TOXICITY ASSESSMENT OF CIPROFLOXACIN ON TRITICUM AESTIVUM

By

Samreen Akhtar

(2011-NUST-MSPhD-Env S-11)

A thesis submitted in partial fulfillment of requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
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It is certified that the contents and form of the thesis entitled

"TOXICITY ASSESSMENT OF CIPROFLOXACIN ON TRITICUM AESTIVUM"

Submitted by

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has been found satisfactory for the requirements of the degree of

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Dedication

I dedicate this thesis to my family

ACKNOWLEDGEMENTS

*All appreciation is for the Almighty **ALLAH**, who's Blessings gave my thoughts and my modest effort in the form of this manuscript. I offer my countless salutations to the Holy **Prophet Muhammad** (PBUH), the bacon of enlightment and forever torch of guidance for humanity.*

*I would like to express my sincere gratitude to my supervisor, **Dr. Muhammad Arshad** (Assistant Professor IESE, SCEE NUST), for his patience, motivation and knowledge sharing. His guidance helped me in all the time of research and writing of this thesis. I am thankful to GEC members **Dr. Muhammad Anwar Baig** (Professor IESE, SCEE NUST), **Dr. Imran Hashmi** (Professor IESE, SCEE NUST) and my external member **Dr. Nasar-um-Minallah** (Assistant Professor ASAB, NUST) for their devotion, support, scholarly guidance and efforts throughout this research work. My sincere thanks also go to **Dr. Ishtiaq Ahmad Qazi** (Associate Dean IESE, SCEE NUST), for his every kind of help and moral support in the completion of this thesis.*

*Last but not the least; I would also like to thank my loving **parents**, my supportive **husband**, sweet **family** members and dearest **friends**. They were always supporting me and encouraging me with their best wishes and prayers.*

SAMREEN AKHTAR

Table of Contents

List of Tables

List of Figures

List of Abbreviations

PPCPs	Pharmaceuticals and Personal Care Products
CIP	Ciprofloxacin
GDP	Gross Domestic Product
NOR	Norfloxacin
RCF	Root Concentration Factor
LCF	Leaf Concentration Factor
PCPs	Personal Care Products
TCS	Triclosan
Al	Aluminum
AlCl	Aluminum Chloride
MI	Mitiotic Index
DNA	Deoxyribonucleic Acid
Cu	Copper
PPM	Parts Per Million
HCl	Hydrochloric Acid
EDTA	Ethylene-diamine-tetra-acetic Acid
NARC	National Agriculture Research Centre

ABSTRACT

Development of human society had led the progression in the field of medicine. To treat the bacterial infections in humans and animals antibiotics are used worldwide but these antibiotics are entering in the environment and making it vulnerable for other living organisms due to discharges of untreated wastewater from pharmaceutical industries as well as via excretion from the living organisms. Pakistan is a developing country and farmers are utilizing the wastewater for irrigation of crops which could be potentially harmful with respect to the productivity and human health. The present study is focused to assess the toxicity of antibiotic Ciprofloxacin (CIP) in *Triticum aestivum* (Wheat*).* For this purpose antibiotic levels used were 1, 10, 20, 30, 40 and 50µg/L and for comparison control experiment was treated with distilled water. Physical growth parameters like plant's biomass, root, shoot and total plant length were determined after exposure to different concentrations of CIP. Genotoxicity was assessed using comet assay while presence of antibiotic in plants was also detected. Ciprofloxacin has negative impacts on plant's biomass after exposure of 48 and 72 hours. Total plant length was also affected when CIP at different concentration was provided to plants. After 24 hours of exposure, the decrease in total plant length was 31% at highest concentration and after 48 and 72 hours of exposure, the decrease was 42% and 56% respectively. The results of comet assay showed that with the increase in exposure concentration and time, the percentage of damaged cells also increased. Tails produced by the damaged cell also positively correlated with the exposed concentration and time and it was observed that uptake of antibiotic is dependent on the concentration of exposed antibiotic. This study showed that antibiotics like Ciporfloxacin in our environment are toxic for the plants being directly irrigated with contaminated wastewater and could potentially be problematic for animals and

humans, consuming these plants. To avoid these issues wastewater should be treated properly before irrigation. New, simple and cheap methods of degrading antibiotics in wastewater should be developed. Proper legislation in environmental quality standards regarding antibiotics should be formulated and implemented.

Chapter 1

INTRODUCTION

1.1. Background

The development of human society led to a progression in the field of medical science which resulted in an increased use of different chemical compounds day by day. Annually, the treatment and prevention of illness in humans and animals is carried out through the use of pharmacological active substances in huge amount (Diaz-Cruz *et al*., 2006; Sarmah *et al*., 2006). Antibiotics, a class among these chemicals are widely used for treatment and control of bacterial attacks on humans and animals. The fates of these chemicals have widely been analyzed on humans and animals but limited assessments have been done for the risks involved due to exposure of these substances to the environment (Halling-Sørensen *et al*., 1998). In late 1990s, the effects of Pharmaceuticals and Personal Care Products (PPCPs) on environment came into the attention of scientific community (Daughton and Ternes, 1999; Halling-Sørensen *et al*., 1998) and since after that the research related to the concentration and effects of these substances on the environment has been increased. Up till now, ecological risk assessment of various antibiotics has been conducted worldwide which includes assessment of antibiotic's toxic effects on aquatic organisms. The effects of these chemical compounds on plants are not very well documented specially on agricultural plants although agriculture system is identified as the most likely receiver of these compounds in the environment. These chemicals are present in animal manure, bio-solids from wastewater treatment plant and excretions from free-ranging livestock, which are applied as organic fertilizers in agriculture system and with the surface run-off they are leached into other environmental systems.

1.2. Antibiotics

Antibiotics or antibacterial originated from two Greek words, “anti” means against and “bios” means life. These are the chemical compounds which are used to destroy or slow down the bacterial growth. Antibiotics are used to treat the diseases caused by the bacteria. The first antibiotic was discovered by Alexander Fleming in 1928 and that was Penicillin, which is naturally found in fungus. With the discovery of penicillin, new doors were opened in the field of medicine.

Early the antibiotics were found naturally but now a days, these are synthetically produced by pharmaceutical industry. Among these antibiotics, a class named Quinolone is one of the most significant families with broad synthetic spectrum of antibacterial drugs. Quinolones are further subdivided in different groups and flouroquinolones is one of the most important groups.

1.2.1. Fluoroquinolones

Fluoroquinolones is the class which covers a wide range of quinolones that are clinically used. In this class, a fluorine atom is attached to central ring system of quinolones. Figure 1.1 is showing a typical structure of Fluoroquinolones.

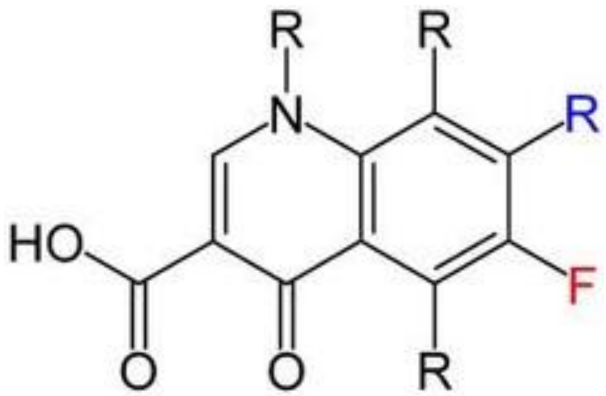

Figure 1.1: Fluoroquinolone

1.2.1.1. Ciprofloxacin

Ciprofloxacin is second generation of fluoroquinolones. In United States, the most frequently prescribed fluoroquinolone is Ciprofloxacin. It is used to treat different kinds of bacterial infections. It is effective against gram negative bacteria. Mostly it is used to treat the urinary tract and upper respiratory tract infections. Figure 1.2 shows the molecular structure of Ciprofloxacin.

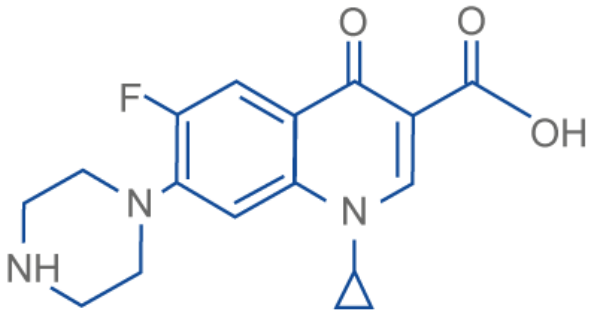

Figure 1.2: Chemical structure of Ciprofloxacin

It has been frequently found in the waste water of hospitals and sewage of wastewater treatment plants and is rarely biodegradable (Garcia-Käufer *et al*., 2012).

1.3. Ciprofloxacin in Wastewater

Antibiotics are being used for treatment of different microbial diseases and they are entering in the environment through intentional and unintentional sources like many of the drugs are disposed directly to the wastewater. They are also reaching in the wastewater by the release of sewage having urine and feces, leachate from landfills, waste from animal house etc. The bioavailability of Ciprofloxacin is almost 69% and it could enter in the wastewater through fecal and renal route (Hayder *et al*., 2012). As it is used widely, it is frequently detected in the environment.

Many studies had been done to detect the concentration of these antibiotics in wastewater. A study has been conducted by Verlicchi et al. (2012) in Italy, in which concentration of different antibiotics in urban wastewater were determined. The range of concentration of antibiotic was from 0.001 to 32 µg/L. The most frequently found antibiotics were trimethoprim, sulphamethoxazole and ciprofloxacin. The maximum concentration of ciprofloxacin found was 14 µg/L according to the research carried out. Another study was conducted by Hartmann et al. (1999) in Germany which showed that the concentration of ciprofloxacin in hospital wastewater was between 0.7 and 124.5 µg/L. In India, a study was conducted by Diwan et al. (2009) in which they detected the concentration of different antibiotics in hospital effluents of India. In this study, it was found that hospital wastewater contained many antibiotics like norfloxacin, levofloxacin, ceftriaxone, ciprofloxacin and others which ranged from 1.4 – 236.6 µg/L. Results of the study showed that ciprofloxacin is the most common antibiotic found in almost all samples. In Lahore, Pakistan a study was conducted by Ahmad et al. (2013). In this study, they investigated the amount of ciprofloxacin and norfloxacin in river Ravi. It was showed in the study that at downstream the concentration of ciprofloxacin was increasing from 0.032 to 0.125 µg/L by mixing of first to last wastewater drains.

1.4. Agriculture in Pakistan

Agricultural industry in Pakistan is the main industry on which the economy is based. According to Agriculture Department, Punjab, 21% of GDP is based on agriculture and agro-based products are producing 80% of the country's total export earnings. Country's 48% labour force is engaged in this industry. The main crops of the country are wheat, rice and cotton.

Table 1.1: Different Varieties of Wheat cultivated in Pakistan

Varieties	Area recommended
Pak – 81	Throughout country
Inqalab-91	Irrigated areas of Punjab
Auqab – 2000	Irrigated areas of Central & southern parts of Punjab
Bhakkar 2002	Initially recommended for Thal area, now recommended for irrigated Punjab
Zarlashta 99	Irrigated areas of Baluchistan
Zamindar 80	Irrigated areas of Baluchistan
Zarlashta 99	Irrigated areas of Baluchistan
Manthar 03	Punjab irrigated
Punjnad 01	Punjab irrigated
Pasban 90	Punjab irrigated
Ufaq 02	Punjab irrigated
Anmol 91	Recommended for Northern & Southern Sindh
Marvi 2000	Irrigated areas of Sindh
Bhittai 2003	Cotton belt of Sindh
TD 1	Recommended for Sindh

In Pakistan wheat is the most important crop which is being cultivated almost in all parts of the country. It contributes about 3%of country's GDP. There are various wheat varieties in Pakistan which are being cultivated; table 1.1 provides the overview of varieties available in Pakistan and their areas of cultivation. During this study, one of the listed variety i.e. Inqalab 91was used.

