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In Vitro Evaluation of Antibacterial Chemicals and Bioagents against *Ralstonia solanacearum* Infecting Bacterial Wilt in Ginger

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A B S T R A C T

An experiment was conducted to find out the effective antibacterial chemicals and bioagents 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 highest average inhibition was recorded in the antibiotic Streptocycline (20.05 mm). This was followed by the antibiotics *viz.*, Gentamycin (17.5 mm), Tetracycline (16.5 mm) and Copper oxychloride + Streptocycline (11.95 mm). All the bioagents evaluated exhibited antibacterial activity against *R. solanacearum*. The antagonistic microorganism *Pseudomonas fluorescens* resulted in maximum inhibition of the *Ralstonia solanacearum* with an inhibition zone of 24.33 mm which was found significantly superior over other treatments. The second and third best antagonists found were *Trichoderma viride* and *Bacillus subtilis* with an inhibition zone of 21.17 mm and 19.33 mm, respectively.

Introduction

Bacterial wilt caused by *R. solanacearum* is deemed to be one of the most important plant diseases in tropical agriculture (Hayward, 1990; Milling *et al.*, 2011). It has a large host range of more than 200 species in 50 families (Aliye *et al.*, 2008). Bacterial wilt disease is one of the major constraints of ginger in small and marginal farming communities. The strain causing bacterial wilt of ginger in India belongs either to biovar 3 or 4; the former being the most virulent in India (Kumar and Sarma, 2004; Kumar and Hayward, 2005). Sambasivam and Girija (2005) reported host resistant and loss in ginger cultivation by *R. solanacearum* in Kerala. Many a times this important cash crop is subjected to premature wilting resulting in 100% crop loss. *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. A Kelman's 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 characteristic symptoms of bacterial wilt of ginger include green leaves roll and curl due to water stress caused by bacteria blocking the water-conducting vascular system of the ginger stems, leaf yellowing and necrosis (Nelson, 2013; White *et al.,* 2013).

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

Materials and Methods

Isolation of *R. solanacearum* from bacterial wilt affected ginger plant 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 (Kelmen, 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).

Pathogenicity test

Pathogenecity test was attempted to established host-pathogen interaction by pseudostem inoculation method (Kumar, 2006). The ginger sprouts were raised by planting 30 g bits of seed rhizomes in steam sterilized standard potting mixture with soil, sand, and FYM in 3:1:1 ratio. Forty five days old plants were used for inoculation and a control treatment without inoculation was maintained.

The pathogenicity was conducted by preparing aqueous suspension of the bacterium grown on CPG or NA broth medium with a concentration of $5x10^8$ cfu/ml. Twenty micro liters of suspension was poured at the base of ginger plants by making injury to the pseudostem of ginger plants in pots. The pots were maintained at 25 per cent moisture holding capacity. The nutrients required for the plant growth were supplied through nutrient solution at an interval of fifteen days. The plants were watered

regularly and observations on appearance of wilt symptoms were recorded. The plants expressing wilt symptoms were selected and bacterium was re-isolated as explained under above isolated pathogen showing typical characteristic of *R. solanacearum* so as to satisfy the Koch's postulate.

In vitro evaluation of antibacterial chemicals

Six antibiotics (each @ 400 and 500ppm), three fungicides (each @ 1500 and 2000 ppm) and two combinations of fungicide + antibiotic [(1000:500) and (1500:500)] by inhibiton zone assay method were evaluated *in vitro* against *R. solanacearum*. The mass multiplied broth culture of the test bacterium (2×10^8 cfu/ml) was seeded to autoclaved Nutrient agar medium, mixed thoroughly and poured into sterilized glass Petri plates allowed to solidify.

