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Evaluation of antibiotics and bactericides against the bacterial wilt of chilli through *in vitro* condition

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Abstract
Chilli (*Capsicum annuum* L.) is one of the major vegetables growing in Manipur. Bacterial wilt caused by *Ralstonia solanacearum* is responsible for the reduction of its yield production. Thirty chilli plants which show the bacterial wilt symptoms were isolated. The organism produced small milky white fluidal colonies on Sucrose Peptone Agar after incubated at 28±1 °C for 48 hours. The organism was 0.5 x 1.5 µm in size, motile, rod in shape. Evaluation of antibiotics and bactericides resulted that streptocycline gave maximum inhibition zone 25.6 mm in diameter followed by ciprofloxacin, tetracycline, cephalexin, streptomycin and bacterinashak at 100 ppm. Tetracycline showed maximum inhibition zone of 27.2 mm in diameter followed by ciprofloxacin, streptomycin, bacterinashak, cephalexin and streptocycline at 200 ppm. At 300 ppm ciprofloxacin showed maximum inhibition zone of 33.4 mm in diamter followed by tetracycline, bacterinashak, streptomycin, cephalexin and streptocycline. However, erythromycin and amoxicillin were found to be non effective against bacterial wilt pathogen. The result of the paper disc method indicated that there is no significant difference between ciprofloxacin and tetracycline at all level of concentration which indicates these two antibiotics are equally effective. But ciprofloxacin cannot be recommended for plant disease management. According to the result of turbidimetric method, all the antibiotics and bactericides were unable to inhibit the growth of bacterial wilt pathogen. However, least density of turbidity have been recorded in bacterinashak treated inoculums solution followed by ciprofloxacin treated inoculums. The result of statistical analysis indicated that there is no significance difference between ciprofloxacin and bacterinashak treatments.

Keywords: Bacterial wilt, chilli, *Ralstonia solanacearum*, antibiotics, bactericides

Introduction
Chilli (*Capsicum annum* L) is one of the cultivated vegetable crops in India. It belongs to Solanaceae family. It was originated from South America and was introduced for the first time in India from Brazil in 16th century by Portuguese. It is mainly used in culinary adding flavour, colour, vitamins and pungency. The pungency of chilli is due to the presence of capsicin and captivating red colour is due to the pigment capxanthin. India is the largest producer and exporter of chilli which is grown over an area of 775 thousand hectares with a production of 1492 metric tons (2014-15 estimates) contributing about 40% of the World`s chilli production. In India, Andhra Pradesh has been leading both in area and production of chili followed by Karnataka, West Bengal and Odisha. In Manipur, the total production of green chilli during 2016-17 is 2560 MT.

Bacterial wilt of chilli which is caused by *Ralstonia solanacearum* is most lethal disease. It causes severe economic loss to the farmers. Bacterial wilt disease attacks over 450 plant species (Daushtrey *et al.*, 1995) [5]. Several researchers reported the occurrence of the devastating disease from west Bengal (Das and Chattopadhyay, 1955; Mukherjee and Chattorpadhyay, 1955; Chattopadhyay and Mukerjee, 1968; Sharma and Mukherjee, 1970; Chaudhuri and Khatua, 1982; Chatterjeet *et al.*, 1997 Samaddar *et al.*, 1998, Mondal, 2003; Mondal *et al.*, 2004) [4, 16, 1, 12, 3, 2, 14, 10, 11]. In Kerala, the yield loss due to the bacterial wilt incidence range from 20 per cent depending upon the varieties (Sadhankumar 1995) [13]. In Himachal Pradesh, bacterial wilt has now assumed serious concern in different areas of Kangra, Kullu, Hamirpur and Mandi districts. The target control measure has been limited due to its broad distribution, vascular nature, wide host range, great variability and ability to survive in soil and water (Haywads, 1991) [9].

According to Sood and Singh (1993) [17], bacterial wilt is one of devastating bacterial diseases affecting vascular bundles of plants and sometimes it may result in complete failure of the crop. Thus, the management of chilli against bacterial wilt caused by *Ralstonia solanacearum* is an important in order to maximize the production of the crop yield and hence, the present study was carried out for first time in Manipur to isolate, purified and evaluate some antibiotics and bactericides against the bacterial wilt of chilli through *in vitro* condition.

