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Evaluation of Bio Control Efficacy of *Pseudomonas fluorescens* AS15 against Banded Leaf and Sheath Blight Pathogen (*Rhizoctonia solani*) in different Carbon and Nitrogen Sources

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A B S T R A C T

Banded leaf and sheath blight disease causes major yield loss of maize. Bio control agents control the disease without any harmful effect. *Pseudomonas fluorescence* is a potential bio control agent due to production lytic enzymes and various secondary metabolites such as DAPG, Phenazine, pyoluteorin, pyrollnitrin. In dual plate assay *P. fluorescens* AS15 culture and it supernatant inhibit the growth of *Rhizoctonia solani* with 35.5% and 86.6% percent inhibition respectively. The λ max for DAPG was detected in all carbon and nitrogen sources except tween 20 and Pyoluteorin λ max was also detected in all carbon and nitrogen sources except arabinose and tween 20. Whereas λ max for pyrrolnitrin was detected in the presence of serine, arginine, L-phenylalanine, threonine, arabinose, ducitol, sucrose, glucose and galactose. The best sources for the growth of *P. fluorescens* AS15 were sucrose and glucose. Good percent inhibition of *P. fluorescens* AS15 against *Rhizoctonia solani* was observed in the presence of inulin, dulcitol, glucose, galactose, serine and sucrose. In present study we conclude that glucose is the best carbon source for the growth, production of antibiotic and antagonistic behaviour of *P. fluorescens* AS15.

Introduction

Among all diseases of maize, Banded Leaf and Sheath Blight (BL and SB) disease is considered as most damaging (Payak and Sharma, 1985). BL and SB is disease caused by *Rhizoctonia solani*. It leads to yield loss of upto 40%. Banded Leaf and sheath blight is one of the major disease of maize causes upto 40% yield loss. Infected leaves and sheaths that are discoloured and there is appearance of irregular to roundish spots on both the surfaces (Saxena, 1997). BL and SB disease be controlled by two main methods, chemical and biological. The use of chemical pesticides is hazardous and environmentally damaging. Biological control is an important control measure because it controls the disease without any harmful effects. *Pseudomonas* is ideal antagonists as they have ability to produce various inhibitory compounds (Schroth and Hancock, 1982). Bio control agent which isolates from the rhizosphere of

maize performed as better bio control agent against maize pathogen because it easily colonize on the plant root and survive for long term. This study aimed to finding best carbon and nitrogen sources that would allow maximum growth and antibiotic production of *P. fluorescens* AS15 for improving its bio control potentiality against banded leaf and sheath blight pathogen. The growth medium has a profound effect on growth and active metabolite production. The accurate incorporation of nutrients enhanced growth as well as antagonistic behavior of bio control agents (Slininger *et al.,* 1996).

Materials and Methods

Isolation of culture

Ten different bacterial strains were isolated using King's B medium from rhizospheric soil of maize obtained from CRC GBPUA and T Pantnagar. Out of ten, only one bacterial strain (AS15) was selected as bio control agent on the basis of its antagonistic potential. Banded leaf and sheath blight pathogen (*AS5*) was isolated from infected leaves of maize and evaluated for its virulence by pathogenicity testing.

Pathogenecity test of fungal pathogen

Isolated *Rhizoctonia solani* used in this study was assessed for its pathogenic potential through *in vivo* assay on maize plants carried out in a glasshouse. Pathogenicity test was conducted in a completely randomized (CR) design with four replicates.

The 5 kg capacity pots were filled with sterilized soil and three surface sterilized seed were sown in each pot at temperature range from 30-40°C with high humidity. Pathogen was inoculated on 30 day old maize plant between the stem and leaf sheath (Vidhyasekaran *et al.,* 1997). The plants were observed for development of disease symptoms and infected leaves were used for re-isolation of *Rhizoctonia solani*.

