CYTOTOXIC AND GENOTOXIC EFFECTS OF TEXTILE EFFLUENT DILUTIONS ON *Zea mays* (MAIZE PLANT)

BY

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CERTIFICATION

This is to certify that this project work entitled: **"Cytotoxic and Genotoxic Effects of Textile Effluent Dilutions on Maize Plant (*Zea mays*),"** was carried out by **AUGUSTUS KELECHI AHAMEFULE** (**20134870658)**, an M.Sc student of the department of Biotechnology, School of Biological Sciences, Federal University of Technology, Owerri, Imo State, Nigeria**.**

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DEDICATION

This work is dedicated to God Almighty, the author and finisher of my destiny.

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I wish to acknowledge the almighty God first, for His unreserved grace in my life.

My undiluted gratitude goes to my supervisor, Dr. E. U. Ezeji, for his candid advice and friendly relationship throughout the research work. I am also grateful to the Head of Department, Dr. T. E. Ogbulie and all my departmental lecturers: Prof. P. T. E. Ozoh, Prof. H.C. Nwigwe, Dr. I.O. Onyeocha, Dr. T. I. N. Ezejiofor, Dr. J. N. Okereke, Dr. A. C. Udebuani, Dr. N. C. D. Ukwandu, Dr. I. C. Mgbemena, Dr. S. O. Anyadoh-Nwadike, Dr. E. A. Anyalogbu and Dr. I. Emeka-Nwabunnia. I also want to appreciate Mr David Igwe of Biotechnology Research and Development Centre, Ebonyi State University, Abakaliki, for his contributions.

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The trend of my sincere recognition moves to my good friends and colleagues; Mr Okonkwo, Samuel Ugochiturum, Mr Nimrod Pwaduvi, amongst others too numerous to appear on this tiny page. I owe you people a lot and pray that God will help me to live up to your good expectations.

TABLE OF CONTENTS

CHAPTER THREE

CHAPTER FOUR

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This research investigated the cytotoxic and genotoxic effects of textile mill effluent on the maize plant (*Zea mays*). In this research, seeds (4/treatment) of maize (*Zea mays*) were grown in wood shavings (sawdust) irrigated with different concentrations of textile effluent (0%, 25%, 50%, 75% and 100%) for 15 days. Most of the physicochemical parameters of the effluent, analyzed using specific instrument for each, were above permissible limits, examples are the COD (4208mg/L against 90mg/L), BOD (171mg/L against 50mg/L), Nitrate (71.2mg/L against 10mg/L), etc. There was complete loss of viability at concentration 100%, while germination reduced by 75%, 50% and 25% in 75%, 50% and 25% textile effluent concentrations respectively. Plant growth rate was inversely proportional to concentration increase; growth of the control significantly differed with other treatments at $p<0.05$. The cytotoxic effects were investigated using Automated Image Analyses Software and RAPD; there was a concentration dependent significant ($p<0.05$) decrease in cell proliferation (Mitotic Index) and increase in chromosomal aberrations, compared to the control. The RAPD profile obtained showed textile effluent had genotoxic effects on the plants. This was evident with the appearance and disappearance of bands in the treatments compared with the control. In all, 64 bands were scored, 31 (48.4%) of these were polymorphic. Altogether, 13 new bands were formed while 15 were lost. A dendrogram of the four accessions using Weighted Neighbour-Joining (WNJ) procedure clustered the accessions into two major groups. The control (Maize-1) and treated 25% effluent (Maize-2) samples were clustered in one group with 67% bootstrap value. Group II, 50% effluent (Maize-3) and 75% effluent (Maize-4), were separated in another cluster, with 88% bootstrap value. The above results show that high concentrations of textile mill effluent have adverse cytotoxic and genotoxic effects on the maize plant.

KEYWORDS; Textile Effluent, *Zea mays*, RAPD, Cytology, Mitotic Index, Pollution, Chromosome Aberration.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

Enormous industrial and economic development is taking place. An example of such development is seen in the chemical industries, leading to the manufacture of a large number of products. These products have enjoyed a worldwide use in recent times, particularly clothing of various designs, fertilizers, insecticides, etc, then followed. This, together with the development of new high-yield grains, led to dramatic increases in local food production. These developments, concomitant with improved medicine and medical technology, has helped to improve public health. While many people were enjoying the benefits of these advancements, just little became aware that this extraordinary development was not without cost.

The significance of water to human and other biological systems cannot be over emphasized, and there are numerous scientific and economic facts that, water shortage or its pollution can cause severe decrease in productivity and deaths of living species (Bhattacharjee *et al*., 2013). Reports by Food and Agricultural Organisation (FAO) revealed that in African countries, particulaly Nigeria, water related diseases had been interfering with basic human development (FAO, 2009). The common sources of water that are available to local communities in Nigeria are fast being severed by a number of anthropogenic factors (eg, industrial waste dump site), of which pollution remains the most dominant problem. Water pollution occurs when unwanted materials with potentials to threaten human and other natural systems find their ways into rivers, lakes, wells, streams, boreholes or even reserved fresh water in homes and industries. The pollutants are usually pathogens, suspended solid particles, automobile emissions, industrial effluents, construction debris, etc. Some of these pollutants are decomposed by the action of microorganisms through oxidation and other processes. During these processes, natural bacteria and protozoa in the water source utilize the oxygen dissolved in the water. This could significantly reduce the oxygen level to less than two parts per million (<2ppm), therefore the respiratory conditions of aquatic species would be seriously affected. Consequently, fishes, bottom-dwelling animals and even marine plants can be contaminated and/or killed, creating significant disruption in the food chain. On the other hand, when this contaminated water is directly used by farmers (with no form of treatment) or invade farmlands on its own, it could bring about severe effects, including changes in the genetic makeups of plants.

Environmental pollution is considered to be the world's most dangerous threats (Spielvogel, 2006). We live in a world exposed to hazardous pollutants and chemicals, being generated from different sources every day. Industries, domestic and other human activities are among the anthropogenic factors that spread pollutants around our world on a daily basis. Although pollution had been known to exist for a very long time (at least, since people started using fire thousands of years ago), it has seen a growth of global proportions only since the onset of the industrial revolution during the 19th century. Environmental pollution is a worldwide problem (Khataee and Denghan, 2011), both in developed and developing countries. It is known to cause a lot of distress not only to animals but to our sustainers (plants), driving many species to endangerment and probably extinction.

Industrial waste water is one of the major sources of pollution to the environment, especially water bodies. During the last century, a huge amount of industrial waste water was discharged into rivers, lakes and coastal areas (Spielvogel, 2006). This resulted to serious pollution problems to the water bodies, and subsequent negative implications to the ecosystem. There are many types of industrial waste water, industrial and contaminants wise, each producing its combinations of pollutants. Generally, industrial waste water could be classified into two, viz; organic and inorganic industrial waste water (Sawyer and McCarty, 1998). Organic waste water is mainly composed of suspended solids, fats, oils, grease, high levels of organic pollutants, and are often classified as high strength. They maybe either excessively acidic or alkaline in nature, and may contain varying concentrations of coloured matter. High strength effluents, when discharged into the water bodies, deplete the dissolved oxygen of such water, and thus a potential threat to both flora and fauna. Hence, these industrial effluents ought to be adequately treated before discharging into rivers, lakes,etc (Gupta *et al.*, 2012).

Likewise, domestic activities which occur daily are good sources of organic and inorganic wastewater. Wastewater from bathrooms, kitchens, laundries and toilets contain human waste (containing pathogens), soap residues, etc. Sewage effluent may contain disease-causing organisms (bacteria, viruses, intestinal worms, and protozoa), degradable organic matter that depletes dissolved oxygen in water, causing foul odours, suspended solids and sediment, nutrients like nitrogen and phosphorous (that could foster algal blooms in water ways and wetlands), household chemical residues such as cleansers and disinfectants, detergent residues (which can harm aquatic beings), trace metals from plumbing fittings, any substance flushed into the waste management system (APHA, 2015). In most rural areas, these waste

waters are directly poured away, preferably on nearby farmlands without proper consideration of the effects on the plant life.

Advances in molecular biology techniques have provided the basis for uncovering virtually all levels of pollution induced damages done to living things (Botstein *et al*., 2008). Over the last decade, polymerase chain reaction (PCR) has become a widespread technique for several novel genetic assays based on selective amplification of DNA (Erlich, 2009). This popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Williams *et al*., 2011, Welsh and McClelland, 2014).

RAPD (pronounced 'rapid'), for *R*andom *A*mplification of *P*olymorphic *D*NA, is a type of PCR reaction, but the segments of DNA that are amplified are random. The RAPD analysis described by Williams *et al*. (2011) is a commonly used molecular marker in genetic diversity studies. No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Perhaps the main reason for the success is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question.

RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way (Roholf, 2008). It is used to analyze the genetic diversity of an individual entity by using random primers.

1.2 PROBLEM STATEMENT

The textile production industry is one of the oldest and technologically complex of all industries (Chen *et al*., 2011). The fundamental strength of this industry flows from its strong production base of a wide range of synthetic/man-made fibers like polyester, viscose, nylon, and acrylic from natural fibers like cotton, jute, silk, and wool. The act of local painting and designing of these fabrics have been taken by many Nigerians, especially those in the western hemisphere. These people, most of them not aware of the actual contents of what they use, involve large quantity of water and discharge the resultant effluents in their surroundings, without proper treatment. With increasing demand for customized textile products, the activities of tie and dye producers, if left unchecked, may pose big environmental and health hazards.

It is worthy of note that worldwide environmental problems associated with local tie and dye industry are typically those of water pollution caused by the discharge of untreated effluent and those because of use of toxic chemicals especially during processing. Note that dyes are considered important pollutants (Khataee and Dehghan, 2011). This effluent is of critical environmental concern since it drastically decreases oxygen concentration due to the presence of hydrosulfides and blocks the passage of light through water body which is detrimental to the water ecosystem (Ayoola *et al*., 2012).

Textile effluents are causes of significant amounts of environmental degradation and human illnesses (Lin *et al*., 2012). About 40 % of globally used colourants contain organically bound chlorine, a known carcinogen (Chen *et al*., 2011). Chemicals evaporate into the air we breathe or are absorbed through our skin; they show up as allergic reactions and may cause harm to children even before birth. Due to this chemical pollution, the normal functioning of cells is disturbed and this, in turn, may cause alteration in the physiology and biochemical mechanisms of plants and animals resulting in impairment of important functions like respiration, osmoregulation, reproduction, and even mortality (Ayoola *et al*., 2012). Thus, untreated or incompletely treated textile dye effluent can be harmful to both fauna and flora by adversely affecting the natural ecosystem and causing long-term health effects.

Most of the time our attentions are drawn to animals adverse effects than those of plants, probably because the former have a better way of narrating their ordeals, then we neglect those of plants - the bedrock of our existence.

1.3 AIM AND OBJECTIVES OF THE RESEARCH

The aim of this research is to evaluate cytotoxic and genotoxic effects of textile mill effluent dilutions on the maize plant (*Zea mays*).

Specific objectives of the research are:

1. To determine the characteristics of the textile dye effluents
2. To determine the effect of textile effluents on germination of maize grains.
3. To determine the effect of textile effluents on growth characteristics of maize plant.
4. To ascertain the possibility of DNA damage in *Zea mays* caused by textile effluents using Random amplified polymorphic DNA (RAPD) techniques.

1.4 HYPOTHESIS

Null Hypothesis (H_0): There is no significant effect of Textile Effluent Dilution on *Zea mays.*

Alternative Hypothesis (H_1): There are significant effects of Textile Effluent Dilution on *Zea mays.*

1.5 JUSTIFICATION OF THE STUDY

Studies have shown that many chemicals used in the textile industry cause environmental and health problems. Among these chemicals in textile waste water, dyes are considered important pollutants (Khataee and Dehghan, 2011). Most of the people that engage in tie and dye are not aware of the content of what they are working with. They end up discarding the waste water around the environment, including nearby gardens. It is therefore wise to find the effects these effluents may have on agriculture. DNA distortion in plants will no doubt affect plants characteristics, hence the necessity to establish the fact on whether textile effluents are capable of inducing mutations in *Zea mays.*

1.6 SCOPE OF THE STUDY

This research work only investigated the germination, growth and the possibility of DNA damage in *Zea mays* caused by textile effluents dilutions, using Random amplified polymorphic DNA (RAPD) techniques. The effluent was collected from a textile industry in Lagos State, while the laboratory works were carried out at Biotechnology Research and Development Centre, Ebonyi State University, Abakaliki, Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE CELL CYCLE AND MITOSIS (CELL CYTOLOGY)

The cell is the biological unit that contains the entire set of genetic information of an organism in the form of DNA (McPherson *et al*., 2013). The transmission of this information from generation to generation is required for continued existence and is accomplished through the cell cycle, the periodic process by which one cell becomes two. There are four main parts of the cell cycle, as described by Innis *et al*. (2012), viz S, G2, M, and G1 (Figure 2.1). S (synthesis) is the period of the cell cycle when the DNA is replicated. M (mitosis) is the period of the cell cycle when a cell splits the two sets of copied chromosomes into two new cells. G1 and G2 (gap) are the periods following M and S respectively during which the cell recovers from either M or S phase; the cell then performs various functions according to the cell type and prepares for the next period of the cell cycle. Often G1, S and G2 are grouped into one phase of the cell cycle; interphase. This very general description applies to most cell types from unicellular organisms, as shown below.

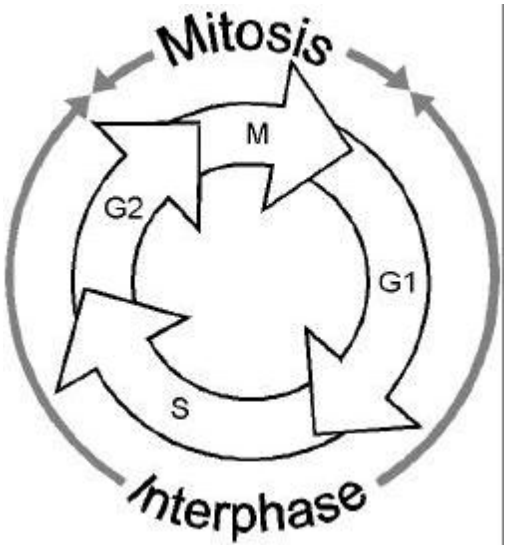


Figure 2.1. The cell cycle grouped into four periods: S (synthesis), G2 (second gap), M (mitosis) and G1 (first gap). Interphase refers to the three phases S, G1 and G2. SOURCE: Yang *et al*., (2014).

Mitosis is traditionally divided into 6 parts: Prophase, Prometaphase, Metaphase, Anaphase, Telophase and Cytokinesis (Unceer *et al.*, 2003). Each of these periods of mitosis is roughly defined by what is observed in the light microscope (Figure 2.2). Prophase occurs when the cell¸s two sets of chromosomes (which were duplicated earlier in S phase) begin to condense and appear as distinct, worm-like objects in the light microscope. The nuclear envelope remains intact around the worm-like chromosomes. Prometaphase begins when the nuclear envelope disappears. The chromosomes continue to condense as microtubules move them around the cell. Metaphase begins as the chromosomes are aligned along a plane, known as the "metaphase plate," which is located midway through the cell. The cell then waits for a period of time while the chromosomes continue to oscillate. Anaphase begins as the two sets of chromosomes move toward opposite poles. After the chromosomes are separated, telophase occurs: the chromosomes decondense and the nuclear envelope reappears. Finally, during cytokinesis, the cell cleaves itself into two new cells, each with an identical set of the parent cell¸s chromosomes. A movie of mitosis can be found at http://safarsquid.phy.uic.edu/~mpoirier/experiments/mitosis.mpg. Clearly, many complicated processes must take place to enable that mitosis be successfully completed.

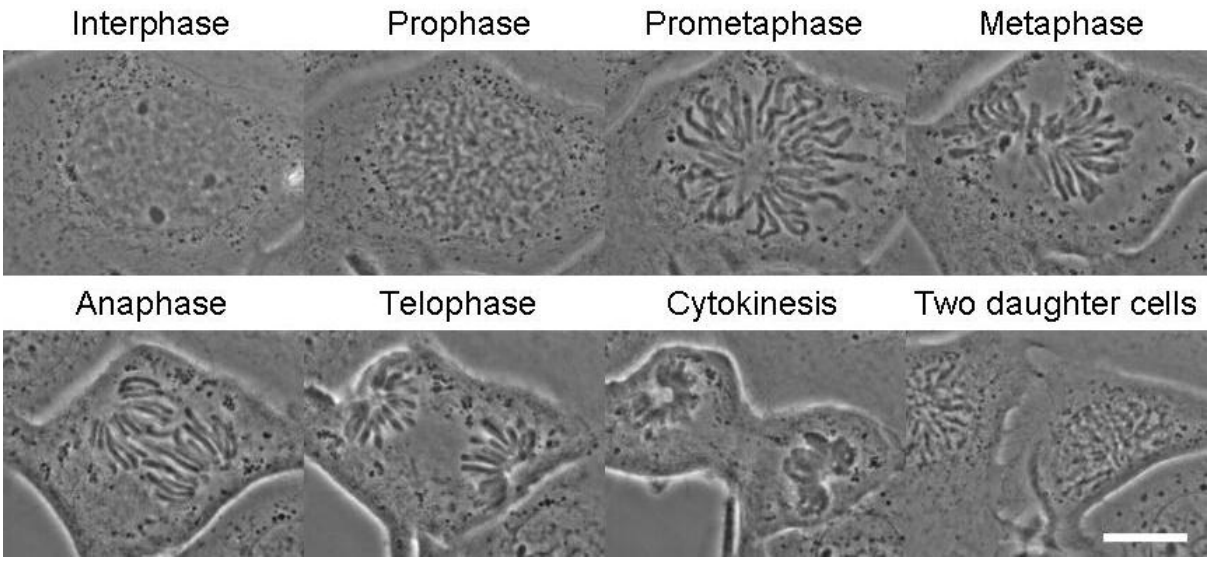


Figure 2.2. Photomicrographs of a new lung cell during different phases of mitosis. Bar = 20 ìm (adapted from Unceer *et al.*, 2003).