Materials and Methods

The present investigations were undertaken in the Department of Plant Pathology, School of Agriculture, Pandit Deen Dayal Upadhyay institute of Agricultural Sciences, Utlou, Bishnupur during 2017-19. The experimental approaches and procedures adopted during the course of investigations are given below:

Collection and isolation of bacterial wilt of chilli:

The chilli plants showing the typical symptoms of bacterial wilt were collected from different chilli growing areas in Bishnupur district, Manipur.

Table 1: Disease scale (0-5) developed by Winstead and Kelman, 1952 [18] to record the degree of resistance against bacterial wilt has been followed.

Disease scale	Expanded value	Disease incidence
0	Highly resistance(HR)	0%
1	Resistance (R)	1-20%
2	Moderately resistance(MR)	21-40%
3	Moderately susceptible(MS)	41-60%
4	Susceptible(S)	61-80%
5	Highly susceptible(HS)	>80%

Disease Incidence % = No. of wilted plants / Total no. of plants × 100

(a) Selection from infected stem: Partially infected stem segments were cut into small pieces of 10 cm, surface sterilized with 70% ethanol for 1 min. and rinsed in 2 or 3 changes sterile distilled water in test tube.

(b) Bacterial ooze: The disinfected tissue was cut into tiny pieces with sterile scissors from the boundary between healthy and diseased tissue and placed in 2 or 3 drops of sterile physiological water in a clean, sterile glass-slide and allow the bacteria in these tissues to ooze out for 5 min and examined under the stereolight microscope.

(c) Preparation of inoculums for isolation: After confirming the presence of bacterial ooze, disease infected stem was cut into small pieces with the help of sterile razor blade and suspended in 3 ml of sterile physiological water in test tube. The cut pieces of infected stem diffused bacterial ooze cells at room temperature for at least 60 minutes.

(d) Streaking on SPA plates: A loopful bacterial suspension has been taken from the test tube and streaked onto SPA plates.

(e) Incubation: The streaked plates were kept upside down at 28±1^{0}C for 48h.

After streaking, observation was made daily to check whether saprophytic bacteria appeared after 24hr. Small pin head size single milky white colonies appeared after 48hr of incubation which were selected for purification.

Purification and maintenance of *Ralstonia solanacearum* pathogen

The growth on the isolation plates exhibited a mixture of different colony types. The different colony types were picked up and restreaked in three right angle direction onto SPA plates. The plates were then incubated again for 96 hours at 28±1 °C. Milky white and fluidal colonies from purified plates were selected, touched with the wire loop and transferred aseptically in slants of SPA by making a single vertical streak on the agar surface in order to allow the spread horizontally and evenly. The slants were incubated at 28±1 °C for 72 hours and stored at 4 °C.

Hayward`s medium or SPA was used during the process of isolation, purification and maintenance of bacterial wilt pathogen because it is suitable for isolation and for determination of distinctive colony formation of *Ralstonia solanacearum* pathogen (Hayward,1960) [8].

Composition of Sucrose Peptone Agar (SPA)\ Hawyard`s Medium

Sucrose - 20g
Peptone - 5g
K_2HPO_4 - 0.5g
$MgSO_4.7H_2O$ - 0.25g
Agar - 15g
Water (distilled) - 1 L
pH - 7.2 – 7.4

Pathogenicity Tests

For conducting the pathogenicity test, healthy chilli seedlings were used. The test was conducted under pot culture conditions in the department of plant pathology. For the experiment, the artificial root inoculation method was followed (Elphinstone, 2005) [6]. The bacterial suspension was prepared with sterile water. The concentration of the suspension was adjusted to 10^6 cfu/ml which read its turbidity as O.D= 0.17 at 520 nm. The roots of seedlings were cut and dipped in the bacterial suspension for 10-15 minutes. Then, the treated seedlings were planted in sterile soil taken in pots. A set of untreated plants served as control. The inoculated plants were covered with perforated polythene bags to maintain high humidity inside for 24hrs. Observations on incidence of bacterial wilt were recorded.

Evaluation of antibiotics and bactericides against bacterial wilt pathogen in *in vitro*

Eight antibiotics and bactericides of 100, 200 and 300 mg namely, streptocycline, bacterinashak, streptomycin, ciprofloxacin, amoxicillin, cephalexin, erythromycin and tetracycline were dissolved first in small quantity of 60% ethanol and diluted with sterile distilled water to get the required concentration of 100, 200 and 300 ppm solutions. These concentration were evaluated for their efficacy in *in vitro* against bacterial wilt pathogen in two different method.

