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In Vitro Screening for Antagonistic ability of Plant Growth Promoting Strains of *Pseudomonas, Azotobacter* and *Azospirillum* Spp.

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Abstract

Plant growth promoting bacteria used as biofertilizers for nutrient solubilization and nitrogen fixation also has the potential to suppress disease causing pathogens. This study aims to investigate the antagonistic action of plant growth promoting *Pseudomonas* (P), *Azotobacter* (Azb) *and Azospirillum* (Asp) to suppress the growth of agriculturally important five fungal phytopathogens viz. *Macrophomina phaseolina*, *Sclerotium rolfsii, Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *ricini* and *Alternaria tenuissima*. Out of 50 strains of *Pseudomonas*, 38 strains of *Azotobacter* and 41 strains of *Azospirillum* tested seven strains of *Pseudomonas* (P88, P95, P101, P105, P107, P115, P124) Five strains of *Azotobacter* (Azb 2, 6, 10, 16, 18) and six strains of *Azospirillum* (Asp2, 10, 22, 30, 32 and 39) showed the inhibition of mycelium development of all the five major soil borne phytopathogens. Therefore, invitro screening for antagonistic ability provided the basis for identification and selection of plant growth promoting strains with potent biocontrol ability.

Keywords: Antagonistic Activity; *Pseudomonas*; *Azotobacter*; *Azospirillum*

Abbreviations

MDA: Maltose-Dextrose Agar.

Introduction

Biotic stress in agroecosystems has a significant impact on the quantity and quality of global agricultural production. Plants are vulnerable to variety of biotic agents, like fungi, bacteria, nematodes, viruses, and arachnids. These organisms inhibit plant growth and also are the reason for plant mortality as they induce stress in their hosts by interfering with normal metabolism. In addition, they also cause pre- and post-harvest losses in crop plants [1]. According to Yaman, et al. [2], biotic stress can lead to yield losses of 28.2% in wheat, 37.4% in rice, 31.2% in maize, 40.3% in potatoes, 26.3% in soybeans, and 28.8% in cotton. In order to improve the crop yield and quality, agricultural crops must be protected from microbial pathogens.

The soil borne fungal phytopathogens like *Macrophomina phaseolina*, *Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *ricini* causes serious widespread losses to agricultural crops worldwide. *Macrophomina phaseolina* is distributed worldwide and causes charcoal root rot on

more than 500 species of crops. Southern blight, caused by *Sclerotium rolfsii* is a devastating disease of vegetable, fruit and ornamental crops. *Fusarium oxysporum* causes vascular wilt on a wide range of plants. Phytopathogens like *Sclerotium rolfsii* displays strong ability to survive in soil through the formation of dark brown spherical sclerotia that have strong resistance to both chemical and biological degradation. To reduce the negative impact of disease-causing microbes on plant health and productivity biological control, utilizing beneficial microbes, is an excellent approach. Excessive usage of fungicides exposes the living organisms and the environment to the high toxicity of chemical compounds.

Hence management of the plant diseases caused by this soil borne pathogens is very difficult with fungicides alone. In recent years, the biological control of soil-borne pathogens such as *S. rolfsii* and *Rhizoctonia solani* have been investigated. The best solution to overcome these problems is to incorporate biocontrol agents. Although lot of work has gone into finding microbial biocontrol agents that can suppress phytopathogens, particularly those that cause soilborne illnesses, and that can increase agricultural output [3]. Agriculture industry still needs new isolates with potential for use in Integrated Pest Management programs.

Plant growth promoting microbes such as *Pseudomonas*, *Azospirillum*, and *Azotobacter* are some of the prevalent strains, that possess biocontrol abilities due to the production of antibiotics. *Pseudomonas* produces toxins or substances that have a direct impact on the existence of phytopathogens. The most well-known of them are siderophores, low-molecular-weight compounds that may reversibly chelate iron. Specific strains of these pseudomonads have an ability to colonize the rhizosphere at high densities, produce secondary metabolites with antifungal activities, produce phytostimulatory compounds and degrade toxic contaminants [4]. *Azotobacter* spp. produces anti-fungal antibiotics which inhibit the growth of several pathogenic fungi in the root region thereby preventing seedling mortality to a certain extent [5]. Hence the objective of the current study is to discover and isolate promising and effective strains of *Pseudomonas*, *Azotobacter* and *Azospirillum* in different crop production systems of various agro-ecological zones of India that have biocontrol potential against *Macrophomina phaseolina*, *Sclerotium rolfsii, Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *ricini* and *Alternaria tenuissima*.