Chromosome structure and its modulation are critical to many aspects of cell function. During interphase, control of chromosome structure at the chromatin level is important for the success and regulation of transcription, replication and recombination (Sunnucks, 2010). As the cell enters mitosis, larger-scale chromosome structure becomes central to disentanglement and compaction of the two chromosome copies, for successful segregation of chromosomes into the daughter cells (Alimba *et al.*, 2015). Chromosome structures at these different periods of the cell cycle are intensely studied, yet no clear picture of large-scale structure exists for either interphase or mitotic chromosomes (Alimba *et al.*, 2015; Ahbay *et al.*, 2014).

2.2 MITOTIC INDEX STUDY

Mitosis is the process by which eukaryotic cell separates. It replicates the chromosome in the same nucleus into two identical nuclei, immediately followed by cytokinesis. This helps the cell to share chromosomes equally. The separation of nucleus (karyokinesis) and that of the cytoplasm (cytokinesis), together form the mitotic phase of cell division. The Mitotic index of a cell population has long been regarded as an important criterion of the growth and multiplication of the cells and tissues (Sunnucks, 2010). It is commonly measured in fixed and stained specimens, and therefore represents the stage of the material at the time of fixing only. One of the reasons for the mitotic indexing of species is to generate data which is important for breeding purposes. Some of such data are the chromosomal attributes. Chromosome details are best studied on cells with optimal chromosome contraction or condensation, i.e. metaphase cells. These are best studied using root tips as well as floral buds (Freeland, 2015). For most farm crops like *Zea mays*, the most convenient materials for such study are young root tips of germinating seeds. However, cells grown in tissue culture may be observed and photographed for a considerable period before any measurement is made; but such observations, which have been reported elsewhere (Olorunfemi *et al.*, 2011), show that certain cytochemical results may require reinterpretation.

2.3 GENETIC MARKERS

A genetic marker is an easily identifiable piece of genetic material, usually DNA, that can be used in the laboratory to separate cells, individuals, populations, or species. The use of genetic markers begins with extracting proteins or chemicals (for biochemical markers) or DNA (for molecular markers) from tissues of the plant (for example, seeds, foliage, pollen,

sometimes woody tissues). Laboratory protocols (often well developed, but may need adjustments for certain species) are then applied, resulting typically in visual representations from staining or tagging techniques, which are then converted into data - usually allele types and frequencies, or presence/ absence data. Genetic markers thus allow us to characterize genetic diversity, detect mutations, etc.

The choice of the most appropriate genetic marker for a study will depend on the characteristics of that marker, species characteristics (generation time, information already available for the species, tissue types available, existing protocol development for markers for that species, and so on), and genome characteristics (for example, which genome is most appropriate - cpDNA, mtDNA, nuclear DNA?).

Among the defining and distinguishing characteristics of the various genetic markers are:

- **VARIABILITY AND RESOLUTION THAT CAN BE DETECTED BY THE MARKER**: This will often depend on how much protocol development there has been for that species (for example, the number of probes developed to sample the genome), as well as the cost.
- **DOMINANCE OR CODOMINANCE:** That is, whether the marker reports on diversity at both codominant or only one dominant allele for any particular gene or locus.
- **COST:** The expenses involved in the analysis.
- **TIME:** Time required from data collection through analysis to results.
- **EXPERTISE:** The technology needed at all stages from sampling through analysis.
- **REPLICABILITY:** That is, how consistent the test results are when repeated in the same or different laboratories.
- **THE OBJECTIVES BEST SERVED BY THE MARKERS:** For example, molecular markers are generally considered to measure neutral DNA variation and consequently are useful in studies of species (phylogenetic) relationships, gene flow, hybridization, fingerprinting, genetic structure of populations, and other objectives. Allozymes, however, are mixed in their utility - often assumed to measure neutral genetic diversity (and therefore suitable for similar studies as molecular markers) but also including some enzymes that are known to be influenced by selection. So there are some instances in which allozymes can be useful in studying certain types of adaptive genetic variation. Also, some markers are specific to, or better developed for,

certain genomes (e.g., mtDNA or nuclear DNA). In addition, different genomes and even different sections of DNA within the genome are known to reflect different mutation rates, and thus the information derived from them is interpreted differently, as reflecting different time dimensions of the population's or species' genetic history.

In summary, no single genetic marker is inherently good or bad, markers vary in attributes and in their most appropriate application. Many of these features may change over time. For example, costs may go down as the technological tools are better developed, automated, or otherwise become more cost effective. In concert with this trend, less expertise or time may be required for the procedures. New markers may be developed in the future, making some of the current markers less attractive. Finally, DNA sequencing is increasingly common and as this advances for various species, there may be less need to use markers that give insights based on just a small sample of the genome.

Table 2.1. Characteristics of Genetic Markers

CRITERION	**AFLP**	**RAPD**	**SSRs**	**nRFLP**	**ALLOZYMES**
Number of Possible Loci Detected	Many	Many	Many	Many	Perhaps 10-20
Replicability	High	Variable	High	High	High
Resolution	High	Moderate	High	High	Moderate
Nature of Markers	Dominant	Dominant	Codominant	Codominant	Codominant
Lab Time, Ease of Assay	Short, Moderate to difficult	Short Easy to moderate	Moderate Easy to moderate	Long, Difficult	Short, Easy to moderate

Adapted from, The United States Department of Agriculture (USDA, 2012).

RAPD: Random Amplified Polymorphic DNA

AFLP: Amplified Fragment Length Polymorphism

RFLP: Restriction Fragment Length Polymorphism

SSRs: Simple Sequence Repeats (also known as Microsatellites).

2.4 PRINCIPLE OF RAPD TECHNIQUE

The principle is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome (Sambrook *et al.,* 2009). The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualised by ethidium bromide staining.

The use of a single decamer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band (Fig. 2.3). This means that RAPDs are dominant markers and, therefore, cannot be used to identify heterozygotes.

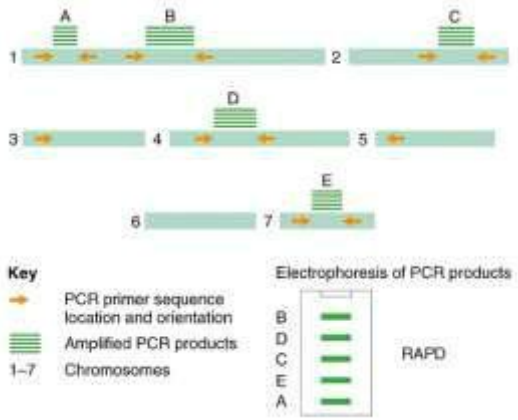


Figure 2.3. RAPD Analysis General Model (Adapted from Griffiths *et al*., 2006)

The standard RAPD utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Primers are commercially available from various sources

(e.g. Operon Technologies Inc., California; Biosciences, Eurofinns, Bangalore; GCC Biotech, Kolkata, amogst others).

Welsh and McClelland (2014) independently developed a similar methodology using primers about 15 nucleotides long and different amplification and electrophoretic conditions from RAPD and called it the arbitrarily primed polymerase chain reaction (AP-PCR) technique. PCR amplification with primers shorter than 10 nucleotides [DNA amplification finger-printing (DAF)] has also been used to produce more complex DNA fingerprinting profiles (Caetano *et al.*,2015). Although these approaches are different with respect to the length of the random primers, amplification conditions and visualization methods, they all differ from the standard PCR condition (Erlich, 2009), in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required (Griffiths *et al.*,2006).

At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products, if these priming sites are within an amplifiable distance to each other (Fig. 2.4). The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Recently, sequence characterised amplified regions (SCARs) analysis of RAPD polymorphisms (Paran and Michelmore, 2003; Bardakci and Skibinski, 2009), showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile, which is similar to that of low stringency mini-satellite DNA fingerprinting patterns and is therefore, also termed RAPD fingerprinting. On average, each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns.

2.5 DIFFERENCES BETWEEN STANDARD AND RAPD PCR

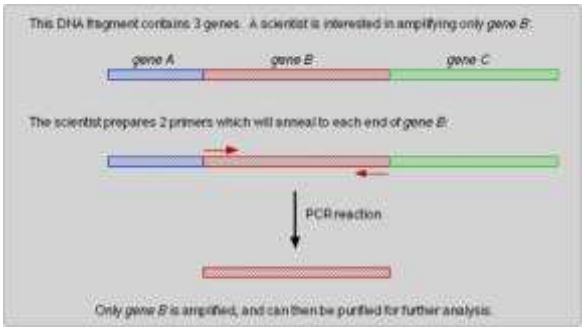


Fig. 2.4, Standard PCR Source: Paran and Michelmore 2003

In RAPD analysis, the target sequence(s) (to be amplified) is unknown. A primer is designed with an arbitrary sequence. In order for PCR to occur; the primers must anneal in a particular orientation (such that they point towards each other) and, they must anneal within a reasonable distance of one another. Figure 2.5 depicts a RAPD reaction, a large fragment of DNA (genome A) is used as the template in a PCR reaction containing many copies of a single arbitrary primer.

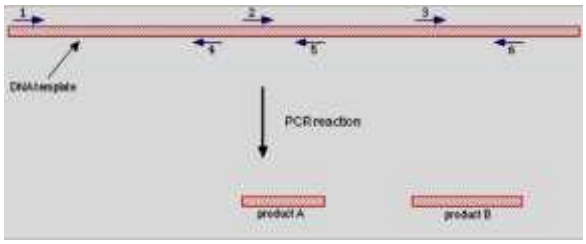


Figure 2.5. RAPD PCR. Source: Paran and Michelmore 2003

RAPD reaction for genome A. The arrows represent multiple copies of a primer (all primers have the same sequence). The direction of the arrow also indicates the direction in which DNA synthesis will occur. The numbers represent locations on the DNA template to which the primers anneal. Primers anneal to sites 1, 2, and 3 on the bottom strand of the DNA template and primers anneal to sites 4, 5, and 6 on the top strand of the DNA template.

RAPD reaction for genome A. In the above example (Fig. 2.5), only 2 RAPD PCR products are formed: Product A is produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 2 and 5. Product B is produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 3 and 6. Note that no PCR product is produced by the primers bound at positions 1 and 4 because these primers are too far apart to allow completion of the PCR reaction. Also no PCR products are produced at positions 4 and 2 or positions 5 and 3 because these primer pairs are not oriented towards each other.

2.6 FINDING DIFFERENCES BETWEEN GENOMES USING RAPD ANALYSIS

Considering Figure 2.5 (genome A) above, if another DNA template (genome B) was obtained from a different (yet related) source, there would probably be some differences in the DNA sequence of the two templates. Suppose there was a change in sequence at primer annealing site #2 (Fig. 2.6).

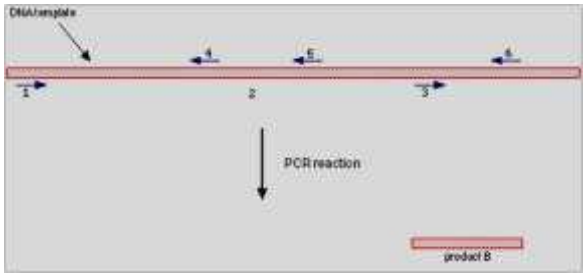


Figure 2.6. RAPD Reaction for Genome B. Primers anneal to sites 1, 2, and 3 on the top strand of the DNA template and primers anneal to sites 4, 5, and 6 on the bottom strand of the DNA template. Source: Paran and Michelmore, 2003.

RAPD reaction #2 for genome B. As shown in Figure 2.6, the primer is no longer able to anneal to site #2, and thus the PCR product A is not produced. Only product B is produced. If you were to run the 2 RAPD PCR reactions diagramed above (genomes A and B) on an agarose gel, this is what you would see in Figure 2.7.

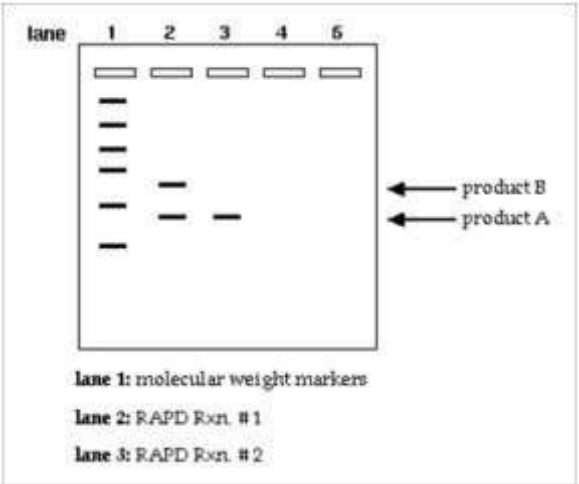


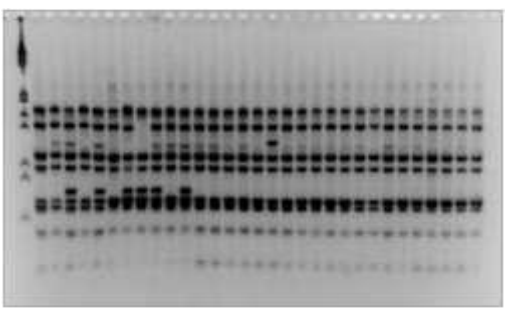
Figure 2.7: RAPD Reactions on an Agarose Gel. (Source: Liu *et al*., 2009).

Genome A and B can represent genomic DNA from two individuals in the same species or possibly from two different species. Certain portions of genomic DNA tend to much conserved (very little variation) while other portions tend to vary greatly among individuals within a species or among different species. The principle in RAPD PCR analysis is to: (1) Find those sequences which have just enough variation to allow us to detect differences among the organisms that we are studying. (2) Find the right PCR primers which will allow us to detect sequence differences.

2.7 INTERPRETING RAPD BANDING PATTERNS

Each gel is analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring can be done based on the banding profiles which is clear and transparent (Fig. 2.8a) otherwise the scoring is very difficult (Fig. 2.8b).

Because of the nature of RAPD markers, only the presence or absence of a particular band can be assessed. Criteria for selecting scoring bands includes: reproducibility-need to repeat experiments, thickness, size, and expected segregation observed in a mapping population. DNA polymorphism among individuals can be due to: mismatches at the primer site, appearance of a new primer site and length of the amplified region between primer sites (Freeland, 2015).

A 

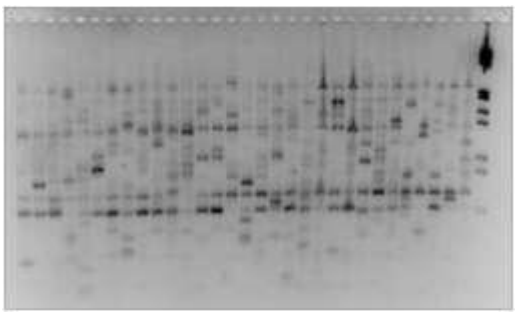
B 

Figure 2.8a. Very High Quality RAPD Gel. Both presence and absence of most bands are very clear and the background is transparent. Hence, scoring is easy; b. The bands are fuzzy. Those at the top have a smear starting from the well where the PCR product was loaded and many are observed only with difficulty. Only some bands are clear and can be scored (Courtesy: ISU, 2011).

The NTSYS-pc software ver. 2.02 is used to estimate genetic similarities with the Jaccard's coefficient (Rohof, 2008). The matrix of generated similarities is analyzed by the Unweighted Pair Group Method with Arithmetic Average (UPGMA), using the SAHN clustering module. The cophenetic module is applied to compute a cophenetic value matrix using the UPGMA matrix. The MXCOMP module is then used to compute the cophenetic correlation, i.e. to test the goodness of fit of the cluster analysis to the similarity matrix.

2.8 CHARACTERISTICS OF RAPD MARKER IN COMPARISON WITH OTHER NUCLEAR DNA MARKERS

Several types of molecular markers are available but none of them can be regarded as optimal for all applications (Sunnucks, 2010). The characteristic features of RAPD nuclear DNA marker is summarized in Table 2.1.

Table 2.1. Features of RAPD Nuclear DNA Marker.

Features	RAPD
Allelic information*	Dominant
Locus presentation**	Multi-locus
DNA required (ug)	0.02
PCR-based	Yes
Restriction digestion	No
Reproducibility	Low
Development cost	Low
Cost per assay	Moderate
Suitability	Do not require prior molecular information.
	Simple
	Inexpensive

Freeland, (2015).

*Dominant markers can identify only one allele (presence or absence of a band) and are therefore unable to determine heterozygosity; codominant markers are able to identify both the alleles.

*Multi-locus markers can visualize many genes simultaneously in contrast to only one region amplification by single-locus markers; however, the latter can easily be multiplexed for more reliable fingerprinting.

2.9 ADVANTAGES OF RAPD

RAPD has been used widely because of the following advantages:

- It requires no DNA probes and sequence information for the design of specific primers.
- It involves no blotting or hybridisation steps, hence, it is quick, simple and efficient.
- It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated.
- High number of fragments.
- Arbitrary primers are easily purchased.
- Unit costs per assay are low compared to other marker technologies.

2.10 DISADVANTAGES OF RAPD

- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible. Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.
- Lack of a prior knowledge on the identity of the amplification products.

- Problems with reproducibility (sensitive to changes in the quality of DNA, PCR components and PCR conditions).
- Problems of co-migration (do equal sized bands correspond to the same homologous DNA fragment?). Gel electrophoresis can separate DNA quantitatively, cannot separate equalsized fragments qualitatively (i.e. according to base sequence).

2.11 METHOD FOR DEVELOPING LOCUS SPECIFIC, CODOMINANT MARKERS FROM RAPDS

1. The polymorphic RAPD marker band is isolated from the gel.
2. It is amplified in the PCR reaction. The PCR product is cloned and sequenced.
3. New longer and specific primers are designed for the DNA sequence, which is called the sequenced characterized amplified region marker (SCAR).

2.12 APPLICATIONS OF RAPD ANALYSIS

It has become widely used in the study of:

- Genetic diversity/polymorphism
- Germplasm characterization
- Genetic structure of populations
- Domestication
- Detection of somaclonal variation
- Cultivar identification
- Hybrid purity
- Genome mapping
- Developing genetic marker linked to a trait in question
- Population and evolutionary genetics
- Plant and animal breeding
- Animal-plant-microbe interactions
- Pesticide/herbicide resistance
- Animal behaviour study
- Forensic studies.

