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In-vitro and *In-vivo* evaluation of anti-bacterial chemicals and bio-agents to manage the bacterial wilt of gherkins caused by *Ralstonia solanacearum* (E. F. Smith) Yabuuchi *et al.*

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Abstract
In vitro evaluation of eight different chemicals and their combination *viz.* Copper oxychloride, Bleaching powder, Streptocycline + Copper oxychloride, K-cycline+ Copper oxychloride, Bacteriomycin+ Copper oxychloride, Streptocycline + Copper oxychloride + Bleaching powder, K-cycline + Copper oxychloride + Bleaching powder, Bacteriomycin + Copper oxychloride + Bleaching powder was carried out against *Ralstonia solanacearum,* among these Strptocycline (500 ppm) combined with Bleaching powder (3000 ppm) and Copper oxychloride(3000 ppm) showed best among all the treatments with mean inhibition zone of 26.66 mm and Bleaching powder alone had showed least mean inhibition zone of 10.33 mm. Six different antagonist organisms *viz. Trichoderma harzianum, Pseudomonas fluorescens and Bacillus subtilis* from Institute of Organic Farming, Dharwad (IOF Dharwad) and *Trichoderma viridae, Pseudomonas fluorescens and Bacillus subtilis* from local market were selected for *in vitro* evaluation. Among these *Pseudomonas fluorescens* of IOF Dharwad showed maximum inhibition zone of 15.33 mm, whereas *Trichoderma viridae* has no effect on the growth of test organism i.e., *Ralstonia solanacearum.* The anti-bacterial chemicals and bio-agents found effective under *in vitro* were evaluated under glass house. Among these Streptocycline 500 ppm + Copper oxychloride 3000 ppm + Bleaching powder 3000 ppm showed minimum per cent disease incidence with 16.66 per cent while *Bacillus subtilis* of local market was observed with maximum incidence of 61.11 per cent. The results supported that the use of antibiotics combined with Copper oxychloride and Bleaching powder was more effective and suitable than the other treatments for the management of bacterial wilt gherkins.

Keywords: Gherkins, *Ralstonia solanacarum,* K-cycline, Bleaching powder, Antagonist

Introduction
Gherkin (*Cucumissativus* L.) is also called Bur gherkin or West Indian gherkin, an annual trailing vine of gourd family (cucurbitacae), grown for its edible fruits. The Gherkin plant is native to Southern Africa and is grown in warm climates around the world. Fruits are served as raw and in cooked or pickled form. Its nutritional value is similar to cucumber. There is a growing demand worldwide for pickled gherkins, more and more food companies have started to explore opportunities for producing gherkins. Gherkins cultivation and exports started in India during the early 1990s with a modest beginning in Karnataka state and later extended to the neighbouring states of Tamil Nadu and Andhra Pradesh. Karnataka accounts for almost 60 per cent of the gherkin production. Currently, there are more than 1,00,000 small and marginal farmers who are engaged in the gherkin production. Gherkin industry in Indiais well established with exports reaching 2, 25, 000 metric tons per annum. Gherkin is exported to major countries like USA, France, Germany, Australia, Spain, South Korea, Canada, Japan, Belgium, Russia, China, Srilanka, Israel, and Estonia (Santhoshreddy, 2011) [8].
Ralstonia solanacearum is a soil borne aerobic, non sporing, Gram negative bacterial plant pathogen and a major limiting factor in the production of many crop plants all over the world. As a highly diversified pathogen, *R. solanacearum* is considered as a species complex, a heterogeneous group of related strains. *R. solanacearum* has developed an extremely broad host range throughout the world, including more than 450 host species representing 54 plant families causing wilt disease (Wicker *et al.*, 2007) [10]. The loss of turgidity of the leaves followed by drooping, drying of lower leaves and sudden wilting of plants were the symptoms of the disease noticed. Considering the economic importance of gherkins losses caused by Bacterial wilt in northern Karnataka the present study was undertaken to find out the effective management for bacterial with of gherkins.

Material and Methods

The *in vitro* study was conducted at the Department of Plant Pathology Laboratory, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India during 2020 to 2021. The *in vivo* experiment was carried out in glass house of Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India during 2020 to 2021.

