highway in the evening hours and return back to the forest area during early morning hours.

Besides, elephants also utilize the Gaziwali bridge, Shyampurwali bridge and Pili bridge situated peripheral to the canal road over to Ganga canal for their movements towards western direction and to feed on the cultivated crops in nearby villages. During the study period all the villages suffering from crop raiding have been investigated. The affected villages are Jagjeetpur, Mishrpur, Panjneri, Ajeetpur and Jaipota in the western side of the conservation area and all these villages are situated peripheral to river Ganges. Villages Kangri, Ghaziwali, Shyampur, Sajanpura, Pili and Rasiabad are located peripheral to forest area and national highway whereas villages Gaindikhata, Lahadpur, Chiriapur, Vasuchandpur and Naurangabad are also situated adjacent to the forest area and national highway on south western direction of the conservation area.

The villages along the river Ganges are situated on land that was once part of the elephant's home range. Therefore, the increasing elephant – man conflict is unfortunate but inevitable. The electric fence erected along with these villages and river Ganges has presently got damaged due to lack of proper maintenance. It was observed that elephants are utilizing their traditional feeding grounds in few of the areas, which are presently denied to them and are replaced by human settlements.

The present study reveals that elephants utilizes whole of the park area for their movement, but mostly they leave some of the areas for few months, as part of their seasonal migrational activities. The local movement and long term migration of elephants within the RNP shows a definite range use pattern. After the isolation of Chilla forest and Motichur forests the elephants population of the RNP has divided into two parts. Presently, elephants of Chilla and adjoining areas in the eastern part of river Ganges show the better migration between the Chilla area and Kotdwar (LFD) whereas the elephant populations of Hardwar, Motichur, Kansrao and Dholkhand has been isolated. Again due to large scale developmental activities inside the Dogadda forest area has caused the hindrance in their corridor area. Slowly seasonal movements and migratory routes have also undergone to minor changes. Elephants in North Bengal are pocketed but these pockets have

increased in number and also changed their locations with the passage of time. Elephants are trying to adopt themselves to the changing environment by changing their ranges, moving on to new areas and by adopting new routes (Barua and Bist, 1996).

The reasons for migration of elephants can be annual fire, drought, non-availability of fodder, paucity of drinking water and absence of cool green shades in their respective areas (Ramachandran, 1990). In Chilla, the elephants, which were deep in the hilly terrain of north in the rainy season, gradually start moving towards the south due to scarcity of water winter season in the hilly areas. The study further reveals that the animals are directly affected by water availability and availability of fodder species inside the park area. Presence of river Ganges in Chilla area further ensures the migration of animals at the onset of summer. Most of the seasonal migratory routes through which elephants performed their long-term migration have been shrinked presently as the result of which elephants of Rajaji are restricted to move only in internal ecological units, whereas bull elephants occasionally were observed to move within such long corridors like Chilla - Motichur and Khara – Anjani (Joshi and Singh, 2007).

India's elephant populations are currently threatened by habitat deterioration, developmental activities, anthropogenic pressure inside the deeper forest regime and unregulated exploitation of natural resources. Effective human-elephant conflict mitigation cannot take place in the long-term without the involvement and true support of the local communities. Similarly, rural livelihoods depend on a flow of natural resource benefits, many of which cannot be sustained without active protective measures. To build these partnerships requires greater understanding about working with local communities in designing programs to realize joint benefits. It also requires effective community empowerment to allow the communities to plan for wildlife management and conservation.

The Rajaji National Park, Lansdowne and Hardwar forest divisions are important biological areas and have great potential for wildlife and its conservation. One line of evidence is that the Rajaji National Park harbours important populations of species on the IUCN Red list, the Asian elephant. In recent years, human activities have expanded in the boundaries of the protected areas and as a result most of the wildlife corridors have been shrinking rapidly and elephant's long-term migration became restricted, which can influence their population persistence.

Recommendations

1) As pointed out from the present investigation that the same herds were utilizing the Chilla (RNP), Shyampur and Chiriapur (HFD) and Laldhang (LFD) forest alternatively round the year and their movements are entirely seasonal, therefore, proper census should be carried out, which will provide us the exact population composition database of elephants of this entire forest stretch.
2) During the course of elephant movement nearer to the national highways (Hardwar – Dehradun and Hardwar - Bijnor), traffic should be stopped at a safe distance. At the same time people are not allowed to deter the elephants.
3) In Chilla forest elephants interchange the forest through a small bridge over to Ganga canal commonly known as Soni shroth. The bridge should be widened to some extent so that elephants may cross easily.
4) Ghasiram shroth is a traditional corridor in Chilla area and elephants utilize this track especially during dry months. During that period traffic of the Chilla – Rishikesh road should be stopped in evening hours. Similarly, heavy traffic should not allowed in Laldhang – Kotdwar forest road.
5) Dudhia forest beat (island) and the islands situated in between river Ganges should be restored from any anthropogenic disturbances.
6) Grazing may be banned at least from the crucial areas of the corridors.
7) Habitat restoration may be planned to enhance forest cover in degraded areas. Besides, plantation of fodder plant species is also required.

8) Artificial water holes must be created, spread within the park area at short distance. For solving the problem water uplifting pumps will be used to uplift the well water during day hours, which will help during dry periods. Waterholes can also be connected with Ganga canal and management related practices should be carried out regularly.
9) Relocation of villages and Gujjar deras (shelters) those are in the corridor area.
10) It was observed during the study period that the population of elephants of Gohri, Chilla, Shyampur, Chiriapur and Laldhang forests are same and their movements in these forest zones are entirely seasonal. Therefore, proper monitoring of elephant's populations is required and few research studies are also required, which will help us in documenting the appropriate management plan.
11) Besides, managed tourist activities, minimizing the forest fires effect and proper thinning of trees under high-tension lines, which passess through the protected habitats are highly recommended.

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References

Ali S, Daniel JC, Sivaganesan N, Bhushan B. Study of the ecology of some endangered species of wildlife and their habitats (elephants in Andhra Pradesh). Technical Report. 1986.

Barua P, Bist SS. Changing patterns in the distribution and movement of wild elephants in North-Bengal. In: Daniel JC, Datye HS, eds. A week with elephants. BNHS and Oxford University Press. 1996:66-84.

Daniel, JC. Ecology of the Indian elephant. Technical Report. Bombay Natural History Society, India. 1988.

Daniel JC. Conservation of Asian elephant. Gajah 1996;16:9-16.

Datye HS, Bhagwat, AM. Home range of elephants in fragmented habitats of central India. Journal Bombay Natural History Society 1995; 92(1): 1-10.

Dawson S, Dekker AJFM. Counting Asian elephants in forests. A technique manual, F.A.O., Bangkok. 1992.

Easa PS. Movement pattern of Asiatic elephant, *Elephas maximus* in Parambikulam Wildlife Sanctuary, Kerala. Technical Report. Kerala Forest Research Institute, Peechi, India. 1988.

Eisenberg JF, Lockhart M. An ecological reconnaissance survey of Wilpattu National Park, Ceylon. Smithson Contrib. Zool., Washington DC. 1972;101:1-118.

Hirst SM. Road-strip census techniques for wild ungulates in African woodland. Journal of Wildlife Management 1969;33(1):40-48.

Javed S. Elephants in Dudhwa. Gajah 1996;16:17-22.

Johnsingh AJT, Narendra P, Goyal SP. Conservation status of the Chilla-Motichur corridor for elephant movement in Rajaji-Corbett National Park area. Biological Conservation 1990; 52:125-138.

Johnsingh AJT, Williams AC. Elephant corridors in India: lessons for other elephant range countries. Oryx 1999;33(3):210-214.

Joshi R. Population dynamics and general behavioural pattern of Indian Elephant (Elephas maximus) in the Shivalik foothills of the Rajaji National Park, India. Ph.D. thesis, Gurukul Kangri University, Hardwar, India. 2002.

Joshi R, Singh R. Asian Elephants are loosing their seasonal traditional movement tracks: A decade study in and around the Rajaji National Park, India. Gajah 2007;27:15-26.

Joshi R, Pande GS. Rehabilitation of Gujjar community from the Rajaji National Park: An approach for biological diversity conservation through restoration ecology. Nat. Acad. Sci. Lett. 2007;30(9&10):263-267

Joshi R, Singh R. Asian elephant (*Elephas maximus*) and riparian wildlife corridors: A case study from lesser-Himalayan zone of Uttarakhand. J. Am. Sci. 2008a;4(1):63-75.

