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Comparison of selected fungicides and *Pseudomonas fluorescens* on brown spot disease incidence and plant growth parameters of paddy

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Abstract
Emerging fungicides evaluation will help in determining efficacy against the target pathogens and avoid its indiscriminate use and economic losses. The use of bio-agents *Pseudomonas fluorescent* can effectively suppressed the pathogen through mycoparasitism, antibiosis and plant immunity through induce resistance and find best alternative to chemical use in sustainable crop pest management system. In our present studies 5 fungicides *viz*. Thiophanate, Myclobutanil, Carbendazim, Propineb, Propiconazole and bio-agent *Pseudomonas fluorescent* isolates were used against brown spot disease of rice caused by *Helminthosporium oryzae* (Breda de Haan). *In-vitro* studies in broth media revealed most significant effect on fungal biomass production was recorded in Propiconazole (0.05 g) followed by Propineb (0.07 g). On solid media minimum radial growth was recorded in Propiconazole (2.73 cm) followed by Propineb (2.86 cm) in compared with others including Control. The antagonistic effect of *Pseudomonas fluorescent* on dual culture test found most significant reduction on linear growth of *Helminthosporium oryzae* was found at *P. fluorescent* cell conc. of $1.3x10^8$/ml (4.29 cm) with percent inhibition 52.23 over the untreated Control. Pooled data of the two consecutive *in-vivo* trials revealed that all the fungicides and *P. fluorescent* significantly reduced incidence of *H.oryzae* in compared with untreated Control. Among the treatment significantly reduced the disease incidence was recorded in T_6 (7.39) followed by T_5 (7.91), T_2 (9.63), T_4 (9.97), T_3 (10.77), T_1 (16.00) from Control (27.12), whereas T_4-*Pseudomonas fluorescent* significantly reduced disease incidence from T_1 and T_3. However (T_4&T_2) are not significant on each other. Fungicides and bio-agent *P.fluorescent* were found to have a significant increase on plant parameters being investigated *viz.*, plant high, flag leaf, number of filled grain and the overall grain yield parameter.

Keywords: Fungicides, *Pseudomonas fluorescens*, brown spot disease incidence, plant growth parameters, paddy

Introduction
Brown spot disease caused by *Helminthosporium oryzae* is the most serious disease of rice because of devastation and widespread distribution and existence of several physiological races of the causal organism *Arshad et al.*, (2008) [2]. In India the disease is most severe in direct seeding rice in the state of Bihar, Jharkhand, Chhattisgarh, Madhya Pradesh, Orissa, Assam and West Bengal (Sunder, *et al.*, 2014) [20]. Evaluation of the efficacy of available chemicals against this disease will prevent tremendous economic losses and indiscriminate use of chemicals. On the other hand use of bioagent *Pseudomonas fluorescens* a growth promoting rhizobacteria (PGPR), with grams negative in nature can play a major role in inducing systemic resistance and control of plant pathogens, Voisard *et al.*, (1988) [22]. The antagonistic activities are due to the production of sidderophores, iron-chelating compounds Kloepper *et al.*, (1980) [6]. Production of antibiosis is the most commonly suggested trait responsible for their activity against plant pathogens and a number of antimicrobial compounds such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), hydrogen cyanide (HCN) and biosurfactant antibiotics etc. Picard, (2008) [16]. Thus proper harnessing abundant, freely available non pathogenic bacteria strain *Pseudomonas fluorescens* will remain an important component in integrated pest management system approaches.

Materials and methods
In-vitro broth and solid media test of selected fungicides on growth of *Helminthosporium oryzae*
Five fungicides, Myclobutanil 10% WP, Thiophanate 70% WP, Propiconazole 25% EC, Carbendazim 50% WP, and Propineb 70% WP were under taken for analysis of their effect on the growth of fungus.

Potato dextrose broth was prepared and dispenses 50 ml each in 150 ml capacity conical flasks and autoclaved at 121 ºC for 20 minutes. After cooling down each treatment fungicides were added and shaken gently in circular motion for homogeneous spread of the test fungicides, then 4 mm mycelial disc taken from 5 days old pure fungal culture were aseptically inoculated to each flask with the help of sterilized cork borer and inoculating needle, the medium without fungicides act as control. Each treatment was replicated four times. The inoculated flasks were incubated at 27±1 ºC for 10 days. The flasks were shaken gently in a BOD shaker every day. The mycelial mats were harvested after 10 days incubation and filtered through pre-weighted filter paper Whatman No.1 (11 cm diameter) then dried at 60 ºC for 72 hrs in hot air oven and after cooling in desiccators for 24 hrs it is reweight and actual mycelium dry weight were determined.

