The Pharma Innovation Journal 2023; 12(7): 1539-1543

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(7): 1539-1543

www.thepharmajournal.com
Received: 13-04-2023
Accepted: 29-05-2023

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In vitro evaluation of fluorescent *Pseudomonas* and *Trichoderma* isolates against *Rhizoctonia solani* and their compatibility with fungicides

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Abstract
Rhizoctonia solani (Teleomorph: *Thanatephorus cucumeris*) is a Soilborne fungus belongs to class Basidiomycetes. *Rhizoctonia solani* is a complex of 100 species which has wide host range attack all horticultural crops, pastures, cereals and all known crops. *Rhizoctonia solani* is divided into 14 anastomosis groups i.e., (AG1 to AG13 and AGBI). Among *Rhizoctonia solani* AG1 has 3 main intra-specific groups (ISG), one of the most important plant pathogens groups is AG1 IA, which cause diseases like sheath blight, aerial blight, brown patch and banded blight in more than 27 plant families of monocots and dicot, including many plants. Most common practice among farmers for the effective management of *R. solani* is application of fungicide which is adopted worldwide. However, it is necessary to evaluate or screen the new molecules of fungicides for the management of the disease very effectively. Fungicide molecules are hazardous to natural ecosystem an answer to this problem application of fungicides is replaced by use of bio-control agents which is more eco-friendly in the long term. The present investigation was therefore undertaken with the evaluation and identification of potential bio-agents against *R. solani*. Confrontation assays involving 131 bio-agents (121 fluorescent *Pseudomonas* + 10 isolates of *Trichoderma* spp.) identified two potential fluorescent *Pseudomonas* (P86 and P74) and 10 isolates of *Trichoderma* spp. effective against *R. solani*. Isolates of *Trichoderma* spp. (IRRI2, 94a and T14) were showed compatibility with and Thifluzamide 24% SC. The fluorescent *Pseudomonas* isolates (P5, P8 and P10) were compatible at all the tested concentration with Thifluzamide 24% SC.

Keywords: *Rhizoctonia solani*, evaluation, bio-agents, compatibility, fungicides

Introduction
Rhizoctonia solani (Teleomorph: *Thanatephorus cucumeris*) is a soilborne fungus belongs to class Basidiomycetes. *Rhizoctonia solani* is a complex of 100 species which has wide host range attack all horticultural crops, pastures, cereals and all known crops. In nature, *Rhizoctonia solani* usually reproduce asexually by vegetative mycelium or sclerotium. As the causal agent of sheath blight of rice, *Rhizoctonia solani* AG1 IA mainly considered an asexual fungus on rice (*Oryza sativa* L.), sexual structure of its teleomorph has been observed occasionally in the field conditions. The mycelium produced by fungus is Septate, colorless in young stage but they become yellowish brown in older, 8-12 in micrometer. Sclerotia are superficial, more or less globose flattened, white in young become brown and dark brown which is serve as primary inoculum. The fungus considered complex of many species rather than a single species (Adams 1988; Anderson 1982) [2, 3]. Species complex has been divided into many homogeneous groups based on their Hyphal anastomosis. *Rhizoctonia solani* is divided into 14 anastomosis groups i.e., (AG1 to AG13 and AGBI). Among *Rhizoctonia solani* AG1 has 3 main intra-specific groups (ISG), one of the most important plant pathogens groups is AG1 IA, which cause diseases like sheath blight, aerial blight, brown patch and banded blight in more than 27 plant families of monocots and dicot, including many plants. Most common practice among farmers for the effective management of *Rhizoctonia solani* is application of fungicide which is adopted worldwide. However, it is necessary to evaluate or screen the new molecules of fungicides for the management of the disease very effectively. If the fungicide molecules used judiciously in a proper way, chemical control can provide long way for effective management of the disease (Prakasam *et al.*, 2013) [11]. Chemical molecules induce long-term resistance against large number of plant diseases. Fungicide molecules are hazardous to the natural ecosystem an answer to this problem application of fungicides is replaced by the use of bio-control agents which is more eco-friendly in the long term.

