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Research Article

ISOLATION, SCREENING OF RHIZOSPHERE FUNGI ANTAGONISTIC TO RICE STEM ROT DISEASE PATHOGEN *SCLEROTIUM ORYZAE CATT*

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ABSTRACT

Objective: The production of antifungal substances by rhizosphere fungi has long been recognized and this knowledge is entering practical life through the use of fungal antagonists to protect crops against their fungal pathogens.

Methods: In this study rhizosphere fungi isolation in serial dilution plate technique, and their antimicrobial properties against plant pathogen tested by dual culture, disc diffusion method. Effect of these fungi in seed germination and growth promoting activity was measured in pot culture method.

Results and conclusion: Rhizosphere fungi it has become clear that in addition to diffusible substances evidence has accumulated that these bioactive compounds are not only able to promote seed germination and plant growth, but also to strongly inhibit pathogenic fungal growth. As the demand for organic products and the need to render agriculture more sustainable are rising, finding new environmentally friendly crop protection strategies is essential. In this perspective, the newly discovered capacity of fungal bioactive compounds to efficiently repel phytopathogenic fungi in laboratory experiments holds great promise.

Keywords: Rhizosphere, Biological control, *Sclerotium oryzae,* Seed germination.

INTRODUCTION

Rice (*Oryza sativa*) is an important crop worldwide, with over half of the world population dependent on it for food [1]. Rice plants are attacked by many diseases such as stem rot, blast, sheath blight, bakanae disease caused by various phytopathogens, which result in low yield and quality of the crop [2]. Stem rot caused by *Sclerotium oryzae* is one of the major diseases of rice in India. It is prevalent in Andhra Pradesh, *S. oryzae* is a polyphagous soil borne facultative parasite and induces stem rot disease. Though the fungus is plant debris, and soil-borne, soilborne inoculum is more important in causing infection and disease development. It was considered desirable to evaluate the efficacy of some chemical fungicides against the disease Application of chemical fertilizer to control the disease is not only very much effective, but also hazardous to environment and host plant resistance, which is often based on a single gene, may not be durable in the field, leading to frequent resistance breakdown. Fungicides can cause acute toxicity, and some cause chronic toxicity as well [3]. The use of chemical pesticides has been known to cause various environmental and health problems. The International Labor Organization (ILO) estimates that as much as 14 percent of all occupational injuries are due to exposure to pesticides and other agrochemical constituents [4]. The World Health Organization (WHO) and the United Nations Environment Programme estimates that each year, three million workers in agriculture in developing world experience severe poisoning from pesticides, about 18,000 of whom die [5]. Appropriate technological improvement, which results in the more effective use of natural resources, is required in agriculture. One of them is the use of microbial antagonists.

Naturally, the majority of the microorganisms distributed around plant root surface have a role in the decomposition of organic matter, and some may suppress deleterious microorganisms, which could inhibit plant growth. A few of the root-associated microorganisms can promote plant growth, and they have been called "plant growth promoting fungi." Rhizosphere microorganism refers to a bacterial or a fungal microorganism that colonizes the region of the soil immediately adjacent (within 1 mm) to plant roots [6] are different from those living in the non-rhizosphere surrounding soil, both in gross numbers of cells and the variety of strains. The rhizosphere microbial communities influence growth, resistance to disease or even death of the plant host depending on the degree of parasitism and pathogenicity.

The exploration of alternative methods has been a global effort to attain food security because of the public concern on pesticide use in crops. A number of antagonists have been reported to inhibit the growth of rice blast pathogen *Magnaporthe grisea* [7] or sheath blight pathogen *Rhizoctonia solani* [8,9]. Many microbial antagonists have been reported to posses' antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtils*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Trichoderma virens*, *Burkholderia cepacia*, *Sacharomyces* sp., *Gliocadium* sp. The successful control by these antagonists mainly against the diseases caused by following genera of pathogens: *Alternaria*, *Pythium*, *Aspergilus*, *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Botrytis*, *Pyricularia* and *Gaeumanomyces* [10] In particular, an antagonistic *Bacillus subtilis* strain NSRS 89-24 has been found to inhibit the growth of rice pathogens [11]. However, little information is available on the simultaneous effect of fungal antagonists on stem rot pathogens of rice.

