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In-Vitro Management of *Erwinia amylovora* caused by Fire Blight of Apple with Synthetic Chemicals

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Apple (*Malus pomila* L) belongs to family Rosacea and it is an edible fruit; mostly utilized by different age groups. Apple trees are grown worldwide. It is considered an important fruit in the temperate region of Balochistan, Pakistan. Fire blight is caused by the pathogen *Erwinia amylovora* the most damaging disease of apple and other pomes orchards in the world. The aim of the present study was conducted to record the incidence of the disease and management through the use of chemicals. For this purpose, survey was conducted for disease samples from the various districts of Balochistan Viz., Quetta, Ziarat, Killa Saifullah and Kalat. Among all the districts Ziarat was found to be most severe by pathogen with 20% incidence followed by Quetta having 18.67%, Kalat14.31% and Killa Saifullah 13.44% incidence, and different chemicals, i.e. *Streptomycin, Tetracycline, Gentamicin, Kasugomycin, Oxytetracycline* at different doses were tested against the pathogen and all the chemicals shown great effectiveness against *Erwiana amylorora In-vitro* conditions. The maximum inhibition zone made by chemical streptomycin with value 15.50 and minimum value of tetracycline with 10.90.and there was no inhibition zone observed in control treatment. All the data was statistically analyzed.

Keywords: *Erwinia amylovora*, Identification, In-vitro management, synthetic chemical, Plant disease control, Disease management, Disease resistance, Pathogen inhibition, Antibacterial agents.

INTRODUCTION

The apple tree, (*Pyrus malus*) belong to the rose family (Rosaceae). About 5000 to 6000 apple fruit varieties have been discussed in different literatures, but just a few are most significant for a particular area. Apple fruits contain vitamins A, B and C. Different apple varieties have different concentrations of vitamin C ranging from 1.0 to 20.8 milligrams per 100 grams. The famous proverb "an apple a day keeps the doctor away" clearly explains its healing powers as a food since ancient times. It is used in various ways, such as vinegar, fresh apple juices, and processed jams and jellies (Kuns and Haug, 2006). Apple cultivation in Pakistan is limited to the mountainous regions of the province of Balochistan, the hilly regions of Punjab and Khyber Pakhtoonkhwa. Balochistan produces a wide variety of different fruits and vegetables. The production is of good quality and, from a horticultural point of view, in relatively considerable volumes. Balochistan can rightly be famous for its fruits hence it is called the Pakistan's fruit basket. It stands out for the producing a large number of fresh and dry fruits, some of which do not grow anywhere in our country. Balochistan is one of the major contributors to apple production, producing 34% of the country's production. Pakistan produces almost 1335000 tons. It ranks 10th superscript in the world in apple production (Pakistan Statistical Survey, 2016 – 2017). Fire blight has been a serious threat for fruit growing farmers and scientific researchers for about 200 years since the preliminary symptoms were discovered in the Hudson valley and New York. The rapid progress of epidemics in effected orchards without a history of the disease demolished some recent crops and killed many big limbs or whole trees within a few months. A minor affair lead by the epidemics causes no significant economic losses, even in the previous season, orchards suffers severe blight. Variation in the incidence and severity of fire blight between

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these extremes that seem to follow no particular pattern from orchard to orchard and season to season is characterised (Paul and Stainer, 2000). Fire blight is caused by gram negative bacteria known for its devastation by causal agent *Erwinia amylovora* the bacterial pathogen of apple (*Malus domestica*) and pear fruit (*Pyrus communis*) in the world. In 1873 in New York State since the disease appeared first the number of countries where the disease has been reported has increased rapidly. Now the disease is found in at least 40 countries crosswise New Zealand, Europe, North America and the Middle East. The pathogen injury occurs in the early stage through natural openings in the blooms or on aerial vegetative parts through wounds of the effected tree. Once the pathogen established, the bacteria multiplies into the host and move into small intercellular spaces between parenchyma cells, leading to rapid necrosis of infectious plant tissues and mucus production (Thomson, 2000). The chemicals are very important for controlling the fire blight disease caused by the pathogen *E. amylovora* on core crops that include copper compounds and the use of antibiotics. Even though, the fruit ripening could be often resulting of copper treatment. Therefore, using antibiotics in crop production is highly controversial because of potential risk of developing the progress of antibiotic resistance in human pathogens (McManus *et al*., 2002). The first observation of *Erwinia amylovora* in Switzerland was done on C*otoneaster* sp. The first outbreak occurred in 1991 in apple and pear orchards because of the pathogens nature to remain highly destructive. Eradication and quarantine measures were taken immediately. After initial observations, the disease spread over the majority of the northern and central areas of Switzerland, reaching its highest point in 2007. As a result, about 100 hectares of dwarf barren orchards and 40000 slandered plant trees were removed at the cost of 50 million Swiss fanc. (Pezzatti *et al*., 2008). *Erwinia amylovora* is the main focused pathogen causing fire blight in Balochistan, Pakistan. The disease spread has occurred over the recent decades and therefore captured the attention of the country due to the supply drop line. Consequently, there was an absolute need for conducting a promising and comprehensive study for adding informative documentation about the incidence of fire blight disease in apple fields sorted out through morphological tools. Working on efficient methods through synthetic chemicals considering a rather safer methodology for management functionality.

