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Development and evaluation of native biocontrol microbial consortia for effective management of *Ralstonia solanacearum* of ginger

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Abstract
As many as 4 *Pseudomonas fluorescens* and 4 *Trichoderma* isolates were isolated from the soil samples collected from the ginger rhizosphere and the pathogen *Ralstonia solanacearum* were also isolated for the infected rhizomes of ginger and used for further studies. *In in vitro* dual culture screening studies, the *Pseudomonas fluorescens*- 4 showed maximum inhibitions of 55.00 per cent and *Trichoderma* sp - 4 showed 60.70% of inhibition over control *Ralstonia solanacearum.* Further, the efficient biocontrol consortia of *Pseudomonas fluorescens*- 4 and *Trichoderma* sp- 4 were developed and evaluated against *Ralstonia solanacearum* under greenhouse condition in combination with antibiotic [Streptocyclin] at the rate of 0.5 g and fungicide [Copper oxychloride] 3g/l and the percentage of *Ralstonia solanacearum* causing wilt disease incidence of ginger was recorded at 15 days interval. The incidence of *Ralstonia solanacearum* wilt was found to be severe in the pathogen control (80.33%) were the direct inoculum of pure culture of *Ralstonia solanacearum* was imposed. However the treatments receiving the combined inoculation of Streptocyclin, Copper oxychloride, *Pseudomonas fluorescens*- 4 and *Trichoderma*- 4 recorded less percent disease incidence was 20% at the end of crop indicating the integrated use of chemical control along with the effective biocontrol consortia.

Keywords: Ginger, *Ralstonia solanacearum*, biological control, microbial consortia

Introduction
Green revolution is one of the biggest success stories of India globally, which enabled the country to convert the "begging bowl" status to that of "self-sufficiency" in agricultural production. It also brought about an element of resistance in agriculture toward off the threat of famines. The green revolution obviously ushered in an era of overall rural prosperity. Its impact was so dramatic that India was a model for many developing nations. Success always has its costs and the green revolution has been no exception. With the onset of green revolution, the use of synthetic chemical insecticides increased phenomenally contributing to substantial yield increase for some time, but boomeraged with several ecocidal consequences as documented by Richael Carson in Silent Spring like insecticide resistance, resurgence of crop pests, poisoning of bees, birds, fish *etc.*, Now a days, biological means for production of agricultural commodities is gaining lot of importance, among biological means; microorganisms an integral component of soil ecosystem play a prestigious role by making the soil truly living. These organisms have evolved many mechanisms such as antibiosis, competition, parasitism, resistance induction in plants, N_2 fixation, phosphorus solubilisation and phosphorus mobilization *etc.*, to provide effective disease suppression and plant growth promotion.

Ginger (*Zingiber officinale Rosc*) (Family: *Zingiberaceae*) is an herbaceous perennial crop, the rhizomes of which are used as a spice. The ginger has a spicy yet aromatic taste and smell. The strong taste is due to the fact that it contains a mixture of phenolic compounds and essential volatile oils. It is widely used as a spice in foods and because of its medicinal qualities, has been used in medication too. India is a leading producer of ginger in the world. Ginger is cultivated in most of the states in India. However, states *viz.*, Kerala, Meghalaya, Karnataka, Arunachal Pradesh, Sikkim, Nagaland and Orissa together contribute 70 per cent to the country's total production though the area under ginger has been increasing every year; the productivity is declining due to imbalanced use of chemical fertilizers, poor quality of water and higher incidence of pests and diseases. Among many factors and constrains responsible for low yields of ginger, the diseases are the major ones.

The ginger crop is affected by many fungal and bacterial plant pathogens throughout its life cycle. Among the major diseases of ginger, the bacterial wilt is divesting diseases during the crop stand which reduces the yield more than 30 to 40 per cent.

In bacterial wilt caused by *Ralstonia solanacearum*, water soaked patches or linear steaks were observed on the collar region of the pseudostem. Later, leaves became flaccid with intense yellowish bronze colour noticed and such plants dropped down. The leaves showed rollup symptoms and the whole plant dried up. Pseudostem came off easily with a gentle pull such rhizomes when pressed bacterial exudates oozes out [17].

