GROWTH RESPONSE AND TOXICITY ASSESSMENT OF CIPROFLOXACIN IN *ZEA MAYS*

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CERTIFICATE

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DEDICATION

I dedicate this thesis to my lovely parents for their unconditional love, care, support, and efforts for me, that I will always be indebted to them for.

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LIST OF ABBREVIATIONS

CIP	Ciprofloxacin
FQs	Fluoroquinolones
EC	Electrical Conductivity
NARC	National Agriculture Research Centre
DMSO	Dimethyl Sulphoxide
mg/kg	Milligram per Kilogram
µg/mL	Micrograms per milli liter
µS	Micro Siemens
mg/g	Milligrams per Gram
nm	Nanometer
NEQS	National Environmental Quality Standards
MN assay	Micronucleus Assay
Vas	Veterinary Antibiotics
DNA	Deoxyribose Nucleic Acids
TKN	Total Kjeldahl Nitrogen
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

ABSTRACT

Development of human society has led to the progression in the field of medicine. To treat the bacterial infections in humans and animals, antibiotics are used worldwide, but these antibiotics are being released to the environment through different sources and creating problems for other living organisms. Pakistan is a developing country and farmers are utilizing wastewater for irrigation of crops that might have antibiotics which could be potentially harmful with respect to the productivity and human health. The main aim of this work was to determine plant growth responses upon exposure to ciprofloxacin, their uptake concentration and effects on plant's composition. Different levels of ciprofloxacin antibiotics were tested, and the control were treated with distilled water only. Physiological growth parameters and composition were determined after exposure to different concentrations of the antibiotic. The results warrant the potential negative effects of the presence of antibiotics in wastewater and stress on plants declining the productivity and quality of the products. Irrigation with contaminated water resulted in accumulations of ciprofloxacin in plant tissues. Accumulation of the chemicals in plant tissues such as leaves and kernels is a problem, since they are used to feed animals and humans. Moreover, these chemicals are of potential toxicological concern, principally due to residue accumulations in the food chain. Specially, the antibiotic residue accumulations in maize tissues can assist the induction of antibiotic resistance in bacteria. Therefore, there is need for monitoring the quality of water being used for crop irrigation to avoid economic and food-quality losses.

Chapter 1

INTRODUCTION

1.1. Background

Antibiotics could be circumscribed as naturally effectuate compounds which target accomplished features of a bacterial physiology by either destroying the cell or stunting the growth all together. In a broad spectrum, antibiotic can be termed as an agent that inhibit s the acceleration of microbes along with algae fungi protozoa and bacteria. From hundreds of years, antibiotics have been in used, but in 1928, Alexander Fleming (a chemist) discover ed it first. He perceived the inhibitory effects of *Pencillium* on bacterial growth. Penicillin and streptomycin indicate the origin of natural antibiotics from pencillium fungus and streptomycetes bacteria with the succeeding discovery of penicillin and many of the anti-microbial agents separated from microbes. With the partiality of these chemicals as test subjects, the present generations of antibiotics were introduced.

The outcomes of human society led to the procession in the domain of medical science which affected in an elevated use of distinct chemical compounds. Annually, the averting of illness in animals and humans, and treatment is achieved by the use of active pharmacological substances in a large amount. A class of antibiotic chemicals is extensively used to control the bacterial attacks and treatment of humans and animals. The effects of these chemicals have extensively been anatomized on humans and animals, but restricted evaluations have been done for the danger involved due to the revelation of these things to the environment (Halling-Sorensen et al., 1998).

In the late 1990s, the consequences of Pharmaceuticals and Personal Care Products (PPCPs) on environment came into the observance of scientific community (Deughton and Ternes, 1999; Halling-Sorensen et al., 1998) and because after that the research affiliated to the effects and concentration on the environment of these substances has been increased.

On global scale, due to the annually increasing number of aquaculture and livestock industries, a large number of antibiotics are being used as food additives and veterinary

drugs (Du and Liu, 2012). As the consequences of human activities and discharge of wastewater sewage into the streams and environment, the antibiotics could be found both in the agricultural and aquatic systems. After consumption, these antibiotics mixed into the rivers, streams and crop lands through hospital wastes, sewage and wastewater effluents and industrial wastes in their bioactive forms (Kim and Agha, 2007; Kummerer, 2009). This continuous addition of antibiotic drugs through different sources into the streams makes them pseudo-persistent (Carvalho et al., 2014). The wastewater containing these antibiotic drugs is being used on the agricultural lands, sometimes without pre-treatment or treated through common treatment methods in many areas of the world (Fatta-Kassinos, 2011). Through common treatment methods of wastewater, the chemicals or antibiotic drugs couldn't clean or treated properly (Grassi et al., 2013). Many researches have been done for the effects on human being, aquatic life, animal arable land and many other ecological risks due to these antibiotics. But there is very limited research for the effects on plants and environment (Halling-Sorensen et al., 1998). The subsistence of extensive range of antibiotics in source water at low concentration purposes that the net consequences of antibiotics as environmental pollutant should not be administered (Tandon et al., 2013).

1.2. Antibiotics

'Antibacterial' or 'Antibiotics' arise from two different Greek words as anti means 'against' ant bios means 'life'. With the introduction of first antibiotic 'Penicillin' by Alexander Fleming in 1928, many new accessions were directed up in the sector medicine and health. Antibiotics are the results of increasing inaugurations in the field of health and their use has changed the pattern of current day life. Since the discovery, antibiotics have been used as the healing drugs that are applied in the prevention and treatment of different infections. Their marketable value is increasing at the exponential pace. Independent of their human and veterinary use, their services has also been come out in many different fields (Gothwal and Shashidhar, 2015).

Antibiotics are the chemicals that are produced artificially and semi-artificially to destroy or slow down the growth of micro-organisms (Thiele-Bruhn, 2003). Before handedly, these antibiotics were present naturally. But with the time, they are also produced

synthetically by pharmaceutical industries. There are many classes of antibiotics that are separated on the base of their path of administration, mode of action and chemical structure (Gothwal and Shashidhar, 2015).

1.3. Classification

Antibiotics can be classified on the basis of bacterial spectrum, bactericidal activity, administration route and the origin, whether manufactured or natural. The most extensively used and substantial classification method even so lies in the chemical structure of the drug. In a specific class, the antibiotics evolved on the basis of structure commonly contain similar features like effectiveness pattern, toxic effects and allergic effects. Generally, the antibiotics are found naturally, but now a days these are also manufactured artificially in the pharmaceutical industries. Antibiotics consists of many classes. Quinolone is the most important class of antibiotic with an extensive spectrum of antibacterial drugs. Quinolone is further subdivided into many groups. Fluoroquinolone is the most important subgroup of quinolone.

1.3.1. Fluoroquinolones:

Fluoroquinolones is the most important class of quinolones, as it covers a broad range of chemically used quinolones. Fluoroquinolone stays in the environment for a longer time period because of strong adsorption on to the soil and slow degradation process (Tandon et al, 2013). Households, hospitals and veterinary applications are the main cause of broad-spectrum fluroquinolones. Globally, these contain the 17% of market shares and are third largest class throughout the antibiotics (Doorslaer et al., 2014). Up to 70% of fluroquinolones are defecated in their non-metabolic form and can raise grounds in the environment for microbial resistance. In the typical structure of fluoroquinolone, a fluorine atom is attached to the quinolone central ring system.

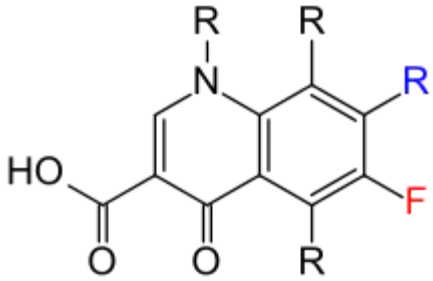

Figure 1.1: Molecular structure of Fluoroquinolones

Fluoroquinolones are the antibiotics of concern because of their common detection in the environment, effects on human health and biota, and increase in bacterial resistance. Although, very scarce information on any specific subgroup of antibiotics is available (Bouki et al, 2013; Hashmi et al., 2017).

1.3.1.1. Ciprofloxacin:

Ciprofloxacin, an antibiotic, is the subgroup of fluoroquinolones. Ciprofloxacin is the most commonly used prescribed fluoroquinolone in the United States. In the present days, there is an increasing interest in the scientific community relating to the environmental fate of antibiotics, particularly those used in the human medications (Turiel et al., 2005). It is commonly used for the treatment of different respiratory diseases.

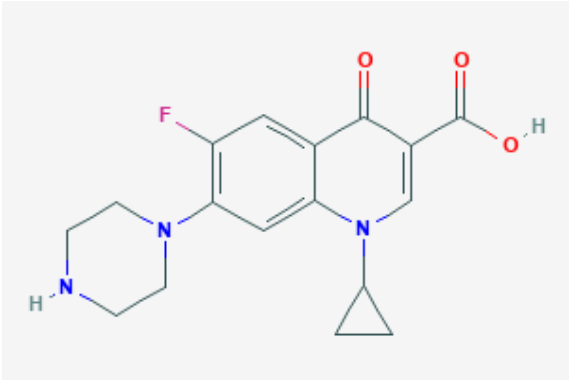

Figure 1.2: Molecular structure of Ciprofloxacin

Ciprofloxacin is very effective for the inhibition of DNA gyrase reliable for the preservation of DNA, in opposition to the Gram-negative bacteria. The existence of

Ciprofloxacin has been reported in the effluents of hospital wastewater, in the effluent of treatment water plants and in surface waters (Garcia-Kaufer et al., 2012).

1.4. Ciprofloxacin in Wastewater

Antibiotics are used for the treatment of various microbial diseases. They are entering the environment through witting and unwitting sources, like most of the drugs are deposited directly into the wastewater. They are also striking in the wastewater with the flash of sewage having feces and urine, waste from animal houses, leachate from landfills etc. Ciprofloxacin is approx. 69% available for the physiological activities and it could release in the wastewater through renal and fecal route (Hayder et al., 2012). It is frequently determined in the environment, as it is used over a broad range.

Many investigations have been done to determine the concentration of ciprofloxacin antibiotics in wastewater. In Italy, a study directed by Verlicchi et al. (2012) was conducted to determine the concentration of various antibiotics in wastewater. The series of concentration of antibiotics were from 0.001 to 32 ug/L. The commonly found antibiotics were ciprofloxacin, sulphamethoxazole and trimethoprim. As found in the research, 14 ug/L was the maximum concentration of ciprofloxacin. In India, a study by *Diwan* et al., (2009) was conducted to determine the concentration of different antibiotics in Indian hospital effluents. The antibiotics that were found in the hospital wastewater were of many types like ciprofloxacin, levofloxacin, norfloxacin, ceftriaxone and many others, which ranged from 1.4 to 236.6. Ciprofloxacin was most commonly found antibiotic in all samples from the above-mentioned studies. In Lahore, a study by Ahmed et al., (2013) was conducted, they studied the amount of norfloxocin and ciprofloxacin in river Ravi. It was determined in the study that the concentration of ciprofloxacin at downstream was increasing from 0.032 to 0.125 ug/L by the combining of first to last wastewater filters.

1.5. Use of Antibiotics in different Fields

Antibiotics are utilized in massive amount to cope with different infections in animals, aquaculture and humans. The data at the global scale on the consumption rate of antibiotics is heterogeneous and limited. The application pattern also vary significantly in

different countries and regions, as an example, in USA streptomycin is used extensively for growing fruits, although it iss illegal to use it for the same purpose in Germany (Kummerer, 2008). At the global scale, around 100,000 to 200,000 tons of antibiotics are being utilized annually (Wise, 2002).

1.6. Use of Antibiotics in Human Medicine

The amount of antibiotics consumed by each person in different countries varies. In markets of different countries, intake of antibiotics with or without the prescription also varies significantly (Molstad et al., 2002). The utilization rate for every single compound also varies. After the sub-group of penicillin B-lactam antibiotics, fluoroquinolones, sulphonamides and macrolides are mostly used antibiotics in many countries. In conflict with the general view the significant sources of pharmaceuticals are the communities itself not the hospitals in municipal wastewater that is only 5 – 20% of antibiotics were released by European hospitals into the environment. While 70 – 75% of antibiotics were released by communities in USA and UK (Wise, 2002). These components are metabolized into the liver. Sometimes, these components excreted through urine as they get more soluble in the water as compared to the parent compounds. Often times, metabolite formation preeminent to the formation of such compounds that are adversely toxic for the human health (Kummerer, 2009).