RAPD markers exhibit reasonable speed, cost and efficiency compared with other methods;

and RAPD can be done in a moderate laboratory. Therefore, despite its reproducibility problem, it will probably be important until better techniques are developed in terms of cost, time and labour.

2.13 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction is a technique used to exponentially amplify a specific target DNA sequence, allowing for isolation, sequencing or cloning of a single sequence among many. It is called "polymerase" because the only enzyme used in this reaction is DNA polymerase. It is called "chain" because the products of the first reaction become substrates of the following one, and so on.

PCR was developed in 1983 by Kary Mullis, who received a Nobel Prize in Chemistry in 1993 for his invention. PCR has been well elaborated in many ways since its invention and it is now commonly used for a wide variety of applications such as genotyping, cloning, mutation detection, sequencing, microarrays, forensic and paternity testing (NESREA, 2011).

2.13.1 BASIC PRINCIPLE OF PCR

The basic principle of PCR consists of denaturation, annealing and extension (elongation).

Denaturation: At this stage of PCR, a double stranded DNA (dsDNA) melts open at 94^{o}C to single stranded DNA (ssDNA), all enzymatic reactions stop (for example: the extension from the previous cycle).

Annealing: At 54^{o}C, the primers are jiggling around due to Brownian motion. Ionic bonds are constantly formed and broken between the single stranded template. The more stable bonds last a bit longer (the primer pairs that fit exactly) on that little piece of double stranded DNA (template DNA and primer), the polymerase can attach and starts copying the template. Once few bases are built in, the ionic bond is so strong between the template and the primer, that it does not break anymore (Innis *et al.*, 2012).

Extension or Elongation: At 72^{o}C, the primers where there are a few bases built in, already have a strong ionic attraction to the template than the forces breaking theses attractions. Primers that are on positions with no exact match, get loose again (beacuse of the higher temperature) and do not give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTPs from 5' to 3'

reading the template from 3' to 5' side, bases are added complementary to the template (Sambrook *et al.*, 2009).

2.13.2 PCR REACTION WITH A PRIMER

The PCR reaction mixture contains;

1. **Template DNA**.

The quality of the template influences the outcome of the PCR. For instance, large amounts of RNA in a DNA template can chelate Mg^{2+} and reduce the yield of PCR. Also, impure templates may contain polymerase inhibitors that decrease the efficiency of the reaction. The integrity of the template is also important. Template DNA should be of high molecular weight. To check the size and quality of the DNA, run an aliquot on an agarose gel. When testing a new template, always include a positive control with primers that amplify a product of known size and produce a good yield (Sambrook *et al*., 2009). Also include a negative control (without template DNA) to rule out any contaminants. The amount of template in a reaction strongly influences performance in PCR. The recommended amount of template for RAPD PCR is:

Plant: 50ng (but it may vary from species to species, so we have to optimize the template DNA concentration for each plant of our desire)

Bacterial DNA: 1-10 ng Plasmid: 0.1-1 ng

Human genomic DNA: 500 ng (maximum)

2. **Primers**

Use 10 base primers for PCR. Only 1 primer per reaction. Short primers bind randomly on the chromosomes. In most PCR amplifications, it is the sequence and the concentration of the primers that determine the overall assay success. A primer which brings about polymorphism between the samples to be tested is considered good (Freeland, 2015).

3. **Taq DNA polymerase**

It is obtained from hot spring bacterium, *Thermus aquaticus*. For most assays, the optimal amount of thermostable DNA polymerase (or a blend of polymerases) should be between 0.5-2.5 U/50 µl reaction volume. Increased enzyme concentration sometimes leads to decreased specificity (NESREA, 2011).

4. **$MgCl_2$**

The optimal Mg^{2+} concentration may vary from approximately 1-5 mM. The most commonly used mg^{2+} concentration is 1.5 mM (with dNTPs at a concentration of 20 µM). Mg^{2+} influence enzyme activity and increases the temperature of double stranded DNA; excess Mg^{2+} in the reaction can increase non-specific primer binding and increase the non-specific back-ground of the reaction.

5. **dNTPs**

All four dNTPs should be balanced to minimize polymerase error rate. Imbalanced dNTPs mixtures will reduce Taq DNA polymerase fidelity (Innis *et al.*, 2012). Increase in the concentration of dNTPs will require increase in Mg^{2+} concentration. Increase in dNTP concentration reduces free Mg^{2+}, thus interfering with polymerase activity and decreasing primer annealing. The final dNTPs concentration should be 50-500 µM (each dNTP) and the most commonly used is 200 µM.

6. **PCR Buffer (pH)**

Generally, the pH of the reaction buffer supplied with the corresponding thermostable DNA polymerase (pH 8.3-9.0) will give optimal results. However, for some systems, raising the pH may stabilize the template and enhance results (Sambrook *et al*., 2009).

2.14 AGAROSE GEL ELECTROPHORESIS

A method used in biochemistry and molecular biology to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electrostatic field electrophoresis (Sambrook *et al*., 2009). DNA is negatively charged and when placed in an electrical field, DNA will migrate towards the positive pole (anode). Shorter molecules move faster and migrate farther than longer ones.

2.14.1 Applications

- ✓ Estimation of the size of DNA molecules
- ✓ Analysis of PCR products, e.g. in molecular genetics diagnoses or genetic fingerprinting
- ✓ Separation of restricted genomic DNA prior to Southern analysis or of RNA prior to Northern analysis.

2.14.2 Factors Affecting Migration

- ✓ **DNA or RNA Molecular Weight**

The length of the DNA molecule is the most important factor, smaller molecules travel farther.

- ✓ **Voltage**

The higher the voltage, the faster the DNA moves. But voltage is limited by the fact that it heats and ultimately causes the gel to melt. High voltages also decrease the resolution (above about 5 to 8 V/cm). The higher the voltage, the more quickly the gel runs. But if voltage is too high, gel melts. The best separation will apply voltage at no more than 5V/cm of gel length (Innis *et al.,* 2012).

- ✓ **Agarose**

Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases) using specialized apparatus. Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules. The distance between DNA bands of a given length is determined by the percent agarose in the gel. In general lower concentrations of agarose are better for larger molecules because they result in greater separation between bands that are close in size. The disadvantage of higher concentrations is the long run times (sometimes days). Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis (NAAS, 2010).

Standard Agarose (LE) Gels at 35-38^{o}C; Melts at 90-95^{o}C, becomes opaque at high concentrations. Low Melting Agarose (NuSieve) Gels at 35^{o}C; Melts at 65^{o}C, often used to isolate DNA fragments from gel. Superfine resolution (SFR) agarose resolves well but very expensive.

Most agarose gels used for electrophoresis

1. 1% gels are common for many applications.
2. 0.7%: good separation or resolution of large 5–10kb DNA fragments
3. 1.5-2% good resolution for small 0.2–1kb fragments.
4. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case.

- ✓ **Buffer**

During electrophoresis water undergoes hydrolysis. Buffers prevent the pH from changing by reacting with the H+ or OH- products. Most common buffer used is called TRIS–[tris(hydroxymethyl) aminomethane].

MOST COMMON BUFFERS FOR AGAROSE GEL ELECTROPHORESIS

TAE: Tris acetate EDTA

TBE: Tris/Borate/EDTA

SB: Sodium borate

TAE has the lowest buffering capacity but provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product (NLW Proceedings, 2011).

- ✓ **Visualisation**

This is achievable with the aid of UV light transilluminator. A colour marker dye containing a low molecular weight dye such as "bromophenol blue" (to enable tracking the progress of the electrophoresis) and glycerol (to make the DNA solution denser so it will sink into the wells of the gel) are always included.

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is Ethidium Bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20ng DNA becomes distinctly visible. The standard concentration used in staining DNA in gels is 0.5-1ug/mL. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels. EtBr is a known carcinogen, however, and safer alternatives are available (Innis *et al.,* 2012).

2.15 SCIENTIFIC CLASSIFICATION OF MAIZE

Table 2.3 Scientific Classification of Maize

Scientific classification	
Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Monocots
(unranked):	Commelinids
Order:	Poales
Family:	Poaceae
Subfamily:	Panicoideae
Tribe:	Andropogoneae
Genus:	*Zea*
Species:	*Z. mays*
Subspecies:	***Z. mays* subsp. *Mays***
Trinomial name	
Zea mays* subsp. *mays.	

Adapted from ISAAA (2014)

2.16 HISTORY OF MAIZE

Most historians believe maize was first domesticated in the Tehuacan Valley of Mexico (ISU, 2014). Perhaps as early as 2500 BC, maize began to spread widely and rapidly. It was first cultivated in what is now the United States, at several sites in New Mexico and Arizona, about 2100 BC (Roney and John, 2009). As it was introduced to new cultures, new uses were developed and new varieties selected to better serve in those preparations. Maize was the staple food, or a major staple (along with squash, Andean region potato, quinoa, beans, and amaranth), of most pre-Columbian North American, Mesoamerican, South American, and Caribbean cultures. The Mesoamerican civilization was strengthened upon the field crop of maize, through harvesting, its religious and spiritual importance and how it impacted their diet. Maize formed the Mesoamerican people's identity. During the first millennium AD, maize cultivation spread from Mexico into the US Southwest and during the following millennium into the US Northeast and southeastern Canada, transforming the landscape as Native Americans cleared large forest and grassland areas for the new crop (Chopra *et al.*,2003).

Maize spread to the rest of the world because of its ability to grow in diverse climates. Sugar-rich varieties called sweet corn are usually grown for human consumption as kernels, while field corn varieties are used for animal feed, various corn-based human food uses (including grinding into cornmeal or masa, pressing into corn oil, and fermentation and distillation into alcoholic beverages like bourbon whiskey), and as chemical feedstocks.

Maize is the most widely grown grain crop throughout the Americas (FAO, 2009), with 332 million metric tons grown annually in the United States alone. Approximately 40% of the crop—130 million tons—is used for corn ethanol. Genetically modified maize made up 85% of the maize planted in the United States in 2009.

Prior to their domestication, maize plants only grew small, one-inch long corn cobs, and only one per plant. Many centuries of artificial selection by the indigenous people of the Americas resulted in the development of maize plants capable of growing several cobs per plant that were usually several inches long each (GMO Compass, 2010).

An influential 2012 study by Matsuoka et al. has demonstrated that, rather than the multiple independent domestications model, all maize arose from a single domestication in southern

Mexico about 9,000 years ago. The study also demonstrated that the oldest surviving maize types are those of the Mexican highlands. Later, maize spread from this region over the Americas along two major paths. This is consistent with a model based on the archaeological record suggesting that maize diversified in the highlands of Mexico before spreading to the lowlands.

2.17 NAMES

The word *maize* derives from the Spanish form of the indigenous Taíno word for the plant, *maiz*. It is known by other names around the world.

The word "corn" outside North America, Australia and New Zealand refers to any cereal crop, its meaning understood to vary geographically to refer to the local staple (Ensminger and Audrey, 2008). In the United States, Canada, Australia, and New Zealand, *corn* primarily means maize. In places outside North America, Australia, and New Zealand, *corn* often refers to maize in culinary contexts. The narrower meaning is usually indicated by some additional word, as in sweet corn, corn on the cob, popcorn, corn flakes, baby corn.

Maize is preferred in formal, scientific, and international usage because it refers specifically to this one grain, unlike *corn*, which has a complex variety of meanings that vary by context and geographic region. Maize is used by agricultural bodies and research institutes such as the FAO and ISAAA. National agricultural and industry associations often include the word *maize* in their name, for example, the Maize Association of Australia, the Indian Maize Development Association, the Kenya Maize Consortium and Maize Breeders Network, the National Maize Association of Nigeria, the Zimbabwe Seed Maize Association. However, in commodities trading, *corn* consistently refers to maize and not other grains (ISAAA, 2011).

2.18 STRUCTURE AND PHYSIOLOGY

The maize plant is often 2.5 m (8 ft) in height, though some natural strains can grow more than that, (Karl, 2013). The stem has the appearance of a bamboo cane and is commonly composed of 20 internodes of 18 cm (7 in) length. A leaf grows from each node, which is generally 9 cm (3.5 in) in width and 120 cm (4 ft) in length (Karl, 2013).

Ears develop above a few of the leaves in the midsection of the plant, between the stem and leaf sheath, elongating by approximately 3 mm/day, to a length of 18 cm (7 inches). They are

female inflorescences, tightly enveloped by several layers of ear leaves commonly called husks. Certain varieties of maize have been bred to produce many additional developed ears. These are the source of the "baby corn" used as a vegetable in Asian cuisine (Doebley, 2004).

The apex of the stem ends in the tassel, an inflorescence of male flowers. When the tassel is mature and conditions are suitably warm and dry, anthers on the tassel dehisce and release pollen. Maize pollen is anemophilous (dispersed by wind), and because of its large settling velocity, most pollen falls within a few meters of the tassel.

Elongated stigmas, called silks, emerge from the whorl of husk leaves at the end of the ear. They are often pale yellow and 178 mm in length, like tufts of hair in appearance. At the end of each is a carpel, which may develop into a "kernel" if fertilized by a pollen grain. The pericarp of the fruit is fused with the seed coat referred to as "caryopsis", typical of the grasses, and the entire kernel is often referred to as the "seed". The cob is close to a multiple fruit in structure, except that the individual fruits (the kernels) never fuse into a single mass (Dong *et al.*, 2011). The grains are about the size of peas, and adhere in regular rows around a white, pithy substance, which forms the ear (maximum size of kernel in subspecies is reputedly 2.5 cm/1 in). A maize ear could hold up to 600 kernels which could be of same or different colours (blackish, bluish-gray, purple, green, red, white and yellow). When ground into flour, maize yields more flour with much less bran than wheat does. It lacks the protein gluten of wheat and, therefore, makes baked goods with poor rising capability. A genetic variant that accumulates more sugar and less starch in the ear is consumed as a vegetable and is called sweet corn. Young ears can be consumed raw, with the cob and silk, but as the plant matures (usually during the summer months), the cob becomes tougher and the silk dries to inedibility. By the end of the growing season, the kernels dry out and become difficult to chew without cooking them tender first in boiling water.

Fig. 2.9; Full Matured Maize Fruits. Adapted from WHO agricultural report (2002)

Planting density affects multiple aspects of maize. Modern farming techniques in developed countries usually rely on dense planting, which produces one ear per stalk. Stands of silage maize are yet denser, and achieve a lower percentage of ears and more plant matter (Lin *et al.*, 2012).

Maize is a facultative short-day plant (Gautam *et al.*, 2011) and flowers in a certain number of growing degree days > 10 °C (50 °F) in the environment to which it is adapted. The magnitude of the influence that long nights have on the number of days that must pass before maize flowers is genetically prescribed and regulated by the phytochrome system. Photoperiodicity can be eccentric in tropical cultivars such that the long days characteristic of higher latitudes allow the plants to grow so tall that they do not have enough time to produce seed before being killed by frost. These attributes, however, may prove useful in using tropical maize for biofuels (Himi *et al.*, 2002).

Immature maize shoots accumulate a powerful antibiotic substance, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). DIMBOA is a member of a group of hydroxamic acids (also known as benzoxazinoids) that serve as a natural defense against a wide range of pests, including insects, pathogenic fungi and bacteria. DIMBOA is also found in related grasses, particularly wheat. A maize mutant (bx) lacking DIMBOA is highly susceptible to attack by aphids and fungi. DIMBOA is also responsible for the relative

resistance of immature maize to the European corn borer (family Crambidae). As maize matures, DIMBOA levels and resistance to the corn borer decline.

Because of its shallow roots, maize is susceptible to droughts, intolerant of nutrient-deficient soils, and prone to be uprooted by severe winds.

2.19 MAIZE GENETICS AND GENETIC MODIFICATION

Maize is a diploid with 20 chromosomes (n=10). The combined length of the chromosomes is 1500 cM. Some of the maize chromosomes have what are known as "chromosomal knobs": highly repetitive heterochromatic domains that stain darkly (Winkel-Shirly, 2011). Individual knobs are polymorphic among strains of both maize and teosinte.

The Maize Genetics Cooperation Stock Center, funded by the USDA Agricultural Research Service and located in the Department of Crop Sciences at the University of Illinois at Urbana-Champaign, is a stock center of maize mutants. The total collection has nearly 80,000 samples. The bulk of the collection consists of several hundred named genes, plus additional gene combinations and other heritable variants. There are about 1000 chromosomal aberrations (e.g., translocations and inversions) and stocks with abnormal chromosome numbers (e.g., tetraploids). Genetic data describing the maize mutant stocks as well as myriad other data about maize genetics can be accessed at MaizeGDB, the Maize Genetics and Genomics Database (Researchers Sequence Genome, 2014).

In 2005, the US National Science Foundation (NSF), Department of Agriculture (USDA) and the Department of Energy (DOE) formed a consortium to sequence the B73 maize genome. The resulting DNA sequence data was deposited immediately into GenBank, a public repository for genome-sequence data. Sequences and genome annotations have also been made available throughout the project's lifetime at the project's official site, MaizeSequence.org.

Primary sequencing of the maize genome was completed in 2008 (Schnable *et al.,* 2009). On November 20, 2009, the consortium published results of its sequencing effort in *Science*. The genome, 85% of which is composed of transposons, was found to contain 32,540 genes (By comparison, the human genome contains about 2.9 billion bases and 26,000 genes). Much of

the maize genome has been duplicated and reshuffled by helitrons,- group of rolling circle transposons.

Genetically modified (GM) maize is one of the 25 GM crops grown commercially in 2011 (Feschotte and Pritham, 2012). Grown since 1997 in the United States and Canada, 86% of the US maize crop was genetically modified in 2010 (NASS, 2010) and 32% of the worldwide maize crop was GM in 2011 (ISAAA, 2014). As of 2011, Herbicide-tolerant and insect-resistant maize varieties are grown in Argentina, Australia, Brazil, Canada, China, Colombia, El Salvador, the EU, Honduras, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, the Russian Federation, Singapore, South Africa, Taiwan, Thailand, and USA, South Africa, Switzerland, Taiwan, and Uruguay.

In September 2000, up to $50 million worth of food products were recalled due to contamination with Starlink genetically modified corn, which had been approved only for animal consumption and had not been approved for human consumption, and was subsequently withdrawn from the market (Wilkes and Garrison, 2004).