In order to control bacterial wilt complex diseases and to increase yields of ginger, farmers are using many of the bactericides and fungicides throughout the year of cultivation. However, increased concern for environmental awareness of chemical hazards has evoked a worldwide interest in biofertilizer and microbial control of plant pathogens. In this context, many microorganisms have been exploited as biocontrol agents for the management of plant pathogens and a number of them have been registered and/or are commercially available for use against pathogens. Among many biocontrol agents the *Trichoderma* spp. *Pseudomonas fluorescens* and *Bacillus subtilis* are the most commonly used ones and have long been known as effective antagonists against plant pathogens. These bioagents are found in all agricultural soils and are easy to isolate and mass multiply the affect wide range of plant pathogens. In spite of enormous scientific literature on biological control of plant pathogens with *Trichoderma* spp. *Bacillus* spp. and *Pseudomonas* spp. are the most effective species against wide range of ginger pathogens.

Trichoderma spp. has been found as an effective Biocontrol Agents (BCA) against many soil borne pathogens [7] *Trichoderma* controls pathogens in an indirect way by producing several groups of antibiotics that inhibit the growth of pathogen. Apart from that, there are direct methods showing antagonism against the pathogen which is called mycoparasitism. *Trichoderma* species can also inhibit or reduce the growth of plant pathogens especially fungi, through competition for space, enzyme substrates, nutrients and oxygen [16].

Fluorescent *Pseudomonas* often predominate among the bacteria of plant rhizosphere and some can have beneficial effects on plants, either by direct stimulation of plant growth of by exerting antagonism towards soil borne pathogens [20,14].

Based on the past work done by different researchers and in view of greater need for developing microbial consortia for biological management of *Ralstonia solanacearum* in Ginger the present investigation under taken.

Materials and Methods

The present investigation was conducted in the Department of Agricultural Microbiology, College of Agriculture, Shivamogga. The details of materials and methodology followed during the course of investigation are highlighted herein.

Isolation of pathogen

Rhizome samples showing typical symptoms were collected during survey and used for isolation of *Ralstonia solanacearum* using Nutrient Agar media containing 0.1% of 2,3,5 Triphenyle tetrazolium chloride [11].

Collection of soil sample

Rhizosphere soil was collected from ginger plants in Savalanga area for isolation of *Pseudomonas fluorescens* and *Trichoderma* sp.

Isolation of *Pseudomonas fluorescens* and *Trichoderma sp.*

The rhizosphere soil were serially diluted and plated on specific media *viz.*, King's B media for *Pseudomonas fluorescens* and *Trichoderma* selective media for isolation of *Trichoderma* and the colonies showing the characteristic of fluorescence under UV transilluminator were selected and confirmed as *Pseudomonas fluorescens* and the fungal colonies showing the uniform concentric green sporulation were selected and confirmed as *Trichoderma* and the pure cultures of both *Trichoderma* and *Pseudomonas fluorescens* were used for further studies.

Characterization of *Ralstonia solanacearum*, *Pseudomonas fluorescens* and *Trichoderma sp.*

The *Ralstonia solanacearum* and *Pseudomonas fluorescens* were identified and characterized based on various morphological and biochemical characteristics [2, 3]. Whereas for characterization of *Trichoderma sp.* the isolates showing the circular ring sporulation were observed based on initial mycelial colour, spore colour, mycelial growth and shape of the conidia. For microscopic observations specimens were prepared according to the sticky tape method [8].

In-vitro screening of *Trichoderma* and *Pseudomonas fluorescens*

The pure culture of *Trichoderma* and *Pseudomonas fluorescens* were evaluated for their antagonistic effect against *Ralstonia solanacearum* by dual culture method [6]. The extent of antagonistic activity by bioagents *i.e.*, growth after contact with *Ralstonia solanacearum* was recorded on 5th day by measuring the growth of *Ralstonia solanacearum*, pathogen in dual culture plate and control plate. The% inhibition of *Ralstonia solanacearum* was calculated using the formula [19].

$$I = \frac{(C-T)}{C} \times 100$$

Where,

I = Per cent inhibition

C = Growth of *Ralstonia solanacearum* control plate (mm)

T = Growth of *Ralstonia solanacearum* in dual culture plate (mm)

Compatibility studies and development of biocontrol microbial consortia

Dual culture method was followed by using solidified PDA plates [6]. Antagonistic bacteria was streaked on one side of the petriplates, similarly antagonistic fungi was placed on the other side of the petriplates at an angle of 180^0 and incubated at 28 ± 2 ^{0}C for 2-3 days. Further based on the compatibility results the effective liquid biocontrol microbial consortium is formulated for *in vivo* studies [18].

