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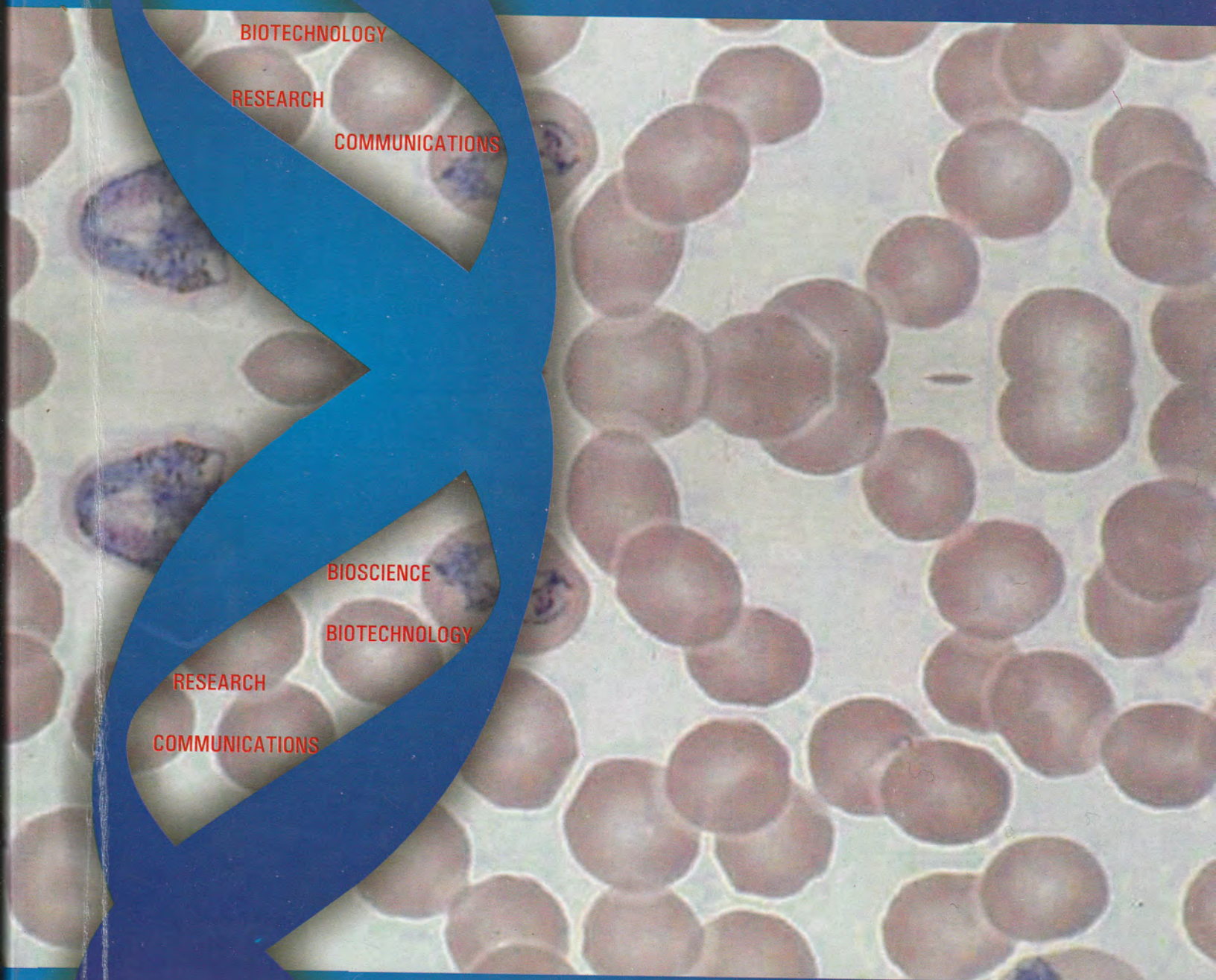
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Structural and functional behaviour of Cyanobacteria

Gunapati Oinam, L. Sophiya, S. Deepa Devi, Laxmipriya Koijam, O.Avijeet Singh, H. Boboy Singh, M.K. Leingaklemba, O.N. Tiwari* and M. Rohinikumar Singh

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Abstract:

Cyanobacteria are capable of adapting several different modes of metabolism with respect to their sources of carbon and energy. All of the known species have the ability to grow in light with carbon dioxide as the principal, if not sole, carbon source. When provided with organic compounds in addition to carbon dioxide, many cyanophytes utilize organic carbon in cell synthesis. The contribution of the organic substrate to cell synthesis varies widely from negligible amounts to the situation where enough of the organic compound is utilized to account for all of the carbon in newly synthesized cell material. Finally, a few species are reputed to be capable of growth in the dark on organic media; under these conditions, the organic compounds presumably act as a source of energy as well as a major source of carbon. During the last decade, cyanobacteria have become generally recognized as a major group which provides experimental material for all aspects of microbiology-with the commendable exception of invasive animal pathogenicity. Although there are predominant traits in their metabolism and ultrasturcture, cyanobacteria do exhibit a wider range than was previously thought. There are several other attributes of cyanobacteria which, though restricted to minority of species, can still be considered as important features of the group. These include complementary chromatic adaptation, the presence of gas vacuoles, morphological changes, particularly the formation of heterocysts associated with the onset of nitrogen deficiency and formation of tapered hairs in whole sections of filament associated with the onset of phosphate deficiency. Only the changes associated with nitrogen fixation are unique to the group. The present paper provides an overview of structural and functional behaviour of Cyanobacteria.

Key words: Structural functional biodiversity, Cyanobacteria.

Introduction

Cyanobacteria are photosynthetic prokaryotes possessing the ability to photosynthesize chlorophyll a. Cyanobacteria have until recently also been characterized by their ability to form the phycobilin pigment, phycocyanin. It is the high concentration of this pigment occurring under some conditions which leads to the bluish colour of the organisms and hence both of the names by which the organisms are commonly known, cyanobacteria or blue-green algae. Cyanobacteria show considerable morphological diversity. They may be unicells or filaments and these may occur singly or grouped in colonies. Unicells may divide in one, two or three planes. Some unicells dividing in one plane show asymmetric division; in *Chamaesiphon* the smaller cells glide down to the base of the larger cell and repeats of this process eventually give rise to a colony of many cells. The resulting colonies can often be seen as brownish spots on submerged rocks. Branching of filaments may be false or true. False branching is when division in a filament with a sheath leads to the formation of two separate sheathed filaments and one of these grows separately from the other. Maintenance of the colonial structure in many of

these forms is aided by the presence of exopolysaccharides such as mucilage and / or a firm sheath. The presence or absence of a heterocyst is an important feature separating genera. However, gas-vacuoles, structures which aid buoyancy, are found in species of many different genera.

The taxonomic system which developed during the 19th century was based almost entirely on morphology and the international code of botanical nomenclature takes various papers published during the period 1886-1892 as the starting point for the valid publication of names of filamentous forms. Rather surprisingly, the starting point for the morphologically less complex forms is much earlier, although there was of course little understanding of their diversity at that time. A number of "floras" summarizing the known species of cyanobacteria in particular regions have been published during the 20th century. Several of these provide a lot of information about species occurring elsewhere in the world. In any case it is clear that many species have a very wide distribution. The best known flora is that of Geitler (1932), which focused on Central Europe, but is still an essential source of information for those wanting to name the organisms in any part of the world. Desikachary's (1959) flora for India could have reached a very wide audience because it is written in English, but unfortunately has long been out of print.

A review by Castenholz (1992) and the sister volume (Bryant,1994) to the present one make clear how important is an understanding of cyanobacterial molecular biology not just as an aid to taxonomy, but for interpreting ecological phenomena in general. Suboptimal light and nutrient conditions result in a number of responses that strongly influence the physiology of the cell. The responses can be striking or subtle and subsequent changes take place rapidly or very slowly. Cyanobacteria tend to show resistance to multiple environmental stresses, and it is probable that the response pathways to the different stimuli overlap. Caution is therefore needed in assuming that, because a particular stimulus brings about a characteristic morphogenetic response in the laboratory, it is the same stimulus which does so in nature. The ability of some cyanobacteria to withstand extremes of UV radiation and dessiccation is aided by their capacity for efficient DNA repair. It will be of interest to understand if, and how, such repair plays a role in other responses to environmental stimuli such as nutrient limitation and cyanophage infection.

Origin and early evolution of Cyanobacteria

Any attempt 20 years ago to delineate the geological history of the cyanobacteria would have been brief indeed, for very little of the now-known fossil record of the group had as yet been discovered. To a considerable extent, this earlier lack of knowledge is a reflection of the fact that 'plant-like' fossils of this type fall within the purview of palaeobotany, a field that traditionally has been concerned largely (indeed, almost exclusively) with the fossil record of megascopic, vascular plants, rather than with that of microscopic 'thallophytes' as cyanobacteria were then regarded.

The evolution of the cyanophytes:

Although the evidence for the time and manner of origin of cyanophytes is still meagre, and most of it subject to varying interpretation, it seems likely that the cyanophytes arose between 2 and $3X10^9$ years ago (Schopf, 1970) probably from a photosynthetic bacterium. After the discovery of gliding photosynthetic bacterium *Chloroflexis* (Pierson and Castenholz, 1971) is a reasonable candidate as a forerunner of the cyanophytes. It is virtually certain that the cyanophytes were probably responsible for a marked increase in the O_2 concentration of the atmosphere during the Precambrian. Another reason why a cyanophytes is the best candidate for the first O_2-evolving photosynthetic organism is that at least some cyanophytes are able to grow anaerobically, a property not normally associated with eukaryotic algae (Stewart and Pearson, 1970). If the bulk of the atmospheric O_2 is indeed of biogenic origin, the organisms first involved in O_2 formation would of course have developed in an anaerobic environment and would have had to be able to live anaerobically.

Stromatolites and the evolution of the cyanophytes:

Stromatolites are rocks consisting of many layers, most commonly developing from the mats of cyanophytes. The rocks may be either siliceous or calcareous, although the later are most common. The layers probably build as a result of sediment trapping and carbonate precipitation by unicellular and filamentous cyanophytes. In shallow marine habitats, calcareous sediments deposit continuously on the surface of the mat, and the cyanophytes move up through the sediments during the day (Monty, 1967; Gebelein, 1969). In some cases the layers are diurnal, resulting from phototrophic movement of the cyanophytes. Stromatolitic formations became less and less frequent and diverse after the Cambrian, and the most common explanation for this is that the

evolution in the Cambrian of microbe-eating metazoans made it impossible for the large cyanophytic mats to build up (Garrett, 1970).

Ecology of cyanophytes:

To what kind of habitats are the cyanophytes as a group adapted, in the precambrian they may have been found in a very wide variety of habitats but today their distribution is more restricted, since in many habitats they must compete with eukaryotes. There is only one known type of habitats on earth today where cyanophytes are the exclusive O_2-evolving photosynthetic organisms, and that is in thermal spring (Brock, 1970). However, even in the thermal springs cyanophytes are not always successful. In acid springs with pH less than 4.0, cyanophytes are never found, irrespective of temperature. In neutral and alkaline thermal springs, cyanophytes are the exclusive O_2-evolving photosynthetic organisms at temperatures above 55-60°C, and are far in the dominance at temperatures down to about 40°C. The upper temperature limit for cyanophytes is about 72-73°C and at temperatures above this only non-photosynthetic bacteria are present. In other habitats, even in those in which cyanophytes are widely held to be successful, they are never exclusive and often not dominant. For instance, in extremely hyper saline environments, *Dunaliella* is usually more common, diatoms are usually dominant in cold water and ice, and even in dry environment such as rock and soil eukaryotic algae are found as well. Cyanophytes are the only group of O_2-evolving photosynthetic organism which fixes nitrogen. The fixation of nitrogen is found under aerobic conditions in heterocystous cyanophytes and under anaerobic condition also in some non-heterocystous form (Stewart and Lex, 1970). This means that under conditions of limitation of combined nitrogen, cyanophytes could successfully compete with eukaryotic algae. The ability of a variety of cyanophytes to fix nitrogen in natural aquatic systems is well established (Stewart, 1969). The presences in cyanophytes of phycobilins as accessory photosynthetic pigments may confer a considerable ecological advantage to them under conditions of low light intensity. Perhaps one of the most important factors controlling cyanophyte development may be the ability of this group to grow under conditions of low O_2 concentration. It might be in such a niche that the first eukaryotic alga could have developed. In such an alga, with its photosynthetic apparatus concentrated in a chloroplast, the cytoplasm surrounding the chloroplast could have provided an effective pH buffer which would have served to keep H^+ from the very acid-sensitive chlorophyll molecule. The cyanophyte, with its photosynthetic apparatus in the cell periphery, may be less well adapted for keeping H^+ from chlorophyll.

Mechanisms of movements in cyanobacteria

Motility in cyanobacteria is gliding motility. Gliding in contrast to swimming is movement in contact with a solid or semi-solid substrate, but without a visible change in the shape of the organism. A filament may have considerable passive flexibility, but there does not seem to be any possibility of steering. The relatively slow progress of gliding is accompanied in some species by a rotation of the trichome along its axis which is either right-handed or left-handed. This means simply that any point on the surface of the trichome will trace either a right-handed or left-handed helix, but whichever it is, it will be species specific and non-interchangeable. In liquid microscope mounts with or without a cover slip, free ends of trichome are seen to jerk back and forth, wave to and fro, or oscillate by tracing a cone. It appears that all of these oscillations are passive responses to the rotation of the trichome when any part of it is able to bear against a substrate and glide. Consequently, the whole trichome rotates and, since they are seldom straight threads they will appear to be swinging their free ends around. Rotation and accompanying oscillations are the usual features of gliding trichome of most Oscillatoriaceae, but not of most hormogonia and gliding trichome of heterocystous groups.

Gliding behaviour:

Gliding is movement in contact with a solid or semi-solid surface without flagella-like propulsive organs. In most cases it is a smooth, non-jerking movement resembling somewhat the progress of a snail. In many cases the movement is continuous in one direction for prolonged periods; in others frequent or regular reversals occur. Gliding of trichome in almost all circumstances probably occurs within a thin sheath casing or film of mucus which adheres to the substrate and is shed and left behind as a 'mucous' trail. When measured microscopically, the velocity of movement without reversals varies with species and environmental conditions. In the Oscillatoriaceae rates of well over 2μm sec^{-1} are command and range up to 11 μm sec^{-1} (Halfen, 1970; Halfen and Castenholz, 1971). There is no consistent correlation between trichome diameter of different species and the velocity of gliding. Other non-photosynthetic, filamentous

prokaryotes and myxobacteria glide at rates usually less than 1.0 µm sec^{-1} (Costerton *et al.*, 1961). In contrast, flagellated rod and spirilla bacteria commonly attain speeds of 20-30 µm sec^{-1} and vibrios apparently reach speeds as high as 50-200 µm sec^{-1}. When measurement are made on a macro-scale, such as distance covered on an agar surface over a period of a few hours, less than maximum rates are usually recorded. This is presumably because of the more or less frequent stops and reversals of individual trichome together with the devious path followed by some on the surface of the substrate. Such phenomena are common even when the organism is oriented towards an external stimulus such as unidirectional light (Drews, 1959; Haupt, 1959).

The mechanism of motility:

One of the earliest suggested mechanism for gliding motility (Doetsch and Hageage, 1968) has been supported and modified by Halfen and Castenholz (1971) the current proposal for *Oscillatoria* is that numerous microfibrils strung uninterrupted in helices around the exterior portion of the trichome are the propulsive organelles which move the chain of cell against an elastic sheath or other suitable substrate by a rapid succession of unidirectional waves with submicroscopic amplitude. The origin of wave propagation may shift from one pole to the other, reversing the direction of wave movement and of gliding. Unicellular species or those with trichome of more disjointed cell could have a similar system but continuous for only a single cell length. Species that do not rotate as they glide should have longitudinal microfibrils which are not helically strung.

Orientated movements:

Phototaxis is only tactic response of cyanobacteria that has received much attention. Photo-topotaxis is the orientation and movement towards (+) or away from (-) the incident light. Photo-phobotaxis is the reversal of direction of movement following a sudden change from high to low light intensity (+) or from low to high light (-) (Nultsch 1965). The general term phototaxis can mean any of these responses. It is often difficult to distinguish topotactic and phobotactic reactions. For example, a spot of bright light in an otherwise darkened agar plate may attract trichome which have a topotactic response; they may remain collected in the bright spot because when the tip of a trichome passes from light into shadow the direction reverses, capturing the filament in the light spot.

Gas vacuoles in cyanobacteria

Fine structure of gas vesicles:

The fine structure of gas vacuoles has been investigated in a total of eleven species from the genera *Microcystis*, *Oscillatoria* (Jost 1965; Smith and Peat, 1967; Whitton and Peat, 1969), *Trichodesmium* (Van Baalen and Brown, 1969), *Anabaena* (Bowen and Jensen, 1965), *Aphanizomenon*, *Nostoc* (Waaland and Branton, 1969; Bisalputra *et al.*, 1969) and *Gloeotrichia* and in each case they have been found to comprise gas vesicles of the same basis morphology originally described by Bowen and Jensen (1965). The vesicles have the form of hollow, cylindrical tubes with conical caps at each end. The tubes are possibly of uniform diameter, usually given as being about 70 nm the reported extreme being 65 nm for *Oscillatoria rubescens* and 75-85 nm for *Trichodesmium erythraeum* (Van Baalen and Brown, 1969).Various synonyms have been used to describe gas vesicles in cyanobacteria but it is suggested that the original term should be retained. Gas cylinder (Smith and Peat, 1967) aptly described these structures in cyanobacteria but is inapplicable to homologous structure of different shape found in various bacteria (Cohen-Bazire *et al.*, 1969; Walsby 1972). 'Gas vacuole subunits' (Walsby, 1969) might be confused with the proteinaceous subunits of which the gas vesicles membrane is formed. However, it is felt that it is often useful to be able to distinguish the composite structures, seen under the light microscope, form the elements which comprise them. In many species the gas vacuole are irregular in form and distributed throughout the cell but in some algae they have a characteristic shape and position.

Chemical composition of gas vesicles:

Chemical analyses have now been made on gas vesicles isolated from cyanobacteria and the results have confirmed the idea, suggested by electron microscopy, that the gas vesicles membrane is fundamentally different from typical unit membrane. Quantitative preparations of highly purified intact vesicles have been obtained by Walsby and Buckland (1969) from the filamentous alga *Anabaena flos-aquae*. To avoid collapsing the gas vesicles, the cell were lysed by method which did not involve exposing the preparations to pressure, the filamentous alga being disrupted by osmotic shrinkage in hypertonic sucrose solution and the unicellular form by osmotic shock after weakening the cell wall by penicillin treatment. The intact vesicles were then purified by a process of repeated, centrifugally accelerated flotation, followed by combination of membrane filtration, molecular

sieving and liquid polymer partitioning (Buckland and Walsby, 1971).

Functions of gas vesicles:

Of the three functions that gas vacuoles might fulfill that of storing gas is no longer tenable. The gas vesicle membranes are far too permeable to retain any particular gas. It has been pointed out that the two other functions, providing light shielding and buoyancy do not depend on the presence of gas in the vesicles, but rather on these structures being kept free of liquid or solids.

Light shielding:

The peculiar optical quantities of gas vacuoles have suggested (Waaland *et al.*, 1971) that they might provide light shielding for cells exposed to high light at water surfaces. Fuhs (1968) has pointed out that in light microscope gas vacuoles are poor amplitude objects on account of their low absorptivity, but the large refractive-index difference between their contents and the surrounding cytoplasm makes them excellent phase objects. The path-length of light passing through a gas vacuole may differ from that passing through the neighbouring cytoplasm by more than ¼ wavelength (the normal retardation of the objective phase plate) and result in 'false reversal' so that the vacuole looks brighter instead of darker than the background . With high resolution optics, gas vacuoles appear almost invisible under bright field illumination but with low numerical apertures they are characterized by diffraction fringes. Spectrophotometric analysis confirm that gas vesicles show strong light scattering properties, and that over most of the visible spectrum the intensity of the scattered light shows the relationship $i \alpha \lambda^{-4}$ which is characteristic of Tyndall scattering (Walsby, 1970). By refraction, scattering and internal reflection gas vacuoles may so redirect the path of light from the cell as to provide shielding of underlying, light-sensitive structures such as photosynthetic pigments (which are sensitive to photo oxidation) and the genetic material (which may be damaged by ultra-violet light). However, (Walsby, 1972) has suggested on the basis of similar measurements carried out with another alga, *Anabaena flos-aquae* that the perturbations are largely, though perhaps not totally, the result of a measurement artifact in which the absorption peaks are flattened when the pigments are confined to cells in suspension, rather than being homogeneously dispersed in solution (Duysens, 1956).

Light intensity in cyanobacteria:

Within certain limits, an inverse correlation between light intensity and photo pigment content is general among photosynthetic organisms. As might be expected, the ratio of phycocyanin to chlorophyll can vary widely in response to simultaneous change in light intensity, temperature and carbon dioxide partial pressure. In chromatically adapting strains, growth in green light stimulates the synthesis of the red-coloured phycoerythrin (absorbing in the green) whereas when growth occurs in red light it is the blue protein, phycocyanin (absorbing in the red), which is the dominant biliprotein. Fujita and Hattori (1960) discovered that the pattern of biliproteins synthesized in the dark depends on the wavelength of light received by the suspension immediately prior to dark incubation. After a brief 'red' suspension immediately (of the order of minutes), phycocyanin alone is synthesized whereas after a brief 'green' irradiation, phycoerythrin and phycocyanin are both synthesizes. These effects are repeatedly reversible; the last 6 irradiation determines the pattern of biliprotein synthesis in the dark. The chromatic effects are clearly photochemical, being independent of the temperature used during the irradiations (Fujita and Hattori, 1962).

Cytology and supra molecular organization of Cyanobacteria

Cell wall:

The fine structure of the cell wall of cyanobacteria is of the Gram-negative type (Edwards *et al.*, 1968; Allen 1968). The innermost layer of the cell wall, the electron-opaque or 'peptidoglycan layer' overlays the cytoplasmic membrane. The peptidoglycan layer is separated from the cytoplasmic membrane by an electron-transparent space. The peptidoglycan layer of most cyanobacteria is thicker than in most Gram-negative bacteria and the width varies between 1 and 10 nm and can go to 200 nm in *Oscillatoria princeps* (Halfen and Castenholz, 1971). The peptidoglycan layer is resistant to treatment with hot sodium dodecylsulphate (4%, 100ºC) whereas both the outer membrane and the cytoplasmic membrane are solubilized by this detergent. By contract, the peptidoglycan layer is completely or partially removed by lysozyme treatment (Jensen and Sicko, 1971). Isolated peptidoglycan layers show a smooth surface and conserve the shape of the cell (Hocht *et al.*, 1965). Regularly arranged discontinuities have been observed in peptidoglycan layers of many cyanobacteria. Pores which average 13 nm in diameter are located in single circumferential rows on either side of every cross wall and are formed from the

inside outward (Frank *et al.*, 1962; Lamont, 1969). Pit-like pores which average 70 nm diameters are uniformly distributed over the cell surface with an average centre-to-centre spacing of 200 nm.

External layers:

The Gram-negative cell wall may be surrounded by one or more additional layer of considerable thickness (up to 1 µm). These layers have been named cell wall layer, sheath, capsule, or slime. The preparations obtained by various techniques may influence the delicate structure of the cell envelope in different ways and difficulties in terminology have been discussed recently (Drews, 1973). Pleurocapsalean cyanobacteria synthesize a fibrous material deposited outside but closely adherent to the outer membrane. The material is produced and laid down after cell division upon the whole surface of the outer membrane. Consequently, after some generations the cells are surrounded by one or several fibrous external layers. Some cyanobacteria excrete slime or mucilage which becomes dispersed around the organism and, to an extent, partially dissolved in the culture medium. The cells or trichome are not regularly arranged in the slime mass. Slimy investments are frequently produced by planktonic cyanobacteria; they can be visualized on agar plates by Indian ink either as broad, amorphous shrouds insoluble in water or as regular zones around the cells with a definite delimitation. Slimy investments do not reflect the shape of the cell and have no well-defined structure.

Biochemical properties of peptidoglycan:

The presence of peptidoglycan in cyanobacteria was indicated by the sensitivity of cyanobacteria cells to specific enzymes and antibiotics. Treatment with penicillin or lysozyme inhibits their growth (Foter *et al.*, 1953; Frank *et al.*, 1962) and induces lysis or formation of spheroplasts (Fuhs, 1958; Crespi *et al.*, 1962; Drews and Meyer, 1964; Biggins, 1967; Jensen and Sicko, 1971). Cell envelopes or murein sacculi (Hocht *et al.*, 1965) isolated from cyanobacteria was degraded by lysozyme. Analytical-chemical data on peptidoglycan from cyanobacteria were first obtained and found diaminopimelic acid in cyanobacteria cells and later by quantitative analysis of isolation peptidoglycan from *Phormidium unicinatum* cells (Frank *et al.*, 1962). Hocht *et al.*, (1965) purified peptidoglycan by phenol extraction, and treatment with proteases and polysaccharide hydrolases. Molar ratios of glucosamine: muramicacid: alanine: glutamic acid: diaminopimelic acid of 1:1:2:1:1 were found with *Phormidium foveolarum* and *Tolypothrix tenuis*. The peptidoglycan from some cyanobacteria including that of a halophilic strain has higher glucosamine content than the A-type of Gram-negative bacteria. Ratios up to 2:1 glucosamine : muramic acid were observed, although larger fragments of lysozyme treated peptidoglycan of *Anacystis nidulans* still showed this high glucosamine:muramic acid ratio, smaller fragments had respectively 1.5:1 or 1:1 ratios (Hocht *et al.*, 1965).

Arrangement and structure of thylakoids

Variation within vegetative cells:

The most widespread arrangement of thylakoids in vegetative cells is that where some membranes at least can be seen in any region of the cell but where, nevertheless, there is a marked tendency for concentration towards the cell periphery. The thylakoids may, however, be almost entirely peripheral as in *Anacystis nidulans* (Allen, 1968) or, at the other extreme, scattered almost through the cell as in meristematic cells of *Gloeotrichia* (Miller and Lang, 1968) or dark grown cell of *Chlorogloea fritschii* (Peat and Whitton, 1967) some, but not all, other species capable of dark growth. In species with cells in which the great majority of thylakoids are peripheral, they are generally oriented parallel to the longitudinal cell wall. In fact, most species show a tendency for more thylakoids to run parallel to the wall rather than either perpendicular to it, or some other irregular arrangement. In *Spirulina platensis* and *S. geitleri* (Busson, 1971), however perpendicular orientation was found to be most common, although in another study of *S. platensis* the arrangement of thylakoids was much more irregular. Thylakoids running predominantly at right angle to the plasmalemma have been shown in a section of *Gloeocapsa* sp. During cell division in some species, the thylakoids are severed immediately by developing cross walls; where as in other they invaginate with wall in growth and part only just before completion of the walls.

Cell division and separation:

Three types of cell division have been observed among cyanobacteria: in the first, simultaneous invagination of all cell wall layers occurs (constrictive type, similar to that of Gram-negative bacteria); in the second, a septum is formed by invagination of the outer membrane (septum-type) .The difference between both types is more gradual than clear-cut. Cell division of chroococcacean and pleurocapsalean cyanobacteria, which are reproduced by binary fission, is initiated by invagination of the

cytoplasmic membrane and peptidoglycan layer. Outer membrane and external layers follow immediately, or delay this constrictive invagination (Ingram and Thurston, 1970; Thurston and Ingram, 1971). In the constrictive type, cell division and cell separation are combined. A stimulation of peptidoglycan synthesis was observed during cross-wall formation. There was, however, no correlation between septum formation or cell separation and the rate of turnover of the peptide portion.

Specialized cells in cyanobacteria:

Cells which appears with the light microscope to be obviously different from typical vegetative cells are terminal cells of some filamentous forms (especially *Oscillatoriaceae*), the akinetes (spores) and the heterocyst. Electron micrographs of terminal cells in the form of calyptras (Lamont, 1969) with no attention called to any distinction of the intracellular membrane. Bailey-Watts (1968) showed that the end region of a very long-celled form of *Synechococcus* sp. possessed a set of microtubules about 15 nm in diameter and 300 nm long and thus quite unlike thylakoids. Although there have been only a few studies (Wildon and Mercer, 1963; Clark and Jensen, 1969; Miller and Lang, 1968) illustrating the ultrastructure of akinetes, in general, the arrangement of thylakoids is similar to that in vegetative cells prior to their enlargement and differentiation. During germination of the akinete of *Cylindrospermum* sp. increase in thylakoids in the germling filament appears to occur by fusion of small vesicles in the cytoplasm in a somewhat similar fashion to that seen in hormogonia of *Oscillatoria* (Jost, 1965).

The heterocyst: nitrogen fixation

Morphology and occurrence:

Heterocyst is produced by a group of cyanobacteria, particularly nostocalean and stigonematalean members which display an unbranched or branched filamentous vegetative structure. In most cases, heterocyst is readily recognized under the light microscope by their characteristic feature- a) rounded shape and relatively large size as compared with the ordinary vegetative cells; b) partial separation from the neighbouring vegetative cells by means of constriction; this only leaves a narrow waist-like connection between the two types of cell; c) presence of a conspicuous, thick envelope which surrounds the heterocyst, except at the polar region. These characteristic features of heterocyst are not always apparent; for example in *Chlorogloea fritschii* heterocyst is small, show a variety of structures (Whitton and Peat, 1967) and are therefore not easy to recognize. Heterocyst may appear in terminal position, i.e. at one end of the filament as in *Gloeotrichia* species and are then designated 'basal' heterocysts, or at both ends of the filament as in *Anabaenopsis*. Alternatively they may be situated between the vegetative cells of the filament in an 'intercalary' position as in *Anabaena* or *Nostoc* species. In the later case they are sparsely scattered between the vegetative cells showing a neither more nor less regular distribution.

Contact between heterocyst and vegetative cell is mediated by the pore channel. This is the constricted, narrow extension of the heterocyst protoplast surrounded by an enormously thickened part of the heterocyst envelope with a septum separating heterocyst from adjacent cell , this whole structure is the 'pore', 'polar nodule' or 'polar thickening' of light microscopic. Terminal heterocyst appear 'one pored' while intercalary heterocyst are generally 'two pored'. In species with branched filaments heterocyst eventually may display three pores when they form at the branch base as in *Brachytrichia balani* (Iyengar and Desikachary, 1953) or in *Mastigocladus laminosus* (Venkataraman, 1957); such heterocyst are in direct contact with three vegetative cells. Heterocyst usually appears solitary but frequently double heterocysts are encountered. In exceptional cases they may be formed in series (Singh and Tiwari, 1969). Heterocyst in *Anabaenopsis* as a rule form in pairs; the filament afterwards breaks between the two newly formed heterocyst; the new filaments produced bear terminal heterocyst at both ends.

Structure:

Early light-microscopic observations have provided vague and often conflicting information on the content and structure of heterocyst. It is only since the application of the electron microscope to the study of heterocyst (Grilli, 1964; Lang, 1965) that more consistent and detailed information has been obtained on the cellular organization of these cells (Fay and Lang 1971; Kulasooriya *et al.*, 1972). In the electron microscope the heterocyst of *Anabaena cylindrica* appears as most characteristic, highly differentiated cells, possessing dense cell contents and furnished by a massive surface structure. The heterocyst protoplast is distinguished by the presence of an extensive membrane system and by the absence of granular inclusion typical of the vegetative cells. The thylakoids, which in the vegetative cells are arranged in concentric layers and show by and

large a peripheral distribution, are replaced by a reticulate system of membranes, more evenly distributed within the cytoplasm.

The membranes may locally exhibit a densely appressed or coiled configuration. In mature heterocyst some polarization of the membrane system is usually present, accompanied by an increasing contortion resulting of thylakoids and their concentration towards the pole region. This results in the formation of typical semi-crystalline lattice-like structures which resemble the so-called 'prolamellar bodies' common in the proplastids of higher plants. It appears that in the late period of heterocyst development these structures may break down contributing thereby to the formation of a plug-like material gradually filling the pore channel .On the whole, the heterocyst membrane system appears to be more extensive, constituting a substantial increase of the total membrane surface area, as compared with that represented by the thylakoids in the vegetative cells. This suggests that membrane synthesis accompanies heterocyst differentiation (Kulasooriya *et al.*, 1972). The only granular structures which are seen to be retained in the electron micrographs of mature heterocyst following osmium fixation are the ribosomes. These appear less densely clustered than in the vegetative cells, though not necessarily reduced in numbers as they are more evenly scattered throughout the cytoplasm in the heterocyst. Another distinguishing feature of heterocyst structure is the vast envelope around the cell. It is composed of three distinct layers, the outermost fibrous, the middle homogenous and the innermost laminated layers.

