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Antagonistic activity of fungal cultures filtrate and their enzyme activity against fusarium moniliforme causing bakanae diseases in paddy

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Abstract
The present investigation deals with the antagonistic activity of several microfungi (*Aspergillus fumigatus, A. ochraceus, A. terreus, A. niger, Cephalosporium* sp., *Fusarium oxysporum, F. solani, Helmithosporium* sp., *Trichoderma* sp., *Memonimila* sp., *Penicillium granulatum* and *Penicillium* sp.) on the growth of *Fusarium moniliforme.* Among them *Trichoderma viride* showed excellent performance when compared with other soil microfungi. *Penicillium* sp. recorded minimum zone of inhibition by against *F.moniliforme.* Surprisingly, all the microfungi possess various degrees of antifungal properties against *Fusarium moniliforme*. The effect of culture filtrate of antagonistic fungi by using different concentrations (5, 10, 15 and 20%) treated against *F.moniliforme* by *in-vitro* method. Among the different concentration, the 20% concentration seems to be nil growth, whereas 5% concentration of potential fungal extract (filtrate) has enormous action against *F.moniliforme*. The antagonist potential fungal culture filtrate not only controls the *F.moniliforme* growth but also the fungal enzymes can suppress its growth and other parameters. The fingi of *A. fumigatus* fungal enzymes have an excellent performance of antagonistic activity. The effect of *Trichoderma* sp. fungal enzymes of amalyse, cellulose, lipase, pectinase and protease showed significant results than the *A. fumigatus*.

Keywords: dual culture, culture filtrate, enzyme activity

1. Introduction

Paddy (*Oryza sativa* L.) is an important crop worldwide with over half of the world population dependent on it for food. Paddy 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. Bakanae disease is one of the most important diseases affecting Paddy (*Oryza sativa* L.). It was first identified in 1828 in Japan, and is present in the all of the major Paddy cultivation areas throughout the world. The most striking disease symptoms are yellowing and anomalous elongation of infected plants due to the production of plant fungal gibberellic acid which has resulted in the disease being called the Japanese word 'bakanae' which means the foolish seedling. Wetanabe (1974) reported bakanae disease caused by *Fusarium moniliforme* shield and the pathogen was soil borne. Antagonistic activity on a wide ranging group of pathogens such as *Sclerotium rolfsii, S.cepivorum, Rhizoctonia solani, Pythium ultimum, Phytophthora parasitica* and *M. phaseolina* has seen investigated by several authors (Bell *et al*.; 1982; Balasundaram and Sarbhoy, 1988; Harrison and Stewart 1988 ; Hussain *et al*., 1990; Adekunle *et al*., 2006)[3, 2, 7, 9, 1].

The fungal pathogen is also known to cause stalk rot and leaf blight of corn, stalk rot of sorghum, endosepsis and crown rot of Asparagus. On rice, *F. moniliforme* induces seedling elongation, foot rot, seedling rot, grain sterility, and grain discoloration (Ou, 1985) [15]. The pathogen can be both seed-borne and soil borne. Generally, the seed-borne inoculum provides initial foci for secondary infection. Under favorable environmental conditions, infected plants in different foci have the capacity to produce numerous conidia that subsequently infect proximate healthy plants resulted in yield loss (Ou, 1985) [15]. In recent years, in Japan raising rice seedlings in seed boxes for mechanical transplanting has created many seedling disease problems that were not considered important with open-field nursery for manual transporation. Among the fungal diseases, bakanae is frequently encountered and considered as an important (Ogawa, 1988) [13]. Even in open-field nurseries, one of the problems encountered by farmers in the control of bakanae disease is difficulty in distinguishing infected seedlings from healthy plants because there are no obvious symptoms of infected seedlings except the height or slight pale yellowing at the stage of transplanting (Kim, 1981) [11]. The fungus not only causes considerable damage on many plants, but also is parasitic on plants without producing visible symptoms (Hsieh, 1977) [8]. It can be isolated even from kernels that are healthy in appearance. Rice seedlings that grow from these infected seeds tended to display bakanae symptoms (Padwick, 1950) [16].

Materials and Methods

Dual culture method (Skidmore and Dickinson, 1976)

Potential; antagonist's culture blocks of 5mm diameter cut from the margin of 8 days old cultures of both test pathogen and antagonists were placed opposite to each other on PDA in 90mm glass petriplates. The distance between inoculum block was 50mm. The inoculated plates were incubated at room temperature (28±2 °C). The colony growth and radial growth of the pathogen was measured at both sides i.e. towards and opposing each other from their central loci. Intermingled or inhibition zone was also measured at the same period. The assessment of interaction was made following the model of Skidmore and Dickinson, 1976 [19].

