GROWTH RESPONSE AND TOXICITY INDUCED IN WHEAT IRRIGATED WITH ANTIBIOTIC CONTAINING WATER

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By

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CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Growth Response and Toxicity Induced in Wheat Irrigated with Antibiotic Containing Water"** submitted by Ms. Hira Imam Jamil has been found satisfactory for partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

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I dedicate this thesis to my beloved parents and siblings who would always be a source of inspiration for me and stood beside me at every moment in my life


بسم الله الرحمن الرحيم

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Hira Imam Jamil

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LIST OF ABBREVIATIONS

Cip	Ciprofloxacin
Lev	Levofloxacin
Amox	Amoxicillin
Amp	Ampicillin
Oflox	Ofloxacin
FQs	Fluoroquinolones
OTC	Oxytetracycline
CTC	Chlortetracycline
RCF	Root Concentration Factor
LCF	Leaf Concentration Factor
PCPs	Personal Care Products
MN assay	Micronucleus Assay
MI	Mitotic Index

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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 changes in physical growth parameters of plant and composition of its vegetative parts and to assess DNA damage in wheat plant through micronuclei assay. Five different antibiotics were selected namely ciprofloxacin, levofloxacin, amoxicillin, ampicillin and ofloxacin. Antibiotic levels used were 2, 4, 6, 8 and 10 mg L^{-1} and for comparison the control was treated with distilled water only. Physical growth parameters like germination, plant's biomass, root and shoot length were determined after exposure to different concentrations of all the antibiotics. Genotoxicity was assessed using MN assay while vegetative composition of plants was analyzed through quantification of total phosphorus, iron, carbohydrate and protein concentration in plant. Germination of seeds was inhibited up to 20% upon applying 10 mg L^{-1} concentration of ciprofloxacin, levofloxacin, amoxicillin, ampicillin and ofloxacin. The concentration of total phosphorus, carbohydrate and protein declined significantly at 10 mg L^{-1}. Iron's concentration was slightly inhibited by all the five antibiotics. The results of MN assay indicated that number of micronuclei produced was increased with increasing concentrations of antibiotics. At the highest antibiotic concentration, i.e. 10 mg L^{-1} micronuclei (MN) produced were in the following order: ampicillin> ofloxacin> amoxicillin> levofloxacin> ciprofloxacin. The significant differences were observed at 10 mg L^{-1} (46 MN per 100 cells) and 8 mg L^{-1} (34 MN per 100 cells) of ampicillin and at 10 mg L^{-1} (33 MN per 100 cells) of ofloxacin. Formation of MN upon application of antibiotics highlighted the potential toxicity in addition to reduction in growth. Above mentioned results warrant about the potential negative effects of the presence of antibiotics in wastewater and stress on plants declining the productivity and quality of the product.

Chapter 1

INTRODUCTION

1.1 Background

At global scale, apart from human medicine, large amount of antibiotics are used annually as veterinary drugs and feed additives with the increasing growth of aquaculture and livestock industry (Du and Liu, 2012). All such applications have made possible the discharge of antibiotics in larger quantities into natural ecosystems. As a result of human activities and release of wastewater effluents into the environment, antibiotics could be found both in aquatic and agricultural systems. After consumption, these antibiotics are released in to nearby streams, rivers and agricultural lands through medical wastewater, industrial and sewage effluents and animal waste in their bioactive forms (Kim and Agha, 2007; Kummerer, 2009). The continual introduction of low concentration of these drugs from different sources makes them pseudo-persistent (Carvalho et al. 2014). This wastewater containing antibiotic drugs and other chemicals is applied onto agricultural lands without any pre-treatment or after treatment in wastewater treatment plants in many regions of the world (Fatta-Kassinos et al., 2011). These chemicals are not degraded or removed fully even through commonly used wastewater treatment methods (Grassi et al., 2013).

A lot of research has been done on the effects of antibiotics on humans, animals, aquatic organisms, arable land and the other ecological risks, but the effect of these chemicals on plants and environment are not well documented (Halling-Sorensen et al., 1998). The existence of wide range of antibiotics at low concentrations in source water proposes that net effect of antibiotics as environmental contaminant should not be overlooked (Tandon et al., 2013).

1.2 Antibiotics

With the discovery of first antibiotic i.e. Penicillin, by Alexander Fleming, many new doorways were opened up in sector of health and medicine. Antibiotics are the consequence of growing innovations in the health department and their use has transformed the pattern of modern day life. Since the discovery, antibiotics have been used as the therapeutic drugs that can be utilized in the treatment and prevention of various infections. Their commercial value is increasing at an unprecedented pace. Apart from their human and veterinary use, their utility has also been proved in many other fields (Gothwal and Shashidhar, 2015).

Antibiotics are chemicals manufactured artificially or semi-artificially that inhibit microorganisms from growing (Thiele-Bruhn, 2003). There are different classes of antibiotics that can be differentiated on the basis of their chemical structure, mode of action and pathway of administration (Gothwal and Shashidhar, 2015).

1.3 Fluoroquinolones and penicillins

Fluoroquinolone is a class of antibiotics that stays in environment for longer time periods due to slow degradation process and strong adsorption on to soil (Tandon et al., 2013). Hospitals, households and veterinary applications are the major sources of broad spectrum FQs. These are of interest being the third largest class among antibiotics and holding 17% of market share globally (Doorslaer et al., 2014). Upto 70% of FQs are egested in their non-metabolic form that can build grounds for microbial resistance in the environment.

On the other hand, Penicillins having antimicrobial characteristics due to the β-lactam ring, are also widely used drugs in human and livestock medicine. Many studies have reported the presence of antibiotics of this group e.g. ampicillin and amoxicillin in wastewater (Elmolla and Chaudhuri, 2010). These are manufactured by chemical synthesis or modification of the original

compound. β-lactam antibiotics along with the other subgroups of penicillins contribute to the largest market share in human used medicine in most of the countries i.e. accounting for roughly 50 to 70 percent of total use of antibiotics (Kummerer, 2009).

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

1.4 Use of antibiotics in different fields

Antibiotics are used in enormous amounts to treat various infections in humans, animals and aquaculture. At a global scale, the data on the consumption rate of antibiotics is limited and heterogeneous. Usage patterns also vary considerably in different regions and countries, for example, streptomycin is used widely in USA for growing fruits, however it is illegal to use it in Germany for the same purpose (Kümmerer, 2008). Approximately 100,000 to 200,000 tons of antibiotics are consumed annually in the world (Wise, 2002).

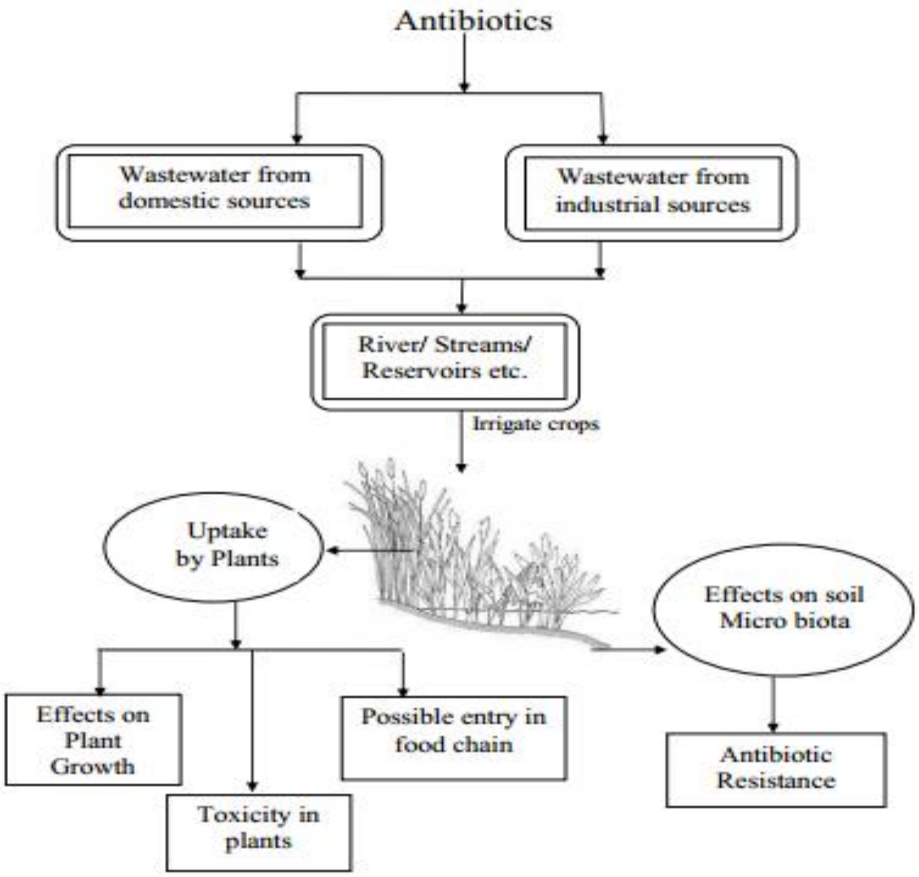


Figure 1.1: Illustration of routes of antibiotics and their possible effects (Adopted from Tandon et al., 2013).

1.4.1 Use of antibiotics in human medicine

The amount of antibiotics consumed by each person or all humans in total differs from country to country. Intake with or without prescription also varies considerably between markets of different countries (Molstad et al., 2002). The usage rates also differ even for a single compound. After β-lactam antibiotics, which is sub-group of penicillins, sulphonamides, macrolides and fluoroquinolones are among mostly used antibiotics in most of the countries. Contrary to the general view, the major source of pharmaceuticals in municipal waste water are not the hospitals but community itself i.e. only 5 to 20% of antibiotics are released into environment by European hospitals. Whereas 70 and 75% by community in UK and US (Wise, 2002). These compounds are metabolized in the liver. Often times, the metabolites of such

compounds are more soluble in water in comparison with the parent compounds and get excreted with urine. Sometimes metabolite formation leads to the compounds that are dangerously toxic to human health (Kummerer, 2009).

1.4.2 Use of antibiotics for animals and agricultural crops

Only rough estimates are available internationally that represent the antibiotic use in agri-food sector, however the true and actual values are unknown. The volume of antimicrobials consumed by animals as a prevention, cure or therapy is usually established by advance animal breeding methods (Gaskins et al., 2002). These are used in lower amounts as a growth promoter for animals and also for a better final product i.e. with less fat and more protein content in meat (Cromwell, 2002).

In USA, of the overall antibiotic use, less than 0.5 percent are applied on plants (McManus et al., 2002). These antibiotics are applied to prevent bacterial attacks, on high-value tree fruits, other food and ornamental plants. On one hand, these drugs promote the plant tolerance towards extreme temperatures, rainfall and other similar factors and on the other such properties pose great havocs to the environment (Kummerer, 2009).

1.5 Significance and scope of study

Large quantities of antibiotics are being used worldwide in agricultural fields through wastewater irrigation and manure application. They cause damage to the ecosystem when discharged into the environment, but there is a lack of information on their toxicity to plants. Crops are an important component of the terrestrial environment and serve as a potential pathway for transport of antibiotics into the food chain. Low antibiotic concentrations found in plants could cause antibiotic resistance when these levels are consumed. This study is designed to understand the toxicity profile for these pollutants on plants.

Agricultural countries like Pakistan need advance research in plant and environmental sciences to cope up with the emerging environmental challenges. This study would help in assessment of effects of antibiotics on *Triticum aestivum* and also would give new insights on their impacts on environment. If antibiotics affect the plant growth and cause phytotoxicity that can pose serious threats to agricultural sector, human health and food security than this study can help to draw boundaries on the use and release of antibiotics in the environment.