Differentiation of heterocyst:

Heterocyst is invariably produced during active growth of the cyanobacteria (Fogg, 1949) by the transformation of a normal undifferentiated vegetative cell. This process can be followed under the light microscope (Fritsch, 1951) and the intermediate stages of heterocyst development are normally present side by side in a growing culture. Although there is species variation, the development of heterocyst may be described in general terms. The first visible signs of a change are the elongation and the rounding off of the transforming cell accompanied by a decrease of granulation characteristic of the vegetative cells. This indicates the dissolution and mobilization of storage products. In the next stage the constriction between developing heterocyst and the vegetative cell deepens and the deposition of a new surface structure becomes obvious. The cell content appears nearly homogenous, and the pigmentation less bluish-green. In the last stage of development, heterocyst appears to be furnished by a copious envelope, especially thick near the poles. The appearance of highly refractive polar thickenings indicates the completion of heterocyst differentiation. Pigmentation, though change to a more yellowish tint, definitely persists in the mature heterocyst (Fritsch, 1951).

Nitrogen-fixing cyanobacteria:

The ability to reduce the dinitrogen molecule to amino is restricted to prokaryotes where it is relatively scarce but spread widely through the systematic groups. Among these organism, a prominent role is occupied by the cyanobacteria; the N_2 both in the free-living state and in symbiosis with a wide range of partner. Free-living N_2-fixing organisms are often said to contribute rather little to the nitrogen input in the global nitrogen cycle. Their resistance to extremes of temperature and dessiccation and to their modest nutrient requirements; most nitrogen-fixing species thrive photoautotrophically, the contributions of the liverworts and particularly of the lichens to the global nitrogen cycle are virtually unknown but probably of major importance, whereas those of the symbioses with higher plants (*Gunnera*, cycads) are more or less negligible. The important of the water-fern *Azolla* containing the cyanobacterium *Anabaena azollae* has been recognized by farmers of the Southeast Asian countries for centuries. N_2-fixation by unicellular cyanobacteria is exceptional. The first report came from Wyatt and Silvey (1969) for *Gloeocapsa*.

Biochemical properties of Cyanobacteria

Proteins and lipoprotein:

Amino acids, indication protein, were found in varying amounts in cell envelope fractions of cyanobacteria (Frank *et al.*, 1962; Drews and Meyer, 1964; Hocht *et al.*, 1965). It is difficult, however, to confidently attribute them to the cell wall or more specifically to the outer membrane. Breaking of the cells with high mechanical power and treatment with detergents may result, on the other hand, in a partial loss of outer-membrane material. Evidence for outer-membrane protein of cyanobacteria was obtained from SDS-polyacrylamide gel electrophoresis of SDS or phenol extracts from cell envelope of *Anacystis nidulans*. Four major protein bands were detected. One of them (44 k daltons) ran in the same position as a major protein of the *Escherichia coli* outer membrane; another one had an apparent molecular mass of 10 k daltons,

similar to the lipoprotein of *E. coli* cell walls. There is no evidence as yet for the presence of lipoprotein in cyanobacteria cell wall. No fatty acids were present in the SDS-insoluble cell envelope fraction of *Synechocystis* PCC 6714 and *Synechococcus lividus* OH-53S.

Lipids:

High lipid content was found in cell envelope of *Anacystis nidulans* (Drews and Meyer, 1964; Drews and Gollwitzer, 1965). Isolation cell envelopes of *Synechococcus lividus* OH-53S and *Synechocystis* PCC 6714 both contain approximately 4% fatty acids. The fatty acid and lipid analyses on whole-cell extracts (Kenyon, 1972) do not allow specific assignments to lipids of the cell wall. Interestingly, the phosphate content of cell envelopes of cyanobacteria is low (0.1%) of cell envelope dry weight in *Anacystis nidulans* (Drews and Meyer, 1964; Drew and Gollwitzer, 1965); 0.1 and 0.2% in *Synechococcus lividus* OH-53S and *Synechocystis* PCC 6714, respectively.

Lipopolysaccharides:

Hocht *et al.*, (1965) described an 'amorphous' fraction obtained from crude cell wall of *Anacystis nidulans* by phenol extraction. It resembles the lipopolysaccharide of Gram-negative bacteria in physico-chemical properties; although no lipid and glucosamine were detected in this material which is rich in the neutral sugars mannose, galactose and glucose. They succeeded in the isolation of a highly aggregated polymer which could be reversibly disaggregated on treatment with 1% SDS and which was clearly shown to consist of polysaccharides with covalently bound lipid A. Mild acid hydrolysis cleaved off lipid A with the concomitant release of 2 –keto-3-deoxyoctonate known to be the molecule connecting lipid A with the polysaccharide moiety of O-antigens of Gram-negative bacteria.

Polysaccharides:

Heteropolysaccharides are regular constituents of cell walls of cyanobacteria. In a number of strains they have been shown to be part of lipopolysaccharides. Although with other strains no fatty-acid analyses have been performed, *Anacystis nidulans* (Drew and Gollwitzer, 1965; Hocht *et al.*, 1965), *Phormidium foveolarum* and *Tolypothrix tenuis* (Hocht *et al.*, 1965), *Anabaena cylindrica* (Dunn and Wolk, 1970), it is reasonable to assume that some of the sugars found are constituents of lipopolysaccharides. In *Spirulina platensis*, a homoglucan was found in cell wall fractions (Van Eykelenburg, 1978).

Phycobilisomes: composition and structure

Phycobiliproteins:

Phycobiliproteins are accessory pigments for the operation of photosystem II in three groups of oxygenic phototrophs: the eukaryotic cryptomonads and rhodophyta and the prokaryotic cyanobacteria (O'Carra and O'hEocha, 1976; Glazer, 1981). Each species of cryptomonad contain one type of biliprotein, either phycocyanin or phycoerythrin (Gantt, 1979). Phycobiliproteins represent a consider fraction (up to 50%) of the total cellular proteins of red algae and cyanobacteria (Myers and Kartz, 1955; Giraud, 1963; Bennett and Bogorad, 1973; Gantt and Lipschultz, 1973, 1974). Before 1965, cytological observations (Brody and Vatter, 1959; Wildon and Mercer, 1963; Giraud, 1963) of thin section had revealed the peculiarity distinctive to cyanobacteria and rhodophyta that the thylakoid membrane were never stacked but separated from one another by a regular space of approximately 80 nm.

Structure of phycobilisomes in the intact cell:

Electron-opaque granules identifiable as phycobilisomes have been described in many red algae and practically all cyanobacteria examined (Bryant *et al.*, 1981; Gantt, 1979). In some cyanobacteria (particularly the small unicellular strains) the interthylakoidal space may appear uniformly electron-dense. This can be attributed to the close packing of the phycobilisomes. Phycobilisomes are arranged in short or long rows spaced on the stromal surface of thylakoid membranes. The unique exceptions are the bundle-shaped phycobilisomes of the structurally atypical cyanobacterium *Gloeobacter violaceus*. The cellular biliprotein composition does not determine the shape of phycobilisomes; however, in red algae, phycoerythrin is the main component of all globular types.

Isolation of phycobilisomes:

A technique for the isolation of phycobilisomes was first devised Gantt and Lipschultz (1974) for *Porphyridium cruentum*, and later slightly modified for *Nostoc* sp.; Gantt, 1979). A simplified procedure applicable to most red algae and cyanobacteria for the preparation of high yields of energetically well-coupled phycobilisomes.

Protein composition:

Method for the isolation of intact phycobilisomes has allowed studies of their protein composition. The major protein components of phycobilisomes are the phycobiliproteins which account for approximately 85-90% of the total phycobilisome protein (Yamanaka and Glazer., 1980).

Biliproteins: Nomenclature and distribution

Phycocyanins and phycoerythrins were originally designated with a prefix, C or R, to indicate the

origins of spectroscopically distinct proteins form rhodophyta or cyanophyta respectively (Svedberg and Katsurai, 1929). For example, C-phycoerythrins have a single absorption maximum at about 565 nm, while R-phycoerythrins exhibit absorption maxima at about 498, 540 and 565 nm. The prefix B is derived from Bangiales, a primitive order of red algae, and was used by Airth and Blinks (1956) to indicate yet another spectroscopically distinct phycoerythrin which has absorption at 540 and 565. As it has been recognized that biliproteins of a given spectral class can be isolated from both red algae and cyanobacteria, the designation R, C and B are now applied to biliproteins to indicate visible absorption spectral properties and do not indicate the type or organisms from which they are derived. The marine cyanobacterium *Trichodesmium thiebautii* (Fujita and Shimura, 1974) and the unicellular cyanobacterium *Gloeobacter violaceus* contain phycoerythrins which are spectroscopically similar to the red algal R-phycoerythrin. Certain cyanobacteria capable of chromatic adaptation have been shown to contain two types of phycocyanin (Bryant and Cohen-Bazire, 1981). One phycocyanin is synthesized constitutively, while the production of the second phycocyanin is specifically induced during growth in red light. The subunits of these two phycocyanins differ with respect to charge, molecular mass and amino-acid composition. *Agmenellum quadruplicatum* (Gray *et al.*, 1976) and *Phormidium luridum* (Brown *et al.*, 1976) have each been reported to contain two spectroscopically distinct phycocyanins; however, the structure basis for the differences in these two cases is not known. Several cyanobacteria have been reported to contain chromatographically distinct allo-phycocyanins (Gysi and Zuber, 1974, 1976; Zilinskas *et al.*, 1978, 1980). The basis for the observed differences in these cases is not clear; the presence of an additional subunit may explain the origin of one of these forms (Zilinskas *et al.*, 1978). Amino-terminal sequencing of three such allo-phycocyanin forms has thus far revealed no structural differences (Troxler *et al.*, 1979). The phycobiliproteins owe their intense visible absorption properties to the presence of open-chain tetrapyrrole chromophores which are related to the animal bile pigments biliverdin and bilirubin (Lemberg and Bader, 1933). These prosthetic groups are commonly referred to as phycobilins, or simply bilins. Unlike the chlorophyll and carotenoid chromophores which are extractable with organic solvents, the phycobilins are bound to the apo-proteins by covalent linkages.

Effects of environment factors on phycobilisomes:

The phycobiliprotein content and composition of cyanobacteria can be affected by numerous environmental factors. These factors include temperature, carbon dioxide concentration (Miller and Holt, 1977), phosphate concentration (Ihlenfelt and Gibson, 1975), sulphate starvation, nitrogen depletion (Allen and Smith, 1969; Yamanaka and Glazer, 1980), light intensity and light wavelength.

Phycobilisomes and thylakoid membrane organization:

Efficient energy transfer from the phycobilisomes to the reaction centres of PS II requires that the phycobilisomes, in particular the allo-phycocyanin-allo-phycocyanin-B of the core, be precisely oriented with and in close proximity to the thylakoid-bound chlorophyll a of this photosystem. In cyanobacteria and red algae, a small fraction of the total chlorophyll a is associated with PS II-16% in *Anacystis nidulans* (Wang *et al.*, 1977) and only 5% in *Porphyridium cruentum* (Ley and Butler, 1976) whereas the bulk of the chlorophyll is associated with PS I.

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Nitric oxide (NO) as a modulator of Periodontitis- A Review

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Abstract:

Periodontitis, a chronic inflammatory disease, is a major cause of tooth loss. Proinflammatory cytokines and prostaglandins are essential components of this disease. Cytokines such as Interleukin -1 (IL-1) and tumour necrosis factor-α (TNF-α) stimulate synthesis of nitric oxide (NO) from osteoblasts and their progenitors. This NO manifests inhibitory action on bone resorption, by inducing apoptosis of osteoclasts. Cytokine combinations were much more potent stimulators of NO production than individual cytokine. NO donors such as 3-morpholinosydnoimine (SIN-1), isosorbide also manifest antiosteoclastic action. Apart from antiosteoclastic action NO also possess antimicrobial action against *Porphyromonas gingivalis* and interacts with free radicals like superoxide ion (O_2^-) to form Peroxynitrate ($ONOO^-$), which is capable of eliciting lipid peroxidation and cellular damage. It also plays complex role in modulation of inflammatory response as the induction of iNOS (inducible nitricoxide synthase) can lead to high concentration NO, which exert proinflammatory effects and induction of eNOS (endothelial nitric oxide synthase) serve as protective or anti inflammatory functions. NO can also activate proinflammatory enzymes such as cyclo oxygenase and metallo proteinase which contribute to periodontal tissue destruction. NO has inhibitory effect on platelet aggregation and adhesion leading to increased gingival bleeding. The gingivomucosal antioxidant defence comprises of Superoxide dismutase (SOD) which prevents the formation of peroxynitrate by catalyzing the dismulation of the superoxide. Low concentration of basally produced NO maintains normal homeostasis and protective under physiological condition in circumdental tissue. Selective inhibition of the inducible isoform of nitric oxide synthase (iNOS) and maintenance of constitutive NO production may have therapeutic utility in periodontitis.

Key words : Periodontitis, Cytokines, Nitric oxide, Osteoblasts, osteoclasts, Isosorbide.

Introduction:

Periodontitis, a chronic inflammatory disease, is a major cause of tooth loss in an adult (William, 1980). The proinflammatory cytokines and prostaglandins are essential components for the pathogenesis of this disease (Offenbacher *et al.*, 1986; Agarwal *et al.*, 1995). Cytokines such as Interleukin-1 (IL-1), Tumour necrosis factor-α and Interferon-γ stimulate nitric oxide production in many cell types and it has been implicated in vast array of inflammatory conditions (Lappin *et al.*, 2000; Lohinai *et al.*, 2001).

Nitric oxide (NO) is a short- lived radical that plays important role in several biological processes (Moncada *et al.*, 1993), it is produced in vascular endothelium and nervous tissue constituvely by calcium-calmodulin dependent form of nitric oxide synthase (NOS). Other cells including macrophages, neutrophils, chondrocytes, osteoblasts, osteoclasts and bone marrow can also be induced to produce NO after exposure to endotoxin or cytokines (Schimidt *et al.*, 1992; Mc Call *et al.*, 1989; Palmer *et al.*, 1993; Kukkarem *et al.*, 1995; Ralston *et al.*, 1994; Punjabi *et al.*, 1992). NO production by these cells are catalysed by an inducible form of nitric oxide synthase (NOS). NO plays a detrimental as well as a beneficial role. Detrimental effects may include a cytotoxic action towards the adjacent host tissue including alveolar bone (Lin *et al.*, 2003). This effect may result from the formation of reactive nitrogen species, such as peroxynitrate anion ($ONOO^-$), that results from combination of nitric oxide (NO) and superoxide anion (O_2^-) (Murphy *et al.*, 1999).

Components of microbial plaque have the capacity to induce the initial infiltrate of inflammatory cells including polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages. Microbial components especially lipopolysaccharides (LPS) play the role to activate macrophages to synthesize and secrete a wide array of molecules including prostaglandins especially PGE-2 and hydrolytic

enzymes. Bacterial subtances activate T-lymphocytes and they produce IL-1 and Lymphotoxin (LT), a molecule having properties similar to TNF-α. These cytokines manifest potent proinflammatory and catabolic activities which play key roles in periodontal tissue destruction (Page RC, 1991).

IL-1, TNF-α and INF-γ have been shown to stimulate NO production from osteoblasts and osteoblast progenitors. NO is a potentially osteoblast-osteoclast coupling factor which has potent inhibitory effect on bone resorption. These actions, inturn are mediated by inhibition of osteoclast formation due to NO induced apoptosis of osteoclast progenitors and by inhibiton of resorptive activity of mature osteoclasts. NO maintains a central control on bone resorption in both avian and mammalian species by a powerful tonic restraint of osteoclast number and its activity. NO also produces rapid osteoclast detachment and contraction in-vitro and this effect is accompanied by profound inhibition of bone resorption. Cytokines- induced nitric oxide inhibits bone resorption by inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity (Brandi *et al.*, 1995; Vant *et al.*, 1997). Cytokine combinations were much more potent stimulators of NO production than individual cytokines. Dramatic stimulation of NO production and inhibition of bone resorption resulted when INF-γ was combined with IL-1 or TNF-α. INF-γ had no effect on bone resorption and little effect on NO production when used alone. IL-1 and TNF-α acted together to stimulate NO production but to a lesser degree that when compared with INF-γ. Nitric oxide synthase inhibitor, L-N(G)-monomethylarginine (LMMA) inhibited bone resorption induced by IL-1 and TNF-α, suggesting that lower concentration of NO stimulate bone resorption. Experiments with NO donor S-nitroso-acetyl-penicillamine (SNAP) showed generalized suppression of bone resorption at high SNAP concentrations, but potentiation of IL-1 induced resorption at lower SNAP concentration. Hence it was concluded that cytokines are potent inducers of NO in bone and that cytokine induced NO production has bi-phasic effect on bone resorption (Ralston *et al.*, 1995). It was shown in the study that nitric oxide produced a decrease of up to 50% of original osteoclast spread area. Similar effects were produced by 3-Morpholinosydnoimine or Sodium nitroprusside, reagents that spontaneously release NO. These shape changes are associated with reduction of bone resorption after a 24 hour incubation of isolated osteoclasts on devitalized bone slices (Mac *et al.*, 1991). It was found that NO donor 3-morpholinosydnoimine (SIN-1) profoundly depressed replication in human pro-osteoclast line (Brandi *et al.*,1995). Leitao *et al.*, (2004) reinvestigated the effect of local administration of NO donor isosorbide in Wister rats that were subjected to ligature placement around the cervix of right second upper molar and sacrificed after 11 days. Alveolar bone loss was measured in one quadrant as the sum of distance between the cuspid tip and the alveolar bone along the axis of each molar root which was subtracted from contra lateral side used as unligated control. The semi quantitative histopathological scale of periodontium was based on cell infiltration along with alveolar bone and cementum integrity. It was concluded that locally applied exogenous NO source reduce local inflammatory cell infiltration, cementum resorption and alveolar bone resorption. The lack of effect with 1% isosorbide and a significant effect with 5% isosorbide shows that the effect of NO depends on its concentration. Apart from antiosteoclastic action nitric oxide also posses antimicrobial activity as it shares an important element of host defense against *Porphyromonas gingivalis* (Gyurko *et al.*, 2003).

Discussion

Nitric oxide is a ubiquitous intercellular messenger molecule with important cardiovascular, neurological and immune functions (Nathan, 1992). The endogenous production of nitric oxide by neurons and endothelial cells play vital role in the regulation of physiological process like, blood vessel tone regulation and neurotransmission as well as host defence and immunity (Clancy et al., 1995; Gonzalez *et al.*, 1998). However excess of NO can exert cytotoxic effect (Stefenovic *et al.*, 1993). This may involve both direct toxicity, for example the reaction of NO with iron containing enzyme of respiratory cycle and in the synthesis of DNA and by the interaction of NO with other free radicals like superoxides ion (O_2^-) to form peroxynitrate ($ONOO^-$), which is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage. (Micheal and Feron , 1997)

In mammalian cell, NO is generated via the oxidation of terminal guanidine nitrogen atom of L-arginine by enzymes nitric oxide synthase (NOS). There are three isomer of mammalian NOS enzyme, named as nNOS or TypeI, iNOS or TypeII and eNOS or TypeIII, reflecting the tissue of origin for the orignal protein, which are neuronal tissue immunoactivated macrophages and vascular and endothelial cells respectively (Fostermann *et al.*, 1991). Nitric oxide plays a complex role in modulation of inflammatory response. The induction of iNOS in the tissue

can lead to sustained production of high concentration of NO, which exerts proinflammatory effects including vasodilation, edema, cytotoxicity and mediation of cytokine dependent process (Palmer *et al.,* 1987). Conversely, the production of NO by endothelial cell may serve as a protective, or anti-inflammatory function, as it modulate blood flow to various tissue and regulates interactions between vascular endothelium and circulating inflammatory cells, it inhibits neutrophil and platelet aggregation and adhesion to endothelial monolayer (Rodomski *et al.,* 1987).

Nitric oxide may activate proinflammatory enzymes such as cyclooxygenase and metalloproteinase which contribute to periodontal tissue damage. The effect of excess NO in the gingivomucosal tissue may contribute to the development of the frequently found clinical symptoms of periodontitis in human. The gingival redness can be explained by vasodilatory effect of NO, and the gingival swelling by the vascular permeability-increasing effect of NO. The increased tendency of the soft tissue to bleed on gentle probing is due to the inhibitory effect of NO on platelet aggregation and adhesion. Lastly the increased alveolar bone resorption may be due to the stimulatory effect of NO on the activity of osteoclasts. (Salvemini *et al.,* 1993, Murrel *et al.,* 1995)

A delicate balance exists between the pro-oxidant mechanism of tissue destruction and antioxidant repair system .The gingivomucosal antioxidant defense mechanism appears to act in concert (Chapple , 1997). Human periodontium contains superoxide dismutase (SOD), with its activity decreasing with age. SOD may play a role in preventing the formation of peroxynitrate by catalyzing the dismutation of the superoxide (Jacoby and Davis , 1991). Exogenous application of Liposome encapsulated superoxide dismutase is beneficial in a dog model of periodontitis (Petelin *et al.,* 1997). The antioxidant ascorbic acid, which is a scavenger of reactive nitrogen species can be detected in gingival crevicular space in concentration at three times higher than in plasma (Whiteman and Halliwall, 1996; Mayla and Kaptiza, 1990). Mercaptoethylguanide (MEG) which is a selective inhibitor of iNOS and scavenger of peroxynitrate, significantly reduces the plasma extravasation in gingivomucosal tissue and decreases the degree of alveolar bone destruction in a rat model in periodontitis (Southan *et al.,* 1996, Szabo *et al.,* 1997).

NO can also be produced non-enzymatically in the circumdental area by the reduction of nitrite under acidic conditions, similar to the situation in the stomach (Benjamin *et al.,* 1994; McKnight *et al.,* 1997).

Conclusion

Inflammatory disease such as periodontitis is associated with increased production of NO via cytokine activation and increased bone loss, features difficult to reconcile with inhibitory effects of NO on bone resorption. Cytokine production in periodontitis is well documented particularly Interleukin-1 and tumor necrosis factor-β (TNF-β). (Salvemini *et al.,* 1993) TNF-synthesis inhibitors such as Chorpromazine have been shown to reduce bone loss in experimental periodontitis (Lima *et al.,* 2000). IL-1 and TNF-α act together to stimulate NO production and bone resorption, however when interferon-γ (INF-γ) is combined with these cytokines, a dramatic stimulation of NO production and inhibition of bone resorption have been observed (Ralston *et al.,* 1995). Thus it is possible that periodontitis is associated with dramatic bone loss because in this condition IL-1 and TNF-α production are both increased, but INF-γ production is relatively deficient as in rheumatoid arthritis. It is also reported that NO inhibits the expression of adhesion surface molecules in the endothelium. (Kubes *et al.,* 1991) Leitao *et al.*, (2004) have shown that locally applied exogenous NO source reduces local inflammatory cell infiltration, cementum resorption and alveolar bone loss in experimental periodontitis model. The lack of effect of 1% isosorbide, a pharmacological NO donor and an antianginal drug, but a significant effect with 5% shows that the effect of NO is concentration dependent. Low concentration of basally produced NO maintains normal homeostasis and is protective under physiological conditions in circumdental tissue. However, NO may be detrimental when produced in excess in inflammation and may destroy the host tissue as well as the invading microorganism. Selective inhibition of the inducible isoform of nitric oxide synthase and maintenance of constitutive NO production may be of therapeutic utility in periodontitis (Lohinai and Czabo , 1998).

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Evaluation of chemical control measures adopted against the German cockroach (*Blattella germanica* L.) in Jeddah province,Saudi Arabia.

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Abstract:

Three chemical formulations, liquid (lambda-cyhalothrin 2.5% CS), dust (boric acid) and gel formulation (imidacloprid 2.15% gel bait) were evaluated singly and in combination with each other for the control of the German cockroach (*Blattella germanica* L.). After 12 weeks, the highest cockroach catch reduction was obtained when using the combination of boric acid and gel bait (98.8%), than the boric acid treatment (94.4%), the combination of lambda-cyhalothrin and Boric acid (89.1%), the combination of lambda-cyhalothrin and imidacloprid gel bait (80.9%), imidacloprid gel bait (54.8%), and finally Lambda-cyhalothrin (-31.1%). These results indicate that the use of chemical combinations has improved the performance of both the liquid and the gel formulations and that boric acid seems to have a long residual effect since the top three treatments include boric acid.

Key words: Cockroach, boric acid.

Intoduction

Cockroaches are among the most common insects which are found in nearly all parts of the world. They are considered by some of the biologists to be one of the most adaptable and successful groups of animals (Bennett *et al.* 1997). Cockroaches are one of the most serious food and residential pests worldwide and infest the places where food is stored, prepared, or served. They are notoriously resilient and difficult to control (Service 2004).Cockroaches are capable of transmitting many pathogens (Agbodaze and Gwusu 1989, Fotedar *et al.*1991, Vythilingam *et al.*1997). In addition, cockroaches not only spoil food but cause allergic reactions and psychological distress (Brenner1995).Control strategies should therefore be redirected to emphasize the knowledge of the biology and ecology of the target cockroaches in addition to insecticide use, if any, and should be more selective and less environmentally polluting (WHO 1996).Heavy infestations of cockroaches can be effectively managed by chemical control measures (residual spray, aerosol, dust, baits, gels), followed by environmental management to deprive them of food and shelter (Schal 1988). Control of German cockroach by use of chemical insecticides has a long history going back to at least the early of this century (Mallis 1969).The main insecticide groups that are currently being used for cockroach control are the organophosphates, carbamates and pyrethroids (Koehler and Patterson 1988, Reierson *et al.* 1988, Rust and Reierson 1978. Schal, 1988). Chemicals in several other classes are also being used to a lesser extent (Fisher, 1990). Insecticide formulations which can be mixed with water and applied as a spray have been the most popular formulations for cockroach control (Mallis, 1990). Type II synthetic pyrethroids have been used widely for cockroach control because of their good residual life and flushing ability (Wickham, 1988). Most microencapsulated insecticides have low odor and can be used with confidence around most chemically sensitive people and in apartment complexes or other public facilities where there may be considerable concern about the perceived toxicity of insecticides (Koehler *et al.* 1995).

In recent years, chemists have become increasingly aware of the fascinating versatility of boron compounds, some of them remarkably complex for inorganic compounds, and the wide range of their application in industry (Borax review 1988). Boric acid is often recommended because of its low mammalian toxicity (Ebeling1975, Piper and Frankie 1978a, Slater *et al.* 1979).Baits are starting to gain wide acceptance in the management of cockroaches (Reierson 1995). Gel bait formulation may be more effective than dry baits (Denzer *et al.* 1988). Traditional insecticides have not always been effective as baits and this may be in part due to their inherent repellency (Ebeling *et al.* 1967). Gel baits can be selectively used in sensitive areas, such as premises used for the preparation of food, hospitals and kindergartens (Benson and Zungoli, 1997). Imidacloprid is an insecticide belonging to the chloronictonyl class of compounds, produced commercially by Bayer Crop Science , and its use as a new crop protection agent was first proposed in 1991(Leicht 1993). Because it exerts its effects after oral ingestion, imidacloprid is also suitable for use in bait formulations (Londershausen 1996). A number of laboratory studies have revealed efficacy of imidacloprid gel in control of cockroach infestation but only a few field studies have been done so far. Since basic studies on cockroaches in Saudi Arabia are still lacking, and to date, no any study on the control measures against German cockroach have been published. This study was conducted with the aim of exploring different cockroach control strategies including the use of a liquid spray, dust and gel bait formulations singly or in combinations with each other.

Materials and Methods

1- General Materials and Methods:

1-1 Study area and duration

The study was carried out in a residential compound in an urban area at Al-Ajwad district in east of Jeddah city from September 2007 to January 2008.

Houses selected were similar in area (50 m^2 each). Each house had kitchen, hall, two rooms (bed and dinning room), and bathroom.

Cockroach density was estimated in all houses of the compound. Of the 76 similar houses included initially, 48 had high cockroach infestation. Of these 24 houses were selected for control treatments by simple random sampling method. Six treatments were applied and each treatment was replicated in 4 randomly selected houses.

Treatments applied were: spraying by Lambda-cyhalothrin 2.5% CS, boric acid dust, imidacloprid 2.15% gel bait, combination of lambda-cyhalothrin and boric acid dust, combination of lambda-cyhalothrin and imidacloprid 2.15% gel bait, and combination of boric acid dust and imidacloprid 2.15% gel bait.

1-2 Residents cooperation:

The individuals whose houses were selected for the different control methods were briefed about the study to get their full cooperation and participation. The precaution to be followed post-treatment in the treated houses were explained to the concerned residents and it was ensured that the same were complied with during the entire trial period.

1-3 Pre-treatment assessment in field:

In every house included in the study, 5 sticky traps were placed around each of the usual harborages of cockroaches (stove, fridge, under the sink, bathroom, and dinning room). Traps were removed next morning to estimate the average cockroach density for every house.

1-4 Post-treatment density:

Post-treatment density was assessed for every house by placing the sticky traps in the same cockroach harborages of pre-treatment. The assessment was done at two, four, eight and twelve weeks after application of control measures. The percentages reduction of cockroach infestation in the respective houses were calculated using the following formula:

$$\% \text{ Reduction} = \frac{\text{Counts of pre-treatment} - \text{Counts of post-treatment}}{\text{Counts of pre-treatment}} \times 100$$

1-5 Statistical analysis:

Data was statistically analyzed by using SAS (2001) software program.

2-Control measures against German cockroaches:

2-1 Lambda-cyhalothrin 2.5% CS:

Chemical used was lambda-cyhalothrin 2.5% CS (Demand) in a form of Capsulated Suspension (CS), Syngenta, Switzerland. Lambda-cyhalothrin 2.5% CS was used at a rate of 50ml per 5 litre water. The residents were required to empty all cabinets and closets. Once the residents had left the

houses, a through insecticide treatment with Lambda-cyhalothrin 2.5% CS was applied with a B&G sprayer system operated at 1.4 kg/cm2 (20psi) with a fan spray nozzle . The spray was applied to areas around kitchen cabinets, closets, appliances, and toilet facilities and on all baseboards, door and window frames and moldings. In addition, insecticide was directed into cracks and crevices in the kitchen, dinning room, and bathroom, as well as, any additional areas of the house specified by the residents.

2-2 Boric acid dust:

Chemical used was orthoboric acid dust 99.90% (H_3BO_3) min., produced by Inkobar Company, Peru.

Boric acid dust was applied lightly (thin layer), by using a hand duster in: cracks and crevices of clothes closet shelves , hollow legs of chairs and tables, and wall or floor cracks and crevices throughout the house. Boric acid dust was lightly spread under the sink, in the dead space between the sink and wall, and around utility pipes. Also along the back edges and in corners of shelves in cabinets, cupboards, under/behind the refrigerator, stove, into the opening where plumbing pipes enter walls (such as behind the shower and washing machine), into openings around drainpipes and electrical conduits.

2-3 Imidacloprid 2.15% gel bait:

Chemical used was Imidacloprid 2.15% gel bait, Bayer AG Leverkusen, Germany.

Imidacloprid 2.15% gel bait (single application at 0.1 gel spots of 2-3 per linear meter) was used in the selected houses at the primary harborages such as the cabinet, under the kitchen sink, and around the stove and refrigerator (Appel and Reid 1992, Lund and Bennett 1978). In addition, gel spots were placed at floor–wall junctions, in corners, next to cracks and crevices (Ebeling *et al.*1966, Ebeling and Reierson 1974a, Ebeling 1991). Other treated sites included lower kitchen cupboards, upper kitchen cupboards, the infested sites and potential harborages in the bathrooms and dinning rooms.

2-4 Lambda-cyhalothrin 2.5% CS and boric acid dust:

Except for crack and crevice treatment which were done by lambda-cyhalothrin 2.5% CS in the kitchen, dinning room, and bathroom, as well as, any additional areas of the house specified by the residents, the other area of the houses were treated by boric acid dusts as previously mentioned.

2-5 Lambda-cyhalothrin 2.5% CS and imidacloprid 2.15% gel bait:

All the infested harborages in the houses were treated by Imidacloprid 2.15% gel bait spots as previously mentioned, except crack and crevice treatment which were done by Lambda-cyhalothrin 2.5% CS in the kitchen, dinning room, and bathroom, as well as, any additional areas of the house specified by the residents.

