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In-vitro evaluation of bioagents and antibiotics against *Ralstonia solanacearum* causing brinjal wilt

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Abstract
Bacterial wilt of brinjal and other solanaceous vegetables caused by *Ralstonia solanacearum* is the most destructive disease. The bacterial wilt disease is wide spread, affecting many solanaceous vegetable crops in India, especially in Karnataka and continue to pose serious problem to farmers. The *In vitro* studies carried out to find the effectiveness of antibiotics and antagonists in inhibiting the growth of *R. solanacearum*. The antibiotics *viz.*, Amoxycilin, Cefixime, Ciprofloxacin, Tetracyclin, Norfloxacin and Streptomycin were highly effective at 500 – 750 ppm with the maximum inhibition. Among bacterial antagonists, *Bacillus subtilis* was very effective in inhibiting the growth of pathogen followed by *Pseudomonas fluorescens* and *Bacillus megaterium*. Among the antibiotics evaluated under pot culture in the green house, the treatments *viz.*, Streptomycin, Cefixime and Tetracycline at 500 – 750 ppm were found very effective in reducing the population of *R. solanacearum* ($0.0x10^4$cfu/g of soil). Bacterial antagonists *viz.*, *P. fluorescens; B. megaterium* and *Bacillus subtilis* reduced the pathogen population from ($686.66 x 10^4$cfu/g of soil) to $3.33x10^4$, $4.33x10^4$ and $7.33x10^4$cfu/g of soil respectively.

Keywords: Bioagents, antibiotics, *Ralstonia solanacearum*, brinjal wilt

Introduction
Bacterial wilt of brinjal and other solanaceous vegetables caused by *Ralstonia solanacearum* (Smith) is the most destructive disease in the tropical, subtropical and temperate regions of the world, causing heavy economic loss. The bacterial wilt disease is wide spread, affecting many solanaceous vegetable crops in India, especially in Karnataka. The major hosts affected by this disease in India include tomato, potato, brinjal, chilli, ginger, groundnut, tobacco, banana and other floricultural plants. In India, brinjal is cultivated in an area of 4, 74,400 ha with a production of 76, 61,510 tonnes and in Karnataka it is grown in an area of 22,481 ha with production of 4, 49,620 tonnes (Anon., 2004) [1]. One of the major factors limiting the cultivation of brinjal crop in Karnataka is the incidence of bacterial wilt caused by *Ralstonia solanacearum*. The soil-borne pathogen causes substantial economic loss to crops.

Although some progress have been made in understanding the biology of the pathogen and measures for control of bacterial wilt, but the disease continue to pose serious problem to farmers. At present, no effective control measures are available except resistant cultivars, crop sanitation, crop rotation and other cultural practices. The use of resistant varieties is the most popular way of controlling the disease, but the development of resistance has been hampered by high degree of variability of the pathogen (Aspiras and Cruz, 1985).

In recent years, owing to the increased awareness to the hazards of chemical control, the biological control is gaining popularity. Various fungi, actinomycetes and bacteria exhibited antagonistic effects against *R. solanacearum* (Kelman, 1953) [11]. Many kinds of beneficial antagonistic bacteria inhabit the soil and the rhizosphere of plants and survive within the plant. These antagonistic bacteria may reduce the effect of pathogen, may exceed the number and weight in soil with their rapid growth and ability to utilize varied substrates under different conditions. While some bacteria are antibiotic producers and others are effective fast growing colonizers. Since, *Bacilli* and *Pseudomonas* are abundant in the rhizosphere and they could prove to be important competitors with the root pathogens (Baker and Cook, 1974) [4].

Some of the studies conducted in India and abroad (Ganesan and Gnanamanickam, 1987; Savithry and Gnanamanickam, 1987 and Vasantha Devi *et al.*, 1988) have shown the potential of root colonizing microorganisms that inhibit or displace rhizosphere pathogenic microorganisms and thereby protect the health of perennial and annual crops (Anuratha and Gnanamanickam, 1990) [2]. Considering all these factors, the biological control of *R. solanacearum* would be ideal if a suitable antagonist is identified, which is very effective,

economical and eco-friendly and highly specific in the context of the bacterial diseases such as bacterial wilt.

Material and Methods

The plant and soil samples from wilt affected brinjal plants showing typical symptoms of bacterial wilt were collected from infested fields. The collected plant samples were packed in polythene bags and kept at 4 ^{0}C for isolation of causal organism. To isolate the pathogen from soil, the soil samples were serially diluted and the pathogen was isolated using TZC medium.

In-vitro evaluation of antibiotics and bactericides, bioagents against *R. solanacearum*

Antibiotics and bacteriocides were evaluated at different concentrations (Table 1) to test their efficacy in inhibiting the growth of *R. solanacearum*, by inhibition zone assay method. The bacterium was multiplied by inoculating the culture into the 250 ml of CPG broth taken in flask. The inoculated flasks were incubated at 28 ^{o}C for 48 hours. The bacterial suspension was then seeded to the CPG agar medium. The seeded medium was poured onto the sterilized Petri Plates and plates were allowed to solidify.

