

Original Research Article

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***In-vitro* and *In-vivo* Efficacy of Antibacterial Compounds against *Xanthomonas oryzae* pv. *oryzae*, A Cause of Bacterial Leaf Blight of Rice**

Durga Prasad^{1*}, Ramji Singh² and Sonika Deep³

¹Department of Plant Pathology, Banda University of Agriculture and Technology,
Banda-210001, UP, India

²Department of Plant Pathology, S.V.P. University of Agriculture and Technology,
Meerut-250110, UP, India

³Department of Plant Pathology, Bihar Agril College, Bihar Agril University, Sabour-813210,
Bihar, India

**Corresponding author*

ABSTRACT

Present study deals with laboratory and field efficacy of six antibiotics and three fungi toxicants against *Xanthomonas oryzae* pv. *oryzae* (Xoo) to evolve a package of practices for efficient and reliable management of BLB. Among the antibiotics, Streptomycin @ 0.05% found most effective against Xoo with inhibition zone of 27.00 mm which was at par with 26.83 mm zone of inhibition exhibited by Streptomycin @ 0.03%. Streptocycline @ 0.05% was found to be next effective in inhibiting the growth of Xoo and it resulted in 24.25 mm zone of inhibition. In combination of antibiotics + fungi toxicants; maximum inhibition zone (24.42 mm) against Xoo was exhibited by Streptocycline @ 0.3% + Carbendazim @ 0.15% and it was at par with 24.25 mm and 23.67 mm zones of inhibition exhibited by Streptomycin Sulphate @ 0.03% + Copper hydroxide @ 0.25% and Streptocycline @ 0.03% + Copper hydroxide @ 0.25%, respectively. The antibiotics and fungitoxicants found highly effective against Xoo *in-vitro* were further evaluated against BLB under artificial epiphytotic conditions in field. Combination of streptomycin sulphate @ 0.03% + copper hydroxide @ 0.25% was found most effective with least BLB severity (57.45%), highest yield (36.53 q/ha) and 1000 grains weight (28.36g). Streptocycline @ 0.03% + copper hydroxide @ 0.25% was found next effective combination against BLB with 59.26% severity, 35.24 q/ha yield and 27.43g 1000 grain weight. These combinations were significantly superior to the respective similar parameters observed in recommended control measure i.e. spray of combination of Copper Oxy-chloride @ 0.25% + Streptocycline @ 0.01%.

Keywords

Antibacterial compounds,
Antibiotics, Bacterial leaf
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Introduction

India has the largest area (44.11 m ha) under rice with 105.48 mt total production and 2.40 tons per ha of average productivity in 2014-15

(Anon, 2016). The total grain demand by 2025 and 2050 has been projected to be around 291 and 377 million tonnes, respectively against 201 m tonnes in 2000 (Khatkar *et al.*, 2016). To meet the growing food needs of increasing

population in the country, there is a need to raise rice production and productivity. Efforts for enhancing the productivity are limited by a number of biotic and abiotic stresses like drought flooding, diseases and insect pests. In India, annual crop losses are estimated about Rs. 6000-7000 crores, losses incurred due to diseases are 26 per cent, weeds 23 per cent, insects 20 per cent and rest by birds and rodents (Raju, 2000). Bacterial leaf blight (BLB) is one of the most serious diseases of rice (Ou, 1985) and it is one of the oldest recorded rice diseases. BLB caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the most destructive diseases of rice in Asia (Mew *et al.*, 1993). Since the introduction and cultivation of new, high-yielding but susceptible rice varieties over a large acreage in recent years, the disease has become one of the most serious problems of rice cultivation in India (Srivastava *et al.*, 1967). BLB caused by *Xanthomonas oryzae* pv. *oryzae* has been ranked in the top-10 list of bacterial plant pathogens (Mansfield *et al.*, 2012). In India, the yield loss due to this disease is up to 81.3% (Srivastava, 1967; Sonti, 1998; Gnanamanickam *et al.*, 1999; Veena *et al.*, 2000). Some of the earlier studies have identified some chemicals and antibiotics with relative field efficacy against the BLB. However, earlier recommended and effective control measures have now become less efficacious probably due to changing ecosystem and pathogen virulence (Reissig *et al.*, 1986). At present in fact, no efficient and reliable control measure is available for BLB of rice so far (Mary *et al.*, 2001b and Singh, 2009). Rice varieties resistant to BLB are not covering large area in India and host resistance is also not stable due to variability and emergence of new virulence in pathogen. Keeping in view the above mentioned status and facts about management of bacterial leaf blight of rice and taking into account the magnitude of disease and its unbearable losses, the current study was performed with

