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Antagonistic Activity of Bacterial Endophytes Isolated from Millets Against *Rhizoctonia solani* in Foxtail Millet

M. Raveendra Reddy and M. K. Shivaprakash
Department of Agricultural Microbiology, College of Agriculture, UAS, GKVK, Bengaluru-560 065
E-mail: raveendra56@gmail.com

Abstract

An investigation was carried out *in vitro* to study the efficiency of endophytic bacteria isolated from millets for biocontrol of *Rhizoctonia solani*. Kuhn, a causal organism of sheath blight in millets. Out of the total isolates obtained from small millets, 12 isolates inhibited mycelial growth of *Rhizoctonia solani*. Isolates KMS5 and KMS1 recorded highest antifungal activity (62.22 and 60.00% inhibition) and took 10.95 and 11.55 days for 50 per cent germination compared to control which received only pathogen. Lowest pre emergence disease incidence was observed with KMS5 (11.16 %) followed by KMS1 (12.50 %) where as control with pathogen recorded highest pre emergence disease incidence (44.44). Least post emergence disease was observed with KMS5 (11.36) and highest biocontrol efficiency with KMS5 (72.22%). Apart from showing antagonistic activity, KMS5 has recorded maximum vigour index (2670.33) followed by KMS1 (2527.87) compared to other treatments.

Keywords: Endophytic bacteria, foxtail millet, *Rhizoctonia solani*, antagonistic activity

Small millets (barnyard, foxtail, finger, kodo, little and proso millet) are one of the oldest foods known to humans and possibly the first cereal grain to be used for domestic purposes. These are small seeded grasses that are hardy and grow well in dry zones as rain-fed crops, under marginal conditions of soil fertility and moisture and are also unique due to their short growing season and when properly stored, whole millets will keep for two or more years. Small millets as a group include several grain crops that are popular as nutria – cereals owing to their high calcium, iron fibre and other quality aspects. Millets are highly nutritious, non-glutinous and non acid forming foods. Hence they are soothing and easy to digest. Millets are particularly high in minerals like iron, magnesium, phosphorous and potassium.

Foxtail millet is one of the important protein rich and food security small millet crop. Foxtail millet (*Setaria italica* L.) also known as German, Italian, Siberian millet is one of the oldest crops cultivated for hay, pasture and grains. It is called by different colloquial names as kangni, navane, tenai, korra and rata. It has the longest history of cultivation among the millets, having been grown in China since sixth millennium BC. At present, its cultivation is confined to semi arid regions in the states of Andhra Pradesh, Karnataka, Chattisgarh and Tamil Nadu. Although no major diseases, a few diseases like blast, rust, smut, brown spot, downy mildew and udbatta have been reported on this crop (Patro and Madhuri, 2014). Under water logging conditions, it was found infected with sheath blight disease caused by a soil borne necrotrophic fungi *Rhizoctonia solani*. Kuhn causing considerable loss in grain yield under favorable environmental conditions.

Endophytic bacteria colonizes the interior parts of the plant without causing any harmful effect on host plant. (Backman and Sikora, 2008). They interact more closely with the host, with less competition for carbon sources and are in a protected environment. The widely recognized mechanisms of biocontrol mediated by plant growth promoting bacteria (PGPB) are competition for an ecological niche or a substrate, production of inhibitory allelochemicals and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Bloemberg and Lugtenberg, 2001) and / or abiotic stresses. The current research focuses on developing endophytic bacteria as potent biocontrol agents, since they can elicit antagonism at the site of infection due to colonization abilities. An

endophyte is an endo symbiont that lives inside the host plants without causing any disease. In addition to the basic biocontrol mechanisms like the production of siderophores, HCN, volatile and nonvolatile organic compounds and lytic enzymes, endophytic bacteria are thought to be capable of triggering plant defense mechanisms by a phenomenon known as induced systemic resistance (ISR). ISR by endophytic bacteria is known to be triggered by various signaling molecules followed by pathogen invasion. Plants respond to infectious agents by recognizing an array microbial signals. Siderophores, lipopolysaccharides, exopolysaccharides, volatiles, flagella, salicylic acid, pyocyanins, etc. are among the reported ISR determinants of endophytic and rhizospheric bacteria. (Akram *et al*., 2015). The present investigation was carried out for the identification of best bacterial endophytes showing antagonism on *R. solani* causing sheath blight in Foxtail millet grown in seedling trays under green house conditions.