In solid media test 50 ml of PDA were dispensed in 100 ml capacity conical flasks and autoclaved at 121 ºC for 20 minutes, after cooling down and before solidify, the required dose of test fungicides were added and mix thoroughly by shaking gently in circular motioned, then 15 ml of this poisoned media were aseptically pour in sterilize 9 cm diameter petri plates and allow to solidify, then 4 mm mycelial discs taken from 5 days old pure fungal culture were aseptically inoculated at the middle of the media plate by using sterilized cork borer and inoculating needle. Each treatment was replicated four times. The inoculated plates were incubated at 27±1 ºC. The radial growth was recorded in 24 hours intervals till the fungus covered the whole plate. The differences in the growth rate were calculated for all the treatments in compare with the control.

Isolation, identification of *Pseudomonas fluorescent*

The rhizospheric soils of paddy plant were collected from the SHUATS experimental field, Allahabad. The collected soil were shade dry and finely powdered, then 10 g of this powdered soil were added into 90 ml sterile distilled water to make 1:10 dilution (10^{-1}). This solution is shaken in magnetic shaker for 10-20 minutes, then transfer 1 ml of the suspension (1:10 dilution) to another 9 ml sterilized distilled water to make 1:100 dilution 10^{-2}, likewise prepared serial solution 10^{-3} upto 10^{-7} as earlier, then a loopfull of this last dilution suspension was spread on sterilized King's B agar medium plate and incubated inverted at 37±1 ºC for 24 hrs. Detect and work individual colonies with yellow-green and blue pigments under U.V. light (366 nm). Pick up the individual colony with sterilized loop & transfer on to fresh King's medium plates, then single colony of bacteria were transfer into a King's B medium slants to obtain pure culture and store in refrigerator at 4 ºC and sub-culture periodically at 15 days intervals on the same medium.

Preparation of different cfu concentration

Pseudomonas fluorescent suspension and its concentration were prepared by following *Lucas et al.,* (2009) [9] methods, where *P. fluorescent* isolates were grown in nutritive King's B broth reaching $1x10^9$ cfu /ml. Inoculation was carried out with bacterial culture media diluted with water to achieve the desired bacterial density.

In-vitro* test on effect of different concentration of cell *P. fluorescent* on linear growth of *H. oryzae

Antagonistic effect of different concentration *i.e.,* number of *P. fluorescent* cell per ml suspension *viz.,* $1.3x10^4$/ml, $1.3x10^6$/ml and $1.3x10^8$/ml against *H. oryzae* was carried out by dual culture technique. The petri plates were poured with 15 ml sterilized PDA without antibiotic and then fresh loopfull of different *P. fluorescent* cell concentration were streaked leaving 1 cm from the margin. Then 5 mm mycelial disc of *H. oryzae* from 5 days old culture were placed at centre of each petri plates. The control plates were inoculated only with pathogen and streak only with sterile distilled water. Each treatment was replicated into four. The whole experiment set up was incubated at 28±1 ºC for 4 days. The distance between fungal growth and bacterial colonies were measured and recorded as inhibition zone. Percent growth inhibition of test fungal growth for all the in-vitro test over the untreated Control was calculated by using the formula Vincent, (1927) [23].

$$I = \frac{C - T}{C} X\ 100$$

Where, I = Percent inhibition
C= Growth in control
T= Growth in treatment

Based on the *in-vitro* test results the most effective cell concentration against the growth of test fungi was selected for *in-vivo* test.

***In-vivo* test**

Field trial was conducted at the experimental plot of Department of Plant Pathology, Allahabad School of Agriculture, SHUATS, Allahabad, U.P., during kharif 2014-15 and 2015-16 by using a susceptible Manipur cultivar *viz.,* Daram-phou. Field layouts were made in Randomized Block Design (RBD) with plot size (2x3) m². 25 days old seedlings were transplanted with spacing 20 cm (row x row) and 15 cm (plant x plant), and 2-3 seedlings/hill. Five fungicides *viz.,* Thiophanate, Carbendazim, Myclobutanil, Propineb, Propiconazole at 1000 ppm and bio-agent *P.fluorescent*, cell conc. $1.3x10^8$/ml were sprayed at 10 days intervals from 48, 58 and 68 days after transplantation, on appearance of prominent disease symptoms. Observations on disease severity were recorded one day ahead of each time of spray and 9 days after the final spray i.e., at 47, 57, 67 and 77 days after transplantation. The percent disease incidence was calculated by using the following formula:

$$PDI = \frac{\text{Summation of numerical ratings}}{\text{Total number of leaves observed} \times \text{Maximum rating grade}} \times 100$$

The disease scoring scale used was 0-4**,** (Kalloo & Banerjee, 2000) [5], where, 0=No symptom, 1=1-25%, 2=26-50%, 3=51-75% and 4=75% and above of the leaf area affected. Disease rating were taken from 5 randomly tagged plant per plot and from top 3 leaves in each time of observation by using above scoring scale.