Materials and Methods

Maltose-dextrose agar was used for evaluating the antagonistic activity of all isolates of *Pseudomonas, Azotobacter* and *Azospirillum* against major plant pathogens, namely, *Macrophomina phaseolina*, *Sclerotium rolfsii, Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *ricini* and *Alternaria tenuissima*. Screening for antagonistic activity was followed by carrying the dual culture method as described by lim, et al. for identifying potential isolates possessing antagonistic activity against test pathogens.

Dual Culture Method

The initial screening of the rhizobacterial isolates for their antifungal activity was carried out by dual culture method. Maltose-dextrose agar (MDA), prepared by adding the ingredients as given below, was used for assessing the antagonistic activity of all isolates of *Pseudomonas.*

Maltose-dextrose agar composition (gL^{-1})

Peptone	:	2
Malt extract	:	20
Yeast extract	:	2
Dextrose	:	5
Agar	:	20
pH	:	7

For primary screening, the fungal discs were cut from fully grown fungal plates by using cork borer of 5 mm. One disc per plate was carefully placed centrally on the MDA plates. Four different bacterial cultures were streaked on four sides of the fungal disc on the MDA plate. Bacterial streaking was done with utmost care so that they would not intersect each other. The fungal disc alone without bacteria was considered as control. The plates were incubated for 3-5 days at 28±2°C until the control fungal plate is well grown. Isolates showing inhibition were carried for the secondary screening. Isolates inhibiting the growth of all test pathogenic fungi were further evaluated for quantification following bangle method.

Bangle Plate Method

Efficacy of isolates was tested against the test pathogens by dual plate assay on petriplates containing maltose-dextrose agar using the bangle method where the bangle (70mm dia) was dipped for 2 min in the culture of bacterial antagonist, multiplied in tryptone soya broth and placed on the solidified medium in a petriplate. Five mm discs of pathogen cut from the periphery of the actively growing cultures were kept in the middle of the bangle. Control plates were inoculated with only fungus. Petriplates were sealed with parafilm and incubated at 28±2°C in a BOD incubator for 6 days. Radial growth of fungus was recorded and percent inhibition was calculated. Antagonistic activity was expressed as percent inhibition of fungal growth.

Results

The biocontrol ability of 50 *Pseudomonas*, 41 *Azospirillum* and 38 *Azotobacter* isolates was tested by adopting dual culture method. In Dual culture method 20 isolates of

Pseudomonas inhibited *Macrophomina phaseolina* and **15** isolates inhibited *Rhizoctonia solani*. *Sclerotium rolfsii* and *Fusarium oxysporum f.sp. ricini* were suppressed by 14 and 12 isolates respectively. The growth of *Alternaria tenuissima* was inhibited by 16 isolates of *Pseudomonas.*

Among the **38** ***Azotobacter*** **(Azb)** isolates tested, **18** successfully inhibited the growth of *Macrophomina phaseolina*, while **23** isolates suppressed the growth of *Sclerotium rolfsii*. **24** isolates were able to inhibit *Rhizoctonia solani*, **10** suppressed *Fusarium oxysporum f.sp. ricini*, and **12** inhibited *Alternaria tenuissima*. 5 isolates (*Azb2, Azb6, Azb10, Azb16,* and *Azb18*) were effective against all five fungal pathogens.

For the **41** ***Azospirillum*** isolates examined, **25** inhibited *Macrophomina. phaseolina*, **32** suppressed *Sclerotium. rolfsii*, and **26** inhibited *Rhizoctonia. solani*. **23** isolates prevented the growth of *Fusarium. oxysporum f.sp. ricini*, while **19** inhibited *Alternaria. tenuissima*. 6 *Azospirillum* isolates (*Asp2, Asp10, Asp22, Asp30, Asp32,* and *Asp39*) were able to suppress the growth of all five tested phytopathogens.

