

Original Research Article

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In vitro* Evaluation of Botanicals, Bioagents and Antibacterial Chemicals against Rhizome Rot of Ginger Caused by *Ralstonia solanacearum

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ABSTRACT

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Bacterial wilt caused by *R. solanacearum* is one of the most widely spread and destructive disease of ginger (*Zingiber officinale* Rosc.). 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 nine botanicals tested neem leaf extract was found highly effective with the maximum mean inhibition of 10.31 mm than other botanicals. Among the bioagents *Pseudomonas fluorescens* (Shivamogga and Bangalore isolate) recorded maximum inhibition zone of 16.85 mm and 16.49 mm respectively, compared to *Bacillus subtilis* and *Trichoderma harzianum*. Whereas, results revealed from the evaluation of anti-bacterial chemicals *viz.*, streptomycin + copper oxychloride showed maximum mean inhibition of 29.38 mm followed by streptomycin at 500 ppm (27.07 mm).

Introduction

Ginger (*Zingiber officinale* Rosc) is an important commercial crop grown for its aromatic rhizomes, used both as spice and medicine. It is herbaceous tropical perennial plant belonging to the family Zingiberaceae. The rhizomes are white to yellowish brown in colour. It is usually grown as an annual crop.

The major constraint in the cultivation of ginger is because of rhizome rot disease and caused by the bacterium *Ralstonia solanacearum* E.F. Smith (Yabbuchi *et al.*, 1995) is a devastating pathogen after the production of many economically important crops such as ginger, tomato, brinjal, chilli, tobacco, potato and banana (Kelman *et al.*,

1994). The yield loss due to this disease in ginger ranged from 15 to 95 per cent (Javier, 1994).

R. solanacearum is a gram negative, rod shaped, strictly aerobic bacterium that is 0.5-0.7 x 1.5- 2.0 µm in size, with a single polar flagellum. Individual bacterial colonies are usually visible after 36 to 48 hrs of growth at 28°C and colonies of ginger strains were highly fluidal with characteristic spiral pink centre whereas in the case of other strains fluidity and pink centre was less conspicuous (Kumar and Sarma, 2004; Sambasivam and Girija, 2006). Occasionally colonies of the mutant or non-virulent type appear uniformly round, smaller and butyrous or dry. Selective nutrient tetrazolium chloride (TZC) medium

(Kelman, 1954) can differentiate the two colony types on this medium. Strains of *R. solanacearum* have been classified into five biovars (Kumar *et al.*, 1993) and five races (Buddenhagen *et al.*, 1962; Bin Li *et al.*, 2010). The symptoms of bacterial rhizome rot of ginger are characterized by the appearance of water soaked patches or linear streaks on the collar region of the pseudo stem of ginger plants. These symptoms are followed by yellow or bronze coloration on margins of the lowermost leaves which gradually progress upwards, at later stages the leaves become flacid with intense yellow bronze colour and droop exhibiting typical wilt symptoms. Pseudostem can be easily separated from rhizome with a gentle pull (Dohroo, 1982).

The aim of present investigation was to study the effect of botanicals, bioagents and antibacterial chemicals on growth of *R. solanacearum* under *in vitro* conditions.

Materials and Methods

The experiment was designed to screen different commercially available chemicals for its antibacterial activity against *R. solanacearum* under *in vitro* using inhibition zone assay.

Isolation and purification of *R. solanacearum* from bacterial rhizome rot affected ginger crop and soil

The diseased plant and soil samples were collected from the farmer's field. The diseased plant samples were washed under tap water to remove the soil particle and air dried. The pseudostem of diseased plant of length 10 to 15 cm was first surface-disinfected with 70 % ethanol for 2 minutes and 1% sodium hypochloride for 5 minutes followed by repeated washing in sterile water for 5 minutes to remove traces of sodium hypochloride. The surface sterilized bits were suspended in the

five-milliliter sterile distilled water taken in test tube for ten minutes. After the water in test tube becomes turbid due to oozing of bacterial cells from cut ends of diseased tissue, the bacterial suspension was serially diluted in nine ml sterile water. One hundred microliter (1 ml) of the bacterial suspension was poured onto the surface of solidified Triphenyl tetrazolium chloride agar (TZC) medium (Kelman, 1954) containing (g/L) peptone 10; casein hydrolysate 1; glucose 5; agar 20; and distilled water 1L; pH 7.0 (1 % TZC will added to a final concentration of 5 ml/L after autoclaving) using spread plate technique. A loopful of bacterial suspension was streaked into TZC medium and incubated at 28±2°C for 48 hours. To isolate the pathogen from soil, the soil samples were serially diluted and pathogen was isolated using TZC medium. At the end of incubation period, the plates were observed for the development of both the virulent and avirulent colonies of *R. solanacearum*. The virulent colonies were irregularly shaped, fluidal, dull white colonies with pink center, whereas, avirulent colonies small, round, convex, butyrous with large red pigment and white fluidal colonies without pink center described by Kelman (1954).

