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Antagonistic Effects of *Bacillus subtilis* and *Pseudomonas fluorescens* Against Seed-Borne Mycoflora of *Pennisetum americanum*

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ABSTRACT

Antagonistic activity of two bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* against *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus and Fusarium oxysporum* of *Pennisetum americanum* was studied. *In vitro* studied were carried out in both dual culture technique and blotter test method. In dual culture technique the*Bacillus subtilis* was most antagonistic ones to the seed-borne mycoflora *in vitro*, while the other isolate *Pseudomonas fluroescens* did not show any antagonistic activity on any seed-borne mycoflora. *Bacillus subtilis* antagonistic isolate as well as the commercial biocide was applied as seed treatment for controlling seed-borne mycoflora under Blotter test *in vitro* and Pot experiment *in vivo* conditions. It was observed that maximum seed germination and maximum shoot and root length recorded with *Aspergillus flavus* and *Bacillus subtilis* combination in Pot experiment. Experiment shows, that *Bacillus subtilis* antagonistic isolate was able to significant reduction in seed-borne mycoflora than *Pseudomonas fluorescens* in *Pennisetum americanum*

1) INTRODUCTION

Pennisetum americanum is the staple diet of vast population of the drought pronesemi arid region of the world. Several seed-borne mycoflora have been reported as internally and externally seeds [1, 2, 3, 4, 5] which cause spoilage of seeds and produce many mycotoxin. Through, seed-borne mycoflora can be reduced by seed treatment with fungicides but they do not persist for the whole cropping season.

The use of chemical fungicides is being discouraged in recent year due to environmental pollution and rising costs. Methyl bromide is a good example for a very efficient soil fumigant that has a great impact on the environment and has been recently phased out to the public concern and international agreements [6]. Therefore, the use of bio fungicides and an integrated approach to pathogenic fungi control have become necessary. Bio fungicides are biodegradable (environment-friend), non-toxic, cost-effective and helps in increasing the nutritional value of soil.

The use of antagonistic microorganisms against seed-borne mycoflora like *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus and Fusarium oxysporum* has been investigated as one of the alternative control methods. Both bacteria are wild spread throughout the world and have been recognized as the most successful biocides agents for pathogenic mycoflora several mode of action of efficient bioagents on reducing diseases have been described, including competition for nutrients, antibiosis, resistance, mycoparasitism, plant growth promotion and rhizospheric colonization capability [6, 7, 8, 9, 10]. Studies on the antagonistic effect of bacteria (*Bacillus subtillus* and *Pseudomonas fluorescens*) were employed against seed-borne mycoflora on seeds of *pennisetusm americanum.* It was investigated that *Bacillus subtillis* showed most effective antagonistic effect against seed-borne mycoflora while *Pseudomonas fluorescens* showed no inhibition against the above seed-borne mycoflora. Management of toxigenic mycoflora associated with seeds of *pennisetusm americanum* through biocontrol agent *Bacillus subtillis* may be safe, long lasting and ecofriendly. Therefore, in the present investigation, relative efficacy of biocontrol of seed-borne mycoflora was assessed under laboratory conditions.

2) MATERIALS AND METHODS

2.1 Isolation and purification of bacterial strains

Seed samples of *pennisetusm americanum* (HHB-67) were

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collected Bikaner, Jaipur, Jodhpur, Nagaur and Sikar districts of Rajasthan. Detection of internal and external seed-borne mycoflora was carried out by standard Blotter techniques and Agar plate (PDA) methods [11]. Preliminary microscopic examination of the mycoflora isolated showed that they could be classified under two genera, *i.e. Aspergillus* and *Fusarium*. *Aspergillus* and *Fusarium* isolates were purified by plating single conidial spores [12].

2.2 Isolation, purification bacteria

Antagonistic microorganisms were isolated from soil rhizosphere samples of healthy *Pennisetum americanum* plants producing area at Jaipur districts of Rajasthan. The used bioagents were isolated on selected medium nutrient agar media and minimal media to the methods recommended by [13]. The bacterial isolates were identified as *Bacillus subtilis and Pseudomonas fluorescence* according to the morphological and biochemical activities in standard tests [14].