the firm attitude to evaluate the effectiveness of most efficient antibiotic and fungi toxicants either single or in combination against BLB in rice.

Materials and Methods

Collection of BLB samples

The experiments were conducted in laboratory of Department of Plant Pathology and at Agriculture Experimental Farm of Mandan Bharti Agriculture College (MBAC), Agwanpur, Saharsa (Bihar) during *Kharif* 2014 and 2015. The diseased leaves of rice cv. Rajendra Sweta showing typical bacterial blight (BB) symptoms were collected in brown paper bags from Agriculture Experimental Farm, MBAC, Saharsa and brought to the Laboratory for further processing.

Isolation and pathogenicity test

Isolation of the bacterium *Xanthomonas oryzae* pv *oryzae* was carried out using infected leaves of rice plant collected from Agriculture Experimental Farm of MBAC, Saharsa. The sample showing typical leaf blight and bacterial oozing from the cut section during microscopy were used for isolation of bacterium. The diseased portion with healthy tissues was cut into 0.5 to 1 cm pieces. These diseased pieces were disinfected in 1% sodium hypochlorite solution for 30 seconds, followed by three subsequent washing with sterilized distilled water in aseptic condition to remove the traces of NaOCl. The diseased bits were then suspended in a test tube containing 3 ml of sterilized distilled water and squeezed gently with sterilized scalpel. When the water became slightly turbid due to oozing of bacterial cells, the suspension was serially diluted upto 10^3 dilutions in 9 ml sterile water blanks. This suspension was streaked on

nutrient agar (NA) medium with the help of sterilized wire loop. The inoculated plates were incubated at room temperature ($27\pm 2^{\circ}\text{C}$) for 48 hrs. After the incubation period, observations were made for the development of well separated, typical, light yellow coloured bacterial colonies resembling Xoo. The typical colony of Xoo was sub-cultured on NA plates to get pure culture. Cultures on NA slants were preserved for longer duration at 4°C . The isolated Xoo proved pathogenic to rice (TN-1) using Koch's postulate, which confirmed that the culture isolated was of *Xanthomonas oryzae* pv *oryzae*.

***In-vitro* evaluation of antibiotics alone and combination of antibiotics + fungi toxicants against Xoo**

In the current study, six commonly available antibiotics viz., Streptocycline, Streptomycin, Streptomycin Sulphate, Plantomycin, Tetracycline Hydrochloride and Oxytetracycline Hydrochloride were evaluated for their efficacy against the growth of Xoo. All these antibiotics were evaluated at three different concentrations i.e. 0.01, 0.03 and @ 0.05% except Plantomycin which was tested @ 0.1, 0.2 and 0.3% following inhibition zone assay method. In another experiment, single concentration @ 0.03% of these six antibiotics except Plantomycin which was tested @ 0.2% were also evaluated in combination of three fungi toxicants i.e. Copper Oxy-chloride 50% WP (Blitox 50% WP) @ 0.25%, Copper hydroxide (Kocide 2000 53.8 DF) @ 0.25% and Carbendazim 50% WP (Bavistin 50% WP) @ 0.15%; separately for their efficacy against growth of Xoo following inhibition zone assay method.