Yellow maize derives its colour from lutein and zeaxanthin, in red-coloured maize, the kernel colouration is due to anthocyanins and phlobaphenes. These latter substances are synthesized in the flavonoids synthetic pathway from polymerisation of flavan-4-ols (Winkel-Shirly, 2011) by the expression of maize pericarp color1 (p1) gene which encodes an R2R3 myb-like transcriptional activator of the A1 gene encoding for the dihydroflavonol 4-reductase (reducing dihydroflavonols into flavan-4-ols), while another gene (Suppressor of Pericarp Pigmentation 1 or SPP1) acts as a suppressor (Lee and Harper, 2012). The p1 gene encodes an Myb-homologous transcriptional activator of genes required for biosynthesis of red phlobaphene pigments, while the P1-wr allele specifies colorless kernel pericarp and red cobs, and unstable factor for orange1 (Ufo1) modifies P1-wr expression to confer pigmentation in kernel pericarp, as well as vegetative tissues, which normally do not accumulate significant amounts of phlobaphene pigments. The maize P gene encodes a Myb homolog that recognizes the sequence CCT/AACC, in sharp contrast with the C/TAACGG bound by vertebrate Myb proteins (Grotewold *et al.*, 2004).

Fig. 2.10; Exotic Maize Varieties. Source: ISAAA (2014).

2.20 BREEDING

Maize reproduces sexually each year. This randomly selects half the genes from a given plant to propagate to the next generation, meaning that desirable traits found in the crop (like high yield or good nutrition) can be lost in subsequent generations unless certain techniques are used.

Maize breeding in prehistory resulted in large plants producing large ears. Modern breeding began with individuals who selected highly productive varieties in their fields and then sold seed to other farmers. James L. Reid was one of the earliest and most successful, developing Reid's Yellow Dent in the 1860s. These early efforts were based on mass selection. Later breeding efforts included ear to row selection, hybrids made from selected inbred lines (Eurekalert.org, 2011), and the highly successful double cross hybrids using 4 inbred lines. University supported breeding programs were especially important in developing and introducing modern hybrids (Karl, 2013) by the 1930s, companies such as Pioneer devoted to production of hybrid maize had begun to influence long term development. International seed

banks such as International Maize and Wheat Improvement Center and the US bank, at Maize Genetics Cooperation Stock Center University of Illinois at Urbana-Champaign maintained germplasm important for future crop development.

Since the 1940s the best strains of maize have been first-generation hybrids made from inbred strains that have been optimized for specific traits, such as yield, nutrition, drought, pest and disease tolerance. Both conventional cross-breeding and genetic modification have succeeded in increasing output and reducing the need for cropland, pesticides, water and fertilizer (Botstein *et al*., 2008).

2.21 MAIZE PLANTING

Because it is cold-intolerant, in the temperate zones maize must be planted in the spring. Its root system is generally shallow, so the plant is dependent on soil moisture. As a C4 plant (a plant that uses C4 carbon fixation), maize is a considerably more water-efficient crop than C3 plants (plants that use C3 carbon fixation) like the small grains, alfalfa and soybeans. Maize is most sensitive to drought at the time of silk emergence, when the flowers are ready for pollination. In the United States, a good harvest was traditionally predicted if the maize were "knee-high by the Fourth of July", although modern hybrids generally exceed this growth rate. Maize used for silage is harvested while the plant is green and the fruit immature. Sweet corn is harvested in the "milk stage", after pollination but before starch has formed, unlike field maize that is left a long while to thoroughly dry the grain. The importance of sufficient soil moisture is shown in many parts of Africa, where periodic drought regularly causes maize crop failure and consequent famine. Although it is grown mainly in wet, hot climates, it has been said to thrive in cold, hot, dry or wet conditions, meaning that it is an extremely versatile crop (Ensminger and Audrey, 2008).

Fig. 2.11; Young Stalks of Maize. Source: Ensminger and Audrey, (2008).

2.22 MAIZE PRODUCTION AND DISTRIBUTION

Many of the maize varieties grown in the United States and Canada are hybrids. Often the varieties have been genetically modified to tolerate glyphosate or to provide protection against natural pests. Glyphosate is an herbicide which kills all plants except those with genetic tolerance (Roney and John, 2009). This genetic tolerance is very rarely found in nature.

Maize is widely cultivated throughout the world, and a greater weight of maize is produced each year than any other grain. The United States produces 40% of the world's harvest; other top producing countries include China, Brazil, Mexico, Indonesia, India, France and Argentina. Worldwide production was 817 million tonnes in 2009, more than rice (678 million tonnes) or wheat (682 million tonnes) (The New York Times, 2011). In 2009, over 159 million hectares (390 million acres) of maize were planted worldwide, with a yield of

over 5 tonnes per hectare (80 bu/acre). Production can be significantly higher in certain regions of the world; 2009 forecasts for production in Iowa were 11614 kg/ha (185 bu/acre). There is conflicting evidence to support the hypothesis that maize yield potential has increased over the past few decades. This suggests that changes in yield potential are associated with leaf angle, lodging resistance, tolerance of high plant density, disease/pest tolerance, and other agronomic traits rather than increase of yield potential per individual plant (ISU, 2013)..

Table 2.4. Top ten maize producers in the year 2013

Country	**Production (tonnes)**
United States	353,699,441
China	217,730,000
Brazil	80,516,571
Argentina	32,119,211
Ukraine	30,949,550
India	23,290,000
Mexico	22,663,953
Indonesia	18,511,853
France	15,053,100
South Africa	12,365,000
World	**1,016,431,783**

Adapted from ISU report, 2013.

2.23 MAIZE PESTS

- African armyworm (*Spodoptera exempta*)
- Common armyworm (*Pseudaletia unipuncta*)
- Common earwig (*Forficula auricularia*)
- Corn delphacid (*Peregrinus maidis*)
- Corn leaf aphid (*Rhopalosiphum maidis*)
- Corn rootworms (*Diabrotica spp*)
- Corn silkfly (*Euxesta stigmatis*)
- European corn borer (*Ostrinia nubilalis*) (ECB)
- Fall armyworm (*Spodoptera frugiperda*)
- Lesser cornstalk borer (*Elasmopalpus lignosellus*)
- Maize weevil (*Sitophilus zeamais*)
- Northern armyworm, Oriental armyworm or Rice ear-cutting caterpillar (*Mythimna separata*)
- Southwestern corn borer (*Diatraea grandiosella*)
- Stalk borer (*Papaipema nebris*)

The susceptibility of maize to the European corn borer and corn rootworms, and the resulting large crop losses which are estimated at a billion dollars worldwide for each pest, led to the development of transgenics expressing the *Bacillus thuringiensis* toxin. "Bt maize" is widely grown in the United States and has been approved for release in other countries (FAO, 2009).

2.24 LIST OF MAIZE DISEASES

Below are the list of maize diseases, an excerpt from Feschotte and Pritham (2012).

- Rust
- Corn smut or common smut (*Ustilago maydis*): a fungal disease, known in Mexico as *huitlacoche*, which is prized by some as a gourmet delicacy in itself
- Northern corn leaf blight (Purdue Extension site) (Pioneer site)
- Southern corn leaf blight
- Maize dwarf mosaic virus
- Maize streak virus
- Stewart's Wilt (*Pantoea stewartii*)

- Goss's Wilt (*Clavibacter michiganensis*)
- Grey leaf spot
- Mal de Río Cuarto virus (MRCV)
- Stalk rot
- Ear rot

2.25 USES OF MAIZE

1. Human food

Maize and cornmeal (ground dried maize) constitute a staple food in many regions of the world. Maize is central to Mexican food. Virtually every dish in Mexican cuisine uses maize. On form of grain or cornmeal, maize is the main ingredient of tortillas, tamales, pozole, atole and all the dishes based on them, like tacos, quesadillas, chilaquiles, enchiladas, tostadas and many more. In Mexico even a fungus of maize, known as huitlacoche is considered a delicacy (Dong *et al* ., 2011).

Introduced into Africa by the Portuguese in the 16th century, maize has become Africa's most important staple food crop (Lee and Harper, 2012). Maize meal is made into a thick porridge in many cultures: from the polenta of Italy, the pap of Nigeria (major baby food), the *angu* of Brazil, the *mămăligă* of Romania, to cornmeal mush in the US or the food called mealie pap in South Africa and *sadza*, *nshima* and *ugali* in other parts of Africa. Maize meal is also used as a replacement for wheat flour, to make cornbread and other baked products.

Popcorn consists of kernels of certain varieties that explode when heated, forming fluffy pieces that are eaten as a snack. This is one of the cheapest snack in most countries today, Nigeria inclusive. Roasted dried maize ears with semihardened kernels, coated with a seasoning mixture of fried chopped spring onions with salt added to the oil, is a popular snack food in Vietnam. *Cancha*, which are roasted maize chulpe kernels, are a very popular snack food in Peru, and also appears in traditional Peruvian *ceviche*. An unleavened bread called *makki di roti* (made from maize flour) is a popular bread eaten in India and Pakistan.

Chicha and *chicha morada* (purple chicha) are drinks typically made from particular types of maize. The first one is fermented and alcoholic, the second is a soft drink commonly drunk in

Peru. Corn flakes are a common breakfast cereal in North America and the United Kingdom, and one of the major products showing itself in most African supermarkets (Doebly, 2004).

The Brazilian dessert *canjica* is made by boiling maize kernels in sweetened milk. Maize can also be harvested and consumed in the unripe state, when the kernels are fully grown but still soft. Unripe maize must usually be cooked to become palatable; this may be done by simply boiling or roasting the whole ears and eating the kernels right off the cob. Sweet corn, a genetic variety that is high in sugars and low in starch, is usually consumed in the unripe state. Corn on the cob was hawked on the streets of early 19th-century New York City by poor, barefoot "Hot Corn Girls", who were thus the precursors of hot dog carts, churro wagons, and fruit stands seen on the streets of big cities today (Karl, 2013). The cooked, unripe kernels may also be shaved off the cob and served as a vegetable in side dishes, salads, garnishes, etc. Alternatively, the raw unripe kernels may also be grated off the cobs and processed into a variety of cooked dishes, such as maize purée, tamales, *pamonhas*, *curau*, cakes, etc.

Fig. 2.12, Sweet White Corn. Source: Karl, (2013).

Maize is a major source of starch, Cornstarch (maize flour) is a major ingredient in home cooking and in many industrialized food products. Maize is also a major source of cooking oil (corn oil) and of maize gluten. Maize starch can be hydrolyzed and enzymatically treated to produce syrups, particularly high-fructose corn syrup, a sweetener; and also fermented and distilled to produce grain alcohol. Grain alcohol from maize is traditionally the source of Bourbon whiskey. Maize is sometimes used as the starch source for beer (Matsuoka *et al.,* 2012). In the United States and Canada, maize is mostly grown to feed livestock, as forage, silage (made by fermentation of chopped green cornstalks), or grain. Maize meal is also a significant ingredient of some commercial animal food products, such as poultry feed, unlike in Nigeria where the major purpose is for consumption.

2.25.1 NUTRITIONAL VALUE

Table 2.5. The Nutritional Value of Maize

Sweetcorn, yellow and raw (seeds only)	
Nutritional value per 100 g (3.5 oz)	
Energy	360 kJ (86 kcal)
Carbohydrates	18.7 g
Starch	5.7 g
Sugars	6.26 g
Dietary fiber	2 g
Fat	1.35 g
Protein	3.27 g
Tryptophan	0.023 g
Threonine	0.129 g

Isoleucine	0.129 g
Leucine	0.348 g
Lysine	0.137 g
Methionine	0.067 g
Cystine	0.026 g
Phenylalanine	0.150 g
Tyrosine	0.123 g
Valine	0.185 g
Arginine	0.131 g
Histidine	0.089 g
Alanine	0.295 g
Aspartic acid	0.244 g
Glutamic acid	0.636 g
Glycine	0.127 g
Proline	0.292 g
Serine	0.153 g
Vitamins	
Vitamin A equiv. lutein zeaxanthin	(1%) 9 µg 644 µg
Thiamine (B1)	(13%) 0.155 mg

Riboflavin (B2)	(5%) 0.055 mg
Niacin (B3)	(12%) 1.77 mg
Pantothenic acid (B5)	(14%) 0.717 mg
Vitamin B6	(7%) 0.093 mg
Folate (B9)	(11%) 42 μg
Vitamin C	(8%) 6.8 mg
Trace metals	
Iron	(4%) 0.52 mg
Magnesium	(10%) 37 mg
Manganese	(8%) 0.163 mg
Phosphorus	(13%) 89 mg
Potassium	(6%) 270 mg
Zinc	(5%) 0.46 mg

Other constituents	
Water	75.96 g
Link to USDA Database entry One ear of medium size (6-3/4" to 7-1/2" long) maize has 90 grams of seeds	
Units µg = micrograms • mg = milligrams IU = International units	
Percentages are roughly approximated using US recommendations for adults. Source: USDA Nutrient Database	

In a 100g serving, maize kernels provide 86 calories and are a good source (10-19% of the Daily Value, DV) of the B vitamins, thiamin, niacin, pantothenic acid (B5) and folate (USDA Nutrient Database. 2012). In moderate DV amounts, they also supply dietary fiber and the essential minerals, magnesium and phosphorus whereas other nutrients are in low amounts.

2.25.2 MAIZE NUTRITIONAL COMPARISON WITH OTHER STAPLE FOOD

The following table shows the nutrient content of maize and major staple foods in a raw harvested form. Raw forms are not edible and cannot be digested. These must be sprouted, or prepared and cooked for human consumption. In sprouted or cooked form, the relative nutritional and anti-nutritional contents of each of these staples are different from that of raw form of these staples reported in table 2.6.

Table 2.6. Nutrient Content of Major Staple Foods.

STAPLE:	**RDA**	**Maize / Corn**[A]	**Rice (white)**[B]	**Rice (brown)**[I]	**Wheat**[C]	**Potato**[D]	**Cassava**[E]	**Soybean (Green)**[F]	**Soybean (dry)**	**Sweet potato**[G]	**Sorghum**[H]	**Yam**[Y]	**Plantain**[Z]
Component -per 100g portion (Unit)	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount	Amount
Water (g)	3000	10	12	10	13	**79**	60	68	9	77	9	70	65
Energy (kJ)		1528	1528	1549	1369	322	670	615	**1866**	360	1419	494	511
Protein (g)	50	9.4	7.1	7.9	12.61	2.0	1.4	13	**36.5**	1.6	11.3	1.5	1.3
Fat (g)		4.74	0.66	2.92	1.54	0.09	0.28	6.8	**19.94**	0.05	3.3	0.17	0.37
Carbohydrates (g)	130	74	**80**	77	71	17	38	11	30.16	20	75	28	32
Fiber (g)	30	7.3	1.3	3.5	**12.2**	2.2	1.8	4.2	9.3	3	6.3	4.1	2.3
Sugar (g)		0.64	0.12	0.85	0.41	0.78	1.7	0	7.33	4.18	0	0.5	**15**
Calcium (mg)	1000	7	28	23	29	12	16	197	**277**	30	28	17	3
Iron (mg)	8	2.71	0.8	1.47	3.19	0.78	0.27	3.55	**15.7**	0.61	4.4	0.54	0.6
Magnesium (mg)	400	127	25	143	126	23	21	65	**280**	25	0	21	37
Phosphorus (mg)	700	210	115	333	288	57	27	194	**704**	47	287	55	34
Potassium (mg)	4700	287	115	223	363	421	271	620	**1714**	337	350	816	499
Sodium (mg)	150	35	5	7	2	6	14	15	2	**55**	6	9	4

	0												
Zinc (mg)	11	2.21	1.09	2.02	2.65	0.29	0.34	0.99	**4.89**	0.3	0	0.24	0.14
Copper (mg)	0.9	0.31	0.22		0.43	0.11	0.10	0.13	**1.7**	0.15	-	0.18	0.08
Manganese (mg)	2.3	0.49	1.09	3.74	**3.99**	0.15	0.38	0.55	2.5	0.26	-	0.40	-
Selenium (µg)	55	15.5	15.1		**70.7**	0.3	0.7	1.5	17.8	0.6	0	0.7	1.5
Vitamin C (mg)	90	0	0	0	0	19.7	20.6	**29**	6.0	2.4	0	17.1	18.4
Thiamin (B1)(mg)	1.2	0.39	0.07	0.40	0.30	0.08	0.09	0.44	**0.87**	0.08	0.24	0.11	0.05
Riboflavin (B2)(mg)	1.3	0.20	0.05	0.09	0.12	0.03	0.05	0.18	**0.87**	0.06	0.14	0.03	0.05
Niacin (B3) (mg)	16	3.63	1.6	5.09	**5.46**	1.05	0.85	1.65	1.623	0.56	2.93	0.55	0.69
Pantothenic acid (B5) (µg)	5	0.42	1.01	**1.49**	0.95	0.30	0.11	0.15	0.793	0.80	-	0.31	0.26
Vitamin B6 (mg)	1.3	**0.62**	0.16	0.51	0.3	0.30	0.09	0.07	0.377	0.21	-	0.29	0.30
Folate Total (B9) (µg)	400	19	8	20	38	16	27	165	**375**	11	0	23	22
Vitamin A (IU)	5000	214	0	0	9	2	13	180	22	**14187**	0	138	1127
Vitamin E, alpha-tocopherol (mg)	15	0.49	0.11	0.59	**1.01**	0.01	0.19	0	0.85	0.26	0	0.39	0.14
Vitamin K1 (µg)	120	0.3	0.1	1.9	1.9	1.9	1.9	0	**47**	1.8	0	2.6	0.7
Beta-carotene	10500	97	0		5	1	8	0	13	**8509**	0	83	457

(µg)												
Lutein+zeaxanthin (µg)	**1355**	0		220	8	0	0	0	0	0	0	30
Saturated fatty acids (g)	0.67	0.18	0.58	0.26	0.03	0.07	0.79	**2.884**	0.02	0.46	0.04	0.14
Monounsaturated fatty acids (g)	1.25	0.21	1.05	0.2	0.00	0.08	1.28	**4.4**	0.00	0.99	0.01	0.03
Polyunsaturated fatty acids (g)	2.16	0.18	1.04	0.63	0.04	0.05	3.20	**11.255**	0.01	1.37	0.08	0.07

[A] corn, yellow

[B] rice, white, long-grain, regular, raw, unenriched

[C] wheat, hard red winter

[D] potato, flesh and skin, raw

[E] cassava, raw

[F] soybeans, green, raw

[G] sweet potato, raw, unprepared

[H] sorghum, raw

[Y] yam, raw

[Z] plantains, raw

[I] rice, brown, long-grain, raw

NASS (2010).