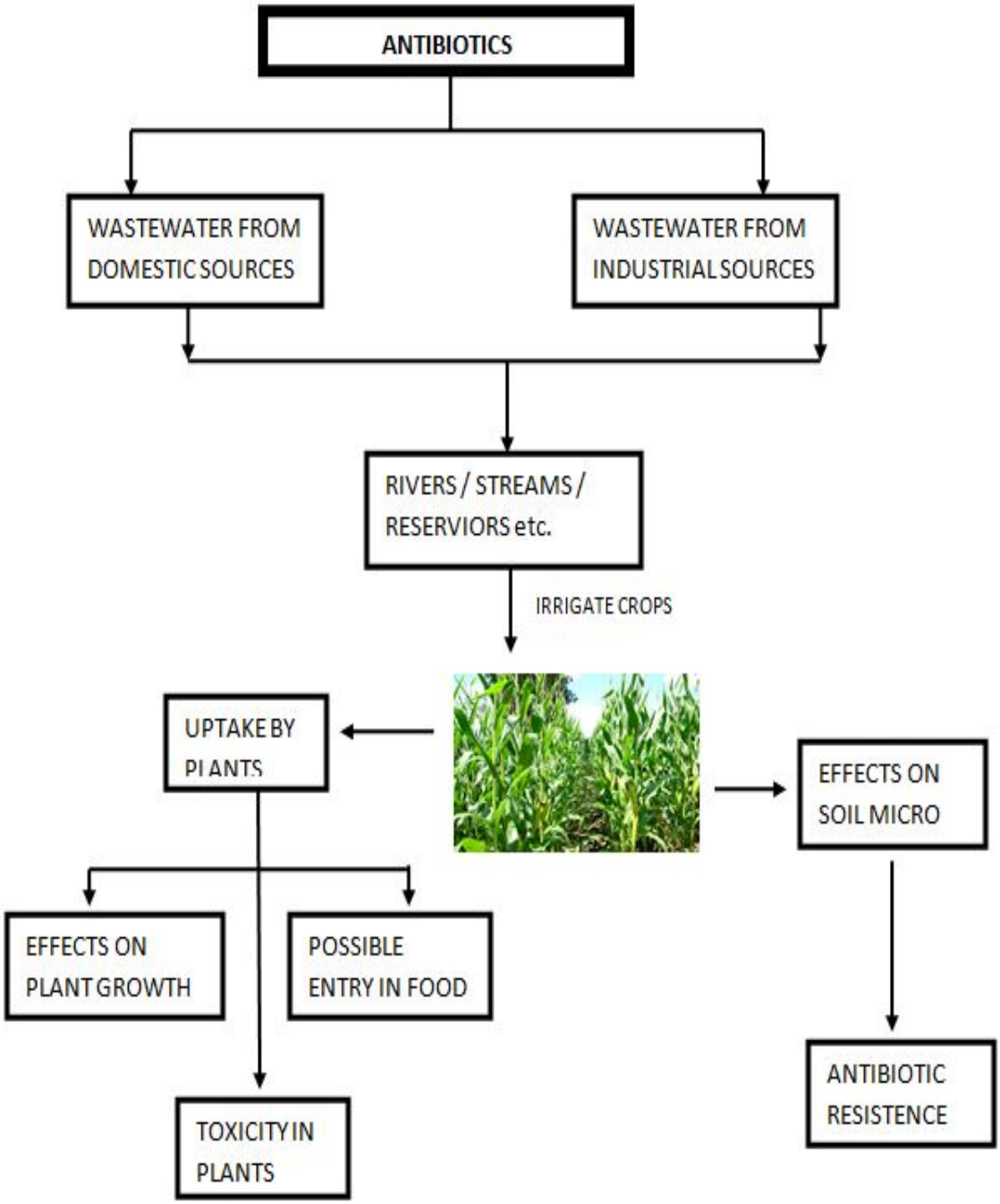

Figure 1.3: Illustrations of route for Antibiotics and their possible effects (Tandon et al., 2013)

1.7. Use of Antibiotics for Animals and Agricultural Crops

Internationally, only rough figures are available to depict the use of antibiotics in agri-food sector, although the correct and actual values are unknown. The quantity of antimicrobials consumed by the animals is usually accepted by advance animal breeding methods as a prevention cure or therapy (Gaskins et al., 2002). These are used as growth promotors in lower amounts for animals for a better end product, as an example, meat contents are with more protein and less fat (Cromwell, 2002).

In USA, overall, 0.5% antibiotics are applied on crops (McManus et al., 2002). To prevent bacterial attacks on ornamental plants, high-value tree fruits and other food antibiotics are applied. These antibiotics increase the plant tolerance towards rainfall, extreme temperatures, and other comparable factors and on the other characters pose great havocs to the environment (Kummerer, 2009).

1.8. Environmental threat

The proposed research has a huge spread of applications in various fields, such as agriculture, biotechnology, food industry, etc. The abundant release of drugs into the environment acts a threat that is not still fully recognized (De Graaff et al., 2011). Upon entering into the soil, these drugs may harm plant growth, when absorbed by the roots (Du et al., 2012). Considerable work has not yet been done on these antibiotics' plant uptake from soil; although many researches show the uptake of these drugs in the plants through the soil and mention minute or significant damages in the physiology and growth.

Fluoroquinolones that enter into the environment through manure applications accumulate because of their extreme adsorption to organic matter and low degradability in the soil (Leal et al., 2012). Antibiotics are given special attention as manure contaminated with antibiotics, being used for the treatment of different animal diseases specifically for the respiratory disorders (ANSES, 2012; European Medicines Agency, 2006). Excessive use of these antibiotics and their metabolites leads to resistance in soil bacteria through contaminated manure (Ghosh and LaPara, 2007).

1.9. Relevance to National Needs

Pakistan for the most part is an agricultural country and definitely requires a good supply of water resources for that reason. Therefore, needs advanced research to deal with the arising environmental challenges in plants and environmental sciences. Due to ever-increasing widespread use of antibiotics and their incomplete elimination in wastewater treatment plants, antibiotics have been introduced to the environment and their residues have been found both in the waste and urban water, which further are used for irrigational purposes is affecting country's crop production, leading to food security concerns. Thus, there is an urgent need to device strategies to help improve the situation for better that are both environment friendly and cost effective.

1.10. Significance and Scope of Study

A large amount of antibiotics are used throughout the world for different purposes i.e. manure applications and wastewater irrigation. These antibiotics results in the damage of ecosystem when they execute into the environment, but there is very limited information for the toxicity on plants. Ciprofloxacin is a class of antibiotics that stays in environment for longer time periods due to slow degradation process and strong adsorption on to soil. Large quantities of antibiotics can affect crop productivity and growth if they reach plants. Crops are the significant part of terrestrial environment and serve as the potential pathway into the food chain for the transport of antibiotics. This research is an effort to understand the uptake concentration and negatively affects plant's physiological growth parameters on *Zea mays* by providing convenient solutions to the soil.

Various advantages that can be obtained by the proposed study include:

- Improving food security.
- Providing agriculture industry with better insight into the methods and mechanisms that can help improve the growth and yield productivity.
- Promotion of reuse of waste organic matter as an amendment.
- Solution provided will be environmentally friendly and cost effective.

1.11. Objectives

In the present study, ciprofloxacin was used to determine the effects of antibiotic on *Zea mays* plant. The objectives for this study were:

- To assess plant growth responses upon exposure to ciprofloxacin.
- To determine uptake of ciprofloxacin in *Zea mays.*
- To explore toxicological effects of ciprofloxacin via micronucleus assay technique.

Chapter 2

LITERATURE REVIEW

This chapter is focused to deliver the possible information about the presence of antibiotics in wastewater, their impacts on the receiving crops and impacts on the health of end-users. Hundreds of medications are used to address the human infections and veterinary medicines. Because of incomplete metabolism, more than half of these medications enter into the environment in the form of bioactive entity. Drug polluted manure applications to terrestrial environment is the most common way of antibiotics into the environment. In sustainable and organic farming system, livestock manure is the most important ingredient as a fertilizer, either it is applied in raw or compost form. The concentration of drugs found in the soil depends on the applied manure conditions. Fluoroquinolones are recognized in the environment resulting from sorption to organic matter because of their low degradability rate in the soil.

2.1. Antibiotics

Matter for toxic substances contribute into the environment was initiated almost thirty-five years ago when 'Silent Spring' the book of Rachel Carson was published (Carson, 1962). When the antibiotics were reputed as "Wonder Drugs" they were broadly used and the issues of their use and misuse were debated worldwide (JETACAR, 1999). In 1969, it was concluded in the Swann Committee that the use of growth supplements for the animals which causes resistance and for humans must not be used, after this a continuous discussion started about resistance instigation (Swann Committee, 1969).

Even after the EIA of veterinary medicine in the European Union (EU) and USA, standards for antibiotic concentrations were not established (EMEA, 1997; Thiele-Bruhn, 2003). In 1999, in EU and Switzerland, the usage of antibiotic was roughly estimated to be 13,288 tons of antibiotics that year, of which 65% were given for human medication, 29% were for veterinary medicine and 6% antibiotics were given as growth supplements to animals (FEDESA, 2001). Latest studies showed that in the streams of Germany and

USA, 15 types of antibiotics are present in the receiving wastewater from industrial and urban sources and agricultural run-off (Ternes et al., 2002; Kolpinet et al., 2002).

Due to the bioactive nature of antibiotics, they are not able to be removed completely from the organism's body. They are super effective at low doses (Kummerer et al., 2000; Thiele-Bruhn, 2003). Quantity of antibiotics elimination depends upon the nature of compound, the type of genus, administration time and the application method (Berger et al., 1986; Haller et al., 2001). Metabolites of antibiotics have ability to change back to their pro form after the elimination from the body of organism (Langhammer, 1989).

When sulphonamides and fluoroquinolones adsorbed to the feces are very strong and rich in organic matter, so do not transformed. Even change in temperature and aeration of manure cannot transform them and they get distributed in the environment in parental form. Most of the drugs used in the food making industries of animals are adsorbed in the animal's stomach inadequately, and 30- 90% of antibiotics are excreted from animal's body in parental form (Christian et al., 2003).

2.2. Sources of Antibiotic in Environment

Antibiotics could be expanding in the environment through agriculture and human resources. These sources include wastewater treatment facilities discharge, waste from medical sources, wash out of old and expired prescriptions, leakage of septic tanks, excretions of animals and agricultural waste storage structures. Uses of waste from agricultural and domestic sources are the ways for spread of antibiotics on land and surface run off. When antibiotics come into the environment their effectiveness depends upon various conditions e.g. chemical and physical nature of antibiotics, soil type, climate of the area and other environmental conditions, like other organic compounds. When the antibiotics in our environment are not totally metabolized, there is attainability that their metabolites will help in the population and stabilization of microorganisms that are resistance to antibiotics will be accelerated (Witte, 1998). So the constantly use of manure on the same area could help the frequently contact of antibiotics remain to the soil microorganisms and the population of bacteria will be increased that are resistant to

antibiotics. It could have significant impacts on the environment, particularly when these remains leached and arrive at adjoining water bodies and travel through surface run off.

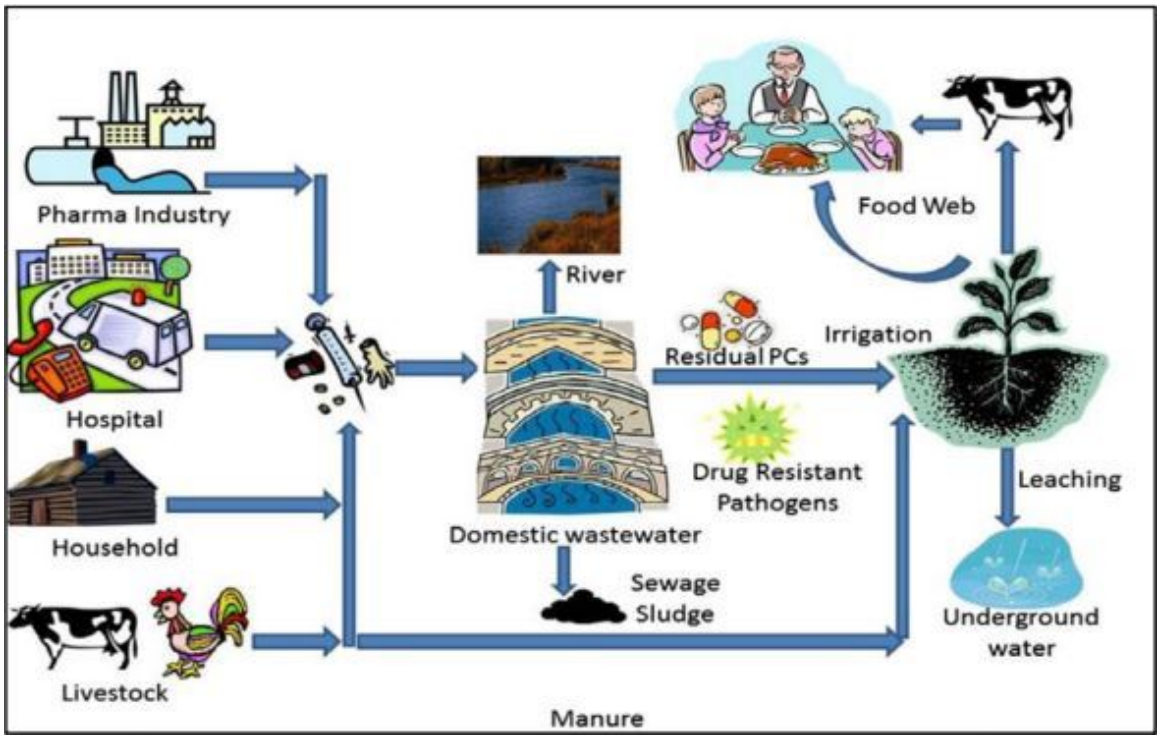

Figure 2.1: Possible pharmaceutical routes to the environment (Rehman et al., 2013)

When these antibiotics come into the environment through various sources, there is feasibility that they have harmful effects on aquatic, terrestrial and human's ecosystem but the effects are still to be determined. In the last few years, the consequences of drugs on environment has raised as a significant area of research for the researchers (Velagaleti, 1997; Halling-Sorensen et al., 1998; Montague, 1998; Daughton and Ternes, 1999; Hirsch et al., 1999; Jensen, 2001; Dietrich et al., 2002). Through the middle of 1990's to late 1990' s, a lot of studies were concerned to the happening and division of veterinary and human medicines in the environment. When these researches exhibit that these drugs are moved through ground and surface water from agricultural and urban sources, scientists have initiated to study the effects of these drugs on environment. (e.g Patten et al., 1980; Cole et al., 2000; Sengelov et al., 2003; Richards et al., 2004; Loften et al., 2005). Quite there is very rare information accessible on fortune of these compounds and their behavior of transport in the soil-water environment.

