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In Vitro Screening for Abiotic Stress Tolerance and Biocontrol Ability of Plant Growth Promoting Strains of *Azotobacter* and *Azospirillum* Spp.

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Abstract

The selection and deployment of microorganisms in stressed ecosystems with biocontrol ability is a major challenge. In this investigation, we sought to isolate and identify strains of *Azotobacter* and *Azospirillum* spp., which could withstand abiotic stresses and possess the potential to serve as biological control against five phytopathogenic fungi. Stress tolerance was evidently less obvious in *Azospirillum* strains than in *Azotobacter* strains, when bacterial strains were screened for high temperature (50 °C), salt (7% NaCl), and drought (1.2 MPa). Strains Asp30 and Asp 32 of *Azospirillum* and Azb 19, Azb20 and Azb27 of *Azotobacter* were found tolerant to temperature, drought and salinity stresses. Five strains of *Azotobacter* viz. Azb2, Azb6, Azb10, Azb16 and Azb18 and six strains of *Azospirillum* viz. Asp2, Asp10, Asp22, Asp30, Asp32 and Asp39 inhibited all the five fungal phytopathogens studied. Therefore, *in vitro* screening provided the basis for identification and selection of strains with abiotic stress tolerance and biocontrol ability.

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Biocontrol Agent;
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Introduction

In rainfed agriculture, abiotic stresses viz. high temperature, salinity and drought lead to substantial crop losses worldwide.[4,14,16] Among the abiotic factors influencing plant evolution, water availability is the most significant one.[13] Water stress in its broadest sense includes both drought and salt stress. Soil salinity affects extensive areas of land in both developed and developing countries. The agricultural intensification, combined with unfavorable environmental factors, has increased the likelihood that these abiotic stresses will worsen in the near future. In this context, rigorous research is being conducted all over the world to explore a variety of rhizobacteria with traits like abiotic stress tolerance;[18,15] biological control of phytopathogens and insects; and plant growth-promoting properties.[10,22,11,17] Other intracellular

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and intercellular microorganisms colonize plants in their natural habitats.[7] By producing and secreting a variety of substances that promote plant growth, rhizosphere microorganisms, primarily helpful bacteria and fungi, can enhance yield both directly and indirectly.[6] *Azospirillum* and *Azotobacter* bacteria are free-living, surface-colonizing, diazotrophic rhizobacteria that stimulate plant growth. Even in challenging environmental conditions, root elongation rate, macro (N-P-K), and micronutrient uptake have been shown to improve after the inoculation of *Azospirillum* and *Azotobacter*.[2] The world's reliance on dangerous agricultural chemicals, which undermine agroecosystems, could be reduced by such plant-beneficial rhizobacteria.

Abiotic stresses like high temperatures, salinity, and drought are prevalent issues in rainfed agro-ecosystems, making it challenging for bioinoculants to survive. The performance of the bioinoculants varies from laboratory to field. Different abiotic stressors that exist in the field could be the cause of variations in the results. Thus, objective of the current study was to discover and isolate promising strains of *Azotobacter* and *Azospirillum* that are stress resistant and have biocontrol potential under different crop production systems of various agro-ecological zones of India.

Materials and Methods

Screening of Isolates for Abiotic Stress Tolerance

Forty strains of *Azospirillum* and 38 strains of *Azotobacter* were evaluated under abiotic stresses, including high temperature (50°C), salinity (1.2M), and drought (-1.2MPa), using tryptone soya broth (TSB). Using an uninoculated medium as a blank, the growth of all isolates was measured using a spectrophotometer (Make and Model?) at 600 nm. Bacterial isolates were considered stress tolerant if the OD (Optical density) was less than 0.1.

Tolerance of High Temperature

In 30 mL screw cap tubes, 10 mL of TSB was dispensed before the tubes were autoclaved. Test strains were cultivated from fresh cultures in a shaking incubator for 6 h before the bacterial population was adjusted to 2 ×10^5 CFU per mL and utilized as the first inoculum. The OD of the inoculated tubes was measured after 24 h of incubation at 50°C.