Results

The data presented on Table 1, and depicted on Fig.1, 2, 3 & 4, revealed *in-vitro* test all selected fungicides shows significant reduction on growth of fungi. In broth media among the treatments lowest mean fungal biomass production was recorded in Propiconazole (0.05 g) with percent inhibition 85.71 followed by Propineb (0.07 g) with 80% over

the Control. However, (T_4, T_5), (T_2, T_4), (T_1, T2, T3) were not significant among themselves. On solid media significant inhibition on radial growth was found at par between Propiconazole (2.73 cm) with percent inhibition of 74.26 and Propineb (2.86 cm) with 73.04% inhibition were found at par with each other which was followed by Mycobutanil (5.11 cm) with 51.83% inhibition compared with other including Control.

The results data presented on Table 2, depicted in Fig. 5 & 6, *P. Fluorscent* in dual culture test found significant linear growth inhibition of *H. oryzae* on *P.fluorescent* cell concentration $1.3x10^8$/ml (4.29 cm) with percent growth inhibition of 52.24 and $1.3x10^6$/ml (4.37 cm) 50.95% growth inhibition was found at par with each other, but significant with lower cell conc. $1.3x10^4$/ml (5.69 cm). However, all treatment were found significantly inhibit on linear growth of *H. oryzae* in compared with Control.

The pooled data of two successive cropping periods presented on Table 3**,** fig.7 & 8 revealed that among the selected fungicides and bio-agent *P. fluorescent,* significant disease reduction was found in Propiconazole (7.39) with pre cent disease reduction of 72.24 closely followed by Propineb (7.91) with 71.24% reduction over the control in compared with Control. The test results also revealed *Pseudomonas fluorescent* effectively reduced the disease incidence (9.69) with percent disease reduction of 63.23, was found at par with fungicides Myclobutanil (9.63) and showed better results in compared with others fungicides *viz.,* Carbendazim and Thiophates. The test results also found T_4 and T_3 not significant to each other.

Results data presented on, Table 4, is the effect of fungicides and *Pseudomonas fluorescent* on different parameters of rice plant being investigated. Among the treatments maximum plant height was recorded in Propiconazole (71.12 cm) followed by Propineb 68.42 cm), the bio-agent *P. fluorescent* also found significant on plant height (65.42 cm) and was at par with Myclobutanils (66.86 cm), maximum flag leaf length and breadth was recorded in Propiconazole (25.39)/(1.7) cm followed by Propineb (24.74)/
(1.67) cm, Myclobutanil (22.41)/(1.59), *Pseudomonas fluorescens* (21.92)/(1.51) cm. Maximum number of filled grain per panicle was recorded in Propiconazole (135.66) followed by Propineb (125.25), bioagent *P. Fluorescent* (120.25) was found significant and found at par with myclobutanils, highest grain yield/ha., in Propiconazole (5.6 t/ha), followed by Propineb (5.46 t/ha), Myclobutanil (4.68 t/ha) and *P. fluorescent* (4.63 t/h) with percent increase of 61.74, 58.36, 35.62 and 34.14 respectively over the untreated Control (3.46t/ha.). Whereas, (T_5, T_6), (T_4, T_2), (T_3, T_1) were not significant among themselves. However, all treatment was found significant in compared with Control. The test also revealed no significant was observed in the investigated growth parameters on panicle length and number of tillers between treatments and Control (data not exhibited).

Discussion

Our present *in-vitro* studies found, among the selected fungicides most effective against the growth of *Helminthosporium oryzae* in terms of biomass production and radial growth of mycelium was recorded in Propiconazole and Propineb followed by Myclobutanil, our finding agreed with Sunder *et al*., (2005) [19] during *in-vitro* analysis of 9 fungicides Propiconazole (EC50, 0.42ppm *a.i*) was found most inhibitory effect against the growth of *H.oryzae*. Sandeep, (2015) [13], reported that Propineb (Antracol) 70 WP, and Aureofungin sol, were found at par in checking the mycelia growth of *H.oryzae* during *in vitro* test. Whereas, Shabana *et al*., (2008) [18] reported that Hinosan, Salicylic acid (mM) and Benzoic acid (mM) can effectively inhibit the growth of brown spot pathogen of rice *Bipolaris oryzae*.

The *in-vitro* dual culture test of *Pseudomonas fluorescent* isolate found maximum percent inhibition on linear growth of fungus at higher cell concentration ($1.3x10^8$)/ml with 52.23 in compared to lower concentration, our finding corroborate with that of Harish *et al*., (2015) [3], who recorded that in dual culture technique *Pseudomonas fluorescens* can inhibit the radial growth of *H.oryzae* (56.50%) at 72 hours of incubation. Prasana *et al*., (2009) [17], also reported that out of 10 isolates of *Pseudomonas fluorescens* tested for antagonistic effect on growth of rice fungus *Rhizoctonia solani*, found *Pseudomonas fluorescens* isolate-003 has recorded best inhibition on linear growth of fungus upto 58%.