Materials and Methods

The small millet samples (barnyard, foxtail, finger, kodo, little and proso millet) were collected during *kharif* and *rabi* season of 2016-17 from the millet research plots of ZARS, University of Agricultural Sciences, GKVK, Bangalore. Ten plants of each small millet samples (Barn yard, Foxtail, Finger, Kodo, Little and Proso millet) were collected and from each plant and 10 of each leaf segments, shoot segments and root segments were analyzed. The samples were surface sterilized, placed on nutrient agar and incubated at 30 ^{0}C for two days. After incubation, the isolates were transferred to fresh nutrient agar media and incubated for 30 ^{0}C for 2 days. The procedure was repeated 2 -3 times to get pure culture.

Per cent inhibition: Antifungal activity was screened using dual culture method in which both endophytic bacteria and test fungi (*Rhizoctonia solani*) were inoculated in single Potato Dextrose Agar (PDA) media plate. The zone of inhibition was measured and the per cent inhibition of the pathogen was calculated using the formula :

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

Where, I = Per cent inhibition,

C = Growth of fungal plant pathogens in control (mm),

T = Growth of fungal plant pathogens in dual culture plate (mm).

Evaluation of bacterial endophytes on growth inhibition of Rhizoctonia solani (Opgenorth and Endo, 1983).

The endophytic isolates showing high inhibition of the pathogen in plate assay were tested in liquid media (Potato dextrose broth). Each flask containing 100 ml broth was inoculated with 8 mm disc of the pathogenic fungi along with 1 ml of 24 hour old endophytic bacterial culture. Control flasks with only the fungus (*R. solani*) and only bacteria were maintained. The flasks were incubated at 30 ^{0}C under static conditions for 10 days. After the incubation period, the contents were filtered through a pre-weighed Whatman filter paper and the fresh weight was recorded. The filter papers were dried in an oven at 105 ^{0}C for 48 hours and reweighed along with the mycelium to get the dry weight. The weight of the mycelium was calculated by subtracting the weight of the filter paper from the weight of the filter paper + mycelium. The reduction in weight of co inoculated flasks was determined by comparing with the control flasks.

Seedling tray experiment: A seedling tray experiment was conducted to evaluate the antagonistic and growth promoting effect of bacterial endophytes in substrate enriched with bacterial endophytes as biocontrol agents against pathogen under green house condition in the Department of Agricultural Microbiology. The substrate for the experiment included 10 kilograms of coir pith enriched with 2.5 kilograms each of red earth, vermi compost and pongamia cake, which were sterilized. Selected bacterial endophytes were grown in sterile nutrient broth in one litre conical flask containing 500 ml of the medium aseptically and placed on rotary shaker for 24 hours. Bacterial inoculum was added at the rate of 10 ml/kg of substrate.

Preparation of pathogen inoculums: A mixture of 940g sand and 60g crushed Sorghum (94:6) were

mixed and the mixture was sterilized. Five mycelial discs of 5 mm size of pathogen on the PDA plate and transferred aseptically to the polybags containing sterilized Sorghum and sand mixture and were incubated at 27 ± 1°C for 15 days.

Preparation of seedling trays and sowing: The mass multiplied pathogen inoculum *Rhizoctonia solani* was added to substrate mixture @ 100 grams / kg to each polybags and bacterial endophytes were added @ 100 ml per kg of seedling mixture and mixed properly one week prior to sowing. The mixed substrate was added at the rate of 100g per tray at the time of sowing.