Collection of bacterial culture and inoculum preparation:

The bacterium was isolated and sub-cultured on nutrient agar (NA) medium (per 1 L: beef extract, 3g; peptone, 5g: agar, 15g) at 28°C for 48 h. Bacterial suspension on nutrient broth was used for *in vitro* tests as well as for inoculation of healthy plants during *in-vivo* experiments.

Culture of bacteria on TTC medium

2, 3, 5- triphenyl tetrazolium chloride (TTC) medium was produced based on standard microbiological procedure (Kelman, 1954) [4]. A loopful of bacteria cultured spread on the TTC media plate by sterile loop and incubated for 30 °C for 48 hours. Virulent bacteria were selected on the basis of colony morphological characteristics.

Pathogenicity tests

Pathogenicity of *R. solanacearum* was confirmed by inoculating five weeks old gherkins plants with a bacterial suspension (5 × 10^8cfu/mL). The plants were kept in glass house (average RH = 80%, temperature, 25-30°C, dim light for 12 h daily) for up to three weeks. Typical symptoms of the disease were noticed on plants. The bacterium was re-isolated from stem on nutrient agar and TTC media (Salvi, 2020) [7].

In-vitro evaluation of anti-bacterial chemicals against *Ralstonia solanacearum*

Eight different chemicals and their combination *viz*. Copper oxychloride, Bleaching powder, Streptocycline + Copper oxychloride, K-cycline + Copper oxychloride, Bacteriomycin + Copper oxychloride, Streptocycline + Copper oxychloride + Bleaching powder, K-cycline + Copper oxychloride + Bleaching powder, Bacteriomycin + Copper oxychloride + Bleaching powder was carried out using inhibition zone assay method. The mass multiplied broth culture of the test bacterium was seeded to autoclaved lukewarm nutrient agar medium (NA) mixed thoroughly and poured into sterilized petriplates and allowed to solidify. The solutions of the desired concentration of the test antibiotics was prepared separately. The Whatman filter paper discs of five mm in diameter were soaked separately in respective chemical solutions for 5 to 10 minutes and transferred on to the solidifying bacterium seeded NA medium in Petri plates. The untreated control plate inoculated with filter paper disc soaked in distilled water was maintained and then the plates were incubated at 28℃ for 48 hours and observed for the production of inhibition zone around filter paper disc (Raghu, 2013) [6].

In-vitro evaluation of bio-agents against *Ralstonia solanacearum*

Six different antagonist organisms *viz*. *Trichoderma harzianum, Pseudomonas fluorescens and Bacillus subtilis* from Institute of Organic Farming, Dharwad (IOF Dharwad) and *Trichoderma viridae, Pseudomonas fluorescens and Bacillus subtilis* from local market were selected for *in vitro* evaluation. A heavy suspension of three days old bacterial culture 1x10^8cfu/ml of *R. solanacearum* multiplied in nutrient broth (20 ml) was mixed with lukewarm nutrient agar medium (1000 ml) contained in flask. Twenty ml of seeded medium was poured into the sterilized petriplates and allowed to solidify. Previously sterilized filter paper disc measuring 5 mm in diameter were soaked in different antagonist broth for 10 minutes and placed in a marked position on the surface of the seeded agar medium (Bawari and Naredrappa 2019) [2]. In case of fungal antagonists the mycelial disc of five mm (diameter) size was taken from actively growing culture were placed in the centre of the plates (Raghu, 2013) [6]. The inoculated plates were then incubated at 28 ℃ for 48hrs. Observations were recorded for the zone of inhibition produced by antagonistic microorganisms around the growth of the pathogen.