The solutions of the desired concentrations of the test antibiotics and fungicides were prepared separately. The filter paper discs (Whatman No. 42) of 5 mm in diameter were soaked separately in the respective chemical solutions for 5-10m minutes and transformed in center onto the solidified bacterium seeded NA medium in Petri plates. The inoculated plates were kept in the refrigerator at 4^0 C for 4 hours to allow diffusion of the chemical into medium. The untreated control plate containing with the test bacterium seeded NA and inoculated with filter paper disc soaked in distilled water was also maintained then the plates were incubated at 28^0 C for 48 hours and observed for the production of inhibition zone around filter paper discs.

In vitro evaluation bioagents/antagonists

Bacterial antagonists

Two isolates of bacterial antagonist's *viz.*, *Pseudomonas fluorescence* and *Bacillus subtilis* collected from Department of Plant Pathology were tested for their efficacy in inhibiting the growth *R. solanacearum* by paper disc method. The virulent isolates *R. solanacerum* was multiplied on Nutrient broth. The 48 hours old culture of *R. solanacerum* containing 2×10^8 cfu/ml was mixed with molten (50°C) Nutrient agar, so as to get a thick lawn of bacteria on the surface of agar medium. The seeded medium poured into sterilized Petri-plates and allowed to solidify. Previously sterilized filter paper (Whatman No. 42) measuring 5 mm in diameter were soaked in different antagonist broth for 10 minutes and placed in the Petri-plates. The excess solution from the filter paper disc was removed by touching slide of the paper discs to the lid of Petri dishes containing broth of the same organism. Then the filter disc was placed in a marked position on the surface of the seeded agar medium. The inoculated plates were incubated at 28±2˚C for 48 hours. The observations for the production of inhibition zone around the filter paper discs were recorded at 24, 48 and 72 hours of incubation respectively. The obtained results were analyzed statistically. Filter paper discs dipped in sterile water served as check.

Fungal antagonists

Six fungal isolates collected from the Department of plant pathology were tested for their inhibitory effect on *R. solanacearum in vitro* by inhibition zone assay method. All the fungal isolates were grown separately on Potato Dextrose Agar. The virulent isolate of *R. solanacearum* was multiplied on Nutrient broth. The 48 hours old culture of *R. solanacearum* containing 2×10^8 cfu/ml was mixed with molten (50°C) sterilized PDA (20 ml), then seeded PDA poured in sterilized Petri-plates and allowed solidify. Fungal discs of 5 diameters from margin of actively growing four days old culture removed and

placed in the center of the plates containing PDA. The plates were incubated at 28±2˚C for 4 days. The observation on the zone of inhibition around the mycelial disc against *Ralstonia solanacearum* was recorded after the incubation period.

Results and Discussion

Isolation of the pathogen

Isolation was made from the soil samples and bacterial ooze (Fig.1) obtained from the infected discolored pseudostem of the plants by serially diluting the bacterial suspension in sterile distilled water and planting on TZC media (Kelman, 1954). Typical virulent colonies of *R. solanacearum* developed within 48 hours. The virulent colonies appeared well-separated, irregular fluidal, dull white colored with slight pink centre (Fig.2) and non-virulent colonies appeared dark red on TZC media (Fig.3). The Colonies were irregular fluidal, creamy white color on Casamino acid peptone glucose agar (CPG) media. The well separated colonies were picked up and purified further by single colony isolation technique and then suspended in sterile distilled water in sterile plastic ependorf tubes and stored at room temperature this served as stock culture for further use. Similar results were also reported (Lemessa and Zeller, 2007A; Chakravarty and Kalita, 2011; Chaudhry and Rashid, 2011; Sagar *et al.*, 2014).

Pathogenicity test

The bacterium was inoculated to the host plant (Ginger) under artificial condition by pseudostem inoculation method in screen house. The inoculated plant showed wilting symptoms 15 days after the inoculation. The isolate was found to be pathogenic to host plant, expressing wilt symptoms. The inoculated plant lost turgidity; leaves started dropping and plant wilted suddenly (Fig.4).