The bacteriocides solutions were prepared at different concentrations as mentioned in the list. The filter paper discs (Whatman No. 42) measuring 5 mm in diameter were soaked in the respective chemical solution for 5 – 10 minutes and transferred onto the surface of the bacterium seeded medium in petriplates. The inoculated plates were kept in the refrigerator at 4 ^{o}C for 4 hours to allow the diffusion of chemical into the medium. Then plates were incubated at 28 ^{o}C for 48 hours and observed for the production of inhibition zone around the filter paper discs. The results obtained were analyzed statistically.

Evaluation of bioagents

Bacterial antagonists against *R. solanacearum*

Three isolates of bacteria *viz.*, *Bacillus subtilis*, *B. megaterium*and *Pseudomonas fluorescens* isolates collected from Department of Agricultural Microbiology were tested for their efficacy in inhibiting the growth of *Ralstonia solanacearum* by the paper disc method. The virulent isolate of *R. solanacearum* was multiplied in Casmino acid Peptone Glucose broth (CPG). The 48 hour old culture of *R. solanacearum* containing 7x10^{8}cfu/ml and was mixed with molten (50 ^{o}C) CPG agar, so as to get a thick lawn of bacteria on the surface of agar medium. The seeded medium was poured onto sterilized petridishes and allowed to solidify. Previously sterilized filter pater (Whatman No. 42) measuring 5 mm diameter was soaked in different antagonist broth for 10 minutes and placed in the petriplates. The excess solution from the filter paper discs was removed by touching side of the paper discs to the lid of petridishes containing broth of the same organism. Then the filter discs were placed in a marked position on the surface of the seeded agar medium. The inoculated plates were incubated at 28 ^{o}C for 48 hours. The zone of inhibition produced around the filter paper disc was recorded. Filter paper discs dipped in sterile water served as check.

Fungal antagonists against *R. solanacearum*

Three fungal isolates collected were tested for their inhibitory effect on *R. solanacearum in vitro* by inhibition assay method. All the fungal isolates were grown separately on Potato Dextrose Agar. Molten sterilized PDA (15 ml) was poured in sterilized peteriplates and allowed to solidify. Fungal culture discs of 5 mm diameter from margin of actively growing four days old culture were removed and placed in the center of the plates containing PDA. The plates were incubated for three day at 30^{o}C. Discs (5 mm) were cut from these plates and used in the experiment.

A heavy suspension of *R. solanacearum* (6.8 x 10^{8}cfu/ml) was mixed with molten (50 ^{o}C) CPG Agar contained in 500 ml conical flask so as to get a thick growth of bacteria on the medium. The seeded medium was poured into sterilized petriplates and allowed to solidify. The actively growing 5 mm size of agar mycelial discs of fungal isolate was placed at the center of petridishes containing the seeded medium. These plates were incubated at 28 ^{o}C for four days. The observations on the zone of inhibition around the mycelial disc against *Ralstonia solanacearum* were recorded after the incubation period.

Results and Discussion

In vitro evaluation of antibiotics on the growth of *R. solanacearum*

The bacterial wilt caused by *R. solanacearum* (Smith) Yabuchiis a major constraint in the cultivation of brinjal and other solanaceous crops in tropical, subtropical and warm temperature regions of the world (Yabuchi, *et al*., 1995) [15]. The yield loss in brinjal due to this disease ranges from 15 to 95 per cent (Javier, 1994) [10].

At present, there is no effective control measure to manage the bacterial wilt. However, several antibiotics are being used in the field and several other non-antibiotics which are effective against other Gram negative bacteria are being tested in different concentrations against *R. solanacearum*. These antibiotics are effective under *in vitro* but fail to control the diseases under field condition. Also, continuous application of antibiotics to soil is known to affect other beneficial micro flora. Further, most of the antibiotics tested/ used are not developed for agricultural use and there is a need to evaluate the antibiotics which are less hazardous to soil micro-flora or the environment.

Biocontrol is regarded as one of the important IDM strategy for control of bacterial wilt. There are contradictory reports about the efficiency of various biological agents in controlling bacterial wilt under field conditions. Hence, in the present investigation attempts were made to evaluate the new antibiotics and potential antagonistic bacteria and fungi under *in vitro*.

The results obtained on isolation of the causal agent from the wilt affected brinjal plant and mass production, formulation and *in vitro*evaluation of selected antibiotics and biocontrol agents against the pathogen/ disease are discussed below.

Seven antibiotics were evaluated at different concentrations (Table 1) to test their efficacy to inhibit the growth of *R. solanacearum*, by inhibition zone assay method.