Joshi R, Singh R. Feeding behaviour of wild Asian elephant (*Elephas maximus*) in the Rajaji National Park, India. J. Am. Sci. 2008b;4(2):34-48.

Joshi R. Behavioural biology of Asian elephant (*Elephas maximus*) in and around the Rajaji National Park, India with special reference to the impact of railway track on their seasonal traditional migration. Technical Report. G. B. Pant Institute of Himalayan Environment and Development, Almora, India. 2008.

Joshua J, Johnsingh AJT. Ranging patterns of elephants in Rajaji National Park: Implications for reserve design. In: Daniel JC, Datye HS, eds. A week with elephants. Bombay Natural History Society and Oxford University Press. 1995:256-260.

Mckay GM. Behaviour and ecology of the Asiatic elephants in south-eastern Ceylon. Smithson. Contrib. Zool., Washington DC. 1973.

Ramachandran PV. Migration of elephants in Wayanad Wildlife Sanctuary. In: Karunakaram CK, ed. Ecology, behavior and management of elephants in Kerala, Kerala forest Department, India.1990:109-113.

Ramakrishnan U, Santosh JA, Sukumar R. Censusing elephants in forests. Technical report No.2, AESG–IUCN / SSC. 1991.

Santiapillai C, Suprahman H. The ecology of the elephant (*Elephas maximus* Linn.) in the Way Kambas Game Reserve, Sumatra. WWF / IUCN- Final report, Bogor. 1986.

Santiapillai C, Wijeyamohan S, Wijesundara C, Vandercone R. Population structure, composition and abundance of elephants Elephas maximus in Minneriya National Park, Srilanka. Journal of Bombay Natural History Society 2003;100(2and3):308-321.

Singh VB. The elephant (*Elephas maximus* Linn.) in Uttar Pradesh, India. Journal Bombay Natural History Society 1969; 66(2): 239-250.

Singh AP, Sharma RC. Conflicts between linear developments and Asian elephants in sub-Himalayan zone of Uttaranchal. In: Irwin CL, Garrett P, McDermott KP, eds. Ecology and transportation. Centre for Transportation and the Environment, Raleigh, North Carolina State University. 2001:423-432.

Sinha MK. Elephant migration in Kaziranga. Tiger Paper 1981;8(1):16-18.

Sukumar R. The Asian elephant: Ecology and management. Cambridge University Press, Cambridge. 1989.

Williams AC, Johnsingh AJT. Status survey of elephants and their habitats in Garo Hills, north east India. Gajah 1996;16:43-60.
Williams AC. Elephants (*Elephas maximus*), their habitats in Rajaji – Corbett National Parks, north-west India. Ph.D. thesis, Saurashtra University, Rajkot, India. 2002.

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Quantitative analysis of fallen lichen vegetation in eleven forest sites of a *Quercus semecarpifolia* forest of Garhwal Himalaya, India

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ABSTRACT: The present study was carried out on eleven forest sites dominated by *Quercus semecarpifolia* forest to assess fallen lichen (fall from trees) diversity between 2500m to 3500m elevation in Garhwal Himalaya. A total of ten fallen lichens were recorded from the study area. [Nature and Science. 2009;7(2):95-100]. (ISSN: 1545-0740).

Keywords: Fallen lichens, study sites, distribution pattern, Garhwal Himalaya

Introduction

The Himalayan Mountain (27´38 º N latitude and 72´98 º E longitude) is the youngest, largest, highest and most complex mountain system in the world covering east to west (Gupta, 1963). On the basis of altitudinal variation the Himalayan ranges are divided into sub-tropical, temperate and alpine zone representing a variety of forest types.

Garhwal Himalaya is extremely rich in lichen diversity, it is about 69% of the Uttarakhand and 35% of the Himalayas and more than 16% of Indian lichen diversity (Kumar, 2008), and its climate factors, temperature variations, rainfall pattern, soil support, strong fauna and flora. Kumar (2008) reported 106 species of lichens from the area and also reported ten regularly fallen lichen species. Studies of the Northwest Pacific forests indicate that lichens are important component of food chain, and they play a significant role in forest nutrient cycling (Pike 1978; Maser et al. 1985).

In this article author describe the diversity of fallen lichen genera and their distribution pattern in different forest sites of a brown oak (*Quercus semecarpifolia*) forest.

MATERIALS AND METHODS

Study area Chopta was located at altitude between 2500-3500m elevations of Garhwal Himalayas India. Altitudinally Chopta is located in temperate zone. For the detailed study of fallen lichen (fall from trees) diversity, the area was divided into eleven different sites. All the sites broadly have similar major tree species. *Quercus semecarpifolia* and *Rhododendron arboretum* trees was the major tree species present in all the eleven investigated sites. In all the sites the forest cover were recorded between 32-58% (Kumar, 2008).

The phytosociological analysis of the fallen lichen vegetation was done by sampling of 40, $2M^2$ ground quadrats on each site. All the individuals of fallen lichen genera were recorded carefully in each sampled quadrat. The collected lichen samples were identified in the Lichen Laboratory, IBRI Lucknow.

The data on fallen lichen vegetation were quantitatively analyzed for abundance, density, and frequency and A/F ratio by the following formulas given by Curtis and Mc Intosh (1950).

$$\text{Abundance} = \frac{\text{Total number of individuals}}{\text{Number of quadrat occurrence}}$$

$$\text{Density} = \frac{\text{Total number of individuals}}{\text{Total number of quadrats studied}}$$

$$\text{Frequency (\%)} = \frac{\text{Number of quadrats occurrence X 100}}{\text{Total number of quadrats studied}}$$

Distribution of population: The ratio of abundance to frequency is a relative measure to present the distribution of fallen lichen vegetation in a community. Curtis and Cottam (1956) suggested the following for regular (less than 0.025), contagious (0.025-.05) and random (more than 0.05) distribution of the population.

RESULTS

Quantitative analysis of fallen lichen vegetation at different study sites are given in Table 1. A total of 10 fallen lichen genera were recorded from the study area. The density of fallen lichen genera was recorded to be maximum 13175 individuals of *Everniasteru* ha¹ at site 1st and 3rd and the minimum density 125 individuals of *Everniastrum* ha¹ was recorded at site 9th. Among the lichen vegetation maximum density was recorded for 26900 individuals of lichen ha¹ was recorded for site 1st, and the minimum density also recorded 6350 individuals of lichen ha¹ at site 6th (Table 1). *Everniastrum* was the most dominantly fallen lichen in all the eleven investigated sites followed by species of *Usnea*. The other common fallen lichen genera of the study area were *Parmotrema* spp, *Cetrariopsis* spp, *Heterodermia* spp, *Ramalina* spp, *Leptogium* spp, *Parmelia* spp, *Lobaria* spp and *Cladonia* spp.

There was 6.06% fallen lichens displayed regular distribution pattern in the study area as maximum lichens genera (56.06%) displayed their random distribution pattern at different sites and 37.87% genera of fallen lichens contagious distribution pattern at different sites of the study area.

Table 1: Vegetational parameters for fallen lichens at different forest sites

Forest sites	Fallen lichen taxa	Frequency (%)	Density (Ind ha⁻¹)	Abundance (Ind ha⁻¹)	A/F
1	*Usnea*	85	575	2.7	0.031
	Everniastrum	92.5	13175	5.7	0.061
	Parmotrema	62.5	5675	3.64	0.058
	Cetrariopsis	42.5	1375	1.29	0.03
	Heterodermia	32.5	925	1.15	0.035
2	*Usnea*	42.5	3300	3.11	0.073
	Everniastrum	67.5	6500	3.85	0.057
	Parmotrema	57.5	2875	2	0.034
	Heterodermia	17.5	675	1.57	0.089
	Cetrariopsis	15	425	1.16	0.077
3	*Usnea*	55	4300	3.13	0.056
	Everniastrum	87.5	13175	6.02	0.068
	Parmotrema	72.5	5000	2.28	0.031
	Heterodermia	0.35	1175	1.35	3.857