Pathogenicity of *R. solanacearum* causing bacterial wilt was proved earlier by several workers (Winstead and Kelman, 1952; Schell M. A., 2000; Williamson *et al.,* 2002; Rajan *et al.,* 2002; Kumar and Sarma, 2004; Umesha *et al.,* 2005; Hikichi, 2007; Rashmi *et al.,* 2012; Artal, 2012; Thomas and Upreti, 2014; Zulperi *et al.,* 2014). Kumar A. (2006) proved the pathogenicity of *R. solanacearum* using susceptible ginger cultivar '*Himachal*'. Mathews *et al.,* (2008) also proved the pathogenicity of *R. solanacearum* using ornamental ginger species, the final pathogenicity assessment was recorded 21 DAI.

In vitro evaluation of antibacterial chemicals against *R. solanacearum*

Present investigation was carried out to evaluate antibacterial chemicals to find out their effectiveness against the growth of *R. solanacearum* under *in vitro* condition and the results were presented in Table 1 and Fig. 5.

Total six antibiotics *viz.,* Sreptocycline, Cephalexin, Neomycin, Tetracycline, Dicrystacin, Gentamycin, and three antibacterial fungicides *viz.,* Blitox (Copper oxy chloride) Kocide (Copper hydroxide) and Amistar individually and in combination Blitox + Streptocycline, Blitox + Tetracycline were evaluated *in vitro* by inhibition zone assay method against *R. solanacearum.*

Antibiotics

At 400 ppm, inhibition was ranged from 6.9 mm (Dicrystacin) to 18.4 mm (Streptocycline). However, significantly highest inhibition was recorded in the antibiotic Streptocycline (18.4 mm). This was followed by the antibiotics *viz.,* Gentamycin (15.4 mm), Tetracycline (14.2 mm) and Neomycin (8.1 mm). Antibiotics, Cephalexin

and Dicrystacin were found less effective with 7.5 and 6.9 mm inhibition, respectively.

At 500 ppm, inhibition was ranged from 7.0 mm (Dicrystacin) to 21.7 mm (Streptocycline). However, significantly highest inhibition was recorded in the antibiotic Streptocycline (21.7 mm). This was followed by the antibiotics *viz.,* Gentamycin (19.6 mm), Tetracycline (18.8 mm) and Neomycin (8.3 mm). Antibiotics, Cephalexin and Dicrystacin were found less effective with 7.6 and 7.0 mm inhibition, respectively.

Average inhibition was ranged from 6.95 mm (Dicrystacin) to 20.05 mm (Streptocycline). However, significantly highest average inhibition was recorded in the antibiotic Streptocycline (20.05 mm). This was followed by the antibiotics *viz.,* Gentamycin (17.5 mm), Tetracycline (16.5 mm) and Neomycin (8.2 mm). Antibiotics, Cephalexin and Dicrystacin were found less effective with 7.55 and 6.95 mm inhibition, respectively.

Antibacterial fungicides

At 1500 ppm, inhibition was ranged from 6.1 mm (Copper hydroxide) to 11.9 mm (Copper oxychloride + Streptocycline). However, significantly highest inhibition was recorded in the combination of antibacterial fungicide and antibiotic Copper oxychloride + Streptocycline (11.9 mm). This was followed by the antibacterial fungicides *viz.,* Copper oxychloride (10.6 mm), Copper oxychloride + Tetracycline (10.5 mm). Antibacterial fungicides, Amistar and Copper hydroxide were found less effective with 7.2 and 6.1 mm inhibition, respectively. At 2000 ppm, inhibition was ranged from 6.3 mm (Copper hydroxide) to 12.0 mm (Copper oxychloride + Streptocycline). However, significantly highest inhibition was recorded in the combination of antibacterial fungicide and antibiotic Copper oxychloride + Streptocycline (12.0 mm). This was followed by the antibacterial fungicides *viz.,* Copper oxychloride (11.6 mm), Copper oxychloride + Tetracycline (10.7 mm). Antibacterial fungicides, Amistar and Copper hydroxide were found less effective with 7.3 and 6.3 mm inhibition, respectively.

