# Impacts on Growth Rate of Wheat, Tomato and Tobacco by Using Different Antibiotics Against Agrobacterium Tumefaciens

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## *Abstract*

*Plants has great nutritious value and extensively used as feast and famine for the development of agriculture sector of any economy. Researchers are trying to produce many genetically modified plants for improving their nutritional value. Many transformation techniques are used for this purpose, but agrobacterium mediated transformation system is very efficient and reliable method for production of transgenic plants. Agrobacterium strain EHA105 was found to be the most efficient with higher rate of transformation frequency, because it provides stable integration at lower copy number. Bacterial contamination is main problem when EHA105 is used for transformation. Antibiotics are only source available source for elimination of Agrobacterium. The main purpose of this study is to determine effect of different antibiotics on the growth of Agrobacterium tumefaciens and selected plant species. In this study, examined that effects of these antibiotics (CTX, LVX, AMX, AMC) on Agrobacterium as well as their effects on root and shoot germination of wheat, tobacco and tomato. It was observed that CTX > AMX > AMC >LVX rate was seen in vitro wheat germination, estimated that all four antibiotics gave good results at 250mg/L. But, AMX > CTX > AMC > LVX rate was observed in vitro tomato and tobacco germination, AMX gave maximum growth rate at all 250mg/L, 500mg/L ,1000mg/L concentrations in tomato, remaining CTX and AMC gave best results at 250mg/l in both plants. The O.D measurements and inhibitory activity presented that β-Lactam antibiotics including AMX, CTX and AMC can use for suppressing of Agrobacterium tumefaciens. But AMX gave maximum growth of inhibition. The findings of this study suggests that AMX is best antibiotic which has less effect of growth rates in plants and has maximum control on Agrobacterium tumefaciens.*

**Key Words:** Wheat; Tomato; Tobacco; CTX; AMX; AMC; LVX

## Introduction

Numerous transformation systems have been reported (Shillito *et al*, 1985; Potrykus, 1991) like; microinjection (de la Pena *et al*., 1987), polyethylene glycol-mediated transfer (Uchimiya *et al*., 1986), protoplast and intact cell electroporation (Fromm *et al*., 1985, 1986; Lörz et al., 1985; Arencibia 1995) and gene gun technology (Sanford, 1988). But, Agrobacterium-mediated transformation has amazing advantages over direct transformation techniques. Because it is most efficient, reliable and it decreases the copy number of the transgene and it reduces the problems with transgene cosuppresion and variability (Koncz et al., 1994, Hansen et al., 1997). Furthermore, it is a single-cell transformation system not developing mosaic plants, when direct transformation is used, this problem is more common (Enríquez-Obregón et al 1997, 1998). Also, it has been mostly used for research in plant molecular biology and for genetic improvement of crops since 1983.

Plant transformation through *Agrobacterium tumefaciens,* a gram negative plant pathogenic bacterium, a member of the eubacterial family Rhizobiaceae. This technique has developed for the introduction of foreign genes into plant cells and the successive regeneration of transgenic plants. In dicotyledonous plants the *A. tumefaciens* naturally infects the wound sites and it

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producing the crown gall tumors (Smith and Townsend, 1907). *A. tumefaciens* has the unique capability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester et al., 1984; Binns and Thomashaw, 1988). T-DNA holds two types of genes: the oncogenic genes, encoding for enzymes involved in the formation of auxins and cytokinin and responsible for tumor formation; and the genes encoding for the production of opines. By condensation between amino acids and sugars these compounds are synthesized and excreted by the crown gall cells and used up by *A. tumefaciens* as carbon and nitrogen sources. Some genes are present at outside of T-DNA, responsible for opine catabolism and also involved in the process of T- DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995).

Agrobacterium mediated transformation procedure has been recognized in several crops like rice (Hiei et al. 1997; Dong et al. 1996), maize (Ishida et al. 1996), and barley (Tingay et al. 1997; Wu et al. 1998). Although the application of this method has increased progressively in common wheat (Cheng et al. 1997; Weir et al. 2001; Wu et al. 2003), the efficacy of successful transformation via Agrobacterium has lagged behind that of other crop species.

**Table 1. Classes and mode of action of different antibitoic**

| Antibiotics | Class | Mode of action |
|---|---|---|
| Cefotaxime sodium | cephalosporin | Cell wall synthesis inhibitor |
| Amoxicillin | penicillin | Cell wall synthesis inhibitor |
| Clavamox | penicillin | Cell wall synthesis inhibitor |
| Levofloxacin | flouroquinolon | Nucleic acid inhibitor |

But, our study had focused on the germination of seeds of different plants in the presence of antibiotics for checking the growth rate and selected an antibiotic which has maximum efficacy to suppress the *Agrobacterium* EHA 105 and has lesser effect on the plant growths. For this purpose, four plants are used (wheat, tobacco, tomato) in the presence of four antibiotics (cefotaxime sodium, amoxicillin, clavamox and levofloxacin). Before attempting to germinate a seed, it is important to know whether the seed (or fruit) will imbibe water. In the laboratory this is determined by placing the seeds on moist filter paper at room temperature and then at hourly intervals for 8-10 hours, blotting the seeds dry and weighing them (Baskin C. and Baskin J., 2001). A gradual increase in seed weight indicates that the seed is absorbing water and is therefore 'water permeable'.