MATERIALS AND METHODS

***Survey and sample collection*:** Various orchards were surveyed to observe the number of infected trees affected by the pathogen and for sample collection. During survey, four districts were selected for sample collection of apple trees including Ziarat, Killa Saifullah, Quetta and Kalat. At each district examined three orchards consisting upon 155 trees at ziarat, 210 at Killa Saifullah, 130 at Quetta and 160 trees at kalat. Five samples were taken from each three orchards of each districts randomly. And brought at labourtry Laboratory at Department of Plant Pathology, Balochistan Agriculture College, Quetta for isolation and identification of pathogens Similar work was practiced by Cochran (1986). Percentages of infected trees of apple showing fire blight diseases were recorded and disease incidence (%) were calculated by using following formula.

$$\text{Disease incidence (\%)} = \frac{\text{Apple trees showing disease parts}}{\text{Total number of tested apple trees}} \text{ x } 100$$

Table 1. Survey and collection of samples as (Khamis, *et al*., 2020).

Localities/ Districts	No. of Orchards	Total no. of trees	No. of tested trees/ samples
Ziarat	3	155	5
Killa Saifullah	3	210	5
Quetta	3	130	5
Kalat	3	160	5

***Isolation, Purification and preservation of the isolates*:** The sampling were cut with the help of sample cutter sassier and wrapped in newspaper. Isolation of *Erwinia amylovora* was carried out by using nutrient agar (NA) medium, that was prepared by dissolving 1gm of yeast extract 2.5g of NaCL, 2.5g of peptone 2.5g of sucrose in a volume of 500 ml of distilled water. The PH was then adjusted to a range of 7.0-7.2 and then to sterilize by placing in autoclave at 121C at 15 Psi for about 15 minutes. After microscopic study a single pathogenic colony was then transferred from the NA plate with a sterilized loop prepared by adding 20 grams of peptone. The plates after that were incubated at 27^0C for about 2-3 days and the colony of bacteria was observed daily as pathogenic *E. amylovora* colonies with white, circular, mucoid. This procedure was then repeated each after 3 to 5 times to obtain pure culture for tests identifications and to preserve them for future observations. Similar procedure was performed by (Islam *et al*., 2014).

***Biochemical characterization*:** All isolates of biochemical characteristics were necessarily performed according to the method Mashwari and Dubey (2006). The assay was performed as detailed below.

***Gram Staining*:** To check whether the test pathogen is gram positive or gram negative we conducted gram staining tests were conducted in the laboratory. It was confirmed that the tested bacteria were *Erwinia amylovora* as the stain color showed pinkish to red and appeared Rod shaped under microscopic study. The procedure was followed for gram staining was described by Vincent (1970). He used staining method to differentiate species of bacteria into gram negative and gram positive. This was achieved by using the Chemical and Physical compositions of their cell walls. About 1-2 drops of the culture were used to prepare a smear on a sterilized clean glass slide and then heat fixed. The crystal

violet solution was applied by dripping 1-2 drops onto the fixed smear for 1 minute. The smear with distilled water was then washed. Solution B gram iodine for one minute was applied and then with 95% concentrated alcohol washed. For 30 seconds with safaranin the smear was stained following by washing with sterile distilled water. The coating was air dried, using oil immersion examined under a light microscope. Bacterial cells appeared purple of gram positive, while bacterial cells of gram negative were pinkish to red (Vincent, 1970). For five minutes the slide was washed with water then washed and counterstained for 30 seconds with safari in. Again the slide was washed and blotted dry. The slide was then kept observing under an oil immersion microscope objective (Vincent, 1970).