Macrophomina phaseolina	**Sclerotium rolfsii**	***Rizoctonia solani***	***Fusarium oxysporum*** **f.sp. *ricini***	***Alternaria tenuissima***
P78, P83, P88, P92, P95 to P97, P101, P102, P104, P106 to P108, P115, P118, P121 to P125 **(20)**	P76, P87, P88, P93, P95, P97, P101, P106, P107, P115, P119, P121, P122, P124 **(14)**	P79, P82, P88, P90, P95, P98, P99, P101, P106, P107, P112, P115, P117, P120, P124 **(15)**	P78, P88, P93, P94, P95, P101, P106, P107, P114, P115, P121, P124 **(12)**	P78, P79, P80, P88, P89, P90, P93, P95, P100, P101, P103, P106, P107, P115, P120, P124 **(16)**

Table 1: Antifungal activity of *Pseudomonas* against phytopathogens.

Macrophomina phaseolina	***Sclerotium rolfsii***	***Rizoctonia solani***	***Fusarium oxysporum*** **f.sp. *ricini***	***Alternaria tenuissima***
Azb 2, 6, 7, 10, 12, 13, 14, 15,16, 18, 19, 20, 26, 28, 32, 33, 35, 36 (**18**)	Azb 1, 2, 3, 4, 6, 7, 10, 12, 16, 18, 19, 21, 22, 23, 24, 29, 30, 31, 32, 33, 34, 35, 38 (**23**)	Azb 1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 16, 18, 19, 20, 22, 23, 24, 29, 30, 31, 32, 36, 38 (**24**)	Azb 2, 6, 8, 10, 16, 18, 20, 25, 27, 29, (**10**)	Azb 2, 6, 7, 10, 16, 17, 18, 19, 20, 25, 27, 29 (**12**)

Table 2: Antagonistic activity of *Azotobacter* isolates against phytopathogenic fungi.

Macrophomina phaseolina	***Sclerotium rolfsii***	***Rizoctonia solani***	***Fusarium oxysporum*** **f.sp. *ricini***	***Alternaria tenuissima***
Asp 1, 2, 3, 5, 7, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22, 29, 30, 32, 33, 34, 36, 39, 40, 41(**25**)	Asp 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 30, 31, 32, 34, 36, 38, 39, 40, 41 (**32**)	Asp 2, 6, 8, 10, 12, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 29, 30, 32, 33, 35, 36, 37, 38, 39, 40 (**26**)	Asp 2, 3, 4, 7, 8, 9, 10, 11, 20, 22, 23, 24, 29, 30, 32, 36, 38, 39, 41 (19)	Asp 1, 2, 4, 5, 6, 7, 10, 11, 13, 15, 16, 17, 19, 20, 22, 29, 30, 32, 34, 38, 39, 40, 41 (**23**)

Table 3: Antagonistic activity of *Azospirillum* isolates against phytopathogenic fungi.

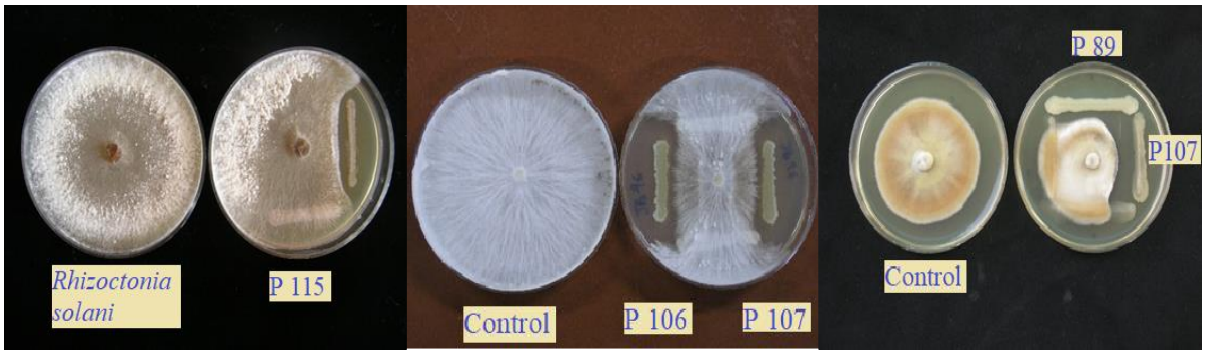

Figure 1*: In vitro* antifungal activity of *Pseudomonas* isolates (dual culture assay).