2-6 Boric acid dust and imidacloprid 2.15% gel bait:

Boric acid dusts were used as a crack and crevice treatment, while, the other areas of the houses were treated by Imidacloprid 2.15% gel bait as previously mentioned.

Results

Table (1) and Fig.(1) summarize the results of the three chemical formulations when applied singly. The pre-treatment population counts of *B. germanica* concerning the three treatments when compared with each other, showed no significant differences among them ($P > 0.05$).

The best results were obtained when applying boric acid dust followed by the imidacloprid gel and then the liquid formulation of lambda-cyhalothrin . The Boric acid dust treatment reduced the cockroach population significantly at all the intervals ($P > 0.05$), with a percentage reduction maintained at a constant level ranging from 96% to 88%. The imidacloprid gel treatment showed that there was a gradual increase in the calculated percentage reductions at week two, week four, and week eight (58%, 83% and 85.7% respectively), but the percentage reduction at week twelve declined to 54.8%. The effect of the lambda-cyhalothrin 2.5% CS started with a 73% reduction in the population counts, and then declined gradually to 69.7% reduction at week four, 30.3 % at week eight post-treatment till it was no longer effective and the counts of *B. germanica* increased by one third over the 24h pretreatment counts in week twelve.

Table (1): The effect of Lambda-cyhalothrin 2.5% CS , Boric acid dust, Imidacloprid gel when applied singly on the population of *Blattella germanica*

Intervals	Treatments					
	Lambda-cyhalothrin (Mean*± Std. Error)	% Red	Boric acid (Mean*± Std. Error)	% Red	Imidacloprid gel bait (Mean*±Std. Error)	% Red
24 hours before treatment	30.5 ± 18.2414 [a]	0.00	35.75 ± 17.1385 [a]	0.00	31.5 ± 18.7194 [a]	0.00
2 weeks post-treatment	8.25± 6.2899 [a]	73	1.25 ± 0.9465 b	96.5	13 ± 5.5528 [a]	58.7
4 weeks post-treatment	9.25 ± 2.4958 [a]	69.7	4.25 ± 2.9826 b	88.1	5.25 ± 2.0156 [a]	83.3
8 weeks post-treatment	21.25 ± 8.6639 [a]	30.3	2.75 ± 0.9465 b	92.3	4.5 ± 1.7078 [a]	85.7
12 weeks post-treatment	40 ± 10.2551 [a]	- (31.1)	2 ± 0.7071 b	94.4	14.25 ± 8.4001 [a]	54.8
	LSD =31.866		LSD =23.539		LSD =28.874	

*Number of cockroaches per house
Means with the same letters are not significantly different
LSD = Least Significant Difference.

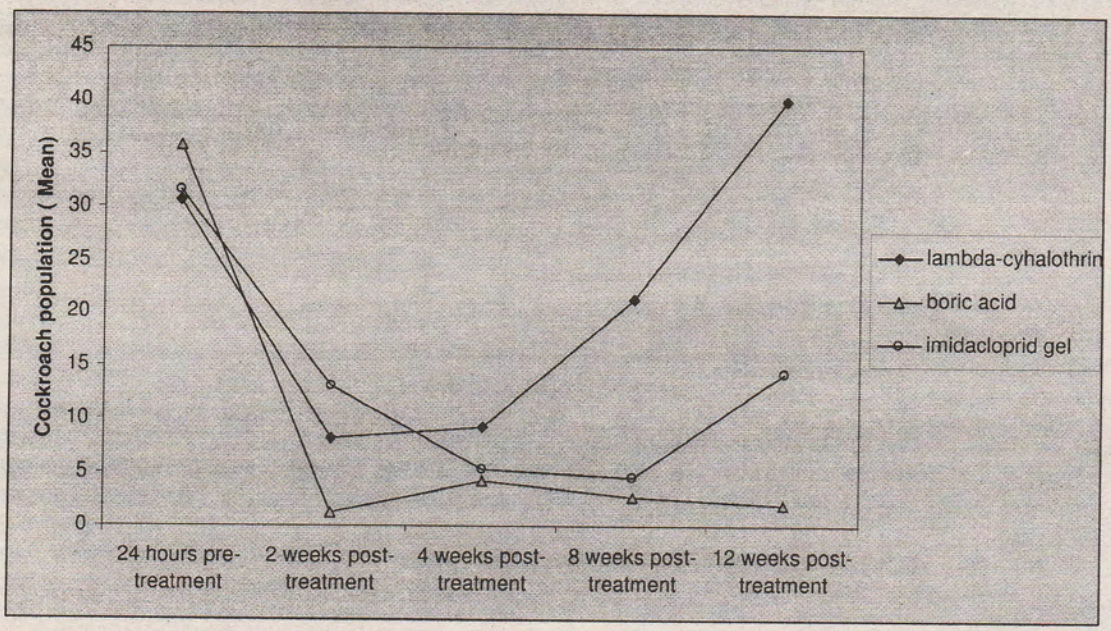


Fig. (1): The effect of lambda – cyhalothrin 2.5 CS , Boric acid dust and Imidacloprid gel when applied singly on the population of *Blattella germanica*

The effect of the three chemicals combinations on the population of *Blattella germanica* :

The combination of Lambda-cyhalothrin 2.5% and boric acid dust treatments gave a significant decline in the cockroach populations ($P < 0.05$), (table 2 and Fig.2) and indicated a gradual increase in the population reduction. This reduction started at the second week (72.6%), continued at the fourth week (83.4%), and eighth week (85.1%), and finally at twelfth week (89.1%). The combination enhanced the efficacy, of the spray solution than when it was used alone

All post treatments population counts throughout the experimental period gave significantly less cockroach's counts than the untreated 24h pre-treatment counts ($P < 0.05$) when applying Lambda-cyhalothrin and the Imidacloprid gel, (table 3 and Fig.3) . The higher percentage reduction, when compared to pretreatment counts, was obtained at the fourth week after treatment (91.8 %) followed by the eighth week (86.4%), then the second week (83 %), and finally the twelfth week after treatment (80.9%). It is obvious that the combination between the two chemicals improved the performance of both of them.

Boric acid dust when used together with Imidacloprid 2.15% gel bait significantly reduced the German cockroach (*B. germanica)* population counts at week two, week four, week eight and week twelve after application compared with the 24h pretreatment counts ($P < 0.05$), (table 3 and Fig.3). The order of the cockroaches population declining was gradually increased with time. This started with a 72.5% reduction in the cockroach population at week two after treatment, then at week four it jumped to 77.5% followed by a 97.5% reduction at week eight, and ended with 98.8 % reduction at week twelve compared with the 24h pretreatment. It is clear that the combination of imidacloprid gel bait with boric acid dust significantly enhanced the efficacy of the imidacloprid gel bait (table 4 and Fig.4).

Table (2) The effect of the combinations on the performance of Lambda-cyhalothrin

Intervals	Treatments					
	Lambda-Cyhalothrin singly (Mean*± Std. Error)	% Red	Lambda-cyhalothrin + boric acid (Mean*± Std. Error)	% Red	Lambda-cyhalothrin + imidacloprid (Mean*± Std. Error)	% Red
24 hours before treatment	30.5 ± 18.2414 [a]	0.00	43.75 ± 12.7761 [a]	0.00	36.75 ± 14.6081 [a]	0.00
2 weeks post-treatment	8.25± 6.2899 [a]	73	12 ± 1.5811 b	72.6	6.25 ± 3.6142 b	83
4 weeks post-treatment	9.25 ± 2.4958 [a]	69.7	7.25 ± 1.6008 b	83.4	3.75 ± 1.9311 b	91.8
8 weeks post-treatment	21.25 ± 8.6639 [a]	30.3	6.5 ± 1.3229 b	85.1	4.25 ± 1.3149 b	86.4
12 weeks post-treatment	40 ± 10.2551 [a]	- (31.1)	4.75 ± 2.1360 b	89.1	7 ± 0.9129 b	80.9
	LSD =31.866		LSD = 17.813		LSD = 20.566	

*Number of cockroaches per house
Means with the same letters are not significantly different
LSD = Least Significant Difference.

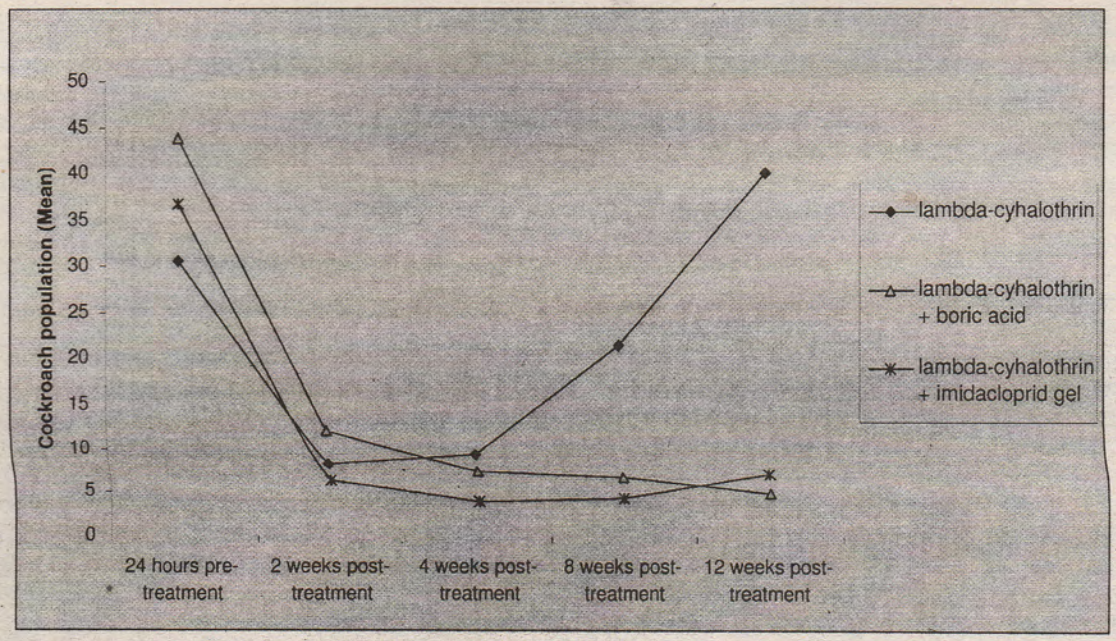


Fig. (2) The effect of the combinations on the performance of Lambda-cyhalothrin

Table (3) The effect of the combinations on the performance of Imidacloprid gel

Intervals	Treatments					
	Imidacloprid gel singly (Mean*± Std. Error)	% Red	Imidacloprid + lambda–cyhalothrin (Mean*± Std. Error)	% Red	Imidacloprid + boric acid (Mean*± Std. Error)	% Red
24 hours before treatment	31.5 ± 18.7194 [a]	0.00	36.75 ± 14.6081 [a]	0.00	40 ± 12.8906 [a]	0.00
2 weeks post-treatment	13 ± 5.5528 [a]	58.7	6.25 ± 3.6142 b	83	11 ± 4.1028 b	72.5
4 weeks post-treatment	5.25 ± 2.0156 [a]	83.3	3.75 ± 1.9311 b	91.8	9 ± 5.0166 b	77.5
8 weeks post-treatment	4.5 ± 1.7078 [a]	85.7	4.25 ± 1.3149 b	86.4	1 ± 0.4082 b	97.5
12 weeks post-treatment	14.25 ± 8.4001 [a]	54.8	7 ± 0.9129 b	80.9	0.5 ± 0.2887 b	98.8
	LSD =28.874		LSD = 20.566		LSD = 19.461	

*Number of cockroaches per house
Means with the same letters are not significantly different
LSD = Least Significant Difference.

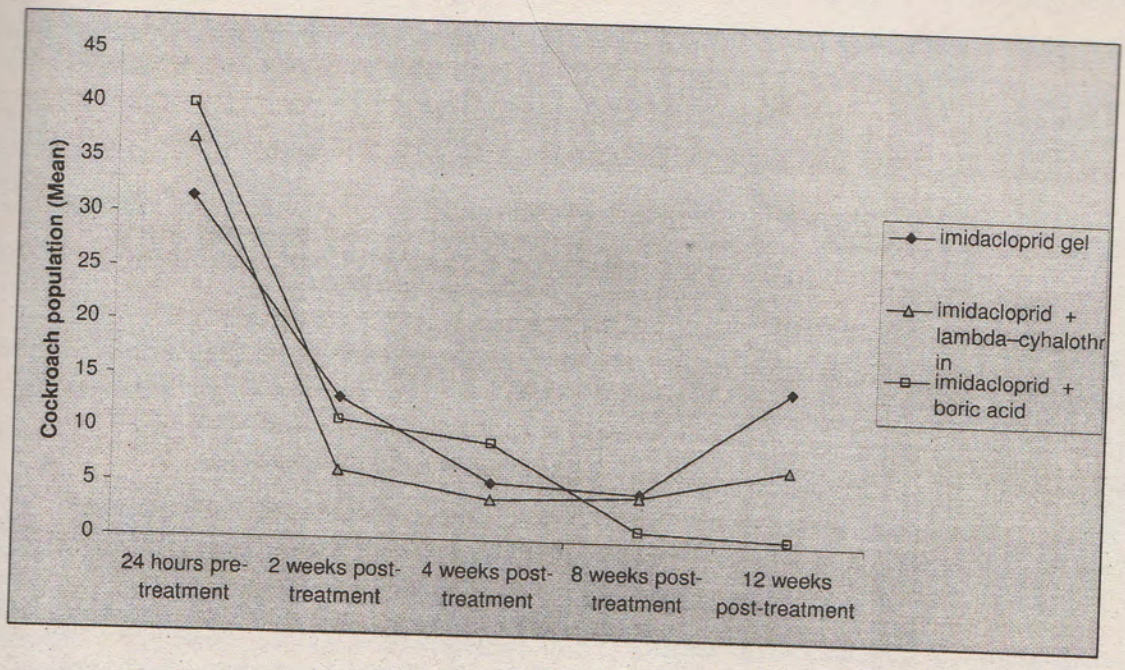


Fig. (3) The effect of the combinations on the performance of Imidacloprid gel

Table (4) The effect of the combinations on the performance of Boric acid dust

Intervals	Treatments					
	Boric acid singly (Mean*± Std. Error)	% Red	Boric acid +lambda–cyhalothrin (Mean*± Std. Error)	% Red	Boric acid + Imidacloprid (Mean*± Std. Error)	% Red
24 hours before treatment	35.75 ± 17.1385 a	0.00	43.75 ± 12.7761 a	0.00	40 ± 12.8906 a	0.00
2 weeks post-treatment	1.25 ± 0.9465 b	96.5	12 ± 1.5811 b	72.6	11 ± 4.1028 b	72.5
4 weeks post-treatment	4.25 ± 2.9826 b	88.1	7.25 ± 1.6008 b	83.4	9 ± 5.0166 b	77.5
8 weeks post-treatment	2.75 ± 0.9465 b	92.3	6.5 ± 1.3229 b	85.1	1 ± 0.4082 b	97.5
12 weeks post-treatment	2 ± 0.7071 b	94.4	4.75 ± 2.1360 b	89.1	0.5 ± 0.2887 b	98.8
	LSD =23.539		LSD = 17.813		LSD = 19.461	

*Number of cockroaches per house
Means with the same letters are not significantly different
LSD = Least Significant Difference.

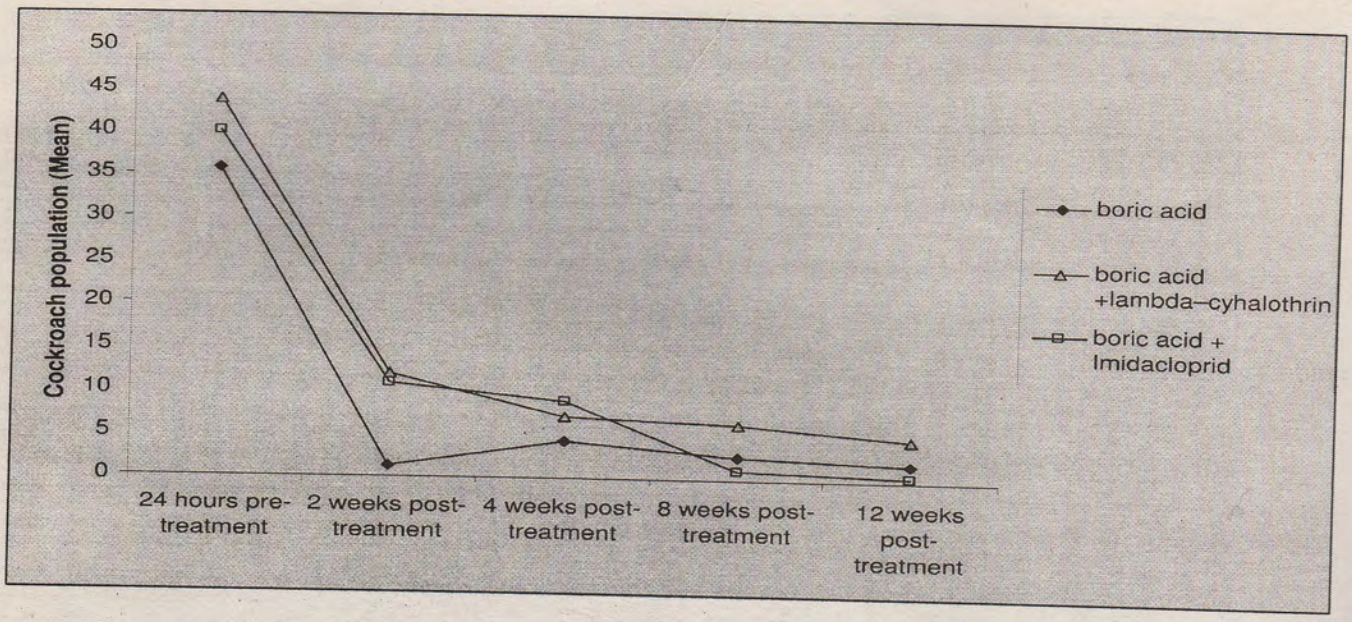


Fig. (4) The effect of the combinations on the performance of Boric dust

Discussion

The use of Lambda- cyhalothrin 2.5% CS singly to control German cockroach in this study did not result in a significant reduction of the insect populations.

The best performance of the chemical was noticed at week two which may be due to the fast knockdown effect of the liquid formulation (table 1).

Nonetheless, the relatively low percent reduction in German cockroach populations through the 12 weeks of this study may be resulted from one or more of the following reasons: (1) German cockroach may developed resistance to lambda-cyhalothrin . It has been noticed that this pyrethroid was used excessively in Jeddah province throughout the last 4 years especially with its EC and WP formulations. Lee *et al.* 1996a, Cochran 1997, Ladonni 2001, Nasirian *et al.*2006 reported that extensive use of insecticides has led to the development of resistance in German cockroach to a wide range of insecticides including organochlorines ,organophosphates, carbamates and pyrethroids. Hemingway *et al.* (1993) collected thirty strains of *Blattella germanica* (L.) reported to be pyrethroid resistant from three continents, and found greater than 2-fold resistance to the pyrethroids cyfluthrin, fenvalerate, cypermethrin, and lambda cyhalothrin appeared in 15 of these strains. (2) Repellent effects, which can lead to cockroaches avoiding exposure to lethal doses of toxicant, have been associated with dry deposits of pyrethroid insecticides (Ross and Cochran, 1992). (3) Inactivation of the chemical formulation due to adhesion of the formulation particles to substrate. It worth mentioning that the floor of the treated houses were a ceramic tile. Some laboratory bioassays using *B. germanica* indicated that Demand CS (Lambda-cyhalothrin) and other products such as deltamethrin SC were inactivated when applications were made to ceramic tile surfaces contaminated with cockroach faeces (Wege *et al.* 1999). Inactivation was immediate suggesting that insects were unable to pick-up of the formulations, possibly due to adhesion of the formulation particles to substrate. Inactivation by cockroach faeces does not appear linked to any formulation type or insecticide chemistry: Braness and Bennett (1989) demonstrated similar results for EC and CS formulations of chlorpyrifos and WP and EC formulations of cyfluthrin. (4) Leaching of the chemical deposits from the treated surfaces by routine cleaning practices undertaken by residents.

Results on table (1) showed that the population counts of *B. germanica* declined at all the post treatment counts except the last count at week twelve when it witnessed more than 30% increase indicating a

recovery of the suppressed population even more than the respective pretreatment levels. This result parallels the finding of Agrawal *et al.* (2005) who used pyrethroid (deltamethrin 0.02% + allethrin 0.13%) and propoxur (2%) aerosols in a field trial to control German cockroach, *B. germanica* and reported that an increase of 127.04% by the third week, and further to 157.86 % by eight week in the cockroach populations, was obtained in the pyrethroid aerosol. One possible explanation for that is what was reported by Grothaus *et al.* (1981) who found that at least 95% control of an initial infestation of *B. germanica* needs to be attained in order to reduce the population below a visual threshold and to avoid complaints just two weeks post-treatment. Theoretically, 90% control of a population of 500 *B. germanica* allows for 170 live cockroaches one month after treatment; 80% allows for 341; and 70% control allows for 513, more than was present at the start. Therefore, just a few survivors and some immigrants can result in numerous numbers of cockroaches later (Reierson 1995).
The relatively low percent reduction in German cockroach populations through the 12 weeks of this study is similar to other control failures in some field population that have been reported by Cochran1989, Scott *et al.* 1990, Atkinson *et al.*1991, Valles and Yu 1996, Dong *et al.* 1998, Valles 1999, Valles *et al.* 2000, Wei *et al.* 2001.

Vythilingam *et al.* (1997) carried out Control measures using lambda cyhalothrin against American cockroach (*Periplaneta americana*) and Smoky brown cockroach (*Periplaneta brunnea*) found that there was no significant difference between treated and control sites.

In this study, boric acid significantly reduced the population of the German cockroach throughout the twelve weeks, and the higher percentage reduction was obtained at week two post-treatment (96.5 %,), (table 1and Fig.1). This may be explained by the fact that there were more cockroaches that encountered the dust in the treated areas (the mean of the pretreatment cockroach counts were 43.75) resulted in more cockroaches being affected and died by the insecticide at this period. While the percentages reduction achieved at week four were (88.1%), week eight (92.3%), and week twelve (94.4%) indicating a gradual suppression in the cockroach population towards the end of the experiment. In addition, it suggests the long residual effects of boric acid over time.
Boric acid is an inexpensive inorganic insecticide with a favourable safety record and no known cases of resistance in arthropods. Various boric acid formulations have been used for more than a century for effective management of German cockroach infestations (Ebeling 1995). However, its use has declined in the last several decades primarily because much faster acting organic insecticides are preferred by consumers and pest management professionals.
Nonetheless, in the recent years, the insecticidal and toxicological profiles of boric acid are becoming increasingly favourable in the urban environment because of its efficacy and safety record.

The percentage reduction in cockroach density when using imidacloprid gel bait are presented in table (1). Imidacloprid is the first active substance from chloronicotinyl class of compounds to be used in a gel bait formulation for the control of cockroach infestations .The decisive factor with the gel is its ingestion by the insect in sufficient quantity to achieve oral efficacy. For this to occur, the gel must be highly attractive and must remain so for a lengthy period. The active ingredient should not have repellent properties, should be active during all stages in the cockroach life cycle, and should not induce resistance (Agrawal and Tilak, 2006). With its lack of contact activity against cockroaches imidacloprid is predestined for use in bait form alone (Pospischil *et al.* 1999).
Laboratory and field studies have demonstrated the high efficacy of imidacloprid cockroach gel against economically important cockroach species. Mortality rates of 95-100 % were achieved within 6 days under laboratory conditions (Miller and Peters, 1999a). Even tiny amounts of the gel bait are sufficient to kill cockroaches. Under the described conditions, application of 0.1 g gel spots at a rate of 2-3 per linear meter was found to be sufficient for causing mortality in cockroach species tested. Nevertheless, with very high infestations, sufficient gel bait must always be available to the cockroaches, which means carrying out further treatment if the spots have all been eaten or have otherwise disappeared (Durier and Rivault 2003).

Appel and Tanley (2000) reported that the imidacloprid gel bait was toxic to all stages of the German cockroach.

Baits affected cockroaches differently in relation to their developmental stage, small nymphs died more rapidly than large nymphs and adults. This may be related to differences in size, action on metabolism, or physiology (Cornwell 1976, Rust *et al.* 1995).

Gel baits have been the main method for German cockroach, *B. germanica* control in the United States for at least 5-8 years (Harbison *et al.*2003). This popularity was a result of the availability of a variety of highly effective and economical bait products such as Siege (1992), Maxforce (1997), Combat (1997), Avert (1997), Maxforce FC (1998), and Pre-Empt (1999). The active ingredients used in these cockroach baits are hydramethylnon, fipronil, abamectin, and imidacloprid.

The results in imidacloprid treated areas indicated a reduction of 58%, 83% and 85.7% at week two, week four, and week eight respectively. Since the decisive factor with a gel is its ingestion by the insect in sufficient quantity, percentage reduction was more after 5-8 weeks. A reduction in cockroach infestation by 85.7% at the end of week eight post-treatment in this study was comparable to earlier findings (Agrawal and Tilak 2006, Durier and Rivault 2003, Appel and Tanley 2000, Bhandari and Makkapati 2001).

Based on numbers of cockroaches trapped, under best conditions in the field Reierson (1995) has rarely attained greater than 80% reductions of *B. germanica* with bait. His experience has been that only under optimal conditions ,especially under considerable and unusual food or water stress such as might eventually occur in a vacant apartment , can bait reduce cockroach populations > 95%, and lead to the population being eradicated. Bait usually reduces the number of cockroaches present, but other measures are needed to keep the population suppressed.

Under dietary stress, cockroaches will try to survive by eating just about anything, including other cockroaches that have died. This phenomenon of eating carcasses or the remains of dead is called necrophagy and, of course, toxicants responsible for killing others may be ingested if live cockroaches eat toxified dead ones.

Relevant amounts of toxicant may be ingested by *B. germanica* feeding on the excrement of poisoned cockroaches. Although cockroaches eat the excrement of other cockroaches (coprophagy), the significance of that behavior is not understood. It may be related to behaviors associated with mating and reproduction (Cochran 1985b, Mullins and Keil 1980). Silverman *et al.* (1991) observed coprophagous behavior among all stages of *B. germanica* and found that feces from cockroaches that fed on hydramethylnon bait was eaten by nymphs and killed them within 14 days. In the case of hydramethylnon, the effectiveness of coprophagy is apparently related to the passage of unmetabolized parent material in the feces .

Imidacloprid is relatively new and acting at new target sites, which are currently not affected by resistance than other previous insecticides that will be used for pest control especially against German cockroach in Saudi Arabia.

In this study, the effect of the combination of lambda-cyhalothrin liquid formulation and the boric acid dust on the populations of German cockroach was significant and better than the liquid formulation alone (table 2 and Fig. 2) but lower than the boric acid treatment. This may be attributed, to a marked extent, to the residual and long lasting effects of boric acid.

Likewise, the combination of the liquid formulation and the imidacloprid gel bait enhanced the efficacy of both chemical formulations and showed a significant reduction on the German cockroach populations at all assessment periods (table 2 and Fig. 2).

This result may be due to the fact that the mode of action of Lambda- cyhalothrin and imidacloprid gel bait is different and thus imidacloprid gel bait has potential for use in the management of biochemical resistance to pyrethroids, and vice versa. Incipient behavioral resistance has been noted with baits (Silverman and Bieman 1993) and cockroaches certainly can detect, and may be repelled by some insecticides (Ebeling *et al.*1967). Alternating two classes of insecticides has been proposed for managing pyrethroid resistance in German cockroach (Cochran 1990).

These results are similar to that obtained by Miller and Peters (1999b) who used fipronil and deltamethrin to control German cockroach and found them to significantly reduce the populations of German cockroach throughout the assessment periods.

The combination of the boric acid dust and the gel formulation has achieved the best results of all the three chemical combinations (table 3 and table4).

It worth mentioning that the differences between the six treatments became more

significant towards the end of the experimental period, which means the efficacy of treatments became more pronounced at this stage. After 12 weeks, the 3-top highest catch reductions of the German cockroach populations were obtained when using the combination of boric acid and Gel treatment (98.8%),(table 3), then the boric acid treatment (94.4%),%),(table 4), followed by the combination of lambda-cyhalothrin and Boric acid (89.1%),%),(table 2). This result indicates that boric acid seems to have a long residual effect since the top three treatments include boric acid.

These findings concurs with that of Ballard and Gold 1983, Ebeling *et al.* 1968, Robinson 1985, and Rust *et al.* 1983 who stated that when boric acid had been properly applied, the German cockroach population tended to continuously decline for a period of time up to at least three months; while with most conventional organic insecticides the opposite trend occurred .

One of the explanations for the retard and long residual action of boric acid was reported by Eidman (1922) who found the cuticular lining of the crop to be 5 to 8 µ thick three or four times thicker than that of the hind gut . Penetration of acid and alkaline aqueous solutions through the wall of the crop required about 24 hours, compared with 10-15 minutes for the hind gut. This appears to be one of the factors that accounts for the long time required for boric acid to exert its toxic action, when compared with insecticides that pass rapidly and copiously into the hindgut.

Another explanation was indicated by Ebeling *et al.* (1966) who reported that boric acid powder was less repellent and remained effective for longer periods than most insecticide powders used at that time.

The mode of action of boric acid against insects remains unknown, Bennett *et al.* (1988) stated that by cuticular penetration and ingestion cockroaches slowly succumbed to boric acid due to compound's interference with energy conversion in the insect's cells.

Destruction of the gut wall of *B. germanica* after boric acid ingestion has been suggested (Ebeling, 1995).Based on a histological study of the digestive tract of *B. germanica* after ingestion of boric acid, Cochran (1995) concluded that destruction of the foregut epithelium might result in death from starvation .

Comparing the performance of the three chemical formulations when applied singly the results clearly indicated that the boric acid dust was the best throughout the experimental period, which extended to twelve weeks indicating a long residual effect. The gel bait was second to the boric acid but with a moderate effect in the early (two weeks) and late (twelve weeks) stages of the control program. The residual effect of the liquid spray formulation (Lambda-cyhalothrin) was very low compared to the two other formulations.

Based on the findings of the insecticidal combinations in other studies, and the published reports on cockroach control, Tilak *et al.* (2005) recommended to implement an "integrated insecticide strategy".

In this study, and based on the above, recommendation is made to implement an "integrated insecticide strategy" between any two of the three chemical formulations for German cockroaches with initial spray of lambda-cyhalothrin followed by imidacloprid gel or boric acid dust for an effective and long-lasting control of German cockroaches in houses in urban areas. This strategy will ensure a quick initial knockdown with Lambda-cyhalothrin followed by a prolonged maintenance phase offered by imidacloprid gel (with its unique cascade effect) or boric acid dust (with its long residual effect). This "integrated insecticide strategy" may offer a synergistic control of German cockroaches in houses in urban areas.

Conclusion

Boric acid proved to have an excellent efficacy on the cockroaches, and all treatments including this chemical gave a highly significant suppression of the cockroaches over a longer period of time. It is therefore, recommended to be used in any control programs against cockroaches. Treatments with boric acid dust and imidacloprid cockroach gel bait were found to be highly efficacious for control of cockroaches in houses and safe for the user and the local environment. The application of the two formulations was not only found more convenient to both operators and clients, but was also easy to apply (and was very stable under a wide range of conditions) and there was minimal disruption of the home or workplace. This study demonstrated clearly that combinations of more than one control method against German cockroach

are more effective and better than using a single one.

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Effect of fluoride intoxication on spermatogenesis in Rat.

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Abstract:

The present study was undertaken to evaluate testicular damage in experiment fluorosis. Fifty five Sprague-Dawley male albino rats were divided into nine experimental groups and one control group. The control animals were given double distilled water 1cc/kg bw/day subcutaneously. The experimental groups were given 30, 45, and 75 mg sodium fluoride/kg bw/day for 30 days. The experimental groups were divided into three phases at the interval of 7, 17, and 30 days. Animals were sacrificed from each group at the end of 8, 18, and 31 days. Pathological examination of testis revealed, reduction in number of tubular cells in Group I, focal areas of necrosis and subsequent increase in interstitial tissues in Group II, formation of multinucleated giant cells, shrinkage of seminiferous tubules in Group III, fusion of spermatids and presence of dark pyknotic nuclei in Group IV, loosening of disintegrating germinal epithelium in Group V, and atrophy of cells, thickening of germinal epithelium in Group VI. The intensity of hypertrophy of seminiferous tubules had increased in Group VII, cell infiltration occurred in interstitium and nuclei were scattered in necrotic region of tubules in Group VIII, Leydig cells increased in size and the amount of interstitial tissue was found to be elevated in Group IX.

