ROLE OF ORGANIC AMENDMENTS IN ALLEVIATION OF ANTIBIOTIC TOXICITY IN PLANTS (*ORYZA SATIVA* L.)

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By

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CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Role of organic amendments in alleviation of antibiotic toxicity in plants (*Oryza sativa* L.)"** submitted by Ms. Ayesha Mukhtar has been found satisfactory for the 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 have always been a source of inspiration for me and stood beside me at every moment in my life

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Ayesha Mukhtar

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

UV	Ultra Violet
OTC	Oxytetracycline
CTC	Chlortetracycline
RCF	Root concentration factor
VAs	Veterinary antibiotics
PCPs	Personal care products
NOR	Norfloxacin
TCS	Triclosan
PPCPs	Pharmaceuticals and Personal-Care Products
DNA	Deoxyribose Nucleic Acid
TUNEL	Terminal deoxynucleotidyl transferase Nick-End Labeling

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Extensive use of antibiotics and direct discharge of their residues in hospital and industrial wastewater streams result in significant antibiotics pollution in the environment. A limited number of studies have been conducted to assess the role of organic amendments in alleviation of antibiotics' stress on plants. The main aim of this study was to gain insight into impacts of five antibiotics (Ciprofloxacin, Levofloxacin, Ofloxacin, Amoxicillin and Ampicillin) on plants and antibiotic stress alleviation through application of different organic amendments. A pot experiment was conducted with rice (*Oryza sativa* L.) as model plant. Effect of five antibiotics was observed in the presence of three organic amendments (Rice husk, farmyard manure and poultry liter) upon application of antibiotics @10 mg kg^{-1} of soil. In pot experiment, antibiotics and organic amendments were applied to 3-week old seedlings and after four months, plants were harvested. After that physical growth parameters like root shoot length, plant biomass and nutritional composition including grain protein content, carbohydrates, phosphorous and iron were determined. At germination stage, germination rate, seedling root-shoot length, seedling biomass and vigor index were negatively impacted. Approximately, 1-2 days delay in germination was also observed. Upon exposure to antibiotics, seedling length was decreased by 71%, seedling dry biomass by 61% and vigor index by 59%. Application of organic amendments alleviated the antibiotic stress in seedling dry biomass, length and vigor index by 1.3, 3.8 and 2.1 folds, respectively as compared to the antibiotic controls. Concentration of phosphorous, iron, carbohydrates and proteins declined by 62%, 23%, 30% and 22% respectively, upon application of different antibiotics. Genotoxicity was assessed by comet assay that showed antibiotic toxicity in the following order: levofloxacin > ofloxacin> ciprofloxacin > ampicillin >amoxicillin. Poultry litter helped in alleviation of genotoxicity by 59% collectively. In general, application of amendments alleviated phytotoxicity as well as genotoxicity in the presence of different antibiotics.

Chapter 1

INTRODUCTION

1.1 Background

Rice (*Oryza sativa* L.) is an important cash crop and is a staple food in many countries in Asia. It is third largest crop of Pakistan in terms of cultivated area. Pakistan is the leading producer and exporter of rice (Hamid et al., 2018). According to a survey in 2012, about 162.3 million hectares land was dedicated for rice cultivation with a production yield of 738.1 million tons annually. On average in 2012, 4.5 tons per hectare of the total arable land was associated with rice production globally, making it the second largest cultivated crop of the world (FAO, 2014). Asian countries are the largest consumers of the rice as this serial crop is the staple food in half of the world. Rice is a rich source of fiber, carbohydrates, vitamins, minerals and even proteins, which are essential for the growth and development of our bodies (Aguilar-Garcia et al., 2007). The most successful family of the drugs that deals with the improvement of human health is antibiotics. Beside its large applications in human treatment, antibiotics are also used in treatment of animal infections and cattle farming as well as for infection prevention in plants (McManus et al., 2002; Singer et al., 2003; Cabello, 2006). Extensive use of antibiotics has increased the concentration of these drugs in natural ecosystem (Sarmah et al., 2006). Result of such human activities and discharge of wastewater into the nearby rivers and streams without treatment spread these drugs in to the ecosystem. Later on, this water is used to irrigate the agricultural lands and taken by the plants (Kim and Carlson, 2007). These drugs are continually added to the ecosystem from a variety of sources which make them persistent and very stable in the environment (Carvalho et al. 2016). Use of wastewater for arable purposes is a common practice all over the world (Fatta-Kassinos et al., 2011) and serious threat for food chain contamination.

Another source of these antibiotics is direct application of animal manure as fertilizer, which contains certain amount of veterinary antibiotics that accumulate into the soil and further taken up by the plants (Knapp et al., 2009). Effects of the antibiotics on human health, animals' health and aquatic life have been studied frequently but there is a research gap in assessing the impacts of these environmental pollutants on cash crops, moreover the mechanism of uptake by plants and change in plant physiology by these agents, is yet to be documented (Xu et al., 2018).

1.2 Antibiotic classes

1.2.1 Penicillin and fluoroquinolones

Fluoroquinolones is a class of antibiotics which are very persistent in nature due to their strong adsorbing capacity and slow degradation (Tandon et al., 2013). Fluoroquinolones are frequently added to the environment by hospitals, veterinary applications and household activities. It is the 3rd largest class of the antibiotics holding 17% of global medicine market (Doorslaer et al., 2014). Almost 70% of these antibiotics are released in their non-metabolic form that introduce microbial resistance in the environment.

Penicillin is a widely-used drug in human and livestock medicine, because it is having antimicrobial properties due to its β-lactam ring. Extensive studies have been conducted to confirm the presence of this group of drugs in wastewater, for example, ampicillin and amoxicillin (Elmolla and Chaudhuri, 2010). Original compound β-lactam is chemically modified in manufacturing of these drugs. β-lactam and some other subgroups of penicillin cater the largest market approximately 50 to 70 percent of total antibiotics worldwide (Kummerer, 2009).

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. Penicillin group is used to treat different types of gram-negative and gram-positive bacterial infections. Due to the presence of beta lactam group in their structure, they are also called beta lactam antibiotics. Beta lactamase enzyme produced by gram-negative and gram-positive bacteria is hydrolyzed by this beta lactam ring. Figure 1.1 is showing a typical structure of Fluoroquinolones and penicillin.

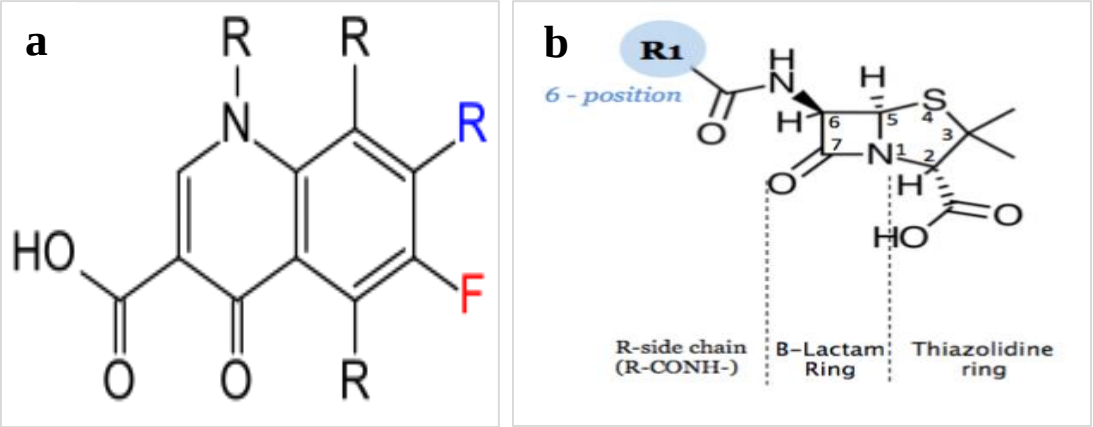

Figure 1.1: Typical structure of Fluoroquinolones and penicillin. a. Fluoroquinolone b. Penicillin

Antibiotics are a matter of concern due to their frequent detection in waste and ground waters, induction of resistance in microbial communities, effects on biota and human health. However, very little information is available regarding any subgroup of these antibiotics (Bouki et al. 2013).

1.3 Plant growth and organic amendments

Biosolids are added in soil as a source of minerals and organic compounds to restore organic matter, improve soil fertility, improve soils' biological and physicochemical features, enable the resettlement of plants and restore altered soil microbial communities (Cogger et al., 2013).

Biosolids in the soil act as glue to prevent compaction of soil particles, which increase root growth of the plants. With an improvement in soil structure, soil water absorption and water holding capacity is increased, chances of erosion by wind or rain are reduced. They also hold the soluble nutrients for many plants in the soil to make them available for plant growth. When soil microorganisms break down the organic matter, it becomes easier for plant to uptake. Soil organic matter also leads to increase the number of microorganisms in the soil, which leads to reduce plant pathogenic nematodes, diseases and pathogens. All these together make the soil food web (Thiele-Bruhn et al., 2004).

1.4 Antibiotic use in different fields

Since 1940, antibiotics have been a major part of health care industry. Their vital role in treating serious infections during human surgeries, cancer treatment, livestock and other food animal treatments is unavoidable. In the treatment and prevention of human and animal illness, tons of pharmacologically active substances are annually used (Dıaz-Cruz et al., 2003; Sarmah et al., 2006). Main purpose of antibiotics is to cease harmful bacterial activities in human and animals to protect their health. At the end of treatment, most of antibiotics are being excreted from the body as metabolites, some of them are still bioactive (Sarmah et al., 2006). This is the fact which makes them potentially harmful for the environment, other organisms and for the soil microorganisms (Baguer et al., 2000).

1.5 Sources of antibiotics

Antibiotics have become a major concern due to their large environmental impacts and huge demand. In soil, municipal and industrial wastewater, sediments, animal manure, surface and groundwater bodies. A large amount and range of Veterinary antibiotics are detected (Igiehon and Babalola, 2017). Whereas, in agro-ecosystems, the contamination of fluoroquinolones,

tetracyclines and sulfonamides antibiotics is serious concern globally (Spence et al., 2014). 75% of these drugs passed through animal digestive tracts unaltered, then they are egested in the form of urine and feces in the fields or directly applied as fertilizer. Irrigation of crops by antibiotic contaminated water is another source of antibiotic contamination in agricultural land. Due to their persistent nature, they get accumulated in the soil. Level of exposure also depends on physicochemical properties of the antibiotics and their sorption potential (Hou et al., 2014). Under contaminated conditions plants (potato, carrot, tomato, cucumber, spinach, cabbage, lettuce, corn, wheat and rice) can uptake several toxic compounds (ofloxacin, tylosin, sulfamethoxazole, tetracycline, ciprofloxacin, amoxicillin and sulfamethazine) from the growth media through roots where they get accumulated. Diffused antibiotic pollution in to the environment makes their way to the food supply system (Igiehon and Babalola, 2018).

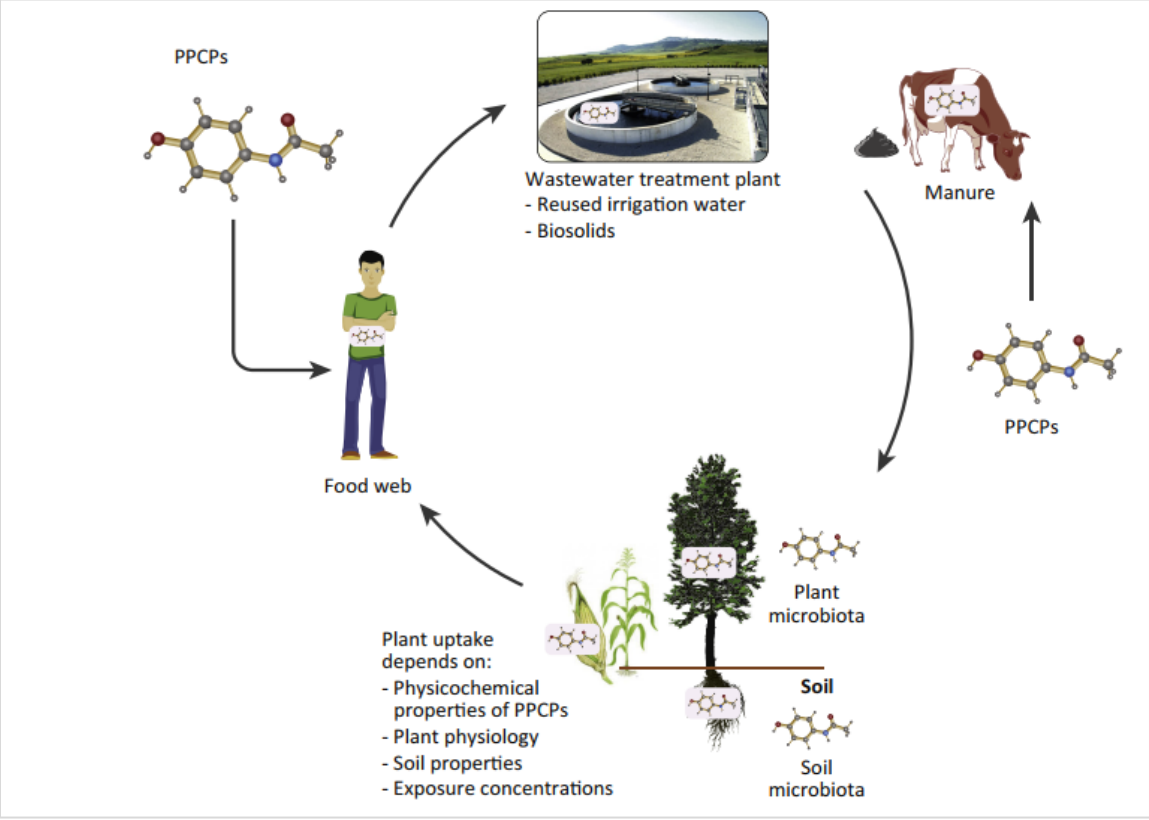

Figure 1.2: Main sources and fates of pharmaceuticals and personal care products in plants and environment. Human activities and livestock farming are main sources of pharmaceuticals in the environment. Treated water from wastewater treatment plants is used as irrigation water from where antibiotics are taken up by plants depending upon plant physiology and physiochemical properties of antibiotics. In this way, antibiotics enter into food chain and ultimately affect human beings.

1.6 Impacts of antibiotics

1.6.1 Impacts on environment

De Graaff et al. (2011) investigated the extensive release of these drugs in the environment that poses such large-scale threats that are not being realized completely. One of the concerns regarding these drugs is, they damage the plants and affect their growth when taken up by the roots (Du and Liu, 2012). Several researches showed some major or minor effects of these drugs on the plants, yet an extensive work has to be done.

1.6.2 Impacts on plants

Antibiotics penetration in the arable land can impact the vegetation growth. It may also cause harm to soil microbial activities (Jjemba, 2002). Few studies have been conducted to assess the phytotoxicity of the plants. Seed germination and plant growth tests can be used to assess phytotoxicity of certain chemicals like enrofloxacin, sulphadimethoxine and oxyetracycline (Kong et al., 2007). Antibiotic effects differ between compounds and between types of plant species (Farkas et al., 2007; Jjemba, 2002). It is observed that tetracycline has phytotoxic effects on different plant species by enhancing antioxidant enzyme activity (Xie et al., 2011). Whereas chlortetracycline showed an increased stress on plant proteins (such as peroxidases and glutathione S-transferases) in maize plant but not in pinto beans (Farkas et al., 2007).

Very limited knowledge is available on antibiotics' potential effects on plants genotoxicity and nutritional composition. Most of the studies focus on the physical growth parameters or the accumulation rate in the plants.

1.7 Significance of the study

Antibiotics are extensively used worldwide, which accumulate in agricultural fields through irrigation and manure application. As crops are major component of terrestrial environment and a major source of antibiotics in food chain, therefore even a little concentration can cause harm to the plants. Main focus of this study was to understand the toxicity profile of these pollutants on plants. Currently, no work has been done to alleviate antibiotic stress with organic amendments. This study was designed to gain insight into the impacts of different antibiotics and organic amendments.

1.8 Objectives

Based on literature review and previous studies conducted within institute, this study was designed with the following objectives:

1. To determine the changes at germination stage in physical growth parameters and germination rate upon exposure to different antibiotics.
2. To determine the changes in physical growth parameters and nutritional composition at harvesting stage.
3. Role of different organic amendments in alleviation of antibiotic toxicity in *Oryza sativa* L.

1.9 Scope of the study

To deal with the emerging environmental and agricultural issues, a country like Pakistan needs an advance research in environmental and plant studies. This study helped to investigate and measure the adverse effects of antibiotics on rice crop. These agents caused phytotoxicity in

crops, it is a major threat to agricultural sector, human health and food security. This study might help in developing methods to combat food security issues.

Chapter 2

REVIEW OF LITERATURE

2.1 Sources of antibiotics

2.1.1 Pharmaceuticals

Per capita use of medicines varies worldwide. A medicine which is in a high demand in one region might be less used or banned in another region. Antibiotics or other medicines which are being used by the humans or animals are excreted from their bodies in the environment in bioactive forms (Thai et al., 2018).

Previously no or very little attention has been paid to pharmaceutical products, but in recent past, it has become an issue of serious concern. In Asian countries, antibiotic residues in wastewaters of pharmaceutical industries have been detected up to levels of 10,000 mg L^{-1} (Cardoso et al., 2014; Caldwell, 2016). About 31 mg L^{-1} concentration of ciprofloxacin has been detected in effluents released by few Asian countries (Larsson et al., 2007; Li et al., 2008). Díaz et al. (2018) investigated that even the effluent of sewage treatment plants of manufacturing facilities, in developed countries also have a significant amount of antibiotics in it.

2.1.2 Livestock farming

In livestock farming, use of therapeutic and sub-therapeutic antibiotics is a common practice worldwide. Moreover, uptake of these compounds becomes relatively easy due to physiological interactions. Their continuous interaction for longer time gives rise to higher solubility rates and continuous introduction makes them very persistent in the environment (Tasho and Cho, 2016). Intensive farming proposes significant use of antibiotics for treatment

of animal diseases and in feed additives. Residues of these drugs are frequently detected in animal feces, which at the end is used as manure infields (Migliore et al., 2003).

At present, manure-based fertilizers and animal manure is one of the biggest sources of antibiotic effluence from agricultural landscapes (Samrah et al., 2006). Runoff from agricultural lands enters to the aquatic ecosystems. In this way, antibiotics become a part of surface water, seawater and ground water bodies (Santos et al., 2010). Due to the continuous discharge and inherent biological activity in water systems, antibiotics may impact non-target organisms (Nie et al., 2013).