Analysis of consecutive two crop seasons (2014-15) and (2015-16) trials revealed that all selected fungicides and *Pseudomonas fluorescent* isolate has significant reduction on disease incidence, in all the three schedule of spray, however, among the treatments highest significant inhibition on percent disease incidence was recorded in Propiconazole, followed by Propineb, Myclobutanil, and *Pseudomonas fluorescent* in compared with other treatments. Our finding was in support with Percich (1989) [15] and Pannu *et al*., (2003) [14], in their studies on comparison of Propiconazole rate for control of fungal brown spot, found Propiconazole treatment reduces the disease intensity of rice brown spot, similarly Moiletti *et al*., (1996) [10] also report that Propiconazole treatment reduces the disease incidence of rice *Akiochi* brown spot diseases. However, Kumar and Rai (2008) [7] reported that Propineb/Antracol-75 WP, and RIL-FA, 200 Sc, effectively reduced brown spot incidence.

Our present investigation also found selected fungicides and *P.fluorescent* significant effect on plant parameters *viz.,* plant height, flag leaf length and breadth, number of filled grain and grain yield/ha. Among treatments Propiconazole recorded lowest disease incidence and highest grain yield followed by Propineb, Myclobutanil, *P. fluorescent,* Carbendazim and Thiophanate respectively. *H. oryzae* being a seed and air borne foliar pathogens, affect the morphological and physiology of rice plant such as photosynthesis, photorespiration and other physiology and metabolic activities. The disease also reduces the rate of photosynthesis as the infected leaves destroyed the chloroplast and chlorophyll content directly or through the enzymes concerned photosynthesis Aldesuquy *et al.,* (1992) [1], therefore reducing disease condition will enhances the growth and development of the plant. Lore *et al*., (2007) [8] reported spraying of crops with Hexaconazole and Propiconazole at early booting and at 50% panicle emergence stage considerably reduced both leaf spot and stalk rot phase of disease along with significant increase in the grain yield. Ou, (1985) [12], reported that heavy infection significantly reduce the number of tillers and grains and lowered the quality and weight of individuals grains resulting in a loss of 30-43%. Terashima *et al.,* (1962) [21] isolated ergosterol from mycelium of the fungus, Cochliobolin extracted and purified from filtrate and Ophiobolin detected in diseased leaves inhibited the growth of roots, coleoptiles and leaves. Nakamura and Oku, (1960) [11], Hegazy *et al.,* (1992) [4] reported that partially purified toxins from the pathogen

inhibited seed germination and reduced root and shoot length in rice cultivars. The present studies therefore found plant growth and developments were directly affected by the fungal pathogen and its infection. Therefore, reducing the disease condition and its intensity will enhance physiological, morphological and metabolic activities leading to increase yielding capacity of the rice plant.

Table 1: Effect of different selected fungicides on biomass production and radial growth of *Helminthosporium oryzae*

Sl. No.	Treatment	Dosage	Average mycelium dry wt. at 240 hrs incubation (g)*	% growth inhibition	Average radial growth at 144hrs incubation (cm)*	% growth inhibition over Control
1.	T0 Control	-	0.35	-	10.61	-
2.	T1 Thiophanate	1000ppm	0.12	65.71	5.73	45.99
3.	T2 Myclobutanil	„	0.10	71.42	5.11	51.83
4.	T3 Carbendazim	„	0.11	68.57	5.66	46.65
5.	T4 Propineb	„	0.07	80.00	2.86	73.04
6.	T5 Propiconazole	„	0.05	85.71	2.73	74.26
	S.Ed (±)		0.01	3.6	0.35	1.67
	CD (0.05%)		0.03	10.44	1.71	4.85

*Mean value of four replication

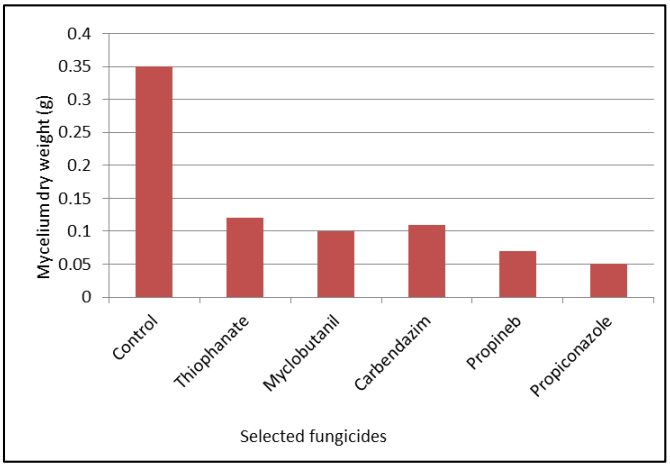

Fig 1: Selected fungicides and biomass production of *H. oryzae* at 10 days incubation