Key words: Albino rats; Fluorosis, Germinal epithelium, Spermatic, Spermatocyte, Spermatogenesis.

Introduction

Spermatogenesis is a complex process by which spermatogonia differentiate into mature sperm, and this process involves remarkable structural and biochemical changes. The suppression of spermatogenesis (Sprando *et al.*, 1997), deficient maturation and differentiation of spermatocytes, degenerated spermatogenic cells devoid of spermatozoa (Shashi, 1990), inhibition of testicular steriodogenesis endocrine function (Narayana and Chinoy, 1994), decline in sperm viability, a significant increase of sperm abnormalities (Wan *et al.*, 2006), decrease in Ledig cells, spermatogonia and spermatocytes (Cui *et al.*, 2003; Wan *et al.*, 2006) in fluoride intoxication has been reported. Chinoy and Sequeria (1989) investigated that reproductive organs of mice were affected by sodium fluoride ingestion resulting in decrease in sperm density and sperm motility. Susheela and Jethanandani (1996) have reported male infertility with abnormality in sperm morphology due to fluoride toxicity. The present study was designed to find out the effect of fluoride on testis of rat.

Material and Methods

Young Sprague Dawley male albino rats in the age group of 2-3 months weighing in ing 100-250 g were allowed to acclimatize to laboratory conditions for one week. Animals were divided into one control and nine experimental groups. They were maintained on standard rat pellet diet obtained from experimental fluorosis. Hindustan Lever, Ltd. Mumbai. The water was supplied *ad libitum*. All the animals were weighed prior to the start of experiment. The rats were injected with NaF dissolved in double distilled water subcutaneously in the doses of 30, 45, and 75 mg NaF/kg bw/day for 30 days. The highest concentration of NaF was saturated with water at 100° C and was used directly. The animals of control group received 1cc double distilled water/kg bw/day for the same period.

These rats were sacrificed under ether anesthesia and testes were taken out in normal saline. The body weight of each animal was noted before treatment. The weight of testis of respective groups of animals was recorded. For histopathological studies, the testicular tissues were fixed in Bouin's fluid for 24 hours and Carnoy's fixative for 6 hours, washed in 70% alcohol, dehydrated in tertiary butyl alcohol, cleared in amyl acetate, infiltrated and embedded in paraffin wax. They were serially sectioned at 7µm and stained with Iron haematoxylin and eosin studied under research binocular microscope and subsequently photomicrographed.

Results

Organo-somatic index: The organo-somatic index (OSI) of testis was calculated as follows :

$$\text{Organo-somatic index} = \frac{\text{Weight (g) of the organ}}{\text{Total body weight (g)}} \times 100$$

The organo-somatic index of testis in control rats was 54%. The OSI has been found to decline to 48% in phase 1 of experiment, 35% in second phase of experiment and 22% in third phase of experiment. The weight of testis decreased with all three treatments as compared to control.

Table 1. Summary of treatment

	Experimental Group	n	Dose (mg/kg bw/day)	Treatment days	Day of Autopsy
	Control	10	1cc double distilled water	30	Sacrificed along with treated
Phase 1	I	5	30 mg NaF	6	7
	II	5	45 mg NaF	7	8
	III	5	75 mg NaF	9	10
Phase 2	IV	5	30 mg NaF	15	16
	V	5	45 mg NaF	17	18
	VI	5	75 mg NaF	19	20
Phase 3	VII	5	30 mg NaF	30	31
	VIII	5	45 mg NaF	30	31
	IX	5	75 mg NaF	30	31

n=number animals used

Pathological alterations:

Control: In testis of control rat, the seminiferous tubules were compactly arranged, and have normal shape and size (Fig. 1, Plate-1). The spermatogonia were present in various stages of development. Interstitial cells were well defined. Each seminiferous tubule was bounded by a distinct germinal epithelium. The spermatids in the cap phase of acrosome formation were present (Fig. 2, Plate-1).

Phase -1: In the first phase of experiment, the animals were treated with sodium fluoride in the dosage of 30, 45, and 75 mg/kg bw/day. The histopathological changes observed are shown in Figures 3, 4, and 5.

Experimental Group – I (30 mg NaF/kg bw /day for 6 days):

In this group, the seminiferous tubules were hypertrophied. Focal areas of necrosis and formation of pyknotic nuclei occur in secondary spermatocytes. There was slight increase in interfollicular connective tissue. The spermatogenic cells have two to three nuclei. The spermatids in the cap phase of acrosome formation were also visible. Vacuolization in cytoplasm was evident (Fig. 3, Plate-2).

Experimental Group – II (45 mg NaF/kg bw /day for 7 days):

The testis of experimental rat in this group treated with 45 mg NaF for 7 days, showed focal areas of necrosis in seminiferous tubules. An increase in number of interstitial cells and a subsequent increase in connective tissues occurred. The seminiferous tubules revealed hypertrophy as compared to control and experimental group I. The spermatogenic cells contained pyknotic nuclei and depict vacuolization of cytoplasm (Fig. 4, Plate-2).

Experimental Group –III (75 mg NaF/kg bw/day for 9 days):

In rat testis fluoridated for 9 days, the degenerative changes especially the formation of multinucleated giant cells by coalescing spermatids were prominent.

Seminiferous tubules shrink and gradually come to contain only sertoli cells and spermatogonia. Abnormal spermatogenic cells were present. Majority of spermatogenic cells were eliminated by maturation and differentiation. Some cells contained crescent shaped nuclei residual bodies were also present (Fig. 5, Plate-3).

Phase - 2: In the second phase of experiment, animals were treated with NaF in the dosages of 30, 45, and 75 mg/kg bw for 16, 17, and 19 days. The histopathological changes observed are shown in Figures 6, 7, and 8.

Experimental Group – IV (30 mg NaF/ kg bw/day for 16 days):

Exposure of the testis to fluoride caused an extensive degeneration of spermatogenic cells. The formation of multinucleated cells by fusion of spermatids increased as compared to experimental group-III. The germinal epithelium showed presence of dark pyknotic nuclei (Fig. 6, Plate-3).

Experimental Group – V (45 mg NaF/ kg bw /day for 17 days):

In hypertrophied seminiferous tubules, there was loosening of germinal epithelium and extensive disintegration of cells occurred in the centre of tubules (Fig. 7, Plate-4).

Experimental Group – VI (75 mg NaF/ kg bw /day for 19 days):

The seminiferous tubules showed thickening of germinal epithelium, focal area of necrosis, and abnormal spermatogenic cells (Fig. 8, Plate-4). An increase in interstitial tissue along with cell filtration was more as compared to experimental group V.

Phase – 3: In the third phase of experiment animals were treated with NaF in the dosage of 30, 45, and 75 mg/kg bw for 30 days each. The histopathological changes observed are shown in Figures 9, 10, and 11.

Experimental Group – VII (30 mg NaF/ kg bw /day for 30 days):

Exposure of testis to 30 mg NaF for 30 days showed condensed nuclei, increase in inter- follicular tissues as compared to experimental group VI. Hypertrophy of seminiferous tubules and necrotic areas were also seen (Fig. 9, Plate-5).

Experimental Group – VIII (45 mg NaF/ kg bw /day for 30 days):

There was an increase in intracellular spaces between tubules. There was degeneration of germinal epithelium along with clumping of spermatogenic cells. The extensive cell infiltration occurred in interstitium. The dark black pyknotic nuclei were found scattered in necrotic areas (Fig. 10, Plate-5).

Experimental Group – IX (75 mg NaF/ kg bw /day for 30 days):

In this dose group, the seminiferous tubules showed loosening and disintegration of germinal epithelium. The Leydig cells showed an increase in size and amount of interstitial tissue elevated. Complete atrophy of all spermatogenic cells was visible. A few nuclei were found scattered and empty cells underwent disintegration. In disintegrating central region, a few nuclei were present (Fig. 11, Plate-6).

Discussion

The present study was undertaken to investigate the testicular damage in experimental fluorosis. The albino rats were administered NaF as (30, 45, and 75 mg/kg bw/day) for variable periods (6, 7, 9, 15, 17, 19, and 30 days). The results revealed that fluoride treatment caused testicular damage. There was hypertrophy of seminiferous tubules, reduction in the number of testicular tubule epithelial cells, and increase interstitium. These alterations resulted in deficient differentiation and maturation of spermatocytes, abnormal spermatogenic cells, formation of multinucleated giant cells, fragmentation of nuclei, vacuolization and infiltration of cells. The above findings are consistent with the study of Kour and Singh (1980), which reported necrosis of the seminiferous tubules, and lack of differentiation and maturation of the spermatocytes in mouse testis. The findings of the present study are in consonance with Chinoy and Sequiera (1989) in which there was arrest of spermatogenesis due to extensive damage to the seminiferous tubules leading to denudation of cells, vacuolization in their cytoplasm, and nuclear pyknosis. A single micro dose (50µg /50 µL) injection of NaF dissolved in water in male rats caused defective spermatogenesis and absence of spermatozoa in the lumina of the seminiferous tubules of the testes, which consequently led to a decline in sperm count. The sperm deflagellation and tail abnormalities were related to the alterations in the internal milieu of testis which rendered the spermatozoa immotile and consequently caused fertility impairment in the experimental animals (Chinoy, 1991).

The findings of present study coincide with the study of Shashi (1990) which presented definite relationship between fluorosis and the testicular damage in rabbits given different doses of NaF (5 to 50 mg/kg/day) for three and a half months. There had been deficient maturation and differentiation of spermatocytes, and increase in interstial tissue. In higher dosage groups, spermatogenesis stopped and seminiferous tubule had become necrotic. Susheela and Kumar (1991) found that in rabbits treated with 10 mg NaF/kg bw for 18 to 29 months, also observed that in the testis spermatogenic cells were disrupted, degenerated and devoid of spermatozoa. Spermatogenesis had ceased and they concluded that ingestion of high dose of fluoride had harmful effects on male reproductive system.

In the present study on rats, in the higher F-intoxicated group, there was atrophy of seminiferous tubules. Similar findings were observed by Kumar and

Susheela (1995) who noted structural changes in the testis of rabbit treated with 10 mg NaF /kg bw/day for 20 and 23 months. They found decrease in the tubule diameter of testis and epithelial cell height. In patients living in fluoride endemic areas, Susheela and Jethanadani (1996) found that the decreased testosterone concentrations adversely affect the male reproductive system. Ghosh *et al.* (2002) reported dilation of seminiferous tubule, fewer mature luminal spermatozoa, diminutation in the relative weight of testis in rats treated with 20 mg/ kg bw/day of NaF for 29 days. Chinoy *et al.* (2002) reported giant cell formation with disturption of spermatogenesis and steroidogenesis in testis of mice treated with fluoride and arsenic. They observed histological changes in the testis of mice treated with 10 mg NaF/kg bw/ day for 30 days, in the form of disorganized epithelium and denudation of cells in the lumen of seminiferous tubules that hampered spermatogenesis (Chinoy *et al.*, 2005).

A significant decrease in testicular index, sperm count, sperm motility, sperm viability, and sperm function (HOS positive) with increased sperm abnormalities has been reported in adult male albino rats administered NaF at a level of 4.5 ppm and 9.0 ppm for 75 days in the drinking water (Pushapalatha *et al.*, 2005). Our studies are also in accordance with the findings of Wan *et al.* (2006) who found that NaF caused disorganization, denudation and reduction in germinal epithelial cells of the seminiferous tubules and an accompanying absence of sperm in the lumina in testes of rat administered 150 mg NaF/L(68 ppm F^-) in their drinking water for 50,80,100, and 120 days. Sperm motility is one of the most important indexes in evaluating male reproductive function (Zhao *et al.*, 1995) and is closely associated with impaired spermatogenesis which can cause a significant decline in sperm motility in cauda epididyma of male rat given 100 mg NaF/L in their drinking water for eight weeks (Zhang *et al.*, 2006).

In conclusion, the results of present investigation revealed that fluoride treatment cause testicular damage. There was hypertrophy of seminiferous and structural changes in the form of necrosis of the seminiferous tubules, arrest of spermatogenesis due to extensive damage to the seminiferous tubules due to fluorosis.

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EXPLANATION OF FIGURES

PLATE-1

Fig. 1. Transverse section of testis of rat of control group showing compactly arranged seminiferous tubules (ST).HE (X 100.

Fig. 2. Magnified view of Figure 1 HE (X 100).

PLATE-2

Fig. 3. Transverse section of testis of rat given 30 mg NaF /kg bw /day for 6 days showing pyknotic nuclei in the germinal epithelium (GM), hypertrophy of seminiferous tubule, and focal area of necrosis. HE (X 400).

Fig. 4. Transverse section showing necrotic areas, increased in connective tissues in the seminiferous tubules given 45 mg NaF / kg bw /day for 7 days. HE (X 100).

PLATE-3

Fig. 5. Transverse section of testis showing deficient differentiation and maturation of spermatocytes, and formation of multinucleated giant cells in the testicular cells of rat treated with 75 mg NaF / kg bw /day for 9 days. HE (X 400).

Fig. 6. Transverse section of testis of rat given 30 mg NaF / kg bw /day for 15 days showing extensive vaculization and loosening of seminiferous tubule. HE (X 100).

PLATE-4

Fig. 7. Transverse section of testis of rat given 45 mg NaF / kg bw /day 17 days showing degenerated spermatogenic cells. There is formation of multinucleated cell by fusion of spermatids. HE (X 100).

Fig. 8. Transverse section of testis of rat given 75 mg NaF / kg bw /day for 19 days showing thickening of germinal epithelium, increase in interfolliculer connective tissue and focal areas of necrosis. HE (X 100).

PLATE-5

Fig. 9. Transverse section of testis of rat given 30 mg NaF / kg bw /day for 30 days showing condensed nuclei and hypertrophy of seminiferous tubules. HE (X 100).

Fig. 10. Transverse section of testis of rat given 45 mg NaF / kg bw /day for 30 days showing degenerative germinal epithelium and clumping of spermatogenic cells. HE (X 100).

PLATE-6

Fig. 11. Transverse section of testis given 75 mg NaF / kg bw /day for 30 days showing fusion of germinal epithelium of the tubules and cell disintegration. HE (X 100).

PLATE 1



Fig. 1



Fig. 2

PLATE 2

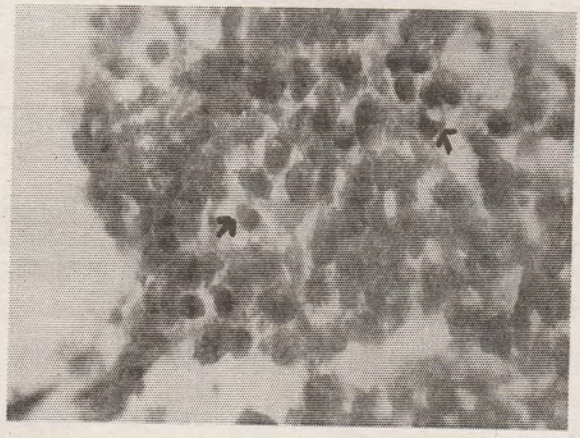


Fig. 3



Fig. 4

PLATE 3

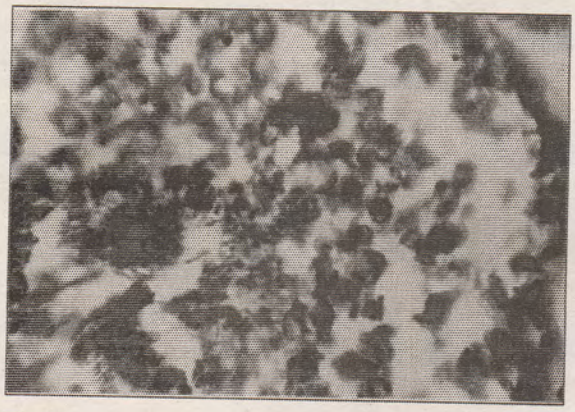


Fig.5

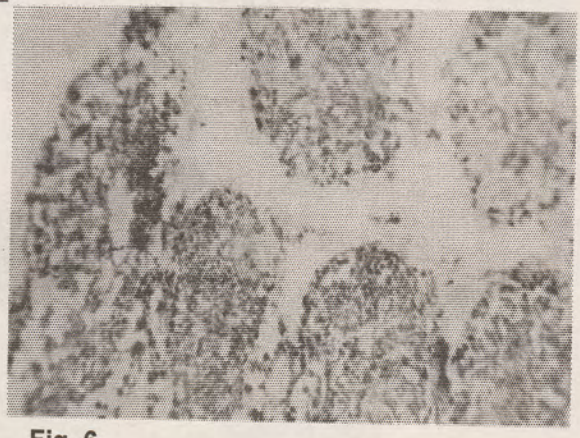


Fig. 6

PLATE 4

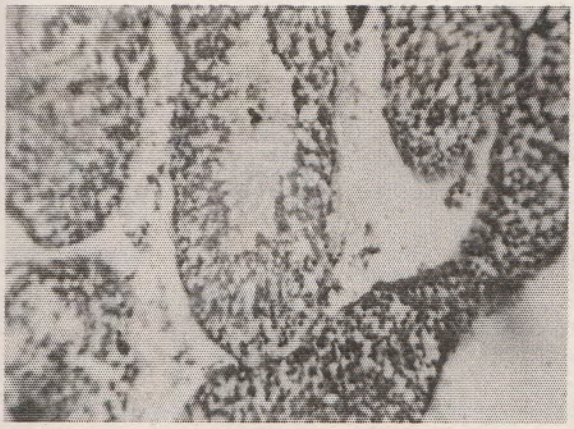


Fig. 7

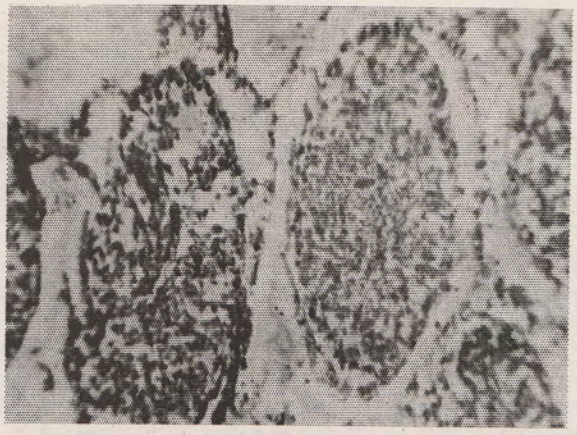


Fig. 8

PLATE 5

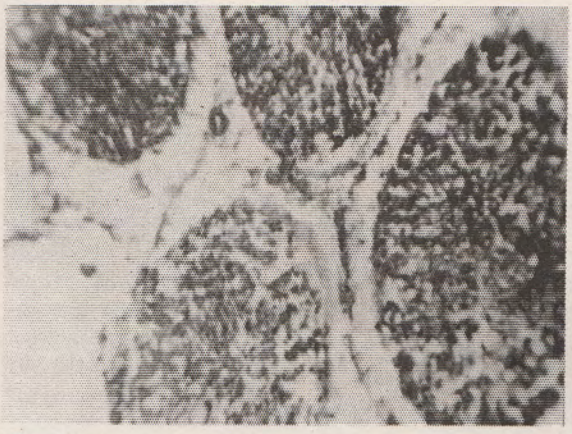


Fig. 9

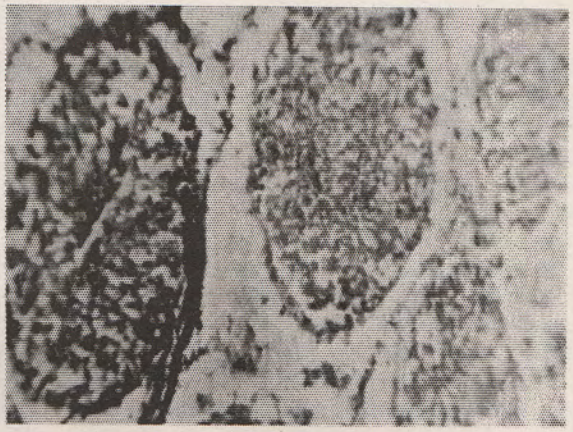


Fig. 10

PLATE 6



Fig. 11

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Neuropathological changes in cerebellum of rat in experimental fluorosis.

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Abstract:

The neuropathological effect of fluoride on cerebellum of rat was assessed during experimental fluorosis. NaF at 30, 45 and 75 mg/kg bw/day was injected subcutaneously for 20 and 35 days into 60 Sprague Dawley albino rats of both sexes. After 20 and 35 consecutive days of exposure to fluoride, chromatolysis occurred in Purkinje neurones and adenocarcinomatous islands having highly degenerative areas with pyknotic cells and neurones with globose type tangle inside the perikaryon was observed in the cerebellar cortex of experimental rats. Hypertrophy of neurones in the granular layer while atrophy of neurones in molecular cell layer was observed. The neuroglial cells showed more advanced disorganization with retention of only a small portion of vacuolated neuroplasm along with disintegrated nuclei in brain of rats treated with 75 mg NaF/kg bw/day for 35 days. In Purkinje cell layer, the nucleus size has increased while in granular cells, the nucleus become dot like in appearance.

Keywords: Albino rat, Cerebellum, Chromatolysis, Purkinje neuron, Sodium fluoride.

Introduction

Toxic neuronal injury in the form of tetaniform convulsions due to ingestion of excessive amount of fluoridated water has been reported by Waldbott (1957). Fluoride is known to cross the blood brain barrier (Geereats *et al.*, 1978). Long term intake of high levels of fluoride in humans causes neurological complications such as paralysis of limbs, vertigo, spasticity in extremities and impaired mental acuity (Waldbott *et al.*, 1978). Accumulation of fluoride in hippocampus of brain caused degeneration of neurones and decreased aerobic metabolism (Spittla, 1994). Mullenix *et al.* (1995) reported that fluoride interrupts normal brain development in babies and children and it affects hippocampus. Neuropathological changes in the form of loss of molecular layer and glial cell layer, chromatolysis in the brain tissue of rabbit exposed to varying doses of fluoride has been reported by Shashi (Shashi, 2003).

The aim of present investigation is to assess the neuropathological alterations in cerebellum of rat exposed to varying doses of fluoride for variable periods.

Materials and Methods

Animals: Sixty Sprague Dawley albino rats of both sexes weighing 100-300 g were maintained on standard rat pallet diet and water provided *ad libitum*.

Experimental design: Rats were divided randomly into seven groups. Group I was kept as control and administered double distilled water as 1 ml/kg bw/day. The remaining six experimental groups were treated with 30, 45 and 75 mg NaF/kg bw/day. The animals from each group were sacrificed at an interval of 20 and 35 days respectively. The experiments were terminated after 35 days. At day 20, three rats from control group and 9 rats from fluoride treated group were randomly selected, weighed and sacrificed and at 35 day three rats from control group and 9 rats from fluoride treated group were sacrificed under ether anesthesia. The brain was carefully removed, blotted free of blood, and fixed in Carnoy's fixative and Bouin's fluid. The tissues were dehydrated in 95% alcohol for 45 minutes, tertiary-butyl alcohol for 6 hours, cleared in amyl acetate for overnight and embedded in paraffin wax (temperature 60-62 ºC). Wax blocks were then prepared and 7µm thin serial sections were cut with rotary microtome and stained with Haematoxilin and eosine, and Mallory's Triple stain. Neuropathological alterations in cerebellum were studied by using Luxol fast blue method (Kluver and Brerra, 1953), Ninhydrin-Schiff's method using Haematoxilin (Yasuma and Itchikawa, 1953) and Feulgen nuclear stain (Pearse, 1968).

Results

Group-1 (Control)

The cerebellum was divided into an outer cortex of gray matter and an inner white matter. The gray mater constitute the cortex, which has three distinct cell layers, an outer molecular layer with few cells and horizontally directed fibres, an inner granular layer with small neurons with intensely stained nuclei and a central layer of Purkinje cells (Fig. 1, Plate-1). Purkinje cell nuclei are located at the junction of outer and inner cerebeller layers. The Purkinje axons extend from the base of cell into granular layer to enter into the white mater (Fig. 2, Plate-1).

Neurodegeneration in Cerebellum

Phase –I

During the first phase of experimentation, the rats were treated with 30, 45, and 75 mg NaF/kg bw/day for 20 days.

Group II - 30 mg NaF/kg bw /day for 20 days

In cerebeller cortex, a few neurons showed "ballooned" chromatolysis (Fig. 3, Plate-2). The Purkinje neuron lost their angular shape and acquired a plump, rounded, ovoid or 'ballooned' type appearance. The nucleus was displacing to the periphery of axonal base and was shrunken, pyknotic, hyper chromatic or absent. Nissl's substances also showed various degree of decrease or complete loss. The intracellular neurofibrils exhibited neurodegenerative changes. The neuroplasm was filled with granular, amorphous substances and contained residual erythrocytes. Glial cells were dilated and their swollen cell processes surrounded neurones as well as microvessels (Fig. 4, Plate-2).

Group III - 45 mg NaF/kg bw/day for 20 days

The cerebeller cortex exhibited adenocarcinomatous islands, which contained highly degenerative areas with pyknotic cells and shrunken nuclei. A few neurones in the molecular layer have globose type tangle inside the perikaryon (Fig. 5, Plate-3).

Group IV- 75 mg NaF/kg bw/day for 20 days

An increase in cell number occurred in the granular layer. Some neurones showed hypertrophy, their nuclei shifted to the periphery and appeared dot like. In other cells, hypertrophied nucleus occupied large areas in perikaryon. Some cells became vacuolated with complete loss of neuroplasm and nucleus. In molecular layer there was decrease in number of cells. The atrophied nuclei stained deeply (Fig. 6, Plate-3).

Phase – II:

During the second phase of experimentation the rats were treated with 30, 45, and 75 mg NaF/ kg bw/day for 35 days.

Group V- 30 mg NaF/kg bw/day for 35 days

A great increase in the number of neurones in the granular layer and a large decline in cell number in the molecular layer has been observed (Fig. 7, Plate,4).

Group VI - 45 mg NaF/kg bw/day for 35 days

In the molecular layer of cerebeller cortex, the number and size of neurones were decreased. A few neurones were deeply stained in comparison to others. The fibrillar network formed tangles around the glial cells (Fig. 8, Plate-4).

Group VII - 75 mg NaF/kg bw/day for 35 days

In cerebeller cortex, hypertrophy of nuclei in Purkinje neurones was visible. A few neurones contained atrophied nuclei, which appeared dot like. Purkinje neurones contained various sized spheroid bodies (Fig. 9, Plate-5).

Discussion

In the present investigation, the neurodegenerative changes in the form of moderately damaged and grossly abnormal cells in various parts of brain, chromatolysis in Purkinje neurones, reduction in thickness of granular layer in cerebellum, gliosis, hypertrophy, necrosis, and cystic degeneration of neurones, occurred in fluoride intoxicated groups of rat. The perikaryon was filled with numerous small vacuoles. The above findings are consistent with the study of Wadhwani and Ramaswami (1953) which reported the cellular chromatolysis and replacement with fibrous tissue has also been reported in monkeys provided with 4.5 mg of F/day for 24 weeks. Neuronal abnormalities were observed in the NaF treated animals especially in the deeper cell layers. These abnormalities include distortion of cells and in some rats, cell losses in particular brain regions, and vascular inclusions were also observed (Isaacson *et al.,* 1997). The findings of the present study are also in consonance with study of Liu (1989) who recorded increase in nerve cell density, mild degeneration of organelles in nerve cells in the 60 ppm fluoride treated groups of rat.

Mullenix *et al.* (1995) found that fluoride levels increased in the medulla oblongata in rats of both sexes and in the hippocampus of females exposed to 100 ppm fluoride in drinking water for 6 weeks compared to the controls. In addition, the fluoride levels were higher in the cerebellum, basal ganglia, mid brain, and hippocampus of male rats and in the cortex of female rats. Trebelsi *et al.* (2001) noted increase in the cell deaths, reduction or absence of external granular layer and poor differentiation of Purkinje cell layer and presence of apoptotic Purkinje cells in the rats administered to 0.5 g/L fluoride in the drinking water. Fluoride accu -mulation has been reported in the brain of experimental animals exposed to chronic high fluoride intake, and this accumulation increased as the drinking water fluoride levels increases. The neurotoxic changes in the present study are in consonance with our earlier study in which neurodegenerative changes in Purkinje neurones of brain of rabbits treated with 5-50 mg NaF/kg bw/day for 16 weeks were reported. Chromatolysis, and disintegration of nuclei, and in some cells the nucleus was displaced to the periphery or at the base of axon. It was shrunken, pycnotic and hyperchromatic. The neuronal loss was accompanied by increased number of glial cells. Among the remaining neurones, pear shaped or "ballooned" forms were frequently prominent. The nissle substance underwent various degree of changes (Shashi, 2003).

In the present study, pyknosis, vacuolation and chromatin clumping, reduction in number of Purkinje cells, and degenerative and apoptotic Purkinje neurones in cerebellar cortex of fluoridated rats were present. The hypertrophied neurones and increase in number of neurones in granular layer and atrophy of neurones in molecular layer of cerebeller cortex was observed. Earlier Zhavoronkova (1981) reported foci of demylination in the cortex and subcortical areas, a decrease in the number of Purkinje cells in the

cerebellum, swelling and irregular staining of the nissl substances and pyknosis of neurones in experimental animals subjected to fluoride intoxication.

During present investigation apoptosis pyknosis and axonal swelling in Purkinje neurones was observed. Shivarajshankara *et al.* (2001) mentioned decrease in the number of neurons in all regions of brain, decrease in number of Purkinje cells in the cerebellum and sign of chromatolysis and gliosis in the motor cortex, neurodegenerative changes in the hippocampus motor cortex and cerebellum.

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PLATE 1



Fig. 1. T.S. of cerebellum of control rat showing Molecular layer and granular layer, H & E (X 100).