The bacterium was multiplied by inoculating the bacterial culture into the Erleyenmayer's flask containing 20 ml of nutrient broth. The inoculated flasks were incubated at 30°C for 72 hours. The bacterial suspension

(10^8 cell/ml) was then seeded to the lukewarm nutrient agar medium (1000 ml). The seeded medium was poured into the sterilized petriplates and plates were allowed to solidify. Solution of test antibiotics either alone or in combination with fungi toxicants at different concentrations (Table 1) were prepared separately. The filter paper discs (Whatman No. 42) measuring 5 mm in diameter were soaked in the respective chemical solutions of different concentration for 5 minutes and transferred to the surface of the medium seeded with bacterial culture in petriplates. Six replications of each treatment were maintained. The inoculated plates were kept in the refrigerator at 5°C for 4 hours to allow the diffusion of chemical into the medium. Then plates were incubated at $28\pm 1^{\circ}\text{C}$ for 72 hours. At the end of incubation period these plates were observed for the production of inhibition zone around the filter paper discs. Inhibition zone in each plate was measured in terms of diameter (mm) and the results analysed statistically.

Field evaluation of antibiotics and combination of antibiotics + fungi toxicants against Bacterial Leaf Blight

The antibiotics alone or their combination with fungi toxicants which inhibited the growth of Xoo significantly were screened for their efficacy to check the severity of bacterial blight in rice under artificial epiphytotic conditions in field. The experiments were conducted in *Kharif* 2014 and 2015 at MBAC, Saharsa. The trials were laid out in Randomized Block Design (RBD) with three replications using TN-1 as a test variety. The TN-1 plants were inoculated at maximum tillering stage. For inoculation, the tip of 4-5 leaves/plant were clipped with scissor dipped in the bacterium suspension (10^8 cells /ml) followed by dipping the cut ends of leaf in the same suspension. Thereafter, 4-5 cm water level was maintained in the field for two

weeks to create high humidity (85-90%) which favours disease development. After inoculation, the antibiotics alone or their combination with fungi toxicants were sprayed twice on TN-1 plants. First spray was done at 1st appearance of BLB symptoms and 2nd after two weeks of first inoculation. Post-inoculation BLB severity was measured up to one month period using 0-9 SES scale (IRRI, 1996) and accordingly data were recorded as percent severity. Crop yield and 1000 grain weight were also recorded after harvesting and data were analyzed statistically (Sukhatme and Amble, 1985).

Results and Discussion

***In-vitro* effect of antibiotics on growth of Xoo**

Various antibiotics (Table 1 and Plate 1) with three levels of concentrations were evaluated against Xoo *In-vitro*. Inhibition zones for the growth of bacteria were appeared to be very clear around the paper disks. Streptomycin @ 0.05% was found to be the most effective antibiotic against Xoo as it potentially inhibited (27.00mm) the growth of Xoo. It was significantly superior to rest treatments including control but at par with inhibition zone (26.83mm) exhibited by Streptomycin @ 0.03%.

Streptocycline @ 0.05% was found to be the next effective antibiotic with 24.25mm inhibition zone against Xoo. Plantomycin was the least effective antibiotic against Xoo *in vitro*. The findings are quite in conformity with the previous report that best inhibition of virulent isolate of Xoo exhibited by Streptomycin followed by Kanamycin, Ampicillin, Sinobionic, Benzylpenicillin and Chloramphenicol. Sensitivity of Xoo isolates against antibiotics was progressively increased with increase in the concentration of antibiotics (Khan *et al.*, 2012; Ashrafuzzaman,

1987). According to Mahto *et al.*, (1988), Streptocycline (1000ppm) exhibited the widest zone of inhibition (27.83 mm) against Xoo after 72 hrs of incubation. It inhibited Xoo growth at all three concentrations (10, 100 and 1000ppm).

***In-vitro* effect of combination of antibiotics and fungi toxicants on growth of Xoo**

The screening of antibiotics and fungi toxicants *in vitro* provides preliminary information in short time about the efficacy of fungicides and antibiotics against the pathogens in standing crop in field. It is economical and time saving in planning of chemical control of plant disease in the field. In present investigation, the combination of six antibiotics and three fungi toxicants were assessed against Xoo (Table 2 and Plate 2).

Single concentration of each antibiotic was assessed in combination with three different concentrations of fungi toxicants for their efficacy against Xoo. Among the combinations of antibiotics and fungi toxicants, combination of Streptocycline @ 0.03% + Carbendazim @ 0.15% was found most effective with zone (24.42 mm) of inhibition against Xoo.

