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RESEARCH ARTICLE

Effect of combined application of biofumigant, *Trichoderma harzianum* and *Pseudomonas fluorescens* on *Rhizoctonia solani* f.sp. *sasakii*

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Abstract
Banded leaf and sheath blight disease of maize is incited by *Rhizoctonia solani* f. sp *sasakii* (RSS) and it can be effectively managed by biointensive management package, such as use of biofumigation of soil with mustard plant material in combination with potential *P. fluorescens* and *Trichoderma harzianum* for enhanced yields. The viability of RSS inoculum decreased in all the biofumigation treatments as compared to untreated control. The decrease in the viability ranged from 81.7 to 99%. Maximum reduction in the viability was recorded in treatment biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens*. The radial growth of the inoculum in different treatments showed significant differences when compared to control which ranged from 44.7 to 90 mm. Total bacteria, *Trichoderma* spp. and *Pseudomonas* spp. population were high in all bio-fumigant treatments 50 days after incorporation of mustard plant material in both green house and field studies.

Keywords Biocontrol agents · BLSB · Maize · Total bacteria · Total fungi

Introduction

Maize (*Zea mays* L.) is being extensively grown across varied agro-climatic conditions due to its wide adaptability. Among many potential factors that limit maize production, fungal diseases are reported to cause extensive crop yield reduction. Of which, Banded leaf and sheath blight (BLSB) incited by *Rhizoctonia solani* f.sp. *sasakii* Exner (*Thanatephorus sasakii* (Shirai) Tu and Kimbrough) (Tu and Kimbrough 1978) is an economically important disease causing huge losses in all maize growing areas of the world. Control measures used can be only partly effective because *R. solani* is able to produce sclerotia that can persist in the soil for at least 2 years (Ou 1985). Absence of host plant resistance, expensive chemical control measures and environmental pollution due to extensive fungicide usage have forced the researchers to think of viable alternative management practices such as use of biocontrol agents with few limitations. To overcome the limitations in use and to improve the activity of biocontrol agents, their integration with other practices is desirable. One such concept is biofumigation, a process in which soil-borne pathogens are suppressed by the incorporation of glucosinolate containing plant materials as green manures into the soil (Angus et al. 1994). Application of *Pseudomonas fluorescens* as seed treatment, soil application along with FYM and foliar spray is effective in minimizing the sheath blight and in rice and maize crops, respectively (Rajput and Harlapur 2015; Biswas and Datta 2013) hence integration of biofumigant (mustard crop incorporation) and beneficial microorganisms such as *Trichoderma* and *P. fluorescens* were studied in the present research work against the viability of *R. solani* in green house and field.

Materials and methods

Green house experiment

The potential PGPR and fungal antagonist isolated from different maize fields of Andhra Pradesh (Madhavi et al. 2015; unpublished data) were used in the present study for suppressing the BLSB pathogen. Mustard is the effective biofumigant material identified in vitro and incorporation of mustard plant as green manure was further tested in green

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house and field conditions for one season in biofumigant treatments (Madhavi et al. 2015). Susceptible maize hybrid Pioneer 30v 92 was used throughout the study. Mustard crop was grown and incorporated into the soil at standard dose (5 kg m^{-2}) prior to 15 days before maize seed sowing. Azoxystrobin fungicide was applied at standard dose for seed treatment @ 1.5 g kg^{-1} seed, soil drenching @ 1.5 mL L^{-1} and foliar spray @ 1.5 mL L^{-1}. *P. fluorescens* and *T. harzianum* were applied at recommended rates 10 mL kg^{-1} seed (1×10^8, CFU mL^{-1}) as seed treatment, 2 kg $acre^{-1}$ as soil application and 5 mL L^{-1} as foliar spray. Soil drenching was done at 30 days after sowing and foliar sprays were given at 45 and 60 DAS for each of the treatments wherever applicable. For T 3, T 6, T 7 i.e., combination of *P. fluorescens* and *T. harzianum,* the individual bioagent dose was reduced to half and then combined for all the seed treatment, soil application and foliar spray methods. The green house experiment was conducted in randomized block design and replicated thrice with the treatments.