Tolerance for Salinity

In 30 mL screw cap tubes, 10 mL of TSB that had been modified with 7% NaCl were distributed and autoclaved. Fresh cultures of test strains were adjusted to 2 × 10^5 CFU per mL population and grown for 6 h on a shaking incubator. OD was measured after 24 h of incubation at 28°C for the inoculated tubes.

Tolerance for Drought

To characterize drought tolerance, a known volume of TSB medium that had been amended with 32.6% polyethylene glycol-6000 (326 g of PEG in 1 L of media results in an osmotic pressure of about 1.2 Mpa) was heated on a hot plate until it was completely dissolved. The remaining volume was then filled to 1 L with PEG unamended medium. In 30 mL screw-cap tubes, the liquid medium was distributed and autoclaved. Fresh cultures of test strains were adjusted to 2 × 10^5 CFU per mL population and utilized as the initial inoculum after growing for 6 h on a shaking incubator. OD was measured after 24 h of incubation at 28 °C.

Screening for Antagonistic Activity

All *Azotobacter* and *Azospirillum* isolates were tested for their antagonistic activity against the major plant pathogens viz. *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *ricini* and *Alternaria tenuissima* using maltose-dextrose agar. The dual culture approach, as described by was used to screen for antagonistic activity and identify prospective isolates with antagonistic activity against test pathogens.[9] Following the aforementioned approach, isolates that inhibited the development of all test pathogenic fungi were subsequently evaluated by quantitative methods. By using the bangle method and a dual plate assay on Petri plates with maltose-dextrose agar, effectiveness of the isolates was evaluated against the test pathogens. Five mm discs cut from the periphery of the actively growing pathogenic fungal cultures were kept in the centre of the bangle. Control plates had only fungus. Parafilm was used to seal the Petri plates, which were then incubated for 6 days at 28.2 °C in a BOD incubator. Fungus radial growth was measured, and % inhibition was calculated after the incubation. Antagonistic activity was expressed as percent inhibition of fungal growth.

Results

Abiotic Stress Tolerance

All the isolates were screened for their ability to tolerate *in vitro* abiotic stresses like high temperature (50 °C), salinity (2.0 M) and osmotic stress (−1.6MPa) *in vitro*. Out of 38 *Azotobacter* isolates, eight (Azb 7, Azb 8, Azb 9, Azb 16, Azb 18, Azb 19, Azb 20 and Azb 27) could tolerate 50°C, five (Azb9, Azb18, Azb19, Azb20 and Azb27) could tolerate the tested salinity and 6 isolates (Azb 7, Azb 8, Azb 10, Azb 19, Azb 20 and Azb27) tolerated −1.6MPa osmotic stress (Table 1). Multiple abiotic stress tolerance was noticed in some of the isolates of *Azotobacter*. Azb 19, Azb 20 and Azb 27 showed temperature, salinity and drought tolerance; whereas, Azb7 and Azb 8 showed tolerance to high temperature and drought. Similarly, out of 41 *Azospirillum*, seven isolates (3, 19, 20, 29, 30, 32, 36) tolerated 50°C, four isolates (19, 20, 29, 32) could tolerate salinity levels of 2.0M (22 × 10^2 dS/ m) and three isolates (29, 30, 32) tolerated osmotic stress of -1.6MPa. *Azospirillum*32 showed tolerance for all the three tested stresses.