The purpose of study is to select an antibiotic which has maximum control on *Agrobacterium tumefaciens* growth and less effect on the growth of plants and to improve transformation frequency for obtaining desirable transgenic plants in future.

## Materials and Methods

This research was conducted in Biochemistry and Molecular Biology lab, NSMC, UOG, Gujrat, Pakistan.

**Collection of Seeds and Chemicals**

Seeds of different plants such as wheat (watan variety), tobacco (burley) and tomato (reograndy) were collected from local market of different areas of Gujranwala and Gujrat. Chemicals were provided by the Department of Biochemistry and Molecular Biology, University of Gujrat.

**Germination of Wheat Seeds (with antibiotic)**

**Antibiotic stock preparation**

The antibiotics Cefotaxime sodium, Amoxicillin, Levofloxacin, clavamox, Carbenicillin were used in this study. These were freshly prepared after dissolving in dis.H2O and filter sterile.

**Germination medium with antibiotics**

Medium was prepared as mentioned before. But before pouring into the autoclaved test tubes it was first cooled down enough to not change the nature of antibiotic but still warm enough to be poured into the test tubes. Antibiotics with different concentrations were added to this cooled medium. This was then poured in test tubes and solidified.

**To Check the Growth Rate of *A. tumefaciens* through O.D Measurements**

**Purification of *Agrobacterium tumefaciens* strain EHA105**

*Agrobacterium tumefaciens* strain EHA105 was provided by the Department of Biochemistry and Molecular Biology Department, University of Gujrat.

**Media Preparation**

YEP medium was used in this research. Media were prepared both in liquid and solid form and stored at room temperature in a conical flask.

Bacterial Purification

Glycerol stock was provided by the Department of Biochemistry and Molecular Biology. Culture was streaked on a plate with rifampicin. Pure colony was picked and cultured on liquid medium. Fresh glycerol stocks were prepared.

**Optical Density Measurement (Without and without Antibiotics)**

Culture was initially inoculated overnight. Next day when OD was 0.6, 1 ml of this culture was inoculated in 50 ml fresh medium. Reading were taken on hourly bases until the OD has reached 0.6. This same experiment was repeated with the addition of antibiotics and reading were again taken on hourly basis.

**Disc Diffusion Assay**

Disc diffusion assay was performed as described by Kirby Bauer (1996). All experiments were performed in sterile conditions. For disc diffusion assay, media was prepared by weighing 1.6g of nutrient broth and 1.6 gm of bacterial agar through weighing balance, dissolved in 100ml of dis.H2O. Then media was autoclaved at 121$^{o}$C for 15 minutes. Media was poured into these plates, left it to solidify then evenly spread *Agrobacterium. Tumefaciens EHA105* strain on entire surface of agar plates. Antibiotic disc were placed over agar plates seeded with bacterial strain and 1 petri plate was without antibiotic, served as control. These were incubated at 28$^{o}$C for 24 hours and zone was observed for zone of inhibition. The diameter of zones was measured in millimeters of different antibiotic discs and compared these with control that was without antibiotic. Assay was performed in triplicates with each antibiotic disc.

## Results

The main objective of this research was to determine such antibiotic which has maximum control on *Agrobacterium tumefaciens* and less effect on selected plants (wheat, tomato, tobacco). For this purpose, *in vitro* plants germination was performed without antibiotics and with antibiotics. Growth rate of different plants were observed on daily basis and selected one concentration of one antibiotic which has less effect on plant growth for each plant.

***In vitro* Germination of Wheat Seeds (Without Antibiotics)**

After inoculation of 24 wheat seeds in one test tube rack, the growth rate of *in vitro* germination of wheat seeds were observed on daily basis to determine how many seeds were germinated in 24 test tubes. This showed that 13 seeds were grown out of 24 seeds. In the absence of antibiotics, approximately 50-60% seeds were grown.

| Inoculation of no. of seeds | After 1 day | After 2 days | After 3 days | After 4 days | After 5 days | After 6 days | After 7 days |
|---|---|---|---|---|---|---|---|
| 24 | 2 | 5 | 8 | 11 | 12 | 13 | 13 |

**Table-2. (a) *In vitro* wheat seed germination growth rate on daily basis.**

After 1 week, picked each seed through forceps and measured shoot length and root length in centimeters through scale antibiotics. Because it was very lethal for wheat plant.