1.6 Objectives

Keeping in view the above information, it was hypothesized that antibiotics present in waste water can negatively affect the plant growth. The objectives of this study were:

- To determine changes in physical growth parameters of plant and composition of its vegetative parts.
- To assess DNA damage in wheat plant through micronuclei assay.

Chapter 2

Review of Literature

Key focus of this chapter is to provide information on the presence of antibiotics in wastewater, their possible action and impacts on receiving crop yield and health of the end-users.

2.1 Antibiotics in wastewater

Water pollution is most often due to human activities. The major ones are indiscriminate disposal of industrial, municipal and domestic wastes in water channels, rivers, streams and lakes, etc. In developing countries, the situation is worse where over 90% of raw sewage and 70% of untreated industrial wastes are dumped into surface water sources (Azizullah et al., 2011). According to Sial et al. (2006), in Pakistan out of 6634 registered industries 1228 are considered to be highly polluting. Due to the high load of organic and toxic materials in their waste effluents, industries have become a major source of water pollution in Pakistan (Nasrullah et al., 2006).

The major industries contributing to water pollution are textile, pharmaceuticals, ceramics, petrochemicals, food industries, steel, oil mills, sugar industries, fertilizer factories, and leather tanning (Sial et al., 2006). These industries produce several hundred thousand of wastewater containing huge quantities of pollutants like nitrates, nitrites, cations and anions such as Ag^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} Cl^{-}, CO_3^{2-}, HCO_3^{-} and toxic metals like arsenic, iron, lead, mercury, chromium, cadmium, copper, nickel, zinc, cobalt and magnesium (Ali et al., 1996; Sial et al., 2006; Ullah et al., 2009). Most of the industries in Pakistan are located in or around major cities. They dispose their waste effluent directly into the nearby drains, rivers, streams, ponds, ditches and open or agricultural land (Ullah et al., 2009; Ashfaq et al., 2017; Rivera-Jaimes et al., 2018).

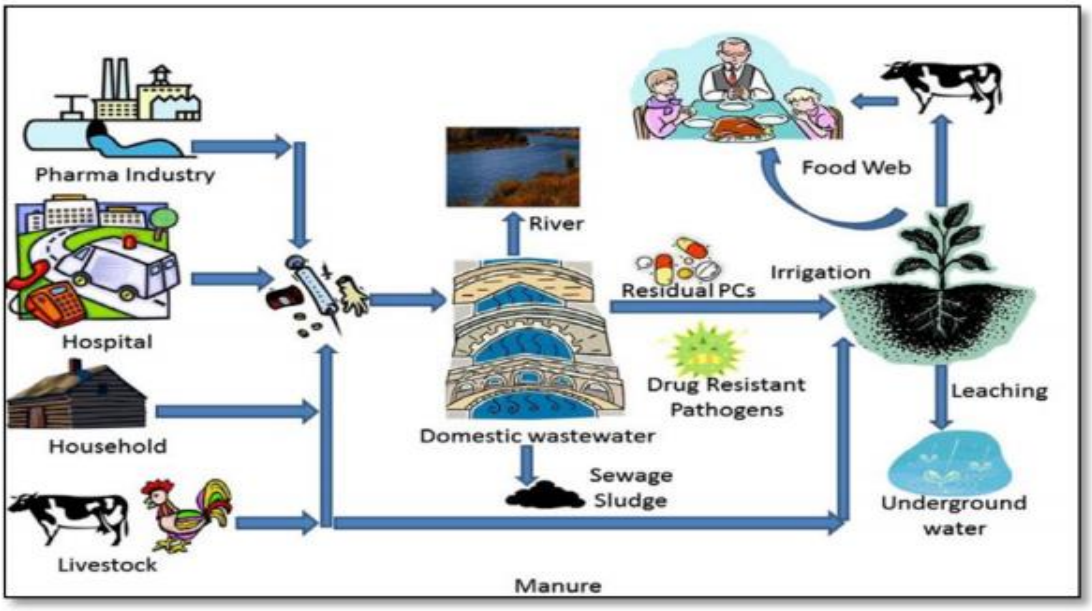


Figure 2.1: Possible pharmaceutical routes to environment (Adopted from Rehman et al., 2013).

Antibiotics are manufactured for curing different biological effects and are present widely in the environment. Unfortunately, these antibiotics have quite a long half-life period that allows them to persist in the environment for long time (Liang et al., 2016). So there is a possibility that these drug residues may enter the food chain and may result in potential human health and environmental consequences upon entering aquatic and terrestrial ecosystems (Fent et al., 2006). Low doses of antibiotics are present in municipal sewage, hospital wastewater, surface and ground water ranging from ng L^{-1} to few µg L^{-1}.

Different concentrations of antibiotics in the environment have been reported by researchers over the years. No limit has been set for risk analysis of drug contaminated manure or sludge if it reaches a certain level in the fields. However, the European Agency for Evaluation of Medicinal Products (EMEA) recommends analysis of the potential risk caused by any drug that exceeds the threshold concentration 0.01 mg L^{-1} in surface waters (EMEA, 2006; Grung et al., 2008).

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 presence of antibiotics in wastewater treatment facility in Wisconsin. They screened 21 antibiotics compounds in the treatment facility. Their results showed that six major groups were found in the wastewater 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 include metronidazole, norfloxacin, sulpha-methazole, ceftriaxone, ofloxacin, ciprofloxacin, levofloxacin and tinidazole in the range from 1.4 to 236.6 μg L^{-1}. 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 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 up to 3.35, 5.30 and 2.10 μg L^{-1} in animal wastewater, river and pond water respectively. Similarly, the concentration of Enrofloxacin ranged from 0.05 μg L^{-1} in pond water to 4.24 μg L^{-1} in river water and concentration of Florfenicon ranged from 0.95 to 2.84 μg L^{-1}. Their results showed that river water contained higher concentration of antibiotics which will ultimately affect the river ecosystem and will enter in the environment.

Antibiotics are damaging our environment but the extent of damage 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. (2010) 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 Ofloxacin in hospital was at highest concentration i.e. ranged from 1.660 to 4.240 µg L^{-1} in all water environments. Second highest was Norfloxacin ranged from 1.36 to 1.62 µg L^{-1}, then Ciprofloxacin ranged from 0.011 to 0.136 µg L^{-1} in all samples. Trimethoprim was at the lowest concentration ranged from 0.061 to 0.174 µg L^{-1}. According to this study, the removal efficiency of treatment plants for antibiotics was less than 65 percent which means they may enter in our environment and make it vulnerable for other living organisms.

Many studies have reported that fluoroquinolones and tetracyclines can possibly interfere with the synthesis of DNA, proteins, plastids and mitochondria in plants (Opris et al. 2013). However, the potential affects and risks of these antibiotics in food crops are still not known completely (Sarmah et al. 2006). In this scenario, there is a need to understand the

environmental consequences of prevailing concentrations of antibiotics and their metabolites in near future.

In Lahore, a study was conducted by Ahmad et al. (2013) to determine the role of untreated wastewater in spreading antibiotics and antibiotic resistant bacterial species in the main river of the 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 the 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. Another study by Khan et al. in 2013 reported concentrations of different antibiotics in waste water from 2 major hospitals and river Ravi in Lahore. These concentrations are given in table 2.1.

Table 2.1: Antibiotic levels in Pakistan (Adopted from Khan et al., 2013)

Antibiotics	**Concentration (µg L^{-1})**
Moxifloxacin	224
Ofloxacin	66
Ciprofloxacin	18
Sparfloxacin	58
Levofloxacin	27
Sulphamethoxazole	49

2.2 Pathway of antibiotics in plants

Many experiments have 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 another study conducted, Boxall and his coworkers 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 taken up by plants. There is a growing public concern that antibiotics may 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).

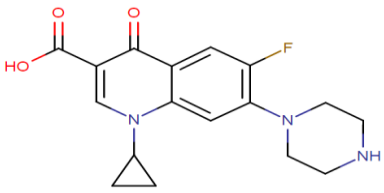
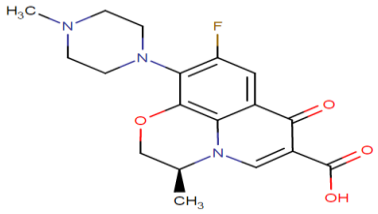
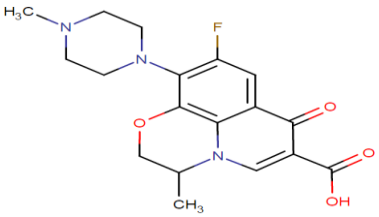
Boxall et al. (2006) conducted a study to evaluate plant uptake of 7 antibiotics by lettuce and carrots grown in a soil that was spiked at concentrations of 1 mg kg^{-1}. Florofenicol and trimethoprim were detected in lettuce leaves and enoxacin, florfenicol and trimethoprim were detected in carrot root 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.

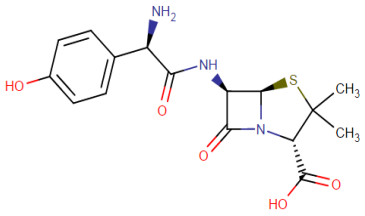
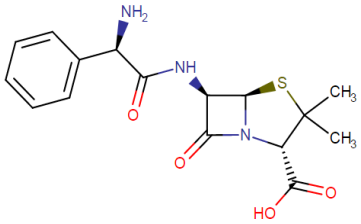
2.3 Effects of antibiotics on plants

Plants play a crucial role in maintaining the integrity of ecosystem. The introduction of toxic substances into the environment causes changes in the structure and function or even genetics of these primary producers (Yi and Si, 2007; Yin et al., 2008). The pollutants, once they enter the ecosystem, are taken up by plants. Accumulation of certain veterinary drugs in different

parts of plants has been observed in a number of studies. Rice, wheat, lettuce, soybeans and alfalfa have been used extensively as test species to study the accumulation of antibiotics in certain parts of these plants. This accumulation occurs through the water transport system and via passive absorption. When these pollutants enter the primary producers, they can cause serious damage to the physiology of plants and their biochemical activities (Liu et al., 2009; Boonsaner and Hawker, 2010; Hillis et al., 2011; Li et al., 2011; Luo et al., 2011). CAS number, structural and molecular formulas of various antibiotics and their affects are given in table 2.2.

Table 2.2: CAS number, structural and molecular formulas of antibiotics and their affects.