- **Surface Waters:**

In England, more than twenty years ago, a case reported of contaminated surface water through antibiotics. Watts and his team (1982) observed a compound from sulphonamide, tetracycline and macrolide group of antibiotics in river water and the concentration observed was 1ug/L. Proceeds to this, a range of various pharmaceutical were also observed in surface water and the concentrations determined was up to 1ug/L (e.g Richardson and Bowron, 1985; Pearson and Inglis, 1993; Ternes, 1998). A group from Germany, detected remains of chloramphenicol in a small river in southern part of Germany and in effluent of sewage treatment plant with the concentration of 0.06 and 0.56 ug/L respectively (Hirsch et al., 1999). Chloramphenicol is an antibiotic used to treat acute meningitis in human but in very distinct medical cases. Since 1995 in the European Community, the use of chloramphenicol for veterinary purposes has been banned (BGVV, 1996).

- **Ground Water and Marine Sediments:**

The antibiotics used for animals are present in ground water was observed in different studies (Holm et al., 1995; Hirsh et al., 1999 and Hamscher et al., 2000). But most of the drugs are found in ground waters to kill bacteria and were used in agricultural lands but their concentrations didn't cross the value of quantization range from 0.02-0.05 ug/L (Hirsh et al., 1999).

- **Drugs Manure and Agricultural Soils:**

The use of drugs in body surely results in the left-over concentrations that move into waste material (Thiele-Bruhn, 2003). Therefore, it is considered to detect remaining drugs either in pro form or in the form of metabolites in manure and dung and then in agricultural lands (Patten et al., 1980; Hoper et al., 2002). A soil fertilized with liquid manure was analyzed by Hamscher et al., (2002) the determinations of their study cleared that in liquid manure, the concentration of chloro-tetracycline and tetracycline was 0.1 and 4.0 mg/kg respectively while the concentration of these antibiotics in soil were ranged from 86.2 ug/kg (in the top soil of 0-10 cm) to 171.7 ug/kg (in the soil of 20-30 cm layer).

- **Manufacturing Industries/Human Medicine:**

A slight attention had been given precedent to the pharmaceutical products evolved from manufacturing plants but in the latest years, concentrations were high as 31 mg/L, as determined in case of ciprofloxacin, were detected in the effluents coming out in some of the Asian countries (Larsson et al., 2007; Li et al., 2008a,b). Even the developed countries can have inventing facilities as the important source of antibiotics for sewage treatment plant (Thomas, 2008).

The per capita usage of human medicines changes prominently across national boundaries (Molstad et al., 2002). It is the possibility that the medicine used widely in one country might be banned completely or sparsely in another country. Similarly the use of specified and non-specified medicines varies at a great range in different countries. A broad portion of the medicines absorbed are excreted out from the body in bioactive form and find their way to the environment.

2.3. Antibiotic Resistance:

The potential of life to accommodate and modify enabled it to defeat the challenges faced by it since the start and come out stronger than before. This potential is the intellectual that microbes, the most familiar of all species, can be introduced in the harshest of environments, severely high pressures, at a large pH range, from hot springs to ice sheets. This flexibility for adaption of microbes is mostly featured to their huge population and the ability to deliver desirable genes intra species. This flexibility is also the reason microbes have approached resistance to anthropogenic compounds refined for their destruction (Rojas et al., 2013).

The results of antibiotics entering the terrestrial and aquatic environment are broad; however the loss that effected to the microbial communities can be supposed by far the biggest concern caused by the contamination of these pharmaceutical products of environment. The vicinity of these antibiotics in environment directed to the selection of antibiotic-resistance genes (ARGs) in forced conditions invariant of the concentrations that are supposed harmless in environment and directed towards the fate of resistance in

microbes (Mojica and Aga, 2011). The fate of antibiotic resistance in microbes can be followed back to the 20th century where *fluoroquinolone, methicillin* and *vancomycin* resistance *Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus* issued in a speedy increase in infections (Cooper et al., 2011). Now a day, at least 70% of pathogenic bacteria are evolved with the resistance against antibiotics present in the environment. These antibiotics are causing an increase in the mortality rate to 2.0 million people each year because of microbial infections (Berdy et al., 2012). The development of antibiotic resistance paired with decrease in production of new antibiotics is reason of severe discomfort for human health.

Development of resistance in bacteria is the major reason for the utilization of veterinary and human medicine which shows a health risk for both animals and humans. The antibiotics are of particular concern for the reason of used for poultry and they have a big chance of in the choice of resistant strains to pharmaceuticals used as human medicine. The shifting of resistance microbial strain can happen either by ingesting the animal meat or directly from contact. Bacterial strains of some human pathogens in the environment and in the animals have refined resistance to antibiotics and their resistance genes have been removed (Angulo et al., 2004).

A study was done by Xu et al. (2015), in which they worked on the plenty of ARGs and antibiotics in sewage treatment plants in China. Quinolone were observed with concentration of 3866 ng/L and four resistant genes (gryA, parC, qnrC and qnrD) of quinolone class were observed using quantitative PCR. The process of resistance development is described briefly in the fig 2.2.

Pathogenic bacteria are present in the wastewater of both animals and humans origin. Antibiotics polluted this water and results in the promotion of ARGs and also causes particular pressure on microbial communities (Baquero et al., 2008; Farrell, 2009). Different water sources are containing antibiotic resistance bacteria (Caplin et al., 2008; Vanneste et al., 2008). Many studies have concluded that the major reservoirs of ARB and ARGs are present in the water sources even in the drinking water (Nonaka et al., 2007; Hoa et al., 2008).

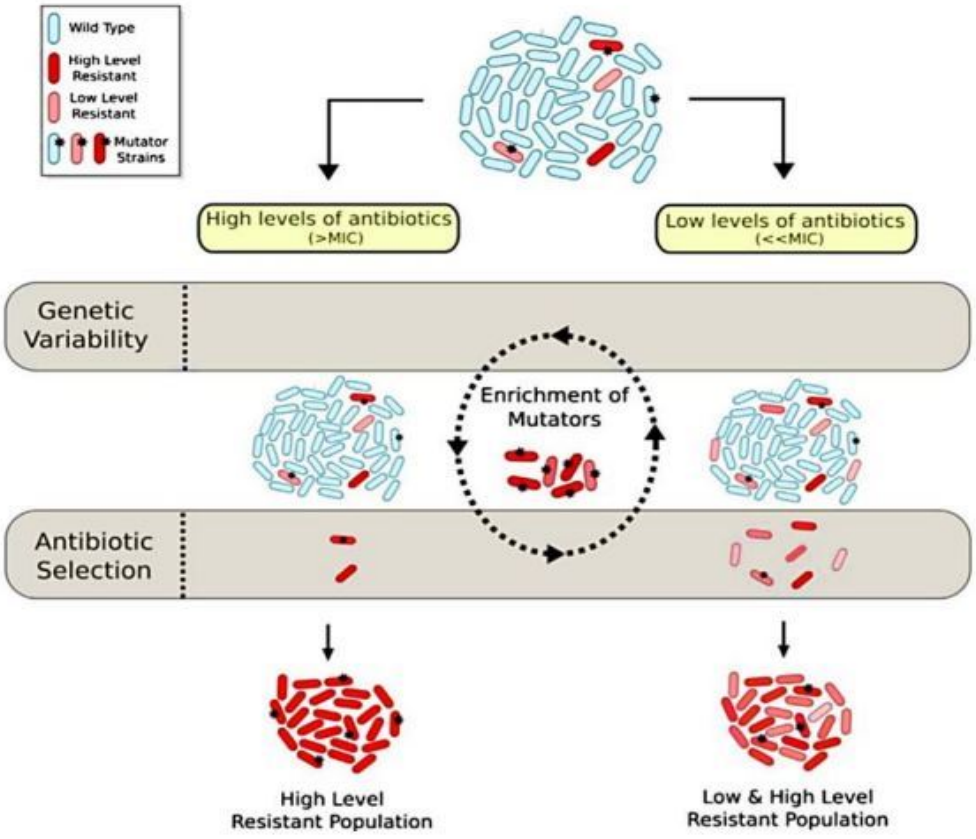

Figure 2.2: The above figure displays the process of bacterial resistance development against antimicrobials. High antibiotic concentrations lead to the selection of developing high resistance bacterial population and resistant strains already existing. Low concentration of antibiotics lead to enhanced chances of high and low level antibiotic resistance and increased genetic variability of population (Rojas et al., 2013).

2.4. Quinolones

In the early 1960s, quinolones were developed as non-fluorinated drugs. These are anti-bacterial agents. This effective class of current day medicine was firstly developed for the treatment of human and veterinary urinary tract infections. First introduced antibiotic was nalidixic acid was specialized for the treatment of urinary tract infections, due to its inability to reach various organs and tissues in required concentration (Nava, 2007). In 1980s with the introduction of first 6-fluorinated derivative, modified to form broad range antimicrobials thereby yielding to the class of fluoroquinolones (Silva, 2004; Stahlmann,

2002). The evaluated use of generic quinolones was approximately of 70 tons in European Union, Japan, South Korea and USA while about 50 tons was formed as proprietary products. In China, the evaluated quinolone consumption concentration for humans and animals were 1350 tons and 470 tons respectively (Sukul and Spiteller, 2007).

2.4.1. Fluoroquinolones:

Compounds of fluoroquinolones (FQs) consist of developing group of synthetic antimicrobial agents. The compound consists of a fluorine molecule attached at the 6-position of quinolone nucleus. Basic structure of both compounds is similar, but their physiochemical properties, pharmacokinetic characteristics and microbial activities differ significantly (Martinez et al., 2006). Since 1980s, fluoroquinolones have become a severe cause of concern in environmental pollution due to their extreme use in the treatment of animals and human diseases (ANSES, 2012; European Medicines Agency, 2012; Pico and Andreu, 2007). Fluoroquinolones are the third largest group of antibiotics globally. They have a global market share of 17% and a net share of 7.1 billion US dollars (Hamad, 2010). The fluoroquinolones that consumed are mostly excreted and join the sewage system. From that way, these affected persistent compounds find their path from water bodies to soil (Sturini et el., 2012); fluoroquinolones also enter into the soil through manure application. They accumulate into the manure having characteristics of strong adsorption to organic matter and low degradation rate (Alexy et al., 2004).

2.4.1.1. Ciprofloxacin:

Ciprofloxacin (CIP) is a part of fluoroquinolone group of antibiotics (Bayer et al., 1983). It possesses the ability to treat a broad range of humans and animal diseases, against gram-negative and positive bacteria (Davis et al., 1996). In 2007, WHO determined the half-life of ciprofloxacin human body is 4h as a significant antibacterial human medicine. ciprofloxacin is the most often used in European Countries (Ferech et al., 2006) and is prescribed in the treatment of joint and bone infections, typhoid fever and tuberculosis to name a few diseases (Rocha et al., 2011). It is a demonstrated genotoxic drug and found in the natural environment on regular basis (Kummerer et al., 2000).When its concentration found in the pharmaceutical remnants of health care center in the range up to 0.083 mg/L,

and then it has been included in a EU project as a critical substance to abolish from pharmaceutical remnants (Hartmann et al., 1998).

There are about 15 – 25 % of drugs excreted from human's body in the form of feces and 45 – 62 % of them removed through human urine (Golet et al., 2003). Consequently, ciprofloxacin finds its way into the environment through water treatment plant, drug manufacturing facilities and sewage systems. Some other ways of ciprofloxacin to interact with the environment includes irrigation of land with contaminated water, application of livestock manure and by leaching in landfills (Boxall et al., 2006; Topp et al., 2008).

- **Mechanism of Action:**

Ciprofloxacin presents bacterial activities against a broad range of gram-positive and negative bacteria. In gram-positive bacteria, it avoids bacterial multiplication by targeting DNA gyrase. DNA gyrase is a topoisomerase II, which takes part in super coiling of circular DNA. In case of gram-negative bacteria, it targets topoisomerase IV, which takes part in unwinding of super coiling of circular DNA (Sharma et al., 2009). Hence, disrupting enzymes are compulsory for the repair, replication, transcription and recombination of DNA (Behal, 2006).

- **Clinical particulars:**

Ciprofloxacin is used as a therapeutic agent against infections of respiratory tract (Jones et al., 1994), urinary tract, kidney (Mahamat et al., 2006), genital organs, abdominal cavity and middle ear. Some more diseases expounded by CIP enclose infections of skin, soft tissues, eyes, joints, bones and diarrhea. It is also used to deal with the pneumonias affected by bacterial species including *Enterobacter spp.*, *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus* (Medical pamphlet).

- **Side Effects:**

Results are particularized from clinical trials demonstrate certain side effects including rash (1%), vomiting (1.0%), abnormal liver function tests (1.3%), diarrhea (1.6%) and nausea (2.5%) cases (Medical pamphlet).

- **Environmental Presence:**

Over the last few years, 30% of oral consumption of ciprofloxacin has increased (Batt et al., 2007). Most of the data in soil relating to the presence of fluoroquinolones is on enrofloxacin (ENR) and ciprofloxacin, with the ciprofloxacin ranging from 0.65 to 2.1 mg/kg in poultry manure and 4 – 40.7 mg/kg in chicken manure (Leal et al., 2012). In a study by Wu et al (2014) determined the presence of four quinolones in the soils consist of five organic vegetables farms in southern China. The antibiotics were detected at the concentrations ranging from 0-42 ug/kg at a frequency of >97%. The degree of frequency decreased in the order of ENR>CIP>NOR of drug appearance. The ciprofloxacin concentrations found in different environment are mentioned in the table 2.1:

Table 2.1: Concentrations of Ciprofloxacin in the Environment

Matrix	Detected conc.	Regions	References
WWTP Effluents	31	India	Larrson et al., 2007
Agricultural Soils	0.75*	Austria	Carballo et al., 2007
River	$6.7x10^{-3}$-$1.02x10^{-3}$	Portugal	Pena et al., 2007
Sewage Treatment Plants (STPs)	0.056-0.211	Coimbra	Seifrtova et al., 2008
Ground Water	$7x10^{-3}$-$14x10^{-3}$	India	Fick et al., 2009
Surface Water	0.36	US	Sadezki et al., 2010
Chicken Dung	0.7 to 45.6*	China	Zhao et al., 2010
Production Facility Wastewater	4.9	Korea	Sim et al., 2011

Hospital Effluents	0.007-0.1245	Swiss	Fink et al., 2012
Poultry Manure	0.65-2.1*	Brazil	Leal et al., 2012
River	$1.25x10^{-3}$	Pakistan	Ahmad et al., 2013

Concentrations are mentioned as: mgL^{-1}; values with * indicate $mg\ kg^{-1}$

Veterinary Medicine International Coordination Commission defined the Eco toxic risk value for antibiotics is 100 ug/kg. Golet et al., (2002) determined the presence of ciprofloxacin in the environment on land even after 13 months of biosolid application. Their findings were later authenticated by Wetzstein et al., (2009) and Walters et al., (2010).