1.5. Wastewater Used In Agriculture

As the population of world is increasing exponentially, the need of water is also increasing and in some part of the world the water availability with reference to demand is at alarming stage. To use the water in sustainable way many approaches are being adopted by the world in which one is reuse of wastewater for agriculture purposes. As the sources of wastewater are different it may contain the mixture of different types of chemicals. This wastewater may contain several types of antibiotics which are coming from hospital and domestic sources. The chemicals which are present in wastewater may be helpful for the agricultural use but the antibiotics present in the wastewater may be harmful for the plants. These antibiotics could damage the physical properties and could cause toxicity in crop plants which ultimately affects the crop yield (Hussain et al., 2002). Figure 1.3 illustrates the route of antibiotic release in the wastewater streams and their uptake by crop plants due to extensive irrigation by wastewater.

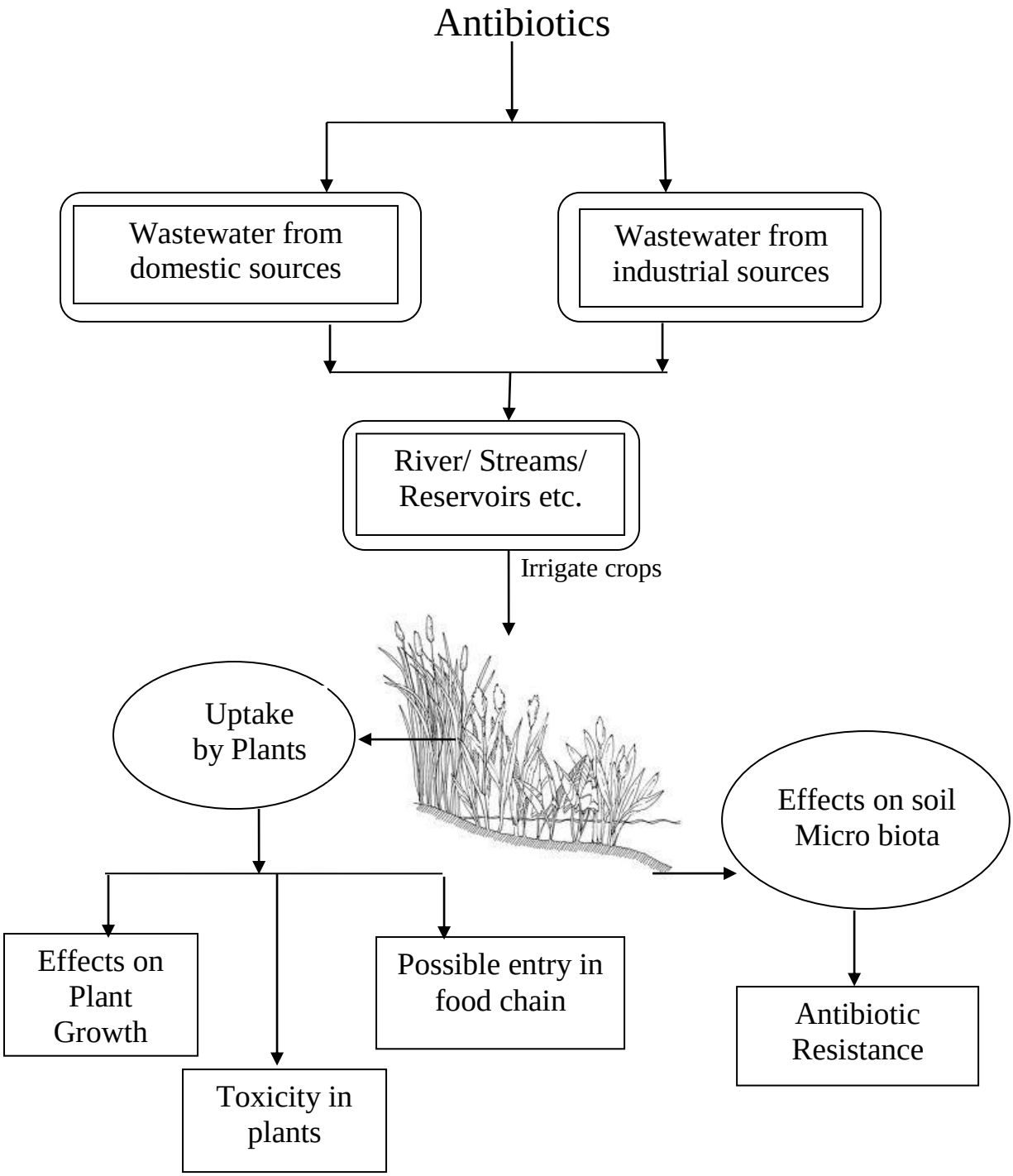

Figure 1.3: Illustration of route of antibiotics and their effects

1.6. Present Study

In this study, the antibiotic Ciprofloxacin from class fluoroquinolones was selected for determining the toxicity caused by this antibiotic on wheat. The objectives of the study were

- Toxicity assessment of ciprofloxacin in wheat plant by comet assay
- To determine the uptake of antibiotic by plant

Chapter 2

LITERATURE REVIEW

2.1 Antibiotics

Concern for toxic substances present in the environment was started almost 35 years ago when Rachel Carson's book the Silent Spring was published (Carson, 1962). When the antibiotics are famed as "Wonder Drugs" they were extensively used and the effects of their use and misuse were discussed worldwide (JETACAR, 1999). In 1969, it is concluded in the Swann Committee that use of drugs (antibiotics) for humans or for those which cause resistance must not be used for animals as growth supplements, after that a constant discussion was started about resistance encouragement (Swann Committee, 1969).

Standards for concentration of antibiotics in environment are still not established, even after EIA of veterinary medicine in the United States and European Countries (EMEA, 1997; Thiele-Bruhn, 2003). In 1999 a survey had been done and antibiotic usage was calculated roughly in EU and Switzerland. The report of survey showed that in the EU and Switzerland 13,288 tones of antibiotics were used in a year, in which 29% antibiotic was veterinary medicine, 6% antibiotics were used as growth supplements in animals and 65% were used for human medication (FEDESA, 2001). Recent studies showed that there are 15 different types of antibiotics are present in the streams of US and Germany which are receiving wastewater from urban and industrial source and agriculture run off (Ternes et al., 2002; Kolpinet al., 2002).

Antibiotics are bioactive therefore they are not completely eliminated from body of organism. They are highly effective at low doses and have short residence time that's why they will excrete from the body after very short time. (Kummereret *et al*., 2000;

Thiele-Bruhn, 2003). Rates of excretion of antibiotics depends upon the nature of compound, the application method, the type of genus which excrete and administration time. In a study it was showed that excretion rates of tetracycline and sulphonamides vary between 40 and 90% (Berger *et al*., 1986; Haller *et al*., 2001; Halling- Sørensen, 2001). If the degradation of antibiotic takes place in the body it will excrete in the feces but if they didn't metabolized it will remain present in the environment (Kummerer *et al*., 2000). Metabolites of antibiotics could be altered back to their pro form after they excrete from the body of organism (Langhammer, 1989). When fluorchinolones and sulphonamides adsorbed to the feces, they are rich in organic matter and are strong that's why they didn't transformed. Even after aeration of manure and increase in temperature could not transform them and they are distributed in parental form in the environment. Most of the drugs used in the food-manufacturing industry of animals, adsorbed inadequately in animal's stomach and 30–90% of the antibiotic are excreted from animal body in parental compound (Christian *et al*., 2003).

2.1.1. Ciprofloxacin

The fluoroquinolones (enrofloxacin and ciprofloxacin) are broad spectrum synthetic antibiotics. They are used for humans and veterinary medicine because they show strong against bacteria. Ciprofloxacin is a chief metabolite of enrofloxacin for animals (Cester and Toutain, 1997). These compounds are relatively strong resistant to biotic and abiotic degradation (Goodman Gilman, 1997). As quinolones are extensively used in aquaculture that's why they are studied as contaminants of marine ecosystem and it is found that their impacts was at benthic level (Migliore *et al*., 1996). Some work had been done on third generation of quinolones i.e., fluoroquinolone and studies showed that they have negative impacts of some plant species when thin the environment

(Migliore *et al*., 2000). A study showed that Lythrum plants uptake the antibiotic under laboratory conditions and it will accumulate in the plant body. Results of the study showed the bioaccumulation and phytotoxicty if the antibiotic. Quinolone were not studied as contaminants of terrestrial ecosystem. (Cester and Toutain, 1997).

2.2. Antibiotics In Environment

Antibiotics could be spread in the environment via human and agricultural sources, which include excretions of animals, wash out of old and expired prescriptions, waste from medical sources, wastewater treatment facilities discharge, leakages of septic tanks and agricultural waste storage structures. Other passages for spread are through use of waste from domestic and agricultural sources on land and surface runoff. When antibiotics enter the environment their efficiency depends upon different conditions e.g. physical and chemical nature of antibiotic, climate of the area, soil type and other environmental conditions, similar to other organic compounds. When the drugs present in our environment and are not completely metabolized there is possibility that their metabolites will help in stabilization and population of microorganisms that are resistant to antibiotics will be increased (Witte, 1998). So repeated use of manure on the exact area could help the constant contact of soil microorganisms to such antibiotic remains and population of bacteria that are resistant to antibiotics will be increased. It could have potential impacts on the environment, specifically when these remains travel through surface runoff or they are leached and arrive at neighboring water bodies.

When these antibiotics are enter in the environment through different sources there is possibility that they have adverse effects on humans, terrestrial or aquatic ecosystems but these effects are still to be understood. In previous few years the effects of drugs on environment has appeared as an important area of research among the researchers

(Velagaleti,1997; Halling-Sørensen *et al*., 1998; Montague, 1998; Daughton and Ternes, 1999; Hirsch *et al*., 1999; Jensen, 2001; Dietrich *et al*., 2002). The majority of studies during middle of 1990s to late 1990s were concerned to the occurrence and division of human and veterinary medicines in the environment. When these researches showed that these pharmaceuticals are moved through surface water and ground water from urban and agricultural sources, scientist have started to study their effects on environment (e.g. Patten *et al*., 1980; Cole *et al*., 2000; Halling-Sørensen *et al*.,2002; Sengeløv *et al*., 2003; Richards *et al*., 2004; Loftin *et al*., 2005). Still there is a very little information available on fortune of these compounds and their behavior of transport in the soil–water environment.

2.2.1. Surface Waters

The case which was firstly reported of contaminated surface water through antibiotics was in England more than twenty years ago, when Watts and his team (1982) found a compound from the macrolide, sulfonamide, and tetracycline group of antibiotics in river water and the amount detected was 1 µg/L. Follow to this, a range of different pharmaceuticals were also found in surface water and the concentrations found was up to 1 µg/L (e.g. Richardson and Bowron, 1985; Pearson and Inglis, 1993; Ternes, 1998 and Hirsch *et al*., 1999). A group from Germany found remains of chloramphenicol in effluent of sewage treatment plant and in a small river in southern part of Germany with amounts of 0.56 and 0.06 µg/L respectively (Hirsch *et al*., 1999). Chloramphenicol is a antibiotic which is used to treat severe meningitis in human but in very rare medicals cases, and its use for veterinary purposes has been banned in the European Community since 1995 (BGVV, 1996).