	Cetrariopsis	0.35	1050	1.21	3.457
4	*Usnea*	70	4000	2.28	0.032
	Everniastrum	90	8300	3.69	0.041
	Parmotrema	47.5	2050	1.73	0.036
	Cetrariopsis	37.5	1175	1.26	0.033
	Heterodermia	27.5	750	1.09	0.039
	Ramalina	25	675	1.1	0.044
5	*Usnea*	40	1425	1.43	0.035
	Everniastrum	72.5	6125	3.37	0.046
	Parmotrema	67.5	3125	1.85	0.027
	Ramalina	17.5	425	1	0.057
	Heterodermia	10	650	1	0.1
	Cetrariopsis	12.5	300	1	0.08
	Leptogium	12.5	375	1.2	0.096
	Parmelia	10	2500	1	0.1
6	*Usnea*	22.5	1500	2.66	0.118
	Everniastrum	32.5	2750	3.38	0.104
	Parmotrema	22.5	925	1.66	0.073
	Ramalina	12.5	425	1.4	0.112
	Cetrariopsis	7.5	375	2	0.266
	Heterodermia	12.5	375	1.2	0.096
7	*Usnea*	50	5925	4.75	0.095
	Ramalina	55	1375	1.46	0.026
	Parmotrema	32.5	1625	2	0.061
	Everniastrum	70	7000	4	0.057
	Heterodermia	32.5	1050	1.3	0.04
	Cetrariopsis	22.5	1300	2.33	0.103
8	*Usnea*	50	5625	4.5	0.09
	Everniastrum	85	11750	5.52	0.064
	Parmotrema	35	4050	4.64	0.132
	Ramalina	22.5	1175	2.11	0.093
	Cetrariopsis	10	500	2	0.2
9	*Usnea*	42.5	2125	2	0.047
	Everniastrum	52.5	5925	4.52	0.086
	Parmotrema	40	2000	2	0.05
	Heterodermia	10	500	2	0.2
	Cetrariopsis	5	125	1	0.2
	Ramalina	7.5	250	1.33	0.177
10	*Usnea*	67.5	3050	1.81	0.026
	Everniastrum	82.5	8300	4.03	0.048
	Parmotrema	82.5	4500	2.18	0.026
	Heterodermia	17.5	625	1.42	0.081
	Cetrariopsis	22.5	675	1.22	0.054
	Ramalina	22.5	750	1.33	0.059
	Lobaria	10	300	1.25	0.125
	Leptogium	15	625	1.66	0.11
11	*Usnea*	47.5	2925	2.47	0.052

	Everniastrum	65	5875	3.61	0.055
	Parmotrema	70	5425	3.1	0.044
	Heterodermia	32.5	1175	1.46	0.044
	Cladonia	7.5	250	1.33	0.177
	Ramalina	25	750	1.2	0.048

DISCUSSION

Lichen fall is a relatively more continuous process in the temperate oak forest of the Garhwal Himalaya. In the Central Himalayan forests, water stress and extremes of temperature are probably not the dominant causal factors of wood fall. The abscission of wood is promoted by higher temperatures in the annual cycle (summer and rainy seasons) although abscission continues, though irregularly, through out the year as a mechanism of canopy-clearing by self-pruning (Singh and Singh, 1992). According to the concept of Stone (1989) allogenic factors caused by outward growth of oak canopy, including changes in microclimate and thickening and sloughing of bark, appear to be far more important to most species than changes brought on by the epiphytic species. However, within the framework of the allogenic tree canopy factors, the same sorts of interspecific interactions take place as more found in autogenic type of succession.

The fallen density depends on the forest cover and tree density, site 5^{th} & 10^{th} represented by 8 fallen lichen genera followed of 6 at site 4^{th}, 6^{th}, 7^{th}, 9^{th} & 11^{th}, and 5 genera at 1^{st}, 2^{nd}, 3^{rd} & 8^{th}. Lichen genera *Parmelia*, *Leptogium* and *Stcta* of the study area were found rare.

According to Kumar (2008) the lichen fall in a particular area may be affected by a number of climatic factors and activities of the inhabitants of the area. The common factors responsible for lichen fall in the study area were type of fauna (jumping of Languor's from one tree to other), birds, heavy snow fall, hails, heavy rainfall, human activities and wind condition, direction.

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REFERENCES

Curtis, J.T. and Mc Intosh, R.P. The interrelation of certain analytic and synthetic phytosociological characters. *Ecology* (1950), 434-455.

Curtis, J.T. and Cottam, G. The use of distance measure in phytosociological sampling. *Ecology* (1956), 37:151-160.

Gupta, A.C., 1963. Annual precipitation and the vegrtion of the dry temperate coniferous region of North-West Himalaya. *Jour. Inl. Bot. Soc.* (1963), 42 (2).

Kumar, B. Lichen diversity of Baniyakund-Chopta region of region of Garhwal Himalaya, India. *A national seminar on globalwarming* (2008), SMJN College Haridwar. p 46.

Kumar, B. Lichen species distribution, cover and fall in a *Quercus semecarpifolia* (J E Smith) forest of Garhwal Himalaya, Ph. D. Thesis (2008), HNB Garhwal University, Srinagar (Garhwal), India.

Maser, Z., Maser, C. and Trappe, J.M. Food habits of the northern flying squirrels (*Glaucomys sabrinus*) in Oregon. *Canadian Journal of Zoology* (1985) **63**: 1085-88.

Pike, L.H. The importance of epiphytic lichens in mineral cycling. *The Bryologist* (1978) **81**(2): 247-57.

Singh, J.S. and Singh, S.P. Forest of Himalaya: Structure, Functioning and impact of Man. Gyonodaya Prakashan, Nainital (India) (1992).

Stone, D.F. 1989. Epiphytic succession on *Quercus garryana* branches in Willamette valley of western Qregon. *Bryologist* (1989) **92:** 81-94.

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Lichens Resource Use Pattern and its Socio-economic Status in Temperate Region of Garhwal Himalaya, India

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ABSTRACT: The resource use pattern of some macrolichens and their socioeconomic status in temperate region of Garhwal Himalayas has been discussed. Out of five blocks, stakeholders of Narayanbagar and Dewal block are found highly dependent on lichen (macrolichens) based activity to conduct their livelihood. [Nature and Science. 2009;7(2):101-106]. (ISSN: 1545-0740).

Key Words- Macrolichen biomass, *Quercus semecarpifolia*, Garhwal Himalaya

INTRODUCTION

According to the concept of Upreti et al (2005) the lichens have been household items of Indians since ancient times. In India, lichens collected from the temperate regions of the Himalayas are used indigenously and are explored. The Uttarakhand hills and Himanchal Pradesh are the main areas of the lichen collection in India. The lichens are very slow growing plants. Because of their unique thallus composition, which is made of fungus and alga, they can not be cultivated in large scale like other plants. Thus, lichens growing in nature provide a basic raw material required for various uses of lichens. The lichens weigh very little when dry, thus a vast bulk of these plants is required.

Mountain and hillside areas hold a rich variety of ecological systems. Because of their vertical dimension, mountain creates gradient of temperature, precipitation, and insulation. In Uttarakhand nine of the thirteen districts comprise the expansion of lesser Himalaya. But with the pace of rapid modernization and increasing anthropogenic pressure on vegetation in general and on forest in particular coupled with natural disasters, the Himalayan vegetation is rapidly deteriorating in its richness as well as diversity. However, in recent past there has been a deep concern and realization for the conservation of the fragile Himalayan ecosystem.

Lichen exploitation is a common practice among the villagers and the rivals in moist temperate regions of the Garhwal Himalaya to collect the lichens together with tree twigs as oak and other trees bears luxuriant growth of lichens. Kumar (2008) reported Parmelioid lichens belonging to family Parmeliaceae are commercially trading lichens from Garhwal Himalaya i.e. *Everniastrum, Parmotrema, Cetrariopsis, Bulbothrix, Hypotrachyna and Rimelia* collected by rivals together with two fruticose genera, *Ramalina* and *Usne.*

Lichens in India are collected from the temperature regions of Himalayas and used indigenously for preparation of perfumes, dyes, and condiments (Kumar and Upreti, 2008). Approximately 750 metric tons of lichens are collected from Uttarakhand hills, 800 metric tons are imported from other regions of India, including Himachal Pradesh, Sikkim and Assam and out of which about 50-80 tons are exported (Shah, 1997).

Upreti (1995) assessed the different factors responsible for loss of lichen diversity in India. Important factors include the change in the ecological conditions, forest cover, and loss of habitat and increase of the urban and industrial areas. The various activities of man in hilly regions of India such as 'Jhoom' cultivation, agriculture, mineral extraction, tourism, hydroelectric and road building projects are other factors leading to the rapid deterioration of lichen rich habitats. Overexploitation and selective removal of economically important lichens by local people have now become the major threat to the lichen flora of India.

