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Chapter - 1

Plant Protection using Cold Frame

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Abstract

A cold frame is an enclosed structure that is used primarily for the protection of plants and seedlings. The four walls are generally made of glass or wood with a removable glass or wooden top. Heat can be used in a cold frame, and that source of heat can come from a bottle of warmed water to an actual heater. In agriculture and gardening, a cold frame is a transparent-roofed enclosure, built low to the ground, used to protect plants from adverse weather, primarily excessive cold or wet. The transparent top admits sunlight and prevents heat escape via convection that would otherwise occur, particularly at night. Essentially, a cold frame functions as a miniature cold frame to extend the growing season. Cold frames provide an easy and economical way to establish greater control over your growing environment, and are used to increase crop yields and extend growing seasons. They are ideal for starting seedlings or transitioning (hardening off) greenhouse plants prior to outdoor planting. Cold frames offer a simple way to increase your crop yield and extend your growing season. By protecting plants from the elements and providing insulation, the ground takes much longer to freeze inside the cold frame and frost damage is reduced for up to a month on each end of your growing season.

Keywords: agriculture, gardening, cold frame and environments

1. Introduction

Historically, cold frames were built to be used in addition to a heated cold frame. The name itself exemplifies the distinction between the warm cold frame and the unheated cold frame. They were frequently built as part of the cold frame's foundation brickwork along the southern wall (in northern latitudes). This allowed seeds to be germinated in the cold frame and then easily moved to the attached cold frame to be "hardened- off" before final planting outside. Cold frames are similar to some enclosed hotbeds, also called hotboxes. The difference is in the amount of heat generated inside [1]. This is

parallel to the way that some cold frames are called "hothouses" to emphasize their higher temperature, achieved either by the solar effects alone or by auxiliary heating via a heater or HVAC system of some kind.

In Figure 1, cold frames are found in home gardens and in vegetable farming. They create microclimates that provide several degrees of air and soil temperature insulation, and shelter from wind. In cold-winter regions, these characteristics allow plants to be started earlier in the spring, and to survive longer into the fall and winter.

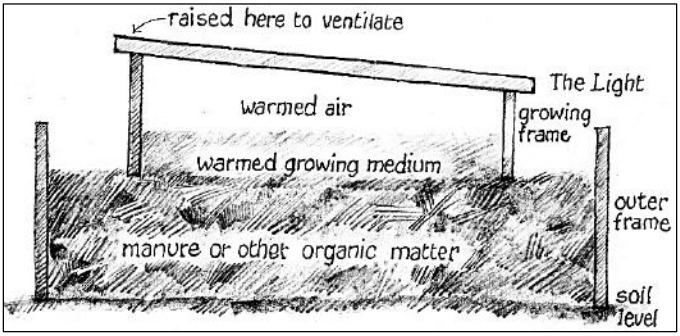

Fig 1: Cold frame

1.1 History of a cold frame

The idea of growing plants in environmentally controlled areas has existed since Roman times. The Roman emperor Tiberius ate a cucumber- like vegetable daily. The Roman gardeners used artificial methods (similar to the cold frame system) of growing to have it available for his table every day of the year. Cucumbers were planted in wheeled carts which were put in the sun daily, then taken inside to keep them warm at night [2-3]. The cucumbers were stored under frames or in cucumber houses glazed with either oiled cloth known as specularia or with sheets of selenite (A.K.A. lapis specularis), according to the description by Pliny the Elder.

Cold frames in which the temperature could be manually manipulated first appeared in 15th century Korea. The 15th century treatise, the Sanga Yorok, contains descriptions of cold frames designed to regulate the temperature and humidity requirements of plants and crops. One of the earliest records of the Annals of the Joseon Dynasty in 1438 confirms growing mandarin orange trees in a traditional Korean cold frame during the winter and installing an ondol system to provide heat.

The concept of cold frames also appeared in the Netherlands and then England in the 17th century, along with the plants. Some of these early

attempts required enormous amounts of work to close up at night or to winterize. There were serious problems with providing adequate and balanced heat in these early cold frames. Today, the Netherlands has many of the largest cold frames in the world, some of them so vast that they are able to produce millions of vegetables every year.

The French botanist Charles Lucien Bonaparte is often credited with building the first practical modern cold frame in Leiden, Holland, during the 1800s to grow medicinal tropical plants. Originally only on the estates of the rich, the growth of the science of botany caused cold frames to spread to the universities. The French called their first cold frames orangeries, since they were used to protect orange trees from freezing. As pineapples became popular, pineries, or pineapple pits, were built.

Experimentation with the design of cold frames continued during the 17th century in Europe, as technology produced better glass and construction techniques improved. The cold frame at the Palace of Versailles was an example of their size and elaborateness; it was more than 150 metres (490 ft) long, 13 metres (43 ft) wide, and 14 metres (46 ft) high.

The golden era of the cold frame was in England during the Victorian era, where the largest glasshouses yet conceived were constructed, as the wealthy upper class and aspiring botanists competed to build the most elaborate buildings. A good example of this trend is the pioneering Kew Gardens. Joseph Paxton, who had experimented with glass and iron in the creation of large cold frames as the head gardener at Chatsworth, in Derbyshire, working for the Duke of Devonshire, designed and built The Crystal Palace in London, (although the latter was constructed for both horticultural and non-horticultural exhibition). Other large cold frames built in the 19th century included the New York Crystal Palace, Munich's Glaspalast and the Royal Cold frames of Laeken (1874-1895) for King Leopold II of Belgium.

In Japan, the first cold frame was built in 1880 by Samuel Cocking, a British merchant who exported herbs. In the 20th century, the geodesic dome was added to the many types of cold frames. Notable examples are the Eden Project, in Cornwall, The Rodale Institute in Pennsylvania, the Cimarron at the Missouri Botanical Garden in St. Louis, Missouri, and Toyota Motor Manufacturing Kentucky.

Cold frame structures adapted in the 1960s when wider sheets of polyethylene film became widely available. Hoop houses were made by several companies and were also frequently made by the growers themselves. Constructed of aluminium extrusions, special galvanized steel tubing, or even

just lengths of steel or PVC water pipe, construction costs were greatly reduced. This resulted in many more cold frames being constructed on smaller farms and garden centres. Polyethylene film durability increased greatly when more effective UV-inhibitors were developed and added in the 1970s; these extended the usable life of the film from one or two years up to 3 and eventually 4 or more years.

Gutter-connected cold frames became more prevalent in the 1980s and 1990s. These cold frames have two or more bays connected by a common wall, or row of support posts. Heating inputs were reduced as the ratio of floor area to exterior wall area was increased substantially. Gutter- connected cold frames are now commonly used both in production and in situations where plants are grown and sold to the public as well. Gutter- connected cold frames are commonly covered with structured polycarbonate materials, or a double layer of polyethylene film with air blown between to provide increased heating efficiencies.

2. Construction and Design of a cold frame

2.1 Construction of a cold frame

Cold frame construction is a common home or farm building project, although kits and commercial systems are available [4]. A traditional plan makes use of old glass windows: a wooden frame is built, about one to two feet tall, and the window placed on top. The roof is often sloped towards the winter sun to capture more light, and to improve runoff of water, and hinged for easy access. Clear plastic, rigid or sheeting, can be used in place of glass. An electric heating cable, available for this purpose, can be placed in the soil to provide additional heat.

2.2 Design of cold frame

Fig 2: Design of Cold frame

In Figure 2, analysis of issues of near-infrared radiation in a cold frame with screens of a high coefficient of reflection concluded that installation of such screens reduced heat demand by about 8%, and application of dyes to transparent surfaces was suggested. Composite less- reflective glass, or less effective but cheaper anti-reflective coated simple glass, also produced savings.

Ventilation

Ventilation is one of the most important components in a successful cold frame, especially in hot and humid tropical climate condition. If there is no proper ventilation, cold frames and their growing plants can become prone to problems. The main purposes of ventilation are to regulate the temperature, humidity and vapor pressure deficit to the optimal level, and to ensure movement of air and thus prevent build-up of plant pathogens (such as Botrytis cinereal) that prefer still air conditions. Ventilation also ensures a supply of fresh air for photosynthesis and plant respiration and may enable important pollinators to access the cold frame crop. Ventilation can be achieved via use of vents - often controlled automatically via a computer - and recirculation fans.

Heating

Heating or electricity is one of the most considerable costs in the operation of cold frames across the globe, especially in colder climates. The main problem with heating a cold frame as opposed to a building that has solid opaque walls is the amount of heat lost through the cold frame covering. Since the coverings need to allow light to filter into the structure, they conversely cannot insulate very well. With traditional plastic cold frame coverings having an R-value of around 2, a great amount of money is therefore spent to continually replace the heat lost. Most cold frames, when supplemental heat is needed use natural gas or electric furnaces.

Passive heating methods exist which seek heat using low energy input. Solar energy can be captured from periods of relative abundance (day time/summer) and released to boost the temperature during cooler periods (night time/winter). Waste heat from livestock can also be used to heat cold frames, e.g., placing a chicken coop inside a cold frame recovers the heat generated by the chickens, which would otherwise be wasted. Electronic controllers are often used to monitor the temperature and adjusts the furnace operation to the conditions. This can be as simple as a basic thermostat but can be more complicated in larger cold frame operations.

Carbon dioxide enrichment

The possibility of using carbon dioxide enrichment in cold frame cultivation to enhance plant growth has been known for nearly 100 years. After the development of equipment for the controlled serial enrichment of carbon dioxide, the technique was established on a broad scale in the Netherlands. Secondary metabolites, e.g., cardiac glycosides in Digitalis lanata, are produced in higher amounts by cold frame cultivation at enhanced temperature and at enhanced carbon dioxide concentration. Commercial cold frames are now frequently located near appropriate industrial facilities for mutual benefit. For example, Corner ways Nursery in the UK is strategically placed near a major sugar refinery, consuming both waste heat and CO2 from the refinery which would otherwise be vented to atmosphere. The refinery reduces its carbon emissions, whilst the nursery enjoys boosted tomato yields and does not need to provide its own cold frame heating.

Enrichment only becomes effective where, by Liebig's law, carbon dioxide has become the limiting factor. In a controlled cold frame, irrigation may be trivial, and soils may be fertile by default. In less- controlled gardens and open fields, rising CO2 levels only increase primary production to the point of soil depletion (assuming no droughts, flooding, or both), as demonstrated prima facie by CO2 levels continuing to rise. In addition, laboratory experiments, free air carbon enrichment (FACE) test plots, and field measurements provide replicability.

3. Different methods to build a cold frame

3.1 How to build a cold frame

Summer is winding down and the evenings are getting cooler. Trees are beginning to show colour. You loved your summer vegetables. Why not think of extending your growing season with a cold frame. This way you can grow vegetables well into the winter or even all winter.

Here we are going to show you how to build a cold frame using three methods that will help you do just that [5-6].

Things to Consider Before Building A Cold Frame:

- You need to find a site that is fairly protected such as against the house or fence
- The site should be south facing so that it takes full advantage of the Winter sun
- You should check the winter sun patterns so that you will know your site gets sufficient sun

- Decide how large a bed you want. This can even be a raised bed that you have already built
- If you don't already have a raised bed in this location dig out the space you have selected an amendment as you would for any growing bed
- Now take your cold frame and set it on the site

3.2 Three cold frame methods-the hoop method

In Figure 3, is the easiest method but will not be suitable for some of the colder areas of the country. However, it has been used successfully in areas where the temperatures get down to about 20 to 25° (or you can try to push your luck).

To make this cold frame you will need:

- 1/2 or 3/4-inch PVC pipe cut to 6ft lengths
- You will also need 1-foot pieces of PVC pipe at that is one size larger (2 pieces for each of the 6 ft-length (Make sure the narrower PVC pipes can fit into the wider one)
- Large clamps
- 6 mil plastic sheeting (enough to cover the cold frame)

Fig 3: Hoop Method

To make this cold frame you will need:

1/2 or 3/4-inch PVC pipe cut to 6ft lengths You will also need 1-foot pieces of PVC pipe at that is one size larger (2 pieces for each of the 6 ft-length (Make sure the narrower PVC pipes can fit into the wider one). Large clamps 6 mil plastic sheeting (enough to cover the cold frame). Putting this Cold Frame together starting at one end of the raised bed insert 1 ft pieces of PVC pipe into the ground on each side of raised bed (inside the bed). Do this

every 18 inches 2 ft until you get to the other end of the raised bed. Insert one end of the 6 ft sections of PVC pipe into the 1 ft pieces of PVC you just inserted into the ground. Insert the other end into the short PVC pipe on the other side of the raised bed so that it forms an arch. Do this for each 6ft section of PVC pipe. Cover the framework you have just made with the 6mil plastic. Anchor the plastic in place with the large clamps. Lap the ends of the plastic together and secure with a clamp.

In Figure 4, have a completely covered cold frame that you can access by temporarily removing the clamps and pulling back the plastic. You will also want to occasionally leave the ends of the cold frame open so that your plants don't get too hot.

Fig 4: Design of a cold frame theory of operation

The warmer temperature in a cold frame occur because incident solar radiation passes through the transparent roof and walls and is absorbed by the floor, earth, and contents, which become warmer. As the structure is not open to the atmosphere, the warmed air cannot escape via convection, so the temperature inside the cold frame rises. This differs from the earth-oriented theory known as the "cold frame effect". Quantitative studies suggest that the effect of infrared radiative cooling is not negligibly small and may have economic implications in a.

3.3 Applications

Cold frames allow for greater control over the growing environment of plants. Depending upon the technical specification of a cold frame, key factors which may be controlled include temperature, levels of light and shade, irrigation, fertilizer application, and atmospheric humidity [7].

Cold frames may be used to overcome shortcomings in the growing qualities of a piece of land, such as a short growing season or poor light levels,

and they can thereby improve food production in marginal environments. Cold frames in hot, dry climates used specifically to provide shade are sometimes called "shade houses".

As they may enable certain crops to be grown throughout the year, cold frames are increasingly important in the food supply of high-latitude countries. One of the largest complexes in the world is in Almería, Andalucía, Spain, where cold frames cover almost 200 km2 (49,000 acres).

Cold frames are often used for growing flowers, vegetables, fruits, and transplants. Special cold frame varieties of certain crops, such as tomatoes, are generally used for commercial production. Many vegetables and flowers can be grown in cold frames in late winter and early spring, and then transplanted outside as the weather warms. Bumblebees are the pollinators of choice for most pollination, although other types of bees have been used, as well as artificial pollination. Hydroponics can be used to make the most use of the interior space.

The relatively closed environment of a cold frame has its own unique management requirements, compared with outdoor production. Pests and diseases, and extremes of heat and humidity, have to be controlled, and irrigation is necessary to provide water. Most cold frames use sprinklers or drip lines. Significant inputs of heat and light may be required, particularly with winter production of warm-weather vegetables.

Cold frames also have applications outside of the agriculture industry. Glass Point Solar, located in Fremont, California, encloses solar fields in cold frames to produce steam for solar-enhanced oil recovery. For example, in November 2017 Glass Point announced that it is developing a solar enhanced oil recovery facility near Bakersfield, CA that uses cold frames to enclose its parabolic troughs.

An "alpine house" is a specialized cold frame used for growing alpine plants. The purpose of an alpine house is to mimic the conditions in which alpine plants grow; particularly to provide protection from wet conditions in winter. Alpine houses are often unheated, since the plants grown there are hardy, or require at most protection from hard frost in the winter. They are designed to have excellent ventilation.

4. Conclusion

Cold frames protect plants from strong winds and retain heat. Gardeners use cold frames to extend their gardening season-both in the autumn to protect plants for a few more weeks and in the spring to get a jumpstart on sowing

seeds. Cold frames are also used to “harden off” seedlings that were started indoors. Cold frames allow growers to start planting earlier and harvest longer, while producing better quality plants. They protect your plants from wind, heavy rain, irrigation and pest problems, reducing stress and improving overall plant health. Cold frames provide the economical and functional benefits of a growing shelter, without the need for investing in a fully equipped greenhouse.

The biggest difference between a cold frame and a greenhouse is that a cold frame does typically use a heat source and might only stand a few feet tall; whereas a greenhouse is a tall structure that has heating and ventilation systems for a year-round controllable climate.

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Chapter - 2
Polymorphism of MSTN Gene (T2230C and A2232G) and Its Relationship with Some Physiological Traits and Growth of Common Year 2108 Carp (*Cyprinus carpio* L)

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Chapter - 2

Polymorphism of MSTN Gene (T2230C and A2232G) and Its Relationship with Some Physiological Traits and Growth of Common Carp (*Cyprinus carpio* L) Year 2108

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Abstract

Polymorphism of the Myostatin genetic is an important role in the process of selection and genetic improvement in aquatic organisms, especially fish, and knowledge of the allele repetition of the different alleles of the gene and the extent of its effect on this gene expression and that the changes in the locations T2230C and A2232G had a significant impact in increasing the rate of total weight and daily weight increase, it exceeded the rate of relative and qualitative growth. The mutant genotypes increase in the percentage of protein, a decrease in the percentage of fat in the components of the body, an increase in the white blood cells, the size of plasma cells, hemoglobin and blood protein. The study concluded that the possibility of adopting polymorphism in the Myostatin gene for the site A2232G as well the location of the T2230C mutation in setting, genetic improvement methods in fish to achieve the largest economic return from breeding projects by electing and striking the genotypes that achieved the best performance, and the application of the study to a larger sample of fish in different locations would give more accurate results in the application of (Gene-Assisted Selection-GAS methods).

Keywords: myostatin gene, genotype, growth traits, body composition, blood picture, common carp

Introduction

Common carp fish (*Cyprinus carpio* L.) has a long history of aquaculture in earthen ponds, cages, canals and other breeding systems, which has led to the emergence of a large many of local breeds in several countries of the world and is well adapted to environmental and is considered a fish Carp is an economically important resource for its high nutritional value. The process of muscle growth in fish is subject to some

factors, the most important of which are the genes responsible for the expression process and building muscle tissue through coding to build the proteins involved in muscle formation (Kuradomi *et al*., 2011). The myostatin protein that genetic code the myostatin gene is one of the main factors responsible for regulating skeletal muscle growth in fish at several different levels including epigenetic genetics during fetal development stages (Elliott *et al*., 2012), as well its role in regulating growth muscle. Structure and building muscle tissue and increasing the number of muscle fibers in fish, the myostatin gene is involved in regulating the expression of many tissues as it a role in the growth of the brain, eyes, gill strings, spleen, ovaries and intestine, and its lowest expression is in the male reproductive system, especially the testes (Rodgers *et al*., 2001).

Associated the myostatin gene with many related to the overall animal performance such as feed conversion factor, chest muscle depth, carcass weight without viscera, blood oxygen level, and volumetric standard antibody in poultry (Chandan *et al*., 2014), and in the same context the body's activity in fish in Muscles because they are the largest components of the body and represent 50-70% of the total body mass. Therefore, controlling the genes that control muscle mass and their growth in fish such as the myostatin gene may result in improvement in growth rates, and may contribute to electing the genetic structure that is traits by high productivity, details physiological traits (Elkatatny, 2016).

Therefore the study of the relationship of the genetics of the gene with the productive and growth, traits and in some physiological traits of a sample of common carp is considered one of the tasks for the person in which distinct individuals are elected, as well as the study of the different genotypes and their distribution ratios for the myostatin gene in the studied samples and the allele frequency in them.

Myostatin

Called the Growth Differentiation Factor (GDF-8) and writes an abbreviation MSTN, and the size of the myostatin gene is 6.4 kilobytes (Cheng and Gang, 2003). The gene is located on chromosome 11 in common carp and consists of three exons. There are two introns (Yanhong *et al*., 2012) and there are two versions of the Myostatin MSTN-1 gene and MSTN-2 in some fish species such as solar Salmo, Zebrafish (*Brachydanio rerio*) and sea bream (*Sparus aurata*) (Helterline *et al*., 2007). Myostatin gene works primarily on the regulation and expression of skeletal muscles through its important role in growth, and despite maintaining its function in

the negative muscle regulation, the myostatin gene works to regulate the cloning process from the response factors in the DNA that play the role of Enhancer-BOX, a phrase About sequences of DNA within the promoter of the gene can correlate with transcription factors to regulate the RNA transcription process, this a role in the transcription process and translation process (Li *et al*., 2012).

Effect of mutation of the myostatin gene on fish growth regulation

The myostatin gene is a negative regulator of the growth and development of skeletal muscles and growth in general and works to inhibit the reproduction of muscle aromatics (Myoblast) and its differentiation, as the myostatin gene works to control the cell cycle by its effect in inhibiting the cell cycle that includes a group of sequenced events accomplished in The cells of the eukaryotic organisms that lead to the formation of two similar cells of the parent cell are called structural cells and are usually divided into two basic stages, the growth phase (G1), in which the contents of the cell are multiplied and prepared to split the second phase (S-phase), which includes two overlapping processes represented by the division of the nucleus (Karyokinesis) is followed by the division of the cytokinesis (Cytokinesis), and there is a group of genes that work through their expression to regulate the cell cycle and its role in the manufacture of proteins and have an effect at every stage of the cell cycle that is symbolized by Cell division kinase-Cdk (Huang *et al*., 2007). Liu *et al*. (2012) indicated that the myostatin gene has multiple shapes related to some growth characteristics of Aristichthys nobilis, and that the mutation that occurred in the regulatory region g. 2770C> A correlates significantly with body weight and overall length. The standard length has led to an increase in growth and improved performance for these fish, as Tang *et al.* (2010) demonstrated by his study of Nile tilapia (*Oreochromis niloticus*).

The polymorphism of SNP of the myostatin gene resulting from a mutation in the MSTN gene sequence of normal carp correlated with several traits such as weight, characteristics of total weight gain, daily weight increase, relative growth, and specific growth, since the mutation at site 2230T> C and location 2232A> G in normal carp fish, had a significant association ($P < 0.05$) with the mentioned characteristics (Al-Alnjawi and Al-Khshali, 2018).