Use of bio-agents is found effective against rice diseases as enhancing the plant growth promotion and increasing the yield of grains. Among the several antagonistic agents, *Trichoderma* spp. and fluorescent *Pseudomonas* have been found effective in management of rice sheath blight. Against *Rhizoctonia solani*, introduction of several antagonistic agents provides effective reduction in disease (Prasad and Kumar 2011) [12]. *Trichoderma* and *Pseudomonas* has Chitinase, Glucanase, peroxidase, increased N uptake, phosphate solubilization, siderophore and synthesis of phytohormone production.

Materials and Methods

Isolation of pathogen

Isolation of *R. solani* is made by isolation from infected host plant tissues. Infected plant tissues are cut into 5 cm small pieces, washed in running tap water to eliminate any attached debris, and blotted to dry. Small Sample of leaf lesions tissue which was containing healthy as well as diseased parts were cut in to 0.5 cm small pieces by using sterilized blade. Pieces was surface sterilized with 1% of sodium hypochlorite solution for 1 minutes followed by 3 subsequent changes in sterilized water to remove traces of the chemical. The pieces were then transferred aseptically to an isolation media (e.g., alkaline water agar or Potato Dextrose Agar (PDA) media) or selective (e.g. modified Ko & Hora) medium and incubated at 28±2ºC under BOD incubator. Alkaline water agar medium provides a faster way of isolating the fungus *R. solani* can obtained after 24 hrs. of transfer. The transferred sample was regular examined for fungal growth radiation on petri plates.

Purification

Petri plates poured with 20 ml of PDA medium or alkaline water agar medium, with addition of 300 ppm of streptomycin sulphate to avoid bacterial contamination. A small about 5-8 mm of Mycelial disc from a freshly isolated culture plates cut with the help of cork borer and transferred aseptically to the solidified PDA medium or alkaline water agar medium in each petri plates. Then petri plates were incubated at 28±2 ºC in BOD incubator for growth of the fungus. Adequate numbers of sub culture transformation were separately made for further purification.

Collection of soil samples

Soil samples were collected from crop fields like Mustard, Onion and different Rice fields of Raipur district during 2018 season. Soil samples from the rhizosphere of Mustard, Onion and Rice plants were collected. The collected samples were brought to the laboratory for further experimentation.

Isolation and identification of Bio-agents

The Bio-agents were isolated from collected soil samples of different crops rhizosphere by using serial dilution plate technique. *Trichoderma* specific medium (TSM) (Elad *et al.* 1980) [5] were used for isolation of *Trichoderma* species. The isolated cultures were purified and maintain on PDA for further experiments. For isolation of Fluorescent *Pseudomonas* King's B medium (King *et al.*, 1954) [7] were used. Isolated *Trichoderma species* were identified morphologically according to type of mycelium (Aerial or non-aerial), growth rate and color of mycelium. Fluorescent *Pseudomonas* was identified by pigmentation of colonies.

Evaluation of bio-agents against *Rhizoctonia solani*

Dual culture technique

The isolated bio-agents were evaluated for their antagonistic potential against *R. solani* by dual culture technique (Dennis and Webster, 1971) [4]. 5 mm mycelium disc of the *Trichoderma* and *Rhizoctonia* culture were cut with the help of cork borer and placed on petri plates containing PDA medium. The mycelium disc was placed opposite to each other at equal distance from the periphery. *Trichoderma* inoculated at one side of the petri plate and *Rhizoctonia* was placed at the opposite side of the bio-agent and incubated at 28 ºC in incubator. Petri dishes inoculated with *Rhizoctonia* alone served as control. 3 replications were maintained for each isolate. Percent inhibition of radial growth of pathogens by *Trichoderma* isolates over control is calculated by using the formula.