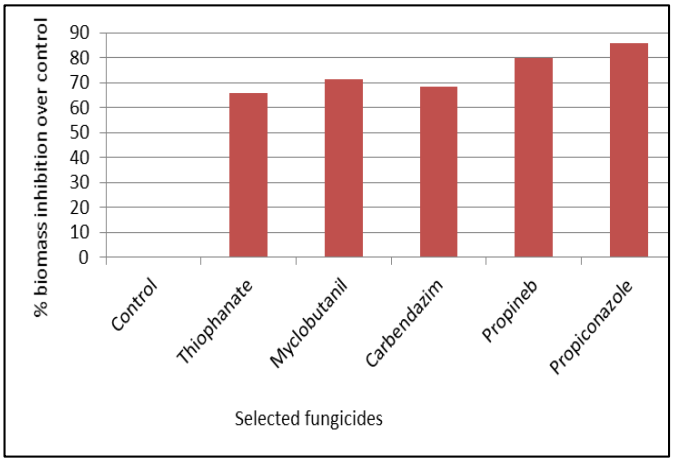

Fig 3: Percent growth inhibition of fungal biomass over Control.

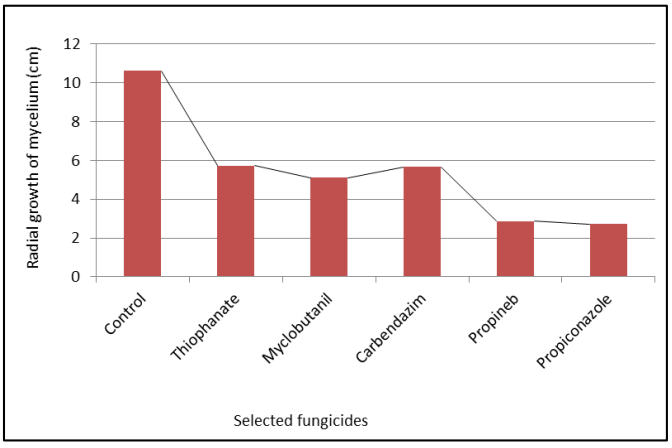

Fig 2: Selected fungicides and percent growth inhibition over control

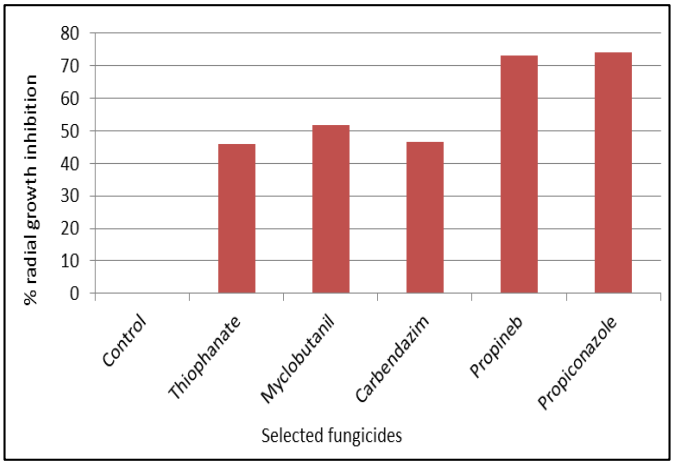

Fig 4: Percent radial growth inhibition over control.

Table 2: Effect of different cell concentration of *P. fluorescent* on linear growth of *H. oryzae*

Sl. No.	Treatment	Dose cell conc./ml	Average linear growth of mycelium at 96 hrs. incubation (cm)*	Percent linear growth inhibition over control
1.	T0 Control	-	7.47	-
2.	T1 *P. fluorescent*	$1.3x10^4$/ml	5.69	29.80
3.	T2 *P. fluorescent*	$1.3x10^6$/ml	4.37	50.95
4.	T3 *P. fluorescent*	$1.3x10^8$/ml	4.29	52.24
	S.Ed (±)	-	0.47	0.12
	CD (0.05%)	-	1.23	1.28

*Mean of five replication

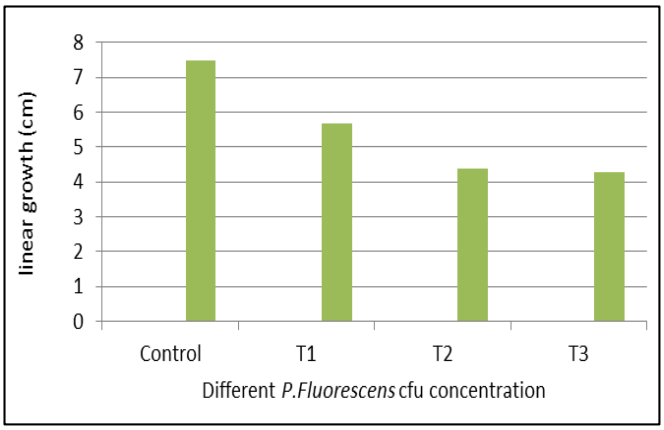

Fig 5: Efficacy of diff. *P. fluorescent cell* conc. on linear growth of *H. oryzae*