- **Bacterial Resistance to Ciprofloxacin:**

The dissimilarities of fluoroquinolone from other medicines in structure and properties and their diverse mode of action indicates that resistant microbes to various medicines like cephalosporin, macrolides, aminoglycosides and tetracycline might be susceptible to ciprofloxacin. The sequence of microbial resistance against ciprofloxacin and other fluoroquinolones is usually concludes from mutation in DNA gyrases and reduced outer membrane permeability (Cipro: Retrieved from http://www.rxlist.com/cipro-drug/clinical-pharmacology.htm, retrieved on August 28, 2014).

- **Degradation:**

Antibiotics after taken by the aquatic environment are confronted with natural elements which can cause their degradation. The degradation of ciprofloxacin and fluoroquinolones can be understood by considering both biotic and abiotic factors. Biotic factors mostly involved biodegradation by micro-organisms present in the aquatic system while the abiotic factors in the surface layer play a significant role in degradation by photo-chemical reactions. The mechanism basically involved the replacement of piperazinyl ring present in the seventh position (Nowara et al., 1997).

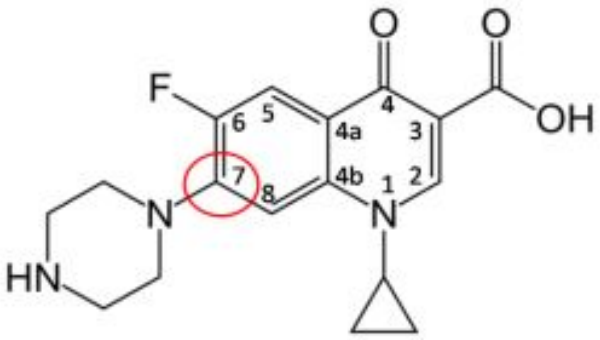

Figure 2.3: Degradation Mechanism of Ciprofloxacin by removal of Peprazinyl Ring at the seventh position.

The ciprofloxacin is of very low biodegradability (Kummerer et al., 2000). The ciprofloxacin usually attaches readily through cation exchange with the soil (Pico et al., 2007; Vasu devan et al., 2009) and therefore could remain there for a periods as soil behave like its reservoir (Rooklidge et al., 2004). In a biodegradation study Mougin et al., (2013) detected the ciprofloxacin mineralization was less than 0.01% after an incubation period of 84 days. In another study by Girardi et al., (2011) observed the degradation rate of 0.03% with the six days of experiments and after six days the degradation rate started to slow down at the rate of 0.008% per day. Kummerer et al., (2000) did the same experiment in a closed bottle; he observed no degradation of ciprofloxacin even after forty days experiment period. In a study by Walters et al., (2010) showing the high resistance of ciprofloxacin to biotic and abiotic degradation, after the calculated half life time of 1-3 years in a mesocosm soil. Dalkmann et al., (2012) worked for showing the presence the ciprofloxacin in environment sequestered in Maxican soils over long term untreated wastewater irrigation.

Degradation of ciprofloxacin in river and pure water conducted in a study, It was determined that the degradation cannot be occurred in the absence of light as degradation depends on light. The rate of photo-degradation is very low initially for the two months because of no additional biochemical degradation but after sometimes it increases rapidly. Therefore in the pure samples, the rate of degradation did not increase exceptionally. The degradation of ciprofloxacin can also be explained by the presence

of chemical degradation by various river matrices and microbial communities in river water.

2.5. Zea mays

Frequently detected residuals of antibiotics in crops has drawn increasing attention from research community and the general public. This study was conducted under the controlled environmental conditions to investigate the uptake, translocation and distribution of three different veterinary antibiotics (VAs) in plants of Zea mays L. (maize, the third largest crop in the world, especially in China) and the associated mechanisms (Cheng et al., 2019). Results provide a novel insight into CIP uptake by plant roots, and improve the strategy of minimizing CIP accumulation in crops for food safety by fertilization management.

2.6. Effects of antibiotics on plants

Plants play a significant role for the sustainability of ecosystem. The emerging toxic substances into the environment results in the change of structure, function and genetics of these primary products (Yi and Si, 2007; Yin et al., 2008). The pollutants enter into the environment, formerly taken up by the plants. Through various studies it was observed that certain veterinary drugs accumulate into the different parts of plants. Wheat, rice, soybeans alfalfa and lettuce have been widely used as test species for the observation of accumulation of antibiotics in the certain parts of these plants. This accumulation was occurred through passive absorption and water transport system. When these pollutants come into the primary producers, they can cause severe damages to their biochemical activities and physiology of the plants (Liu et al., 2009; Boonsaner and Hawker, 2010; Hillis et at., 2011; Li et al., 2011a,b; Luo et al., 2011). Different studies conducted to estimate the toxic effect of various antibiotics on different species, as the antibiotics are known as minor wastewater pollutant. Aquatic species are mostly included. The consequences of these antibiotics are not fully documented. When the plants are fed with wastewater containing pollutants are exposed to these compounds. Bio-solids and animal manure are the sources of these antibiotics from wastewater treatment plants, when they are given as fertilizers in agriculture (Hillis et al., 2011).

A study had been directed by Liu et al., (2009) to determine the toxic effects on selected plant species of six antibiotics (Tetracycline, Sulfamethazole, Sulfamethazine, Tylosin, Chlorotetracycline, and Trimethoprim). The effects of these antibiotics were checked by using sweet oat, rice and cucumber on plant growth, enzyme and soil microbial activities. Rice was recognized as the most sensitive plant species in competition with antibiotics. The study found out that these antibiotics had negative impacts on plant growth. The impacts of these antibiotics varied from species to species from each other. The study resulted that the antibiotics present in the manure and soil affect the plant growth or plant yield and also have harmful effects on the enzyme activities and soil microbes.

Tetracycline is a newly formed pollutant present in wastewater. It is introducing into the environment from waste discharge of humans and animals as it is used in the veterinary and human medicine. When different concentration of tetracycline were exposed to wheat, crop, there were impacts on genetics and physiological parameters of plants (Xie et al,. 2010).

Different group of antibiotics occurr naturally in the environment and some are synthetically manufactured in pharmaceutical industries. Quinolone is the most important group of antibiotics. When a fluorine atom is added into the chemical structure of quinolone, they formed fluoroquinolone both have great importance in the medical field as they used to treat the upper respiratory tract and urinary tract infections. They are also effective against gram-negative bacteria. When antibiotic mixed wastewater is exposed to plants, they show harmful effects on the plants. A study was done by Khadra and his co-workers, determined that antibiotics have genotoxic effect on *Vicia faba* plant. They used nalidixic acid (quinolone) Ciprofloxacin and levofloxacin (fluoroquinolones) to find out the genotoxicity in *Vicia faba.* They conclude from this study that the lower concentration of antibiotics have no major effects on the plants while the exposure of higher concentrations of these antibiotics results in significant genotoxicity in the plant (Khadra et al., 2012).

A study was directed by Eggen et al., (2011) by using the crop and forage plants by the uptake of antibiotics in the plant body. They preferred barley and carrot plant for the study. The results showed that the RCF (route concentration factor) is larger than the LCF

(leaf concentration factor). They conclude that when the antibiotics exposed to plants, they have negative impacts on the development of carrots and on plant growth. They determined from their study that human pharmaceutical compounds might have negative impacts on humans when they strike from crops through livestock. In another study, a wet land plant named as *Phragmitesaustralis* also known as reed was used to determine the effects of ciprofloxacin and oxytetracycline. They found LCF less than RCF in this plant. They also conclude that with the increase in the concentration of these products, they negatively effects on many plant activities like Catalase activity and super oxide dismutase activity (Liu et al., 2013b).

2.7. Pathway of Antibiotics in plants

Various experiments have been done to find out the uptake concentration of different antibiotics by plant with different ways like pot experiment and spiked soil experiment etc. (Gao et al., 2005; Boxall et al., 2006; Dolliver et al., 2007; Aslund et al., 2008; Winker et al., 2010; Wu et al., 2010). A study directed by Trine et al., 2011, in this study they exposed the forage crops with three antibiotics and ciprofloxacin was one of them. They find out that the ciprofloxacin was on second number to the uptake by forage crop. The influential concern for the study was that the antibiotics are becoming the part of food chain system by taken up from the food crops (Boxall et al., 2006). The bioaccumulation of chlortetracycline in green onion, corn and cabbage was observed from manure amended soil ranged between 2 – 17 ug/kg fresh weights. However, due to the large size of tylosin molecule, it was not absorbed (Kumar et al., 2005).

Boxal et al., (2006) determine the uptake of seven different antibiotics by plants. Carrots and lettuce were grown in the soil spiked at the concentration of 1 mg/kg. Enoxacin, trimethoprim and florfenicol were observed in the carrot roots at the concentration of 3 – 38 ug/kg fresh weight while trimethoprim and florfenicol were observed in the lettuce leaves. Although the potential adverse health risks may exist but the health implication of antibiotic leftovers are not known in plants. Gentamicin is a kind of small molecule with the molecular weight of 477.6, a water soluble aminoglycoside, used as an effective antibiotic against pseudomonas, gram-positive and gram-negative bacteria. This antibiotic

is heat-stable and remains active even after autoclaving (Macdougall et al., 2007). Streptomycin is a kind of large molecule with the molecular weight of 581.6, a water soluble aminoglycoside, used as an effective antibiotic in humans and animals and also used as pesticide to compete some fungal and bacterial diseases in plants. A significant use is in the control of fire blight on pear and apple trees.

Growing concern over antibiotic presence, toxicity and fate in the soil, which may pose adverse effects on plant growth and productivity was researched by Pan and Chu (2017) and the findings of the study shows their uptake and accumulation in crops. Trine et al. (2011) studied the uptake and translocation of ciprofloxacin in forage and crop plants and indicated the reduced growth of primary root, hypocotyl, cotyledons and growth/ number of leaves of crop plants. Turiel et al. (2005) studied the degradation products of ciprofloxacin in river water samples by HPLC. The result of their finding shows that the photodegradation takes place via substitution on the piperazinyl ring in position 7 and degraded after 3-4 months of storage. Hina Imtiaz (2014) researched impacts of ciprofloxacin on plant growth and soil microbial biomass and found the effects of ciprofloxacin stress at various concentrations on plant growth and soil microbial biomass.

2.8. Toxicity assessment techniques

To determine the rate of toxicity by different harmful chemicals and antibiotics, there are many techniques followed by the researchers. As for example, seed germination technique, comet assay and micronucleus assay are commonly used. Physiological parameter also determined to assess the toxicity.

2.8.1. Physiological Parameters:

To study the effects of antibiotics on plants, physiological parameters are commonly observed. Liu et al. (2009) determined the plants physiological parameters after exposed the plant with six selected antibiotics found in the environment. They find out the effects of antibiotics on the roots and shoots of plants and come out with the results of an increase in antibiotic concentration causes decrease in roots and shoots length. They

conclude that the antibiotics present in the environment having negative impacts on theplants growth.

In a study by Xie et al. (2011) found that the Tetracycline is an emerging environmental pollutant because of its extensive use in the human and veterinary medicines. They determined the physiological effects of tetracycline on wheat plant. They conclude that the lower concentration of tetracycline had positive effects on the plant growth as it is stimulated. But with the increase in concentration, inhibition starts in the plant.

Hillis et at. (2011) determined the plants physiological parameters after expose the three selected plants with ten selected antibiotics present in the environment. They find out the effects of antibiotics on the roots and shoots of plants and showed the results that an increase in antibiotics causes decrease in roots and shoots length. It was also showed by the study that the roots are the more sensitive end points to such pollutants.

2.8.2. Seed Germination Technique:

Tetracycline is an antibiotic used to control various bacterial infections in animals and humans worldwide. Concentration of tetracycline is increasing in the environment due to extensive use of this antibiotic. Xie et al. (2011) determined the effects of this antibiotic on wheat at concentrations ranging from 0.5 – 300 mg/L. They conclude from the experiment that at lower concentration 0.5 – 10 mg/L, the seed germination stimulates while at higher concentrations from 10 – 300 mg/L, the seed germination is significantly inhibiting in concentration dependent manner.

Hillis et al. (2011) assessed toxicity by exposing the three selected plants with ten selected antibiotics present in the environment. They used different concentration ranging from 3.6 ug/L to 10,000 ug/L. They find out that the antibiotics had no effects on the seed germination even at the higher concentrations.

Jing et al. (2009) studied the effects of two personnel care products (PCPs) i.e., Triclosan (TCS) and glaxolides on the seedlings of wheat. Seed germination, roots and shoots were studied to determine the effects of these PCPs. The consequences for the effects of PCPs show negative impacts in the seed germination, as at higher concentrations

the seed germination of wheat is inhibiting. But at lower concentrations, seedling growth wasn't affected for the less exposer time. When the exposer time is higher the results can't be negligible.