PLATE 1

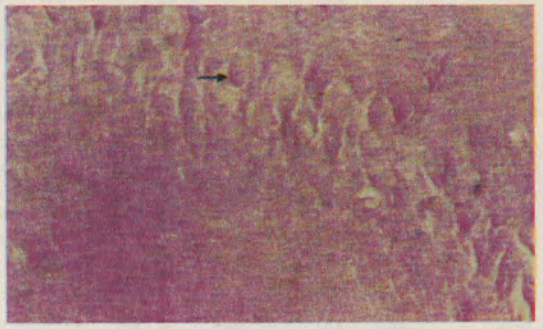


Fig. 2. T.S. cerebellum of rat of control group showing Purkinje neurones, Mallory triple stain (X 400)

PLATE 2

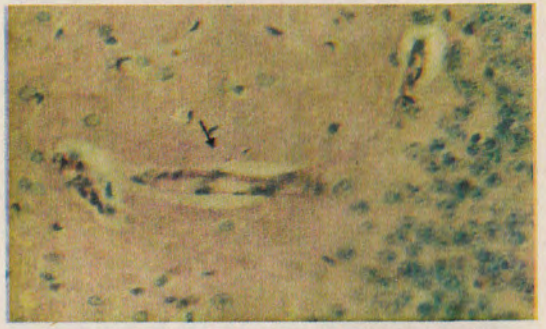


Fig. 3. T.S. cerebellum of brain treated with 30 mg bw/day for 20 days showing adenomatous foci.H E stain, (X 400)

PLATE 2

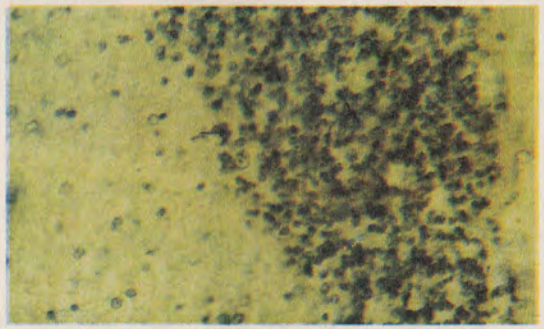


Fig. 4. T.S. of cerebellum of rat treated with 30 mg NaF/Kg NaF/Kg bw/day for 20 days showing arge, rounded, ovoid Purkinje cell with shrunken nuclei. Ninhydrin schiff's stain (X 400)

PLATE 3

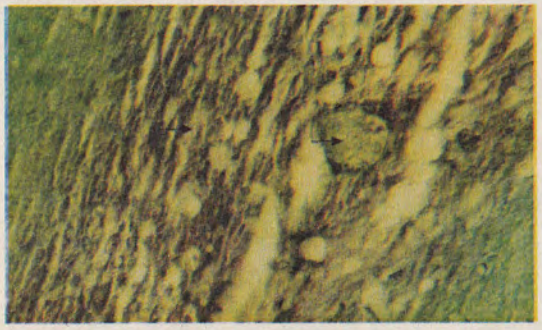


Fig. 5. T.S. of cerebellum of rat treated with 45 mg NaF/kg bw/day for 20 days Showing neurones having tangled neurofibrils. Feulgen nucleal stain (X 400)

PLATE 3

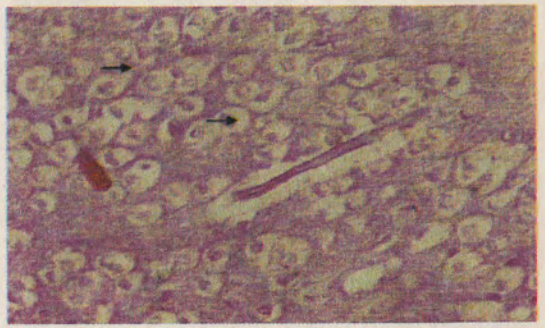


Fig. 6. T.S. of cerebellum of brain rat treated with 75 mg NaF/kg bw/day for 20 days Showing hypertrophy of glial cells in granular layer, Luxol fast blue. (X 400)

PLATE 4

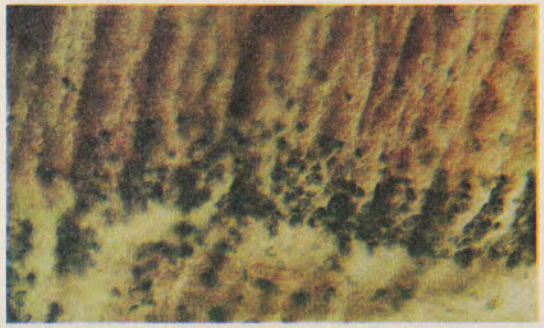


Fig. 7. T.S. of cerebellum of brain of rat treated with 30mg NaF/Kg bw/day for 35 days showing hypertrophied axon of molecular layer. H E stain, (X 400)

PLATE 4

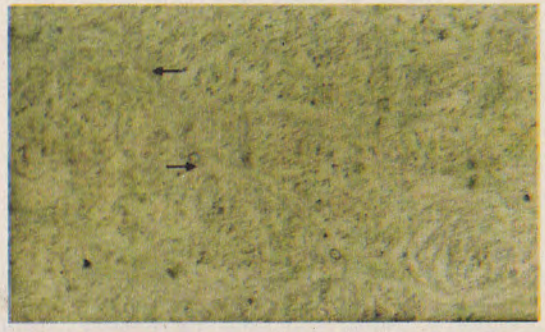


Fig. 8. T.S. of cerebellum of rat treated with 45mg NaF/ Kg bw/day for 35 days showing formation of fibrillary network and axonal disintegrationm Feulgen nucleal stain (X 40)

PLATE 5

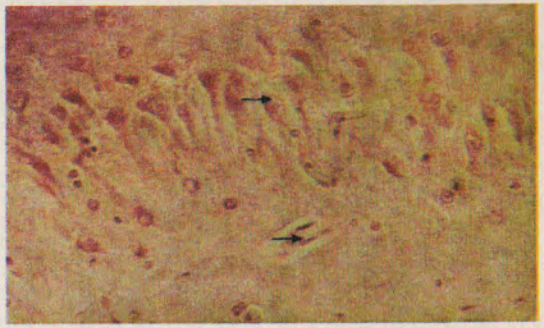


Fig. 9. T.S. of cerebellum of rat treated with 75mg NaF/ Kg bw/day for 35 days showing polymorphic Purkinje neurones.Mallory Triple stain (X 400)

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Marketing of Non-Timber Forest Products: A Case Study of Tumrakheda Village in Raisen District of Madhya Pradesh

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Abstract:

The present case study was undertaken in Tumrakheda village of Raisen District in Madhya Pradesh state to find out the existing marketing system, channels and dependence of households on Non Timber Forest Products (NTFPs) for their survival. The existing three marketing channels for NTFPs movement from production site to end user was identified in Obaidullahganj tehsil of Raisen district (a) subsistence use (direct consumption or barter at the local level), (b) sale to private trader/middleman and (c) sale through co-operative. A composition of the purchase price and sale price of the trader indicated that there is a big spread with a very nominal portion of end user selling price accounting into the primary collectors pocket and the rest is effortlessly consume by the middlemen especially in the case of NTFPs like Achar (*Buchananla lanzan*), Aonla (*Emblica officianlis*), Imli (*Tamarindus Indica*), Safed Musli(*Chlorophytum spp.*), Lac, Mahua (*Madhuca latifolia*) flowers and fruits. The present study suggests that co-operative society at village (primary collector) level can play a crucial role as an alternative marketing channel for efficient handling of NTFPs as well as to ameliorate the socio-economic conditions of the local tribals. The local tribal people can get a substantial portion of their total (income) survival requirement in form of both cash and in kind from NTFP collection and sale.

Key words: Non timber forest products, sustainable development, marketing channels.

Introduction

Non Timber Forest Products (NTFPs) represent one of the most challenging product groups from a marketing point of view because of their number, versatility, end-use variation, and dissimilarities of the producer base and resources richness. A particular feature related to non-wood products is their great number and versatility. Producers of non-wood forest products may be individual gatherers, including subsistence farmers and industrial plantations supplying either primary consumer goods or raw materials for further processing. Marketing for NTFPs range simple local village –level consumers market to the most sophisticated industrial niche markets in numerous end-uses sectors in both developed and developing countries. In terms of end-use, the markets for non-wood raw materials and primary processed products are numerous and may be extremely varied even for a single product. NTFPs are a vital source of livelihood for a large proportion of the poor living in or close to the forest in most tropical countries. In West Midnpore districts in West Bengal, many village communities derive as much as 17 % of their annual household incomes from NTFPs (Malhotra *et al.*, 1991). Other estimates suggest that up to 35% of the income of tribal household in India come from the collection of unprocessed NTFPs also since NTFPs involve a large variety of seasonal products, returns are frequent and relatively continuous. Moreover, local processing of NTFPs can increase off-farm rural employment opportunities. Small scale forest based enterprise, many of them based on NTFPs, provide upto 50% of income for 20-30% of the rural labour force in India (Campbell, 1988). In addition to subsistence and income generating potential, NTFPs also provides food security to large lower income populations, their cattle and other domestic animals particularly droughts or famines (FAO, 1989). According to Raintree and Francisco (1994), who analyzed the market for small-scale multipurpose tree products in the Philippines, most producers depended on the buyers (mostly wholesalers) as their source of price information. Only 10 % had actual access to prevailing market prices. This, of course, left the producers largely at the mercy of intermediaries as to the availability and accuracy of information on which they had to base their marketing decisions. India is primarily a rural country with 74.29% of its people residing in rural

areas. India has also one of the largest tribal populations in the world. There is rapid build up of evidence (from numerous micro studies) to suggest that NTFP play an important role in the life of forest fringe communities, especially, the tribals and other marginalized groups. One study indicates that Baiga tribal living in Madhya Pradesh collect the largest number of NTFP species among forest dwelling communities in the world (Kennedy,1991). Similarly, study conducted in 07 villages spread over four districts of Orissa illustrates that of the situation prevalent in many poor and tribal regions of the country. It was found that household having an annual income of less than Rs.3,000.00 derived 50% of their earnings from NTFP, whereas those earnings over Rs.6000.00 derived 21% from NTFPs (Nabi,S.,1997).There is a large scale private trade of NTFPs, according to Mahalingam (1989) this trade is often exploitative with cheating and under payment. The large number of forest dwellers brings their produce to local market and sell or barter these NTFPs quickly at the prices fixed by the shrewd traders. Extraction, processing and marketing of NTFPs is the sources of employment and income to a vast majority of the tribal people. NTFPs are collected haphazardly without looking into the quality and sustainability of the product. It is also common that many of the collectors do not know where and whom to sell the NTFPs, for want of information and partly without realizing the need for quality. They sell the product locally at whatever price is offered to them with the consequently revenue to the collectors (Ganapathy *et al.,* 2000).

A study conducted in Bastar reveals that a family earns on average Rs. 1500/- a year from the sale of NTFP against a total income of Rs 1750/- annually. In the Madhya Pradesh state, 40-60% yearly income of a family comes from the sale of NTFPs. It is also very interesting fact that the collection of these NTFPs are mostly done in the forest area which are in the fringe of the village (Prasad and Bhatnagar, 1991). Murthy *et al* (2005) evaluated the flow of NTFPs in Uttar Kannada district, Western Ghats collected from four zones. They estimated value of NTFPs per household which varies between Rs. 3445/household in the evergreen zone and Rs. 1233/household in the dry zone. They also observed financial value realized per hectare varies across the four zone ranged from Rs.634 in dry deciduous to Rs.1801 in the evergreen zone with a mean of Rs.1159/ha/yr. Some studies on NTFP harvest and trade suggest that an emphasis on NTFP as a major source of income for forest communities might be problematic for several reasons: markets are frequently unstable (Padoch 1992); trade is often controlled by the elite, both locally and regionally (Ribot 2000); access to NTFPs is socially mediated and inequitable (Kumar 2002); most NTFPs occur at low densities (LaFrankie 1994); and the potential for trade is usually low, with only few species being traded (Saw et al. 1991). Furthermore, the lack of security of tenure, a situation commonly encountered in state-controlled forests, often results in such adverse ecological impacts as damage during harvest and suppressed regeneration. Ecological effects have been shown to be higher than expected (Padoch 1992) and economic returns are low (Godoy *et al.* 2000). Bista and Webb (2006) investigated the intensity of NTFP collection, financial benefits gained through the collection of a species and factors influencing NTFP collection and sale in 12 villages of the Baitadi district of Nepal. Approximately 39% of the economically active population collected a total of 24 NTFPs from 23 species, which contributed 0-90% of the total household income. Marketing chain and financial analysis of *Valeriana jatamansi* revealed that various trading and marketing channels existed from the village to district-level NTFP outlets. Study also suggests that increasing access to NTFP-selling outlets may be achieved through information dissemination, empowerment of collectors and establishment of linkages between collectors and traders. They also found that Middlemen, traders are important stabilizers of prices as long as fair trading practices are followed.

Materials and Methods

The Madhya Pradesh state is very rich in NTFPs collection and trade. Most of the tribal peoples living in fringe forest villages are dependent on NTFPs for their livelihood. Raisen district is selected due to main market place and Obaidullahgunj act as centre point for the movements of wild products collected from the natural forests of adjoining districts also. The present study is an attempt to find out the marketing systems/ channels for NTFPs in Tumrakheda village in Raisen District. Raisen District is situated in the centre part of the present Madhya Pradesh State. The district is having the boundaries of Sagar and Vidisha districts in North, Bhopal district in the West, Sehore and Hoshangabad districts in the south and Sagar and Narasinghgarh districts in the east. The district is located 220 45' North Latitude to 23 45' North Latitude and 77 21' East Longitude to 78 49' East Longitude. The District is situated about 465 to 470 meters above the Mean Sea Level. All development blocks of the district are having a considerable area under forest. Total forest area in the district is about 333236.856 hectare out of which about 31.7% forest land is in Goherganj tehsil.

The present study was conducted in Tumarkhera village in Raisen district of M.P. State during January-December 2006-07. Tumrakhera village was selected for the study because of vast forests and large tribal population living in and around the forest. The simple random sampling technique was used to select the households. The sample sizes are 12 households and 4 marketing channel members. The study was conducted systematically to ascertain the dependence of the forest dwellers in the study area.

Fig.1. Map of Raisen district showing study area.



Questionnaire survey:

A questionnaire survey was conducted to collect information on – diversity of NTFPs extracted, season of collection, harvesting period, number of persons, household involved in NTFP collection, total quantity collected per day and per person, Quantity collected per household, time spent for collection etc.

Table 1: Collection/ harvesting period of some of the common NTFPs in Tumrakhera village.

SN.	Name of NTFPs	Jan	Feb	Mar	Apr	May	June	Jul	Aug	Sept	Oct	Nov	Dec
1.	Mahua Fls			*	*								
2.	Mahua Fr.				*	*							
3.	Aonla												
4.	Achar				*	*				*	*		
5.	Imli				*	*							
6	Musli								*	*			
7	Lakh				*	*							
8	Tendu leaves					*	*						

The above table 1 depicts collection/harvesting periods of important commonly found NTFP species in the study area. It can be inferred that maximum and important NTFPs like Mahua, Imli, Tendu leaves and achar are harvesting period being March-May which is peak season NTFP collection in natural forests. The time period between March-May not only enriches the income of the local people but also help them store for future (rainy season) essential requirement. The selling prices are different in NTFPs, between primary collector to end-user in selected study site. The primary collector gets 33% of the price paid by end user to NTFP while middlemen and trader gets more profit of the NTFPs. The village shop keeper gets much profit because they purchase forest produce from primary collector and sold to big trader.

Table : 2 showing NTFP quantity collected per day and total time spent in collection and wage rate of Tumrakheda village range_Dahod and Division-Obaidullaganj, District- Raisen (M.P.)

SN.	Name of NTFPs	Total number of persons involved	Person involved in per house hold	Quantity collected /day/person/kg (dry)kg	Quantity collected/kg/ HH (dry) kg	Number of days harvesting	Annual collection/ Kg./HH (dry)	Total quantity collected /person	Time spent for collection (Hrs/Day)	Wages rate in Rs.	P.C rate in Rs.
1	Mahua Fls	54	5.40	2.40	12.99	20	259.8	130	5/day	33.72	5/kg
2	Mahua Fr.	42	4.20	0.3	3.49	20	69.8	35	4/day	22.48	12/kg
3	Aonla	29	2.90	1.41	4.09	15	61.35	14	8/day	33.72	25kg
4	Achar	50	5.00	1.30	6.50	10	65	65	7/day	28.10	25/kg
5	Imli	4	0.40	3.75	1.50	15	22.5	15	5/day	16.86	5/kg
6	Musli	3	0.30	3.33	0.99	8	7.92	10	8/day	33.72	15/kg
7	Lakh	5	0.50	0.50	0.50	9	4.5	5	6/day	22.48	50/kg
8	Tendu leaves	58	5.80	77.58	449.99	7	3149.93	4500	7/day	50.58	45/100 bundle

P.C= Primary Collector.

Total number of house hold surveyed-12.

The table 2 depicting several aspects, such as total number of person involved in collecting various NTFPs, average family numbers, quantity collected per person, per house hold, total collection p.a. time spent by collectors in collecting NTFP, number of days of NTFP collection wage rate vis-à-vis income from NTFP collection. It can be inferred that Mahua flower and fruits are the major NTFPs in the study site the total number of persons involved for both 96 persons, the total collection is 329.6 kg average time spent by person per day is 5 hours, total inco

Table 3 Source of income of the villagers house hold in study area.

Name of NTFP	Annual collection in Kg/H.H	PC Rate	Annual income Rs by NTFPs/ HH (A)	Annual income in Rs. From agriculture /HH (B)	Annual income From labors (C)	Annual income from livestock (D)	Annual income from fuel wood (E)	Total
Mahua Fls	259	5	1295	Rs. 2800/ year	Rs. 2200/ year	Rs. 1200/ year	Rs. 1300/ year	
Mahua Fr.	69.8	12	837.6					
Aonla	61.35	25	1533.75					
Achar	65	25	1625					
Imli	22.5	5	112.5					
Musli	7.92	15	118.5					
Lakh	4.5	50	225					
Tendu leaves	3149.93	45	1417					
Total			Rs. 7118.65	Rs. 2800	Rs. 2200	Rs. 1200	Rs. 1300	**Rs 14618**

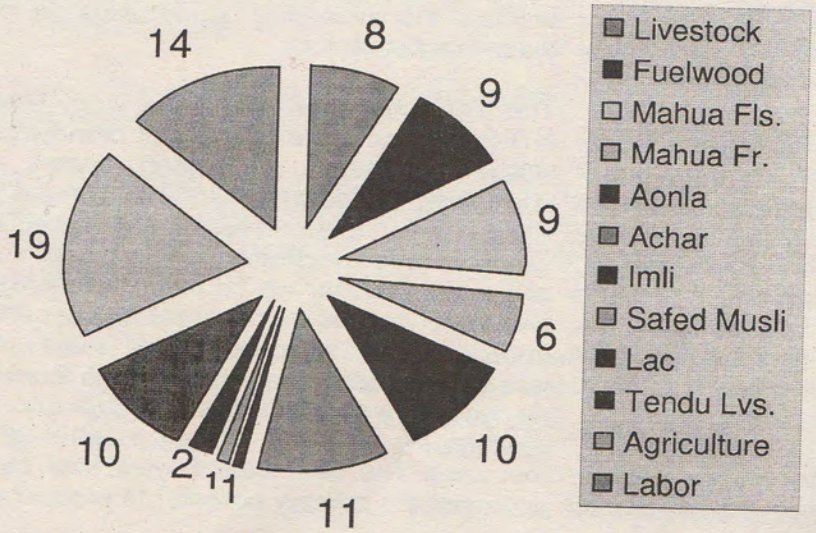
Total income/year = A+B+C+D+E (7118.65+2800+2200+1200+1300)
= Rs. 14618/-

Table 3 depicting annual income from NTFP and other sources of income. Various NTFP products that are collected and their collection period are also depicted in the table 3. it can be inferred from the table 3 that almost 50% of the total income (survival) per house hold in the study area accounted form NTFP, collection and sale and this signifies the importance of NTFPs in meeting the requirements of the local people in the study area.

Marketing Channel

There are number of middlemen and traders dealing of the marketing on NTFPs. Numbr of local middlemen in village level, Tehsil level and districts levels. The marketing channel of village to district and out of the state is also observed along with number of middlemen and traders.

Figure 2. Percentage contribution of forest produce in Tumrakhera village.



The above figure showing percentage of total income for house hold in the study site from various sources, viz. NTFPs (50%), agriculture (19%), labor (14%), fuel wood (9%) and livestock (8%). It can be inferred from the above figure that NTFPs accounted for major chunk of total income per house hold, that signifies the role of NTFPs in meeting the requirement of fringe village.

Results and Discussion:

It was observed that March to May being peak months for NTFP harvest in the study area. In the same period the villagers do not have agriculture labor work hence they are highly rely on NTFPs collection & consumption and sale. April and May are summer months followed by rainy season during these two months the villagers collect NTFPs and store for the next rainy season.

It has also reported that the villagers barter NTFPs for commodities like salt etc. which will not be easily available to the fringe villagers. The most abundantly available NTFPs in the study area was Achar followed by Aonla and Tendu leaves. It has been reckoned that about 50% income accounts from NTFPs and rest 50% from other sources including agriculture labor etc. The hours spent by the villagers for NTFPs collection varied from product to product. The maximum time per family hours spent accounted for Tendu leaves harvesting followed by Mahua where as Imli accounted for minimum flowers and minimum from lack. Mishra *et.al.* (2002) have reported barter system and primary collector's exploitation by middleman of three districts of Orissa state. They had found much more dependency of villagers on forest products as out of the total income, more than 50% were found to earn from NTFPs and fuel wood sale.

The most prevalent channel for movement of NTFPs in the study area includes: primary collector → village trader → tehsil level trader→ district level trader →retailer →end user.

The study reveals that total income of the villagers per family was worked out to per Rs.14619 out of which Rs. 2800 was from agriculture and fuel wood formed Rs.1300/year. Similar study has been done by Nabi, (1997) and Malik and Radhamohan (1994) in the seven villages of Orissa state which revealed that household having an annual income of less than Rs.3,000.00 derived 50% of their earnings from NTFP, whereas those earnings over Rs.6000.00 derived 21% from NTFPs.

The marketing system/channels for NTFPs is rather highly unorganized. There are no well-established marketing channels for the NTFPs from the existing method of exploitation and marketing practices of NTFP in Tumrakheda village, the three major channels was identified-(a) Subsistence use (Direct consumption);(b) Sale to private trader/middlemen;(c) Sale through co-operative (Village forest protection committee-VFPCs). Mishra (2000) also reported huge profit margins between various level middlemen's to retailers in the market in case of *Rauvolfia serpentina* species in the state of Madhya Pradesh i.e. village level middlemen to processor (132.38%) and processor to commission agent at big cities(43.22%). Later on Mishra (2003), Teki *et.al.* (2003) reported marketing of selected NTFPs of Orissa state and observed that primary collectors were selling their produce at throw away prices to the local middlemen. Villagers were reluctant to do value addition of the raw produce collected from forests and there is complete absence of local level institutions (NGOs) for value addition, training and marketing etc. The study recommends that a committed NFGO can play as catalyst in forming village level NTFP syndication for collective harvest and collected sale of NTFPs.

Conclusion

It has been observed in the study area that in most cases NTFPs are sold by the primary collectors without any value addition that could have been otherwise elevated the earnings of the primary collectors. Simple value addition in forms of peeling skin, grading, storage, drying to some species like Safed musli, Aonla, Imli, Achar etc. can be done easily without having any sophisticated technology, thereby enhancing the selling price of the NTFPs at primary collectors level. Due to lack of market information they are forced to sell their raw produce to thirsty middleman at throw away prices. A committed local level NGO should educate and train the villagers about the benefits of value addition and facilitate the availability of remunerative market to the primary collectors.

The distribution network in the study area for all NTFPs by and large consists of primary collectors, village shopkeeper, middleman, traders and also end users. Village and market level institutions like village level cooperatives/mutual collections and sale of NTFPs syndication will help both the villager and trader. Villager will be benefited by higher price and the trader will be benefited by cost efficiencies, materializing on account of bulk quantity and thus less transportation cost etc. Through this kind of set up long network, channel of distribution can also reduces to required efficient number of channel members. The wholesale traders then can directly approach the primary collector to procure the NTFP

harvests, which will ultimately result in increase of the earnings of the primary collectors.

Promotion of Self Help Groups (SHGs) should be focused. In fact primary collectors sell off his NTFPs as soon as harvested, because of his working capital (meeting basic needs) requirements, if this is provided through SHGs, then the primary collectors would be in a better position to store his harvest till he gets remunerative price. Bartering of NTFPs especially for salt etc., should be discouraged to arrest undue exploitation of the villagers. There should be an NTFP price regulatory agency in the study area to make and implement minimum support price (MSP) for different NTFPs as is the case of Agricultural produces.

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A cross sectional investigation on Contagious Caprine Pleuro Pneumonia among goats of Wayanad district of Kerala, India.

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Abstract:

The prevalence of contagious caprine pleuro pneumonia (CCPP) in goat population of Wayanad district of Kerala was studied, using Slide Agglutination Test (SAT). The overall antibody prevalence among the 446 goat sera tested was 28.92%. Males had lower prevalence (24.07%) than females (30.47%). Higher prevalence rate noticed on advancement of age.

Key words: Contagious caprine pleuro pneumonia, slide agglutination test, goats

Introduction

Goat rearing is the major source of income for the jobless women tribes of Wayanad district of Kerala. In addition the goat milk forms a good protein source for their children. According to 2003 census, the region has a sizable population of about 41, 921 goats. Due to visiting tourists, there is a good demand for meat in this region. Contagious caprine pleuro pneumonia (CCPP) is a major scourge and is considered to be the world's most devastating goat disease in many goat-producing countries. It is a highly contagious fatal disease causing 100% morbidity, severe respiratory distress and 60-100% mortality. (Radostits *et al.* 2000), resulting in major economic losses in countries of Africa and Asia (FAO, 1993). So far no systematic survey has been undertaken for CCPP in Wayanad region of Kerala. Hence to ascertain the status of this disease in the region, it was envisaged to carry out a serological investigation of CCPP in goats of the region.

Material and Methods

The study was conducted for a period of one year from May 2007.

COLLECTION OF SERA SAMPLES

About 2ml of blood was collected from 446 goats from different parts of Wayanad district of Kerala. The sample constitutes 1% of total goat population in the region. Age and sex of the animals were recorded for comparison. Of these 446 samples, 108 were from males and 338 from females with 143 samples in age group of 1-2 years, 130 in 2-3 years, 93 in 3-4 years and 80 samples above 4 years of age. The sera was separated, inactivated at 56° C for 30 minutes and stored at -20° C until tested.

SLIDE AGGLUTINATION TEST

All the samples were screened by Slide Agglutination Test (SAT) as described by Srivastava *et al.* (1991) using colored antigen of *Mycoplasma mycoides subsp capri* (stained with rose bengal dye). The colored antigen was kindly provided by Dr. V.P.Singh, Principal Scientist and Head, Division of Bacteriology and Mycology, IVRI, Izatnagar, Bareilly (U.P), India. Twenty microlitres each of colored antigen and test serum were mixed on a clean glass slide and the agglutination reactions were graded as 1+ to 4+ according to the size of the agglutinate and speed of their formation. A rapid coarse flocculation occurring in 15-30 seconds was given 4+, a similar but less flocculation and complete in 2 minutes as 3+, a definite reaction but far from complete 2+ and a very fine flocculation detectable only in a good light 1+. Any agglutination after 2 minutes was taken as non-specific. Statistical analysis of data was done according to the method described by Martin *et al.* (1988).

Results and Discussion

Out of 446 sera tested, 129 were found to be positive for CCPP antibodies. The percentage positivity being 28.92. Age and sex- wise analysis of data are summarized in Table 1 and 2. Higher prevalence of CCPP was recorded in female goats (30.47%) in comparison to males (24.07%). Age-wise analysis of data revealed that there was increase in prevalence rate with the advancement of age. Age group of 1-2 years showed seropositivity of 23%; 2-3 years with 24.6%; 3-4 years with 35.4% and above 4 years recorded 38.7%.

The present investigation clearly revealed the presence of CCPP antibodies among goats of Wayanad district of Kerala. Higher prevalence rate noticed on advancement of age agreeing to the findings of El-Manakhly (1995), Jain *et al.* (2006) and Agrawal *et al.* (2007), who opined that it could be due to lowering of immune status in older animals. However, Pradhan (1997) and Monda and Pramanik (2002) reported higher prevalence in kids and lambs compared to adults.

Data on sex-wise distribution of CCPP antibodies revealed higher prevalence in females compared to males could be attributed to the lowering of immunity in females due to pregnancy / lactation – induced stress. (Abegunde *et al.* 1981, Jain *et al.* 2006 and Agrawal *et al.* 2007).

The seropositivity of 28.92% obtained in the present study was comparable with earlier reported 28% in adult sheep and goat and 87% in kids and lambs by Pradhan (1997). Another survey on CCPP in kids revealed mycoplasma in 31.37% cases in an intensive farm management. (Shaheen *et al.* 2001). Among goats in Goa region, Barbuddhe *et al.* (2005) reported 26.43% and a prevalence of 11.31% were recorded by Jain *et al*, (2006) in goats of the sub himalayan garhwal region. Also Ghosh *et al.* (1989) noticed 9.09 to 63.64% prevalence in goats of Tripura. A prevalence of 3.9% to 31% was observed against various members of 'mycoide cluster' in goat sera samples collected from Rajasthan, Uttarpradesh and Delhi. (Singh *et al.* 1999).

Slide agglutination test was found to be quick, simple, and low cost with ease of application in the field without the need for any specialized training and equipments. Srivastava *et al.* (1991) also opined that colored antigen was more economical and easy to prepare than latex antigen.

In the present study the results indicated the CCPP positive status of the animals screened in the part of Kerala state. Hence, it is the need of the hour to plan effective control measures against CCPP to prevent economic losses among goat rearing groups of Wayanad district.

Acknowledgements

The authors are grateful to The Kerala State Council for Science, Technology and Environment (KSCSTE) for financial support and to the Associate Dean, College of Veterinary and Animal Sciences, Pookot for the facilities provided and the Director of Research and The Comptroller, KAU for the support to carry out the work. Thanks are due to the Government Veterinarians of Wayanad district for their help rendered to us in collecting blood samples.

Table-1: Age - wise distribution of CCPP antibodies

Age group	No. of samples tested	No. of samples positive	Percent positive
1-2 years			
Males	40	9	
Females	103	24	
Total	**143**	**33**	**23**
2-3 years			
Males	35	7	
Females	95	25	
Total	**130**	**32**	**24.6**
3-4 years			
Males	25	7	
Females	68	26	
Total	**93**	**33**	**35.4**
Above 4 years			
Males	8	3	
Females	72	28	
Total	**80**	**31**	**38.7**

Table-2: Sex - wise distribution of CCPP antibodies

Sex	No. of samples tested	No. of samples positive	Percent positive
Males	108	26	24.07
Females	338	103	30.47
Total	**446**	**129**	**28.92**

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Cytochemical alterations in pancreas of fluoride intoxicated Rat.

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Abstract:

Sprague Dawley albino rats were given sodium fluoride subcutaneously as 30, 45, and 75 mg NaF/kg bw/day for 36 days to study cytochemical changes in glycogen and phospholipids of pancreas. The glycogen showed uniform distribution in exocrine and endocrine cells of control rats. The level of glycogen in the exocrine glands, islets of Langerhans and intercaleted ducts exhibited marked elevation in all test rats. The intensity of phospholipids in control rats revealed uniform distribution in exocrine glands, islets cells and interlobular connective tissue. There was abnormal accumulation of phospholipids in all cellular components of pancreas in fluoridated rats.

Keywords: Exocrine glands, Fluoride, Glycogen, Pancreas, Phospholipids.

Introduction

Disorders of energy metabolism in the form of abnormal anaerobic and aerobic respiration are among the key effects of fluoride intoxication (Kolodziejczyk *et al.* 2004) The endocrine effects of fluoride toxicity can produce glucose intolerance and abnormalities in insulin secretion (Trivedi *et al.* 1993). Riggali *et al.* (1990, 1995) investigated in rats, decrease in insulin, and increase in plasma fluoride concentration. The intraperitoneal administration of 10 mg NaF/kg bw/day resulted in hyperglycemia in rats due to increased hepatic glycogenolysis (Varadacharyulu and Rao, 1997). Male Japanese white rabbits given tributylin fluoride with a single dose of 100 mg/kg body weight exhibited remarkable increase in plasma lipids (Matsui *et al.* 1982). Sodium fluoride in the concentration of 5-20 mol/L in the intracellular space inhibited secretion by isolated Langarhans islets in the pancreas of rat (Manoyo *et al.* 2005).

The aim of present study was to examine the influence of fluoride toxicity on the cytochemical localization of glycogen and phospholipids in pancreas of fluoridated rats.

Materials and Methods

Sixty adult Sprague Dawley albino rats of both sexes in the weight ranging from 100-300 g, were given NaF in the dose of 30, 45, and 75 mg/kg bw/day for36 days. Animals were sacrificed under ether anesthesia. The pancreas was dissected out and fixed in Bouin's fluid and calcium formaldehyde for 24 hours, embedded in paraffin wax, and 7μ thin sections were stained with Best's caramine stain (Best, 1905) for glycogen and with acid heamtein (Baker, 1946) for phospholipids. The stained section for cytochemical lesions was studied under the research microscope. The microphotography was done in the bimolecular electronics and nanotechnology laboratory of Central Scientific Instrument Organization (CSIO), Chandigarh, India. The microphotographs were taken with a digital camera attached on inverted research fluorescent microscope (Axivort-200) interphase with computer.

Results

Results of experiment are given in Plates 1-3

Glycogen

Control-

The acini and islet's cell were darkly stained showing uniform localization of glycogen in all cellular components. The nuclear membrane stained positive for glycogen (Fig.1, Plate1).

Group I- 30 mg NaF/kg bw/day for 36 days

There was increase in the concentration of glycogen in the acini. The islets showed proliferation and nuclei were deeply stained. The granules were present in the islet showing deposition of glycogen (Fig. 2, Plate1).

Group II- 45 mg NaF/kg bw/day for 36 days
The disintegrated acinar cell showed increased level of glycogen. The ducts increased in size revealing of deposition of glycogen in margins (Fig. 3, Plate1).
Group III- 75 mg NaF/kg bw/day for 36 days
There was increase in the concentration of glycogen. The nuclear membrane and cell membrane were intensely stained showing marked elevation of glycogen (Fig. 4, Plate 2).
Phospholipids
Control-
The acini and islet showed uniform distribution of phospholipids in pancreas of control rats (Fig. 5, Plate 2).
Group I- 30 mg NaF/kg bw/day for 36 days
The acini showed accumulation of phospholipids in the form of granules. There was decrease in the concentration of phospholipids in the connective tissues. The islets of Langerhans showed intense activity of lipids. The nuclear membrane intensely stained. The cytoplasmic vacuolated areas near the acini showed moderate level of phospholipids (Fig. 6, Plate 2).
Group II- 45 mg NaF/kg bw/day for 36 days
The interlobular connective tissue showed increased localization of phospholipids as compared to 30 mg NaF group. The intercalated ducts were filled with mucinous secretion and showed intense activity of lipids (Fig. 7, Plate 3). There was increase in concentration of phospholipids in the acini. The islet cell stained lessly showing moderate level of concentration of lipids. The nuclei stained intensely (Fig. 8, Plate 3).
Group III- 75 mg NaF/kg bw/day for 36 days
This highest dose group showed increase in the localization of phospholipids deposition in the lobulated acini (Fig. 9, Plate 3).