These factors have led to the search for new and innovative approaches for plant disease management. Biological control has attained importance in modern agriculture to minimize the residual effects due to the continuous and indiscriminate use of toxic chemicals for the disease control. The addition of organic amendments to soil exerted a favorable effect on disease reduction due to its suppressive nature. The organic amendment not only increases the activity of biocontrol agents but also acts as a source of nutrients to growth promote in crop plants. Present study isolation and identification of rhizosphere fungi and their antagonistic, seed germination activities were tested.

METHODS

Isolation and Identification of the pathogen

S. oryzae was isolated from stem rot infected paddy plants collected from Nellore District using tissue segment method [12]. Small pieces of tissues about 3 mm^2 from infected collar region with some healthy tissue were cut with a sterile scalpel. Then the pieces were surface sterilized with one percent sodium hypochlorite solution for 30 seconds. The tissue pieces were subsequently washed in three changes of sterile distilled water to eliminate excess sodium hypochlorite and then pieces were transferred to potato dextrose agar (PDA) plated Petri dishes.

Plates were incubated at 28±2°C and were observed periodically for the growth of the fungus. The culture was purified by single hyphal tip method and maintained throughout the present investigation by periodical transfer onto PDA.

The pathogen was identified as *S. oryzae* based on its mycelial and sclerotial characters [13].

Isolation of native antagonistic mycoflora from rhizosphere of paddy

Serial dilution technique [14] was used to isolate mycoflora from rhizosphere soil of Paddy. Composite soil sample collected from the rhizosphere of healthy paddy plants was shade dried and then used for serial dilution. 10 g of this soil was dissolved in 100 ml of sterile distilled water to get 10^{-1} dilution. From this 1 ml of soil, suspension was taken and added to 9 ml of sterile distilled water to get 10^{-2} dilution. This is repeated until a final dilution of 10^{-4} was obtained. Antagonistic mycoflora were isolated on Rose Bengal Agar medium by using a dilution of 10^{-4}. 1 ml of soil suspension was taken in sterilized petriplates, melted and cooled medium was poured. Plates were rotated gently to get uniform distribution of soil suspension into the medium. Then the plates were incubated at 28±2°C and observed at frequent intervals for the development of colonies. 3-day-old colonies of mycoflora were picked up and purified by single hyphal tip method. Rhizosphere mycoflora were identified based on mycological keys described by Barnett and Hunter [13]. Mycoflora was maintained by periodical transfer on PDA.

Dual culture technique

This method [15] is used to study the efficacy of biocontrol agent, against plant pathogens under laboratory conditions. Prepare PDA and sterilize the medium in an autoclave at 121°C for 15 minutes pour the medium (20 ml) into sterilized Petri-plate (90 mm diameter) when the medium is in lukewarm state and allow it to solidify at room temperature. Cut the culture discs (7-day-old) of the bioagents and pathogen separately with the help of sterilized cork bores (5 mm). Transfer the culture discs of pathogen and bioagent aseptically and place them at periphery of the Petri plate containing the medium Inoculate with culture disc of the pathogen alone in the Petri plates containing PDA, which serves as control. Transfer the inoculated Petri-plates to an incubator and incubate at 27±2°C. Observe periodically for the growth of the pathogen and antagonist in Petri plates and measure the colony growth (diameter) in each Petri plate. Calculate the per cent inhibition of the pathogen by the bioagent when the growth of the pathogen is full in the control plates. Percent inhibition of growth of the pathogen can be calculated by using following formula;

Disc diffusion method

Preparation of crude fungal extracts

Crude extracts of fungi were prepared as described by Wang [16], All antagonistic culture were cultivated on potato dextrose broth by placing agar blocks of actively growing pure culture in 250 ml Erlenmeyer flaks containing 100 ml of the medium, the flasks were incubated at 26±2°C for 1-week with shaking 120 rpm incubator. Fungal cultures were filtered using filter paper to separate the culture broth and mycelia. All filtrates were transferred to separating fennel filled with equal amount of ethyl acetate stirred fully and left overnight, separating for the organic layer and then further concentrated in a vacuum rotary evaporator to dryness to remove organic solvents. EtOH extracts were dried by freeze drying, and then diluted with dimethyl sulfoxide to a concentration of 10 mg/ml and 5 mm disc were prepared for the antifungal activity assay.