Evaluation of botanicals, bioagents and antibacterial chemicals

Collection and preservation of botanicals

The nine botanical species used in the present study viz., Onion (*Allium cepa*), Garlic (*Allium sativum*), Neem (*Azadirachta indica*), Turmeric (*Curcuma longa* L), Lantana (*Lantana camara*), Pongamia (*Pongamia pinnata*), Noni (*Morinda citrifolia*), Glyricidia (*Gliricidia sepium*) and Nilgiri (*Eucalyptus obliqua*) were collected locally. The plant leaves were collected from the fields around college campus shivamogga 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., 5 per cent, 10 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

Four bioagents viz., *Trichoderma harzianum*, *T. viride*, *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 and Bangalore.

A heavy suspension of bacterial old culture 1×10^8 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 Erlenmeyer'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

Seven different chemicals viz., Kasugamycin, Streptocycline, Copper oxychloride, Copper hydroxide, Strptocycline + CoC, Plantomycin and Bleaching Powder were evaluated for their efficacy against the growth of *R. solanacearum* by inhibition zone assay. The chemicals were prepared at different concentrations. The bacterium was multiplied by inoculating the culture into the 20 ml of nutrient broth taken in 'Erlenmeyer's' 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.

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 0 C for 48 h and observed for the production of inhibition zone around the filter paper discs. The results obtained were analysed statistically.

Paper disc method

The sterilized circular discs (5mm dia.) of Whatman No. 44 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 0 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± 10 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 nine 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, neem leaf extract was found significantly superior than rest of the plant extract with mean inhibition zone of 10.31 mm followed by glyricidia (7.19 mm) and least inhibition zone was found in Turmeric (0.31 mm) respectively. The effect of botanicals on inhibition zone assay of *R. solanacearum* at 5, 10, 20 per cent concentrations differed significantly. Similar observations are made by (Raghu, 2011).

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 Plate 2. Among the three bio

agents, *P. fluorescens* (Shivamogga + Bangalore isolate) was found significantly superior in inhibiting the growth of pathogen (16.85 mm and 16.49 mm) followed by *Bacillus subtilis* (11.70 mm). Whereas, among the fungal antagonists *Trichoderma viride* (Shivamogga isolate) recorded maximum inhibition zone of 8.08 mm followed by *T. viride* of Bengaluru isolate (7.96 mm), *T. harzianum* (7.57 mm) of Shivamogga isolate and *T. harzianum* (Bengaluru isolate) of 7.50 mm were on par With each other. The least inhibition zone of 2.50 mm formation was observed in *Verticillium lecani*. Bacterial bioagents such as *P. fluorescens* and *B. subtilis* were significantly more effective compare to fungal bioagent, *T. harzianum* and *T. viride* 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). Bioagents viz., *P. fluorescens* and *B. subtilis* were reported efficient antagonists against *R. solanacearum* earlier by many workers (Gupta and Razdan, 2013; Raghu *et al.*, 2011 and Roop Singh, 2017).

Evaluation of antibacterial chemicals

Sl. No.	Chemical Name	Trade Name	Concentration (ppm)
1.	Biomycin	Kasugamycin	100, 500, 1000
2.	Streptocycline	Streptomycin sulphate 90% + tetracycline hydroxide 10%	300, 400, 500
3.	Blue copper/ blitox	Copper oxy chloride	2000, 3000, 4000
4.	Kocide	Copper hydroxide	100, 500, 1000
5.	Strptocycline + Blitox	(Streptomycin sulphate 90% + tetracycline hydroxide 10%) + Copper oxy chloride	300, 400, 500
6.	Plantomycin	Streptomycin sulphate 9% + tetracycline hydroxide 1%	300, 400, 500
7.	Bleaching Powder	40 % Chlorine	1000, 1500, 2000

Table.1 *In vitro* evaluation of botanicals on the growth of *Ralstonia solanacearum*