2.3 In vitro screening antagonistic effect

The study was carried out employing a 'Dual Culture Test' method [15]. Seed-borne fungi were grown separately on Petri-plate containing PDA medium for 14 days at room temperature. An aliquot of 10 ml of sterile distilled water was added to each of the above Petri-plate and conidia were gently freed from the culture by shaking. The remaining conidia were dislodged with a sterile brush and the suspension was collected in a test tube. It was passed through cheese cloth, centrifuged at 2500 rpm for 10 minutes and re suspended in sterile distilled water.

The isolates of two antagonistic bacteria viz., *Bacillus subtilis* and *Pseudomonas fluorescence* were grown in nutrient broth culture with 1.0 optical Density was further analysed for spore count. The viable cells count of *Pseudomonas fluorescence* and *Bacillus subtilis* were approx $236X10^6$ and $205X10^6$, respectively which were estimated by the serial dilution technique.

The suspension of seed-borne mycoflora was spread on the PDA medium after 12 hours. O.2 ml of antagonistic bacteria having 1.0 OD was inoculated in the centre of Petri-plate. These plates kept under 28^oC for 8 days. After 8 days, the inhibition zone between the two bacteria and mycoflora was estimated with the help of microscope.

2.4 Effect of biocontrol agents on seed-borne mycoflora

In this experiment, technique for suspension preparation in the same as used in dual culture test.Seed pelleting method – fungal spore were count using hemocytometer and spore concentration adjusting to $15X10^3$ conidia/ml 10 seeds were pelleted with 3 ml. Spore suspension for each seed-borne fungi for 30 minutes following by carboxyl methyl cellulose (0.2%w/v) for 50 second and them dried in shade, After drying, the seeds were pelleted with 1 ml of bacterial suspension (1.0 OD) containing gum Arabic [16]. In case of control unionculated seeds were dipped only in carboxyl methyl cellulose solution,

One hundred seeds of *Pennisetum americanum*(for each treatment and uninoculated control) were placed on moisture blotter paper in sterilized Petri-plate@ 10 seeds *et al*per plate and incubated at 28^oC for 10 days [11]. After incubation percent germination of seeds, root and shoot length of seedling were measured.

2.5 Experiment

Pennisetum americanum seeds were pelleted by the seed-borne fungi individually and in combination with the antagonistic bacterium as described earlier. Treated seeds were sown in earthen pots containing garden soil. The soil was sterilized by autoclaving. The antagonistic treated seeds (four per pot) were shown in each pot at a depth of 3 cm.pot were out treatment served as control. Four replicated pots were for each treatment. Pots were water daily to maintain the field capacity. Effect of seed coating was recorded on seed germination. The plants were harvest after 90 days and growth parameter like root and shoot length, root and shoot dry weight were recorded.

Simultaneously, population colony forming unit (cfu) of seed-borne fungi and antagonistic bacterium individually, per gm of soil was determined at a dilution of 10^{-3} and 10^{-6} by dilution plate technique on PDA medium and nutrient agar. The number of individually colonies appearing on each culture plate on the 4^{th} day determined the number of colony forming unit (cfu) per gm of soil.

3) RESULTS AND DISCUSSION

In vitro studies indicated that only antagonistic bacterium *Bacillus subtilis* inhibited the growth of the seed-borne fungi with different degrees of inhibition. The maximum inhibition zone created by *Bacillus subtilis* against *Aspergillus flavus* and *Aspergillus terreus* was 1.0 cm and against *A.niger* was 0.9 cm and the minimum zone of 0.8 cm was recorded against *Aspergillus fumigatus* and *Fusarium oxysporum*by the bacterium. The *Pseudomonas flureoscence* used to study its antasgonistic behaviour against the seed-borne fungi did not show any inhibition (**Table 1**). The above study is in agreement with reports of produced certain antibiotics, responsible for the inhibition of the growth of *Aspergillus* species and *Fusarium* species During the course of this study, inhibition of various fungi by the *Bacillus subtilis* could be due to some such effect. Many investigators reported that many microorganisms are able to inhibit growth of the pathogenic fungi [9, 17, 18, 19]. Elad [20] stated that mechanisms of the antagonism of many microorganisms like fungi and bacteria against different pathogens may be due to mycoparasitism, competition and antibiosis.