In vitro antagonism assay

Pseudomonas fluorescens AS15 was screened for *in vitro* antagonistic activity against *Rhizoctonia solani* f. sp. *sasakii* by dual culture plate assay (Anith and co-workers, 2003) using both whole cell and cell free supernatant against phytopathogenic fungi. A disc of *Rhizoctonia solani* f. sp. *sasakii* growing on PDA was placed at the centre of the Petri plate. Log phase culture of *Pseudomonas fluorescens* AS15 was streaked directly at the edge of the Petri plate containing 1/4th strength of King's B + PDA. Log phase culture of bio control agent was centrifuged and cell free supernatant used for *in vitro* antagonistic assay by disc method. Plates were incubated at 28±1°C for three days and examined for inhibition of fungus by the bacterium. Thereafter, sizes of inhibition zone of fungus (in cm) were determined by measuring the distance between the fungal mycelia and the bacterial culture. The percent inhibition was calculated as described by Hagedorn *et al.,* (1989). The percent inhibition (PI) was calculated by the following formula:-

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

Where, C is the growth of test pathogen (in cm) in the absence of the antagonistic strain, T is the growth of test pathogen (in cm) in the presence of the antagonistic strain.

Antibiotic production assay

Stock solution 10% of each carbon and all nitrogen sources were prepared in sterilized TDW or 1N NaOH and filtered through 0.22 µm bacteriological filter and stored at 4°C. The production of antibiotics was assayed in

liquid basal medium M1 (K_2HPO_4=1g, KH_2PO_4=1g, $FeCl_3.6H_2O$=0.01g, $MgSO_4.7H_2O$=0.2g, $CaCl_2$=0.1g, $(NH_4)_2SO_4$=1g, Agar=15g) amended with different carbon and nitrogen sources separately with three replications. Carbon and nitrogen sources were added in Erhlenmeyer flask containing 40 ml M1 basal medium to the final concentration of 2%. Each flask was inoculated with single colony of *P. fluorescens* AS15 and incubated at 28±1°C, 120 rpm on rotary shaker.

The sample were withdrawn twice at 36 and 72 h respectively and analysed for growth and antibiotic production. Growth was measured at 600 nm and antibiotics such as DAPG, pyoluteorin, pyrollnitrin were detected with its λ max at 225, 227 and 325 nm respectively by UV-Visible spectrophotometer. Same samples were also analysed for *in vitro* antagonistic activity against *Rhizoctonia solani*.

Results and Discussion

Characterization of Microbial Isolate

Bacterial strain AS15 is gram negative (Fig. 1a), short rod and appeared as white mucoid colony on King's B Agar (Fig. 1b). It molecularly characterized as *Pseudomonas fluorescens* on the basis of 16SrDNA sequencing. It possessed antifungal attributes viz., production of fungal cell wall lytic enzymes such as chitinase, lipase, cellulase, β-1, 3-glucanase and protease as well as antibiotics (2, 4 diacetylphloroglucinol, pyoluteorin, pyrrolnitrin) (Rajwar, 2015).

Disease symptoms were appeared on leaf and sheath after 48h of pathogen inoculation (Fig. 2) during pathogenecity test. Fungal mycelium initially appears white but turns brown after 4 days of incubation at 25±1°C. It forms sclerotium that appears as dark brown color spots on the PDA plates (Singh and Sharma, 1976; Maiti, 1978 and Akhtar *et al.*, 2009). Vegetative hypha is branched near distal septum at angle of 90°, and constricted near the point of branching (Fig. 3).

In vitro antagonism assay

The whole cell culture and cell free supernatant of *Pseudomonas fluorescens* AS15 inhibits the fungal mycelium of *Rhizoctonia solani in vitro*. The percent inhibition with whole cell culture and cell free supernatant was 35.5% and 86.6% respectively. The higher percent inhibition in case of supernatant assay indicates that secondary metabolites produced by *P. fluorescens* AS15 play a significant role in inhibition (Fig. 4).

The medium components (carbon and nitrogen sources, their ratios, mineral factors) and conditions (pH, temperature and agitation) used to grow bio control agents influence their growth as well as subsequent properties like secondary metabolite production (Zhang *et al.*, 2005). The aim of the study was to find out carbon and nitrogen sources that provide maximum biomass production for *Pseudomonas fluorescens* strain AS15 and enhanced antibiotic production.