Sl. No.	Name of the Botanical	Botanical Name	Part/s used	Mean diameter of inhibition zone (mm) at different concentrations			Mean
				5	10	20	
1.	Onion	<i>Allium cepa</i>	Bulb	1.50 (1.58)*	2.47 (1.86)	3.38 (2.09)	2.45
2.	Garlic	<i>Allium sativum</i>	Bulb	4.95 (2.44)	5.85 (2.62)	6.87 (2.80)	5.89
3.	Neem	<i>Azardictra indica</i>	Leaves	8.33 (3.06)	10.17 (3.34)	12.43 (3.67)	10.31
4.	Turmeric	<i>Curcuma longa</i> L	Rhizome	0.00 (1)	0.43 (1.20)	0.50 (1.22)	0.31
5.	Lantana	<i>Lantana camera</i>	Leaves	1.00 (1.41)	3.40 (2.10)	4.00 (2.24)	2.80
6.	Pongamia	<i>Pongamia pinnata</i>	Leaves	0.73 (1.32)	1.77 (1.66)	1.98 (1.73)	1.49
7.	Noni	<i>Morinda citrifolia</i>	Leaves	0.83 (1.35)	1.70 (1.64)	1.83 (1.68)	1.46
8.	Glyricidia	<i>Gliricidia sepium</i>	Leaves	6.43 (2.73)	7.13 (2.85)	8.00 (3.00)	7.19
9.	Nilgiri	<i>Eucalyptus obliqua</i>	Leaves	0.50 (1.22)	1.67 (1.63)	1.90 (1.70)	1.36
10.	Control			0 (1)	0 (1)	0 (1)	
Factors				S.Em±	CD at 1 %		
Botanicals				0.09	0.24		
Concentration				0.03	0.08		
Interaction (B x C)				0.28	0.73		

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

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

Sl No.	Bioagents	Inhibition zone (mm)
1	<i>Trichoderma harzianum</i> (Shivamogga Isolate)	7.57 (2.93)*
2	<i>T. harzianum</i> (Bengaluru Isolate)	7.50 (2.84)
3	<i>T. viride</i> (Shivamogga Isolate)	8.08 (3.01)
4	<i>T. viride</i> (Bengaluru Isolate)	7.96 (2.99)
5	<i>Pseudomonas fluorescens</i> (Shivamogga Isolate)	16.85 (4.22)
6	<i>P. fluorescens</i> (Bengaluru Isolate)	16.49 (4.18)
7	<i>Verticillium lecanii</i>	2.50 (1.87)
8	<i>Bacillus subtilis</i>	11.70 (3.56)
9	Control	0.00
S.Em±		0.15
CD at 1%		0.60
CV		2.58

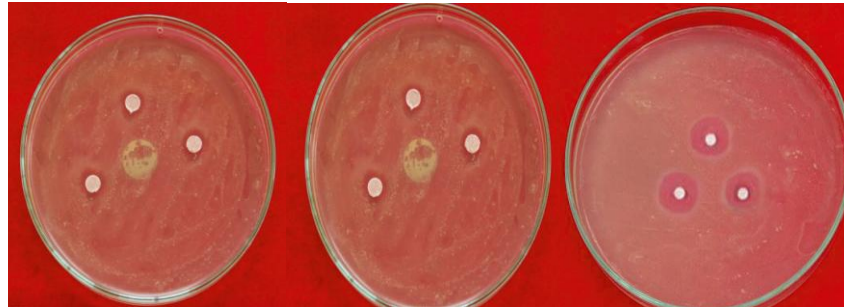
* Figures in the parenthesis are $\sqrt{x+1}$ transformed values.

Table.3 *In vitro* evaluation of antibacterial chemicals on the growth of *R. solanacearum*

Treatment	Concentration (ppm)**	Mean diameter of the inhibition (mm)
Kasugamycin	100	6.05 (2.66)*
	500	6.17 (2.68)
	1000	6.87 (2.80)
Streptocycline	300	16.83 (4.22)
	400	17.90 (4.35)
	500	27.07 (5.30)
Copper oxy chloride	2000	11.27(3.50)
	3000	11.80 (3.58)
	4000	12.30 (3.65)
Copper hydroxide	100	5.96 (2.64)
	500	6.15(2.67)
	1000	6.45 (2.70)
Strptocycline + COC	300 + 3000	17.44 (4.29)
	400 + 3000	18.53 (4.42)
	500 + 3000	29.38 (5.51)
Plantamycin	300	6.56 (2.75)
	400	7.20 (2.86)
	500	8.04(3.01)
Bleaching Powder	1000	1.22 (1.49)
	1500	1.26 (1.50)
	2000	1.30 (1.52)
Control		0.00
S.Em±		0.05
CD at 1%		0.19
CV %		2.6