Average inhibition was ranged from 6.2 mm (Copper hydroxide) to 11.95 mm (Copper oxychloride + Streptocycline). However, significantly highest average inhibition was recorded in the combination of antibacterial fungicide and antibiotic Copper oxychloride + Streptocycline (11.95 mm). This was followed by the antibacterial fungicides *viz.,* Copper oxychloride (11.1 mm), Copper oxychloride + Tetracycline (10.6 mm). Antibacterial fungicides, Amistar and Copper hydroxide were found less effective with 7.25 and 6.2 mm inhibition, respectively.

Thus, all the antibiotics/antibacterial chemicals tested were found effective against *R. solanacearum*. However, antibacterial chemicals found most effective in the order of merit were Streptocycline, Gentamycin, Tetracycline, Copper oxychloride + Streptocycline, Copper oxychloride, Copper oxychloride + Tetracycline, Neomycin, Cephalexin, Amistar and Dicrystacin.

These results are in conformity with the findings of those reported earlier by several workers (Hidaka and Murano, 1956; Dutta and Verma, 1969; Indersenan *et al.,* 1981; Khan *et al.,* 1997; Singh *et al.,* 2000; Devnath *et al.,* 2002; Dubey, 2005; Sunder *et al.,* 2011; Gupta and Razdan, 2013; Owoseni and Sangoyomi, 2014).

In vitro evaluation of bioagents/antagonists against *R. solanacearum*

The six fungal antagonistic microorganism's *viz., Trichoderma harzianum*, *Trichoderma*

viride, *Trichoderma koningii*, *Gliocladium virens*, *Trichoderma longibrachiatum*, *Aspergillus niger* and two bacterial antagonistic microorganisms *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated against *R. solanacearum* under *in vitro* condition by inhibition zone method as explained in the material and methods.

Table.1 *In vitro* evaluation of antibacterial chemicals against *R. solanacearum*

Tr. No.	Antibiotics	Inhibition zone (mm)* Concentration at		
		400 ppm	**500 ppm**	**Av. (mm)**
T_1	Sreptocycline	18.4	21.7	20.05
T_2	Cephalexin	7.5	7.6	7.55
T_3	Neomycin	8.1	8.3	8.2
T_4	Tetracycline	14.2	18.8	16.5
T_5	Dicrystacin	6.9	7.0	6.95
T_6	Gentamycin	15.4	19.6	17.5
	Antibacterial fungicides	**Inhibition zone (mm)* Concentration at**		
		1500 ppm	**2000 ppm**	**Av. (mm)**
T_7	Blitox (Copper oxy chloride)	10.6	11.6	11.1
T_8	Kocide (Copper hydroxide)	6.1	6.3	6.2
T_9	Amistar (Azoxystrobin)	7.2	7.3	7.25
T_{10}	Blitox + Streptocycline	11.9	12.0	11.95
T_{11}	Blitox + Tetracycline	10.5	10.7	10.6
T_{12}	Control (Untreated)	0.0	0.0	0.00
S.E. ±		**0.27**	**0.31**	-
CD (P 0.01)		**0.80**	**0.92**	-

*-Mean of three replications

Table.2 *In vitro* evaluation of biocontrol agents against *R. solanacearum*

Tr. No.	Treatments	Inhibition zone (mm) *
T_1	*Trichoderma viride*	21.17
T_2	*T. harzianum*	16.33
T_3	*Gliocladium virens*	9.67
T_4	*T. koningii*	8.33
T_5	*T. longibrachiatum*	7.67
T_6	*Pseudomonas fluorescence*	24.33
T_7	*Bacillus subtilis*	19.33
T_8	*Aspergillus niger*	10.33
T_9	Control (untreated)	0.00
S.E. ±		**0.58**
CD (P 0.01)		**1.73**

*-Mean of three replications

Fig.1&2 Bacterial ooze from pseudostem of wilted ginger plant and Virulent colonies of *R. solanacearum* on TZC media