Observations recorded

During the experimental period, germination percentage was recorded.

Germination percentage (%) = (No of seeds germinated / No. of seeds sown) x 100

Per cent pre-emergence disease incidence

100 (GA-GT)/GA

Where, GA-Germination percentage in absolute control, GT- Germination percentage in treatment

Per cent post-emergence disease incidence

100 (GP – ND)/ND

Where, GP-Number of healthy plants left in control, ND- Number of healthy plants left in treatment.

Seedling Vigour Index (SVI): The vigour index of seedlings were calculated by adopting the method suggested by Abdul-Baki and Anderson (1973) and expressed in number by using the below formula.

SVI = Germination (%) x [shoot length (cm) + root length (cm)]

Shoot length (cm): The shoot length was measured from collar region to the tip of the plant with the help of a scale and the mean shoot length was expressed in centimeters.

Root length (cm): The root length measured from collar region to the tip of primary root with the help of a scale and the mean root length was expressed in centimeters.

Biological control efficacy (BCE): Biological control efficacy was calculated using the following formula given by Guo *et al.* (2004).

BCE = (DIPC-DIT/ DIPC) x 100

Where DIPC- Disease incidence in pathogen control, DIT- disease incidence in treatment group

Results and Discussion

Antagonistic activity of bacterial endophytes against mycelial growth of Rhizoctoniasolani

The per cent inhibition of *Rhizoctonia solani* by endophytic bacteria is presented in Table I. The best isolates showing antagonism against *Rhizoctonia solani in vitro* were selected. The isolate KMS5 showed significantly highest per cent inhibition (62.22 %) followed by the isolate KMS1 (60.00 %) and BML1 (56.66 %). The isolate BMR7 showed poor antagonistic activity (38.89 %).

The per cent reductions in dry weight of the mycelium mat of *Rhizoctonia solani* by the isolated

Table I

Antagonistic activity of endophytic bacterial isolates on growth of Rhizoctonia solani

Crop	Parts	Isolates	Percent inhibition (%) on *R. solani*
Barnyard Millet	Root	BMR7	38.89 ^{i}
	Leaf	BML1	56.66 ^{c}
Finger Millet	Root	FMR7	50.00 ^{d}
	Root	FMR12	41.11 ^{h}
Foxtail Millet	Shoot	FTMS4	43.33 ^{fg}
	Shoot	FTMS5	42.22 ^{gh}
Kodo Millet	Shoot	KMS1	60.00 ^{b}
	Shoot	KMS5	62.22 ^{a}
Little Millet	Root	LMR4	44.44 ^{f}
	Leaf	LML4	47.77 ^{e}
Prosomillet	Root	PMR6	46.66 ^{e}
	Leaf	PML3	44.44 ^{f}

Note: Each value is the mean of three replications

Different letters in superscripts indicate significantly different values @ Pd ≤ 0.05 as per DMRT

endophytes are presented in Table II. The highest per cent reduction of dry weight was shown by the isolate PML3 (82.05 %), followed by FMR12 and KMS1 (80.76 %) and KMS5 (79.49 %). The least per cent reduction in mat dry weight was by the isolate BMR7 (70.51 %). Many works have reported positive findings using bacterial endophytes for controlling different plant pathogens. The suppression of mycelial growth of fungal pathogen by bacterial endophytes may be due to the production of inhibitory allelochemicals, volatile and non volatile compunds, hydrogen cyanide and cell wall degrading enzymes by antagonists. These results were in accordance with the work of Lalhali *et al*. (2007) where they evaluated 220 bacterial strains isolated from different organs of healthy potato plants and rhizospheric soils, out of which 25 isolates were selected using screening methods based on *in vitro* dual culture assays. The mycelial growth inhibition rate of the pathogen ranged from 59.4 to 95.0 per cent. Also seven fungal strains isolated from the rhizospheric soil and potato roots showed a high mycelial growth inhibition of *R. solani*. The mycelial growth inhibition rate obtained with these fungi between 60.0 and 99.4 per cent. This result is in accordance with Shabanamol *et al*. (2017) where they isolated *Lysinibacillus sphaericus*, a diazotrophic endophyte from rice against the sheath blight pathogen *R. solani* and also reported that the endophytic isolate showed 100 per cent growth inhibition of the fungal pathogen compared to chemical fungicide treatments via volatile organic compound production and was positive for the production of siderophores, biosurfactants, HCN, and ammonia under green house conditions and also showed that foliar and soil application of *L. sphaericus* significantly decreased the percentage of disease incidence. Ji *et al*. (2014) isolated endophytic bacteria from Korean rice which shows antagonistic affect against *Rhizoctonia solani* in plate assay.