Eight isolates of *Azotobacter* and seven isolates of *Azospirillum*, which could grow at 50°C were further tested for their ability to grow between 45-50°C (Table 2). Increase in the temperature significantly reduced the number of viable cell count. At 45 °C, except Azb8, Azb16 and Asp36, growth of all the remaining isolates was higher. At 46 °C, growth of Azb9, 19, 20 and Asp3, 20, 29, 32 was higher compared to other isolates. However, at 47 and 48 °C Azb20, viable counts were the highest. Asp 29 showed higher number of viable cells at 47 °C, but at 48 °C Asp29 reduced when compared to Asp19. At 49 oC, Asp19 outnumbered other isolates followed by Azb 27, Azb 20 and Azb 9. Increase in the temperature significantly reduced the population. Among all the isolates, Azb27 and Asp19 survived better at 50°C and formed 10 ×10^6 CFU/ mL.

Five isolates of *Azotobacter* and four isolates of *Azospirillum*, which tolerated 1.0M (11 × 10^2 dS/m) salt concentration were tested for their ability to grow further up to 2.0M (22 ×x 102 dS/m). At 1.0M concentration, Azb20 and Asp32 showed higher colony counts than other isolates (Fig. 1). Increase in the salinity significantly reduced the cell viability. At 1.2M concentration, along with Azb20 and Asp32, growth of Asp20 was also higher. At 1.4M concentration growth of Azb20 declined, whereas number of cells of Azb19 increased and Asp 32 outnumbered other isolates. Azb19 and Azb20 growth was more up to 1.6M salt level and reduced at 1.8M salt concentration. Asp32 strain growth was higher than other isolates at tested salt concentrations. Asp32 outnumbered other isolates at all tested salt concentrations. At 1.0M salt concentration, viable count was 249 × 10^6 CFU/ mL, which gradually decreased with increase in salt level in the medium. At 2.0M (22 ×10^2 dSm) salt concentration the viable count of Asp32 was 3 ×10^6 CFU/ mL.

Five isolates of *Azotobacter* and four isolates of *Azospirillum* tolerated osmoticum stress up to -1.6MPa (Fig. 2). With increase in stress there was a reduction in populations of all the isolates, however, Asp19 maintained reasonably high population levels as compared to the other isolates. At -1.2MPa maximum growth of Asp19 was recorded followed by Azb18; whereas, Asp20 showed the least growth. The population levels reached the lowest in case of Asp32, when the stress was increased to -1.4 MPa and the trend remains the same with further increase in the stress.

In Vitro Antagonistic Activity

The biocontrol ability of 41 *Azospirillum* and 38 *Azotobacter* isolates was tested by adopting dual culture method. The test pathogens included major

Table 1: List of *Azotobacter* and *Azospirillum* isolates showing tolerance to various abiotic stresses.

Treatments	High temperature (50 °C)	Salinity tolerance (14 × 10^2 dS/m)	Drought (1.2MPa)
Azotobacter spp	Azb 7, 8, 9, 16, 18,19, 20, 27	Azb 9, 18, 19, 20, 27,	Azb 7, 8, 10,19, 20, 27
Azospirillum spp	Asp 3, 19, 20, 29, 30, 32, 36	Asp 19, 20, 29, 32,	Asp 29, 30, 32

Table 2: Temperature tolerance of selected *Azotobacter* and *Azospirillum* isolates beyond 45 °C (CFU × 106 per mL)

Treatments	45 °C	46 °C	47 °C	48 °C	49 °C	50 °C
Azotobacter						
7	124	69	24	16	12	3
8	98	86	33	21	9	4
9	205	128	89	72	40	7
16	83	77	56	38	19	9
18	102	83	61	42	13	2
19	201	120	93	62	60	8
20	210	113	108	98	40	8
27	216	93	85	77	41	10
Azospirillum						
3	146	130	91	63	38	7
19	200	96	80	75	49	10
20	200	101	95	38	18	6
29	200	134	101	63	38	7
30	114	72	41	33	9	1
32	136	106	59	23	16	3
36	86	64	39	24	13	4