***Catalase test and Amylase test*:** Catalase test for performed similarly as it was prescribed by (Kumar, *et al.,* 2015). For amylase test a sterile swab or sterile loop was used to pick a few colonies from pure cultured Petri dish, then a starch sheet was drawn in a line across the width of that sheet. Incubation process was fixed at 37°C for 48 hours, then added 10% Iodine solution directly to the edge of the colonies with quantity of 2-3 drops and Kept for 10-15 minutes to record the result's positive test. The medium showed darkness at the areas where isolated colonies were covered. The starch had been hydrolyzed by amylase exhibited clear appearance. Besides, where in case of amylase negative test the stained dark edge of the medium to the edge of the isolated colonies, two different species onto a starch plate were inoculated and incubated at 30°C until growth was seen. Then the Petri plate was filled with aid of prepared iodine solution and photographed after 10 minutes of duration. Amylase-positive species showed a clear halo around growth (Kumar *et al.,* 2015).

***In-vitro application of fungicides*:** The chemicals used at three different concentrations i.e. 0.5% 1% and 1.5%. streptomycin, Tetracycline, Kasugomyc, Gentamycine, Oxytetracyclinee and control in which chemicals were not added as given in table.2. All the treatments at various concentrations were tested against *Erwinia amylovora* and their effects were being compared with sixth treatment, the control.

Table 2. Chemicals against *Erwinia amylovora* and their concentrations.

Treatment	Chemicals	Concentrations		
T1	Streptomycin	0.5%	1.0%	1.5%
T2	Tetracycline	0.5%	1.0%	1.5%
T3	Kasugomycin	0.5%	1.0%	1.5%
T4	Gentamycin	0.5%	1.0%	1.5%
T5	Oxytetracycline	0.5%	1.0%	1.5%
T6	Control	0.0%	0.0%	0.0%

***Chemical susceptibility test*:** Chemicals susceptibility test was conducted according to Charteris *et al.* (2001). In this test a number of anti-microbial compounds were applied at different chemical concentrations to the autoclaved Petri plates making four holes of 8mm diameter into the plates. The plate holes were made by using sterilized cork borers inserting into slide media to cut part of the media in circular form in sterilized conditions. These wells were then filled with different antibiotics using different concentrations. The plates were then observed after 6 days and found that inhibition zones were produced by the antibiotics against the pathogenic smear (Charteris *et al.,* 2001).

***Statically Analysis*:** In the analysis of data, we determined the severity and occurrence of the disease to determine, how many trees were affected and what percentage made it through the tree survey field. In case, all infected and healthy trees were mentioned (Steel *et al.,* 1999) was used for the data analysis at 0.05 P Value.

RESULTS AND DISCUSSION

***Disease Incidence*:** The pathogen under study causes losses in apple and pear over the globe where these fruit trees are grown. Survey results showed that the Incidence of disease percentage in Ziarat was found highest amongst the all four districts i.e. 20% followed by Quetta 18.67%. Kalat had third place in incidence with 14.3%. The least incidence percent occurred in Killa Saifullah 13.44%. In the whole survey of four districts of Balochistan the disease incidence was inter pretted which resulted with other researchers, it was also explained that the bacteria are responsible to spread in dew and rainy areas. Due to ratio of rainfall in Ziarat heigher thAn other three districts because of natural forests of juniper. On the regular basis of rainfall in the spring on blossom stage of apple the pathogen spread due to the avilability of heavy rainfall dew ratios. These enviornmental factors provide favorable conditions for the pathogens which cause the disease reaching at most severe stages. Some of the researchers have also indicated that the factors like Dew, rainfall, high temperature provide chances to cause the disease (Cochran, 1986).

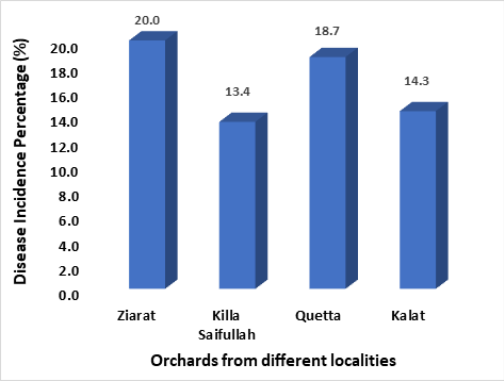

Figure 1. Disease incidence of four districts from different localities.