Efficiency of bacterial endophytes in enhancing seedling vigour of Foxtail Millet grown in seedling trays under green house condition

The effects of seed bacterization of Foxtail millet variety Si A 3085 TL with endophytic bacterial isolates on the control of *Rhizoctonia solani* disease are

TABLE II

Effect of bacterial endophytes on inhibition of mycelial growth of Rhizoctonia Solani

Isolates	Mycelial Mat Wet weight (g)	Mycelial mat dry weight (g)	Reduction in wet weight (g)	Reduction in dry weight (g)	Percent reduction in dry weight (%)
Control	11.43 ^{a}	0.780 ^{a}	0.00 ^{g}	0.000 ^{f}	0.00 ^{h}
BMR7	7.87 ^{b}	0.230 ^{b}	3.56 ^{f}	0.590 ^{e}	70.51 ^{g}
BML1	7.92 ^{b}	0.210 ^{c}	3.51 ^{f}	0.610 ^{e}	73.08 ^{f}
FMR7	6.76 ^{fg}	0.220 ^{b}	4.67 ^{b}	0.640 ^{d}	71.79 ^{fg}
FMR12	7.23 ^{cd}	0.150 ^{h}	4.20 ^{cd}	0.660 ^{cd}	80.76 ^{ab}
FTMS4	7.21 ^{cd}	0.180 ^{e}	4.22 ^{c}	0.700 ^{ab}	76.92 ^{de}
FTMS5	7.43 ^{c}	0.190 ^{d}	4.00 ^{e}	0.680 ^{bc}	75.64 ^{e}
KMS1	6.79 ^{fg}	0.150 ^{h}	4.64 ^{b}	0.690 ^{ab}	80.76 ^{ab}
KMS5	6.56 ^{g}	0.160 ^{g}	4.87 ^{a}	0.710 ^{a}	79.49 ^{bc}
LMR4	7.35 ^{cd}	0.170 ^{f}	4.08 ^{de}	0.690 ^{ab}	78.20 ^{cd}
LML4	7.16 ^{d}	0.190 ^{d}	4.27 ^{c}	0.700 ^{ab}	75.64 ^{e}
PMR6	6.86 ^{ef}	0.180 ^{e}	4.57 ^{b}	0.710 ^{a}	76.92 ^{de}
PML3	7.11 ^{de}	0.180 ^{e}	4.32 ^{c}	0.650 ^{d}	82.05 ^{a}

Note: Each value is the mean of three replications

Different letters in superscripts indicate significantly different values @ Pd≤ 0.05 as per DMRT

TABLE III

Biocontrol efficiency of bacterial endophytes on Foxtail millet grown in seedling trays under green house condition