Glass house evaluation of effective anti-bacterial chemicals/antibiotics and bio-agents against *Ralstonia solanacearum*

Effective anti-bacterial chemicals and bio-agents were screened for their efficacy to check the incidence of gherkins bacterial wilt. This experiment was conducted in April-June 2021 in Glass house, Department of Plant Pathology, UAS Dharwad. Totally three replication of seven treatments were maintained. The details of the treatments were given below. The chemicals and bio-agents were first applied at 30 days after sowing in the pot as soil drench at 10 days interval up to the final harvest. Plants were inoculated by scrapping the lower part of the stem with scissor dipped in fresh bacterial suspension of 5×108 cfu/mL and then one ml of bacterial suspension was spread on the wounded stem (Mohsin *et al.* 2016) [5]. Data were recorded on disease incidence. The experiment was done using completely randomized design (CRD) with three replications. Data collected during experimental period were tabulated and analyzed using Statistics method.

Table 1: Treatment details

Treatment details	Concentration (ppm) and amount of chemicals
Streptocycline + Copper oxychloride + Bleaching powder	500+3000+3000, 50 ml/pot
K-cycline + Copper oxychloride + Bleaching powder	500+3000+3000, 50 ml/pot
Streptocycline + Copper oxychloride	500+3000, 50 ml/pot
K-cycline + Copper oxychloride	500+3000, 50 ml/pot
Pseudomonas fluorescens(IOF Dharwad)	5x10^8 cfu/ml, 40ml/plant
Bacillus subtilis (Market sample)	5x10^8 cfu/ml, 40ml/plant

Results

Morphology and pathogenicity of *R. solanacearum*

On nutrient agar, the bacteria produced white/cream colored, irregular, fluidal and non-transparent colonies after 48 hours of incubation at 28 ℃. The bacterial pathogen produced highly fluidal, slightly raised and creamy white colonies with

light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30℃ on TTC medium. Plants inoculated with *R. solanacearum* showed symptoms of bacterial wilt disease fifteen days after inoculation. The bacteria were re-isolated from the infected plant samples to confirm Koch postulates.

In-vitro* evaluation of anti-bacterial chemicals and bio-agents against *Ralstonia solanacearum

The results indicated that, among the different treatments T_6 (Streptocycline 500 ppm + Copper oxychloride 3000ppm + Bleaching powder 3000 ppm) showed statistically singnificant result with mean maximum inhibition zone of 26.66 mm, followed by T_7 (K cycline 500ppm + Copper oxychloride 3000ppm + Bleaching powder 3000ppm) with inhibition diameter of 24.0 mm and Bleaching powder showed minimum inhibition zone of 10.33 mm.

Among six bio-agents *Pseudomonas fluorescens*(IOF Dharwad) showed maximum inhibition zone of 15.33 mm followed by *Bacillus subtilis*(market sample) with mean inhibition zone of 13.33 mm and *Trichoderma viridae* has no effect on the growth of test organism which didn't show any inhibition zone.

Table 2: *In vitro* evaluation of anti-bacterial chemicals against *Ralstonia solanacearum*

Treatment No.	Chemicals	Concentration (ppm)	Mean diameter of inhibition zone (mm)
T_1	Copper oxychloride	3000	12.66 (3.69)*
T_2	Bleaching powder	3000	10.33 (3.36)
T_3	Streptocycline + Copper oxychloride	500 + 3000	24.66 (5.06)
T_4	K cycline + Copper oxychloride	500 + 3000	22.66 (4.86)
T_5	Bacterimycin + Copper oxychloride	500 + 3000	18.33 (4.39)
T_6	Streptocycline + Copper oxychloride + Bleaching powder	500 + 3000 + 3000	26.66 (5.26)
T_7	K-cycline + Copper oxychloride + Bleaching powder	500 + 3000 + 3000	24.00 (4.99)
T_8	Bacterimycin + Copper oxychloride + Bleaching powder	500 + 3000 + 3000	19.66 (4.54)
T_9	Control		0.00 (1.00)
		S.Em. ±	0.050
		CD @ 1%	0.129

*- Figure in the parenthesis are √ x + 1 transformed value

Table 3: *In vitro* evaluation of bio-agents against *Ralstonia solanacearum*

Treatment No.	Bioagent	Mean diameter of inhibition zone (mm)
T_1	*Trichoderma harzianum*(IOF Dharwad)	9.33 (3.24) *
T_2	*Pseudomonas fluorescens* (IOF Dharwad)	15.33 (4.04)
T_3	*Bacillus subtilis*(IOF Dharwad)	7.66 (2.94)
T_4	*Trichoderma viridae*(ALDERM)	0.00 (1.00)
T_5	*Pseudomonas fluorescens*(ALOMONAS)	8.33 (3.05)
T_6	*Bacillus subtilis*(ABACIL)	13.33 (3.78)
T_7	Control	0.00 (1.00)
	S.Em. ±	0.010
	CD @ 1%	0.030