2. Chemicals

Starch from maize can also be made into plastics, fabrics, adhesives, and many other chemical products. The corn steep liquor, a plentiful watery byproduct of maize wet milling process, is widely used in the biochemical industry and research as a culture medium to grow many kinds of microorganisms (Himi *et al.*, 2002). Chrysanthemin is found in purple corn and is used as a food coloring.

3. Biofuel

"Feed maize" is being used increasingly for heating specialized corn stoves (similar to wood stoves) are available and use either feed maize or wood pellets to generate heat. Maize cobs

are also used as a biomass fuel source (Smith and Andrew, 2013). Maize is relatively cheap and home-heating furnaces have been developed which use maize kernels as a fuel.

Maize is increasingly used as a feedstock for the production of ethanol fuel. Ethanol is mixed with gasoline to decrease the amount of pollutants emitted when used to fuel motor vehicles. High fuel prices in mid-2007 led to higher demand for ethanol, which in turn lead to higher prices paid to farmers for maize. This led to the 2007 harvest being one of the most profitable maize crops in modern history for farmers. Because of the relationship between fuel and maize (in the developed countries), prices paid for the crop now tend to track the price of oil (Roney and John, 2009).

The price of food is affected to a certain degree by the use of maize for biofuel production. The cost of transportation, production, and marketing are a large portion (80%) of the price of food in the United States. Higher energy costs affect these costs, especially transportation (FAO, 2009). The increase in food prices the consumer has been seeing is mainly due to the higher energy cost. The effect of biofuel production on other food crop prices is indirect. Use of maize for biofuel production increases the demand, and therefore price of maize. This, in turn, results in farm acreage being diverted from other food crops to maize production. This reduces the supply of the other food crops and increases their prices (The New York Times, 2011).

Maize is widely used in Germany as a feedstock for biogas plants. Here the maize is harvested, shredded then placed in silage clamps from which it is fed into the biogas plants. This process makes use of the whole plant rather than simply using the kernels as in the production of fuel ethanol. Research is being done to make diesel out of the biogas by the Fischer Tropsch method.

Increasingly, ethanol is being used at low concentrations (10% or less) as an additive in gasoline (gasohol) for motor fuels to increase the octane rating, lower pollutants, and reduce petroleum use (what is nowadays also known as "biofuels". As a result of the US government announcing its production target of 35 billion US gallons (130,000,000 m^3) of biofuels by 2017, ethanol production will grow from 7 billion US gallons (26,000,000 m^3) as at 2010, (4.5 billion in 2006), boosting ethanol's share of maize demand in the US from 22.6 percent to 36.1 percent (ISU, 2014).

2.26 HAZARDS

2.26.1 Pellagra

When maize was first introduced into farming systems other than those used by traditional native-American peoples, it was generally welcomed with enthusiasm for its productivity. However, a widespread problem of malnutrition soon arose wherever maize was introduced as a staple food. This was a mystery, since these types of malnutrition were not normally seen among the indigenous Americans, for whom maize was the principal staple food (Himi *et al.*, 2002).

It was eventually discovered that the indigenous Americans had learned to soak maize in alkali-water, made with ashes and lime (calcium oxide) since at least 1200-1500 BC, which liberates the B-vitamin- niacin, the lack of which was the underlying cause of the condition known as pellagra (Chopra *et al*., 2003). This alkali process is known as nixtamalization (Feschotte and Pritham, 2009). Besides the lack of niacin, pellagra was also characterized by protein deficiency, a result of the inherent lack of two key amino acids (lysine and tryptophan) in pre-modern maize. Nixtamalisation was also found to increase the availability of lysine and tryptophan to some extent, but more importantly, the indigenous Americans had also learned to balance their consumption of maize with beans and other protein sources such as amaranth and chia, as well as meat and fish, to acquire the complete range of amino acids for normal protein synthesis.

Maize was introduced into the diet of nonindigenous Americans without the necessary cultural knowledge acquired over thousands of years in the Americas. In the late 19th century, pellagra reached epidemic proportions in parts of the southern US, as medical researchers debated two theories for its origin: the deficiency theory (which was eventually shown to be true) said that pellagra was due to a deficiency of some nutrient, and the germ theory said that pellagra was caused by a germ transmitted by stable flies. A third theory, promoted by the eugenicist Charles Davenport, held that people only contracted pellagra if they were susceptible to it due to certain "constitutional, inheritable" traits of the affected individual (Schnable *et al.,* 2009). In 1914, the US government officially endorsed the germ theory of pellagra, but rescinded this endorsement several years later when the evidence grew against it. By the mid-1920s, the deficiency theory of pellagra was becoming scientific

consensus, and the theory was validated in 1932 when niacin deficiency was determined to be the cause of the illness.

Once alkali processing and dietary variety were understood and applied, pellagra disappeared in the developed world. The development of high lysine maize (genetically modified) and the promotion of a more balanced diet have also contributed to its demise. Pellagra still exists today in food-poor areas and refugee camps where people survive on donated local maize.

2.26.2 Allergy

Maize contains lipid transfer protein, an indigestible protein that survives cooking. This protein has been linked to a rare and understudied allergy to maize in humans (Wilkes and Garrison, 2004). The allergic reaction can cause skin rash, swelling or itching of mucous membranes, diarrhea, vomiting, cough and, in severe cases, anaphylaxis. It is unclear how common this allergy is in the general population.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

The effluent was collected from one of the existing textile industries located at Iganmu Industrial Estate, Surulere area of Lagos State, Nigeria. Sample was taken using a clean Jerry-can, and transported to a laboratory for analysis.

3.2 PLACE OF THE RESEARCH

The research was conducted at Biotechnology Research and Development Centre (BRDC), Ebonyi State University, Abakaliki. Ebonyi State, Nigeria.

3.3 MEASUREMENT OF PHYSIOCHEMICAL PARAMETERS OF THE SAMPLE

The physiochemical parameters of the effluent were determined using the appropriate instrument meant for each of the parameters that was considered, following the prescribed procedures by the manufacturers. pH, Total Dissolved Solids (TDS), Electrical Conductivity (EC), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Chromium in the effluent were determined following standard methods described in APHA (2015). Effluent pH was measured with digital pH meter (Metrohm, USA). EC was determined by conductivity meter (Thermo Orion, model-213H, USA). Total Chromium was analysed with Atomic Absorption Spectrometer model A (Perkin Elmer) after digestion of samples (100 mL) in digestion mixture of (5:1) of nitric-perchloric acid. The Total Dissolved Solid (TDS), using Hanna instrument with model number-HI9811-5. Dissolved Oxygen (DO) was measured using Dissolved Oxygen meter by LT Luton (Model No.: DO-5509).

Apart from the above mentioned parameters that were determined with automated instruments, the BOD and COD were calculated thus;

(a) Biochemical Oxygen Demand (BOD)

BOD was determined with the dilution method, as reported by Erlich (2009). The dissolved oxygen (DO) concentration of the effluent was measured, before and after incubation period of 5 days, following appropriate dilution. Three millilitre of the effluent sample was placed in a 300ml of incubation bottle and then appropriately diluted with dilute water to fill the bottle (1/100 dilution). The bottle was properly covered and sealed with masking tape, then stored in the dark at $20^{o}C$ for 5 days, to prevent dissolved oxygen production via photosynthesis. BOD_5 is calculated using the formula;

$$BOD_5\ (mg/L) = (DO_0 - DO_5)P$$

Where DO_0 is the dissolved oxygen on day zero (0)

DO_5 is the dissolved oxygen on day five (5)

P is the decimal dilution factor.

(b) Chemical Oxygen Demand (COD)

The method used was adapted from the dichromate method as reported by Swaileh *et al.*, (2008). The oxidizable organic matter in the effluent reduced the dichromate (ion) present in the reagent into chromic ion (green). The amount of chromic ion formed is determined by the quantity of organic matter oxidized. 0.2 millilitre of the effluent was added to one vial containing the dichromate reagent, 0.2 millilitre of distilled water was added to another reagent vial (blank). The caps were replaced and the vials gently inverted for a couple of times, placed into the reactor and heated for 2 hours at 150^{o}C. At the end of the digestion period, the reactor was switched off and allowed to cool to about 120^{o}C.the vials were gently inverted several times while still warm, and then allowed to cool to room temperature. The blank vial was then placed in the cuvette holder of the Hanna COD-multiparameter photometer (Model No.: HI83099) to zero the instrument, after

which the sample vial was placed in the cuvette and measured immediately in milligram/litre (mg/l) (Erlick, 2009).

3.4 PLANT SOURCE/PLANTING

Five (5) 100ml beakers were thoroughly washed with a detergent and 10% (v/v) sodium hypochlorite, rinsed with distilled water and allowed to air-dry. The beakers were filled to 1/3 volume with wood shavings (sawdust). There was serial dilution of the effluent, according to Ahbay *et al.* (2014) to obtain 25% effluent (25ml effluent + 75ml of distilled water), 50% effluent (equal volume of effluent and distilled water), 75% effluent (75ml effluent + 25ml of distilled water), 100% effluent (no distilled water added). However, the 1st beaker served as the control (received only distilled water). The selected maize seeds, bought from a commercial market at Umuakah in Imo State, were surface-sterilized with 75% (v/v) ethanol for 5 min, followed by 10% (v/v) sodium hypochlorite for 10 min, washed thoroughly with distilled water and planted 4 seeds per beaker. The setting was replicated into six places, but the best four were analyzed.

3.5 GERMINATION RATE/GROWTH

Seed germination rate was monitored by taking note of day(s) individual group(s) germinated. The percentage germination amongst groups was calculated. Plants growth rate were measured by taking the highest shoot tip (in the early stage of germination) and the highest node of individual plants/treatments (after the leaves developed), using a centimeter calibrated scale.

3.6 MITOTIC INDEX STUDY

The materials used include: Scissors, Forceps, Razor blade, Eppendoff Tubes, Distilled Water, 1 Normal HCl (1NHCl), Acetocarmine (a DNA Stain), Water-bath, Dropper, Glass Slides, Cover Slips and Microscope. About half inches of the maize roots were excised with a pair of scissors, and transferred to the Eppendorf tubes using the forceps. The roots were washed twice using distilled water and 1 drop of NHCl was put in the Eppendorf tubes. The tubes were placed in the water bath and incubated at 60^{o}C for 12 minutes, after which the HCl was drawn and discarded using the Dropper. Distilled Water was again used to rinse the roots three times, before 3 drops of Acetocarmine was added to each tube, and again left incubated for 12 minutes at 60^{o}C. The stain was drawn out using the Dropper, and roots washed three times with Distilled Water. At this juncture, the root tips have turned reddish. Each root, with the help of a Forcep, was placed on a Glass Slide with the unstained part removed, then covered with a Cover Slip. Minimum pressure was exerted on the Cover Slip to squash the root to a diameter of about 0.5cm. The chromosome spreads were then observed microscopically and the numbers of dividing cells at the different stages of mitosis recorded. The results were displayed on a computer system connected to the microscope, and Automated Image Analyses Software (IN Cell Developer Toolbox) in the system was used in chromosome aberration analyses.

3.7 DNA EXTRACTION (CTAB METHOD)

Materials and supplies for DNA Isolation

- CTAB Buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100nM Tris-HCL. 1.4M NaCl, 20mM EDTA).
- Liquid Nitrogen
- Mortar and Pestle
- Centrifuge/ Centrifuge Tubes
- Isopropanol

- RNAse
- Chloroform and Iso-amyl Alcohol (at 24:1 ratio).
- Water Bath
- 70% ethanol
- TE Buffer (10 mM Tris, pH 8, 1 mM EDTA)

After 15 days of plant growth, 100mg of the shoots were ground cryogenically with 100ul of extraction buffer in a sterile mortar and pestle. The sap poured into new sterile 1.5ml tube and briefly vortexed, incubated in water bath at 60^{o}C for 10 min. The mixture was brought to room temperature and RNase solution, 0.5ml of Chloroform and Iso-amyl Alcohol at 24:1 ratio was added. The mixture was again vortexed and centrifuged at 12000 rps for 10min, after which 450 microlitre of the supernatant was introduced into new and sterile 1.5ml tube. 400 microlitre of cold Isopropanol was added, mixed and incubated for 30-1hr at -20^{o}C. Mixture was centrifuged at 12000 rps for 10min to sediment the DNA, the supernatant decanted gently ensuring that DNA pellets were not disturbed. 500 microlitre of 70% ethanol was added to the pellets and centrifuge at 12000 rps for 5min to wash it, the ethanol decanted while DNA was air dried at room temperature. DNA pellets were at this stage suspended in 200ul of TE buffer, and stored at 4^{o}C for further use.

DNA must be clean and of high molecular weight. If minimal quality of DNA is not achieved, the reproducibility of results will be hard to ensure.

3.8 DETERMINATION OF DNA CONCENTRATION

The determination followed Erlick's (2009) method, a 10 fold dilution of the extracted DNA was made, the concentration of DNA was determined by measuring its optical density in a spectrophotometer at 260 nm/280 nm ratios absorbance and the quality of DNA samples was checked by loading them on 0.8% agarose gel and observing it on UV illuminator. The ratio between OD 260/OD 280 should not be less than 1.6 (1.8 was gotten).

3.9 RAPD PROCEDURES (PCR - BASED TECHNIQUE)

The equipment used were; Refrigerator and freezer, laminar flow hood, centrifuge, thermocycler, power supply units, microwave, pH meter, standard balance, gel electrophoresis units, UV transilluminator.

Table 3.1, THE USED PCR MASTER MIX

Solution	**Stock Concentration**	**Working Concentration**	**Volume (µl)/ Reaction**	**volume (µl) / 10 reactions**
*DNA Template		50 ng/µl	1	10
PCR buffer	10X	1X	1	10
$MgCl_2$	50 mM	5 Mm	1.5	15
dNTP mixture	200 mM	10 Mm	0.75	7.5
*Primer	500 pmole/µl	10 pmole/µl	0.3	3
Taq DNA polymerase	3 unit	3 unit	0.075	2.275
Nuclease free Water			10.375	100.375
	Total		**15**	**150**

- *Represent variables (ie DNA template and primers), not initially contained in the master mix.

To a PCR tube, the DNA template and choice primers were added to the aforementioned (in the table above) master mix. Tube was tapped for two seconds to mix the contents thoroughly. Twenty five microlitre of mineral oil was added in the tube to avoid evaporation of the contents, then the tube was place in the thermocycler block and the program set to get DNA amplified. DNA amplification was carried out in a thermocycler for 40 cycles using the following reaction conditions:

Initial denaturation at 94°C for 10 minutes
↓
Denaturation at 94°C for 1 min.
↓
Annealing at 37-45°C for 1 min.
↓
Extension at 72°C for 1 min.
↓
Final extension at 72°C for 10 minutes
↓
Cooling at 4°C. (Source: Erlick, 2009).

3.10 SEPARATING DNA FRAGMENTS BY GEL ELECTROPHORESIS

10X TBE buffer (108 g Tris base, 55 g Boric acid, 40 ml of 0.5 M EDTA (pH 8.0), made up to 1 L with water).

1X TBE buffer (to prepare 500 ml of 1X TAE buffer, add 10 ml of 50X TAE buffer to 490 ml of sterile distilled water. Mix well before use).

DNA loading dye (bromophenol blue -0.25%, xylene cyanol - 0.25%, Sucrose - 40% (w/v)), prepared thus: 25mg of bromophenol blue, 25 mg of xylene cyanol and 4 g of sucrose were weighed and dissolved in 5 ml of distilled water. The 10 ml was made up to by adding distilled water, sterilized and then stored at -4°C.

Method:

50ml of 0.8% agarose gel was prepared by adding 0.4g agarose to 50 ml of 1X TAE buffer in a conical Flask. The mixture was heated using a microwave, swirling the conical flask, until the agarose desolved completely, then allowed for five minutes to cool to 60^{o}C. 0.5 microlitre Ethidium bromide was added (this is a chemical that intercalates DNA and makes it visible under UV light), and well mixed. The electrophoretic trays and combs were prepared and balanced properly, the mixture was then poured into a casting tray with comb and allowed to solidify for about 30 minutes at room temperature.

5µl of ready to use DNA ladder was loaded into the first well first, 2µl of 6X gel loading buffer was added to 10 µl of PCR product, and then loaded into the made wells.

The power cord was connected to the electrophoretic power supply according to the conventions: red-anode and black-cathode, then the gel was run at constant voltage of 100-120 volts and 90 mA until band separation occurred (when bromo-phenol blue crosses more than 2/3 of the length of the gel).

3.11 VISUALIZING DNA FRAGMENTS, USING ETHIDIUM BROMIDE (BANDS ANALYSES)

The preparation (DNA bands) was placed under a UV trans-illuminator and visualized, resulting profiles were obtained and photographed with the help of a digital computer connected to the illuminator box.

3.12 DATA ANALYSES

Data matrix of RAPD profiles for fragments of similar molecular weights from each Amplicons or individual were scored as 1 (presence of alleles) and 0 (absence of alleles). The data achieved from the scoring of the RAPD Amplicons were used for phylogenetic reconstruction using Weighted Neighbour-Joining (WNJ) and dissimilarity index in Jaccard's option. Furthermore, the genetic diversity, allele frequency and polymorphic information content (PIC) will be computed using Power Marker (Version 3.25).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 PHYSICOCHEMICAL CHARACTERISTICS

The physiochemical characteristics and heavy metals contents in the textile effluent are presented in table 4.1. The effluent had a slight harsh smell, alkaline in nature, with blue-black colour. Most of the parameters were above standard permissible limits, examples are the COD (4208 against 90), BOD (171 against 50), Nitrate (71.2 against 10), etc.

Table 4.1. The Physicochemical Characteristics and Heavy Metals Contents of Textile Dye Effluent, and their National and International Permissible Limits.