It was at par with zones of inhibition exhibited with combination of Streptomycin Sulphate @ 0.03% + Copper hydroxide @ 0.25%/ Tetracycline Hydrochloride @ 0.03% + Copper Oxochloride @ 0.25%/ Streptocycline @ 0.03% + Copper hydroxide @ 0.25%/ Oxytetracycline Hydrochloride @ 0.03% + Copper Oxochloride @ 0.25%; and significantly superior to zones of inhibition recorded with others combination of antibiotics and fungi toxicants including control. The least effective combination was Plantomycin @ 0.2% + Carbendazim @ 0.15% which showed 12.75mm zone of inhibition against Xoo.

Table.1 <i>In vitro</i> evaluation of antibiotics against <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	
Antibiotics	Inhibition zone (mm)
Streptocycline @ 0.01%	19.00 (4.41)*
Streptocycline @ 0.03%	23.67 (4.91)
Streptocycline @ 0.05%	24.25 (4.97)
Streptomycin @ 0.01%	22.08 (4.75)
Streptomycin @ 0.03%	26.83(5.23)
Streptomycin @ 0.05%	27.00(5.24)
Streptomycin Sulphate @ 0.01%	21.67(4.71)
Streptomycin Sulphate @ 0.03%	21.75(4.72)
Streptomycin S Sulphate @ 0.05%	23.33(4.88)
Plantomycin @ 0.1%	12.33(3.58)
Plantomycin @ 0.2%	13.25(3.71)
Plantomycin @ 0.3%	15.58(4.01)
Tetracycline Hydrochloride @ 0.01%	16.75(4.15)
Tetracycline Hydrochloride @ 0.03%	21.33(4.67)
Tetracycline Hydrochloride @ 0.05%	21.42(4.68)
Oxytetracycline Hydrochloride @ 0.01%	17.25(4.21)
Oxytetracycline Hydrochloride @ 0.03%	23.00(4.85)
Oxytetracycline Hydrochloride @ 0.05%	23.83(4.93)
Control (Untreated)	0.00(0.71)
CD ($P_{0.05}$)	0.13

*Figures in Parenthesis are square root transformation value. Each value is the mean of six replications

Table.2 <i>In vitro</i> evaluation of antibiotics + fungi toxicants against <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	
Antibiotics	Inhibition zone (mm)
Streptocycline @ 0.03% + Copper Oxy-chloride @ 0.25%	19.25 (4.44)*
Streptocycline @ 0.03% + Copper Hydroxide @ 0.25%	23.67 (4.91)
Streptocycline @ 0.03% + Carbendazim @ 0.15%	24.42 (4.99)
Streptomycin @ 0.03% + Copper Oxy-chloride @ 0.25%	19.67 (4.49)
Streptomycin @ 0.03% + Copper Hydroxide @ 0.25%	21.42 (4.68)
Streptomycin @ 0.03% + Carbendazim @ 0.15%	21.33 (4.67)
Streptomycin Sulphate @ 0.03% + Copper Oxy-chloride @ 0.25%	20.58 (4.59)
Streptomycin Sulphate @ 0.03% + Copper Hydroxide @ 0.25%	24.25 4.97)
Streptomycin Sulphate @ 0.03% + Carbendazim @ 0.15%	22.50 (4.80)
Plantomycin @ 0.2% + Copper Oxy-chloride @ 0.25%	14.00 (3.81)
Plantomycin @ 0.2% + Copper Hydroxide @ 0.25%	14.17 (3.83)
Plantomycin @ 0.2% + Carbendazim @ 0.15%	12.75 (3.64)
Tetracycline Hydrochloride @ 0.03% + Copper Oxy-chloride @ 0.25%	24.25 (4.97)
Tetracycline Hydrochloride @ 0.03% + Copper Hydroxide @ 0.25%	13.75 (3.77)
Tetracycline Hydrochloride @ 0.03% + Carbendazim @ 0.15%	19.42 (4.46)
Oxytetracycline Hydrochloride @ 0.03% + Copper Oxy-chloride @ 0.25%	23.33 (4.88)
Oxytetracycline Hydrochloride @ 0.03% + Copper Hydroxide @ 0.25%	15.75 (4.03)
Oxytetracycline Hydrochloride @ 0.03% + Carbendazim @ 0.15%	19.75 (4.50)
Treated control: Streptocycline (@ 0.01% + Copper Oxy-chloride @ 0.25%	14.67 (3.89)
Control (Untreated)	0.00 (0.71)
CD ($P_{0.05}$)	0.14