**Comparison of all antibiotics with standard**

| Plant | No. of seeds | After 1 day | After 2 days | After 3 days | After 4 days | After 5 days | After 6 days | After 7 days |
|---|---|---|---|---|---|---|---|---|
| Wheat (Without Antibiotic) | 24 | 2 | 5 | 8 | 11 | 12 | 13 | 13 |
| Wheat (CTX) | 8 (1000mg/l) | -- | -- | 2 | 3 | 3 | 3 | 3 |
| | 8 (500mg/l) | -- | 2 | 2 | 3 | 4 | 6 | 6 |
| | 8 (250mg/l) | -- | 1 | 3 | 5 | 6 | 7 | 7 |
| Wheat (AMX) | 8 (1000mg/l) | 1 | 2 | 2 | 3 | 3 | 3 | 3 |
| | 8 (500mg/l) | 1 | 3 | 4 | 4 | 5 | 5 | 5 |
| | 8 (250mg/l) | 2 | 3 | 4 | 5 | 6 | 6 | 6 |
| Wheat (AMC) | 8 (1000mg/l) | -- | -- | 1 | 2 | 2 | 3 | 3 |
| | 8 (500mg/l) | -- | 1 | 2 | 2 | 3 | 3 | 3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 8 (250mg/l) | 1 | 3 | 3 | 4 | 4 | 4 | 4 |
| Wheat (LVX) | 8 (100% levo) | -- | 1 | 2 | 2 | 2 | 2 | 2 |
| | 8 (50% levo) | -- | 2 | 2 | 3 | 3 | 3 | 3 |
| | 8 (25% levo) | 1 | 1 | 2 | 4 | 4 | 4 | 4 |

**Table-3.2 (a) Comparison of all Antibiotics with Standard *in vitro* Wheat Seed Germination**

After obtaining data through whole experiment, graph was drawn for comparison of all antibiotics with standard.

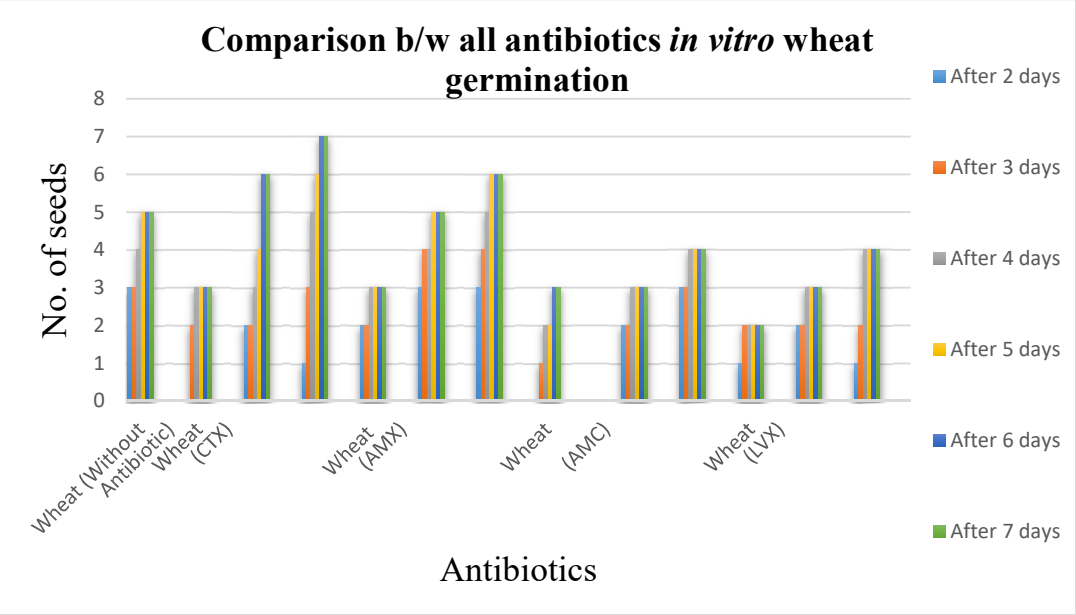



**Figure-3.1 comparison between all antibiotics in vitro wheat seed germination**

This graph explained that among all antibiotics the CTX was an appropriate choice for eliminating *A. tumefaciens* because it showed maximum growth at 500mg/l and 250mg/. Good results were also obtained in case of AMX, because both CTX and AMX showed maximum growth compared with standard (control). While remaining AMC and LVX showed lower growth in case of wheat germination because both were lethal for plant growth.

***In vitro* Germination of Tomato Seeds (Without Antibiotics)**

After inoculation of 24 tomato seeds in one test tube rack, the growth rate of *in vitro* germination of tomato seeds were observed on daily basis to determine how many seeds were germinated in 24 test tubes.

| Inoculation of no. of seeds | After 1 day | After 2 days | After 3 days | After 4 days | After 5 days | After 6 days | After 7 days |
|---|---|---|---|---|---|---|---|
| 24 | 10 | 14 | 17 | 19 | 20 | 20 | 20 |