Lichens are sold at rates of approximately half a dollar/kg in the local markets (Upreti et al 2005). The price however doubles when these lichens reach the central market areas. A trained

collector can easily collect 6-8kg of lichens with twigs from the ground (collecting lichens from attached twigs slow down the collection as the entire branches are cut or the lichens are scraped off along with the bark and portion of sapwood). A collector for the major part of the year can earn a reasonable income by collecting the fallen lichens without being destructive with some knowledge of the fall and seasonal pattern.

A number of lichen patches in the forests 'hot spots' were identified together with the study viz. Bramtal, Jhaltal, Suptal, Bhekaltal, Didina forest, Kuling forest, Ghesh-Balan, Badeni forest, and Gairsain forest patches are in Chamoli district. Similarly Chopta-Tunganath, Khod-Bakseer, Badhanital, Devariyatal, Madhmaheshwar peak, and Tirjuginarayan forest patches were the major hot spots in Rudraprayag district. These all identified forest patches are similar in lichen diversity as well as for lichen biomass resource availability. These all forest patches (lichen hot spots) are purely dominated by the *Quercus semecarpifolia* (brown oak) trees and some time associated with *Rhododendron arboreum* (Burans) trees and associated shrubs *Barberis* spp and *Cotoneaster spp* occurs in these regions.

METERIALS AND METHODS

District Rudraprayag and Chamoli Garhwal of Uttarakhand state are the remotest areas in terms of lifestyle and also rich in botanical resources like lichen resource. A total of five blocks has been covered in two districts Rudraprayag and Chamoli. Ukhimath and Jakholi blocks in Rudraprayag and Dewal, Tharali and Narayanbagar in Chamoli district have been studied. From each selected block of Chamoli district, selected three village randomly villages on the basis of the temperate region, availability of lichen resource, lichen exploitation by local collectors and the areas were open for lichen collection. But the district Rudraprayag was totally band for lichen collection since ten years; from this district only two blocks (Ukhimath and Jakholi) were selected. The three selected blocks of district Chamoli were similar in lichen diversity and resource use pattern, but different in its collection and trading system.

A. Reconnaissance Survey: The reconnaissance survey was conducted for knowing the traditional method of lichen collection and involvement of lichen stakeholders of different rivals of the area. The traditional method of lichen collection is locally called 'Makku Tipan'. The method has been traditionally followed by lichen collectors of some lichen exploiting areas of Deval and Tharali block of Chamoli district of Uttrakhand state. In Chamoli district, lichens collected by the villagers or lichen collectors of Ratgawn, Bursol, Dungari, Man, Kolpuri, Mundoli, Vaan, Kuling and Ghes villages of the Tharali and Deval block. These areas come under the Badrinath forest division. These areas falls within the Garhwal Himalaya region and the forests are dominated with *Quercus semecarpifolia* (brown oak) and these areas lies between 2000m to 3000m altitudes in west Pinder range of Tharali Tehsil. Brown oak trees of the area harbors luxuriant growth of epiphytic lichens.

The traditional collectors of the villages are collects these plants and sale in local market at Tharali, Deval and Narayanbagar. Some small villagers sold it at Kerabagar and Vaan village of the area.

During the field visit author have interviewed with some lichen collectors and local contractors to asses the information on traditional method of lichen collection, extraction, resource use pattern and socioeconomic status of lichens (macrolichens) in the area.

B. Questionnaire Design: An ideal questionnaire was prepared after complete search of available literature on the lichen ecology and its economic role in our vital needs. The questionnaire was designed with keeping in mind of some tasks related to socio-economic and ecological impacts of lichens, which are always ignored by various workers.

C. Questionnaire Sampling and Selection of the Respondent: The survey was carried out during May-June 2007. The questionnaire was used to gather information on resource use pattern and assessment of earn money from lichen sector at different level of stakeholders. The respondents from the area were selected randomly on the basis of their involvement in the lichen sector as traditional collector, store keepers, packers loaders, horse trackers (transpiring lichens from forest to collection point/store house), local traders etc. were the respondents of the ideal questionnaire.

D. Process Questionnaire Filling: All questionnaires were filled throughout a long discussion along with the respondent.

E. DATA ANALYSIS: The data has been analyzed by using the SPSS software.

RESULTS

Households of Narayanbagar block depends highly on macrolichen based activity to conduct their livelihood represented by 93.65% followed of 63.32% households of Dewal and 18.38% of Tharali block of Chamoli district. Households of Rudraprayag district (Ukhimath & Jakholi block) was found less dependent on lichen sector (Table 1). In both the districts lichen sector found highest contribution to generate income as compare to other sources (Table 2). In both the districts lichen transporters and traders get maximum benefit from lichen sector as compared to other sources like agriculture, labor and shop etc. (Table 3). Earned money of the stakeholders from lichens sector was mostly used to provide foods like rice, wheat, pulses and vegetables etc. and it was less used in other daily needs (Table 4). The lichens collected/extracted from different substratum by the collectors maximum (51.53%) from tree bark followed by 43% and 4% from ground (fallen lichens) and rock substratum and only 1.37% extracted from soil (Table 5).

In the district Chamoli a lichen collector was collected average 254.5 kg lichens per year and its estimated income was Rupees 7668.08 per year @ 30.13 Rs. /kilogram. Similarly, it was in district Rudraprayag the average annual lichen collection was 78 kg and its estimated annual income was only Rupees 2393.82 @ Rupees 30.69/kilogram. Table 5 showed in Chamoli district, April-May (summer season) provided the maximum lichen material (313.4-267.15 kg/month/collector) followed of lowest (12.8kg/month/collector) in rainy season. In the district Rudraprayag, winter season (November to February) showed the maximum collection of lichens and throughout the year it was provided 414 kilogram /collector and provided Rupees 12705.66/ collector/year (Table 6).

Table1. Percentage of households engaged in lichen activity

District	Block	Number of households engaged in lichen activity (% of the total households)
Chamoli	Deval	62.32
	Tharali	18.38
	Narayanbagar	93.65
Rudraprayag	Jakholi	3.4375
	Ukhimath	14.375

Table 2. Contribution of lichens in income generation of lichen stakeholders

Sources of income	Contribution of lichens in income generation (%)	
	Chamoli	Rudraprayag
Service	1.08	0.90
Agriculture	33.82	31.59
Agriculture labor	1.56	1.30
Other labor	5.93	11.66
Lichen collection	56.31	53.02
Shopkeeping	1.29	1.53

Table 3. Shearing benefit from lichen sector at different level of stakeholders

Different level of Stockholders of lichens	Shearing benefit from lichens (%)	
	Chamoli	Rudraprayag
Collector	29.14	30.96
Tracker	15.36	0.00
Transporter & Traders	46.13	58.59
Storekeeper at village	0.00	0.03
Grader & shorter at village	0.84	2.17
Loader (Nepalis at local market)	8.54	8.16
Packer	0.00	0.09

Table 4. Percentage wise use of earned money (from lichens) in different needs of stakeholder

Needs of stakeholder	Percentage wise (%) use of earned money from lichens	
	Chamoli	Rudraprayag
Food	44.00	75.29
Medicines	10.93	6.47
House construction	6.86	4.71
Schooling of children's	1.00	1.18
Agriculture	1.40	0.00
Clothing	18.72	7.06
Assets creation	8.37	5.29
Marriage celebrations	7.21	0.00
Purchasing grams (feed) for horse	1.51	0.00

Table 5. Percentage wise extraction or collection of lichens from different substratum

Substratum	Lichen extraction or collection (%)
From trees	51.53
From rock	4
Fallen lichen collection	43
From soil	1.37

Table 6. Month wise collection of lichens by collectors in Chamoli and Rudraprayag districts

Months	Collection of lichen material kg/month/collector	
	Chamoli	**Rudraprayag**
Jan	190.4	97
Feb	78.36	51
March	62.53	34
April	313.4	23
May	267.15	0
June	74.41	8
July	15.82	0
Aug	12.8	5
Sep	33.54	24
Oct	66.96	52
Nov	75.6	74
Dec	42.1	47
Total	1233.07	414

DISCUSSION

Well resource use pattern of lichens was situated in diatrict Chamoli Garhwal and the collectors were mostly depends on lichen sector. It was the interesting feature of the study, the involvement of outsiders like Nepali's labours are completely restricted in lichen harvesting activity, which were involved only in few cases as loading of lichens and some time grading and sorting of the lichen species. But through the economic point of view, the outsiders are interfering in the income of lichen stockholders at the time of sorting and grading, loading-unloading and transporting from forest to collection point. The lichen traders (local traders) were the highest beneficiaries in lichen sector because they were well aware about this sector.

