Assessing the Effect of Antibiotics on *Lactuca sativa* Cultivated in Soil Amended with Organic Residues

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Dedication

To My Family

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CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Assessing the Effects of antibiotics on *Lactuca sativa* Cultivated in Soil Amended with Organic Residues"** submitted by Ms. Fakhria Wahid 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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List of Abbreviations

UNEP	United Nations Environment Programme
EPA	Environmental Protection Agency
AIMS	Agriculture Marketing Information Service
WWTP	Wastewater Treatment Plants
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
AMP 5	Ampicillin 5 mg kg^{-1}/L^{-1}
AMP 10	Ampicillin 10 mg kg^{-1}/L^{-1}
OFX 5	Ofloxacin 5 mg kg^{-1}/L^{-1}
OFX 10	Ofloxacin 10 mg kg^{-1}/L^{-1}
kDa	kilo Daltons

Abstract

The global increase in the use of antibiotics has resulted in the contamination of different ecosystems such as soil and water, which can have severe implications for crop productivity. This study investigates the effect of antibiotics, ampicillin and ofloxacin, on *Lactuca sativa* grown in antibiotic-contaminated soil (control, 5 and 10 mg kg^{-1}) and treated with three organic amendments: compost, rice husk and vermicompost. In the germination test, compost as well as rice husk significantly reduced the inhibitory effect of antibiotics on root elongation by 51% and 75% respectively. In the pot experiment, enhanced plant biomass was observed with the use of rice husk. Furthermore, a synergistic effect between antibiotics and compost as well as vermicompost was observed in shoot and root length in the germination test. A similar effect was observed on the rubisco large subunit and soluble protein content of plants cultivated in rice husk and vermicompost. On the contrary, an antagonistic effect of the rice husk and antibiotics at 5 mg kg^{-1} was observed on the chlorophyll content. This study concludes that the effect of antibiotics on different plant traits vary, depending on the antibiotic concentration as well as type of amendment used to alleviate the antibiotic stress.

Keywords: *Lactuca sativa*, antibiotic stress, biochemical plant traits, ampicillin, organic amendments, plant nutrients.

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Chapter 1

Introduction

Since the last few decades, due to increase in the antibiotics' consumption, concerns have been rising about their environmental implications. There has been a 65% rise in the use of antibiotics, mainly driven by the countries of low and middle income, between the year 2000 and 2015 (Klein et al., 2018). Since antibiotics are designed to be highly effective, they are excreted shortly after ingestion (Kong et al., 2007). Hence antibiotics tend to persist in the environment, entering various environmental matrices such as soil and water bodies (Wang & Wang, 2015).

Pakistan is the third major contributor to the reported 65% increase in the antibiotics' consumption globally (Klein et al., 2018). National environmental quality standards are generally not followed by the various multinational pharmaceutical industries in countries such as Pakistan, Bangladesh, India and China (Rehman et al., 2015). Antibiotics in high concentrations have been detected in wastewater, soil and plants due to the untreated industrial effluents being discharged into the sewage (Hussain et al., 2016a, 2016b).

Examples include Kahuta Industrial area in Islamabad and Hattar industrial area in Haripur. In both of these locations, antibiotic contamination is prevalent (Riaz et al., 2017). Ofloxacin ranging from 7 to 39 μg L^{-1} was detected in wastewater from hospitals in Lahore. In another study, concentrations as high as 224 and 129 μg L^{-1} were found in the wastewater and sludge samples from 2 different hospitals in Lahore (Ahmad et al., 2012; Ashfaq et al., 2016).

1.1 Effect on Humans and Plants

Antibiotics can have adverse effects on human health since they play an important role in the development as well as spread of antibiotic resistant genes. Furthermore, many nontarget species, including plants, are affected as well (Kumar at al., 2012; Bártikova et al., 2016; Minden et al., 2017; Jechalke et al., 2014). Several studies have been reported that indicate the ability of plants, such as lettuce, carrot, cucumber, spinach, wheat corn to take antibiotics, through the root system (Ahmed et al., 2015; Chowdhury et al., 2016; Herklotz et al., 2010; Kang et al., 2013; Franklin et al., 2015; Hussain et al., 2015; Pan et al., 2014).

In plants, antibiotics are known to result in decreased root and shoot biomass as well as length, photosynthetic rate, stomatal conductance and delayed germination (Puckowski et al.,2016., Bartikova et al., 2016; Minden et al., 2017; Carvalho et al., 2014).

1.2 Use of Organic Amendments in Agriculture

For decades, organic amendments have been used as fertilizers since they provide a way of recycling the organic waste produced as result of various agricultural processes. They serve as a source of important plant nutrients such as carbon, nitrogen, phosphorus and sulfur, with the carbon forming the major proportion of 50% that become available to be taken up by plants upon mineralization (Kirkby et al., 2011; Pribyl, 2010).

In many countries, using animal manure for agricultural purposes, has been a common practice in order to condition as well as nourish the soil with important nutrients (Churchman and Landa, 2014). Inorganic fertilizers along with a source of organic carbon i.e. crop residues or organic amendments can be used enhance the organic matter content in soil (Alvarez, 2005). Improved biological properties and soil structure as well as cation

exchange and water holding capacity are some of the other advantages associated with the use of organic amendments, which can lead to improved crop yields (Lal, 2006; Deacons and Montemurro, 2010).

The modification of the adsorptive capacity of the soil organic matter through the addition of organic amendments for the immobilization of antibiotics is a complicated process, that depends on several factors. The presence of organic matter in soil has been reported to provide adsorptive sites for antibiotics to bind with. There is a large quantity of COOH bonds present in organic matter, to which the positively charged antibiotic ions can bind through the hydrogen bonding (Sibley et al., 2008; Gu et al., 2007). The adsorptive sites present in soils such as clay loam can be shielded the additions of organic matter, thus making these sites unavailable for the adsorption of antibiotics. Thus, organic matter present in soil cannot be considered as the only factor affecting antibiotic adsorption (Wang and Wang, 2015).

With the increase in the global population, the waste generation rate is projected to increase. For the middle to low income countries, an increase of 38% to 67% of the present rate has been predicted by the year 2050, with the major proportion of this waste being organic in nature (Hoornweg and Bhada-Tata, 2012). Furthermore, developing countries lack the necessary technology as well as financial ability to safely dispose the increasing quantity of solid waste, such as in a landfill, as opposed to developed countries (UNEP, 2011). Recycling seems to be the better choice here, being ecofriendly as well as energy efficient. The organic waste can be recycled with the help of 2 common processes i.e. composting and vermicomposting, to produce compost and vermicompost to be used as

organic amendments. Both these processes have known to be more environmentally friendly and feasible in comparison to incineration as well as landfilling (Saer et al., 2013).

In composting, either aerobic or anaerobic, the biological decomposition of the organic matter is carried out with the help of mesophilic as well as thermophilic microorganism. When certain worms decompose the organic waste into a stable product, the process is known as vermicomposting. Both these processes require optimum carbon and nitrogen ratio as well as optimal temperature i.e. 23 to 42ºC (Chen et al., 2011). The resulting end products i.e. compost and vermicompost are used as source of organic matter in soil to enhance crop yield.

Rice husk is byproduct resulting from rice production and has poor nutritional value. Rice husk has various applications, one of which is the use as a soil amendment. The global production of rice was 741.48 million tons in 2014, with approximately 150 million tons of rice husk (Lertwattanaruk et al., 2018; Zou and Yang, 2019). Although many studies have been carried out on treated rice husk as adsorbent, organic fertilizer and soil amendment for various purposes, there is a limited literature reported on the use of untreated rice husk.

This can serve as a significant route, due to which antibiotics are released into the environment (Minden et al., 2018). Typically, antibiotics are found in animal manure in concentrations up to 10 mg kg^{-1}, however, higher concentrations of 200 mg kg^{-1} have also been reported (Kumar et al., 2005). The presence of antibiotics, varying from µg kg^{-1} to g kg^{-1}, has been reported in soil, cereals and vegetable (Boxall et al., 2002).

1.3 Test Crop

The lettuce (Loose-leaf variety) was selected as the test crop for this research since it is cultivated in large quantities and consumed in raw form. Moreover, lettuce has been extensively used in toxicity assessment tests, given its sensitivity to toxic chemicals (US EPA, 1994). In Pakistan around 441 tons of lettuce is produced annually as of 2016. A total of 414 hectares of land has been dedicated for this purpose (AMIS, 2016). Given its considerable production, it is crucial to investigate the possible effects of the antibiotics on this crop.

1.4 Significance of this Study

Since antibiotic consumption has increased, so has its proliferation into various environmental compartments. Given the reported studies on the adverse effect of different antibiotics on crops, based on the importance of agriculture. Few studies have been conducted in Pakistan, where untreated wastewater is the major source of irrigation (Khalil et al., 2011). Moreover, it is very important to alleviate the stress induced by these antibiotics in plants and limited studies have been reported on the role of cheaper organic amendments in alleviating antibiotic-induced stress in plants.

1.5 Objectives

Keeping in view the scientific context and literature survey, the scientific objectives of the current study are:

- To assess the effect of antibiotics (ampicillin and ofloxacin) on the physical growth parameters of *Lactuca sativa.*
- To determine the effect of nutritional composition in *Lactuca sativa* under antibiotic stress.
- To assess the effect of antibiotics on rubisco activity in leaves of *Lactuca sativa*.

Chapter 2

Literature Review

A considerable number of antibiotics, belonging to different classes, have been developed up to this day. These antibiotics utilize different mechanisms to combat bacterial infections. Given the scope of the current study and their widespread global consumption, the following classes of antibiotics have been discussed:

2.1 Beta-Lactams

Known for the four-ringed member found in their molecular structure, β-Lactams are among the earliest manufactured antibiotics. Penicillin, the very first member of this class, was discovered by Sir Alexander Flemming in 1928 from a culture of mold and its discovery was the beginning of the golden era of antibiotics. Currently, a larger number of beta-lactam antibiotics have been produced to meet the ever changing and emerging challenges.

2.1.1 Mode of Action

The β-lactams cause the lysis of the bacterial cell by targeting the penicillin binding proteins (PBP) present in the cell wall. Peptidoglycan forms the basic structure of bacterial cell wall and PBP plays a significant role in its formation. The lactam ring of these antibiotics makes the penicillin-binding proteins unavailable for further production of peptidoglycan, hence inhibiting the cell wall synthesis. This way the bacterial cell is destroyed.

An important β-lactam, ampicillin belonging to penicillin group, is used to treat as well as prevent gram-negative bacteria and infections caused by bacteria, in human beings as well

as animals (Kapoor et al., 2017). Being acidic in nature, ampicillin inhibits the synthesis of cell wall as well as proteins just like the other members of this class. It owes its widespread use in medical treatments due to its acid stability, lower toxicity and high effectiveness (Džidic et al., 2008). Figure 2.1a shows the different groups of beta lactam class of antibiotics, as indicated by the β-lactam ring. Figure 2.1b shows the chemical structure of ampicillin.

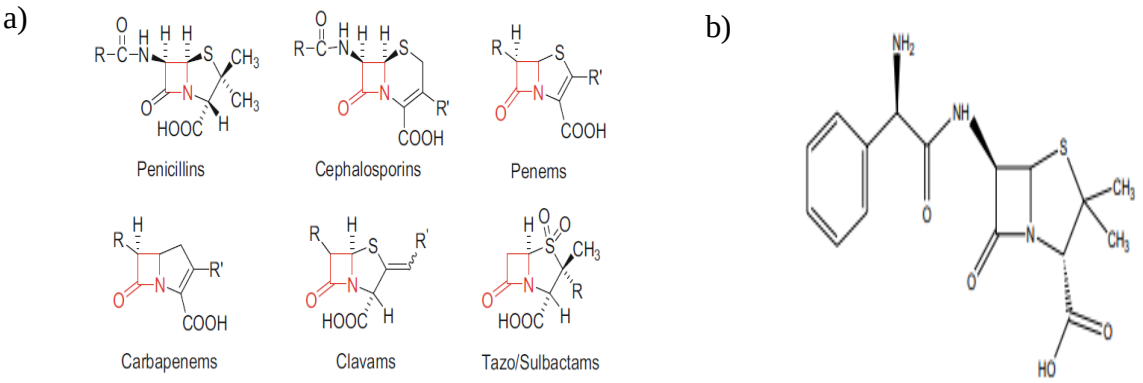


Figure 2.1. Beta Lactams and the chemical structure of ampicillin (a) The beta lactam class of antibiotic (Bhalla et al., 2017) (b) The chemical structure of ampicillin (Absalan et al., 2014).

2.2 Fluoroquinolones

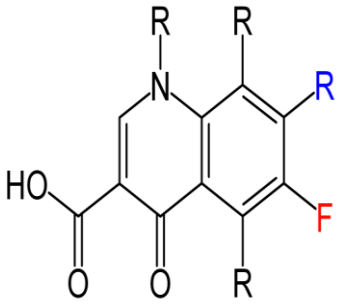
Produced from quinolones through modifications to its chemical structure by the addition of fluorine atom, these are the 2nd generation antibiotics. (Mulas et al., 2018). With the addition of fluorine, the potency of these antibiotics has increased (Andersson and MacGowan, 2003). Some common fluoroquinolones are ofloxacin, ciprofloxacin, levofloxacin, norfloxacin and moxifloxacin that are used for humans therapy as well as for veterinary purposes. The basic structure of fluoroquinolones is shown in figure 2.2a.

2.2.1 Mode of Action

Under normal conditions, the bacterial DNA gyrase initiates with cutting of the double stranded DNA followed by the introduction of negative supercoils and then the cut ends are resealed. It is a very significant process for transcription as well as replication. This enzyme consists of 2 subunits i.e. large and small. These antibiotics stop the bacterial growth by binding to the A subunit, thus inhibiting the function of this enzyme. The potency of fluoroquinolones in gram positive bacteria is due to inhibiting another enzyme, topoisomerase. This enzyme is crucial for segregating the daughter DNA, following the replication process (Kapoor et al., 2017).

Ofloxacin, a commonly used fluoroquinolone, is used to treat bacterial infections related to prostatitis, eye, and ear as well as diarrhea in some cases (Barza, 1991; Cox, 1981; DuPont, 1997). It is an amphoteric compound i.e. it can act both as an acid and base. Its pKa values include: pKa_1 = 5.98, pKa_2 = 8.00 (Gao et al., 2019). Figure 2.2b shows the chemical structure of ofloxacin.

a)



b)

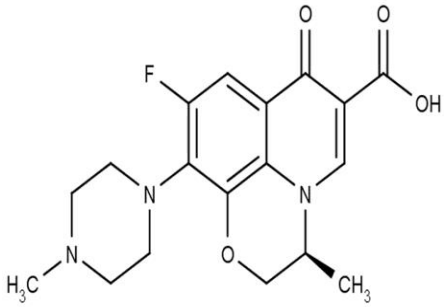


Figure 2.2. a) the basic structure of fluoroquinolones. **b)** Chemical structure of ofloxacin (Rusu et al., 2016)

2.3 Pathways of Antibiotics in the Environment

Since antibiotics consumed by human beings as well as animals are metabolized only to some extent, large amounts are excreted that enter the environment through one way or another (figure 2.3). Below are discussed the presence of antibiotics in various environmental matrices.

2.3.1 Water Bodies

There exist several courses through which the antibiotics enter the environment, the major being wastewater from household, hospital and pharmaceutical companies that either enters the sewage treatment plants (STPs) and wastewater treatment plants (WWTPs) or is discharged directly into water bodies (figure 2.3). Since these contaminants are not removed completely in the afore mentioned facilities, concentrations ranging from ng L^{-1} to µg L^{-1} have been detected in recovered surface water. This treated waste water, from sewage system or septic tank, is often used for the irrigation for agricultural purposes which provides a significant pathway into the environment. Other times, the treated effluent is released directly into fresh water bodies hence contaminating the aquatic environment. Furthermore, the groundwater can be contaminated as a result of leaching from the soil. Surface runoff serves another important route for the release of antibiotics into the aquatic environment.