***Identification of bacteria isolates*:** The samples under observation were tested after incubation of the pathogens for seven days and their presence in the grown media was confirmed from the following performed studies. Our study is aimed at focusing the cause of pathogen spreading in the area and finding efficient solution to control the pathogenic spread. We have conducted research using chemicals that have shown promising results with some modifications in our research i.e. using different chemicals and their concentrations. Other researchers have also incorporated chemicals with different concentrations as described by (Bareswill *et al.,* 1997). For identifying the pathogen of fire blight *Erwinia amylovora*, the morphological colony was studied when it was appeared in (NA) media. To identify the colony morphology of *Erwinia amylovora* was studied under microscope when it was appeared on NA media. In addition, the gram staining was performed by measuring at 39°C temperature and 4% NaCl concentration containing Nutrient Agar (NA) medium. For further confirmation, a fluorescence assay was performed at 366 nm after 48 hours of incubated plates under UV light. Similar method was performed as mentioned by (Bareswill *et al.*,1997).

Table 2. Catalase and Amylase test.

Strain No.	Catalase Test (+/-)	Amylase Test (+/-)
S No.1	+	-
S No.2	+	-
S No.3	+	-
S No.4	+	-
S No.5	+	-
S No.6	+	-
S No.7	+	-
S No.8	+	-
S No.9	+	-
S No.10	+	-
S No.11	+	-
S No.12	+	-
S No.13	+	-
S No.14	+	-
S No.15	+	-
S No.16	+	-
S No.17	+	-
S No.18	+	-
S No.19	+	-
S No.20	+	-

***Catalase and Amylase test*:** Table 2 revealed that the main aim of the study was to develop a method utilizing a simple extraction manner for the simple rapid and correct identification and differentiation of the fire blight *Erwinia amylovora* that is positive or negative. In catalase test the status of bacteria was checked the catalase was positive or catalase negative Slide having bacterial smear was tested on various bacterial strains and a drop of hydrogen peroxide was placed on a microscope in catalase test. The mixture producing bubbles or froth were indicated the organism as 'catalase-positive'. The mixture not producing bubbles or froth was represented the organism as 'catalase-negative'. After applying hydrogen peroxide it was proved that bacteria are catalase positive *Erwinia amylovora* because of making bubbles on slide containing bacterial smear.

Amylase test: In amylase test I applied iodine in bacterial mixture in this case the color of the mixture remained dark The purpose of the test was to see whether the bacteria is amylase negative or not and it seemed that the bacteria is amylase negative and is responsible for causing disease of fire blight pathogen *Erwinia amylovora* which is very harmful to apple orchards.

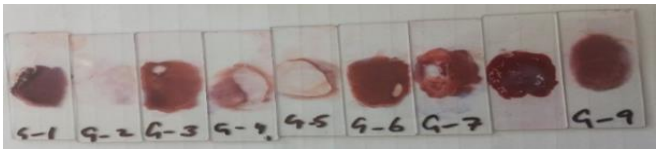

Figure 2. Slides for Catalase test.

***In- vitro* application of synthetic chemical:**

***Efficacy of different chemicals on growth inhibition at 0.5 concentrations*:** Table 3. Represents the effect of different chemicals on growth inhibition at 0.5% concentration. The comparison between different chemicals the conducted research represents that streptomycin with 0.5% showed maximum control over pathogen with value 13.83 while tetracycline was found less effective with minimum value of 10.90. Kasugomycin with value 11.58, Gentamycin was the second highest value with 12.01, Oxytetracycline with 11.50 and control remained nil. And means were compared in overall chemicals showing significantly different result to each other.

***Efficacy of different chemicals on growth inhibition at 1% concentration*:** Table 4. represents the effect of different chemicals on growth inhibition at 1% concentration in which inhibition zones which were measured in millimeters with days' intervals in hours. The comparison between different chemicals the conducted research represents that the streptomycin with 1% showed maximum control over pathogen with value 14.42 while Tetracycline was found less effective with minimum value 11.01. Kasugomycin with value 11.85, Gentamycin was the second highest value with 12.54, Oxytetracycline with 11.66 and control remained nil. Overall chemicals show non-significant different results to each other's by comparing chemicals means.