Relationship of the myostatin gene in the formation of fish muscles

Muscle formation is one of the important factors for the success of commercial projects for agricultural animal husbandry, especially fish

because the muscles represent the largest components of the body and constitute 50-70% of the total body mass, so controlling genes that control muscle mass in fish such as the myostatin gene may result in improved rates growth, and may contribute to the election of genotypes with high productivity (Jiang *et al*., 2002).

Du *et al*. (2003) mentioned in his study on genetically modified Zebrafish fish, and after measuring the level of gene expression in the growth stages of these fish, that gene expression on days 7, 45 and 245 in both the Myogenic factor and the Myogenic Enhancer Factor-5) And the origin of the muscle (Myogenic) was similar in GM and non-GMO fish. When comparisons were made at the age of 3 and 5.5 months, GMOs showed a weight increase of 15-10% higher than non-GM fish. Zhong *et al*. (2016), when analyzing a cluster of common carp fish, were able to modify the genetic material of cells using an enzymatic compound called CRISPR-Cas9 that cuts the DNA by inserting the compound onto a nanoparticle of DNA strands that connect the compound to the nucleus, revealing a pattern The myostatin gene and the correlation of its genetic manifestations with the characteristics of body weight and height in the age stages of fish and comparison with wild common carp and then the detection of gene coordination with PCR technique.) A mutation in the gene was found that led to an increase in body weight and a decrease in SMAD pathways by 25% And increase in the number and size of muscle fibers and its organs the results showed a significant increase in muscle regulating factors ($P < 0.01$) when compared to wild types within the same type.

Results of the study of Al-Alnjawi and Al-Khshali (2018) showed the superiority of mutant genotypes in the first site T2230C and the second site A2232G in the ratio of total and daily weight gain and the rate of relative and specific growth compared to the hybrid and wild genotypes, and this is consistent with what, Pang *et al*. (2018) found. In their study of large-head carp fish, the analysis of DNA indicated the presence of several mutations in the myostatin gene that were closely associated with the characteristics of growth, as the mutant genotype in the overall weight increase ratio was significantly ($P < 0.01$) over the genetic and hybrid genotype. The polymorphism of a nucleotide an SNP, which is located within coding regions, is widely used in the development of genetic markers and genomes in common carp fish that have a relationship with the multiple genetic manifestations of the myostatin gene associated with growth traits for use in breeding and fish improvement programs (Sun *et al*., 2012), this was demonstrated by Khalil *et al*. (2017) in their study of Catfish (*Ictalurus*

punctatus) genetically modified fish for myostatin gene. Genetically modified fish gave a significantly higher total and daily weight gain (P <0.01) to unmodified fish and also gave an average increase in weight of modified larvae. Genetically 29.7% compared to untreated larvae (wild composition). The results of the current study agreed with Liu *et al*. (2012) in their study on large-head carp fish, like fish in which a change occurred in one of the nitrogenous bases showed a high weight gain (P <0.01) compared to the wild composition of the myostatin gene, myostatin gene is a major goal for researchers in the aquaculture sector because one of the main goals of this sector is to achieve the largest increase in weight in the lowest possible period with an increase in muscle mass.

It is noted from these results that the individuals carrying the mutant genotype (CC, GG) in the study of Al-Alnjawi and Al-Khshali (2018) outperformed most of the studied growth characteristics due to the important role that the myostatin gene role in increasing expression and muscle growth, through which it is possible to elect Individuals carrying this composition, as the traits of growth in fish are among the important economic characteristics that are selected based on them since the mutation occurring at the site T2230C and A2232G led to a change in the nucleotide sequence and may have inhibited the action of the gene and thus an increase in muscle and body growth in general In mutation-bearing fish this his Elkatatny *et al*. (2016) in their study on tilapia Nile, as individuals carrying the mutation achieved an increase in the rate of weight was significantly (P <0.01). Sun *et al*. (2012) that the multiple forms of the myostatin gene have a significant impact on the characteristic head length, body weight, and body height of common carp fish, as their study results showed two mutations in the myostatin gene sequence that were associated with the main growth characteristics and achieved the best performance of the studied traits.

Gene polymorphism

There is much evidence indicating the multiple manifestations of the myostatin gene through natural selection, several studies have been conducted on many poultry and aquatic organisms including fish (Baylan *et al*., 2015), cows and sheep (Boman *et al*., 2009) and in domestic birds (Zhang *et al*., 2011) and rabbits (Fontanesi *et al*., 2011) as well as pigs (Yu *et al*., 2007), as they confirmed the presence of multiple manifestations of the myostatin gene, and the reason is due to the different setting of alleles that maintain the multiplicity of the gene and there are several effective technologies for detecting multiple manifestations of analysis Genes in animals, in the myostatin gene it was adopted PCR technology included

analysis of special sequences for Oligonucleotide (Sayd, 2014), single-strand conformational polymorphism-SSCP (Liu and Cordes, 2004), RFLP technology using Southern stigma analysis (Balon, 1995) and genetic sequences or sequences Guo *et al*., 2011).

Genotypes of the myostatin gene

The study of Al-Alnjawi and Al-Khshali (2018) showed the results of sequencing for the myostatin gene and the presence of a change in a single nucleotide polymorphism-SNP) in the second intron, specifically at the location of 2230bp of the myostatin gene, as the base changed from T The genotypes were extracted by Geneious Software (version 10.1.3), as the fish sequences were compared with the wild genotype of the common carp found in the World Gene bank. Thoughtful of the myostatin gene (Figure 1) and the presence of one green curve indicates for TT-Wild genotype, there is no mutation in both stripes, and the presence of green and blue curves indicates the Heterozygous-TC genotype, that is, a mutation in one of the two bands. The presence of one blue curve and the base change below the curve indicate C mutant genotype (C). Both strips (CC) have changed. The results of sequencing also showed a mutation at the site of 2232bp, as the nitrogenous base A changed to A (G) (A2232G), and the results of the comparison between samples of experiment fish showed that two genotypes were obtained (Figure 2) and the presence of one yellow curve indicates the mutant genotype (GG) while the appearance of yellow and red curves indicates a hybrid genotype (AG). It is noted that there is a complete absence of wild genotype (AA) at this genetic site in the studied fish samples.

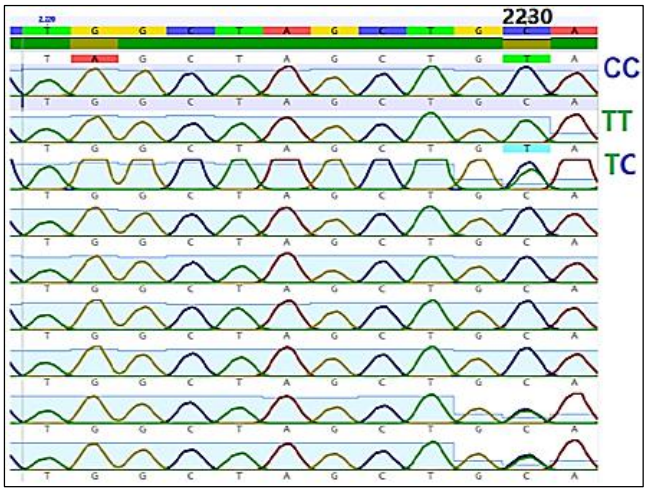

Fig 1: Genotypes in the first location of the myostatin gene

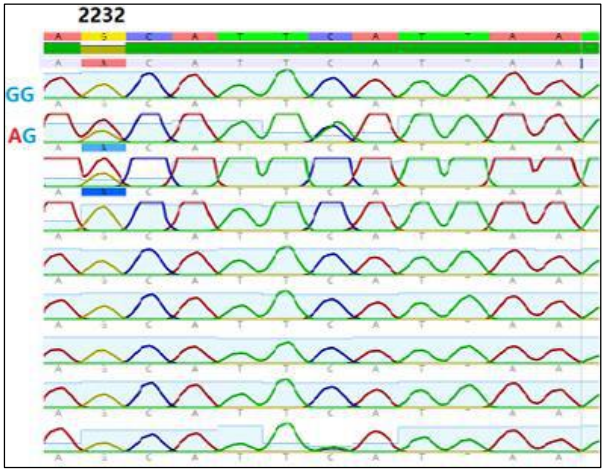

Fig 2: Genotypes in the second location of the myostatin gene

Relationship of genotypes and allele repetition to myostatin gene

Al-Alnjawi and Al-Khshali (2018) made it clear in a study on Common carp that there are two mutations in Intron 2 and the numbers and proportions of genetics distribution of fish have reached 5.88% for fish carrying the wild composition TT and 38.24% for fish Which bear the hybrid composition TC and 55.88% for fish that carry the mutant structure CC and the results showed highly significant superiority of fish with the CC structure and the lack of carrier of the genetic makeup TT, the law has been applied to calculate the night repetition according to the Hardy-Weinberg for equilibrium, as the frequency of the allele T 0.25% and C allele were 0.75%, while numbers and proportions of genotypes distribution were The thickness of the second site, as the percentage reached 17.65% for fish hybrid composition AG and 82.35% for fish with the mutant composition GG. The Hardy Weinberg base for equilibrium, as the frequency of alleles A reached 0.09% and allele G 0.91%, while in previous studies the distribution ratios of genotypes of Nile tilapia (*O. niloticus*) for AB and BB were 80.00 and 20.00%, respectively, and the nocturnal repetition of both A and B in that study were 0.4 and 0.6%, respectively (Elkatatny, 2016). In the same context, previous studies indicated the distribution ratios of the common carps for AA, AG and GG 20.37, 59.88 and 19.75%, respectively, and the nocturnal repetitions of A and G were 0.51 and 0.49%, respectively, (Yanhong *et al*., 2012). Another study on Atlantic salmon (*Salmo salar*) showed the presence of three influential genotypes resulting from the analysis of the MSTN-1b gene, represented by TT, TC and CC, with rates of

39.11, 27.49 and 33.40%, respectively, and the night frequency of T and C was 0.29 and 0.71%, respectively, Penaloza (2013).

Relationship of the myostatin gene with some productive traits

The myostatin gene is one of the most important genes that have an effect on growth and in some productive traits as it has a role in regulating the growth of skeletal muscles (Liu *et al*., 2012), and that the multiple forms of the myostatin gene have several effects on some important traits of different types of fish, especially commercial ones. Muscle mass and weight gain rate (Gahr and Weber, 2012). Yanhong *et al*. (2012) indicated two mutations in exon 3 using PCR-RFLP technology to detect the presence of multiple myostatin gene shapes that correlate with some productive characteristics of Common carp, the results showed three genotypes at the mutation site 371+749 A> G, which are AA, AG and GG, and the differences between these structures were highly significant (P <0.01), as the genetic makeup GG outperformed the rest of the structures in the body weight and condition factor (Condition Factor) The second mutation in the C1371 + 781T site showed three genotypes represented by TT, CT and CC, as the genotype CT significantly (P <0.01) was significantly superior to the rest of the combinations in the body weight and condition factor, whereas the total length did not differ. The standard length in any of the combinations of the two mutations, and when comparing the two mutations, the first mutation was significantly superior (P <0.05) over the mutation second. Results of a study of Liu *et al*. (2012) of a group of large-head carp fish on the myostatin gene showed that there are two mutations in Exon 3 and that there are three genotypes of this gene through the application of PCR-RFLP technology which is CC, CA and AA and it was clear by making comparisons in a weight characteristic Body, total length, and standard length between genotypes significantly outweighed the CC genotype (P <0.01) over the rest of the genotypes.

Cheng and Sun (2015) explained that the multiple forms of the myostatin gene have a significant effect on the characteristic of body length, total length, standard length, body weight, and body height when studying 190 finned radial fish (*C. alburnus*). The results show that there is heterogeneity in four locations and each the site of three genotypes is the site of the first heterogeneity c.6T> C the TT and CT and CC combinations. The second site c.152G> A was the genotypes GG, GA and AA, the third site c.155G> AGG, GA and AA as well as in the fourth heterogeneous site c. 162G>A After conducting the statistical analysis between the genotype, it was revealed that the genotype CT was significantly superior (P <0.01) over

the rest of the genotypes in the studied traits. Elkatatny (2016) indicated the role of the myostatin gene in regulating skeletal muscle growth and that the multiple forms of the gene had a significant effect on the body weight characteristic of Nile tilapia using PCR-RFLP technique as it produced the BsmlIntron1 and Exon2 cutting enzyme and the number of bases resulting from the cut was 607pb base pair, results showed that there are two genotypes, AB which has several bases bp507 base-pair lower than the original sequence with bp100 base pair per segment and genotype BB that contains some bases bp507 base-pair less than the original sequence bp507 base pair per segment in When the AA genotype was absent, showed the AB genotype was significantly ($P < 0.05$) on the BB genotype in the weight-gain trait.

Explain Al-Alnjawi and Al-Khshali (2018) through their study that there were significant differences ($P < 0.05$) in the final weights of common carp with different genotypes of the myostatin gene, as the highest final weight in the first site T2230C 217.84±5.27g/A fish when mutating the genotype CC, while the weight was 172.15±5.73g/fish in the hybrid genotype TC and 173.50±3.75g/fish in the wild genotype TT, as well as the characteristics of total weight gain, daily weight increase, relative growth, and specific growth of fish, showed differences. Significant ($P < 0.05$)), as the overall weight increase of fish reached 88.11g/fish in the genotype CC and 48.38 M/fish in the genotype TC and 53.50g/fish in the wild genotype TT, while the rate of daily growth reached the highest value in the genotype CC (1.04 g/fish/day) and the lowest value was in the genotype TC (0.57 g/fish/day, CC structure differed significantly $P < 0.05$) compared to the rest of the combinations, as the results showed that the relative growth was significantly affected by $P < 0.05$)) according to the different genotypes of the myostatin gene and reached 44.58, 39.09 and 67.91% for the TT, TC and CC genotypes respectively. Likewise, for specific growth, the results were significantly affected ($P < 0.05$) and the values were 0.190, 0.170 and 0.267% g/day, respectively, of the same genotypes.

While the results in the second site A2232G indicated that there is a significant difference ($P < 0.01$) in the average body weight of common carp fish with different genotypes of the myostatin gene, as the highest final weight of fish, reached 205.57±4.48 g/fish in the genotype mutant GG during the experiment While the final weight was 161.33±4.67g/fish in the hybrid genotype AG, the characteristics of total and daily weight increase and the relative and specific growth of fish also showed significant differences ($P < 0.01$), as the total weight increase of fish reached 76.75

g/fish in genotype. GG and 43.50g/fish in the genotype AG, while in the daily growth rate, it is The highest value in the genotype GG was 0.981g/day/fish and the lowest value was in the genotype AG 0.54 g/day/fish, and differed significantly (P <0.01) in favor of the genotype GG, as the results showed that the relative growth was significantly affected (P <0.01) (With different genotypes of the myostatin gene, the value was 59.57 and 39.91% for the two genotypes GG and AG sequentially, as well for specific growth, as the results were affected significantly (P <0.01).

The importance of the myostatin gene as one of the main genes associated with the characteristics of growth and candidate is reflected in the tract of breeding and genetic improvement programs. It has been observed that the state of polymorphism is associated with the characteristics of growth in fish and that the polymorphism of single nucleotide (SNP) that falls within the coding regions is exploited on Widely used in the development of genetic and genome markers (Tong and Sun, 2015), this was demonstrated by Sun *et al*. (2017) in *Ancherythroculter nigrocauda*, as the mutant genotype the overall and daily overweight ratio. The results of the current study were consistent with the results of Sawatari *et al*. (2010) on Japanese rice fish (*O. latipes*), as it was noted that mutant genotypes outperformed wild composition in terms of total weight gain and daily weight gain and agreed with the results of Liu *et al*. (2012) in their study of carp. It has a large head (*A. nobilis*), as the fish in which there was a change in the nitrogenous base showed a high weight increase compared to the land composition of the Myostatin gene, for this sector achieved the largest weight increase in the least possible time period with an increase in muscle mass (Galt *et al*. 2018), and in these cases, the polymorphisms in the Myostatin gene were revealed through the technology of polymerase chain reaction (PCR), where polymorphism is revealed Single nucleotide (SNP) only when there is a mutation at a specific site.

Polymorphism in the Myostatin gene were revealed in this study by identifying single nucleotides (SNPs) using Sequencing Direct, after the target piece of the gene was isolated by (PCR) and the sequence comparison of all fish and extraction of genotypes and differences were found in the composition of alleles In the second intron of the Myostatin gene, we note from these results the superiority of individuals carrying mutant genotypes (CC, GG), in the study of Al-Alnjawi and Al-Khshali (2018) in most of the studied growth traits due to the important role that the myostatin gene role in the increased expression and muscle growth, and these results Individuals carrying this composition can be elected, as the traits of growth in fish are

among the important economic characteristics on which the selection is made, and then the adoption of the genetic expression improving these traits seems to be beneficial the mutation in the location T2230C led to a change in the nucleotide sequence and may have inhibited the action of the gene and increase in muscle and body growth in general in fish carrying the mutation and this is shown Yanhong *et al*. (2012) found in their study on Common carp fish, as the individuals carrying the mutation achieved an increase in the average weight and gave the fish the best condition factor and the traits of total and standard body length. Cheng and Sun (2015) show that the multiple forms of the myostatin gene have a significant effect on the head length, body weight, and body height characteristics of (*Culter alburnus*) fish, the results of their study showed that there were five individual patterns based on four SNP sites where Four groups of H1H3 individual patterns used the best performance for the studied traits. In the same topic, Terova *et al*. (2006) found in their study of sea bream fish that a number of sequences of the myostatin gene were linked to the rate of growth and weight gain, as the carriers carrying the mutation were superior to body weight, total length, and condition factor over fish carrying wild composition, the results of the current study were consistent with the results of Lee *et al*. (2009), which linked the multiple genetic manifestations of the myostatin gene with the characteristics of growth in characteristic body weight, total and standard length in their study on Zebrafish fish.

Relationship of the myostatin gene with some physiological traits

Relationship of genotypes with the number of Blood cells, white cells, Packed cell volume, and haemoglobin

Al-Alnjawi and Al-Khshali (2018) the effect of polymorphism of the myostatin gene at the site of the first mutation T2230C in the Red Blood Cells-RBC number), white blood cells (WBC) and the size of the compressed cells % Packed Cells Volume-PCV and Haemoglobin-Hb, the results showed that there were no significant differences between the genotypes in the number of red blood cells of fish at a rate of 1.06, 1.08 and 1.05 cells x 610 for the genotypes TT, TC and CC, respectively, this did not agree with the results of the study of Garikipati *et al*. (2006), as he demonstrated in his study on (*Salmo gairdneri*) that the red blood cells increased significantly and did not explain the reasons for the rise in their numbers, while there were significant differences in the numbers of white blood cells, as they exceeded CC mutant composition on the rest of the genotype significantly ($P < 0.01$) at a rate of 26.16 cells$\times^3$10 compared to the hybrid TC and TT combination by 14.24 and 20.38 cells$\times^3$10 respectively,

and this may be due to an increase in the number of macrophages cells at a higher rate than the wild and hybrid synthesis, and this is what he said Garikipati *et al*. (2006) on the increase in the number of white blood cells as compared to the wild composition, as evidenced by the results in the increase in the packed cell volume for the genotype mutant CC over the rest of the genotype significantly (P <0.01) as the percentage of cells reached 27.70% while it was 20.30 and 17.70% sequentially for TT and TC installations. The increase in the size of the packed cell's volume may be due to the high rate of growth that the gene performs through its role in increasing the gene expression to build proteins and muscles. This was shown by Rescan *et al*. (2001) on trout fish (*O. mykiss*), as for hemoglobin or hemoglobin, it has reached the genetic makeup. Mutant CC 10.80g/100 mL and scored significant differences (P <0.05) with TT 8.60 and TC combinations 5.50g/100 mL, increased hemoglobin level in a mutant composition may be due to increased gene expression of the myostatin gene in a number of tissues responsible for forming blood cells and that the process Building proteins requires high energy and high oxygen concentration and this requires it a higher percentage of oxygen in the blood, which leads to an increase in hemoglobin concentration, and this is what he explained Eames *et al*. (2010), by studying Zebrafish fish, observed some improvements in the respiratory system, cardiac performance, surface area of the gills, and increased hemoglobin concentration in mutant fish.

The physiological processes of any fish need a high rate of increased oxygen consumption that occurs during swimming and eating, which represents the total energy spent on activities related to eating, ingestion, digestion, absorption and manufacture of special proteins for growth (Fu *et al*., 2011) and in the same framework all indicated Al-Alnjawi and Al-Khshali (2018) there is also the effect of multiple genetic polymorphisms of the myostatin gene at the site of the second A2232G mutation in RBC number), white blood cell count (WBC) and compressed cell size (% PCV), and hemoglobin (Hb), as the results showed a significant difference between me N genotypes in the red blood cells count of fish, and a significant difference was recorded (P <0.01) in the genotype mutant GG at a rate of 1.34 cells, x ^{6}10 compared to the genotype hybrid AG at a rate of 0.74 cells x ^{6}10, and this may be due to the high oxygen consumption for the purpose of sustaining the processes of building muscle fibres and promoting their growth, this was indicated by Garikipati *et al*. (2006). In his study on *Salmo gairdneri*, he showed a significant increase in the number of erythrocytes, and the reason for that rise was not mentioned, and significant differences (P <0.01) of the genotype mutant GG in the numbers of white blood cells were

significantly increased at a rate of 24.89 cells x $^{3}10$ compared to the genotype. Hybrid AG has a ratio of 16.87 cells x $^{3}10$, due to an increase in the number of accusative cells At a higher rate than the hybrid genotype, and this was indicated by Garikipati *et al*. (2006) to double the number of white blood cells in the mutant genotype by a factor of twice compared to the wild genotype, as evidenced by the presence of a significant difference (P <0.01) of the GG mutant size the size of the PCV on the hybrid AG, as the percentage of cells 24.70%, while in the hybrid genotype 13.10%, the increase in the size of the compressed cells may be due to the high rate of growth that the gene causes in increasing the gene expression to build proteins and muscles. This was demonstrated by Rescan *et al*. (2001) in their study on fish (*O. mykiss*) and hemoglobin Blood. The mutant genotype GG recorded a significant difference (P <0.05) bm 8.20mg/100 ml of the hybrid synthesis AG at a rate of 4.50mg/100 mL, the increased hemoglobin level in the mutant composition may be due to increased gene expression of the myostatin gene in a number of tissues, including the spleen responsible for forming blood cells, and that the process of building proteins requires high energy and concentration High in oxygen and this requires a higher concentration of oxygen in the blood, which leads to an increase in hemoglobin concentration. This is what Eames *et al*. (2010) explained in his study on Zebrafish fish.