Chemical name	**CAS number**	**Molecular formula**	**Structural Formula**	**Impacts on Plants**	**References**
Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$		Photosynthe-sis and chlorophyll content in *Triticum aestivum*	Opris et al. (2013)
Levofloxacin	100986-85-4	$C_{18}H_{20}FN_3O_4$		Reproductio-n rate, chlorosis and root length in *Lemna minor* and *Medicago sativa*	Robinson et al. (2005) and Hillis et al. (2011)
Ofloxacin	82419-36-1	$C_{18}H_{20}FN_3O_4$		Reproductio-n rate, chlorosis in *Lemna minor*	Robinson et al. (2005)

Amoxicillin	26787-78-0	$C_{16}H_{19}N_3O_5S$		Photosynthe-sis and plant growth in *Triticum aestivum* and *Daucus Carota*	Opris et al. (2013) and Hillis et al. (2011)
Ampicillin	69-52-3	$C_{16}H_{19}N_3O_4S$		Photosynthe-sis in *Triticum aestivum*	Opris et al. (2013)

Accumulation of antibiotics in plants has been reported in a number of studies. In 2005, a study on green onion, cabbage and corn reported the uptake of pharmaceutical products proportional to their presence in the environment. The increasing antibiotic containing manure increased the uptake of antibiotics (Kumar et al., 2005). Boxall et al. (2006) observed the uptake of several different medicines in different plants. The study showed that the uptake of antibiotics florfenicol, enrofloxacin, trimethoprim and diazinon by carrot roots while trimethoprim, levamisole, and florfenicol were detected in the lettuce plant. Ciprofloxacin (Cip) has been shown to be absorbed by lettuce and cucumber (Lillenberg et al., 2010) while sulfadimetoxin has been taken up by barley (Brambilla et al., 1996). The uptake of these drugs by plants can lead to impact on human health by microbial infections that cannot be treated by pharmacotherapy (Auerbach et al., 2007; Jia et al., 2008; Du et al., 2012). It can also impact their growth either by direct uptake or by affecting symbiotic relationship with soil bacteria (Riaz et al., 2017). In lettuce and potato, sulfametazine uptake has been reported by Dolliver et al. (2007) while a study by Lillenberg et al. (2010) showed uptake of ciprofloxacin and enrofloxacin by lettuce, cucumber and barley.

Various studies have been carried out to ascertain the toxic effects of pollutants on plants. A decrease in the growth of roots and shoots of alfalfa by 85% and 61% was observed on exposure to oxytetracycline (OTC) (Kong et al., 2007). Migliore et al. (2003) observed toxicity of enrofloxacin at 5 mg L^{-1} when taken up by the crop plants. On the other hand, no significant stress resulting from the two sulfonamides was observed by the shoot apparatus. Maize plants were exposed to chlortetracycline (CTC) at concentrations of 0.05, 0.5, 5 and 50 mg L^{-1} for 10 days and its uptake and effect was observed. Root length, shoot length and fresh biomass of maize were shown to suffer with the highest stress observed by root apparatus (Wen et al., 2012).

The effect of tetracycline (TC) on wheat at different concentrations (0.5, 1, 5, 10, 25, 50, 100, 150, 200, 250, and 300 mg L^{-1}) was studied by Xie et al. (2011) and it was concluded from their study that wheat showed both positive and negative response to TC. At lower TC concentrations, enhanced root growth, seed germination and cell mitosis were observed. At higher concentrations, reverse trend was observed. Another study on wheat highlighted the negative effect of OTC on fresh and dry biomass of root and shoot. The root and shoot biomass decreased by 12% to 90.2% and 21.7% to 88.6% respectively.

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 ones. 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 (chlortetracycline, tetracycline, tylosin, sulfamethazole, sulfamethazine and trimethoprim) on selected plant 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 physiologically or genetically. From this study, it was concluded that tetracycline contamination is a potential risk which may cause cytogenetic damage in living organisms.

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 *Vicia faba* plant. They used nalidixic acid (quinolone) ciprofloxacin and levofloxacin (fluoroquinolones) to determine the genotoxicity in *Vicia faba* 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) determined 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 they had 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 and oxytetracycline on a wetland plant namely *Phragmites australis*, 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 were negative impacts on many plant activities like superoxide dismutase and catalase activity.

2.4 Wheat

The economic stability of Pakistan is highly dependent on agriculture and contributes 21.4% to GDP providing employment to 45% of working class. Wheat (*Triticum aestivum*) is one of the most widely consumed staple foods of the world especially in Pakistan with an average yield of 2714 kg ha^{-1} (Shahbaz et al., 2017) and a growth rate of 2.08%. Pakistan is ranked as ninth largest producer and distributer of wheat by the Food and Agriculture Organization. The presence of pollutants in agricultural field can have negative impacts on the growth of the crop plants. Even if the residual period of these compounds is short, they can cause serious damage to plant growth in a short time. The effects of 9 antibiotics on the physiology and secondary metabolites of wheat at the concentrations of 0.5 and 1.5 mg L^{-1} were analyzed by Opris et al. (2013). Cip and cephalosporins caused a stomatal reduction thereby influencing the net assimilation. A reduction in its photosynthetic responses in chlorophyll, pigments and carotids was also observed by application of Cip, tetracycline and erythromycin.

2.5 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.

2.5.1 Seed germination technique

An 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 concentrations of the PCPs were increasing, the seed germination of wheat was inhibiting. At lower concentrations of these PCPs, seedling

growth wasn't affected when the exposure time was 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^{-1} to very high concentrations i.e. 10,000 µg L^{-1} 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^{-1} by Xie et al. (2010), it was observed that lower concentrations i.e. 0.5 to 10 mg L^{-1} were stimulating the seed germination. The higher concentrations of tetracycline from 10 – 300 mg L^{-1} significantly inhibited the seed germination in concentration dependent manner.

2.5.2 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 Ostling and Johanson in 1984 and then with the passage of time this technique was 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 study showed that 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 pollutants 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 mg kg^{-1} for 24 and 48 hrs. The result of the study showed that genotoxicity was increased with the increase in the concentration.

2.5.3 Micronuclei assay

The micronucleus test is a key biomarker both *in vivo* and *in vitro* to determine the cytogenetic abnormality in different types of cells and populations that are exposed to genotoxic agents (Araldi et al., 2013; Araldi et al., 2015). MN assay is representative of chromosomal losses along with the results of DNA amplification (Samanta and Dey, 2012). It is usual in oncogenic processes generally to detect DNA amplification which results in double minute chromosomes (DM), which are then excluded from the main nucleus forming MNs (Shimizu and Tanaka, 2000). This exclusion is related to the loss of allele dose that results in carcinogenesis (Terradas et al., 2010).

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^{-1}. The results of the study showed that the lower concentration of tetracycline caused increase 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 $AlCl_3$ ranging from 0.01 – 10 mM for 12 hrs, it showed that number of micro-nucleated cells were increased with the increase in concentration. The number of mitotic cells in all treated group was also dependent on the pH. It was concluded in the study that $AlCl_3$ is genotoxic for the *Vicia faba* and this plant can be 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 *Vicia 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^{-1} of antibiotics had no significant genotoxic effect on *Vicia faba* but when the mixture of these antibiotics was exposed to plant, it induced significant micronuclei induction. Above mentioned techniques are important to assess genotoxic potential of emerging pollutants, industrial chemicals and pharmaceuticals. Micronucleus assay is a quick, relatively

efficient and simple method. Therefore, in this study MN assay was used to assess the effects of antibiotics on germination and micronuclei production in wheat.

Chapter 3

MATERIALS AND METHODS

This chapter describes the experimental framework adopted during the conducted research work. The work was divided into three major levels. At the first level, soil was prepared and its general characteristics were determined. At the second level after the completion of seed germination test, seeds were grown and antibiotic dose was applied. The whole experiment was conducted in locally made greenhouse at IESE, NUST. And finally, at the last step, the assessment of toxicity through micronuclei assay was done and vegetative composition was determined. All the methodologies followed throughout the study are described here in detail.

3.1 Soil treatment (physical and chemical analysis)

Soil was taken from NUST and it was spread and air dried for four days to remove moisture content. Soil was then prepared in the ball mill at Particulate Technology Lab SCME-NUST (figure 3.1) and passed through the sieve to achieve fine and homogenized soil having particle size of < 2mm. Plastic pots that could sustain 1 kg of soil were used for experimentation. 1 kilogram of prepared soil was then added to all pots.

Figure 3.1: Ball Mill at SCME (NUST).

3.1.1 Soil texture

Soil texture is the relative proportion of sand, silt and clay. Its type is critical for mobility and bioavailability of pollutants in soil. Texture of soil was determined on the basis of saturation percentage (Malik et al., 1984).

0-19% Sand

20-29% Sandy loam

30-45% Silt Loam

46-60% Clay Loam

More than 60% Clayey

3.1.2 Soil pH and EC

The pH of the soil was measured to check acidity or alkalinity of soil. For this purpose, 5 g of air dried soil with a particle size of less than 2 mm was taken in 50 mL beaker and 25 mL of distilled water was added into it with the help of measuring cylinder. The mixture was placed at a mechanical shaker at 180 rpm for 30 min for proper mixing and left to stand for 1 hour (McLean, 1982). Reading was taken with the help of pH and EC meter (inoLab pH/Cond 720, Germany). The soil pH came out to be 7.1 and EC was 424 µs cm^{-1}.

3.1.3 Moisture content

Ten grams of air dried soil with a particle size < 2 mm was weighed through weighing balance (Phoenix E-balance, BTG-303, China) and placed in a petri dish. The lid was removed and it was then dried in oven for 24 hours at 105 °C. The Petri dish was then removed from the oven carefully and was left for 30 minutes to cool. Afterwards, it was re-weighed to determine dry weight.

Following formula was used to calculate moisture content:

% moisture in soil = (Wet soil-dry soil) / (dry soil) ×100

3.1.4 Soil water holding capacity

Ten grams of air dried soil was weighed through weighing balance. Whatman filter paper no. 42 was then placed in a funnel. Weighed soil was then placed on the filter paper. After this, 10 mL of distilled water was poured onto the soil. The filtrate was collected in a graduated cylinder. The final volume of filtrate was noted (Harding and Ross, 1964).

3.1.5 Soil digestion

Soil sample of 0.5 g was taken. For digestion, 8 mL of perchloric acid and 4 mL of nitric acid were added into it. The sample was then digested at a hot plate with the initial temperature of 50 °C and then raising the temperature gradually to 100 °C and then to 150 °C.

The sample was digested until all the soil disappeared and the color changed to light grey. Volume of sample was then raised to 50 mL by distilled water. It was then passed through filter paper. The resulting filtrate was then stored in a plastic bottle in refrigerator to use for further testing.

3.1.6 Nitrate-nitrogen

For quantification of nitrate- nitrogen, 10 g of air dried soil was weighed in to Erlenmeyer flask and 50 mL of 0.02 N $CuSO_4.5H_2O$ was added into it. It was then shaken for 15 minutes and filtered through a double Whatman no. 42 filter paper. Subsequently, 3 mL of filtrate was pipetted into a 50 mL conical flask and 1 mL of 0.1 % chromotropic acid solution was added drop by drop into it and was allowed to cool.

Then 6 mL of concentrated sulfuric acid was added and yellow color was developed after 45 minutes. Standards were prepared by dissolving 3.6092 g KNO_3 in 500 mL 0.02 N copper

sulfate solution. Absorbance was taken through spectrophotometer (SPECORD, 200 PLUS, Analytikjena, Germany) at 430 nm (Ryan et al., 2007).

3.1.7 Total phosphorus in soil

Phosphorus is one of the most important nutrients required for the fertility of soil and growth of plants. To determine the amount of total phosphorus, 22.5 g of ammonium hepta-molybdate was dissolved in 400 mL distilled water and 1.25 g of ammonium metavandate was dissolved in 300 mL of hot distilled water. Both of these solutions were mixed and 250 mL of nitric acid was added slowly into it. Final volume was raised to 1 litre. For preparing standard stock solution, 2.5 g of potassium di-hydrogen phosphate was oven dried at 105 °C for one hour. An amount of 0.4393 g was taken from this and dissolved in 1 litre of distilled water.

Standards were made by taking 1, 2, 3, 4 and 5 mL from the stock in 50 mL flask. Ten milliliters of prepared reagent were added into each and the final volume was raised to 50 mL. Blank was prepared by taking 10 mL of reagent in a 50 mL volumetric flask and the final volume was raised with distilled water. For preparing sample, 5 mL of digested soil solution was taken. 10 mL of reagent was added into it and final volume was raised with distilled water up to 50 mL. After 10 min, the blank, standards and sample were run on spectrophotometer (SPECORD, 200 PLUS, Analytikjena Germany) at a wavelength of 410 nm and readings were noted (Rayan et al. 2007).