Discussion

In the present study, the cellular distribution of glycogen in pancreas revealed increase in rats treated with 30, 45, and 75 mg NaF/kg bw/day for 36 days. Decrease in insulin, increase in plasma glucose and disturbances of glucose tolerance, associated with increased plasma fluoride concentrations has been observed by Rigalli *et al.* (1990, 1995). Earlier we reported that elevation of glycogen in the pancreas of albino rabbits administered with 5, to 10, 20, and 50 mg NaF/kg bw/day for 4 months, results in diabetic hyperglycemia (Shashi, 1993). Menoyo *et al.* (2005) observed that NaF in the concentration of 5-20 µmol/L in extracellular space inhibited insulin secretion by isolated islets Langerhans stimulated with glucose. Lin *et al.* (1976) observed effects of fluoride on insulin biosynthesis and inhibition in the oxidation of glucose by islets of Langerhans isolated from rat pancreas.

In the present study, fluoride treatment caused accumulation of phospholipids in pancreas of rat treated with 45 and 75 mg NaF/kg bw/day for 36 days. Fluoride is known to cause deposition of lipids in most of the soft organs like liver, pancreas and muscles of rabbits, fluoride results in accumulation of phosphoglyceric acid and decreased production of ATP (Dirkson, 1972). Single oral administration of triphenyltin fluoride to rabbits induces transient diabetes and diabetic lipemia by inhibiting insulin secretion from morphological normal pancreatic β-cells (Manbe and Wada, 1981; Wada *et al.* 1982)

Manabe and Wada (1981) demonstrated that oral administration of 100 mg/kg bw/day of triphenyltin fluoride to rabbits evoked hypertriglyceridemia. A single oral dose of triphenyltin fluoride evoked glucose intolerance due to insulin deficiency. Pancreatic β-cells contained normal amounts of granules at the maximum hyperlipemic stage. Triphenyltin fluoride decreased the sensitivity of islets to release insulin in response to increasing level of block glucose. Matsui *et al.* (1982) in male Japanese white rabbits given a single dose of 100 mg tributyltin fluoride/kg bw, investigated lipids and glucose metabolism. A remarkable increase in plasma lipids, particularly of triglycerides and total cholesterol was observed. Lipoprotein lipase activity reduced significantly to the levels of about 50% of the control. Fasting blood glucose level was elevated and a significant inhibition of insulin caused hyperglycemia. The hyperlipidemia was induced in rabbits by tributyltin fluoride due to decreased LPL (Lipoprotein Lipase) activity. The decreased in Lipoprotein activity seems to be related to inhibition of insulin release from islet.

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EXPLANATION OF FIGURES

Plate 1

Figure 1. T.S. of pancreas of rat of control group showing intense localization of glycogen in all cellular components. Best' carmine stain (X 100).

Figure 2. T.S. of pancreas of rat treated with 30 mg NaF/kg bw/day for 36 days showing deposition of glycogen in the form of granules in the islet's of Langarhans and marked deposition in acini. Best' carmine stain (X 100).

Figure 3. T.S. of pancreas of rat treated with 45 mg NaF/kg bw/day for 36 days showing hypertrophy of ducts and deposition of glycogen on margins of intercalated ducts. Best' carmine stain (X 100).

Plate 2

Figure 4. T.S. of pancreas of rat treated with 75 mg NaF/kg bw/day for 36 days showing intensive accumulation of glycogen in acinar cells. Best' carmine stain (X 100).

Figure 5. T.S. of pancreas of rat control group showing uniform distribution of phospholipids in acinar cell and islet's of Langarhans. Acid Haematein stain (X 100).

Figure 6. T.S. of pancreas of rat treated with 30 mg NaF/kg bw/day for 36 days showing marked deposition of lipid granules in exocrine glands. The islets show high intensity of phospholipids. Acid Haematein stain (X 100).

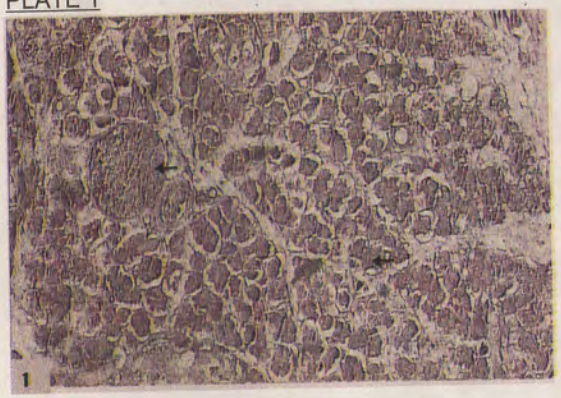
Plate 3

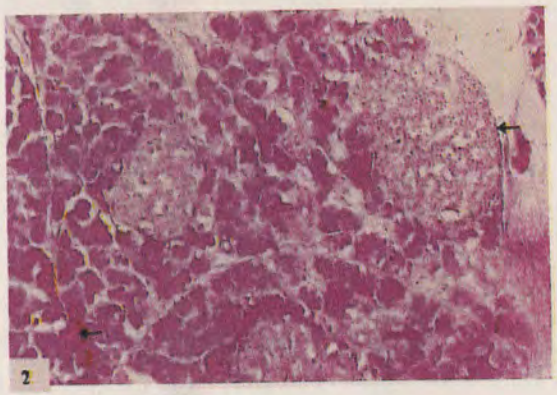
Figure 7. T.S. of pancreas of rat treated with 45 mg NaF/kg bw/day for 36 days showing elevation in localization of phospholipids in connective tissue and intercalated ducts show intense activity of phospholipids. Acid Haematein stain (X 100).

Figure 8. T.S. of pancreas of rat treated with 45 mg NaF/kg bw/day for 36 days showed elevation in lipid deposition in the acini while islets showing moderate level of phospholipids. Acid Haematein stain (X 100).

Figure 9. T.S. of pancreas of rat treated with 75 mg NaF/kg bw/day for 36 days showing marked elevation of lipids in all cellular components. Acid Haematin stain (X 100)

PLATE 1





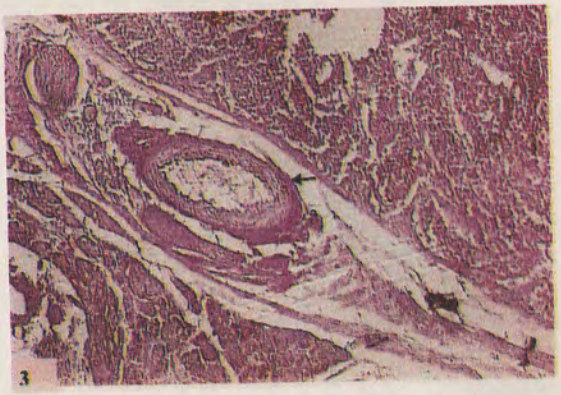


PLATE 2

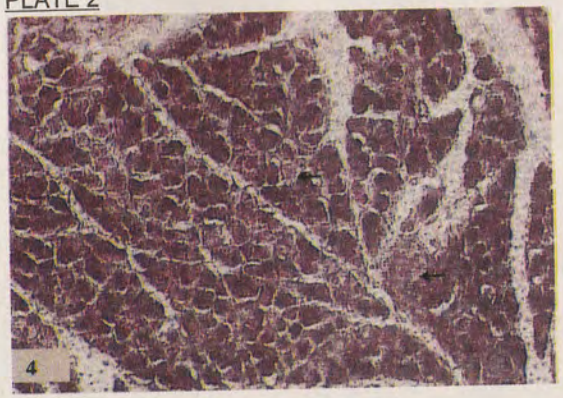
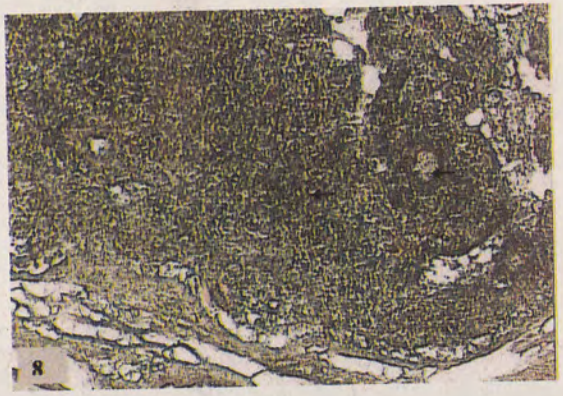


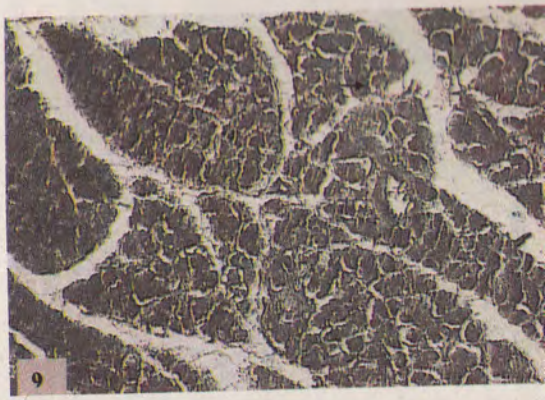




PLATE 3







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Development of indirect ELISA to screen EDS-76 virus antibodies in Ducks.

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Abstract:

Egg drop syndrome – 76 (EDS – 76), caused by an avian adenovirus is a major etiological agent for reduced egg production in domestic birds during the peak production. Ducks are considered as asymptomatic carriers of the virus, which necessitates the screening in the species; however ELISA kits for use in this species was not available. Hence an attempt was made to develop ELISA for detection of antibodies in ducks. Salting out technique using saturated ammonium sulphate solution was chosen to precipitate globulins from duck sera. After testing the purity and concentration, the globulins recovered were used to raise antigammaglobulins in rabbits. Direct two-step glutaraldehyde method was employed to label the antigammaglobulin with horse radish peroxidase (HRPO). Indirect ELISA was done using the conjugate. There was good correlation between the test results and the results obtained from Haemagglutination – inhibition (HI) test done simultaneously using the same sera and ELISA could detect more number of positive cases.

Key words: avian adenovirus, EDS –76 infection, duck

Introduction

The drop in egg yield in domestic birds is due to multiple etiological factors of which egg drop syndrome (EDS –76) virus is of major importance. Although the virus mainly infects laying hens, it has been suggested that ducks and geese were the main natural host of infection (McFerran and Adair, 2003) and the virus basically originated from ducks (Benko, 2000). More over EDS-76 viral antibodies is widespread in domestic ducks and well documented by Mohanty *et al* (1985), Sulochana and Sudharma (1987) and Reddy and Raghavan (1987) in India. In a state like Kerala where backyard system of poultry keeping is widely practiced, the chance of mingling of chicken and ducks and sharing feeders and waterers are more common. This necessitates the screening of available duck population. Though several serological techniques have been employed for the diagnosis of EDS –76 infection, Haemagglutination test (HI) and Enzyme linked immunosorbant assay (ELISA) are more sensitive (Monreal and Dorn, 1981) and the later is used for differential diagnosis of other avian adenoviruses. However, an ELISA kit specifically for ducks is not available. Therefore the present study was aimed to develop an indirect ELISA to detect EDS –76 virus antibodies in ducks.

Materials and Methods

Virus: The reference virus (strain 127 of UK) of EDS –76 was obtained from the Department of Animal Biotechnology, Madras Veterinary College, Chennai. It was passaged three times in nine- day-old embryonated duck eggs.

Purification of virus: The infected allantoic fluid was collected and purified as per Chandramohan (1994) by 36 percent sucrose cushion ultracentrifugation technique.

Production of hyperimmune serum to EDS virus: Antiserum to the above virus was raised separately in apparently healthy cockerels (free from EDS viral antibodies by HI test) and rabbits as per the method of Polly (1977) and Chetty (1985) respectively and were used for HI and ELISA.

Production of antiduck serum: Two healthy rabbits were immunized by the following schedule.

One millilitre of whole duck serum with a protein concentration of 68 mg per ml was homogenized with one millilitre of Freund's complete adjuvant (1: 1) and 1 ml of this emulsion was given intramuscularly to each rabbit. Thereafter, three booster doses of 0.5 ml of serum

without adjuvant were given at weekly intervals by the same route. When sufficient level of antibody was found in the serum by AGPT and IEP, blood was collected by cardiac puncture. Serum was separated and stored at – 20º C until used.

Preparation of conjugate: Salting out technique using saturated ammonium sulphate (SAS) solution (33 %; pH 7.0) was chosen to precipitate duck globulins from its sera (Garvey *et al*, 1977). The solution was added drop by drop to avoid localized concentration of salt and precipitation of nonimmunoglobulins. The purity of recovered globulins was tested by AGPT and IEP and its concentration was determined by Biuret method.

Antiduck gammaglobulins were raised by the same procedure as for antiduck serum in rabbits, using SAS precipitated globulins with a concentration of 10 to 15 mg per ml.

Conjugation of antigammaglobulins with Horse radish peroxidase (HRPO) was done according to Avrameas (1969) with slight modifications. Direct two – step glutaraldehyde method was employed. The antigammaglobulin was reconstituted with the borate buffer to obtain 5 to 7 mg of globulins per ml. pH was adjusted to 6.9 by addition of 0.1 M solution of Potassium phosphate. For each ml of the above solution 10 to 14 mg of HRPO enzyme was added (2500 units of HRPO per ml of antigammaglobulin) and after its complete dissolution 0.05 ml of glutaraldehyde was added. The mixture was shaken for 2 h at room temperature by end-over-end rotation. The product thus obtained was then dialysed overnight at 4 º C againt physiological saline, pH 7.4. Next day the solution was emulsified for 15 min at 2000 x g. The supernatant was collected and stored in small aliquots at –20º C.

Sera samples: A total of 281 sera samples were randomly collected from ducks (28 to 30 weeks of age) from five districts of Kerala.

Haemagglutination and Haemagglutination – inhibition test: HA and HI tests were performed in U shaped microtitre plates as per Sulochana and Sudharma (1987) with minor modifications. Titres of 3 $\log_2$ and above were taken as positive.

Enzyme linked immunosorbant assay: An indirect ELISA was done using the prepared conjugate as described by Voller *et al.* (1976) with slight modifications.

The purified EDS reference virus was suspended in PBS and used as antigen. The optimum concentration of antigen (1:200), positive control serum (1:200) and HRPO conjugate (1:1000) were arrived at by preliminary checker board titrations. While standardizing the test, the optimum dilution of the test sera was found to 1: 40 .This dilution of serum was used for screening the field sera samples. Samples with more than twice the mean optical density (OD) value of the negative serum was taken as positive (Garret *et al.* 1984).

Sera samples: A total of 281 sera samples were randomly collected from ducks (28 to 30 weeks of age) from five districts of Kerala.

Results and Discussion

With 281 duck sera samples, 75 were positive by HI (26.69 %) as compared to 93 positive samples by ELISA (33.10 %) (Table). In the present study, it has been possible to detect more number of positive cases with ELISA compared to HI.

While developing ELISA, the choice of enzyme and method of conjugation depends on number of factors. The enzyme HRPO ("Bangalore Genei" Pvt. Ltd.) was used for conjugation. Several workers suggested peroxidase as a good choice for conjugation among the three most commonly used enzymes (Avrameas., 1969; Nakane and Pierce., 1966). Because it was more stable, cheapest and had a much faster turnover time than other enzymes.

The prepared conjugate was then stored in small aliquots at –20 º C. Voller et al. (1976) reported that peroxidase conjugate stored in a lyophilized state had retained its full activity for one and half years . However, they opined that this technique and storage of conjugate leads to wastage of some conjugate as it is usually stored in large amounts than required at a time.

Eventhough more positive samples were detected by ELISA; statistically there was no significant difference between the two tests employed. The probable reason for this lower efficacy of ELISA might be due to the use of prepared conjugate rather than a commercial one. Further attempts in purification of globulins or use of ELISA kits might have resulted in statistically significant difference.

Presence of EDS – 76 viral antibodies in ducks indicated that the normally apathogenic virus for ducks had gained virulence on infecting chicken, thereby affecting their egg production. The risk is that these introductions could become endemic.

Table. Screening of EDS –76 virus antibodies in ducks in Kerala

District	Total number of samples screened	HI		ELISA	
		Number positive	Percentage positive	Number positive	Percentage positive
Alleppey	65	12	18.46	14	21.54
Kottayam	70	23	32.86	29	41.43
Palakkad	36	8	22.22	11	30.56
Thrissur	72	28	38.89	33	45.83
Trivandrum	38	4	10.53	6	15.79
Total	**281**	**75**		**93**	

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Bio-physical characterization of two nostocalean cyanobacterial isolates of rice fields of Manipur, India

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Abstract:

The rice field soils of Manipur valley are dominated by species of filamentous, sheath forming cyanobacteria. Out of 126 cyanobacterial isolates two dominant cyanobacterial strains *Cylindrospermum gorakhpurense* and *Nostoc commune* were examined for growth attributes and tolerance to weedicide and pesticides. The utility of these isolates as sources of pigments and as biocide tolerant biofertilizers is discussed.

Key words: Nostocales, taxonomical characterization, biocides, pigments, rice fields

Introduction

Cyanobacteria are a group of ubiquitous photosynthetic prokaryotes, which are capable of performing oxygenic plant-like photosynthesis with autotrophy as their dominant mode of nutrition. These organisms have a widespread distribution which reflects their broad spectrum of physiological properties and tolerance to environmental stress (Tandeau de Marsac and Houmard, 1993). Many cyanobacteria are capable of reducing the atmospheric nitrogen by the process called biological nitrogen fixation. Many workers (Kamat, 1974; Goyal *et al.*, 1984; Bisoyi and Singh, 1988; Nayak *et al.*, 2001; Tiwari and Singh, 2005) have explored the cyanobacterial floras of rice fields from different parts of country and the range of morphological variations have been studied systematically and extensively under standard conditions (Pandey, 1965; Tiwari, 1979; Staley *et al.*, 1989). The cyanobacterial biofertilizer usage in India is much hampered by the diversity in rice ecologies, which favor the growth of selective forms, with native forms outnumbering the inoculated strains. This has led to a general feeling among researchers regarding their inefficiency in terms of their establishment in soil and nitrogen ability. Hence, a need exists to isolate region-specific strains and characterize them, before employing them as bioinoculants in rice crop. The present study was therefore directed towards examining cultural, physiological, weedicidal and pesticidal tolerance characteristics of two dominant diazotrophic rice cyanobacterial isolates namely *Cylindrospermum gorakhpurense* Singh, R. N. and *Nostoc commune* Vaucher ex Bornt.et flah. from Imphal West, Manipur.

Materials and Methods

Unialgal strains of two diazotrophic cyanobacterial genera namely *Cylindrospermum gorakhpurense* and *Nostoc commune* were isolated from low land rice fields of Manipur during rainy season of 2005 and maintained in BG-11 medium (Stanier *et al.*, 1971) under controlled illumination (4K lux) and 14/10 light and dark cycle at 28± 2º C temperature. These strains were examined for specific growth rate and generation time (Guillard,1973), pigments like chlorophyll, phycobiliproteins and carotenoids (Mackinney, 1941; Bennett and Bogord, 1971; Jenson, 1978). Nitrogenase activity was examined by acetylene reduction assay (Hardy *et al.*, 1973) and total nitrogen was estimated (Allen, 1957). Photosynthetic oxygen evolution was

measured with a Clark type oxygen electrode (YSI model 53) at 26ºC with a light intensity of 3000 lux, the illumination being provided by a 100 W in candescent bulb and 5.0 ml of algal suspension was used to measure O_2 evolution.

Results and Discussion

Cyanobacteria can be found in almost every conceivable habitat, including soil, rocks, fresh water, and salt water. They are among the most successful in extreme environments including high temperatures, high levels of UV light, and high salinity. In aquatic environments that lack direct photosynthesis, cyanobacteria often form thick microbial mats, making cyanobacteria crucial in such ecosystem. These mats also contain the primary producers at the basis of the microbial food web. The role of cyanobacteria in the sustained fertility of flooded/irrigated rice field soils is well established (Mandal *et al.*, 1993; Roger *et al.*, 1987). Many photosynthetic free-living strains of cyanobacteria capable of fixing nitrogen have been isolated and used in biofertilizer consortia in South East Asian countries, and the potential impact of these organisms on agriculture through their use as biofertilizers, soil conditioners, plant growth regulators and soil health ameliorators is well recognized (Amma *et al.*, 1966; Bastia *et al.*, 1993; Whitton, 1992).

In India, rice is cultivated in about 45 million hectares area and accounts for the largest share of chemical fertilizers. Although, recurrent combinations of cyanobacterial species appear frequently, the generalization that practically all cultivated soils and grasslands support an abundant growth of these forms is not validated and a need exists to inoculate cyanobacterial strains through algalisation (Roger and Kulasooriya, 1980; Whitton, 2000; Nayak *et al.*, 2001). Despite such efforts, the cyanobacterial biofertilizer technology has not provided uniform and reliable results and a need has been felt to enrich the native populations as against using a common consortium for all types of rice ecologies. The present investigation is focused in a similar direction and preliminary investigations have recorded extensive cyanobacterial diversity. The species composition of cyanobacterial community in these soils has been documented (Tiwari and Singh, 2005) and the dominant cyanobacteria in the rice field soil of Manipur valley were found to be rich in carotenoid pigments. Among the 126 cyanobacterial strains isolated during our investigations, heterocystous forms were maximum in number (12 genera, comprising 85 strains, followed by non-heterocystous filamentous (7 genera and 31 strains) and unicellular/colonial forms (4 genera and 10 strains). Form this germplasm, two cyanobacterial strains namely, *Cylindrospermum gorakhpurense* and *Nostoc commune*, which were abundant in terms of their prevalence were selected for further studies.

Characterization in terms of morphological, cultural, growth and physiological attributes: In the present investigation, the thalli of *Cylindrospermum gorakhpurense* was blue green in color, which later turned yellowish, globular and mucilaginous under cultural conditions. Microscopic examination revealed straight trichome with cylindrical cells, ellipsoidal heterocysts, terminal spores which were more or less parallel to each other and were radiating. Cells were 6.5 – 17µ long, heterocysts were 13.2-16.4 µ long and akinetes were 5-8 µ broad and 13-16 µ broad. Normally, basal heterocysts are formed from the terminal vegetative cells at one or both ends of the filament however cultured specimens may have both intercalary and terminal heterocysts. The genus *Cylindrospermum* has curved or slightly coiled cylindrical filaments composed of square or rectangular vegetative cells. The gray or blue-green colored cells lack gas vesicles but sometimes have visible granules. The trichomes are enclosed in loose, colorless, homogeneous mucilage. The 50 known species of *Cylindrospermum* are found mostly in the benthic, epiphytic, or epilithic communities of unpolluted or eutrophic waters, or in soils. Mainly periphytic species, growing in benthos, on water plants and submerged woody and stony substrates, usually in not polluted and slightly eutrophized waters have also been recorded. *Cylindrospermum* usually inhabits soft, acidic freshwater lakes and is known to form dark patches on moist soils and submerged vegetation. The filaments form fine or compact benthic mats or colonies and may be epiphytic and filaments are found in fine or compact, mucilaginous mats, slightly curved or irregularly coiled, cylindrical along the whole length or slightly narrowed in the middle, without sheaths, but with very fine, colourless, homogeneous, diffluent mucilage, symmetrical,

constricted at the cross walls (Anagnostidis and Komarek, 1988).

Nostoc commune has been collected from habitat of the upland rice fields, in the present study. The filaments are composed of moniliform cells, embedded in a gelatinous substance with the thallus being firm, gelatinous and blue green, olivaceous, filaments flexuous and entangled. Heterocysts were spherical and 7.5 µ broad, flexuous, entangled, sheath distinct and thick. Trichome 5-6.5 µ broad and cells barrel shaped. Akinetes were spherical, big as the vegetative cells. Under extended cultural conditions, perennating cells became thick walled and brownish in colour. The genus *Nostoc* is one of the ubiquitous forms present in diverse habitats, and observed to form mutualistic associations with higher plants, which provide a selective advantage from getting washed away by running water in fields. *Nostoc commune* is observed in both aquatic and terrestrial habitats, with flat membranous thalli having firm outer layer (Desikachary 1959).

The two cyanobacterial isolates differed significantly with respect to various physiological parameters examined namely dry weight, generation time, total nitrogen and pigments. Photosynthetic rate was higher in *Cylindrospermum gorakhpurense* as compared to that of *Nostoc commune*. The dry weight accumulation was also higher in *Cylindrospermum gorakhpurense,* and its generation time was much lower (Table-1). Both the strains were observed to accumulate high concentrations of the pigments examined. Hence, these strains can be studied for their commercial viability through biotechnological interventions (Emodi, 1978). Dainippon Ink and Chemicals of Japan have commercialized phycocyanin from *Spirulina* under the trade name Lina Blue that gives brilliant blue colour with faint reddish fluorescence. Total nitrogen accumulation was however much higher in *Nostoc commune*. Nitrogenase activity was more or less similar amongst two strains, however; respiratory activity was more than double in *Nostoc commune*. The high respiratory activity may be an indicator of the higher oxygen scavenging potential of this strain, which may lead to greater accumulation nitrogen in the organism. Such strains may be useful in mass culturing strategies involving bioreactors wherein aeration (enhanced oxygen tensions) sometime proves problematic to the growth of strains. These forms certainly merit attention for future in depth studies regarding kinetics and mode of growth and use in value addition.

Weedicide and Pesticide tolerance:

One weedicide, 2, 4-D (18% WP), and two pesticides namely Malathion (50% EC) and Dimecron (50% EC) were used for studying the behaviour of heterocystous diazotrophic forms of cyanobacteria. The recommended field dozes of 2, 4-D ranges between 17 and 22 µg/ml $^{-1}$, but in the present study, both *Cylindrospermum* and *Nostoc* could tolerate up to 500 ppm of 2,4-D. The growth of algae at higher concentration of 2, 4-D has been reported by earlier workers (Venkataraman and Rajyalakshmi, 1972; Singh 1974; Das and Singh, 1977; Kar and Singh, 1978; Sarada, 1992; Tiwari *et al.*, 2000; Tiwari *et al.*, 2001; Tiwari *et al.*, 2005). Lower concentrations have been found to be stimulatory to the growth of cyanobacteria (Table-2). 28 strains of cyanobacteria against a range of weedicides found that *Tolypothrix tenuis, Aulosira fertilissima* and *Anacystis nidulans* could tolerate 2, 4-D up to application rates of 4.5 kg/ha (Venkataraman and Rajyalakshmi, 1971). Malathion has been shown to be inhibitory at 10-25 µg/mL $^{-1}$ for many cyanobacteria (De Silva *et al.,* 1975; Tandan *et al.,* 1988), although *Anabaena* sp. and *Hapalosiphon* tolerated up to 500 µg/mL^{-1}. Malathion is a commonly utilized pesticide and a dose of 4-8 mg/mL $^{-1}$ is recommended as field doze. . Dimecron is generally recommended at a dose of 0.8-1.2 µg/mL $^{-1}$ for field application in rice crops, but cyanobacterial isolated used in the present study were able to grow well up to 100 ppm of dimecron concentration. Heterocystous cyanobacteria tolerated up to 250 µg/mL^{-1} of Dimecron as indicative of heterocystous forms which were hardier and tolerant (Anand and Veerappan, 1980).

It has been suggested that cyanobacteria may utilize the breakdown products of agrochemicals as nutrient energy sources although diverse processes such as biosorption, accumulation in the sheath/outer layers and also the enzymatic degradation (Anand and Veerappan, 1980; Higazy and Fayez, 1989; Bhunia *et al.,* 1991). At low doses, stimulation of growth in terms of chlorophyll, nitrogenase activity and excretion of ammonia has been observed (Anand and Veerappan, 1980; Maghraj *et al.,* 1988). The interaction of weedicides and pesticides and

their degradation products with soil algae has also been reported (Stratton, 1984; Singh and Tiwari, 1988). Monocrotophos, Lindane and Quninalphos have been found to be degraded by green and blue green algae (Meghraj *et al.*, 1988). The selection of cyanobacterial strains capable of not only tolerating the adverse effects of a wide range of weedicides and pesticides, but also able to utilize the breakdown products for increasing their growth/biomass is a formidable task, which provide rich dividends (Yanni, 1992).

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Table 1: Growth potential and physico-chemical characterization of diazotrophic cyanobacterial strains from rice fields of Manipur, India

Strains	Dry weight (µg mL^{-1})	Generation Time (h)	Total nitrogen (µg mL^{-1})	Chl-a (%)	Carotenoids (%)	Phycocyanin (%)	N_2ase activity (nmole C_2h_4 µg chl^{-1} h^{-1})	Photosynthetic rate (m mol o_2 evol. µg chl^{-1} h^{-1}	Respiratory activity (m mol O_2 abs.µg chl^{-1} h^{-1}
Cylindrospermum gorakhpurense	370	105	6.98	5.40	7.34	87.26	6.80	180.20	34.72
Nostoc commune	460	95	7.40	4.20	5.80	90.00	6.70	170.80	120.10

Tables 2: Weedicides and pesticide tolerance level (Chl-a µg/mL) of diazotrophic cyanobacterial strains from rice fields of Manipur, India

Cylindrospermum gorakhpurense:
(In brackets are the values for *Nostoc commune*)

Concentration (ppm)	2,4-D (18%WP)	Malthion (50% EC)	Dimecron (50% EC)
Control	2.12 (2.20)	2.10 (2.60)	2.24 (2.74)
1	2.45 (3.00)	2.40 (2.98)	2.56 (3.04)
5	2.98 (3.10)	2.90 (3.56)	2.98 (3.44)
10	3.12 (3.40)	3.80 (4.10)	3.90 (4.14)
25	3.30 (3.90)	4.96 (4.68)	2.00 (2.22)
50	3.78 (4.20)	1.98 (2.10)	1.92 (1.98)
100	4.04 (4.80)	1.40 (1.90)	1.58 (1.44)
200	4.86 (5.30)	0.98 (1.64)	1.30 (1.40)
500	1.98 (2.04)	0.60 (1.10)	0.70 (0.98)

WP: Wettable powder; EC: Emulsifying concentration

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Structural coloration by bio-optical nanostructures in Butterfly wings.

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Abstract:

Correlation between structural colour and scale morphology of butterfly scales has been investigated in the present study. The arrangement of scales on the blue spot of the wings has been studied at 20µm magnification. The 2D Fourier analysis of the butterfly scales of *Papilio demoleus* showed an array of elliptical or circular air spaces (microcells). They showed either oval /circular distributions of Fourier power. The homogeneity and energy values are found to be positively exponential during the analysis. The digital SEM micrographs of structurally coloured butterfly scales were analyzed using the matrix algebra program MATLAB (Version R2008a); on a IBM computer. Thus from the present observations of SEM images, it is inferred that the nanostructures in butterfly scales are responsible for structural colour production. Further the arrangement of nanostructures in butterfly scales is so typical and accurate that it forms a low pass filter protecting the butterflies from low frequency rays from sunlight. The nanostructure arrangement in scales is natures' own creation of several years and has evolutionary value. It is suggested that if cotton fibres are manufactured with the same arrangement like that in butterfly scales, human population can have the protection from low frequency wavelengths from sunlight.

Key words: Butterfly scales, bio- optical, nanostructures.

Introduction

Butterflies have vivid, colourful wing patterns, created by rows of partly overlappingscales with intricate spatial structures that scatter incident light. The scattered light can interfere coherently or incoherently. When the scale structures have spatial periodicity, the interference of coherent light waves often results in striking iridescences. The displayed colours are called structural or physical. In white scales without periodicity, light scattering is incoherent or random. They contain pigment that selectively absorbs in a certain wavelength range that results in the latter case a pigmentary or chemical colour. (Vukusic and Sambles 2003; Kinoshita and Yoshioka 2005). Many butterfly species feature structural as well as pigmentary colours (Rutowski *et al.,* 2005; Yoshioka and Kinoshita 2006).Yoshioka and Kinoshita (2006) investigated the phenomenon of colour production in *Morpho* wings. Absolutely no work has been done on the wing colouration of Indian butterflies. Therefore, it was thought to study the morphological characteristics of wing scales of the lemon butterfly, *Papilo demoleus.* Lepidoptera have evolved extraordinary anatomical diversity and complexity in nanostructure. Many of these anatomical variations create additional optical effects beyond hue itself, including iridescence, highly or partially polarized reflections, and colour mixing effects Ghiradella (1991); Vukusic *et al.*, (2000).Prum and Torres, (2003) stated that one major reason for this apparent mechanistic diversity is, the traditional physical tools used to analyze structural colour production vary with the anatomy of the colour producing nanostructure, i.e. its laminar, crystal like, or quasi-ordered organization. The mechanisms proposed includes interference, diffraction, reinforcement, multiplayer reflection, Bragg scattering, Tyndall scattering, Mie scattering, etc. Structural colours are an important component of the phenotype of butterflies and diurnal moths (Ghiradella, 1998; Parker, 1999; Vukusic and Sambles , 2003; Vukusic *et al.*, 2000).

