



***In vitro* antibacterial activity of some antibiotics against *Xanthomonas campestris* pv. *musacearum*: *Ensete ventricosum* (Welw.) Cheesman Pathogen**

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Abstract

Enset bacterial wilt caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) is a destructive disease of *Ensete ventricosum* (Welw.) Cheesman) in south and southwestern Ethiopia. There is no known Bactericide against Xcm and the disease is systemic in nature, making the management very difficult. The objective of the study was to evaluate the antibacterial activity of amoxicillin, cephalixin, chloramphenicol, streptomycin sulphate and tetracycline *in vitro* against Xcm. The pathogen was isolated from infected *Enset* pseudostem and its identity was confirmed by pathogenicity test. The antibacterial activity of antibiotics was evaluated using disc diffusion method. Minimum inhibitory concentration (MIC) was determined using agar dilution method. Sub-culturing the contents of MIC onto growth medium was used to know the Minimum bactericidal concentration (MBC). All antibiotics showed antibacterial activity against Xcm, but with varied potency. Significant differences were recorded between test concentrations and antibiotics. Streptomycin sulphate was found to be the most effective antibiotic in inhibiting the growth of Xcm followed by amoxicillin and tetracycline. Moreover, the MIC and MBC values of antibiotics indicate the potential to use in *Enset* bacterial wilt control. Accordingly, tetracycline showed the lowest MIC (0.02 mg/mL) and MBC (0.049 mg/mL) values against Xcm as compared to others. Thus, it could be used in the management of *Enset* bacterial wilt. However, further studies need to be conducted on the effectiveness and method of application of tetracycline under field condition.

Key words: Antibiotics, *Ensete ventricosum*, *Enset* bacterial wilt, *Xanthomonas campestris* pv. *musacearum*.

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Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a perennial staple food crop widely cultivated in the South and Southwestern Ethiopia. It supports the lives of approximately 20 million people (Addis, 2005). It is a multipurpose crop, providing a range of services such as food, forage, medicine, and environment protection (Fekadu, 2009). As a food crop *Enset* has several advantages. First, the plant can be harvested at any time during the year and be harvested at any stage over a period of several years. Second, *Enset* foods can be stored for long periods (Dereje Fekadu, 2009). Third, compared to cereals, it gives high yield per unit area. *Enset* growing regions of Ethiopia are well known for their high population density and the land holding of each farmer is very small and yet the population rarely faces food shortage due to the high productivity of *Enset* (Tsegaye & Struik, 2001). Fourth, *Enset* grows in a wide range of environments, extending from about 1200 to 3100 meters above sea level (Addis, 2005). This allows farmers to grow the crop in all parts of the country, including areas not suitable for cereal cultivation. Fifth, it is considered tolerant to environmental conditions (Fekadu, 2009). However, *Enset* cultivation is impeded by a disease commonly called *Enset* bacterial wilt (*Xanthomonas* wilt). It is caused by the Gram negative phytopathogenic bacterium *Xanthomonas campestris* pv *musacearum* (Ashagari, 1985; Yirgou & Bradbury, 1968). The disease is a major constraint of *Enset* based agriculture, affecting the lives of millions of people in Ethiopia (Welde-Michael et al., 2008;

Afza et al., 1996). In addition to *Enset*, the disease also affects other *Musa* species like banana, which is the main staple food source in the whole of East Africa. The disease poses serious food insecurity in the region (Mwangi et al., 2007). Unlike other diseases, *Enset* bacterial wilt is both extreme and rapid, causing gradual increasing losses over years. The economic impact of bacterial wilt is due to death of the mother plant that would otherwise contribute to the continuation of *Enset* production cycles. Fields infested with *X. campestris* pv *musacearum* cannot be replanted for at least 6 months due to carryover of soil borne inoculum (Tripathi et al., 2009). The major transmission means of the disease across or within fields are insects, contaminated tools and infected plant materials (Mwangi et al., 2007). The spread of the disease is prevented by cultural disease management practices such as burying infected plants, restricting movement of infected plant materials and sterilizing farming tools (Addis et al., 2010; Tripathi et al., 2009; Wolde-Michael et al., 2008; Biruma, et al., 2007; Mwangi et al., 2007). However, these methods are not effective as farmers are inconsistent and reluctant to employ labor-intensive disease control measures (Tripathi et al., 2009). Moreover, the disease is systemic and hence surface application of chemicals has little or no use to control the disease (Smith et al., 2008). Thus, investigations for alternative disease controlling strategies that are effective, eco-friendly, long-lasting, low cost, and easy to prepare have prime importance. Antibiotics have been used since the 1950s to control certain bacterial diseases of high-value fruit, vegetable,