Relationship between the genotypes of the myostatin gene with both total protein and glucose

The results of the study of Al-Alnjawi and Al-Khshali (2018) showed significant differences in the total blood protein ratio in different genotypes of the gene at the site of the first mutation T2230C, as both the TC and wild genotypes recorded a significant difference (P <0.01) with a concentration of 4.51 and 4.20 g/100 ml, respectively, compared to the CC mutant 3.10g/100 ml. The low protein concentration in the blood of the mutant genotype CC may be due to increased protein demand due to the building of muscle proteins and the increase in the number of muscle fibres, which leads to a decrease in the concentration of protein in the blood. This was explained by Eames *et al*. (2010) in their study on Zebrafish fish, and its results agreed with what Gao *et al*. (2016) found that protein concentration in the blood decreased by studying it in fish within the same species. As for the level of glucose in the blood, no significant differences were recorded between the genotypes, as the highest level in the hybrid genotype TC reached 105.61 mg/100 ml, followed by the mutant CC 100.09 mg/100 ml, then the wild genotype TT 87.05 mg/100 ml, although there were no significant

differences at the level of glucose, except that the highest values were in the hybrid and mutant genotype, and this is explained by Gao *et al*. (2016) about the high level of glucose in the mutant model higher than it is in wild shape and this is due to the exploitation of glucose in building muscle tissue and increase the synthesis of glucose in the muscles and the continuous need of the body to request glucose and this process accelerates the increase in glucose in the blood compared to With wild shape.

Moreover, He *et al*. (2015) indicated in their study of common carp fish that the processes of regulating glucose and fatty acids are subject to the control of many genes as well as related receptors such as the hormone-insulin-like and the myostatin gene, and this shows that glucose was used to build muscle tissue, and this is what Seiliez *et al*. (2012) in his study on trout, that the myostatin gene caused an increase in the concentration of glucose in the blood and the distribution of fat within the muscles in the mutant composition of the gene and this result indicates the use of glucose in metabolism and muscle building compared to the wild genotype, as it was low glucose content. Al-Alnjawi and Al-Khshali (2018) also showed that there were significant differences in the total protein ratio according to the genotypes of the gene at the site of the second mutation (A2232G), as the hybrid genotype AG recorded a significant difference ($P < 0.05$) at a rate of 3.08 g/100 mL compared to GG mutant composition 2.70 g/100 mL The reason for the low level of protein in the blood for the genetic makeup of the GG mutant may be due to the constant need to order protein in building muscle proteins that leads to low protein in the blood. This was shown by Li *et al*. (2010) in a study on fish. Zebrafish.

As for the level of glucose in the blood, there were no significant differences between the genotypes, as the highest rate in mutant GG was 110.63 mg/100 ml, followed by the hybrid genotype AG 92.35 mg/100 ml, and despite the absence of significant differences in the level of glucose, the highest values It was in mutant, and this result is Agree with what Eames *et al*. (2010) created from the high level of glucose in the mutant genotype and attributed this to the exploitation of glucose in building muscle tissue and the increase in the synthesis of glucose in the muscles by the process of Gluconeogenesis and this process increases the production of glucose in the blood compared to the genotype Wild and Hybrid.

Relationship of the genotypes of the myostatin gene in the chemical composition of body components

The results of Al-Alnjawi and Al-Khshali (2018) showed significant differences in the components of the body according to the different

genotypes of the gene at the first site T2230, as the humidity was affected by the different genotypes of the myostatin gene, and it reached 80.72, 79.13 and 79.60% at the first site of the T2230C mutation For TT genotypes, and TC and CC, respectively, and recorded significant differences (P <0.05) for the wild composition TT with the rest of the structures, as well as there were significant differences with protein percentage, as the genotype mutant CC significantly increased (P <0.05) by 16.25% compared to the hybrid composition T and wild TT by 15.06 percent, 13.64%, respectively, this may be due to the effect of the gene on growth and increasing muscle size in the genotype CC mutant, This is indicated by Tseng *et al*. (2011) that the myostatin gene may play an important role in promoting the growth and differentiation of muscle cells and then in increasing the percentage of protein in muscle fibers, which indicates the superiority of mutant over the rest of the genotype, as shown by the results that there are differences in The percentage of fat according to different genotypes, as the values reached 4.12% in the genotype TT and 4.41% in the genotype TC and a significant difference (P <0.05) from the genotypic genotyping CC 3.15% may be attributed to the decrease in the percentage of fat in the body composition in fish that carry the mutant composition TT the high representation of fat and this indicates the high ionized energy fat inside muscles in the mutant genotype (Zheng *et al*., 2015), as fat is called from the body's storage areas to the muscle tissue for the purpose of using it as a source of energy for muscle building, so fat is low in mutant genotype, and this is what Gao *et al*. (2016) found on Zebrafish (*Brachydanio rerio*) fish. Whereas Fraher *et al*. (2015) in his study on Zebrafish fish, the process of controlling the pathways of lipid metabolism is one of the important things in the processes of demolition and construction, especially fats, which are an important source of energy and used in building processes and muscle formation, as fat is used, especially the fatty acids in Muscle fat building, as found in the mutant composition, the distribution of fat between the muscles, in contrast to that in the wild, is concentrated under the skin. The ash content in both the wild genotype TT and the hybrid TC differed significantly (P <0.05) with rates of 1.76 and 1.68% with the mutant. CC (1.22%).

Results of the second site A2232G also indicated that there were significant differences in the components of the body according to the different genotypes, as the humidity was affected by the different genotypes of the Myostatin gene and reached 79.73 and 78.35% respectively, significant differences (P<0.05) for the mutant genotype (GG) were recorded on hybrid genotype (AG), as well as significant differences appeared with protein ratio, as mutant genotype (GG) significantly outperformed (P <0.05)

by 15.86% compared to hybrid AG by 14.61%, this may be due to the effect of the gene on growth and the increase in muscle size in the mutant genotype (GG). This was indicated by Tseng *et al*. (2011) in their study on sea bream (*S. aurata*) that the Myostatin gene role an important role in promoting growth and differentiation. Muscle cells and then increase the percentage of protein in the muscle fibers, which indicates the preference for mutant over the rest of the genotype, as it is clear from the results that there are differences in the percentage of fat in different genotypes, as the values reached 5.56% in the hybrid genotype AG and a significant difference was recorded ($P < 0.05$) On mutant genotype GG% 3.29, the low percentage of fat in the body composition Fish that carry the mutant genotype is due to the high metabolism of fat, which refers to the high ionized energy of the fat inside the muscles in the genotypic composition, as the fat moves from different areas of the body to the muscle tissue for the purpose of using it as a source of energy in the muscle building, In addition to using it to perform vital activities for the body, the fat percentage is low in the mutant genotype, and this is what Gao *et al*. (2016) found on Zebrafish, ash did not differ significantly between the genotypes as it reached 1.80% in the hybrid genotype AG and 1.35% in Composition the of mutant genotype GG.

Conclusions and Recommendations

From the above and in light of the results of the study, a set of conclusions and recommendations can be presented regarding the use of the Myostatin gene to improve the productive and physiological performance of common carp.

Conclusions

1. Genotype, according to the analysis of the Myostatin gene, has a significant effect on the growth of common carp
2. The fish with genetically mutant genotype (CC) in the first site T2230C and the second site A2232G (GG) achieved positive results with respect to the total and daily weight increase and the rate of relative and specific growth compared with the fish containing wild composition, which means that the mutation in these sites linked positively with the above traits
3. Increase the protein content in fish muscles that carry the mutant genotype CC and GG, which leads to improving the quality of fish meat
4. The mutation was associated morally with the number of white blood cells, which means an increase in the natural immunity of

fish, as well as the number of red blood cells in the genetic makeup (GG) and an increase in the volume of compressed cells and hemoglobin, as well as an increase in the level of blood glucose in the mutant genotypes, as it leads to the release of components More energy, which makes fish more active

5. Election of individuals with the first mutation in the second intron at the site T2230C and exclusion of fish containing the allele T, the exploitation of mutant individuals in improving growth characteristics, as well as the election of individuals carrying the second mutation in the second intron at site A2232G with a positive impact on economic characteristics, including growth characteristics

Recommendations

1. Use of polymorphism in the Myostatin gene by selection the genotypes (CC) and (GG) that have a positive effect on economic characteristics including growth traits
2. Selection of carriers carrying alleles C, at site T2230C as well as increasing frequency of alleles G at site A2232G for this allele with the aim of improving growth traits of common carp
3. Expanding the use of biotechnologies for molecular genetics in animal production to select the best fish as parents for future generations
4. Conducting further studies in other locations of the Myostatin gene and other genes directly related to the characteristics of growth, food transformation and disease resistance in common carp
5. The possibility of studying the genetic expression of a number of genes responsible for growth, such as the Myostatin gene, insulin-like growth factor and the growth hormone gene in Iraqi economic fish

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Chapter - 3
Role of Biotechnology in Crop Improvement

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Chapter - 3

Role of Biotechnology in Crop Improvement

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Abstract

Biotechnology has emerged as the most dynamic force of this century and is virtually reshaping every other industry, it intersects and redefining our lives. In a broad sense, biotechnology has been practiced for centuries for curd making, food preservation, pickle making and fermentation. However, biotechnology received a boost during the 1970s with the discovery of restriction enzymes, which led to the development of a variety of gene technologies and thus, considered the greatest scientific revolution of the 20th century. In a true sense, it deals with changing and improving, more efficiently than conventional technologies can, the characteristics of an organism at cellular and molecular levels for the benefit of mankind. Unlike conventional plant breeding, the biotechnological techniques for genetic modifications largely operate at organ, tissue, cell, protoplast, and molecular levels. During the past 15 years, remarkable achievements have been made in the production, characterization, field evaluation, and release of transgenic varieties/hybrids in several crops. These innovative techniques are considered an adjunct to the conventional methods for efficient and precision plant breeding.

Keywords: biotechnology, transgenic, genetic modified, DNA, crops

Introduction

The increase in human population worldwide has become a major threat to food security. Population growth, particularly in countries with developing economies, will result in the need for a 70% increase in food production by the year 2050 (Delaney, 2015), making the significant enhancement of agricultural productivity in the next several decades a priority. In this respect, efforts of biotechnology have been concentrated on creating technologies that can increase crop yields.

During the last few decades, significant progress has been made in the development and application of new biotechnological approaches to agriculture and plant breeding (Altman and Hasegawa, 2012). Biotechnology

can be considered as a series of enabling technologies, which each involve the manipulation of living organisms or their subcellular components to develop useful products, processes and services. Plant biotechnology encompasses applications of a wide range of scientific discoveries involving the elucidation and manipulation of genetic and developmental systems in plants (Murphy, 2011).

One of the most significant impacts of recent plant biotechnologies is seen in breeding of crops with improved pest and disease resistance and in the production of disease-free plants (Walters, 2009). This is true for almost every aspect of plant biotechnology such as development of molecular markers to speed up plant breeding practices and using knowledge of genes and their expression to generate and commercialize transgenic crops.

In general, the role of biotechnology in crop improvement can be divided into two categories:

1. Those directed towards same goals as conventional plant breeding like improved yield, quality, resistance to pests and diseases, tolerance to abiotic stresses etc. by molecular breeding or production of transgenic crops
2. Novel applications such as use of plants as bio reactors to generate pharmaceuticals, vaccines or biodegradable plastics

Biotechnological development of agriculture

Agriculture has been the backbone of the human food supply directly and indirectly. However, agricultural land is limited, and it has decreased over time due to the increasing world population. Therefore, global agricultural productivity must increase in order to meet the increasing food demands (FAO, 2013). Agriculture is considered one of the oldest activities practiced by human kind. In the beginning, it was manually practiced; later, primitive technologies based on the use of the plow and the harrow allowed an increase in agricultural productivity.

To increase agricultural production and meet consumer and producer needs, the use of machinery on farms was imperative, progressing through remarkable improvements. The introduction of chemical fertilizers around the same period of time enabled crop protection against disease and attainment of higher yields. One of the first chemicals employed was nitrogen based, although its use was restricted due to high costs.

The next important documentation regarding the development of plant hybridization was provided by quarter in 1776. Gregor Mendel's postulates related to how different traits were passed from one generation to the next, and

his paper “Experiments on plant hybridization”, published in 1866 (Persley, 1991), marked the beginning of new technologies designed to improve vegetable species.

In 1970, plants genetics allowed obtaining plant species that were more productive and exhibited resistance to some pests. In 1960, a new movement changed farming: The green revolution, which is a term used for the rapid increase in food production, especially in underdeveloped and developing nations, via the introduction of high-yield crop varieties and the application of modern agricultural techniques (FAO, 2004). The green revolution arose as the technological response to a worldwide food shortage, which became threatening in the period after World War II. The technologies developed during this period, which usually involve bioengineered seed that worked in conjunction with chemical fertilizers and heavy irrigation, had a huge impact on three main cereals (maize, wheat and rice).

A particularly important finding was the discovery how the biological molecule of DNA was responsible for inheritance. In the 1960s, the genetic code was cracked, and subsequent studies began the transfer of genetic material from one organism to another by genetic engineering techniques (Dash *et al.*, 2016). The intersection between genetic engineering and biotechnology was the main factor in the creation of genetically modified organisms (GMOs).

The use of nanotechnology can boost agricultural production, and it is becoming one of the most important tools in modern agriculture. Nanotechnology provides new agrochemical agents and new delivery mechanisms to improve crop productivity, and it promises to reduce pesticide use through nanoformulations of agrochemicals. Nanotechnology also allows the application of nanosensors/nanobiosensors in crop protection (Bhupinder, 2014).

Methods of biotechnology to crops

In the 1970s, a series of complementary advances in the field of molecular biology provided scientists with the ability to readily move DNA between more distantly related organisms. The application of “recombinant DNA technology” frequently has been referred to as genetic engineering. An organism that has been modified, or transformed, using modern techniques of genetic exchange is commonly referred to as a genetically-modified organism (GMO).

However, the offspring of any traditional cross between two organisms also are "genetically modified" relative to the genotype of either of the contributing parents. Plants that have been genetically modified using

recombinant DNA technology to introduce a gene from either the same or a different species also are known as transgenic plants and the specific gene transferred is known as a transgene. Not all GMOs involve the use of cross-species genetic exchange, recombinant DNA technology also can be used to transfer a gene between different varieties of the same species or to modify the expression of one or more of a given plant's own genes, e.g., to amplify the expression of a gene for disease resistance.

The application of recombinant DNA technology to facilitate genetic exchange in crops has several advantages over conventional breeding methods. The exchange is far more precise because only a single (or at most, a few), specific gene that has been identified as providing a useful trait is being transferred to the recipient plant. As a result, there is no inclusion of ancillary, unwanted traits that need to be eliminated in subsequent generations, as often happens with traditional plant breeding. Application of recombinant DNA technology to plant breeding also allows more rapid development of varieties containing new and desirable traits.

Further, the specific gene being transferred is known so the genetic change taking place to bring about the desired trait also is known, which often not the case with traditional breeding methods is where the fundamental basis of the trait being introduced may not be known at all. Finally, the ability to transfer genes from any other plant or other organism into a chosen recipient means that the entire span of genetic capabilities available among all biological organisms has the potential to be genetically transferred or used in any other organism. This markedly expands the range of useful traits that ultimately can be applied to the development of new crop varieties.

As a hypothetical example, if the genes that allow certain bacteria to tolerate high external levels of salt can serve the same purpose when transferred into crops such as potatoes, wheat, or rice, then the production of such improved food crops on marginally saline lands may be possible. Given that the acreage of such saline lands is estimated to be equal to 20 to 25% of the land currently under cultivation worldwide, this would be a significant contribution toward global food security.

Two primary methods currently exist for introducing transgenic DNA into plant genomes in a functional manner. For plants known as dicots (broad-leaved plants such as soybean, tomato, and cotton), transformation is usually brought about by use of a bacterium, *Agro-bacterium tumefaciens*. *Agrobacterium* naturally infects a wide range of plants and it does so by inserting some of its own DNA directly into the DNA of the plant. By taking out the undesired traits associated with *Agrobacterium* infection and inserting

a gene of interest into the *Agrobacterium* DNA that will ultimately be incorporated into the plant's DNA, any desired gene can be transferred in to a dicot's DNA following bacterial infection. The cells containing the new gene subsequently can be identified and grown using plant cell culture technology into a whole plant that now contains the new transgene incorporated into its DNA.

Plants known as monocots (grass species such as maize, wheat, and rice) are not readily infected by *Agrobacterium* so the external DNA that is to be transferred into the plant's genome is coated on the surface of small tungsten balls and the balls are physically shot into plant cells. Some of the DNA comes off of the balls and is incorporated into the DNA of the recipient plant. Those cells can also be identified and grown into a whole plant that contains the foreign DNA. The ability to easily incorporate genetic material from virtually any organism into many different crop plants has reached the stage of commercial applicability. The major technical limitation on the application of recombinant DNA technology to improving plants is insufficient understanding of exactly which genes control agriculturally important traits and how they act to do so.

The study of genes involves the rapidly developing field of “genomics”, which refers to determining the DNA sequence and identifying the location and function of all the genes in an organism. It appears that many traits are conserved between species, i.e., the same gene confers the same trait in different species. Thus, a gene for salt tolerance in bacteria may confer salt tolerance if it is transferred and expressed in rice or wheat.

The advent of large-scale sequencing of entire genomes of organisms as diverse as bacteria, fungi, plants, and animals, is leading to the identification of the complete complement of genes found in many different organisms. This is dramatically enhancing the rate at which an understanding of the function of different genes is being achieved. From the stand point of agricultural biotechnology, advances in genomics will lead to a rapid increase in the number of useful traits that will be available to enhance crop plants in the future.

Responses to biotechnology in crop improvement

Bioinformatics

Another important factor in the successes of the genetic improvement of crops was the development of fast and more reliable computers, which allowed easier management and analysis of data as well as publication of scientific reports. More publications and easy means for retrieving this

information accounted for such growth of knowledge dissemination in plant genetics and breeding. Computers are deciphering, and organizing the huge genetic information that may become "the raw resource of the emerging biotech economy" in the next century (Rifkin, 1998). Scientists working in the new field of "bioinformatics" are developing biological data banks to download the genetic information accumulated during millions of years of life evolution, and perhaps reconstruct some of the living organisms of the natural world.

Plant genomics

This new term, defined by the development of biotechnology, refers to the investigations of whole genomes by integrating genetics with informatics and automated systems. Genomic research aims to elucidate the structure, function and evolution of past and present genomes (Liu, 1997). Some of the most dynamic fields concerning agriculture are the sequencing of plant genomes, comparative mapping across species with genetic markers, and objective assisted breeding after identifying candidate genes or chromosome regions for further manipulations. As a result of genomics, the concept of gene pools has been enlarged to include transgenes and native exotic gene pools that are becoming available through comparative analysis of plant biological repertoires (Lee, 1998).

Understanding the biological traits of one species may enhance the ability to achieve high productivity or better product quality in another organism. DNA markers and gene sequencing provides quantitative means to determine the extent of genetic diversity and to establish objective phylogenetic relationships among organisms. 'Gene chips' and transposon tagging will provide new dimensions for investigating gene expression molecular biologists will study not only individual genes but how circuits of interacting genes in different pathways control the spectrum of genetic diversity in any crop species. For example, more information will be available on why plant resistance genes are clustered together, or what candidate genes should be considered when manipulating quantitative trait loci (QTL) for crop improvement (Paterson, 1997).

Farming in environmentally friendly systems

The aims of applied plant science research for agriculture are to enhance crop yields, improve food quality and preserve the environment where human beings and other organisms live. The best way for conservation of plant biodiversity and its environment, would be to achieve high crop productivity per unit area. In this regard, Briggs (1998) reported that as yields treble, soil

erosion per ton of food decreases by two-thirds. There has been a significant yield improvement owing to enhanced crop husbandry, but in the next years progress will be achieved by changing plants that could be more suitable to sustainable and environmentally friendly farming systems.

Agro-chemical corporations are developing pest and disease resistant transgenic crops to avoid pollution with pesticides in the farming system. Furthermore, food quality will become more important than crop productivity in a wealthy society. Consumers will prefer transgenic crops if they have the desired characteristics.

In the next decades meiotic-based breeding will still generate cultivars for farmers. Genetic improvement through biotechnology needs conventional breeding because

1) The elite cultivars will be the parents of the next generation of improved genotypes
2) Field testing across locations or cropping systems and over years will be needed to determine the best selections due to the genotype-by environment interaction (Kang and Gauch, 1996)

As stated by Briggs (1998), "transgenes must be viewed as improvements rather than replacements for elite germplasm".

Indeed, genetic engineering may provide a means to add value by introducing synthetic or natural genes that enhance crop quality and yield, as well as protect the plant against pest and diseases. Farmers will pay more for transgenic crop propagules if they obtain extra-income after adopting biotech-derived products. For example, seeds of insect resistant transgenic crops will be more expensive than those of available cultivars but the farmer will not need to apply pesticides in their transgenic fields.

Gene banks, DNA banking and virtual plant breeding

The sequencing of crop genomes opened new frontiers in conservation of plant biodiversity and its genetic enhancement. The advances in gene isolation and sequencing in many plant species allows to envisage that within a few years, gene-bank curators may replace their large cold stores of seeds with crop DNA sequences that will be electronically stored. The characterization of plant genomes will ultimately create a true gene bank, which should possess a large and accessible gene inventory of non-characterized crop gene pools. Seed banks of comprehensively investigated stocks should remain because geneticists and plant breeders, the main users of gene banks, will need this germplasm for their work.