3.1.8 Total organic carbon

Total organic carbon was determined through Walkley and Black (1934) method. Soil was ground and passed through a 0.5 mm mesh sieve and was placed in a 500 mL Erlenmeyer flask. The amount of soil used in the determination of TOC was calculated based on initial information on the C concentration in the soil and ranged from 0.1 to 0.5 g. Ten milliliters of

0.167 potassium dichromate ($K_2Cr_2O_7$) and 20 mL of concentrated sulfuric acid were added to the soil while stirring it to make sure that the soil was mixed with reagents. After 30 min rest, 200 mL of distilled water, 10 mL of concentrated H_3PO_4 and 1 mL of 0.16% diphenylamine were added. The excess dichromate that was not reduced in the reaction was determined by volumetric titration using ammonium ferrous sulfate (Mohr's salt).

3.1.9 Sodium in soil

Sodium was measured by introducing the extracts from soil into a Flame Photometer; which emitted light with a wavelength (color) specific to the element and of intensity proportional to the concentration (Richards, 1954). The extracting solution was ammonium acetate solution (NH_4OAc). Stock solution (1N) was prepared from which series of sodium standards were made. Standards were run on Flame Photometer at 589nm wavelength.

3.1.10 Potassium in soil

Potassium was also determined by using the method which was developed by Richards (1954). Ammonium acetate solution, 1N, was used as an extracting solution. Stock solution was prepared from which series of potassium standards were made. Standards were run on Flame Photometer at 767 nm wavelength.

3.2 Seeds preparation

Wheat (*Triticum aestivum*) was selected as test plant species to analyze the effects of antibiotics. Healthy seeds of wheat (Galaxy 70) were selected and sterilized in 5% solution of calcium hypochlorite for 5 minutes (Cannell, 1990).

3.3 Nutrient application

The wheat plants were fertilized with NPK: 0.1-0.05-0.05 g per kg of soil (Arshad et al. 2011). To achieve this dose 18% of nitrogen from DAP (diammonium phosphate), 46% of nitrogen

from urea, 46% of phosphorus from DAP and 50% of potassium from potash were calculated. Nitogen was applied in three intervals into the soil. The doses contained 0.196 g of nitrogen from DAP and 18.3 g of nitrogen from urea, 11.41 g of phosphorus and 10.5 g of potassium.

3.4 Preparation of solutions

Five antibiotics namely ciprofloxacin, levofloxacin, amoxicillin, ampicillin and ofloxacin were selected for the preparation of stock solutions. For this purpose, commonly available medicines Mercip (ciprofloxacin 500 mg), Levomerc (levofloxacin 500 mg), Oflox (ofloxacin 200 mg), Penbritin (ampicillin 500 mg) and Ospamox (amoxicillin 500 mg) were crushed into powder and dissolved in one litre of distilled water (figure 3.2 A). The solutions were then placed on a magnetic stirrer for mixing at 500 rpm for two hours. Aluminum foil was used to completely cover the solutions to avoid photochemical reactions (figure 3.2 B). These stocks were then stored in one litre plastic bottles covered with aluminum foil at room temperature. Concentrations of 2, 4, 6, 8, and 10 mg L^{-1} were prepared from the stock solution by dilution.

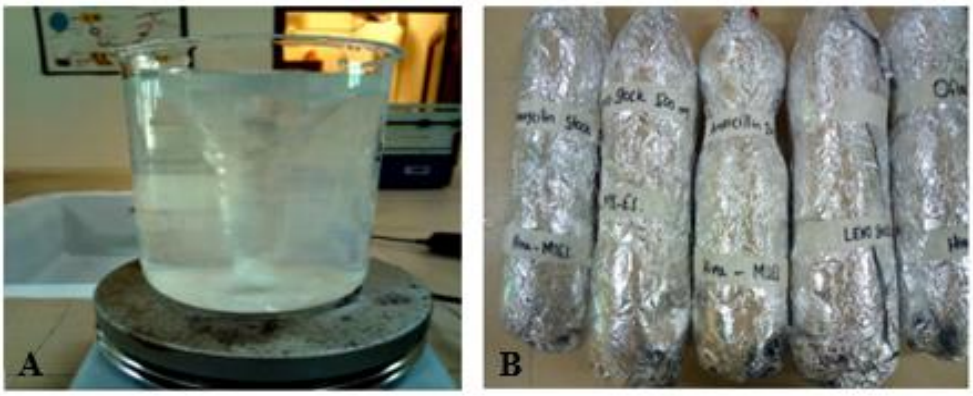


Figure 3.2: Preparartion of antibiotic stock solutions. A) Solution on stirrer for proper mixing; B) Stock solution stored in containers covered with aluminium foil.

3.5 Seed germination test

The wheat seeds were obtained from Ayub Agricultural Research Institute (AARI). The variety of wheat used for experimentation was ‘Galaxy 70’. Healthy seeds were separated first and then

they were washed with distilled water. These seeds were then sterilized in 5 percent calcium hypochlorite solution. After sterilization, they were washed again with distilled water three times. Petri dishes were washed and left to be air dried at room temperature. Filter papers were placed in these dishes and five seeds were placed in each Petri dish. The filter paper was then moistened with different concentrations of antibiotics and of control with the distilled water as shown in figure 3.3. These petri dishes were then placed in an incubator (Stuant orbital incubator S150, UK) at 25°C for germination for 5 days.

Two replicates were used for each concentration. After 5 days, the number of seeds germinated in each dish was counted and percentage formula was applied to determine the effect of all antibiotics on wheat germination.

% Seed Germination = No. of seeds germinated/Total number of seeds* 100

Figure 3.3: Sterilized seeds placed on filter paper treated with different concentrations of selected antibiotics for germination.

3.6 Plant cultivation

In plastic pots, 1 kg of test soil was weighed. Pots were labeled accordingly. The experiment was performed in quadruplicates. To each pot, five seeds were added and the pots were watered at regular intervals to maintain moisture level (figure 3.4). After one week of seed germination, thinning of plants was done and maximum of three plants were allowed to grow through the full experimental period. The soil was tilled time to time for proper growth and development of seedlings.

Figure 3.4: Pot experiment in progress exposing *Triticum aestivum* to different levels and types of antibiotics.

3.7 Application of antibiotic water on plants

Suspensions of desired concentrations were prepared by weighing the calculated amounts and by the addition of distilled water. A total of six different concentrations including control (0, 2, 4, 6, 8 and 10 mg kg^{-1}) of selected antibiotics were applied to the soil in each pot. The antibiotic dose was given in two splits.

3.8 Growth parameters (length and biomass)

Whole plant was taken from the pot at the time of harvest. Roots were washed with tap water gently. Root and shoot lengths were measured using measuring tape (cm) and the fresh biomass was noted using portable weighing balance. Roots and shoots were separately placed in an oven at 65 °C for 48 h and then dry biomass was recorded. Both the roots and shoots were ground by using electric grinder and then kept in sampling bags for further analysis.

3.9 Composition of vegetative parts

3.9.1 Total phosphorus and iron

The effect of all the five antibiotics on total phosphorus and iron in wheat plant was analyzed. The ground plant samples were used for analysis. The dried material was digested in concentrated nitric acid perchloric acid (HNO_3 $HClO_4$) mixture (2:1) on hot plate (Rashid,

1986). The analysis was done on shoots of test plants. The phosphorus contents were measured at 430 nm by spectrophotometer (SPECORD, 200 PLUS, Analytikjena, Germany) with the help of vanadomolybdo-phosphoric acid colorimetric method. The iron contents in digested wheat samples were analyzed through (AA-7000 Schimadzu) Atomic Absorption Spectrophotometer shown in figure 3.5 (Ryan et al., 2007).

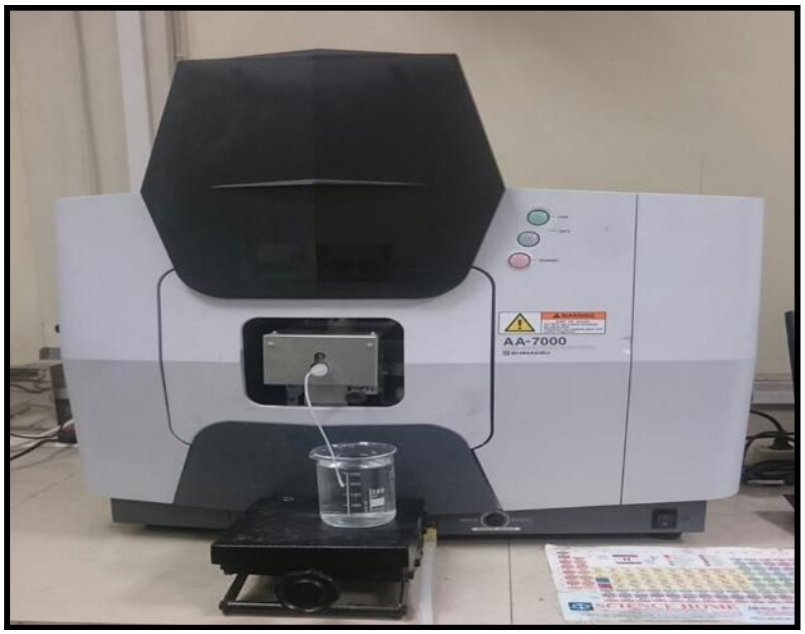


Figure 3.5: Atomic absorption spectrophotometer (AA-7000, Shimadzu, Japan).

3.9.2 Total carbohydrates

For the quantification of total carbohydrate content in wheat plant, "Morris anthrone method" was used (Ludwig and Goldberg, 1956). The shoot material was grounded into fine powder. 100 mg of sample was taken into a test tube and was hydrolyzed using 2.5N HCl in a boiling water bath for 3 hrs. Hydrolyzed samples were then centrifuged at 4000 rpm for 10-12 minutes. The supernatant was collected and was used as an extract for further analysis. Anthrone reagent was prepared by adding 2 grams of anthrone chemical into 1 L of 95% H_2SO_4. 8 mL of this reagent was added through pipette into each test tube containing 5 mL of sample solution. The solution was mixed thoroughly and was allowed to stand for 10 min to develop green color.

Blank containing distilled water and reagent, and standards of glucose were prepared and amount of carbohydrates in test sample was measured through a spectrophotometer (SPECORD 200 PLUS, Analytikajena, Germany) at 620 nm.

3.9.3 Total proteins

The amount of total proteins in wheat plant was quantified through the determination of total nitrogen content of plant (Martin et al. 1983). The first step was mineralization in which 1 mL of 36N H_2SO_4 was used to digest 50 mg of plant sample. This was carried on for 10 min at 150°C and then for 30 min at 310°C. Samples were removed from the mineralization block and were allowed to cool. Then 0.1 mL of H_2O_2 was added into it and sample was again digested at 310°C until the evaporation of H_2O_2. This step gave a colorless extract. The sample was then diluted by adding 10 mL of H_2O. From this diluted sample, 0.1 mL was taken and 3.5 mL of H_2O was added into it. For preparation of first reagent, 2.5 mL Phenol and 12.65 mg Na Nitroprusside were added in to 250 mL H_2O. Then 25 g NaOH, 16.75g $Na_2HPO_4.7H_2O$ and 2.5 mL NaOCl (12%) were added in to 250 mL of H_2O to prepare second reagent. After this, 0.1 mL of sample was taken into Eppendorf's. With the help of pipettes 0.5 mL of reagent 1 and 0.5 mL of reagent 2 was added into it and the sample was incubated for 30 minutes at 37 °C. Reading was taken with the spectrophotometer at 625 nm.