The isolated Fluorescent *Pseudomonas* was for their antagonistic potential against *R. solani* by Funnel technique (Kotasthane *et al.*, 2017) [9]. 5 mm mycelium disc of the *Rhizoctonia* culture were cut with the help of cork borer and placed on the center of the petri plates containing equal quantity of PDA and King's B medium. Then the edge of funnel was dip in Fluorescent *Pseudomonas* culture which was multiplied on King's B Brath medium for making a ring around the fungal disc. The petri plates then incubated at 28ºC in incubator. Petri dishes inoculated with *Rhizoctonia* alone served as control. 3 replications were maintained for each isolate. Percent inhibition of radial growth of pathogens by Fluorescent *Pseudomonas* isolates over control is calculated by using the formula.

$$\text{Percent inhibition} = \frac{C - T}{C} \text{ X } 100$$

Where,
I = Percent inhibition over control
C = Radial growth of pathogen in control (mm)
T = Radial growth of pathogen in treatment (mm)

Compatibility of *Trichoderma* and fluorescent *Pseudomonas* isolates against Thidluzamide 24% SC, WCPL6060 and BAS 750 02 F 400g/L SC

For *in vitro* compatibility of *Trichoderma* isolates (IRRI2, T14 and 94a) by poison food technique, the fungus was grown on PDA medium for 7 days for experiment. PDA medium was prepared and melted and required quantity of fungicide was added to the melted medium to obtain required concentration. 20 ml of poisoned medium was poured in the sterilized petri plates and control maintained without addition of fungicide. To avoid bacterial contamination 300 ppm streptomycin sulphate was added at the time of pouring. 5 mm mycelial disc was taken from the 7 days old culture of *Trichoderma* isolates inoculated at the center of the petri plate aseptically. Then plates were incubated at 28 ºC and 3 replications were maintained for each treatment. Diameter of the colony was measured when maximum growth of the *Trichoderma* isolates was reached in any of the treatments and the observations were recorded and percent inhibition was calculated by using the formula of Vincent (1947) [19].

For *in vitro* compatibility of fluorescent *Pseudomonas* isolates (P5, P8 and P10) by poison food technique, the bacteria were grown on King's B medium for 2 days for experiment. King's

B medium was prepared and melted and required quantity of fungicide was added to the melted medium to obtain required concentration. 20 ml of poisoned medium was poured in the sterilized petri plates and control maintained without addition of fungicide. Then bacterial culture was streaked on the plate aseptically. Then plates were incubated at 28ºC and 3 replications were maintained for each treatment. After two days of inoculation the observations were recorded and percent inhibition was calculated by using the formula of Vincent (1947) [19].

Where,

$$\text{Percent inhibition} = \frac{C - T}{C} \text{ X } 100$$

I= Percent inhibition
C= Mycelial growth in control
T= Mycelial growth in treatment

Results

Evaluation of fluorescent *Pseudomonas* against *Rhizoctonia solani*

Bipartite interactions were performed following a simple confrontation assay developed by Kotasthane *et al.*, 2017 [9] to identify prospective bio-agent. The technique involves the use of edge of glass funnel to deploy the bio-agent inoculum deposition surrounding pre-inoculated fungal pathogen. Inoculation was done by gently touching the edge of the funnel (containing fluorescent *Pseudomonas*) which encircled the pre-inoculated plant pathogenic fungi on agar plug equidistantly. Inhibition zone was measured after 72 hrs of incubation at 28ºC. Percent inhibition of pathogens by fluorescent Pseudomonas isolates over control was calculated using the formula of (Vincent 1947) [19]:

Percent inhibition of pathogens = [(Growth of pathogen in control - growth of pathogen with Pseudomonas isolate) / growth of pathogen in control] *100