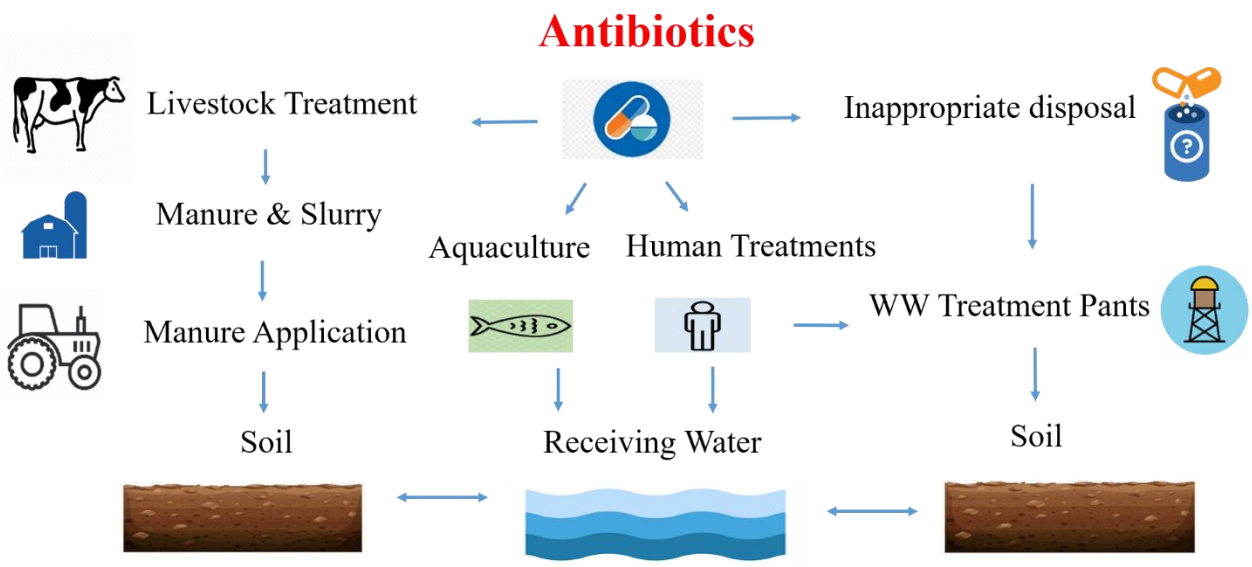


Figure 2.3. Various Pathways of antibiotics into the environment

Extensive literature exists on the presence of antibiotics in freshwater bodies, with their concentration varying around the globe. Clofibric acid was the first pharmaceutical to be detected in the wastewater in the range of 0.8 to 2 µg L^{-1} in Kanas City, US back in 1971 (Fent et al., 2006).

In 1981, the presence of 25 pharmaceuticals at concentration of 1 µg L^{-1} was detected in river Lee which served a potable water source for North London. Following this, many researches were carried out in this regard. The presence of erythromycin, trimethoprim, tamoxifen, ibuprofen and propranolol was reported in a range of 4 to 2370 ng L^{-1} in surface water samples of lower River Tyne in UK. In Spain, samples tested along the four main Iberian River basins showed that tetracycline was present at a concentration of 6 ng L^{-1} (Richardson and Bowron, 1985; Roberts and Thomas, 2005; Lopez-Serna et al., 2012).

Assessment of groundwater in Montana, US indicated the presence of sulfamethoxazole at a concentration of 490 ng L^{-1}. Ciprofloxacin, sulfamethoxazole and clindamycin were

detected in the stream receiving wastewater effluents, ranging from 0.043 to 0.076 µg L^{-1} in East Aurora and Holland, New York at a distance of 100 m. A study carried out in Maryland, USA found the presence of antibiotics in a major agricultural watershed. Sulfadimethoxine and sulfamethoxazole, ranging from 0.005 to 0.007 µg L^{-1}, were the two frequently detected antibiotics. Moxifloxacin was also reported to be among the frequently detected pharmaceuticals in the upstream of a WWTPs, in a study carried out by Minnesota Pollution Control Agency (Miller and Meek, 2006; Batt et al., 2006; Arikan et al., 2008; Ferry, 2013, 2015).

The presence of ciprofloxacin as high as 31 mg L^{-1} was reported in an effluent sample from a WWTP, where wastewater from a pharmaceutical company in Hyderabad, India was received. A similar study carried out in India reported the existence of ciprofloxacin at a concentration of 6.5 mg L^{-1} in ground, surface as well as drinking water, near an industrial area (Larson et al., 2007; Fick et al., 2010). In Man Kyung River, South Korea the presence of levofloxacin and erythromycin at concentrations as high as 87.4 and 137 ng L^{-1} was reported (Kim et al., 2009). Another study carried out in China found the presence as well as transport of antibiotics classes quinolone, tetracycline, macrolide and sulfonamide in the Haihe River Basin (Luo et al., 2011).

A study carried out on Pearl River, China reported the presence of ofloxacin at a high concentration of 1560 µg L^{-1} in the sediments, among other antibiotics from sulfonamide, tetracycline and macrolides classes of antibiotics (Yang et al., 2010). The highest reported concentrations of ofloxacin, sparfloxacin ciprofloxacin, and gemifloxacin was found to be 66, 58, 18 and 0.2 µg L^{-1} respectively, in the wastewater and sludge samples from two of the major hospitals in Pakistan (Ashfaq et al., 2016).

2.3.2 Soil Ecosystem

Antibiotics, such as fluoroquinolones, are extensively used for the treatment as well as growth promotion and disease prevention in livestock, by adding them as feed additives. A large amount of antibiotics is manufactured and used for non-therapeutic purposes globally, including United States, China & European Union.

Since these antibiotics are neither metabolized, nor absorbed, a large portion (30 to 90%) occurs in manure (Herberer, 2002). Furthermore, these antibiotics are particularly stable under the absence of any light, i.e. up to 120 days (Wu et al., 2005). Antibiotics in manure have been detected in numerous studies reported (Zhao et al., 2010; Zhou and Kang, 2013).

Zhao et al. (2010) reported the presence of antibiotics enrofloxacin and norfloxacin in poultry litter, at higher concentrations 1 mg kg^{-1}, a period of two days following ingestion. Therefore, precautionary measures need to be taken in using poultry manure as a soil fertilizer, to avoid spread into the environment (Janusch et al., 2014). The utilization of manure as land fertilizer has served as a major source through which antibiotic are released into the agricultural soils and is a major concern around the World (Kumar et al., 2005; Hu et al., 2010; Li et al., 2011; Hou et al., 2015; Wei et al., 2016).

The occurrence of various classes of antibiotics such as β-lactams, sulfonamides, macrolides quinolones and tetracyclines has been reported many times, in activated and digested sludge as well as biosolids from WWTPs. When used as soil fertilizers, these serve as a major source of antibiotics into the agro-ecosystems (Zhang & Li, 2011; Qiao et al, 2018; Wu et al, 2015; Zhao et al., 2018a).

2.4 Effect on Plants

Plants have the ability to uptake contaminants like antibiotics, therefore, contaminating the food chain and increasing the risk of exposure to human beings as well as other non-target species. In plants, many negative impacts induced by antibiotics have been reported. Some of these are summarized in the table given below:

Table 2.1: Effect of antibiotics on different plant species

Antibiotic(s)	Concentration(s)	Crop(s)	Effects	Reference
Tetracycline, Oxtetracycline, Chlortetracycline	0 to 8 mg kg^{-1}	*Pisum sativum* (Pea)	Decrease in, root length, Peroxidase activity.	Ziolkowska et al., (2015)
Sulfadimethoxine, Sulfamethazine	11,200 µg L^{-1}	*Hordeum vulgare L.* (Barley)	Damage of the membrane permeability, root shortening, increased root development.	Michelini et al., 2013.
Ofloxacin, tetracycline	10 µg L^{-1}	*C. alternifolius, T. angustifolia, L. salicaria, A. calamus*	No effect on chlorophyll content, increased nitrogen uptake	Tong et al., 2019
Ciprofloxacin, levofloxacin, enrofloxacin	5, 100 and 300 mg L^{-1}	*Triticum aestivum* (Wheat)	Decrease in growth due to oxidative stress	Riaz et al., 2017
Ciprofloxacin	0 to 2 mg L^{-1}	Zea mays L.,	Stimulated root elongation	Gomes et al., 2019

Penicillin, sulfadiazine, tetracycline		*Apera spicaventi, Brassica napus, Triticum aestivum*	No effect of Penicillin and tetracycline, sulfadiazine reduced plant growth	Pufal et al., 2019
Amoxicillin	0.1 to 100, 000 µg L^{-1}	*Lactuca sativa*	No effect on germination and seedling growth	Rede et al., 2019.
Sulfathiazole	10 and 100 mg kg^{-1}	*Lactuca sativa*	Significant decrease in growth at 100 mg kg^{-1}	Caban et al., 2018
Amoxicillin	45.44 to 110.94 mg kg^{-1}	*Lactuca sativa, Zea Mays, Pisum sativum, Lycopersicon esculentum, Cucurbita pepo, Festuca arundinacea.*	Lettuce growth stimulated	Litskas et al., 2018

2.5 Use of Organic Amendments for Antibiotic Adsorption

Due to increasing concern about the release of antibiotics in the environment, a number of solutions have been proposed and applied to reduce their spread as well as their toxicity to the plant. Adsorption though increased soil organic matter has been one of these.

Rajapaksha et al. (2014) studied the inhibition of sulfamethazine uptake in lettuce with the addition of biochar. The uptake was reduced by 86% and 63% at 5 mg kg^{-1} and 50 mg kg^{-1} of the antibiotic concentration. The uptake of carbamazepine, a common pharmaceutic, along with a number of other emerging organic contaminants, was reduced by 20% to 76% with the addition of biochar in lettuce (Hurtado et al., 2017). Similarly, the use of biochar for the retention of antibiotic sulfamethazine has been carried out (Vithanage et al., 2015).

Since a large amount of thermal energy is required for the production of biochar, new, less energy extensive methods are being devised for the removal/adsorption of antibiotics. The use of cheaper organic amendments such as compost and agricultural waste like rice husk can provide a feasible alternative. Few studies have been reported in this regard.

Rice husk as well as coffee husk have also been used to remove norfloxacin, a fluoroquinolone, from urine, municipal and deionized water. Similarly, sulfuric acid treated termite feces have been used for the sorption of norfloxacin and it proved to be highly efficient. In another study, the addition of hairy vetch and compost significantly reduced the uptake as well as phytotoxicity of sulfathiazole in lettuce, the concentrations being 10 and 100 mg kg^{-1} (Laverde et al., 2018; Caban et al., 2018; Chahm et al., 2019).

Chapter 3

Methodology

3.1 Soil Preparation

Soil from the NUST nursery was used for the purpose of this research. Soil was prepared by air drying it for two days, followed by sieving in order to achieve a particle size< 2mm.

3.2 Analysis of Soil and Organic Amendments

Various physical and chemical properties of the prepared soil and organic amendments were used i.e. compost, rice husk and vermicompost were analyzed as explained below.

3.2.1 Physical Parameters

3.2.1.1 Classification of soil

The soil was classified based on its texture by using the saturation percentage method (Malik et al., 1984). Given below is the table that lists various soil textures based on saturation percentage.

Table 3.1 Soil classification according to saturation percentage

Soil Texture	Saturation Percentage (%)
Sand	0-19
Sandy Loam	20-29
Silt Loam	30-45
Clay Loam	46-60
Clayey Loam	> 60

3.2.1.2 Determination of pH

For measuring the soil pH, 10 g of soil was taken in a flask and 50 mL of distilled was added to it with a measuring cylinder. The flask was placed on a rotatory shaker for 30 minutes at a speed of 150 RPM. This mixture was then left to settle down for an hour and a pH meter was then used to check the pH (McLean, 1982).

3.2.1.3 Water Holding Capacity

The water holding capacity was measured by weighing ten grams of soil and placing it on a filter paper that was set in a funnel. A graduated cylinder was placed right below the funnel. Slowly, 10 mL of distilled water was poured over the soil. The volume of the resulting filtrate collected in the measuring cylinder was noted (Harding and Ross, 1964).

3.2.1.4 Moisture Content

Ten grams of soil was weighed and placed in a china dish. The oven was set at 105°C and the china dish was placed in it. After 24 hours, the china dish was removed from the oven to cool it down. The soil was then reweighed. The moisture content was measured using the following formulae:

$$\text{Moisture Content (\%)} = \frac{\text{Initial soil weight} - \text{Final soil weight}}{\text{Initial soil weight}} \times 100$$

3.2.2 Chemical Parameters

3.2.2.1 Extractable Phosphorus

Extractable phosphorus present in soil and organic amendments was determined using the method of Olsen et al. (1954). Extraction was carried out by adding 100 mL of 0.5 M $NaHCO_3$ to the flasks containing 5 g of soil and the organic amendments, separately. The flasks were placed on a shaker and mixed for thirty minutes and then passed through a filter

paper. Stock solution was prepared from KH_2PO_4 for making a number of standard solutions for generating a calibration curve. 10 mL of each filtrate was taken in 4 flasks and their pH adjusted to around 5 with 5 N H_2SO_4. To these flasks, 42 mL of the distilled water and 8 mL of the colorimetric reagent was added with the total volume being of 50 mL. The colorimetric reagent used included ascorbic acid dissolved in a mixture of ammonium heptamolybdate, antimony potassium tartrate and sulfuric acid. Standards and samples were run on a spectrophotometer after color development after 10 minutes at a wavelength of 882 nm. The following formulae was used for calculating extractable phosphorus

$$\text{Extractable P (ppm)} = \text{ppm P (from the calibration curve)} \times \frac{V}{Wt.} \times \frac{V2}{V1}$$

V = Total Volume of the Extract

V1 = Extract volume used for measurement

V2 = Volume of flask used for measurement

Wt. = Weight of the soil/organic amendments used

3.2.2.2 Nitrate Nitrogen

The soil and organic amendments were analyzed for nitrate nitrogen through the chromotropic acid method. Extraction was performed by mixing 10 g of the prepared soil/organic amendments with 0.02 N $CuSO_4$ on a shaker at 150 RPM. The resulting mixture was then filtered, and the filtrate used for further analysis. Oven-dried KNO_3 was dissolved in a liter of 0.02 N $CuSO_4$ and 10 mL of this solution was used for making the diluted stock solution. Seven solutions were prepared from the diluted stock solution with the concentration in the range of 0.5 to 3.5 ppm. For making the standards, 3 mL of each of the solutions was pipetted in a flask. Chromoptropic acid (0.1%) and concentrated

sulfuric acid were used as the colorimetric reagents. After the incubating the standards for 45 minutes at room temperature, the absorbencies were checked on a spectrophotometer at a wavelength of 430 nm and a calibration curve was generated. Similar process was used for the preparation of samples.

3.2.2.3 Organic Matter in Soil

The organic matter in soil was measured using the Walkley-Black method. In a 500 mL flask, 1 g of the prepared soil was taken and 10 mL of 1 N potassium dichromate ($K_2Cr_2O_7$) was added to it. After that, 20 mL of the concentrated sulfuric acid (H_2SO_4) was added to this mixture. After 30 minutes, 200 mL of distilled water and 10 mL concentrated phosphoric acid (H_3PO_4) was added to the flask. Diphenyl amine was used as the indicator and 15 drops of it were added to the sample. Two blanks were prepared as well, containing all the mentioned chemicals but no soil. The sample was then titrated with 0.5 M ferrous ammonium sulfate solution. When the color of the sample changed from violet-blue to green, titration was stopped and the readings were noted.

3.2.3 Organic Matter in Amendments

For the determination of organic matter in the amendments, loss on ignition method was used. 10 g of each organic amendment was weighed in separate china dishes and were placed in the muffle furnace. The temperature of the furnace was adjusted at 250°C and the samples were left for 12 hours. The samples were then removed from the furnace and reweighed. The difference was noted down.

Figure 3.1 Samples after they were removed from the furnace

3.3 FTIR Analysis of the Organic Amendments

Samples of compost, rice husk and vermicompost were sieved to achieve a uniform particle size. For control, no antibiotic treatments were applied. 10 g of each amendment was taken in separate falcon tubes and were treated with 5 and 10 mg L^{-1} ampicillin and ofloxacin solutions. These samples were then placed in the incubator at 25°C for 72 hours. After that, the antibiotic solutions were removed and the samples were transferred to petri plates and dried in the oven at 35°C for 48 hours. All the samples were then analyzed through to FTIR.