Genomics may accelerate the utilization of candidate genes available at these gene banks through transformation without barriers across plant species or other living kingdoms. Nonetheless, genetic engineering should be seen as one of the methods of plant breeding that permits the direct alteration and re-building of a crop population. "shutting-off" genes coding for undesired characteristics may be another application of transgenic in crop improvement. Plant breeders will change their modus operandi with the development of objective marker-assisted introgression and selection methods.

Back cross breeding will be shortened by eliminating undesired chromosome segments (also known as linkage drags) of the donor parent or selecting for more chromosome regions of the recurrent parent. Parents of elite crosses may be chosen based on a combination of DNA markers and phenotypic assessment in a selection index, such as best linear unbiased predictors (Bernardo, 1998). To achieve success in these endeavours, cheap, easy, decentralized, and rapid diagnostic marker procedures are required. There are many areas of basic and strategic research in plant breeding and genetics that are being facilitated by marker-aided analysis (Paterson, 1996).

With molecular markers, plant biologists are reviewing crop evolution and gathering new knowledge. Such information should be incorporated into genetic enhancement programmes, especially those with an evolutionary breeding scheme. Likewise, plant ideotypes for each crop should drive the work of plant breeders. Specific plant morphotypes have been defined in rice and wheat based on accumulated knowledge of crop physiology and crop protection. The needed characteristics required to develop improved plant prototypes ensuing from such a 'virtual breeding' approach may be available in gene banks of the crop or in those of other species. Otherwise, breeders may obtain novel transgenes to develop the required ideotype.

Now a days, the finding of new genes that add value to agricultural products seems to be very important in the private agri-business. Unique gene databases are being assembled by the industry with the massive amount of data generated by genomics research. A new term 'Biosource' was coined recently to refer to a fast and effective licensed technology of pinpointing genes. With this method, a 'benign' virus infects a plant with a specific gene that allows researchers to observe directly its phenotype. Biosource replaces the standard time-consuming approach of first mapping a gene to subsequently determine its exact function. Gene identification in DNA libraries coupled with Biosource technology and an enhanced ability to put genes into plants will be routine for improving crops in the next decade.

Genomics may provide a means for the elucidation of important functions that are essential for crop adaptedness (Wallace and Yan, 1998). Regions of the world should be mapped by combining data of geographical information systems, crop performance, and genome characterization in each environment. In this way, plant breeders can develop new cultivars with the appropriate genes that improve fitness of the promising selections. Fine-tuning plant responses to distinct environments may enhance crop productivity.

Development of cultivars with a wide range of adaptation will allow farming in marginal lands. Likewise, research advances in gene regulation, especially those processes concerning plant development patterns, will help breeders to fit genotypes in specific environments. Photoperiod insensitivity, flowering initiation, vernalization, cold acclimation, heat tolerance, host response to parasites and predators, are some of the characteristics in which advanced knowledge may be acquired by combining molecular biology, plant physiology and anatomy, crop protection, and genomics.

Pharming and Farmer-ceuticals

Growth of cities in the developed world has already replaced farmland with shopping malls, parking lots, and housing developments. Peri-urban agriculture and home gardening are also becoming very important for national food security in the developing world as a result of rapid urban expansion. Hence, new cultivars will be needed to fit into intensive production systems, which may provide the food required to satisfy urban world demands of the next century.

Specific plant architecture, tolerance to urban pollution, efficient nutrient uptake, and crop acclimatization to new substrates for growing are, among others, the plant characteristics required for this kind of agriculture. Genes controlling these characteristics may be available in gene banks for further cross breeding, which can be assisted by genomics. For example, food crops with low fats, and high in specific amino acids may be needed to satisfy people who wish to change their eating habits. If genes controlling these characteristics do not exist in a specific crop pool they may be incorporated into the breeding pool using transgenics.

Tissue culture of certain parts of the plant may provide a means to achieve success in this endeavour. For example, edible portions of fruit crops could be grown *in vitro*. A steady and cheap supply of these edible plant parts will be required in this new agri-business. It will take some time before such a process can be scaled up for commercial output. This biotechnique, as well as other new farming methods, offers a means for new ways of producing food, feed or fibre.

Often plants provide the raw materials for agro-industry, and not only for food or fibre processing. Active ingredients of plants have been transformed into commercial products such as medicines, solvents, dyes, and non-cooking oils for many years. Hence, it would not be surprising to see, in few years from now, entire farms without food crops but growing transgenic plants to produce new products, e.g. edible plastic from peas or plant oils to manufacture hydraulic fluids and nylon (Grace, 1997).

For example, oral vaccines appear to be a convenient delivery system for vaccination throughout the world. Biotechnology has been used to engineer plants that contain a gene derived from a human pathogen (Tacker *et al*. 1998). An antigenic protein encoded by this foreign DNA can accumulate in the resultant plant tissues. Results from preclinical trials showed that antigenic proteins harvested from transgenic plants were able to keep the immunogenic properties if purified. These antigenic proteins caused the production of specific antibodies in injected mice.

Arakawa *et al*. (1998) recently demonstrated the ability of transgenic food crops to induce protective immunity in mice against a bacterial enterotoxin such as cholera toxin B subunit pentamer with affinity for GMI-ganglioside. Also, potato tubers have been used successfully as a biofactory for high-level output of a recombinant single chain antibody (Artsaenko *et al*. 1998).

Applications

Genetic engineering: All crops improved with transferred DNA to date have been developed to aid farmers to increase productivity by reducing crop damage from weeds, diseases or insects.

Molecular markers: Scientists can use molecular markers to select plants or animals that possess a desirable gene, even in the absence of a visible trait. Thus, breeding is more precise and efficient.

Molecular diagnostics: Molecular diagnostics are methods to detect genes or gene products that are very precise and specific. Molecular diagnostics are used in agriculture to more accurately diagnose crop/livestock diseases.

Vaccines: Biotechnology-derived vaccines are used in livestock and humans. They may be cheaper, better and/or safer than traditional vaccines. They are also stable at room temperature, and do not need refrigerated storage.

Tissue culture: Tissue culture is the regeneration of plants in the laboratory from disease-free plant parts. This technique allows for the reproduction of disease-free planting material for crops Examples of crops

produced using tissue culture include citrus, pineapples, avocados, mangoes, bananas, coffee and papaya.

Flowers: There is some simply aesthetic application and an example of this is the use of gene recognition and transfer techniques to improve the colour, smell, size and other features of flowers biotech has been used to make improvement to other common ornamental plants, in particular, shrubs and trees.

Benefits

Following are a few examples of benefits resulting from applying currently available genetic engineering techniques to agricultural biotechnology.

Increased crop productivity: Biotechnology has helped to increase crop productivity by introducing such qualities as Enhanced crop protection It is provide cost-effective solutions to pest problems crops such as corn, cotton, and potato have been successfully transformed through genetic engineering to make a protein that kills certain insects when they feed on the plants.

Improved nutritional value: It has allowed new options for improving the nutritional value, flavour, and texture of foods. Transgenic crops in development include soybeans with higher protein content, potatoes with more nutritionally available starch content and beans with more essential amino acids.

Better flavour: Flavour can be altered by enhancing the activity of plant enzymes that transform aroma precursors into flavouring compounds. Transgenic peppers and melons with improved flavour are currently in field trials. Fresher produce genetic engineering can result in improved keeping properties to make transport of fresh produce easier, giving consumers access to nutritionally valuable whole foods and preventing decay, damage, and loss of nutrients.

Environmental benefits: When genetic engineering results in reduced pesticide dependence, we have less pesticide residues in foods, we reduce pesticide leaching into groundwater, and we minimize farm worker exposure to hazardous products.

Benefits for developing countries: Genetic engineering technologies can help to improve health conditions in less developed countries. Growth of crops in industrial and developing countries.

Herbicide tolerance: Chemical herbicides are frequently used to control weeds. Researchers realized that if a crop plant is genetically engineered to be

resistant to a broad-spectrum herbicide weed management could be simplified and safer chemicals could be used. It is often argued that such GE varieties reduce soil erosion, because they make adoption of soil-conserving practices such as "no-till" easier.

Virus resistance: Many plants are susceptible to diseases caused by viruses, which are often spread by insects (such as aphids) from plant to plant across a field. It can be very difficult to control and crop damage can be severe. Insecticides often have little impact on the spread of the disease. Scientists have discovered new genetic engineering methods that provide resistance to viral disease.

Delayed fruit ripening: Delaying the ripening process in fruit is of interest to producers because it allows more time for shipment of fruit from the farmer's fields to the grocer's shelf, and increases the shelf life of the fruit for consumers. Fruit that is genetically engineered to delay ripening can be left to mature on the plant longer, will have longer shelf-life in shipping, and may last longer for consumers.

Conclusion

Within the next 10 or 20 years, five research areas may become very important for crop improvement:

i) Apomixes to fix hybrid vigour
ii) Male sterility systems with transgenics for hybrid seed in self-pollinating crops
iii) Parthenocarpy for seedless vegetables and fruit trees
iv) Short-cycling for rapid improvement of forest and fruit trees
v) Converting annual into perennial crops for sustainable agricultural systems

The development of perennial crops will be especially important to protect the soil from erosion. Plant biotechnology will play an important role in achieving research and development success in these areas. However, rapid and cost-effective development, and adoption of biotechnology-derived products will depend on developing a full understanding of the interaction of genes within their genomic environment, and with the environment in which their conferred phenotype must interact.

Plant biotechnology with focus on seed-varietal improvement, such as Genetic Modified (GM) technology and molecular-assisted breeding, has generated products that help agriculture to achieve enhanced yields in a more

sustainable manner. GM technology has brought significant improvements to earned income, life quality, and per acre productivity. Innovative approaches of cellular and molecular biotechnology have emerged as a valuable adjunct to supplement and complement the conventional methods for precise and efficient breeding of a wide variety of crop plants.

Governments and other responsible parties should effectively communicate with the public about the nature of new crop types and new crop varieties, about the unity of life processes in all organisms, and about the risks and benefits of agricultural biotechnology in their own country. There also is a need to continually improve the transparency and broad participation in the decision-making processes in relation to biotechnology, the release of genetically modified organisms into the environment, and the approval of genetically modified foods for commercial use.

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Chapter - 4
Histological Study of Seasonal Cyclicity in the Ovary of *Lepidocephalichthys irrorata* (Hora)

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Chapter - 4

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Abstract

L. irrorata were monthly sampled throughout a year and the histological analysis of their ovaries was done to determined the changes occurring in ovarian development. On the basis of changes in the cytoplasm and nucleus, the oocyte development can be divided into 6 stages i.e.

1) Chromatin Nucleus Stage
2) Early Perinucleolar Stage
3) Late Perinucleolar Stage
4) Yolk Vesicle Stage
5) Yolk Stage
6) Ripe egg stage

Keyword: *L. irrorata*, Histology, Ovary etc.

Introduction

L. irrorata is an endemic loach of Manipur. This fish has been associated with the culture of Meitei communities in this state from immemorable time. During the recent years capture fishery of *L. irrorata* in the state have been deteriorated to a great extent, gradually disappearing from the waters of Manipur Valley in spite of their abundance in the past. Although, they have less commercial value they have high traditional and nutritive value (Vishwanath, W and Sarojnalini, Ch., 1988), information on the reproductive physiology and histomorphological changes of ovary of *L. irrorata* remains limited. *L. irrorata* occupy an important part of Meitei communities. Therefore, keeping in the view of food value and traditional value of *L. irrorata*, histological study of seasonal changes in ovaries and the process of game togenesis have been undertaken for the development and management of *L. irrorata.* A detailed study of gonad maturation is important for proper management of aquaculture practices, since such studies are aimed in understanding the annual changes of the population

(Thorpe *et al*., 1990, Jobling *et al*., 2002, Tomkiewicz *et al*., 2003, Shein *et al*., 2004).

Many investigators have also described the seasonal histological changes in the ovary and oogenesis of fishes viz, *Labeo capensis* (Van-der Merwe *et al*., 1988), *Channa orientalis* (Brajakeshwor, Th., 1993), *Schizothorax plagiostomus* (Naresh Kumar Agarwal, 2009).

The detailed information on changes in the ovaries of the *L. irrorata during* the reproductive cycle is important to provide useful data for the management of this species, Hence, this study was performed to examine the ovarian developmental stages during its annual maturation cycle.

Materials and Methods

Fresh specimens of *L. irrorata* were collected for every month for a period of 2 years. The fishes were weighed individually and also measured their length. Gonads were dissected out, blotted to remove excess body fluid and weighed. The gonads after weighing were fixed in Bouin's fluid for 24 hours, then dehydrated and embedded in paraffin wax. Deparaffinised, hydrated, 6-7 thick sections were dehydrated through upgraded series of ethanol upto 70 percent, stained in eosin, wash in 2 changes of 90 percent ethanol, dehydrated in absolute alcohol, and cleared in xylene and mount in DPX. The stained sections of gonad were examined and the different Oogenic stage were identified by following the method of Van der Merwe *et al*., 1988 and Naresh Kumar Agarwal, 2009. Diameter of cells were measured using a micrometer.

Results

The Ovary in *L. irrorata* is paired, lying in the abdominal cavity ventral to kidney and cyst ovarian type. Each Oogonia passes through a series of stages to form ripe egg involving complicated changes occurring in the cytoplasm and nucleus. Oogonium varies in shape and possesses a large nucleus and a thin layer of ooplasm. Nucleus to cytoplasm ratio is usually high. On the basis of changes occurring in the Cytoplasm and nucleus, the oocyte development can be divided into the following stage:

Stage 1: Chromatin Nucleolus Stage (November, December, January) (Fig. 1 A & B).

Here, Oogonia grew in size to form the earliest primary oocytes. The primary oocytes are easily distinguished by their nuclei with little ooplasm. Some oocytes were seen with several deeply stained nucleoli while other have a large nucleolus.

At this stage diameter of the oocytes ranged from 0.00117mm-0.0156mm.

Stage 2: Early perinuclear stage: (January, February) (Fig. 2).

Oocytes increase in size and the amount of ooplasm increased correspondingly. A characteristics features of this stage is the appearance of a large number of nucleoli of different size arrange on the periphery of the nuclear membrane. These nucleoli are spherical in form. The diameter of the oocyte ranged from 0.035 mm to 0.0624 mm.

Stage 3: Late perinuclear stage: (March) (Fig. 3).

This stage is accompanied with further increased in the amount of ooplasm of the oocyte. Several nucleoli are situated adjacent to the nuclear membrane, appearance of yolk nucleus in some oocytes as a circular body in the cytoplasm adjacent to the nuclear membrane. The oocytes measures 0.062 mm to 0.117 mm in diameter.

Stage 4: Yolk vesicle stage: (March, April May) (Fig. 4).

This is characterized by further increase in the size of oocyte and is designated mainly by the first appearance of minute vacuoles in the cortex of cytoplasm which are termed as yolk vesicles. The vesicles later on increase in size by fusion of several smaller ones. The nuclear membrane becomes irregular and several nucleoli are seen but their size is reduced compared to nucleoli of previous stages. The diameter of the oocyte at this stage ranged from 0.15 mm to 0.23 mm.

Stage 5: Yolk Stage: (April, May, June) (Fig. 5).

During this stage, an extensive development of yolk globules is observed, increasing in the size and occupying whole of ooplasm around the nucleus. The rapid accumulation of yolk globules results in rapid growth of oocytes. The nucleoli are scattered in the periphery of the nuclear membrane. The diameter of oocytes in this stage ranged from 0.26mm to 0.35mm.

Stage 6: Rip egg stage (May, June, July) (Fig. 6).

At this stage the eggs attain the largest sizes. The egg is fully grown and completely packed with yolk mass. The nucleus is not seen in the ooplasm of such oocytes. The oocytes measure 0.36 mm to 0.39mm in diameter.

In late July atretic follicle and absorption of yolky oocytes (Fig. 7) were seen. The asynchronous development if oocytes occurred in *L. irrorata*. In the ovary of a ripe fish, three groups of oocytes can be distinguished, viz, fully ripe oocytes, yolk vesicles or early yolk globule oocytes and yolkless oocytes.

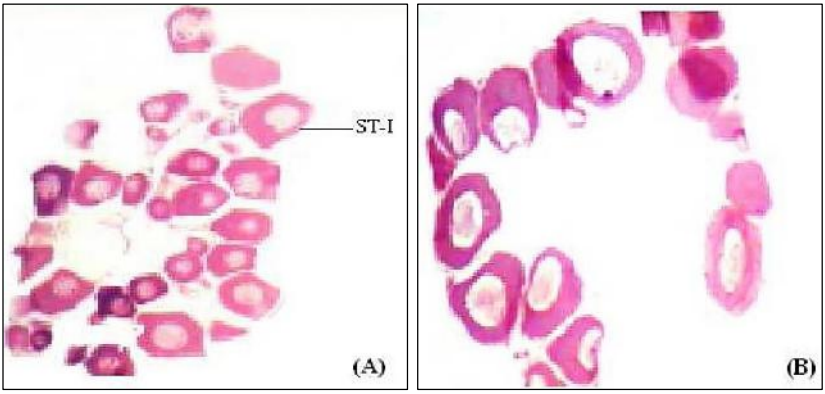

Fig 1: Photomicrograph of TS of ovary at Stage-I. H.E. X100

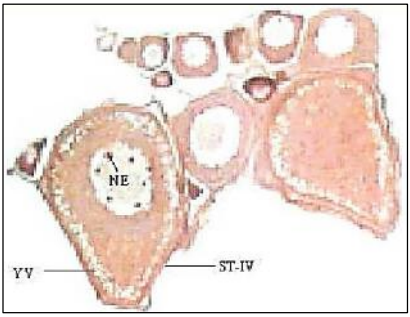

Fig 2: Photomicrograph of the Vitellogenic follicle at Stage-IV (yolk vesicle stage). H-E. X100

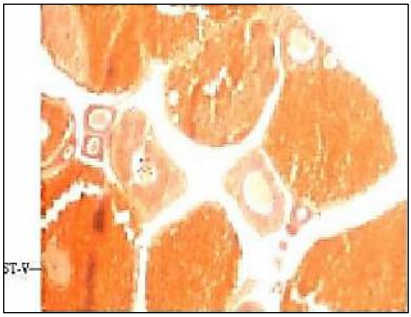

Fig 3: Photomicrograph of Oocytes at Stage-V (yolk stage). H-E. X 100

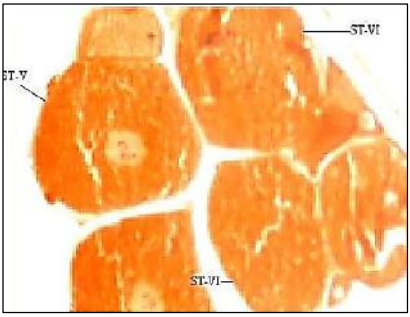

Fig 4: Photomicrograph of the Vitellogenic follicle at Stage-IV, completely filled up with yolk. H-E. X100

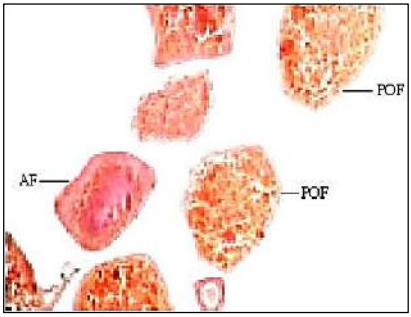

Fig 5: Photomicrograph showing postovulatory follicle and atretic follicle H-E. X 100

Discussion

Naresh Kumar Agarwal (2009) described seven stages in the ovary of *Schizothorax plagiostomus*. In a majority of teleost fishes, five-eight stages of oogenesis have been reported (Nagahama, 1983; West, 1990; Arockiaraj *et al*., 2004) described five stages in the gonad of *Mystus montanus*. Similarly, Kader *et al*., (1988) made similar observations in *Odontamblyopus rubicundus*. James Milton *et al*. (2018) also described five stages in *Channa gachua* Marza (1938), described three types of rhythms in maturation of cytes:

a) Total Synchronism
b) Partiat Synchronism
c) Asynchronism

Prabhu (1956), Classified four types of spawning:

1) Spawning once a year with a short duration
2) Spawning once a year with a long duration
3) Spawning more than once a year
4) Spawning throughout the year intermittently

The fully ripe ovary of *L. irrorata* contains oocytes of stage V and VI. In addition to these, few immature oocytes of stage II, III are also present. The ovarian histology reveals that the eggs are spawned in two or more batches during the same spawning season, which extends from May to July. The breeding season is similar to that of the report given by Ranabir (1991). Thus, this fish belongs to the category of asynchronism of Marza (1938) and to the second category of Prabhu (1956).

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Chapter - 5
Environmental Contamination: Pesticides and Toxins

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Chapter - 5

Environmental Contamination: Pesticides and Toxins

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Abstract

In recent scenario, the pollution is increased by increasing in the population value. There are many parameters, which can affect the environmental life decisively. Nature is minimising the hazards, while human beings are maximising them. There are many organic and inorganic materials, which can easily contaminate the environmental cycle. Environmental contamination is the result of the irrational use of resources at the wrong place and at the wrong time. Environmental contamination has changed the lifestyle of the people in many ways all around the world. It has also reduced the extent of life on earth. Today, human beings are bound to compromise with such environmental conditions, which was not anticipated for the sustenance of humanity and other life forms. There are many ways through water; air and land are being contaminated by. Most contaminants enter the environment from the industrial and commercial facilities. Pesticides and Toxins are the main environmental contaminations, which affected the lifestyle of human being by harming human health. By the acute toxicity or chronic toxicity, the pesticides and toxins are frequently harming the human health with several major causes. There has been many ways, through the pesticides and toxins are affecting to human health and cause cancer. In this chapter, the basic information of pesticides and toxins are explained for better understanding of their usages. It also represents different types of pesticides and toxins in brief. The chapter also explains the benefits and hazards of pesticides and toxins.