There were differences in the antagonistic abilities of fluorescent *Pseudomonas* isolates against *R. solani* pathogens (Table 1). All of the 121 isolates of fluorescent *Pseudomonas* showed differences in growth inhibitions of *R. solani*. Based on antagonistic abilities fluorescent pseudomonas isolates, 1) nineteen isolates were not able to inhibit; 2) twenty-one isolates exerted poor inhibitory effect (1-25%); 3) Fifty-eight isolates expressed medium inhibitory effect (26-50%); 4) twenty-three fluorescent *Pseudomonas* isolates expressed strong inhibitory (more than 50%). Confrontation assays revealed 23 potential antagonists against *R. solani* which exerted strong inhibitory effects on the mycelia growth of *R. solani.*

Table 1: Average % inhibition of *Rhizoctonia solani* by fluorescent *Pseudomonas* isolates

S. No.	No Inhibition (0%)	Low Inhibition (1-25%)	Medium Inhibition (26-50%)			High Inhibition (More than 50%)
1	P39	P2	P76	P114	P107	P53
2	P40	P10	P47	P117	P119	P15
3	P42	P48	P106	P46	P26	P16
4	P51	P29	P85	P63	P6	P1
5	P62	P105	P98	P24	P4	P72
6	P64	P113	P80	P23	P18	P97
7	P67	P91	P118	P7	P5	P100
8	P69	P81	P60	P20	P19	P41
9	P70	P66	P65	P52	P36	P44
10	P73	P49	P43	P111	P27	P68
11	P77	P32	P56	P3	P21	P31
12	P88	P75	P71	P12	P35	P103
13	P89	P57	P94	P17		P13
14	P90	P58	P59	P34		P30
15	P102	P78	P84	P37		P25
16	P112	P93	P87	P33		P104
17	P116	P95	P96	P109		P38
18	P120	P83	P92	P28		P55
19	P121	P101	P14	P54		P79
20		P108	P22	P8		P45
21		P82	P50	P110		P11
22			P99	P115		P74
23			P61	P9		P86

Evaluation of *Trichoderma* spp. against *Rhizoctonia solani*

The 10 isolates of *Trichoderma* spp. are evaluated for their antagonistic effect on the mycelium growth of *Rhizoctonia solani* under *in vitro* condition. At 7th day after inoculation the data, (Table 2) shows that isolates *viz*., (IRRI2, T14, 94a, T4, T5, T6, T7, T8, T9 and T10) significantly reduced the mycelium growth of *Rhizoctonia solani* over control. Out of 10 isolates T4 showed minimum mycelium growth (15 mm) which is at par (statistically) with isolates IRRI2 (16 mm), T8 (19 mm), T6 (20 mm), T10, 94a (21 mm), T14 (23 mm), T7 (24 mm), T5 (25 mm), and T9 (36 mm). The *Trichoderma* spp. isolates which resulted maximum average % mycelium inhibition i.e., T4 (83.7%), IRRI2 (82.2%) and isolate T9 showed minimum average % mycelium inhibition.

Table 2: Average % inhibition of *Rhizoctonia solani* by *Trichoderma* isolates

S. No.	Isolate	Average % Inhibition
1	IRRI2	82.2
2	T14	74.4
3	94a	76.6
4	T4	83.2
5	T5	72.2
6	T6	77.7
7	T7	73.3
8	T8	78.4
9	T9	60
10	T10	76.6

Several lines of evidence indicate the evaluation and identification of potential bio-control agents viz., fluorescent *Pseudomonas* and *Trichoderma* spp. against different plant pathogens in general and specifically soil-borne plant pathogens like *R. solani* and *S. rolfsii* following confrontation assays in petriplates (Kotasthane *et al.*, 2017, Sharma, Muneeshwar, Razdan, and Mohd Rajik., 2017, Toppo and Tiwari, 2015, Yadav *et al.*, 2018) [9, 14, 15, 20].