2.2.2. Ground Water And Marine Sediments

The presence of antibiotics used for animals in groundwater was reported in different studies (Holm *et al*., 1995; Hirsch *et al*., 1999 and Hamscher *et al*., 2000). But majority of the drugs to kill bacteria found in ground waters were used in agricultural lands but they did not cross the maximum value of quantization (0.02–0.05 µg/L; Hirsch *et al*., 1999).

2.2.3. Drug, Manure And Agricultural Soils

The use of drugs in body certainly results in remaining concentrations that goes into waste material (Thiele-Bruhn, 2003) Therefore it is expected to find remaining drugs either in the form of metabolite or pro form in dung, manure and then in agricultural lands (Patten *et al*., 1980; Hamscher *et al*., 2002; Hoper *et al*., 2002). Hamscher et al. (2002) analyzed the soil which is fertilized with liquid manure and the results of their study showed that in liquid manure the amount of tetracycline and chloro-tetracycline was 4.0 and 0.1 mg/kg respectively while in soil the concentration of these antibiotics were ranged between 86.2 µg/ kg (in the top soil (0–10 cm)) to as high as 171.7 µg/ kg (in the 20–30-cm layer).

2.3. Veterinary Antibiotics

Veterinary antibiotics are used to treat the infections cause by microorganisms or bacteria in animals. Therefore they are potentially harmful to other living species present in our environment (Warman, 1980). Amount of antibiotics present which cause toxicity in the environment are more toxic for the micro-algae, microorganisms, and bacteria living in the environment (Wollenberger *et al*., 2000). In recent few years, the studies were based on the effects of antibiotics on soil and aquatic organisms, and plant species under artificial controlled conditions in lab (Halling-Sørensen *et al*., 2003). Batchelder (1981) studied the impacts of Chlorotetracycline

and Oxytetracycline on plants of pinto bean which were grown in controlled environment and the results of the study demonstrated that low concentrations of antibiotic have negative impact the plant development and growth. When the plants were grown in the soil, the sensitivity varied among the plant species and it was noticed that pinto beans are more sensitive than edible radish. Migliore et al. (1995) studied the biological accumulation of sulfamethoxine in roots and shoots of different and the result of the study showed that, and bioaccumulation in the roots was often higher than shoots (Migliore *et al*., 1996). But, the concentrations used in this study are not found in the soil actually (Jjemba, 2002) therefore concentrations used of this kind of contaminants for such type of the studies should be realistic.

Reproductive effects and adverse impacts were investigate in different studies on starting stages of life of different living organisms present in the aquatic environment and it is shown that the presence of antibiotics in the environment are harmful for aquatic organisms (Kumpel et al., 2001).

2.4. Antibiotics In Wastewater

Wastewater is a worldwide issue and a major concern of most countries. Our water resources are polluted by several ways but the main reason of water pollution is discharging of untreated domestic and industrial wastewater in water bodies (Mashiatullah *et al*., 2005). Different types of pharmaceuticals and personal care products (PPCPs) are being in used since several decades. Antibiotics are one of the groups from PPCPs which is used to treat several bacterial infections in animals and humans and they are also used as growth promoter in animals. When these antibiotics are entered in the organism's body almost 25 to 75% of the active substance are excreted and the reason is incomplete metabolism and by this route they enter into the environment. These antibiotics are the getting attention of the scientist as a

wastewater pollutant and effects of these pollutants on environment and living organisms are still unknown (Ahmad *et al*., 2013).

After the recognition of antibiotics as a pollutant several studies had been done to determine the amount of antibiotics in wastewater thorough out the world. A study has been conducted by Karthikeyan and Meyer (2006) in USA to determine the the presence of antibiotics in wastewater treatment facility in Winsconsin. They screened 21 antibiotics compounds in the treatment facility. Their results showed that six major groups were found in the waste water that were sulfonamides, tetracycline, fluoroquinolones, macrolide and trimethoprim.

In 2009 a study by Wan et al. was conducted in India to investigate the presence of antibiotics in ground water, incoming safe water and hospital effluents in two hospitals of Ujjain district. They had concluded that the incoming safe water and ground water is free of antibiotics while hospital effluent contained a large no of antibiotics which includes metronidazole, norfloxacin, sulphamethazole, ceftriaxone, ofloxacin, ciprofloxacin, levofloxacin and tinidazole in range from 1.4µg/Lto 236.6 µg/L. They had concluded that presence of this large amount of antibiotics in hospital effluent may have serious implications on public health and environment.

Wei et al. (2012) determined in their study the occurrence of three antibiotics (Ciprofloxacin, Enrofloxacin and Florfenicol) in wastewater coming from animal sources and other water sources of in a city of eastern China. They monitored animal wastewater residue and surface water resources like river water and pond. The results of their study showed that the amount of Ciprofloxacin was upto 3.35, 5.30 and 2.10 µg/L in animal wastewater, river and pond water respectively. Similarly the concentration of Enrofloxacin ranged from 0.05 µg/L in pond water to 4.24 µg/L in

river water and concentration of Florfenicon ranged from 0.95 to 2.84 µg/L. their results showed that river water contained higher concentration of antibiotics which will ultimately effects the river ecosystem and will enter in the environment.

Antibiotics are damaging our environment but up to what extent it is still unknown. Different studies are conducted to determine the presence of antibiotics in wastewater and water resources. A study had been conducted by Harris and Cummins (2012) to predict the fate and effects of Ciprofloxacin after wastewater treatment. They found the concentration of Ciprofloxacin above the expected concentrations in wastewater treatment plant. They concluded in their study that hospital wastewater should not be mixed with municipal wastewater because the presence of such chemical compounds in wastewater makes treatment very difficult and residues of such compounds are big risk for the environment.

A study had been conducted in China by Chang et al. in 2012 to determine the presence of antibiotics in sewage coming from hospitals, nurseries and slaughter houses in the region of three Gorge reservoirs. The sampling was conducted from 4 hospitals, 1 nursery, 1 slaughter house and 1 wastewater treatment plant of the region. Six antibiotics were analyzed in this study in which floxacin in hospital was at highest concentration i.e ranged from 1.660 to 4.240 µg/L in all water environments. Second highest was Norfloxacin ranged from 1.36 to 1.62 µg/L, then Ciprofloxacin ranged from 0.011 to 0.136 µg/L in all samples. Trimethoprim was at lowest concentration ranged from 0.061 to 0.174 µg/L. According to current study the removal efficiencies treatment plants for antibiotics ranged from 80 to 100% which means they may enter in our environment and make it vulnerable for other living organisms.

In Lahore a study was conducted by Ahmad et al. (2013) to determine the role of untreated wastewater in spreading antibiotics and antibiotic resistant bacteria species in the main river of city i.e River Ravi. The study showed that many wastewater drains are emptying into this river in which untreated and treated wastewater is coming from whole city. They determined the concentration of two antibiotics in the river water i.e Ciprofloxacin (CIP) and Norfloxacin (NOR). The results showed that as the river flows downstream the amount of these antibiotics was increasing. The sensitivity test of experimental bacterial species showed that resistance of the species to CIP and NOR was increased. They had concluded that untreated wastewater from the city of Lahore is contaminating the River Ravi with antibiotics and antibiotic resistant bacteria which may cause severe human health problems if they consumed that water.

2.6. Antibiotic Resistance In Bacteria

Extensive use of antibiotics for humans and animals is increasing resistance in bacteria that's why use of such type drugs is increased and increase amount of antibiotics are entering in the environment. Other bacteria living in the environment serve as the reservoir of genes disease causing bacteria which are resistant to such drugs (Wegener *et al*., 1996; Al-Ahmad *et al*., 1999).

The pathway how antibiotic drugs helps in developing and maintaining the bacterial population which is resistant to such drugs, is still to be investigate in further studies. And also there is a hole of studies which describe the relation between antibiotic remains in environment and presence of resistant population of bacteria. Even a link between use of antibiotic and percentage of resistant bacteria is calculated at general, the threshold concentration of antibiotic is still unknown at which resistant bacteria

are produced (Nwosu, 2001). These resistant strains of bacteria could be transfer to the organisms either through direct exposure or via food chain and ultimately decrease the effects of antibiotics in human and animals (Richter *et al*., 1996). Work has to be done to find out the new pathways of exposure for transport of multidrug-resistant bacteria from animals to human beings (Chapin *et al*., 2005).

Resistance is produced in the bacteria of soil when agricultural land is continuous fertilized by the manure containing feces which is contaminated by lethal dose of antibiotics (Gavalchin and Katz, 1994). When pig slurry is stored experimentally it is revealed that some antibiotics like trimethoprim and chlortetracycline and their metabolites which are active are partially degraded (Grote *et al*., 2004). In June 2001, the limit for concentration of antibiotics was increased from 10 to 100 mg/kg by the Steering Committee of the Veterinary International Committee on Harmonization (VICH) even then this higher value is sometime crossed by some antibiotics like tetracycline when manure is continuously use for fertilization of soil (Hamscher *et al*., 2002). That is why the continuous use manure on agricultural land can cause serious contamination specially when antibiotics like tetacycline is present in the manure, it will accumulate in the environment. Different studies has already being done by scientists which showed that when plants are grown hydroponically they will uptake antimicrobial compounds (Schwake-Anduschus *et al*., 2003) and it is confirmed in latest researches (Grote *et al*., 2006). Banning of antibiotics which are used as growth promoters in animals is a precautionary step by European countries in 2006 because use of manure containing antibiotic in agricultural land could be a source of resistance in bacteria which are affecting humans (EC, 2003).

2.6.1. Resistance In The Aquatic Environment

In aquatic environment (Kümmerer, 2004; Kim and Aga, 2007; Schlüter *et al*., 2007; Watkinson *et al*., 2007; Caplin *et al*., 2008; Vanneste *et al*., 2008) and soil bacteria are found which are resistant to antimicrobial drugs (Schmidt and Römbke, 2008). On the other hand, there is proves the in environment antibiotic resistance is present in nature (Davison, 1999). In a study by Schlüter et al. (2007) it is concluded that when bacteria from different environments are isolated from animal, human and plant pathogens and others, they can share a single pool of resistance determinants which could be easily exchanged. In result of some studies it is concluded that transfer and selection of resistant bacteria could not be occurred in environment which have higher concentrations of antibiotic (Ohlsen *et al*., 1998, 2003).

2.7. Effects Of Antibiotic Resistant Bacteria On Human

Presence of antimicrobial drugs residue and resistant bacteria is due to use of animal manure in agriculture but humans are the bigger source of these contaminants than animals. Wastewater treatment plants are the main source of human antibiotic release in the environment because these antibiotics are not completely removed in the treatment process. This antibiotic resistance will be used in new methods to find out the source from where fecal pollution is coming by analyzing the bacteria (Kaspar *et al*., 1990; Wiggins, 1996; Pillai *et al*., 1997; Hagedorn *et al*., 1999). The conclusion of different studies showed that the effects of antibiotic resistant bacteria on humans is still unknown.

2.8. Effects Of Antibiotics On Plants

When the antibiotics are recognized as minor wastewater pollutant, different studies conducted to evaluate the toxic effects of many antibiotics on different species. These

species mostly include the aquatic species. The effects of these antibiotics are not very well documented. The plants are exposed to such compounds when they are fed with the wastewater containing these compounds. Animal manure and bio solids from wastewater treatment plant are also sources of these antibiotics, when they are used as fertilizer in agriculture (Hillis *et al*., 2011).