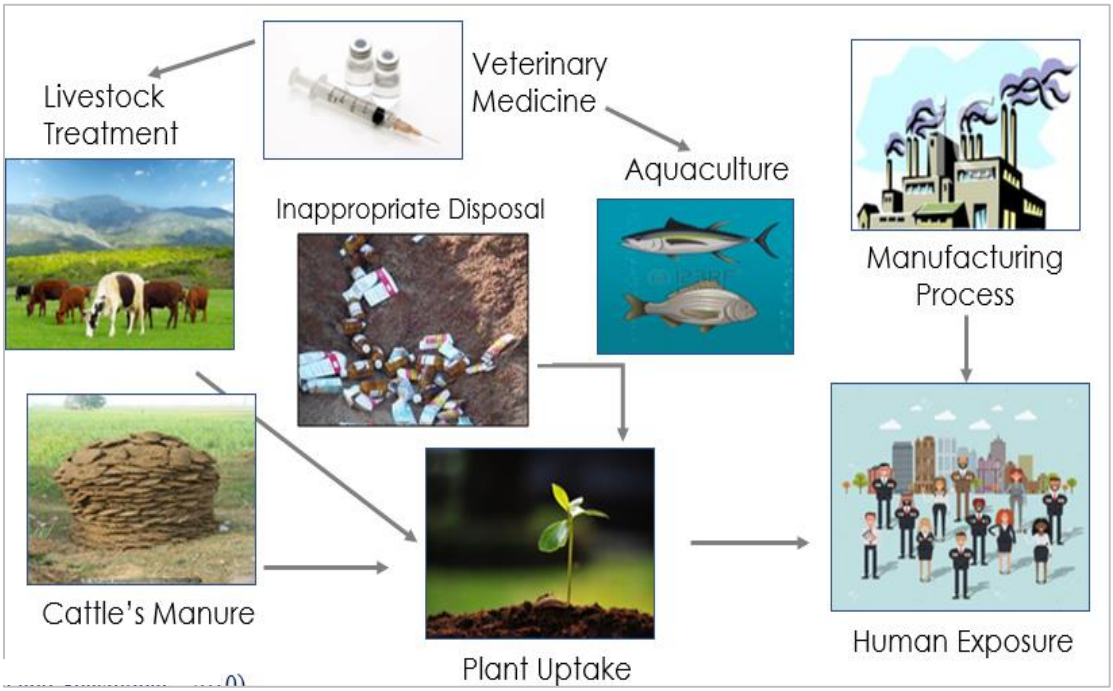

Figure 2.1: Possible sources and pathways of antibiotics in the environment (Tasho and Cho, 2016).

2.1.3 Presence in wastewater

A study conducted by Diwan et al. (2010) in India, to investigate antibiotics' presence in ground water, drinking water and wastewater of two different hospitals in Ujjain district. They found that the ground and drinking waters were safe. Whereas, hospital effluent was full of

multiple types of antibiotics such as: norfloxacin, ceftriaxone, metronidazole, ofloxacin, levofloxacin, ciprofloxacin and tinidazole, ranging between 1.4 – 236.6 µg L^{-1}. Presence of such large amount of antibiotics in the hospitals wastewater will be a serious threat to the plants irrigated with this water. Antibiotic residues have also been detected in digested sludge, activated sludge and sewage sludge (Ashfaq et al., 2017).

Wei et al. (2012) determined that in a city of eastern China, wastewater coming from animal sources and pharmaceuticals contained three antibiotics i.e. Ciprofloxacin, Florfenicol and Enrofloxacin. Results showed that enrofloxacin was present in range of 0.05-4.24 µg L^{-1} in pond and river water, respectively. Similarly, ciprofloxacin was found up to 3.35 µg L^{-1} in animal wastewater, 5.30 µg L^{-1} in pond water and 2.10 µg L^{-1} in river water. River contained high antibiotic amounts which were affecting river ecosystem and environment.

A study was conducted by Harris and Cummins (2012) that showed ciprofloxacin concentration was higher than expected concentrations in wastewater treatment plant. Moreover, they concluded that hospital waste water should not be mixed with municipal wastewater which reduces treatment efficiency. Such compounds remained untreated and when discharged in to the environment, they become a big risk.

In China, a study was conducted by Chang et al. (2010) to determine antibiotic presence in upcoming sewage from hospitals, slaughterhouse, treatment plant and nurseries in the region of three Gorge reservoirs. Sampling was done from four hospitals, one wastewater treatment plant, one nursery and one slaughter house of the local area. Six antibiotics were detected in the wastewater with ofloxacin having higher concentration ranged from 1.660 to 4.240 µg L^{-1} in all sources. Second highest detected concentration was of norfloxacin from 1.36 to 1.62 µg L^{-1}. Ciprofloxacin concentration was ranging from 0.011 to 0.136 µg L^{-1}. The lowest found

concentration of trimethoprim was 0.061 and the highest was 0.174 µg L^{-1}. Moreover, results showed that antibiotic removal efficiencies were ranged from 80% to 100%. It means that these antibiotics even after treatment can introduce into the environment to make it vulnerable for other living organisms.

2.2 Use of wastewater in irrigation

Wastewater is a major concern of many countries and it is a global issue. There are many causes of water pollution but the major source is the domestic and industrial wastewater. The water from these sources is discharged directly into the fresh or clean water bodies without any treatment (Ibekwe et al., 2018).

Ahmad et al. (2012) conducted a study to understand the role of untreated wastewater in the River Ravi (main river of Lahore) in spreading the antibiotics and resistant bacteria species. The river was contaminated with the wastewater of the whole city. Two antibiotics i.e. Ciprofloxacin (CIP) and Norfloxacin (NOR) were investigated in the river and the results showed, towards downstream, the concentration of these antibiotics was increased. The reason was untreated wastewater with a huge load of antibiotics. This situation could result into antibiotics resistance in bacteria and severe human health issues.

2.3 Antibiotic pathway in plants

Plants are one of the excellent tracers of global pollution (Lin, 2015) because they are present everywhere on the planet. They uptake chemical compounds from the water in which they are irrigated, from atmosphere and from the soil in which they are grown. Thousands of tons of pharmaceutical products produced each year, from them several have been documented and detected in plant bodies (Manzetti and Ghisi., 2014).

Some common veterinary and human antibiotics that found in plant tissues include: clarithromycin, norfloxacin, enrofloxacin erythromycin, azithromycin, ciprofloxacin, sulfamethazine, sulfapyridine, tylosin, ofloxacin, levofloxacin, doxycycline, lincomycin, chlortetracycline, trimethoprim, salinomycin, methronidazole, oxytetracycline, sulphadimethoxine, tetracycline, roxithromycin and sulfamethoxazole. These antibiotics are taken up by plants, they start accumulated and metabolized by plants. They have several negative impacts including effecting microbiota, phytotoxicity and genotoxicity. Currently very limited research has been reported on lifecycle of these compounds and their effects on environment (Bartrons and Peñuelas, 2017).

2.3.1 Plant uptake, bioaccumulation, and metabolism

Antibiotic uptake by plats have received increased attention. In various plant species and tissue large amounts of PPCPs have been found (Hyland et al., 2015; Wu et al., 2015) with varying concentrations from no detection to 487 µg kg^{-1}. Physiochemical properties like hydrophobicity and ionization behavior play a major role in the extent of uptake, accumulation, translocation and transformation of antibiotics. Other influencing factors include exposure concentration and duration and water quality. Pathways of uptake and bioaccumulation of these compounds are currently not well understood (Miller et al., 2016). Plants uptake these antibiotics through roots and aerial tissues (figure 2.2). Roots take up these antibiotics via diffusion of dissolved compounds or by mass flow (Miller et al., 2016). Ionized compounds enter roots by diffusion and electrostatic interactions of the ionic fraction (Trapp and Legind, 2011).

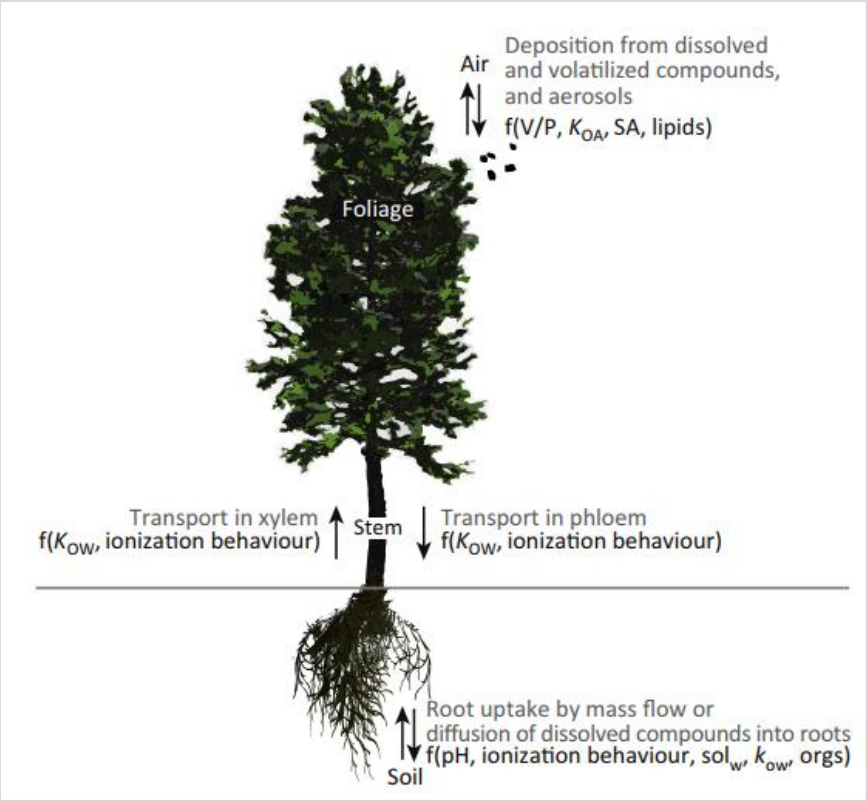

Figure 2.2: Principal Pathways for the Uptake of Pharmaceuticals

(Bartons & Penuelas, 2017)

2.4 Antibiotics' effect on plants

Plants play a vital role in maintenance of ecosystem. Introduction of pollutants in the environment pose a serious threat not only to human beings but also on plants. Antibiotics seriously alter the physiology of plants and their biochemical activities. Figure 2.1 illustrates some negative impacts caused by antibiotic pollution in different plant species (Yi et al., 2016).

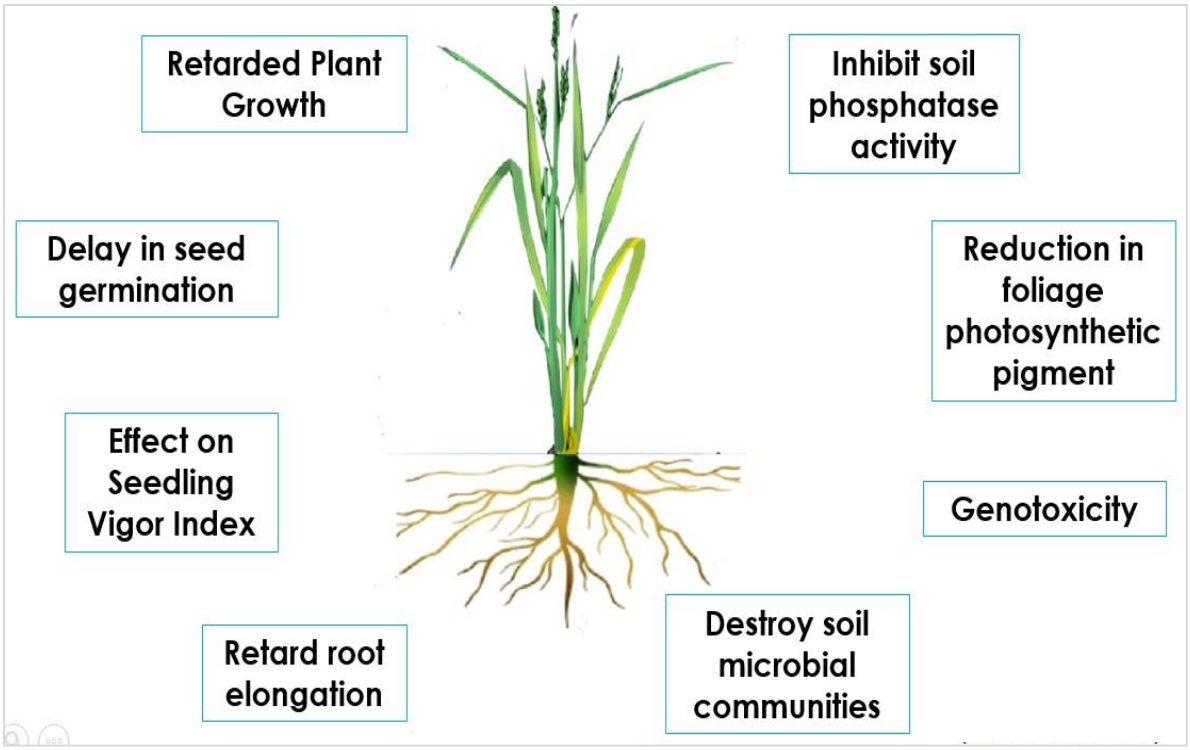

Figure 2.3: Effects of antibiotics on plants

Pollutants once introduced in the environment are entered in to the food chain when taken up by primary producers. Many studies have been conducted to investigate the effect of antibiotics on plants and their uptake (Li et al., 2011; Luo et al., 2011). Accumulation of certain antibiotics in different plant species like rice, wheat, lettuce, soybeans and alfalfa have been reported in a number of studies. Antibiotic toxicity at minimum and maximum concentrations have been documented in table 2.1.

Table 2.1: Maximum and minimum antibiotic concentrations and subsequent Phytotoxicities of Each Compound under Realistic Field Conditions

Compound	Plants	Min-max [a]	Plant and Algae	Effect	Min-max [b]	Reference
Amoxicillin	Chinese white cabbage (*Brassica rapa*), water spinach (*Ipomoea aquatica*), rice (*Oryza sativa*), Chinese radish (*Raphanus sativus*), corn (*Zea mays*)	2.6–22.4 mg kg^{-1} dry weight	Alfalfa (*Medicago sativa*), carrot (*Dacus carota*), lettuce (*Lactuca sativa*)	Growth and germination reduction	0.001–10 mg L^{-1}	(pan et al., 2014) (Hillis et al., 2011)
Sulfadiazine	Winter wheat (*Triticum aestivum*)	ND–487 mg kg1 dry weight	Maize (*Zea mays*)	**Death of plant**	0 and 200 mg kg dry weight, spiked soil	(Grote et al., 2007)
Chlortetracycline	Corn (*Zea mays*), green onion (*Allium cepa*), cabbage (*Brassica oleracea*)	2–17 mg kg^{-1} fresh weight	Alfalfa (*Medicago sativa*), carrot (*Dacus carota*), lettuce (*Lactuca sativa*)	Growth and germination reduction	0.001–10 mg L^{-1}	(Kummar et al., 2005) (Hillis et al., 2011)

Tetracycline	Chinese white cabbage (*Brassica rapa*), water spinach (*Ipomoea aquatica*), rice (*Oryza sativa*), Chinese radish (*Raphanus sativus*), corn (*Zea mays*)	4.0–10.1 mg kg^{-1} dry weight	Alfalfa (*Medicago sativa*), carrot (*Dacus carota*), lettuce (*Lactuca sativa*)	Growth and germination reduction	0.001–10 mg L^{-1}	(Pan et al., 2014) (Hillis et al., 2011)
Amoxicillin	Radish (*Raphanus sativus*), rape (*Brassica napus*), celery (*Apium graveolens*), coriander (*Coriandrum sativum*)	ND–330 mg kg^{-1} dry weight	Cucumber (*Cucumis sativus*), rice (*Oryza sativa*), sweet oat (*Cichaorium endivia*)	Growth and germination reduction	0–500 mg L^{-1}	(Hu et al., 2010) (Liu et al., 2009)

a Min–max concentration in plants.
b Concentrations in irrigation water (mg L^{-1}) or in soil (mg kg^{-1}).

Motwani, and Mehta (2018) studied effect of three antibiotics (tetracycline, amoxicillin and trimethoprim) on three different rice (*Oryza sativa* L.) varieties. Antibiotic applied concentration was 0, 1, 10, 100, 1000, 10000 mg L^{-1} for each antibiotic. None of the antibiotic showed a significant effect on seed germination for three types of rice plant species. Whereas, root elongation, shoot measurements and seedling vigor index was highly affected at the

highest concentration. Most sensitive end-point for phytotoxicity was root elongation. EC50 varied among different varieties i.e. ranged from 0.2 > 10,000 mg L^{-1}.

A study was conducted by Riaz et al. (2017) for examination of short term (twenty days) toxicity of fluoroquinolone class on wheat. Selected antibiotics (concentrations were 5, 100 and 300 mg L^{-1}) were levofloxacin, ciprofloxacin, enrofloxacin and their combinations. Sand culture study was conducted in greenhouse and effect was assessed on antioxidants stress products. Results showed that the highest concentrations 100 and 300 mg L^{-1} declined the plant growth by causing oxidative stress. Catalase and peroxidase activity was also decreased as compared to the controls. This stress damaged the physiological structure of young plants. In long term, it could reduce crop yield.

Veterinary antibiotics in large quantities are introduced in agricultural fields by manure application and wastewater irrigation. Antibiotic toxicity on plants and animals is yet not well documented. A study conducted by Pan and Chu, (2016) highlighted phytotoxic effect of five major antibiotics sulfamethazine, tetracycline, norfloxacin, chloramphenicol and erythromycin on cucumber, lettuce, tomato and carrot. Effect was checked on seed germination and root elongation. Selected antibiotic concentrations were 0.00, 0.01, 0.10, 1.00, 10.0, 100 and 300 mg L^{-1}. Results showed that root elongation was significantly inhibited. Most sensitive crop specie was lettuce as compared to others. Root elongation inhibition was different among different pant species as 70% for lettuce, 32% for carrot, 48% for cucumber and 42% for tomato. EC50 of sulfamethazine was above 300 mg L^{-1} in carrot, cucumber and tomato and 157 mg L^{-1} in lettuce which shows that lettuce was more vulnerable to antibiotic toxicity.

Opris et al. (2013) gained insight in to the impacts of nine antibiotics (amoxicillin, ampicillin,

ceftazidime, penicillin G, tetracycline, doxycycline, ceftriaxone, ciprofloxacin and erythromycin) on key secondary metabolites and physiological characteristics in wheat plant. Effects of different antibiotics were assessed at realistic environmental concentrations (0.5 to 1.5 mg L^{-1}). Antibiotics were applied in the form of solutions. Results revealed that net assimilation rate was inhibited most strongly by cephalosporins and ciprofloxacin. Reason of this inhibition was reductions in stomatal conductance. Penicillin, cephalosporins and tetracyclines inhibited photosynthetic electron transport rate. Moreover, antibiotics alter plant physiology and cause in reduction of chlorophyll pigments and carotenoids by 10-20% on a dose of 1.5 mg L^{-1}.

In another study, uptake of antibiotics (metformin, an antidiabetic II medicine) and their translocation was examined in plant body by using the forage and crop plants. Tested crops were carrot and barley, chosen antibiotic concentration was 5 mg kg^{-1}. Results of the study showed that high uptake and translocation was observed by metformin in rape (oily seeds) having bioconcentration factor of 21.72. Bio-Concentration Factors (BCF) for other crops barley, wheat, and oat were in the range of 0.29-1.35. BCF of other fruits and vegetables were lower than rape. Reduced growth and biomass were also observed in carrot crop at metformin soil concentration 5 mg kg^{-1} (Eggen and Lillo, 2012).

Many antibiotics are specifically designed to exhibit DNA damage in bacterial as well as eukaryotic cells. Few studies have been conducted to assess the genotoxic effects of antibiotics on different plant species. In a study genotoxicity of quinolone and fluoroquinolones was assessed. The used technique was micronucleus (MN) assay. *Vicia faba* roots were directly exposed to nalidixic acid ciprofloxacin and enrofloxacin. Tested antibiotic concentrations were different for both treatment groups: for nalidixic acid 0.01, 0.1, 1 and 10 mg kg^{-1} and for

ciprofloxacin and enrofloxacin tested concentrations were 0.005, 0.05, 0.5 and 5 mg kg^{-1}. In both groups, at the highest two concentration levels, the MN induction was significant. Whereas, the lowest concentrations had insignificant genotoxic impact. Combination (mixture) of antibiotics having concentration 0.02 to 20 mg kg^{-1} had significant MN induction (Khadra et al., 2012).

2.5 Antibiotic resistance and persistence

Antibiotics are used to cure different biological effects and are widely spread in the environment. Unfortunately, these compounds have long half-life which permits them to persist in nature for very long times (Liang et al., 2016).