Antibiotic production and antagonistic behavior of *P. fluorescens* AS15 against *R. solani* with different amendment

All the three antibiotics, DAPG, pyrrolnitrin, pyoluteorin were detected in basal medium supplemented with glucose, galactose, L-phenylalanine, threonine and arginine. In glucose and galactose the production started at 36 h and continued till 72 h. The production of DAPG was detected in basal medium incorporated with all carbon sources except tween 20. Pyoluteorin was detected in all carbon and nitrogen sources except arabinose and tween 20 (Table 1).

Table.1 Influence of carbon and nitrogen source amendment in Liquid basal medium on Antibiotics production of *Pseudomonas fluorescens* AS15

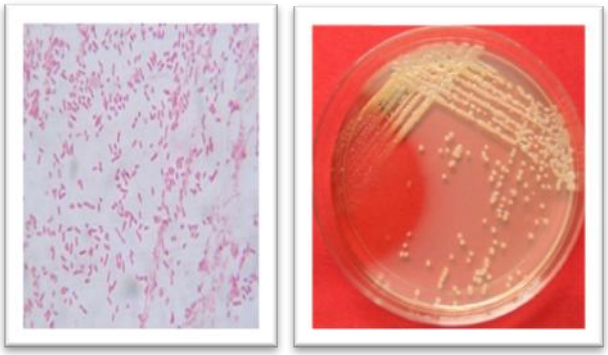
S.No.	Carbon sources	Detection of various antibiotics spectrophotometrically					
		DAPG λmax 225		Pyoleutorin λmax 272		Pyrolnitrin λmax 325	
		36h	72h	36h	72h	36h	72h
1	Serine	+	+	+	+	+	-
2	Arginine	+	+	-	+	+	+
3	Glutamic acid	-	+	-	+	-	-
4	L-Phenyalanine	+	+	+	+	+	-
5	L- Threonine	+	+	+	+	+	-
6	L-Alanine	-	+	-	+	-	-
7	Arabinose	+	+	-	-	+	+
8	Inulin	+	+	+	+	-	-
9	Adonitol	+	+	+	+	-	-
10	Fructose	+	+	+	+	-	-
11	Ducitol	+	+	-	+	+	+
12	Sucrose	+	+	+	+	+	-
13	Glucose	+	+	+	+	+	+
14	Galactose	+	+	+	+	+	+
15	Maltose	+	+	-	+	-	-
16	Glycerol	+	+	+	+	-	-
17	Ribose	+	+	+	+	-	-
18	Dextrin	+	+	-	+	-	-
19	T 20	-	-	-	-	-	-
20	T 80	+	+	+	+	-	-

Table.2 Effects of different sources of carbon and nitrogen on growth and Antagonistic efficacy of *Pseudomonas fluorescens* AS15

S.No.	Name of carbon source	Zone of inhibition (cm)	OD at 600nm
1	Control	0.1 ± 0.05	0.01
2	Serine	1.0 ± 0.11	1.8
3	Arginine	0.7 ± 0.05	1.8
4	Glutamic acid	0.3 ± 0.10	1.8
5	L- Phenyalanine	0.9 ± 0.10	0.8
6	L- Threonine	0.4 ± 0.05	1.4
7	L-Alanine	0.6 ± 0.10	1.4
8	Arabinose	0.9 ± 0.10	1.6
9	Inulin	1.2 ± 0.05	1.6
10	Adonitol	0.7 ± 0.05	1.6
11	Fructose	0.9 ± 0.10	1.6
12	Dulcitol	1.1 ± 0.10	1.8
13	Sucrose	1.2 ± 0.07	2.0
14	Glucose	1.3 ± 0.05	2.0
15	Galactose	1.2 ± 0.05	1.6
16	Maltose	0.6 ± 0.10	1.5
17	Glycerol	0.8 ± 0.07	1.8
18	Ribose	0.5 ± 0.05	1.3
19	Dextrin	0.2 ± 0.05	1.2
20	Tween 20	0.0 ± 0.00	0.9
21	Tween 80	0.3 ± 0.05	0.4

Cor- 0.35082 between zone of inhibition and OD at 36 hrs

Fig.1 Growth of *P. fluorescens* AS15 on King'S B medium (a) and gram staining (b)



(a) (b)

Fig.2 BL and SB disease symptom in maize plant inoculated with *Rhizoctonia solani* in pathogenecity test