Figure 3.2 FTIR Analysis of the Samples

3.4 Seed Germination Test

The protocol provided by the International Seed Testing Association (ISTA) was used for carrying out the seed germination test. For this purpose, petri plates of 10 cm diameter were used. Filter paper was paced in each of the plates and the plates were labelled. The

treatments were kept similar as in the pot experiment to ensure the same concentrations as used in the pot experiment, although calculations were adjusted to be appropriate for the germination test.

For each treatment, 10 seeds were placed on the filter paper. 5 mL of the antibiotic solution was added to the plates, or distilled water in case of the control. The plates were placed in the incubator, set at a temperature of 25°C in the dark. Each treatment was carried out in triplicates. A root length greater than 5 mm was used as the endpoint i.e. 5 days later. The seeds were considered germinated when the length of the radical was approximately 0.2mm. The following parameters were measured:

- Root Length and Shoot Length (mm)
- Germination percentage: No. of germinated seeds/ No. of total seeds x 100
- Seed Vigor Index: Total plant length x Germination Percentage
- Germination Index (%): Germination percentage x root length/100

3.5 Pot Experiment

Seed Sterilization

Seeds were sterilized by soaking them in a solution of 6 % calcium hypochlorite $Ca(ClO)_2$ for 25 minutes. The solution was then removed, and the seeds were rinsed with distilled water multiple times and allowed to dry. The sterilized seeds were then stored at 25°C under dark conditions.

3.5.1 Cultivation of Plants

The pot experiment was carried out at the green house located near IESE, NUST, under natural weather conditions. Pots were filled with 1 kg of the sieved soil. Organic

amendments were mixed at this stage in the required pots at a concentration of 1%. Afterwards the sterilized seeds were sown, with each pot receiving 5 seeds. Each treatment was replicated 3 times.

Figure 3.3: The greenhouse experiment showing growth of lettuce in pots filled with soil

3.5.2 Application of Antibiotics

Antibiotic solutions were prepared by dissolving each antibiotic i.e. ampicillin and ofloxacin in a liter of distilled water. The antibiotic doses used for this experiment were 5 and 10 mg kg^{-1} of soil. Solutions of antibiotics were applied to the pots when the seedlings were 4 to 5 weeks old. For achieving a concentration of 10 mg kg^{-1}, the antibiotic solutions were applied in split doses. One dose being applied when the seedlings were 4 to 5 weeks old, while the other one in the 7^{th} week.

3.5.3 Plant Harvesting

The plants were harvested after 3 months of growth period. Different measurements were made for various parameters. The details are presented in upcoming section.

3.5.4 Measurement of Physical Growth Parameters

Fresh Biomass

The fresh weight of the plant samples was determined right after harvesting, using a weight balance.

Root Length

The root length was measured from the hypocotyl to the tip of the primary root.

3.5.5 Nutritional Analysis of the Plant Samples

3.5.5.1 Plant Sample Preparation

The oven dried plants were crushed in a blender to get a uniform size. These samples were stored in the zip bags at room temperature, to be used for further analysis.

Digestion

Plant samples were digested by taking 200 mg of root and shoot, separately, in a 25 mL flask and pipetting nitric acid and perchloric acid (2:1) into each of the flasks. These samples were placed on a hot plate and the temperature was adjusted at 230ºC. The digestion process was considered complete after the acid solution became clear and white precipitates were formed, following the formation of white dense fumes. All the samples were then made to volume with distilled and filtered. The resulting digest was then used for iron and phosphorus analysis.

Figure 3.4 The digestion process

3.5.6 Phosphorus Content

To determine the amount of phosphorus present in the digested samples, the colorimetric method was used.

Preparation of Reagents

The colorimetric reagent was prepared by dissolving 22.5 g of ammonium heptamolybdate in 400 mL of distilled water. In a separate beaker, 300 mL of distilled was heated and 1.25 g of ammonium metavandate was dissolved in it. Both of these solutions were added to a 1-liter volumetric flask. Slowly, 250 mL of the concentrated nitric acid (HNO_3) was added to the flask and allowed to cool at room temperature. The solution was made to the volume with distilled water.

For preparation of the standard stock solution, 2.5 g of KH_2PO_4 was dried in the oven for an hour at a temperature of 105°C. To 400 mL distilled. Six standard solutions were prepared by pipetting 1, 2, 3.4 and 5 mL from the standard solutions and mixed with distilled water in a 100 mL flask. The concentrations of phosphorus in these standards were 5, 20, 40, 60, 80 and 100 ppm respectively.

Preparation of Standards

From the standard solutions, standards were prepared by mixing 2 mL of each solution with the colorimetric reagent in a flask. The final volume of each standard was made 20 mL with distilled water. A blank was also prepared by pipetting 2 mL of the colorimetric reagent i.e. ammonium heptamolybdate-ammonium metavandate reagent. The standards, along with the blank, were run on a spectrophotometer at wavelength of 410 nm after waiting for thirty minutes for color development. A standard curve was generated this way.

Preparation of Samples

The procedure for preparation of samples was similar to that of standards. 2 mL of each digested sample was pipetted in a flask and then mixed with 2 mL of colorimetric reagent and made up to volume with distilled water and were run on a spectrophotometer after thirty minutes.

Calculations

The equation obtained from the standard curve graph was used to calculate the concentration of phosphorus in each sample. The resulting value was then subject to further calculations by using the following formulae:

$$\text{Phosphorus (\%)} = \text{ppm P (from the calibration curve)} \times \frac{V1}{Wt.} \times \frac{20}{V2} \times \frac{1}{10000}$$

Where P stands for phosphorus, V1 stands for the total volume of the digest, 20 is the total volume of the sample prepared, Wt. is the weight of the plant dry matter used for digestion and V2 is the volume of the digest used for sample preparation. For conversion into percentage, the equation is multiplied by 1/10000.

3.5.7 Carbohydrates Analysis

For the determination of total soluble sugars present in the samples, the Anthrone method was used (Hedge and Hofreiter, 1962).

Reagents

2.5 N HCL

50 mL of distilled water was taken is a 250 mL flask. 52 mL of 37% hydrochloric acid (HCl) was added to the flask and was filled up to the mark with distilled water.

Concentrated H_2SO_4

The 500 mL of sulfuric acid was taken in a beaker and stored in the freezer for making the anthrone reagent.

Anthrone Reagent

Anthrone, 100 mg, was weighed and added to a 100 mL flask. 100 mL of concentrated sulfuric acid was added to the flask and was then placed on the stirrer until complete dissolution.

Glucose Stock Solution

To a 100 ml flask, 100 mg of glucose was added and dissolved in 100 mL of distilled water.

10 mL of this solution was pipetted and mixed with 90 mL of distilled water in a flask to prepare the working stock solution. 100 mg of the plant samples were weighed in test tubes and labeled.

Sample Hydrolysis

100 mg of each sample was weighed in glass test tubes and labelled accordingly. 5 mL of the 2.5 N HCL was added to each test tube. These samples were then incubated for 3 hours in a boiling waterbath. After hydrolysis was complete, these samples were filtered and were neutralized with Na_2CO_3 until effervescence was finished.

Figure 3.5 The hydrolysis of the samples in the water bath

Preparation of the Standard Solutions

From the working solution of glucose, 0.2, 0.4, 0.6, 0.8 and 1 mL were pipetted into different test tubes. To each, 4 mL of the anthrone reagent was added. The blank was prepared with distilled water. The absorbance of the blank and the standard solutions was checked on a spectrophotometer at 630 nm and a calibration curve was generated.

Preparation of the Samples

To 1 mL of the sample, 4 mL of the anthrone reagent was added. All the samples were then run on a spectrophotometer. The following formulae was used for determining the carbohydrates concentration:

$$\text{Carbohydrates(sample)} = \frac{\text{value of x from the graph (mg)}}{\text{volume of sample used (mL)}} \times \frac{\text{extract total volume (mL)}}{\text{sample weight (mg)}}$$

3.6 Chlorophyll Content

The method of Hiscox and Israelstam (1979) was used for the determination of the chlorophyll content. 0.5 grams of the fresh plant samples were weighed and placed in a test tube. To this, 7 mL of the Dimethyl Sulfoxide (DMSO) was added and incubated overnight in a water bath at a temperature of 65ºC.

The resulting extracts were transferred to clean test tubes and DMSO was added to each one of them in order to achieve a final of 10 mL. These samples were then run on a spectrophotometer at wavelengths of 645 and 663 nm.

The following equations were then used to determine the chlorophyll content:

Chlorophyll a (µg/mL) = 12.7 (A_{663}) – 2.69 (A_{645})

Chlorophyll b (µg/mL) = 22.9 (A_{645}) – 4.68 (A_{663})

Total Chlorophyll (µg/mL) = 20.2 (A_{645}) + 8.02 (A_{663})

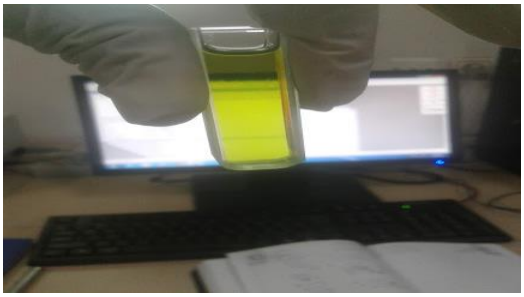


Figure 3.6 Spectrophotometric determination of the chlorophyll content

3.7 Soluble Protein Content

Sample Extraction0.5 g of the fresh leaves were weighed and crushed in a pre-chilled mortar, using the extraction buffer. The recipe for the extraction buffer is given below:

2-mercaptoethanol (5 mmol)

Tris Base (50 mmol) pH 8.80

Glycerol (12.5%)

The extracts were then collected in Eppendorf tubes and centrifuged at 2°C at a speed of 1500 RPM for 17 minutes. The supernatant was transferred to a clean tube and stored in the -20 freezer until further analysis. Bradford assay was used to measure the protein content in the samples extracted through the above-mentioned method. The following reagents were used:

Bradford Reagent: To a 500 mL conical flask, 50 mg of coomassie brilliant blue G-250 was added and dissolved in 25 mL of 95% methanol. To this solution, 50 mL of 80% phosphoric acid was added and the solution was placed on a magnetic stirrer. Afterwards, it was made to the volume with distilled water and placed in the refrigerator at 4°C and protected from light. To remove any precipitates, the solution was filtered multiple of times using Whatman No.1 filter paper.

Bovine Serum albumin (BSA): Stock solution of BSA was prepared by dissolving 10 mg of BSA in 10 mL of distilled water. This solution was prepared fresh, although can be stored in a -20 or -80ºC freezer for future use.

Standard solutions of the concentration 0.05, 0.1, 0.2, 0.4, 0.8, 0.12, 0.16 and 1 mg mL^{-1} were prepared by taking 1, 2, 4, 8, 12, 16 and 20 µl of the BSA solution respectively, and adjusting final volume to 20 µl with distilled water. The blank solution was prepared with

distilled water. A replicate of each standard was also prepared. Bradford reagent, 1 mL was added to each of the standards and were then incubated at room temperature for 5 to 10 minutes and the absorbance were checked on a spectrophotometer at a wavelength of 595 nm. A standard curve was generated.

Similar method was used for preparing the samples, except that 20 µl of the samples were used without adding any distilled water. The protein concentration in the samples were determined with the calibration curve.

3.8 Rubisco Content

The supernatant from the soluble protein extraction was used for the determination of rubisco content.

3.8.1 SDS-PAGE

Rubisco content was measured using SDS PAGE. For this purpose, the following solutions were prepared:

Sodium Dodecyl Sulfate (SDS) 10%: It was prepared by dissolving 10 g of SDS in 60 mL of distilled water. The total volume of the solution was made 100 mL with distilled water.

Tris (1.5 M, pH 8.80): This solution was prepared by dissolving 18.18 g of tris in 50 mL of distilled water. The pH of the solution was adjusted to 8.80 with 1N HCl. The final volume was made 100 mL with distilled water.

Tris (0.5 M, pH 6.80): 3 g of tris was dissolved in 20 mL distilled water and its pH was adjusted to 6.80 with sodium hydroxide (NaOH) pellets. The total volume was made 50 mL with distilled water.

Ammonium persulfate (APS) 10%: This solution was prepared fresh each time. 1 g of ammonium persulfate was dissolved in 10 mL of the distilled water in amber flask and stored in the freezer until use.

Polyacrylamide (PAA) 29:1, 30%: 29 g of acrylamide and 1 g of N, N'-Methylene bisacrylamide in 50 mL of distilled water. Afterwards, the total volume of the solution was made to 100 mL with distilled water.

2-mercaptoethanol 0.5%: 0.5 mL of the 2-mercaptoethanol was pipetted in a beaker and 95.5mL distilled water was mixed with it, the final volume being 100 mL.

Glycerol 20%: 20 mL of glycerol was mixed with 80 mL of distilled water, with a total volume of 100 mL.

Bromophenol Blue (0.5%): 5 g of bromophenol blue was dissolved in 100 mL of distilled water and stored in a bottle.

Staining Solution: The staining solution was prepared by dissolving 0.5 g of Coomassie brilliant Blue-R 250 in of 20 mL acetic acid, 90 mL methanol and 90 distilled water. The final volume of the solution was 250 mL.

Destaining Solution: This solution was prepared by mixing 450 mL of distilled water, 450 mL of methanol and 100 mL of acetic acid in a 1000 mL beaker.

Preparation of the Resolving and Stacking Gel

The solutions used for preparing of stacking and resolving gel are given in table 3.2.

Table 3.2: a) Chemical composition of the resolving gel b) Stacking gel

Chemical/solution	Quantity
PPA 30%	12 mL
Distilled Water	9.9 mL
Tris 1.5 M (pH 8.8)	7.5 mL
SDS 10%	300 µl
TEMED	30 µl
APS 10%	300 µl

Composition of 12% Resolving Gel

Chemical/solution	Quantity
PPA 30%	2.5 mL
Distilled Water	8.45 mL
Tris 0.5 M (pH 6.8)	3.75 mL
SDS 10%	150 µL
TEMED	15 µL
APS 10%	150 µL

Composition of 5% Stacking Gel

Preparation of the samples

20 µL of the supernatant was mixed with the sample buffer which consisted of bromophenol blue (5%), glycerol (20%), SDS (10%) and 2-mercaptoethanol (0.5%). The samples were placed on a heat block and incubated at a temperature of 95°C for 7 minutes. The samples were then allowed to cool at room temperature for 10 minutes and loaded into the wells.

Running Conditions

The setup for SDS PAGE was assembled and the resolving gel was poured between the glass plates. After its solidification, the stacking gel was poured and the comb was inserted. The comb was removed after gel solidification and the samples were pipetted into the wells, with the first well containing the molecular marker and the second one containing BSA as a positive control. For the negative control, distilled water was used. The voltage was set

at 60 V as the samples moved through the stacking gel and was then changed to 110 V through the resolving gel. The runtime was approximately 3 hours.

Figure 3.7 SDS-PAGE Assembly

Gel Staining and Destaining

The gel was removed from between the plates and placed in the beaker. After several washes with distilled water, the staining solution was added. The beaker was placed on the shaker, set at 39 RPM and was the gel was stained for two and a half hours. The dye was removed and the destaining solution was added to the beaker and was left overnight on the shaker to achieve complete destaining.

Colorimetric Determination

The band for rubisco large subunit was identified with the help of molecular marker. These bands were then cut with a surgical blade and placed in a test tube, separately. 2 mL of formamide was added to these tubes and were incubated in a water bath for 8 hours at 50°C. The obtained solution was transferred to a cuvette and the absorbance was checked on spectrophotometer, using BSA to generate a calibration curve and the background gel was used as the blank.

3.9 Statistical Analysis

One-way ANOVA was conducted to analyze the data, using Statistix 8.1 software. Treatments with amendments at each antibiotic concentration were compared with their respective controls, in which no amendments were used. Tukey's HSD was performed as a post hoc test, where significant difference was found between the treatment groups ($p<0.05$). The bars represent mean of the 3 replicates ± standard error.

Chapter 4

Results and Discussion

Some of the physical and chemical properties i.e. pH, texture, electrical conductivity, moisture, phosphorus, nitrogen and organic matter content of the soil are given in **table 4.1.**

Table 4.1 Physicochemical properties of the soil

Parameter	**Result**
pH	8.5
Electrical Conductivity (µS/cm)	250
Extractable Phosphorus (ppm)	50.96
Nitrate Nitrogen (ppm)	135.6
Organic Matter (%)	1.52
Moisture Content (%)	7.4
Texture	Clay Loam

The chemical properties i.e. pH, electrical conductivity, phosphorus, nitrogen and organic matter content of amendments compost, rice husk and vermicompost are shown in table 4.2.