Materials and Methods

For the present study, butterfly *Papilo demoleus* from Papillionidae family is used. For scanning electron microscopy SEM samples were prepared by cutting pieces of the wing to the site needed by using sharp edge cutter. Then the pieces of the wings were attached to double side conductive tape, followed by the deposition of 15nm of sputtered gold, to allow examination in the SEM without charge build up. Images of scales were taken on different range of micrometer scale such as 50µm, 2 µm and 1µm.

The digital SEM micrographs of structurally coloured butterfly scales were analyzed using the matrix algebra program MATLAB (Version R2008a); on a IBM computer. The scale of each image (nm/pixel) was calculated from the number of pixels in the scale bar of the micrograph. A 1024 X 698 pixel portion of each array was selected from each image for analysis. The average refractive index of the scales in each image was estimated by generating a image with adaptive histogram equalization. The frequency distribution of darker and lighter pixels was used to estimate the relative frequency of chitin and air in the tissue.

The Fourier transforms were calculated with the 2D Fast Fourier Transform (FFT2) algorithm after developing a program for the desired images. I then calculated the 2D Fourier power spectrum, or the distribution of the squares of the Fourier coefficients. The 2D Fourier power spectra were expressed in spatial frequency (nm-1) by dividing the initial spatial frequency values by the length of the matrix.

Computer Program designed for Fourier Analysis:

```
% Fourier Transform of an Image
I = imread('Sikha521.jpg');
I_imadjust=imadjust(I);
I_histeq=histeq(I);
I_adapthisteq=adapthisteq(I);
pause
clf,
figure,
imshow(I)
title('Original Image');
pause
figure,
imshow(I_imadjust);
title('Image Adjustment');
pause
figure,
imshow(I_histeq);
title('Histogram equalization');
pause
figure,
imshow(I_adapthisteq);
title('Adaptive Histogram equalization');
pause
%I2 = im2double(I_adapthisteq);
%J = fft2(I2);
%figure,
%Jshift = fftshift(J);
%J1 = abs(Jshift);
%J2=log(J1);
%imshow(J2, []);
%figure,
%J3 = angle(Jshift);
%imshow(J3,[]);
fourier2(I_adapthisteq);
fprintf('Statistics of an Image \n\n')
avg = mean2(I_adapthisteq)
sd = std2(I_adapthisteq)
entr = entropy(I_adapthisteq)
M = graycomatrix(I_adapthisteq);
stats = graycoprops(M)
fprintf('average: %f \n',avg)
fprintf('standard-deviation: %f\n', sd)
fprintf('entropy: %f\n', entr)
```

Results

A fast flying Papilionid, *Papilio demoleus* (Lime butterfly) is a very colourful butterfly (Fig 1) and has different colour spots on forewings as well as on hind wings. The arrangement of scales on the blue spot of the wings is studied at 20µm magnification. Scales from the discal region of forewing (dorsal side) has alternate arrangement of nondentate and dentate scales (Fig.3). The ribs of these scales are arranged regularly, but cross ribs form a mesh. Overlapping lamellae are present on ribs. Pigments are totally absent.

A very small spot of blue colour is present at the discal area on the ventral side of hindwing. The nondentate and dentate scales are arranged at randomly overlapping each other. Dentate scales at 2µm, show parallel arrangement of ribs and crossribs are not uniformly arranged. They form somewhat pepper-pot type of structure. The mesh formed by crossribs is very irregular in arrangement. Microribs are not seen. Pigment granules are absent (Fig .4). Nondentate scale also shows pepper-pot structure. Ribs are uniformly arranged and have overlapping lamellae on it. The crossribs form a mesh. Microribs are

clearly seen and they are well developed. trabaculae are interminantly present.

Fourier Transform images of SEM of butterfly scales:

The pixels of SEM images were made sharper with a tool from MATLAB and the images with adaptive histogram equalization were used for Fourier Transform Analysis. The 2D Fourier analysis of the butterfly scales of *Papilio demoleus* showed an array of elliptical or circular air spaces (microcells). They showed either oval /circular distributions of Fourier power (Fig. 4).

Table 1, gives the statistical data of Fourier Transform images of *Papilio demoleus* butterfly species. The data indicates that the entropy of all scales under study is around 8 and the energy is 0.0407. The average homogeneity of nanoparticals in scales is 0.6754. The homogeneity and energy values are found to be positively exponential during the analysis.

Discussion

The aim of the present study was to investigate the correlation between structural colour and scale morphology of butterfly scales. It is known that the coloration of butterfly wings has two main sources. Colour arises either from pigmentation or from structure of the wing scales. The first one is also called chemical colour, the second one physical or structural colour. Pigments that are in butterfly scales (melanins, pterins), can produce yellow, orangeyellow, red, black, and brown colours. In butterflies pigments cannot produce blue, violet and green colours. Blue, violet and green colours are based on the scales' micro- and nanostructure (Stavenga, *et al.*,2004)). Scale has a featureless reverse surface and a complex obverse surface with elements like longitudinal ridges and cross ribs (Fig. 4). These longitudinal ridges and cross ribs form microcells called windows. Biro *et al.* (2003) stated that colouration in butterfly wings resulted from three dimensional microstructures and nanostructures of the scales that cover the wings. Light interacts with three-dimensional micro- and nanostructures of the scales, to produce colours. The *Papilio demoleus* butterfly has scales that are characterized by parallel layers of cuticles filling the microcell of the individual scale. These layers have circular holes (Fig.3).

The difference between the nanostructures of different species and subfamilies is mainly determined by a filling factor, i.e. the ratio of chitin walls and holes in the microcell (Vertesy *et al.* 2006). The microcell is defined as the volume formed by two consecutive ridges, two consecutive cross-ribs and the basal membrane of the scale. The cuticle form concave surfaces inside the microcells. The circular pores in microcellular cuticles probably in manipulating the light. Until recently, the biological literature on structural colour has obscured the most fundamental physical distinction among all mechanisms of structural colour production: incoherent vs coherent scattering (Prum and Torres, 2003a; Prum and Torres, 2003b; Prum and Torres, 2004). Incoherent scattering is the differential scattering of light wavelengths by individual scatterers, and it is determined by the size, shape and refractive index of individual scatterers without regard to the phase relationships among multiple waves scattered by different objects (van de Hulst, 1981; Bohren and Huffman, 1983). In contrast, coherent scattering is differential scattering of light wavelengths from multiple objects, and it is determined by the phase relationships among scattered light waves (Joannopolous *et al.*, 1995; Prum and Torres, 2003a; Prum and Torres, 2003b). Incoherent light scattering requires that the light scattering objects are spatially independent, or randomly distributed over spatial scales of the same order of magnitude in size as the wavelengths of visible light. Spatial independence insures that the phase relationships among scattered waves are random, and can thus be ignored in the calculations of light scattering (Bohren and Huffman, 1983). Rayleigh and Tyndall scattering (Prum and Torres, 2003a) describe incoherent scattering by particles the size of visible light or smaller, and they predict the production of short wavelength hues: blue, violet and ultraviolet. Coherent scattering occurs when spatial variation in refractive index is periodic, resulting in predictable phase relationships among light waves scattered by different objects. Interference, reinforcement, diffraction, multilayer and thin- film reflection, and Bragg scattering are all forms of coherent scattering. A wide variety of different nanostructures with periodic spatial variation in refractive index over one, two or three dimensions can result in coherent scattering. Unlike incoherent scattering, coherent scattering can produce the phenomenon of iridescence – a prominent change in hue or

brilliance with angle of observation or illumination – because changes in angle of observation and illumination may affect the phase relationships among the scattered waves that determine the hue. Consequently, since 1923, iridescence has often been inaccurately synonymised with coherent scattering (Mason, 1923), leading to the indiscriminant assignment of noniridescent blue structural colours to incoherent, Rayleigh or Tyndall scattering (Fox, 1976; Nassau, 1983; Herring, 1994). Recently, however, it has been shown that a previously unappreciated class of nanostructures, called quasiordered arrays, can produce noniridescent or weakly iridescent colours by coherent scattering alone (Prum *et al.*, 1998; Prum *et al.,* 1999a; Prum *et al.*, 1999b; Prum and Torres, 2003a; Prum and Torres, 2003b; Prum and Torres, 2004).

The present results provide an important insight into how diversity in nanostructure and optical function may have evolved in lepidopterans through selection on novelties in optical function. The Fourier transforms (Fig. 4) of the intersections of the concave multilayer in windows in *Papilio demoleus* demonstrate how internal laminar and internal crystal-like nanostructures can intergrade into one another. These scale concavities function and have likely evolved by selection to reduce iridescence and have produced the periodic establishment across the scale of an intermediate form of nanostructure between laminar and crystal-like arrays. Analysis makes easy to imagine how selection on optical function could lead to an evolutionary transition between these two types of nanostructures. Furthermore, the deeper concavities of *Papilio demoleus* that produce optical colour mixing of yellow and blue (Vukusic *et al.,* 2000; Vukusic *et al.,* 2001) likely evolved as derived elaborations of the type of iridescence reducing concavities found in *Papilio demoleus.* As previously recognized (Vukusic *et al.,* 2001), many of the species with colour producing, laminar nanostructures within the body of the scales also have superficial ridges with periodic ornamentation that quite likely also function in coherent scattering.

The Fourier Transfer Images; Log of Magnitude images; (Fig 4) probably indicates that the nanostructures in scales might be forming a low pass filter protecting the butterflies from UV radiations. The Fourier Transform images indicate that the reflectance is high in the visible wavelength range, but it is low in the ultraviolet range.

From the visual inspection of the log magnitude spectra of the nanostructures, it could be conjectured that these nanostructures demonstrate bilateral as well as radial symmetry so far as the inherent geometric structure in spatial domain is concerned, spectrum also shows dominating directions. It contains components of all frequencies; however, their magnitude gets smaller, for higher frequencies. Therefore the nanostructures are forming, a low pass filter in some sense. It can be infrared that the reflectance of nanostructure in the scale is high in the low frequency range (possibly visible wave length range) but it is low in the high frequency range (possibly UV range).

Thus from the present observations of SEM images and the earlier work of scientists from Biophysics, it is inferred that the nanostructures in butterfly scales are responsible for structural colour production. Further the arrangement of nanostructures in butterfly scales is so typical and accurate that it forms a low pass filter protecting the butterflies from low frequency rays from sunlight. The nanostructure arrangement in scales is natures' own creation of several years and has evolutionary value. It is suggested that if cotton fibres are manufactured with the same arrangement like that in butterfly scales, human population can have the protection from low frequency wavelengths from sunlight.

Table 1, Statistical data of Fourier Transform images of *Papilio demoleus* butterfly species [from Hind Wing –Ventral Side -Discal region (Blue colour)]

Entropy	7.9739
Contrast	1.0425
Correlation	0.8728
Energy	0.0407
Homogeneity	0.6754
SD	72.179088

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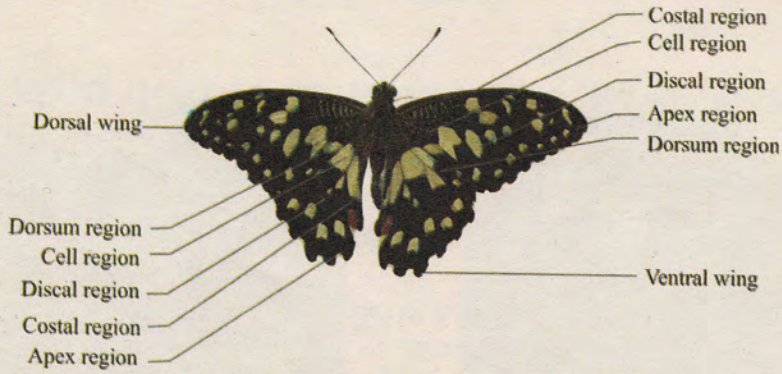


Fig.1, Showing different region of fore wing and hind wing of butterfly (*Papilio demoleus*)

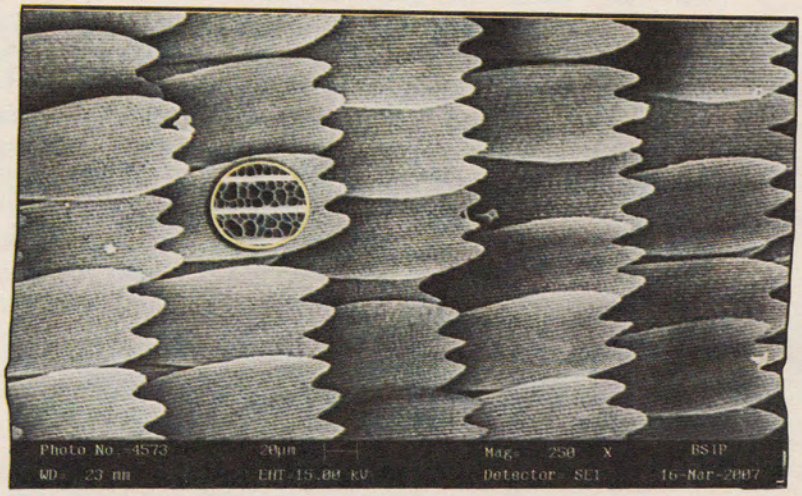


Fig. 2, Scales from ventral side of hindwing of *Papilio demoleus* from discal colour patch of discal region.

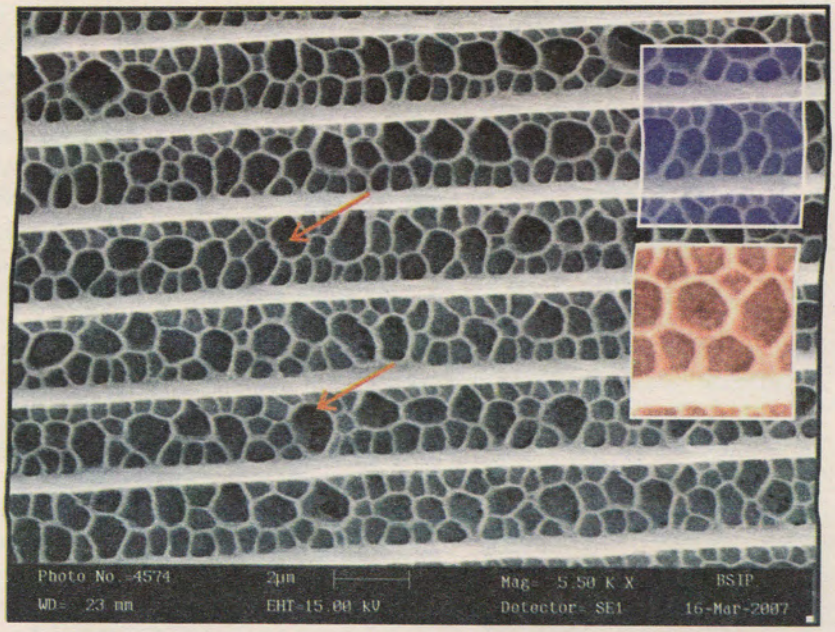


Fig. 3, Scanning Electron Micrograph of scale from ventral side of hindwing of *Papilio demoleus* from blue colour patch of discal region.

↑ Indicates Nanopores in microcell forming pepper pot strutcure

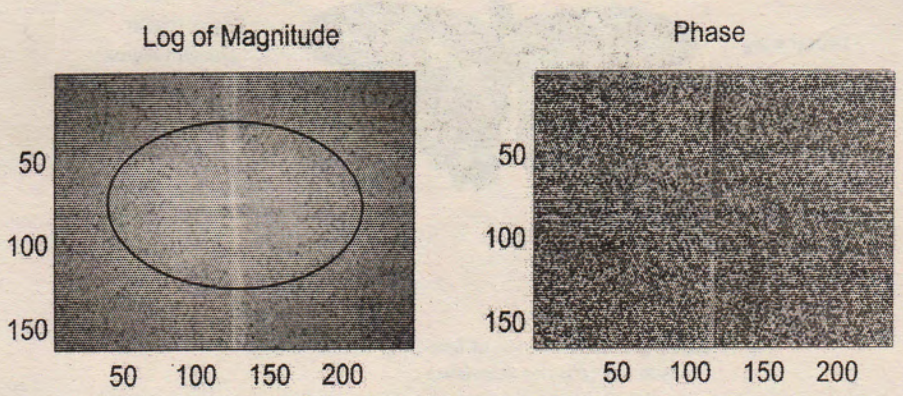


Fig.4, Two dimensional Fourier Power spectra (Log of magnitude and phase) of Scanning Electron Micrographs of butterfly Scale from ventral side of hindwing of *Papilio demoleus* from blue colour patch of discal region

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Some wild edible mushrooms from Melghat Tiger Reserve Forest and Amravati Region.

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Abstract:

For mycofloristic study purpose, many periodical surveys were conducted of Melghat Tiger Reserve Forest and Amravati region including Pohara Forest, Wadali Forest Nursery, S.G.B. University Campus and Tapovan area. Surveys were conducted during 2004-2007 monsoon seasons. During the period, a rich diversity of wild macromycetes including edible and non-edible fleshy mushrooms was encountered. Identification and edibility of collected fleshy forms was noted by referring the standard literature.The critical study of literature shows that, out of fifteen fungi presented in this paper, five are new additions to the wild edible mushrooms of Maharashtra.

Key words: Wild macromycetes, edible mushrooms, Melghat and Amravati region.

Introduction

Collecting larger fungi should not be considered a haphazard pursuit. It is to emphasize that the task of identification and proper record of larger fungi is of prime importance and can only be fulfilled by extensive surveys of different zones of the country. Amravati is endowed with Satpuda ranges. It is surrounded by Melghat Tiger Reserve Forest and Pohara Forest. Due to suitable geographical and favourable climatic conditions, wide and rich mycoflora is hidden in these regions. In this regard, none contributed to explore this untouched wealth of Vidarbha region. Hence the attempts are being made to undertake macrofungi study in Vidarbha region.

Some wild edible mushrooms have been reported from South-West India by Sathe and Kulkarni (1987). The mushroom flora of Kerala has been investigated by Bhavani Devi and Nair (1988). Many Agarics were reported from Andhra Pradesh by Manoharachary and Vijay Gopal (1991). From several bio-geographical regions of India, at least 2000 edible species of larger fungi were reported. But Central India region has not been investigated extensively for mushroom flora (Kaul, 1999). A list of 37 wild mushroom species of Sundarijal and Kathmandu region has been recently published by Pandey and Budhathoki, 2008.

Material and methods

Healthy specimens were collected, cleaned and brought to the laboratory. Standard techniques for collection, preservation and description have been followed. Morphological observations were based on fresh specimens. The microscopic observations and spore prints were done immediately (Kaul, 1999). Colour terminology used is that of Kornerup and Wanscher (1967). The specimens has been deposited at the Mushroom Herbarium of the P.G.Botany Dept. Shri Shivaji Sci.College, Amravati.

Identification and edibility was noted by referring the standard literature (Atkinson, *1961; Lange and Hora, 1981; Purkayastha and Chandra, 1985; Patil et. al., 1995; Swanton, 2002). Edibility was confirmed by interacting* with the local and tribal people, who were habitual to consume the specific mushroom of that region.

Description of studied wild edible mushrooms:

***Agaricus campestris* L. ex Fr.** (Field mushroom)
Sporophores- Solitary, stipilate, white when young. Pileus- 4.0-8.0 cm. in diameter, first rounded, then convex and more or less flattened at maturity, sometimes with light brown triangular scales towards centre of cap. Gills- crowded, distinctly formed, free from stem, at first white then pink and finally purple brown. Stipe- central, cylindrical, 5.0 - 8.0 cm. long, 8 – 12 mm. in thickness, white and fleshy. Annulus- single, white. Flesh- white, firm. Basidiospores- purple brown, spherical to ellipsoidal.
Collected from- S.G.B. University campus, amongst grass, by roadsides, Tapovan.
Edibility- Atkinson (1961), Walting (1973).

***Auricularia mesenterica* Pers.** (Jews Ear)
Sporophore- growing on dead wooden logs, 3.0-7.5 cm. wide, 0.2- 0.4 cm. thick, sessile, cup or ear-shaped, lobed, gelatinous and somewhat translucent, the upper surface velvety, hairy and concentrically zonate, lower surface reddish-purple or deep purple. Six distinct hyphal zones in transverse section of sporophore, bone-hard when dry. Basidiospores- sausage-shaped.
Collected from: Wadali forest, Amravati, on log of *Eucalyptus*, Melghat on log of *Tectona,* College on dead branch of *Alstonia.*
Edibility: Cheng and Tu (1978).

***Calocybe indica* P. & C.** (Milky Mushroom)
Sporophores- usually robust in size, centrally stipitate, fleshy, white. Pileus- 10.0-14.0 cm. in diameter, at first convex, later expanded and flattened, cuticle easily peeled, margin regular, incurved, smooth. Gills- crowded, separable, white, unequal. Stipe- central to eccentric, cylindrical, without annulus, with subbulbous base, up to 10.0 cm. long, white, cartilaginous, base solid. Flesh-white. Spores-white.
Collected from: Tapovan, College campus, Shivarpan colony, at the bottom of Gulmohor.
Edibility: Purkayastha and Chandra (1980).

***Calvatia cyathiformis* (Bosc) Morg.** (Cup-shaped puffball)
Sporophores- growing solitary or scattered on the ground, in meadows, grassy lands, pear-shaped, rounded above and tapering below to the stout base, 6.0-12.0 cm. high. Peridium- 5.0-12.0 cm. wide, white when young, pinkish or brownish when old, surface covered with warty patches. Gleba- white when young and purplish at maturity, wall cracking into irregular fragments and purplish mass of the spores is exposed. Capillitium and Spores- purple-brown. Old sterile cup-like bases of sporophore are often found in the field after spore dispersal. Basidiospores- round, purple brown.
Collected from: Tapovan Goshala, on grassland.
Edibility: Atkinson (1961), Krieger (1967), Jong (1978).

***Coprinus comatus* (Muell ex Fr.) S. F. Gray** (Shaggy ink caps)
Sporophores- singly or in clumps on compost, refuse dumps. Pileus- 5.0 –10.0 cm. long (before expansion), oblong, companulate fleshy, fragile, white, covered with woolly, shaggy scales, the center smooth and yellowish-brown, whitish between the scales, the margin becomes pinkish then black as autodigestion proceeds. Stipe- central, tapering at the top, smooth, hollow, 12.5- 17.5 cm. long, 10-12 mm thick. Gills- at first white, then pink and finally black, deliquising into an inky fluid. Spores- black. Common on rich ground, road-sides.
Collected from: Wadali road, College campus, on dung rich grassy land, Shivaji Agri; on compost, S.G.B. Amravati University on compost.
Edibility: Atkinson (1961), Kaul (1971), Phillips (1981).

***Leucocoprinus capaestipes* (Sow. ex Fr.) Patouillard** (Onion-stemmed *Lepiota*)
Sporophores- in groups on rotten wood, straws, decomposed vegetable matters, with powdery veil and collapsing at maturity. Pileus- ovate to bottle shaped when young, later expanded or broadly conical with an umbo, usually white, squamules often appear. Stipe- slender 5.0 – 8.0 cm. long, 2.0 – 6.0 mm. thick, bulbous at the base, white, smooth, hollow, ring thin. Gills- crowded, free, narrow, white. Flesh- white and very soft. Spores- white.
Collected from: Tapovan, Tapovaneshwar on rotten wood of *Butea monosperma*, Agriculture field on Soybean straw, Camp at the bottom of *Butea.*
Edibility: Krieger (1967).

***Lycoperdon pusillum* Persoon** (Puff-ball)
Sporophores- scattered on grazed sandy soil. Peridium- globose or subglobose, about 2 cm.

in diameter, with a strongly developed basal rhizomorph, yellowish when young, becoming brown with age, sterile base and diaphragm absent. Exoperidium- with minute squamules, fragile. Endoperidium- membranous, smooth, dehiscing by a small irregular plane stoma. Gleba- yellowish, becoming brown at maturity. Capillitium- branched, brownish. Spores- globose, olivaceous.
Collected from: Wadali, Pohara forest, Yogiraj nagar, on grazed land.
Edibility: Cunningham (1979).

***Macrolepiota procera* (Scop. ex Fr.) Sing.** (Parasol mushroom)
Sporophores- growing solitary or in clusters in woods, in pastures, 12.0-20.0 cm. high. Pileus- 8.0-15.0 cm. in diameter, at first ovate, then companulate, finally becoming expanded, surface cracking into large, brown scales which are concentrically arranged except at the umbo, the margin thin, usually turning downwards. Gills- free, not forked, white when young and pinkish at maturity, broad in the middle. Stipe- cylindrical, with prominent bulbous base, 15.0-20.0 cm. long, covered with minute darker scales, annulus large, conspicuous, thick, white, movable. Flesh- white, remaining unchanged when bruised, soft. Spores- white, ovate to elliptical.
Collected from: Pohara forest road-side.
Edibility: Atkinson (1961), Krieger (1967), Jong (1978).

***Macrolepiota rhacodes* (Vitt.) Singer** (Shaggy Parasol mushroom)
Sporophores- in clusters, forming fairy rings in open forests, centrally stipitate.
Pileus- 8.0-15.0 cm. in diameter, globose when young, then convex to expanded, reddish brown, scales formed by the rupture of the cuticle, scales concentrically arranged. Gills- crowded, free from stipe, whitish to pinkish, broad. Stipe- 8.0-16.0 cm. long, thick, surface glabrous, base enlarged, annulus not movable, edge not red. Flesh- white, soft, turning red on cutting or when broken. Spores- white, elliptical-oval.
Collected from: Yogiraj Nagar.
Edibility: Ghosh and Pathak (1965), Krieger (1967).

***Morchella conica* Pers.** (Conic morel)
Sporophores- scattered on sandy loam organic rich soil, yellowish when young. Pileus- 3.0 – 6.0 cm. long, 3.0 – 5.0 cm. in diameter at the base, with narrowing tip, conical in shape, pits are usually longer and arranged more or less in distinct rows, ridges extend longitudinally from the base to top, buff to brownish. Stipe- cylindrical, 4.0 –7.0 cm. long, 1.0 –3.0 cm. thick, hollow fragile. Ascospores – lacking internal oil drop.
Collected from: Chikhaldara garden and Sipna college on organic rich soil amongst Coffee plantation.
Edibility: Christensen (1955), Atkinson (1961).

***Podaxis pistillaris* (L. ex Pers.) Morse** (Khumbi Mushroom)
Sporophores- growing abundantly on sandy soil. Peridium- ovate-oblong, 5.0-10.0 cm. tall, 1.0-3.0 cm. in diameter, apex rounded, reaching up to 15.0-25.0 cm. in height. Exoperidium- covered with scales. Endoperidium- membranous, dehiscing by longitudinal fissuring. Stipe- 6.0-8.0 cm. tall, 6-12 mm. thick, covered with white, brittle fibrils, stipe may be provided with a bulbous rooting base. Gleba- dense, reddish brown to black, capillitium threads deeply coloured, aseptate, sparingly branched. Spores- reddish brown, apically perforated by a conspicuous pore. Common on road-side, near bricks factory, sandy soil, semi compost.
Collected from: College campus, Rampuri camp, Wadali road, on roadside.
Edibility: Kannaiyan and Ramasamy (1980).

***Russula claroflava* Pers.** (Yellow *Russula*)
Sporophores- often solitary, brightly coloured. Pileus- up to 8.0-10.0 cm. across, convex then flattened or a little depressed, smooth, cadmium yellow margin slightly striate with age. Stipe- 5.0- 9.0 cm. long, 1.0-2.5 cm. thick, white at first, but turning grayish at length, longitudinally furrowed. Gills- pale-yellow, broad, free. Flesh- white, becoming grayish with age. Spores- pale ochraceous in the mass, coarsely warted, elliptical.
Collected from: College campus, Tapovan, Camp at the bottom of *Tamarindus indica.*
Edibility: Lange and hora (1981).

***Sparassis crispa* Wulfen ex Fries.** (Cauliflower fungus)
Sporophores- large, 20.0-25.0 cm. across, creamy to pale buff, much branched and forming a dense mass, cauliflower like shape, branches compact, fleshy, brittle, flattened, lobed, wavy or curled, the edges tinted

brownish with age. Stem- whitish, short. Spores- pale ochraceous, egg-shaped.
Collected from: Chikhaldara garden at the base of conifer.
Edibility: Lang and Hora (1963), Garcha (1980), Phillips (1981).

Termitomyces heimii **Natarajan (Dumbersathi Mushroom)**
Sporophores- in association with termite nests, fleshy, centrally stipitate.
Pileus- 6.0-10.0 cm. in diameter, plano-convex with an umbo; whitish gray at the umbonal region, margin inflected and cracking. Gills- crowded, free, whitish but pinkish with age. Stipe- 12.0-16.0 cm. long, white, annulate, cylindrical, pseudorhiza fleshy. Basidiospores- pinkish gray, ellipsoidal.
Collected from: Shivaji College garden, S.G.B.University campus.
Edibility: Bose (1923), Sathe and Kulkarni (1980).

Termitomyces mammiformis **Heim (** Dumbersathi Mushroom)
Sporophores- growing in groups in association with termite nests, fleshy, centrally stipitate. Pileus- 7.0-9.0 cm. in diameter, with a very prominent umbo and inflexed margin. Gills- crowded, white, free. Stipe- 5.0-8.0 cm. long and 1.0 cm. wide, persistant annulus present, pseudorhiza up to 10.0 cm. deep. Flesh- white. Basidiospores- pinkish, hyaline, ellipsoidal.
Collected from: S.G.B. University campus, on humus rich soil.
Edibility: Zoberi (1972).

Discussion and Conclusion:

During survey, edible macrofungi from various sub-groups have been collected and identified *viz.* gill fungi, stomach fungi, jelly fungi, pore fungi and cup fungi. Out of fifteen wild edible fungi studied and recorded so for fourteen fungi belongs to sub-division Basidiomycotina, and one to Ascomycotina.

In the present study, *Sparassis crispa, Russula claroflava, Termitomyces heimii, Macrolepiota procera* and *Macrolepiota rhacodes* are the new additions to the edible wild fungi of Maharashtra. Melghat-Amravati region is found to be one of the important biodiversity rich-area for varied wild fleshy fungi. It is expected that edible mushroom industry if properly organized; can ease the problem of food (malnutrition) in Melghat, medicine, employment generation and farmers suicide in Vidarbha region to some extent.

Acknowledgements

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Biochemical studies of crude extracts of roots and leaves of *Withania somnifera*.

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Abstract:

Biochemical investigation of Ashwaghanda, *Withania somnifera*, a shrub commonly found in the Indian subcontinent has been done as this plant is increasing becoming popular for its medicinal uses. Various phytochemical like alkaloids, carbohydrates, proteins, saponins, flavonoids and phenols have been identified. Using HPLC and LC/MS the main active ingredients like Withaferin A, Withanolide A, Withanolide D and tropine have been reported.

Key words: Withania Somnifera, HPLC, LC/MS.

Introduction

The plant kingdom has provided an endless source of medicinal plants first used in their crude forms as herbal teas, syrups, infusions, ointments, liniments and powders. With the development of chemistry and western medicine, the active substances of many species have been isolated and in some cases duplicated in the form of synthetic drugs. There are various medicinal plants which are known to have several medicinal properties, since ancient times. Among these medicinal plants, *Withania somnifera* is such a wonderful herb having great potential for the treatment of several maladies, various studies indicate that *Withania somnifera* possesess a large number of active ingredients in its parts and is been used as an alternative anti–tumor, anti–inflammatory, anti–Parkinsonian, an anti–stress, anti–venom agent and memory enhancer (Singh *et al.*, 1986). Like other medicinal plants, it contains significant amounts of alkaloids, carbohydrates, proteins, saponins, flavonoids, phenols and other substances that are important in supporting this particular herbs medicinal properties. In order to discover new bioactive compounds from plant sources, which could become new leads or new drugs, plant extracts should be evaluated by fine chemical analyses. In view of the increasing importance of medicinal plants and their ingredients, the present study was undertaken where a qualitative and quantitative analysis of various phytochemical(s) of *Withania somnifera* was done. Also, the presence of some major bioactive compounds in crude roots as well as leaf extracts of *Withania somnifera* were studied by using HPLC and hyphenated technique of Liquid Chromatography and Mass Spectrometry (LC/MS).