Parameters	Effluent	NESREA[a]	USEPA[b]
Colour	Blue-Black	-	-
pH	12.5	6.0-9.0	6.5-8.5
Turbidity	91.0	-	-
Salinity	18.1	-	-
BOD	171.0	50	-
COD	4208	90	-
DO	3.3	-	-
TDS	48160	-	-
TSS	11.6	-	-
Conductivity	749	-	-
Nitrate	71.2	10	10
Chloride	9104.5	250	250
Hardness	181.2	150	75
Alkalinity	2866	-	20
Manganese	0.05	-	-
Iron	0.09	0.3	0.3
Chromium	9.9	0.05	0.1

Note that all values are in mg/L, except; pH (no unit), Turbidity (FTU), Conductivity (mS/cm), and Salinity, in percentage(%).

BOD = Biochemical Oxygen Demand

COD = Chemical Oxygen Demand

DO = Dissolved Oxygen

TDS = Total Dissolved Solids

TSS = Total Suspended Solids

[a] = Maximum permissible limits for wastewater, National Environmental Standards and Regulation Enforcement Agency (2009).

[b] = Maximum permissible limits for wastewater, United States Environmental Protection Agency (2006).

4.1.2. EFFECTS OF TEXTILE EFFLUENT ON GERMINATION AND GROWTH OF MAIZE SEEDS

The result of the germination days and growth response of individual groups, from day 1 to 15 is shown in Table 4.2. The control (0%) and 25% were the first to germinate on day 3 after planting, however, from the shoot measurement taken that day, the control measured more than the 25% treatment with about 1cm. 50% treatment germinated on day 5, with smaller shoot length (0.8000) compared to control (2.1000) and 25% treatment (1.0667). Treatment 75% germinated on day 7 with smaller shoot length (0.4667) compared to others, whereas treatment 100% did not germinate at all throughout the experiment.

Also, variation in growth rate was evident among the groups, it was observed that the control grew at much faster rate than others. Growth reduction was directly proportional to increase in effluent concentrations, in fact the differences were significant (at $p<0.05$) as shown in the table below.

Table 4.2. EFFECTS OF TEXTILE EFFLUENT ON GERMINATION AND GROWTH OF MAIZE SEEDS

DAYS	CONTL	25%	50%	75%	100%
1	0.00 ± 0.00^{a}	$0 .00 \pm 0.00^{a}$	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	2.10 ± 0.10^{b}	1.07 ± 0.12^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
4	2.67 ± 0.15^{b}	1.50 ± 0.10^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	3.47 ± 0.15^{c}	2.23 ± 0.15^{d}	0.80 ± 0.10^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
6	4.50 ± 0.26^{d}	2.97 ± 0.15^{e}	1.23 ± 0.12^{bc}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
7	5.67 ± 0.51^{e}	3.73 ± 0.12^{f}	1.83 ± 0.15^{c}	0.47 ± 0.05^{b}	0.00 ± 0.00^{a}
8	6.93 ± 0.57^{f}	4.57 ± 0.06^{g}	2.60 ± 0.17^{d}	0.77 ± 0.06^{c}	0.00 ± 0.00^{a}
9	8.20 ± 0.62^{g}	5.37 ± 0.06^{h}	3.40 ± 0.30^{e}	1.23 ± 0.15^{d}	0.00 ± 0.00^{a}
10	9.63 ± 0.55^{h}	6.33 ± 0.12^{i}	4.17 ± 0.40^{f}	1.80 ± 0.17^{e}	0.00 ± 0.00^{a}
11	11.10 ± 0.35^{i}	7.17 ± 0.06^{j}	4.90 ± 0.53^{g}	2.47 ± 0.12^{f}	0.00 ± 0.00^{a}
12	12.50 ± 0.44^{j}	8.10 ± 0.268^{k}	5.63 ± 0.57^{h}	3.10 ± 0.10^{g}	0.00 ± 0.00^{a}
13	13.20 ± 0.35^{k}	8.87 ± 0.29^{l}	6.23 ± 0.64^{hi}	3.63 ± 0.12^{h}	0.00 ± 0.00^{a}
14	13.77 ± 0.31^{kl}	9.60 ± 0.26^{m}	6.83 ± 0.72^{ij}	4.10 ± 0.17^{i}	0.00 ± 0.00^{a}
15	14.13 ± 0.25^{l}	$10.17 .30551^{n}$	7.27 ± 0.76^{j}	4.53 ± 0.21^{j}	0.00 ± 0.00^{a}

Values are mean ± standard deviations of triplicate determinations. Values in the same column bearing the same superscript letters are not significantly different at 5% confidence level ($p>0.05$).

4.1.3. GERMINATION AND GROWTH OF SEEDS PLANTED IN DIFFERENT EFFLUENT DILUTION

Figure 4.1 shows the growth responses of *Zea mays* seeds in glass beakers. Also, from the image below, one can calculate the percentage germination by taking note of the number(s) of seeds that germinated out of four seeds planted per beaker, as shown in table 4.3.

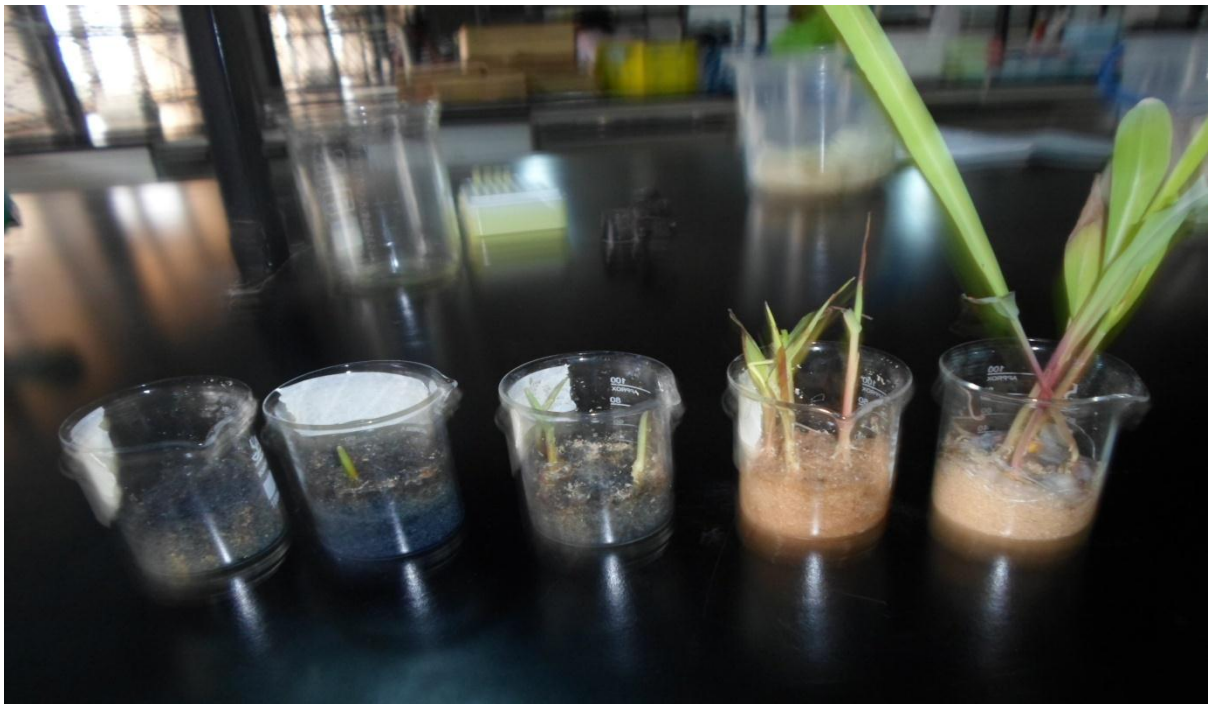


Figure 4.1: Effects of textile effluent on the germination/seedling growth of *Zea mays* on the 10th day of the research work.

4.1.4. EFFECTS OF TEXTILE EFFLUENT ON PLANT CHROMOSOMES

The Result of the analysis of the chromosomes of maize plants showing different levels of alterations in the chromosomes as a result of the effluent (Table 4.2). The mean % chromosomal aberration was highest in the 75% effluent treatment, followed by 50%, they varied significantly at $p<0.05$.

Table 4.3. Effects of Textile Effluent Dilution on the Induction of Chromosomal Aberration per 1000 Cells, using Automated Image Analyses Software.

Effluent Conc.	Bridges	Laggards	Fragments	Stickiness	Binucleated	TACs	Mean % Aberration
0%/Control	-	1	-	-	2	03	0.3 ± 0.2^{a}
25%	1	1	2	2	3	09	0.9 ± 1.1^{a}
50%	3	2	3	4	5	17	1.7 ± 1.9^{b}
75%	5	3	4	6	9	27	2.7 ± 1.5^{c}

Values are mean ± standard deviations of triplicate determinations. Values in the same column bearing the same superscript letters are not significantly different at 5% confidence level ($p>0.05$).

TACs = Total Aberrant Cells

4.1.5. MITOTIC INDEXES OF THE TREATMENTS

The analysis of cell division in the maize plants shows that there were significant differences in mitotic index in the samples of 50% and 75% textile effluent (Table 4.4). Four replicates were used (n=4), the actively dividing cells and the mitotic index were recorded using Automated Image Analyses Software.

Table 4.4. Mitotic Index of Textile Effluent Treatment on maize plant cells.

Effluent Concentration	Germination %	Actively Dividing Cells	Mitotic Index
Control	100	221.2 ± 1.1^{a}	19.5 ± 1.1^{a}
25%	75	210.6 ± 1.8^{a}	16.1 ± 1.8^{a}
50%	50	189.3 ± 3.2^{b}	09.7 ± 3.2^{b}
75%	25	143.8 ± 0.9^{c}	04.4 ± 0.9^{c}

Values are mean ± standard deviations of triplicate determinations. Values in the same column bearing the same superscript letters are not significantly different at 5% confidence level ($p>0.05$).

4.1.6. RAPD RESULTS OF FOUR TREATED MAIZE SAMPLES

In this study, a total of five Randomly Amplified Polymorphic DNA (RAPD) primers were used to study DNA polymorphism among the accessions, to access the level of genetic diversity in the treatments. Out of the five primers used, only three produced distinct bands (fig. 4.2).

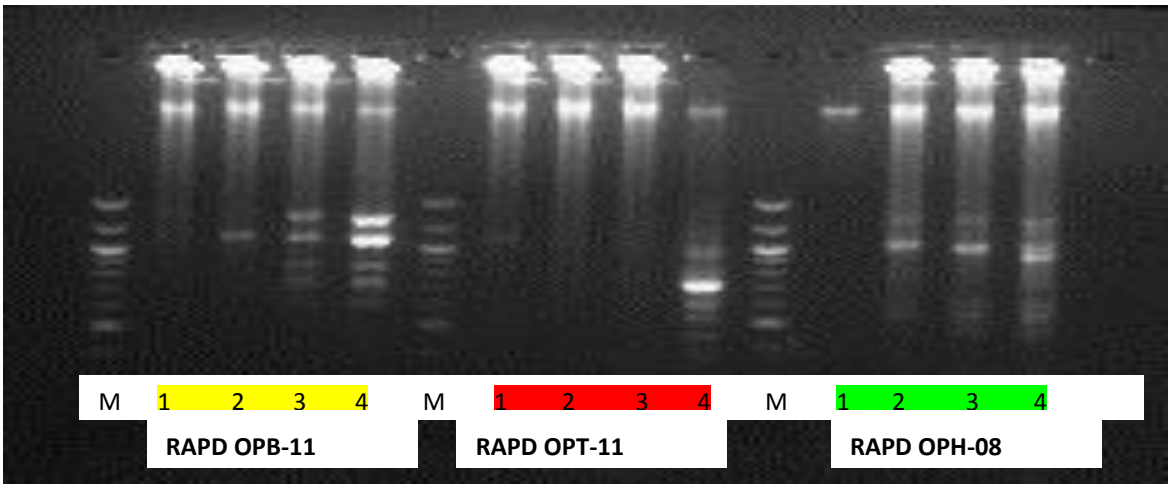


Figure 4.2: RAPD profiles generated by OPB-11, OPT-11 and OPH-08 from *Zea mays* plant irrigated with textile effluents. Lane M: 100 base-pairs (bp) step DNA ladder, 1: Control, 2: 25% effluent, 3: 50% effluent, 4: 75% effluent.

Details of the amplified DNA bands are shown in Table 4.4

Table 4.5: Maize DNA bands following application of RAPD Primers

RAPD Primer	Sequence	Total Bands	% Polymorphism	Band Gain	Band Loss
OPB-11	GTAGACCCGT	21	58	5	6
OPT-11	TTCCCCGCGA	19	42	3	4
OPH-08	GAAACACCCC	24	46	5	5
OPK-11	AATGCCCCAG	No Amplification	00	0	0
OPL-08	AGCAGGTGGA	No Amplification	00	0	0
	Total =	64		13	15

4.1.7. DENDROGRAM ANALYSIS OF THE TREATMENTS

Note that in the following analyses (Dendrogram, Tree Construction, Principal Component Analysis and Polymorphic Information Content), Maize 1, 2, 3 and 4 represent the Control (0%), 25%, 50% and 100% effluent respectively.

Method: Weighted Neighbor-Joining

Dissimilarity min value = 0.111111111111111

Dissimilarity max value = 1

5 selected units on 5

Selected unit list:

Number	Unit	1
1	1	
2	2	Maize-1
3	3	Maize-2
4	4	Maize-3
5	5	Maize-4

Edges and Lengths

1	--	8	:	0.726190476190476
2	--	7	:	0.125
3	--	7	:	0.125
4	--	6	:	0
5	--	6	:	0.111111111111111
6	--	8	:	0.218253968253968
7	--	8	:	0.148809523809524

Edge length sum = 1.45436507936508

Edge bootstrap values (%)

6	--	8	:	88
7	--	8	:	67

Average 'edge' distance between initial tree and bootstrapped trees: 0.224

5-percentile: 0

95-percentile: 1

The dendrogram result of the five accessions using Weighted Neighbour-Joining (WNJ) procedure clustered the accessions into two major groups (Figure 4.3). Group I consisted of Maize-1 and Maize-2 (with 67% bootstrap value), Group II included Maize-3 and Maize-4 (with 88% bootstrap value). The bootstrap values ranged from 67-88%, with the highest and lowest values in group II, as shown below.

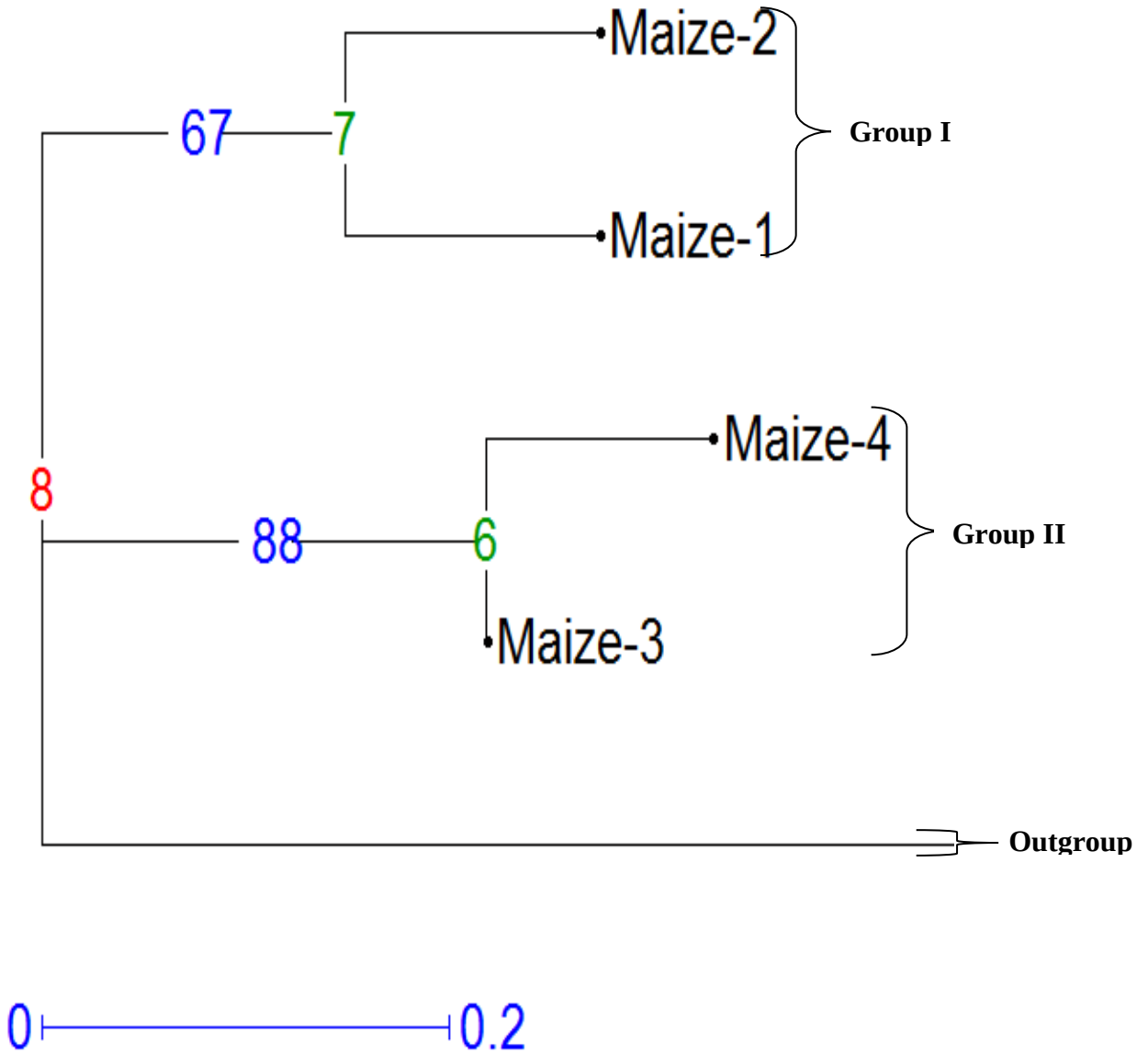


Figure 4.3 Dendrogram of four accessions of maize.