Table 4.2 Chemical properties of the organic amendments

Organic Amendment	OM (%)	Phosphorus (%)	Nitrogen (%)	Electrical Conductivity (microsiemens/cm)	pH
Compost	28.55	0.63	0.109	3.93	6.97

Vermicompost	56.5	0.72	0.093	2.83	6.61
Rice husk	78.65	0.64	0.103	4.61	7.06

4.1 FTIR Analysis of Organic Amendments

Compost Treated with Ampicillin

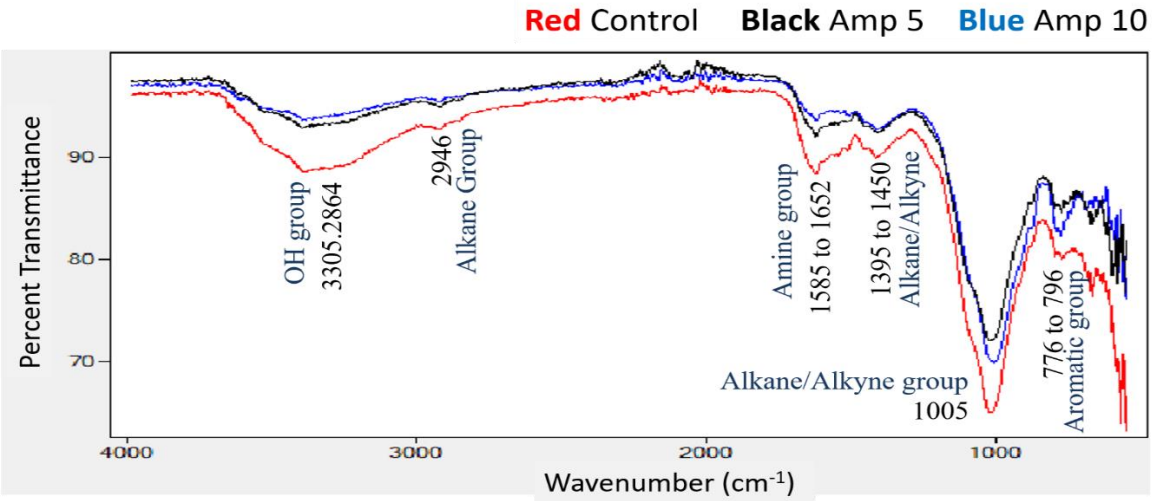


Figure 4.1 FTIR graph of compost treated with ampicillin

The various frequencies and possible functional groups are shown in the graph. The red colored spectrum is of the compost control with no antibiotic treatment, the black one is of compost treated with ampicillin at a concentration of 5 mg L-1, while the blue is of compost treated with 10 mg L^{-1} ampicillin. The peak around 3305.4 wavenumber has been assigned to the hydroxyl (OH) functional group. Alkane group has been assigned to the slight stretching around 2946 wavenumber. The peak around 1585 to 1652 has been attributed to the amide group. Similarly, the peak around 1395 to 1450 and that around 1005 has been assigned to the alkane/alkyne group. Stretching of the aromatic group has been attributed to the formation of peaks around 776 to 796 wavenumbers. The decrease in the intensities

of the functional groups indicates that adsorption/chemisorption did take place. Though the difference is very slight between the treated and the control compost (Coates, 2006; Vargas et al., 2012).

Compost Treated with Ofloxacin

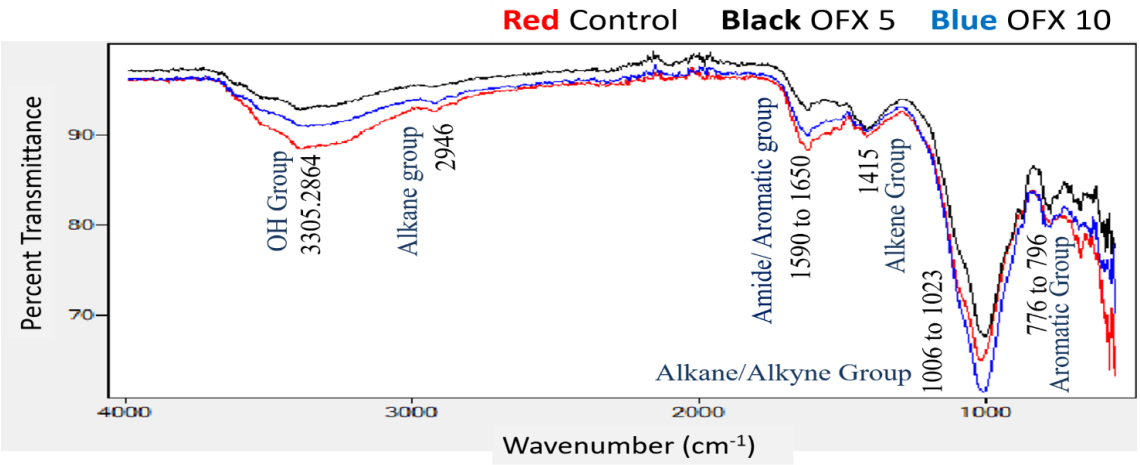


Figure 4.2 FTIR graph of compost treated with ofloxacin

As evident from the graph for compost treated with ofloxacin, more adsorption took place at 5 mg L^{-1} as compared to of antibiotic concentration 10 mg L^{-1}. The stretching around 1000 wavenumber after treatment with 10 mg L^{-1} ofloxacin can indicate electrostatic attraction between the alkyl group of the compost & the amide group present in the chemical structure of the ofloxacin (Brandão et al., 2012).

Rice husk Treated with Ampicillin

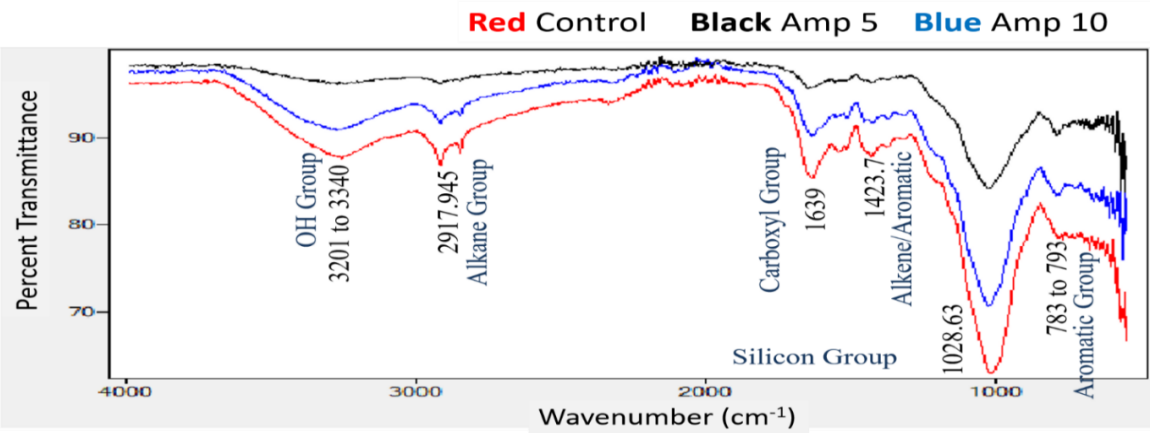


Figure 4.3 FTIR graph of rice husk treated with ampicillin

The graph for rice husk treated with ampicillin indicates the involvement of the different functional groups in removing the antibiotics from the solution. The broad peak around 3201 and 3040 wavenumbers has been assigned to the OH stretching of the alcohols (R-OH), carboxyl groups (-COOH) and the phenols (-Ph-OH). The peak around 2917.945 has been assigned to the vibration of aliphatic group present in cellulose and hemicellulose (Imessaoudene et al., 2015; Hu et al., 2016). The peak around 1639 has been assigned to the C=O stretching of the carboxyl. The peaks around 1423.7 and 1028.63 have been assigned to the aromatic and silicone group (Si-O-Si), respectively (Ebrahimi et al., 2017; Masoumi et al., 2016). The adsorption at 5 mg L^{-1} was greater as compared to the10 mg L^{-1}, indicating slight saturation at that concentration. Similarly, stretching around 783 to 793 wavenumbers can be attribute to the aromatic functional group (Coates, 2006).

Rice husk Treated with Ofloxacin

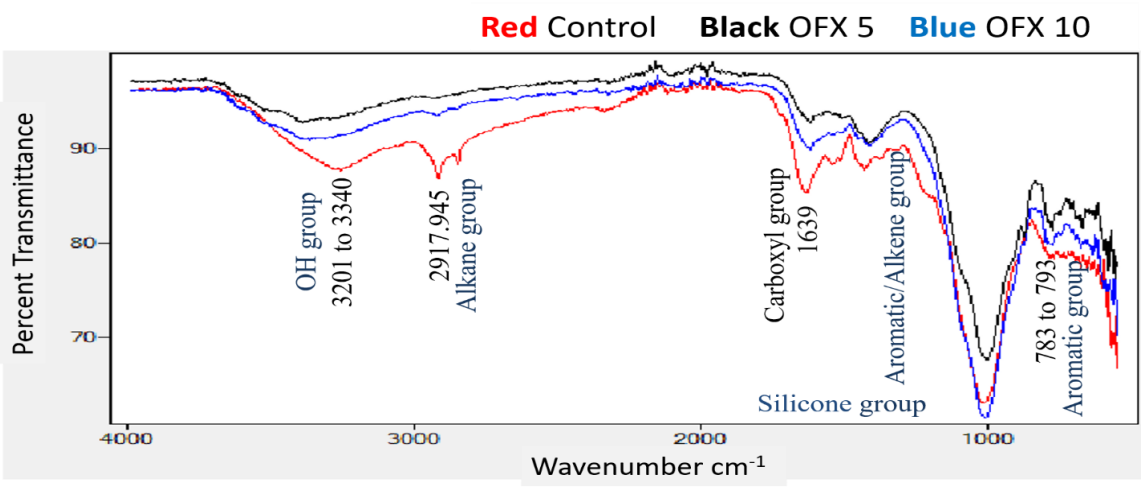


Figure 4.4 FTIR graph of rice husk treated with ofloxacin

When rice husk was treated with ofloxacin 10 mg $^{-1}$, slight stretching was observed around 1028 wavenumber, which can be attributed to the formation of hydrogen bond between SiO_2 of the rice husk & hydrogen of the amide group present in ofloxacin (Paredes-Laverde et al., 2018).

Vermicompost Treated with Ampicillin

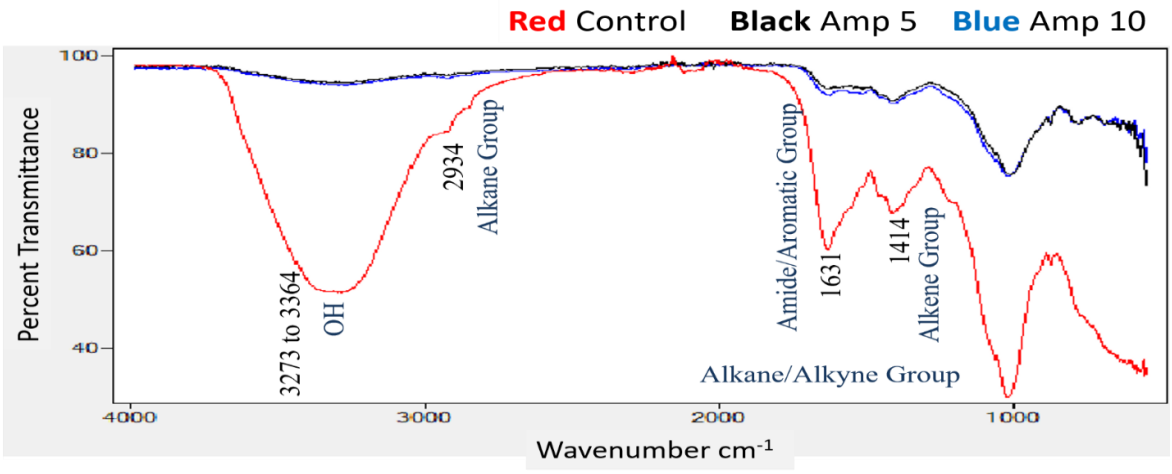


Figure 4.5 FTIR graph of vermicompost treated with ampicillin

The stretching around 3272 to 3364 wavenumbers, as evident from the FTIR analysis of vermicompost control and treated groups, has been assigned to the vibration of OH functional group. Alkane group has been assigned to the peak around 2934 wavenumber as well as around the 1000 wavenumber. Amide/aromatic group has been assigned to the peak around 1631 wavenumber, while alkene group has been assigned to the peak around 1414 wavenumber (Cotes, 2006). Adsorption of antibiotics is observed for vermicompost treated with ampicillin, where the stretching of functional groups such as OH, alkane, amide and alkene reduced with the application of antibiotics, both the 5 mg and 10 mg L^{-1} concentrations as compared to the control.

Vermicompost Treated with Ofloxacin

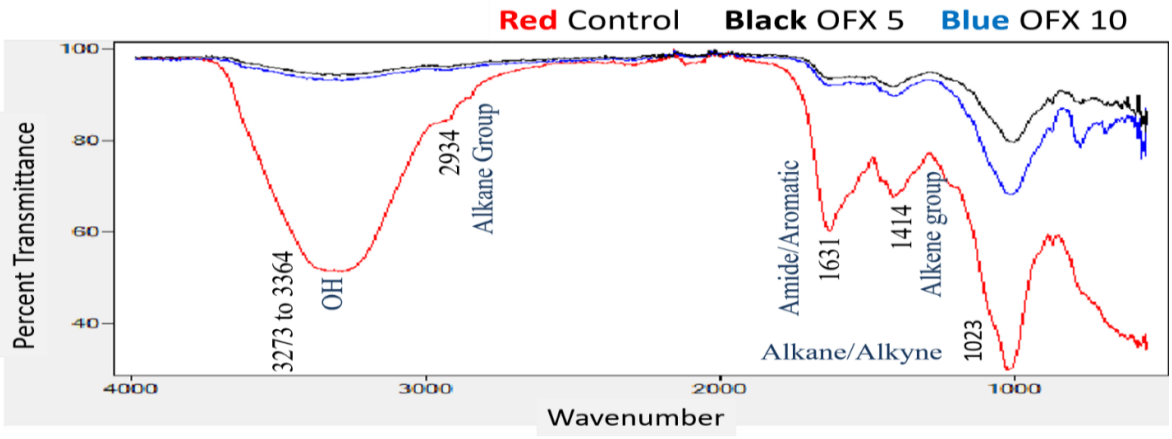


Figure 4.6 FTIR graph of vermicompost treated with ofloxacin

Similarly, for vermicompost treated with ofloxacin, higher adsorption was observed at 5 mg L^{-1} as compared to 10 mg L^{-1}, indicating slight saturation.

4.2 Germination Test

4.2.1 Germination Percentage

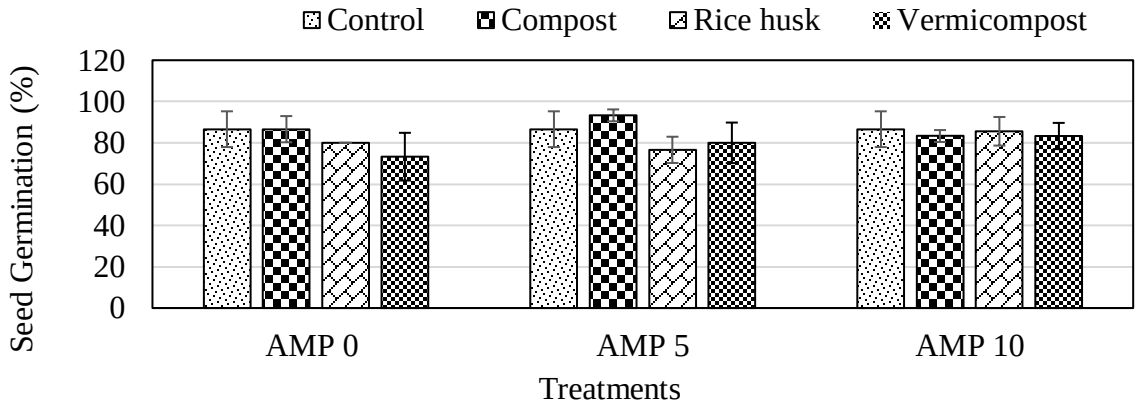


Figure 4.7 Germination percentage of seeds treated with ampicillin

Ampicillin had no significant effect on the germination percentage of the lettuce seeds in the presence of antibiotics. No statistical difference ($P>0.05$) was found between any of the treatments.