**Table-3. (a) *In vitro* tomato seed germination growth rate on daily basis.**

**Comparison of all antibitoics with standard in vitro tomato germination**

| Plants | No. of seeds | After 1 day | After 2 days | After 3 days | After 4 days | After 5 days | After 6 days | After 7 days |
|---|---|---|---|---|---|---|---|---|
| Tomato (Without Antibiotic) | 24 | 10 | 14 | 17 | 19 | 19 | 20 | 20 |
| Tomato (CTX) | 8 (1000mg/l) | 4 | 5 | 6 | 6 | 6 | 6 | 6 |
| | 8 (500mg/l) | 3 | 5 | 6 | 7 | 7 | 7 | 7 |
| | 8 (250mg/l) | 6 | 6 | 6 | 8 | 8 | 8 | 8 |
| Tomato (AMX) | 8 (1000mg/l) | 4 | 6 | 7 | 8 | 8 | 8 | 8 |
| | 8 (500mg/l) | 3 | 5 | 6 | 7 | 8 | 8 | 8 |
| | 8 (250mg/l) | 5 | 5 | 6 | 8 | 8 | 8 | 8 |
| Tomato (AMC) | 8 (1000mg/l) | 3 | 4 | 6 | 6 | 6 | 6 | 6 |
| | 8 (500mg/l) | 4 | 6 | 7 | 7 | 7 | 7 | 7 |
| | 8 (250mg/l) | 3 | 5 | 6 | 7 | 7 | 7 | 7 |
| Tomato | 8 | -- | -- | -- | -- | -- | -- | -- |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (LVX) | (100%levo) | | | | | | | |
| | 8 (50% levo) | -- | -- | -- | -- | -- | -- | -- |
| | 8 (25% levo) | -- | -- | -- | -- | -- | -- | -- |

**Table-4. (a) Comparison of all Antibiotics with Standard *in vitro* tomato Seed Germination**

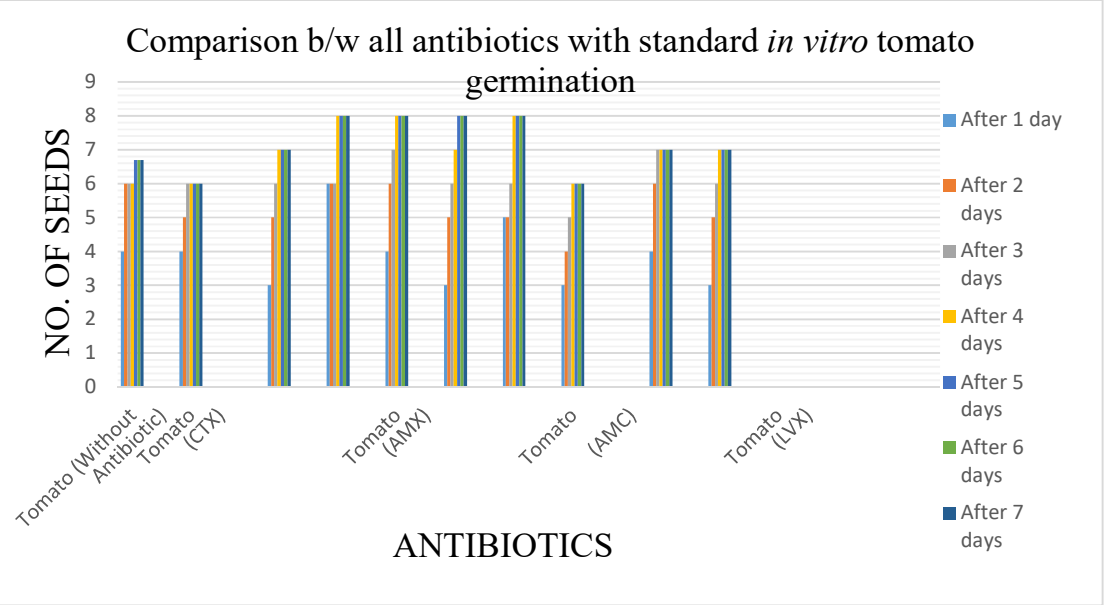



**Figure-5. comparison between all antibiotics in vitro tomato seed germination**

### 3.5. *In vitro* Germination of tobacco seeds (without antibiotics)

After inoculation of 24 tobacco seeds in one test tube rack, the growth rate of *in vitro* germination of tobacco seeds were observed on daily basis to determine how many seeds were germinated in 24 test tubes.

| Inoculation of no. of seeds | After 3 day | After 5 days | After 9 days | After 11 days | After 13 days | After 14 days | After 15 days |
|---|---|---|---|---|---|---|---|
| 24 | 7 | 11 | 13 | 15 | 15 | 15 | 15 |

**Table-6. *In vitro* tobacco seed germination growth rate on daily basis.**

This data showed that 15 seeds were grown out of 24 seeds. In the absence of antibiotics, approximately 62.5% seeds were grown. After 1 week, root and shoot length was calculated.

| No of seeds | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Root length(cm) | 5.5 | 5.5 | 4 | 6.1 | 3 | 5 | 2.5 | 7 | 4.5 | 8 | 6 | 6.5 | 0.6 | 8 | 5 |
| Shoot length(cm) | 11 | 10 | 8 | 9 | 4 | 11 | 5 | 11 | 8 | 9 | 6.5 | 10.5 | 9.5 | 9 | 10.5 |