Figure 2: *In vitro* antagonistic activity of *Azotobacter* isolates against *Rhizoctonia solani* (dual culture assay).

Figure 3: *In vitro* antagonistic activity of *Azospirillum* isolates against *Sclerotium rolfsii* (dual culture assay).

Quantification of antagonistic activity by bangle method

To quantify the biocontrol ability of test pathogens, best performing isolates of *Pseudomonas Azotobacter* and *Azospirillum* were tested using bangle method.

Percent Inhibition by Isolates of *Pseudomonas*

The maximum inhibition i.e.,74.90% was shown by P83 towards *Macrophomina phaseolina* and the minimum inhibition of 4.71% was recorded with P106. The inhibition percent of *Rhizoctonia solani* was in the range of 27.06% to 73.33%, the highest inhibition was exhibited by P121 followed by P115 with 65.49% P101and P94 could inhibit *Sclerotium rolfsii* and *Fusarium oxysporum* f.sp. *ricini* efficiently among the treatments with 60% and 60.35% respectively. In case of *Alternaria tenuissima*, the highest inhibition was recorded as 67.57% with P90 followed by P107 with 66.28% inhibition. Seven isolates, highlighted in the table below have inhibited all the five phytopathogens

Percent Inhibition by Isolates of *Azotobacter* and *Azospirillum*

This study revealed that Azb10 exhibited the strongest antagonism against *Macrophomina phaseolina*, with an inhibition rate of 65%, followed by Azb18, which inhibited 53% of the pathogen's growth. Against *Sclerotium rolfsii*, Azb2 was most effective, achieving a 64% reduction in growth, followed closely by Asp10. For *Rhizoctonia solani*, Azb2 reduced growth by 40%, with Asp2 and Asp22 following at 36% inhibition. Asp30 demonstrated significant activity against *Fusarium oxysporum f.sp. ricini*, inhibiting 47% of its growth, while Azb18 caused a 42% reduction. In the case of *Alternaria tenuissima*, Asp32 inhibited growth by 49%, with Azb18 following at 44% inhibition. Overall, Azb10 inhibited the growth of all five phytopathogens within a range of 30% to 65%.