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

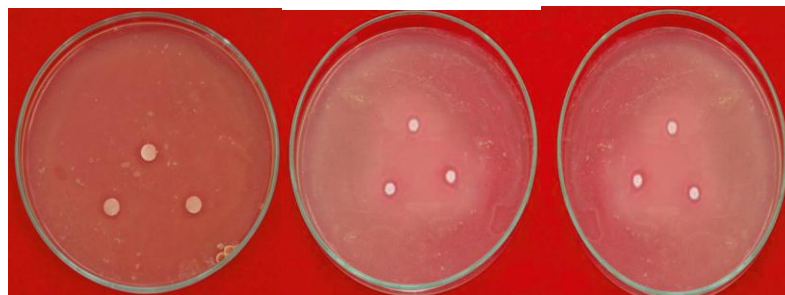
Plate.1 *In vitro* evaluation of botanicals on the growth of *R. solanacearum*



Onion (20 %)

Garlic (20 %)

Neem (20 %)



Turmeric (20 %)

Lantana (20 %)

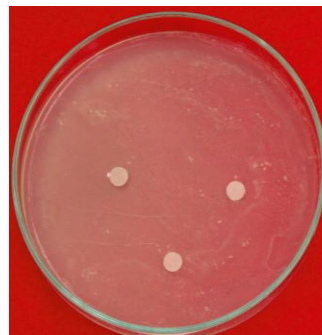
Pongamia (20 %)



Noni (20 %)

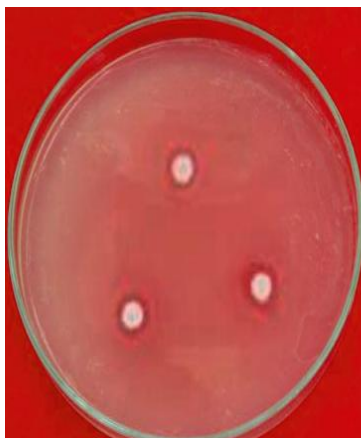
Glyricidia (20 %)

Nilgiri (20 %)



Control (20 %)

Plate.2 *In vitro* evaluation of bioagents on the growth of *R. solanacearum*



Trichoderma harzianum
(Shivamogga isolate)



T. harzianum
(Bangalore isolate)



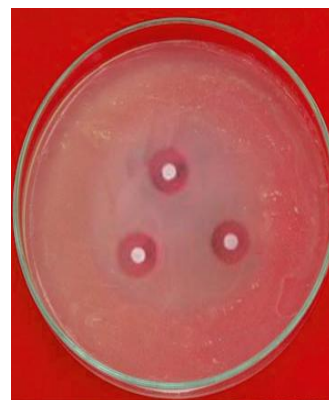
T. viridae
(Shivamogga isolate)



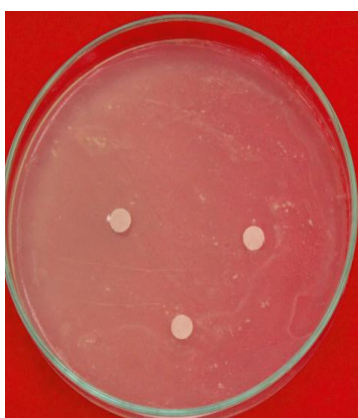
T. viridae
(Bangalore isolate)



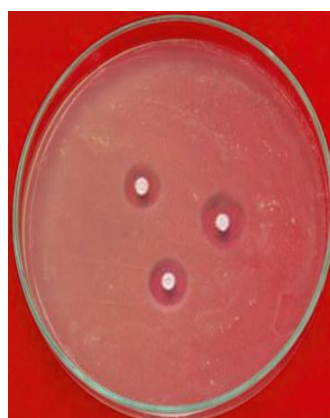
Pseudomonas fluorescens
(Shivamogga isolate)



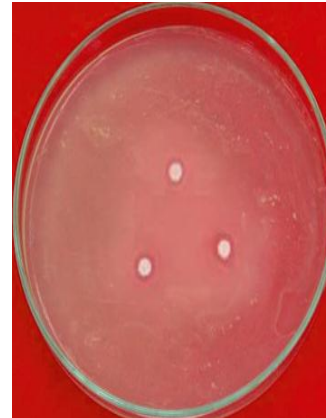
P. fluorescens
(Bangalore isolate)