1. Paper disc method: Paper disc of 8 mm in diameter were dipped separatedly in 100, 200 and 300 ppm solutions of the different antibiotic solutions. After removing the excess antibiotics the discs were placed on the surface of solidified SPA medium plates which contained bacterial pathogen. Paper disc immersed in sterile distilled water were placed on the surface of SPA borne bacterial wilt pathogen as a control. Three paper discs each for each concentration of antibiotics and for sterile distilled water were placed in a petridish. The experiment was replicated

three times. These plates were incubated at 28±1^{0}C. The diameter of inhibition zone around each disc were measured after 48 hours of incubation.

2. Turbidimetric method: Inoculum suspension was prepared in sterile distilled water from 48 hours old culture of bacterial wilt pathogen growth on SPA medium and inoculum suspension was adjusted to standard turbidity (optical density = 0.17 at 520 nm) containing 10^6 cfu/ml. Different concentrations i.e. 100, 200 and 300 ppm of eight antibiotics and bactericides namely, streptocycline, bacterinashak, streptomycin, ciprofloxacin, amoxicillin, cephalexin, erythromycin and tetracycline were used. 1 ml solution of each test chemical of different concentrations was aseptically transferred to the test tubes containing 10 ml of sterilized Hayward`s broth medium. Then, the tubes were inoculated by adding a loopful bacterial wilt pathogen suspension (O.D = 0.17 at 520 nm) to each tube under aseptic condition. The antibiotic free tubes containing the bacterial wilt inoculum were kept as control. Each treatment were replicated three times and the culture tubes were then incubated at 28±1^{0}C for 48 hours. Observations were recorded in terms of optical density with the help of spectrophotometer.

Results

Pathogenicity

The organism produced disease symptoms on king chilli when inoculated with 10^6 cfu/ml by dipping the root zone in inoculums for 20 min. Re-isolations made from infected leaves consistently yielded a bacterium.

In vitro evaluation of bactericides and antibiotics against the bacterial wilt pathogen

a) Paper disc method: Result of the paper disc method showed (table 2) that among the antibiotics and bactericides evaluation, streptocycline gave maximum inhibition zone of 25.6 mm in diameter followed by ciprofloxacin (25.4 mm), tetracycline (25.2 mm), cephalexin (18 mm), streptomycin (17.6 mm) and bacterinashak (16 mm) at 100 ppm. Tetracycline showed maximum inhibition zone of 27.2 mm in diameter followed by ciprofloxacin (26.4 mm), streptomycin (22.4 mm), bacterinashak (20.4 mm), cephalexin (14.2 mm) and streptocycline (14 mm) at 200 ppm. At 300 ppm ciprofloxacin showed maximum inhibition zone of 33.4 mm in diamter followed by tetracycline (30 mm), bacterinashak (24.2 mm), streptomycin (19.4 mm), cephalexin (14.2 mm) and streptocycline(13.8 mm). However, erythromycin and amoxicillin were found to be non effective against bacterial wilt pathogen.

b) Turbidimetric method: According to the result of turbidimetric method (table 3), all the antibiotics and bactericides were unable to inhibit the growth of bacterial wilt pathogen (table 3). The least OD (0.073) was recorded on inoculum dispersed in 100 ppm, 200 ppm and 300 ppm solution of ciprofloxacin. However, bacterinashak at 200 ppm and 300 ppm, showed minimum OD of 0.066 and 0.075 respectively. Tetracycline at 200 ppm and 300 ppm showed less OD as compared to other treatments.

Table 2: Inhibition of growth of *Ralstonia solanacearum* with different antibiotics on SPA medium *in vitro* Inhibition zone (mm) at different concentration #

Antibiotics	100(ppm)	200(ppm)	300(ppm)	C.D.
Bacterinashak	16	20.4	24.2	
Tetracycline	25.2	27.2	30	
Cephalexin	18	14.2	14.2	4.6075
Streptomycin	17.6	22.4	19.4	
Streptocycline	25.6	14	13.8	
Ciprofloxacin	25.4	26.4	33.4	

Mean of three replications

Table 3: *In vitro* evaluation of antibiotics and bactericides against *Ralstonia solanacearum* by turbidimetric method. Difference in OD in 500 nm between inoculated and uninoculated control.