A disc diffusion method pathogenic fungi was inoculated on the center of the PDA plates. The crude extract containing discs were placed on PDA plate with the distance apart from the pathogenic fungi. The control disc was filled with DMSO. The plates incubate 26±2°C for 3 days. The fungal growth was determined by the inhibition distance between crude extract discs and the mycelium of the pathogen compared to the control disc inhibitory zone was measured.

Fungal extract effect on seed germination

Healthy rice (*Oryzae sativa*) seeds were taken into sterile flasks treated with pathogenic fungal suspension, after test antagonistic fungal spore suspension 10, 20% were applied, and control seed were surface sterilized in 2% sodium hypo chloride. These seeds were dried in room temperature then after showing in sterile soil pored polyteen bags. Ten days after planting the percentage of seed germination, rotting infected radicles were recorded there were three replicates of each treatment.

RESULTS AND DISCUSSION

Identification of the pathogen

The pathogen was identified based on mycological characters as *S. oryzae* catt. First the fungal mycelium was silky white in color and later turned to dull white with radial spreading giving fan-like appearance. Microscopic examination of the fungal culture revealed that the mycelium was hyaline, thin walled, septate, and profusely branched with clamp connections. When the fungus attained maturity, small mycelial knots were formed later turned to mustard seed like sclerotia. Initially, sclerotia were deep brown or brownish black shiny, hard, and spherical to irregular in shape. At maturity, the sclerotia showed honeydew like liquid material. The sclerotial bodies were concave on the side attached to the mycelium and were easily detachable from the mycelium. The sclerotia were bigger in size measuring about 1.0-1.3 mm in diameter.

The colony characters and morphological characters of mycelium and sclerotia were in agreement with earlier reports [17]. Thus, the fungus under present investigation was identified as *S. oryzae*.

Identification of native antagonistic rhizosphere mycoflora

Rhizosphere mycoflora were identified based on colony and morphological characters. *Penicillium notatum*, *Rhizopus* sp., *Aspergillus flavus, Aspergillus niger, Fusarium* sp., *Penicillium* sp., *Trichoderma harizanum Aspergillus* sp. *and Fusarium* cultures were isolated, observations regarding the color and number of colonies per gram of rhizosphere soil (Table 1).

In vitro* evaluation of antagonistic mycoflora against *S. oryzae

A total of eight identified fungi *viz., Fusarium* sp., *Aspergillus flavus, A. niger, Penicillium* sp. *Penicillium notatum, Trichoderma harizanum, Alternaria* sp., and *Rhizopus* sp. were isolated from rhizosphere samples of paddy. The antagonistic effect of these isolates was assessed based on their ability to inhibit the pathogen growth and sclerotial population by dual culture technique under *in vitro*. Moreover, the efficacy of antagonists against the test pathogen was assessed on the basis of the ability to form the inhibition zone. The reduction in the growth of the pathogen due to antagonistic mycoflora was calculated and expressed in percent inhibition.

The data pertaining to percent inhibition of mycelial of *S. oryzae* due to antagonistic mycoflora are presented in Table 2, (Fig. 1). The data reveals that the *T. harizanum* was found to be superior followed by *Aspergillus flavus*, *Aspergillus niger* compared to others in reducing the mycelial growth and sclerotial population. The native *T. harizanum* isolate was superior with highest percent inhibition of mycelial growth by 74.50% and the microscopic examination revealed that the hyphae of

Table 1: List of antagonistic mycoflora and percentage of inhibition to pathogenic fungi

