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

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#### Abstract

*Ralstonia solanacearum*, the causal organism of bacterial wilt of solanaceous crops is a major limitation on the production of brinjal. An experiment on *in vitro* studies was carried out to find out the effectiveness of botanicals, bioagents and anti-bacterial chemicals to inhibiting the growth of *R. solanacearum*. It was revealed from the evaluation that among the eight botanicals tested patchouli leaf extract was found highly effective with the maximum meaninhibition of 11.6 mm than other botanicals. Among the bioagents *Pseudomonas fluorescens* showed maximum mean inhibition zone (20.30 mm) compare to *Bacillus subtilis* and *Trichoderma harzianum*. Whereas, results revealed from the evaluation of anti-bacterial chemicals *viz.*, copper hydroxide showed maximum mean inhibition of 12.30 mm followed by copper-oxy chloride (11 mm).

**Keywords:** *Ralstonia solanacearum*, inhibition zone, patchouli leaf, brinjal

#### Introduction

The brinjal (*Solanum melongena* L.) belongs to the family solanaceae, is a common and popular warm weather vegetable crop. Brinjal is also referred as 'eggplant' the unripe fruit of eggplant is primarily used as a cooking vegetable for the various local dishes. In Karnataka it is grown in an area of 15.8 thousand ha with production of 402.5 metric tons with the productivity of about 25.4 MT/ha (Anon., 2016) [1]. Cultivation of brinjal facing different diseases problem among those bacterial wilt of brinjal *R. solanacearum* (Yabuchi *et al.*, 1995) [8] is the most harmful disease causes heavy loss up to 39-40 per cent in Karnataka region. The bacterial wilt symptoms in brinjal are characterized by initial yellowing of upper leaves and within few days followed by complete wilting of the plants. The vascular tissues of the infected stem have brown discoloration and, if the stem is cut crosswise, white or yellowish bacterial ooze may be visible.

The bacterial wilt disease is difficult to manage due to their broad host range of *R. solanacearum* with exceptional ability to survive in the soil and roots of non-hostplants including several weeds. Since the malnad region of Karnataka is rich biodiversity of wild botanicals and aromatic flora, many botanicals can be tried as alternative biodegradable components for the control of bacterial wilt. 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) [2]. *R. solanacearum* was effectively inhibited by the lower concentrations of antibiotics under *in vitro* conditions.

#### Material and methods

##### Isolation and purification of *R. solanacearum* strains from wilt affected brinjal plants

Brinjal plants showing typical symptoms *viz.*, wilting, stunting and yellowing of plants infected by bacterial wilt were collected and diagnosis of the disease was done by ooze test. The pathogen was cultured on TZC medium and well isolated typical colonies of isolate of *R. solanacearum* were picked and streaked separately on TZC medium. The well separated colonies of *R. solanacearum* were picked up with sterile inoculation loop and suspended in

sterile distilled water in sterile propylene culture tubes and stored at 4 °C in refrigerator for further use as stock culture.

### Evaluation of botanicals

#### Collection and preservation of botanicals

The eight botanical species used in the present study viz., Neem (*Azadirachta indica*), Lantana (*Lantana camara*), Pongamia (*Pongamia pinnata*), Marigold (*Tagetes erecta*), Tridax (*Tridax procumbens*), Patchouli (*Pogostemon cablin*), Glyricidia (*Glyricidia sepium*) and Eucalyptus (*Eucalyptus obliqua*) were collected locally. The plant leaves were collected from the fields around college campus shimoga during November-2016.

The leaves of all the tested plants were collected and washed in tap water and then in distilled water, 100 g of fresh sample was chopped and macerated in a surface sterilized pestle and mortar by adding 100 ml of sterile water (1:1 w/v). The extract was filtered through two layers of muslin cloth, filtrate thus obtained was used as a stock solution.

To study the antibacterial mechanism of plant extracts at three different concentrations viz., 10 per cent, 15 per cent, 20 per cent was made by adding sterilized distilled water proportionately and stored in the refrigerator for the further use.

#### Evaluation of bio agents

Three bioagents viz., *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated for their efficacy against the growth of *R. solanacearum* by inhibition zone assay method. The cultures/formulations of these

bioagents were obtained from department of Agricultural Microbiology, University of Agricultural and Horticultural Sciences, Shivamogga.