Keywords: environmental contamination, pesticides, toxins, human health

1. Environmental contamination

Environmental pollution by heavy metals like lead is a worldwide public health problem; this type of poisoning has several adverse health impacts, which particularly affects the children. Where it has been specifically linked to neurological, neuro-behavioural and developmental problems and iron

deficiency anaemia, whose prevalence among pregnant women and children. This type of heavy metals was entering into food, water and contaminated air and creates health problems in human. Different types of pesticides are most widely used herbicide in the world. Food and Agriculture Organization (FAO) reported that different types of hazardous materials like pesticides and its major metabolites are of potential toxicological concern, and mainly as a result of accumulation of residues in the food chain. Heavy metals, Pesticides and Toxins are present in the soil, water and food will create the toxicity and also contaminate the environment, animals and human being also (Magoha *et al.*, 2008), (Bai and Ogbourne, 2016).

2. Introduction of Pesticides and Toxins

Pesticides occur in detectable amounts throughout the environment in virtually all inhabited areas of the world and in some, if not all, of the uninhabited portions. If our methods of detection were sufficiently sensitive and definitive, there is no part of the earth where we could not now find at least a few molecules of many pesticides in plants, man, animals, soil, water, and air. Pesticides are introduced into the environment in a variety of ways, including direct application in agriculture, in forest pest control and for control of pests affecting human health. Comparatively small areas of the world are so treated, but transport by wind, water, and movement of food and feed in commerce results in universal distribution of minute amounts of the compounds. For example pesticides may be used in the prevention of malaria, which kills up to 1 million children per year and for preventing other vector-borne diseases such as dengue, leishmaniasis and Japanese encephalitis. Pesticides are toxic by design-they are BIOCIDES, designed to kill, reduce or repel insects, weeds, rodents, fungi or other organisms that can threaten public health and the economy. Their mode of action is by targeting systems or enzymes in the pests which may be identical or very similar to systems or enzymes in human beings and therefore, they pose risks to human health and the environment. Pesticides are ubiquitous in the environment and most are synthetic. There is growing concern about children's exposure to pesticides and their special susceptibility (Westlake and Gunther, 1966), (Waksom, 2017).

Pesticides have different distribution and persistence patterns in the environment, even if all of them are distributed in some way through air, soil and water. This should be addressed to gain an understanding of how acute and chronic exposure may occur because air, water and soil are the media of exposure. Some pesticides are characterized by being very persistent in the environment. They may represent long-term dangers as they bio magnify up

the food-chain. Pesticides are widely used to protect crops and livestock from losses due to insects, weeds, and diseases. These chemicals have helped to increase agricultural production with reduced labour. However, problems associated with improper pesticide use have led to human illness, wildlife losses, and water quality degradation. Since pesticides are an important tool for most farming operations, and cleaning up contaminated groundwater is extremely difficult, producers need to evaluate their use of pesticides and adopt BMPs that are appropriate for their crops and site (Costa *et al.*, 2008), (Johnston, 2001).

Toxins are natural poisons that include the most toxic substances known. Toxins are sometimes classified as chemical warfare agents and sometimes as biological warfare agents. Bacteria, fungi, dinoflagellates, algae, plants, and animals produce toxins. Toxins have been used to develop drugs, such as digitalis and physostigmine; as research probes to "dissect out" mechanisms of biological action and to treat neurological disorders and cancer. Toxins have also been associated with public health and agricultural problems, primarily contamination of food and more recently, health problems arising from airborne contamination. They may be distributed as aerosols, liquids, or powders, with attacks capable of covering tens to hundreds of square kilometres and can be delivered by air- or ground-bursting munitions; aircraft spray tanks or ground-based aerosol generators. There are no volatile toxins (WESTLAKE and GUNTHER, 1966). Some toxins are capable of sustained surface contamination and may also represent a secondary aerosol hazard as soil is disturbed. Most toxins are odourless and their aerosols are not visible. Their potency and diversity have, to date, precluded the deployment of specific detector systems, although there are military systems that can detect aerosol clouds. Technology, such as microencapsulation, has the potential of altering delivery systems to permit skin intoxications, tailoring particle sizes and making agents more resistant to environmental degradation. It is not easy to demonstrate toxins in biological tissues or the environment, which may account for some of the controversy about their suspected use (Four, 1990), (States *et al.*, 1972).

3. History of pesticides

The Early Years Pesticides have been used to a limited degree since ancient times. Even in this century until the mid 1930's, pesticides were mainly of natural organic or inorganic compounds. The use of arsenical insecticides in agriculture has greatly decreased following the introduction of modern insecticides; the use of arsenical herbicides has increased. The first recorded recommendation of sulphur for the control of diseases are at the

beginning of the last century and around 1850 its fumigant properties were recognized. Until 1903, 95% of the world supply of sulphur came from Sicily. Despite the introduction of organic sulphur fungicides such as captan, maneb and others in the late 1950's, because of the toxicological problems associated with some of them, inorganic sulphur remains one of the most important fungicides. Its major advantage is its low toxicity to man, wildlife and the environment. Nicotine has been widely utilized as an insecticide all over the world, as has been rotenone, used as a fish poison in South America since 1725. Mercury chloride was extensively used as a fungicide since 1891 and was slowly replaced by its organic forms such as phenylmercury (1915), alkyloxy alkylmercury (1920's) and alkylmercury (1940's). The first synthetic organic insecticides that appeared for public use were probably the dinitro compounds and the thiocyanates in the early 1930's. Beginning with those years, significant discoveries occurred, which led to the proliferation of new synthetic pesticides including DDT, organophosphates and pyrethroids (Bencko, Yan and Foong, 2017), (Costa, 1987), (Tweedy, 1981).

Table 1.1: Historical Development of Pesticides

Year	Pesticides
1000BC	Sulphur is Used by the Greeks
900	Arsenicals are used by the Chinese
1763	Nicotine, as crude tobacco, used as insecticide
1800s	Frist usage of pyrethrin’s in Asia
1848	Frist usage of rotenoids
1939	Discovery of the insecticidal properties of DDT
1940-50	Development of organochlorine insecticides (aldrin, dieldrin, etc.)
1944	Synthesis of parathion
1950s	Development of insecticidal carbamates
1963	Chlordimeform, the first of the formamidine pesticides is synthesized
1970s	Development of modern pyrethroids

3.1 The development of modern insecticides

The period between 1935 and 1950 was characterized by the development of DDT and other chlorinated hydrocarbon insecticides. In the late 1930's, the Swiss firm J.R. Geigy A.G. was very active in researching a contact poison for flies, mosquitoes and other insects. Although first synthesized by Zeidler in 1874, it was not until 1939 that Dr. Paul Muller found that dichlorodiphenyltrichloroethane (DDT) acted as a contact poison on flies, mosquitoes, and other insects. In 1940 the first patent was obtained, and from the beginning of 1942 the preparation appeared on the market

under the names Gesarol and Neocid, among others. During the first half of this century, it is estimated that some 300 million people suffered from malaria each year, and of those 3 million died of it. Before the development of DDT, most of the attempts to control or eliminate malaria were unsuccessful. As an example of the effectiveness of DDT, in the province of Latina (Italy) there were 50-60 cases of malaria per 1,000 inhabitants in 1944. This incidence was reduced to zero by 1949, after DDT spraying program was started in 1945 (Costa, 1987). The first one, hexachlorocyclohexane (also called benzene hexachloride, BHC) had been synthesized in 1825 by Faraday, but its insecticidal properties were not discovered until more than 100 years later. In the early 1940's, scientists in England and in France recognized the gamma isomer of BHC, commonly known as lindane, as a highly potent insecticide and introduced it to the market. The cyclodiene-type chlorinated hydrocarbons were introduced to the market starting from the mid 1940's. The insecticidal properties of chlordane were described in 1945, heptachlor was introduced for agricultural use in 1948 and in the next five years dieldrin and aldrin were also marketed. But a few minutes after inhaling, marked pressure develops in the larynx combined with breathlessness. Then, mild disturbances of consciousness set in and also a feeling of being dazzled and painful hypersensitivity of the eyes to light. The symptoms decrease only after several hours and very small quantities produce the effects (Tweedy, 1981).

While working in the synthesis of organic fluorine and sulphur compounds, one day in December 1936, Schrader noticed that, on my way home my Visual acuity was somewhat reduced. This substance was isolated, but it was too toxic to warm-blooded animals to be used in agriculture compounds, which have greater toxicity than parathion, such as sarin, Soman and Tabun were synthesized as potential chemical warfare agents, but these discoveries were kept secret by the German government. The most important group of insecticides of recent discovery is that of synthetic pyrethroids. These compounds derive from molecules originally isolated from pyrethrum flowers which were used by Caucasian tribes and in Persia since the early 1800's to control body lice. Some of the most commonly used pyrethroid insecticides, such as permethrin, cypermethrin, decamethrin and fenvalerate, were synthesized in the 1970's. Because of their low mammalian toxicity and low environmental persistence, their use is expected to continue to increase in years to come (Casida, 1980).

3.2 Historical development of pesticide regulations

As later drugs, food additives and toxic chemicals in general, the development of pesticides was paralleled or followed by a series of laws and

regulations in order to ensure their safe and effective use and to ensure that the health of the users and of the general population is not jeopardized. Pesticide regulation in the United States began with the Insecticide Act of 1910. This act prevented fraudulent efficacy claims and authorized the seizure and banning of compounds dangerous to human health. The increase in variety and use of synthetic pesticides in the 1940's created new problems and resulted in the passage in 1947 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) which deals with the marketing aspects of pesticides. FIFRA broadened the scope of products regulated, and established a products registration procedure administered by the U.S. Department of Agriculture (USDA). It also prohibited the distribution and sale of chemicals in interstate commerce unless known to be safe when used as directed and effective for the purpose shown on the label. In 1948, the Food and Drug Administration (FDA) began establishing safe levels of residue tolerances in foods. In 1954, the "Miller Amendment" of the FFDCA formalized the tolerance-setting procedure of FDA. USDA registered only pesticide uses that resulted in no residues or in residue levels declared safe by the FDA (Ross, 1980). In the late sixties, increased awareness of the effects of pesticides on fish, wildlife and the environment, contributed to the creation of the U. S. Environmental Protection Agency (USEPA) by Executive Order on December 2, 1970. In 1972 legislation was enacted for the Federal Environmental Pesticide Control Act (FEPCA). Among them are the World Health Organization (WHO) and the Food and Agricultural Organization (FAO). In 1962, these two organizations created the Codex Alimentarius Commission with the purpose of stabilizing standards for food products, which included a Codex Committee on Pesticide Residues (Ehler, 2006), (Ota, 2011).

4. History of toxins

A toxin is any chemical toxic substance that can be produced by a biological organism including animal, plant or microbe. Some toxins can also be produced by molecular biological techniques or by chemical synthesis. Although toxins are produced by self-replicating biological systems, they are themselves essentially chemical in nature and do not replicate. Examples are widespread in nature and range from envenomation following snake or insect bites to the production of toxins by bacteria following an infection. Clostridium botulinum produces botulinum toxin which causes a severe form of food poisoning from eating poorly canned food. It should be noted that although bacteria cause damage in the body via toxins, this is not the case with viruses which interfere directly with the internal metabolism of the body cells. “Toxin” is a word that has no

commonly accepted meaning in the scientific literature. It may then be important to understand how toxins are treated in the Biological and Chemical Weapons Conventions since, to differing degrees, these two international treaties are potential sources of such assistance (Olsnes, 2004). The 1972 Biological and Toxin Weapons Convention covers "toxins whatever their origin or method of production". It does not define toxins, but its *travaux preparatoires* show that the term is intended to mean toxic chemicals produced by living organisms. This states that: the term "toxin" means the toxic material of plants, animals, microorganisms, viruses, fungi, or infectious substances, or a recombinant molecule, whatever its origin or method of production, including-

A) Any poisonous substance or biological product that may be engineered as a result of biotechnology produced by a living organism

B) Any poisonous isomer or biological product, homolog, or derivative of such a substance

This includes all such chemicals, regardless of their origin or of their method of production, and regardless of whether they are produced in facilities, in munitions or elsewhere. So, although there is no consensus on the term among scientists, international law regards a wide range of substances as "toxins". They are high-molecular-weight proteins that can at present be produced on a significant scale only by the methods of industrial microbiology (Markowitz, 2018).

Although histamine might not itself be made into an effective weapon, the same cannot necessarily be said for other bioregulators. Indeed, now that large-scale production processes for biologically active peptides and similar substances are undergoing rapid commercial development, bioregulators and other toxins constitute a field rich in potential weapons as well as pharmaceuticals and in particular weapons of intense disabling or incapacitating power. It is fortunate, therefore, that this advance in biotechnology should have coincided with the adoption of the Chemical Weapons Convention, since it places its States Parties under the express obligation to ensure that bioregulators and other toxins, like all other toxic chemicals, are used only for the purposes that the Convention does not prohibit. A few plants besides Ricinus communis and Abrus precatorius contain two-chain toxins termed RIP II. Modeccin (from Modeca or Adenia digitata) is found in the roots of the plant growing in Southern Africa. Extracts of mistletoe have been used against a variety of diseases and are still in use in alternative medicine, in particular in treatment of cancer. As

these extracts are mostly preparations of varying composition, it is difficult to know to which compounds any beneficial or harmful effects should be ascribed (Guide, 2016), (Whitcup, 2019).

The main product obtained from the castor bean is castor oil. Intoxication by castor beans is not rare in countries where the plant is growing in abundance. At least 700 cases of human intoxication have been described. This represents a considerable amount of toxin, but being a protein, ricin is to a large extent destroyed in the intestinal tract. Therefore, the toxin is about 100 times more toxic when administered parenterally. Abrus seeds are very uniform in size, they are hard and durable and they have, therefore, played an important role in South-East Asia as weights (rati) for weighing gold and jewels. The seeds of Abrus precatorius have been used in Asian medicine since ancient times. In Arabic countries they are known as coq's eye and have been used as an aphrodiasiac. Extract from the seeds have been used in South America against eye diseases and was in 1882 introduced by deWecker into European medicine. For some years Jequiriti-infus was used against chronic eye diseases and particularly against trachoma. In many cases severe inflammation of the eye appeared (Jequiriti-ophthalmia) and even necrosis of the eye was observed. The complication was first related to alleged Jequiritibacilli, but it was later realised that the inflammation was of toxic origin and the treatment was abandoned (Balint, 1974).

5. Types of pesticides

5.1 Classification based on mode of entry

The ways pesticides come in contact with or enter the target are called modes of entry. These include systemic, contact, stomach poisons, fumigants, and repellents.

5.1.1 Systemic pesticides

Systemic pesticides are pesticides which are absorbed by plants or animals and transfer to untreated tissues. Systemic herbicide moves through the plant and can reach to untreated areas of leaves, stems or roots. They are capable in killing of weeds with partial spray coverage. They can effectively penetrate in the plant tissues and move through plant vascular system to kill specific pests. Some systemic insecticides are also applied and move through animals to control pests such as warble grubs, lice, or fleas. The movement of pesticides in plant tissues may be unidirectional or multidirectional. Some pesticides may only move in one direction either up or down within the plant while other pesticides may only move upwards in plants. If applied to the

root zone, it will travel throughout the plant, but if applied to the leaves it will not move throughout the plant. Furthermore, few pesticides are considered locally systemic and move only to a short distance in a plant from the point of contact. Examples of systemic pesticides include 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and glyphosate (Yadav and Devi, 2017).

5.1.2 Non-systemic (Contact) pesticides

The non-systemic pesticides are also called contact pesticides as it acts on target pests when they come in contact. Pesticides must come into physical contact with the pest to be effective. The pesticide enters the body of pests *via* their epidermis upon contact and causes death by poisoning. These pesticides do not necessarily penetrate the plant tissues and consequently not transported through the plant vascular system. Examples of contact pesticides are paraquat and diquat dibromide (Yadav and Devi, 2017).

5.1.3 Stomach poisoning and stomach toxicants

Stomach poisoning pesticide enters the pest's body through their mouth and digestive system and causes death by poisoning. Stomach poisons are acquired during feeding of pests, when they ingest the insecticide applied in the leaves and other parts of the plant. Stomach toxicants may also enter the body of insects through the mouth and digestive tract, where they are absorbed into the insect's body. This is more appropriate especially in vector control including bacteria, or their toxins, applied to the water where filter feeding mosquito or black fly larvae will consume the poison. These insecticides kill the vector by destroying the midgut (or stomach) of the larvae. Example: Malathion (Yadav and Devi, 2017).

5.1.4 Fumigants

Fumigants are such pesticides which acts or may kill the target pests by producing vapor. These pesticides form poisonous gases when applied. These pesticides in vapor form enter the body of pests *via* their tracheal system (respiratory) through spiracles and causes death by poisoning. Some of their active ingredients are liquids when packaged under high pressure but change to gases when they are released. Other active ingredients are volatile liquids when enclosed in an ordinary container and are not formulated under pressure. Fumigants are used to remove stored product pests from fruits, vegetables and grains. They are also very useful in controlling of pests in soil (Yadav and Devi, 2017).

5.2 Classification based on pesticide function and pest organism they kill

Under this method, pesticides are classified based on target pest's organism and pesticides are given specific names to reflect their activity. Pesticides are also classified according to their function. For examples: growth regulators, which stimulate or retard the growth of pests; defoliants, which cause plants to drop their leaves; desiccants, which speed the drying of plants for mechanical harvest or cause insects to dry out and die; repellents which repel pests; attractants, which attract pests, usually to a trap; and chemosterilants, which sterilize pests (Isbn, Publication and The, 2011).

Table 1.2: Pesticide classification by target pests

Type of pests	Target pests/Function	Example
Insecticides	Kill insects and other arthropods	Aldicarb
Fungicides	Kill fungi (including blights, mildews, molds, and rusts)	Azoxystrobin
Bactericides	Kill bacteria or acts against bacteria	Copper complexes
Herbicides	Kill weeds and other plants that grow where they are not wanted	Atrazine
Acaricides	Kill mites that feed on plants and animals	Bifenazate
Rodenticides	Control mice and other rodents	Warfarin
Algaecides	Control or kill growth of algae	Copper sulphate
Larvicides	Inhibits growth of larvae	Methoprene
Repellents	Repel pests by its taste or smell	Methiocarb
Desiccants	Act on plants by drying their tissues	Boric acid
Ovicides	Inhibits the growth of eggs of insects and mites	Benzoxazin
Virucides	Acts against viruses	Scytovirin

Also, there are pesticides that control more than one class of pests and may be considered in more than one pesticide class. Aldicarb, which is widely used in Florida citrus production, may be considered an acaricide, insecticide, or nematicide because it controls mites, insects, and nematodes, respectively (Isbn, Publication and The, 2011).

5.3 Classification based on chemical composition of pesticides

The most common and useful method of classifying pesticide is based on their chemical composition and nature of active ingredients. It is such kind of classification that gives the clue about the efficacy, physical and chemical properties of the respective pesticides. The information on chemical and physical characteristics of pesticides is very useful in determining the mode of application, precautions that need to be taken

during application and the application rates. Based on chemical composition, pesticides are classified into four main groups namely; organochlorines, organophosphorus, carbamates and pyrethrin and pyrethron. The chemical-based classification of pesticides is rather complex. However, some inorganic compound is also used as pesticides. Insecticides are important pesticides that can be further classified into several sub-classes. The sub-classification of insecticides is given in Fig 1.1 (Yadav and Devi, 2017), (Isbn, Publication and The, 2011).

5.3.1 Organochlorine

Organochlorines pesticides (also known as chlorinated hydrocarbons) are organic compounds attached with five or more chlorine atoms. They represent the one of the first group of pesticides ever synthesized and used in agriculture and in public health. Most of them were widely used as insecticides for the control of a wide range of insects, and they have a long-term residual effect in the environment (Eddleston *et al.*, 2002). These insecticides may disrupt the nervous system of the insects leading to convulsions and paralysis followed by eventual death. Most common examples of these pesticides includes: DDT, lindane, endosulfan, aldrin, dieldrin and chlordane. Though, the production and application of DDT was banned in most developed countries including United States many years ago, it is still being used in most tropical developing countries for vector control (particularly where malaria occurs) (Isbn, Publication and The, 2011).

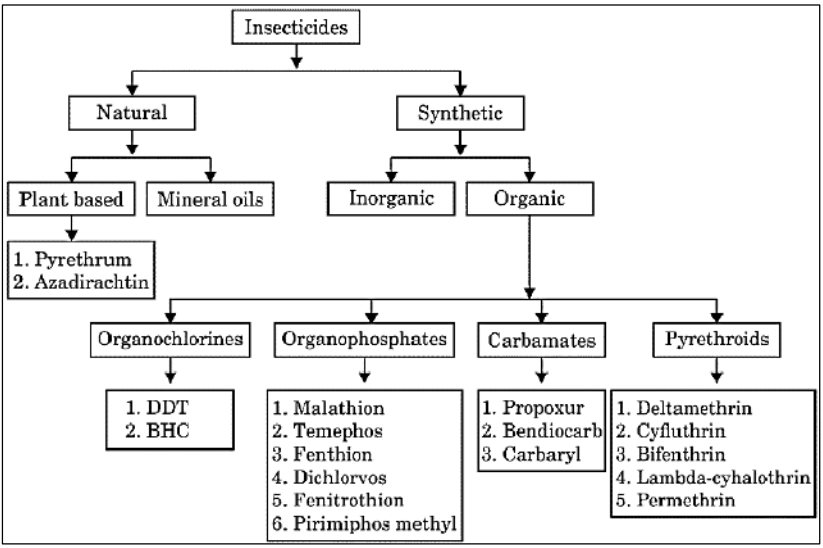

Fig 1.1: Flow chart of the types of Insecticides

5.3.2 Organophosphates

Organophosphate pesticides are considered to be one of the broad-spectrum pesticides which control wide range of pests due to their multiple functions. They are characterized with stomach poison, contact poison and fumigant poison leading to nerve poisons. These pesticides are also biodegradable, cause minimum environmental pollution and are slow pest resistance. Organophosphorus insecticides are more toxic to vertebrates and invertebrates as cholinesterase inhibitors leading to a permanent overlay of acetylcholine neurotransmitter across a synapse. As a result, nervous impulses fail to move across the synapse causing a rapid twitching of voluntary muscles, hence, leading to paralysis and death. Some of the widely used organophosphorus insecticides include parathion, malathion, diazinon and glyphosate (Isbn, Publication and The, 2011).