O'Brien (2002) studied that the repeated use of antibiotics by co-selection and cross selection leads to the adaption of microbes towards certain antibiotics. Heavy metals presence in farmyard manure has also increased the antibiotic resistance in bacteria by co-selection.

The presence of veterinary antibiotics (VA's) in a certain environment depends upon various factors such as: temperature, pH, UV light, animal excreta and soil type. A decrease in temperature may also decrease the degradation rate of antibiotics. Gavalchin and Katz (1994) used seven different types of antibiotics at three different temperature and showed that the antibiotics become more persistent with decrease in temperature. After that such antibiotics will remain unaltered even if temperature is increased or manure is aerated (Winckler and Grafe, 2001).

The half-life of the antibiotics varies between few days (ceftiofur, chloramphenicol) to 300 days sarafloxacin, oxytetracycline). Hektoen et al. (1995) investigated and concluded that the half-life of the antibiotics is considerably increased in cold and dark weather conditions. Ingerslev and Hallising-Sørensen (2001) and Weerasinghe and Towner (1997) have reported

the half-life of antibiotics in marine sediments, water, soil manure, manure and soil, respectively.

2.6 Toxicity assessment techniques

Multiple techniques have been developed to assess the toxicity of different harmful compounds in the environment. Such as, comet assay, seed germination technique, wheat bioassay, micronuclei assay and physiological parameters are used commonly. Seed germination and plant physiological parameters can be used to assay the phytotoxicity of chemicals. There are just a few studies have been conducted to investigate the toxicity of antibiotics in plants (Migliore et al., 1998 and 2003). Genotoxicity induced by antibiotics in *Vicia faba* was assessed by using micronucleus assay (Khadra et al., 2012).

There is a very limited knowledge present on the potential effects of antibiotics on nutritional composition and plants growth. Most of the studies which are being carried out, focus on the detection of antibiotic, ability to accumulate in plants or the evaluation of toxicity of antibiotics (Igiehon and Babalola, 2018).

2.6.1 Seed germination technique

An et al. (2009) investigated the effect of triclosan (TCS) and glaxolide, the two potential personal care products (PCPs), on wheat and found that these PCPs were affecting the seed germination rate as well as root and shoot length. As the PCPs concentration was increased, the seed germination rate was decreased. Whereas at low concentration of these PCPs, seedling growth was not affected, but with an increase in exposure time, the seedling growth was negatively affected by PCPs, even at low concentrations.

In another study, ten antibiotics were selected and their toxicity was assessed on three different plants. Concentration levels (i.e. 3.9 – 10,000 µg L^{-1}) from very low to extremely high were

used for these antibiotics. The study revealed that even at higher concentrations of these antibiotics, there was no significant effect found on germination stage (Hillis et al., 2011).

Tetracycline is intensively use in both human and animal infection prevention worldwide. Xie and his fellows (2010) investigated the effects of tetracycline on wheat, they found that at lower concentration (i.e. 0.5 – 10 mg L^{-1}) tetracycline stimulates the seed germination. Whereas, at higher concentrations (i.e. 10 – 300 mg L^{-1}) this compound significantly inhibits the seed germination.

2.6.2 Comet assay

Antibiotic induced toxicity is increasing in the environment due to new chemicals continuously entering the natural ecosystem, new techniques are also being developed for toxicity measurements. One of the techniques is known as comet assay. It was first introduced by Ostling and Johanson (1984). With the passage of time, advancements occurred and this technique was further improved to assess the damage in the DNA of living cells. At present, this technique is so much developed and improved that it is seen as one of the best, simple, sensitive and rapid technique to assess damage in DNA, both in eukaryotic and prokaryotic cells (Dhawan et al., 2009). In another study, comet assay was used to assess the genotoxic effects of environmental pollutants (Methyl Methanesulfonate, Spent Potliner; Cadmium; Atrazine; Arbitrary Units). They found that comet assay was most significant in determining the difference between the treatments (Silveira et al., (2017).

Zhang et al. (2013) used comet assay technique by using rice as a model plant. They checked the effect of high hydrostatic pressure, synthesis of DNA, RNA and proteins. Results showed that exposure to 25-100 MPa high hydrostatic pressure for 12-hour time-period, resulted in DNA strand breakage in germinating seeds and may have been the source of mutations.

Gichner et al. (2006) assessed toxicity and DNA damage in two different plants (i.e. potato and tobacco). Study was performed in heavy metal spiked soil. DNA damage was significant in spiked samples as compared to the control plants. Zhang et al. (2013) studied the genotoxic effects of copper (Cu) in the wheat crop by comet assay. They concluded that with the increase in the concentration of Cu, the DNA damaged cells were also increased. The study also revealed that toxicity in the roots was greater than the shoots.

2.6.3 Micronucleus assay

Micronucleus assay is one of the emerging techniques frequently used in genotoxicity assessment. According to a study by Bolt and his fellows (2011), revealed that micronucleus test was performed by 2000 studies in 1992 and the number increased up to 6000 and 13,000 in 2004 and 2010, respectively. Since then, it was the most frequently used technique than others all over the world.

Khadra et al. (2012) investigated genotoxicity of quinolone and fluoroquinolones in *Vicia faba* by using micronucleus (MN) assay. Plant roots were directly exposed to nalidixic acid, ciprofloxacin and enrofloxacin. Results showed that at higher two concentration levels (5 mg kg^{-1} and 10 mg kg^{-1}), the MN induction was significant. Whereas, the lowest concentrations had insignificant genotoxic impact. Combination (mixture) of antibiotics having concentration 0.02 to 20 mg kg^{-1} had significant MN induction.

Yi et al. (2010) used the micronucleus assay technique to determine the effects of aluminum (Al) heavy metal on *Vicia faba* and concluded that the increase in the concentration leads to increase in micro-nucleated cells.

2.6.4 TUNEL assay

Silveira et al. (2017) conducted a study on *Allium cepa* L. and *Lactuca sativa* L. to check cytogenotoxic effect of different environmental pollutants. Death process in the cells exposed to the different environmental pollutants was verified through TUNEL (Terminal deoxynucleotidyl transferase Nick-End Labeling) assay. In this assay apoptotic cells were detected that undergo extensive DNA degradation. The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template.

Chapter 3

MATERIALS AND METHODS

3.1 Overview

This section consists of all the procedures and protocols which were followed throughout this research. All the experimental work was carried out in the Environmental Biotechnology Lab of Institute of Environmental Sciences and Engineering (IESE), NUST, Islamabad. Plants were grown in glasshouse at IESE-NUST with the objective to assess the role of organic matter and fertilizers in alleviation of antibiotics toxicity in plants.

i. At first stage, via pot experiment, the changes in physical growth parameters and nutritional composition were measured.
ii. At the second stage toxicity was assessed by comet assay. Major and minor details are mentioned in this chapter.

3.2 Selection of plant, soil and treatment group

3.2.1 Plant (*Oryza Sativa)*

Rice (*Oryza sativa*) was selected as a test crop because it is the second staple food and third largest crop of Pakistan. This test plant was analyzed throughout the lifecycle to test the formulated hypothesis i.e. antibiotics may have negative impact on plant growth and nutritional composition.

3.2.2 Soil preparation

The soil was purchased from a nearby local nursery. The soil was kept open for 3 days for air drying, all the moisture content was removed. This air-dried soil was then passed through the sieve to obtain fine soil free of pebbles, roots and other coarse matter. Obtained soil was of < 2 mm size particles. This fine soil was further used in all experiments.

3.2.3 Treatment Group

Treatment group was comprised of antibiotics and organic amendments. Plants were given exposure to five antibiotics (Levofloxacin, Ofloxacin, Ciprofloxacin, Ampicillin and amoxicillin) and three organic amendments (Rice husk, farmyard manure and poultry litter) were used. In total, eighty-four pots were prepared, there were five sets of pots, each set contained sixteen pots treated with one particular antibiotic. In each antibiotic group first, four plants were amended with rice husk, next four with farm yard manure and other four with poultry litter.

Figure 3.1: Application of antibiotics in pot experiment. a. crushing of antibiotics by mortar pestle, b. plant exposure to antibiotic solutions.

3.3 Control group

There were two types of controls, one control group was that one having no treatment, other one was treatment control having just antibiotics and no organic amendment was there. Later on, the comparison was carried out between the control group and treated group to assess the role of organic matter and fertilizer in the alleviation of antibiotic toxicity in plants.

3.4 Factorial design of the experiment

keeping in view the both antibiotics and organic amendments, factorial design was made for the pot experimentation.

Table 3.1: Factorial design of the study

	Organic Amendments		
	Farmyard Manure	**Rice Husk**	**Poultry Litter**
Ampicillin 10 mg kg^{-1}	AMP 10 x FYM	AMP 10 x RH	AMP 10 x P
Amoxicillin 10 mg kg^{-1}	AMX 10 x FYM	AMX 10 x RH	AMX 10 x P
Ofloxacin 10 mg kg^{-1}	OFX 10 x FYM	OFX 10 x RH	OFX 10 x P
Ciprofloxacin 10 mg kg^{-1}	CFX 10 x FYM	CFX 10 x RH	CFX 10 x P
Levofloxacin 10 mg kg^{-1}	LFX 10 x FYM	LFX10 x RH	LFX10 x P

AMP = Ampicillin, AMX = Amoxicillin, OFX = Ofloxacin, CFX = Ciprofloxacin, LEV= Levofloxacin, FYM = Farmyard manure, RH = Rice husk, P = Poultry litter

3.5 Plant cultivation

1 kg soil was weighed for each pot. Organic amendments (Rice husk, poultry liter and farmyard manure) at the rate of 5 g kg^{-1} soil were added and mixed in the pot's soil. Rice seedlings (16 days old) of similar size were shifted in the pots. Plant cultivation was done in June followed by harvesting in mid-December.

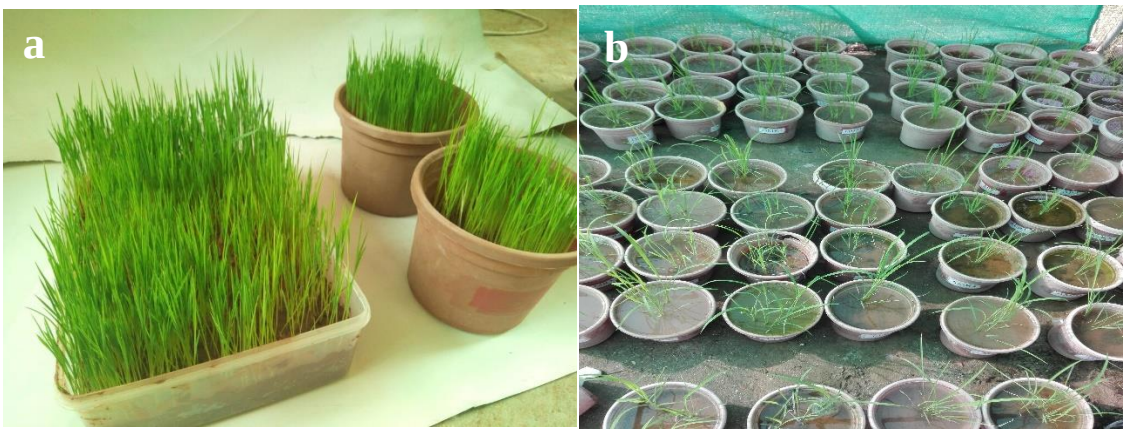

Figure 3.2: Steps of plant cultivation. a. Preparation of seedlings b. cultivation of crop

3.6 Soil characterization

3.6.1 Physical parameters

3.6.1.1 Moisture content

20 g air dried soil was taken in the petri dish. Further placed in the hot oven at temperature 105°C overnight while lid was removed. After 24-hour petri dish was taken out of oven and kept in desiccator for minimum 30 minutes and then re-weighed. Moisture content was calculated by using formula:

$$\% \text{ Moisture in soil} = \frac{\text{wet soil- dry soil}}{\text{dry soil}} \times 100$$

3.6.1.2 Soil texture

Method followed for the determination of texture of soil was saturation percentage (SP) method. By using this method, soil texture and water holding capacity were measured. For this purpose, in a 100-mL beaker, 50g of air-dried soil was taken. Then, distilled water was slowly added and mixed consistently until a saturated paste of uniform thickness was attained. Saturation percentage was calculated by:

$$\text{Saturation Percentage} = \frac{\text{Weight of water required to saturate the dry soil sample}}{\text{Weight of the dried soil}} \text{ X } 100$$

3.6.1.3 Water holding capacity

It is the amount of water detained in the soil subsequently when the excessive water moving under the action of gravity has drained away and when the proportion of descending movement of water has substantially terminated. To find the WHC, 1:1 of soil and water were taken. 50 g air dried soil was placed on the filter paper fixed in the funnel. 50 g water from the measuring cylinder was poured on that soil. Filtrate was measured. Water holding capacity was measured by using the formula.

$$\text{Water Holding Capacity} = \frac{\text{Initial vol. of water - final vol. of water}}{\text{Mass of soil}} \times 100$$

3.6.2 Chemical parameters

3.6.2.1 Soil pH

It is important to determine the soil pH to indicate numerous chemical activities within the soil. To determine the soil type and characteristics (acidic or basic), soil pH was investigated. To measure soil pH, a suspension of soil and water was prepared. The ration of water and soil in this suspension was 1:5, the soil used for this purpose was air dried soil (< 2 mm). 10 g soil was taken in a 250-mL glass beaker. 50 mL distilled water was added in this soil by using a measuring cylinder. This mixture was placed on mechanical shaker (at 180 rpm for 30 minutes) for homogenization. The pH of suspension was measured using combined electrode, it was measured for 3 replicates of the soil sample and average was taken.

3.6.2.2 Electrical conductivity

EC of a soil is a characteristic of soil which impacts crop yield. Into a 100-mL glass beaker, 50 g of air-dried soil was taken. 50 mL DI water was added with a graduated cylinder. Mixture was mixed well and allowed to stand for 30min. This suspension was stirred after every 10 minutes throughout this period. Later an hour, suspension was filtered by using filter paper

Whatman No. 42. Conductivity meter was calibrated then placed in filtrate. Reading was taken for all three replicates and average was calculated.

3.6.2.3 Organic matter

Soil organic matter (SOM) characterizes the remnants of plant matter, roots, and soil organisms in numerous phases of synthesis and decomposition and is flexible in configuration. Organic matter has a principal effect on nutrient reserve and its availability, soil aggregation, moisture holding capacity and biological activity.

Reagents

A. Potassium Dichromate Solution ($K_2Cr_2O_7$), 1N

- $K_2Cr_2O_7$ was dried at 105 °C for 2 hours in oven. It was kept in a desiccator (silica gel) for cooling and stored in a stoppered bottle.
- In DI water, 49.04 g $K_2Cr_2O_7$ was dissolved and brought to 1 L volume.

B. Concentrated orthophosphoric Acid,

C. Concentrated Sulfuric Acid (H_2SO_4)

D. Ferrous Ammonium Sulfate Solution 0.5 M,

In a 1-L flask, 196 g ferrous ammonium sulfate was dissolved in DI water, then 5 mL concentrated H_2SO_4 was added, solution was stirred well, and brought up to volume.

E. Diphenylamine Indicator

100 mL concentrated H_2SO_4 was taken and 1 g diphenylamine indicator was dissolved in it.

Procedure

Air-dried soil (1g) was weighed into a 500-mL beaker. Potassium dichromate solution 10 mL (1 N) was added by using a pipette, then 20 mL concentrated H_2SO_4 was added, suspension

was mixed well by swirling the beaker. Allowed it to stand for 30 minutes. In this solution about 200 mL DI water was added then 10 mL concentrated Orthophosphoric acid was then added and the mixture was allowed to cool. After adding diphenylamine indicator (10-15 drops), the solution was kept on a magnetic stirrer. This solution was titrated with 0.5 M [$(NH_4)_2SO_4.FeSO_4.6H_2O$] solution. End-point was from violet blue to green. All the procedure was repeated for blank. The blank contained same reagents but not the soil and was titrated in the same way (Ryan, 2008).

3.6.2.4 Available phosphorus

As phosphorus is a chief nutrient and is mostly found inaccessible in calcareous and alkaline soils, so it is needed to be measured in the soil laboratories for assessing the need of phosphorus fertilizer for better growth of the crops. The improved method of Olsen (1954) is a quick, simple and reasonable soil test which is usually approved as an appropriate guide of phosphorus accessibility in alkaline soils, wherever the Ca^{2+} precipitated as $CaCO_3$, increasing the solubility of calcium phosphate. Therefore, Olsen's method was performed for checking the phosphorus availability in soil.

A. Extracting Solution

a. Sodium Bicarbonate Solution, 0.5 M

42 g of sodium bicarbonate was dissolved in about 700 mL distilled water and pH of the solution was adjusted to 8.5 with 5N NaOH. The volume was filled up to 1L with distilled water.

b. Sodium Hydroxide Solution, 5 N

50 g of sodium hydroxide was dissolved in 200 mL distilled water and made the volume up to 250 mL with distilled water.

B. **Mixed Reagent**

a. 6 g of ammonium heptamolybdate was dissolved in 125mL distilled water.

b. 0.1455g of antimony potassium tartrate was dissolved in 50 mL distilled water.

The both dissolved reagents mentioned above were added to a 1 L volumetric flask, then 500 mL of 5 N H_2SO_4 (74 mL concentrated H_2SO_4 in 500 mL DI) were added to the mixture. After mixing thoroughly, the volume was filled up to 1L with DI water and stored in a dark and cool place in a Pyrex glass bottle.

C. Color Developing Reagent

For this purpose, 2.64 g of Ascorbic acid ($C_6H_8O_6$) were dissolved in 500 mL Mixed Reagent. Color developing reagent was prepared freshly because it could not be kept for more than 24 hours.

D. Standard Stock Solution

Accurately, 2.5 g potassium dihydrogen phosphate was oven dried for 1h at 105 °C, cooled in a desiccator then stored in air tight bottle. Exactly, 2.197g potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 500 mL distilled water. This solution contained 1000 mg L^{-1} stock solution. Precisely, 10 mL stock solution was diluted to final volume 100 mL with distilled water. A series of standards were made by using this stock solution. These solutions contained 0, 0.25, 0.5, 0.75, 1, 1.25, 1.50, 1.75, 2, 2.25, 2.50, 2.75, 3, 3.5 and 4 mg kg^{-1} phosphorus respectively.

Procedure

Into a 250-mL beaker, 2.5 g air-dried soil was weighed; 50 mL sodium bicarbonate extracting solution ($NaHCO_3$) was added. For 30 minutes at 180 rpm, the mixture was placed on a

mechanical shaker. Blank was also made in one flask containing all reagents except soil. Using Whatmann filter paper No. 42, the solution was filtered. Then 5mL of the filtrate was pipetted out into 25 mL volumetric flask, 5 mL color emergent reagent was added into it and filled the volume up to mark with distilled water. Later bluish color developed. In soil, the concentration of phosphorus is directly proportional to the strength of blue color developed. After 15 minutes, the samples were examined on the Spectrophotometer (SPECORD, 200 PLUS, analytikjena, Germany). The absorbance of standards, blank, and samples were recorded accordingly at a specific wavelength of 880nm. The calibration curve for standards was prepared, plotting the absorbance of the samples on y-axis and phosphorus concentrations on x-axis. From the calibration curve, phosphorus concentration was measured for the unknown sample which was soil used for the experimentation

Phosphorus concentration was measured by following formula.

$$\text{Phosphorus (mg kg}^{-1}\text{)} = \text{P (from calibration curve)} \times \frac{A}{Wt.} \times \frac{25.}{V}$$

Whereas; A = Total vol. (mL) of the extract

Wt. = Wt. (g) of air-dried soil

V = Vol. (mL) of extract used for measurement

3.6.2.5 Nitrate-Nitrogen

To determine the amount of nitrogen present in the soil and need of nitrogen in the form of fertilizer, this test was carried out. Main instrument used for this test was spectrophotometer.