3.10 Micronucleus assay for toxicity assessment

DNA damage was quantified thorough micronucleus assay to assess genotoxicity. Various steps in the process are discussed in detail in upcoming paragraphs.

3.10.1 Plant growth for MN test

Triticum aestivum seeds obtained from Ayub Agricultural Research Institute (AARI) were disinfected with 5% $Ca(ClO)_2$ up to 5 minutes and then these were thoroughly washed with distilled water for 3 to 4 times.

After disinfection seeds were placed in distilled water for 30 minutes and placed in petri dishes over wet filter paper for germination. Petri dishes were then placed in incubator (Suant, orbital incubator S150, UK) at 25 °. These seeds were treated with six different concentrations including 0, 2, 4, 6, 8, and 10 mg L^{-1} of selected antibiotics for 72 hours.

3.10.2 Preparation of Feulgen stain

To prepare Feulgen stain, about 0.3 g of basic Fuchsin powder was taken in 250 mL beaker and dissolved in 60 mL boiling distilled water by constant stirring using hot plate stirrer. After dissolution, it was cooled and filtered through Whatman no.1 paper. Then 4 mL of 1N HCl was added and decolorized with 0.5 grams of potassium meta-bisulphite and was placed in the dark for 24 hours to bleach. The prepared stain was stored in refrigerator in light proof bottle and covered with aluminum foil to protect from light.

3.10.3 Root fixation and staining

After 24 hours of exposure, the root tips were cut about 1-2 cm. They were fixed in a Cornoy solution which was freshly prepared. Cornoy solution is a mixture of ethanol and glacial acetic acid (3:1) and stored in refrigerator overnight. After fixation, the roots were maintained in phosphate buffer saline (PBS) solution for 10 minutes and 1N HCl was used for hydrolyzing the root tips for 30 minutes in Water Bath (MEMMERT UNB-10, Germany) at 60 °C. Roots were then stained with Feulgen (figure 3.6) for 30 minutes in water bath at 60 °C and slides were prepared for microscopic study (protocol was modified). Squash Technique was used for the preparation of slides in 45% acetic acid. In total, 100 cells were analyzed visually (figure

3.7) for each concentration of antibiotics, for micronucleus test under microscope (Model No. Optika, B-350, Italy) at 40 X magnification (Ullah and Arshad, 2014).

Figure 3.6: Root staining with Feulgen stain.

Figure 3.7: Fluorescent microscope for visual analysis (Model No. Optika, B-350, Italy).

3.11 Statistical analysis of data

Statistical significance of findings was checked by using software "Statistics 8.1" applying single factor ANOVA and LSD through all pair-wise comparison. When the probability of the results was less than 0.05 ($p < 0.05$), results were considered statistically significant.

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 usage of antibiotics, they are entering 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 different antibiotics on *Triticum aestivum* (wheat). For this, plant physical growth parameters, nutritional composition and DNA damage caused by selected antibiotics were determined.

4.1 General soil characteristics

The soil used in the study was silt loam. Its physiochemical properties are given in table below.

Table 4.1: Physio-chemical characteristics of the soil used for experimentation.

Parameters	Values
pH	7.14
EC	424 µS cm^{-1}
WHC	66%
Moisture	0.19%
Texture	Silt loam
TOC	0.98%
Total phosphorus	51.3 mg kg^{-1}
Sodium	78.4 mg kg^{-1}
Potassium	62.5 mg kg^{-1}
Nitrate-nitrogen	3.1 mg kg^{-1}
Iron	1.75 mg kg^{-1}

4.2 Effect of antibiotics on plant physiological parameters

4.2.1 Effects on seed germination

Percentage of germinated seeds after treatment with 2, 4, 6, 8 and 10 mg L^{-1} of selected antibiotics was 90.3 %. The result showed that all the five antibiotics had no significant negative effect on seed germination but at highest concentration of antibiotics i.e. 10 mg L^{-1}, seed germination rate was affected significantly. The results are illustrated in figure 4.1. Hillis et al. (2011) 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. The study concluded that seed germination is insensitive to antibiotics even at higher concentrations. Liu et al. (2009) concluded that antibiotics mainly tetracyclines and sulfonamides could hinder seed germination in rice, cucumber and sweet oat but the effects could vary depending upon the plant species and antibiotics use.

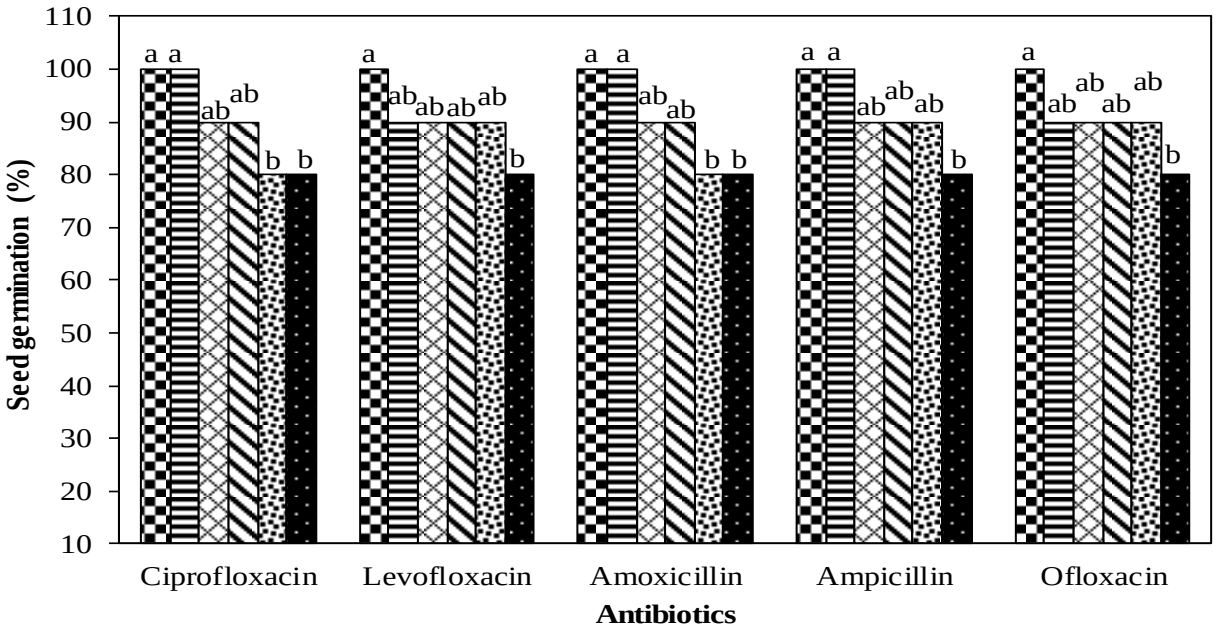


Figure 4.1: Effect of different concentrations of antibiotics on seed germination

= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.3 Effects on plant morphology

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

4.3.1 Effects on root and shoot length

The effects of all selected antibiotics on root and shoot length of wheat plant are presented in figure 4.2 and 4.3. All of the five antibiotics showed a significant decrease, in both root and shoot length with increase in the concentration of dose, in comparison with the control. The effect of Amp and Oflox on root length was higher as compared to other antibiotics; however, on shoot length, Ofloxacin had a greater negative effect than others.

The overall percentage decrease in root length by the highest dose i.e. 10 mg kg^{-1} of Cip, Lev, Amox, Amp and Oflox was in the order of 41%, 67%, 57%, 48% and 63% respectively, compared to the control. Similarly, the percentage decrease caused by these antibiotics in shoot length was 32%, 37%, 36%, 33% and 49% in the same order of antibiotics as mentioned above. The suppression of plant growth due to antibiotic stress has been reported previously by various studies. A decline in growth of roots as well as shoots of alfalfa by 85% and 61% was observed by Kong et al. (2007) on exposure to the antibiotic to oxytetracycline (OTC). A study by Hillis et al. (2011) also showed the similar results that roots are more sensitive than the shoots when different species of plants were exposed to antibiotics at different concentrations. Liu et al. (2009) studied the toxic effects of six antibiotics on length of roots and shoots of sweet oat, cucumber and rice which indicated the varied susceptibility of plant growth to these antibiotics. But in each case, a decrease in growth was observed.

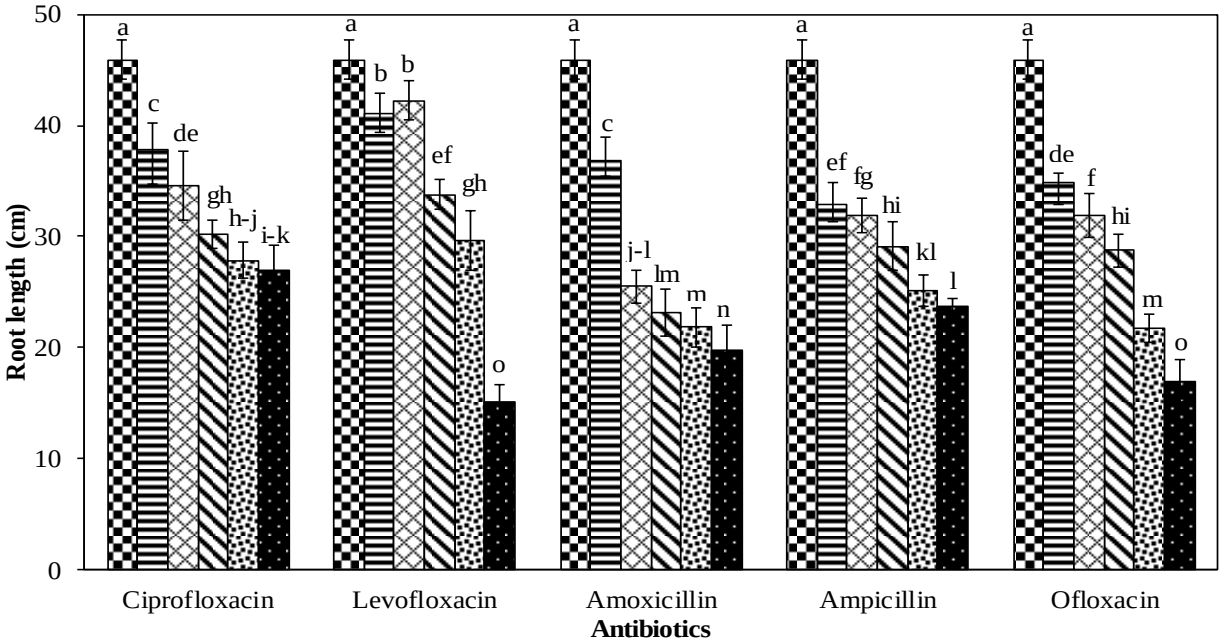


Figure 4.2: Effect of different concentrations of antibiotics on plant root.

= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

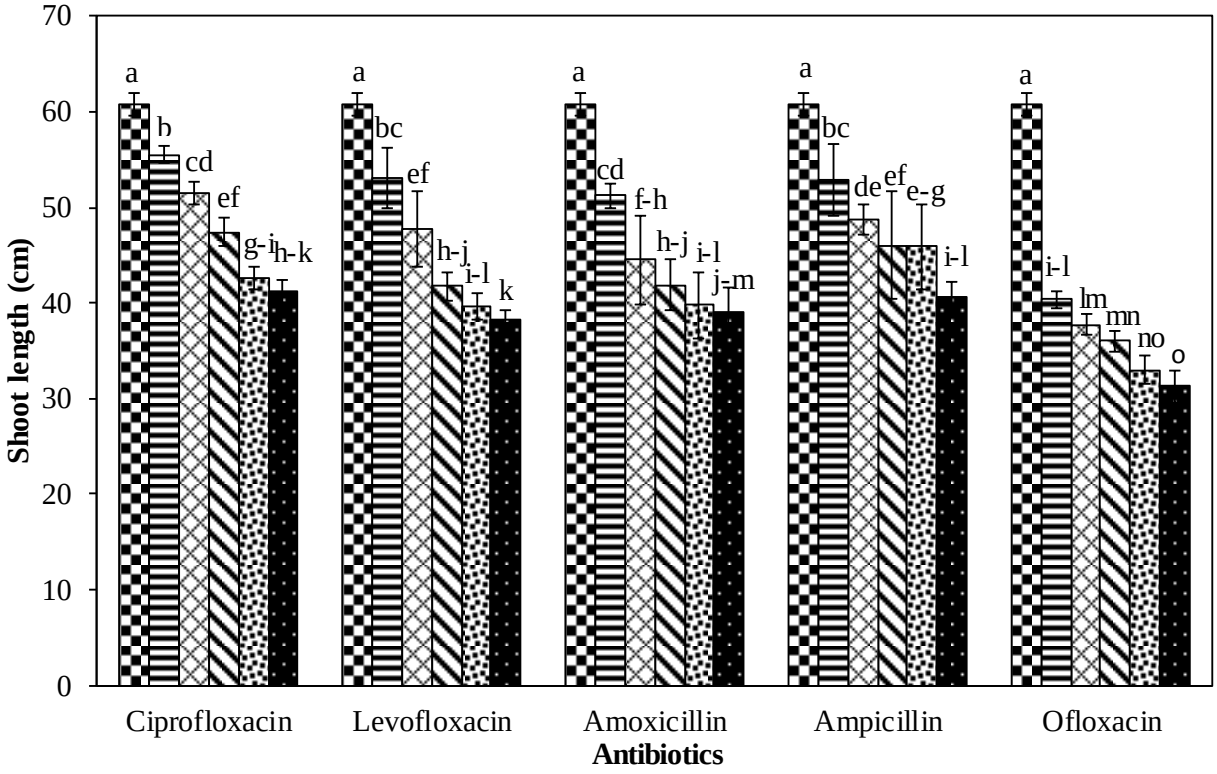


Figure 4.3: Effect of different concentrations of antibiotics on plant shoot.

= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.3.2 Effect on biomass

In the current study, dried biomass of root did not change significantly at initial concentrations i.e. at 2 mg kg^{-1} concentration of Cip and Oflox specifically (Table 4.2) but showed a significant declining trend at higher concentrations. However, the shoot biomass showed a decrease at initial concentration with maximum effect at the highest dose i.e. 10 mg kg^{-1}. The percentage decrease in dry root weight, for the maximum dose of Cip, Levo, Amox, Amp and Oflox as given in table below was 60%, 83%, 71%, 64% and 70%. The percentage decline for dry shoot weight by the highest concentration was 74%, 79%, 87%, 80% and 71% for similar order of antibiotics.

In a study, Li et al. (2011) exposed Oxytetracycline resistant and sensitive wheat cultivars to four different concentrations of 0.01, 0.02, 0.04 and 0.08 $mmol^{-1}$ and noted a decline in shoot length by 3.3-8.57% while shoot biomass was decreased by 5.6-13.75%. In another study conducted by Eggen et al. (2011) Ciprofloxacin and narasin were applied to carrot and barley and their effects were observed. Both antibiotics had an inhibitory effect on root and leaf in case of carrot and for barley, suppression in growth of seed and leaf was noted as a reduced plant biomass. This study corresponds with the present study confirming the negative effect of antibiotics on plant growth.

Table 4.2: Effect of different concentrations of antibiotics on root and shoot biomass.

Sr#	Treatments		Fresh weight		Dry weight	
	Antibiotics	Dose (mg kg^{-1})	Root	Shoot	Root	Shoot
1	Control	0	1.8±0.05	3.3±0.39	$0.6±0.08^{ab}$	$2.3±0.35^{a}$
2	CIP	2	1.7±0.06	2.7±0.24	$0.5±0.06^{bc}$	$0.9±0.17^{de}$
3		4	1.3±0.09	2.0±0.31	$0.3±0.07^{d-f}$	$0.8±0.15^{e-f}$
4		6	0.9±0.09	1.7±0.28	$0.3±0.08^{d-f}$	$0.7±0.17^{e-h}$
5		8	0.5±0.09	1.8±0.41	$0.3±0.06^{d-h}$	$0.7±0.17^{e-h}$
6		10	0.5±0.06	1.7±0.34	$0.2±0.06^{f-i}$	$0.6±0.18^{f-h}$
7	LEV	2	0.5±0.09	2.9±0.22	$0.3±0.06^{de}$	$0.9±0.24^{df}$
8		4	0.4±0.06	2.2±0.39	$0.2±0.05^{e-i}$	$0.8±0.25^{ef}$
9		6	0.4±0.05	1.7±0.24	$0.2±0.09^{h-k}$	$0.7±0.25^{e-h}$
10		8	0.2±0.08	1.4±0.31	$0.1±0.04^{jk}$	$0.7±0.13^{e-h}$
11		10	0.2±0.06	1.2±0.25	$0.1±0.04^{k}$	$0.5±0.13^{g-i}$
12	AMOX	2	1.3±0.06	3.5±0.36	$0.6±0.08^{a}$	$1.5±0.27^{b}$
13		4	1.1±0.13	3±0.36	$0.5±0.05^{c}$	$1.2±0.22^{bc}$
14		6	0.9±0.09	2.2±0.24	$0.2±0.06^{f-i}$	$1.1±0.26^{cd}$
15		8	0.7±0.09	1.9±0.22	$0.2±0.06^{i-k}$	$0.7±0.22^{e-h}$
16		10	0.4±0.08	0.8±0.26	$0.2±0.05^{i-k}$	$0.3±0.08^{i}$
17	AMP	2	1.2±0.08	2.6±0.30	$0.3±0.09^{dg}$	$0.9±0.08^{de}$
18		4	0.9±0.09	2.2±0.31	$0.2±0.07^{f-i}$	$0.8±0.11^{e-g}$
19		6	0.6±0.06	2.6±0.15	$0.2±0.04^{f-i}$	$0.8±0.14^{ef}$
20		8	0.6±0.07	2.4±0.34	$0.2±0.06^{e-i}$	$0.7±0.09^{e-h}$
21		10	0.3±0.09	2.3±0.24	$0.2±0.08^{g-j}$	$0.5±0.19^{hi}$
22	OFL	2	1.4±0.09	3.2±0.33	$0.5±0.06^{bc}$	$1.2±0.08^{bc}$
23		4	1.3±0.05	2.8±0.21	$0.4±0.05^{d}$	$0.9±0.06^{d-f}$
24		6	1.0±0.05	2.5±0.42	$0.3±0.09^{d-f}$	$0.8±0.09^{ef}$
25		8	0.8±0.08	2.3±0.31	$0.2±0.07^{e-i}$	$0.7±0.09^{e-h}$
26		10	0.7±0.09	1.9±0.13	$0.2±0.04^{i-k}$	$0.7±0.17^{e-h}$

The effects of CIP were observed at high concentrations only. This is due to the low bioavailability of CIP which reduces its toxicity against plants. The greater suppression rate in shoot biomass compared to roots can be explained by higher concentration of pharmaceuticals in the above ground parts of plants.

This has been previously reported in different studies including the uptake of carbamacepine (CZB) by cucumber where the concentration detected in stem and roots was low while about 76-84% accumulated in leaves (Shenker et al., 2011). The same case was observed by Wu et al. (2010) with higher CZB uptake in aerial parts of soya bean. Winker et al. (2010) used ryegrass as model plant and found 34% of the drug in aerial plant parts while only 0.3% in roots when treated with CZB contaminated urine.

4.3.3 Effect on number of spikes

Percentage decline in number of spikes after treatment with 2, 4 6, 8 and 10 mg kg^{-1} concentrations is presented in figure 4.4. The difference was not significant at initial concentrations of 2 and 4 mg kg^{-1} for all the antibiotics except for levofloxacin which showed significant negative effect at 2 mg kg^{-1}. The percentage decrease at the highest concentration of 10 mg kg^{-1} for Cip, Lev, Amox, Amp and Oflox was 63%, 73%, 55%, 73% and 63% respectively, with levofloxacin showing the higest overall negative effect compared to the control.

Although there is yet no study available on the effect of antibiotics on the development of spikes of any crop, however many studies have reported the uptake of pharmaceuticals and other antibiotics into the root, stem, and leaves of different vegetables, crop plants and aquatic plants depending upon the compound, its concentration and plant specie (Carvalho et al., 2014). Eggen and Lillo (2012) during a study on uptake of antibiotics on edible parts of plants found that bioconcentration factor of metformin in wheat cereals was 0.29 and highest in rape seeds. However the accumulation factor in fruits and leaves of rape, tomato and squash was less than the grains of wheat, rape, barley and oat.

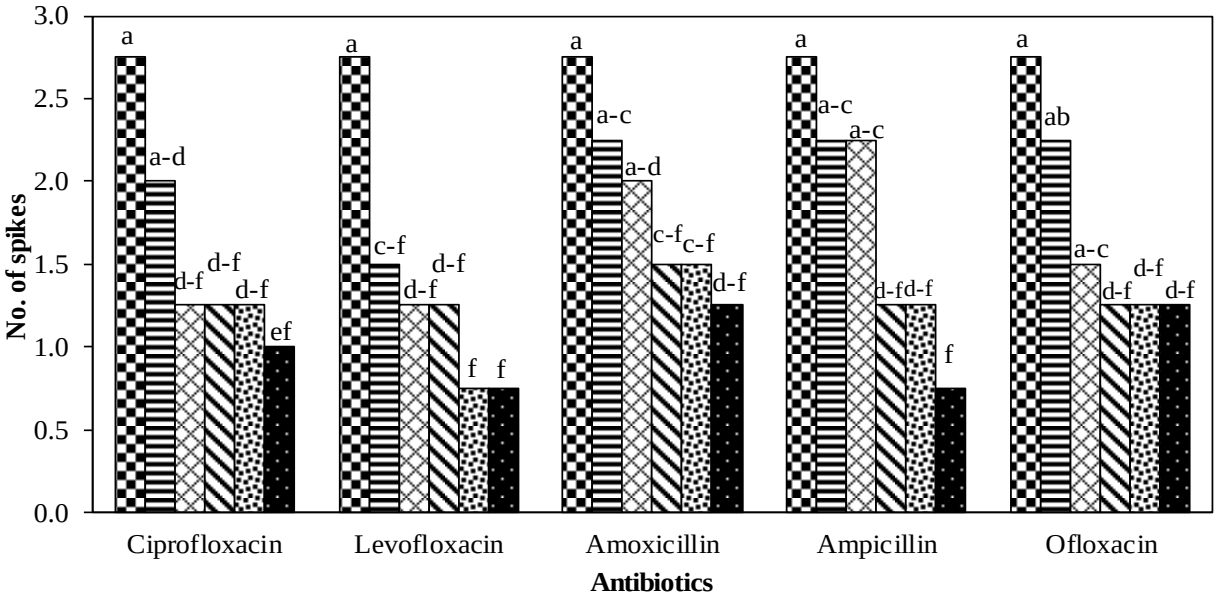


Figure 4.4: Effect of different concentrations of antibiotics on no. of spikes.

= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.4 Effect of antibiotics on composition of vegetative parts

4.4.1 Effect on total phosphorus

Figure 4.5 presents the total phosphorus concentration in shoots of *Triticum aestivum*. The concentration of phosphorus in shoots after treatment with antibiotics declined with the increasing dose, in comparison with control. The initial concentrations of antibiotics did not show a significant declining trend specifically by Cip and Lev. However, inhibitory effect was quite significant at the highest dose i.e. 10 mg kg^{-1} in all antibiotics with Ofloxacin showing greater effect. The percentage decrease at 10 mg kg^{-1} concentration by Cip, Lev, Amox, Amp and Oflox was 16%, 17%, 19%, 21% and 33% respectively.

Batchelder in 1982 reported that contents of calcium, magnesium, potassium and nitrogen contents were significantly reduced in bean plant on exposure to antibiotics as compared to control. The effects of 9 antibiotics on the physiology and secondary metabolites of wheat at the concentrations of 0.5 and 1.5 mg L^{-1} were analyzed by Opris et al. (2013). Ciprofloxacin

and cephalosporins caused a stomatal reduction thereby influencing the net assimilation. A reduction in its photosynthetic responses in chlorophyll, pigments and carotenoids was also observed by application of ciprofloxacin, tetracycline and erythromycin.

Rice, wheat, lettuce, soybeans and alfalfa have been used extensively as test species to study the accumulation of antibiotics in certain parts of these plants. This accumulation occurs through the water transport system and via passive absorption. When these pollutants enter the primary producers, they can cause serious damage to the physiology of plants and their biochemical activities (Liu et al., 2009; Boonsaner and Hawker, 2010; Hillis et al., 2011; Li et al., 2011; Luo et al., 2011).

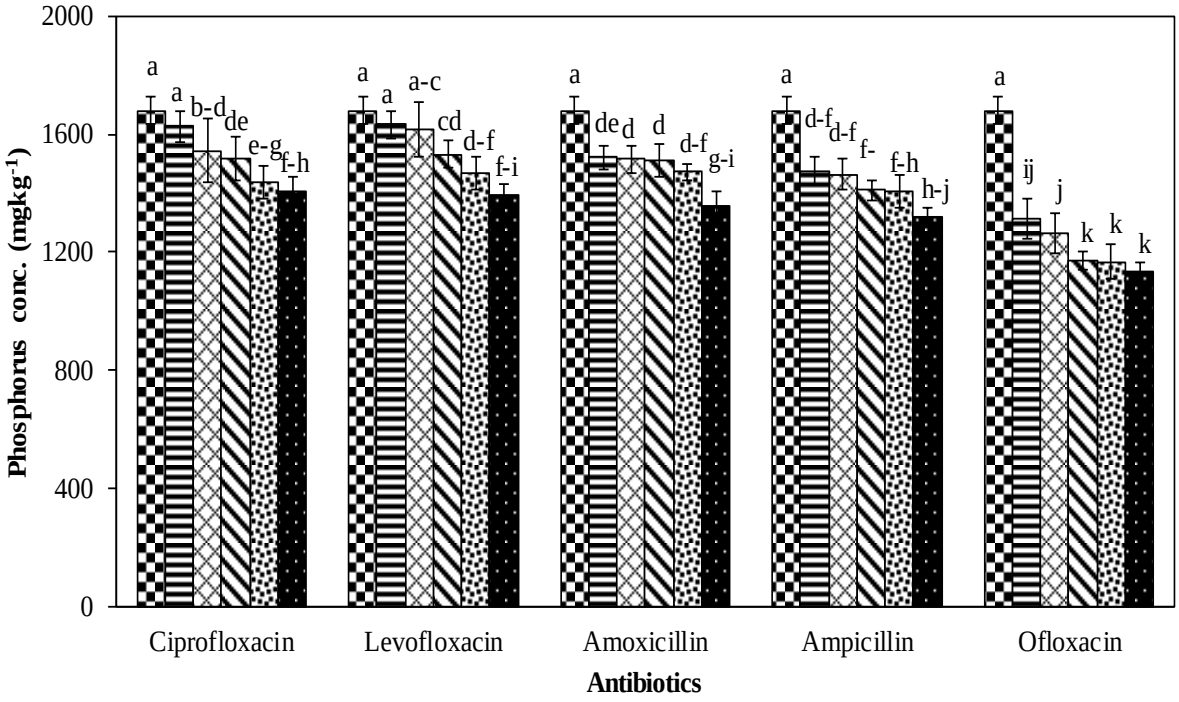


Figure 4.5: Effect of antibiotics on phosphorus concentration in plant shoot.
= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.4.2 Effect on iron

The effects of antibiotics on iron are given in figure 4.6. Among all antibiotics, Oflox and Amp had inhibitory effect at the highest concentration i.e. 10 mg kg^{-1} on plant's iron concentration,

but the difference was not significant at any of the concentration. The percentage decrease at 10 mg kg^{-1} concentration of Cip, Lev, Amox, Amp and Oflox was 12%, 14%, 6%, 25% and 26%, respectively.

No earlier work has been reported on the effect of antibiotics on iron content in plants. However, it has been reported that when these pollutants enter the primary producers, they can cause serious damage to the physiology of plants and their biochemical activities (Liu et al., 2009; Boonsaner and Hawker, 2010; Hillis et al., 2011; Li et al., 2011; Luo et al., 2011).

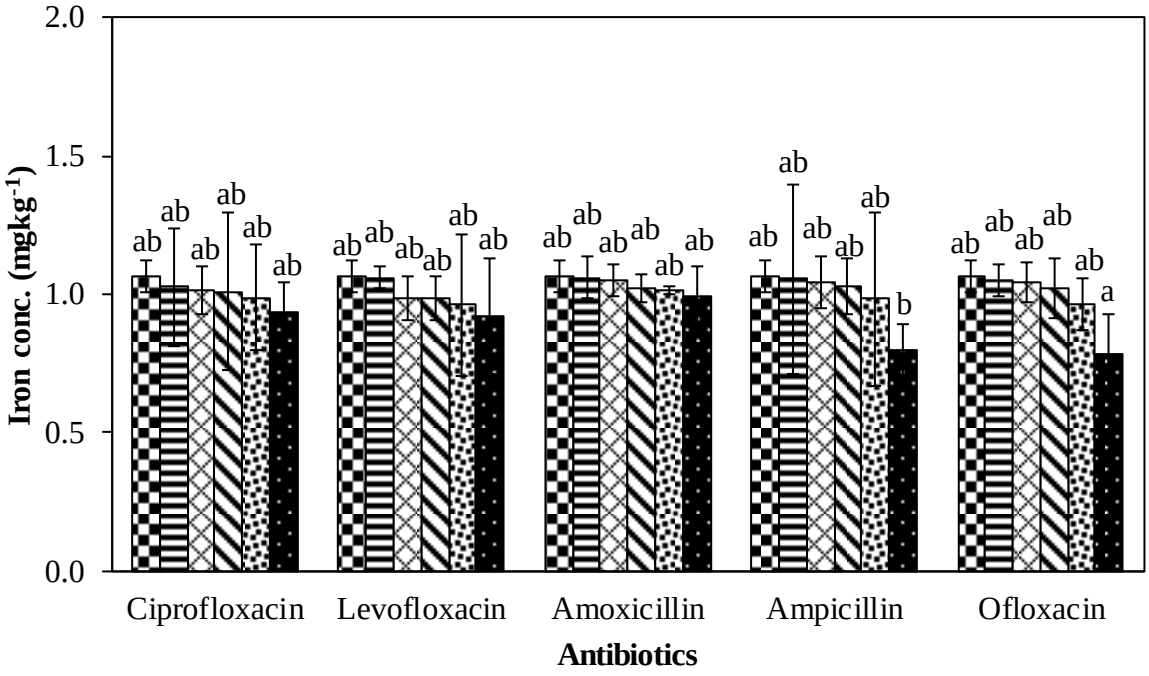


Figure 4.6: Effect of antibiotics on iron concentration in plant shoot.

= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.4.3 Total carbohydrates

All the five antibiotics had an overall declining effect on total carbohydrates in shoot of wheat plant. The percentage decrease at 10 mg kg^{-1} concentration of Cip, Lev, Amox, Amp and Oflox was 14%, 16%, 16%, 16% and 14%, respectively. A significant decline at the highest

concentration i.e. 10 mg kg^{-1} in comparison with control could be seen in figure 4.7. No study has been reported on the effect of antibiotics on carbohydrate content in plants.

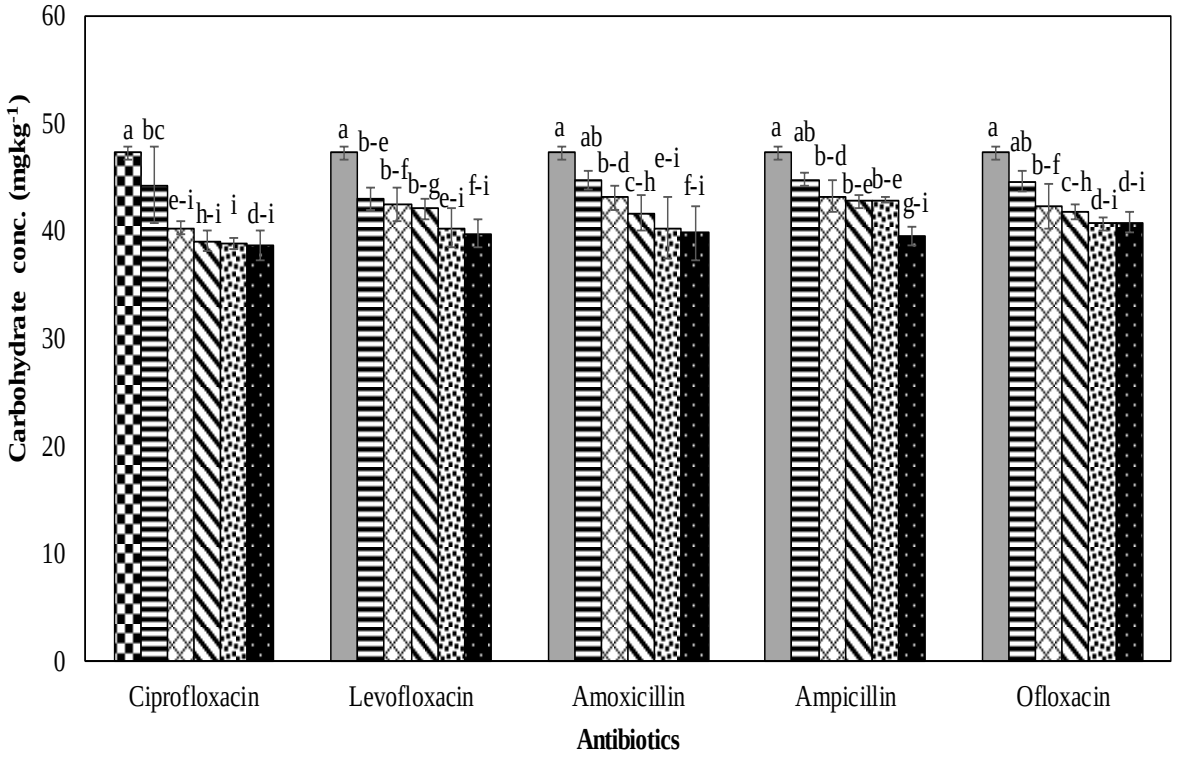


Figure 4.7: Effect of antibiotics on carbohydrate concentration in plant shoot.
= 0 mg kg^{-1}, = 2 mg kg^{-1}, = 4 mg kg^{-1}, = 6 mg kg^{-1}, = 8 mg kg^{-1}, = 10 mg kg^{-1}.