Lichens are house hold items of some local users and used for Garam Masala for providing flavoring taste through lichens etc. The earned money through lichens about 44% has been used in food by the stakeholders of district Chamoli, and in past the earned money of about 75.29% was used in food by the stakeholders of district Rudraprayag, therefore, at presently the activity is directly effects on food requirements of the stakeholders of district Rudraprayag due to the lichen harvesting activity was totally banned by the forest department. Some other needs like clothing, schooling of children's, medicinal treatments, house constructions, and assets creation of lichen stakeholders were directly effected by the process of opening and closing rules of forest department for lichen harvesting from the forests.

Kumar (2008) hypothesis showed only fallen lichen (fall from trees) collection can be allowed to provide livelihood for some stakeholders of high altitude or temperate regions of Uttarakhand, it can be possible about five kilograms per hectare per year from a pure Kharsu Oak forest.

If the grading and sorting process of the lichens would be conducted at villages so it can be increased the income of the collector (primary collector). The lichen sector in the state Uttarakhand has required a proper channel of its tender, collection and trading/marketing system from its collectors to traders (Village to Mandi).

If the lichen based livelihood activity would be started so the migration of some peoples of the area to the plains can be reduced, because they can get the job opportunity in lichens sector.

REFERENCES

Kumar, B. and Upreti, D. K. An account of lichens on fallen twigs of three *Quercus* species in Chopta forest of Garhwal Himalayas, India. *Annals of Forestry* (2008), **15** (1):92-98.

Kumar, B. Lichen species distribution, cover and fall in a *Quercus semecarpifolia* (J E Smith) forest of Garhwal Himalaya. Ph. D. Thesis (2008) HNB Garhwal University, Srinagar (Garhwal), India.

Shah, N.C. Lichens of economic importance from the hills of Uttar Pradesh, India. Journal of Herbs, Spices and Medicinal Plants (1997), 5: 69-76.

Upreti, D. K. Loss of Biodiversity in Indian Lichen Flora. *Environmental Conservation* (1995) 22: 362-365.

Upreti, D. K., P. K. Divakar, and Nayaka, S. Commercial and ethnic use of lichens in 167-India. *Economic Botany* (2005) **59**(3):269 – 273.

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Ecological Importance of Ectomycorrhizae in World Forest Ecosystems.

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Abstract: During the last two decades, mycorrhizal research has received much attention and studies on plant-fungus mycorrhizal association have indicated that applied work on this research may compliment the so called *"green revolution"* to increase plant yield through the use of modern technology. Long-term productivity is measured in terms of sustainable ecosystem processes that maintain fertility and the over all *"ecosystem health"* as well as recovery after adverse disturbances. Over the last several decades in Indian Central Himalaya due to a variety of uncontrolled uses, *Quercus* species are heavily lopped, and grazed resulting in a poor stocking density, poor regeneration and eventually elimination. The forest cover is shrinking significantly. A number of environmental condition (moisture, mineral, nutrients, pollutants) and biological factors (interaction with the soil organisms) affect the root development and ectomycorrhizal formation. In this paper we have explained the ecological benefits of mycorrhiza to the Himalayan Forest Ecosystems and their role in rejuvenating the Himalayan forests. Now-a-days most research on inoculation with ectomycorrhizal fungi has been based on two working premises. First, any ectomycorrhizal association in tree seedlings is far better than no such association at all. Secondly, some species of ectomycorrhizal fungi have proven to be more beneficial to trees under certain environmental conditions than others. [Nature and Science. 2009;7(2):107-116]. (ISSN: 1545-0740).

Keywords: Ectomycorrhizae, Himalaya, Forest Ecosystems.

Introduction: Botanically, the mycorrhiza is a mutualistic association (non-pathogenic association) between soil borne fungi and the root of the higher plants. The term mycorrhiza *(Gr. Mykes=Fungus or Mushroom; Rhiza=Root)* i.e. *"Fungal Root"* was coined by the German Pathologist (Frank 1885) to describe the union of two different beings to form a single morphological organ in which the plant nourishes the fungus and fungus does the same for the plant. Mycorrhiza confers many attributes to plants such as growth stimulation due to increased nutrient uptake, tolerance of plants to adverse conditions and bio-control of root disease (Molina *et al.*, 1992).

The mycorrhizal symbiosis between plant roots and fungus is essential for the survival of both the partners (Harley and Smith, 1983) and it has been suggested that this symbiosis was a prerequisite for the successful colonization of the terrestrial environment by plants some 400 million years ago (Pyronzinski and Mallock, 1975). The term mycorrhiza describes a range of symbiotic structures formed between the fine root and different fungi.

Being highly specialized in their nutritional requirement, the mycorrhizal fungi obtain simple sugars, amino acids and plant growth substances from the host for growth & development. Mycorrhizae also benefit the host directly by influencing important ecosystem properties such as

soil structure (Finley and Söderström, 1992). The biological requirement of many forest tree species in respect of the ectomycorrhizal association was initially observed when attempts to establish plantation of exotic pines routinely failed, and this could only be overcome by the introduction of symbiotic fungal associates (Gibson, 1963; Madhu, 1967). Thus mycorrhizae should be regarded as an integral part of the natural and normally functioning root system of plants, taking part in symbiosis and function as dynamic biological linkages.

Types of Mycorrhizae:

In early days, mycorrhizae were classified into two main groups, based upon the structure of the root fungus association, namely endomycorrhizae and ectomycorrhizae (Payronel *et al.*, 1969), which were subsequently renamed as endotrophic for endomycorrhizae and ectotrophic for ectomycorrhizae. The latest classification of mycorrhizae has seven groups, namely *Vesicular-Arbuscular Mycorrhizae* (VAM), *Ectomycorrhizae*, *Ectendomycorrhizae, Arbutoid mycorrhizae, Monotropoid mycorrhizae*, *Ericoid mycorrhizae* and *Orchid mycorrhizae* (Harley and Smith, 1983).

Morphologically several distinct types of mycorrhizae have been described to be associated with a particular vegetation type (Fig. 1).

(1) Vesiular Arbuscular Mycorrhizae (VAM): In the first group Vesicular Arbuscular Mycorrhizae (VAM) belonging to the fungal order Endogonales of the Zygomycetes with aseptate hyphae (lower fungi) has been included. In the other six groups the endophytes are fungi with septate hyphae (higher fungi) belonging to Ascomycetes or Basidiomycetes. In all these mutualistic types, the mycorrhizal association contributes significantly to the host health, in exchange for photosynthates (Barker *et al.*, 1998).

The most common VAM (vesicular arbuscular mycorrhiza) found in the most herbaceous and graminaceous species. These fungi belong to the family *Endogonaceae*. Vesicular arbuscular mycorrhizal association generally lacks specificity. Individual species of VAM fungi can associate with diverse plants groups from herbs to long-lived woody perennials. Similarly, VAM plants often associate with many VAM fungi. (Molina 1979) found *Festuca* spp. associated with 11 species of Endogonaceae in habitats scattered over the Western United States. Repeated failures to isolate and grow aexenic cultures of VAM fungi indicate the obligate nature of VAM fungi. VAM plants, on the other hand, range widely from facultative to obligate dependency on mycorrhizal association. Many facultative plants are VAM in some ecological settings but may lack mycorrhizae in others (Trappe, 1987).

(2) Ericoid mycorrhizae: Ericoid mycorrhiza develops on the members of Angiospermic order Ericales (*Ericoideae*, *Vacciniodeae* and *Rhododendroidae* of most *Ericaceae* as well as *Epacridaceae* and *Empetraceae*). A septate fungus forms intracellular coils, restricted to the epidermal cells. Each epidermal cell is colonized individually with little change in root morphology. These fungi seem to have broad host range among ericaceae plant family and are restricted to these.

(3) Monotropoid mycorrhizae: Monotropoid mycorrhizae are associated with achlorophyllous members of the *Ericaceae family* (the *Monotropoideae*). The fungi involved in this relationship often have narrow host range. Monotropoid hosts thus appear to share wide receptivity similar to arbutoid hosts, most certainly an ecological advantage given the proven dependency of achlorophyllous plants on carbon of mycorrhizally linked EM plants (Bjorkman, 1960; Furman & Trappe, 1971).

(4) Arbutoid mycorrhizae: Arbutoid mycorrhizae belong to two genera of plant family Ericaceae and these genera are *Arbutus* and *Arctostaphylos.* These mycorrhizae resemble ectendomycorrhizae in that their hyphae colonize both intercellularly and intracellularly. The fungi that form arbutoid mycorrhizae on *Arbustus* and *Arctostaphylos* also form EM on other hosts (Molina and Trappe, 1982a; Zak, 1976a, b). Ericoid mycorrhizae and EM may also occur on Arbutoid hosts (Largent *et al.*, 1980; Mejstrik & Hadac, 1975; Trappe, 1964; Zak, 1973, 1974).