Material and Methods

The fine powder of leaves and roots of *Withania somnifera* was made from plants grown in the College garden after proper identification on the basis of their characteristics as mentioned in the literature (Chopra *et al.*, 1955; Basu, 1975).

The crude extracts roots and leaves of *Withania somnifera* were prepared and quanlitative bio-chemical analysis was done according to the methods described by Mojab *et al.*, (2003).,whereas the quantitative estimation of some identified phytochemicals *viz.* saponins, flavanoids, phenols, alkaloids, carbohydrates and proteins was conducted from both root and leaves, following the methods of Khanna and Kannabiran, (2007).

For the biochemical analysis an aqueous extracts of root and leaf of *Withania somnifera* was prepared using soxhlet assembly by taking 20 gms of root and leaf powder in 200 ml distilled water separately. Alcoholic extracts (80% methanol) of root and leaf of *Withania somnifera* were prepared using soxhlet assembly by taking 20 gms of the root and leaf powder in alcohol (Schliebs *et al.*, 1997).

HPLC analysis for *W. somnifera* roots was done at M.F.P. Processing & Research Centre, Bhopal and *W. somnifera* roots and leaves LC/MS analysis was performed at IIT, Mumbai.

Results and Discussion

The qualitative study of the phytochemicals was carried out on the aqueous / alcoholic extracts of the medicinally important herb, *Withania somnifera.* The list of medicinally active compounds, in roots and leaves of *Withania somnifera* is given in Table-1.

S. No	Phytochemicals	Presence / Absence of Phytochemicals(leaves)	Presence / Absence of Phytochemicals(roots)
1.	Saponins	+ + + + +	+ + + + +
2.	Flavonoids	+ + + +	+ + + +
3.	Alkaloids	++ +	+ + + +
4.	Phenols	+ +	+ + +
5.	Carbohydrates	+ +	+ + +
6.	Proteins	+	+ +
7.	Tannins	+	+
8.	Terpenoids	–	–
9.	Phlobatannins	–	–

+: - Presence of the compound, –: - Absence of the compound

Table 1:Qualitative analysis of the phytochemicals of aqueous root and leaf extracts of *Withania somnifera.*

Quantitative analysis of active compounds present in roots and leaves of *Withania somnifera:*

A well-organized quantitative estimation of the phytochemicals, present in aqueous / alcoholic extracts of dried powder of roots and leaves was also simultaneously carried out with qualitative studies. The estimated phytochemicals in the samples were found to be – saponins, flavonoids, alkaloids, phenols, carbohydrates, and proteins are given in the following (Table 2& 3).

S. No.	Name of phytochemical	Minimum	Maximum	Average	Standard deviation
1.	Saponins	6.28	9.97	8.125	± 1.845
2.	Flavonoids	3.24	4.12	3.68	± 0.44
3.	Alkaloids	0.461	1.020	0.7405	± 0.2795
4.	Phenols	0.32	0.66	0.49	± 0.17
5.	Carbohydrate	0.26	0.45	0.355	± 0.095
6.	Proteins	0.015	0.019	0.017	± 0.002

All values are mentioned in (%) percent

Table 2: Quantitative analysis of phytochemicals present in the root extracts of *Withania somnifera.*

S. No.	Name of phytochemical	Minimum	Maximum	Average	Standard deviation
1.	Saponins	5.53	6.31	5.92	± 0.39
2.	Flavonoids	5.28	7.24	6.26	± 0.98
3.	Alkaloids	1.68	2.05	1.865	± 0.185
4.	Phenols	0.32	0.56	0.44	± 0.12
5.	Carbohydrates	0.023	0.101	0.062	± 0.039
6.	Proteins	0.014	0.016	0.015	± 0.001

All values are mentioned in (%) percent

Table 3: Quantitative analysis of phytochemicals present in the leaf extracts of *Withania somnifera.*

The root and leaf extracts were analyzed and according to their molecular weights following fine chemicals were identified such as - withaferin A, molecular weight 470.6, peak area 1.507e+7 and retention time 0.821 minutes, withanolide A, and D having identical molecular weight of 472.1, peak area 7.990e+8 and retention time 8.594 minutes and tropine, molecular weight 141.211, peak area1.324e+10 and retention time 6.078minutes

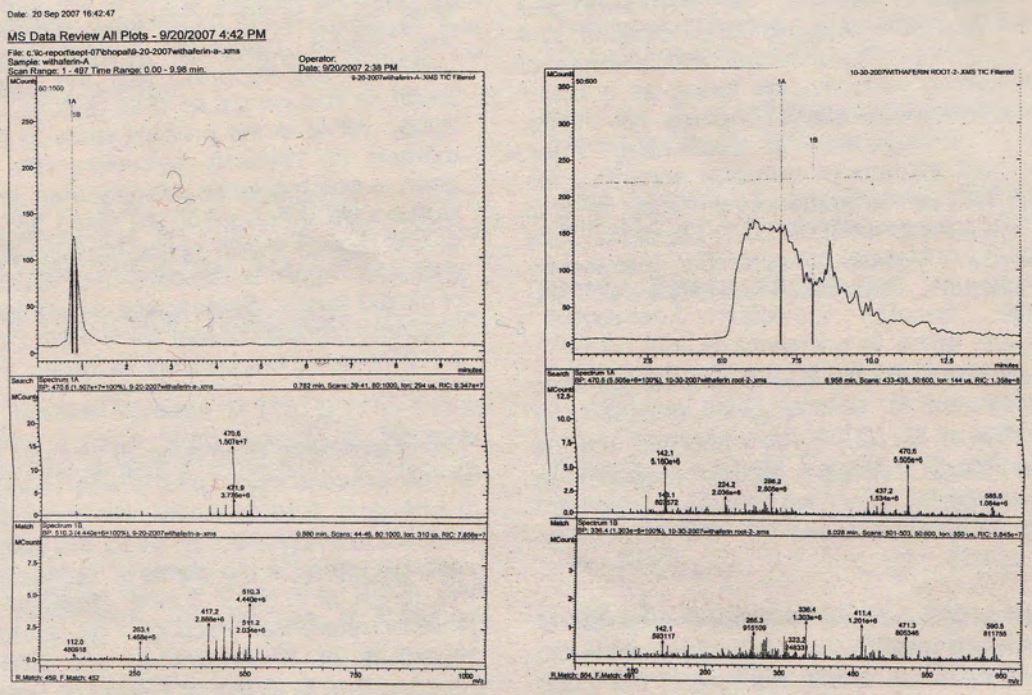


Fig: 1.MS showing peaks areas of compound(s) with molecular weigth along their retention times.

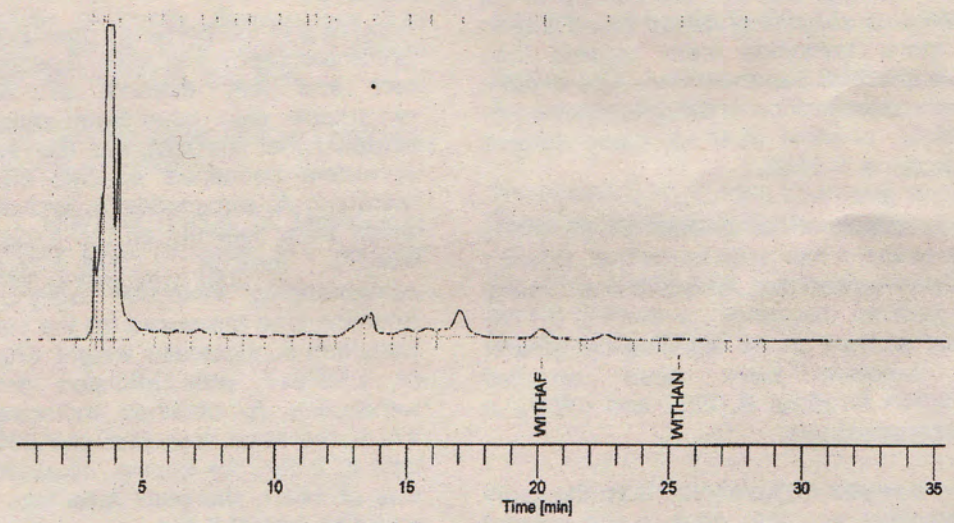


Fig: 2.HPLC showing peak areas of Withaferin A and Witahnolide A. .

The roots extract of *Withania somnifera was* subjected to HPLC analysis and their main ingredient Withaferin A and Withanolide A were identified and their content were found to be 0.108 and 0.0128% respectively.

Looking to the increasing importance of phytochemicals, in the present study plant extracts of *Withania somnifera* (roots and leaves) were subjected to biochemical evaluation both qualitatively as well as quantitatively. Also, the presence of some major bioactive compounds in extracts of *Withania somnifera* were detected and studied by sophisticated techniques of Liquid chromatography and Mass spectrometry (LC/MS).

The qualitative analysis of leaf extracts of *Withania somnifera* revealed the presence of large number of medicinally active phytochemicals like saponins,

flavanoids, alkaloids, phenols, carbohydrates and proteins to be present in varying concentrations. Out of all these phytochemicals in the leaf extracts of *Withania somnifera,* saponin was found as a major constituent followed by alkaloids, phenols, flavonoids, proteins, and carbohydrates in decreasing order. Similarly, the root extracts of *Withania somnifera* too revealed the similar components in more or less similar order of concentrations. Saponins are prominent, being in highest concentration followed by flavanoids, phenols, alkaloids, carbohydrates and proteins.

Qualitatively, it is well known that saponin is found as a major constituent in several plant materials for example – Mojab *et al.,* (2003) have reported that the most of the plant extracts, contain saponin in significant quantities. Thus, the results of present study are in full collaboration with the findings of Mojab *et al.,* (2003).

The quantitative analysis of the root extracts of *Withania somnifera* which is a much more authentic analysis, it has been found that the root of this plant contained highest amounts of saponins (8.125%) followed by flavanoids (3.68%), alkaloids (0.74%), phenols (0.49%), carbohydrates (0.355%) and proteins (0.017%). Similarly, in the leaf extracts of the same plant; *Withania somnifera,* it was found that in leaf extracts more flavonoids were present than saponins, that is (6.26%). Saponins were also present in significant high concentration of (5.92%) followed by alkaloids (1.865%), phenols (0.44%), carbohydrates (0.062%) and proteins (0.015%).

The quantitative analysis of the different plants of this family i.e. *Solanaceae,* it has been found that, Edeoga *et al.,* (2005) have reported that *Physcalis bransilensis* contained saponins in the range of 3.92 ± 0.11%, whereas in the present study significantly greater amounts of saponins have been detected quantitatively, which is about 8.125% and 5.92% in roots and leaves respectively.

The quantitative analysis of flavonoids from the roots of *Withania somnifera* was also done in the present study, and it has been found that the flavonoids were present in 3.68 ± 0.44%. Similarly, the leaf extracts of *Withania somnifera* contained 6.26 ± 0.98%. The presently estimated quantity of flavonoids in the roots and leaves of *Withania somnifera* are not matching with the findings of Akubugwo *et al.*, (2007) who studied some plants of family *Solanaceae* and reported that the flavonoid contents were in the range of 0.81 ± 0.01 to 1.01 ± 0.01%. In another study of Edeoga *et al.,* (2005), on a plant *Physcalis bransilensis* of family *Solanaceae,* it has been found that this plant contained 0.15 ± 0.20% of flavonoids, which is many times less in the comparison of presently found flavonoid content in *Withania somnifera*

With regard to the quantitative analysis of alkaloids in the various plants of *Solanaceae* family, many workers have reported that the alkaloid content is found in the range of 0.40 ± 0.11 (Edeoga *et al,* 2005). While in the present study in the root and leaf extracts of *Withania somnifera*, the alkaloids have been in much greater quantity than reported by these workers i.e. 0.740 ± 0.279% and 1.865 ± 0.185%. One of the reports which come very close to the present data with regard to the content of alkaloids in the plant of similar family; *Solanaceae,* where leaf and seeds of *Solanum nigrum* content were 1.62 ± 0.02% and 1.07 ± 0.05% w/w. respectively recorded by Akubugwo *et al.*, (2007).

The quantitative estimation of phenols was also done in the present study and it was found to be 0.49-± 0.17% and 0.44 ± 0.12% for roots and leaves. In contrast to the present findings, the amount of phenols reported earlier in the plants of *Solanaceae* family is in the range of 0.80 ± 0.22% (Edeoga *et al.,* 2005). Another reference which can be quoted here in this regard is of Akubugwo *et al.,* (2007) who have reported that in *Solanum nigrum* of *Solanaceae* family phenols were found to be in large quantities i.e. about 13.17 ± 0.02% and 14.69 ± 0.01% (w/w) than that of *Withania somnifera.*

According to the presently conducted studies of Liquid Chromatography and Mass Spectrometry (LC/MS) on root and leaf extracts of *Withania somnifera* remarkable data have been obtained. It has been revealed that the root and leaf extracts of *Withania somnifera* contained specific steroidal compounds withaferin A, withanolide A, and alkaloid like tropine; which were detected on the basis of their molecular weights through LC/MS techniques. The corresponding molecular weight, peak area and retention time measured for the compounds were, for withaferin A, molecular weight 470.61, the peak area of 1.507e+7 with retention time of 0.821, for withanolide A, obtained molecular weight was of 471.9, the peak area was of 7.990e+8 and retention time of 8.594, for tropine, obtained molecular weight was of 142.1, the peak area was of 1.324e+10 and retention time of 6.078.

A similar analysis of major bio-active phytochemicals using LC/MS has been reported by Sun *et al.,* (2005) who subjected the leaf extracts of *Ginkgo biloba* of family *Ginkgobiaceae* for LC/MS analysis where they detected the major terpenoids- bilobalide and ginkgolides (A, B, C and J). The obtained molecular weights were 163, 351, 367,367 and 386 respectively whereas their retention time was 7.6, 11.2, 11.5, 9.3 and 9.0 minutes respectively. In the present study similar data has been obtained, which revealed that the wedelolactone and demethylwedelolactone were present and identified by their molecular weight and retention time. (Sun *et al.,* 2005)

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Secondary metabolite enhancement through elicitationin micropropagated plants of Ashwagandha (*Withania somnifera L. Dunal*)

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Abstract:

A methodology has been developed for enhancing the secondary metabolite (Withanolide-A and Withaferin-A) production through efficient shoot proliferation and regeneration of plants from axillary bud explants of *Withania somnifera* by studying the interactive effect of plant growth regulators and carbohydrates as an elicitor for stress induction . Explants cultured on Murashige and Skoog's (MS) medium supplemented with combination of 6-benzylaminopurine (BAP) and Kinetin (KN) in concentration of 0.5 mg/l and 1.0 mg/l. respectively, sprouted with an average of 86.67% shoots, proliferated on 3/4 MS medium supplemented with BAP(0.1 mg/l) and KN(0.05 mg/l) with an average of 8 shoots per explant. Excised shoots from this culture were rooted on 1/2 MS medium with 2.0 mg/L IBA and supplemented with additional carbohydrate source (4% sucrose + 2% mannitol). The complete plantlets thus obtained were successfully transferred to soil. The roots of micropropagated plants showed higher accumulation of Withanolide-A and Withaferin-A which was 0.047% w/w and 0.0401% w/w respectively.

Key Words: Clonal propagation, Axillary bud, Ashwagandha, Withania somnifera

Introduction

Withania somnifera (L.) Dunal (Solanaceae) has been a part of traditional Indian System of Medicine (ISM) since ages. In Ayurveda, roots of *Withania somnifera* are described as *"Tikta, kashaya rasam ushna veeryam, katu vipakam, kapha vata haram"* i.e. it is aphrodisiac, strength giving, complexion improving and is good in treatment of white leprosy, pruritis and fatigue (Nadkarni, 1954). It has been proved scientifically that it acts as anti-tumor, radiosensitizer, anti-stressor, immunomodulatory, anti-inflammatory and anti-bacterial effects (Umadevi, *et al.*, 1996; Ziauddin, *et al.*, 1996; Archana, *et al.*, 1999; Ali, *et al.*, 2001; Rasool, *et al.*, 2006). Being used for its roots, this plant has been a victim of unsustainable over-exploitation. The rich resource is decreasing at an alarming rate as a result of over- exploitation and unsustainable methods.

In *Withania somnifera*, the need for micropropagation is for various reasons explicitly, poor viability and death of young seedlings under natural environmental conditions, the long gestation period (4-6 years) between planting and harvesting without knowing the quality with respect to active principle content and thirdly the plant to plant variation in alkaloid and withanolide yield and quality which differs from source to source. Agronomically, the plants are propagated through seeds, since the plants do not have the natural ability for vegetative propagation therefore the variability generated by sexual recombination and agro-climatic

conditions is very high. Secondary metabolite production in entire plants depends on both endogenous (Hashimoto *et al.*, 1991) and exogenous factors (Boitel-Conti *et al.*, 2000). Several medicinal plants species react against the modifications of their environment by producing secondary metabolites. These compounds protect the plant against environmental stresses. According to Verpoorte (1999), "the secondary metabolite production in plants is a form of differentiation and an obvious correlation exists between the cellular differentiation and the secondary metabolism". Koroch *et al.* (1997) reported that preconditioning at different sucrose concentration prior to acclimatization had no effect on plant establishment, but influenced the plant quality by enhancing the secondary metabolite production. For example, increasing the carbohydrate concentration to 6% (3% sucrose plus 3% mannitol) and adding 25µM abscisic acid significantly increased the accumulation of monoterpenes in axillary cultures of *Lavandula latifolia*. And hence herein stress factor was used to enhance the production of Withanolides in *Withania somnifera*.

Material and Methods

Seeds of *Withania somnifera* were collected, germinated and grown (Sen and Sharma, 1991) in the fields for the screening of the plants with highest withanoidal content through HPLC, which were then used as the source of explant material for micropropagation. After the period of six months, samples were collected by uprooting them and then taken for performing HPLC (Awasthi *et al.*, 2007), to evaluate the maximum Withanolide-A and Withaferin-A contents. Axillary buds were collected from the screened plants with maximum withanoidal content. Twenty to twenty five centimeter (10-15 cm) long branches were collected from the plants. The leaves were excised and the branches cut into segments of 1.5 cm. comprising of single node. Axillary buds were first washed simply with tap water so as to remove dust and then with weak HISPARK (neutral and bio-degradable soap solution) and after which rinsed with distilled water for two to three times, after this explants were treated with 70% ethyl alcohol for a very brief period of 10 seconds followed by dipping in 2% Mercuric chloride just 4-5 times. Finally explants were washed with double distilled sterile water thoroughly. All the axillary bud explants of *Withania somnifera* were inoculated in baby food jars and culture tubes containing full strength MS medium (Murashige and Skoog, 1962) supplemented with 2% sucrose, 0.8% Agar agar as gelling agent and with different concentration and combination of plant growth regulators independently for shoot induction. The cultures were incubated under 16/8 hour photoperiod provided by cool white fluorescent tubes of light intensity 38 $mE.m^{-2}s^{-1}$ at 25°C ± 2°C. The explants were inoculated on the MS basal media with MS vitamins, cytokinins like BAP, KN or 2iP (1.0 mg/l) alone or in combination of two with 1.0 mg/l of each, Sucrose (20 gm/l.) and gelled with agar (4 gm/l.). During the experiment, the axillary buds responded best in the MS basal media with combination of BAP with KN. To optimize the concentrations of BAP and KN, for sprouting, the buds were inoculated on MS basal media supplemented with cytokinins BAP or KN (0.1 to 3.0 mg/l.) alone or in combination and Sucrose (20 gm/l.) and gelled with agar (4 gm/l.). In addition, Auxins like IAA or NAA (0.05-0.1 mg/l.) were added to test for their effects on the number of sprouts per explant. After three weeks, the explants showing response were sub-cultured onto shoot proliferation medium. Number of experiments were carried out to maximize the production of shoots from one axillary bud. This included, low concentrations of cytokinins (BAP and KN; 0.05-0.5 mg/l.) as compared to induction medium. And use off dilutions of MS nutrients with two different combinations of Cytokinins [Medium-A: BAP (0.2 mg/l) + KN (0.2 mg/l) and Medium-B: BAP (0.1 mg/l) + KN (0.05 mg/l)] while keeping organic supplements at full strength. *In vitro* rooting experiments were carried out using shoots of length more than 2.5 cm. in length. Other carbohydrate sources like mannitol (20 gm/l.), glucose (20 gm/l.), maltose (20 gm/l.), sorbitol (20 gm/l.) either alone or in combination were added in the rooting medium to induce the stress due to osmotic pressure to increase the secondary metabolite production. In rooting experiments, two types of media were used in one experiment hormone free MS medium gelled with Agar was used. And in other, MS 1/2 strength supplemented with IAA (0.5, 1.0 and 2.0 mg/l.) was used. The shoots were incubated in dark for 48 hours and then transferred to hormone free MS 1/2 strength in

light conditions with 16 hour photoperiod. The rooted plants were transferred for acclimatization.

Batch of 500 plantlets of micropropagated and non-micropeopagated (i.e. seed raised) were regenerated for comparative study and then grown in the fields. Frequency for the collection of root samples was three months. Among the plants roots of 20-30 plants were collected, washed and dried in the shade and were stored in the airtight glass reagent jars. Out of each collected samples 250 grams of the roots of *Withania somnifera* was taken for the determination of Withanolide-A and Withaferin-A through High Performance Liquid Chromatography (HPLC). HPLC (Perkin Elmer) was performed using RP-18 column with particle size of 5µm (Purospher). Accurately weighed quantities of dried 50% alcoholic extracts were dissolved in known volume of HPLC grade methanol: water (1:1). The mobile phase consisted of methanol: water (60:40) delivered at a flow rate of 1ml/min. The samples were analyzed at 25°C to provide efficiency to the peaks. The UV chromatograms were recorded at 215 nm (Awasthi, et. al., 2007).

Results and Discussion

The buds of newly sprouted branches which were nearer to the apical bud were more responsive and showed 83% sprouting within 7 days of culturing. While after about tenth axillary bud, hardening of stem portion was observed and such buds did not showed any response in cultures.Of the cytokinins tested individually for sprouting, BAP alone showed maximum percentage (76.67%) of cultures with multiple of shoots (2-3 shoots per culture), followed by KN (56.67%), while 2iP alone showed no effect on sprouting. However, combination of BAP and KN was found to be best with induction of 3-5 shoots per explant with about frequency of 73.33% of multiple shoot sprouting. While 2iP in combination either with BAP and KN showed negligible effect of 13-17% sprouting (Table-1).

Table-1 Showing effect of Cytokinin on sprouting of axillary buds of *W. somnifera*

Medium	Cytokinin (1mg/l)	Cultures with multiple shoots (in %)	Number of shoots per culture ± S.D.
MS	BAP	76.67	2-3 ± 0.76
MS	KN	56.67	2-3 ± 0.75
MS	2iP	Nil	Nil
MS	BAP + KN	73.33	2-5 ± 1.45
MS	BAP + 2iP	16.67	1-2 ± 0.37
MS	KN + 2iP	13.33	1-2 ± 0.33

Number of cultures studied = 10 per replicate; Number of replicates = 3.

Since in the preliminary experiments, BAP and KN showed synergistic effect on sprouting of buds, further experiments were carried out to evaluate the optimum concentration of both the cytokinins. Effects of Cytokinins on sprouting after three weeks of incubation on optimized media were shown in Table-2. The explants were incubated in MS basal medium supplemented with MS vitamins and growth regulators BAP and KN either alone or in different combination of both in the range of (0.1 – 3.0 mg/l). It was observed that the combination of BAP (0.5 mg/l) and KN (1.0 mg/l) produced best results in percentage sprouting (86.67%) and the number of sprouts per explant was three (Fig-1). Higher concentration of cytokinins (1.5, 2.0 and 3.0 mg/l) favoured only callusing of buds from the explant base.

Table-2 Showing optimization of media for sprouting of axillary buds of *W. somnifera*

Medium	Cytokinin (mg/l)		Cultures showing sprouting (in %)	Sprouts per explant
	BAP	KN		
MS	0.1	–	16.67	1
MS	0.2	–	26.67	1
MS	0.3	–	53.33	1
MS	0.5	–	73.33	1
MS	1.0	–	40.00	1
MS	1.5	–	30.00	1
MS	2.0	–	23.33	1
MS	3.0	–	10.00	1
MS	–	0.1	26.67	1
MS	–	0.2	30.00	1
MS	–	0.3	33.33	1
MS	–	0.5	50.00	1
MS	–	1.0	73.33	1
MS	–	1.5	33.33	1
MS	–	2.0	16.67	1
MS	–	3.0	13.33	1
MS	0.5	0.5	53.33	2
MS	0.5	1.0	86.67	3
MS	1.0	0.5	43.33	1
MS	1.0	1.0	20.00	1

Number of cultures studied = 10 per replicate; Number of replicates = 3.

Growth Experiments

It was found that in an attempt to increase the number of shoots per explant, initially sprouted buds were transferred to lower concentrations of BAP and KN (0.05 – 0.5 mg/l) in various combinations. It was observed that low concentration was not effective in increasing the number of shoots per explant. However, rapid elongation was observed with lower cytokinin (BAP + KN: 0.1 + 0.05 mg/l) concentration(Table-3).

Table-3 Showing effect of lower concentration of Cytokinins on growth of axillary buds of *W. somnifera*

Medium	Cytokinin (mg/l)		Average length (cm)
	BAP	KN	
MS	0.05	0.05	0.8
MS	0.05	0.1	1.6
MS	0.1	0.05	3.9
MS	0.1	0.1	2.2
MS	0.2	0.2	3.0
MS	0.2	0.5	1.2
MS	0.5	0.5	0.8

Effect of concentration of basal media

Combinations of Medium (B) showed good results as compared to combinations of Medium (A). In Medium (B) number of shoots per explant was 5-8 with length of 2.3-4.0 cm. (Fig-2), while that of Medium (A) showed 5 shoots per explant only on 3/4 MS dilution with average length of 3.5 cm. Dilutions of MS 3/4 concentration was more effective than that of 1/2 concentration, irrespective of cytokinin concentration.

Medium (A)	Strength	Sucrose (in %)	Cytokinin	Average Number	Average length cm)
MS	Full	2	BAP(0.2 mg/l)+KN(0.2 mg/l)	2	2.7
MS	3/4	2	BAP(0.2 mg/l)+KN(0.2 mg/l)	5	3.0
MS	1/2	2	BAP(0.2 mg/l)+KN(0.2 mg/l)	1	1.2
MS	1/4	2	BAP(0.2 mg/l)+KN(0.2 mg/l)	1	1.0

Medium (B)	Strength	Sucrose (in %)	Cytokinin	Average Number	Average length (cm)
MS	Full	2	BAP(0.1 mg/l)+KN(0.05 mg/l)	5	3.5
MS	3/4	2	BAP(0.1 mg/l)+KN(0.05 mg/l)	8	4.0
MS	1/2	2	BAP(0.1 mg/l)+KN(0.05 mg/l)	6	2.3
MS	1/4	2	BAP(0.1 mg/l)+KN(0.05 mg/l)	3	1.6

Rooting

In vitro and *Ex vitro* both type of rooting experiments were carried out using shoots of length more than 2.5 cm. in length. Stress as a elicitor was used to enhance the secondary metabolite production. In case of *in vitro* rooting experiments, Carbohydrate sources like mannitol (20 gm/l.), glucose (20 gm/l.), maltose (20 gm/l.), sorbitol (20 gm/l.) either alone or in combination were added additionally to induce the stress due to osmotic pressure to increase the secondary metabolite production. While in case of *ex vitro* rooting experiments transfer shock as stress factor was to enhance the secondary metabolite production.

(A) In hormone free semi-solid MS medium supplemented with additional Carbohydrate source (4% Sucrose + 2% Mannitol) and gelled with Agar, 57% rooting was observed after 15 days where 2-4 thin long roots developed.

(B) While in MS 1/2 strength liquid + IAA (0.5, 1.0, 2.0 mg/l) supplemented with additional Carbohydrate source (4% Sucrose + 2%

Mannitol) and gelled with Agar, rooting was observed within 8 days of incubation on medium supplemented with IAA 2.0 mg/l, 84% of rooting was observed with one or two strong roots(Fig-3). With IAA 1.0% only 46% rooting was observed, while IAA 0.5 mg/l did not show any response. These plants were transferred to soil showed 94% survival rate.

Acclimatization

Plants with well formed roots were carefully removed from the culture vessels and washed by gently shaking in a beaker with double distilled water to remove the gelling agent and medium sticking to the surface. The plantlets were washed with gentle flow of distilled water. Rooted plantlets were transferred to pre-autoclaved clay pots filled with soil : 'Soilritemix' ("KEL Perlite", India) in 1:1 proportion. These were kept in the mist chambers to prevent the rapid loss of humidity and thereby helping them to regulate opening and closing of stomata. They were kept in the hardening room at 27 $\pm$ 2°C for two weeks. The hardened plants were sprayed with a sterile solution of major salts of MS medium at 1/10 strength, twice a week, for a period of two weeks. Such hardened plants were transferred to a green house with ambient temperature (28-30°C), diffuse sunlight and 70% humidity maintained by overhead sprinklers, where the survival was 100%. Acclimatized plants were shifted to field conditions thereafter.

Comparative HPLC analysis of roots of micropropagated and non-micropropagated (seed grown) *Withania somnifers* plants

During the set of experiments with roots of micropropagated and non-micropropagated raised plants of *W. somnifera*, it was observed that Withanolide-A and Withaferin-A was found to be maximum in the roots of micropropagated plants which was 0.047% w/w and 0.0401% w/w respectively, while in the roots of seed raised plants the Withanolide-A and Withaferin-A contents were 0.0379% w/w and 0.0386% w/w respectively.

Huge quantity of plants with known medicinal properties are used by large number of pharmaceutical industries. Secondary metabolites or bioactive principles occurring in intact plants are employed either directly or indirectly. The continuous supply of these secondary metabolites from natural resource is problematic. Over few decades many strategies like media manipulation, phyto-hormone regulation, precursor feeding, plant cell immobilization, biotransformation and bioconversion, hairy root cultures and genetically modified cells etc. have been tried but failed to synthesize the desired products in appreciable quantity and at competitive economic value. This was due to poor understanding of secondary metabolic regulation in plant cell cultures. "Elicitation" of plant cell culture showed favorable results in production of secondary metabolites in intact plants during *in vitro* regeneration. Though, elicitation enhances secondary metabolism in plants or plant cells *in vitro* but the exact mechanism of elicitation is not exactly understood. This provides an opportunity for intensive research in the field of biosciences for exploitation of plant cells for the production of secondary metabolites. From the results of present studies it can be concluded that higher amounts of Withanolide-A and Withaferin-A in roots of micropropagated plants may be due to the "Elicitation" which is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival, persistence and competitiveness.

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Fig.-1 Shoots of *W. somnifera* axillary bud explants sprouted on MS medium containing BAP (0.5 mg/l) and KN (1.0 mg/l.).

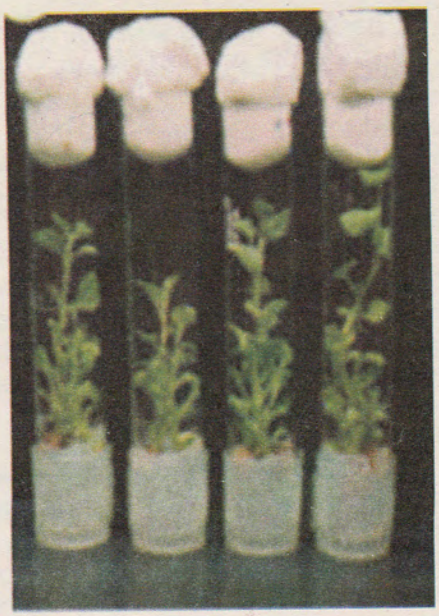


Fig-2 Multiple shoots of *W. somnifera* axillary bud explants developed on 3/4 MS medium containing BAP (0.1 mg/l) and KN (0.05 mg/l.).



Fig-3 Rooted plant of *W. somnifera* on 1/2 MS medium containing IAA (2.0 mg/l) and supplemented with Carbohydrate source (4% Sucrose + 2% Mannitol).