A heavy suspension of bacterial old culture  $1 \times 10^8$  cfu/ml (3 day old) of *R. solanacearum* multiplied in nutrient broth (20 ml) was mixed with lukewarm nutrient agar medium (1000 ml) contained in Erleyenmayer's flask. Twenty ml of seeded medium was poured into the sterilized Petri plates and allowed to solidify. A loopful culture of each of the antagonistic organism was placed in the centre of Petri plates containing the seeded medium. In case of fungal antagonists, mycelial discs of five mm (diameter) size taken from actively growing culture were placed in the centre of the plates. The inoculated plates were then incubated at 28 °C for 48 h. Observations were recorded for the zone of inhibition produced by antagonistic microorganisms around the growth of the pathogen.

#### Evaluation of antibacterial chemicals

Chemicals each at three concentrations (300, 400 and 500 ppm) were evaluated for their efficacy against the growth of *R. solanacearum* by inhibition zone assay method. The bacterium was multiplied by inoculating the culture into the 20 ml of nutrient broth taken in 'Erleyenmayers' flask. The inoculated flasks were incubated at 28 °C for 48 h. The bacterial suspension was then seeded to the lukewarm nutrient agar medium (1000 ml) and seeded medium was poured into the sterilized Petri plates and plates were allowed to solidify. The list of the chemicals as follows.

Sl. No.	Chemical name	Trade name
1	Copper-oxy chloride	Blitox (50% WP)
2	Kasugamycin	Kasu B (3% SI)
3	Copper hydroxide	Kocide (53.8%WP)
4	Streptocycline	Streptocyclin (9% sterptocyclin sulphate + tetracycline 1%)
5	Copper hydroxide+streptocycline	Kocide+streptocycline
6	Copper oxychloride+streptocycline	Blitox+streptocycline

The bactericidal solutions were prepared at different concentrations as mentioned in the list. The filter paper discs (Whatman No. 42) measuring five mm in diameter were soaked in the respective chemical solution for 5 minutes and transferred onto the surface of the seeded medium in petri plates. Then plates were incubated at 28 °C for 48 h and observed for the production of inhibition zone around the filter paper discs. The results obtained were analysed statistically.

#### In vitro evaluation

The aqueous plant extract, bio agents and anti-bacterial chemicals were evaluated under *in vitro* by paper disc method.

#### Paper disc method

The sterilized circular discs (6mm dia.) of Whatman No.1 were dipped in each of the concentration of a plant extract and test chemical for a few seconds. Likewise, the discs dipped in sterilized distilled water served as control. Three discs from each treatment were picked up and placed equidistantly in a Petri plate containing seeded 2, 3, 5-triphenyl tetrazolium chloride (TZC) medium. The three discs in a single Petri plate comprised three replications of each treatment. The Petri plates were then placed in the lower most shelf of a refrigerator (5 °C) for half an hour, thus allowing plant extracts and test chemicals to diffuse into the medium. The

plates were then shifted to the incubator at 28± 1°C. The inhibition zone (mm) was measured with the help of a scale after 48 hours of incubation. The mean inhibition zone was worked out for each treatment and compared with the control.

#### Results and discussion

Effect of eight plant leaf extract was evaluated against *R. solanacearum* by inhibition zone assay through paper disc method, as described in 'Material and methods'. The results are presented in the Table 1 and Plate 1.

The results revealed that among the botanicals, patchouli leaf extract was found significantly superior than rest of the plant extract with mean inhibition zone of 11.6 mm followed by pongamia leaf (10.0 mm) and marigold leaf (9.8 mm) respectively, which were on par with each other. Lantana (8.0 mm) showed lower efficacy among the botanicals tested. The effect of botanicals on inhibition zone assay of *R. solanacearum* at 10, 15, 20per cent concentrations differed significantly.

Patchouli leaf extract showed maximum inhibition because it contains two major compounds such as patchouli alcohol and pogostone about 60 per cent which showed the inhibitory action against *R. solanacearum* was reported earlier (Xian Yang *et al.*, 2013)<sup>[7]</sup>. Bacterial wilt in solanaceous vegetable crop has also been reported the *in vitro* evaluation plant extract against *R. solanacearum* (Pankaj and Pardeep Kumar, 2014)<sup>[4]</sup>.

Study conducted on effect of bio agents on growth of *R. solanacearum* under *in vitro* by inhibition assay and the results were presented in Table 2 and and Plate 2. Among the three bio agents, *P. fluorescens* was found significantly superior in inhibiting the growth of pathogen (20.30 mm) followed by *Bacillus subtilis* (16.70 mm). Whereas *Trichoderma harzianum* was found less effective showed the inhibition zone of 14.00 mm.