5.3.3 Synthetic pyrethroids

Synthetic pyrethroid pesticides are group of organic pesticide that can be synthesized by duplicating the structure of natural pyrethrins. Relatively, they are more stable with longer residual effects than natural pyrethrins. Pyrethrins are grinded to produce active components. The major active components are pyrethrin I and pyrethrin II plus smaller amounts of the related cinerins and jasmolins. Synthetic-pyrethroid pesticides are highly toxic to insects and fish but slightly toxic to mammals and birds. Most of synthetic insecticides are non-persistent, and got broken easily on exposure to light. They are considered to be amongst the safest insecticides for use in food. Cypermethrin and Permethrin are the most used synthetic pyrethroid pesticides (Isbn, Publication and The, 2011).

5.4 Classification based on sources of origin

Pesticide is a chemical or biological substance that aims to destroy the pests or prevent the damage caused by pests. Based on sources of origin, pesticide may be classified into chemical pesticide and bio-pesticides. The main benefits of using biological pesticides are host specificity. They act on the target pest only and strongly linked organisms, whereas chemical pesticides are usually of wide range which affects large group of non-target organisms. Bio-pesticides are usually environmentally friendly as they are less toxic, decomposed easily and required in small quantities. Chemical pesticides cause major environmental pollution as they are quite toxic and not always biodegradable. Another important advantage of using bio-pesticide is the fact that they are less susceptible to genetic modification in plant populations. Chemical pesticides are further divided into

organochlorine, organophosphate, carbamate and pyrethroids and are discussed already in previous section. Bio-pesticides group of pesticides derived from natural materials such as animal, plant and microorganism. They are classified into three groups (Yadav and Devi, 2017).

5.4.1 Microbial pesticides

The active ingredient in microbial pesticides is microorganism such as bacterium, fungus or protozoan. These pesticides kill insects either by toxins released by microbial organisms, or by infection by the organisms. Two most common pesticides that fit within this group include the bacterial toxin produced by *Bacillus thuringiensis* (*Bti*), and the live bacteria, *Bacillus sphaericus* (*Bs*). The mode of action generally is producing a protein that binds to the larval gut receptor which starves the larvae. These two bacterial toxins are used against mosquito larvae and black fly larvae. Most microbial pesticides are more selective than biochemical pesticides (Yadav and Devi, 2017).

5.4.2 Plant incorporated protectants

These groups of pesticides are produced by plants naturally. Also, the gene necessary for production of pesticide is introduced into the plant through genetic engineering. Hence, the pesticide then produced by such plant and the genetic material introduced are together defined as plant incorporated protectants (PIPs) (Yadav and Devi, 2017).

5.4.3 Biochemical pesticides

The third class is Biochemical pesticides which include natural materials that have nontoxic mechanisms to control pests. Examples of Biochemical pesticides are insect sex pheromones (act by interfering in mating), a range of aromatic plant extracts (work by attracting insect pests into traps) (Yadav and Devi, 2017).

5.5 Based on toxicity of pesticides

Depending on the health risk associated with pesticides and toxic behaviour of pesticides. The World Health Organization (WHO) classified them into four categories (WHO, 2008).

Table 1.3: WHO classifications of pesticides

WHO class	Toxicity level	LD50 for the rat (mg/kg body weight) Oral Dermal	Examples
Class Ia	Extremely hazardous	<5 <50	Parathion, Dieldrin
Class Ib	Highly hazardous	5-50 50-200	Eldrin, Dichlorvos

Class II	Moderately hazardous	50-2000 200-2000	DDT, Chlordane
Class III	Slightly hazardous	>2000 >2000	Malathion
Class IV	Unlikely to present acute hazard in normal use	5000	Carbetamide, Cycloprothrin

6. Types of toxins

6.1 Alkaloids

Alkaloids are the bitter components of plants found widely in nature and frequently have pharmacological properties. Mostly acting as secondary plant metabolites, alkaloids are often basic nitrogen-containing compounds able to form salts with acid. Alkaloids have been isolated from the roots, seeds, leaves, or bark of some members of at least 40% of plant families. Families being particularly rich in alkaloids include Amaryllidaceae, Compositae, Leguminosae, Liliaceae, Papaveraceae and Solanaceae. Alkaloids Found in Plants and in Common Foods: One type of alkaloid widely found in the plant kingdom is the pyrrolizidine alkaloids. These are chemicals found in as many as 6000 plant species or 3% of flowering plants. Plants containing these compounds are distributed in all climatic regions of the world. Ingestion of plants containing pyrrolizidine alkaloids are usually through contaminated crops since plants containing these alkaloids may grow as weeds in food crops such as wheat or corn and may be harvested with the grain. Another source of oral exposure to this group of toxins may be via intake of herbal foods and preparations containing these toxins. Prevention of poisoning can be achieved by reducing ingestion of alkaloid-containing foods and herbal preparations, and by applying effective measures in agriculture to reduce contamination of pyrrolizidine containing plants in food crops. Other plant alkaloids such as the glycoalkaloids are found in common food plants acting like a natural pesticide against common pests. Common examples include solanine and tomatine. Solanine is an alkaloid present in small amounts in potatoes while tomatine is found in tomatoes (Studies, 2007), (Food, 2001), (World Health Organization. *et al.*, 1989).

6.2 Glycoalkaloids

Glycoalkaloids consist of a steroidal alkaloid coupled to one or more monosaccharides. All members of the botanical family solanacea produce glycoalkaloid toxins. In potatoes, the major glycoalkaloids are α-solanine and α-chaconine. They are formed in the parenchyma cells of the periderm and the cortex of the tubers. These chemicals are toxic to insects and animals and serve to protect the plant from predators. They are normally present in

all potato tubers in small amounts and are concentrated in the skin of the potato and also in the areas of high metabolic activity such as the eye regions. Its level is increased in greened potatoes or blighted potatoes, and can reach very high levels in the sprouts. This toxin is not decreased by any of washing, soaking or cooking. Cooked potatoes that contain high level of solanine have a bitter taste and cause a burning sensation in the throat (Maga, 1980).

Toxicity: The amount of glycoalkaloids usually found in edible plants that are fresh and undamaged do not normally cause toxicity. However, glycoalkaloids can produce toxic effects at higher doses (Jadhav, Sharma and Salunkhe, 1981).

Acute toxicity: The greatest concern for glycoalkaloid toxicity is its acute toxicity. There have been many reported cases of human poisonings (sometimes fatal) due to the ingestion of greened, damaged or sprouted potatoes as a consequence of high levels of glycoalkaloids. Acute toxicity syndromes in humans have been observed at glycoalkaloid levels of more than 2.8 mg/kg bodyweight. Onset of symptoms has ranged from minutes to 2 days after ingestion of toxic potatoes, with longer incubation periods generally associated with the more severe cases. For low grade poisoning, symptoms are acute gastrointestinal upset with diarrhoea, vomiting and severe abdominal pain. The *solanum* alkaloids have strong anticholinesterase activity on the central nervous system. Glycoalkaloids also have saponin-like properties and can disrupt membrane function in the gastrointestinal tract leading to haemorrhagic damage. This damage can be severe enough to cause death with the extent of necrosis far outweighing the inhibitory effects on acetylcholinesterase activity (Jadhav, Sharma and Salunkhe, 1981).

Chronic toxicity: Because of the lack of chronic toxicity data, an adequate no-observed adverse effect level for potato glycoalkaloids has not been assessed, and a tolerable daily intake for humans has not been determined (Patil *et al.*, 1972).

JECFA evaluation: JECFA considered that, despite the long history of consumption of plants containing glycoalkaloids, the available epidemiological and experimental data from human and laboratory animal studies did not permit the determination of a safe level of intake. Based on the available human data, an intake of total glycoalkaloids of 3-6 mg per kg body weight is considered by JECFA a potentially lethal dose for humans, and total glycoalkaloids of more than 1 to 3 mg per kg body weight is considered a toxic dose for humans. Children may be more sensitive than

adults. Other factors may be present in suspect potatoes and modulate the toxicity of the steroid glycoalkaloids (Baker, Keeler and Gaffield, 1988), (JECFA, 1993).

6.3 Cyanogenic glycosides

Cyanogenic glycosides occur in at least 2000 plant species, of which a number of species are used as food. They are amino-acid-derived constituents of plants produced as secondary metabolites. There are approximately 25 cyanogenic glycosides known. Different kinds of cyanogenic glycosides may be found in different cyanogenic food plants, e.g. taxiphyllin in bamboo shoots, linamarin in cassava (Harvey M.H., Morris B.A., McMillan M., 1979).

Occurrence: Important staple foods for some parts of the world (such as 12 cassava and sorghum) contain cyanogenic glycosides. Other edible plants containing cyanogenic glycosides include bamboo shoot, flaxseeds, and seeds of stone fruits such as apricot and peach, seeds of peas and beans such as lima beans, and shell of soya beans. Other food products that may contain cyanogenic glycosides include some food ingredients with flavouring properties such as ground almonds powder or paste, marzipan, stone fruit preserves (cherry, plum, apricot, peach) and stone fruit juices, and alcoholic drinks made from stone fruits. These foods therefore represent potential sources of hydrogen cyanide (Bolarinwa *et al.*, 2016).

Toxicity: Toxicity of cyanogenic glycosides-containing plant is due to the cyanide produced on ingestion. The plant species that produce cyanogenic glycosides usually also has a corresponding hydrolytic enzyme (β-glucosidase). In the presence of water, the non-toxic cyanogenic glycosides are hydrolysed by the enzyme producing cyanohydrins which quickly decompose to the toxic hydrogen cyanide. In this way, cyanogenic plants are protected against predators. Cyanogenic glycosides, cyanohydrins and hydrogen cyanide are collectively known as cyanogens (Bolarinwa *et al.*, 2016).

Acute toxicity: In humans, the clinical signs of acute cyanide intoxication can include: rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea, mental confusion, stupor, cyanosis with twitching and convulsions followed by terminal coma. Death due to cyanide poisoning can occur when the cyanide level exceeds the limit an individual is able to detoxify. The likelihood of cyanide intoxication from consumption of cyanide-containing food is dependent on body weight. For example, it is possible that a child or person

of smaller body weight would not be able to detoxify the cyanide resultant from a meal of inadequately prepared cassava or bamboo shoots. The acute lethal dose of hydrogen cyanide for humans is reported to be 0.5-3.5 mg/kg body weight. Approximately 50-60 mg of free cyanide constitutes a lethal dose for an adult man (Bolarinwa *et al.*, 2016).

Chronic toxicity: It is uncommon for dietary cyanide intake to cause chronic diseases. Adverse effects may, however, accompany individuals who have underlying dietary deficiency such as inadequate protein and/or iodine intake. For example, individuals already suffering from goiter and cretinism due to iodine deficiency may sometimes experience exacerbation of the condition following continuous dietary cyanide exposure. Neurological diseases such as konzo and tropical ataxic neuropathy (TAN) have been reported in individuals with inadequate dietary intake of protein and/or iodine where cassava is consumed as a staple food. Konzo is an upper motor neuron disease characterised by irreversible but non-progressive symmetric spastic paraparesis with an abrupt onset, whereas tropical ataxic neuropathy (TAN) is a severe neurological syndrome with clinical symptoms of optical atrophy, angular stomatitis, sensory gait ataxia, and neurosensory deafness (Studies, 2007), (Bolarinwa *et al.*, 2016).

6.4 Aflatoxins

This type of toxins is produced by the molds *Aspergillus flavus* and *A. parasiticus*, which commonly contaminate food grains before and after harvest. Their toxicity was recognized in the 1960s, and it was later appreciated that they are a significant health problem for domestic animals and humans. The toxins are stable and survive cooking. Attention has focused on chronic exposure and illness from oral intake, although there have also been acute effect. This review concentrates on AFB1, the most toxic of the aflatoxin family, although the actual mixture Iraq weaponized is unknown. Aflatoxins show delayed acute toxicity (eight hours to several days) because most require metabolic activation. However, most interest in aflatoxins arises from their carcinogenicity. They are implicated in the genesis of hepatocellular carcinoma, which is prevalent in tropical regions (Payne, 1992).

Chemical characteristics: The top four-B1, B2, G and G2 are the four most commonly mentioned. All occur naturally. The “B” and “G” designations of these toxins relate to the fluorescence colour in ultraviolet light (blue or green) while the subscripts refer to chromatographic mobility (Soini *et al.*, 1996).

Mechanisms of action: The concern about aflatoxin producing cancer in humans and animals has produced an extensive literature on the metabolism of aflatoxin and the biochemical reactions of the metabolites. Active metabolites act on several cell structures (e.g., mitochondria, lysosomes, endoplasmic reticulum), but the cancer concern has focused attention on the effects on the nucleus and DNA. Activated AFB1 attacks nucleic acids with the formation of adducts that can act like point mutations, damaging DNA and impairing RNA and protein synthesis. Proteins, including receptors and those with important intracellular functions, may also be non-specifically but irreversibly bound by toxins, producing diverse loss of function. Acute mycotoxin injury inhibits cellular energy production. The aflatoxins act on the electron transport system, interfering with the cytochrome system, depleting ATP, inhibiting ATPase, and causing mitochondrial swelling. The effect of aflatoxin on the electron transport system may not require activation of the toxin. Recent studies draw attention to mitochondrial disease and injury, producing liver failure and associated disorders with brain and liver injury. Carbohydrate and lipid metabolism are impaired, and hepatic glycogen stores are depleted, with a secondary rise in blood sugar. Lipids accumulate in the liver and fatty oxidation decreases, perhaps secondary to mitochondrial injury. These effects occur at levels lower than those producing RNA and growth effects (Shotwell *et al.*, 1966), (McLean and Dutton, 1995).

Activation: Metabolic activation is required to produce toxicity from AFB1. After crossing the cell membrane, the molecule is activated by microsomal mixed-function oxidases involving the cytochrome P450 enzymes and nicotinamide adenine dinucleotide phosphate reductase (NADPH) and oxygen. The active and toxic AFB1 8,9-epoxide (Figure 4.3) is the result. This active molecule has a short half-life and binds to DNA and other structures in the endoplasmic reticulum. Other NADPH reactions can reversibly produce aflatoxicol, which can serve as a sink or source of AFB1 in the cell. The microsomal monoxygenase system may transform AFB1 into more polar molecules, such as AFM1, Q1 or P1, which can be eliminated by liver cells. The situation is complex and there are other concepts of toxicity involving more indirect mechanisms associated with membrane actions involving lipid peroxidases and aldehydes (Eaton and Gallagher, 1994).

Detoxification: Aflatoxins are also detoxified by mechanisms that deal with xenobiotics-leading to conjugation with glucuronic acid, sulphates, or glutathione. The major route for AFB1 detoxification is conjugation of the epoxide with glutathione (through glutathione S transferase) and subsequent excretion in bile (Roy, Sajan and Kulkarni, 1995).

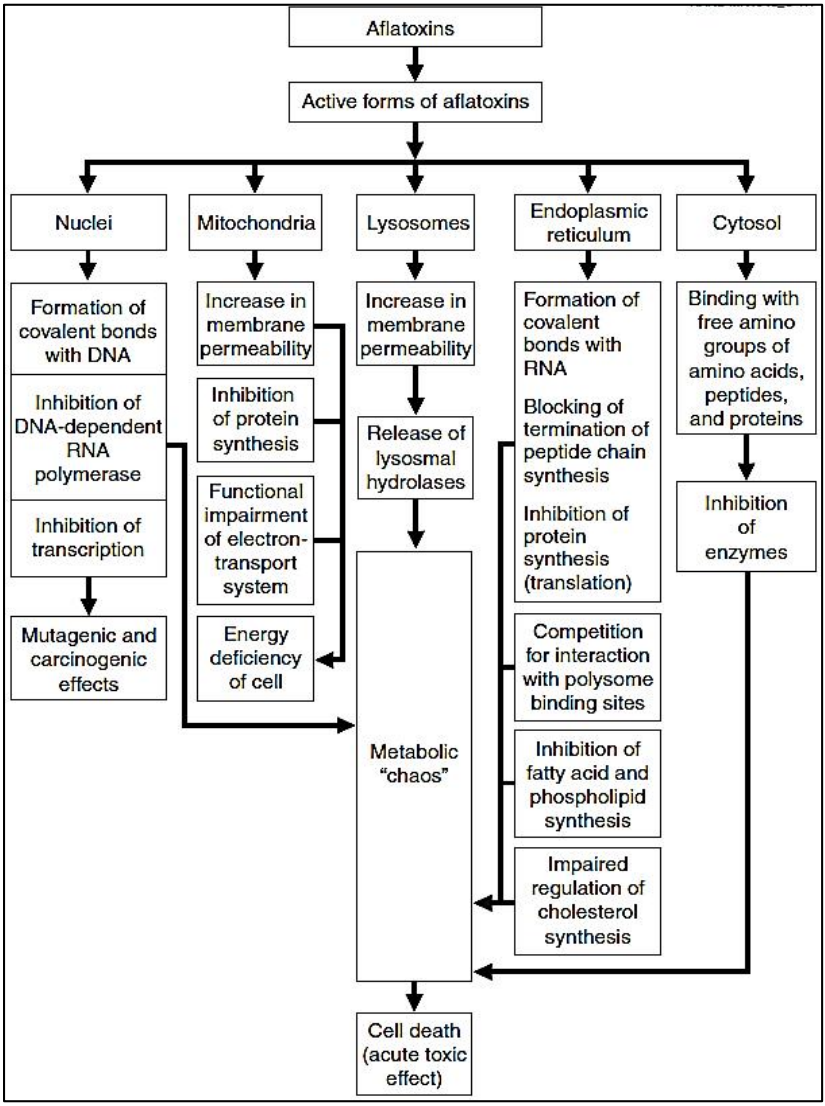

Fig 1.2: Mechanism of Effects of Aflatoxins on Cell

This means that toxicity may vary depending on intracellular glutathione stores in various tissues, which can vary considerably with circadian effects or depletion by other factors diet, smoking, alcohol, and medications. Other aflatoxins Appear to be primarily eliminated via glucuronide or sulphate conjugation. Various species differences in sensitivity to aflatoxins may reflect differences in detoxification mechanisms (Tsutsumi and Miyazaki, 1994).

7. Benefits of pesticides and toxins

The primary benefits are known as consequences of the direct pesticides' effects such as protection of people, animal and crop health and protection of recreational turf. The secondary benefits arise from primary and these are the less immediate, less intuitively obvious or longer-term consequences. Table 1.4 summarizes effects, primary and secondary benefits, and their interactions. The interplay between negative effects and benefits is complex and not easy to follow always (Cooper and Dobson, 2007).

Table 1.4: The complexity of the effects, primary and secondary benefits of pesticides

Primary benefits	Secondary benefits
1. Controlling pests and plant disease vectors • Improved crop/livestock quality • Reduced fuel use for weeding • Reduced soil disturbance • Invasive species controlled	Community benefits • Nutritional and health improved • Food safety/security • Life expectancy increased • Reduced maintenance costs
2. Controlling disease vectors and nuisances organisms • Human lives saved • Human disturbance reduced • Animal suffering reduced • Increased livestock quality	National benefits • National agricultural economy • Increased export revenues • Reduced soil erosion/moisture loss
3. Prevent or control of organisms that harm other human activities and structures • Tree/buss/leaf hazards prevented • Recreational turf protected • Wooden structures protected	Global benefits • Less pressure on uncropped land • Fewer pest introductions elsewhere • International tourism revenue

Over last 60 years, farmers achieved significant progress in production of foodstuff by using pesticides. They have done this principally to prevent or reduce agricultural losses due to activity of pests which resulted in improved yield and greater availability of food, at a reasonable price and over all seasons. By the use of pesticides in agriculture, the productivity has increased dramatically in most countries. For example, wheat yields in the United Kingdom, corn yields in the USA, and total yields in the Russia and other countries were enhanced enormously. It has been long believed that diets containing fresh fruits and vegetables far outweigh potential risks from eating very low residues of pesticides in crops. Improved nutrition and reduced drudgery both improve the quality of life and longevity. Improved medical care and drug treatments along with hygiene have played a significant role in extending lives, but the value of nutritious, safe and

affordable food should not be underestimated as a health promoter that increases life expectancy (Bowles and Webster, 1995), (Johnson, 2010). Control of wide range of human and livestock disease vectors thus reducing the number of infected individuals and deaths accompanied by prevention of international disease spread is among critical obvious benefits of broad pesticide use. Killing of vectors is the most effective method to struggle them. According to the World Health Organization without access to chemical control methods life will be unacceptably dangerous for a large proportion of mankind. Pesticides play an important role in destruction of various organisms which have a negative impact on human activities, infrastructure and the materials of everyday life. In many specific sectors of human activity, pesticides are used to control unwanted organisms, such as prevention of accelerated corrosion of metal constructions, maintain the turf on sport pitches, cricket grounds and golf courses, helping to facilitate a hugely popular pastime that provides fresh air and exercise for millions of people around the world in domestic and ornamental gardening etc (Sekhar, Banji and Rsnakk, 2012), (Kapoor, 2010).

8. Hazards of Pesticides and Toxins

Pesticides have improved the standard of human health by controlling vector-borne diseases, however, their long term and indiscriminate use has resulted in serious health effects. Human beings especially infants and children are highly vulnerable to deleterious effects of pesticides due to the non-specific nature and inadequate application of pesticides. As the pesticide use has increased over the past few decades, the likelihood of exposure to these chemicals has also increased considerably. Pesticides enter the human body through ingestion, inhalation or penetration via skin. But the majority of people get affected via the intake of pesticide contaminated food. After crossing several barriers, they ultimately reach human tissues or storage compartments. Although human bodies have mechanisms for the excretion of toxins, however, in some cases, it retains them through absorption in the circulatory system (Van Der Werf, 1996), (Hakeem, Akhtar and Abdullah, 2016). Toxic effects are produced when the concentration of pesticide in the body increases far more than its initial concentration in the environment (Aktar, Sengupta and Chowdhury, 2009).