Growing of mustard in green house

The locally available mustard (*Brassica juncea*) was used for green house experiment. Each plastic pot of 60 cm height and 30 cm radius was filled with 5 kg black soil. Five mustard plants were raised for each pot. Sixty day old mustard plants were harvested along with the stem, leaves, flowers, and roots, chopped into small pieces and incorporated into the soil immediately to a depth of 15 cm from the top of the pot (Motisi et al. 2009). A little quantity of water was added to hydrolyse the glucosinolates and covered with polythene sheet for 15 days. The quantity of mustard plant parts incorporated into the soil was equivalent to 200 g fresh weight per pot. Control pots were maintained without mustard incorporation.

Soil inoculation of pathogen

RSS multiplied on sorghum grains was incorporated into the pots containing 10 kg of soil @ 5 g after the incorporation of mustard plant parts at a depth of 1 cm from the top of the soil. Following biofumigant incorporation, muslin bags containing 3 g of inoculum were buried in the soil at a depth of 10 cm. Bags were recovered 3 days later. Ten pieces of inoculum of *R. solani* were plated onto PDA media amended with 0.05 g streptomycin to determine their viability. Additionally, the colony diameters of *R. solani* were measured after 3 days incubation at 26 °C in the dark. At 5 and 50 days after biofumigant incorporation, 20 random soil cores (2.5 cm diameter to depth of 10 cm) were taken per plot and pooled. A 10 g sub-sample was taken from the pooled sample, and populations of total fungi, total bacteria, Gram negative Pseudomonads and *T. harzianum* were estimated by using soil dilution and cultural methods (Porter 1991). Plates were incubated at room temperature up to 2 weeks and the number of colony forming units (CFU) per gram of dry soil was determined. For isolation of total fungi, bacteria, Pseudomonads and *T. harzianum* selective media were used viz., malt extract agar, tryptone soy agar, Kings B medium and potato dextrose agar, respectively. Same procedure was followed for field experiment.

Field experiment

A field experiment was conducted at RARS, Lam, Guntur during *rabi* 2015–2016. The farm is geographically situated at 16.10°N latitude, 28.29°E longitude and 31.5 m altitude and falls under the Krishna agro climatic zone of Andhra Pradesh. The soils of the farm are deep black in nature of about 6^1–7^1 depth with good moisture retention capacity with a pH 8.4, EC 0.16 m mhos cm^{-1}. Plot size of 4.5×3 m^2 was prepared following randomized block design with three replications. Local variety of mustard was sown during September 2015 in 12 plots (4.5×3 m^2) at a spacing of 45 × 10 cm for obtaining required biomass for incorporation in biofumigation treatments. The crop was managed well throughout the period of experiment by following recommended agronomic practices.

Soil inoculation of pathogen

Artificially inoculated plot of *R. solani* f. sp. *sasakii* was maintained by applying sorghum grains infested with the pathogen @ 400 g inoculum per m^2 into the top 5 cm depth of the soil.

Incorporation of mustard crop

Mustard crop was harvested from each plot, 8 weeks after sowing the crop along with roots at flowering stage. Freshly harvested plants were chopped along with leaves, stem, flowers and roots and incorporated in situ at a depth of approximately 15 cm in the field. The quantity of freshly harvested mustard residues incorporated into the soil was adjusted equivalent to 4–8 t of fresh weight per hectare. The mustard bio mass was incorporated @ 9 kg per plot for all biofumigant treatments. The plots were covered with black polythene sheath for 15 days. Later maize hybrid pioneer 30v 92 was sown during November 2015. Recommended agronomic practices were followed throughout the crop growth period. Evaluation of population dynamics of microorganisms and plant pathogen were done for all biofumigation treatments. For estimation

Table 1 Viability and growth of buried inoculum of *R. solani* following incorporation of mustard into the soil in different treatments in green house and field experiments

Tr. no.	Treatment	Viability and growth of buried inoculum of *R. solani* 3 days after incorporation of biofumigant							
		In green house				In field			
		R. solani viability (per cent)	Per cent reduction of viability	*R. solani* colony diameter (mm)	Per cent inhibition of growth	*R. solani* viability (per cent)	Per cent reduction of viability	*R. solani* colony diameter (mm)	Per cent inhibition of growth
T1	Seed treatment + soil drenching + foliar spray with *P. fluorescens*	100.0	0	89.7	0.0	100.0	0	89.7	0.0
T2	Seed treatment + soil drenching + foliar spray with *T. harzianum*	99.0	1	89.7	0.0	99.7	0.3	89.7	0.0
T3	Seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	100.0	0	89.7	0.0	99.7	0.3	89.7	0.0
T4	Biofumigation + seed treatment + soil drenching +foliar spray with *P. fluorescens*	81.7	18.3	45.5	49.3	90.0	10	62.3	29.4
T5	Biofumigation + seed treatment + soil drenching + foliar spray with *T. harzianum*	82.3	17.7	46.0	48.7	90.3	9.7	63.0	29.8
T6	Biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	82.7	17.3	44.7	50.2	89.0	11	63.0	30.5