Bacterial bioagents such as *P. fluorescens* and *B. subtilis* were significantly more effective compare to fungal bioagent, *T. harzianum* against *R. solanacearum*. Literature supported for the *in vitro* evaluation, under biological control of plant diseases, various antagonistic organisms have been identified, which fight against the pathogens by different mechanisms viz., competition, lysis, antibiosis, siderophore production and hyper parasitism (Vidyasekaran, 1999) [6].

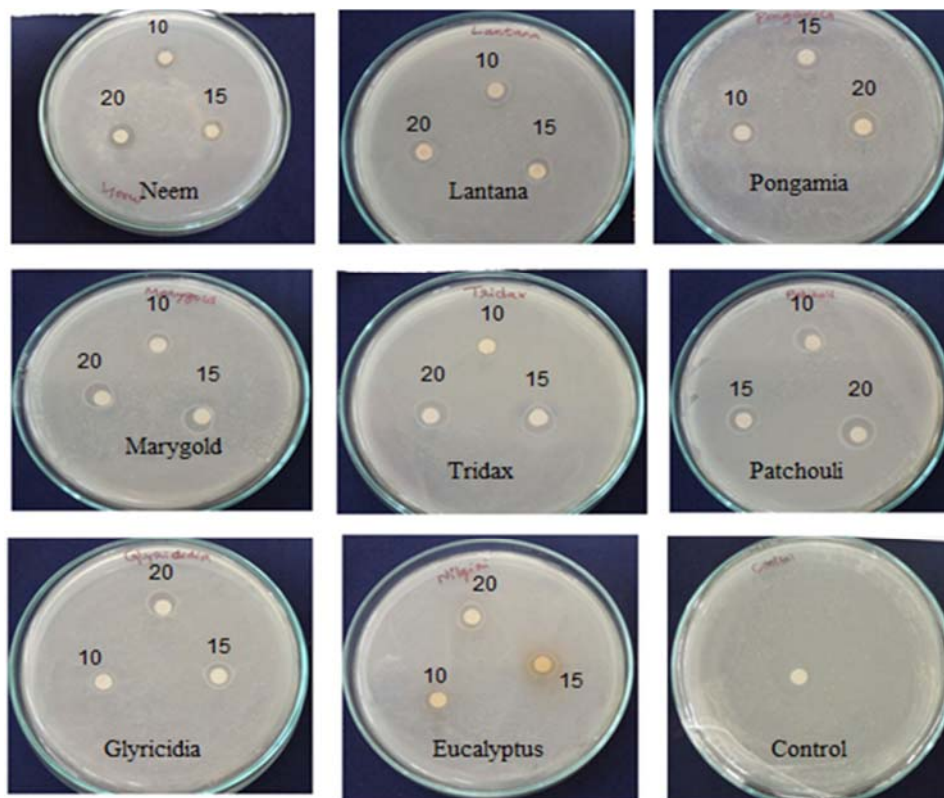
**Table 1:** *In vitro* evaluation of botanicals against *R. solanacearum*.

Sl. No	Botanicals	Mean diameter of inhibition zone (mm)		
		Concentration (%)		
		10	15	20
1.	Neem ( <i>Azadirachta indica</i> )	7.00 (2.82)	8.00 (3.00)	9.50 (3.24)*
2.	Lantana ( <i>Lantana camara</i> )	6.00 (2.64)	8.00 (3.00)	10.00 (3.32)
3.	Pongamia ( <i>Pongamia pinnata</i> )	8.50 (3.08)	9.50 (3.24)	12.00 (3.60)
4.	Marygold ( <i>Tagetes erecta</i> )	8.00 (3.00)	10.00 (3.31)	11.50 (3.54)
5.	Tridax ( <i>Tridax procumbens</i> )	5.00 (2.44)	10.00 (3.32)	12.00 (3.60)
6.	Patchuoli ( <i>Pogostemon cablin</i> )	10.00 (3.32)	12.00 (3.60)	13.00 (3.74)
7.	Glyricidia ( <i>Gliricidia sepium</i> )	5.00 (2.44)	8.50 (3.08)	11.00 (3.46)
8.	Eucalyptus ( <i>Eucalyptus obliqua</i> )	7.00 (2.82)	10.00 (3.32)	11.00 (3.46)
9	Control	0.00	0.00	0.00
Factors		SEm±		CD (1%)
Botanicals(B)		0.06		0.16
Concentration(C)		0.03		0.09
Interaction(BxC)		0.10		0.28