2.8.3. Comet Assay:

As the new chemicals are introducing into the environment, they are disturbing the natural ecosystem by causing toxicity. With the promotion in scientific studies, many techniques are now introduced to assess the toxicity. In 1984, Comet assay technique was first introduced by Ostling and Johanson. With the passage of time, this technique further modified for determining the DNA damage in living cells. Now the comet assay is perceived as sensitive, simple and rapid tool used for assessing DNA damage in eukaryotic cells as well as in prokaryotic cells (Dhawan et al., 2009).

A study by Gichner et al. (2006) found out the DNA damage and toxicity in two plants i.e., potato and tomato. Both plants grown on the heavy metal polluted soil. Then they determine the DNA damage by comet assay technique and recognized that the plants grown on polluted soil having a lot of DNA damage as compared to the plants that grown on the control soil.

Zhang et al. (2007) determine the genotoxic effects of copper in wheat. They also used the comet assay technique to determine the DNA damage. The results for the toxicity showed that an increase proportion of Cu also increases the number of DNA damaged cells. It was also recognized that the roots were more toxic than the shoots on fixed concentration of Cu.

Turkoglu (2012) studied on the determination of genotoxic effects by using comet assay technique. They took two environmental pollutants i.e., Chlorfenvinphos and fenbuconazole for the assessment of toxicity. They exposed the pollutants for 24 and 48 hours on *Allium cepa* specie with different concentration ranging from 10 – 100 ppm. The results showed that the toxicity increases with the increase in concentration of pollutants.

2.8.4. Micronucleus assay:

Micronucleus assay is the most commonly used technique for the genotoxicity assessment. In 1992, micronucleus assay technique was followed in 2000 studies and till 2006 the numbers for the study of this technique were increased up to 6000 and in 2010 the numbers increased to 13,000, which are very large as compare to the other toxicity assessment techniques (Bolt et al., 2011).

The genotoxic effects of tetracycline were observed in the study by Xie et al. (2011). In the study from 0.25 – 300 mg/L concentrations of antibiotics were ranged. The consequences showed that the decrease in concentration of tetracycline results in an increase in mitotic index (MI). There was significant increase in MI with the high concentration of tetracycline. Therefore, it was declared that when tetracycline is exposed to a crop it causes genotoxic effects as it is an environmental pollutant.

Yi et al. (2010) found the effects of aluminum (Al) on *Vicia faba* by using micronucleus assay technique. *Vicia faba* was exposed with $AlCl_3$ in different concentrations ranging from 0.01 – 10 mM for 12 hours. Results showed that an increase in concentration caused increase in micronucleated cells. In all treated groups, the number of mitotic cells dependent on the pH. It is concluded that the *V. faba* plant is a model plant for the toxicity measurement and Alcl3 is toxic for *V. faba*.

Khadra et al. (2012) observed the genotoxicity assessment of two groups of antibiotics i.e., quinolones and fluoroquinolones in *Vicia faba* by micronucleus assay technique. In this study, very low concentrations of antibiotics of 0.001 and 0.005 μg/L were exposed to the *V. faba* and there were no significant genotoxic effects observed. But it induces significant micronuclei induction when the mixture of these antibiotics exposed to a plant.

Micronucleus assay is a key indicator for both *in vivo* and *in vitro* to determine the genotoxicity in various types of cells and populations when the genotoxic agents are exposed (Araldi et al., 2013; Araldi et al., 2015). MN assay is standardized as the chromosomal losses along with the effects of DNA amplification (Samanta and Dey, 2012). DNA amplification detection in oncogenic processes results in double minute

chromosomes, which are then eliminated from the main nucleus forming MNs (Shimizu and Tanaka, 2000). This elimination resulted in the carcinogenesis as related to the loss of alleles (Terradas et al., 2010).

Above mentioned techniques are significant to determine the genotoxic effects of pharmaceuticals, industrial chemicals and emerging pollutants. Micronucleus Assay is very quick, simple and relatively simple method. Therefore, in the following study MN assay was used to assess the effects of antibiotics on germination and micronuclei production in *Zea mays*.

Chapter 3

MATERIALS AND METHODS

This chapter describes the experimental framework that was adopted during the whole research. It explains the steps taken during the whole experiment like soil preparation, soil measurements, seed germination test, seedling and planting, antibiotic application, harvesting and analysis. The whole experiment was set in the locally made in glasshouse in the backyard of IESE, SCEE, NUST, Islamabad, Pakistan. The measurement of growth parameters, toxicity assessment and calculation of uptake concentration of antibiotic in the plants was performed in different labs. Methodology of every step taken during the whole experiment and calculations are described here in details.

3.1. Soil Preparation

Soil was taken from nursery at NUST and then spread and dried in the backyard for four to five days to remove moisture properly. Soil was then prepared by crushing and sieving at the Particulate Technology Lab SCME-NUST. Soil was crushed in the ball mill and then sieved through <2 mm sieve for a very fine and homogenized soil. The columns (pots) that could sustain up to 4.5 kg soil were used. Soil put in the pots with 4.5 kg prepared soil in each pot and fixed them in the glass house in the backyard of IESE-NUST.

Figure 3.1: Ball Mill at SCME, NUST

3.1.1. Soil Texture:

Soil was prepared with the relative proportion of sand and the soil. This type of soil is critical for bioavailability and mobility of pollutants to the plants. Soil texture was determined on the basis of saturation percentage (Malik et al., 1984).

3.1.2. pH:

pH of soil was determined to check the acidity and alkalinity of soil as it influences the soil conditions and plant growth by affecting the significant biological elements. 5 g of prepared soil was taken in a 50 mL beaker. 25 mL of distilled water was then added into the soil by measuring with measuring cylinder. Then soil suspension was put on a shaker and it was shaken for 30 minutes at 180 rpm for fine mixing. After proper mixing, the suspension was placed to rest for an hour till the water and soil get separated. And then it was filtered through Wattman No. 42 filter paper. The pH of filtrate was measured using glass electrode (Mclean, 1982).

3.1.3. Electrical Conductivity:

Electrical conductivity of soil was determined by using conductivity meter. 5g of prepared soil was taken in a 50 mL beaker. 25 mL of distilled water was then added into the soil by measuring with measuring cylinder. Then soil suspension was put on a shaker and it was shaken for 30 minutes at 180 rpm for fine mixing. After proper mixing, the suspension was placed to rest for an hour till the water and soil get separated. And then it was filtered through Wattman No. 42 filter paper. The electrical conductivity of filtrate was measured using conductivity meter (Rayment and Lyons, 2011).

3.1.4. Moisture Content:

Moisture content refers to the amount of water present in the soil. The china dish was taken for measuring moisture content in the prepared soil, and rinsed with distilled water. And then dry in hot air oven at 105^0C for 3 hours. After drying, the china dishes were transferred to the desiccator for cooling and then weighed. 1g of prepared soil was taken and spread into china dish and placed china dish in the hot air oven at 70^0C for 24 hours. After 24 hours, they were set aside for cooling. After cooling the sample, the dish along

with its dried sample was re-weighed. The moisture content in sample was calculated using the following formula:

$$\textbf{Moisture content (\%)} = \frac{W_1 - W_2}{W_2} \times 100$$

As,

W1 = weight gram of sample before drying

W2 = weight gram of sample after drying

3.1.5. Water Holding Capacity:

Water Holding Capacity was measured to observe the capacity of soil to hold the water. 30 g of prepared soil was taken and put on the filter paper that was fixed in the funnel. 30 mL of distilled water was poured over the soil gently. And then excess water was allowed to drain into the beaker below a funnel. After all the water had drained, the filtered water then measured using a graduated cylinder. Water holding capacity was calculated through following formula:

$$\textbf{Water Holding Capacity (40mL/L)} = \frac{\textbf{Retained Water (mL)} \times 100}{\textbf{Volume Sample}}$$

As,

Retained Water = Total volume of water– Drained water

Volume Sample = Total volume of water sample

3.1.6. Total Sodium:

5g of soil sample and 25 mL of ammonium acetate solution was taken in a 100 mL of conical flask as per procedure developed by the Richards (1954). The mixture was then shaken for 5 minutes constantly till it appears as a homogenized mixture and then filtered through the Wattman No. 1 filter paper. Sodium was measured in the extract through flame photometer at 589nm wavelength (Richards, 1954).

3.1.7. Total Phosphorus:

2.5 g of soil was taken in a 100 mL volumetric flask and 0.5g of activated charcoal was added in it. After that 50 mL of 0.5M $NaHCO_3$ was added in it. And fixed the flask on shaker and shaken the solution for 30 minutes. A blank was also run without soil. The mixture was filtered through a Wattman No. 40 filter paper. And 5 mL of filtered extract was separated in a 25 mL of volumetric flask. This extract was then acidified with 5N H_2SO_4. And 4 mL of Ascorbic acid working solution was added in a small quantity of distilled water. The solution was rested for 10 minutes. After 10 minutes, the absorbance (intensity of blue color) was measured in spectrophotometer at 660 nm wavelength. Calculation of the amount of phosphorus was measured using following formula:

$$\text{P (kg/ha)} = \frac{\text{R x (B/A) x 50 x 2.24}}{\text{5 x 2.5}}$$

As,

R = Absorbency

A = Absorbency from standard curve

B = Concentration of Phosphorus for absorbency A

3.1.8. Total Nitrogen:

0.2 g of dried soil sample was put into the digestion tube along with 0.1 g $CuSO_4$ and 3 g K_2SO_4 was added. 20 mL distilled water was also added in it and then dissolved well. 7 mL H_2SO_4 was added in the digestion tube. This mixture was mixed well and put on digester at 350 ^{0}C for 20 minutes.

After the digestion, the digested tube was set into the distillation assembly along with 50 mL borate buffer in a flask. Distillation time was set to 5 minutes during which the N get absorbed in borate buffer from the digested material. After distillation, 6 to 7 drops of mixed indicator was added in the receiving flask. After which the borate buffer solution will turn into yellow. This solution was then titrated with 0.02N H_2SO_4 till the yellow color

disappears and turns into pink. The readings were then noted and the amount of organic nitrogen was calculated using the following formula:

$$\textbf{Organic nitrogen (\%)} = \frac{\textbf{Sample reading} \times 0.02N \times 14 \times 1000}{\textbf{Mass of sample (g)}}$$

3.1.9. Total Potassium:

5g of soil sample and 25 mL of ammonium acetate solution was taken in a 100 m L of conical flask as per procedure developed by the Richards (1954). The mixture was then shaken for 5 minutes constantly till it appears as a homogenized mixture and then filtered through the Wattman No. 1 filter paper. Potassium was measured in the extract through flame photometer at 767 nm wavelength (Richards, 1954).

3.2. Seeds Selection and Preparation:

Maize (*Zea mays*) was selected for the experimentation of effects of antibiotic (ciprofloxacin). Seeds were collected from National Agriculture Research Centre (NARC) Islamabad, Pakistan. Healthy seeds were separated and soaked in 5% solution of calcium hypochlorite for 5 minutes for sterilization. After sterilization, seeds were washed with distilled water for 3 times. Then they were remaining soaked in distilled water for twenty-four hours.

3.2.1. Seed Germination Test

About 12 petri dishes were taken for the seed germination test. All petri dishes were washed properly with distilled water and then dried in oven and sterilized in the autoclave. Sterilized filter papers were put in the dishes. After twenty-four hours of soaked seeds, 8-10 seeds were placed in each petri dish and sprinkle the distilled water just to wet. Place the petri dishes in incubator at 30^0C for three days (Eggen et al. 2011).

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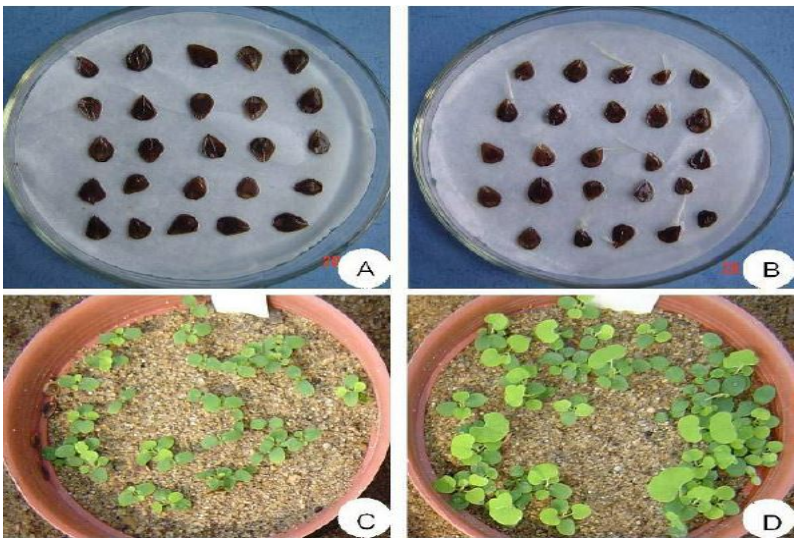

Figure 3.2: Pictorial View of Seed Germination Test

These seeds were sprinkled with distilled water after regular intervals to ensure moisture for growth. After three days, the rate of germination was calculated. The following formula was used to find out the percentage of seeds germination:

$$\text{Seed Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds}} \times 100$$

3.3. Pot Experiment

Germinated seeds were sowed into the small pots of half kg volume till they grew into the small seedling of approx. 8-10 cm length. The germinated seeds were remained there in these pots for 10 – 15 days till they grew into healthy plants which could be separated and shifted in the large pots. After the growth of seedlings to respective height, they were shifted into the columns of about 5kg volume for the further experimentation.