A study had been conducted by Liu et al. (2009) to evaluate the toxic effects of six antibiotics (chlorotetracycline, tetracycline, tylosin, sulfamethazole, sulfamethazine and trimethoprim) on selected plants species. By using rice, sweet oat and cucumber they checked the effect of these antibiotics on plant growth, soil microbial and enzyme activities. Rice was found the most sensitive plant species against antibiotics. The study revealed that these antibiotics had negative impacts on plant growth. The effects of these antibiotics vary from each other and also varied from species to species. They also concluded that antibiotics present in the soil and manure have adverse effects on the soil microbes and enzyme activities, which also ultimately may affect the plant growth or may be plant yield.

Tetracycline is an emerging pollutant in wastewater as it is being used in human and veterinary medicines and is entering into the environment by waste discharge of the animals and humans. When wheat was exposed to different concentrations of this antibiotic, there was impact on the physiological and genetics of plant. To determine the toxicity of tetracycline Xie et al. (2010) conducted a study. This study showed as the concentration of antibiotic was increasing the toxicity caused in plant body was also increasing either it is physiologically or genetically. From this study it was concluded that tetracycline contamination is a potential risk which may cause cytogenetic damage in living organisms. In another study, Xie et al. (2011), studied

the impacts of chlortetracycline on wheat plants and the results of the study were same as the previous study.

Different groups of antibiotics are present in which some are naturally occurring and some are synthetically produced in the pharmaceutical industries. Among these groups quinolone is an important group and when fluorine is added in the chemical structure of quinolone they formed fluoroquinolone. Quinolones and fluoroquinolones have great importance in the field of medicine as they are used to treat urinary tract and upper respiratory tract infections and they are also effective against the gram negative bacteria. When these antibiotics are present in the wastewater and this water is exposed to plants they have adverse effects on the plants. A study by Khadra and his co-workers showed that the antibiotics included in these groups have genotoxic effect on *Viciafaba* plant. They used nalidixic acid (quinolone) ciprofloxacin and levofloxacin (fluoroquinolones) to determine the genotoxicity in *Viciafaba*which is a model plant to study the toxicity. The results of this study showed that lower concentration of these antibiotics have no significant effects on the plant while the higher concentration of these antibiotics when exposed to the plants, they caused significant genotoxicity in the plant (Khadra *et al*., 2012).

A study was conducted by Eggen et al. (2011) determine the uptake of antibiotics and their translocation in plant body by using the forage and crop plants. They selected carrot and barley plant for the study. The results of their study showed that the RCF (root concentration factor) is higher than the LCF (leaf concentration factor). They found that all the selected pharmaceutical compounds exposed to plants have negative impact on plant growth especially on they have negative impact on developments of carrots. They concluded from their study that human pharmaceutical compounds might have effects on humans when they reached to them from crops via livestock. In

another study the impacts of ciprofloxacin (CIP) and oxytetracycline on a wetland plant named Phragmitesaustralis commonly known as reed, were determined by Liu et al. (2012). They found RCF more than LCF in this plant. They also found out that as the concentration of these products was increased in the water there are many negative impacts on many plant activities like superoxide dismutase and catalase activity.

2.9. Pathway Of Antibiotics In Plants

Many experiments has been done to determine the plant uptake with different designs which can be compared like pot experiments and spiked soil experiments at different concentrations of contaminants (Gao *et al*., 2005; Boxall *et al*., 2006; Dolliver *et al*., 2007; Åslund *et al*., 2008; Winker *et al*., 2010; Wu *et al*., 2010). In a study conducted by Trine et all in 2011, they determined the uptake of 3 antibiotics in forage crops, ciprofloxacin was one of them and they found out that Ciprofloxacin was on the second number to be uptake by plants. There is a growing public concern that antibioticsmay be taken up by food crops and make their way into food supply systems (Boxall *et al*, 2006). The bioaccumulation of chlortetracycline in cabbage, corn and green onion from manure amended soil ranged between 2–17 μg kg^{-1} fresh weights. However, tylosin was not absorbed by these crops, most probably due to the large size of the tylosin molecules.(Kumar *et al*, 2005). Boxall et al. (2006) conducted a study to evaluate plant uptake of 7 antibiotics by lettuce and carrots grown in a soil that was spiked at concentrations of 1mg kg^{-1}. Florofenicol and trimethoprim were detected in lettuce leaves and enoxacin, florfenicol and trimethoprim were detected in carrot root at concentration of 3–38 μg kg^{-1} fresh weight. Although the health implications of antibiotic residues in plants are not known, potential adverse health risks may exist.

Gentamicin is a relatively small molecule (molecular weight 477.6), a water-soluble aminoglycoside, used as a broad-spectrum antibiotic against Gram-positive and Gram-negative bacteria and pseudomonas. It is one of the few heat-stable antibiotics that remain active after autoclaving (Macdougall *et al*., 2007). Streptomycin is a large molecule (molecular weight 581.6), a water soluble aminoglycoside, used as a broad spectrum antibiotic in animals and humans and is also used as a pesticide to combat bacterial and some fungal diseases in plants. A major use is in the control of fire blight on apple and pear trees.

2.10. Toxicity Assessment Techniques

To assess the toxicity caused by different harmful chemicals many techniques are being used by the researchers. For example in plants to assess toxicity seed germination technique, comet assay, micronuclei assay, wheat bioassay and physiological parameters are commonly used.

2.10.1. Seed Germination Techniques

Jing et al. (2009) determined the effects of two personal care products (PCPs), i.e triclosan (TCS) and glaxolide on the seedlings of wheat. Effect of these PCPs were determined on seed germination, root and shoot length. The results of this study showed that both the PCPs have negative effects on the seed germination and as the concentration of the PCPs were increasing the seed germination of wheat is inhibiting. At lower concentrations of these PCPs seedling growth wasn't affected when the exposure time is less but for longer exposure the effects of both PCPs weren't negligible.

Hillis et al. (2011) assessed toxicity of ten antibiotics on three different plant species. In this study very low concentrations i.e. 3.9 µg/L to very high concentrations i.e.

10,000 µg/L of antibiotics were used. In this study when seeds of plants were exposed to different concentrations of antibiotics, no significant difference was noted even at higher concentrations**.**

Tetracycline is an important antibiotic which is being in used to control different bacterial infections in humans and animals worldwide. The extensive use of this antibiotic is also increasing its concentration in the environment. When the effects of this antibiotic on wheat were determined at concentration 0.5 to 300 mg/L by Xie et al. (2010) it was observed that lower concentrations i.e. 0.5 to 10 mg/L were stimulating the seed germination. The higher concentrations of tetracycline from 10 – 300 mg/L are significantly inhibiting the seed germination in concentration dependant manner.

2.10.2. Micronuclei Assay

Micronuclei assay is one of the most frequently used technique for genotoxicity assessment. According to a study micronucleus assay was used in 2000 studies in year 1992 and its number was increased up to 6000 in 2004 and in 2010 the number was increased to 13,000 which is very high as compared to other toxicity tests (Bolt *et al*, 2011).

Xie et al. (2011) studied the effects of tetracycline present in environment, in wheat. The genotoxic effects of tetracycline were investigated in this study and the concentration of antibiotic ranged from 0.25 – 300 mg/L. The results of the study showed that the lower concentration of tetracycline caused increased in mitotic index (MI). There was significant increase in MI when the concentrations were high and the results were concentration dependent. The study showed that tetracycline is an

environmental pollutant and when crop plants are exposed, it caused genotoxicity in them.

Yi et al. (2010) determined the effects of aluminum (Al) on *Vicia faba* by using micronucleus assay. When *Vicia faba* was exposed to different concentration of AlCl3 ranging from 0.01 – 10 mM for 12 hrs, it showed that number of micronucleleated cells were increased with the increase in concentration. The number of mitotic cells in all treated group was also dependent on the pH. It is concluded in the study that $AlCl_3$ is genotoxic for the *V.faba* and this plant is used as a model to assess the toxicity in plants.

Khadra et al. (2012) determined the effects of two groups of antibiotics i.e. Fluoroquinolones and quinolones on *V.faba* by using micronuclei assay. In this study, very low concentrations of different antibiotics were used. The results showed that very low concentration i.e. 0.001 and 0.005 µg/L of antibiotics had no significant genotoxic effect on *V.faba* but when the mixture of these antibiotics were exposed to plant, it induces significant micronuclei induction.

2.10.3. Physiological Parameters

To study the effects of environmental pollutant on plants, effects on physiological parameters are commonly determined. Liu et al. (2009) selected six antibiotics which are found in the environment and determined their effects on plants physiological parameters. They investigated the effects on roots and shoots and the result showed as the concentration of antibiotic was increasing the length of root and shoot was decreasing. The study concluded that the antibiotics residues in the environment had negative impacts on the plant growth.

Xie et al. (2011) determined the physiological effects of tetracycline which is an emerging environmental pollutant due to extensive use in human and veterinary medicines, on wheat. The results of the study showed that the lower concentration had positive effects on plant growth that it stimulated it but as the concentration is increased in the experiment it started inhibiting it.

Hillis et al. (2011) determined the effects of ten selected antibiotics on three plant species. They specially investigated the effects on physiological parameters like root and shoot length of plants. The study showed that the as the concentration of antibiotics were increased the root and shoot length were start decreasing. It was also shown in the study that roots are more sensitive end point to such pollutants.

2.10.4. Comet Assay

As the new chemicals are entering in the environment they are creating disturbance in natural ecosystem by causing toxicity. With the development of science many techniques are developed now to assess the toxicity. Comet Assay is one of the techniques which was first introduced by the Ostling and Johanson in 1984 and then with the passage of time this technique further modified for assessing the DNA damage in living cells. Now the Comet assay is recognized as the simple, sensitive and rapid tool widely for assessing DNA damage not only in eukaryotic cells but in some prokaryotic cells too (Dhawan *et al*., 2009) .

Gichner et al. (2006) determined the toxicity and DNA damage in two plants i.e. potato and tobacco grown on soil which was polluted with heavy metals. They determined DNA damage by using comet assay technique. The results showed that the plants grown on the polluted soil had significant increase in DNA damage as compared to the plants grown on control.

Genotoxic effects of Cu were determined in the wheat by Zhang et al. (2010). They used comet assay to determine the DNA damage in the plants. The results of the following study showed that the as the exposed concentration of Cu was increased the number of damaged cells were also increased. It was also shown in the study that roots had more toxicity then the shoots.

Türkoğlu (2012) determined the genotoxic effect of two environmental pollutant i.e. Chlorfenvinphos and fenbuconazole by using different toxicity assessment techniques including comet assay in *Allium cepa*. They exposed different concentration of these pollutants ranging from 10 to 100 ppm for 24 and 48 hrs. The result of the study showed that genotoxicity was increased with the increase in the concentration.

Chapter 3

MATERIALS AND METHOD

3.1. Materials

For the present study Ciprofloxacin (CIP) 250mg manufactured by Bayer Pakistan was purchased from local pharmacy. Sodium Hydroxide, Boric Acid. Tris Base and Ethylenediaminetetraacetic acid (EDTA), were obtained from ScharlauChemie (Spain). Formic acid, Methanol and HCl were obtained from Sigma Aldrich (Germany). Bacterial Culture of *P.aeruginosa* was grown in microbiology laboratory using standard protocols. Distilled water and all other chemicals used during all the experiments were used without further filtration and purification.