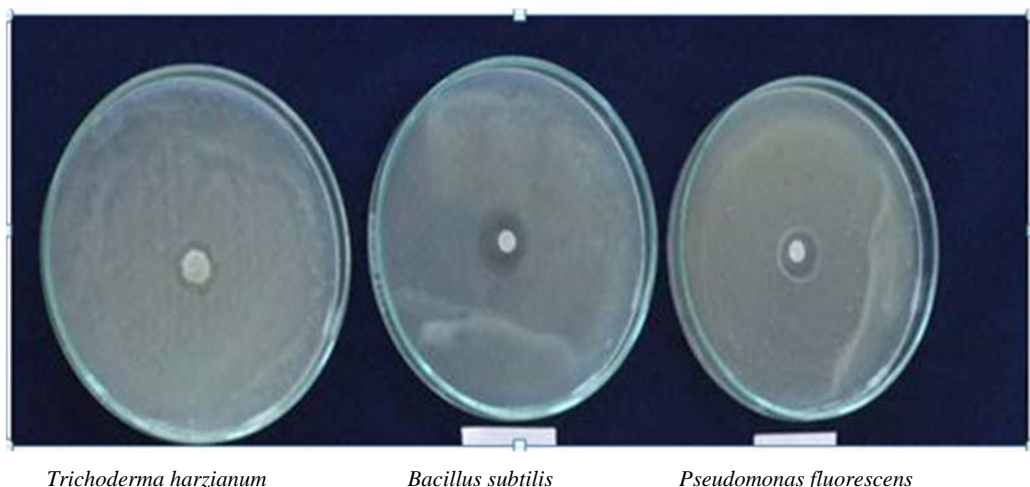
**Table 2:** *In vitro* evaluation of bioagents against *R. solanacearum*.

Sl. No	Bioagents	Mean diameter of inhibition zone (mm)
1.	<i>Bacillus subtilis</i>	16.7(4.21)*
2.	<i>Pseudomonas fluorescens</i>	20.3(4.61)
3.	<i>Trichoderma harzianum</i>	14.0(3.87)
4.	Control	0.00
	SEm±	0.04
	CD (1%)	0.14