In vivo evaluation of biocontrol agents for antagonism against bacterial wilt of ginger

The best isolates tested from the *in vitro* studies were evaluated against bacterial wilt pathogen of the ginger plant under greenhouse condition.

1. Fungal biocontrol agents: *Trichoderma sp.* – 4
2. Bacterial biocontrol agents: *Pseudomonas fluorescens* – 4
3. Pathogen: *Ralstonia solanacearum*

Greenhouse evaluation

A pot experiment was conducted under greenhouse condition at Department of Agricultural Microbiology, College of Agriculture, Shivamogga to evaluate antagonistic effect of selected fungal and bacterial biocontrol microbial consortia against bacterial wilt of ginger.

Experimental details

1. **Crop:** Ginger
2. **Soil:** Black Soil
3. **Treatments:** 5
4. **Replication:** 3 (in each replication three plants were maintained separately one each in pots)
5. **Details of treatments imposed:**

   T1 = Absolute Control
   T2 = Streptocycline @ 0.5 g + Copper oxychloride (3g/lit)
   T3 = Streptocycline @ 0.5 g + Copper oxychloride (3g/lit) + *Trichoderma sp.* – 4
   T4 = Streptocycline @ 0.5 g + Copper oxychloride (3g/lit) + *Pseudomonas fluorescens* – 4
   T5 = Streptocycline @ 0.5 g + Copper oxychloride (3g/lit) + *Trichoderma sp.* – 4 + *Pseudomonas fluorescens* – 4

During experimentation the incidence of disease was recorded at 15 days interval for 5 days based on the mortality of plants and from the observation recorded per cent disease incidence was calculated using the formulae given below

$$\text{Per cent disease incidence} = \frac{\text{No. of plants rotted (Diseased) X 100}}{\text{Total number of plants}}$$

Results and Discussion

A detailed survey was conducted to know the disease severity of *Ralstonia solanacearum* and also to isolate the pathogen from the infected fields of Ginger growing area of Savalanga village, Shivamogga district. As many as 6 infected rhizome samples and 7 soil samples were collected and brought to the laboratory under aseptic condition and used for further isolation purpose (Plate 1).

Plate 1: Ginger rhizosphere soli and *Ralstonia solanacearum* infected rhizome

Isolation and characterization of *Ralstonia solanacearum*

Out of six infected rhizomes collected the *Ralstonia solanacearum* was isolated using specific nutrient agar media supplemented with 0.1 per cent of 2,3,5 Triphenyle tetrazolium chloride salt (Plate 2). The results are in agreement with the findings of [11]. Further, the *Ralstonia solanacearum* isolated were characterized based on morphological and biochemical characters as described by [11]. The *Ralstonia solanacearum* isolate produced fluidal, dull white, convex, round to irregular pink colonies on medium after 24 hours and the cells are Gram negative rods and found positive to casein hydrolysis, indole production, Vogesproskauer's test, citrate utilization, starch hydrolysis and acid and gas production. Whereas it was negative to catalase test, H_2S production, methyl red and KOH test, urease activity and gelatin liquefaction (Table 1). The results are in conformity with the findings of [12] who isolated and characterized the *Ralstonia solanacearum* from the soil and infected plant parts.

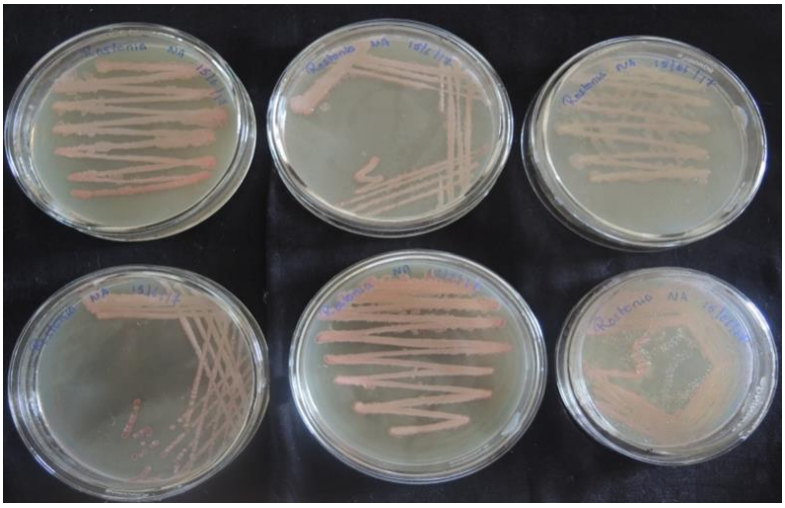


Plate 2: Growth of *Ralstonia solanacearum* on nutrient agar media supplemented with 0.1% TTC