- Pesticide exposure can cause a range of neurological health effects such as loss of coordination and memory, reduced visual ability and reduced motor signalling
- Long-term pesticide exposure damages the immune system and can cause hypersensitivity, asthma and allergies

- Pesticide residues have been found in the bloodstream of cancer patients compared to normal individuals. Pesticides have been associated with leukaemia, brain cancer, lymphoma, cancer of the breast, prostate, ovaries and teste.
- The presence of pesticides in the body for a longer time also affects reproductive capabilities by altering the levels of male and female reproductive hormones. Consequently, it results in stillbirth, birth defects, spontaneous abortion and infertility
- Lon-term exposure to pesticide also damages liver, lungs, kidneys and may cause blood diseases

After application, pesticides can be taken up by target organisms, degraded, or transported to the groundwater; they can also enter the surface water bodies, volatilize to atmosphere, or reach non-target organisms by ingestion, for example. The physical and chemical properties of the pesticide, soil, site conditions, and management practices influence the behaviour and fate of pesticides. Adsorption depends on the chemical and also on the soil type. The volatility of pesticides indicates their tendency to become a gas; the higher the volatility, the larger their loss to the atmosphere. Environmental conditions such as temperature and humidity impact volatility, which can occur from soil, plants, or surface water, and may continue for several days or weeks after pesticide application. In the atmosphere, the chemicals can be transported over long distances. Subsequent atmospheric deposition can contribute to surface water pollution. The degradation of pesticides is determining the behaviour and the fate of these compounds in the environment. Degradation can occur by photodecomposition, microorganisms, and a variety of chemical and physical reactions. Pesticides with low biodegradation are called persistent; they can remain in the environment for a long time (Culliney, Pimentel and Pimentel, 1992).

Toxic agent includes substances that cause both acute and latent effects with both short and long duration. Chronic exposure to low doses of toxic agents has been increasingly recognised as a cause of illness and is the subject of study of clinical, occupational and environmental toxicology. Toxic trauma, however, is the result of acute exposure to hazardous substances that cause life-threatening, seriously disabling acute effects and the intermediate effects that follow. Such life-threatening injury requires early recognition and life-support measures, as well as antidote and other supportive therapy. Toxic agents which affect the peripheral nervous system do so through effects on the synapses of both the autonomic and voluntary

systems and by direct actions on peripheral nerves. Military examples include nerve agents and toxins. Civil examples include pesticides and many marine and animal toxins. A wide range of toxic agents, both CW and TIC, have primary effects on the respiratory system, including airways and breathing. CW agents acting on the respiratory system include the lung-damaging agents, a wide range of toxic agents, both CW and TIC, chlorine, phosgene, organophosphate compounds and also inhaled vesicant agents such as mustard gas at high ambient temperatures. A number of toxic agents have a primary effect on the heart and blood vessels either directly or through disruption of nervous control. Examples include OP and plant alkaloids such as atropine. These agents can cause life-threatening cardiac dysrhythmia with, again, terminal failure of cellular oxygen delivery. Military examples include nerve agents and toxins. Civil examples include pesticides and aliphatic hydrocarbons. In addition to TIC, there are a wide range of animal toxins, particularly from snakes which produce co-agulopathies (UNL Environmental Health and Safety, 2002).

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Chapter - 6
Studies on the Relationship Between Breathing Habit and Total RBC Count and Haemoglobin Percent in Some Freshwater Fishes

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Chapter - 6

Studies on the Relationship Between Breathing Habit and Total RBC Count and Haemoglobin Percent in Some Freshwater Fishes

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Abstract

Our present study focuses on the relationship between breathing habits and total RBC count and haemoglobin (Hb) present in some freshwater fishes, mainly air-breathing and water-breathing fishes. In the study, some accessory respiratory organs were observed like air-sac, labyrinthiform organs, arborescent organ in *Amphipnous cuchia*, *Anabas testudineus* and *Heteropneustes fossilis* respectively. The study also divulges that RBC count of *Cyprinus carpio* is approximately (2.24±0.006) x 10^6 per mm^3 whereas in case of air-breathing fishes the value ranges from (3.36±0.008) x 10^6 per mm^3 to (~ 6.68±0.008) x 10^6 per mm^3. Likewise, the RBC counts if *C. carpio* is found to be 8.2±0.021 g/dl whereas in case of air-breathing fishes it ranges from 14.8±0.014 g/dl to 18.65±0.017 g/dl.

Keywords: haematology, haemoglobin, accessory respiratory organs, air-sac, labyrinthiform organs, arborescent organs, air-breathing fishes, water-breathing fish

Introduction

Fishes are aquatic organisms but in course of evolution organs of aerial respiration developed in some fishes. The organs of aerial respiration may be originally developed for increasingly the area for aquatic respiration and later, under adverse conditions of stagnation and draught, through "Change of function" become adapted for aerial respiration (B.K. Das 1927, S.L. Hora 1935) of aerial respiration *Amphipnous cuchia* is one where air-chambers are present (B.K. Das, 1927; S.L. Hora, 1935; Munshi and Singh, 1968). They are capable of performing aquatic respiration for a relatively much shorter period (B.K. Das, 1927; S.L. Hora, 1935) as their gills are reduced (B.K. Das, 1927; S.L. Hora, 1935; Munshi and Singh, 1968; Hughes and Munshi, 1973). In *Heteropneustes fossilis* both the secondary respiratory organs and gills are well developed. So, in spite of being air-breathing fish they can

respire even when immersed in water (B.K. Das, 1927; S.L. Hora, 1935). In *Clarias batrachus*, the organs of aerial respiration could not by themselves sustain the life of the fish for more than 6 to 8 hours (B.K. Das, 1927; S.L. Hora, 1935). The snakeheads, because of labyrinth-like suprabranchial organs in the upper gill-chamber, breathe atmospheric O_2 concentrations (J.S.D Munshi *et al.*, 1985). Due to labyrinth-like structure snakeheads can live outside of water for a considerable period of time (J.S.D. Munshi and G.M. Hughes, 1992.

Blood is an important component of respiratory system in fishes. One third of the total blood volume in fishes consists of blood cells; the rest is fluid plasma (R.W. Wedemeyer and M.C. Warner, 1976). In spite of systematic diversity all fishes possess two main types of blood cells-RBC and WBC, a property shared by the land-living vertebrates, which are derived from early fish like ancestors (Nelson J.S., 1984). Inside RBC, haemoglobin is present. O_2-tension of the blood is reduced due to binding of O_2-molecules by haemoglobin molecules within erythrocytes and carried in this form to the tissue and the binding capacity of O_2 per unit volume of blood depends on number of RBC, concentration of haemoglobin within RBC prevailing O_2 partial pressure and O_2 binding affinity of haemoglobin molecules (Randall D. Burggren W., K. French, 2002; Rantin F.T. Kalinin, A.L. Glass M.L., 2007). Blood parameters are considered as pathophysiological indicator of the whole body (Adhikari S. Sarkar B. *et al.*, 2004). There should be standard value of RBC count and haemoglobin content in all animals. There is a numerical variation of RBC among mammals, birds and reptiles, where mammals have more RBC than birds and birds in turn have more RBC than reptiles and so on (Hawkey CM *et al.*, 1991). In controlled condition RBC count in water breathing fish *Labeo rohita* is $1.79x10^6$ per mm^3 of blood (Binu Kumari and Vasanthi, 2013) in *Cyprinus carpio* the Rbc count is $(1.42\pm0.05) \times 10^6$ per mm^3 (Zeynab Abedi *et al.*, 2013). In controlled condition RBC count in air-breathing fishes *Channa punctatus* and *Channa striata* ranges form $3.29x10^6$ to $3.24x10^6$ per mm^3 of blood (*K. Malathi, A. kannathasan* and *Rajendran, 2012). Among Mystus tengara, Heteropneustes fossilis, Labeo bata, Labeo cursa, Notopterus notopterus, Mastacembelus pancalus* and *Channa punctatus*, RBC count ranges from $2.74x10^6$ to $4.03x10^6$ per mm^3 in controlled conditions (K.A. Goel, B.P. Mishra, Kalpana Gupta and Sandhya Wadhwa, 1984). It is observed that in active predacious and pelagic fishes RBC count is comparatively higher than in slow, sedentary and benthic species of fishes (Larson *et al.*, 1976; K.A. Goel, B.P. Mishra, Kalpana Gupta and S. Wadhwa, 1984).

It is observed that like RBC, hemoglobin concentration is also an important blood parameter. In controlled condition haemoglobin concentration of water-breathing fish *Labeo rohita* is 3.10 g/dl of blood (S. Binukumari and Vasanthi, 2013). In controlled condition haemoglobin context in air-breathing fish *Heteropneustes fossilis* is 16.10 g/dl of blood (K.A. Goel, B.P. Mishra, Kalpana Gupta and S. Wadhwa, 1984), in *Channa punctatus* and *Channa striata* haemoglobin content ranges from 9.37 to 10.84 g/dl of blood (Malathi K., Kannathasan A and Rajendran K., 2012). In *Cyprinus carpio* haemoglobin content is (8.76±0.68 g/dl) (Zyenab Abedi *et al*., 2013). It is observed that in active, predacious and pelagic fishes haemoglobin content is comparatively higher than the slow moving, sedentary and benthic species (Larsson *et al*., 1976; K.A. Goel, B.P. Mishra, Kalpana Gupta and S. Wadhwa, 1984). Changes in physio-chemical parameters may be reflected haematological parameters of fishes (Hickey, C.R. Jr 1982). The values of blood parameters depend on season and active or slow movement of fishes (R. Yasmin, B.N. Pandey and A. Yasmin, 1993) and also depends on sex, body size, season and age of fishes (R.P. Bhagat and Banerjee, 1986). On the other hand, the physiological properties of blood are very sensitive to environmental changes (G.M. Hughes and J. Nemesok, 1988). In *Channa punctatus*, it is observed that as temperature increases Hb content increases and as temperature decreases Hb content also decreases (Jagtap Ashwini, Ravichandra, 2012).

Methods and materials

Collection and culture of model fishes

Four fishes of four different species (irrespective of sex and almost of some age) were collected in the month of April, 2014 form the ponds and swamps of North Guwahati [Dist. Kamrup(R), Assam] and were maintained in aquaria in the laboratory of Zoology Department, B. Borooah College, Guwahati-07. The fishes were fed with earthworm and pieces of properly cleaned intestine of goat and were acclimatized to the laboratory conditions for 10 days.

Collection of blood sample

After proper acclimatization of the fishes in laboratory conditions, i.e. after about 10 days from the date of capture, blood samples were collected from caudal region after piercing the caudal peduncles. Then the total number of RBC and Haemoglobin content were calculated by suitable methods.

Dissection of respiratory organs

After collection of blood the fish were dissected to observe their accessory respiratory organs.

Determination of haemoglobin content

The Haemoglobin content of different fishes were determined by the following procedure-

- A large drop of blood was taken out from the caudal peduncle.
- The blood was sucked into the haemoglobin pipette up to the mark 20 cubic mm.
- N/10 HCl was taken in the haemoglobin tube.
- To it, the blood from haemoglobin pipette was added and it was blown back into the tube in order to wash the blood of the pipette.
- The contents of the haemoglobin tubes were stirred with the stirrer.
- It was allowed to stand for 10-20 minutes.
- The tube was in the black plastic box.
- N/10 was added to the liquid content drop wise till the colour in the Hb tube matched exactly with that of the standard known plates.
- Dilution of blood was taken as reading in terms of gram percentage.

Total count of RBC

The total RBC of the fishes were counted by the following procedure –

- The RBC tube was cleaved and rinsed with 9% sodium citrate solution to avoid coagulation of blood in the pipette
- A large drop of blood was taken out of the caudal peduncle using a pricking needle
- The tip of RBC pipette was put on the drop of blood and was sucked into the pipette up to 0.5 marks
- RBC diluting fluid was sucked into the pipette up to 101 marks
- The open end of the pipette was closed by the index finger and the pipette was rotated 3-4 minutes to mix the blood and dilution fluid thoroughly
- The fluid from lower stem of the pipette was blown out up to 1.0 mark
- The pipette was hold on the slide at an angle of 45° and a drop of diluted blood was put on the surface of each counting chamber

- The chambers were covered by a cover slip (entry of air bubbles must be avoided)
- Blood cells were allowed to settle keeping the slide undisturbed for 5 minutes
- Counting of RBC is done under microscope by focusing the central squares

Observation and Results

A. Observation and results of dissection

All the air-breather fishes (*Amphipnous cuchia*, *Anabas testudineus*, and *Heteropneustes fossilis*) were dissected to observe their common and accessory respiratory organs and the following structures were observed-

1. Accessory respiratory organs in *Amphipnous cuchia*

After dissection, a pair of air-sacs was observed situated on the lateral sides of the head of *cuchia*. These are the accessory respiratory organs in *cuchia*.

2. Accessory respiratory organs in *Anabas testudineus*

In *Anabas*, after dissection special air-chambers were observed above the gills, where there three concentrically folded bony laminac were observed, which are known as labyrinthiform organs. These are accessory respiratory organs in *Anabas*.

3. Accessory respiratory organs in *Heteropneustes fossilis*

After dissection, in *H. fossilis*, a pair of supra-branchial organs were found each lying on one side and divided into two parts, a highly branched arborescent organ formed from second and fourth branchial arches, and a vascular sac of the branchial chamber which encloses the arborescent organs. These are the accessory respiratory organs in *Heteropneustes fossilis.*

B. Observation and result of haematology

The different results of RBC count and haemoglobin estimation in different air-breathing and water breathing fishes are given in the following table:

Table 1: Showing haematological values of air-breathing and water-breathing fishes

	Water-breathing fish	**Air-breathing Fishes**		
	Cyprinus carpio	***Amphipnous cuchia***	***Anabas testudineus***	***Heteropneustes fossilis***
Total RBC Count (RBC x 10^6 per mm^3)	2.24±0.006	6.68±0.008	3.88±0.009	3.36±0.008
Haemoglobin content (g/dl)	8.2±0.021	18.65±0.017	15.5±0.014	14.8±0.014

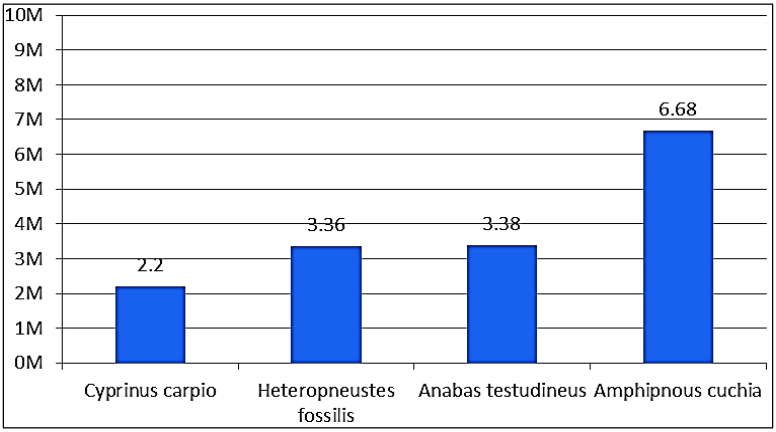
Analysis of the table

From the above table, it is observed that the total RBC count of water-breathing fish *Cyprinus carpio* is $(2.24\pm0.006)x10^6$ per mm^3, whereas in case of the air-breathing fishes *Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis* the total RBC counts are $(6.68\pm0.008)x10^6$ per mm^3, $(3.38\pm0.009)x10^6$ per mm^3 and $(3.36\pm0.008)x10^6$ per mm^3 respectively. From these data it is quite clear that the total RBC is more in air-breathing fishes than the water-breathing fishes.

Similarly, from the above table it is observed that the haemoglobin count of water-breathing fish *Cyprinus carpio* is (8.2±0.021) g/dl, whereas in case of the air-breathing fishes *Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis* the haemoglobin contents are (18.65±0.017) g/dl, (15.5±0.018) g/dl, (14.8 ± 0.014) g/dl respectively. From these data, it is quite clear that the Hb content is more in air-breathing fishes than the water-breathing fishes.

From above table the following graphs can be drawn:

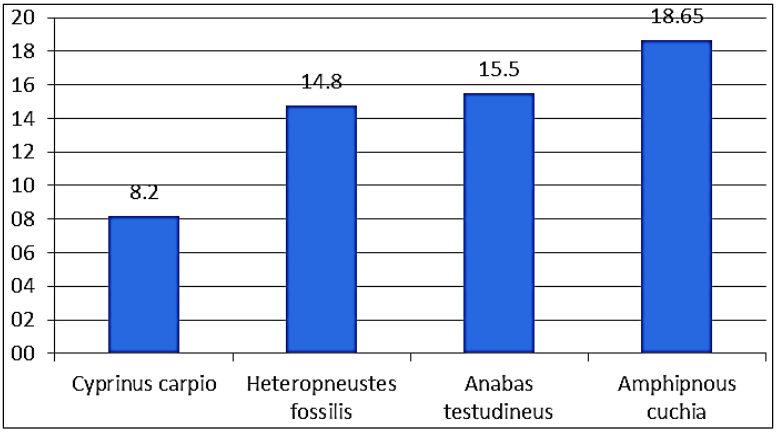
I. Histogram showing total RBC count among *Cyprinus carpio*, *Amphipnous cuchia*, *Anabas testudineus* and *Heteropneustes fossilis*:



[Note: M represents 1 million (10^6)]

In the above graph the total RBC count of *Cyprinus carpio, Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis* are shown by histogram. In this histogram along Y-axis, total count of RBC is considered where 1cm is equal to $1x10^6$ and along X-axis different fish species are considered. The histogram clearly shows that the total RBC count is less in water-breathing fish *Cyprinus carpio* than the air breathing fishes *Heteropneustes fossilis, Anabas testudineus* and *Amphipnous cuchia* and the highest value of total RBC count is shown by *Amphipnous cuchia*.

II. Histogram showing haemoglobin contents among *Cyprinus carpio, Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis*:



In the above graph the haemoglobin content of *Cyprinus carpio, Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis* are

shown by histogram. In the histogram, along Y-axis total haemoglobin content is considered where 1cm is equal to 2 g/dl and along X-axis different fish species are considered. The histogram clearly shows that the haemoglobin content is less in water-breathing fish *Cyprinus carpio* than air-breathing fishes *Amphipnous cuchia, Anabas testudineus* and *Heteropneustes fossilis* and the highest value of Hb content is shown by *Amphipnous cuchia.*

From the graphs, it is observed that the RBC count in *C. carpio* is less than the three air-breathing fish species that is why the Hb content is also lower than the other three species. The highest count of RBC is found in *A. cuchia* where the Hb content is also the highest. From the graphs it is clear that the Hb content of a fish is directly related to the RBC count. The more the RBC count is, the more will be the content of Hb.

Discussion

The accessory respiratory organs in air-breathing fishes help the fish to respire even in absence of water. When they remain in water they respire by means of gills but in absence if water they respire by the means of the accessory respiratory organs. While in some air-breathing fishes like *A. cuchia* where gills are greatly reduced (B.K. Das, 1927; Munshi and Singh, 1968; Hughes and Munshi, 1973) they respire by means of air-chambers (B.K. Das, 1927; S.L. Hora, 1935; Munshi and Singh, 1968). They are capable of performing aquatic respiration for much shorter period of time (B.K. Das, 1927; S.L. Hora, 1935). In this way accessory respiratory organs help the air-breathing fishes to respire even in absence of water.

Blood parameters are considered as patho-physiological indicators of the whole body and therefore important in diagnosing the structural and functional status of the fish under various stresses (S. Adhikari, B. Sarkar *et al.*, 2004). The physical properties of blood are very sensitive to environmental changes (G.M. Hughes and J. Nemesok 1988). Changes in physic chemical parameters may be reflected in haematological parameters of the fishes (C.R. Hickely, Jr. 2011). It has been reported that the blood parameters remarkably vary in different fishes and this is considered to reflect adaptations to the varied environmental conditions (Ramaswamy and Reddy, 1978; Moyle and Cech, 1982). Changes in total count of RBC, Hb concentration are associated with season, sex and size of body and age of fishes (R.P. Bhagat and Banerjee, 1986). It is also dependent on season and slow and active movement of fishes (Larsson *et al.*, 1976; R. Yasmin, B.N. Pandey and A. Yasmin, 1993). The changes of haematological values are also due to chemical stress (Joshi and Tandon, 1977; Mahajan and Dheer,

1979; Srivastava and Agarwal, 1981). The literature reveals that the erythrocyte count among fishes ranges from lowest $0.84x10^6$ per mm^3 in carps (Barron *et al*., 1956) to the highest count of $6.48x10^6$ per mm^3 in *Acanthurus bahianus* (Saunders, 1966), however, Taylor (1960) has reported a count of 0.66 x 10^6 per mm^3 in *Trematomus borchgrevink*, which is the lowest RBC count for a teleost fish. The Hb content in fishes is also dependent on temperature. In *Channa punctatus* it is observed that as temperature. In *Channa punctatus* it is observed that as temperature increases Hb content increases and as it decreases Hb content decreases (Jagtap Ashwini Ravichandra, 2012). Haemoglobin is present in all vertebrates which strongly decreases the circulatory requirements. Hb is considered as a heat carrier which absorbs the O_2 in tissue is liberated in the gills on oxygenation (J. Wyman, 1964). Changes in Hb content have been identified as an efficient stress response mechanism, to compensate for a reduction in O_2 availability (A. Wawrowski, F. Gerlach, T. Hankeln and T. Burmester, 2011). Haemoglobin content is considered as an important diagnostic tool for determination of physiological status in aquatic animal under stress condition (Rehulka J., 2002). In climbing perch (*Anabas testudineus*) the RBC Count and Hb content are significantly higher in male than in females (Mahmud Hasan, Abdullah-Al-Mamum and Md. Golam Rabbane, 2012).