and ornamental plants (McManus et al., 2002). The major reason for the use of antibiotics in crop protection is their highly selective nature in that they are absorbed by plants or seeds, enabling eradication of established infections, even when the pathogen is found deep in the plant tissue. Moreover, antibiotics are less washed off by rain and, upon translocation in the plant. They also protect untreated or newly growing parts of plants (Dekker, 1963). Antibiotics are active on plants for less than a week, and significant residues have not been found in harvested fruit (Stockwell & Duffy, 2012). Antibiotics have been indispensable for crop protection without reports of adverse effects on human health or persistent impacts on the environment (Stockwell & Duffy, 2012). Direct toxic effects of antibiotics on plants, soil microflora and fauna are unlikely because of the low concentrations at which antibiotics are applied on land (Kumar et al., 2005). Therefore, the objective of the study was to evaluate the anti-Xcm activity of some antibiotics *in vitro*.

Materials and methods

Antibiotics: Most of the antibiotics were bought from pharmaceutical shops in Addis Ababa, Ethiopia. Most are prepared to treat human and animal ailments. Streptomycin sulphate was obtained from chemical reagent dealers and is prepared for laboratory activities.

Infected *Enset* material collection: Newly infected *Enset* pseudostem samples were collected from home gardens in major *Enset* growing areas in

Ethiopia. The samples were cut into small pieces of approximately 2-5 mm² in size using sterilized knife. Each piece was placed in separate plastic bags and kept as cool as possible in an ice box to prevent drying, microbial degradation and avoid tissue decomposition. The specimens were then transported to the laboratory for Xcm isolation.

Isolation: Infected pseudostem specimens were further cut into smaller pieces using sterilized scalpel. The pieces were surface disinfected by dipping in 5 % sodium hypochlorite solution for one minute and immediately immersed in distilled water three times to remove the disinfectant. Then, after the cut pieces were immersed into a test tube containing 5 mL of distilled water and allowed to stand for 5 minutes until the bacterial population diffuses out of the cut tissue into the distilled water. Loopful of bacterial suspension was streaked to sterile semi-selective growth medium composed of yeast extract (10 g L⁻¹), peptone (10 g L⁻¹), sucrose (10 g L⁻¹), agar (15 g L⁻¹), cephalixin (50 mg L⁻¹) and amphotericin (150 mg L⁻¹) as developed by Tripathi et al. (2007). Cephalixin and amphotericin were used to inhibit the growth of most saprophytes and fungal contaminants, respectively. The streaked Petri dishes were incubated in an inverted position at 28°C for 72 h. Isolated colonies were selected and streaked on a newly prepared yeast extract, peptone, sucrose and agar (YPSA) growth medium. The sub-culturing of the bacterium was carried out using streak plate method. In this method, loopful of bacteria was directly taken using wire loop from growth plates that contain uniform colonies of Xcm.

Sub-culturing was done several times until pure colonies were produced. The pure culture was stored at 4°C, and every time activated at 28°C before use.

Pathogenicity test: Plastic buckets filled with soil, sand and manure in the ratio of 2:1:1 were prepared in the glasshouse and suckers of a susceptible *Enset* clone were transplanted. After establishment, individual *Enset* plants were inoculated with 10 ml of Xcm suspension adjusted to 1.5×10^8 CFU/mL (0.5 McFarland standard) at the base of midrib in three replications. The negative control was inoculated with the same amount of distilled water using syringes with metal needle. A week after inoculation, symptom development was monitored for every other day. Yellowing of the inoculated leaf was seen after three weeks. Pseudostem of the infected plants was taken and the bacterium was re-isolated using the standard isolation procedure.

Antibacterial susceptibility testing of leaf extracts: The antibacterial activity of antibiotics against Xcm was evaluated by disc diffusion method (EUCAST, 2012). Petri dishes, paper discs, cotton swabs, forceps, test tubes and other materials were autoclaved every time before use. All the tests were performed in three replications and repeated at least three times. Antibiotics were dissolved in distilled water and serial test concentrations were prepared. In separate containers, approximately 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195, 0.098, 0.049 and 0.02 mg/mL serial test concentrations were prepared. Discs with a size of 5 mm in diameter were prepared from Whatman's No 1 filter paper using

paper borer. The discs were sterilized and impregnated in each serial concentration for 12 h. Subsequently, the discs were taken out and dried before application.