Table-1:Antagonastic Behaviour of *Bacillus subtilis*and *Pseudomonas fluorescens*with Seeds of*Pennisetum americanum*

Antagonastic bacteria + Seed-borne fungi	Growth of fungi in (cm)	Inhibition zone(cm)
Basubtilis+Aspergillus flavus	9.0	1.0±0.02
Basubtilis+ Aspergillus fumigates	9.0	0.8±0.05
Basubtilis+ Aspergillus niger	9.0	0.9±0.10
Basubtilis+ Aspergillus terreus	9.0	1.0±0.41
Basubtilis+ Fusarium oxysporum	9.0	0.8±0.05
P. fluorescens+ Aspergillus flavus	9.0	-
P. fluorescens+ Aspergillus fumigates	9.0	-
P. fluorescens+ Aspergillus niger	9.0	-
P. fluorescens+ Aspergillus terreus	9.0	-
P. fluorescens+ Fusarium oxysporum	9.0	-

In the present study, the bioagent evaluated under DCT were further tested in blotter test as biological seed dressing agents against seed-borne mycoflora of *Pennisetum americanum* [21, 22, 23, 24, 25, 26]. Several combinations of *Bacillus subtilis* with *Aspergillus flavus, Aspergillus niger, Aspergillus fumigates* and *Aspergillus terreus* were experimented. Results revealed that the combination of *Aspergillus flavus* and *Bacillus subtilis* were best in terms of seed germination (74.0%) and growth shoot length (8.4 cm) and root length (8.0 cm) as comparison to single inoculation treatment with *Aspergillus flavus*. The second best performance of seed germination (68%) was recorded with combination of *Bacillus subtilis* and *Aspergillus terreus* while the remaining dual combination recorded lesser values of seed germination and growth than that single inoculation and uninoculated control.

Table-2: Effect of Seed Pelleting of Seed-borne mycoflora and Bacterium (*Bacillus subtilis*) on Seed Germination and Growth of *Pennisetum americanum* (Blotter Test)

Treatment	Seed germination (%)	Shoot length (cm)	Root length (cm)
Control (uninoculated)	60	6.1±0.15	7.0±0.88
Aspergillus flavus alone	45	2.3±0.05	2.8±0.26
Aspergillus fumigates alone	40	1.8±0.26	2.5±0.34
Aspergillus niger alone	34	1.5±0.14	2.1±0.95
Aspergillus terreus alone	30	1.3±0.28	1.8±0.14
*Fusarium oxysporum*alone	36	2.0±0.01	1.9±0.07
B.subtilis+Aspergillus flavus	74	8.4±0.31	8.0±0.74
B. subtilis+ A. fumigates	58	5.6±0.84	6.8±0.64
B.subtilis+ Aspergillus niger	62	6.2±0.69	7.4±0.10
B. subtilis+ Aspergillus terreus	68	7.0±0.05	7.9±0.24
B. subtilis+ F.oxysporum	60	5.8±0.02	6.9±0.22

Seed treatment with different seed-borne fungi and biological agent bacterium *Bacillus subtilis* greatly influenced the germination of *Pennisetum americanum* seeds as compared to control (**Table 3**). Maximum average seed germination of (65%), shoot length (69.0 cm), root length (25.80 cm), shoot dry weight (1.5203gm), root dry weight (0.1986 gm) and population of antagonistic bacterium $114X10^6$ was recorded with *Aspergillus flavus + Bacillus subtilis* combination followed by *Aspergillus terreus + Bacillus subtilis, Aspergillus niger + Bacillus subtilis, Fusarium oxysporum + Bacillus subtilis*and *Aspergillus fumigatus + Bacillus subtilis* combination. The maximum population of seed-borne mycoflora of $25X10^3$ was recorded with *Aspergillus fumigatus + Bacillus subtilis* followed by *Fusarium. oxysporum + Bacillus subtilis, Aspergillus niger + Bacillus subtilis* and *Aspergillus flavus + Bacillus subtilis.* This proves that *Bacillus subtilis* is showing to antagonistic effect, which is significantly in suppressing the growth of *Aspergillus* species and *Fusarium oxysporum.*