S. No	Rhizosphere fungi	Number of colonies per gram of soil*
1	*Penicillium* sp.	23
2	*P. notatum*	10
3	*A. flavus*	24
4	*T. harizanum*	10
5	*A. niger*	7
6	*Fusarium* sp.	15
7	*Rhizopus* sp.	11
8	*Alternaria* sp.	12

*Mean of 10 plates, *P. notatum: Penicillium notatum, T. harizanum: Trichoderma harizanum, A. flavus: Aspergillus flavus*

antagonist coiled around the hyphae of the pathogen. *Aspergillus flavus* inhibited mycelial growth by 70.86%, *Pencillum notetum* inhibited mycelial growth by 48.50% The remaining fungi also inhibited mycelia growth indicate that overall percent inhibition of mycelial growth of *S. oryzae* was maximum in case of *T. harizanum* and minimum in case of *Rhizopus* sp. Effect antagonists in dual culture were screened also inhibited to disc diffusion method. Maximum inhibition zone as observed *T. harizanum* extract followed by *A. flavus, Penicillium* sp. And *Fusarium* sp., was most effective inhibited to pathogenic fungi *S. oryzae.*

In pot culture experiments, the effect of seed germination, with fungal antagonists, on the stem rot fungus is shown in Table 3 (Fig. 3). In seeds naturally infected with *S. oryzae*, where seed infection was high, the biological treatment gave maximal germination and survival.

All treatments decreased significantly stem rot and radicle infection as compared with the control. However, seed germination was increased significantly only by *Trichoderma* sp. compared with control. In this test, *S. oryzae* controlled most effective treatments for reducing seed infection were *T. harzianum* and *A. flavus* causing 85% and 76% seed rot reduction. However, *Penicillium* sp. Treatments were the least effective ones for reducing seed infection. The seed treated with antagonists ensure quicker and more effective utilization of the antagonists by the plants than the addition of antagonists to the soil. The action of biological agents at the seed surface seems to be more effective than soil application of fungal antagonists.

Abdelmonem and Rasmy [18] found that seed treated with *Trichoderma* spp. was the best biological treatment for reducing seed and seedling infections of mangrove caused by fungi and bacteria. The stem rots diseased fungus *S. oryzae*. The advantage of biological seed treatment is that protection can be prolonged, whereas chemical protects the seeds, the antagonists protect the seeds and roots. The rate of seed germination was increased only when seeds were either treated with culture filtrate or coated with a spore suspension of *T. harizanum* compared with controls. In this study, *Aspergillus flavus* appeared to be a more promising antagonist, as seed protecting bioagent, than *Trichoderma* spp. because it protected completely rice seeds and radicles against the infection of *S. oryzae*, the causal agent of stem rot disease of rice.

T. harzianum capable of lysing mycelia of *Sclerotium rolfsi* and *Rhizoctonia solani* was isolated from a soil naturally infested with those pathogens [19]. Under greenhouse conditions, incorporation of the wheat-bran inoculums preparation of *T. harzianum* in pathogen-infested soil reduced significantly bean diseases caused by *S. rolfsi, R. solani*, or both, but its biocontrol capacity was inversely correlated with temperature. The wheat-branpreparation of T. *harzianum* increased the growth of bean plants in a non-infested soil and its controlled *S. rolfsi* more efficiently than a conidial suspension of the same antagonist. In naturally infested soils, wheat-bran preparation of *T. harzianum* inoculums significantly decreased diseases caused by *S. rolfsi* or *R. solani* in three field experiments with bean, cotton, or tomato, and these increased significantly the yield of beans [19]. Patale and Mukadam [20] tested the antagonistic activites of there *Trichoderma* species, i.e. *T. viride, T. harzianum*, and *Trichoderma* sp. against seven pathogenic fungi, namely *Aspergillus niger*, *A. flavus*, *Phytophthora* sp., *Fusarium oxysporum*, *Rhizoctonia solani, Penicilium notatum,* and *Alternaria solani.* They found that all three species of *Trichoderma* suppressed effectively the growth of seven pathogenic fungi.