Table 1 (continued)

Tr. no.	Treatment	Viability and growth of buried inoculum of *R. solani* 3 days after incorporation of biofumigant							
		In green house				In field			
		R. solani viability (per cent)	Per cent reduction of viability	*R. solani* colony diameter (mm)	Per cent inhibition of growth	*R. solani* viability (per cent)	Per cent reduction of viability	*R. solani* colony diameter (mm)	Per cent inhibition of growth
T7	Biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum* and foliar spray with azoxystrobin	82.3	17.7	45.3	49.5	89.7	10.3	63.3	29.8
T8	Seed treatment + soil drenching + foliar spray with azoxystrobin	100.0	0	89.7	0.0	100.0	0	89.7	0.0
T9	Sees treatment + soil drenching + foliar spray with commercially available *P. fluorescens* + *T. viride*	99.3	0.7	89.7	0.0	100.0	0	89.7	0.0
T10	Untreated control	100.0	0	89.7	0.0	100.0	0	89.7	0.0
	SEm ±	0.74		0.65		0.51		1.04	
	CD (0.05%)	2.21		1.94		1.52		3.10	

of soil microbial population, the soil samples were taken from each plot before and after sowing of maize. The data obtained in different experiments were statistically analyzed by using completely randomized design (CRD) for laboratory experiments (in vitro) and randomized complete block design (RCBD) for field experiments.

Results and discussion

Effect of biofumigation on the viability of *R. solani* f. sp. *sasakii* inoculum

The viability of inoculum had decreased in all the biofumigation treatments but remained unaffected in other treatments (Table 1). The decrease in the viability ranged from 81.7 to 99%. Maximum reduction in the viability was recorded in T4 and was on a par with treatments T5, T6 and T7. The radial growth of the inoculum in different treatments showed significant differences when compared to control which ranged from 44.7 to 90 mm. Maximum growth (89.7 mm) was recorded in treatment T1, T2, T3, T8 and T9. Minimum growth of 44.7 mm was recorded in treatment T6 and was on a par with treatment T7 (45.3 mm), T4 (45.5 mm) and T5 (46 mm), respectively.

Similar trend was observed in field experiment. All the biofumigation treatments recorded decreased viability of inoculum and the remaining treatments had shown no effect on the viability of *R. solani*. The viability ranged from 89.0 to 99.7% and maximum reduction in the viability was recorded in T6 (11%) and at par with T7, T4 and

Table 2 Populations of soil microflora following incorporation of mustard into the soil in different treatments of greenhouse experiment

Tr. no.	Treatment	Populations of soil microflora (g^{-1} of soil)							
		5 days after incorporation of biofumigant				50 days after incorporation of biofumigant			
		TF × 10^4	TB × 10^5	T × 10^4	P × 10^5	TF × 10^4	TB × 10^5	T × 10^4	P × 10^5
T1	Seed treatment + soil drenching + foliar spray with *P. fluorescens*	8.2	19.6	5.4	7.8	10.7	23.3	5.4	10.2
T2	Seed treatment + soil drenching + foliar spray with *T. harzianum*	8.6	18.8	5.5	7.2	11.8	20.0	7.3	8.5
T3	Seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	9.2	19.9	5.9	7.7	13.1	25.0	7.1	12.1
T4	Biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens*	7.3	18.4	5.7	7.3	15.3	27.0	8.2	15.2
T5	Biofumigation + seed treatment + soil drenching + foliar spray with *T. harzianum*	7.2	18.0	5.4	7.1	15.0	28.0	9.4	14.3
T6	Biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	7.3	18.6	5.4	7.8	18.8	29.2	12.1	18.2
T7	Biofumigation + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum* and foliar spray with azoxystrobin	7.5	18.5	5.3	7.7	19.2	29.5	12.5	18.2
T8	Seed treatment + soil drenching + foliar spray with azoxystrobin	10.5	20.0	5.4	8.4	13.0	20.0	5.4	10.3
T9	Sees treatment + soil drenching + foliar spray with commercially available *P. fluorescens* + *T. viride*	10.7	19.0	5.6	8.0	11.0	13.0	4.4	7.5
T10	Untreated control	11.0	18.7	5.6	6.8	8.5	11.7	4.6	7.3
		SEm ±	CD (0.05%)			SEm ±		CD (0.05%)	
Main		0.27	0.75		Main	0.3		0.83	
Sub		0.17	0.48		Sub	0.19		0.52	
Inter		0.54	1.58		Inter	0.6		1.73	