Reagents

A. Chromotropic Acid Solution, 0.1 %

0.368 g chromotropic acid was dissolved in 200 mL Conc. Sulfuric acid.

B. Copper Sulfate Solution, 0.02 N

4.9936g $CuSO_4.5H_2O$ was dissolved in DI water, volume prepared was 2 L.

C. Sulfuric Acid, concentrated

D. Standard Stock Solution

- At 100 °C in oven 4-g KNO_3 was dried for 2 hours. For cooling kept in desiccator and stored in a tightly stoppered bottle.
- In 500 mL 0.02 N copper sulfate 3.6092 g KNO_3 was dissolved.
- 10 mL Stock Solution was diluted to 200 mL flask.

Procedure

10 g air dried soil was taken in a flask. 0.02 N $CuSO_4.5H_2O$ in 50 mL was added into it. Shaking for 15 min was done. The solution was filtered through Whatman No. 42 filter paper. In a conical flask of volume 50 mL, 3 mL filtrate was taken. 1 mL 0.1% Chronotropic acid was drop by drop added and allowed to cool. H_2SO_4 Conc. 6 mL was then added color of the solution became yellow after 45 minutes. Standards were made by dissolving 3.6092 g KNO_3 in 500 mL 0.02 N copper sulfate solution. Spectrophotometer was used to check absorbance at 430 nm wavelength (Ryan and Estefan et al. 2007).

3.6.2.6 Sodium in soil

By using flame photometer, Sodium in the soil sample was measured. Under the flame, light was emitted from the element which was proportional to the concentration of sodium present in it. Ammonium acetate solution was used for extraction. 1N stock solution was prepared from which series of standards were made and run on Flame Photometer (Sherwood Scientific, Model 360, UK) at 589 nm wavelength.

3.6.2.7 Potassium in soil

Soil potassium was determined by following the protocol developed by Richards (1954). Ammonium acetate solution was used for the extraction. From the stock solution series of

standards were made. Standards were prepared and run after in flame-photometer at 767 nm wavelength.

3.7 Seed Germination test

Laboratory tests were carried to evaluate the associated effect of each antibiotic on seed germination using the filter paper method according to the International Seed Testing Association (ISTA) test protocols (ISTA,1985). Rice seeds were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The variety of seeds was Super Basmati rice. Healthy seeds were screened and disinfected by using 5 percent Sodium hypochlorite solution for 5 minutes. Then washed with distilled water for three times. Petri-dishes were washed and air dried. Filter paper were kept in the petri-dishes and ten seeds were placed in each petri-plate. These petri dishes were then placed in an incubator at 25°C for germination for 7 days. Four Replicates were used to test different parameters at germination stage. Percentage of seed germination was calculated by using formula:

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

(Hillis et al., 2011)

3.7.1 Application of antibiotics

Antibiotic solutions @ 10 mg L^{-1} were applied. For each petri-plate, organic amendment dose was calculated, same dose which was applied to the plants in outdoor pot experiment i.e. 0.05 g/10 mL solution.

3.7.2 Parameter analysis in germination set

Two parameters were analyzed from the germination set.

i. Root/Shoot length.

ii. Germination rate.

iii. Seedling Vigor Index

After a week Number of seeds germinated at each petri-plate were counted. To determine the effect of antibiotic and organic amendments percentage formula was applied.

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

Root shoot length was measure with the help of standard scale.

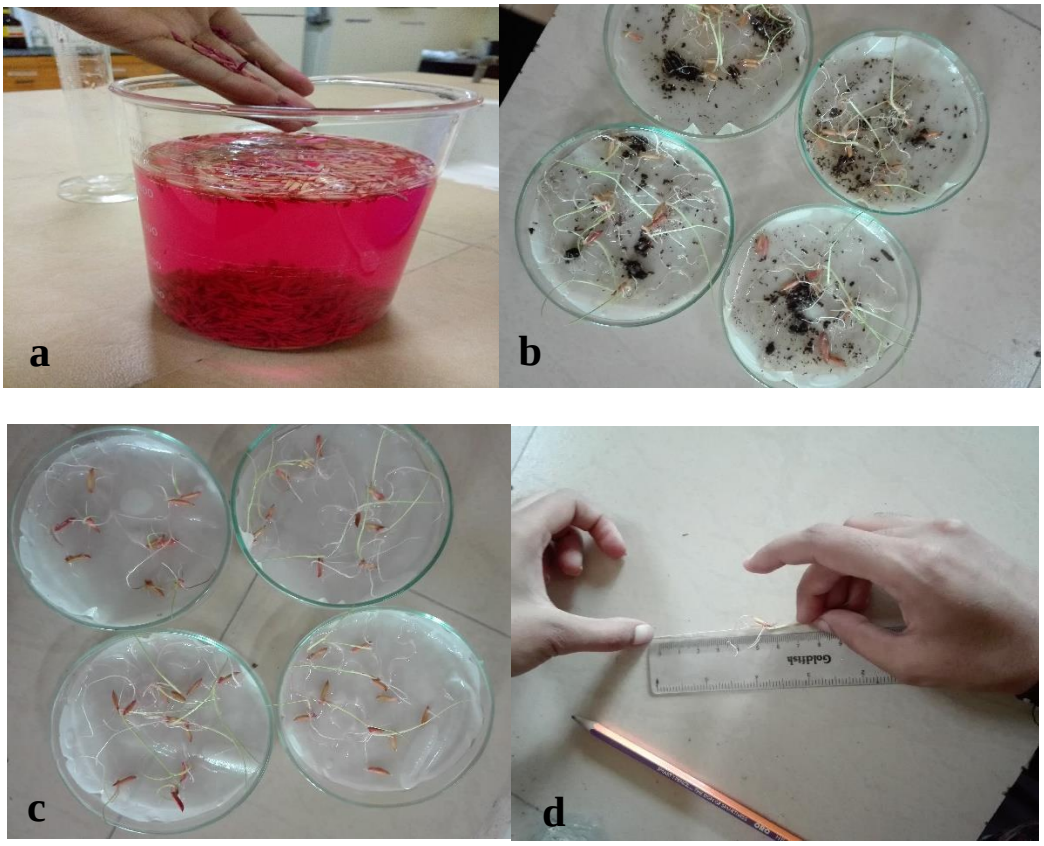

Figure 3.3: Germination test setup and analysis, a. disinfection of the seeds b. application of treatments in petri plates c. controls d. measurement of seedling length

3.7.3 Seedling vigor index

Seedling vigor index is an indicator of seed's physical health, activity and maintenance. Seedling vigor index was calculated by using formula:

(SVI) = [Lr + Ls] × GP

SVI = Seedling vigor index

Lr = Mean root length

Ls = Mean shoot length

GP = Percentage of seed germination

(Zhao et al., 2016).

3.8 Toxicity assessment – comet assay

Comet Assay protocol was followed to quantify DNA damage in order to assess genotoxicity.

3.8.1 Germination set for comet assay

Rice seeds of super Basmati variety were obtained. Disinfection of the seeds was the next step which was done while keeping the seeds in 5% Calcium hypochlorite solution up to 5 minutes followed by direct washing with distilled water three to four times.

For 30 minutes, seeds were placed in distilled water and then kept on petri dishes over wet filter paper. Five antibiotic treatments were given, having dose 10 mg L^{-1}. Controls were also placed along with the treatments for 24 hours.

3.8.2 Preparation of cell suspension

Cell suspension was prepared according to the protocol devised by Gnanam and Kulandaivelu (1969). Seeds were germinated for 7 days on a moist filter paper in incubator at 25°C.10 g roots were cut and chopped in 40 mL grinding medium (20 µmol. Sucrose, 10 µmol $MgCl_2$, 20 µmol tris HCl buffer, pH 7.8) by mortar pestle. Sucrose cushion (320 mM sucrose in 19 PBS and 1 mM Calcium) was used to get intact nuclear fraction. Then centrifugation was done at 2000 rpm for 10 minutes and supernatant was collected (Peycheva et al., 2011).

3.8.3 Comet assay protocol

The assay was performed according to the protocol described by Singh et al. (1988) in 1% Low melting point (LMP) agarose 60 uL cell suspension was mixed with 60 uL gel and added on pre-coated slides with 1% NMP agarose. Coverslip was added and slides were placed at ice bar for solidification of gels at 4°C. After that, slides were immersed in cold lysis buffer (2.5 M NaCl,100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) for 2 h, then rinsed with distilled water. These slides were then placed in electrophoresis tank for DNA unwinding for 20 minutes. This tank contained a solution of electrophoresis buffer (0.3 M NaOH,1 mM EDTA, pH 13). Electrophoresis was performed for 30 min (0.67 V/cm, 110 mA) at 4^0C.After that neutralization was done with 0.4 M Tris buffer (pH 7.5). slides were then subjected to fluorescent microscope equipped with an excitation filter of 515-565 nm and a barrier filter of 590 nm. Images of 30 randomly selected nuclei per slide were analyzed with image analysis software (Comet Assay Software Project v.1.2.2, CASP, http://sourceforge.net/projects/casp/), and the data was processed in Microsoft excel (Zhang et al., 2013)

3.9 Post-Harvesting Analysis

After harvesting, plant was washed with tap water. Following parameters were measured:

i. Physical parameters (Root/shoot length and dry/fresh biomass)
ii. Nutritional Parameters (Carbohydrates, Proteins, Iron, Nitrogen, Phosphorus)

3.9.1 Physical parameters

At the termination of experiment, endpoints were determined as root length, shoot length, root dry biomass and shoot dry biomass. Plant was taken and washed with tap water. Root and shoot lengths were measured using measuring tape (cm), from the tip of the primary root to the

hypocotyl. Shoot length was measured from tip of the shoot to the hypocotyl (Hillis et al., 2011). By using portable weighing balance fresh biomass was taken separately for roots and shoots then for dry biomass they were packed in brown paper and placed in oven at 75°C for 48 h. Dry biomass was recorded until plants completely dried and constant biomass was reached (Zhao et al., 2011).

Figure 3.4: Difference in plant height treated by antibiotics and plant length measurements

3.10 Nutritional composition

3.10.1 Total phosphorus and iron

Total P and Fe were analyzed in the plant in order to observe the effect of antibiotics and organic amendments on these parameters. Plant samples were grinded and digestion was done. Concentrated nitric acid- perchloric acid (HNO_3- $HClO_4$) mixture (2:1) was taken in flask already contained grinded plant material on hot plate (Rashid, 1986). Testing parameters were analyzed in both roots and shoots of tested plants. By using vanadomolybdo-phosphoric acid colorimetric method, phosphorus content was measured at spectrophotometer (wavelength 430 nm). Whereas, Iron content was analyzed by using Atomic Absorption Spectrophotometer (AA-7000, Shimadzu) (Ryan, 2008).

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

3.10.2 Total carbohydrates

Morris Anthrone method was used in order to determine the carbohydrate content (Ludwig and Goldberg, 1956). Material was grinded to powder form. 100 mg sample was taken in a test tube and hydrolyzed. In boiling water bath, by using 2.5N HCl, hydrolyzation was done for 3 hours. Then centrifugation was done at 4000 rpm for 10-12 minutes. Supernatant was taken and used as an extract for further analysis. 2 grams Anthrone powder was mixed in 1 L Sulphuric acid in this way Anthrone reagent was formed. 8mL Anthrone reagent was added in each test tube already contained 5 mL of sample extract. Solution was mixed thoroughly and allowed to develop green color after approximately 10 minutes. By using spectrophotometer at wavelength 620 nm samples, blank (distilled water) and standards were analyzed. Carbohydrate content in the samples was quantified.

3.10.3 Total proteins

By estimating total nitrogen content of plant, the amount of protein was quantified (Martin et al. 1983). First step was mineralization. In this phase, 1 mL of 36N H_2SO_4 was used to digest

50 mg of plant sample. This process was carried out firstly at 150°C for 10 minutes and 310°C for 30 minutes till the evaporation of H_2O_2. A colorless extract was achieved. 10 mL water was added in order to dilute the sample. For further dilution from this diluted sample, 0.1 mL was taken and 3.5 mL of H_2O was added into it.

Reagents

a. In 250 mL water, 5 mL Phenol and 12.65 mg Na Nitroprusside were added.
b. In 250 mL water, 1.25g NaOH, 16.75g $Na_2HPO_4.7H_2O$ and 2.5 mL NaOCl (12%) were added.

In Eppendorf, 0.1 mL sample was taken, 0.5 mL of reagent (a) and 0.5 mL of reagent (b) were added into sample. It was incubated at 37 °C. By using spectrophotometer at wavelength 625 nm readings were taken.

3.11 Statistical analysis of data

Statistical significance of results' data was checked by using software "Statistics 8.1" applying single factor ANOVA and HSD 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 from different sources are released into our environment especially using wastewater for irrigation purposes. Probable human health impacts are intensively investigated. However, there is a huge research gap to find the effects of antibiotics on plants particularly on cash crops. In this study, effect of antibiotics on rice was assessed, furthermore the release in stress of antibiotics was also observed by using organic amendments.

This particular section contains the results and discussion of antibiotics' effects on nutritional composition, physical parameters, germination rates and genotoxicity.

4.1 General soil characterization

Physiochemical properties of experimental soil have been listed in the table 4.1.

Table 4.1: Physicochemical characteristics of experimental soil

Soil Parameters	Values
EC (Electrical conductivity)	30.8 mS cm^{-1}
pH	8.20
Texture	Silt clay
Moisture Content (%)	1.34%
Total Organic Carbon	0.40%
Water Holding Capacity	70 %
Nitrate-Nitrogen	63.1 mg kg^{-1}
Phosphorus	33.0 mg kg^{-1}
Potassium	89.3 mg kg^{-1}
Sodium	40.9 mg kg^{-1}

Electrical conductivity of the soil is a measure of the amount of salts in soil. It can be used as an indirect indicator of many soil chemical and physical properties (Sudduth et al., 2005). EC affects crop suitability, crop yields, activity of soil microorganisms and plant nutrient availability. EC value of experimental soil is high which indicates water-soluble nutrients are

available sufficiently for plant uptake such as nitrate nitrogen. Moreover, rice is one of the moderately salt tolerant crop.

Soil was slightly basic at pH 8.2, rice crop grows best in neutral pH soils. So, pH did not have any significant impact on crop yield. Rice required more water that is why the soil texture (silt clay) was more appropriate for cultivation of rice crop. Water holding capacity of the testing soil is also good in order to retain sufficient water. Recommended dose of phosphorus, potassium and nitrogen for rice is 40 mg kg^{-1}, 35.5 mg kg^{-1} and 70 mg kg^{-1}. Soil nitrogen level was low but phosphorus and potassium levels were almost fine. General characteristics of soil shows that soil was appropriate for plant growth.

4.2 Antibiotics' effects on plant physiological parameters

4.2.1 Seed germination

After 7 days germination period, germination rate was observed. Percentage of germinated seeds was reduced by 80% with the application of antibiotics. However, application of organic amendments released the stress and germination percentage get improved by 75% relative to controls. According to the statistical analysis, antibiotics have significant ($p< 0.05$) negative impact on seed germination rate. Although all organic amendments released antibiotic stressed but, Tukey HSD test showed that organic amendments were not significantly different from each other. Moreover, a delay in seed gemination of 1-2 days was also observed with the application of antibiotics.

Similar results were reported by Wright (1951) who determined the phytotoxic effects of different classes of antibiotics and found that penicillin class of antibiotics significantly reduced the germination rate with an increase in antibiotic dose up to 25 mg L^{-1}. Results are also consistent with a study conducted by Liu et al. (2009). The study showed that sweet oat

and rice seeds were more sensitive to antibiotic and seed germination rate of these two crops were highly affected as compared to the cucumber seeds. Study also concluded that antibiotics mainly tetracyclines and sulfonamides could hinder seed germination in rice.

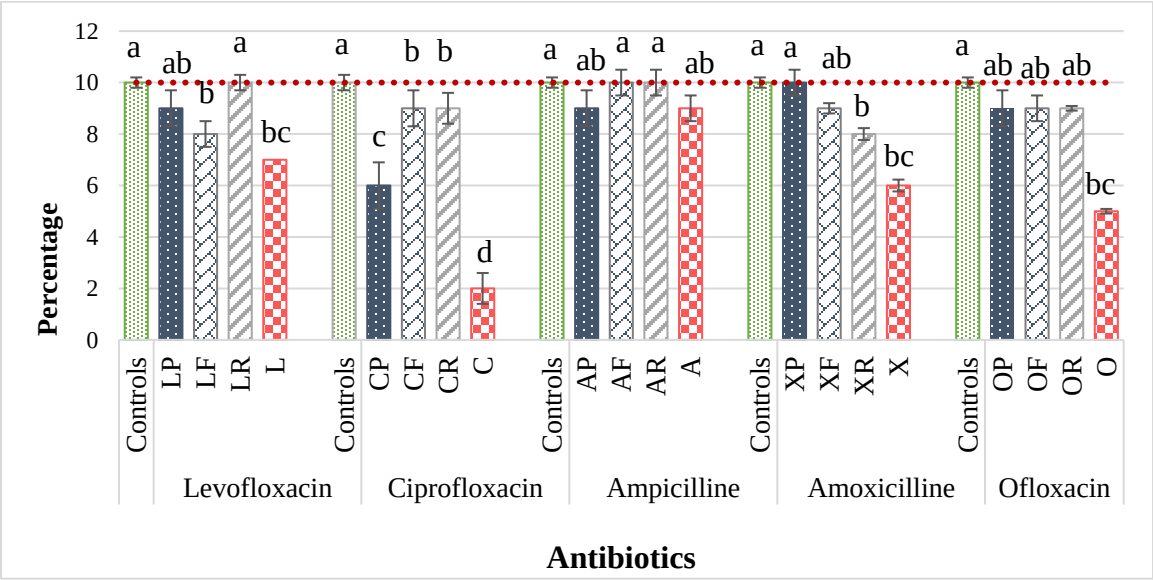

Figure 4.1: Effects of different antibiotics and organic amendments on seed germination rate (after 7 days germination period). Alphabets show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference. Among treatments for different alphabets, there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.2.2 Seedling root shoot length

Instead of number of germinated seeds, the seedling root-shoot length was considered an endpoint of phytotoxicity in statistical analysis. Significant ($p< 0.05$) decrease in seedling root and shoot length was observed. By applying 10 mg kg^{-1} dose of each antibiotic seedling root length was affected because they are direct contact point with pollutants (antibiotics) and accumulation points of antibiotics (Migliore et al., 2010). Root length was majorly affected by ciprofloxacin and shoot length by levofloxacin as compared to the other antibiotics. In the

starting two to three days, the effect was insignificant but later on difference in root shoot lengths was observed.

The overall decrease in percentage by applying Ciprofloxacin, Levofloxacin, Amoxicillin, Ampicillin and Ofloxacin was 82%, 78%, 77%, 54% and 78%, respectively. Uptake and impact difference depend upon the type and class of an antibiotic, plant species and other environmental factors (Migliore et al., 1995). However, application of organic amendments resulted in improving growth as compared to antibiotic controls. Rice husk was the most effective treatment followed by farmyard manure and poultry litter. Tuckey HSD test showed that all organic amendments had different and significant impact on reducing antibiotic stress. Adsorption is one of the most effective and attractive method to remove environmental pollutants and is characterized by reasonable costs and easily controlled process (Inyang et al., 2011, 2014). By using various materials like activated carbons, resins and carbon-nano material, sorption of antibiotics have been conducted (Yang et al., 2011; Chen et al., 2014, 2015). Rice husk is a natural sorbent for the removal of a variety of metal cations and basic dyes (Suemitsu et al., 1986; Low and Lee, 1997).