Verticillium lecani

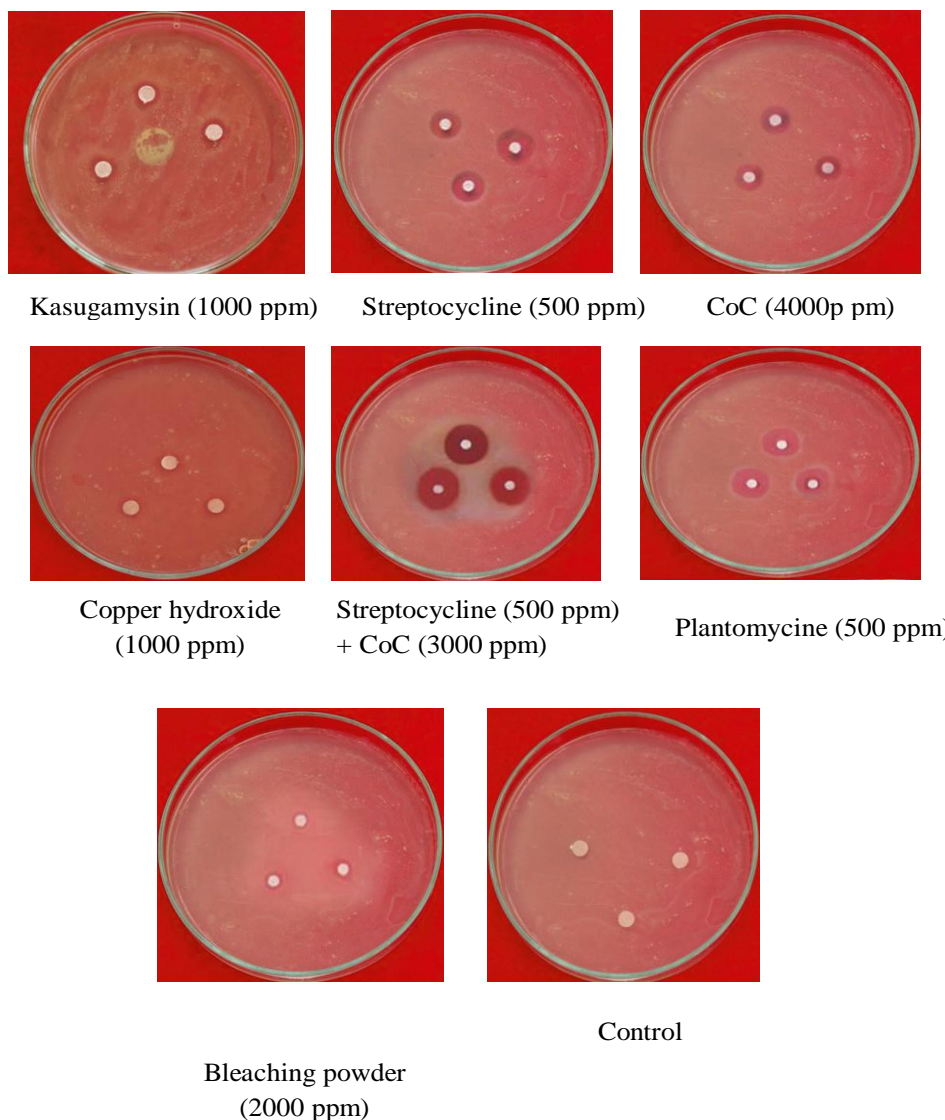


Bacillus subtilis



Control

Plate.3 *In vitro* evaluation of antibacterial chemicals on the growth of *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, streptocycline + copper oxychloride at 500 ppm + 3000 ppm showed the highest inhibition of 29.38 mm followed by Streptocycline 500 ppm (27.07 mm). However all other chemicals *viz.*, kasugamycin, copper oxychloride, copper hydroxide and plantomycin were found effective and which remain on par with each

other. Whereas, bleaching powder was less effective even at higher concentrations. These results are in conformity with the findings of those reported earlier by several workers (Sunder *et al.*, 2011; Gupta and Razdan, 2013; Owoseni and Sangoyomi, 2014, Raghu *et al.*, 2011 and Roop Singh, 2017).

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