Isolates	Percent Germination (%)	Days taken for 50 percent germination	Pre-emergence disease Incidence (per cent)	Post emergence disease incidence (per cent)	Biocontrol efficiency (per cent)	Root length (cm)	Shoot length (cm)	Vigour Index
T_1(Pathogen alone)	50.00 ^{j}	13.50 ^{a}	44.44 ^{a}	40.91 ^{a}	0.00 ^{j}	13.00 ^{h}	8.80 ^{k}	1090.00 ^{j}
T_2 (Pathogen +BMR7)	75.00 ^{cde}	12.00 ^{e}	16.66 ^{f}	24.09 ^{e}	41.11 ^{f}	16.10 ^{de}	10.10 ^{j}	1965.00 ^{i}
T_3 (Pathogen +BML1)	74.58 ^{de}	13.00 ^{b}	17.13 ^{f}	26.36 ^{d}	35.56 ^{g}	15.90 ^{e}	12.40 ^{h}	2110.70 ^{def}
T_4 (Pathogen +FMR7)	72.92 ^{ef}	11.55 ^{f}	18.98 ^{e}	23.18 ^{f}	43.33 ^{e}	15.60 ^{ef}	11.50 ^{i}	1976.04 ^{hi}
T_5 (Pathogen +FMR12)	77.17 ^{bc}	11.55 ^{f}	14.25 ^{g}	18.18 ^{g}	55.56 ^{d}	15.30 ^{f}	12.90 ^{g}	2176.33 ^{d}
T_6 (Pathogen + FTMS4)	72.08 ^{fg}	12.25 ^{de}	19.91 ^{e}	23.18 ^{f}	43.33 ^{e}	14.30 ^{g}	13.60 ^{ef}	2011.12 ^{ghi}
T_7 (Pathogen +FTMS5)	69.42 ^{h}	12.72 ^{bc}	22.87 ^{cd}	27.72 ^{c}	32.22 ^{h}	16.50 ^{cd}	13.20 ^{fg}	2061.67 ^{efg}
T_8 (Pathogen + KMS1)	78.75 ^{ab}	11.55 ^{f}	12.50 ^{h}	14.54 ^{i}	64.44 ^{b}	17.30 ^{b}	14.80 ^{ab}	2527.87 ^{b}
T_9 (Pathogen +KMS5)	79.95 ^{a}	10.95 ^{g}	11.16 ^{i}	11.36 ^{j}	72.22 ^{a}	18.20 ^{ab}	15.20 ^{a}	2670.33 ^{a}
T_{10} (Pathogen +LMR4)	76.76 ^{bcd}	11.95 ^{ef}	14.70 ^{g}	25.91 ^{d}	36.66 ^{g}	16.80 ^{bc}	14.49 ^{bc}	2402.79 ^{c}
T_{11} (Pathogen +LML4)	68.50 ^{hi}	12.55 ^{cd}	23.89 ^{c}	32.27 ^{b}	21.11 ^{i}	16.00 ^{de}	13.80 ^{de}	2041.30 ^{fgh}
T_{12} (Pathogen +PMR6)	70.16 ^{gh}	12.25 ^{de}	22.66 ^{d}	25.91 ^{d}	36.66 ^{g}	15.90 ^{e}	14.10 ^{cd}	2115.00 ^{de}
T_{13} (Pathogen +PML3)	66.75 ^{i}	11.85 ^{ef}	25.83 ^{b}	15.91 ^{h}	61.11 ^{c}	15.80 ^{ef}	14.40 ^{bc}	2015.85 ^{ghi}

Note: Pathogen – *Rhizoctonia solani*

Each value is the mean of three replications

Different letters in superscripts indicate significantly different values @ Pd≤ 0.05 as per DMRT

presented in Table III. Highest per cent germination (79.95 %) was recorded with the isolate KMS5 followed by KMS1 (78.75%) and FMR12 (77.17%). Lowest percent germination (66.75 %) was recorded with the control (50.00%).