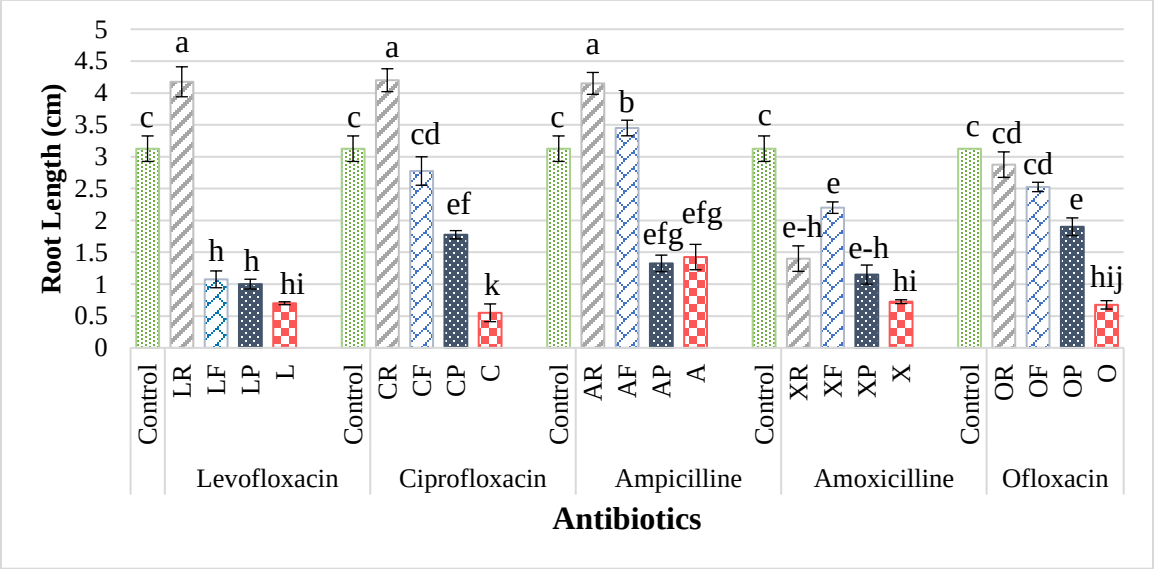

Figure 4.2: Effects of different antibiotics and organic amendments on seedling root length Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

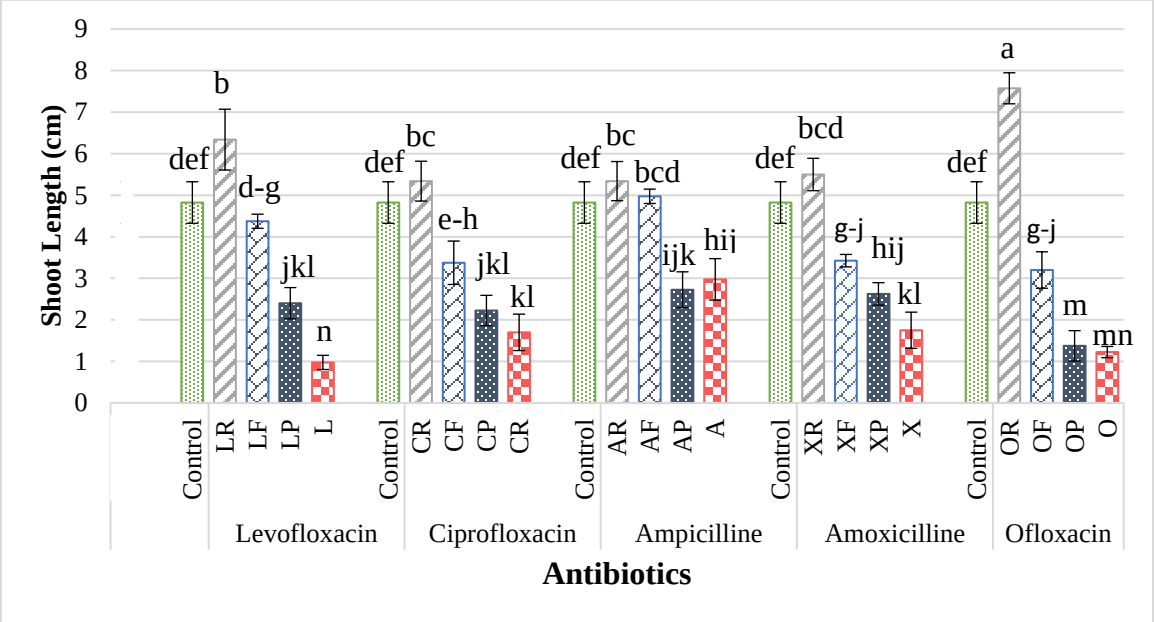

Figure 4.3: Effects of different antibiotics and organic amendments on seedling shoot length. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.2.3 Seedling dry biomass

Like seedling root and shoot length, seedling dry biomass was also negatively affected when antibiotics were applied. Dry root biomass was significantly reduced by Ofloxacin followed by Amoxicillin and Levofloxacin by 67%, 63% and 55%, respectively. Similar trend was also observed in dry shoot biomass with the application of antibiotics. Overall reduction in seedling dry biomass was recorded up to 54% upon exposure to all antibiotics. However organic amendments released this stress of antibiotics, the highest positive impact was seen with rice husk up to 2.4 folds followed by 1-fold decrease in stress by

farmyard manure. The reason is the formation of antibiotic–soil organic matter complexes. These complexes induce high aromaticity and high density of polar functional groups in the soil. Hence, attraction of polar host molecules is favored (Schulten, 1999). Similar study was conducted by Thiele-Bruhn et al. (2004), they found that antibiotic–soil organic matter (farmyard manure) complexes enhanced the sorption of sulfonamide pharmaceutical compounds in the soil by increasing the accessibility of voids, resulting in high contents of sorptive organic mineral surfaces sulfonamide in soil and consequently decrease the risk of uptake of these compounds by plants.

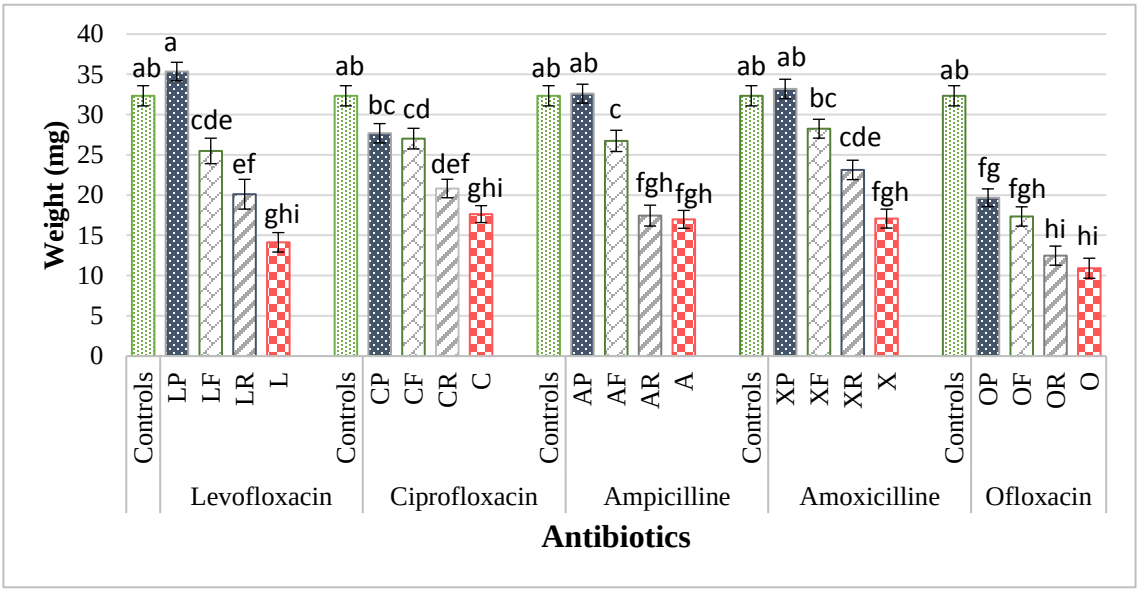

Figure 4.4: Effects of different antibiotics and organic amendments on seedling shoot dry weight. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

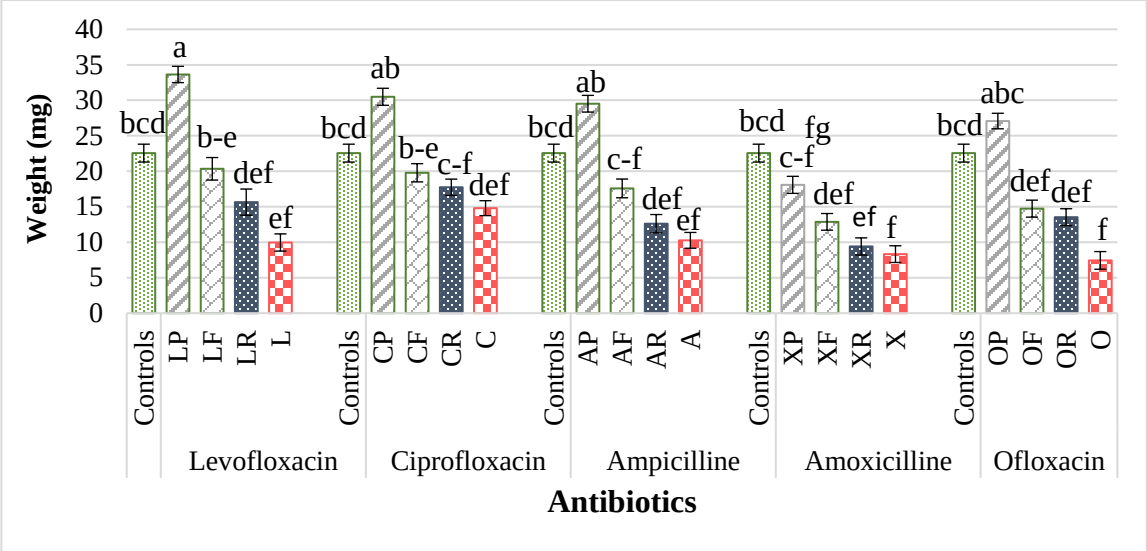

Figure 4.5: Effects of different antibiotics and organic amendments on seedling root dry weight. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.2.4 Seedling vigor index

It is an indicator of seed health, performance and activity. When antibiotics were applied, seedling vigor index was significantly reduced. The highest reduction was observed in those plants who were treated with ciprofloxacin. Reduction in SVI was 87% with this antibiotic. Those plants who were treated with organic amendments showed better performance. Most effective organic amendment was rice husk, it reduced antibiotic stress by 4.72 folds.

Results (shown in figure 4.6) illustrate that as coefficient of variance showed that antibiotics had significant *($p<0.05$)* negative impact on seed vigor index. Tukey HSD showed all five antibiotic groups and all three organic amendments are significantly different from each other. Zhao et al. (2016) estimated seedling vigor index of sunflower treated with heavy metals,

results coincide with the results of this study. In both studies, environmental pollutants negatively affected seed health, maintenance and activity.

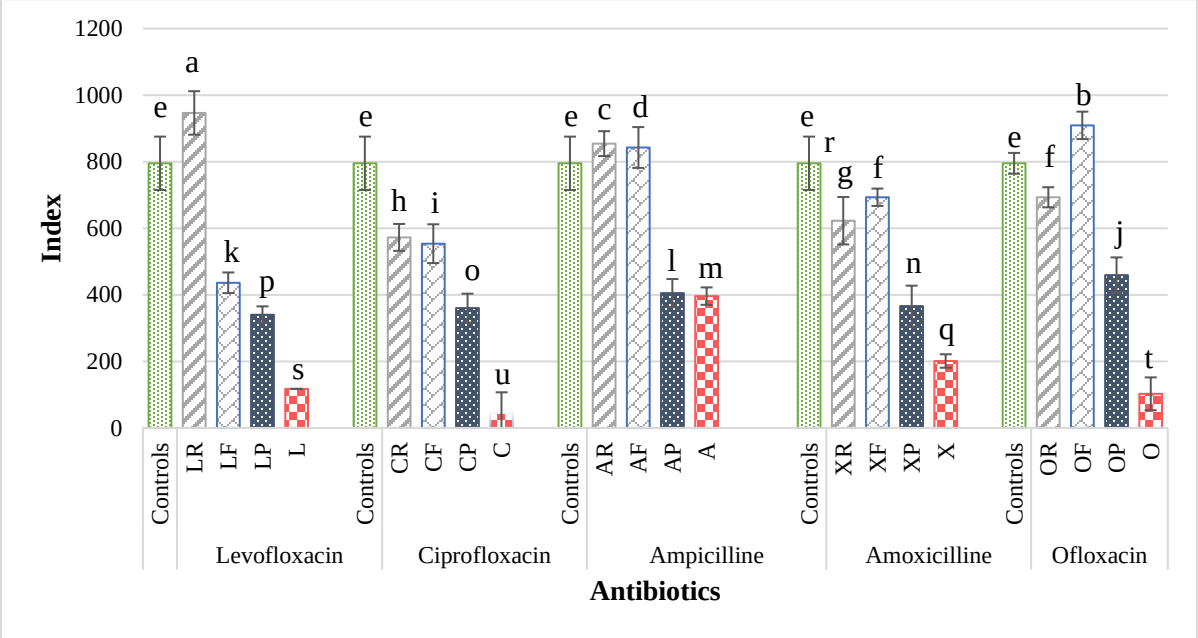

Figure 4.6: Effects of different antibiotics and organic amendments on seedling vigor index Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.3 Plant morphology

At the termination of experiment (harvesting), phytotoxicity endpoints were determined as root length, shoot length, root dry biomass and shoot dry biomass. Effects of all five antibiotics and organic amendments were as follows:

4.3.1 Root and Shoot Length

Upon exposure to all antibiotics, root and shoot length were reduced. Those plants, who contained organic amendments along with antibiotics, showed better growth than antibiotic controls. This effect is illustrated in the figure 4.7. Although, effect varies among different

treatment groups also depends on type and class of antibiotic (Migliore et al., 1995). Variance analysis shows that impact of antibiotics was significant ($p< 0.05$) on plant root-shoot length. Tukey HSD test also showed that the impact of all the antibiotics and organic amendments was not significantly different from each other.

Effect of Ofloxacin and Amoxicillin was higher, as compared to the other antibiotics, both in roots and shoots. Maximum relative decrease in percentage was observed by Ofloxacin up to 65% whereas, rice husk successfully released the stress up to 0.6 folds (65%) in shoots. Maximum decrease in root length was up to 55% by ofloxacin and 105% release in stress was recorded when rice husk was applied to the plants. Farmyard manure and poultry litter also reduced antibiotic stress in plants. Results are consistent with a study conducted by Liu et al. (2009) who found that among three crops (sweet oat, rice and cucumber), antibiotic toxicity varied and effect on root shoot length was different. But each case observed a significant difference.

Similar studies conducted by Yi et al. (2016) concluded that rice husk biochar had a huge potential for the removal of levofloxacin as an effective low-cost sorbent. Results are also consistent with another study by Thiele-Bruhn et al. (2004) which illustrated that farmyard manure enhanced the sorption capacity of sulfonamides pharmaceutical antibiotics in soils by making soil-SOM complexes.

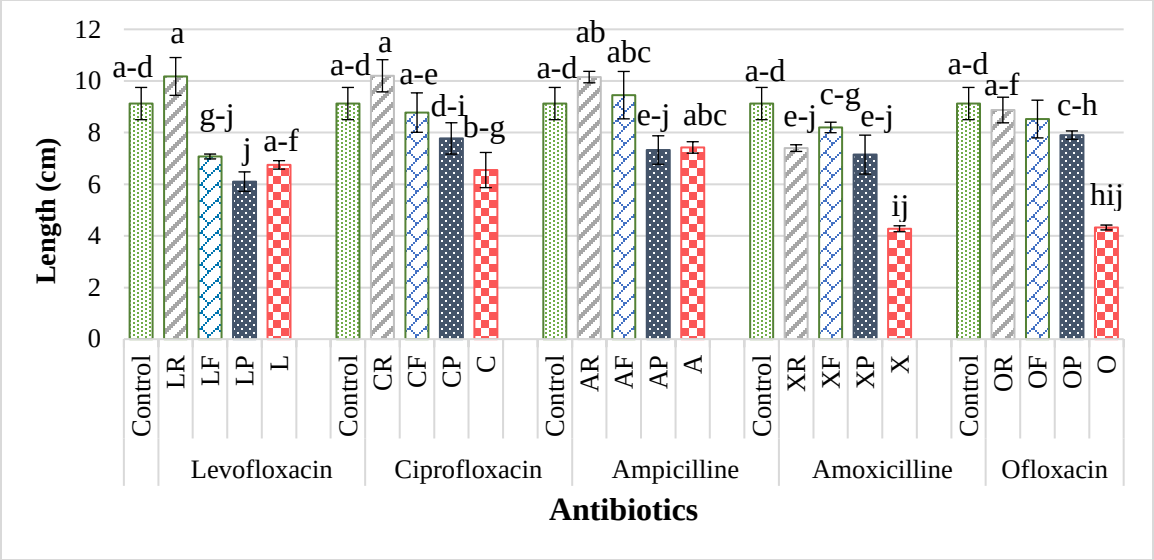

Figure 4.7: Effects of different antibiotics and organic amendments on plant root length. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

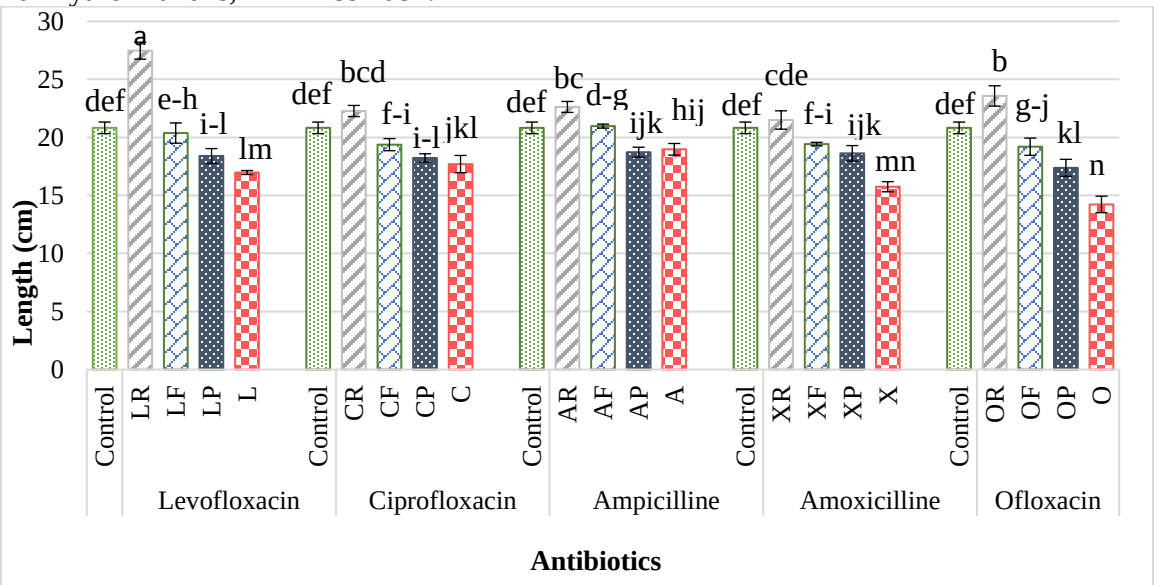

Figure 4.8: Effects of different antibiotics and organic amendments on plant shoot length. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.3.2 Root and Shoot Dry Biomass

The dry weight (mass) was measured for controls and the spiked samples. Plots of all dry biomass of roots as well as of shoots are shown in table 4.2. Although statistical evaluation (ANOVA) showed that plants biomass was significantly ($p< 0.05$) reduced by all antibiotics and significantly ($p< 0.05$) improved by the application of organic amendments. Similarly, all three organic amendments were not significantly different from each other. It means that separate effect of every treatment was recorded but among each group difference was insignificant.