\* Figures in parenthesis are  $\sqrt{x + 1}$  transformed values



**Plate 1:** *In vitro* evaluation of botanicals against *R. solanacearum*

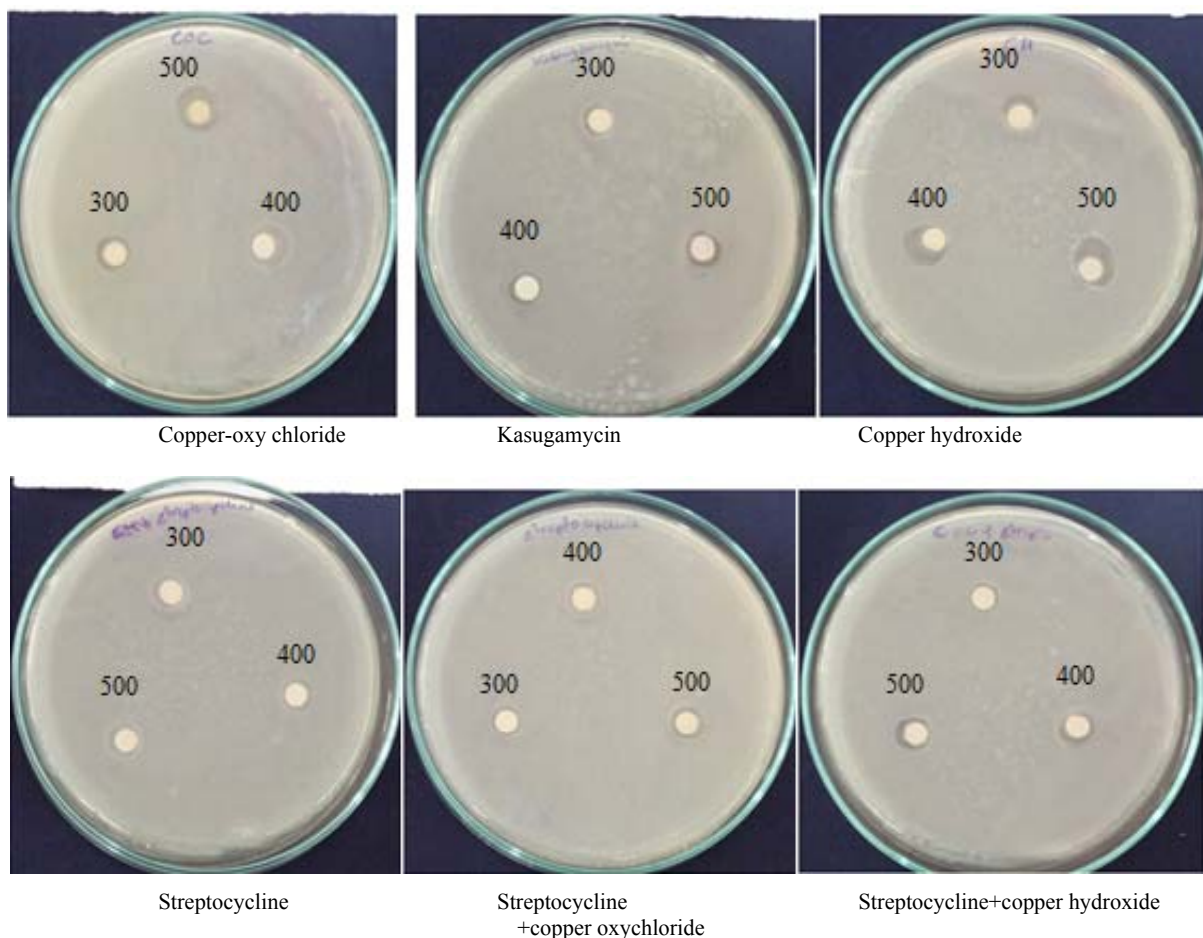


**Plate 2:** *In vitro* evaluation of bioagents against *R. solanacearum*

Data from the table revealed that, the efficacy of different bactericides; concentrations and their interaction on formation of inhibition zone differed significantly tabulate in Table 3 and Plate 3.

Among different chemicals and their combinations, mean maximum inhibition zone (12.30 mm) of *R. solanacearum* was recorded in copper hydroxide which was significantly superior over all other treatments followed by copper-oxy chloride (11.00 mm). Kasugamycin showed moderate effect with inhibition zone of 9.00 mm. whereas, combinations like streptomycin + copper - oxy chloride and streptomycin + copper hydroxide were found less effective (8.00 mm).

Between the concentrations of each chemical, efficacy was significant from lower to higher concentration. At 500 ppm copper hydroxide showed maximum inhibition zone (12.00 mm) followed by copper-oxy chloride with inhibition zone of 11.00 mm. Similar results were found at 400ppm whereas, in case of 300 ppm copper hydroxide and copper-oxy chloride showed maximum inhibition zone of 10.00 mm which were remain on par with each other. The copper compound has the potential to disrupt cell function and excess copper causes a decline in the membrane integrity of bacteria, leading to leakage of specific essential cell nutrients.



**Plate 3:** *In vitro* evaluation of antibacterial chemicals against *R. solanacearum*

**Table 4:** *In vitro* evaluation of antibacterial chemicals against *R. solanacearum*

Sl. No	Chemicals	Mean diameter of inhibition zone (mm)		
		Concentration (ppm)		
		300	400	500
1.	Copper-oxy chloride	10.00 (3.31)	11.00 (3.46)	12.00 (3.60)*
2.	Kasugamycin	8.00 (3.00)	9.00 (3.16)	10.00 (3.31)
3.	Copper hydroxide	10.00 (3.31)	12.00 (3.60)	15.00 (4.00)
4.	Streptocycline	9.00 (3.16)	8.00 (3.00)	8.00 (3.00)
5.	Streptocycline+ Copper-oxy chloride	7.00 (2.82)	9.00 (3.16)	8.00 (3.00)
6.	Streptocycline+ Copper hydroxide	7.00 (2.82)	8.00 (3.00)	9.00 (3.16)
7	Control	0.00	0.00	0.00
Factors		SEm±		C.D (1%)
Chemicals (B)		0.07		0.19
Concentrations (C)		0.05		0.12
Interaction (BxC)		0.12		0.33

\* Figures in parenthesis are  $\sqrt{x + 1}$  transformed values

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