The parameters used for the assessment of colony interaction were the width of inhibition zone of growth intermingled zone and percent inhibition of radial growth, I. e. $100 \times (r_1 - r_2)/ r_1$, where, r_1 denotes the radial growth of the paddy pathogen towards the opposite side and r_2 denotes the radius of the pathogen towards the antagonist to fungus.

Culture filtrate method (Rabindran and Vidyasekaran, 1996) [18]

To assess the effect of culture filtrate, the antagonist fungi were grown on PDA medium. Three mycelia agar discs (each of 5mm diameter) of an individual antagonist, were cut from the actively growing margins of 5 day old culture and were inoculated into a 250ml conical flask containing 100ml Potato Dextrose broth medium. After 10 days of incubation at (30 ± 2 °C) the culture of an antagonist was filtered through a filter paper and then centrifuged at 3000rpm for 20 minutes and finally filter through a micro pore filter paper under vacuum pressure to obtain the cell free culture filtrate. The Culture filtrate of an antagonist containing its non – volatile metabolites was tested in three concentrations (5, 10, 15 and 20%) against rice pathogen. The culture filtrate of a particular concentration was obtained by supplemented it with required amount of sterilized PDA medium. The each Petri plate was inoculated centrally with a 5mm. mycelia agar disc cut from the margin of actively growing culture of a *Fusarium moniliforme* pathogen. All the plates were incubated at room temperature (28 ± 2 °C). The radial growth of the colonies was measured after five days of incubation. The percent growth inhibition of a rice pathogen was calculated by using the formula.

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

Where,
I-Percent inhibition of the pathogen
C-Radial growth of the pathogen in control
T-Radial growth in of the pathogen in treatment

Enzyme Assay

Amylase (Gessner, 1980) [5]

Amylase activity was assayed by growing the fungi on starch medium (starch - 2 g, peptone - 1 g, yeast extract - 1 g, agar - 20 g; distilled water-lL. After 5-10 days, the plates were flooded with 1% aqueous IKI solution. A yellow zone around the colony in an otherwise blue medium was considered as positive test for starch hydrolysis.

Cellulolytic enzymes (Gessner, 1980) [5]

Three methods were used to detect cellulolytic enzymes (cellulase, endoglucanase and β-glucosidase). The detailed methods were described by Pointing (1999) [17]. The cellulolysis basal medium (CBA) consisted of: C4H12N206 - 5 g, KH2P04 - 1 g, MgS04.7H20 - 0.5 g, yeast-extract - 0.1g, CaCl2.2H20 - 0.001 g, distilled water - 1L.

Cellulase (cellulose azure agar) (Pointing, 1999) [17]

Cellulolysis Basal Medium supplemented with 1.8% w/v agar was transferred in 10mL aliquots to glass culture bottles, autoclaved, and allowed to solidify. Then gently and carefully mixed viscous 0.1mL CBM medium supplemented with 1% w/v cellulose azure (azure I dye, C.I.5201O) and 1.8% w/v agar was added to the surface of the solidified agar as an overlay. The BI-layered medium was inoculated with discs of the test fungi and incubated. The migration of dye into the clear lower layer indicates the presence of cellulase.

Lipase (Pointing, 1999) [17]

Lipase activity was determined by growing the isolates on a medium containing a lipid (Tween 20, Sigma Chemical Co.) as the primary source of carbon. The medium was as follows: peptone - 1 g, yeast extract - 0.1 g, agar 18g, Tween 20 - 10 mL (autoclaved separately from the rest of the medium), distilled water - 990 mL. A positive test was the occurrence of precipitated fatty acid crystals around the colony.

Pectolytic enzymes (Hankin and Anagnostakis, 1975) [6]

To detect pectylotic activity, the present study used the/medium contained 500mL of mineral salt solution, 1 g yeast extract, 15 g of agar, 5g of pectin, and 500 mL of distilled water. The mineral salts solution contained per liter: 2 (NH4)2 SO_4, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $FeSO_4.7H_2O$, 1 mg $CaCl_2$, 10 µg H_3BO_3, 10 µg Mn SO_4, 10 µg $ZnSO_4$, 50 µg $CuSO_4$, 10 µg; MoO_3, pH-7 or pH-5 as needed. This medium at pH7 was used to detect pectate lyase production. The same medium at pH5 was used to detect polygalacturonase activity. For all tests, plates were incubated for 5-10 days and then flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. This reagent precipitates intact pectin in the medium and thus a clear zone around the colony in opaque medium indicates the degradation of the pectin.