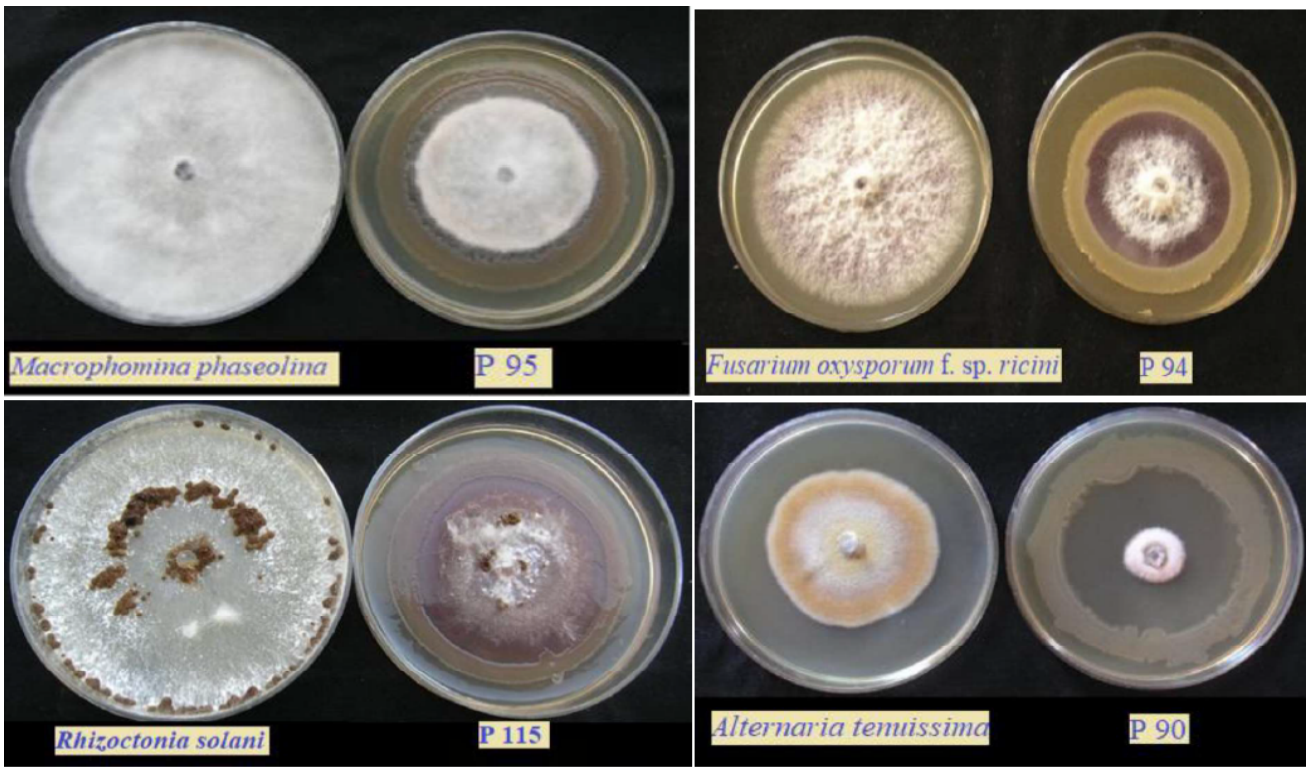

Figure 4: Secondary screening for antifungal activity of *Pseudomonas* isolates.

Treatments	*Macrophomina phaseolina*	*Sclerotium rolfsii*	*Rizoctonia solani*	*Fusarium oxysporum* f.sp. *ricini*	*Alternaria tenuissima*
Pseudomonas					
76	-	-	30.98	-	-
78	56	-	-	43.82	33.85
79	-	3451	-	-	36.45
80	-	-	-	-	53.31
82	-	47.45	-	-	-
83	74.9	36.08	-	-	-
87	-	41.96	27.06	-	-
88	34.9	36.08	40.78	52.9	40.34
89	-	-	-	-	63.68
90	-	41.96	-	-	67.57
92	44.31	-	-	-	-
93	-	-	41.18	20.28	19.58
94	-	-	-	60.35	-
95	62.35	34.12	62.75	23.17	18.29
96	46.67	-	-	-	-
97	66.27	-	43.53	-	-
98	-	29.41	-	-	-
99	-	21.18	-	-	-
100	-	-	-	-	45.53

101	58.82	60	52.94	47.13	20.88
102	17.65	-	-	-	-
103	-	-	-	-	20.88
104	5.49	-	-	-	-
105	4.71	23.92	41.96	37.63	42.93
107	55.69	41.96	56.08	31.02	66.28
108	74.12	-	-	-	-
112	-	27.84	-	-	-
114	-	-	-	10.73	-
115	30.98	58.04	65.49	12.85	13.1
117	-	23.92	-	-	-
118	51.37	-	-	-	-
119	-	-	40.39	-	-
120	-	27.06	-	-	31.26
121	61.18	-	73.33	16.98	-
122	59.61	-	47.84	-	-
123	44.31	-	-	-	-
124	50.98	16.86	38.43	36.93	26.07
125	54.51	-	-	-	-

Table 4: Percent inhibition of selected phytopathogens against isolates of *Pseudomonas.*