3.2. Methods

3.2.1. Preparation of Solutions

In this study different concentrations of antibiotic "Ciprofloxacin" were used. In order to prepare the different concentrations of antibiotic firstly the stock solution were prepared. For this purpose the commonly available medicine named Ciproxin (250 mg) was crushed to powder and added in 100mL distilled water in 250 mL volumetric flask. The flask was shaken well and 0.1 N HCl was added to attain the maximum solubility. Then the solution was filtered with Whattman No. 40 Filter paper and further diluted up to 250mL to prepare 1mg/ml concentration. Different concentrations of ciprofloxacin (CIP) were prepared using the stock solution by dilution. The concentrations of CIP used were 1, 10, 20, 30, 40 and 50 µg/L.

3.2.2. Seed Germination

The wheat seeds were obtained from National Agriculture Research Centre (NARC) Islamabad. The variety of wheat used for experimentation was Inqalaab-91. From the

collected seeds, healthy ones were separated. These seeds are then soaked in distilled water for 12 hours. Then they were placed on moist filter paper for 24 hours (Xie *et al*, 2009).

Figure 3.1: Seeds Soaked in distilled water and placed on filter paper for germination

3.2.3. Seed Germination Test

To determine the effects of the antibiotic on seed germination the seeds were thoroughly washed with tap water followed by distilled water 3 times. Then these seeds were soaked in distilled water for 12 hrs. Petri dishes were washed and rinsed with distilled water and dried at room temperature. Filter papers were placed in the dishes and moistened with different concentrations of antibiotic while the control was treated with distilled water. About 15 seeds were placed on each Petri dish and were placed at room temperature. Three replicates for each concentration were used. Germinated seeds were considered to have 2 mm roots after 24 hours. After 24 hours the number of seeds germinated in each Petri dish, were counted and by using percentage formula the effect of CIP on seed germination of wheat was determined.

$$\%\ \boldsymbol{Seed\ Germination} = \frac{\boldsymbol{No.of\ Seeds\ Germinated}}{\boldsymbol{Total\ No.of\ Seeds}}$$

3.2.4. Antibiotic Exposure

When the roots of the plants were almost 2 mm in length the seedlings were exposed to different concentrations of the antibiotic. After repeated experiments the amount of antibiotic solution fed to the plants was optimized and that was 3mL/day.

3.2.5. Growth Parameters

The effects of CIP on plant growth parameters like plant biomass, root and shoot length were determined. To determine the effects on roots and shoots length, 24, 48 and 72 hours of exposure were considered.

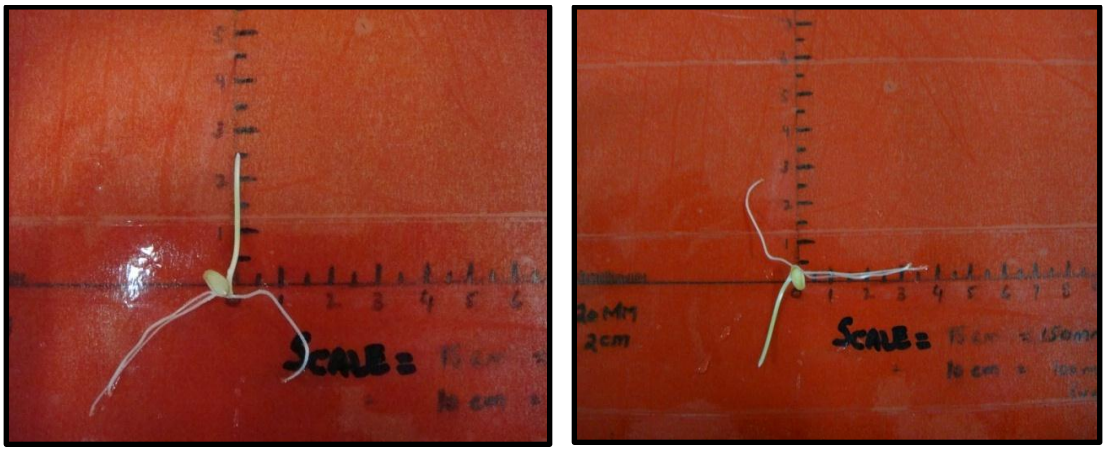

Figure 3.2: Measuring the root and shoot length

By using the following formula the percent decrease in total plant length after 24, 48 and 72 hours was determined w.r.t. control.

$$\%\ \boldsymbol{Decreaseinplantlength} = \frac{\boldsymbol{Initiallength - Finallength}}{\boldsymbol{Finallength}}\boldsymbol{X100}$$

To determine the effects on biomass, the roots and shoots were sampled separately and these were then oven dried at 60^0C for 2-3 hours to remove all the moisture and were weighed using weighing balance.

3.2.6. Toxicity Assessment

For toxicity assessment the comet assay technique was used at Institute of Environmental Sciences and Engineering (IESE) Environmental Microbiology & Biotechnology Research laboratory. Comet Assay is a technique used to detect the breaks in DNA. Type of comet assay used in this study was Alkaline Comet Assay which is comprised of series of steps.

3.2.6.1. Preparation of Reagents

Following reagents were prepared to perform comet assay.

Phosphate Buffer Saline(PBS)

PBS or Phosphate buffer saline was prepared by dissolving a tablet in 100 ml distilled water and it was autoclaved at 110^0C for 15 mins. pH of the solution was maintained at 7.4.

Agarose

I. Normal Melting Agarose

   1% Normal Melting Agarose (NMA) was prepared by dissolving 0.1 g of powder agarose in 10 ml PBS solution and it was placed in water bath for 10– 15 mins at 90^0C temperature.

II. Low Melting Agarose

   2% Low Melting Agarose (LMA) was prepared in same manner as NMA was prepared.

Alkaline Buffer Solution

Stock solution of 10N NaOH and 200 mmole EDTA were prepared. To prepare alkaline buffer solution 30 ml of 10N NaOH and 5 ml of 200 mmole EDTA were taken in 1000 ml flask and make the volume up to the mark with distilled water.

Neutralizing Buffer

Neutralizing buffer was prepared for comet assay. For this purpose 0.4 M Tris base was prepared by dissolving 6.06 g of the base in 100 ml distilled water. The pH of the solution was maintained at 7.5 by using HCl.

Staining Solution

For the staining of slides solution of Ethidium Bromide was prepared by dissolving 10 mg of EtBr in 50 ml distilled water. To avoid exposure to fluorescent light, it was stored in dark condition at room temperature.

3.2.6.2. Comet Assay Procedure

1. Slides Preparation

   For Comet Assay double concave microscope slides (Cat.NO.7104) were used. Slides were dipped in 70% ethanol and burnt on blue flame to disinfect and remove all the dust and oil particles.

2. Slides Pre-coating

   Slides were then coated with 1% NMA and dried at room temperature in flat tray.

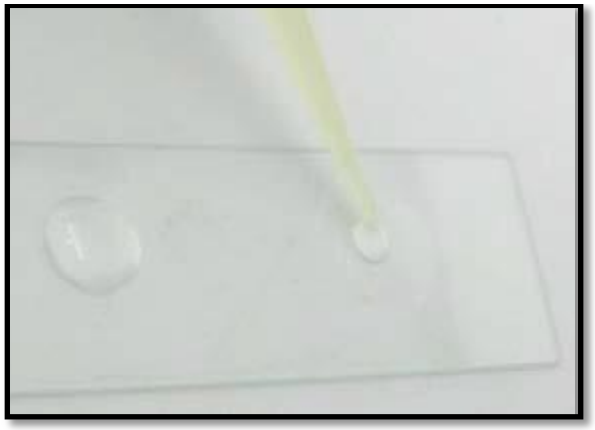

Figure 3.3: Slides Pre-coating

3. Isolation of Plant Nuclei

   Roots of plants (exposedwith different concentrations of CIP and control) were taken and washed thoroughly with tap water, followed by distilled water. For isolation of nuclei 320 µL of 0.4MTris Base were used in Petri dish and placed on ice. The roots were dipped in the solution and gently sliced with the help of sharp blade. The dish is kept tilted to collect all the separated nuclei.

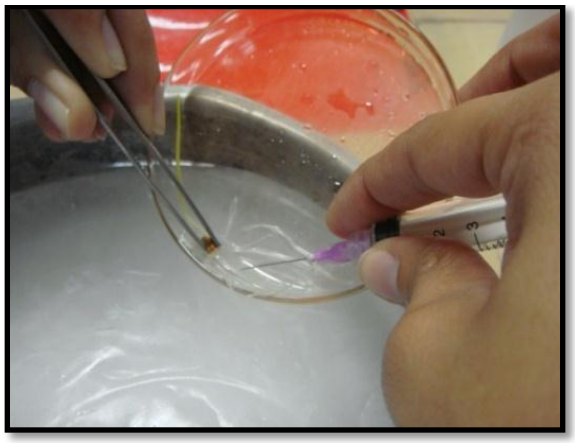

Figure 3.4: Isolation of Nuclei

4. Sample Pouring

   On pre-coated slides 65 µl of LMA were poured and 10 µl of separated nuclei sample was added and dried at room temperature.

5. Layering

After drying of slides another layer of 2% LMA (80 µl) was added. This layer should be smooth and slides were kept at room temperature for drying.

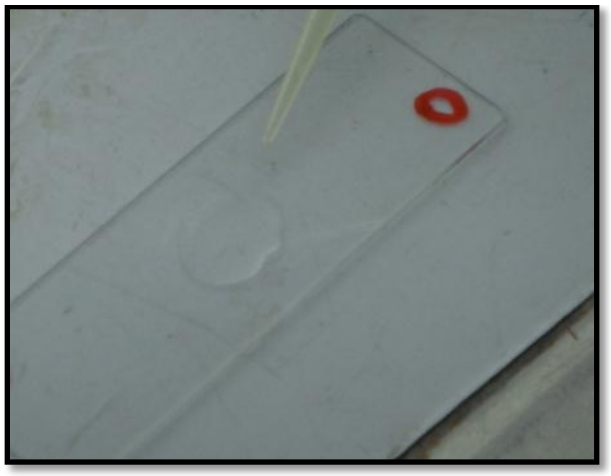

Figure 3.5: Layering of 2% LMA

6. DNA Unwinding

Dried slides were then kept in alkaline buffer for DNA unwinding. The time required for this step is 15-20 mins at room temperature.

Figure 3.6: DNA Unwinding

7. Electrophoresis

After DNA unwinding electrophoresis was done by using alkaline solution. Prepared slides were gently placed in horizontal gel electrophoresis tank

(MUPID PNE. NO. 07912) and the tank was filled with the solution. Electrophoresis of the comet slides was done at 25V for 30 mins.

Figure 3.7: Electrophoresis

8. Neutralizing

After electrophoresis the slides were removed carefully from the tank and rinsed with the neutralizing buffer. This step is repeated 3-4 times to remove all the background interferences and air dried over night.

Figure 3.8: Neutralizing

9. Staining

After neutralizing the slides were stained with 50 µL EtBr stained. The slides were stained twice at room temperature for better results.

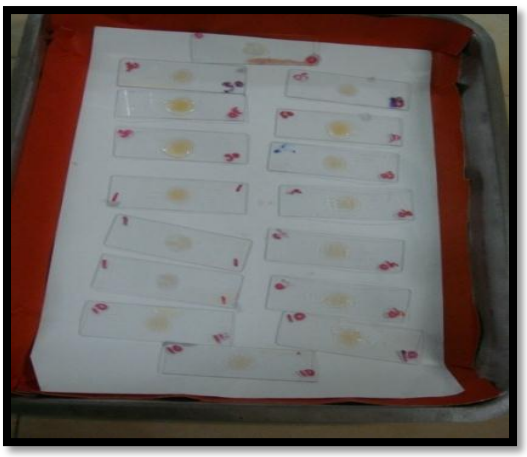

Figure 3.9: Stained slides with EtBr

10. Drying

    After staining the slides were dried in oven at 40^0C for 30 mins to remove all the moisture.