Similar results were reported by Li et al. (2011) where wheat dry biomass was significantly reduced with an exposure to OTC (Oxytetracycline) in two wheat cultivars. Stunted plant growth was observed where shoot biomass was decreased by 5.6%-13.75%. Another study by Eggen et al., (2011) corresponds with the present study confirming the negative effect of antibiotics on plant growth.

Table 4.2: Effects of different antibiotics and organic amendments on plant root and shoot dry biomass

Treatments	Root Dry Biomass (g)	Shoot Dry Biomass (g)
Control	2.25±2.2 ^{bcd}	3.23±0.1 ^{bcd}
LP	3.363±3.4 ^{def}	3.53±0.2 ^{ef}
LF	2.034±2.0 ^{bcde}	2.54±0.4 ^{cde}
LR	1.564±1.5 ^{a}	2.01±0.07 ^{a}
L	0.995±0.9 ^{ef}	1.41±0.1 ^{ghi}
CP	3.04±3.0 ^{cdef}	2.76±0.1 ^{def}
CF	1.97±1.9 ^{bcde}	2.70±0.2 ^{cd}
CR	1.77±1.7 ^{ab}	2.08±0.1 ^{bc}
C	1.47±1.4 ^{def}	1.76±0.3 ^{ghi}
AP	2.95±2.9 ^{def}	3.26±0.1 ^{fgh}
AF	1.75±1.7 ^{cdef}	2.67±0.2 ^{c}
AR	1.26±1.2 ^{ab}	1.74±0.2 ^{ab}
A	1.02±1.0 ^{ef}	1.69±0.2 ^{fgh}
XP	1.80±1.8 ^{ef}	3.31±0.3 ^{cde}

XF	1.28±1.2 ^{def}	2.82±0.1 ^{bc}
XR	0.94±0.9 ^{cdef}	2.31±0.1 ^{ab}
X	0.83±0.8 ^{f}	3.31±0.2 ^{fgh}
OR	2.70±2.7 ^{def}	1.96±0.05 ^{hi}
OF	1.47±1.4 ^{def}	1.734±0.20 ^{fgh}
OP	1.35±1.3 ^{abc}	1.24±0.2 ^{fg}
O	0.74±0.7b ^{f}	1.09±0.2 ^{hi}

Where, means for the factors within a column followed by the same letter are not significantly different at $p < 0.05$ by Tukey's Honestly Significant Difference (HSD) Test. A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin, O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.3.3 Number of tillers

The results of analysis of variance showed that number of tillers was significantly ($p<0.05$) influenced by all five antibiotics. Whereas, significant ($p< 0.05$) positive potential was observed in each organic amendment to reduce antibiotic stress in the plant. Figure 4.9 illustrates that the highest number of tillers plant^{-1} (7) was found in plants treated with rice husk. Whereas, those plants who contained only antibiotic and no organic amendments were significantly affected in terms of reduced tillering. The lowest number of tillers were recorded in plants treated by Amoxicillin followed by ciprofloxacin. Antibiotics stunted plant growth (tillering) by slowing down their ability to convert sun light into useful energy (Munne´-Bosch and Alegre, 2000). Moreover, it is observed that antibiotics are responsible for the reduction of photosynthetic pigments (chlorophyll and carotenoids) (Opris et al., 2013).

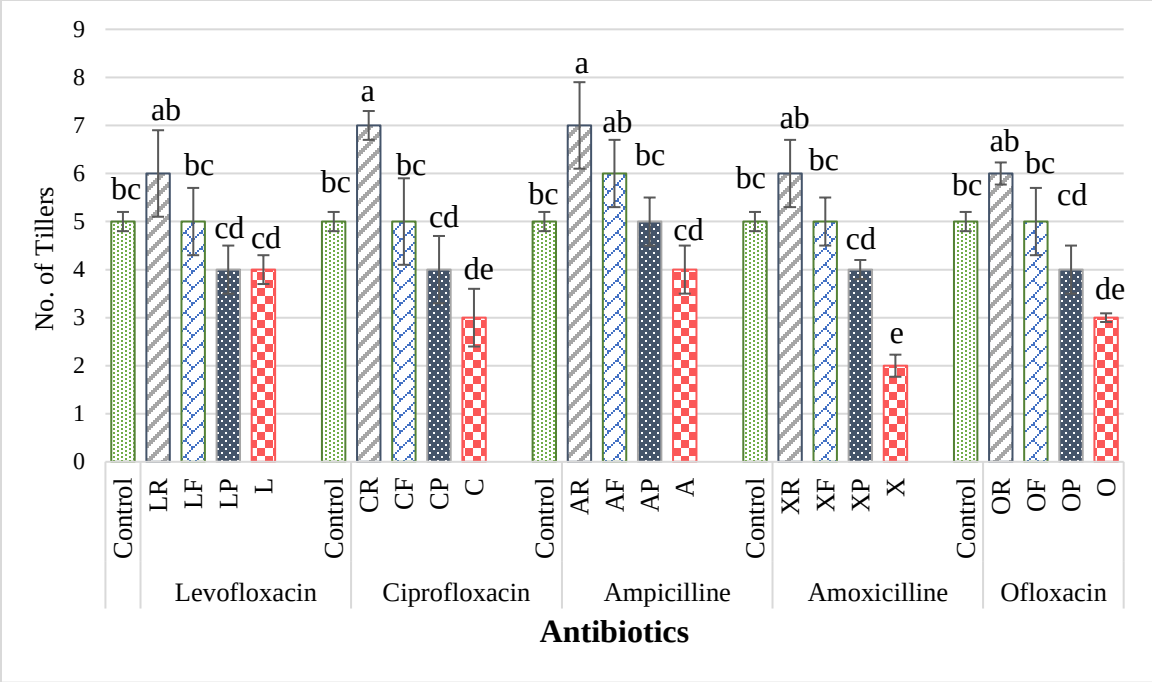

Figure 4.9: Effects of different antibiotics and organic amendments on Tillering. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.4 Antibiotic Effect on Nutritional Composition of Vegetative Parts

4.4.1 Total phosphorous

Phosphorous concentration declined both in roots and shoots with the application of antibiotics. Organic amendments specially poultry liter helped in recovery of phosphorous content in plant. Table 4.3 presents the total phosphorus concentration in shoots of *Oryza sativa.* In comparison to the control.

Analysis of variance revealed that antibiotics have significant ($p < 0.05$) effect on phosphorous uptake both in roots and shoots. All three treatments (rice husk, farmyard manure and poultry litter) also have significant ($p < 0.05$) effect in alleviation of antibiotic stress in plants. Maximum Phosphorous (2700 mg kg^{-1}) was measured in plants exposed to poultry litter. Moreover, Tukey's HSD test showed that all 5 antibiotics had significantly different impact on

plant phosphorous. The most effective treatment was poultry litter. Ofloxacin most significantly reduced plant phosphorous uptake by 86%. With help of poultry liter, this stress was released by 4.78 folds.

Antibiotics negatively impact soil microbial communities and kills not only pathogenic but also beneficial soil microbes (Pini et al., 2012). These microbial associations make phosphorous available to the plant through solubilization and mineralization (Richardson, 2007). Furthermore, when these antibiotics enter into the plants, they alter the physiology of plants and their biochemical activities negatively (Luo et al., 2011; Boonsaner and Hawker; 2010; Hillis et al., 2011; Li et al., 2011; Liu et al., 2009). Batchelder (1982) reported that potassium, calcium, magnesium and phosphorus content in bean plant were reduced on exposure to antibiotics.

Table 4.3: Effects of different antibiotics and organic amendments on plant root and shoot phosphorous

Treatments	Root phosphorus (mg kg^{-1})	Shoot Phosphorus (mg kg^{-1})
Control	675±49.7 ^{a}	383±0.0 ^{a}
LP	587±61.8 ^{cd}	271±23.2 ^{c}
LR	500±91.7 ^{f}	184±3.4 ^{f}
LF	464±24.9 ^{g}	156±8. ^{fghi}
L	286±41.9	126±13.0
CP	635±52.1 ^{b}	204±48.0 ^{ef}
CR	425±61.3 ^{f}	167±11.5 ^{fg}
CF	431±27.8 ^{gh}	161±16.3 ^{fgh}
C	340±44.3 ^{k}	96±12.5 ^{l}
AP	553±37.2 ^{e}	323±21.2 ^{b}
AR	466±36.8 ^{g}	166±16.4 ^{fg}
AF	409±56.5 ^{i}	140±12.5 ^{hij}
A	384±51.4 ^{h}	123±22.1 ^{jk}
XP	639±50.2 ^{b}	383±18.9 ^{a}
XR	604±31.9 ^{c}	318±9.9 ^{bc}
XF	487±36.7 ^{d}	273±20.2 ^{c}
X	451±58.4 ^{gh}	202±36.3 ^{ef}
OP	605±50.2 ^{c}	234±8.2 ^{d}

OR	444±36.4 ^{gh}	209±21.7 ^{de}
OF	382±5.5 ^{ij}	174±20 ^{f}
O	297±36.5 ^{l}	130±19.9 ^{ijk}

Where, means for the factors within a column followed by ± standard deviation. A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

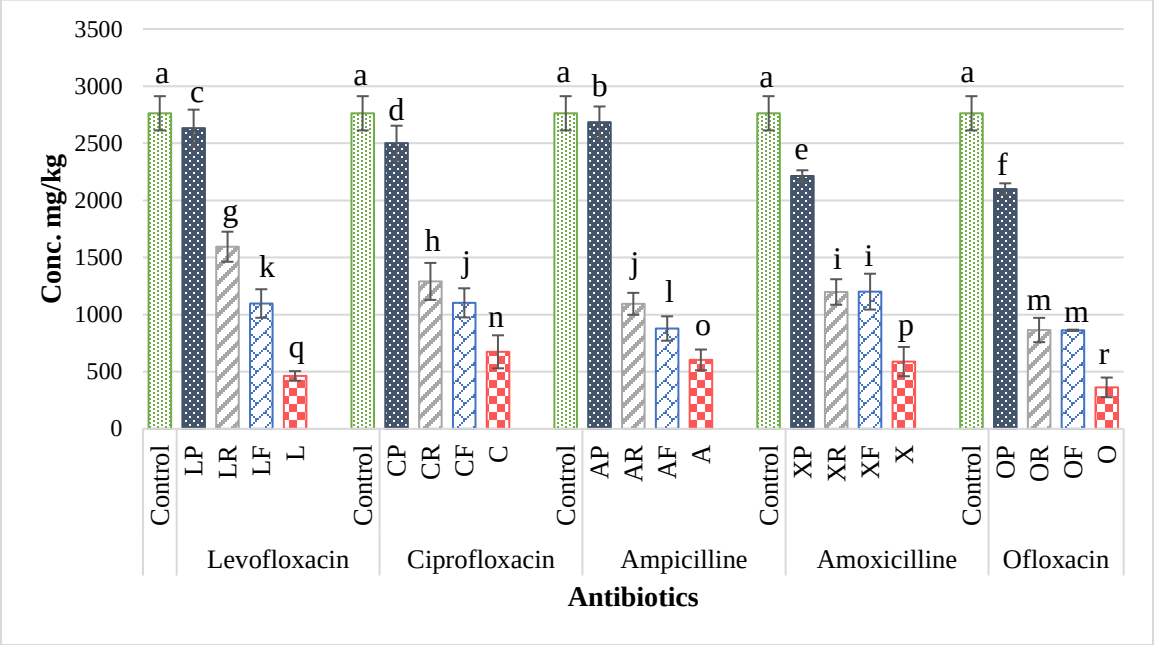

Figure 4.10: Effects of different antibiotics and organic amendments on total P uptake. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.4.2 Iron

Effects of all five antibiotics (Levofloxacin, Ofloxacin, Ciprofloxacin, Ampicillin and amoxicillin) on plant root and shoot iron were measured. Respective results are shown in figure 4.11. Iron contents both in root and shoot significantly ($p < 0.05$) decreased with the application of antibiotics. In figure 4.11 different alphabets on graph bars illustrate that rice husk, farmyard manure and poultry litter had different effects from each other. Moreover, all the organic amendments significantly alleviated the antibiotic stress on iron uptake as shown by analysis of variance (p value is smaller than 0.05). In roots, iron percentage decreased 23%, 22%, 21%,

35% and 25% by Levofloxacin, Ofloxacin, Ciprofloxacin, Ampicillin and Amoxicillin, respectively. Total average reduction in roots' iron was 25%. Similar trend was observed in shoots.

No research has been reported yet on the effect of antibiotics on iron content in plants. But many studies show that when these antibiotics enter into the plants, they alter plants physiology and negatively impact their biochemical activities (Luo et al., 2011; Boonsaner and Hawker; 2010). Antibiotics increased oxidative stress, thus cell redox balance get disturbed. It results in production of peroxides and free radicals, consequently reduction in proteins and lipids is observed along with breakage of DNA strands (Riaz et al., 2017).

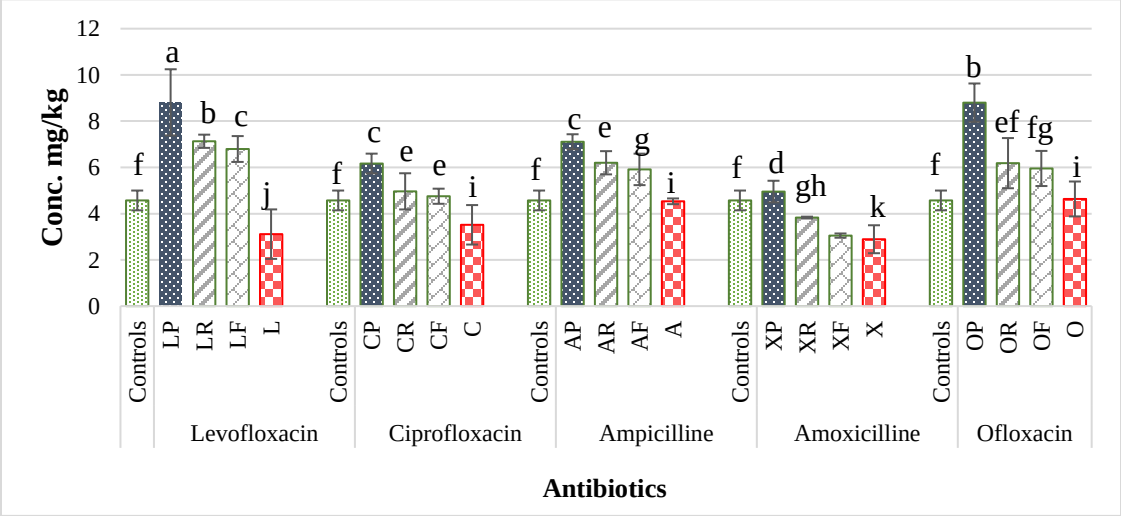

Figure 4.11: Effects of different antibiotics and organic amendments on plant Iron. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.4.3 Total carbohydrates

Total carbohydrates concentration declined with the application of antibiotics. The highest reduction was recorded in plants treated with ampicillin i.e. 41%. Overall reduction in carbohydrate content, by all antibiotics, was 30%. Whereas, organic amendments released the

stress on nutrient uptake and plant growth improved. With poultry litter, carbohydrate content was increased by 47% (0.47 folds).

Analysis of variance proved that antibiotics have significant ($p<0.05$) negative impact on plant growth. Organic amendments significantly ($p<0.05$) lessened antibiotic stress in rice plants. However, TUKEY HSD test showed that all five antibiotics were not significantly different from each other in overall impact. But, the potential of stress releasing in all three organic amendments was significantly different from each other.

Figure 4.12 illustrates that ampicillin had most negative impact on plant carbohydrate content (41% decrease. Poultry litter alleviated the antibiotic stress by 45% collectively.

At present, there is a huge research gap in estimation of antibiotics' effect on plant nutritional composition. Effect of antibiotics on different nutrient uptake are yet to be documented. The probable reason of organic amendments' positive effect (stress alleviation) could be the addition of nutrients in soil, improving soil fertility and restoration of soil microbial communities. These microbes play a vital role in making nutrients available to the plant for easy uptake. Moreover, organic substances provide medium for the adsorption of environmental pollutants (antibiotics).

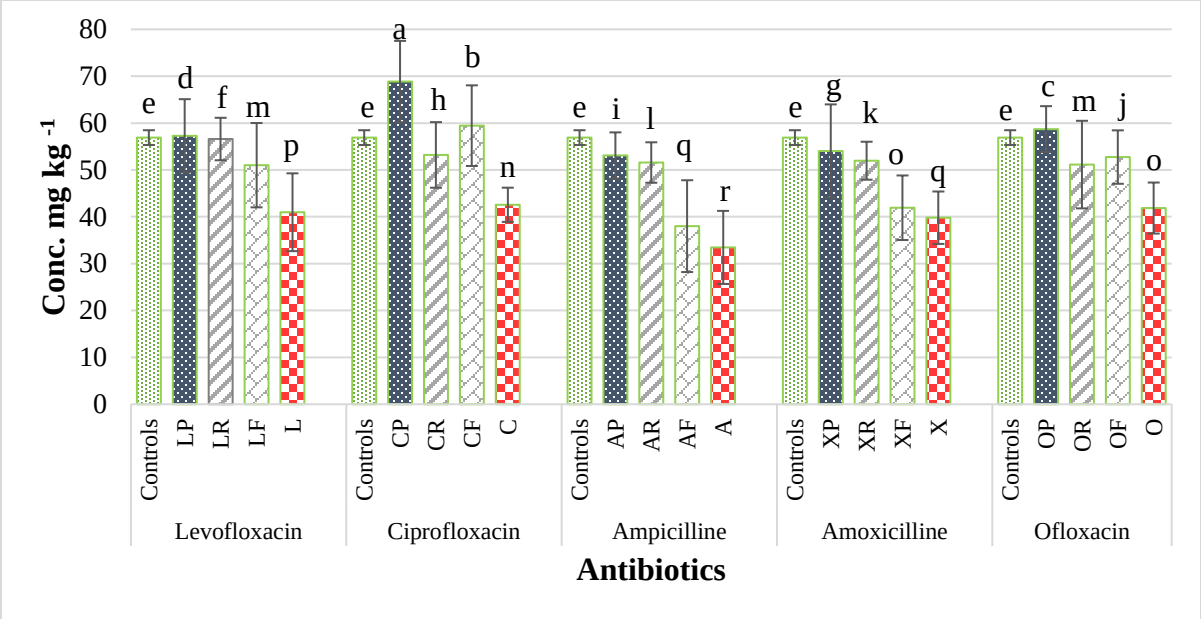

Figure 4.12: Effects of different antibiotics and organic amendments on plant shoot carbohydrates. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.4.4 Protein

All the selected antibiotics had an overall declining effect on the protein content of rice grains. Percentage of protein was majorly affected by ampicillin (decrease by 30%) followed by Levofloxacin 29% and least effect was on Amoxicillin i.e. 8%. As shown by TUKEY HSD test, most effective organic treatment was poultry litter followed by rice husk and farmyard manure. It is also proved by this test; all five antibiotics are not significantly different from each other in impact. But rice husk, farmyard manure and poultry litter are significantly different from each other in releasing antibiotic stress.