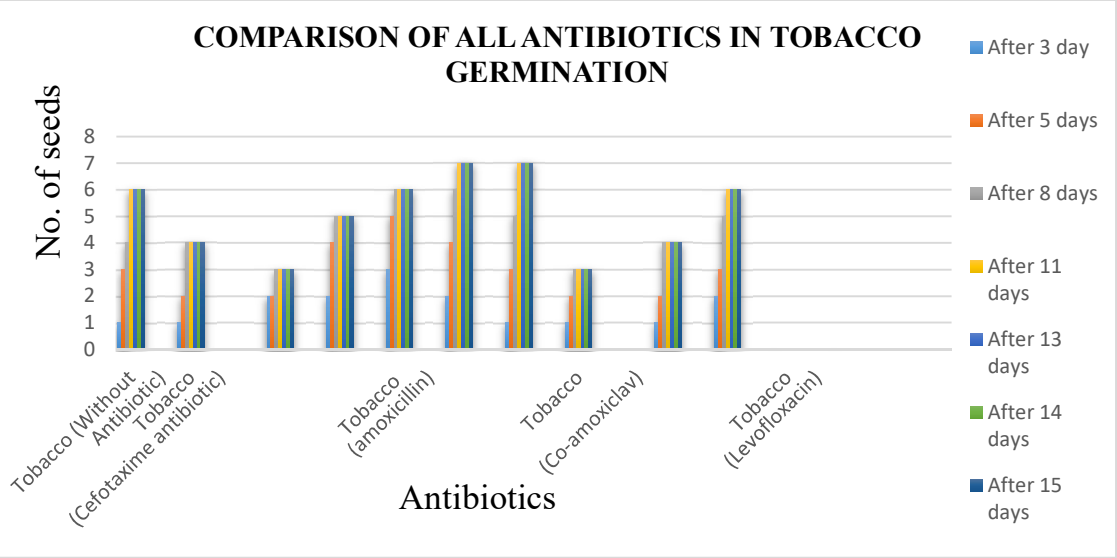



**Figure-3. comparison between all antibiotics in vitro tobacco seed germination**

**To Check the Growth Rate of *A. tumefaciens* through O.D Measurements**

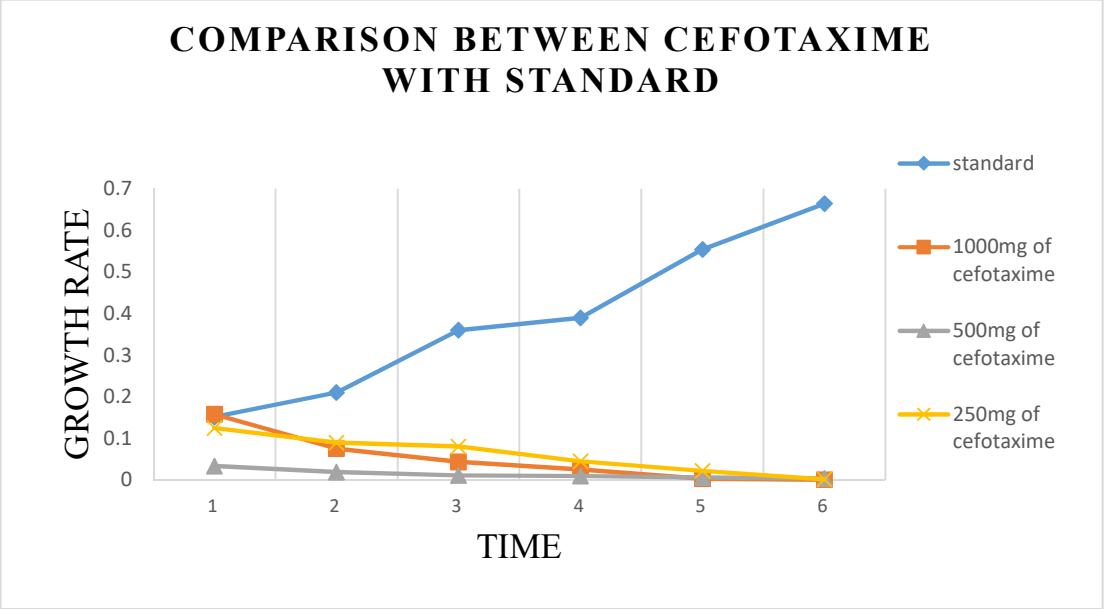
Growth rate of *A.tumefaciens* was checked after different intervals through O.D measurements in spectrophotometer apparatus in the absence of antibiotics and recorded all readings. Then *A.tumefaciens* growth rate was also observed in presence of different antibitoics at different conc. and selected one best antibiotic which has maximum ability to eliminate *A.tumefaciens* at lower conc.