TF total fungi, *TB* total bacteria, *T Trichoderma harzianum*, *P Pseudomonas fluorescens*

T5 treatments with 10.3, 10, 9.7%, respectively. The radial growth of the inoculum in different treatments showed significant differences when compared to control. The radial growth recorded in different treatments ranged from 44.7 to 90 mm. Maximum growth inhibition of 30.5% was recorded in T6 and was at par with T7, T4 and T5 with 29.8, 29.8, 29.4%, respectively.

The decrease in the viability of *R. solani* may be due to the volatiles released from the soil incorporated mustard and this finding was in conformity with the report of Friberg et al. (2009) who reported that the incorporation of mustard (*Brassica juncea*) as a green manure decreased the inoculum density of *R. solani* in the soil. In the present study the viability was not much affected as high concentrations of isothiocyanates are needed to successfully suppress the pathogen growth. *R. solani* produced pseudosclerotia or thick-walled hyphae as survival bodies and these survival structures appeared to be less susceptible to GSL hydrolyzed products than young hyphae, restricting the biocidal potential of green manure to inhibit fungal pathogens and this finding was in accordance with the report of Yulianti et al. (2006).

Effect of biofumigation on soil microbial population

Green house experiment

The results of estimation of soil microbial populations 5 days after incorporation revealed that the lowest total fungal and bacterial population was observed in biofumigation treatment T5 (biofumigation + seed treatment + soil application + foliar spray of *T. harzianum*) which were 7.2×10^4 CFU g^{-1} of soil and 18.0×10^5 CFU g^{-1} of soil, respectively. Total fungal and total bacterial population was at par in the treatments T4 (7.3×10^4 CFU g^{-1} of soil and 18.4×10^5 CFU g^{-1} of soil), T6 (7.3×10^4 CFU g^{-1} of soil and 18.6×10^5 CFU g^{-1} of soil) and T7 (7.5×10^4 CFU g^{-1} of soil and 18.5×10^5 CFU g^{-1} of soil). Population of *Trichoderma harzianum* varied from 5.3×10^4 to 5.9×10^4 CFU g^{-1} of soil in different treatments whereas

population of *P. fluorescens* varied from 6.8×10^5 to 8.4×10^5 CFU g^{-1} of soil in different treatments 5 days after incorporation of biofumigant. The total fungal population was low in biofumigation treatments when compared to control might be due to the decrease of *R. solani* population by volatiles released by the incorporation of mustard. 50 days after incorporation of biofumigant, population of total fungi, total bacteria, *Trichoderma harzianum* and *P. fluorescens* increased significantly in all treatments fumigated with mustard plants i.e. in T4, T5, T6 and T7 (Table 2).

Field experiment

The results of soil microbial populations 5 days after incorporation revealed that the lowest total fungal and total bacterial population was observed in treatments where one of the common treatment component was biofumigant i.e. T4 (7.0×10^4 CFU g^{-1} of soil and 16.4×10^5 CFU g^{-1} of soil), T5 (7.1×10^4 CFU g^{-1} of soil and 16.7×10^5 CFU g^{-1} of soil), T6 (7.2×10^4 CFU g^{-1} of soil and 17.0×10^5 CFU g^{-1} of soil) and T7 (7.3×10^4 and 16.7×10^5 CFU g^{-1} of soil). The total fungal population was low in biofumigation treatments when compared to control might be due to the decrease of *R. solani* population by volatiles released by the incorporation of mustard. This finding was in agreement with the report of Friberg et al. (2009) who reported that the incorporation of mustard (*Brassica juncea*) as a green manure decreased the inoculum density of *Rhizoctonia solani* in the soil (Table 3).

Total bacteria, *Trichoderma* spp. and *Pseudomonas* spp. population were also slightly less in biofumigant treatments compared to control and this may also be due to the tolerance of *Trichoderma* and *Pseudomonas* spp. to the volatiles released from mustard when compared to *R. solani*. These findings are in accordance with the report of Smith and Kirkegaard (2002) as the bacteria and actinomycetes are more resistant to toxicants than fungi and oomycetes.