*Figures in Parenthesis are square root transformation value. Each value is the mean of six replications

Table.3 *In-vivo* evaluation of antibiotics and combination of antibiotics + fungi toxicants on bacterial leaf blight of rice

Treatments	Average disease severity (%)				Yield q/ha				1000 grain wt (g)		
	2014	2015	Pooled	% reduction over control [†]	2014	2015	Pooled	% increase over control [†]	2014	2015	Pooled
Streptocycline @ 0.03%	91.11 (75.90)*	88.14 (70.78)	89.63 (73.34)	10.37	29.62	29.53	29.58	3.89	26.29	26.10	26.19
Streptocycline @ 0.05%	83.7 (66.53)	79.26 (63.70)	81.48 (65.12)	18.52	31.42	31.56	31.49	9.72	26.78	26.89	26.84
Streptomycin @ 0.03%	82.22 (65.06)	76.30 (61.97)	79.26 (63.51)	20.74	31.13	31.04	31.09	8.56	26.69	26.64	26.67
Streptomycin @ 0.05%	77.78 (62.07)	73.33 (59.09)	75.56 (60.58)	24.44	31.61	31.52	31.56	9.92	26.72	26.78	26.75
Streptomycin Sulphate @ 0.03%	94.07 (78.80)	90.30 (71.88)	92.19 (75.34)	7.81	29.47	29.50	29.49	3.59	26.33	26.28	26.30
Streptomycin Sulphate @ 0.05%	88.15 (70.79)	79.93 (64.58)	84.04 (67.69)	15.96	30.75	30.42	30.58	7.03	26.58	26.65	26.62
Streptocycline @ 0.03% + Carbendazim @ 0.15%	68.89 (56.13)	67.41 (55.20)	68.15 (55.67)	31.85	33.00	33.28	33.14	14.21	27.05	26.97	27.01
Streptomycin Sulphate @ 0.03% + Copper Hydroxide @ 0.25%	60.00 (50.78)	54.89 (47.84)	57.45 (49.31)	42.55	36.12	36.95	36.53	22.17	28.33	28.39	28.36
Streptocycline STL 0.03% + Copper Hydroxide @ 0.25%	61.48 (51.80)	57.04 (49.13)	59.26 (50.46)	40.74	35.14	35.33	35.24	19.32	27.39	27.46	27.43
Streptomycin Sulphate @ 0.03% + Carbendazim @ 0.15%	73.33 (59.12)	71.85 (58.25)	72.59 (58.68)	27.41	32.64	32.44	32.54	12.63	27.00	26.95	26.97
Streptomycin @ 0.3% + Copper Hydroxide @ 0.25%	61.48 (51.69)	62.96 (52.56)	62.22 (52.12)	37.78	35.12	35.37	35.25	19.35	27.29	27.41	27.35
Streptomycin @ 0.3% + Carbendazim @ 0.15%	64.44 (53.42)	62.96 (52.55)	63.70 (52.98)	36.30	33.82	34.06	33.94	16.23	27.12	27.19	27.16
Streptomycin @ 0.3% + Copper Oxy-chloride @ 0.25%	68.89 (56.13)	64.44 (53.42)	66.67 (54.78)	33.33	33.19	33.24	33.22	14.42	27.08	27.03	27.05
Treated control (Streptocycline @ 0.01% + Copper Oxy-chloride @ 0.25%)	86.67 (69.43)	85.19 (68.44)	85.93 (68.94)	14.07	31.73	31.23	31.48	9.69	26.84	26.69	26.77
Control (Untreated) [†]	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	0.00	28.64	28.22	28.43	0.00	26.00	25.75	25.87
CD (P _{0.05})	10.95	11.04	1.8	—	4.38	4.43	0.47	—	0.40	0.44	0.16