*- Figure in the parenthesis are √ x + 1 transformed value

Evaluation of anti-bacterial chemicals and bio-agents against bacterial wilt under glass house condition

Results revealed that, bacterial wilt incidence was significantly lower in all the treatments. Among these, T_1 showed minimum per cent disease incidence with 16.66 per cent followed by T_2 and T_3 with 27.77 per cent disease incidence respectively. While T_6 was observed with maximum incidence of 61.11 per cent.

Table 4: Testing of effective antibacterial chemicals and bio-agents in pot condition for the management of bacterial wilt of gherkins

Sl. No.	Treatments	Concentration	PDI (%)
1	Streptocycline + Copper oxychloride + Bleaching powder	500 + 3000 + 3000	16.66 (24.08) *
2	K-cycline + Copper oxychloride + Bleaching powder	500 + 3000 + 3000	27.77 (31.52)
3	Streptocycline + Copper oxychloride	500 + 3000	33.33 (35.25)
4	K-cycline + Copper oxychloride	500 + 3000	33.33 (35.25)
5	*Pseudomonas fluorescens*(IOF Dharwad)	5g	50.00 (44.98)
6	*Bacillus subtilis* (Market sample)	5g	61.11 (54.71)
7	Control	-	100.00 (89.96)
		S.Em. ±	1.407
		CD @ 1%	4.310

*- Figure in the parenthesis are arc sine values or angular transformations

Fig 1: *Ralstoniasolanacearum* colony on TTC media

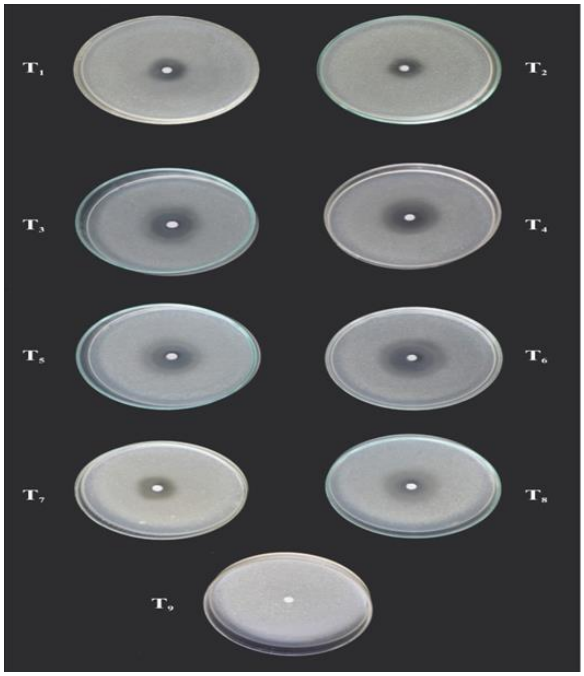

Fig 2: *In vitro* evaluation of anti-bacterial chemicals against *Ralstoniasolanacearum*

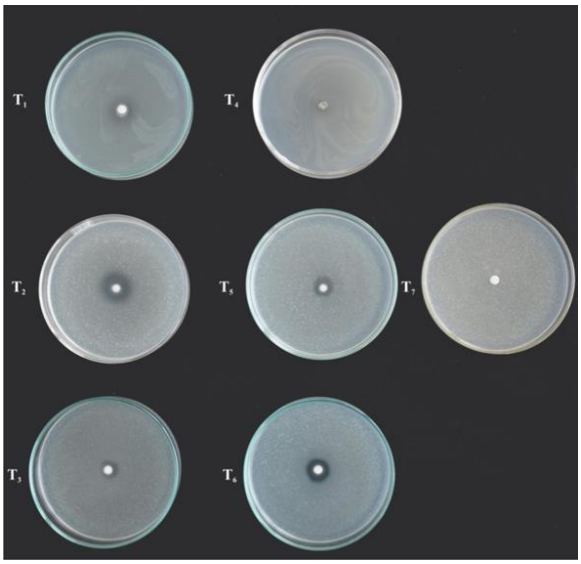

Fig 3: *In vitro* evaluation of bio-agents against *Ralstoniasolanacearum*

Fig 4: Testing of effective antibacterial chemicals and bio-agents in pot condition for the management of bacterial wilt of gherkins