Proteolytic enzymes (Pointing, 1999) [17]

The medium used to detect proteolytic enzyme activity contained gelatin as the protein substrate (Hankin and Anagnostakis, 1975) [6]. This medium consists of nutrient agar plus 0.4% gelatin at pH6. An 8% solution of gelatin in water was sterilized separately and added to the nutrient agar at the rate of 5mL per 100 mL of medium. After incubation, plates were flooded with an aqueous saturated solution of ammonium sulfate which precipitates the protein. A clear zone around colonies indicates the presence of protease.

Results and Discussion

In the previous study, dual culture combinations of *T. viride* with
F. moniliforme showed the inhibition of the pathogen in all the treatments. with the highest percentage growth inhibition in the treatment where the antagonist was introduced 2 days before inoculation of the pathogen. According to Campbell, Okigbo, there are no bio control agents that have enough competitive ability to displace an already established pathogen (Okigbo, 2005) [14].
The present investigation reported that the effect of antagonistic fungi on the growth of inhibition of *Fusarium moniliforme* by beneficial fungi like *Aspergillus fumigatus, A. orchraceus, A. terreus, A. niger, Cephalosporium* sp., *Fusarium oxyporum, F. solani, Helminthosporium* sp., *Memnoniella* sp., *Penicillium* sp. and *Trichoderma viride* were maneuvering results noticed. The maximum zone of growth inhibition from *Cephalosporium* sp to *F. monilifome*. According to the either fungal interactions of beneficial fungi against the *F. moniliforme* by causing

paddy field disease can be reduced respectively and minimum zone of inhibition was observed in *Memnoniella* sp. against *F. moniliforme.* However, the antagonistic potential of fungi has shown an extraordinary performance against pathogenic fungi *F.moniliforme* because fungal secondary metabolites which contain rich amount of bioactive compounds (Table-1).

In the previous study, the time lapse between inoculation of *T. viride* and *F. moniliforme* contributed to the success recorded with the antagonist effect on the pathogenic fungus. Minimum inhibition concentration showed that the antagonist effect of *T. harzianum* has more objective when it was introduced 2 days before inoculation of *F. moniliforme*; this is due to its high competitiveness for nutrient content, mycoparasitism as well as production of antibiosis which is responsible for degradation of fungus cell wall. Since *T. harzianum* has fast growth rate and can easily colonize site, it is recommended that application of the antagonist on yam sets, leaves of growing plant as well as yam tubers in storage can greatly inhibit the fungal growth and development and hence reduce pre harvest and postharvest losses of the crop.

The current study proved that the antagonistic efficacy of fungal culture filtrate of different concentrations of 5, 10, 15 and 20% against *F. moniliforme.* About 20% concentration of culture filtrate shown no growth of *F. moniliforme,* but 5% of culture filtrate expressed moderate growth on *F.moniliforme*. Among the culture filtrate of antagonistic fungi, the *Trichoderma viride* has been observed an excellent suppression of test pathogenic fungi (Table-2).

In this previous study, the effects of abiotic stress factors on *Trichoderma* isolates were observed and the antagonistic activities of native isolates were studied against some *Fusarium* species (*F. solani, F. culmorum, F. moniliforme, F. verticilloides* and *F. chlamydosporum*) *Trichoderma* isolates were produced chitinase in liquid medium. *Trichoderma* isolates have a very tolerance of temperature, NaCl and PEG. Adaptation of *Trichoderma* isolates to environment with different stress factors seems to be important mechanisms of evolution enabling the effective biocontrol activity against plant pathogens. The activity is mainly depends on specific to isolate. The chitinase activity produced by local isolates of *Trichoderma* sp. may be effective in biological control of *Fusarium* species (Sowmya *et al.,* 2014) [20].

In phytopathology, antagonistic refers to the action of any organisms that suppress (or) in the normal growth and activity of a plant pathogen mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites and induction of plant defense system are typical bio control action of these fungi (Kohl *et al.,* 2019) [12].