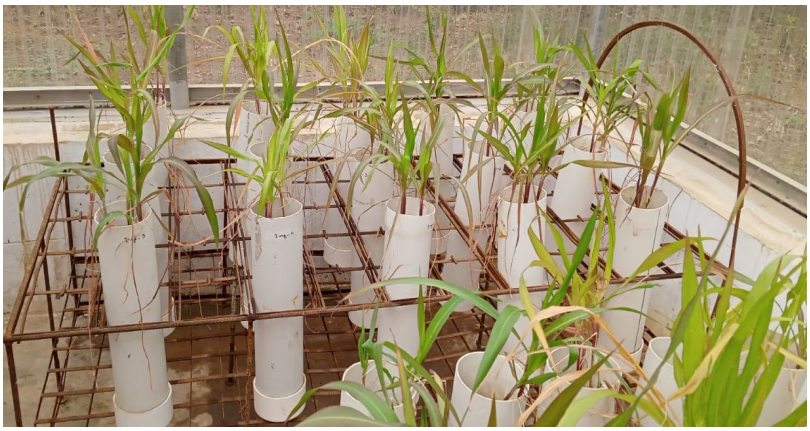

Figure 3.3: Pot Experiment

3.4. Preparation of Antibiotic Solution

In the present study, pure ciprofloxacin (>95%) was used to measure the effects for exposure to the *Zea mays* specie. ciprofloxacin was used in five different concentrations along with replicates and along the controlled. To give a nominal initial concentration of 1, 2, 3, 4, and 5 mg/kg of ciprofloxacin was spiked directly to the pots of given amount as each pot contains 4.5 kg of soil. Stock solution was prepared with required weigh of ciprofloxacin dissolved in distilled water. To acquire the maximum solubility 1M HCl was also added. 350mg of ciprofloxacin was mixed in distilled water in 100 mL volumetric for making the stock solution. Then the stock was filtered through Wattman paper no. 41 and further diluted till to attain the concentration of 1 mg/mL. Different concentrations were prepared by using this stock solution for the application to the pots.

3.5. Antibiotic Application

Required concentrations was made through the stock solution by added distilled water into the solution and given to the plants when they were of two weeks. That antibiotic dose was given once to the plants. Total of five different concentrations were used during this experiment of 1, 2, 3, 4, and 5 mg/kg. Five replicates were tested for each concentration including the control plants.

3.6. Plants Harvesting

Plants were harvested after the period of three months. Samples of roots, shoots and leaves were taken from each pot. Roots were extracted from the pots according to the method described by Macherius and Coworker (2010), washed with the distilled water and stored in the plastic bags in the ultra-freezer (-50^{o}C) until further analysis. Similarly, leaves were also stored into the plastic bags in the ultra-freezer until analysis. Some of the plant material was then dried in an oven before analyzed to assess the possible evaporation at 45^{o} C during the drying process. To prevent from cross contamination, control and exposed plant samples were dried in the separate china dishes (Eggen et al. 2011).

3.7. Growth Parameters (Length & Biomass)

Complete plant was taken from the pot. Shoot and leaves were taken first then roots were removed from the soil carefully. Measuring scale was used for the measurement of plant length the effect of ciprofloxacin on length of plant. Effect of ciprofloxacin on biomass of plant and roots was measured separately by using portable weighing balance by comparing both exposed and control plants. Roots were washed with distilled water and then weighed their biomass. After measuring height and biomass of the whole plant and roots they were packed in the plastic bags and put in the ultra-freezer at -150^{o}C till further experiments. The effect of ciprofloxacin as decrease in length of plant ciprofloxacin exposed vis. control was measured.

3.8. Composition of vegetative parts

3.8.1. Moisture content:

Moisture content refers to the amount of water present in the plant. The china dish was taken for measuring moisture content in the plant sample, rinsed with distilled water a nd then dry in hot air oven at 105^{o}C for 3 hours. After drying, the china dishes were transferred to the desiccator for cooling and then weighed. 1g of plant sample was taken and spread into china dish and placed china dish in the hot air oven at 70^{o}C for 24 hours (Rayment and Lyons, 2011).

Figure 3.4: Wet Plant Samples for Moisture Content Measurement

Figure 3.5: Measurement of Moisture Content through Weighing balance

After 24 hours, they were set aside for cooling. After cooling the sample, the dish along with its dried sample was re-weighed. The moisture content in sample was calculated using the following formula:

$$\textbf{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

As,

W_1 = weight gram of sample before drying

W_2 = weight gram of sample after drying

3.8.2. Chlorophyll content:

This method is used for the extraction of chlorophyll from leaf tissue without maceration. About 100 mg of the fresh plant sample (cut into small pieces) was taken in a

culture tube with addition of 7 mL of dimethyl sulphoxide. It was mixed well and tubes were incubated at 65°C till the leaf extract comes in the solution. After that, DMSO was added into the tube, and it was made up to mark till 10ml.

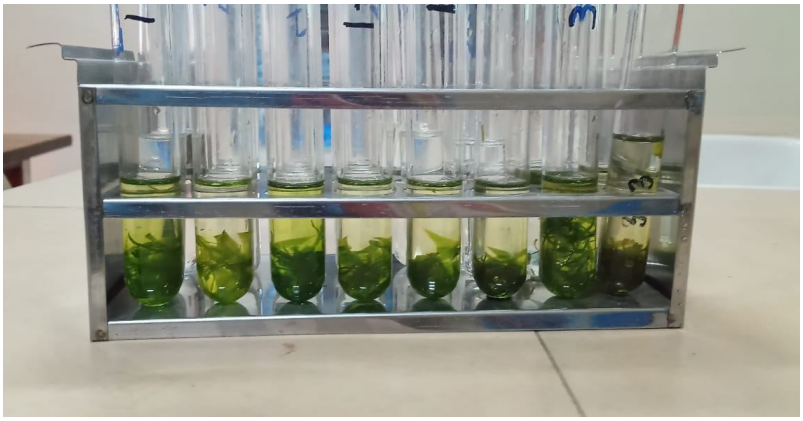

Figure 3.6: Processing for Chlorophyll Content Measurement

The absorption of solution was observed at 645 nm and 663 nm through UV visible spectrophotometer. The value of absorption must be less than 0.7. For samples, where it was more than 0.7, 50% dilution was done with 90 % DMSO and absorption was recorded (Hiscox & Israelstam, 1979). The chlorophyll contents (a, b, and total chlorophyll) in each sample were calculated using following formulas:

Chl. a (µg/mL) = (12.7 × O.D. at 663nm) – (2.69 × O.D. at 645nm)

Chl. b (µg/mL) = (22.9 × O.D. at 645nm) – (4.68 × O.D. at 663nm)

Total Chlorophyll (µg/mL) = (20.2 × O.D. at 645 nm) + (8.02 × O.D. at 663nm)

3.8.3. Organic Nitrogen (Kjeldhal method):

About 0.2 g of dried plant sample was taken in a digestion tube in which 0.1 g of CuSO4 and 3 g of K_2SO_4 was added followed by addition of 20 mL of the distilled water. Additionally, 7 mL of H_2SO_4 was added in that solution and it was mixed well. The digestion tubes were put on digester at 350 °C for 20 minutes.

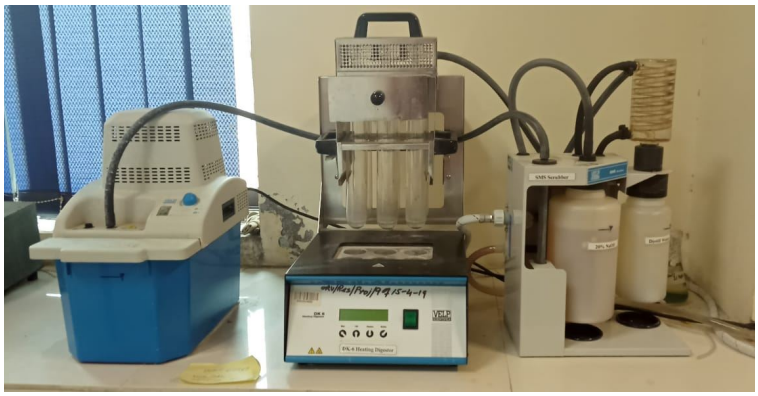

Figure 3.7: Digestion for Total Nitrogen Test

After digestion, the digested tubes were fit into the distillation assembly along with 50 mL borate buffer in a flask and 5 minutes of distillation was carried out during which the nitrogen got absorbed in borate buffer from digested material. 6 to 7 drops of mix indicator were added in the receiving flask upon which the borate buffer solution turned yellow. The solution was them titrated with 0.02N H_2SO_4 till the yellow color disappeared and turned pink (Baird et al., 2017). The readings were noted and the amount of organic nitrogen was calculated by following formula:

$$\textbf{Organic nitrogen (\%)} = \frac{\textbf{Sample reading} \times \textbf{0.02N} \times \textbf{14} \times \textbf{1000}}{\textbf{Mass of sample (g)}}$$

Figure 3.8: Distillation Assembly

Figure 3.9: Titration

3.8.4. Total Phosphorus:

In 100 mL beaker, 0.1g air dried plant samples were added along 14 mL of the distilled water. 6 mL of the concentrated H_2SO_4 and 0.4 g ammonium per sulphate (solid) was also added. The solution was mixed well and beakers were placed on a hot plate for digestion till half of the total solution is left. 40 mL of distilled water was poured in the digested sample and again placed on hot plate till this 50 mL solution got reduced to 10 ml. This digested solution is very acidic, so its pH was reduced to neutral with 2N NaOH.

Figure 3.10: Digestion for Phosphorus Measurement

The digested material was filtered and residues on filter paper were washed with distilled water. The total volume was made up to 20 mL with distilled water in 50 mL beaker. 1 drop of phenolphthalein indicator was added in it and titrated against 3M NaOH until the red color disappeared. The volume of final solution was made up to 50 mL with distilled water and samples were analyzed on UV visible spectrophotometer at the wavelength of 470 nm (Richards, 1954). The total phosphorus was calculated through following formula:

$$\mathbf{y = 0.0161x - 0.0022}$$

As,

y = absorption at 470 nm

3.8.5. Total Potassium:

About 1 g of grounded plant sample was taken in a 100 mL digestion flask and 20-25 mL of acid mixture was added in it. Acid mixture contains comparable ratio (1:2:5) of concentrated H_2SO_4 (150 mL), $HClO_4$ (300 mL), and HNO_3 (750 mL). The digestion flask was put on hot plate and temperature range was kept from low to high, till the liquid became colorless. This point confirmed the complete digestion and solution was cooled down followed by addition of 20-25 mL of distilled H_2O. The samples were filtered in a 100 mL flask and made up to the volume of 25 mL. The samples were analyzed on Flame photometer and readings were noted (Estefan et al., 2013). Following formula was used for the measurement of potassium:

$$\mathbf{K\ (\%)\ =\ X\ .\ 4\ .\ 10^{-3}}$$

Figure 3.11: Flame Photometer used for K Determination

3.9. Ciprofloxacin Uptake Concentration

About 1 g of grounded plant sample was taken in a 100 mL digestion flask and 20-25 mL of acid mixture was added in it. Acid mixture contains comparable ratio (1:2:5) of concentrated H_2SO_4 (150 mL), $HClO_4$ (300 mL), and HNO_3 (750 mL). The digestion flask was put on hot plate and temperature range was kept from low to high, till the liquid became colorless. This point confirmed the complete digestion and solution was cooled down followed by addition of 20-25 mL of distilled H_2O. The samples were filtered in a 100 m L flask and made up to the volume of 25 mL. Three replicates of the sample solution containing ciprofloxacin were prepared and absorbance of each solution was measured at

275 nm on UV spectrophotometer (Parasad et al., 2018). Readings were taken and the amount was calculated.

3.10. Toxicity Assessment through Micronucleus Assay

For the toxicity assessment, the Micronucleus Assay Technique was used in the Biotechnology Research Laboratory at Institute of Environmental Sciences and Engineering (IESE) and DNA damage was measured through micronucleus assay in order to estimate the value of genotoxicity (Bolt et al., 2011; Khadra et al., 2012). Different steps taken throughout the experiment are discussed as follow:

- **Preparation of Root Tips:**

  Five root samples from each antibiotic treatment along with the control were taken and cut in about 1-2 centimeter in length with help of a fine knife from the tips. These samples were rinsed with the distilled water carefully and dipped in aceto-ethanol mixture at $4^{o}C$ for overnight. Aceto-ethanol was made with 1 to 3 ratios (V /V). Next day, these roots samples were rinsed again with the deionized water for ten minutes and then dipped for about ten minutes. The root tips were then transferred to ethanol for the storage till further procedure.

- **Preparation of Feulgen Stain:**

  0.3 g of basic Fuschin powder was taken in a 250 mL beaker and 60 mL of boiling distilled water was added in it with constant stirring, while the beaker was placed on a hot plate stirrer. After complete dissolution, it was cooled down and then filtered through Whatman no. 1 filter paper. After filtration, 4 mL of 1N HCl was added in the filtered solution. 0.5 g of Potassium meta-bisulphite was added in this solution and mixed thoroughly for the decolorization. This solution was placed in dark place for proper decolorization (bleaching) for 24 hours. Prepared stain was then put into a bottle which was properly covered with aluminum foil in order to protect stain from light and placed in the refrigerator till further use.

- **Root tips Fixation and Staining:**

  Prepared root tips were taken and fixed in freshly prepared Cornoy solution. Cornoy solution was prepared by adding ethanol and glacial acetic acid in 3 to 1

ratio. The root tips were left in the Cornoy solution in the refrigerator overnight. After proper fixation, the root tips were put in the phosphate buffer solution and maintained in it for around 10 minutes. The root tips were them hydrolyzed for 30 minutes in the water bath at 60 ^{o}C by using 1N HCl. After hydrolization, the root tips were stained with Feulgen Stain for 30 minutes in the water bath at 60 ^{o}C.