11. Visual Analysis

    After drying the comet slides were then visualized under Trinocular Fluorescent Microscope (Optika B353FL) using 100x objective. An ocular micrometer of 10µm, camera (Aiptek: AED-Z600) and white LED/12V 20W illuminator was equipped with microscope and by using these, the tail length was measured and images were taken.

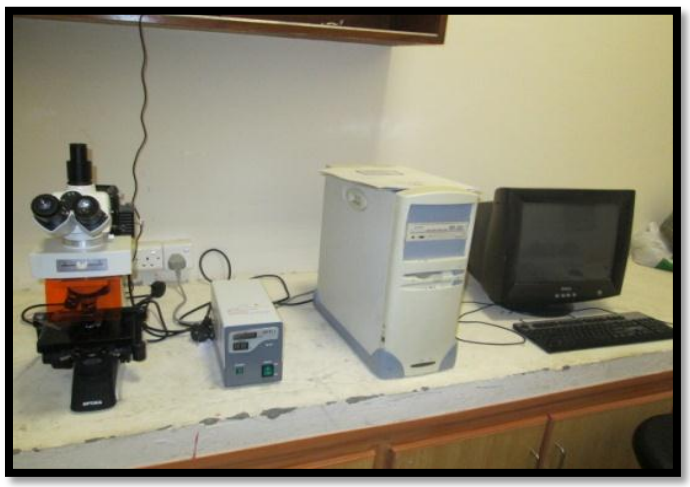

Figure 3.10: Fluorescent Microscope for Visual Analysis

12. Data Analysis

Slides were properly labelled and visualized under the microscope. About 50 random cells were selected per slide and 3 replicate slides were prepared for each sample. Undamaged cells were shaped spherical while damaged cell produced comet like tail. By visual analysis numbers of damaged and undamaged cells were counted on each slide and tail lengths of damaged cell were calculated according to given formula (Heepchantree et al; 2006):

Comet tail length (µm) = Tail length – Head diameter

To determine the genotoxicity using comet assay the number of damaged cells were counted. By using the percentage formula, percent of damaged cell for each concentration were calculated.

$$\%\ \boldsymbol{Damagecells} = \frac{\boldsymbol{No.damagedcells}}{\boldsymbol{Totalno.ofcellscounted}} \boldsymbol{X\ 100}$$

13. Statistical Analysis

All the data collected from the comet slides were then analyzed by using excel worksheet and the relation between concentration of antibiotic and genotoxicity on plant were determined.

3.2.7. Detection of CIP in Plants

To determine the plant uptake in this study, 0.1 g roots and shoots were taken from the experimental plants. They are washed thoroughly with distilled water and crushed with the help of mortar and pestle. 0.2mL of 0.01M HCl-CH_3OH (60 + 40) was added in the glass tube containing grinded roots or shoots. 1.7 mL of ammonium acetate – formic acid (90 + 10) were further added in the glass tube and shaken well. Solution

was then transferred into eppendorf and centrifuged at 3000 rpm for 5 mins. After that supernatant was transferred into another eppendorf and further centrifuged at 10,000 rpm for another 5 mins. To determine the presence of CIP in the plant roots and shoots Kirby Baeur test was used. To perform this test bacterial species *Pseudomonas aeruginosa* was used. Autoclaved discs were dipped in the plant extract and placed on the petri dish containing *P. Aeruginos*streaking. Petri dishes were incubated for 24 hours at 37^0C.

Chapter 4

RESULTS AND DISCUSSION

Antibiotics have great importance in our daily life as they are used to treat bacterial infections in humans and animals. Due to vast use of antibiotics they are entering in our environment either during productions or during consumption by humans and animals, as these are excreted from their body. In wastewater different concentrations of antibiotics were found in various studies. When plants or crop fields are irrigated with this wastewater, the antibiotics get entered to the plant body and accumulate there. Keeping this in view, current study was planned to determine the effects of antibiotic Ciprofloxacin (CIP) on *Triticumaestivum* (wheat). For this purpose seed germination, plant physical growth parameters, presence of antibiotic in plants ad DNA damage caused by CIP were determined.

4.1. Seed Germination

To determine the effects of CIP on seed germination seeds were exposed to different concentrations of CIP i.e. 1, 10, 20, 30, 40, 50 µg/L and control was treated with distilled water. The result showed that CIP had no effects on seed germination even at higher concentration of antibiotic seed germination rate was not affected. The results are illustrated the figure 4.1. Hillis et al. (2010) determined the effects of ten antibiotics (amoxicillin, chlortetracycline, levofloxacin, lincomycin, oxytetracycline, sulfamethazine, sulfamethazole, tetracycline, trimethoprim and tylosin) on seed germination of three plant species i.e. Lettuce, alfalfa and carrot. They concluded that seed germination is insensitive to antibiotics even at higher concentrations.

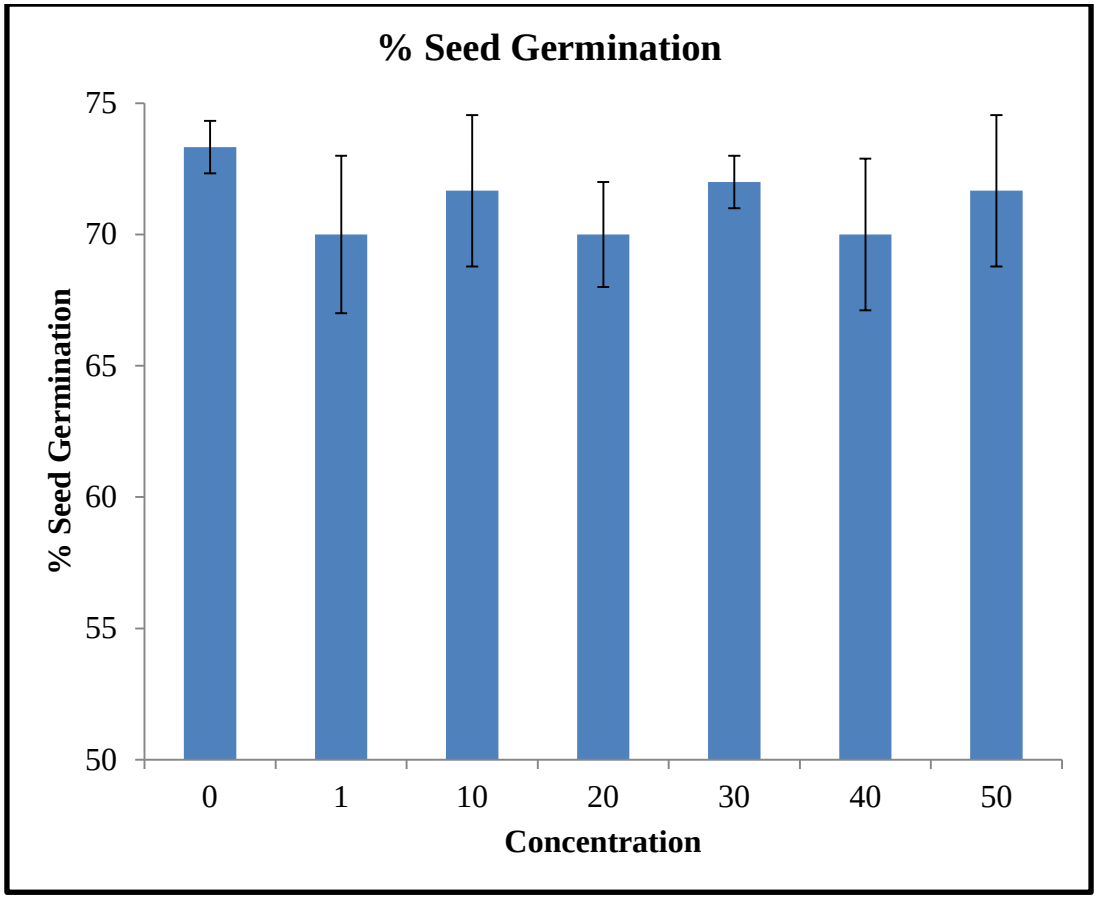

Figure 4.1: Effects of different concentration of CIP on seed germination
*** 0 conc. shows control group**

4.2. Physical Growth Parameters

Physical growth parameters like plant's biomass, root and shoot length were analyzed after plants exposure to CIP. The effects of antibiotic on various growth parameters were as follows.

4.2.1. Plant's Biomass

Results indicating the effects of CIP on biomass of plant are displayed in Figure 4.2. Exposure of different concentrations of antibiotic to *T. aestivum* affected its total biomass. The result showed that in comparison with control the total biomass of plants was not affected by antibiotic after exposure of 24 hours. It was observed that there was a significant decrease in plant's biomass as the exposure time was increased

to 48 and 72 hours and the results were dependent on exposed concentration i.e. plant's biomass decreased with increased in concentration as shown in Figure 4.2.

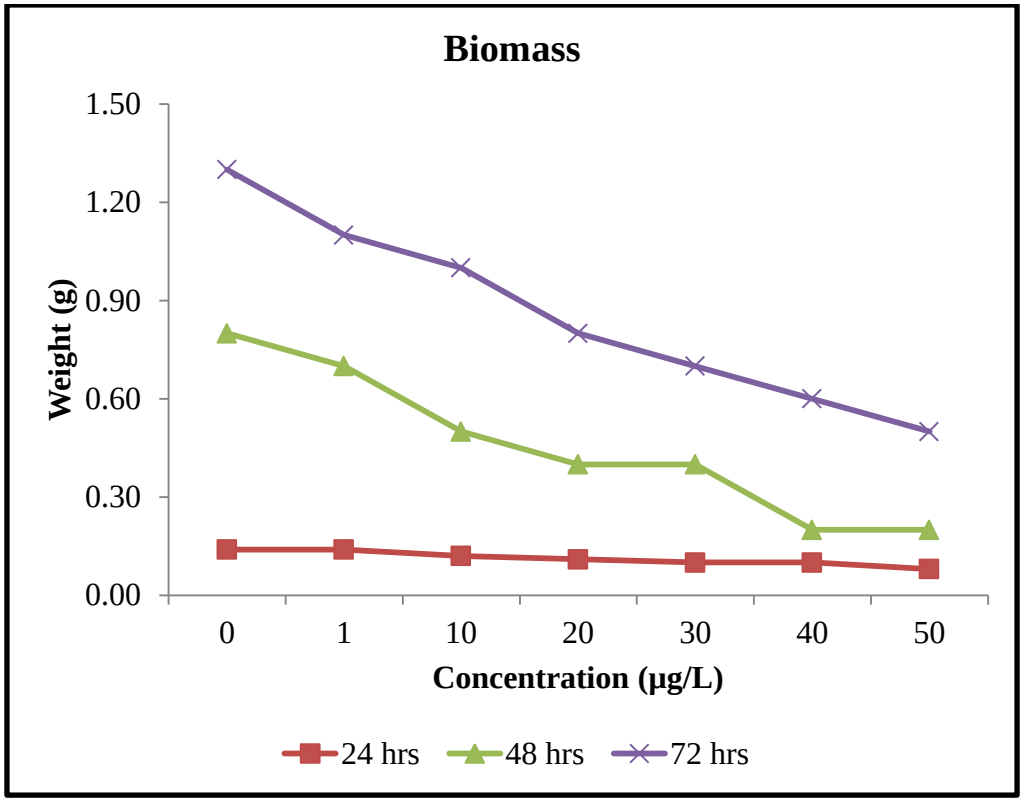

Figure 4.2: Effects of CIP concentration on plant's Biomass after exposure of 24, 48 and 72 hours

4.2.2. Root, Shoot and Total Plant Length

Effects of CIP concentrations on roots, shoot and plant length were determined in the present study. Figure 4.3 shows the results of effects of antibiotic and exposed time on total plant length. The results showed that as the exposure time and concentration were increased the total plant length was decreased. It was observed that after 24 hours of exposure the percent decrease at highest concentration of CIP was 31%. After 48 and 72 hours of exposure it was 42% and 56% respectively, which means exposure time of plants with increase in concentration of antibiotic have negative impact on total plant length.