Compatibility of bio-agents with fungicide Thifluzamide 24% SC

The carboxamides form a group of systemic fungicides which consist of carboxin and the related oxycarboxin, flutolanil and mepronil (which are also close analogues), fenfuram and thifluzamide. Prasanna Kumar *et al*., (2011b) reported that thifluzamide 24% SC @110 ai/ha were highly effective in controlling sheath blight with increased yield when compared to untreated check. Thifluzamide a new fungicide group of carboxynilide was tested for its efficacy against sheath blight in three seasons (Prasanna Kumar *et al*., 2012). The members of this class have a common mode of action. They interfere with fungal respiration by inhibiting succinate dehydrogenase (succinate-ubiquinone reductase). Thifluzamide a carboxynilide was also evaluated and was found highly effective in controlling sheath blight (Kotasthane Personal communication). In view of this Compatibility of Thifluzamide 24%SC with different isolates of *Trichoderma* was evaluated (Table 3). Three *Trichoderma spp.* IRRI2 T14 and 94a were evaluated on Thifluzamide 24%SC fungicide amended PDA media. Compatibility of Thifluzamide 24% SC with all the isolates was observed at different concentrations (1000 to 3000ppm). This formed the basis for field screening of all the three *Trichoderma* isolates in combination with Thifluzamide 24% SC. Fluorescent *Pseudomonas* isolates (P5, P8 and P10) were evaluated for compatibility with the Thifluzamide 24% SC. All the tested isolates were showed compatibility with Thifluzamide 24% SC at the concentration 1500 ppm.

Table 3: Compatibility of Thifluzamide 24%SC with different isolates of *Trichoderma*

S. No.	Isolate	Radial growth (mm) 7 DAI			Control
		Thifluzamide 24% SC			
		1000 ppm	1500 ppm	3000 ppm	
1	IRRI2	90	90	90	90
2	T14	90	90	90	90
3	94a	90	90	90	90

Isolates of *Trichoderma* spp. (IRRI2, 94a, T14 T4 T5 and T6 T7 T8 T9 T10) were also evaluated for compatibility with the new fungicide molecule WCPL6060 and BAS 750 02. None of the isolate was able to grow in PDA plates incorporated with either of the fungicide (WCPL6060 and BAS 750 02) at a concentration of 1000 ppm. The Fluorescent *Pseudomonas* isolates (P5, P8 and P10) were compatible at the concentration (1500 ppm) with Thifluzamide 24% SC (Table 4).

Table 4: Compatibility of Fluorescent *Pseudomonas* with Thifluzamide 24% SC

S. No.	Isolate	Growth of Fluorescent *Pseudomonas* isolates (2 DAI at 1500 ppm) Concentration
1	P5	+ + +
2	P8	+ + +
3	P10	+ + +
	Control	+ + +
Good = + + +; Moderate = + +; Poor = +; No growth = -		

Fungicide resistant or tolerant isolates for use in integrated control are usually readily obtained by selection on pesticide-containing media (Abd-El Moity *et al.*, 1982; Tronsmo, 1986a, 1991) [1, 16, 18]. *Trichoderma* and *Gliocladium* strains differ in their sensitivities to different pesticides (Koomen *et al.*, 1993) [8]. Tronsmo (1989) [17] showed that, on the average, insecticides used at recommended concentrations were more inhibitory to *Trichoderma* spp. than were fungicides; however, the compatibility of a biological control antagonist with a given pesticide should be confirmed before its use in integrated control. Non-resistant isolates may also be used in integrated control programmes, e.g., following fumigation measures that reduce population levels of the indigenous microflora, thus facilitating colonization by the antagonist. The compatibility of *Pseudomonas fluorescens* with 6 fungicides was tested under laboratory condition. Strain *P. fluorescens* 16 was highly compatible with Propiconazole, tebuconazole, trifloxystrobin + tebuconazole, azoxystrobin, Carbendazim and Carbendazim + Mancozeb at 100 ppm and moderately compatible at 500 ppm concentration and poorly compatible with Propiconazole, tebuconazole, trifloxistrobin + tebuconazole and Carbendazim at 1000 ppm by showing poor growth but showed good compatibility with azoxystrobin. (Hanuman and Bindu Madhavi, 2018) [6].

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