Soil microbial population estimated 50 days after incorporation of mustard resulted in high population of total

Table 3 Populations of soil microflora following incorporation of mustard into the soil in different treatments of field experiment

Tr. no.	Treatment	Populations of soil microflora (g^{-1} of soil)							
		5 days after incorporation of biofumigant				50 days after incorporation of biofumigant			
		TF × 10^4	TB × 10^5	T × 10^4	P × 10^5	TF × 10^4	TB × 10^5	T × 10^4	P × 10^5
T1	Seed treatment + soil drenching + foliar spray with *P. fluorescens*	8.0	17.3	4.7	7.8	12.0	21.7	6.8	9.2
T2	Seed treatment + soil drenching + foliar spray with *T. harzianum*	8.0	17.5	4.9	7.3	12.8	22.7	7.5	9.4
T3	Seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	8.2	17.8	5.2	7.3	15.0	23.0	7.8	13.0
T4	Biofumigant + seed treatment + soil drenching + foliar spray with *P. fluorescens*	7.0	16.4	5.0	7.0	15.0	21.0	6.6	11.5
T5	Biofumigant + seed treatment + soil drenching + foliar spray with *T. harzianum*	7.1	16.7	5.2	7.2	15.8	24.0	8.9	10.7
T6	Biofumigant + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum*	7.2	17.0	5.4	7.2	17.1	26.3	9.6	12.1
T7	Biofumigant + seed treatment + soil drenching + foliar spray with *P. fluorescens* and *T. harzianum* and foliar spray with azoxystrobin	7.3	16.7	5.1	7.2	17.7	27.3	9.6	12.3
T8	Seed treatment + soil drenching + foliar spray with azoxystrobin	8.2	17.7	5.4	7.7	8.8	18.3	5.6	8.1
T9	Sees treatment + soil drenching + foliar spray with commercially available *P. fluorescens* + *T. viride*	8.6	17.7	5.6	7.2	10.8	20.0	6.5	8.6
T10	Untreated control	9.1	18.2	4.9	7.7	10.5	20.0	5.8	8.47
		SEm ±	CD (0.05%)				SEm ±	CD (0.05%)	
Main		0.112	0.31		Main		0.39	1.068	
Sub		0.071	0.196		Sub		0.24	0.675	
Inter		0.224	0.65		Inter		0.77	2.236	

TF total fungi, *TB* total bacteria, *T Trichoderma harzianum*, *P Pseudomonas fluorescens*

fungi, bacteria, *Trichoderma* spp. and *Pseudomonas* spp. in all biofumigation treatments compared to control. The maximum fungal, bacterial, *Trichoderma* spp. and *Pseudomonas* spp. populations (17.7×10^4, 27.3×10^5, 9.6×10^4, 12.3×10^5 CFU g^{-1} of soil, respectively) were recorded in treatment T7 compared to control (10.5×10^4, 20×10^5, 5.8×10^4, 8.4×10^5 CFU g^{-1} of soil, respectively) and followed by treatment T6 (17.1×10^4, 26.3×10^5, 9.6×10^4, 12.1×10^5 CFU g^{-1} of soil, respectively).

The increase in microbial population in biofumigation treatments may be due to the addition of green manure to the soil which provided extra biomass and food base for multiplication of the soil microflora and this finding is in conformity with the report that incorporated *Brassica* spp. as plant biomass was found to influence microbial community structures (Cohen and Mazzola 2006; Hoagland et al. 2008; Friberg et al. 2009; Omirou et al. 2010). The incorporation of *Brassica* plant biomass for biofumigation has been shown to increase or decrease the population of the rhizosphere microorganisms such as *Trichoderma* spp., *Pythium* spp., fluorescent Pseudomonads, *Streptomyces* spp., actinomycetes and other antagonists of soil-borne pathogens depending on the plant species and soil type (Mazzola and Manici 2012; Cohen and Mazzola 2006; Mazzola and Zhao 2010). The addition of *Brassica* biomass can aid the native microbial community with competition, parasitism, antagonism and predation against the soil-borne pathogens (Raaijmakers et al. 2009).

Biofumigation resulted in increased population of total bacteria, *Trichoderma* spp. *and Pseudomonas* spp. and reduced the viability of *R. solani* f.sp. *sasakii*, 50 days after incorporation of mustard plant tissue in both green house and field studies.

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