***Efficacy of different chemicals on growth inhibition at 1.5% concentration*:** Table 5. represents the effect of different chemicals on growth inhibition at 1.5% concentration. The comparison between different chemicals the conducted research represents that the streptomycin with 1.5% showed maximum control over pathogen with value 15.50 while Oxytetracycline was found less effective with minimum value 11.68. Kasugomycin with value 12.83, Gentamycin was the

Table 3. Effect of different chemicals on growth inhabitation at 0.5% concentration.

Chemicals	Inhibition Zone Diameter (mm) in Days Interval (Hrs)				Mean
	24	48	72	96	
Streptomycin	12.75	13.83	14.00	14.75	13.83 a
Tetracycline	10.50	10.75	11.00	11.33	10.90 c
Kasugomycin	10.75	11.50	11.58	12.50	11.58 bc
Gentamycin	11.65	11.75	12.25	12.50	12.01 b
Oxytetracycline	10.75	11.50	11.75	12.00	11.50 bc
Control	0.00	0.00	0.00	0.00	0.00 d

Table 4. Effect of different chemicals on growth inhabitation at 1% concentration.

Chemicals	Inhibition Zone Diameter (mm) in Days Interval (Hrs)				Mean
	24	48	72	96	
Streptomycin	13.75	13.75	14.42	15.75	14.42 a
Tetracycline	10.50	11.00	11.25	11.30	11.01 c
Kasugomycin	11.20	11.20	12.25	12.75	11.85 bc
Gentamycin	12.25	12.50	12.65	12.75	12.54 b
Oxytetracycline	9.90	12.00	12.25	12.50	11.66 bc
Control	0.00	0.00	0.00	0.00	0 bc

second highest value with 13.75, Oxytetracycline with 11.68 and control remained almost nil. The overall chemicals showed significant different results to each other by comparing chemicals means.

***Effect of chemical on growth inhibition at all concentration*:** Results revealed as in Fig. 3. Represents the effect of different chemicals on growth inhibition at all concentration in which inhibition zones were measured in millimeters after 6 days. The comparison between different chemicals the conducted research represents that streptomycin with 0.5% showed maximum control over pathogen with value 13.83 while tetracycline was found less effective with minimum value of 10.90. Kasugomycin with value 11.58, Gentamycin was the second highest value with 12.01, Oxytetracycline with 11.50 and control remained nil. And means were compared in overall chemicals showing non-significant different result to each other. The comparison between different chemicals the conducted research represents that the streptomycin with 1% showed maximum control over pathogen with value 14.42 while Tetracycline was found less effective with minimum value 11.01. Kasugomycin with value 11.85, Gentamycin was the second highest value with 12.54, Oxytetracycline with 11.66 and control remained nil. Overall chemicals show non-significant different results to each other's by comparing chemicals means. The comparison between different chemicals the conducted research represents that the streptomycin with 1.5% showed maximum control over pathogen with value 15.50 while Oxytetracycline was found less effective with minimum value 11.68. Kasugomycin with value 12.83, Gentamycin was the second highest value with 13.75, Oxytetracycline with 11.68 and control remained almost nil. The overall chemicals showed non-significant different results to each other by comparing chemicals means. Comparison of means of overall zones through different concentrations of chemicals resulted that Streptomycin was found more effective with highest value of 15.50 and Tetracycline was found less effective with minimum value of 10.90. Overall comparision test of chemicals reaction among various chemicals and their effect on bacterial colony growth on agar plates in vitro condition. Totally six treatment were used in order to show their efficacy in which one of them was kept as control where chemicals were not applied. All chemicals showed better antibacterial activities against the pathogen *Erwinia amylovora* on all three concenrations 0.5%,1%,1.5%. High efficacy showed on 1.5% concenration as compared to lower concentrations. Streptomycin was

Table 5. Effect of different chemicals on growth inhabitation at 1.5% concentration.

Chemicals	Inhibition Zone Diameter (mm) in Days Interval (Hrs)				Mean
	24	48	72	96	
Streptomycin	14.75	15.00	15.50	16.75	15.50 a
Tetracycline	10.85	11.70	12.25	12.95	11.94 c
Kasugomycin	12.50	12.75	12.80	13.25	12.83 bc
Gentamycin	13.20	13.85	13.95	14.00	13.75 b
Oxytetracycline	10.20	11.00	11.75	12.75	11.68 c
Control	0.00	0.00	0.00	0.00	0.00 d

excellent in making of bacterial colony growth when applied at the concentration 0.5%, 1%,1.5% respectively. Althoguh Tetracycline found with heighst 1.5% concentration value 11.94, Kasugomycine 12.83, Gentamycine 13.75 and Oxytetracycline 11.68. But there was found no inhibition zone in control that was kept without applying any chemicals.