Antagonistic activity of fungi appeared at different magnitudes. All rhizobacterial isolates significantly inhibited the growth of pathogens while the inhibition zone varied from 4.09 to 74.97%. Isolate BR15 and BR4 were found most efficient in in-vitro conditions and exhibited 50.82% and 74.97% inhibition of *Fusarium oxysporum* f. sp. cepae respectively. The highest (74.97%) inhibitory effect was found in BRS4 while the lowest 4.09% inhibitory effect was found in BRS3. The plates served as control were found completely covered by fungal mycelia showed no inhibition zone (Javed *et al.,* 2019) [10].

The involvement of enzymes in biological control complicates the distinction between mycoparasitism and antibiosis. The production of a cell wall degrading enzyme may be involved simultaneously in both parasitism and antibiosis. In this regard, Elad *et al*. (1983) [4] demonstrated hyphal penetration by *Trichoderma* sp., mediated by enzyme activity. In addition, *T. harzianum* was able to parasitise *Rhizoctonia solani* hyphae by producing chitinase. In other instances, however, *Trichoderma* sp. produces enzymes without evidence of parasitism.

In the present study the effect of potential fungal enzymes like amylase, cellulase, lipase, pectinase and protease produce more versatile enzymes in order to develop more level, sustainable and economically competitive production process for agricultural industry. However, the *Trichoderma* sp. of the enzymes has enormous quantity of production when compared with *A. fumigatus* fungal enzyme and found to be useful to the agriculture industrial applications (Table-3).

Table 1: Effect of antagonistic fungi on the inhibition of *F.moniliforme* by *invitro* method

S. No.	Name of the fungi	Zone of inhibition (mm) *F.moniliforme*		
		Beneficial organism	Harmful organism	Intermediate
1.	*Aspergillus fumigatus*	12.3±4.01	11.0±3.66	9.05±1.12
2.	*A.ochraceus*	9.33±3.11	6.00±2.33	10.6±1.06
3.	*A.terreus*	15.6±5.02	10.1±1.33	6.03±1.10
4.	*Aspergillus niger*	11.3±3.76	4.66±1.55	8.04±2.04
5.	*Cephalosporium* sp.	12.0±4.00	11.6±3.86	7.05±1.15
6.	*Fusarium oxysporum*	11.3±3.76	2.08±1.52	11.8±3.08
7.	*F.solani*	12.3±4.01	3.91±1.97	10.2±2.10
8.	*Helminthosporium* sp.	13.0±5.01	2.01±1.07	11.5±3.09
9.	*Memnoniella* sp.	17.0±5.66	1.97±0.91	7.02±1.08
10.	*Penicillium granulatum*	12.1±4.04	5.97±2.97	11.6±2.12
11.	*Penicillium* sp.	19.0±6.33	5.02±2.08	8.06±1.05
12.	*Trichoderma viride*	20.0±5.33	6.10±3.17	12.5±3.10

Standard deviation ± Standard error

Table 2: Effect of culture filtrate of antagonistic fungi against *F.moniliforme*

S. No.	Name of the fungi	Zone of inhibition (mm)			
		5%	10%	15%	20%
1.	*Aspergillus fumigatus*	2.10±1.03	0.00±0.00	0.00±0.00	0.00±0.00
2.	*A. terreus*	3.15±2.09	2.10±1.03	1.07±0.98	0.00±0.00
3.	*A. niger*	2.05±1.01	0.00±0.00	0.00±0.00	0.00±0.00
4.	*Cephalosporium* sp.	2.20±1.10	1.15±1.09	0.10±0.03	0.00±0.00
5.	*Memonmila* sp.	3.05±2.01	0.00±0.00	0.00±0.00	0.00±0.00
6.	*Penicillium* sp.	2.13±1.06	1.05±0.98	0.00±0.00	0.00±0.00
7.	*Trichoderma viride*	2.25±1.15	1.20±1.10	0.95±0.50	0.00±0.00

Standard deviation ± Standard error

Table 3: Effect of potential antagonistic fungal enzymes against *F.moniliforme*

S. No.	Name of the fungi	Name of the enzymes	*Fusarium moniliforme*
			Zone of inhibition (mm)
1.	*Aspergillus fumigatus*	Amylase	7.00±2.33
		Cellulase	3.66±1.22
		Lipase	2.66±0.88
		Pectinase	5.00±1.66
		Protease	4.66±1.55
2.	*Trichoderma viride*	Amylase	5.00±1.66
		Cellulase	4.66±1.55
		Lipase	5.00±1.66
		Pectinase	6.00±2.00
		Protease	8.33±2.77

Standard deviation ± Standard error

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