Inoculum preparation and

inoculation: The inoculum was prepared from 72 h old bacteria grown on YPSA medium. The upper surfaces of several pure culture colonies were swabbed with cotton swab and mixed with distilled water in a test tube. The content of the test tube was thoroughly shaken until a homogenous suspension was formed. The optical density of the inoculum was measured with a spectrophotometer (NV202, Sunny) at 600 nm and adjusted to 0.132. This value is equivalent to turbidity of a 0.5 McFarland standard (Sutton, 2011). The bacterial population equivalent to a 0.5 McFarland standard turbidity is approximately 1.5×10^8 CFU/mL. Cotton swab was used for inoculation. The sterilized swab was dipped into the bacterial suspension and the excess fluid was removed by turning the swab against the inside of the test tube. This avoids over inoculation of the Petri dish. The inoculum was spread evenly over the entire surface of the Petri dish by swabbing in three directions. The dried discs were applied to the inoculated Petri dish within 15 minutes of inoculation. During application, the discs were pressed downward and the Petri dish was kept in normal position until the discs got wet. Discs rinsed in distilled water were used as negative controls. All the Petri dishes were inverted and incubated at 28°C for 72 h. Inhibition zone was measured after 72 h of incubation. Zone of complete inhibition was measured using transparent ruler at

the longest possible diameter including the disc (NCCLS, 1997).

Minimum inhibitory concentration:

The minimum inhibitory concentration (MIC) of antibiotics was determined using agar dilution method as described in EUCAST, (2000). One mL of each test concentration of each antibiotic was thoroughly mixed with 19 mL of molten growth medium and poured to Petri dishes. The medium was then allowed to solidify at room temperature. The inoculum adjusted to turbidity of a 0.5 McFarland standard (0.3 μ l) was inoculated at four points on each Petri dish. The inoculated Petri dishes were incubated at 28°C for 72 h. Parallel to this, Petri dishes without extract were used as controls and the results were

compared against these controls.

Minimum bactericidal concentration:

Minimum bactericidal concentration (MBC) of antibiotics was determined as described in Njinga *et al.* (2014). YPSA growth medium was prepared and autoclaved at 121°C for 15 minutes. The medium was poured into sterile Petri-dishes and allowed to cool and solidify. The contents of the MIC Petri-dishes that did not show growth were sub-cultured onto the prepared Petri-dishes. The Petri-dishes were then incubated at 28°C for 72 h. Then after, the Petri-dishes were observed for growth. The Petri-dishes without growth represent the minimum bactericidal concentration (MBC). After 72 h, the results were recorded and taken as MBC.

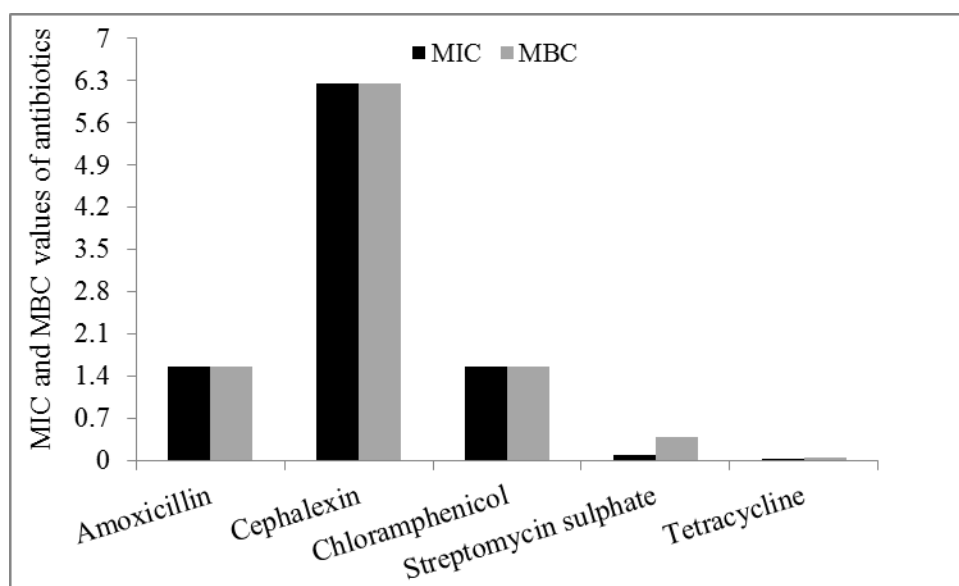


Figure 1: Minimum inhibitory and bactericidal concentrations of antibiotics (mg/mL) against *Xanthomonas campestris* pv. *musacearum*