Results coincide with another study conducted by Jin et al. (2009), they conclude that antibiotics like tetracycline and erythromycin (concentration 1.4-22.4 mg L^{-1}) had an adverse effect on protein synthesis in *Triticum aestivum.* Similar results have also been reported by

Daghrir and Drogui (2013) which concluded that tetracyclines' uptake negatively impacted protein synthesis in aquatic plants. Antibiotics increased oxidative stress, thus cell redox balance get disturbed. It results in production of peroxides and free radicals, consequently reduction in proteins and lipids is observed along with breakage of DNA strands (Riaz et al., 2017).

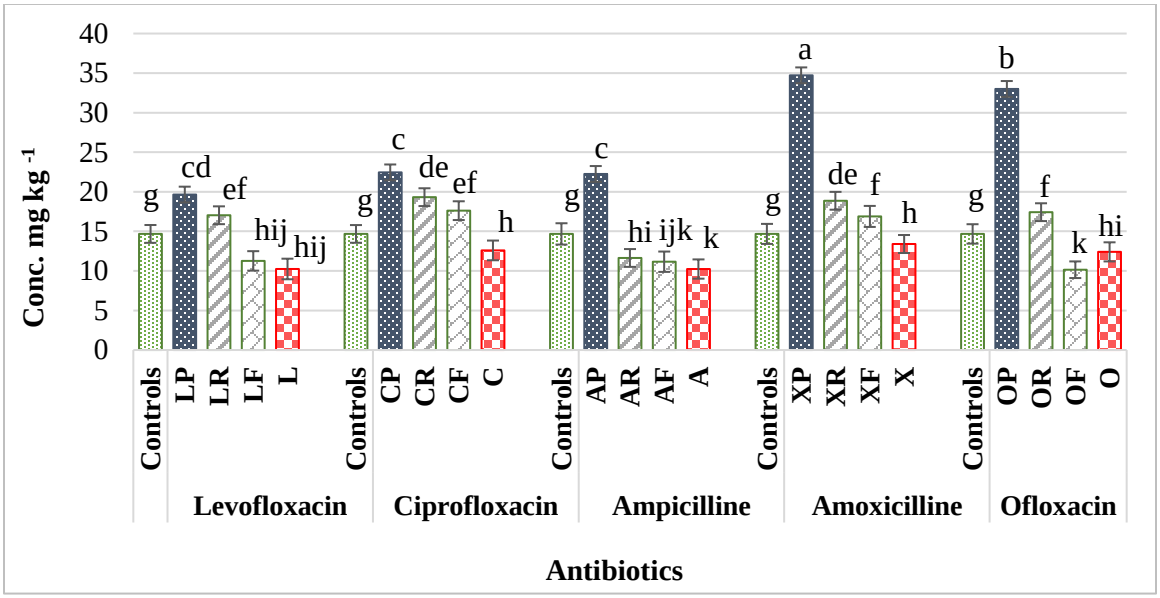

Figure 4.13: Effects of different antibiotics and organic amendments on grain protein content Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

4.5 Genotoxicity assessment – Comet assay

During the study, genotoxicity of all the five antibiotics was assessed by comet assay in the root tips of *Oryza sativa*. The results of comet assay by all five antibiotics and organic amendments have been summarized in the figure 4.15. Each experiment was performed with four replicates. According to the statistical analysis (coefficient of variance), all the antibiotics had significant ($p<0.05$) negative impact and induced genotoxicity in rice plant. These antibiotics are also significantly different from each other approved by Tukey HSD analysis.

Furthermore, the highest genotoxicity was induced by Levofloxacin constituting tail moment up to 121.25 pixels, followed by Ofloxacin 62.25 pixels and ciprofloxacin 59.25 pixels. Whereas, on the other hand, poultry litter in combination with all antibiotics helped to reduced genotoxicity up to 59% relative to antibiotic controls.

DNA strand is a stable structure in eukaryotic cells, it maintains genetic information. Exposure to antibiotics led to DNA strand breakage and gene mutation. Visual observations of this study as well as of Koppen and Vershaeve (1996) showed that minimum DNA migration was observed in controls. They were round red spots and no tail was formed. Whereas, DNA migration towards anode was maximum in those roots which were treated with antibiotics (comets tails were observed). As a result of genotoxity, comets were clearly seen under microscope equipped with an excitation filter of 515-565 nm and a barrier filter of 590 nm.

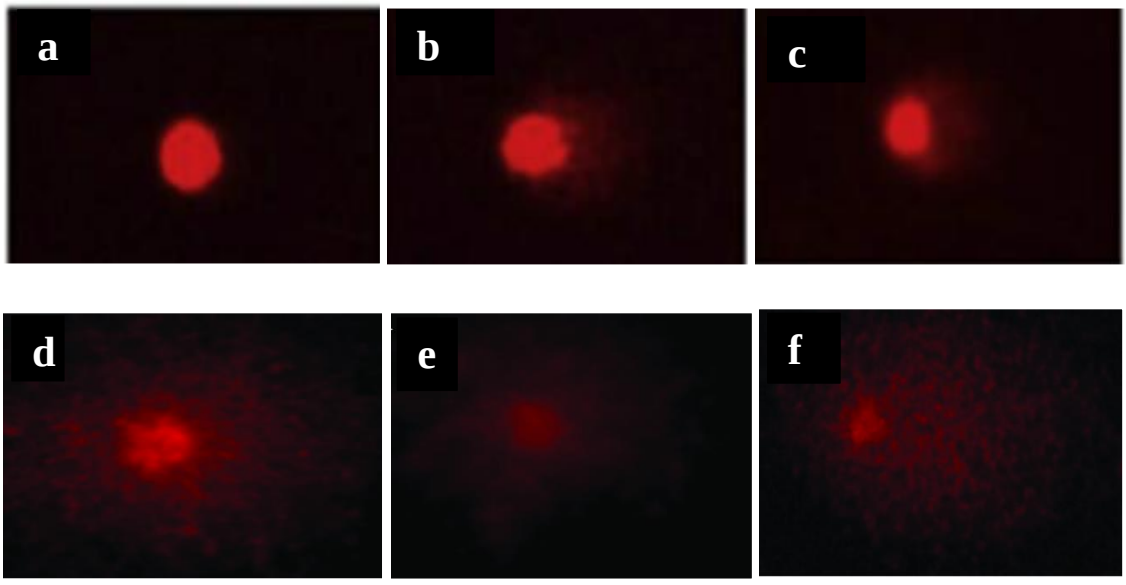

Figure 4.14: Induction of DNA damage, as expressed by comets. Application of antibiotics induced DNA strand breakage in germinating rice roots. Images indicate nuclei after exposure toantibiotics. **a.** control, **b.** Amoxicillin, **c.** Ampicillin, **d.** Ofloxacin, **e.** Ciprofloxacin, **f.** Levofloxacin

Although no literature has been reported on antibiotic induced genotoxicity in plants by using comet assay, but there are other reported studies with different environmental pollutants. A

study by Gichner et al. (2006) was conducted on two plants i.e. potato and tobacco grown in heavy metals spiked soil, which concluded that plant shown a significant increase in DNA damage (genotoxicity) as compared to the control plants. Zhang et al. (2010) also reported that Cu (pollutant) induced significant toxicity in plants. They concluded that number of damaged cells in roots was higher than in shoots.

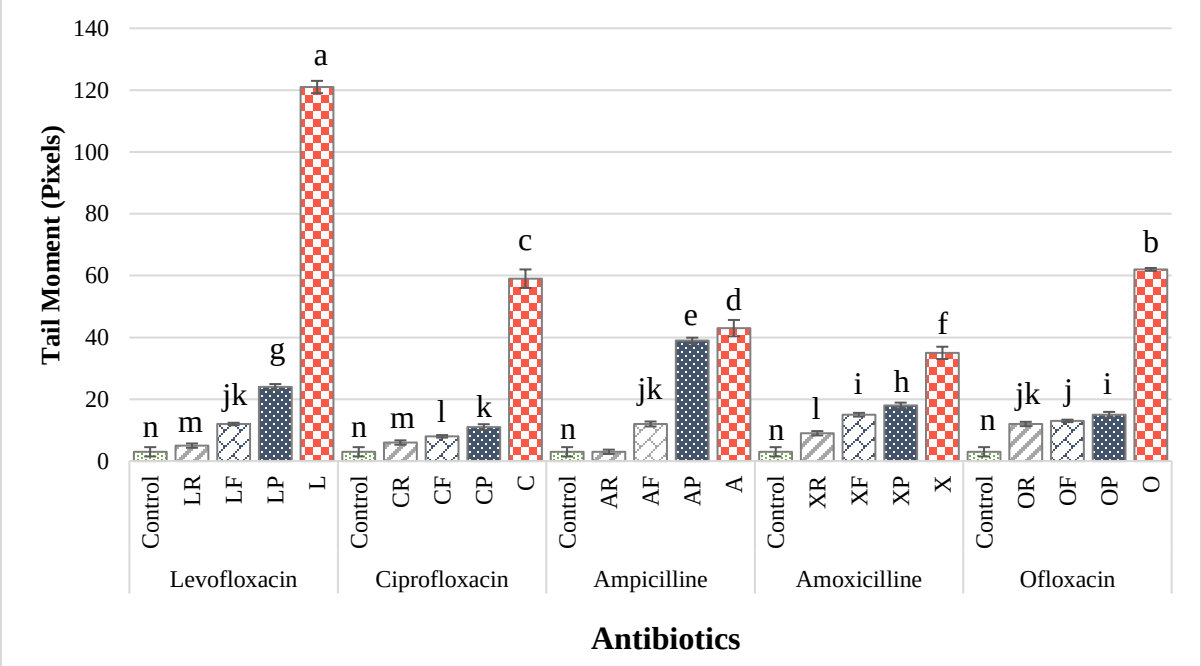

Figure 4.15: DNA damage (tail moment) due to the antibiotics and effect of organic amendments. Alphabets on the bars show the difference among different treatment groups according to Tukey HSD analysis. Where alphabets are same, there is no significant difference among treatments for different alphabets there is significant difference. Whereas, A = Ampicillin, X = Amoxicillin, C = Ciprofloxacin, L = Levofloxacin O = Ofloxacin, P = Poultry litter, F = Farmyard manure, R = Rice husk.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Extensive use of antibiotics globally has resulted in significant diffused pollution in the environment. But effects of different classes of antibiotics on plants' physiology and vegetative nutritional composition have not been investigated in detail so far. This study provided insights into impacts of five antibiotics (Ciprofloxacin, Levofloxacin, Amoxicillin, Ampicillin and Ofloxacin) on plants and investigated the potential of three organic amendments (rice husk, poultry litter and farmyard manure) in alleviation of antibiotic stress in *Oryza sativa.*

Findings of the present study are mentioned below:

- Upon exposure to antibiotics at germination stage, seedling dry biomass was reduced by 53%, seedling length by 71% and seedling vigor index by 59%. Rice husk was most effective treatment that reduced the antibiotic stress by 75%, 138%, 381% and 210%, respectively.
- Delay in germination (1-2 days delay) was observed in response of antibiotic stress.
- When antibiotics were applied, at harvesting stage, decrease in plant biomass (74%), plant length (27%) and tiller development (54%) was observed. Rice husk effectively improved plant growth for all mentioned parameters by 117%, 53% and 138%, respectively.
- Concentration of phosphorous, iron, carbohydrates and proteins declined by 62%, 23%, 30% and 22%, respectively upon application of different antibiotics. Poultry litter was most effective treatment that improved plant nutritional content. Phosphorous was improved by 367%, iron 66%, carbohydrates 47% and proteins by 126%.

- Application of antibiotics induced genotoxicity in plants. Significant alleviation in genotoxicity was observed by poultry liter (reduced by 59%).
- Rice husk successfully alleviated antibiotic stress on phytotoxicity as well as genotoxicity in rice. Whereas, poultry litter improved plants' nutritional content.

Summarizing all the impacts, application of organic amendments alleviated phytotoxicity as well as genotoxicity upon exposure to different antibiotics.

5.2 Future Recommendations

In current study, significant negative impacts of antibiotics on *Oryza sativa* were found. On the other hand, organic amendments showed some good potential in alleviation of these negative impacts. However, there is research gap in this field. Following are the recommendations for the work to be done in future:

- Studies on uptake and transport mechanisms should be conducted as the tendency of accumulation varies among different antibiotic groups.
- Studies on nano- antibiotic degradation can help to minimize the antibiotic pollution in the environment.
- Studies on change in plant physiology and enzymatic activity due to the antibiotics are required for the better understanding of these complex compounds.
- Studies on combination of organic and inorganic fertilizers with antibiotics should be conducted so that potentially good combinations of amendments can be identified to alleviate the impact of these environmental pollutants.

REFERENCES

Aguilar-Garcia, C., Gavino, G., Baragano-Mosqueda, M., Hevia, P., & Gavino, V. C. (2007). Correlation of tocopherol, tocotrienol, γ-oryzanol and total polyphenol content in rice bran with different antioxidant capacity assays. *Food Chemistry*, *102*(4), 1228-1232.

Ahmad, M., Khan, A., Wahid, A., Butt, Z., Farhan, M., & Ahmad, F. (2012). Role of hospital effluents in the contribution of antibiotics and antibiotic resistant bacteria to the aquatic environment. *Pakistan Journal of Nutrition*, *11*(12), 1177-1182.

An, J., Zhou, Q., Sun, Y., & Xu, Z. (2009). Ecotoxicological effects of typical personal care products on seed germination and seedling development of wheat (*Triticum aestivum* L.). *Chemosphere*, 76(10), 1428-1434.

Ashfaq, M., Khan, K. N., Rehman, M. S. U., Mustafa, G., Nazar, M. F., Sun, Q., ... & Yu, C. P. (2017). Ecological risk assessment of pharmaceuticals in the receiving environment of pharmaceutical wastewater in Pakistan. *Ecotoxicology and Environmental Safety*, *136*, 31-39.

Baguer, A. J., Jensen, J., & Krogh, P. H. (2000). Effects of the antibiotics oxytetracycline and tylosin on soil fauna. *Chemosphere*, *40*(7), 751-757.

Bartrons, M., & Peñuelas, J. (2017). Pharmaceuticals and personal-care products in plants. *Trends in Plant Science*, *22*(3), 194-203.

Bartrons, M., & Peñuelas, J. (2017). Pharmaceuticals and personal-care products in plants. *Trends in Plant Science*, *22*(3), 194-203.

Batchelder, A. R. (1982). Chlortetracycline and Oxytetracycline Effects on Plant Growth and Development in Soil Systems 1. *Journal of Environmental Quality*, *11*(4), 675-678.

Bolt, H. M., Stewart, J. D., & Hengstler, J. G. (2011). A comprehensive review about micronuclei: mechanisms of formation and practical aspects in genotoxicity testing. *Archives toxicol.*, *85*(8), 861-862

Boonsaner, M. and Hawker, D. (2010). Accumulation of oxytetracycline and norfloxacin from saline soil by soybeans. *Science of the Total Environment*, 408: 1731–1737.

Bouki, C., Venieri, D., & Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicology and Environmental Safety*, 91, 1-9.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*, *8*(7), 1137-1144.

Caldwell, D. J. (2016). Sources of Pharmaceutical Residues in the Environment and their Control. *Issues in Environmental Science and Technology. Pharmaceuticals in the Environment, The Royal Society of Chemistry, UK*, 92-119.

Cardoso, O., Porcher, J. M., & Sanchez, W. (2014). Factory-discharged pharmaceuticals could be a relevant source of aquatic environment contamination: review of evidence and need for knowledge. *Chemosphere*, *115*, 20-30.

Carvalho, I. T., & Santos, L. (2016). Antibiotics in the aquatic environments: a review of the European scenario. *Environment International*, *94*, 736-757.

Chang, X., Meyer, M. T., Liu, X., Zhao, Q., Chen, H., Chen, J.-a., Qiu, Z., Yang, L., Cao, J., & Shu, W. (2010). Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. *Environmental Pollution*, 158(5), 1444-1450.

Chen, H., Gao, B., & Li, H. (2014). Functionalization, pH, and ionic strength influenced sorption of sulfamethoxazole on graphene. *Journal of Environmental Chemical Engineering*, *2*(1), 310-315.

Chen, Hao, Bin Gao, and Hui Li. "Removal of sulfamethoxazole and ciprofloxacin from aqueous solutions by graphene oxide." *Journal of Hazardous Materials* 282 (2015): 201-207.

Cogger, C. G., Bary, A. I., Kennedy, A. C., & Fortuna, A. M. (2013). Long-term crop and soil response to biosolids applications in dryland wheat. *Journal of Environmental Quality*, *42*(6), 1872-1880.

Daghrir, R., & Drogui, P. (2013). Tetracycline antibiotics in the environment: a review. *Environmental Chemistry Letters*, 11(3), 209-227.

De Graaff, M. S., Vieno, N. M., Kujawa-Roeleveld, K., Zeeman, G., Temmink, H., & Buisman, C. J. N. (2011). Fate of hormones and pharmaceuticals during combined anaerobic treatment and nitrogen removal by partial nitritation-anammox in vacuum collected black water. *Water Research*, *45*(1), 375-383.

Dhawan, A., Bajpayee, M., & Parmar, D. (2009). Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biology and Toxicology*, *25*(1), 5-32.

Díaz, C. A., Hernández-Chávez, J. F., Ulloa-Mercado, G., Gortáres-Moroyoqui, P., Macías, R. M., Meza-Escalante, E., & Serrano-Palacios, D. (2018). Simultaneous quantification of antibiotics in wastewater from pig farms by capillary electrophoresis. *Journal of Chromatography B.1092* (1),386-393.

Díaz-Cruz, M. S., de Alda, M. J. L., & Barcelo, D. (2003). Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. *TrAC Trends in Analytical Chemistry*, *22*(6), 340-351.

Diwan, V., Tamhankar, A. J., Khandal, R. K., Sen, S., Aggarwal, M., Marothi, Y., ... & Stålsby-Lundborg, C. (2010). Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. *BMC Public Health*, *10*(1), 414.

Doorslaer, X. V., Dewulf, J., Langenhove, H. V., & Demeestere, K. (2014). Fluoroquinolone antibiotics: An emerging class of environmental micropollutants. *Science of The Total Environment,500-501*, 250-269.

Du, L., & Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development*, *32*(2), 309-327.

Eggen, T., & Lillo, C. (2012). Antidiabetic II drug metformin in plants: uptake and translocation to edible parts of cereals, oily seeds, beans, tomato, squash, carrots, and potatoes. *Journal of Agricultural and Food Chemistry*, 60(28), 6929-69310.

Eggen, T., Asp, T. N., Grave, K., & Hormazabal, V. (2011). Uptake and translocation of metformin, ciprofloxacin and narasin in forage- and crop plants. *Chemosphere*, 85(1), 26- 33.

Elmolla, E. S., & Chaudhuri, M. (2010). Photocatalytic degradation of amoxicillin, ampicillin and cloxacillin antibiotics in aqueous solution using UV/TiO_2 and UV/H_2O_2/TiO_2 photocatalysis. *Desalination*, 252(1), 46-52

FAO (Food and Agriculture Organization of the United Nations). (2014). How to feed the world in 2050. Proceedings of the Expert Meeting on How to Feed the World in 2050. 24–26. FAO Headquarters, Rome.

Farkas, M. H., Berry, J. O., & Aga, D. S. (2007). Chlortetracycline detoxification in maize via induction of glutathione S-transferases after antibiotic exposure. *Environmental Science & Technology*, *41*(4), 1450-1456.

Fatta-Kassinos, D., Meric, S., & Nikolaou, A. (2011). Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. *Analytical and Bioanalytical Chemistry*, *399*(1), 251-275.

Gavalchin, J., & Katz, S. E. (1994). The persistence of fecal-borne antibiotics in soil. *Journal of AOAC International (USA) 77: 481–485.*

Gichner, T., Patková, Z., Száková, J., & Demnerová, K. (2006). Toxicity and DNA damage in tobacco and potato plants growing on soil polluted with heavy metals. *Ecotoxicology and Environmental Safety*, *65*(3), 420-426.