Table 1: Morphological and biochemical characteristics of *Ralstonia sp.*

Organism	Morphological tests		Biochemical Tests												
	Colony	Gram's reaction and cells shape	CH	IP	VP	CU	SH	AG	CT	H2S	MR	KOH	UA	GL	PG
Bacteria	Fluid, dull white convex round to irregular pink colonies	G -ve Rods	+	+	+	+	+	+	-	-	-	-	-	-	*Ralstonia* sp.

Note:
CH= Casein hydrolysis test
IP= Indole production test
VP= Vogesproskauer's test
CU= Citrate Utilization test
SH= Starch Hydrolysis test
AG= Acid and Gas production test
CT= Catalase test
H_2S= Hydrogen sulphide production test
MR= Methyl red test
KOH= Potassium hydroxide test
UA= Urease activity test
GL = Gelatin Liquefaction test

Isolation and characterization of *Trichoderma and Pseudomonas fluorescens*

Out of seven samples collected, as many as 4 *Trichoderma* and 4 *Pseudomonas fluorescens* isolates were obtained and further all the isolates were characterized and confirmed as *Trichoderma* and *Pseudomonas fluorescens* by morphological, biochemical and microscopic characters (Table 2 & 3 plate 3). The results get support from the findings of [9, 15, 4] who isolated and characterized *Trichoderma* and *Pseudomonas fluorescens* from different soil and infected parts.

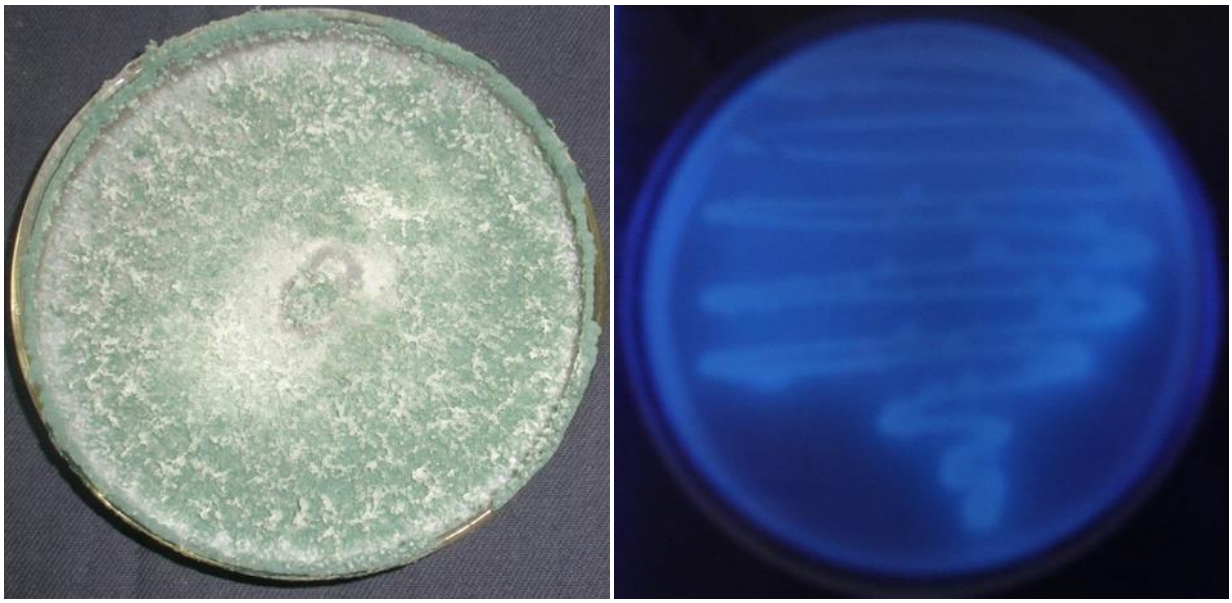
Table 2: Morphological and biochemical characteristics of *Pseudomonas fluorescens*