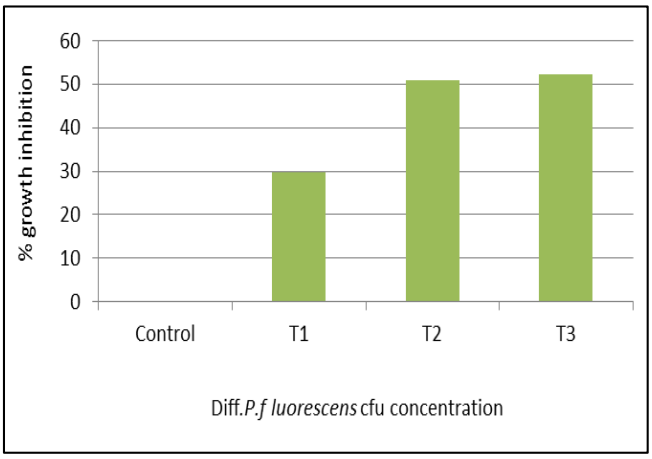

Fig 6: Diff. *P. fluorescent* cell conc. on linear growth inhibition of *H. oryzae*

Table 3: Effect of selected fungicides and *Ps. fluorescent* on percent disease incidence of brown spot disease of rice

lSl. Sl. No.	Treatment	PDI										Pooled mean	% disease reduction over control
		2014-2015					2015- 2016						
		%57 DAT* b	67 DAT* c	77 DAT* d	Mean (bcd)	% reduction overcontrol	57 DAT* b	67 DAT* c	77 DAT* d	Mean (bcd)	% reduction over control		
1.	T0 Control	22.43	29.15	32.76	28.11	0	20.68	26.42	31.29	26.13	0	27.12	0
2.	T1 Thiophanate	12.01	18.17	20.91	17.03	39.41	9.21	16.36	19.37	14.98	42.67	16.00	41.00
3	T2 Myclobutanil	6.91	15.01	13.33	11.75	58.19	5.94	7.59	9.04	7.52	71.22	9.63	64.49
3.	T3 Carbendazim	11.01	11.54	11.54	11.36	59.58	6.91	12.55	11.1	10.18	61.04	10.77	60.28
4.	T4 *Pseudomonas fluorescence*	6.96	11.83	11.69	10.16	63.52	8.40	10.39	10.55	9.78	62.57	9.97	63.23
5.	T5 Propineb	4.69	10.72	10.39	8.6	69.40	4.70	8.31	8.70	7.23	73.09	7.91	70.83
6.	T6 Propiconazole	4.44	9.92	8.94	7.76	72.39	4.23	8.76	8.12	7.03	73.09	7.39	72.12
7.	S.Ed (±)	0.43	0.21	0.22	0.71	1.9	0.17	0.22	0.19	0.19	0.75	0.45	1.32
	CD(0.05%)	0.61	0.29	0.32	0.40	5.60	0.51	0.68	0.57	0.58	2.32	0.49	3.96

*Mean of three replication,
DAT- No.of days after transplantation

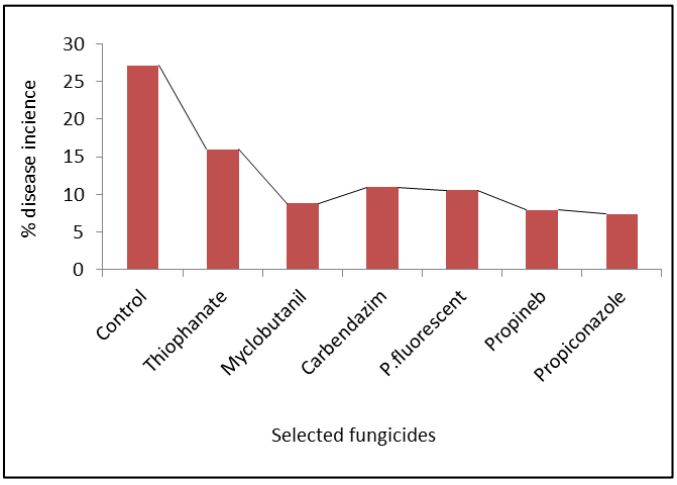

Fig 7: Fungicides and bio-agent (*P. fluorescent*) on PDI, pooled of two cropping periods.