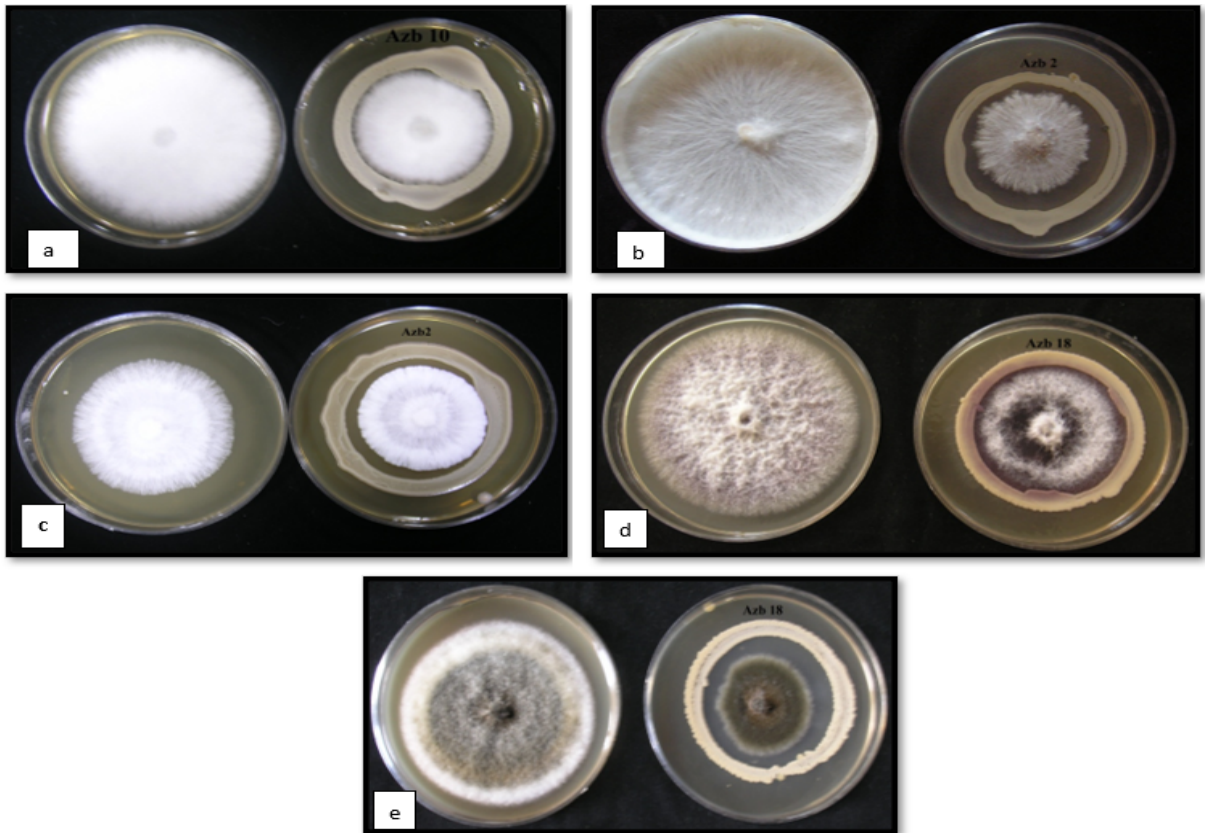

Figure 5: *In vitro* antagonistic activity of *Azotobacter* strains against a) *M. Phaseolina* b) *S.rolfsii* c) *R.solani* d) *F. oxysporum* and e) *A.tenuissima* (dual culture assay).