Antibiotics	100(ppm)	200(ppm)	300(ppm)	C.D.
Bacterinashak	0.230	0.066	0.075	
Tetracycline	0.213	0.148	0.179	
Cephalexin	0.581	0.697	0.697	0.0758
Streptomycin	0.520	0.223	0.381	
Streptocycline	0.170	0.304	0.254	
Ciprofloxacin	0.073	0.145	0.104	

Note: Higher numerical value indicates more growth of organism. Data are average of 3 replication.

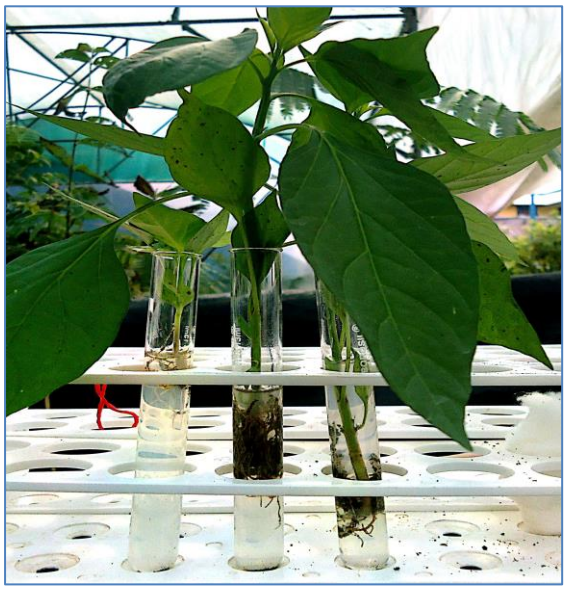
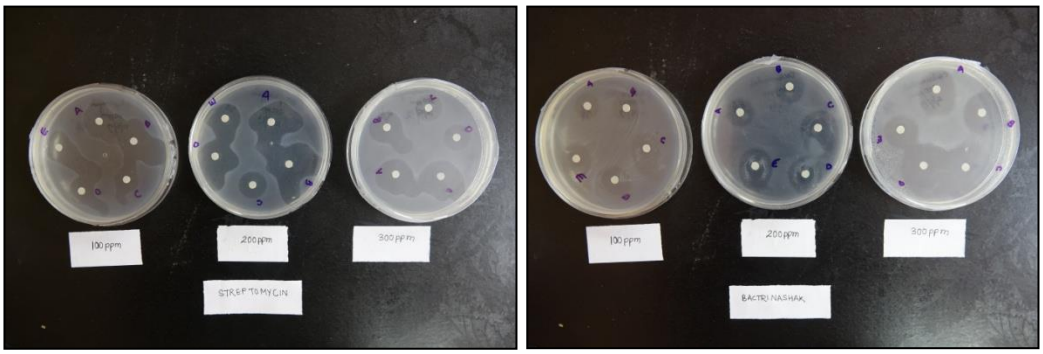


Fig 1: Root dipping inoculation



A. B

Fig 2: Inhibition zone formed by using paper disc method (A. Streptomycin and B. Bacterinashak)

Discussion and Conclusion

Chilli (*Capsicum annum L*) is one of the cultivated vegetable crops in India. It is grown over an area of 775 thousand hectares with a production of 1492 metric tons (2014-15 estimates) contributing about 40% of the World`s chilli production. Yabuuchi *et al.* (1995) [19] found *Ralstonia solanacearum* causing bacterial wilt of chilli is formerly known as *Pseudomonas solanacearum* (EF Smith). It is one of the most important soil borne disease which causes severe economic loss to the farmers. In this study, several antibiotics and bactericides have been evaluated for their effectiveness in controlling the disease *in vitro.* Streptocycline gave maximum inhibitory effect at 100 ppm, tetracycline showed most effective against bacterial wilt at 200 ppm and at 300 ppm ciprofloxacin showed maximum inhibition zone followed by tetracycline. However, ciprofloxacin cannot be used in plant disease management. The result of statistical analysis indicated that there is no significance difference between ciprofloxacin and bacterinashak treatments. Sangayomi *et al.* (2011) [15] showed inhibitory effects of Ciprofloxacin, Ofloxacin, Pefloxacin, Drovid, Cotrimozazole, Norfloxacin and Clindomycin against *Ralstonia solanacearum*. Subsequently Gupta and Razdan (2013) [7] reported that among the antibiotics tested against *Ralstonia solanacearum*, Plantomycin was found to be very effective against the test pathogen. Hence, this investigation revealed that streptomycin and/or bacterinashak may be used to manage the bacterial wilt of chilli caused by *Ralstonia solanacearum*.

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