4.4.4 Protein

The selected antibiotics had an overall declining effect on the protein concentration in grains (figure 4.8). The percentage decrease in protein concentration by Cip, Lev, Amox, Amp and Oflox was 17%, 27%, 36%, 25% and 34%, respectively at the highest antibiotic concentration i.e. 10 mg kg^{-1}. Amoxicillin had the highest negative effect followed by Oflox, Lev, Amp and Cip. Batchelder (1982) reported that contents of calcium, magnesium, potassium and nitrogen contents were significantly reduced in bean plant on exposure to antibiotics as compared to control.

Jin et al. (2009) concluded that pharmaceuticals specifically antibiotics, e.g. tetracycline and erythromycin, between the concentration of 1.4 and 22.4 mg L^{-1} had an adverse effect on protein synthesis in wheat crop. Similar results were obtained by Jing et al. (2009), with triclosan (which is also an antimicrobial agent) that also had an inhibitory effect on protein synthesis in wheat. Daghrir & Drogui (2013) studied the effects of tetracyclines also concluded that tetracyclines could also inhibit protein synthesis in aquatic plants.

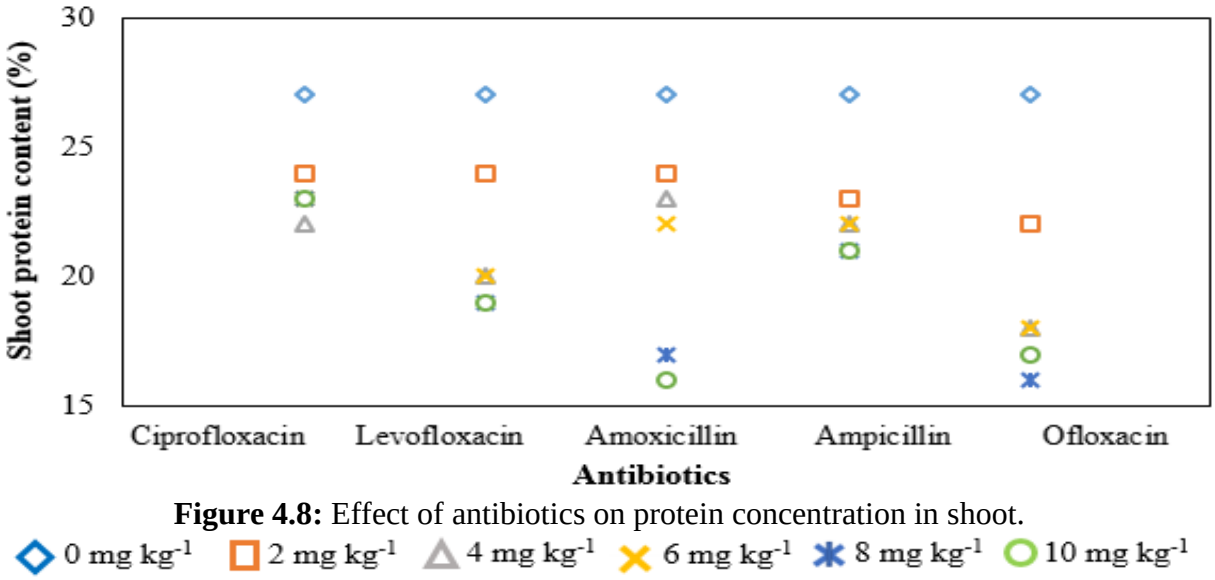


Figure 4.8: Effect of antibiotics on protein concentration in shoot.

4.4.5 Micronucleus assay

During this study, the toxicity of all the five antibiotics was studied using mitotic index and micronucleus induction in the root tips. The results of the micronucleus assay under different concentrations of Ciprofloxacin, Levofloxacin, Amoxicillin, Ampicillin and Ofloxacin are summarized in the Fig 4.9. Each experiment was performed in triplicate and data represents mean ± standard deviation of all 3 replicates. According to the results, the micronuclei formation in treated cells had shown significance difference as compared to the control having no micronucleus.

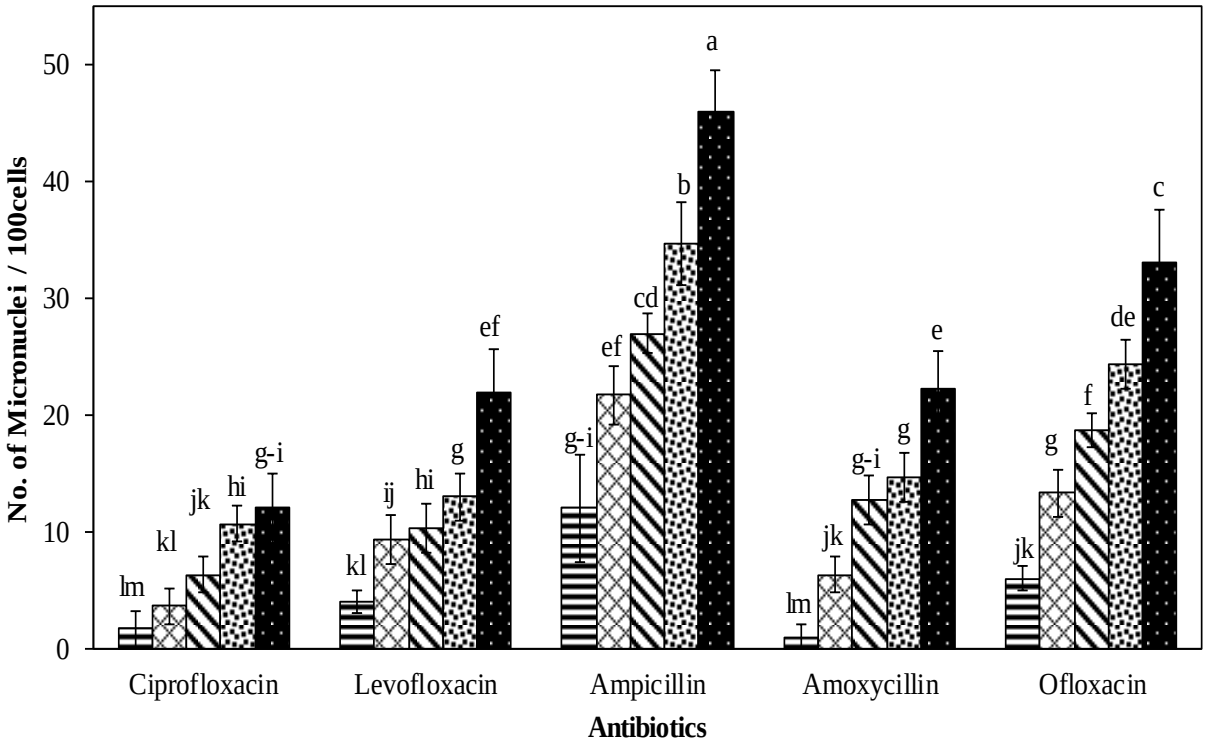


Figure 4.9: Number of micronuclei produced as a result of toxicity by antibiotics.
▤ = 2 mg kg^{-1}, ⊠ = 4 mg kg^{-1}, ▧ = 6 mg kg^{-1}, ⊡ = 8 mg kg^{-1}, ■ = 10 mg kg^{-1}.

The results of MN assay indicated that no. of micronuclei produced was increased with increasing concentration of antibiotics. Only dividing cells were present in root apical meristem of control seeds and no micronuclei was found. At the highest antibiotic concentration, i.e. 10 mg L^{-1}, micro-nuclei were produced in the order of ampicillin> ofloxacin> amoxicillin> levofloxacin> ciprofloxacin.

The significant differences were observed only at 10 mg L^{-1} (i.e. 46 MN per 100 cells) and 8 mg L^{-1} (i.e. 34 MN per 100 cells) of ampicillin and at 10 mg L^{-1} (i.e. 33 MN per 100 cells) of ofloxacin. Minimum effect was found at the lowest concentration i.e. 2 mg L^{-1} in the order of ampicillin> ofloxacin> levofloxacin> ciprofloxacin> amoxicillin. All the tested concentrations had shown micronuclei formation in cells. Many studies have reported that antibiotics into the farmland, can significantly increase the production of micro-nuclei in plant's root tip cells,

causing damage to genetic material and affecting the growth of plants (Migliore et al. 2003; Wang et al. 2016).

Xie et al. (2010) found that chlortetracycline at lower concentrations (5 and 10 mg L^{-1}) stimulated the cell mitotic division whereas the higher concentrations (300 mg L^{-1}) hindered the process. The low concentrations of chlortetracycline resulted in an increase in number of micronuclei, chromosomal aberration and sister chromatid exchange in root tips and the higher concentrations showed significant increase. It was also found that number of micronuclei decreased at 300 mg L^{-1} due to acute toxicity.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Antibiotics are being used since decades for treatment of human and animal bacterial infections. These are constantly entering the environment through various sources and are causing problems for the environment. Present study determined the toxic effects of five antibiotics on *T. aestivum* (wheat) which is an important agricultural crop and is the main food source in most of the countries. The key findings of the present study are as follow:

- All the five antibiotics had no significant negative effect on seed germination except at higher concentration of antibiotics i.e. 8 and 10 mg kg^{-1}, seed germination rate was significantly affected.
- A significant decrease occurred, both in root and shoot length with increase in the concentration of dose, in comparison with the control. The effect on root length was greater than that on shoot. However, dry biomass of root and shoot showed a significant declining trend at higher concentrations i.e. 8 and 10 mg kg^{-1}.
- Number of spikes was also affected significantly by all doses.
- The total phosphorus and total carbohydrate concentration in plant shoot declined with increasing antibiotic concentration but the significant decline only occurred at the higher doses of all five antibiotics.
- All the five antibiotics had inhibitory effect at the highest concentration i.e. 10 mg kg^{-1}, on plant's iron concentration. Amp and Oflox showed the significant negative affect at the maximum antibiotic concentration.
- The selected antibiotics had an overall declining effect on the protein concentration in plant shoot. Amoxicillin had the highest negative effect followed by Oflox, Lev, Amp and Cip.

- No. of micronuclei increased significantly with increasing concentration of antibiotics. Ampicillin and Ofloxacin caused greater toxicity than other antibiotics at 10 mg kg^{-1} concentration.

In conclusion, antibiotics can be taken up by plants and can result in reduced crop yield and low nutritional quality. They can also cause genotoxicity and can create a havoc for humans and animals upon entering food chain.

5.2 Future recommendations

From current study, noteworthy effects were found on *Triticum aestivum* upon exposure to antibiotics. Such exposure studies could be helpful to overcome the issues of release of antibiotics in wastewater, making threshold levels, and help in providing better crop yield by reducing use of untreated water for irrigation purpose and can ultimately help in betterment of environment. Following are the recommendations for future work:

- Mechanism of uptake and transport of antibiotics into plants require investigations as the different antibiotics might have different tendencies to get accumulated in different parts of plants.
- Studies can be carried out on phytoremediation, uptake and degradation efficiency of plants regarding pharmaceuticals and antibiotics in order to provide remediation solution.
- Environmental standards should be devised and legislation is required for such type of pollutants to protect release of these into the environment.

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