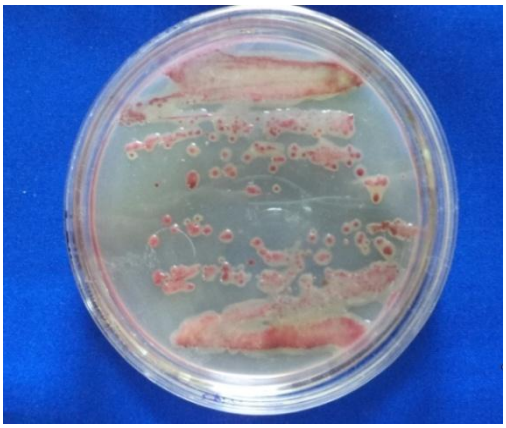


Fig.3 & 4 A virulent colonies of *R. solanacearum* on TZC media and Pathogenicity test of *R. solanacearum* on ginger

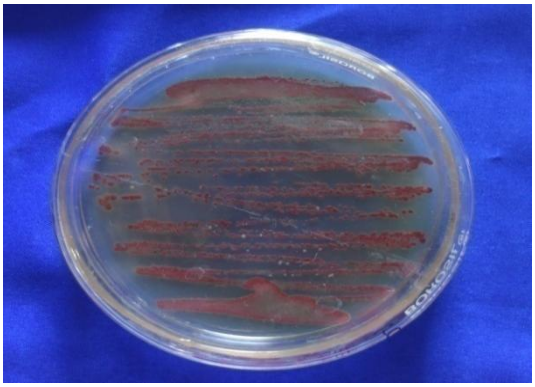


Fig.5 *In vitro* evaluation of antibacterial chemicals against *R. solanacearum*

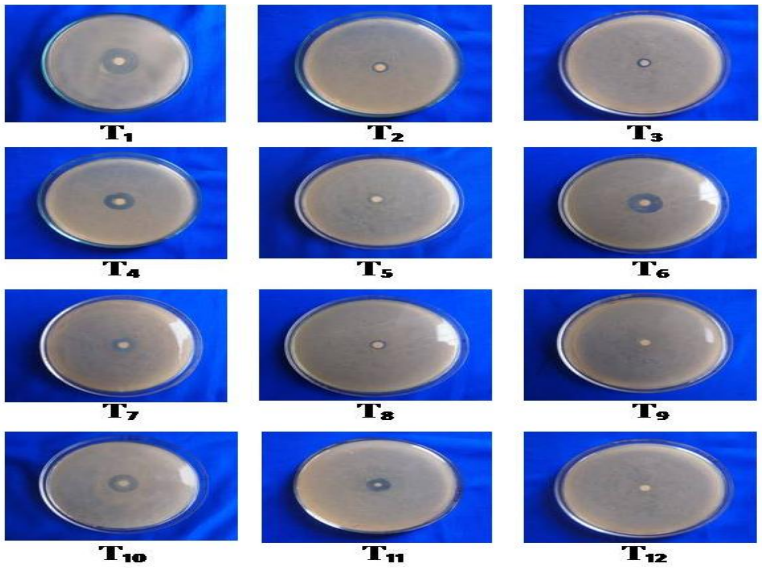
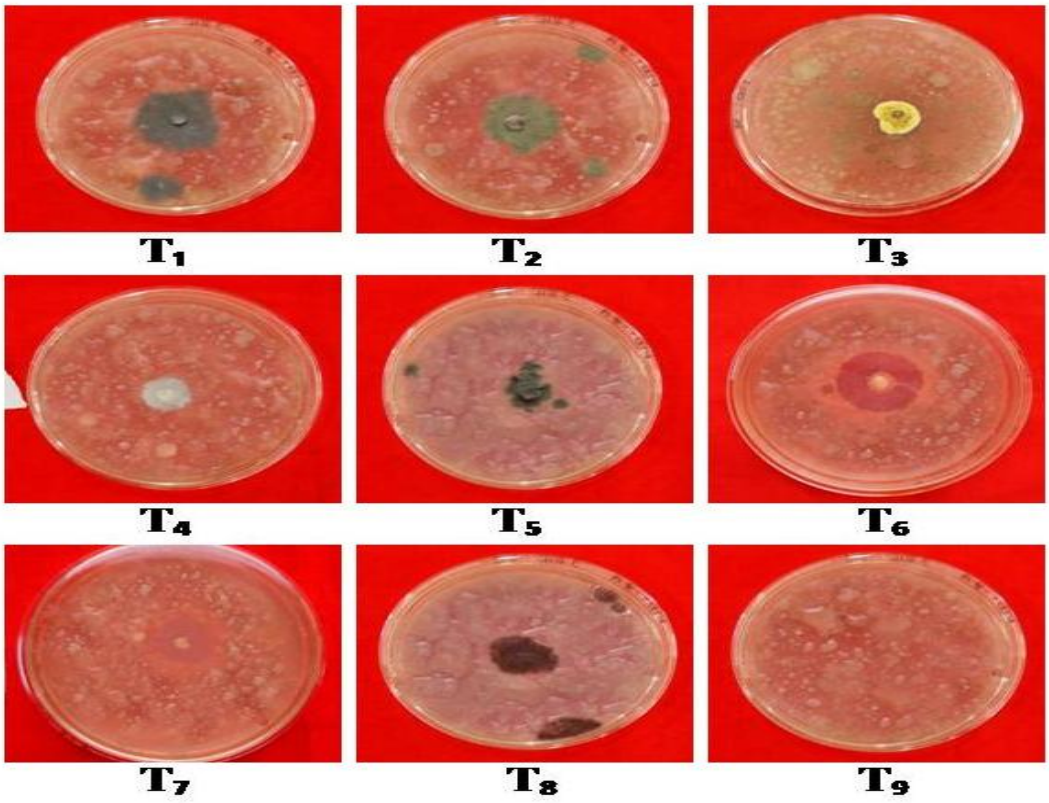