- **Root Tips Squash:**

  Slides were prepared for further microscopic study of root tips through root tips squash technique. For this purpose, root tips were cut to about 5mm length and put on watch glass. A drop of HCl was put on root tips followed by addition of 2 to 3 drops of Acetic Orcein stain. The whole mixture was heated on hot plate for around 5 minutes till the stain gets dry. The root tips were put on clean microscopic slide very carefully followed by addition of a drop of Acetic Orcein stain on them. This slide was covered with another slide and slightly pressed with hand. The prepared microscopic slides were used for further analysis.

- **Visual Analysis of Data:**

  Slides were well prepared, properly labeled, visualized under Fluorescent Microscope. For each concentration of ciprofloxacin, there were 3 replicates and 100 cells were analyzed under Trinocular Fluorescent Microscope (Optika B353FL) with 40X magnification. By visual analysis, numbers of damaged cells were counted and images were taken. For the determination of genotoxicity via micronucleus assay technique, number of damaged cells was counted for each concentration. Percentage of damaged cells was calculated by using percentage formula, as stated:

$$\textbf{Damaged cells (\%)} = \frac{\textbf{No. of damaged cells}}{\textbf{Total no. of cells counted}} \times 100$$

- **Statistical Analysis of Data:**

  All data that was analyzed and collected through micronucleus assay technique was prepared by using Excel Worksheet and the relation between concentration of ciprofloxacin (antibiotic) and toxicity assessment on *Zea mays* plants was determined.

Chapter 4

RESULTS AND DISCUSSION

Antibiotics have significance importance in our daily for the treatment of bacterial infections in humans and animals. Due to over usage of antibiotics, these are entering in our environment either during consumption by humans, animals and production. In wastewater different amount of antibiotics are determined by various studies. When crop fields and plants are irrigated by antibiotic holding wastewater, the antibiotics absorbed by the plants and gather there. Keeping in view, the current study was intended to observe the effects of ciprofloxacin on *Zea mays* (maize). For this purpose, plants physical growth parameters, uptake concentration and DNA damage caused by selected antibiotics were determined.

4.1. General Soil Characteristics

The soil used in this experiment was silty sand. Its physiochemical properties were determined are given in the table 4.1:

Table 4.1: Physicochemical Properties of Soil used for Experimentation

Parameters	**Values**
pH	7.14
EC	185.2 µS
Moisture Content	0.22 %
WHC	40 %
Sodium	78.4 mg/kg
Total phosphorus	51.3 mg/kg
Total Nitrogen	3.1 mg/kg
Potassium	62.5 mg/kg
Soil Texture (%)	
Sand	32
Silt	50.5

Clay	17.5

4.2. Effects of CIP on Plant Physiological Parameters

Plant growth parameters like plant biomass and shoot length was analyzed after plant exposure to CIP of all five replicates. The effect of CIP on different growth parameters are as follows:

4.2.1. Effect on plant shoot length:

Results indicating the effects of CIP on plant shoot length are shown in figure 4.1. Different concentrations were exposed to *Zea mays*, affected the plant length. The displayed graph shows that with increasing concentrations of ciprofloxacin, a decrease in shoot length was seen. The length variation in plant exposed to higher concentrations was not highly variable in comparison to the control, however the higher concentrations affected the plant's shoot length significantly. The results were dependent on the exposed concentration as declared that the increase in concentration of CIP having negative impacts on the plant shoot length as shown in the figure 4.1.

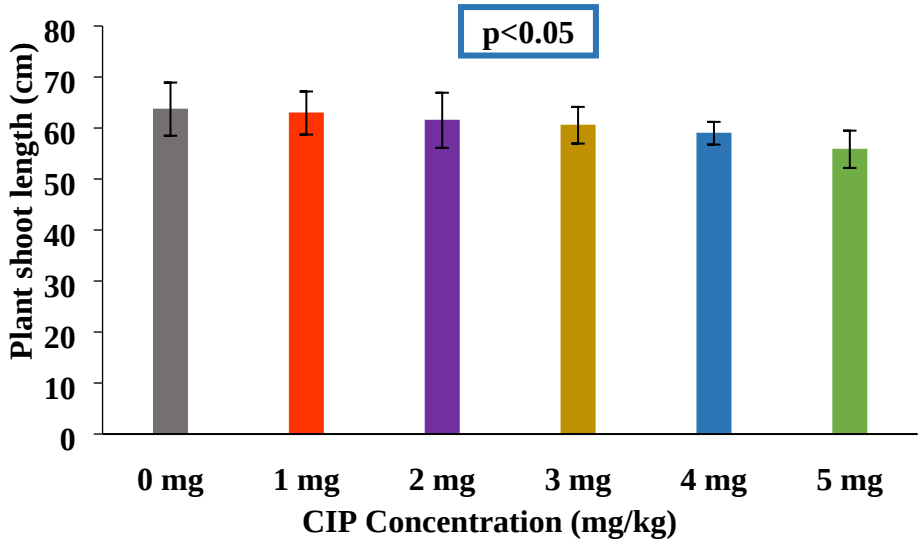

Figure 4.1: Effect of CIP on plant shoot length

4.2.2. Effect on plant biomass:

Results indicating the effects of CIP on plant's biomass are shown in figure 4.2. Different concentrations were exposed to *Zea mays*, affected its total biomass. Results declared that the biomass of plant was not much affected in the lower concentrations in comparison with the control. But a significant decrease was observed in higher ciprofloxacin concentrations. Highest applied concentration of ciprofloxacin lead to a prominent decrease in plant biomass. At 3mg/kg concentration of ciprofloxacin, the biomass reveals the biphasic pattern. In a study, plant's physiological parameters were quantified by adding antibiotics. All experiments showed a biphasic pattern for antibiotic amended soil (Liu et al., 2009; Pan and Chu, 2017) The results were dependent on the exposed concentration as declared that with the increase in concentration of CIP, plant's biomass was decreased as shown in the figure 4.2.

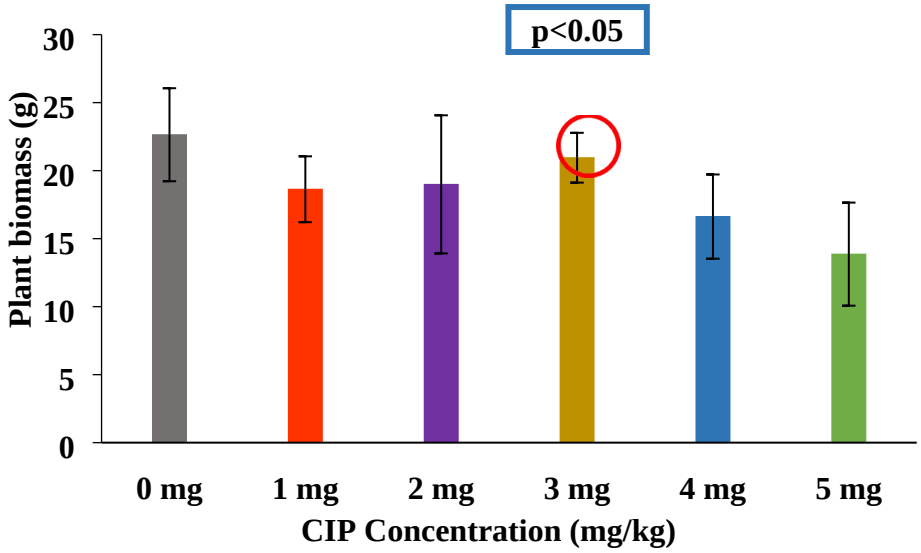

Figure 4.2: Effect of CIP on plant biomass

4.3. Effects of CIP on Composition of Vegetative Parts

4.3.1. Effect on Moisture Content:

Results indicating the effects of CIP on moisture content are shown in figure 4.3. Different concentrations were exposed to *Zea mays*, affected the moisture content. The graph shows that moisture content of the plant shows no significant changes upon the applied ciprofloxacin concentrations. The results were dependent on the exposed concentration as declared that the increase in concentration of CIP having no significant negative impacts on the plant moisture content as shown in the figure 4.3

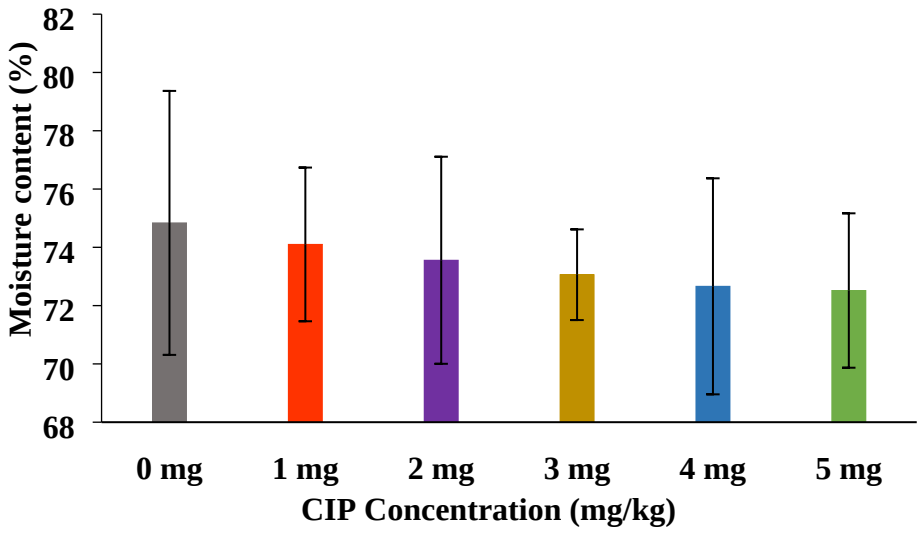

Figure 4.3: Effect of CIP on Moisture Content

4.3.2. Effect on Chlorophyll Content:

Different concentrations were exposed to *Zea mays*, affected the plant's chlorophyll content in the shoots. Chlorophyll a, b and total chlorophyll were separately analyzed. Overall, results declared that all the measurements of chlorophyll, including chlorophyll a, chlorophyll b, and total chlorophyll, shared a similar response to ciprofloxacin exposure, with the most impacted observed for the highest concentration of 5mg/kg having the least amount of chlorophyll that is 9.47 ug/mL. Although, the results suggest there to be no significant variation among the plants exposed to varying ciprofloxacin concentrations in comparison to the control. The results were dependent on the exposed concentration as declared that the increase in concentration of CIP slight negative impacts on the chlorophyll content as shown in the figure 4.4.

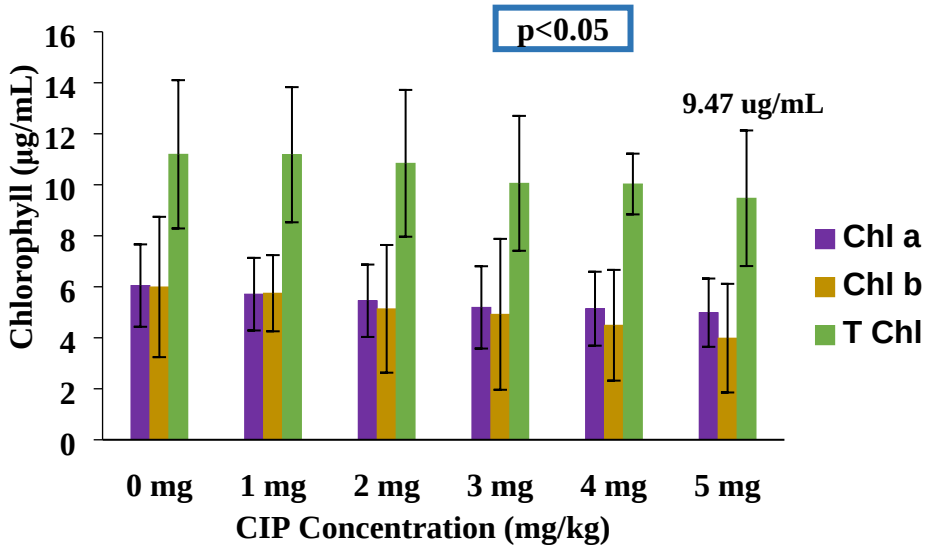

Figure 4.4: Effect of CIP on Chlorophyll Content

4.3.3. Effect on Total Kjeldahl Nitrogen:

Effects of CIP on the total nitrogen in plant shoots are shown in the figure 4.5. The TKN content of the plants did not follow a particular trend in response to increasing concentrations of ciprofloxacin, with highest value of 13.54 mg/g being for the least applied concentration of 1 mg/kg, and the lowest value of 10.72 mg/g being for 3 mg/kg concentration.

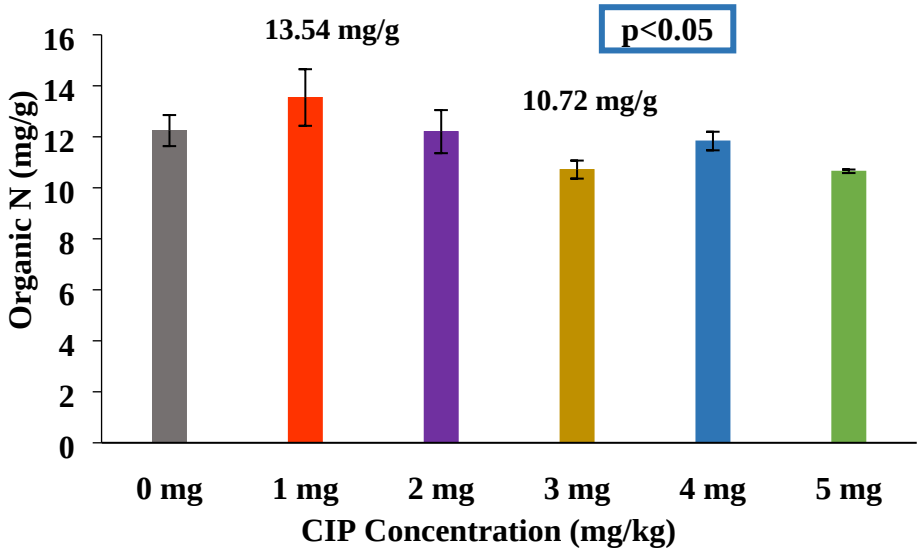

Figure 4.5: Effect of CIP on Total Nitrogen

In 1982, Batchleder reported that the contents of nitrogen, potassium, magnesium and calcium contents were significantly reduced in bean plant on exposure to the antibiotics as compared to the control. Opris et al. (2013) were analyzed the effects of nine different antibiotics on the secondary metabolites and physiology of wheat at the concentrations of 0.5 and 1.5 mg/L. CIP and cephalosporin caused a stomatal reduction, by that mean affecting the net assimilation. By the application of CIP, tetracycline and erythromycin, a reduction in its photosynthetic responses in carotenoids, pigment and chlorophyll was also observed.