Results

The *in vitro* anti-Xcm activities of some commercially available antibiotics were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MICs and minimum bactericidal concentration (MBCs). Visible variations were noticed between the tested antibiotics and test concentrations both within and between antibiotics (Table 1). The differences between amoxicillin test concentrations (50, 25, 12.5 and 6.25 mg/mL) were insignificant at $P < 0.05$. At lower test concentrations; below 0.195 mg/mL, the inhibition zone diameters became nil (Table 1). Unlike others, cephalexin inhibited the growth of Xcm only at higher concentrations (50, 25, 12.5 and 6.25 mg/mL) (Table 1). Besides, the differences between these test concentrations were insignificant ($P < 0.05$) except between 6.25 and others. Chloramphenicol on the other hand, inhibited the growth of Xcm at lower concentrations that extend to 1.56 mg/mL. In chloramphenicol, significant ($P < 0.05$) differences were observed among test concentrations except between two consecutive concentrations in the serial dilution (50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml) (Table 1). Similar to amoxicillin, higher test concentrations of streptomycin sulphate showed wider inhibition zone diameter. However, the differences among these (50, 25, 12.5 and 6.25 mg/mL) test concentrations were insignificant at $P < 0.05$ (Table 1). The various test concentrations of tetracycline also inhibited the growth of Xcm at different levels. The highest inhibition zone diameter was recorded in the highest test concentration (50 mg/mL)

followed by 25 mg/mL (Table 1). The difference between these two was insignificant at $P < 0.05$. Tetracycline showed inhibition up to the lowest test concentration (0.02 mg/mL) and significant differences were recorded between the higher (50, 25 and 12.5 mg/mL) and lower (3.12, 1.56, 0.78, 0.39, 0.098, 0.049 and 0.02 mg/mL) test concentrations (Table 1). Comparison of tested antibiotics revealed the existence of marked variation in potency against Xcm; a plant pathogen. Accordingly, amoxicillin and streptomycin showed significantly ($P < 0.05$) wider inhibition zone diameter than cephalexin, chloramphenicol and tetracycline at 50, 25, 12.5 and 6.25 mg/mL test concentrations (Table 1). The difference between tetracycline and amoxicillin was insignificant in some of the lower (1.56 and 0.39 mg/ml) test concentrations. The significant variation between tetracycline and streptomycin was not restricted to the higher test concentrations. They also showed significant differences at lower (1.56, 0.78, 0.39 and 0.098 mg/mL) test concentrations. Cephalexin and chloramphenicol showed relatively narrow inhibition zone diameter as compared to others (Table 1). In general, the antibacterial activity of all antibiotics increased with increasing concentration. Figure 1 shows the MIC and MBC of antibiotics against Xcm. There was variation in both tests between antibiotics. In the assay, tetracycline showed the lowest MIC and MBC values as compared to other antibiotics (Figure 1). The highest MIC and MBC were recorded by test concentration of cephalexin, while the remaining antibiotics had moderate MIC and MBC (Figure 1).

Table 1: The antibacterial activity of antibiotics against *Xanthomonas campestris* pv. *musacearum* at various consecutive serial test concentrations (n = 6).

Test concentrations (mg/mL)	Amoxicillin	Cephalexin	Chloramphenicol	Streptomycin sulphate	Tetracycline
50	49.7±0.33a	15.7±1.8a	32.2±3.4a	50.3±3.2a	38.7±2.6a
25	48.0±1.0a	12.3±1.2a	25.7±3.2ab	46.0±0.6a	35.3±2.4ab
12.5	46.3±0.9ab	13.7±1.2a	20.3±2.2b	43.7±1.9ab	31.5±1.9bc
6.25	38.7±2.4c	5.5±2.0b	16.7±0.9bc	43.3±1.7ac	28.2±1.5cd
3.12	41.0±0.6bc	0.0±0.0c	5.7±0.3cd	34.7±1.2bd	25.1±0.8d
1.56	31.7±2.7d	0.0±0.0c	5.4±0.0cd	35.0±0.6bcd	21.3±0.5d
0.78	25.3±0.9e	0.0±0.0c	5.3±0.0d	33.0±0.6d	13.7±0.2e
0.39	23.7±0.9e	0.0±0.0c	0.0±0.0d	29.7±0.9d	14.1±0.7e
0.195	6.0±0.0f	0.0±0.0c	0.0±0.0d	23.3±0.7de	13.2±0.3efg
0.098	0.0±0.0f	0.0±0.0c	0.0±0.0d	22.0±1.7e	7.6±0.5fgh
0.049	0.0±0.0f	0.0±0.0c	0.0±0.0d	24.7±4.7d	7.2±0.2fgh
0.02	0.0±0.0f	0.0±0.0c	0.0±0.0d	12.0±1.5e	6.2±0.3fgh

Mean values in each column followed by similar letters are not significantly different at $P < 0.05$.