Conclusion

The variation in the haematological parameters may be due to difference in season, sex, size, habit, habitat and biology of the fish. Therefore, the blood parameters might be considered as potential bio-indicators in assessing the physiological status of fish and the data obtained in this regard might also provide substantial information on the quality of the water body as such. The review of the literature on haematological studies in fishes indicated the data obtained from various fish species by various authors around the globe is not uniform. Since the fishes are the most sensitive fauna, any little change that occurs in their living media might have immediately influenced their physiology. In spite of all known facts and whatever are the realistic there should be need to obtain the uniform data, which is mandatory for every research finding and the data obtained on haematological studies in fishes did not follow a uniform pattern which might be a major setback to this line of scientific investigation. Authenticity of the earlier findings is the vital base for future investigations, not only in this issue but in every scientific endeavor too, however which is not found in this matter, it is opined.

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Chapter - 7
Phylogeny of Green Algae from Tropical Regions of South-East Asia

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Chapter - 7

Phylogeny of Green Algae from Tropical Regions of South-East Asia

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Abstract

With high rainfall, relatively less fluctuation in temperature and ample amount of sunlight throughout the year, tropical regions of lower Himalaya provide perfect conditions for lush growth of green algae. A large number of green algae are reported from regions of Arunachal Pradesh Meghalaya and Assam. The phylogeny of these algae, however, is poorly understood. The molecular phylogenetic approach is rarely adopted as most of the available information regarding their taxonomy is based on morphological studies. *rbcL* gene-based phylogenetic analysis via neighbor joining algorithm reveals that some that species like *Netrium oblongum* shares a common ancestor with members of Cosmarium while *N. digitus* shares a common ancestor with *Zygnema* members. *Cylindrocapsa geminella* shares a common ancestor with Chlorella genera. *Chloromonos rosae* and *Chlamydomonas sp.* have similar origins. *Ulothrix flacca* is more closely related to *Cladophora* sp. than *U. zonata*. Such information may be valuable in the development of a more accurate taxonomic classification system for the green algae of both topical Himalayan region as well as the green algae found in other regions.

Keywords: green algae, phylogeny, neighbor joining, *rbcL* gene, green algae of the tropical region

1. Introduction

Molecular phylogenetic relationships of green algae and primitive land plants and green algae remain largely unaccounted for in the past century (Manhart, 1994), as well as the last few decades (McCourt *et al.*, 2000; McCourt *et al.*, 2004). Taxonomy of members of Green algae (Chlorophyceae) family has been a debated topic for the past few decades (Nakada *et al.*, 2008). The previous attempt to classify the Chlorophycean members is exclusively based on morphological studies made under the light microscope. Members of some Chlorophycean alga were often classified with

the different members based on their morphological characteristics (Melkonian *et al.*, 2000). This, however, fails to satisfy results produced by phylogenetic studies based on 16S rRNA analysis (Pröschold *et al.*, 2007). Lower Himalayas boast of a wide variety of tropical flora due to high rainfall and relatively constant temperatures throughout the year, some of these florae are endemic (Negi, 2007). The lower Himalayan tropical regions include parts of Arunachal Pradesh (Sharma *et al.*, 1992), Meghalaya, Uttarakhand, Himachal Pradesh, Kashmir. The tropical plains under the Himalayan range include parts of Assam, Mizoram, Manipur and Nagaland. Due to the relatively high humidity in these regions harbour a wide range of green algae. The green algae play a vital role in maintaining the ecological food chain in these tropical regions (Niesenbaum, 1988).

The enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) is known to facilitate primary CO_2 fixation during photosynthesis (Figure 1(A)). The enzyme is a heterodimer of large and small subunits (Figure 1(B)). The quaternary structure of the enzyme consists of an octamer large and small subunit attached in a globular structure. In both green plants and green Chlorophycean algae, the large subunit is encoded by *rbcL* gene while the small subunit is encoded by *rbcS* gene (Palmer, 1985) (Figure 1(A)), in other algal species these subunits are coded plastid genes (Valentin *et al.*, 1989) (Kostrzewa *et al.*, 1992).

The *rbcL* gene sequence has been previously used to infer phylogenetic relationships in higher Angiosperm plants (Olmstead *et al.* 1992; Chase *et at.*, 1993; Qiu *et at*., 1993). Reasons for using the *rbcL* gene in angiosperms are:

a) Large size (more than 1000 base pair) which provides various characters for phylogenetic analysis
b) its relatively slow rate of evolution which is appropriate for exploring relationships at higher levels
c) availability of conserved primers allows for rapid amplification and sequencing

Douglas and Destombe (1991) and Maggs *et al.* (1992) have used the spacer sequences between the Large and Small Subunit of the RuBisCO gene to explain the relationship among Rodophycean algal populations of Gracilaria and Gymnogongrus respectively.

Phylogenetic studies of Chlorophyte and Volvocal genera were previously conducted using *rbcL* gene along with 18s rDNA (Hoham *et al.*, 2002) of snow and cold temperature habits emphasizing on *Chloromonas* and *Chlamydomonas* genera. *rbcL* gene sequence data were used for the

phylogenetic relationship analysis within the members of Volvocales (Nozoki *et al.*, 1995).

The present investigation is undertaken to explore the phylogenetic relations between various green algae of the tropical regions of Himalaya. This may help better characterize the algal systematics.

2. Materials and Method

2.1 Collection of nucleotide sequence

Nucleotide sequences of the *rbcL* gene for all the 65 species including the outgroup species were collected from the NCBI GenBank database. The sequences were retrieved in fasta format. The fasta files were used for both pairwise and multiple sequence alignment.

2.2 Alignment of sequences

Pairwise and Multiple sequence alignment was performed using Cluster W software version 2.0 (Larkin *et al*., 2007).

2.3 Formation of the pairwise distance matrix

A pairwise distance matrix was prepared based on kimura 2-parameter model (Kimura, 1980). MEGA 7 phylogenetic analysis software (Kumar *et al*., 2016) was used to compute the pairwise distance matrix with a bootstrap value of 1000 and uniform deletion for missing nucleotides.

2.4 Neighbor joining phylogeny

Neighbor joining phylogeny utilizes a bottom-up clustering method to determine the phylogenetic relationship among the various sequences. The method requires a pairwise distance matrix to calculate the branch points and branching pattern. The distance matrix is used as input information to prepare the tree.

The initial step involves the preparation of the Q-matrix was performed using the distance matrix n taxa are given a Q value based on the distance d between the taxa i and j represented as d(i, j) and distance between j and another taxon k is represented as d(j, k) (Saitou *et al.*, 1987).

$$Q(i,j) = (n-2)d(i,j) - \sum_{k=1}^{n} d(i,k) - \sum_{k=1}^{n} d(j,k)$$

For each new taxa joined in pair i and j the following formula is used calculate the distance to the new node for the pair of taxa where f and u and g are the branch points. δ(f, u) and δ(g, u) are regions of the tree which are gradually being created; they neither affect nor get affected by other neighbor-

joining steps that are to proceed the previous step. (Saitou *et al*., 1987, Studier *et al.*, 1988).

$$\delta(f,u) = \frac{1}{2}d(f,g) + \frac{1}{2(n-2)}\left[\sum_{k=1}^{n} d(f,k) - \sum_{k=1}^{n} d(g,k)\right] \delta(g,u) = d(f,g) - \delta(f,u)$$

2.5 Development of neighbor-joining tree

MEGA 7 phylogenetic analysis software uses the Neighbor joining algorithm to develop a phylogenetic tree from the protein or nucleotide sequences. The aligned nucleotide sequences collected in FASTA format were fed into the MEGA software which provided the distance matrix and the matrix was then used to produce a resolved phylogenetic tree (Figure 2) which is used to draw inference regarding the phylogenetic positions of different taxa.

3. Results and Discussion

3.1 Clustering of different members

In terms of clusters, all the species included in this study can be classified based on their phylogenetic distance into 11 distinct clusters (Figure 2). As is seen in the figure, the first cluster (Cluster I) includes all the studied species of Cosmarium along with *N. oblongum*. While the next closely related cluster *i.e.* cluster II includes all the studied Closterium species. Both these cluster have a common origin for the *rbcL* gene. Cluster III like Cluster II consists of members from a single genus i.e. *Spirogyra*. Similarly cluster IV VI, VII consist of members from a single genus *viz. Zygnema, Oedogonium* and *Volvox* respectively. Cluster V unlike the former clusters has *Cylindrocapsa geminella* in close relation with *Chlorella* members. Cluster XIII have *Chloromonas in roseae* in close relation the members of *Chlamydomonas* genera. Cluster XI, X are heterogenous groups with *Selenastrum, Monoraphidium* and *Ankistrodesmus* belonging to the former cluster while the later cluster has *Pediastrum, Scenedesmus* and *Desmodesmus* members. The last cluster *i.e.* cluster XI have only two members both belonging to different genera *viz. Cladophora* and *Ulothrix*. Species like *Netrium digitus, Mougeotia transeaul, Ulothrix zonata*, *Ankistrodesmus densus* are orphan members as they failed to belong to any of the clusters.

In terms of phylogenetic relation between clusters, cluster I and II are closely related, while cluster III is monophyletically related to both the former clusters. Cluster IV have a unique ancestral sequences independent of all the other clusters. Cluster V, VI and VII are polyphylatically related to each other. Cluster IX and X, even though they have heterogenous members have a

common ancestral sequence. The members cluster XI are unique from all the other members.

3.2 Phylogenetic relation between different members of chlorophyceae

From the phylogenetic tree produced by neighbor joining algorithm (Figure 2), it is seen that all the 64 species grouped into specific groups based on the genera they belong however some inconsistencies were observed with *Netrium oblongum* and *N. digitus*. The nucleotide sequence of *rbcL* gene belonging to *N. oblongum* showed closer phylogenetic affinity to the members of the *Cosmarium* genera. All the studied members of the *Cosmarium* genera form a polyphyletic group with *N. oblongum*. However, the same gene sequence of *N. digitus* resemble more closely the same gene sequence present in the members of the *Zygnema* genera with a closer resemblance to *Z. circumcariantum* and forming a polyphyletic group with other members of *Zygnema* genera, this proposes a closer relation of the gene to the *Zygnema* members than to *Cosmarium* members and in fact to the member of its genera (Figure 2). It is observed that *Cosmarium* and *Clostridium* genera share closer resemblances in the *rbcL* gene sequence as compared to the other genera under study. Among the members of the *Cosmarium* genera, *C. obtusata* and *C. exiguum* have the highly similar nucleotide sequence for the *rbcL* gene as indicated by the 100 bootstrapping value. C. *sphagnicolum* of the shows the least similarity in sequence when compared to other members of the same genera. It forms a polyphyletic group with *N. oblongum*. *Mougeotia transeaui* is polyphyletically associated with studied members of *Cosmarium*, *Closterium*, *Netrium*, *Zygnema* and *Spirogyra* genera indicating a common ancestor between the aforesaid genera and *M. transeaui*, *M. transeaui*, however, has undergone more evolutionary variation when compared to the last common ancestor of both the genera. *Cylindrocapsa geminella* forms a polyphyletic link with the studied members of the *Chlorella* genera. *rbcL* gene nucleotide sequence of *C. germinella*, however, shows the more evolutionary distance from the last common ancestor when compared to the studied members of the *Chlorella* genera which forms a polyphyletic group among themselves. Members of the *Oedogonium* genera form a polyphyletic relation with the members of the genera *Cosmarium*, *Closterium*, *Netrium*, *Zygnema* and *Spirogyra*, *Mougeotia*, *Cylindrocapsa* and *Chlorella*.

Oedogonium bravisingulatum and *O. pakistanense* share a highly similar sequence with the former being more phylogenetically evolved. *O. augustistomum* polyphyletically was related to rest of the members of the genera. The nucleotide sequence of the *rbcL* gene in O. *augustistomum* can be considered to be more closely related to the last common ancestor from the

group diverted. Members of the *Volvox* genera form two monophyletic group with the species *V. ovalis* and *V. tertius* in one group along with *V. reticuliferus* and V. *africanus* in the other both polyphyletically related to *V. ferrisii*. *Chloromonas rosae*, single species belonging to *Chloromonas* genera forms a polyphyletic link with members of *Chlamydomonas* genera (Figure 2). *Chlamydomonas raudensis* and *C. parker* are monophyletically related. *C. pseudogloeogama* forms the polyphyletic link with the rest of the members of the *Chlamydomonas* genus with closer links to *Chloromonas rosae*. *Selenastrum bibraianum* and *S. capricornutum* form a monophyletic group while *Monoraphidium circinale* is polyphylatically related to members of *Selenastrum* genera. Another species of the *Monoraphidium* genera *M. komarkova* formed a monophyletic group with *Ankistrodesmus falcatus* indicating that the *rbcL* gene nucleotide sequence of *A. falcatus* is more close to *Monoraphidium komarkova* rather than *A. stipitatus* is found to be polyphyletically related to members of *Selenastrum* and *Monoraphidium* genera.

Pediastrum duplex and *P. angulosum* have similar nucleotide sequences and hence forms a monophyletic group. *P. simplex* and *P. boryanum* form a monophyletic group. *rbcL* gene sequence in some of the members of *Scenedesmus* and *Desmodesmus* have highly similar sequence and hence are not separated into polyphyletic groups. However, *Desmodesmus multivariabillis* and *D. communis* has more similarity as compared to the other members and hence are monophyletically related. *Cladophora* sp. and *Ulothrix flacca* forms a monophyletic group while the *rbcL* gene sequence of *U. zonata* shows no phylogenetic relation to the member of the same genera. the *rbcL* gene sequence of *Ankistrodesmus densus* is not related to the members of its genus and hence appear as an outgroup to all the Chlorophycean members. The *rbcL* gene sequence of *Anabaena circinalis*, a member of Blue-Green algae is used to determine the root of the Neighbor-joining tree (Figure 2).

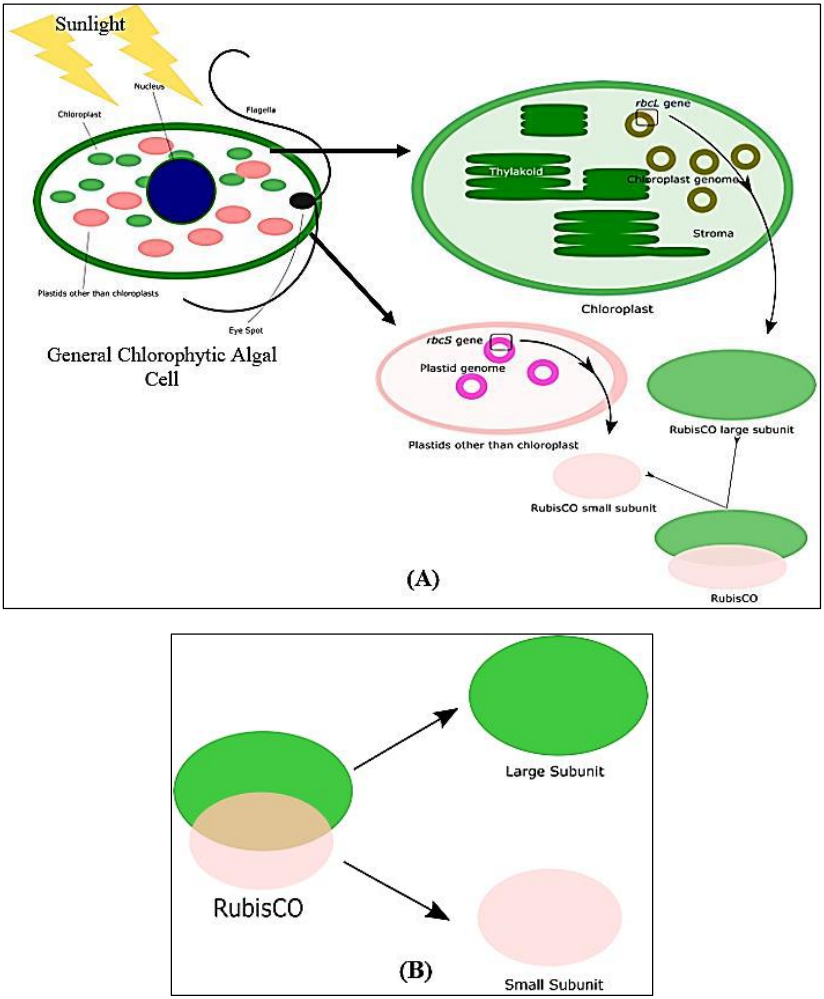

Fig 2: (A) Algal cell producing RubisCO enzyme under the effect of sunlight. (B) Different components of RubisCO heterodimer

4. Conclusion

From the phylogenetic tree based on the neighbor joining algorithm, it is inferred that the *rbcL* gene sequence has undergone a considerable amount of variation among different species and their respective genera. However, the changes are subtle hence helps predict a relationship among the various members. The tree indicates that the changes in some members of the same genera have undergone much variation that they are separated from the members of the same genera. The phylogenetic trees provide a better understanding of the affinities of various Chlorophytic genera with each other.

This may help in better understanding of the systematics of the Chlorophyceae class based on their evolution. *rbcL* being a chloroplast gene undergo lesser modification as compared to the nuclear genes. This helps in better understanding of the diversification of the non-nuclear genes during the long process of evolution. However, a disadvantage of cpDNA concerning phylogenetic estimation at lower taxonomic levels as those of singe celled Thallophytic plants involves the potential occurrence of chloroplast transfer: the movement of a chloroplast genome from one species to another by introgression. This process and the problems it raises in phylogenetic studies have been well-reviewed by Rieseberg and Soltis, 1991; Rieseberg and Brunsfeld, 1992; Harris and Ingram, 1991. Although chloroplast capture, if undetected, will bias estimates of phylogeny, it can, when recognized, be very informative about evolutionary processes.

5. Future prospects

With the advance of sequencing and modern molecular techniques it has become increasingly important to understand the phylogenetic positions of different species in their respective class. Such information is useful in understanding origins and evolution of said species as well as it sheds light on the environmental changes that can be linked to those changes. Chlorophytic algae due to their relatively cosmopolitan distribution and large number of members have been relatively less studied. But with the introduction of new methods like next generation sequencing (NGS) which are becoming more and more affordable, the entire genomic sequences of different Chlorophytic algae can be determined and a better phylogenetic prediction could be made regarding the position of different members. Until then the rbcL based phylogeny (Figure 3) can be used as a map for understanding the relatedness of different members.

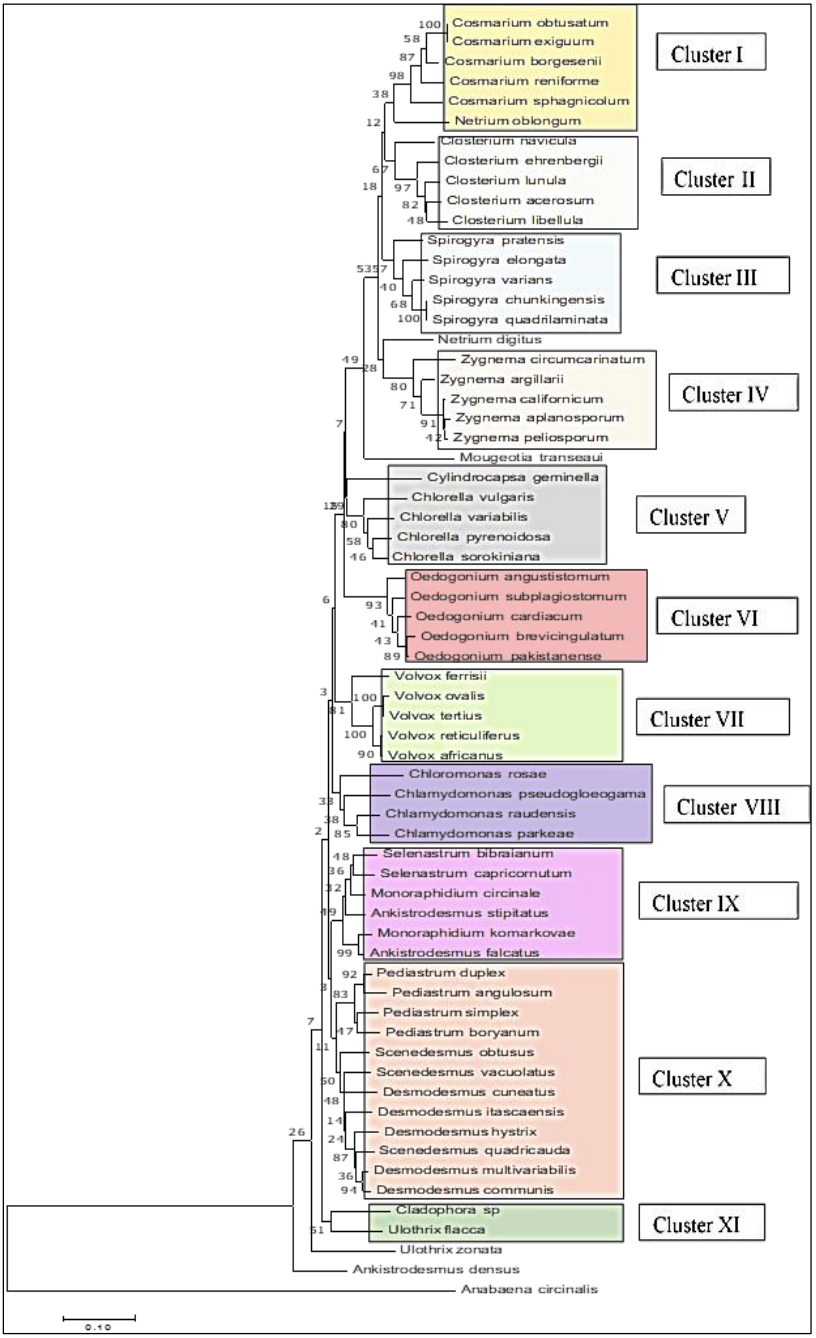

Fig 2: The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test

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