4.3.4. Effect on Total Phosphorus:

Effects of CIP on the total phosphorus in plant shoots are shown in the figure 4.6. Total phosphorus content also followed no trend for 1 mg/kg, but it shows significance

declining in the higher concentrations. The amount of phosphorus was much affected with the increasing concentration of CIP, in comparison with the control. The lowest concentration of antibiotic didn't show the significant declining trend. However, the inhibitory effects were quite significant at the increasing concentrations. Lower concentration of antibiotics has no major effects on the plants while the exposure of higher concentrations of CIP results in significant declining in the plant nutrients (Khadra et al., 2012).

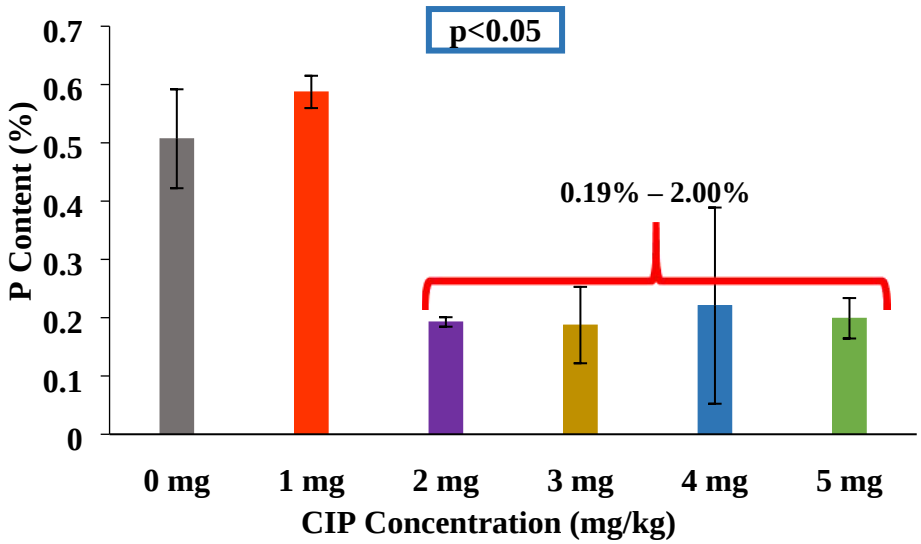

Figure 4.6: Effect of CIP on Phosphorus Content

Rice, wheat, soybean, lettuce, alfalfa and maize has been used extensively as test species to observe the accumulation of certain antibiotics in certain parts of these plants. This accumulation occurs through the passive absorption and via water transport system. These pollutants when enters the primary producers causes significant damages in the biochemical activities and physiology of plants (Liu et al., 2009; Boonsaner and Hawker, 2010, Hillis et al., 2011; Luo et al., 2011).

4.3.5. Effect on Potassium Measurement:

Results indicating the effects of CIP on plant's potassium content are shown in figure 4.7. Different concentrations were exposed to *Zea mays*, affected the amount of potassium present in the plant. Results declared that the potassium concentration of plant was not much affected in the lower concentrations in comparison with the control. But

significantly reduced amount of potassium was observed in plants within response to ciprofloxacin exposure, when compared with the control. Highest potassium content was present in 2 mg/kg exposed plant after the control. The highest concentration of 5 mg/kg was found to have the least amount of potassium, that is 1.11 mg/g.

Antibiotics can also significantly inhibit root activity linked to nutrient absorption and biosynthesis by affecting new cell generation and ammonium production (Liu et al., 2013). The results were dependent on the exposed concentration as declared that with the increase in concentration of CIP, potassium content was decreased as shown in the figure 4.7.

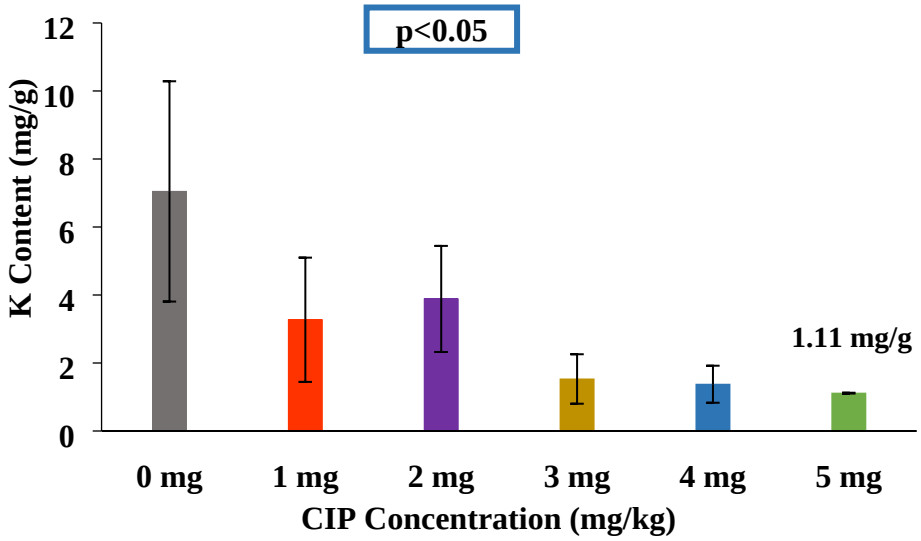

Figure 4.7: Effect of CIP on Potassium Content

4.5. Uptake Concentration of CIP

For the simultaneous estimation of ciprofloxacin, the basic criterion is that the analyte should be soluble in the solvent. Detection wavelength for the analyte was obtained by scanning analytes solution in a spectrophotometer. The spectrum showed the maximum absorbance at 275 nm for ciprofloxacin.

Analysis of ciprofloxacin uptake by plant revealed an increasing trend in response to the increasing concentrations of the applied ciprofloxacin, with control having no uptake,

and 5 mg/kg exposure having the highest uptake of the antibiotic of 2.024. It suggests that the plant tissues would take up higher amount of antibiotics if the plants were exposed to higher concentrations of antibiotics in soil. A study of the capacity of carrot and lettuce to take up antibiotics from irrigated water showed dose response results (Azanu et al., 2016). The overlay graph of uptake of ciprofloxacin is shown in Fig 4.8.

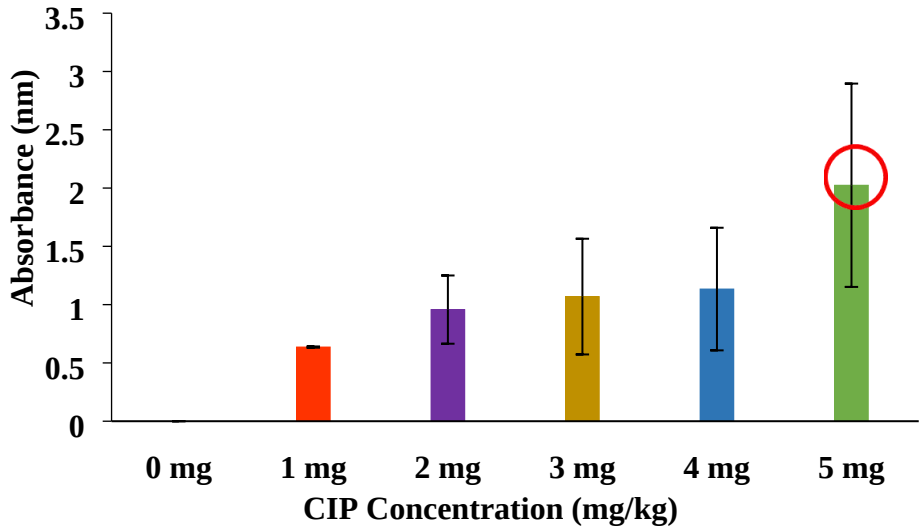

Figure 4.8: Increase in Absorbance Indicating Uptake of CIP

4.6. Micronucleus Assay:

In this study, the toxicity of ciprofloxacin was observed using mitotic index and micronucleus induction in the root tips. The consequences of micronucleus assay under different concentration of ciprofloxacin are summarized in the fig 4.9. Every experiment was achieved in triplicate and data represents in mean ± standard deviation of all 3 replicates. As stated by the results, the micronuclei production in the treated cells had shown much difference as compared to the control having no micronucleus.

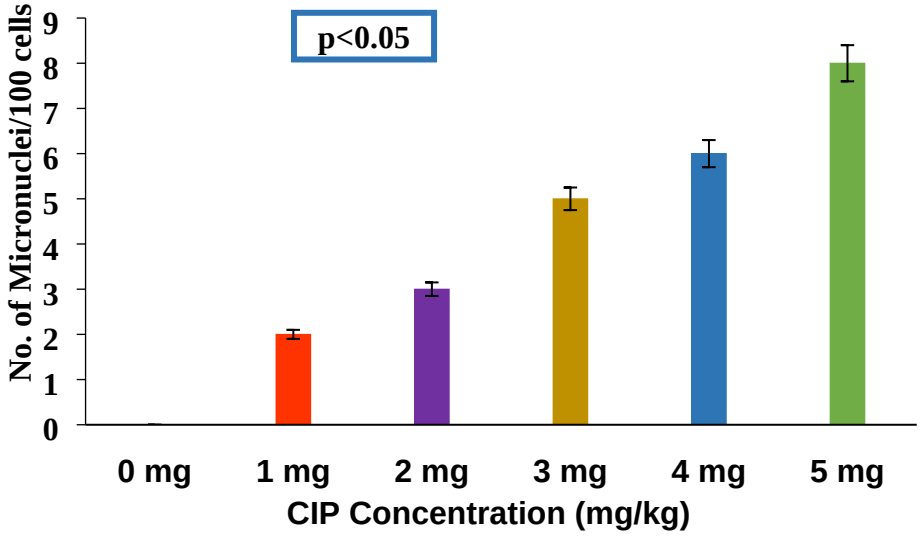

Figure 4.9: No. of Micronuclei due to Toxicity by Antibiotics

The results of MN assay demonstrated that with the increasing concentration of ciprofloxacin, the numbers of micronuclei are also produced. In the root apical meristems of control samples, there were no micronuclei cells were found but only dividing cells were present. At the highest concentration of antibiotics, a large number of micronuclei were observed.

The significant differences were observed only at 5 mg/L (i.e., 8 micronuclei per 100 cells), at 4 mg/L (i.e. 6 micronuclei per 100 cells), 3 mg/L (i.e. 5 micronuclei per 100 cells) and at 2 mg/L (i.e. 3 micronuclei per 100 cells). Minimum effect was found at the lowest concentration i.e., at 1 mg/L, 2 micronuclei per 100 cells. Each the tested concentration has shown micronuclei production in the cells. Many studies have reported the ciprofloxacin and other antibiotics in the farmland can highly increase the production of micronuclei in plant's root tips cells, causing harm to genetic material and affecting the growth of plants (Migliore et al., 2003; Wang et al., 2016).

Another study by Xie et al. (2010) found that Chlortetracycline stimulated the cell mitotic division at lower concentrations (5 and 10 mg/L) whereas hindered the process at higher concentrations (300 mg/L). The low concentration of chlortetracycline causes an increase in number of micronuclei, sister chromatid exchange and chromosomal aberration

in root tips and the higher concentrations produced significant increase. It was also observed that number of micronuclei decreased at 300mg/L due to acute toxicity.

Chapter 5

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Ciprofloxacin as contaminant must be monitored due to its wide presence in the environment, its chemical resistance and the ability to migrate from soil to crop. Ciprofloxacin uptake from soil by food plant was evident. The results suggest that uptake of CIP from the soil may indeed be a potential transport route to plants and this environmental pollutant can reach different edible parts of the selected crops. Measurements of the concentrations of this pharmaceutical in plant materials were used to model potential adult human exposure to this compound. The key findings of the present study are as follow:

- CIP caused negative impacts on plant growth parameters with increasing concentrations.
- CIP uptake in plant was directly proportional to the amount of CIP the plant was exposed to, and showed the dose response effect.
- CIP caused significant genotoxicity with increasing CIP concentrations indicating negative impacts at the highest conc. of 5 mg/kg.

5.2. Future Recommendation

From current study, significant effects were found on *Zea mays* upon exposure to antibiotics. Such studies could be helpful in addressing the issue of release of antibiotics in wastewater, producing better crop yield and making threshold levels by reducing the use of untreated wastewater for irrigation purposes and can surely help in betterment of environment. Based upon the results, following recommendations are being put forward for future research:

- Effect of antibiotics on other important crops may be assessed.

- Behavior of ciprofloxacin in varying soil types and compositions should be studied to better understand it.
- Innovative wastewater treatment techniques should be devised that can help to remove such pollutants including antibiotics that have a tendency to negatively impair crop production and yield.
- The plant uptake of a wide spectrum of commonly used pharmaceuticals from soils fertilized with sewage sludge or its compost are needed with the aim of ensuring food safety.
- Mechanism of uptake and transport of antibiotics into plants required investigations as the different antibiotics might have different tendencies to get accumulated in different parts of plants.
- Environmental standards should be devised, and legislation is required for such type of pollutants to protect the release of these into the environment.

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