(5) Orchid mycorrhizae: Orchid seeds germinate only when infected with endomycorrhizal fungi that subsequently colonize the entire plant (Harley, 1969). Specificity patterns in Orchid mycorrhizae are complicated in that two types of colonization may occur: primary, involving the germinating seed and seedling and secondary, involving new roots (Harley & Simth, 1983).

(6) Ectendomycorrhiza: An intermediate type of mycorrhizal association is also found on coniferous and deciduous trees in nurseries and burned forest sites. The ectendomycorrhiza type forms a typical EM structure, except the mantle is slight or missing and hyphae in the *"Hartig net"* may penetrate root cortical cells. The ectendomycorrhiza is replaced by EM as the seedling matures. The fungi involved in the association were initially designated *"E-strain"* but were later shown to be ascomycetes and placed in the genus *Wilcoxina*. (Sylvia *et al.* 2005).

(7) Ectomycorrhizae: The most important type of mycorrhizal association found in temperate regions is known as Ectomycorrhizae (sheathing mycorrhiza). The ectomycorrhizae characteristic of all colonize temperate to boreal forest trees, are also present in the tropical forest trees. In Central Himalaya, ectomycorrhizal tree species are dominant in most of the forests along an elevation gradient from foothills to the timberline. Most of the ectomycorrhizal fungi belong to Basidiomycetes; some also belong to Ascomycetes. These fungi occur naturally on the plants belonging to some families of angiosperms viz. Dipterocarpaceae (e.g. *Shorea robusta*) and Fagaceae (e.g. *Quercus leucotrichophora* and other spp.). Most of the conifers are also associated with these fungi including all species of the family Pinaceae (e.g. *Pinus roxburghii*). Strict host specificity is rare and one plant may form mycorrhizal associations with different fungi simultaneously. Such sheathing mycorrhizae forming fungi are bio-tropic in their host relationship and ecologically obligate parasites in their mode of carbon (energy) nutrition.

In this mycorrhizal symbiosis the fungus grows into the root of the host plant and the hyphae penetrate between outer cortical cells, forming a typical structure called the "Hartig net". On the root surface the fungus forms a mantle or sheath, a structure, typical of ectomycorrhizae. This structure is typically connected to the hyphae or hyphal aggregates, which penetrate the surrounding soil and often form extensive mycelium. In this way, the mycorrhizal roots acquire access to a much greater soil volume in contrast to uninfected ones and therefore the effective surface area for nutrient absorption is greatly increased. The infected feeder roots undergo morphogenesis. These may be forked, unforked, bifurcated, nodular, multiforked or coralloid. The ectomycorrhizal roots can be distinguished from non-mycorrhizal roots.

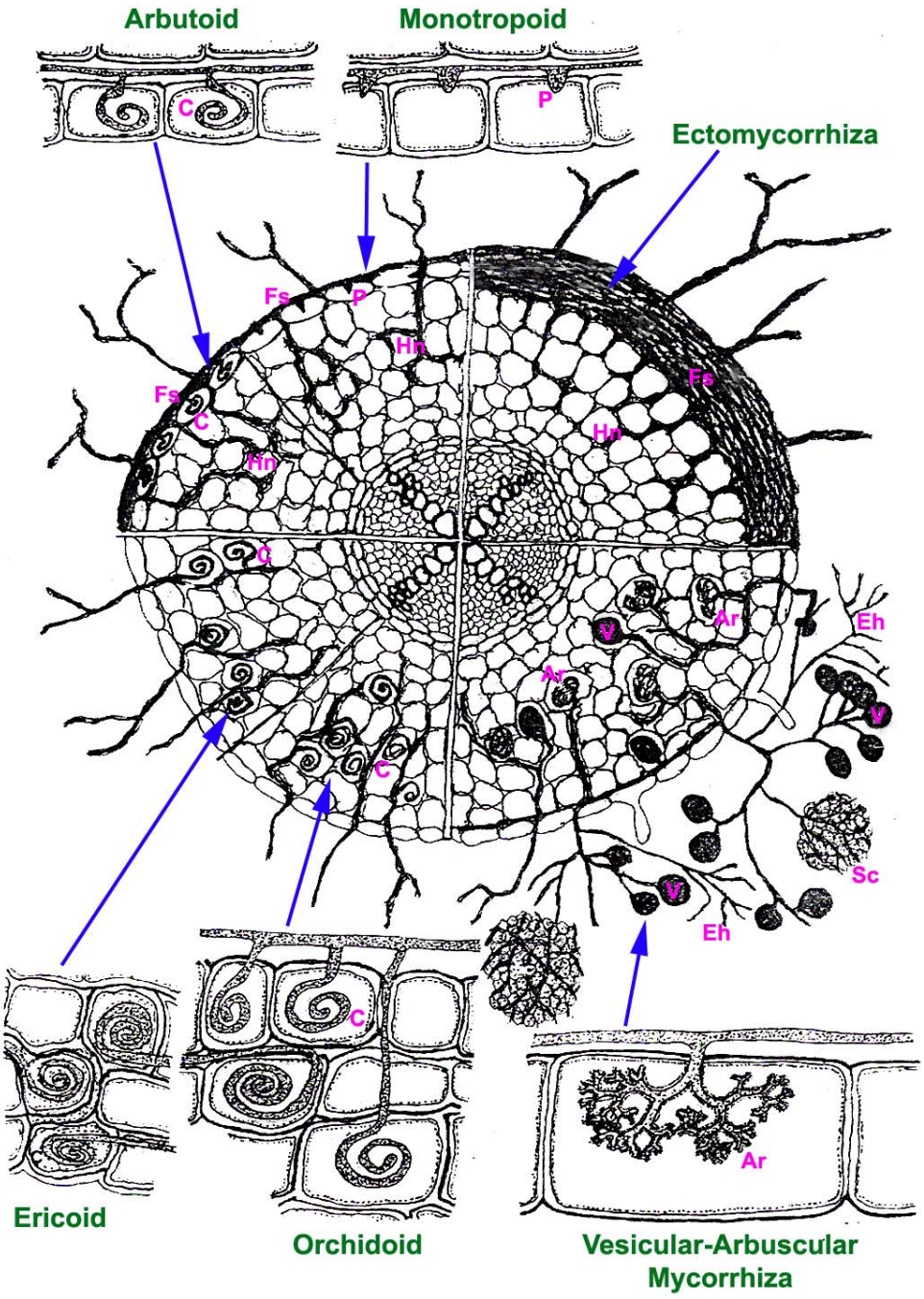

Fig. 1 Diagrams showing different types of mycorrhizae (derived from Mycorrhial Biology by K.G. Mukherji, B.P. Chamola and Jagjit Singh). **Ar** = Arbuscules, **C** = Coiled hyphae, **Eh** = Extrametrical hyphae, **Hn** = Hartig net, **Fs** = Fungal sheath, **P** = Protrusion, **Sc** = Sclerotia, **V** = Vesicles.

Importance of Ectomycorrhizae: Ectomycorrhizal symbiosis is important on a global scale because the dominant trees in most of the world's temperate and boreal forests and in large areas of tropical and subtropical forests are ectomycorrhizal (Allen, 1991; Read, 1991). It is more prevalent in temperate and sub-temperate region than in tropical and sub-tropical region where soil acts as a more important storage compartment for the minerals nutrient and the climatic conditions lead to restricted vegetative activity (Meyer, 1973). Pinaceae and Fagaceae, which dominate temperate forest together with members of Myrtaceae and Dipterocarpaceae from sub-tropical region, are the predominant ectomycorrhizal families (Smith and Read, 1997). It is considered that nearly 95% of wild flora is characteristically mycorrhizal (Barker *et al.*, 1998), although mycorrhizal status has been examined for only about 3% of the total (Smith and Read, 1997). Though the majority of plants show VAM symbiosis, the plants of temperate region are predominantly ectomycorrhizal. Thus ectomycorrhiza is always an integral component of forest ecosystem showing mutual dependency between fungus and the higher symbiont for natural function and survival.

There are over 5,000 fungi belonging to Basidiomycetes, and Ascomycetes involved in forming ectomycorrhizae on about 2,000 plants. However, about 2000 fungi could form ectomycorrhizae with Douglas-fir alone (Trappe, 1977). Already 400 taxa of ectomycorrhizal fungi, mostly belonging to *Russulaceae, Boletaceae, Canterallaceae, Amanitaceae* and *Cortinariaceae* have been described from African forest and woodland, and similar number is likely to be reported in future (Castellanio *et al.*, 2000).