Fig.6 *In vitro* evaluation of bioagents against *R. solanacearum*



The results obtained on inhibition zone produced across the antagonistic microorganisms are presented in Table 2 and Fig.6. Results revealed that all the bioagents evaluated exhibited antibacterial activity against *R. solanacearum*. The results indicated that the antagonistic microorganism *P. fluorescens* resulted in maximum inhibition of the *Ralstonia solanacearum* with an inhibition zone of 24.33 mm which was found significantly superior over other treatments. The second and third best antagonists found were *T. viride* and *Bacillus subtilis* with an inhibition zone of 21.17 mm and 19.33 mm, respectively. This was followed by *T. harzianum* (16.33 mm) and *A. niger* (10.33). Whereas, the antagonists like, *G. virens*, *T. koningii* and *T. longibrachiatum* were moderately effective with slight inhibition zone of 9.67 mm, 8.33 mm and 7.67 mm, respectively. Bioagents *viz.*, *P. fluorescens* and *B. subtilis* were reported efficient antagonists against *R. solanacearum* earlier by many workers (Guo *et al.*, 2001; El-Sayed *et al.*, 2003; Sun *et al.*, 2004; Lemessa and Zeller, 2007 B; Henok *et al.*, 2007; Liza and Bora, 2008; Vanita *et al.*, 2009; Liza and Bora, 2009; Maketon *et al.*, 2010; Choudhry and Rashid, 2011; Khair *et al.*, 2012; Yang *et al.*, 2012; Gupta and Razdan, 2013; Raghu *et al.*, 2013). The species of *Trichoderma viz.*, *viride* and *harzianum* were reported as efficient antagonists against *R. solanacearum* (Ramesh, 2006; Liza and Bora, 2009; Chaudhry and Rashid, 2011, Narsimbha and Srinivas, 2012; Gupta and Razdan, 2013; Raghu *et al.*, 2013).

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