| EHA 105 Growth Rate | | | | | | | |
|---|---|---|---|---|---|---|---|
| Without antibiotics | | After 17 hrs | After 18 hrs | After 19 hrs | After 20 hrs | After 21 hrs | After 22 hrs |
| | | 0.152 | 0.210 | 0.365 | 0.394 | 0.554 | 0.664 |
| With Antibiotics | Concentration | After 1 hr | After 2 hrs | After 3 hrs | After 4 hrs | After 5 hrs | After 6 hrs |
| Inoculum + Cefotaxime | 1000mg/l | 0.158 | 0.076 | 0.044 | 0.026 | 0.003 | 0.001 |
| | 500mg/l | 0.034 | 0.019 | 0.011 | 0.010 | 0.006 | 0.004 |
| | 250mg/l | 0.125 | 0.090 | 0.081 | 0.045 | 0.022 | 0.002 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Inoculum + Amoxicillin | 1000mg/l | 0.092 | 0.061 | 0.043 | 0.026 | 0.017 | 0.009 |
| | 500mg/l | 0.043 | 0.027 | 0.019 | 0.011 | 0.005 | 0.001 |
| | 250mg/l | 0.061 | 0.048 | 0.021 | 0.017 | 0.005 | 0.000 |
| Inoculum + Clavamox | 1000mg/l | 0.146 | 0.023 | 0.016 | 0.009 | 0.005 | 0.003 |
| | 500mg/l | 0.043 | 0.027 | 0.019 | 0.011 | 0.005 | 0.001 |
| | 250mg/l | 0.162 | 0.081 | 0.057 | 0.044 | 0.021 | 0.010 |
| Inoculum + Levofloxacin | 100% levo | 0.167 | 0.111 | 0.100 | 0.089 | 0.067 | 0.047 |
| | 50% levo | 0.108 | 0.091 | 0.074 | 0.061 | 0.045 | 0.034 |
| | 25% levo | 0.121 | 0.097 | 0.071 | 0.047 | 0.031 | 0.029 |

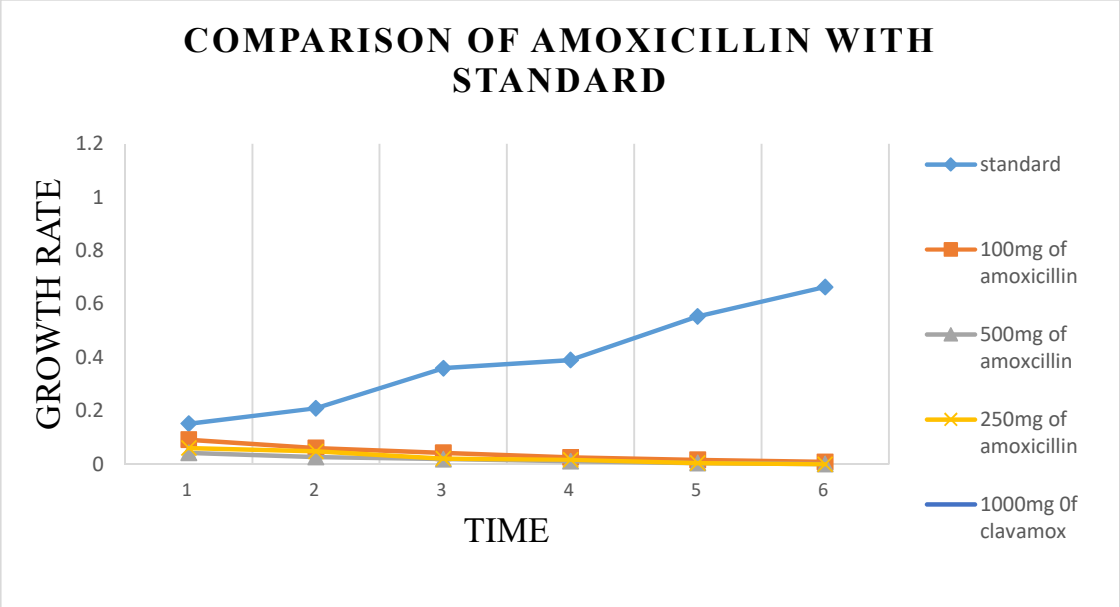
**Table-7. O.D measurement values of all antibiotics against *A.tumefaciens***

This graph showed that different concentrations of CTX compared with standard and concluded that growth rate of *A.tumefaciens* was decreased by the addition of antibiotics. It can use for elimination of *A.tumefaciens EHA105.*