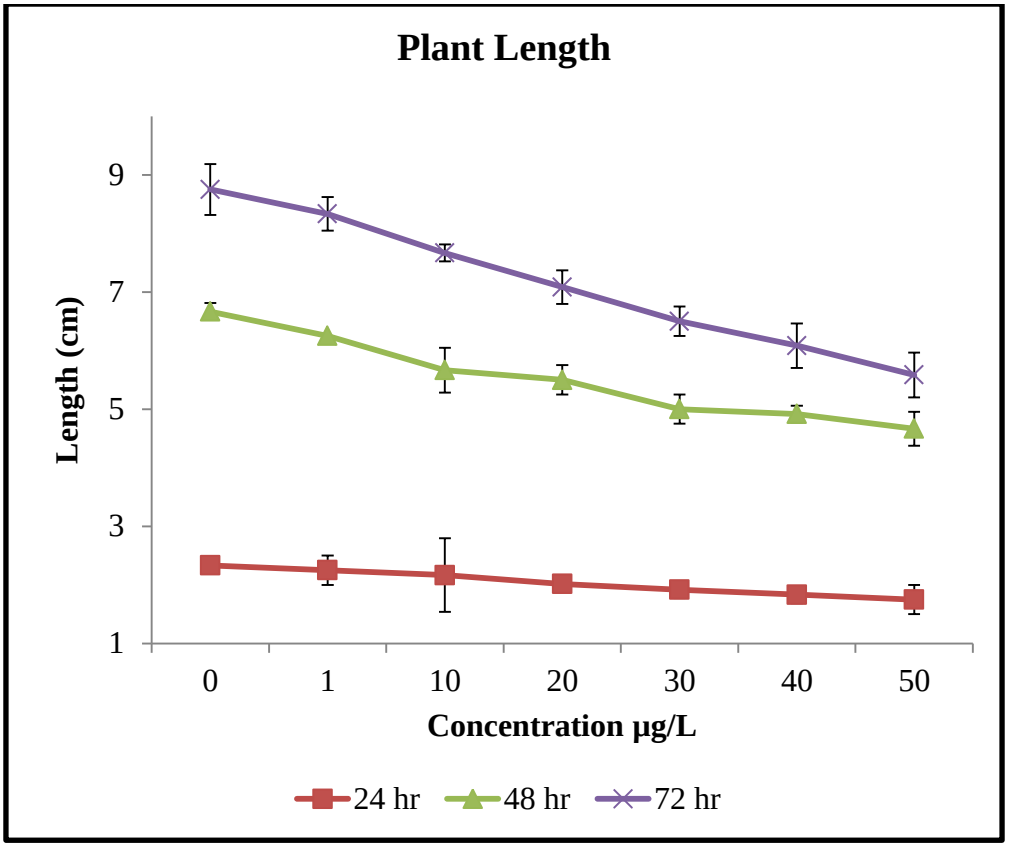

Figure 4.3: Effects of CIP and exposure time on total plant length

The effects of CIP on shoot and root length have been shown in Figure 4.4 and 4.5 respectively. It is observed that roots were more sensitive to increase in concentration of CIP and exposure time as compared to the shoots. As the time and concentration exposed increased the decrease in root length were 40, 60 and 62 % w.r.t. control after 24, 48 and 72 hours respectively. While in shoots it was 40% after 24 hours and 13 and 20% after 48 and 72 hours respectively. A study by Hillis et al. (2010) also showed the same results that roots are more sensitive than the shoots when different species of plants were exposed to antibiotics at different concentrations.

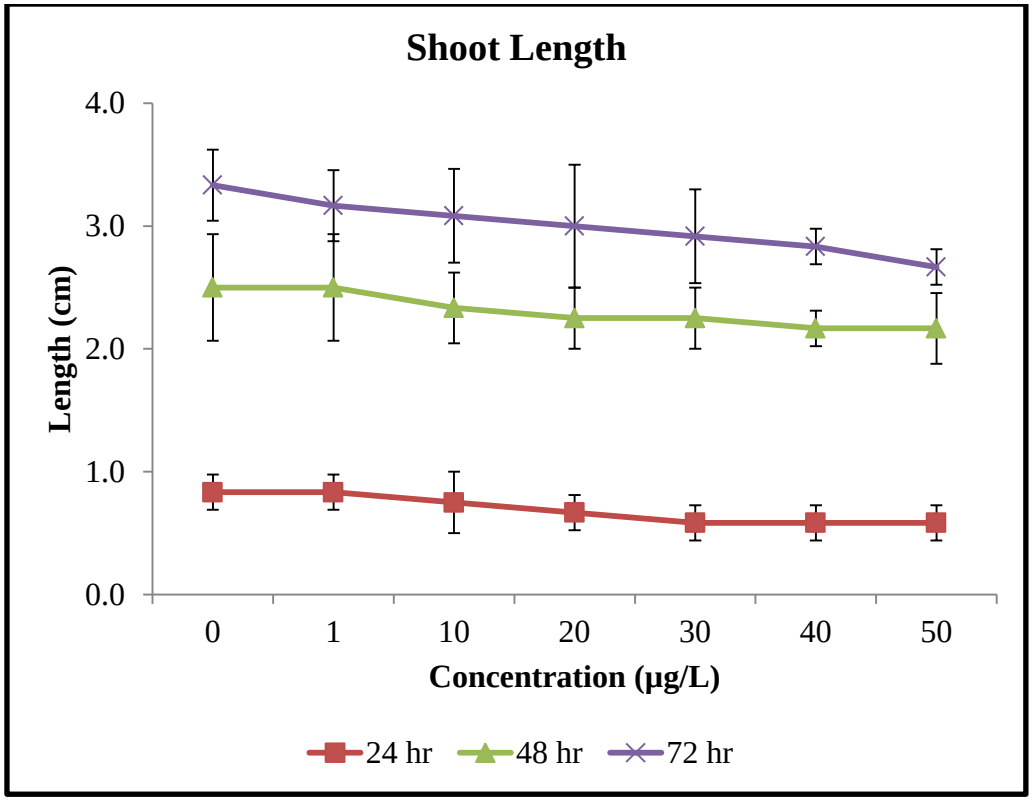

Figure 4.4: Effects on Shoot Length

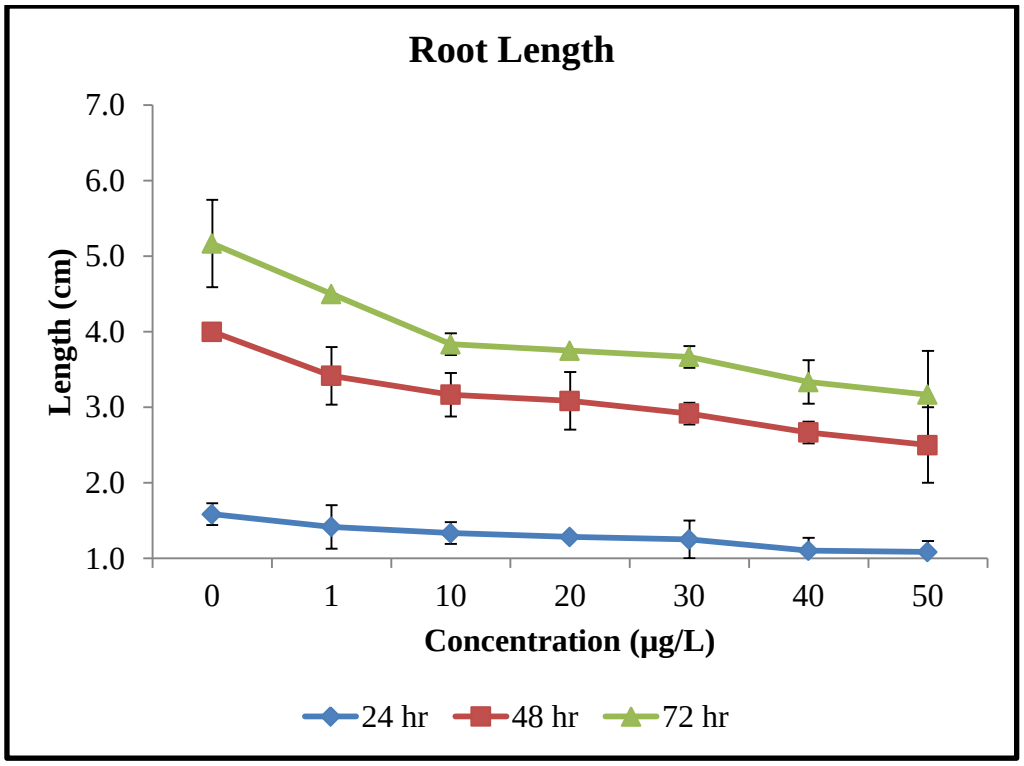

Figure 4.5: Effects on Root Length

4.3. Toxicity Assessment

4.3.1. Optimization of Comet Assay for Plants

For the genotoxicity assessment in *T. aestivum* caused by CIP, comet assay was used at Environmental Microbiology & Biotechnology laboratory, Institute of Environmental Sciences and Engineering. For this purpose conditions were optimized for plants. Standard procedure (Gichner *et al.*, 2009) was used with some modifications after many trials, as described in materials and methods. The modifications made in the procedure were as follow:

Table 4.1: Comparison of Standard procedure (Gichner et al., 2009) and Modified Procedure of Comet Assay

Standard Procedure (Gichner et al., 2009)	Modified procedure
1% LMA was used	2% LMA was used
LMA & NMA prepared in dist. H_2O	LMA and NMA is prepared in PBS
DNA unwinding was done for 5 min	DNA unwinding was done for 15 min
Electrophoresis was done for 15 min	Electrophoresis was done for 45 min
Concentration of EtBr used was 20 µg/L	Concentration of EtBr used was 200 µg/L

For the toxicity assessment in the wheat by comet assay two parameters i.e. average tail length and % damaged cells were selected (Zhang et al., 2010).

4.3.2. No. of Damaged Cells

The results of genotoxicity assessment by number of damaged cells are illustrated in Figure 4.6. It was observed that percent damaged cells increased in exposed roots of plants as compared to control. It was also analyzed in the result that percentage of damaged cells was increased with increase in exposed concentration of CIP. Percent damaged cells were also dependent on exposure time of plant's roots. These results showed that CIP is causing genotoxicity in roots of the exposed plants. Zhang et al., (2010) studied the same results when they exposed the *T. aestivum* plant to different concentrations of copper (Cu).