Similar results were reported by Rede et al. (2019), where no effect on seed germination was observed when lettuce seeds were exposed to amoxicillin at a concentration range of 0.0001 to 100 mg L^{-1}. Hill's et al. (2011) also carried out a study on the effect of antibiotics levofloxacin, chlortetracycline and sulfamethoxazole on lettuce at concentrations ranging from 0.001 to 10 mg kg^{-1} and found no significant effect on germination.

Conversely, a study carried out by Liu et al. (2009) reported a negative effect of the antibiotics chlortetracycline, tetracycline, tylosin, sulfamethoxazole, sulfamethazine & trimethoprim on the germination of the cucumber, rice and sweet oat. This could be because of the high concentrations of antibiotics, up to 500 mg L^{-1}, as well as the different crops & antibiotics used as compared to the current study.

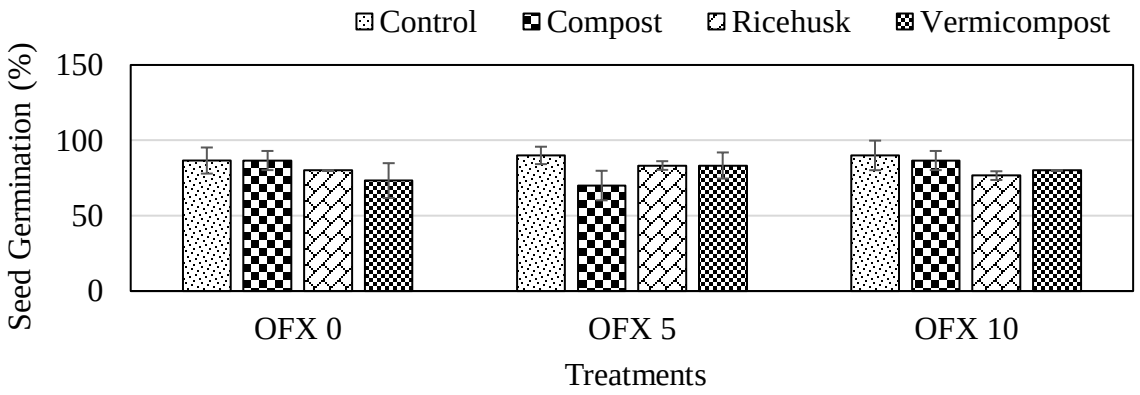


Figure 4.8 Germination percentage of lettuce seeds treated with ofloxacin

Similarly, no effect of the ofloxacin was observed on the germination of the lettuce seeds, as evident by the statistical analysis ($p>0.05$). Neither the presence of amendments, nor the mixture of amendments and antibiotics had any effect on germination of the lettuce seeds.

Similar results were reported by Riaz et al. (2017), where no effect on germination of the wheat seeds was observed in the presence of antibiotics ciprofloxacin, enrofloxacin and levofloxacin at concentrations ranging from 10 to 400 ppm. The seed cover acts as a barrier that protects the seed from the adverse effect of contaminants, such as antibiotics (Vazequez-Roig et al., 2012; Hillis et al., 2011; An et al., 2009).

4.2.2 Root Length

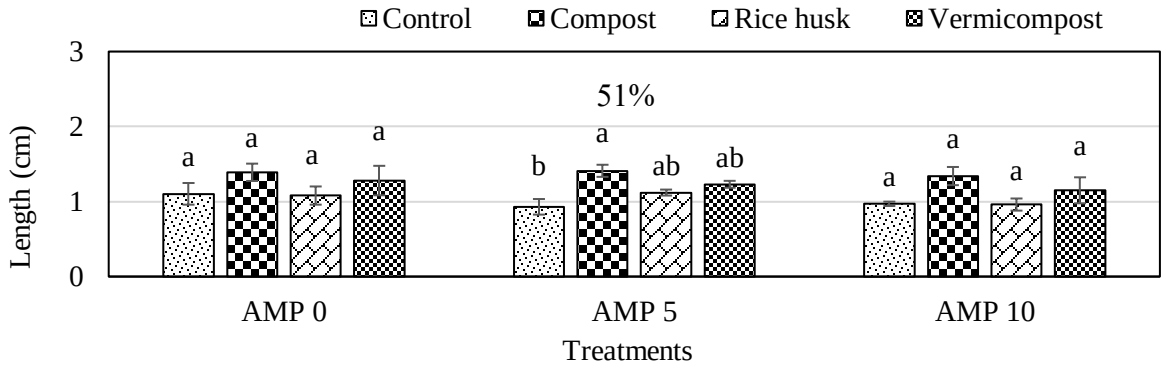


Figure 4.9 Root length of lettuce seedlings treated with ampicillin

With the application of ampicillin, at both 5 and 10 mg L^{-1} concentrations, there was a slight decrease in the root length of seedlings germinated in antibiotics only, although not at statistically significant value (P>0.05). At 5 mg L^{-1} of the antibiotic concentration, antibiotic stress was alleviated by 51% in the presence of compost. The root length of seedlings in compost treatments was significantly higher than those treated with antibiotics only. The presence of the amendments rice husk and vermicompost had no significant effect on the root length as compared to the control with antibiotics only.

At 10 mg L^{-1} of the ampicillin concentration, there was no significant difference between any of the treatments. Compost alleviated the antibiotic stress the most, as evident by the higher root length compared to the other treatments, though this difference wasn't statistically significant (P>0.05). Seremeta et al. (2018) reported that compost prepared from industrial sewage sludge and tobacco had the capacity to adsorb lead Pb^{2+} from the solution. Sewage sludge compost has also been used to remove chromium from aqueous solution (Chen et al., 2017). Furthermore, zinc removal has also been achieved with the use of compost (Al-Mashaqbeh & McLaughlan, 2014).

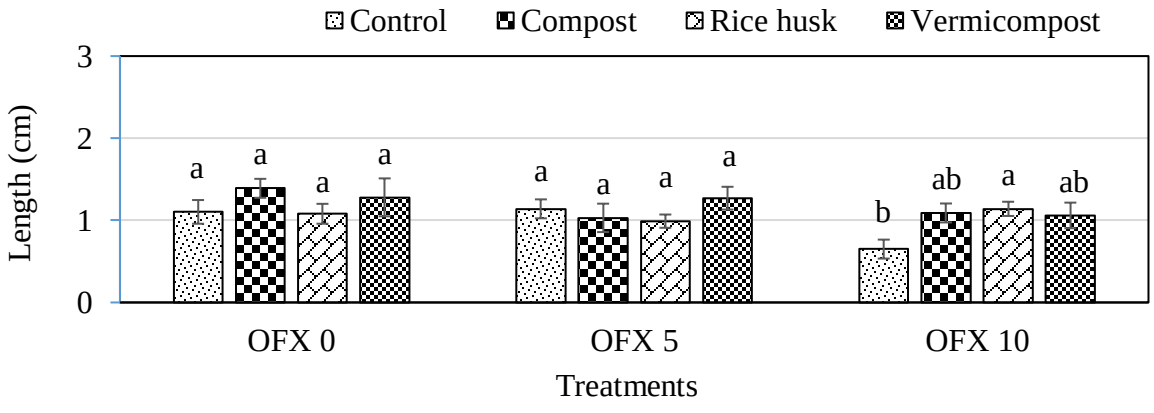


Figure 4.10 Root length of lettuce seedling treated with ofloxacin

In the absence of any antibiotics as well as at 5 mg L^{-1} antibiotic concentration, there was no statistically significant difference ($P>0.05$) in the root length between any of the treatments. At 10 mg L^{-1} of the ofloxacin concentration, the antibiotic stress was alleviated up to 75% in the presence of rice husk, while no significant difference was observed in the presence of either compost or vermicompost. Furthermore, there was significant decrease in the root length at 10 mg L^{-1} of the antibiotic concentration in the absence of any antibiotics.

The adsorptive properties of rice husk have been utilized for a number of purposes, as evident from the literature. Rice husk has been used to remove norfloxain from urine, municipal as well as deionized water (Paredes-Laverde et al., 2018). Similarly, rice husk has also been used for the removal of chromium and phenol from a solution mixture of the 2 chemicals. (Gupta, 2016). Since no research has been carried out on the antibiotic stress alleviation on germination using organic amendments, these results need to be validated.

4.2.3 Shoot Length

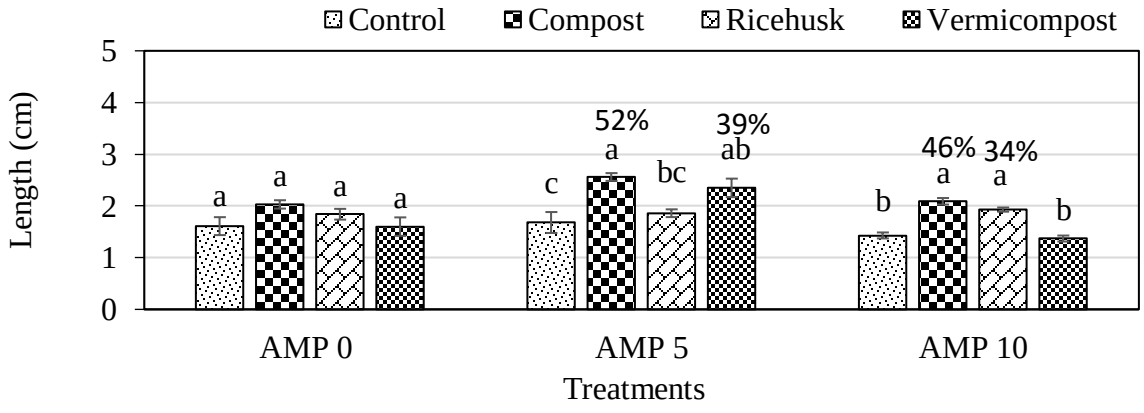


Figure 4.11 Shoot length of lettuce seedlings treated with ampicillin

In the absence of any antibiotics, there was no significant effect on the shoot length with the addition of amendments. With the application of 5 mg L^{-1} of ampicillin, a synergistic effect was observed in treatments with compost and vermicompost, where a significant increase of 52% and 39% respectively, compared to the control was evident. When the antibiotic concentration was increased to 10 mg L^{-1}, a slight decrease was observed in the shoot length of treatment with antibiotic only. This stress was alleviated by 46 and 34% in treatments with compost and vermicompost respectively.

Consistent with the root length, shoot length was also higher in treatments with compost and vermicompost, particularly at 5 mg L^{-1}. Since no such research has been carried out, these results need to be investigated further.

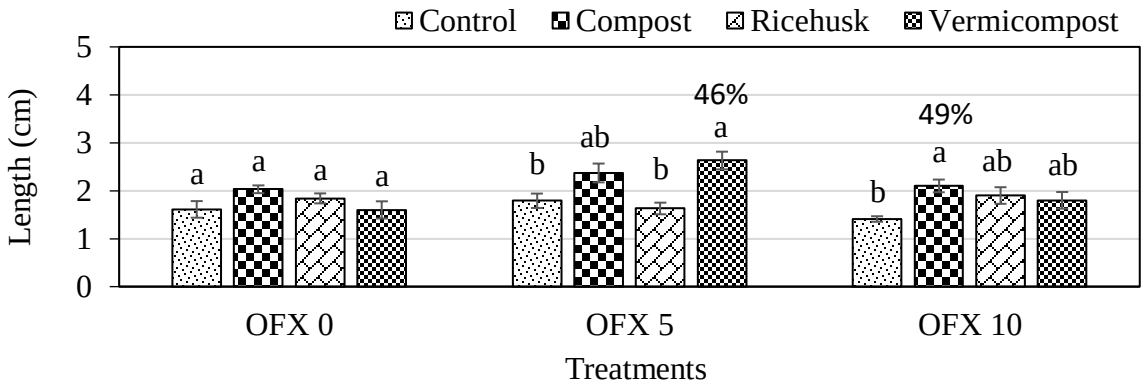


Figure 4.12 Shoot length of lettuce seedlings treated with ofloxacin

Similar to the previous result, at 5 mg L^{-1} of the ofloxacin concentration, a synergistic effect was observed in treatments with compost and vermicompost, where it was more pronounced and significant in case of the later i.e. 46%. At 10 mg L^{-1} of the antibiotic concentration, consistent with the root length, a decrease in the shoot length was observed

in treatment with no amendments. This stress was alleviated by 49% in treatment with compost. Furthermore, the shoot length of the treatments with amendments was higher.

A study carried out on the effect of biogas residues, containing oxytetracycline and sulfadiazine, on lettuce growth showed that the lettuce height increased with the application of the biogas residues as compared to the control with no antibiotics (Han et al., 2019). Bioadsorption using compost as well as vermicompost has proven to be very efficient and ecofriendly. While the adsorption of heavy metals using organic amendments has been reported a number of times, its role in alleviating antibiotic stress in plants yet needs to be investigated since no such work has been reported yet.

4.3 Pot Experiment

4.3.1 Total Biomass

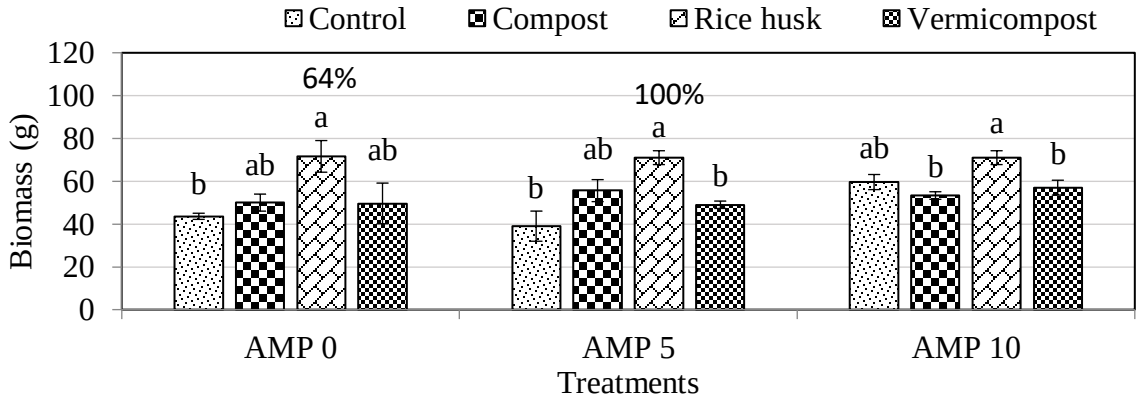


Figure 4.13 Total fresh biomass of lettuce plants treated with ampicillin

In control with no antibiotics, the fresh biomass of plants cultivated in rice husk was significantly higher ($p<0.05$) i.e. 64% as compared to the control with no amendments, while no significant difference was found to between the rest of the treatments. Similar trend was observed at 5 mg kg-1 of the ampicillin concentration, where the fresh biomass of treatments with rice husk was significantly higher as compared to the control with no

amendments i.e. 100 %. Furthermore, there was no significant difference between the treatments with amendments as compared to the control with antibiotic only, when the concentration of ampicillin was increased to 10 mg kg^{-1}.

The higher content of organic matter in treatments with rice husk could be responsible for the higher biomass. An increase was also observed in the biomass at 10 mg kg^{-1}, in the absence of any amendments. A significant increase in the seedling growth of lettuce was observed with the application of amoxicillin at concentration of 110 mg kg^{-1} (Litskas et al., 2018).