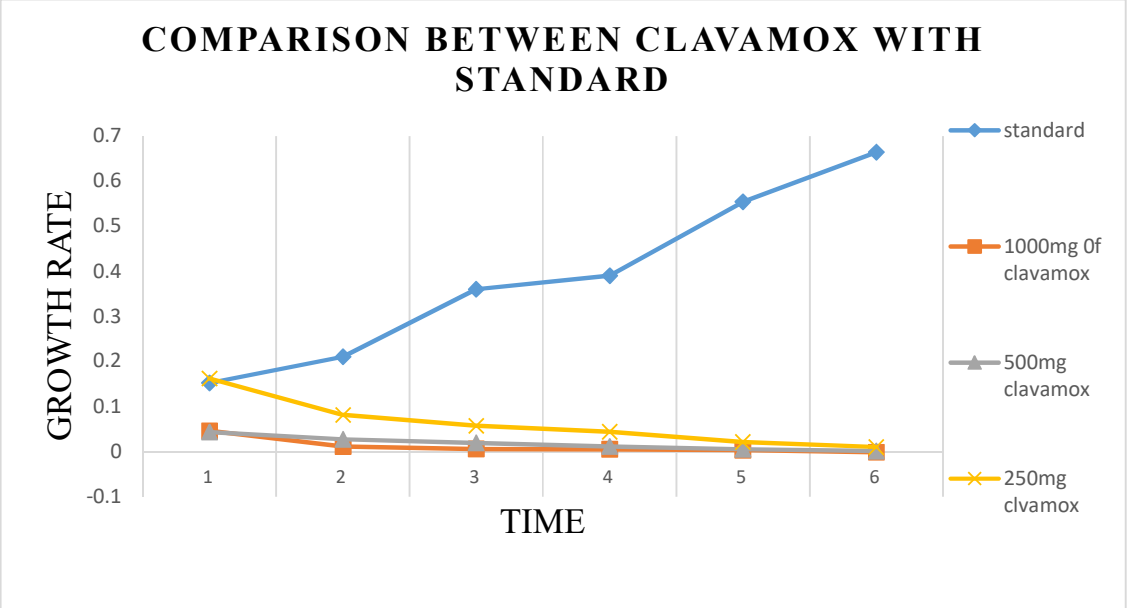

**Firure-4. comparison between different conc. of CTX with standard through O.D measurement.**

This graph showed that different concentrations of AMX compared with standard and concluded that growth rate of *A.tumefaciens* was much more decreased by the addition of AMX antibiotic. It was best choice for elimination of *A.tumefaciens EHA105* among all antibiotics.



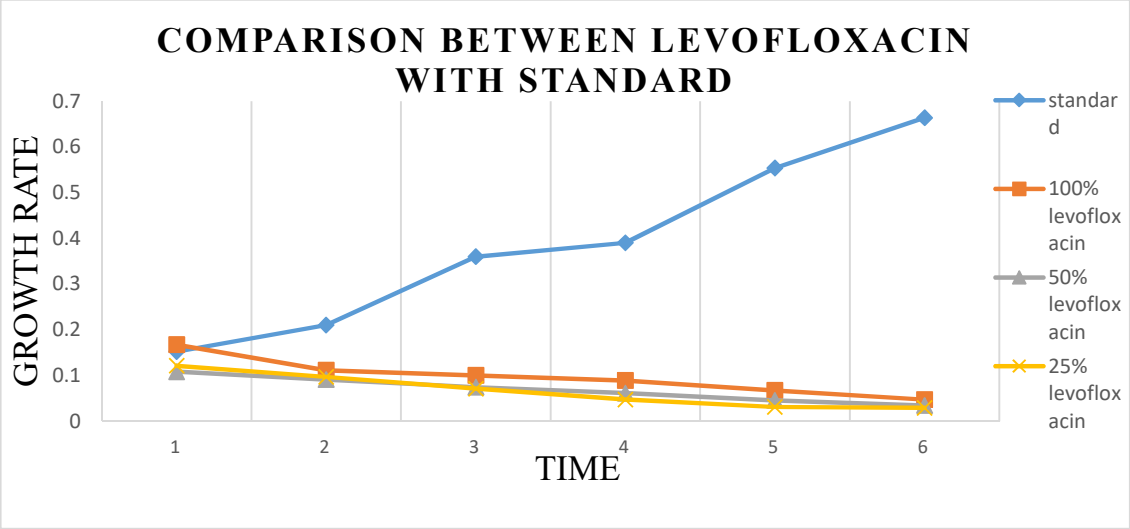

**Firure-5. (a) comparison between different conc. of AMX with standard through O.D measurement.**

This graph explained that different concentrations of AMC compared with standard and concluded that growth rate of *A.tumefaciens* was decreased by the addition of antibiotics. It can also use for elimination of *A.tumefaciens EHA105.*




**Firure-6. comparison between AMC with standard through O.D measurement.**

This graph showed that different concentrations of LVX compared with standard and concluded that growth rate of *A.tumefaciens* was less decreased by the addition of LVX antibiotic. So, It was not best choice for elimination of *A.tumefaciens EHA105*




**Firure-7. comparison between different conc. of LVX with standard through O.D measurement.**

### 3.6. Disk Diffusion Assay

Antibacterial activity of *A.tumefaciens EHA105* was measured by disc diffusion assay.