Isolate number	Morphological tests		Biochemical Tests												
	Colony	Gram's reaction and cells shape	*Fluorescens* under UV light	CH	IP	VP	CU	SH	AG	CT	H2S	KOH	UA	GL	PG
Pf - 1	White convex round colonies	G –ve small rods	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
Pf - 2	Cream round to irregular colonies	G –ve Medium rods	+	-	-	+	+	+	+	-	-	-	-	+	*P. fluorescens*
Pf - 3	Cream convex round colonies	G –ve Small rods	+	-	-	+	+	+	+	-	-	-	-	+	*P. fluorescens*
Pf - 4	White convex round colonies	G –ve Medium rods	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*

Note:
CH= Casein hydrolysis test
IP= Indole production test
VP= Vogesproskauer's test
CU= Citrate Utilization test
SH= Starch Hydrolysis test
AG= Acid and Gas production test
CT= Catalase test
H_2S= Hydrogen sulphide production test
KOH= Potassium hydroxide test
UA= Urease activity test
GL = Gelatin Liquefaction test

Table 3: Microscopic and Morphological characteristics of *Trichoderma* isolates

Sl No.	Isolates	Initial mycelia colour	Spore colour mycelia growth characters	Conidial morphology	Microscopic appearance
1	*Tri-1*	White	Dark green with profused mycelia growth	Shape of conidia and conidiospore are highly branched and branching pattern of phialides	Medium growth with little sporulation
2	*Tri-2*	White	Pale green with little mycelia	Shape of conidia and conidiophores are highly branched and branching pattern of phialides	Less growth with little sporulation
3	*Tri-3*	White	Light green with concentric sporulation	Shape of conidia and conidiophores are highly branched and branching pattern of phialides	Less growth with little sporulation
4	*Tri-4*	White	Dark green with Vigorous mycelia growth with high sporulation	Shape of conidia and conidiophores are highly branched and branching pattern of phialides	Rapid growth, bright green or white conidial pigments, and a repetitive branched, but otherwise poorly defined conidiospore structure



Trichoderma – 4 Pseudomonas fluorescens - 4

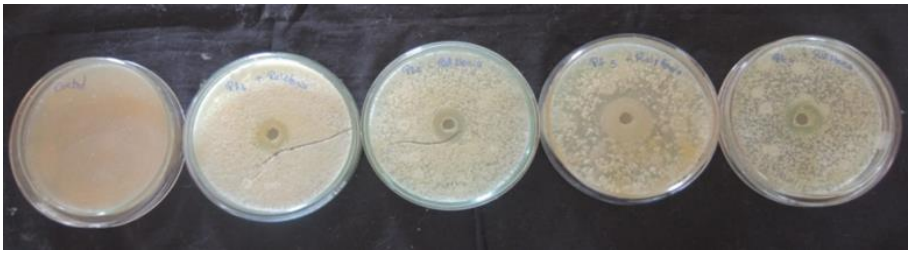
Plate 3: Efficient *Trichoderma* - 4 and *Pseudomonas fluorescens - 4* isolates isolated from ginger rhizosphere soil

In vitro* screening of bioagents against *Ralstonia solanacearum

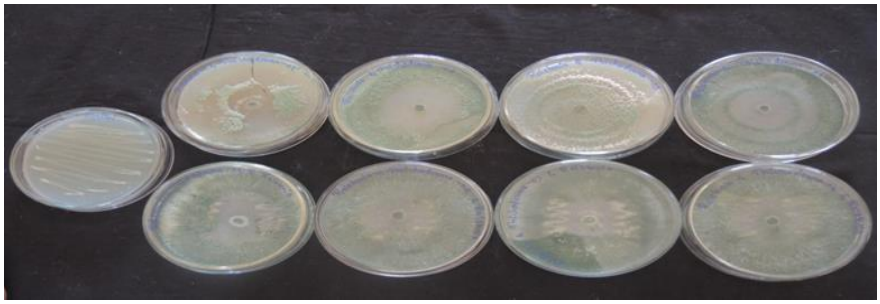
The data on inhibition of *Ralstonia solanacearum* growth by *Trichoderma* and *Pseudomonas fluorescens* isolates in dual cultural experiment are presented in table 4 and plate 4. Out of 4 different native *Trichoderma* isolates tested, the per cent inhibition ranged from 55.20 to 60.70% and statistically the highest per cent inhibition of *Ralstonia solanacearum* of 60% was recorded in the plates where the *Trichoderma* – 4 (Tri - 4) was used on the other hand the inhibition range of 50.20 to 55.00 was observed with *Pseudomonas fluorescens* isolates. However out of 4 *Pseudomonas fluorescens* isolates tested, the *Pseudomonas fluorescens* – 4 (Pf - 4) showed maximum per cent inhibition of *Ralstonia solanacearum* was recorded (55.00%). Hence, the Tri – 4 and Pf – 4 was selected for further *In vivo* evaluation against *Ralstonia solanacearum* under greenhouse condition. The results are in agreement with the finding of [5] who studied the antagonistic activity of *T. viridae*, *Pseudomonas fluorescens* and *Bacillus subtilis* against some soil borne fungal pathogens.