*Figures given in parentheses are angular transformed values

Plate.1 Higher and lowest zones of inhibition against Xoo due to different concentration of antibiotics

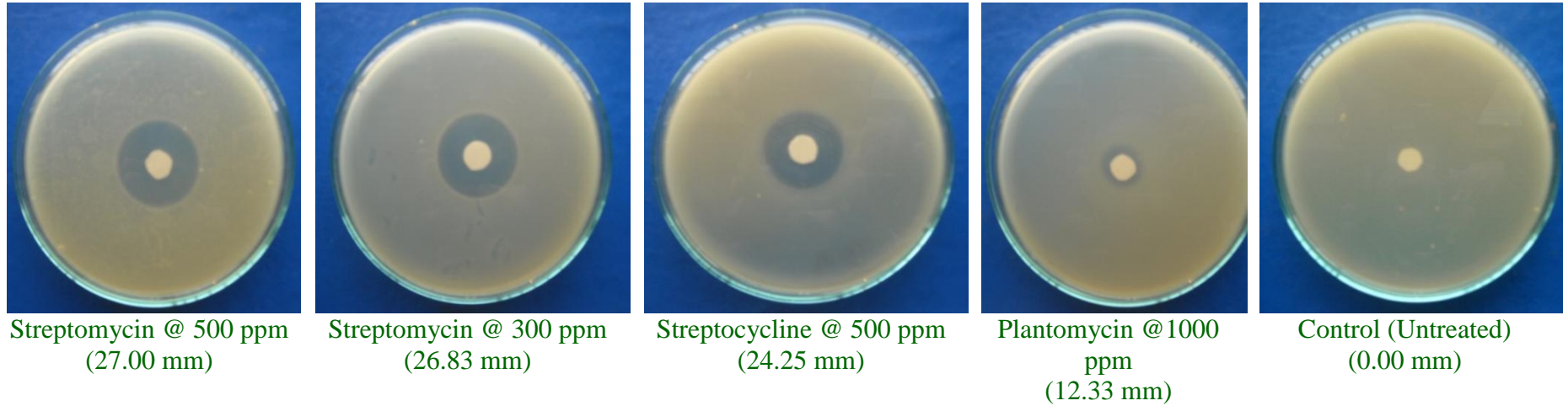
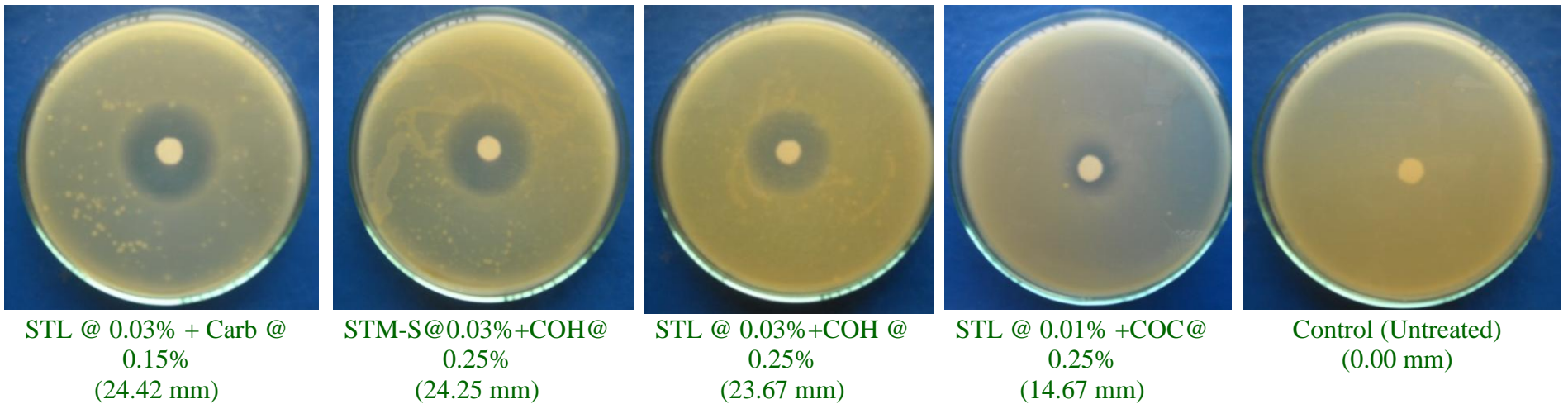