Discussion

The results of the present study showed the potential use of antibiotics in controlling *Enset* bacterial wilt disease. Antibiotics have been used to control certain bacterial diseases of high-value fruits, vegetables and ornamental plants since 1950s in some developed countries (McManus et al., 2002). All antibiotics showed antibacterial activity against Xcm, but with varied potency. Streptomycin sulphate was found to be the most effective antibiotic in inhibiting the growth of Xcm. Similar effects of streptomycin have been reported on *Xylella fastidiosa* isolates that cause pierce's disease of grapevine (Kuzina et al., 2006) and citrus variegated chlorosis in citrus fruits (Lacava et al., 2001). According to Pawar (2015), treating *Xanthomonas campestris* pv. *mangiferaeindicae* (Xcmi) inoculated mango fruits with streptomycin sulphate inhibit the development of mango bacterial canker disease (MBCD). Conversely, SARI, (2013) reported streptomycin sulphate as the least

effective antibiotic against Gurage and Hagere Selam Xcm isolates as compared to amoxicillin, chloramphenicol and tetracycline. Amoxacillin was the second effective antibiotic to inhibit the growth of Xcm at concentrations ranging from 50 – 0.39 mg/mL, which is in agreement with SARI, (2013) that reported higher antibacterial activity of amoxicillin against Gurage Xcm isolate. Tetracycline on the other hand ranked third in its antibacterial activity against Xcm at higher test concentrations ranging from 50 – 0.39 mg/mL and second at lower concentrations below 0.39 mg/mL. It showed inhibition of Xcm growth at the lowest concentration. This result agrees with reports of Cooley et al. (1992), SARI, (2013) and Pawar, (2015). Oxytetracycline treatment; a member of tetracycline antibiotics (Christiano et al., 2010), has significantly reduced the development of symptoms of X-disease caused by mycoplasma-like organism in peach and thus significantly enhance fruit yield 1 year after treatment (Cooley et al., 1992). It is also used as a foliar spray to control *Xanthomonas arboricola*

pv. *pruni* on stone fruits and *Erwinia amylovora* on pome fruits (Christiano et al., 2010). Similarly, SARI, (2013) found out that tetracycline inhibits the growth of both Gurage and Hagere Selam Xcm isolates *in-vitro*. However, it is the most effective antibiotic on Hagere selam isolate. In an *in-vivo* study of antibiotics, tetracycline is found to be more effective to control mango bacterial canker disease than chloramphenicol, gentamicin and streptomycin (Pawar, 2015). Compared to the present study, chloramphenicol has moderate effect on the growth of Xcm (SARI, 2013), *Xylella fastidiosa* (Kuzina et al., 2006) and *Xanthomonas campestris* pv. *mangiferaeindicae* (Pawar, 2015). Moreover, the MIC and MBC values of the present study, especially tetracycline demonstrated the potential to use this antibiotic in controlling *Enset* bacterial wilt. Tetracycline showed the lowest MIC (0.02 mg/ml) and MBC (0.049 mg/mL) against Xcm as compared to others. Similar results even lower MICs and MBCs of tetracycline have been reported by Lacava et al. (2001) against *Xylella fastidiosa*. Comparable result has been reported for streptomycin against *Xylella fastidiosa* (Lacava et al., 2001). Tao et al. (2011) also reported close MIC and MBCs of neomycin against *Xanthomonas campestris* pv. *citri*, *Erwinia carotovora* subsp. *carotovora*, *Xanthomonas oryzae* pv. *oryzae* and *Ralstonia solanacearum*. Therefore, after optimizing the concentration and method of application, antibiotics and extracts of *A. salicifolia* and *P. abyssinica* could be used as a possible alternative controlling agents of *Enset* bacterial wilt.

Acknowledgement

The authors are grateful to Addis Ababa University, Office of Vice President for Research and Technology Transfer for providing the financial support for this research. Bahir Dar University is also acknowledged for financially supporting the research.

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