Table 2: Rhizosphere fungi inhibition of *S. oryzae* in dual culture and disc diffusion methods

S. No	Rhizosphere fungi	% of inhibition dual culture	ZOI
1	*Penicillium* sp.	38.36±0.25	10.2±0.08
2	*P. notatum*	48.50±0.15	10.5±0.26
3	*A. flavus*	70.86±0.22	12.6±0.28
4	*T. harizanum*	74.50±0.24	13.4±0.12
5	*A. niger*	35.12±0.15	10.5±0.14
6	*Fusarium* sp.	48.64±0.18	11.2±0.23
7	*Rhizopus* sp.	26.38±0.24	8.4±0.20
8	*Alternaria* sp.	42.52±0.22	9.5±0.19

S. oryzae: Sclerotium oryzae, P. notatum: Penicillium notatum, T. harizanum: Trichoderma harizanum, A. flavus: Aspergillus flavus, ZOI: Zone of inhibition, *A. niger: Aspergillus niger*

Table 3: The effect of seed treatment with fungal antagonists on *S. oryzae* as measured by percentages of seed germination and infection

S. No	Treatments	Seed germination %	Infection
1	*S. oryzae*	40.50	+
2	*S. oryzae+T. harizanum*	85.06	-
3	*S. oryzae+A. flavus*	76.42	-
5	*S. oryzae+Pencillium* sp.	60.00	-
6	*S. oryzae+Fusarium* sp.	54.50	+
7	*S. oryzae+rhizopus*	46.00	
8	*S. oryzae+A. Niger*	58.04	-
9	*S. oryzae+Alternaria* sp.	55.56	+
	None	94.00	-

*Seeds of rice surface-sterilized with 2% sodium hypochlorite, *oryzae: Sclerotium oryzae, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger*

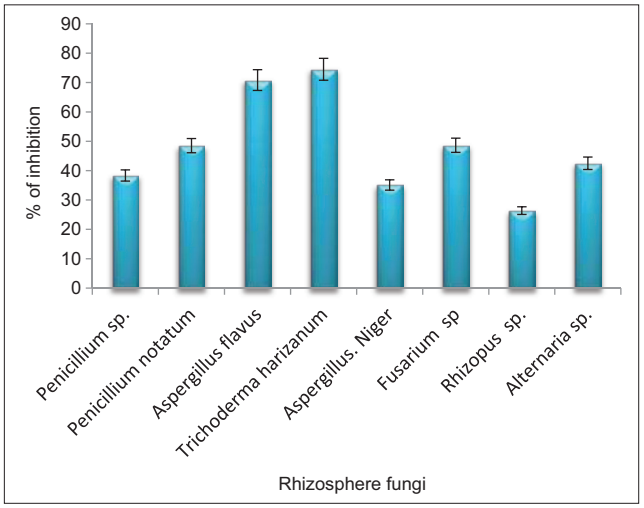

Fig. 1: Rhizosphere fungi Inhibition of *Sclerotium oryzae* in dual culture technique

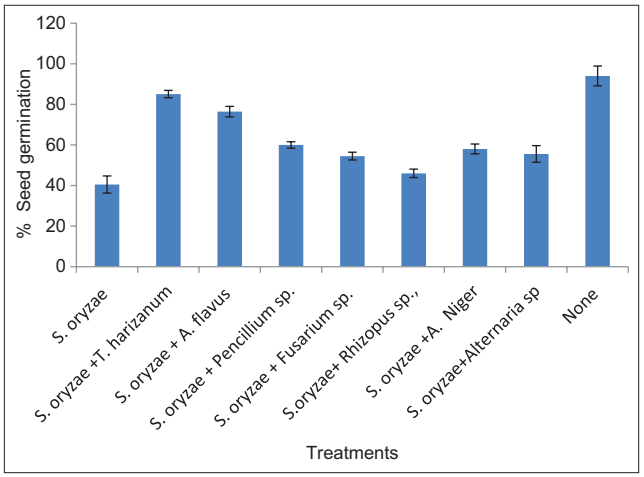

Fig. 2: The effect of seed treatment with fungal antagonists on *Sclerotium oryzae* as measured by percentages of seed germination and infection

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