Plate.2 Zones of inhibition against Xoo due to different combination of antibiotics + fungi toxicants



Abbreviates: STL (Streptocycline), Carb (Carbendazim), STM-S (Streptomycin Sulphate), COH (Copper Hydroxide), COC (Copper Oxy-chloride)

The present findings are also in agreement with earlier reports about good antibacterial property of Streptocycline (Mahto *et al.*, 1988; Chauhan, 1980), Carbendazim 50% WP (Swati *et al.*, 2015), Streptomycin Sulphate (Thimmegowda *et al.*, 2012 and Naqvi *et al.*, 2014), Copper hydroxide (Parthasarathy *et al.*, 2014), Tetracycline Hydrochloride (Balaraman and Rajagopalan, 1978) and Copper Oxychloride 50% WP (Patel, 2008 and Khan *et al.*, 2005) against *Xoo*. Tandan and Chaliganjewar (2016) reported that among the chemicals, copper oxychloride @ 0.25% + streptomycin sulphate (200ppm) was found most effective with highest per cent inhibition against *Xoo* as compared to other chemicals.

Field evaluation of antibiotics and combination of antibiotics + fungi toxicants against Bacterial Leaf Blight

The antibiotics alone or their combination with fungi toxicants exhibited higher zones of inhibition against *Xoo in-vitro* were assessed against BLB under artificial epiphytotic conditions (Table 3). The antibiotics/ fungi toxicants sprayed twice at first appearance of BLB and after 14 days of 1st spray. In field experiment, the pooled data of *Kharif* 2014 and 2015 showed that combination of Streptomycin Sulphate @ 0.03% + Copper hydroxide @ 0.25% was found to be the best with least BLB severity (57.45%), highest yield (36.53 q/ha) and 1000 grains weight (28.36g). It was at par with Streptocycline @ 0.03% + Copper hydroxide @ 0.25% with respect to BLB severity (59.26%) but significantly superior with respect to yield (35.24 q/ha) and 1000 grain weight (27.43g). Streptomycin @ 0.03% + Copper hydroxide @ 0.25% was appeared the next effective combination with BLB severity (62.22%), yield (35.25 q/ha) and 1000 grain weight (27.35g). They were significantly superior to rest treatments including treated control

(recommended control measure) in which crop was sprayed twice with combination of Copper Oxy-chloride @ 0.25% + Streptocycline @ 0.01%. In field experiment, it was found that in general, combination of antibiotics and fungi toxicants performed better than application of antibiotic alone with respect to reduced BLB severity and increased yield/ 1000 grain weight. The treatment i.e. twice spray of combination of Copper Oxy-chloride @ 0.25% + Streptocycline @ 0.01% which is recommended since >30 years for control to BLB, did not perform as good, *in-vitro* and in field conditions. These findings were in accordance with the previous reports of Solanky (1983) and Sinha (2000). Kocide 2000 54 DF (2.5 g/l) was found significantly effective in controlling the BLB severity and increasing the grain yield at all the test doses over control. Kocide 3000 at 2.5 g/l and 3.0 g/l dosage rates were found suitable across the test locations (All India Co-ordinated Rice Pathology Trial) for effective check of the BLB control and increasing the grain yield (Anon., 2007). The farmers growing bacterial blight susceptible genotypes should spray, streptomycin sulphate + copper hydroxide (Kocide 2000 54DF) (50 g/20 liter) to manage bacterial blight and to harvest increased yield and income/hectare in endemic area of bacterial blight (Anon., 2008). The similar results were obtained by Chauhan and Vaishnav (1980). They reported the effectiveness of streptomycin sulphate and copper count against *Xoo*.

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