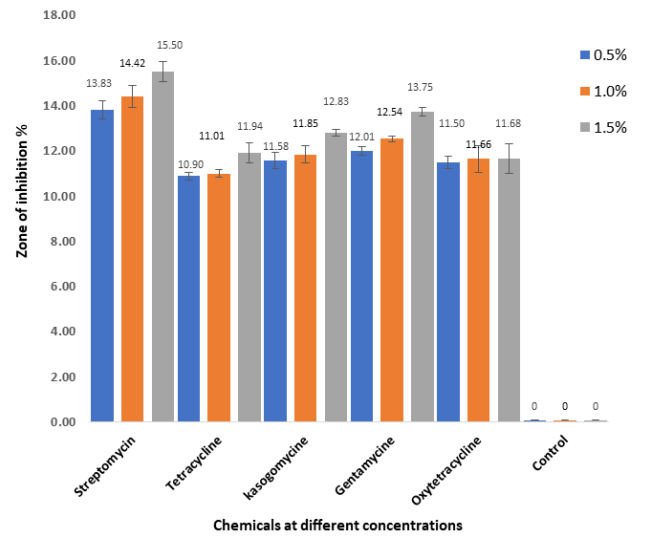

Figure 3. Chemicals with different concentrations.

DISCUSSION

Streptomycin which is an aminoglycoside is naturally producing by actinomycetes of soil that was commercialized for using in plants agriculture in United States of America as early as in 1955. Recently 90% of about the Streptomycin using in plant agriculture crops in the United States of America for controlling of bacterial fire blight pathogen. Using in minor amount of Streptomycin including controlling of bacterial pathogenic diseases in floriculture growing crops and on potato crops of potato tubers, tobacco crops on tobacco seedlings and many other vegetable crops seedlings in green house field. Recently, Streptomycin was registered for controlling fire blight pathogen in Canada, Israel, New Zealand and Mexico. It has been permitted on an emergency basis for utilization in Austeria, Switzerland and Germany subjected to annual reviewing and under strictly restricted conditions (Vidaver *et al.*, 2002). The number of antibiotics used in agriculture crops is in efficient compared with applications through human and veterinary medicines as well as in and animal production, where the United States on their own use more than 30 various drugs from minimum of 14 different classes (Robertson *et al.*, 1999). Antibiotics from herbal plants are typically formulated as powders containing of 17% to 20% of the active ingredient. About 50 to 300 ppm concentrations are used in powdered form by suspension or in dissolved form and are applied as a fine mist to susceptible aerial organs of the susceptible plants. In comparison, the relatively expensive, antibiotics are basically used on high value fruit and vegetables crops and ornamentals, where their costs are most likely to be regained. Using of antibiotics and other chemicals for controlling only fire blight has recently been revived. (Passallidas *et al.*, 2000). In 1950, the antibiotic streptomycin was used for the first time for controlling blossom blight and resulted severe infectious against the pathogen. Besides, proper use of this antibiotic proved in the progressing of Streptomycine in *Erwinia amylovora* populations for resistance. First in the United States in the west and secondly in Michigan and then in rest of the countries including New Zealand, Israel and Canada Oxytetracycline was included. Alternative materials were effective as streptomycin in blossom blight control (Loper and Smith, 1991).

***Conclusion*:** It was concluded that among all five tested chemicals (Streptomycin, tetracycline, Kasugomycin, Oxytetracycline and Gentamycin) The streptomycin was found to be effective in controlling bacterial pathogen *Erwinia amylovora* with highest value of 15.50 while tetracycline found less effective with minimum value 10.90 on the basis of present study, Streptomycin is being recommended and for future studies in-vivo commercial scale treatments of the Streptomycin are being suggested for its effective use.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

***Consent to participate*:** Niamat Ullah, Basheer ahmed and Muhammad Waris conceived the idea and prepared manuscript.

***Consent for publication*:** All authors are giving the consent to publish this research article in Phytopathogenomics and Disease Control.

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