Gnanam, A., & Kulandaivelu, G. (1969). Photosynthetic studies with leaf cell suspensions from higher plants. *Plant Physiology*, *44*(10), 1451-1456.

Grote, M., Schwake-Anduschus, C., Michel, R., Stevens, H., Heyser, W., Langenkamper, G., ... & Freitag, M. (2007). Incorporation of veterinary antibiotics into crops from manured soil. *Landbauforschung Volkenrode*, *57*(1), 25.

Hamid, W., Rashid, M. A., Habib, A., Akbar, N., & Ashraf, E. (2018). Agronomic management of bacterial leaf blight of rice (*Oryza sativa* l.) Disease and yield enhancement through different combinations of nitrogen and potash doses. *Pakistan Journal of Phytopathology*, *30*(1), 01-06.

Harris, S., & Cummins, E. (2012). Simulation model to predict the fate and effect of ciprofloxacin after wastewater treatment. *Biosystems Engineering Research Review 17*, 83.

Hektoen, H., Berge, J. A., Hormazabal, V., & Yndestad, M. (1995). Persistence of antibacterial agents in marine sediments. *Aquaculture, 133*(3-4), 175-184.

Hillis, D. G., Fletcher, J., Solomon, K. R., & Sibley, P. K. (2011). Effects of ten antibiotics on seed germination and root elongation in three plant species. *Archives of Environmental Contamination and Toxicology*, *60*(2), 220-232.

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K., & Bauer, J. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environmental Research*, *113*, 21-27.

Hou, J., Liu, G. N., Xue, W., Fu, W. J., Liang, B. C., & Liu, X. H. (2014). Seed germination, root elongation, root-tip mitosis, and micronucleus induction of five crop plants exposed to chromium in fluvo-aquic soil. *Environmental Toxicology and Chemistry*, *33*(3), 671-676.

Hu, X., Zhou, Q., & Luo, Y. (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environmental Pollution*, *158*(9), 2992-2998.

Hyland, K. C., Blaine, A. C., Dickenson, E. R., & Higgins, C. P. (2015). Accumulation of contaminants of emerging concern in food crops—part 1: Edible strawberries and lettuce grown in reclaimed water. *Environmental Toxicology and Chemistry, 34*(10), 2213-2221.

Ibekwe, A. M., Gonzalez-Rubio, A., & Suarez, D. L. (2018). Impact of treated wastewater for irrigation on soil microbial communities. *Science of the Total Environment, 622*, 1603-1610.

Igiehon, N. O., & Babalola, O. O. (2017). Biofertilizers and sustainable agriculture: exploring arbuscular mycorrhizal fungi. *Applied Microbiology and Biotechnology*, *101*(12), 4871-4881.

Igiehon, N. O., & Babalola, O. O. (2018). Rhizosphere Microbiome Modulators: Contributions of Nitrogen Fixing Bacteria towards Sustainable Agriculture. *International journal of Environmental Research and Public Health*, *15*(4), 574.

Ingerslev, F., & Halling-Sørensen, B. (2001). Biodegradability of metronidazole, olaquindox, and tylosin and formation of tylosin degradation products in aerobic soil–manure slurries. *Ecotoxicology and Environmental Safety*, *48*(3), 311-320.

International Seed Testing Association. (1985). International rules for seed testing. Rules 1985. *Seed science and technology*, *13*(2), 299-513.

Inyang, M., Gao, B., Ding, W., Pullammanappallil, P., Zimmerman, A. R., & Cao, X. (2011). Enhanced lead sorption by biochar derived from anaerobically digested sugarcane bagasse. *Separation Science and Technology*, *46*(12), 1950-1956.

Inyang, M., Gao, B., Zimmerman, A., Zhang, M., Chen, H. (2014). Synthesis, characterization, and dye sorption ability of carbon nanotube-biochar nanocomposites. Chem. Eng. J. 236, 39e46.

Jin, C., Chen,Q., Sun,R., Zhou,Q., Liu,J. (2009). Eco-toxic effects of sulfadiazine sodium, sulfa-mono methoxine sodium and enrofloxacin on wheat, Chinese cabbage and tomato. *Ecotoxicology*, 18: 878-885.

Jjemba, P. K. (2002). The effect of chloroquine, quinacrine, and metronidazole on both soybean plants and soil microbiota. *Chemosphere*, *46*(7), 1019-1025.

Khadra, A., Pinelli, E., Lacroix, M. Z., Bousquet-Mélou, A., Hamdi, H., Merlina, G., ... & Hafidi, M. (2012). Assessment of the genotoxicity of quinolone and fluoroquinolones contaminated soil with the *Vicia faba* micronucleus test. *Ecotoxicology and Environmental Safety*, 76, 187-192.

Kim, S. C., & Carlson, K. (2007). Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environmental sScience & Technology*, *41*(1), 50-57.

Knapp, C. W., Dolfing, J., Ehlert, P. A., & Graham, D. W. (2009). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science & Technology*, *44*(2), 580-587.

Kong, W. D., Zhu, Y. G., Liang, Y. C., Zhang, J., Smith, F. A., & Yang, M. (2007). Uptake of oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.). *Environmental Pollution*, *147*(1), 187-193.

Koppen, G., & Verschaeve, L. (1996). The alkaline comet test on plant cells: a new genotoxicity test for DNA strand breaks in *Vicia faba* root cells. *Mutation Research/Environmental Mutagenesis and Related Subjects*, *360*(3), 193-200.

Kumar, K., Gupta, S. C., Baidoo, S. K., Chander, Y., & Rosen, C. J. (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *Journal of environmental quality*, *34*(6), 2082-2085.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part I. *Chemosphere*, *75*(4), 417-434.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part I. *Chemosphere*, *75*(4), 417-434.

Larsson, D.G., de Pedro, C. and Paxeus, N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials*, 148: 751– 755

Li, D., Yang, M., Hu, J., Zhang, Y., Chang, H. and Jin, F. (2008). Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. *Water Research*, 42: 307–317.

Li, Z. J., Xie, X. Y., Zhang, S. Q. and Liang, Y. C. (2011). Wheat growth and photosynthesis as affected by oxytetracycline as a soil contaminant. *Pedosphere*, 21(2):244–250.

Liang, N., Huang, P., Hou, X., Li, Z., Tao, L., & Zhao, L. (2016). Solid-phase extraction in combination with dispersive liquid-liquid microextraction and ultra-high performance liquid chromatography-tandem mass spectrometry analysis: the ultra-trace determination of 10 antibiotics in water samples. *Analytical and Bioanalytical Chemistry*, 408(6), 1701-1713.

Lin, V. S. (2015). Research highlights: natural passive samplers–plants as biomonitors. *Environmental Science: Processes & Impacts*, *17*(6), 1137-1140.

Liu, F., Ying G.G., Tao R., Zhao J.L., Yang J.F., Zhao L.F. (2009): Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environmental Pollution*, 157: 1636–1642.

Low, K. S., & Lee, C. K. (1997). Quaternized rice husk as sorbent for reactive dyes. *Bioresource Technology*, *61*(2), 121-125.

Ludwig, T. G., & Goldberg, H. J. (1956). The anthrone method for the determination of carbohydrates in foods and in oral rinsing. *Journal of Dental Research*, 35(1), 90-94.

Luo, Y., Xu, L., Rysz, M., Wang, Y., Zhang, H. and Alvarez, P.J.J. (2011). Occurrence and transport of tetracycline, sulfonamide, quinolone, and macrolide antibiotics in the Haihe River Basin, China. *Environmental Science and Technology*, 45: 1827–1833.

Manzetti, S., & Ghisi, R. (2014). The environmental release and fate of antibiotics. *Marine Pollution Bulletin*, *79*(1-2), 7-15.

Martin, F., Winspear, M. J., MacFarlane, J. D., & Oaks, A. (1983). Effect of methionine sulfoximine on the accumulation of ammonia in C3 and C4 leaves: the relationship between NH3 accumulation and photorespiratory activity. *Plant Physiology*, 71(1), 177-181.

McManus, P. S., Stockwell, V. O., Sundin, G. W., & Jones, A. L. (2002). Antibiotic use in plant agriculture. *Annual Review of Phytopathology*, *40*(1), 443-465.

Michelini, L., Reichel, R., Werner, W., Ghisi, R., & Thiele-Bruhn, S. (2012). Sulfadiazine uptake and effects on Salix fragilis L. and Zea mays L. plants. *Water, Air, & Soil Pollution*, *223*(8), 5243-5257.

Migliore, L., Brambilla, G., Cozzolino, S., & Gaudio, L. (1995). Effect on plants of sulphadimethoxine used in intensive farming (Panicum miliaceum, Pisum sativum and Zea mays). *Agriculture, Ecosystems & Environment*, *52*(2-3), 103-110.

Migliore, L., Civitareale, C., Cozzolino, S., Casoria, P., Brambilla, G., & Gaudio, L. (1998). Laboratory models to evaluate phytotoxicity of sulphadimethoxine on terrestrial plants. *Chemosphere*, *37*(14-15), 2957-2961.

Migliore, L., Cozzolino, S., & Fiori, M. (2003). Phytotoxicity to and uptake of enrofloxacin in crop plants. *Chemosphere*, *52*(7), 1233-1244.

Migliore, L., Rotini, A., Cerioli, N. L., Cozzolino, S., & Fiori, M. (2010). Phytotoxic antibiotic sulfadimethoxine elicits a complex hormetic response in the weed *Lythrum salicaria* L. *Dose-response*, *8*(4), dose-response. https://doi.org/10.2203/dose-response.09-033.Migliore

Miller, E. L., Nason, S. L., Karthikeyan, K. G., & Pedersen, J. A. (2016). Root uptake of pharmaceuticals and personal care product ingredients. *Environmental Science & Technology*, *50*(2), 525-541.

Mölstad, S., Lundborg, C.S., Karlsson, A.K. and Cars, O. (2002). Antibiotic prescription rates vary markedly between 13 European countries. Scandinavian *Journal of Infectious Diseases*, 34: 366–371.

Motwani, D., & Mehta, P. (2018). Effect of veterinary antibiotics on the seed germination of indica rice varieties. *Annals of Plant Sciences*, *7*(6), 2321-2327.

Munné-Bosch, S., & Alegre, L. (2000). Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in Rosmarinus officinalis plants. *Planta*, *210*(6), 925-931.

Nie, X. P., Liu, B. Y., Yu, H. J., Liu, W. Q., & Yang, Y. F. (2013). Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole exposure to the antioxidant system in Pseudokirchneriella subcapitata. *Environmental pollution*, *172*, 23-32.

O'brien, T. F. (2002). Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clinical Infectious Diseases*, *34*(Supplement_3), S78-S84.

Olsen, S. R. (1954). *Estimation of available phosphorus in soils by extraction with sodium bicarbonate*. United States Department of Agriculture; Washington. (pp. 110-112).

Opris, O., Copaciu, F., Loredana Soran, M., Ristoiu, D., Niinemets, Ü., & Copolovici, L. (2013). Influence of nine antibiotics on key secondary metabolites and physiological characteristics in *Triticum aestivum*: Leaf volatiles as a promising new tool to assess toxicity. *Ecotoxicology and Environmental Safety*, 87, 70-79.

Pan, M., & Chu, L. M. (2016). Phytotoxicity of veterinary antibiotics to seed germination and root elongation of crops. *Ecotoxicology and Environmental Safety*, *126*, 228-237.

Pan, M., Wong, C. K., & Chu, L. M. (2014). Distribution of antibiotics in wastewater-irrigated soils and their accumulation in vegetable crops in the Pearl River Delta, Southern China. *Journal of Agricultural and Food Chemistry*, *62*(46), 11062-11069.

Peycheva, E., Ivanova, E., Ivanov, S., & Miloshev, G. (2011). Improved procedure for Comet assay on active photosynthetic cells from pea plants. *Oxidation Communications*, *34*(2), 320-325.

Pini, F., Frascella, A., Santopolo, L., Bazzicalupo, M., Biondi, E. G., Scotti, C., & Mengoni, A. (2012). Exploring the plant-associated bacterial communities in Medicago sativa L. *BMC microbiology*, *12*(1), 78.

Rashid, A. (1986). *Mapping zinc fertility of soils using indicator plants and soil analyses* (Doctoral dissertation).

Riaz, L., Mahmood, T., Coyne, M. S., Khalid, A., Rashid, A., Hayat, M. T., & Amjad, M. (2017). Physiological and antioxidant response of wheat (Triticum aestivum) seedlings to fluoroquinolone antibiotics. *Chemosphere*, 177, 250-257.

Richards, L. A. (1954). Diagnosis and improvement of saline and alkali soils. Washington: United States Salinity Laboratory, 1954. 160p. USDA. *Agriculture Handbook*, 60. Robinson, A. A., Belden, J. B., & Lydy, M. J. (2005). Toxicity of fluoroquinolone

antibiotics to aquatic organisms. *Environmental Toxicology and Chemistry*, 24(2), 423-430.

Richardson, A. E. (2007). Making microorganisms mobilize soil phosphorus. In *First international meeting on microbial phosphate solubilization* (pp. 85-90). Springer, Dordrecht.

Ryan, J. (2008). Soil and plant analysis laboratory manual. International Center for Agriculture Research in Dry Areas. *Scientific Publishers Journals Dept.* (ISBN: 978-8172334987).

Ryan, J., Estefan, G., & Rashid, A. (2007). Soil and plant analysis laboratory manual. *ICARDA.* 150-154.

Santos, L. H., Araújo, A. N., Fachini, A., Pena, A., Delerue-Matos, C., & Montenegro, M. C. B. S. M. (2010). Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials*, *175*(1-3), 45-95.

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, *65*(5), 725-759.

Schulten, H. R. (1999). Interactions of dissolved organic matter with xenobiotic compounds: Molecular modeling in water. *Environmental Toxicology and Chemistry*, *18*(8), 1643-1655.

Silveira, G. L., Lima, M. G. F., dos Reis, G. B., Palmieri, M. J., & Andrade-Vieria, L. F. (2017). Toxic effects of environmental pollutants: Comparative investigation using *Allium cepa* L. and *Lactuca sativa* L. *Chemosphere*, *178*, 359-367.

Singer, R. S., Finch, R., Wegener, H. C., Bywater, R., Walters, J., & Lipsitch, M. (2003). Antibiotic resistance—the interplay between antibiotic use in animals and human beings. *The Lancet infectious diseases*, *3*(1), 47-51.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, *175*(1), 184-191.

Spence, C., Alff, E., Johnson, C., Ramos, C., Donofrio, N., Sundaresan, V., & Bais, H. (2014). Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology*, *14*(1), 130.

Sudduth, K. A., Kitchen, N. R., Wiebold, W. J., Batchelor, W. D., Bollero, G. A., Bullock, D. G., ... & Thelen, K. D. (2005). Relating apparent electrical conductivity to soil properties across the north-central USA. *Computers and Electronics in Agriculture*, *46*(1-3), 263-283.

Suemitsu, R., Uenishi, R., Akashi, I., & Nakano, M. (1986). The use of dyestuff-treated rice hulls for removal of heavy metals from wastewater. *Journal of Applied Polymer Science*, *31*(1), 75-83.

Tandon, S. A., Kumar, R., & Yadav, S. A. (2013). Pytoremediation of fluoroquinolone group of antibiotics from wastewater. *Natural Science*, 5(12), 21.

Tasho, R. P., & Cho, J. Y. (2016). Veterinary antibiotics in animal waste, its distribution in soil and uptake by plants: a review. *Science of the Total Environment*, *563*, 366-376.

Thai, P. K., Binh, V. N., Nhung, P. H., Nhan, P. T., Hieu, N. Q., Dang, N. T., ... & Anh, N. T. K. (2018). Occurrence of antibiotic residues and antibiotic-resistant bacteria in

effluents of pharmaceutical manufacturers and other sources around Hanoi, Vietnam. *Science of The Total Environment*, *645*, 393-400.

Thiele-Bruhn, S., Seibicke, T., Schulten, H. R., & Leinweber, P. (2004). Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions. *Journal of Environmental Quality*, *33*(4), 1331-1342.

Trapp, S., & Legind, C. N. (2011). Uptake of organic contaminants from soil into vegetables and fruits. In *Dealing with contaminated sites* (pp. 369-408). Springer, Dordrecht.

Weerasinghe, C. A., & Towner, D. (1997). Aerobic biodegradation of virginiamycin in soil. *Environmental toxicology and chemistry*, *16*(9), 1873-1876.

Wei, R., Ge, F., Chen, M., & Wang, R. (2012). Occurrence of ciprofloxacin, enrofloxacin, and florfenicol in animal wastewater and water resources. *Journal of Environmental Quality*, 41(5), 1481-1486.

Winckler, C., & Grafe, A. (2001). Use of veterinary drugs in intensive animal production. *Journal of Soils and Sediments*, *1*(2), 66.

Wright, J. M. (1951). Phytotoxic effects of some antibiotics. *Annals of Botany*, *15*(4), 493-499.

Wu, X., Dodgen, L. K., Conkle, J. L., & Gan, J. (2015). Plant uptake of pharmaceutical and personal care products from recycled water and biosolids: a review. *Science of the Total Environment*, *536*, 655-666.

Xie, X., Zhou, Q., He, Z., & Bao, Y. (2010). Physiological and potential genetic toxicity of chlortetracycline as an emerging pollutant in wheat (*Triticum aestivum* L.). *Environmental Toxicology and Chemistry*, 29(4), 922-928.

Xie, X., Zhou, Q., Lin, D., Guo, J., Bao, Y., 2011. Toxic effect of tetracycline exposure on growth, antioxidative and genetic indices of wheat (Triticum aestivum L.). *Environmental Science and Pollution Res.* 18, 566–575

Xu, Z., Jiang, Y., Te, S. H., He, Y., & Gin, K. Y. H. (2018). The Effects of Antibiotics on Microbial Community Composition in an Estuary Reservoir during Spring and Summer Seasons. *Water*, *10*(2), 154.

Yang, W., Zheng, F., Xue, X., & Lu, Y. (2011). Investigation into adsorption mechanisms of sulfonamides onto porous adsorbents. *Journal of Colloid and Interface Science*, *362*(2), 503-509.

Yi, S., Gao, B., Sun, Y., Wu, J., Shi, X., Wu, B., & Hu, X. (2016). Removal of levofloxacin from aqueous solution using rice-husk and wood-chip biochars. *Chemosphere*, *150*, 694-701.

Yi, S., Gao, B., Sun, Y., Wu, J., Shi, X., Wu, B., & Hu, X. (2016). Removal of levofloxacin from aqueous solution using rice-husk and wood-chip biochars. *Chemosphere*, *150*, 694-701.

Zhang, D. J., Li, C. X., Zhang, Z. J., Jiang, L. N., & Shao, Y. (2010). DNA damage, copper distribution and element contents in wheat exposed to copper. In *Environmental Science and Information Application Technology (ESIAT), 2010 International Conference on* (Vol. 3, pp. 522-525).

Zhang, W., Liu, X., Zheng, F., Zeng, S., Wu, K., da Silva, J. A. T., & Duan, J. (2013). Induction of rice mutations by high hydrostatic pressure. *Plant physiology and biochemistry*, *70*, 182-187.

Zhao, X., Joo, J., Kim, D., Lee, J., & Kim, J. Y. (2016). Estimation of the Seedling Vigor Index of Sunflowers Treated with Various Heavy Metals. *Journal of Bioremediation and Biodegradation*, *7*(3). doi: 10.4172/2155-6199.1000353.

Zhao-Jun, L. I., Xiao, Xie., Zhang, S. Q., & Liang, Y. C. (2011). Wheat growth and photosynthesis as affected by oxytetracycline as a soil contaminant. *Pedosphere*, *21*(2), 244-250.