Among the fungi belonging to Basidiomycetes, species of Hymenomycetes such as *Boletus, Cortinarius, Suillus, Russula, Gomphedries, Hebelema, Tricholoma, Laccaria,* and *Lactarius* and species of Gasteromycetes, e.g. *Rhizopogon, Scleroderma, Alpara,* and *Pisolithus* form ectomycorrhizae. Certain orders of the Ascomycetes such as, Tuberales and Pezizales have species that also form ectomycorrhizae (Marx, 1991). The importance of mycorrhiza for Pines and Oaks was clearly experienced when these species were planted in the tropics or in grasslands, where the particular types of fungi that they required were not present (Mikola, 1973). Without their fungal species, seedlings did not survive, or even if they grew, they could not perform well (Shemakhanova, 1967). It is reported that trees planted on highly disturbed areas receive high mortality unless given the proper mycorrhizal fungus (Marx, 1991; Singh. 2001). Review of mycorrhizal specificity commonly emphasizes a "lack of specificity" in mycorrhizal associations, i.e., there is no known example of gene-for-gene level of host fungus specificity (Molina *et al.*, 1992).

Numerous studies on the beneficial effects of ectomycorrhizal fungi on seedling growth and development in artificially regenerated forest sites and drastically disturbed land have been conducted (Marx *et al.*, 1991).

The Himalayan region can be divided into three zones on the basis of altitudinal variation, *viz*. Sub-Tropical, Temperate and Alpine representing a variety of forest types (Champion & Seth, 1968; Tewari, 1982; Adhikari *et al.*, 1989; Singh *et al.*, 1994) from the dry tropical deciduous in the foothills to the alpine scrub forest (Adhikari *et al.*, 1989; Garkoti, 1992) near the timberline. Himalayan belt can be divided into three regions Western, Eastern and Central. The Himalayan region of Uttaranchal comprises of about 50,000 km^2 and is often referred to as the Indian Central Himalaya.

The fungal diversity of local region is poorly known in India as well as in the Himalayan region. A few preliminary studies done in these forest sites basically deal with the symbiosis rather than the function of ectomycorrhizae in the forest ecosystem (Dubey *et al.*, 1998; Ginwal 1994) used soil inoculum for introduction of mycorrhizal fungi in *Quercus leucotrichophora* seedlings in nurseries. Recent researches conducted in glass-house experiments from Indian Central Himalaya also showed significant increase in root: shoot ratio and plant biomass in mycorrhizal seedlings as compared to uninfected once (Pande, 2003; Agarwal, 2007).

The importance of mycorrhizae for the Dipterocarp forest ecosystem and its management has only recently been realized. (Smits 1983) suggested that ectomycorrhizae might play an important role for successful regeneration after logging in lowland dipterocarp forests; much more attention was paid to the ecological aspects of dipterocarp ectomycorrhizae. Only a few studies concerning ecological aspects of mycorrhizae in dipterocarp forest ecosystems have been undertaken so far (Becker, 1983; Smits, 1983, 1994; Yasman, 1993). Dipterocarp are among the few species in the tropical forests of which the seedlings can survive and grow under very low light intensities on the forest floor, and may be benefited from interaction of their root system with a mature tree through ectomycorrhizal connection as described for other plants.

Based on the degree of dependency of plant species on mycorrhizae, plants can be grouped into three categories namely Non-Mycorrhizal, Facultative Mycorrhizal and Obligate Mycorrhizal (Bagyaraj, 1989). A number of studies confirm that dipterocarp species are obligatory ectomycorrhizal (Noor and Smits, 1988; Oman, 1994; Smits, 1994). The poor development of ectomycorrhizae on roots of dipterocarps explains the poor regeneration of dipterocarp trees on former skid roads and open areas (Smits, 1983; Julich, 1989; Smits, 1994). For instance, suggested that low availability of phosphorus in the soil and lack of season in a tropical soil might cause tropical species to depend upon mycorrhizal activities for survival and growth (Janos 1985).

Different types of mycorrhizal symbiosis may have different threshold of carbohydrate level. Ectomycorrhizal fungi may require more photosynthetic products (Carbohydrates) from their host than VA-mycorrhizal fungi from similarly dependent hosts for one of the following reasons- first because of a greater biomass of fruiting bodies, hyphae and rhizomorphs in ectomycorrhizae than in VA-mycorrhizae, hence a higher carbon cost if equal hyphae respiration rates of ectomycorrhizal and VA-mycorrhizal fungi are assumed; second, ectomycorrhizal fungi are a stronger sink for photosynthetic products than VA-mycorrhizal fungi, because ectomycorrhizal fungi produce plant hormones that influence translocation of carbon compounds, and convert the host sugars into storage sugars. Therefore in the forest ecosystem when the ectomycorrhizal species predominate as in dipterocarp forest, the role of ectomycorrhizae in the carbon allocation of the ecosystem is expected to be of primary importance (Yasman, 1995).

Much of the information on ectomycorrhizal fungi associated with temperate forest is based on research undertaken on the Northwest Pacific forest of America. Some of the important studies pertaining to the species richness of ectomycorrhizal fungi of forest North America include: spruce and hard wood forest of West Virginia (Bills *et al.,* 1986) and lobally pine (Cibula and Overvo, 1988) and Alder forest in Alaska (Brunner *et al.,* 1992). These forests are characterized by summer dry conditions, most of the area dominated by conifers; in contrast, the climate of Himalayan area has a characteristic monsoon that provides a different situation, such as broadleaf species rather than conifers dominating much of the area. As a result, difference in vegetation and climate would influence the ectomycorrhizal diversity in this region. In India, emphasis is being laid on boosting the afforestation programme in hills, particularly species of *Quercus* (Ginwal, 1994).

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References:

Adhikari, B.S., Ram, J., Sharma, S. and Bhatt, Y.D. 1989. Quantitative vegetational analysis of forest between 2000-3300m altitudes in Nanda Devi Biosphere Reserve. In: Singh, S.P. (ed) *Pattern of soil and vegetation and factors determining their forms and hydrological cycle in Nanda Devi Biosphere Reserve*. First Annual Report, Govt. of India, New Delhi, pp, 7-29.

Agarwal, P. 2007. Ectomycorrhizal Fungi in Three Major Forest Types in Central Himalaya and their Effect on Seedling Growth. Ph.D. Thesis, Kumaun University, Nainital, India.

Allen, M.F. 1991. *The ecology of mycorrhiza* . Cambridge University Press, Cambridge, UK, 184p .

Bagyaraj, D.J. 1989. Mycorrhizas. In "Tropical Rain Forest Ecosystem: Biographical and ecological studies" edited by Leith, H. and Werger, M.J.A. Elsevier, Amsterdam-Oxford-New York- Tokyo. Pp,

Barker, S.J., Tagu, D. and Delp, G. 1998. Regulation of root and fungal morphogenesis in mycorrhizal symbiosis. *Plant Physiology*, 116: 1201-1207.

Becker, P, 1983. Mycorrhizas of *Shorea* (Dipterocarpaceae) seedlings in a lowland Malaysian rain forest. *The Malayan Forester*, 42(2): 146-170.

Bills, G.F., Holtzmann, G.I. and Miller, O.j.Jr. 1986. Comparison of ectomycorrhizal Basidiomycetes communities in red spruce versus northern hardwood forest of West Virginia. *Canadian Journal of Botany*, 64: 760-768.

Bjorkman, E. 1960. *Monotropa hypopitys* L.-an epiparasitic on tree roots. *Physiologia Plantarum*, 13: 308-327.

Brunner, I.; Bunnere, F. and Laursen, G.A. 1992. Characterization and comparison of macrofungal communities in an *Alnus tumuifolia* and an *Alnus crispa* forest in Alaska. *Can. J. Bot*. 1992 70: 1247-1258.

Castellano, M.A., Verbeken, A., Walleyn, R. and Thoen, D. 2000. Some new and interesting sequestrate Basidiomycota from African Woodland Karstenia, 40: 11-21.

Champion, H.G. and Seth, S.K. 1968. *A revised survey of the forest types of India*. Govt. of India Publication, New Delhi, 404p.

Cibula, W. and Ovrevo, C. 1988. Mycosociological studies of mycorrhizal fungi in two loblolly pine plots in Missisipi and some relationship with remote sensing data. In remote Sensing for Resource Inventory Planning and Management (eds, J.D. greer) *American Society for Photogrammetric Remote Sensing*. Falls Church, Va. Pp. 268-307.