The reasons for microbial antagonism has been previously work out by the following workers [21, 22, 23, 24, 25, 26, 27]. According to them bacterium *Bacillus subtilis* treatment reduced seed colonization and root rot caused by *Fusarium solani* and it was suggested in the form of antibiotics that inhibit the seeds-borne mycoflora. In the present study, the lower counts of *Aspergillus* species and *Fusarium* species in the rhizosphere of test seedling indicate the prevalence of some such mechanism operating inhibiting the growth of seed-borne mycoflora.

There are many mechanisms suggested to clarify the role of antagonistic organisms in suppression of growth pathogens and thus to control diseases. Their action could be through antibiosis [28], mycoparasitism [29], competition for nutrients and/or space [30]. Also, the other mechanisms involved are induction of resistance in plants through increased of oxidative enzymes, *i.e.* polyphenol oxidase, peroxidase, enhanced lignifications [31], induction of pathogeneses related protein

Table-3: Effect of Seed Pelleting of Seed-borne mycoflora and Bacterium (*Bacillus subtilis*) on Seed Germination and Growth of *Pennisetum americanum* (Pot Experiment)

Treatment	Seed germination (%)	Shoot		Root		Population of antagonistic bacteria ($cfux10^6/g$)	Population of seed-borne fungi ($cfux10^3/g$)
		Length (cm)	Dry weight (g)	Length (cm)	Dry weight (g)		
Control (uninoculated)	68	69.2±0.04	1.53±0.02	27.2±0.60	0.14±0.95	0	0
***Bacillus subtilis* alone**	72	71.5±0.26	1.68±0.60	29.5±014	0.20±011	195±0.01	0
Aspergillus flavus *alone*	39	36.2±0.21	0.84±0.15	15.0±0.3	0.14±0.02	0	32±0.01
A. fumigates alone	31	29.8±0.15	0.70±0.48	12.8±0.02	0.09±0.55	0	47±0.26
A.niger alone	28	34.2±0.36	0.80±0.95	14.5±0.01	0.09±0.20	0	42±0.00
A.terreus alone	24	20.4±0.18	0.53±0.45	8.9±0.84	0.01±0.00	0	42±0.22
F. oxysporum alone	32	25.2±0.10	0.60±0.04	13.2±0.22	0.09±0.06	0	40±0.52
Bacillus subtilis+Aspergillus flavus	65	69.0±0.02	1.52±0.05	25.8±0.14	0.19±0.01	114±0.02	12±0.60
Bacillus subtilis+ Aspergillusfumigates	50	50.8±0.01	0.98±0.10	20.6±0.59	0.16±0.62	70±0.09	25±0.12
Bacillus subtilis+ Aspergillus niger	56	55.6±0.35	1.01±0.03	23.5±0.95	0.17±0.58	82±0.04	20±0.26
Bacillus subtilis+ Aspergillus terreus	62	67.3±0.01	1.48±0.84	24.5±0.00	0.18±0.04	98±0.21	16±0.05
Bacillus subtilis+ Fusarium oxysporum	54	52.3±0.32	1.10±0.04	20.8±0.02	0.16±0.46	72±0.15	22±0.09

(PR-1), chitinase and β, 1-3, gluconase in addition to increase salicylic acid (SA) level in plants [32].

4) CONCLUSION

On the bases of the above observations it can be concluded that management of seed-borne mycoflora of *Pennisetum americanum* could be based on antagonistic effect of bacterium *Bacillus subtilis* increase of plant growth under field conditions and significant reduction of seed-borne mycoflora. Also, the obtained bioagent *Bacillus subtilis* proved to be a commercial biocide product, but this needs further studies on this bacterium isolates before using in the biological control programs.

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