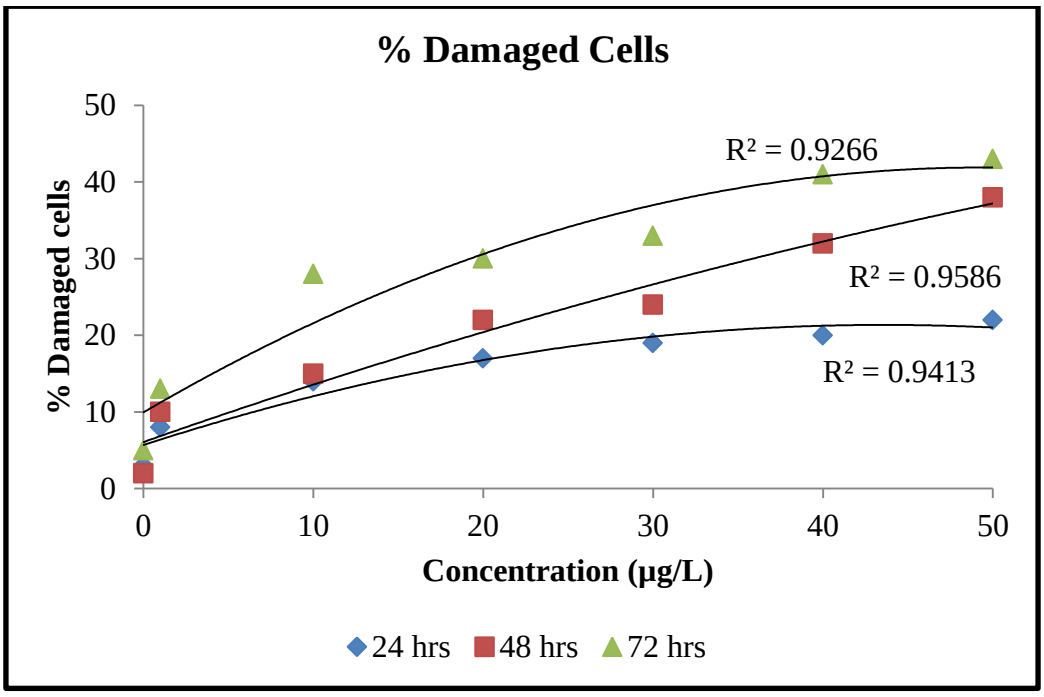

Figure 4.6: % Damaged cell count against each concentration of CIP and exposed time

4.3.3. Average Tail Length

Figure 4.9 shows the average tail length produced against different concentrations at different exposure time.To determine the genotoxicity the average tail length was

calculated for each concentration of CIP exposed to the plants. After 24 hours of exposure the tail length increased with increase in the concentration of CIP and as the exposure time increased to 48 and 72 hours the average tail length also increased. This suggests that antibiotic is causing genotoxicity and exposure time is also mediating it.

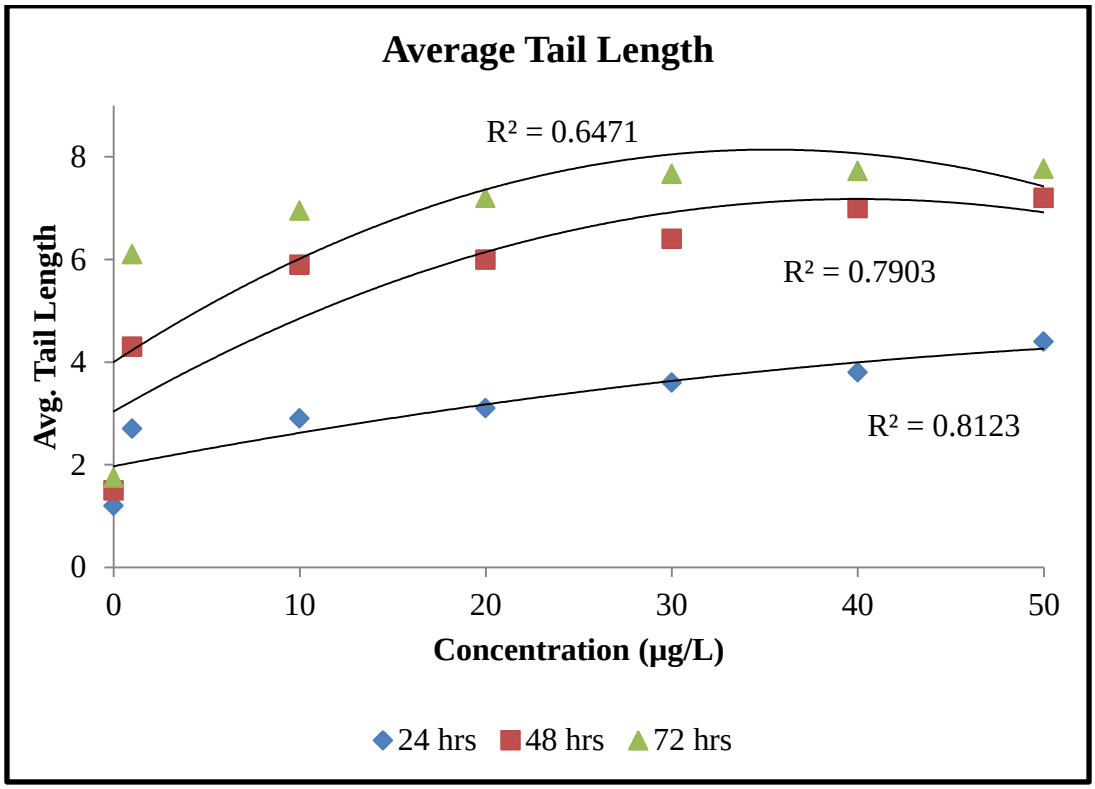

Figure 4.7: Average tail length produced by damaged cells

4.4. Uptake Of Antibiotic By Plant

To determine the uptake of CIP by the plant extract (section 3.2.5) was used for the Kirby Baure test which is performed to check the activity or presence of antibiotic against the bacteria. Figure 4.8 (a, b, c, d) is showing the results of the experiment. The results showedthat zone of inhibition (where there is no bacterial growth is present) were formed against all concentrations including control. But as the concentration of antibiotic was increased the area under zone of inhibition was also

increased. This result showed that chemicals used for the extraction of CIP from plant inhibit the bacterial growth but the area under zone of inhibition increased when exposed concentration of antibiotic was increased which showed the presence of antibiotic in plant. These results show that the plants will uptake the antibiotic when they are exposed to them. A study by Eggen et al., 2011 showed the same results when they analyzed the plant extract with LC-MS.

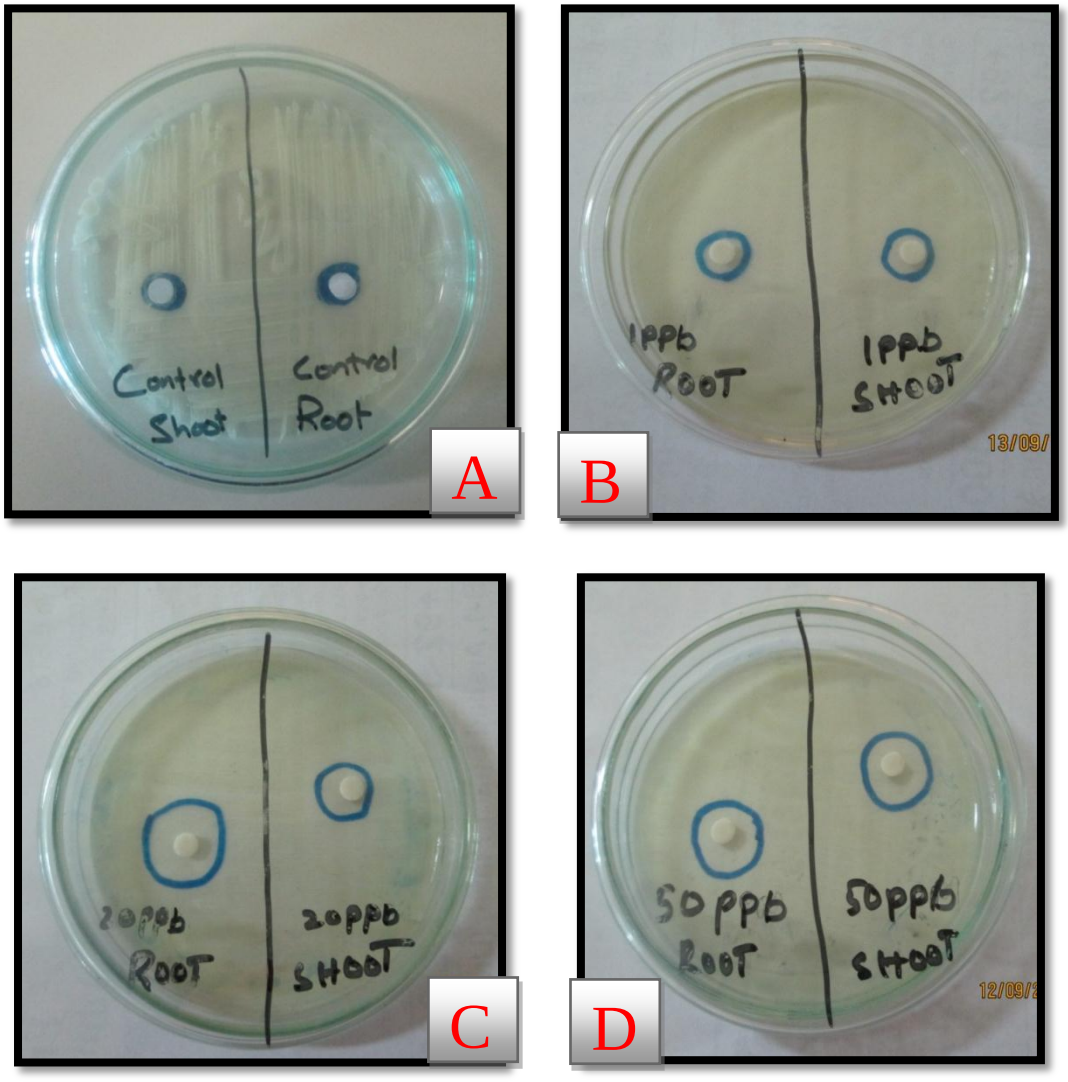

Figure 4.8: Zone of inhibition for different concentrations (A) Control, (B) Conc. 1µg/L (C) Conc. 20µg/L (D) Conc. 50µg/L

Chapter 5

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Antibiotics are being in use since decades for treatment of human and animal bacterial infections. They are constantly entering in to the environment through the excreta of these living organisms and are causing problems for the environment. Present study determined the toxic effects of antibiotic Ciprofloxacin on the *T. aestivum* plants, which is an important agricultural crop and is the main food source in most of the countries of the world. The findings of the present study are as follow:

- Antibiotic CIP did not inhibit the seed germination even at higher concentrations so its presence can never be detected using this parameter.
- CIP inhibits the growth of roots and shoots of the plant and exposure to antibiotic decrease the biomass of plant.
- As the exposure time of plant to CIP increased, it decreased roots, shoots and plant length and plant's biomass.
- CIP induced genotoxicity in the roots of *T. aestivum* which was further augmented with increase in the concentration and exposure time.

5.2. Recommendations

From the present study following recommendations are made:

1. Effects of these antibiotics should be studied on soil microflora and rhizoshpere activities.
2. Effects of antibiotics on other important crops should be studied.
3. Wastewater should be treated before reuse for agricultural crops.
4. Environmental standards should be devised and legislations made for such type of pollutants to protect our environment.

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