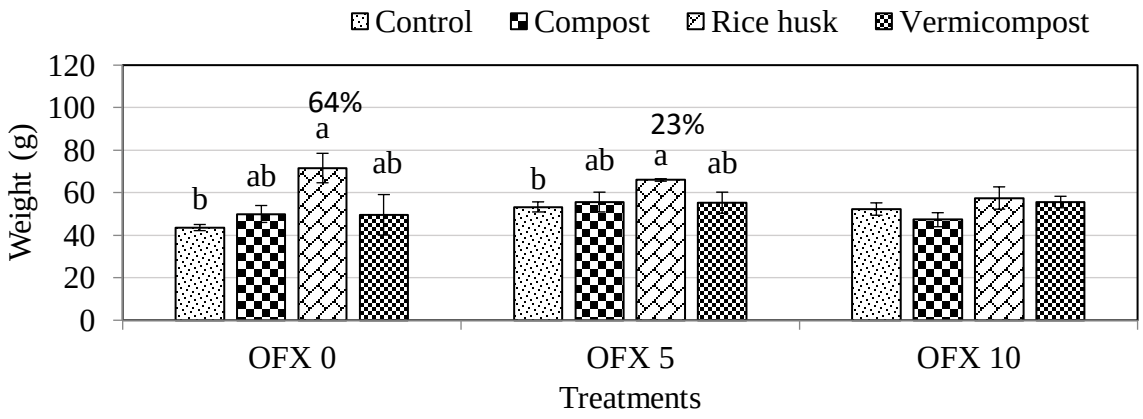


Figure 4.14 Total fresh biomass of lettuce plants treated with ofloxacin

Similar to the control with no antibiotics, the treatments with rice husk had a 23% higher fresh biomass as compared to the control with antibiotics at 5 mg L^{-1} of ofloxacin. A slight increase in the biomass was also observed in treatments with antibiotics only as compared to the control with no antibiotics, although it wasn't significant.

When the antibiotic concentration was increased to 10 mg L^{-1}, no significant difference was observed between any of the treatments ($p>0.05$). It has been reported that the addition of compost as well as hairy vetch can alleviate the antibiotic stress, when lettuce was

exposed to sulfathiazole at a concentration of 10 mg kg^{-1}. Though, a significant decrease in the biomass was observed when the concentration of the antibiotics was increased to 100 mg kg^{-1}, even in the presence of amendments (Caban et al., 2018).

It has been reported that the use of rice husk biochar can reduce the uptake of levofloxacin. Furthermore, it has also been reported that lettuce yield can be increased with the application of rice husk compost, as it increases the soil organic matter as well as the available phosphorus content. (Abubakari et al. 2015; Demir et al., 2015; Yi et al. 2016).

Root Length

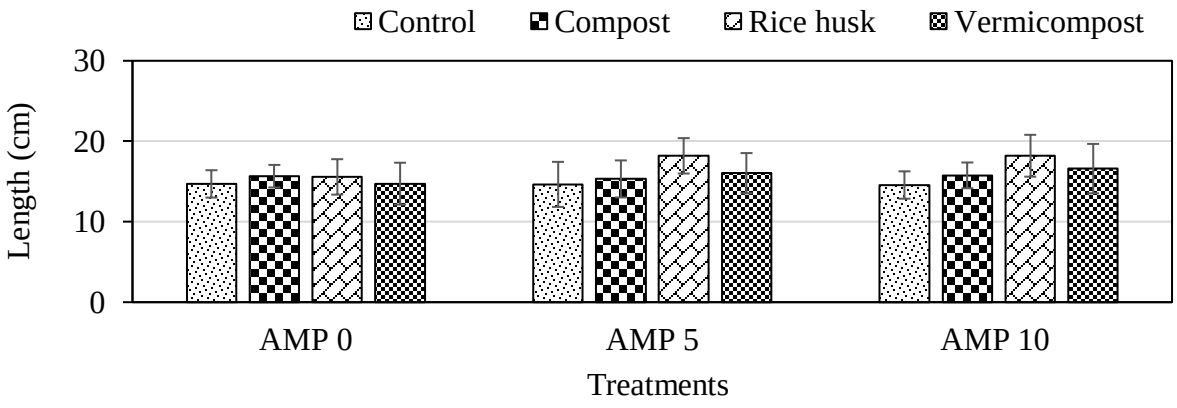


Figure 4.15 Root length of lettuce plants treated with ampicillin

No significant difference could be observed for the root length with the application of ampicillin ($p>0.05$). With the application of antibiotics, the root length was higher in treatments with amendmends as compared to the controls with ntibiotic only. Similar results were reported by Hillis et al., (2011), where no significant effect was observed on *Lactuca sativa*, when treated with amoxicillin at a concentration of 10 mg L^{-1}.

Root eongtion was significantly affected in rice in the germination test with the application of antibiotics tetracycline, chlortetracycline, trimethoprim and tylosin at concentrations

ranging from 0.1 to 500 mg kg^{-1}, while a no significant effect was observed in soil (Liu et al., 2009). The adsorption of the antibiotics to the soil particles as well as soil organic matter could be reponsible or the contrasting effect of antibiotics on rice in the 2 tests. (Mackay and Canterbury, 2005; Pils and Laird, 2007).

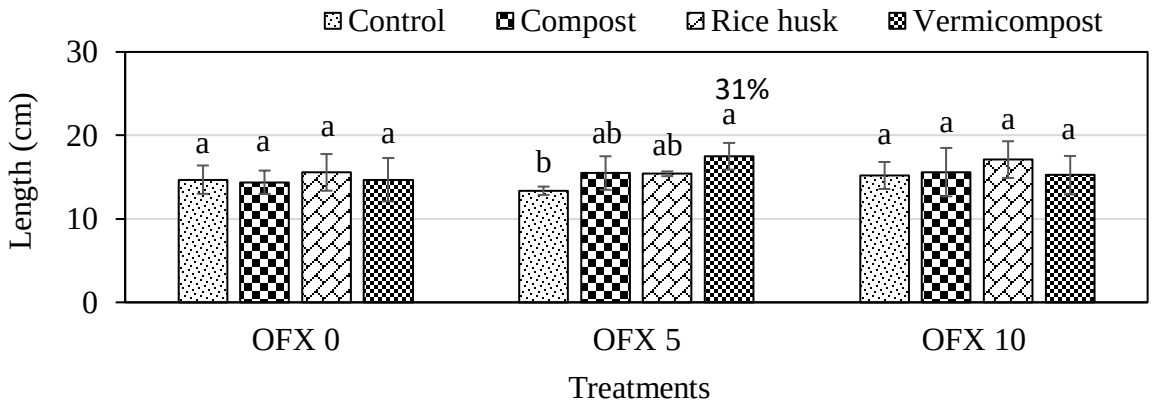


Figure 4.16 Root length of lettuce plants treated with ofloxacin

At 5 mg kg^{-1} of the ofloxacin concentration, there was a slight decrease in the root length in treatment with no amendments. This stress was alleviated significantly by the addition of vermicompost i.e. vermicompost reduced the antibiotic stress by 31%. There was no significant difference between the rest of the treatments ($P>0.05$). When the concentration of antibiotics was raised to 10 mg kg^{-1}, there was no significant difference between the treatments.

Zhang et al. (2019) carried out a study on the sorption of heavy metals using vermicompost and found that heavy metals lead, cadmium and chromium were significantly immobilized with the help of vermicompost. Similarly, tolvene as well as textile dye has been successfully removed using vermicompost (Lusinier et al., 2019; Shirazi et al., 2019). Vermicomposted fly ash improved the soil microbial biomass, enzymatic activity as well

as soil phosphorus and nitrogen content. Furthermore, it improved the germintion of the *L. esculentum* and *S. meongena* (Usamni et al., 2019).

No research has been carried out on the effect of ampicillin and ofloxacin on plants cultivated in soils with organic amendments (used in the current study) so these results need to be validated. **Phosphorus Content in Roots**

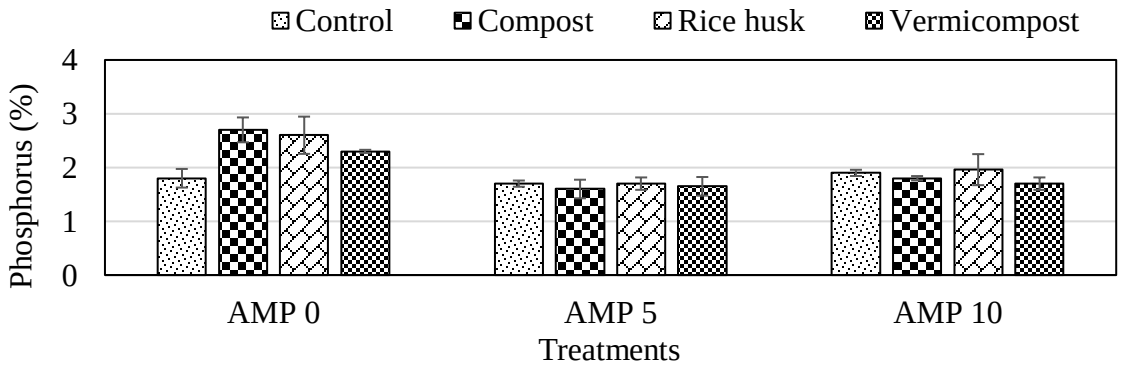


Figure 4.17 Phosphorus content in the root samples of lettuce plants treated with ampicillin

In control with no amendments, the phophorus content was higher in plants cultivated in compost as compared to the control with no amendments though it was not statistically significant. With the appliction of ampicillin, a decrease was observed in the phosphorus content in all of the treatments. Furthermore, no significant difference was found between the phosphorus content of plants cultivated in control with antibiotics only and the rest of the treatment groups with amendments.

Decreae in the phophorus content of *C. bursa-pastoris* was observed with the application of antibiotics penicillin, sulfadiazine and tetracycline at concentrations of 5 and 10 µg L^{-1}. Antibiotics can disturb the normal distribution of nutrients through out different plant

organs i.e. an increase occurs in one part and a decrease in another. This way, the balance of the nutrients in plants is disturbed (Minden et al., 2018).

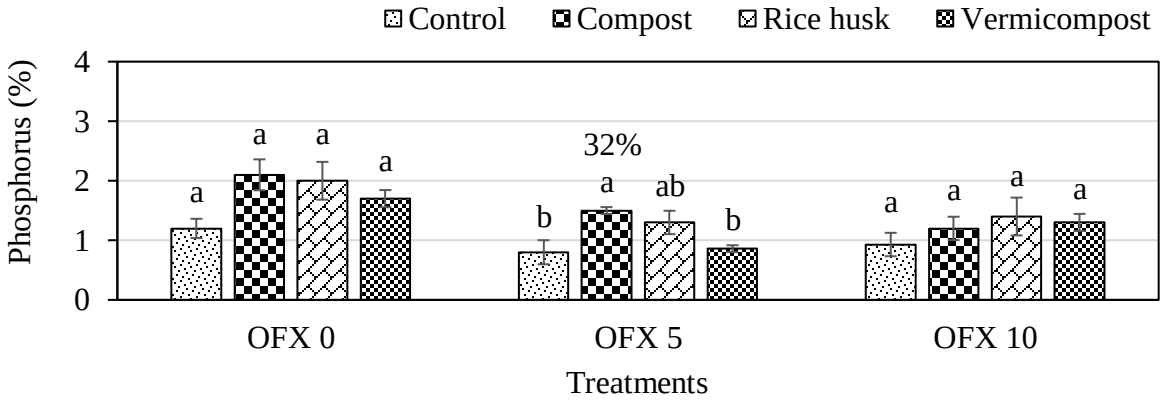


Figure 4.18 Phosphorus content in the root samples of lettuce plants treated with ofloxacin

Similarly, a decrease was observed in the root phosphorus content with the application of ofloxacin. In all the treatments, the phosporus content was higher in plants cultivated in amendments as compared to control without any amendments. No significant difference was found between the treatments in the presence of antibiotics (p-value>0.05).

Antibiotics affect roots more than shoots, since they are in direct contact with the contaminants in soil. Hence phosphorus content in roots showed a higher decrease as compared to the shoot, upon exposure to antibiotics. Limited work has been carried out on the effect of antibiotics on plant nutrient contents, so this aspect needs to be investigated further.

4.4 Phosphorus Content in Shoot

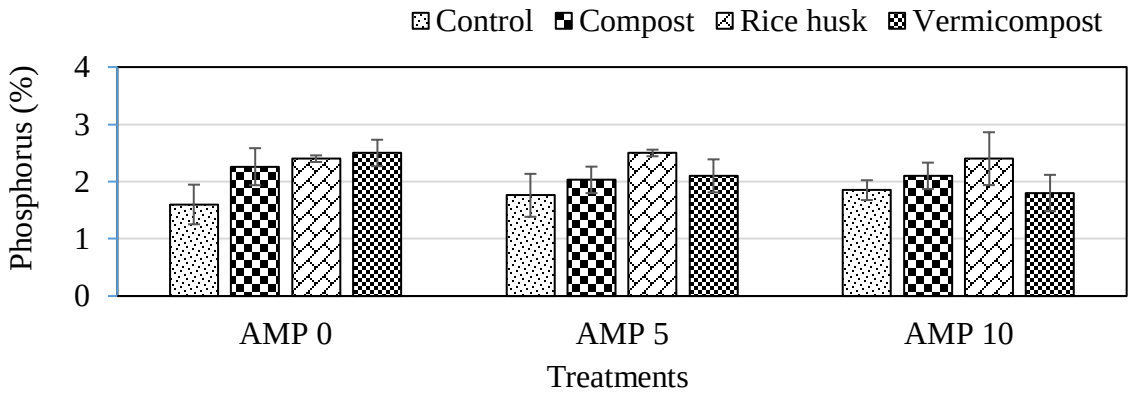


Figure 4.19 Phosphorus content in the shoot samples of lettuce plants treated with ampicllin

The graph for phosphorus content in shoot shows that there was no significant effect on shoot phosphorus content in any of the treatments. In the absence of any antibiotics, the phosphorus content was higher in treatments with vermicompost.

With the application of antibiotics, rice husk treated plants showed higher phosphorus content, although not significantly ($p>0.05$).

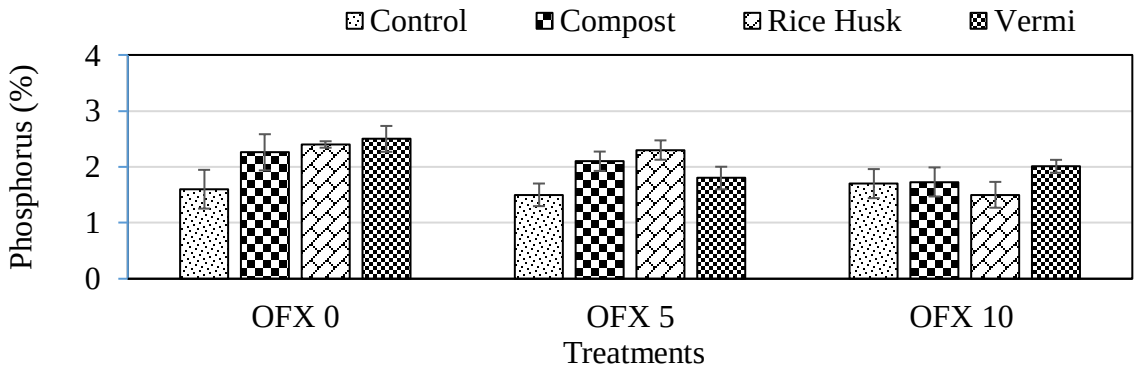


Figure 4.20 Phosphorus content in the shoot samples of lettuce plants treated with ofloxacin

Similarly, no significant difference was observed on the shoot phosphorus content with the application of ofloxacin. A decrease was observed at 10 mg kg^{-1} of the antibiotic concentration, though it wasn't significant. A number of studies have been reported on the negative effects of antibiotics on the soil microbial community i.e. their growth and enzymatic activities. This in turn can negatively impact their ecological functions, such as nutrient transformation, resulting in the loss of functional stability (Pallecchi et al., 2008; Martinez, 2009; Koike et al., 2007; Pauwels and Verstraete, 2006).