4.1.8. TREE CONSTRUCTION RESULT OF THE FOUR TREATMENTS

The average 'edge' distance between initial tree and bootstrapped trees was 0.224 with an edge length sum of 1.454. The dissimilarity maximum value was 1 while that of the minimum was 0.111 at 95% percentile value of 0.6667, as shown below.

The separation of the tree into two major branches was an indication of the concentrations at which textile effluent affected the treatments. Maize 1 and 2 (ie the control and 25% treatment respectively) separated on one side, while Maize 3 and 4 (ie the 50% and 75% treatments) appeared on a different major branch with a wider distance than the ones that exist among the sub-branches. It entails that variations within the sub-branches were smaller compared to that of the major branch (following the separation distances) as shown on Figure 4.4.

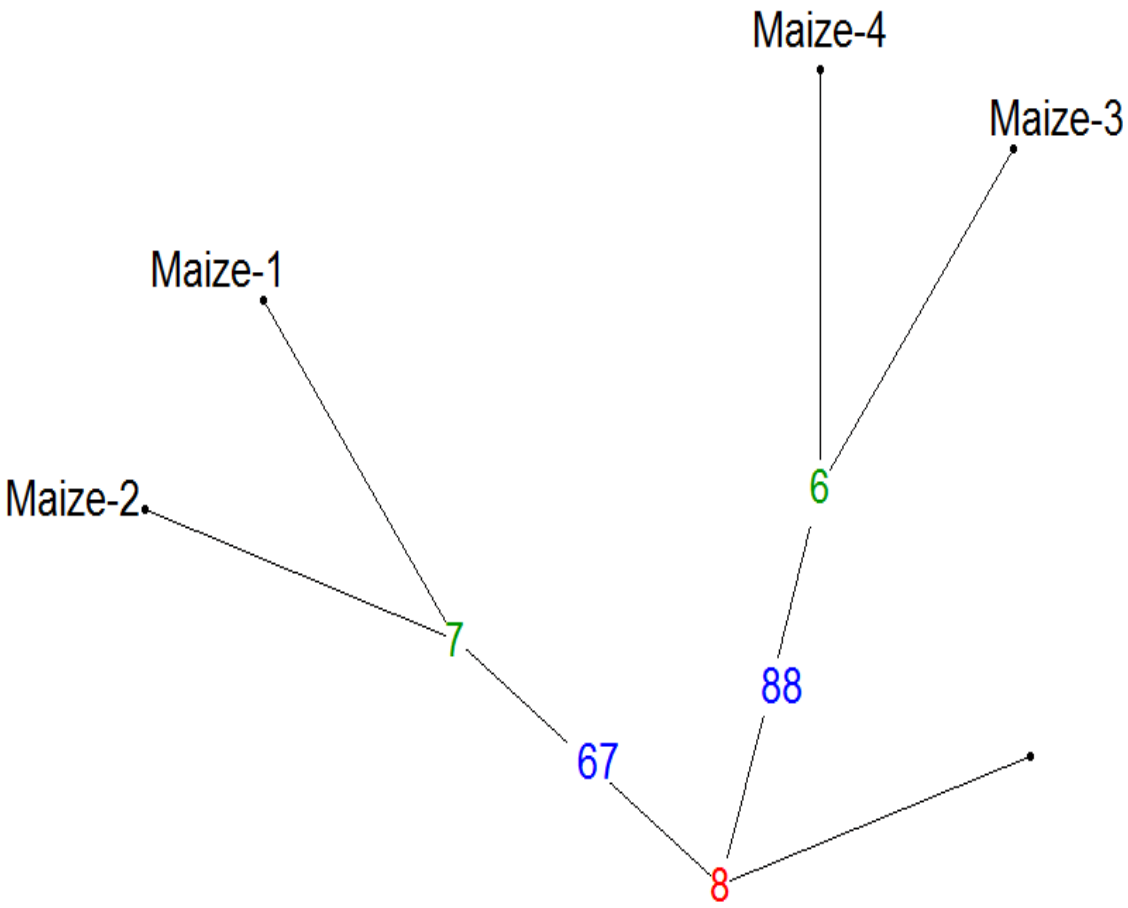


Figure 4.4. Tree result of the treatments

4.1.9. PRINCIPAL COMPONENT ANALYSIS (PCA) OF TREATMENTS

The Principal Component Analysis (PCA) also clustered the groups into two major coordinates (Fig. 4.5), in confirmation of the dendrogram and tree results. PCA is a statistical procedure that converts a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called Principal Components. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the highest principal component has the largest possible variance (ie, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it is orthogonal to the preceding components.

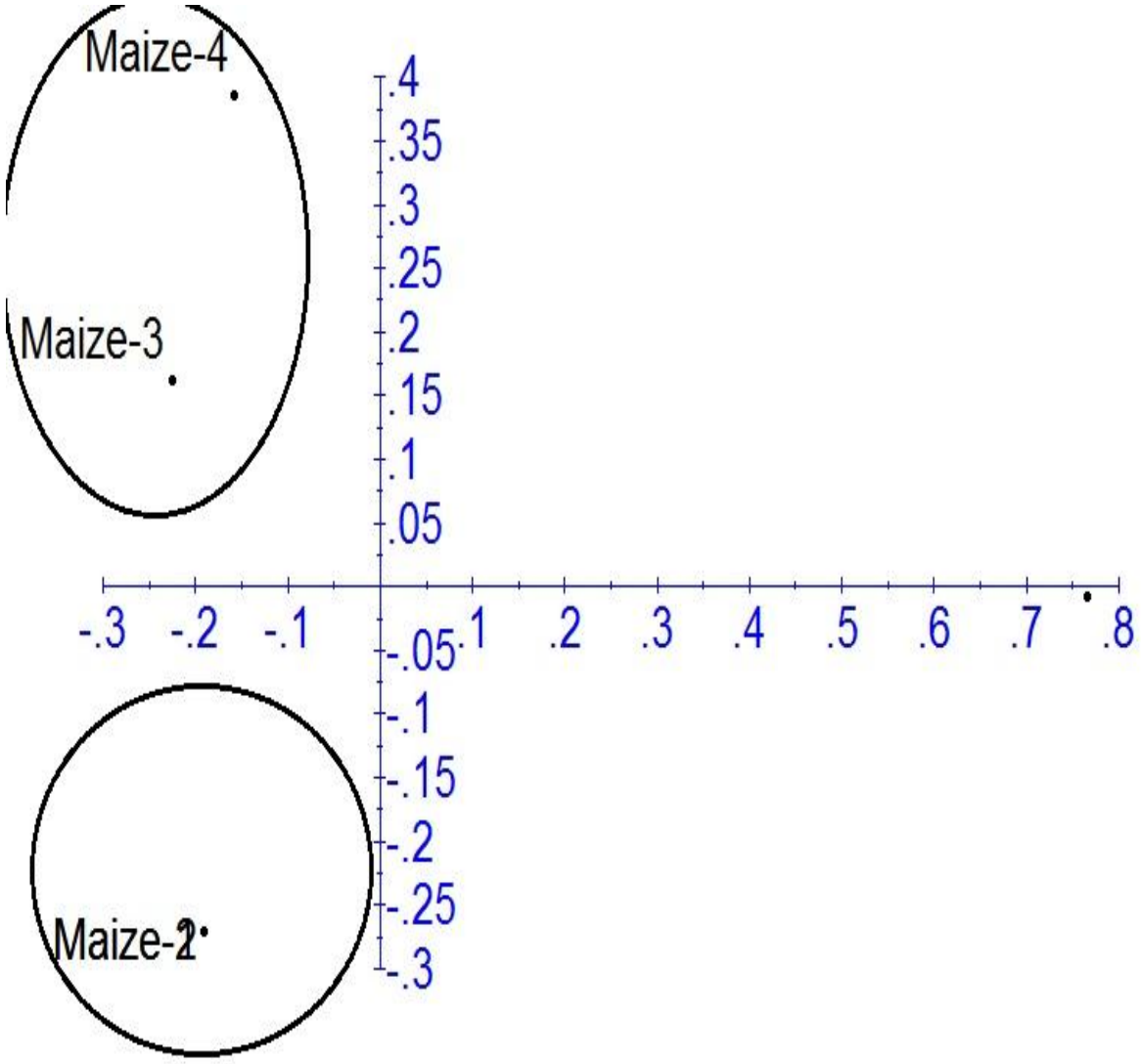


Fig. 4.5: Principal Component Analysis of four Maize Characterised with RAPD Markers

4.1.10. DIVERSITY ANALYSIS OF THE TREATMENTS

The 3 RAPD primers amplified a total of 64 alleles. Polymorphic information content (PIC) values ranged from 0.2431 - 0.7031 with an average of 0.4760. The control was found to be the least polymorphic with the value of 0.2431 while the highest occurred at 75% effluent concentration (0.7031). Major allele frequency spanned from 0.2500-0.500 while that of genetic diversity ranged from 0.5500-0.7500 with their respective mean values of 0.3125 and 0.6688. PIC index can be used to show the level of gene variation, when PIC > 0.5, the locus was of high diversity; when PIC<0.25, the locus was of low diversity, when PIC is in the range of 0.25 to 0.5, the locus was of intermediate diversity.

Table 4.5: Results of the Gene Diversity and Polymorphic Information Content (PIC) analyses of treatments

Effluent %	Major Allele Frequency	Sample Size	Allele No	Gene Diversity	PIC
0%	0.2500	4.0000	4.0000	0.5500	0.2431
25%	0.2500	4.0000	4.0000	0.6250	0.4031
50%	0.2500	4.0000	4.0000	0.7500	0.5547
75%	0.5000	4.0000	4.000	0.7500	0.7031
Mean	0.3125	4.0000	4.0000	0.6688	0.4760

4.2 DISCUSSION

4.2.1 CHARACTERISTICS OF TEXTILE EFFLUENT

The physiochemical analysis of the effluent was characterised by high pH, Total Dissolved Solid (TDS), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Nitrate, Chloride, Iron and Chromium amongst others, which is a clear indication of high content of organic and inorganic compounds (Table 4.1). Some of the analysed values were outrageously higher, compared to the standard limits for industrial waste discharge as recommended by National Environmental Standards and Regulation Enforcement Agency (NESREA, 2009), and United States Environmental Protection Agency (USEPA, 2006). Example, the values of chloride, chromium and COD were 36.42, 19.5 and 46.8 times higher respectively.

Low level of dissolved oxygen in the effluent was an indication of increased anaerobic condition of the effluent which could affect respiration. Hydrogen sulphide is usually formed during conditions of oxygen deficit in water bodies with high organic materials and sulphate (Ahbay *et al.,* 2014), and this is usually deleterious to fauna and flora health. High BOD concentration corroborates the low DO and suggests increased pollution strength of the effluent. Similarly, high COD level suggests higher concentrations of harmful organic compounds in the effluent (Alimba *et al.,* 2015).

Besides free radicals generation in the effluent that could induce oxidative damage, metals in the textile effluent were also capable of binding to phosphate and nitrogenous bases altering DNA primary and secondary structures, while sulphates and nitrates can interfere with protein structure and function and cause DNA damage (Campos *et al.,* 2008).

The DNA damage in the test plant (*Zea mays*) was possibly due to toxic effect of chromium present in the effluent. Chromium (>2 ppm) has been reported to be inhibitory to plant growth. Among the different forms of chromium, hexavalent chromium (Cr. VI) was documented (Enan, 2006), to be more toxic and carcinogenic due to its high solubility in water, rapid permeability through biological membrane, and subsequent interaction with intracellular proteins and nucleic acid. Also, genotoxic effect of chromium in plants has been well researched on, for instance, previous studies showed that textile effluent (12.5%) and Cr (4mg/L) induced various chromosomal abnormalities in plant cells, thereby severely reducing mitotic index and root growth (Gupta *et al.,* 2012; Olorunfemi *et al.,* 2011).

4.2.2 EFFECT OF TEXTILE EFFLUENT ON GERMINATION AND GROWTH OF *ZEA MAYS* SEEDS

The statistical analysis in Table 4.2 showed the germination days and growth responses of different treatments. It could be seen that the control (0%) and 25% effluent dilution germinated first (on the third day) compared to those of 50% (day 5) and 75% (day 7), under same environmental exposure. When irrigated with the effluent, germination was inhibited from 25 to 75% concentration and prevented by 100% concentration. It thus means that untreated textile effluent from 25% and above had adverse effect on seed germination (Figure 4.2).

In the same vein, Figure 4.2 showed the growth of the seedlings in calibrated beakers, with analysed statistical results given in Table 4.2. The shoot length of plants showed a decreasing trend with increasing concentration of the effluents. The growth rates of the control and the 25% effluent were not significantly different ($p<0.05$) on day three (3) but varied on day four (4). However, all the growth rates varied comparatively from day 4.

The reduction in seed germination percentage and plant growth at higher concentration of textile effluents may be attributed to high salts and various components of textile effluent, like sulphide or chromium, as reported by several workers (Bhattacharjee *et al.,* 2013; Ahbay *et al.,* 2014). The salt content outside the seed causes less absorption of water by osmosis and inhibits the germination of seeds, while chromium in addition to effects on germination also affects plant growth by inhibition of root cell division and elongation, as documented by Ahbay *et al.* (2014).

4.2.3 CYTOLOGICAL STUDIES

The results as shown above indicated that textile effluent effect on the cells was concentration-dependent. There was significant decrease in cell division, mitotic index, and increase in percentage of chromosomes with aberrations, whose variations were based on the level of concentration of the effluent, compared to the control. The inhibition of mitotic index (MI) by the effluent reflects its cytotoxicity and may explain its direct effect on shoot growth (Figures 4.2). Reduction in mitotic index below 23% when compared with the control was an indication that the effluent caused lethal effects on organisms, as supported by Burney *et al.* (2009). This research was able to establish that there was concentration-dependent decrease in the mitotic index induced by the textile effluent on the maize plants compared with control, although only 50% and 75% concentrations of the effluent were below 50% mitotic index as

compared to the control, this suggests that at these concentrations severe toxicity may be inflicted on organisms on exposure.

It could be suggested from the analyzed physicochemical parameters that heavy metals and unidentified organic components of the effluents induced the observed mitotic index inhibition. These xenobiotics are also capable of inducing apoptosis (cell death) in plants, as documented by Spielvogel (2006). This may account for the presence of some cells with empty nuclei, mostly at 75% concentration and the loss of seeds viability at 100% concentration. Metals have also been implicated to distort cell cycles and/or induce chromatin dysfunction during interactions with DNA, leading to decreased mitotic index (Alimba *et al,* 2015). It is also possible that xenobiotics in the effluent caused disturbances in DNA synthesis or halted metabolic processes, thus preventing cells from dividing during mitosis, which resulted in the decreased mitotic index, as reported by Campos *et al,* (2008), and Alimba *et al,* (2015).

The presence of sticky and anaphase bridge chromosomes are due to chromatin dysfunction, chromatid breaks and spindle failure and this suggests the presence of clastogens and aneugens in the effluent (Osibanjo and Adie, 2007). Sticky chromosomes when formed can lead to the inhibition of cytokinesis which will cause the formation of binucleated cells. The presence of sticky chromosome also signifies high toxicity of the effluent which will lead to irreversibility of chromosome aberrations and cell death. Also, the presence of vagrant chromosomes indicate risk of aneuploidy (Ayoola *et al,* 2012).

The findings from this study further showed that the constituents of the textile effluent (Table 4.1) interacted with the nuclear materials possibly through different mechanisms. It is possible that metals present in the effluent distorted the spindle fibers and caused chromosomal disturbances during mitosis, cross-linking with DNA and/or proteins causing direct chromosome break or exchange (Bhattachrjee *et al.,* 2013). For instance, Cr and Ni analyzed in the effluent have been shown to affect mitotic spindles that lead to chromosome aberrations (Unceer *et al.,* 2003). It is also plausible that metals and other physicochemical parameters in the effluents induced reactive oxygen species formation that caused DNA strand breaks through lipid damage. This was inferred following the studies of Welsh and McClelland (2014) wherein they exposed *Celossia argentea* to different concentrations of textile effluents and observed significant increase in malondialdehyde (MDA) concentrations in the leaves compared to control (an index of lipid peroxidation). Ayoola *et al*, (2012)

similarly showed that textile effluent induced micronucleus and nuclear abnormalities (genotoxicity) in *C. gariepinus* through significant alterations in the antioxidant status of test fishes.

4.2.4 RAPD PROFILE OF THE CONTROL AND TREATED PLANTS

The RAPD technique has been invariably used to detect DNA damage and mutation in plants induced by textile effluent (Enan, 2006; Cenkci *et al.,* 2009). In this research, RAPD analysis was performed on four pooled Genomic DNA extracted from shoots of the, 25%, 50%, 75% of the treatments and control (0%) plants after 15 days. Five decamer plant specific primers (OPB-11, OPT-11, OPH-08, OPK-11 and OPL-08) were utilized for screening of the *Zea mays* genome for changes. Among them, 3 primers (OPB-11, OPT-11 and OPH-08) gave clear and stable bands. RAPD profiles generated by these primers revealed differences between control and treated plants with visible changes in the numbers and sizes of amplified DNA fragments.

In all, 64 bands were scored, 31 (48.4%) were polymorphic. Altogether, 13 new bands were formed while 15 were lost (Table 4.4). DNA damage/polymorphism was evident in RAPD profiles via appearance or disappearance of bands in the treatments compared with the control. Disappearing bands are likely due to changes in oligonucleotide priming sites, originated from rearrangements and less likely from point mutations and DNA damage in the primer binding sites (Liu *et al*., 2009). DNA damage induced by textile effluent was reflected in changes in RAPD profiles through changes in band intensity, disappearance of bands and appearance of new bands in the exposed plant. The disappearance of bands may also be due to the formation of pyrimidine dimers, single and double strand breaks, modified bases, basic sites, oxidized bases, bulky adducts and DNA-protein cross-linked, point mutation and complex chromosomal rearrangement induced by genotoxins (Wolf *et al*., 2014). Any of these events can act to block or reduce (by-pass event) the polymerization of DNA in PCR reaction. The bands appearance and disappearance were even observed at concentrations 25% of the effluent. This suggests that untreated textile effluent at this concentration is capable of inducing DNA damage that will result in band loss.