Among the antbiotics tested (Table 1), Amoxycillin was found to be highly effective with the maximum inhibition range of 31.25 to 36.00 mm at 500 to 750 ppm and was on par with Cefixime which recorded inhibition zone of 31.25 to 35.50 mm. Ciprofloxacin recorded inhibitory zone of 28.75 to 32.75 mm followed by Tetracycline (24.25 to 27.25 mm), Norfloxacin (20.75 to 22.75 mm) and Streptomycin (14.75 to 16.25 mm). The least inhibitory zone of 7.25 to 10.00 mm was recorded by Bactrimil at 500 and 750 ppm. Dutta and Verma (1969) studied the efficacy of Streptocycline in controlling bacterial wilt of egg plant and found that seedling

treatment of variety Pusa purple long with the antibiotic (1g/1) for 30 minutes before planting gave the best results.

Farag *et al*. (1986) [7] studied the effect of Streptomycin and dihydro streptomycinin relation to potato bacterial wilt and found that both virulent and avirulent forms of pathogen were sensitive to Streptomycin and dihydro streptomycin. Similarly, Das *et al*. (1995) [6] tested 14 antibiotics and amongst them Tetracycline was the most effectiveBetween the concentrations of antibiotics, efficacy significantly increased from lower to higher concentration with greater efficacy of higher concentration.

Table 1: *In vitro* evaluation of antibiotics against *R. solanacearum*

Sl. No.	Antibiotics	Inhibition zone (mm)		
		300 ppm	500 ppm	750 ppm
1	Streptomycin	13.50 (4.70)*	14.75 (4.85)	16.25 (5.04)
2	Tetracycline	21.25 (5.62)	24.25 (5.97)	27.25 (6.21)
3	Amoxycilin	28.50 (6.27)	31.25 (6.60)	36.00 (7.00)
4	Cefixime	28.50 (6.27)	31.25 (6.60)	35.50 (6.94)
5	Ciprofloxacin	26.75 (6.18)	28.75 (6.34)	32.75 (6.72)
6	Norfloxacin	18.75 (5.28)	20.75 (5.51)	22.75 (5.74)
7	Bactrimil	6.75 (3.55)	7.25 (3.74)	10.00 (4.26)
8	control	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
S.Em±		0.06	0.04	0.07
CD @ 1%		0.23	0.15	0.27
CV (%)		1.98	1.24	2.11

*Transformed values

In vitro evaluation of antagonists on the growth and multiplication of *R. solanacearum*

Bacterial antagonists against *R. solanacearum*

The anatagonistic bacteria *viz., Bacillus subtilis, B. megaterium* and *P. fluorescens* were evaluated for their bio efficacy on *R. solancearum* and the data obtained are presented in the Table 2.

Of the three bacterial antagonists tested, *B. subtilus* was found to be the most effective in inhibiting the growth of *R. solanacearum* by producing inhibition zone. The other antagonist *viz., P. fluorsecnes, B. megaterium* also inhibited the growth of *R. solanacearum*. The studies carried out to test the effect of antagonistic bacteria on *R. solanacearum* under *in- vitro* revealed that *B. subtilus*, was found to be most effective in inhibiting the growth whereas, other antagonists *viz., P. fluorsecnes, B. megaterium* also inhibited the growth of *R. solanacearum*.

Gallardo *et al.* (1989) [8] reported the inhibition of *R. solanacearum in vitro* by using *P. fluorescens* strain BC-8. The growth of *R. solanacearum* was inhibited by inoculation with *P. fluorescens*on the medium. Anuratha and Gnanamanickam (1990) [2] reported that, the 125 fluorescent strains of bacteria (which included 117 strains of *P. fluorescens*) screened against *R. solanacearum*, 68 strains (54.4%) inhibited the growth of the pathogen. Ciampi *et al.* (1989) reported that, the biological agent designated as BC-6 caused strong inhibition of *R. solanacearum*in both *in vitro* assay and growth chamber condition.

Table 2: *In vitro* evaluation of bacterial and fungal antagonists against *R. solanacearum*

Sl. No.	Antagonist	Inhibition zone (mm)
1	*Bacillus subtilis*	12.17 (4.49)*
2	*Bacillus megaterium*	9.67 (4.11)
3	*Pseudomonas fluorescens*	9.83 (4.13)
4	*Trichoderma harzianum*	0.00 (1.00)
5	*Trichoderma viridae*	0.00 (1.00)
6	*Trichoderma virens*	0.00 (1.00)
7	control	0.00 (1.00)
S.Em ±		0.03
CD @ 1%		0.12
CV (%)		2.39

*Transformed values

Fungal antagonists against *R. solanacearum*

The results from this experiment revealed that fungal antagonists had no effect on the growth of the *R. solanacearum.* Three fungal antagonists *viz., T. harzianum, T. viridae* and *T. virens* were not effective against *R. solanacearum* under *in vitro* that could be due to the inability of the fungal antagonists to produce antibiotics. However, Nesmith and Jenkins (1985) [12] reported that *Trichoderma* was inhibitory to *R. solanacearum* under *in vitro* conditions.

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