Dubey, R.C., Ginwal, H.S. and Singh, R.P. 1998. Comparative study of ectomycorrhizae of different forest of *Quercus* spp. In Kumaun Himalaya: Vertical distribution along soil depth. In: Rai, P. and Dkhar, M.S. (eds.) *New Trends in Microbial Ecology*. International Society for conservation of natural Resources, Varanasi, Dept. of Botany, BHU, India, pp 306-314.

Finlay, R. and Soderstrom, B. 1992. Mycorrhiza and carbon flow to the soil. In Allen, M.F. (eds.) Mycorrhizal functioning: An integrative plant fungus process, Chapman and Hall, The Netherlands, pp, 134-160.

Frank, A.B. 1885.microbiology. Bishan Singh Mahendra Pal, Dehradun, India. Pp. 83-114.

Furman, T.E. and Trappe, J.M. 1971. Phylogeny and ecology of mycotrophic achlorophyllous angiosperms. *Quarterly Review of Biology*, 46: 219-225.

Garkoti, S.C. 1992. *High altitudinal forest of Central Himalaya: Productivity and nutrient cycling*. Ph. D. Thesis, Kumaun University, Nainital, India, 211p.

Gibson, J.A.S. 1963. "Eine Mitteilung uber die Kiefernmykorrhiza in den waldern Kenias". In: Rawald W. and Lyr. H. (Eds) "Mykorrhiza, fisher, Jena, pp. 49-51.

Ginwal, H.S. 1994. Studies on ectomycorrhizae in different central Himalayan oak forests and their role in nursery practices. Ph.D. Thesis, Kumaun University, Nainital, U.P.

Harley, J.L. 1969. "The biology of ectomycorrhizae" 2nd edition Leonard Hill. London. P. 334.

Harley, J.L. and Smith, S.E. 1983. Mycorrhizal symbiosis, Academic Press, London. 334 pp.

Janos, D. 1985. Mycorrhizal fungi: Agent for symptoms of tropical community composition, in R. Molina (eds.) *Proc. 6th North American Conference on Mycorrhizae*. P, 98-103.

Julich, W. 1989. Dipterocarpaceae and Mycorrhizae. German Forestry Group. GFG-report no. 9. Mulaearman University Samarinda Indonesia. 103 pp.

Largent, D. L., Sugihara, N. and Wisher, C. 1980. Occurrence of ectomycorrhizae on ericaceous and pyroloceous plants in northern California. *Canadian Journal of Botany*, 58: 2274-2279.

Madhu, M. 1967. The biology of ectotrophic mycorrhizal with reference to the growth of pines in Nigeria. Obeche. J. Tree club. Uni. Ibadan. 1: 9-16.

Marx, D.H. 1991. The practical significance of ectomycorrhiza. Use in forest establishment. In: Ecophysiology ectomycorrhizae of forest trees. The Marcus Wallenberg Foundation Symposia Proceedings 7, pp, 54-90.

Marx, D.H., Ruehle, J.L. and Cordell, C.E. 1991. Methods for studying nursery and field response of trees to specific ectomycorrhiza. Method Microbial, 24:383-411.

Mejstrik, V. and Hadac, E. 1975. Mycorrhizae of *Arctostaphylos* uva-ursi. *Pedobiologia*, 15: 336-342.

Meyer, F.N. 1973. Distribution of ectomycorrhizae in native and man-made forests. In: Marks, G.C. and Kozlowski, T.T. (eds) *Ectomycorrhizae: their ecology and physiology*. Academic Press, New York, pp, 79-105.

Mikola, P. 1973. Application of mycorrhizal symbiosis in forestry practice: In: Marks, G.C. and Kozlowski, T.T. (eds) Ectomycorrhizae: their ecology and physiology. Academic Press, New York, pp, 383-411.

Molina, R. 1979. Pure culture synthesis and host specificity of red alder mycorrhizae. *Can. J. Bot*. 57: 1223-1228.

Molina, R. and Trappe, J.M. 1982a. Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. *For. Sci*. 28: 423-458.

Molina, R., Massicotte, H. and Trappe, J.M. 1992. Specific phenomena in mycorrhizal symbiosis; Community ecological consequences and practical implications. In: Mycorrhizal functioning an integrative plant fungi process. Ed. My Allen. Pp. 357-423. Chapman and Hall, New York.

Noor, M. And Smits, W.T.M. 1988. Pengaruh intensities cahaya dan suhu tanah ternadop ektomikoriza dan pertumbuhan anakan *Shorea cf. polyandra*. In Soemarna, K . et. al., (eds) Prosiding symposium hasil-hasil penetration Dipterocarpaceae, pp, 11-28.

Oman, R.M. 1994. Pengaruh jenis cendawan mikoriza dan media tumbuh terhadap perkembangam stek Shorea leprosula Miq. M.Sc. Thesis, Institut Pertanian Bogor 'Unpublished'. 115 pp.

Pande, V. 2003. Diversity and biotechnical applications of ectomycorrhizae associated with oaks of Central Himalaya. Ph.D. Thesis, Kumaun University, Nainital, India.

Payronel, B., Fassi, B., Fontana, A. and Trappe, J.M. 1969. Terminology of Mycorrhizae. *Mycologia*. 61: 410-411.

Pyronzinski, K.A. and Mallock, D.W. 1975. The origin of lands plants: a matter of mycotrophy. *Biosystems* 6: 153-164.

Read, D.J. 1991. Mycorrhiza in ecosystem. *Experientia*, 47: 376-391.

Shemakhanova, N.M. 1967. Mycotrophy of wood plants. USDA and NSF, Washington, DC, Israel Program for Scientific Translation, 329p.

Singh, S. 2001. Role of mycorrhiza in tree planting in the field. II: field inoculation, fungal succession, and effect of climatic and edaphic factors. *Mycorrhiza News*, 12: 2-12.

Singh, S.P., Adhikari, B.S. and Zobel, D.B. 1994. Biomass, productivity, leaf longevity and forest structure along an altitudinal gradient in the Central Himalaya. *Ecological Monograph*, 64(4): 401-421.

Smith, S.E. and Read, D.J. 1997. *Mycorrhizal Symbiosis (2nd edition)*, Academic Press, London.

Smits, W.T.M. 1983. Dipterocarpaceae and Mycorrhizae. An ecological adaptation and a factor in forest regeneration. *Flora Malesiana Bulletin*, 36: 3926-3937.

Smits, W.T.M. 1994. Dipterocarpaceae: Mycorrhizae and regeneration. Tropenuos Series, 9-243 pp.

Sylvia, M. David ; Fuhrman, J.Jeffrey ; Harlet G. Peter and Zuberer A. David. 2005. Principles and Applications of Soil Microbiology (2nd Edition). Prentice Hall.

Tewari, J.C. 1982. *Vegetational analysis along altitudinal gradients around Nainital.* Ph. D. Thesis, Kumaun University, Nainital, India, 570p.

Trappe, J.M. 1964. Mycorrhizal hosts and distribution of *Cenococcum graniforme*. Lloydia, 27: 100-106.

Trappe, J.M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. *Ann. Rev. Phytopath*. 15: 203-222.

Trappe, J.M. 1987. Phylogenetic and ecological aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In Ecophysiology of VA mycorrhizal plants (ed. Safir, G.R.). CRC Press, Boca Raton, Fla., 2-25.

Yasman, I. 1993. Ectomycorrhizal sporocarps appearance in Dipterocarp forest East Kalimantan. Indonesia In Proc. Inter. Workshop Bio-Refor held in Jogyakarta, pp, 179-181.

Yasman, I. 1995. Dipterocarpaceae: Tree-Mycorrhizae seedling connections. Ph. D. Thesis., Wageninger Agricultural University. The Netherlands.

Zak, B. 1973. Classification of ectomycorrhizae. In “ectomycorrhizae” (Eds. G.C. Marks and T.T. Kozlowski) pp, 43-78. Academic Press. New York and London.

Zak, B. 1974. Ectendomycorrhiza of Pacific Madron (*Arbustus menziessi*). *Transaction of the British mycological Society*, 62:201-203.

Zak, B. 1976a. Pure culture synthesis of bearberry mycorrhizae. *Canadian Journal of Botany*, 54:1297-1305.

Zak, B. 1976b. Pure culture synthesis of Pacific Madron of ectendomycorrhizae. *Mycologia*, 68:362-369.

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