Fig.3 Lactophenol cotton blue stained *Rhizoctonia solani* hyphae (40X)

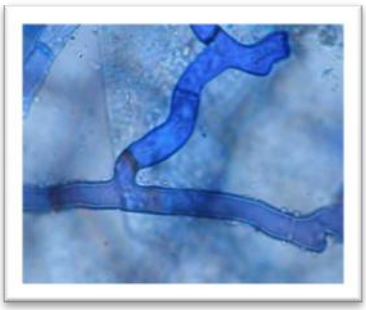
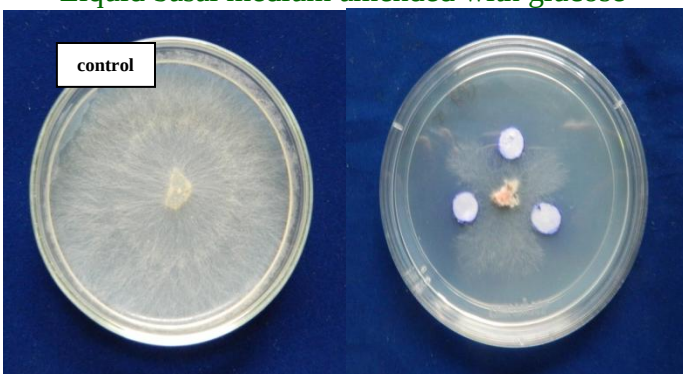


Fig.4 Inhibition of *Rhizoctonia solani* in the presence of supernatant of Liquid basal medium amended with glucose



Single antibiotic pyrrolnitrin was detected when basal medium was supplemented with serine, arginine, L-phenylalanine, threonine, arabinose, ducitol, sucrose, glucose and galactose, at 36 h and its production continue till 72h in the presence of arginine, arabinose, dulcitol, glucose and galactose.

Effects of carbon and nitrogen sources on growth and *invitro* antagonistic activity of *P. fluorescens* AS15

Adonitol, arabinose, arginine, dulcitol, fructose, galactose, glucose, glutamic acids, glycerol, serine, sucrose and Inulin are good sources for growth of *Pseudomonas fluorescens* AS15 as optical density ranges from 1.6- 2.0 at 600 nm (Table 2). Highest optical density 2.0 was observed in the presence of sucrose and glucose while 1.8 optical density was observed with serine, arginine, glutamic acid, dulcitol, glycerol, there was no correlation between the growth and *in vitro* antagonism of bio control agent against pathogen. *P. fluorescens* strain AS15 growing in basal medium amended with sucrose showed highest zone of inhibition against *Rhizoctonia solani* and fairly good inhibition was observed in the presence of inulin, dulcitol, glucose, galactose, serine and sucrose. In these sources the optical density of *P. fluorescens* AS15 was range from 1.6-2.0 at 600 nm. The *in vitro* antagonism of *P. fluorescens* AS15 could be due to threshold population as well production of antibiotics. The use of these carbon and nitrogen sources in the basal medium enhanced the growth of *P. fluorescens* AS15 also enhanced its antagonistic potential.

In this study, the production of antagonistic substances did not correlate with population of the *P. fluorescence* AS15. Earlier Studies also reported that the rate of bacterial growth is not correlated with the antagonistic efficacy (Duffy and Defago, 2000; Costa *et al.,* 2001).

In present study we conclude that glucose is the best carbon source for antibiotic production, growth and antagonistic behavior of *P. fluorescens* AS15.

The ability of *Pseudomonas fluorescens* AS15 to grow on large number of carbon and nitrogen sources facilitates its survival in different habitats and adaptation to changing environmental conditions. Metabolic versatility is linked to a flexible regulation of the metabolic pathways. Global regulation of metabolic networks participates in gene expression programmed under different situations (Sergio Sanchez *et al.,* 2010). Metabolic regulatory processes that allow selection of preferred carbon sources is known as carbon catabolite repression (CCR) or catabolite repression control (CRC). CCR inhibits the expression of the pathways for nonpreferred compounds. CCR plays an important role in utilization of specific carbon, nitrogen sources and determining growth rate of bacterial species thus allowing for a flexible and reversible specialization for a particular carbon source (Gorke and Stulke, 2008).

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