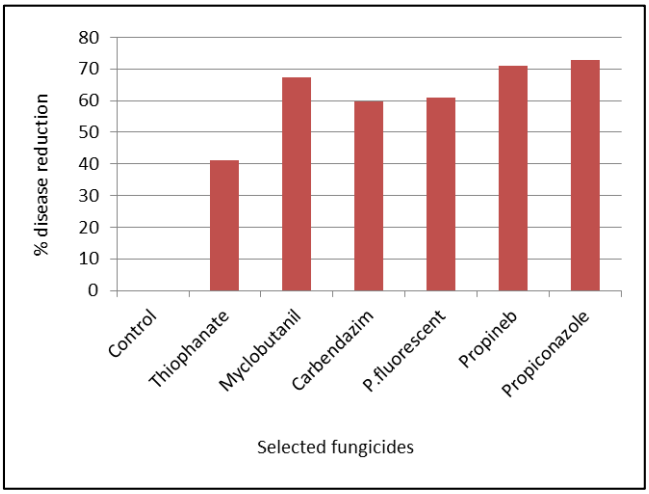

Fig 8: Fungicides and *P. fluorescent* % disease reduction Pooled of two cropping periods

Table 4: Effect of selected fungicides and bioagent *Pseudomonas fluorescent* on various growth parameters and yield of rice

Sl. No.	Treatment	Plant height (cm)*	Flag leaf length (cm)*	Flag leaf Breadth (cm)*	No. of filled grain*	Grain yield tonne/ha.*	% yield increase over Control
1.	T0 Control	59.26	17.63	1.24	93.5	3.46	0
2.	T1 Thiophanate	62.14	20.02	1.5	99.5	4.21	22.09
3.	T2 Myclobutanil	66.86	22.41	1.59	124.25	4.68	35.60
4.	T3 Carbendazim	63.26	21.53	1.51	110	4.27	23.74
5.	T4 *Pseudomonas fluorescent*	65.42	21.92	1.51	120.25	4.63	34.14
6.	T5 *Propineb*	68.42	24.74	1.67	125.25	5.46	58.36
7.	T6 Propiconazole	71.12	25.39	1.7	135.66	5.60	61.74
	S.Ed (±)	0.90	0.2	0.02	1.59	0.5	2.28
	CD (0.05%)	2.78	0.9	0.07	4.9	1.5	6.74

*Mean value of three replication

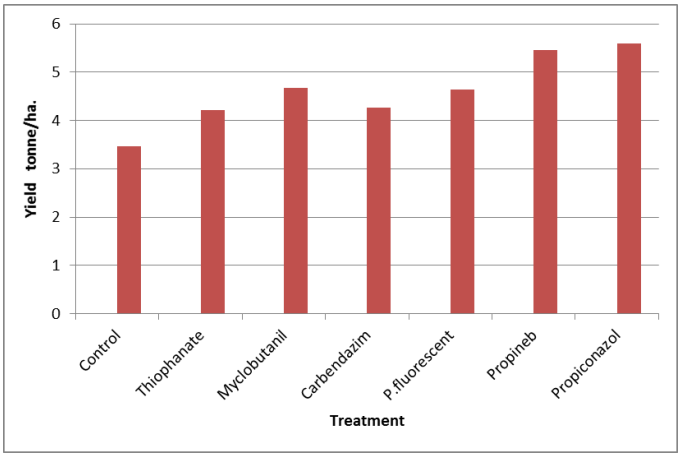

Fig 9: Application of selected fungicides and bioagent *Ps. fluorescent* &yield tonne/ha.