4.5 Iron Content in Roots

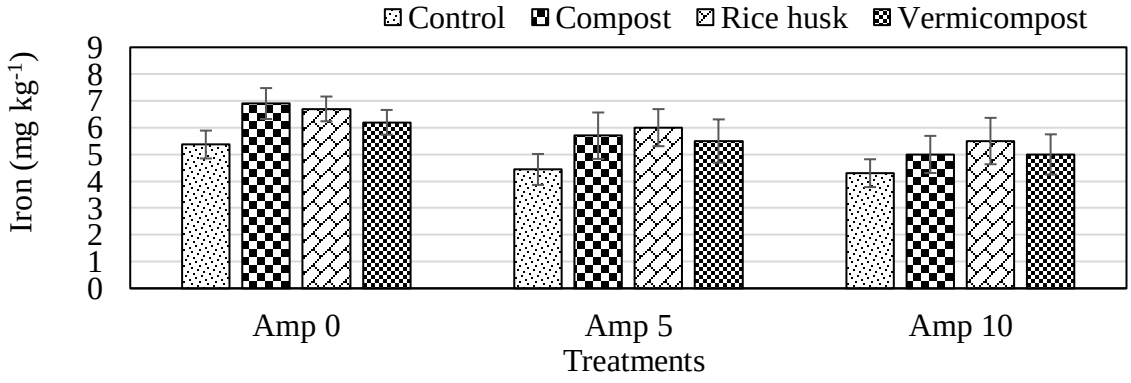


Figure 4.21 Iron Content in the root samples of lettuce plants treated with ampicllin

With the application of ampicillin, there was a decrease in the iron content of roots across all of the treatments and was more visible for plants cultivated in control with antibiotics only. Furthermore, there was no significant difference found ($p>0.05$) between the treatments with and without amendments, at any of the antibiotic concentrations.

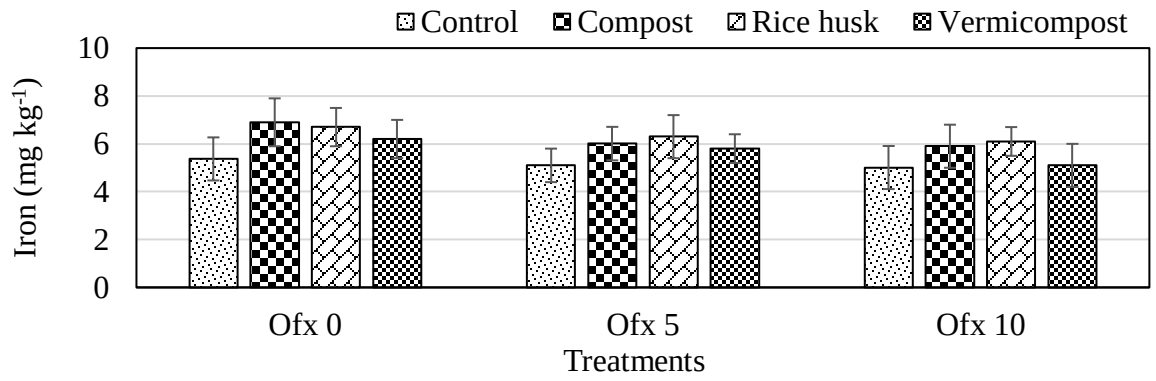


Figure 4.22 Iron content in the root samples of lettuce plants treated with ofloxacin

Similarly, with the application of ofloxacin, there was a slight decrease in the iron content across all the treatments. No significant difference was found ($P>0.05$) between the treatment groups with amendments and those without amendments at any of the antibiotic concentration.

Iron reduction in the soil can be inhibited with the application of antibiotics, and this can lead to significant implications regarding the biogeochemical cycling of the nutrients (Toth et al.,2011).

4.6 Iron Content in Shoot

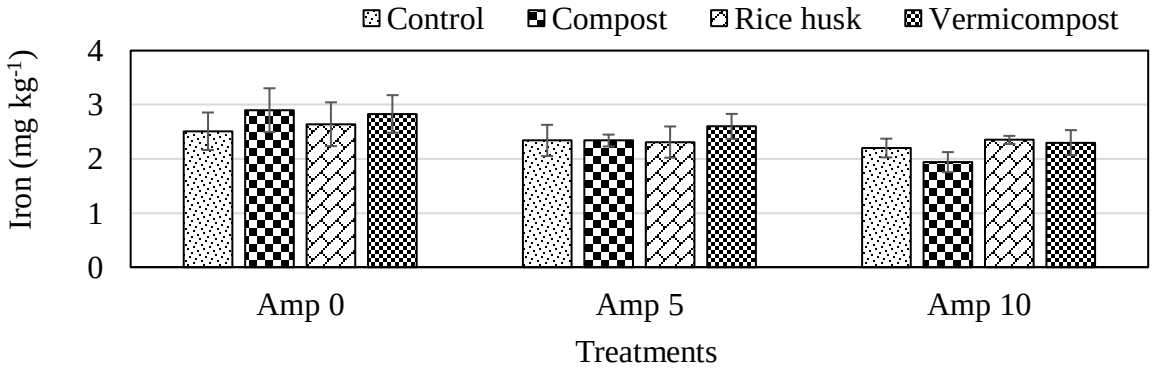


Figure 4.23 Iron content in the shoot samples of lettuce plants treated with ampicillin

A decrease in the shoot iron content was observed as well, with the application of ampicillin and was more evident at 10 mg kg^{-1} of the antibiotic concentration as compared to 5 mg kg^{-1}. Moreover, no significant difference was found between any of the treatments groups, at that specific antibiotic concentration ($p>0.05$).

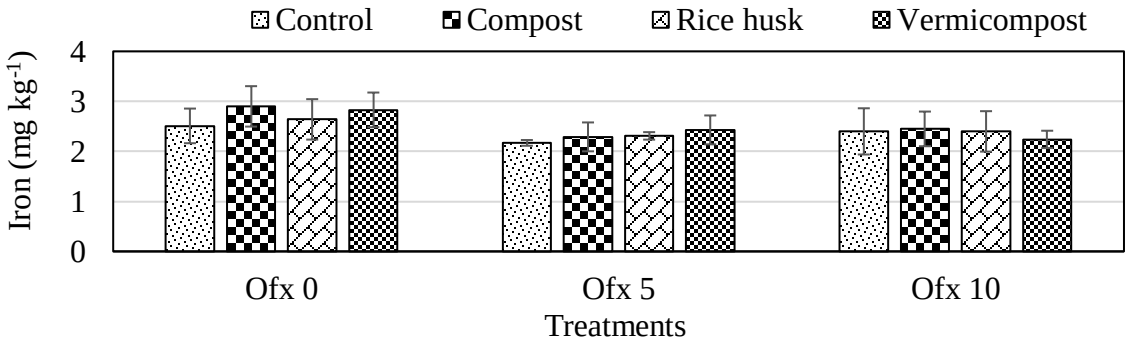


Figure 4.24 Iron content in the shoot samples of lettuce plants treated with ofloxacin

Similarly, no significant difference was found between the the treatments at each of the antibiotic concentration. A decrease in the shoot iron content was also observed in all of the treatments with the application of ofloxacin.

Minden et al. (2018) reported a decrease, though not significant, in the shoot iron content of brassica plants with the application of penicillin and tetracyline at concentrations ranging from 1 to 10 µg L^{-1}. Though the results of this study are only partially comparable to our study, due to the different types as well concentrations of antibiotics used.

Since only one study has been reported on the effect of antibiotics on iron content in plants, more studies need to focus on the effect of antibiotics on iron content given its important role in plant growth.

4.7 Carbohydrates Content

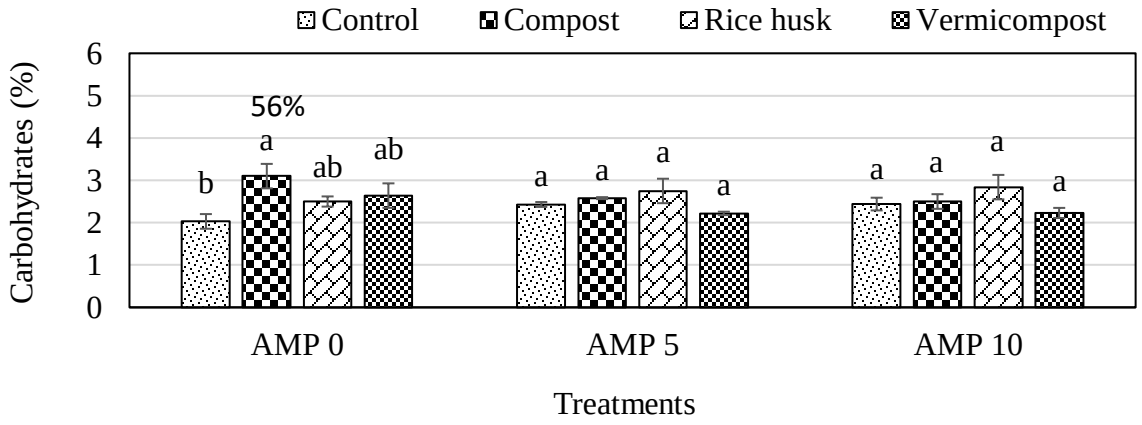


Figure 4.25 Carbohydrates content of lettuce plants treated with ampicillin

In the absence of antibiotics, the carbohydrates content was higher in treatment with compost as compared to the control with no amendments. No significant difference was observed between rest of the treatments ($p>0.05$). Similarly, no significant effect was observed on the the carbohydrates content with the application of ampicillin.

It has been reported that plants under antibiotic stress increase the glucose content for the production of hemiscellulose to strengthen the cell wall, as a defense mechanism. Glucose can also be converted to ascorbic acid, which is an antioxidant (Tasho et al., 2018). The lack of effect observed in the current study could be due to the comparetively lower concentrations of antibiotics used.

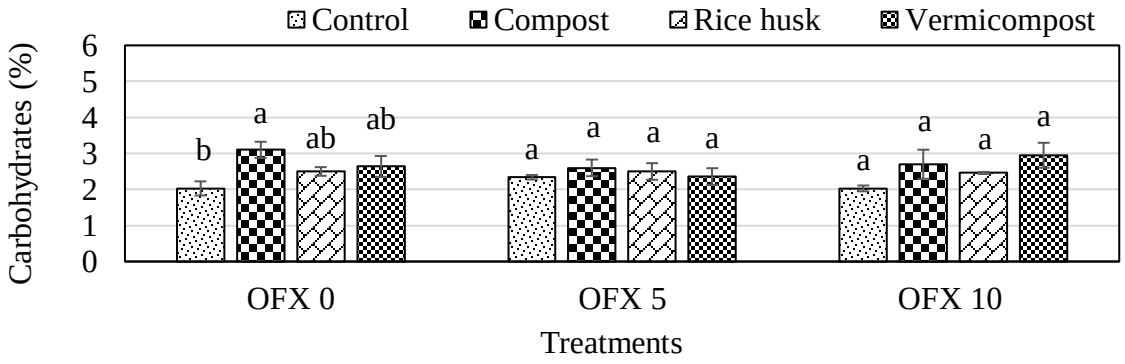


Figure 4.26 Carbohydrates content of lettuce plants treated with ofloxacin

Similarly, no significant effect of ofloxacin was oberved on the carbohydrates content with the application of ofloxacin. Many possible explanations related to the increase as well as decrease in the carbohydrates content have been discussed and reported in the literature. Mortimer et al. (2011) described that glucopyranoside produced as a result of glucose breakdown plays a role in the defense mechanism when plants are exposed to oxidative stress. Price et al. (2004) reported that sugars have the ability to elicit a signal when under stress, as a result of which the induction of the genes, associated with stress response, can take place. Aslund et al. (2012) reported that as a possible response to oxidative stress, the production of reactive oxygen species and the subsequent down regulation of the maltose level can occur.

4.8 Chlorophyll Content

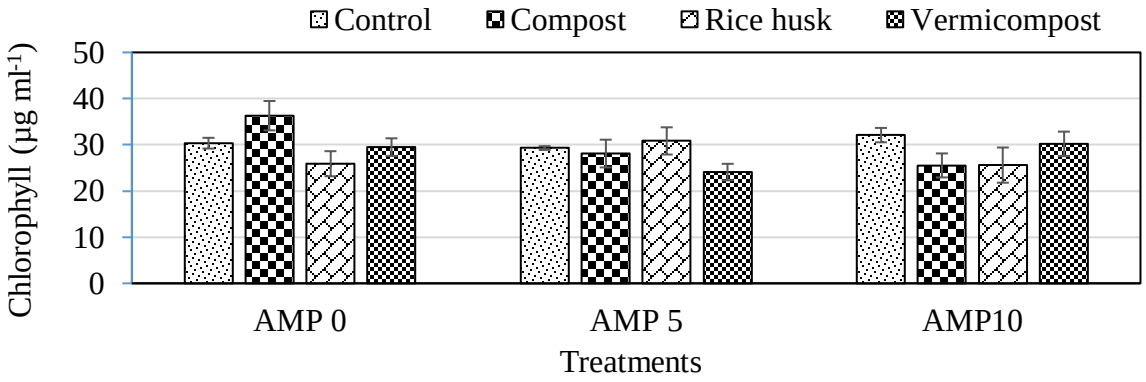


Figure 4.27 Total chlorophyll content of lettuce plants treated with ampicillin

There was no significant difference in the chlorophyll content between any of the treatments, in the absence of antibiotics. Although the chlorophyll content was higher in plants cultivated in compost, this difference wasn't significant. No significant effect of the ampicillin application was observed on chlorophyll content.

Similar results were reported by Michelini et al., 2013, where no effect of sulfamethazine on chlorophyll content was observed in barley plants, at a concentration of 11 mg L^{-1}. Contrastingly, sulfadimethoxine caused a significant decrease in the chlorophyll content. This indicates how the effect of antibiotics on plant paramters vary, depending on the nature of the antibiotics.

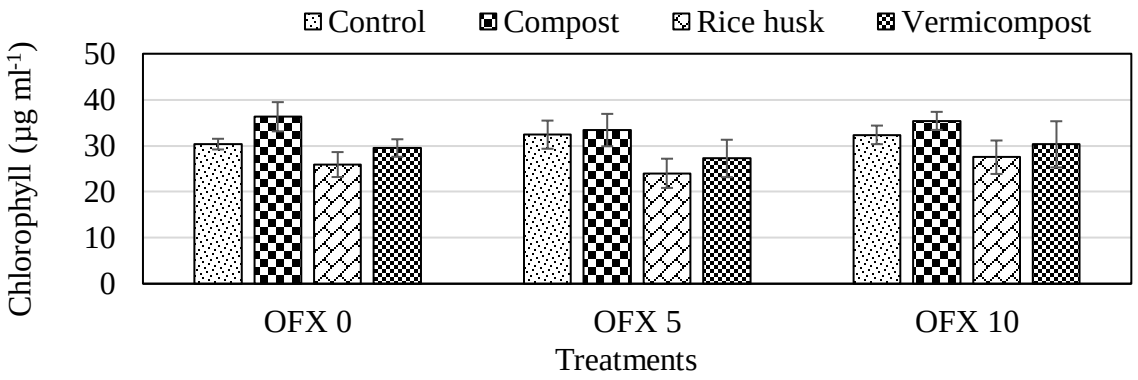


Figure 4.28 Total chlorophyll Content of lettuce plants treated with ofloxacin

No significant difference was observed on chlorophyll content with the application of ofloxacin. Among all the treatments, higher chlorophyll content was observed in plants cultivated in compost, though this difference wasn't significant (P>0.05). The application of sulfadiazine at concentrations of 10 mg kg^{-1} did not have any effect on *Salix fragilis L.*, while a decrease was reported at 200 mg kg^{-1} concentration (Michelini et al., 2012).

4.9 Soluble Protein Content

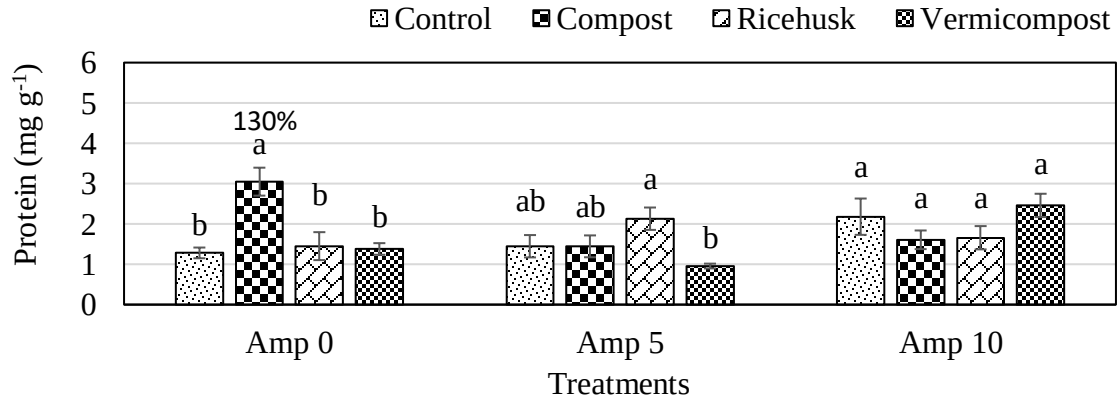


Figure 4.29 Soluble Protein content of lettuce plants treated with ampicillin

In control with no antibiotics, the soluble protein content was significantly higher (p>0.05) in plants cultivated in compost i.e. 130% as compared to the rest of the groups. With the application of ampicillin 5 mg kg^{-1}, no significant difference was found between control

with antibiotics only and the treatments with amendments. However, the protein content of plants cultivated in rice husk was significantly higher as compared to the plants cultivated in vermicompost. A decrease in the soluble protein content of plants cultivated in vermicompost was observed as compared to the vermicompost control with no antibiotics, though it was not statistically significant ($p>0.05$).