Table 4: Inhibition of growth of *Ralstonia solanacearum* by antagonistic *Pseudomonas fluorescens* and *Trichoderma isolates* in dual culture experiment

Sl. No.	*Pseudomonas fluorescence*	% inhibition over control *Ralstonia solanacearum*	*Trichoderma* isolates	% of inhibition over control *Ralstonia solanacearum*
1	*Pf 1*	50.20	*Tri 1*	55.20
2	*Pf 2*	51.00	*Tri 2*	56.20
3	*Pf 3*	52.00	*Tri 3*	58.60
4	*Pf 4*	55.00	*Tri 4*	60.70



Pseudomonas fluorescence against *Ralstonia solanacearum*



Trichoderma against *Ralstonia solanacearum*

Plate 4: *In-vitro* screening of *Pseudomonas fluorescence* and *Trichoderma* isolates against *Ralstonia solanacearum*

Development of biocontrol microbial consortia

Compatibility evaluation

The dual culture method was followed to know the compatibility among the bioagents. In dual culture experiments both the bioagents (Tri – 4 and Pf - 4) was compatible as they grew independently without affecting each other (Plate 5). The investigations are in accordance with the results of [13] who studied the compatibility between fungal and bacterial bioagents on solid as well as liquid media.

Plate 5: Compatibility evaluation of *Pseudomonas fluorescence* - 4 and *Trichoderma* - 4 in dual culture plate

Development and evaluation of effective biocontrol consortia

Based on the compatibility analysis the liquid formulations of biocontrol microbial consortia were formulated and the populations of 10^8 CFU/ml of samples were maintained and were used for further greenhouse studies. In pot studies the pure culture of *Ralstonia solanacearum* was inoculated to all the ginger plants to get disease and further the effective biocontrol microbial consortia were added in combinations with Streptomycin @ 0.5g/l and Copper oxychloride @ 3 g/l and the disease incidence was recorded at 15 days intervals and the per cent disease incidence was calculated. Out of 5 treatments imposed, the maximum of 80.33% disease incidence was recorded in the absolute control where the pure culture of *Ralstonia solanacearum* was imposed whereas, the least percent disease incidence of only 20% was recorded in the treatments receiving the pure cultures of *Ralstonia solanacearum* along with Streptomycin @ 0.5g/l, Copper oxychloride @ 3 g/l, *Trichoderma* – 4 and *Pseudomonas fluorescens – 4* indicating the integrated disease management practices is effective in management of *Ralstonia solanacearum* under greenhouse condition (Table - 5). The salient findings are in agreement with the findings of [10], who concluded the integration of physical, chemical and biological methods are effective in controlling the plant pathogens in field condition. Similarly, the treatment of Rhizomes with Streptocycline @ 0.5 g/l, Copper oxychloride @3 g/l neem cake and bioagents effectively managed the disease incidence of Ginger [1]. Scales up studies are required to use the efficient treatment of the study in the field condition using farmers participatory approach.

Table 5: *In vivo* evaluation of effective biocontrol microbial consortia against *Ralstonia solanacearum*.

Sl. No.	Treatments	Treatment details	% Disease incidence
1	T_1	Control	80.33 [a]
2	T_2	Streptocyclin @ 0.5 g + copper oxychloride (3g/lit)	50.00 [c]
3	T_3	Streptocyclin @ 0.5 g + copper oxychloride (3g/lit) + *Trichoderma - 4*	55.00 [c]
4	T_4	Streptocyclin @ 0.5 g + copper oxychloride (3g/lit) + *Pseudomonas fluorescens - 4*	58.00 [c]
5	T_5	Streptocyclin @ 0.5 g + copper oxychloride (3g/lit)+*Trichoderma – 4 +Pseudomonas fluorescens - 4*	20.00 [d]
SEM ± CD @ 0.01%			3.01 8.27

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