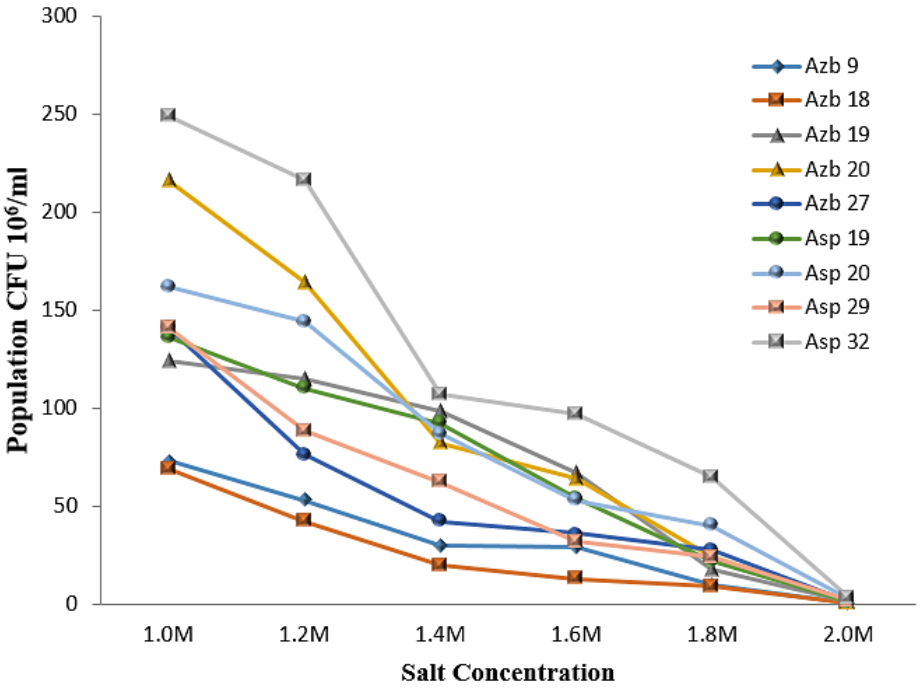

Fig.1: Isolates of *Azotobacter* and *Azospirillum* exhibiting tolerance to increasing levels of salinity

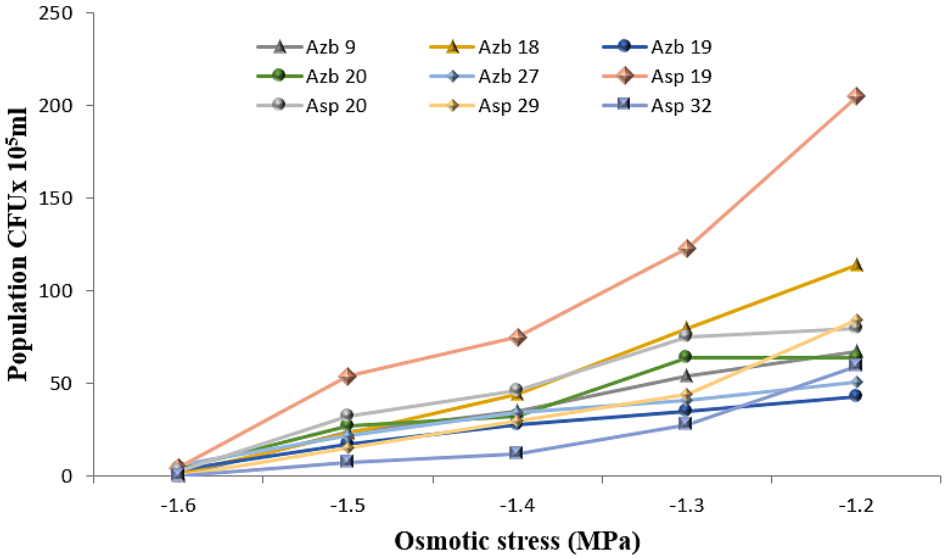

Fig. 2: Osmotic stress tolerance in selected *Azotobacter* and *Azospirillum* strains