Significant differences were observed between the treatments regarding days taken for 50 per cent germination. KMS5 recorded less number of days takenfor 50 per cent germination (10.95) followed by KMS1, FMR7 and FMR12 (11.55) and PML3 (11.85). Uninoculated control without any bacterial isolate (Control) recorded maximum days for 50 per cent germination (13.50). Lowest pre-emergence disease incidence (11.16 %) was observed with the isolate KMS5 followed by KMS1 (12.05%) and FMR12 (14.25%). Control recorded the highest pre emergence disease incidence of 44.44 per cent.

KMS5 recorded lowest post emergence disease incidence (11.36 %) followed by KMS1 (14.54 %) and it was significantly less compare to other treatments. Control (T_1) recorded maximum post emergence disease incidence (40.91%). Highest biocontrol efficiency (72.22%) was recorded in KMS5 followed by KMS1 (64.44%). Uninoculated control did not show any biocontrol efficiency and this may be due to lack of bacterial isolates in the treatments.

Xinqui *et al*. (2012) conducted a pot experiment to assess the *in vivo* disease control efficiency of *Bacillus pumilus* SQR-N43 with green fluorescent protein (GFP) tag and results indicated that *B. pumilus* SQR-N43 induced hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage in *R. solani*. In contrast to applications of only *B. pumilus* SQR-N43 (N treatment), which produced control efficiencies of 23 per cent, control efficiencies of 68 per cent were obtained with applications of a fermented organic fertilizer inoculated with *B. pumilus* SQR-N43 (BIO treatment). After twenty days of incubation, significant differences in the number of colony forming units (CFUs) and the percentage of spores of *B. pumilus* SQRN43 were recorded between the N treatment (2.20×10^7 CFU g^{-1} of soil and 79 per cent, respectively) and the BIO treatment (1.67×10^8 CFU g^{-1} of soil and 52 per cent, respectively). The results indicated that *B. pumilus* SQR-N43 is a potent antagonist against *R. solani*.

The results are in accordance with the studies of Bharathi *et al.* (2013) who evaluated the response of biopesticides and biofertilizers on seed mycoflora and seed quality parameters of Sesame (*Sesamum indicum* L.) and observed that the untreated seeds were found to be associated with maximum per cent incidence of mycoflora and minimum population was recorded in the treatment of *Trichoderma* + *Pseudomonas* formulation followed by *Azotobacter* + *Trichoderma*, *Pseudomonas* and *Azotobacter* in the decreasing order of efficacy. Germination percentage was maximum in the treatment *Trichoderma* + *Pseudomonas* formulation, *Azotobacter* + *Trichoderma*, *Pseudomonas* and *Azotobacter* recording 96, 94, 90 and 88 per cent, respectively.

The data pertaining to the efficiency of bacterial endophytes in enhancing seedling vigour and growth parameters of Foxtail Millet is given in Table III. Significantly highest root length (18.20 cm) was recorded in T_9 (Pathogen + KMS5) followed by T_8 (17.30 cm). Lowest root length (13.00cm) was recorded in control which was treated with only pathogens. Highest shoot length (15.20 cm) was recorded in treatment T_9 (Pathogens + KMS5) and it was significantly higher than T_8 (Pathogens + KMS1) which recorded a shoot length 14.80 cm. Lowest shoot length (8.80 cm) was recorded in T_1 (control). Maximum vigour index (2670.33) was observed in T_9 (Pathogens + KMS5) followed by T_8 (2527.87). Control recorded lowest vigour index (1090.00).

Ramakrishnan and Selvakumar (2012) concluded that plant height and biological yield have been affected significantly by co-inoculation followed by single inoculation because this biofertilizer can enhance absorption of nitrogen by plant. Thus, he concluded that for obtaining maximum grain yield as well as profit from tomato, soil should be inoculated with *Azotobacter* with *Azospirillum.*

The present study revealed that small millets harbored endophytic bacteria which are antagonist against *Rhizoctonia solani* at the same time promoting plant growth. Hence, the isolates could be used for further studies regarding field inoculation, understanding the mechanisms of biocontrol and molecular characterization which will add more information to the study.

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