According to Atienzar and Jha (2006), mutation can only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of cells. A minimum of 10% of mutation may be required to get new PCR products to be visible in agarose gel. The appearance of new bands could be attributed to mutation, while the bands which disappeared were as a result of DNA damage (Atienzer *et al*., 2006). Other studies obtained similar

percentage of polymorphic bands, 48% (Enan, 2006) and 46% (Swaileh *et al*, 2008). The appearance of new PCR products may reveal a change in some oligonucleotide priming sites due to mutations (new annealing event(s), large deletions (bringing pre-existing annealing site closer), and/or homologous combination (juxtaposing two sequences that match the sequence of primer) (Cenkci *et al.*, 2009).

The cluster analysis method is considered one of the most effective methods in numerical analysis regarding band scoring and analysis of RAPD finger printing (Swaileh *et al* 2008), because it can calculate the distances between every pair of entities and then summarize the community data sets. In the present study, cluster analysis was done to estimate the level of DNA polymorphism between the control plants and those irrigated untreated textile effluent. A dendrogram of the four accessions using Weighted Neighbour-Joining (WNJ) procedure clustered the accessions into two major groups (Figures 4.4 and 4.5). The control (Maize-1) and treated 25% effluent (Maize-2) sample were clustered in one group with 67% bootstrap value. Group II, 50% effluent (Maize-3) and 75% effluent (Maize-4), were separated in another cluster, with 88% bootstrap value. This result clearly showed that textile effluent above 25% contains much more genotoxic substances capable of deviations from normalcy, because the control and 25% treatment grouped in one cluster and the plants irrigated with above 25% textile effluent were grouped in another cluster joined at a larger distance.

Also, the Principal Component Analysis clustered them into two major coordinates, with 75% effluent (maize-4), attaining the peak value of more than 4 (Fig. 4.6). This is an indication that the 75% treatment had the largest possible variance, followed by 50% treatment (maize-3). However, variation was almost negligible in the control (maize-1) and 25% effluent treatment (maize-2), the reason why they appeared almost at the same point, as shown in Figure 4.6.

Polymorphic Information Content, a good index for genetic diversity evaluation is an important parameter in RAPD analysis (Swaileh *et al*., 2008). PIC value will be almost zero when there is no allelic variation and can reach a maximum of 1.0 if a genotype has only new allele (which is a rare phenomenon). It is used to assess the diversity of a DNA segment in a population which will throw light to mutation the locus might have undergone over a time period. From the PIC result on Table 4.5, the control had the least PIC below 0.25, an indication of locus low diversity. However, the locus diversity continued to increase with increase in concentration, and reached its peak value of 0.7031 at 75% effluent concentration. These increments were as a result of the textile effluent effects on the genetic loci of the test plants.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Following all the laid down facts, it means that textile effluent has been implicated in the induction of cytotoxic (chromosomal aberration) and genotoxic (DNA polymorphism/damage) effects in *Zea mays*.

The induction of chromosomal aberrations observed in the shoot cytology of treated *Zea mays* compared to control showed the potential genotoxic effects of the textile effluent. Most of these aberrations were lethal and lead to cell death while others caused various degrees of genetic defects, which could even be expressed as congenital abnormalities in case of organisms (including humans), or be transferred from one generation to another if germ cells are affected (Alimba *et al*, 2015). It can also lead to loss of biodiversity by reducing the selective advantage or fitness of these plants to environmental selective pressures.

Besides the cytogenotoxic effects of the textile effluents, colour dyes in the effluents could obscure visibility in aquatic environments, affecting the aesthetic value of water bodies. It may be responsible for low water transparency (turbidity) recorded in the effluent and can lead to poor gaseous solubility in aquatic environment (Ahbay *et al*, 2014). The presence of toxic metals as observed in the effluent can enhance the depletion of dissolved oxygen and destabilize the ability of the water to reduce microbial loads (Osibanjo and Adie, 2007). The analyzed metals in the effluent could be deposited as particulates in the aquatic environment, become bioaccumulated in aquatic forms and pose health risk to humans.

5.2 RECOMMENDATION

The data obtained from this study revealed that untreated effluents from textile industries contain toxic compounds deleterious to plants (using *Zea mays* as a case study). These compounds may contaminate the surface water, thereby making it unfit for irrigation and drinking. Therefore, indiscriminate discharge of textile wastewater into water bodies/farmlands should be prohibited and proper treatment of effluents before discharge into the environment should be enforced. Moreover, laws regulating pollution from all forms of anthropogenic activities should be enforced by appropriate authorities to mitigate its consequences on both plants and animals species. The continual discharge of untreated textile effluents into the environment will undoubtedly cause threat to the ecosystem and to human health.

Environmental pollution from textile effluents have been the subject of much thought and research in recent times. It is expected that these studies will eventually lead to the promulgation and enforcement of regulations that will mitigate pollution of the natural environment by textile industries. I therefore recommend strict legislations in terms of companies effluent disposal systems. All effluents (especially those of textile industries) should be treated (to levels below the standard permissive limit) before releasing them into the environment.

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APPENDIX 1: DUNCAN MULTIPLE TEST STATISTICAL ANALYSES METHODS

Post Hoc Tests

Homogeneous Subsets

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
CONTROL	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	3.00	3	2.1000	.10000	.05774	1.8516	2.3484	2.00	2.20
	4.00	3	2.6667	.15275	.08819	2.2872	3.0461	2.50	2.80
	5.00	3	3.4667	.15275	.08819	3.0872	3.8461	3.30	3.60
	6.00	3	4.5000	.26458	.15275	3.8428	5.1572	4.20	4.70
	7.00	3	5.6667	.51316	.29627	4.3919	6.9414	5.10	6.10
	8.00	3	6.9333	.56862	.32830	5.5208	8.3459	6.30	7.40
	9.00	3	8.2000	.62450	.36056	6.6487	9.7513	7.50	8.70
	10.00	3	9.6333	.55076	.31798	8.2652	11.0015	9.00	10.00
	11.00	3	11.1000	.34641	.20000	10.2395	11.9605	10.70	11.30
	12.00	3	12.5000	.43589	.25166	11.4172	13.5828	12.20	13.00
	13.00	3	13.2000	.34641	.20000	12.3395	14.0605	13.00	13.60
	14.00	3	13.7667	.30551	.17638	13.0078	14.5256	13.50	14.10
	15.00	3	14.1333	.25166	.14530	13.5082	14.7585	13.90	14.40
	Total	45	7.1911	4.90338	.73095	5.7180	8.6642	.00	14.40
25%	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	3.00	3	1.0667	.11547	.06667	.7798	1.3535	1.00	1.20
	4.00	3	1.5000	.10000	.05774	1.2516	1.7484	1.40	1.60
	5.00	3	2.2333	.15275	.08819	1.8539	2.6128	2.10	2.40
	6.00	3	2.9667	.15275	.08819	2.5872	3.3461	2.80	3.10
	7.00	3	3.7333	.11547	.06667	3.4465	4.0202	3.60	3.80
	8.00	3	4.5667	.05774	.03333	4.4232	4.7101	4.50	4.60
	9.00	3	5.3667	.05774	.03333	5.2232	5.5101	5.30	5.40
	10.00	3	6.3333	.11547	.06667	6.0465	6.6202	6.20	6.40
	11.00	3	7.1667	.05774	.03333	7.0232	7.3101	7.10	7.20
	12.00	3	8.1000	.26458	.15275	7.4428	8.7572	7.90	8.40
	13.00	3	8.8667	.28868	.16667	8.1496	9.5838	8.70	9.20
	14.00	3	9.6000	.26458	.15275	8.9428	10.2572	9.40	9.90
	15.00	3	10.1667	.30551	.17638	9.4078	10.9256	9.90	10.50

	Total	45	4.7778	3.40320	.50732	3.7553	5.8002	.00	10.50
	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	3.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	4.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	5.00	3	.8000	.10000	.05774	.5516	1.0484	.70	.90
	6.00	3	1.2333	.11547	.06667	.9465	1.5202	1.10	1.30
	7.00	3	1.8333	.15275	.08819	1.4539	2.2128	1.70	2.00
	8.00	3	2.6000	.17321	.10000	2.1697	3.0303	2.50	2.80
50%	9.00	3	3.4000	.30000	.17321	2.6548	4.1452	3.10	3.70
	10.00	3	4.1667	.40415	.23333	3.1627	5.1706	3.70	4.40
	11.00	3	4.9000	.52915	.30551	3.5855	6.2145	4.30	5.30
	12.00	3	5.6333	.56862	.32830	4.2208	7.0459	5.00	6.10
	13.00	3	6.2333	.64291	.37118	4.6363	7.8304	5.50	6.70
	14.00	3	6.8333	.72342	.41767	5.0363	8.6304	6.00	7.30
	15.00	3	7.2667	.75719	.43716	5.3857	9.1476	6.40	7.80
	Total	45	2.9933	2.64398	.39414	2.1990	3.7877	.00	7.80
	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	3.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	4.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	5.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	6.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	7.00	3	.4667	.05774	.03333	.3232	.6101	.40	.50
	8.00	3	.7667	.05774	.03333	.6232	.9101	.70	.80
75%	9.00	3	1.2333	.15275	.08819	.8539	1.6128	1.10	1.40
	10.00	3	1.8000	.17321	.10000	1.3697	2.2303	1.70	2.00
	11.00	3	2.4667	.11547	.06667	2.1798	2.7535	2.40	2.60
	12.00	3	3.1000	.10000	.05774	2.8516	3.3484	3.00	3.20
	13.00	3	3.6333	.11547	.06667	3.3465	3.9202	3.50	3.70
	14.00	3	4.1000	.17321	.10000	3.6697	4.5303	3.90	4.20
	15.00	3	4.5333	.20817	.12019	4.0162	5.0504	4.30	4.70
	Total	45	1.4733	1.63851	.24426	.9811	1.9656	.00	4.70
	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
100%	3.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	4.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	5.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00

6.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
7.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
8.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
9.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
10.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
11.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
12.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
13.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
14.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
15.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	45	.0000	.00000	.00000	.0000	.0000	.00	.00

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
CONTROL	Between Groups	1053.910	14	75.279	566.483	.000
	Within Groups	3.987	30	.133		
	Total	1057.896	44			
25%	Between Groups	508.751	14	36.339	1287.615	.000
	Within Groups	.847	30	.028		
	Total	509.598	44			
50%	Between Groups	302.701	14	21.622	132.738	.000
	Within Groups	4.887	30	.163		
	Total	307.588	44			
75%	Between Groups	117.788	14	8.413	742.361	.000
	Within Groups	.340	30	.011		
	Total	118.128	44			
100%	Between Groups	.000	14	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	44			

CONTROL

Duncan

TREATMENT	N	Subset for alpha = 0.05											
		1	2	3	4	5	6	7	8	9	10	11	12
1.00	3	.0000											
2.00	3	.0000											
3.00	3		2.1000										
4.00	3		2.6667										
5.00	3			3.4667									
6.00	3				4.5000								
7.00	3					5.6667							
8.00	3						6.9333						
9.00	3							8.2000					
10.00	3								9.6333				
11.00	3									11.1000			
12.00	3										12.5000		
13.00	3											13.2000	
14.00	3											13.7667	13.7667
15.00	3												14.1333
Sig.		1.000	.067	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.067	.228

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

25%

Duncan

TREATMENT	N	Subset for alpha = 0.05													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.00	3	.0000													
2.00	3	.0000													
3.00	3		1.0667												
4.00	3			1.5000											
5.00	3				2.2333										
6.00	3					2.9667									
7.00	3						3.7333								
8.00	3							4.5667							
9.00	3								5.3667						
10.00	3									6.3333					
11.00	3										7.1667				
12.00	3											8.1000			
13.00	3												8.8667		
14.00	3													9.6000	
15.00	3														10.1667
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000

50%

Duncan

TREATMENT	N	Subset for alpha = 0.05									
		1	2	3	4	5	6	7	8	9	10
1.00	3	.0000									
2.00	3	.0000									
3.00	3	.0000									
4.00	3	.0000									
5.00	3		.8000								
6.00	3		1.2333	1.2333							
7.00	3			1.8333							
8.00	3				2.6000						
9.00	3					3.4000					
10.00	3						4.1667				
11.00	3							4.9000			
12.00	3								5.6333		
13.00	3								6.2333	6.2333	
14.00	3									6.8333	6.8333
15.00	3										7.2667
Sig.		1.000	.198	.079	1.000	1.000	1.000	1.000	.079	.079	.198

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

75%

Duncan

TREATMENT	N	Subset for alpha = 0.05									
		1	2	3	4	5	6	7	8	9	10
1.00	3	.0000									
2.00	3	.0000									
3.00	3	.0000									
4.00	3	.0000									
5.00	3	.0000									
6.00	3	.0000									
7.00	3		.4667								
8.00	3			.7667							
9.00	3				1.2333						
10.00	3					1.8000					
11.00	3						2.4667				
12.00	3							3.1000			
13.00	3								3.6333		
14.00	3									4.1000	
15.00	3										4.5333
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX 2: MANUAL STEPS INVOLVED IN CALCULATING THE ANALYSIS OF VARIANCE (ANOVA)

STEP 1:

Compute the correction of mean (CM), given by the formular:

$$CM = \frac{(\text{sum of all observations})^2}{\text{Total number of observations}} \quad \text{ie} \quad \frac{(\Sigma Xij)^2}{N}$$

STEP 2:

Compute the total sum of square (SSt) given by:

Sum of squares from all observations – CM.

STEP3:

Compute the treatment or group sum of squares (SST), with the formulae:

$$SST = \frac{\Sigma Ti^2}{n} - CM$$

where n = number of replicates

STEP 4:

Calculate the sum of square error (SSE), as follows:

SSE = SSt – SST.

STEP 5:

Calculate the mean square for treatment (MST), with the formulae:

$$MST = \frac{SST}{K-1}$$

Where K = number of treatments or groups

STEP 6:

Get the mean square for error (MSE), using the formulae:

MSE = $\frac{SSE}{K(n-1)}$

STEP 7:

s F-ratio = $\frac{MST}{MSE.}$

ANOVA TABLE

SOURCE	SS	Df	MS	F-RATIO	P-VALUE
TREATMENT (T)	SST	K-1	MST	$\frac{MST}{MSE.}$	From statistical table
ERROR (E)	SSE	K(n-1)	MSE		
TOTAL (t)	SSt	N-1			

When Fcalc (ie F-ratio) is greater than Ftab (ie the P-value), it is an indication of significant different. Also when Fcalc is greater than Ftab, it means that the P-value must be less than 0.05 (ie $P<0.05$) and vice versa.

APPENDIX 3: PICTURE OF DIFFERENT EFFLUENT DILLUTION

The figure below shows an equal measurement of different concentrations of the effluent, ranging from the control to that of 100%. Each treatment later received an equal measured amount of wood shavings in readiness of seed planting, after which two 2ml of the corresponding concentrations was being added on a daily basis.

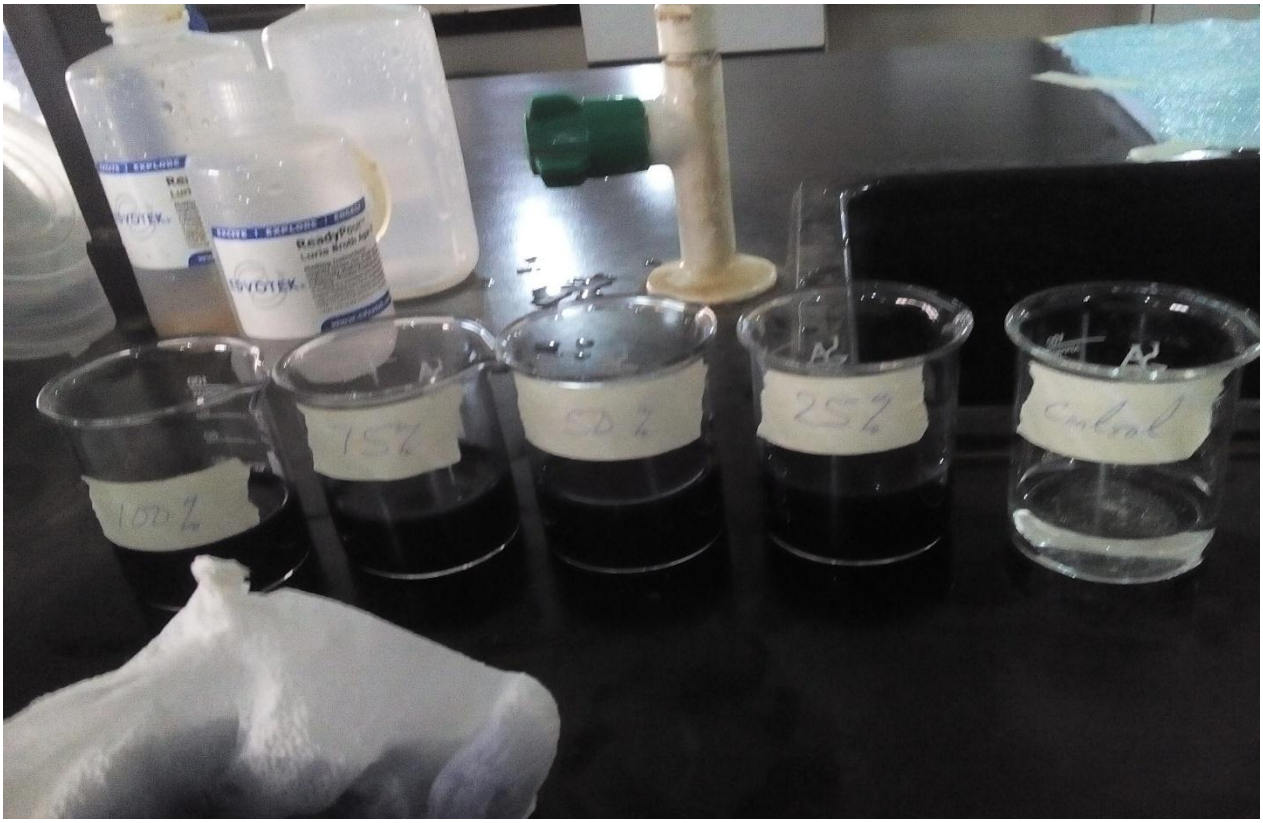

Fig 5.1, Images of different concentrations of the textile effluent, ranging from the control to 100%.