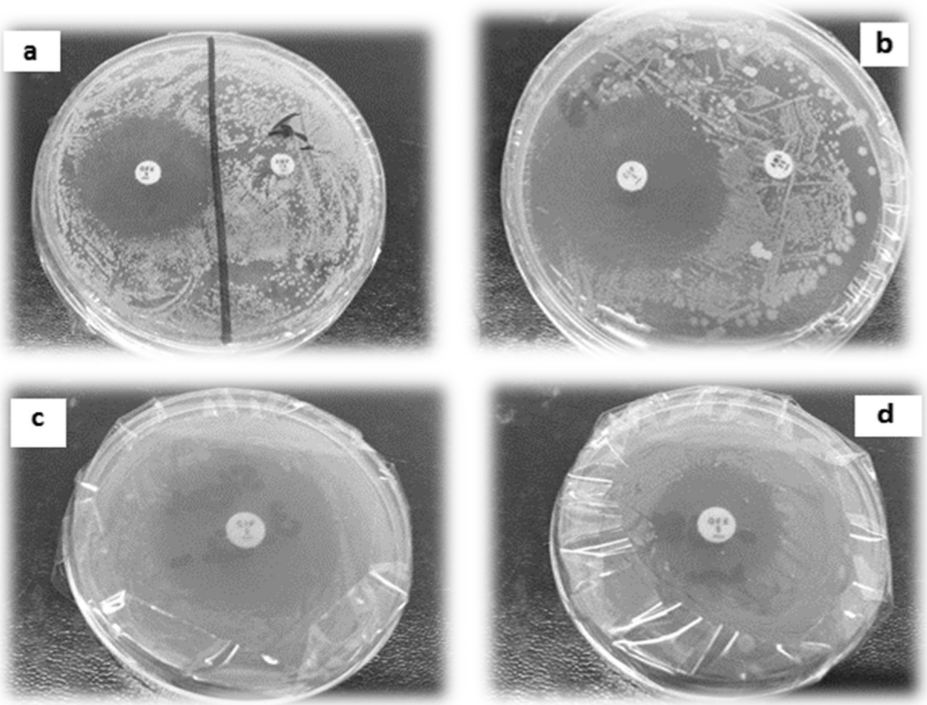



**Figure-8 Antibacterial activity against *EHA105*(a. AMC b. AMX c. CTX d. LVX)**

Greatest zone of inhibition of AMX aginst *A.tumefaciens* was 40mm, and least zone of inhibition was 20mm was recorded in LVX. AMX has high activity against *A.tumefaciens* as compared to LVX.

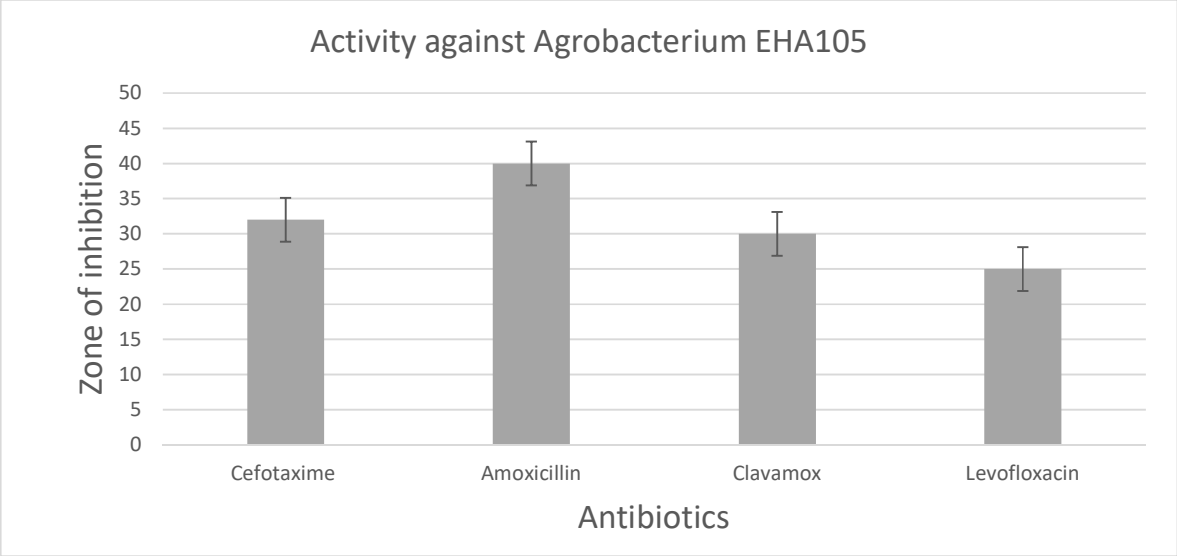



**Figure-9. inhibitory activity of antibiotics against *A.tumefaciens***

This assay concluded that, greatest zone of inhibition of AMX aginst *A.tumefaciens* was 40mm, and least zone of inhibition was 20mm was recorded in LVX. AMX has high activity against *A.tumefaciens* as compared to LVX which has least inhibitory activity.

## Conclusion

The findings of this study are concluded here, *in vitro* germination of three plants (wheat, tomato and tobacco) was performed in the presence of antibiotics (CTX, AMX, AMC and LVX) with different concentrations and in the absence of antibiotics in sterilized conditions. Both results were matched and selected one best AMX antibiotic, which gave high germination rate in both tomato and tobacco plants and CTX gave maximum growth rate in wheat plants at 250mg/l were observed.

Optical density measurement through spectrophotometer for determination of growth rate of *A.tumefaciens* EHA105 in the absence of antibiotics and in the presence of antibitoics. Findings were compared and selected AMX antibiotic which has greatest ability to eradicate *A.tumefaciens*.

Inhibitory activity was checked by disc diffusion method, AMX showed greater zone of inhibition. Our study suggests that β-lactam class of antibiotics for Agrobacterium mediated transformation is an effective strategy to improve transformation methods for a large range of plant species for eliminating this bacterium. Such kind of analysis found in literature which matches with our results.

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