Discussion

In the present investigation, different antibacterial chemicals and their combination was evaluated under *in-vitro* against *R. Solanacearum* using inhibition zone method. Among different treatments Streptocycline 500 ppm + Copper oxychloride 3000 ppm + Bleaching powder 3000 ppm with an inhibition zone of 26.66 mm exhibited superior efficacy followed by K-cycline 500 ppm + Copper oxychloride 3000 ppm + Bleaching powder 3000 ppm with 24.0 mm. Other chemicals were moderately effective whereas, bleaching powder was less effective. Similar observations were recorded by Singh and Jagtap (2017) conducted an experiment to find out the effectiveness of antibacterial chemicals against the growth of *Ralstonia solanacearum* under *in vitro* conditions. Average inhibition was ranged from 6.2 mm (Copper hydroxide) to 20.05 mm (Streptocycline). However, significantly the highest average inhibition was recorded in the antibiotic Streptocycline (20.05 mm).

Antagonistic microorganisms like *Trichoderma harzianum, Pseudomonas fluorescens and Bacillus subtilis* from IOF Dharwad and *Trichoderma viridae, Pseudomonas fluorescens and Bacillus subtilis* from local market were screened for their efficacy against the growth of *Ralstonia solanacearum* under *in vitr*o by using inhibition zone method in case of bacterial antagonist organism and mycelial disc method in case of fungal antagonist. Among the different antagonistic organisms tested, *Pseudomonas fluorescens* (IOF Dharwad) found superior over other antagonists followed by *Bacillus subtilis* (ABACIL). These antagonistic microorganisms act on the pathogen by different mechanisms *viz*., competition, lysis, antibiosis, siderophore production and hyperparsitism. These results were supported by the research findings of Basha *et al.* (2017) conducted *in vitro* evaluation of different antagonistic microorganisms, from this study they revealed that *Bacillus subtilis* was most effective in inhibiting the growth of the *R. solanacearum* followed by *Pseudomonas fluorescens* by producing an inhibition zone of 22.50 mm and 18.00 mm diameter respectively.

The anti-bacterial chemicals and bio-agents found effective under *in vitro* were evaluated under glass house/pot condition. The treatments were imposed through soil drenching and per cent disease incidence of each treatment was recorded. Among these Streptocycline 500 ppm + Copper oxychloride 3000 ppm + Bleaching powder 3000 ppm showed minimum per cent disease incidence with 16.66 per cent while *Bacillus subtilis* (ABACIL) was observed with maximum incidence of 61.11 per cent. Similar work was conducted by (Jaydeep, 2017), evaluated the antibacterial chemicals and bio-agents which were found effective under *in vitro* and in pot condition. Among these, Streptocycline (0.025%) and Plantomycin (0.025%) gave minimum disease incidence

(16.66%) followed by Copper oxychloride (50% WP) at 0.1% + Streptocycline with (250 ppm) 33.33% disease incidence. While all other treatments *i.e.* Copper oxychloride (0.2%), Copper hydroxide (0.2%), *Pseudomonas fluroscens* (5ml/lit) and *T. viride* (5ml/lit) resulted in equal incidence of 50 per cent. The combination of copper compound and antibiotics showed more than the copper compound alone *i.e.* Streptocycline combined with Copper oxychloride and Bleaching powder had showed best result.

Conclusion

Bacterial wilt is a devastating disease of gherkins in recent years. There is no available effective management strategy against this disease. Among the applied treatments highest disease suppression was obtained from the treatment having combination of antibiotics with Copper oxychloride and Bleaching powder.

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