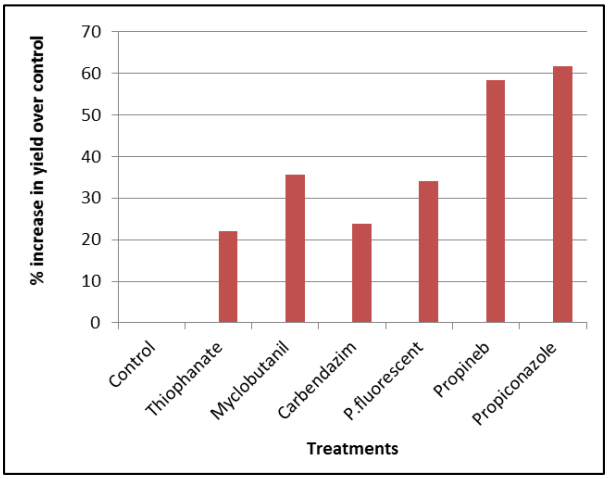

Fig 10: Percent increase in yield over Control plot

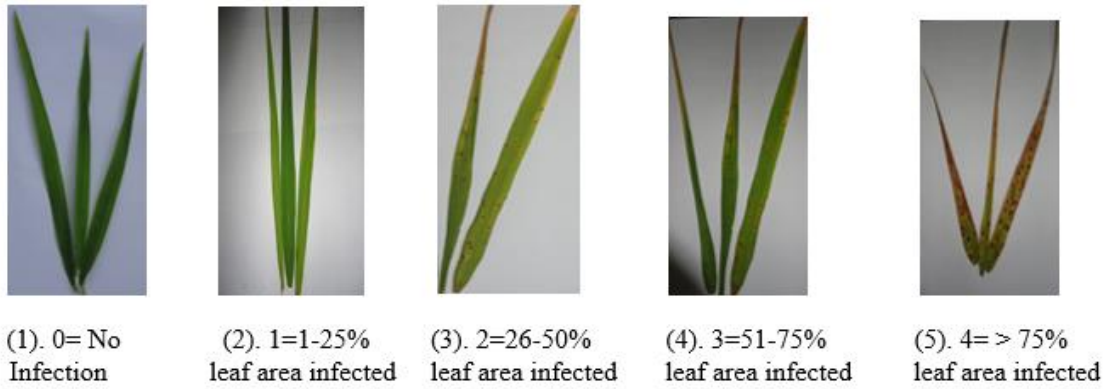

Photo (1, 2, 3, 4, 5): Disease rating scale (0-4) used on the basis of area of leaf infection

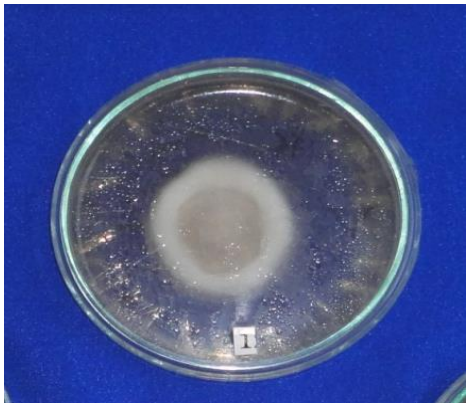

Photo 6: Pure culture of *H. oryzae* a cottony growth developed at 48 hrs.

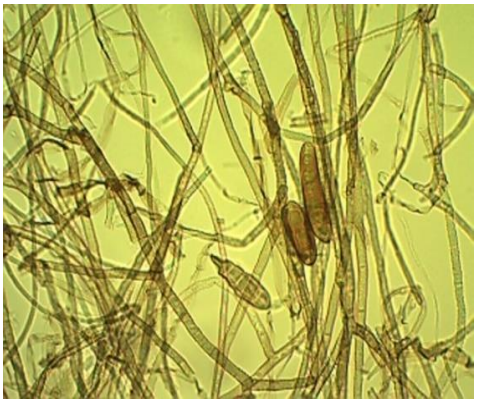

Photo 7: Conidia of brown spot pathogen isolated from infected rice leaf

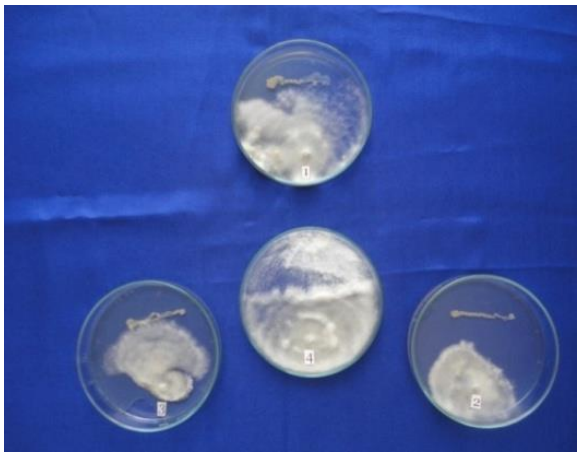

Photo 8: Antagonistic effect of *Ps. Fluorescent* at different cell conc. on linear growth *H. oryzae* (1).1.3x10^{4}/ml, (2). 1.3x10^{6}/ml (3).1.3x10^{8}/ml (4). Control (centre)

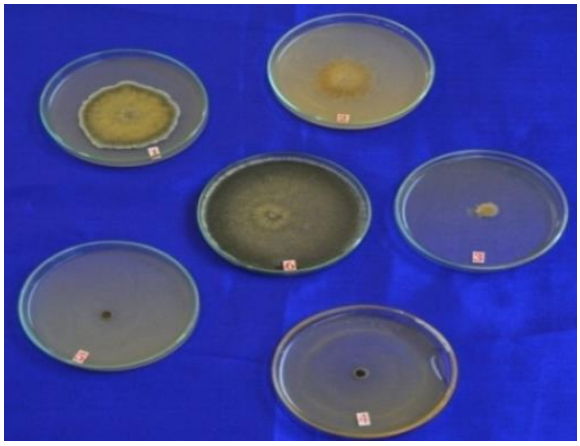

Photo 9: Fungicides against radial growth of *H. oryzae* (1). Thiophanate (2). Myclobutanil, of (3). Carbendazim (4). Propineb (5). Propiconazol (6). Control (centre)

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