Similarly, no significant difference was observed between the different treatments groups as the antibiotic concentration was increased to 10 mg kg^{-1}. An increase in the soluble protein content of plants cultivated in control with antibiotic only as well as those cultivated in vermicompost as compared to their respective controls with no antibiotics.

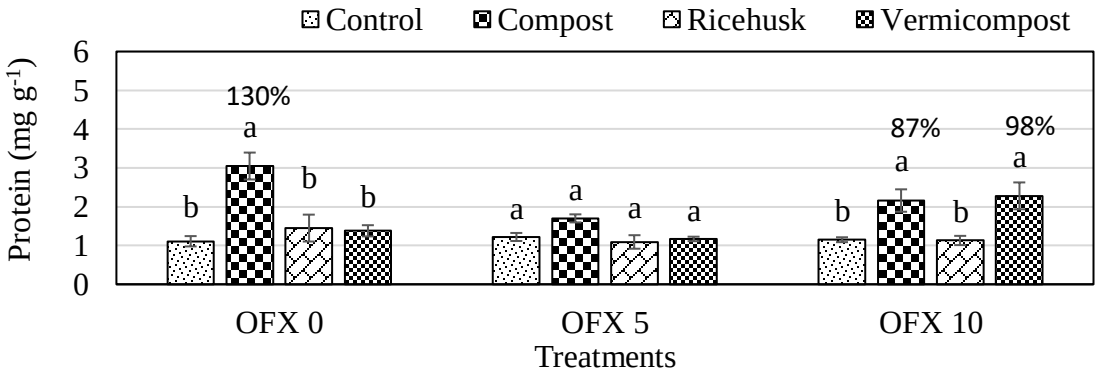


Figure 4.30 Soluble protein content of lettuce plants treated with ofloxacin

With the application of 5 mg kg^{-1} ofloxacin, no significant difference was found between any of the treatment groups ($p>0.05$), while a decrease in the soluble protein content of plants cultivated in compost was observed as compared to the compost control with no antibiotics. As the antibiotic concentration was increased to 10 mg kg^{-1}, a significant increase of 87% and 98% was observed in the soluble protein content of plants cultivated in compost and vermicompost respectively, as compared to the control with antibiotics only.

While many studies have been reported on the effect of antibiotics on plant proteins, their findings are different. A decrease in the total protein content of *Spirodela polyrhiza* was observed with the application of amoxicillin, at concentrations ranging from 0.0001 to 1 mg L^{-1} (Singh et al., 2018). In plants, the ROS and free radicals generated due to oxidative stress can lead to the degradation as well as low production of proteins. This can cause changes in the side chains of the amino acids, reaction of the peptides with carbohydrates and lipid peroxidation products and peptide cleavage (Valavanidis, 2006).

On the other hand, Margas et al. (2019) reported an increase in some of the shoot protein content of pea plants, when exposed to 250 mg L^{-1} tetracycline, including those involved in the defense mechanism of plants, such as diphosphate kinase, superoxide dismutase, peroxiredoxin & glutathione S-tranferase, a protein involved in the degradation of damaged proteins. The highest increase was observed for diphosphate kinase. The overexpression of the gene responsible for this enzyme is related to the increase in the expression of genes responsible for oxidative stress enzymes. Since no research has been carried out on the effect of antibiotics on soluble protein content in the presence of organic amendments, the results of the current study need to be investigated further.

4.10 Rubisco Content

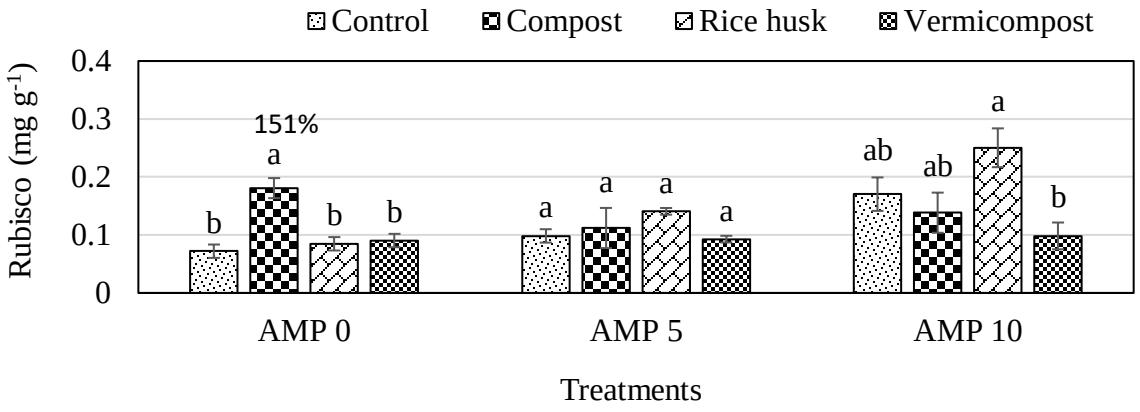


Figure 4.31 Rubisco content in lettuce plants treated with ampicillin

In control group with no antibiotics, the rubisco content was significantly higher i.e. 151% in plants cultivated in compost, as compared to the rest of the treatments. With the application of 5 mg kg^{-1} ampicillin, there was no significant diference found between the treatements with amendments and conrol with antibiotics only.

Similarly, no significant difference was found between the rubisco content of plants cultivated in compost, rice husk and vermicompost, when compared to the control with antibiotics only, as the antibiotic concentration was increased to 10 mg kg^{-1}. Furthermore, an increase in the rubisco content was observed across all the treatments, except compost, and was more obvious in plants cultivated in rice husk.

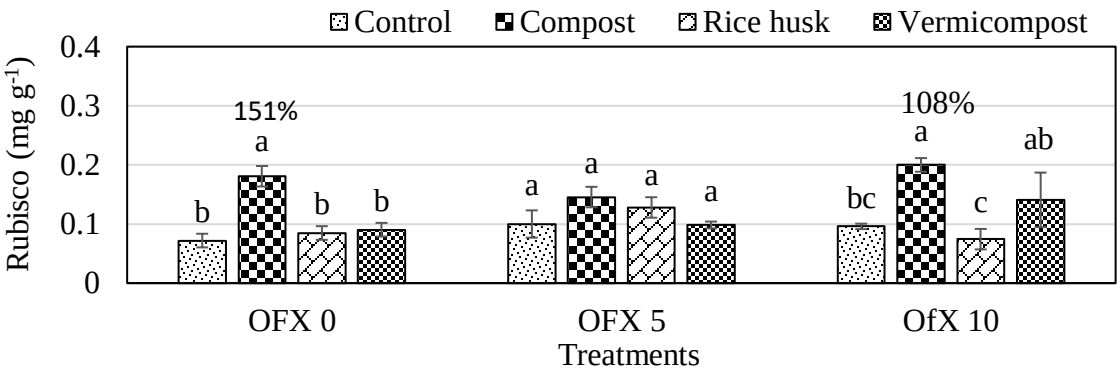


Figure 4.32 Rubisco content in lettuce plants treated with ofloxacin

No significant difference was observed between the treatments with antibiotics and the control with antibiotics only ($p<0.05$), when the plants were exposed to 5 mg kg^{-1} of ofloxacin. With the application of 10 mg kg^{-1} ofloxacin, the rubisco content in treatments with compost was 108% higher as compared to the control with antibiotics only($p>0.05$), while no significant difference was observed between the rest of the treatments.

Liu et al. (2011) reported a decrease in the rubisco content and activity of *Selenastrum capriconutum*, a microalgae, when exposed to increasing concentrations of erythromycin, ranging from 0.06 to 0.30 mg L^{-1}. Ciprofloxacin only reduced the content of rubisco when applied at a concentration of 2.5 mg L^{-1}, while sulfamethoxazole had no significant effect on either rubisco content or activity.

On the contrary, a significant increase in the expression of rbcl gene (responsible for the expression of rubisco large subunit) was observed in *Microcystis aeruginosa* (cynobacteria) when exposed to amoxicillin at a concentration of 8.03 µg L^{-1}. The damage to the D1 protein (present in the photosystem of cynobacteria) could result in its overproduction by the increased expression of psbA as a stress response. This in turn can

lead to increased electron transport, which can result in the overexpression of rbcl gene (Liu et al., 2014).

An increase in the intensity of proteins involved in the photosynthesis was reported, after 2-D electrophoresis of *Pisum sativum* L. shoot protein was performed, following exposure to 250 mg L^{-1} tetracycline (Margas et al., 2019).

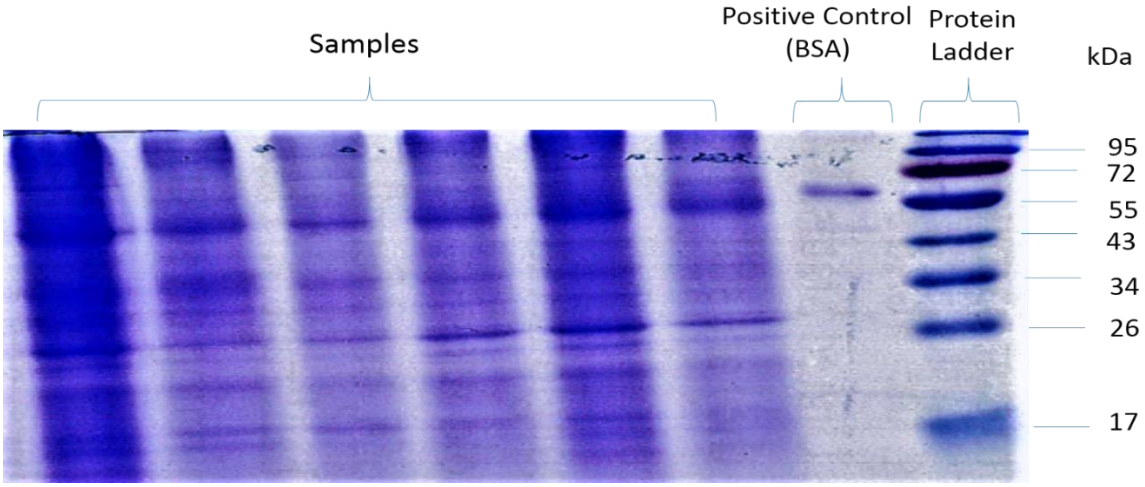
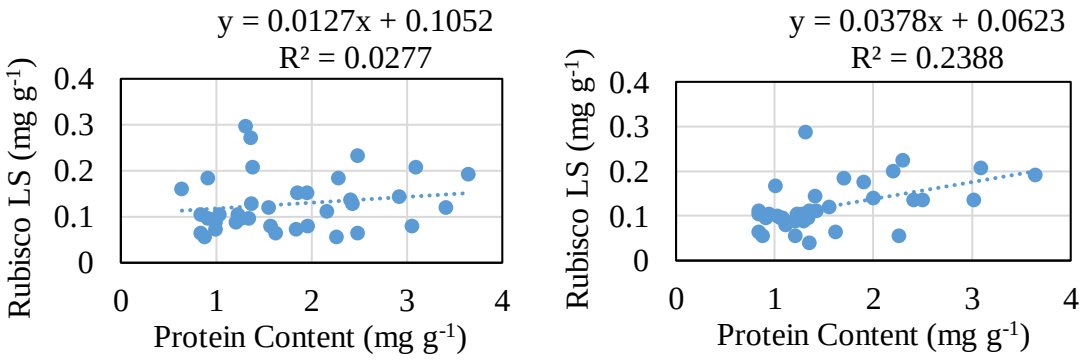


Figure 4.33 PAGE gel. The band for rubiso large subunit is visible around 55 kDa.

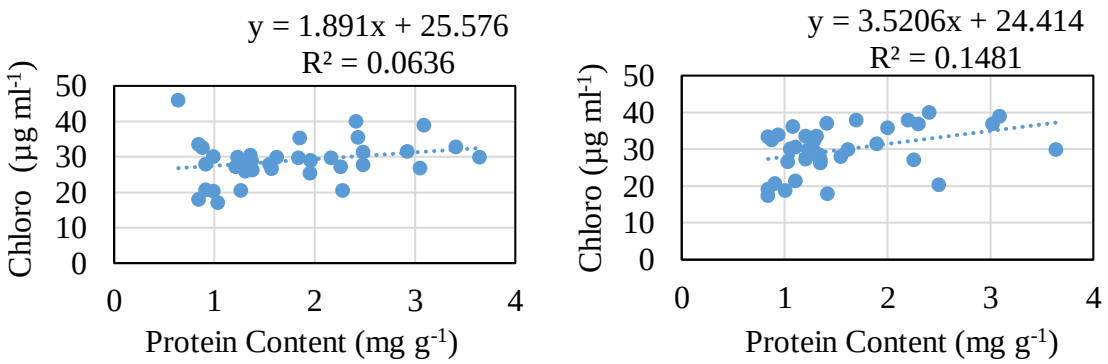
4.11 Correlational Analysis



a) **b)**

Figure 4.34 Correlation between rubisco and soluble protein content in lettuce plants treated with ampicillin **a)** and ofloxacin **b)**

A weak correlation was observed between the rubisco and soluble protein content with no statistical significance differnence (p-value>0.05). Similarly, a weak correlation was observed between rubisco and protein content in lettuce plants treated with ofloxacin, though these results were statistically significant (p<0.05). A weak correlation between the 2 traits in rice has been previously reported, since both are only partially regulated by the same genetics (Ishimaru et al., 2001).



a) **b)**

Figure 4.35 Correlation between total chlorophyll and soluble protein content in lettuce plants treated with ampicllin **a)** and ofloxacin **b)**

A weak and statistically non significant (p-value>0.05) correlation was observed between the soluble protein and rubisco content of lettuce plants treated with ampicllin, while a weak but statisitcally significant (p-value<0.05) correlation was found between the chlorophyll and soluble protein content of lettuce plants treated with ofloxacin.

Although a number of studies have been reported on the correlation between nitrogen and chlorophyll content in plants, limited literature exists on the correlation between soluble protein and chlorophyll content. Guo and Zhao (2018) reported a strong correlation between the 2 parameters in mosses, under the effect of different storage temperatures. The

contrasting results observed in the current study could be due to the different conditions under which the plants were cultivated.

These correlations revealed that rubisco content did not have much difference on photosynthetic activity, which is indicated by biomass results where no visible difference was observed between the treatment groups.

Chapter 5

Conclusion and Recommendations

5.1 Conclusion

The following points can be concluded from this study:

- The application of compost and vermicompost significantly reduced the antibiotic stress in the seed germination test while rice husk performed better in the pot experiment.
- Antibiotics did not have any significant effect on phosphorus and iron content of lettuce plants, as well as the chlorophyll content.
- Both amplicillin and ofloxacin induced significant variations in the rubisco content of the lettuce plant.

5.2 Recommendations

It is recommended that for the future relavant studies, the effect of mixture of antibiotics on plants be assessed since in the environment, antibiotics occur in mixtures rather than separately. Furthermore, changes in the pH needs to be taken into account given changes in pH regulate the adsorptive behavior of the organic matter, concerning antibiotics. It is also recommended that soil of different textures be used since the effect of antibiotics in clay loam soil is minimum due to the clay particles. These particles have higher adsorptive capabilities as compared to other soil particles.

The changes in the rubisco content, due to the antibiotics exposure, also need to be investigated further given the significance of this enzyme. The expression of the *rbcl* gene responsible for rubisco large subunit needs to be assessed in plants under antibiotics stress.

Last but not least, the effect of antibiotics on soil microbial biomass also needs to be taken into account for the future studies.

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