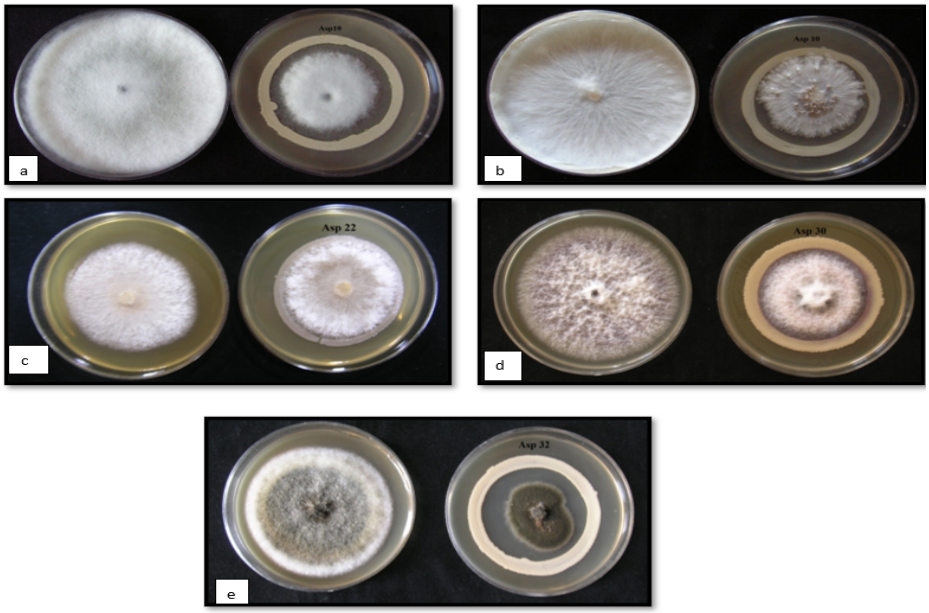

Figure 6: *In vitro* antagonistic activity of *Azospirillum* strains against a) *M. Phaseolina* b) *S.rolfsii* c) *R.solani* d) *F. oxysporum* and e) *A.tenuissima.*

Treatments	*Macrophomina phaseolina*	*Sclerotium rolfsii*	*Rizoctonia solani*	*Fusarium oxysporum* f.sp. *ricini*	*Alternaria tenuissima*
Azotobacter					
2	33	64	40	21	17
6	38	41	24	14	22
10	65	32	33	30	36
16	40	8	33	16	22
18	53	39	33	42	44
Azospirillum					
2	39	12	36	21	39
10	41	50	30	33	22
22	37	0	36	16	42
30	0	21	29	47	22
32	21	0	18	29	49
39	16	23	29	12	32

Table 5: Percent inhibition of selected phytopathogens against isolates of *Azotobacter* and *Azospirillum.*

Discussion

Phytopathogenic fungi are significant contributors to reduced agricultural productivity, but biocontrol agents like *Pseudomonas*, *Azotobacter*, and *Azospirillum* have demonstrated potential in mitigating these impacts. In this study, seven *Pseudomonas* isolates, including P105, were found to significantly inhibit the mycelial growth of several key soil-borne phytopathogens, likely due to the production of siderophores and cyanides. Similarly, five *Azotobacter* and six *Azospirillum* isolates demonstrated effective suppression of phytopathogens, with isolates such as *Azotobacter* 18 and *Azospirillum* 32 exhibiting broad-spectrum antifungal activity. These findings suggest that these microbial species not only promote plant growth but also possess the ability to suppress phytopathogens, making them valuable biocontrol

agents.

The inhibitory mechanisms of these bacteria are varied and multifaceted. *Pseudomonas* strains produce antibiotics such as pyrrolnitrin, phycocyanin, and pseudomonic acid, which have been shown to exhibit antagonistic activity against pathogens like *Candida* species, as observed in both in vitro and in vivo studies [6]. *Azotobacter* strains employ a range of mechanisms for antifungal activity, including the production of hydrolytic enzymes, antibiotics, siderophores, and volatile compounds such as hydrogen cyanide (HCN) and tetra amine polyphosphates. In the case of *Azospirillum*, its antibacterial properties are linked to the production of bacteriocins [7], siderophores [8,9], and phenylacetic acid (PAA).

The results of the present study emphasize the potential of *Pseudomonas*, *Azotobacter*, and *Azospirillum* as biofertilizers and biocontrol agents that can enhance plant growth through nitrogen fixation and suppress a wide range of soil-borne pathogens. However, a significant challenge lies in identifying and optimizing bacterial strains that combine multiple beneficial traits, such as high nitrogen fixation rates, production of growth-promoting substances, and broad-spectrum antifungal activity [10-12]. The dual function of *Azotobacter* 18 and *Azospirillum* 32, which offer both growth promotion and pathogen suppression, make these strains particularly promising for further research and application in fields increasing productivity in rainfed crops.

Conclusion

Microbial bioinoculants with the traits outlined in this study are promising candidates for enhancing plant yield, particularly under stressful environmental conditions. As an effective alternative to chemical pesticides, biological control offers a sustainable approach to managing plant pests. The in vitro results demonstrate the strong antagonistic activities of *Pseudomonas*, *Azotobacter*, and *Azospirillum* against fungal phytopathogens. Harnessing these isolates as a replacement for chemical fungicides could provide significant benefits, particularly in rainfed agricultural systems, promoting both crop health and environmental sustainability.

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