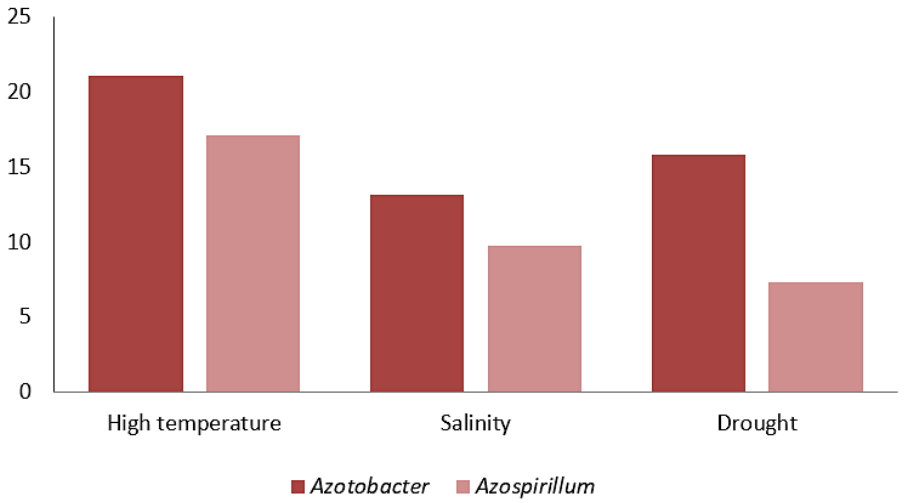

Fig. 3: Percentage of *Azotobacter* and *Azospirillum* strains exhibiting various abiotic stresses.

soil borne plant pathogens mainly *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Rizoctonia solani*, *Fusarium oxysporum* f.sp. *ricini* and also a foliar pathogen *Alternaria tenuissima*. Of the 38 *Azotobacter* isolates that were evaluated, 18 were able to successfully stop *Macrophomina phaseolina* from growing, whereas 23 isolates stopped *Sclerotium rolfsii* from growing. 24 isolates inhibited *Rhizoctonia solani*. Ten isolates prevented *Fusarium oxysporum* f.sp. *ricini* from growing, while twelve isolates stopped *Alternaria tenuissima* from growing (Table 3). All of the test phytopathogenic fungi's growth was inhibited by five isolates, namely Azb2, Azb6, Azb10, Azb16 and Azb18. Twenty-five of the 41 *Azospirillum* isolates that were tested could prevent *M phaseolina* from growing Thirty- two isolates prevented the growth of S. rolfsii, while 26 isolates prevented the growth of R. solani. Nineteen isolates inhibited the growth of *Alternaria tenuissima* and 23 isolates inhibited *Fusarium oxysporum* f. sp. *ricini*. Asp2, Asp10, Asp22, Asp30, Asp32 and Asp39 were six isolates that were able to inhibit all

five phytopathogens.

Quantification of Antagonistic Activity by Bangle Method

To quantify the biocontrol ability of test pathogens, best performing isolates of *Azotobacter* and *Azospirillum*

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

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 4: Antagonistic activity of *Azospirillum* 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 5: Percent inhibition of selected phytopathogens against isolates of *Azotobacter* and *Azospirillum*

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

were tested using bangle method. It was observed that Azb10 was highly antagonistic towards *Macrophomina phaseolina* showing an inhibition of 65% followed by Azb18 inhibiting 53% growth. Azb2 was effective with an inhibition of 64% against *Sclerotium rolfsii* followed by Asp10 (Table 5). Azb2 reduced the growth of R. solani by 40% followed by Asp2 and Asp22 (36%). Asp30 inhibited *Fusarium oxysporum* f.sp. *ricini* by 47%, while it was reduced by 42% in Azb18. In case of *A. tenuissima*, Asp32 inhibited the growth by 49% followed by Azb18 (44%). Overall inhibition of five phytopathogens by Azb10 was in the range of 30 to 65%.

Discussion

Ability of the microorganisms to withstand abiotic stresses would be a boon as often cropping systems face stresses like drought, high temperature,

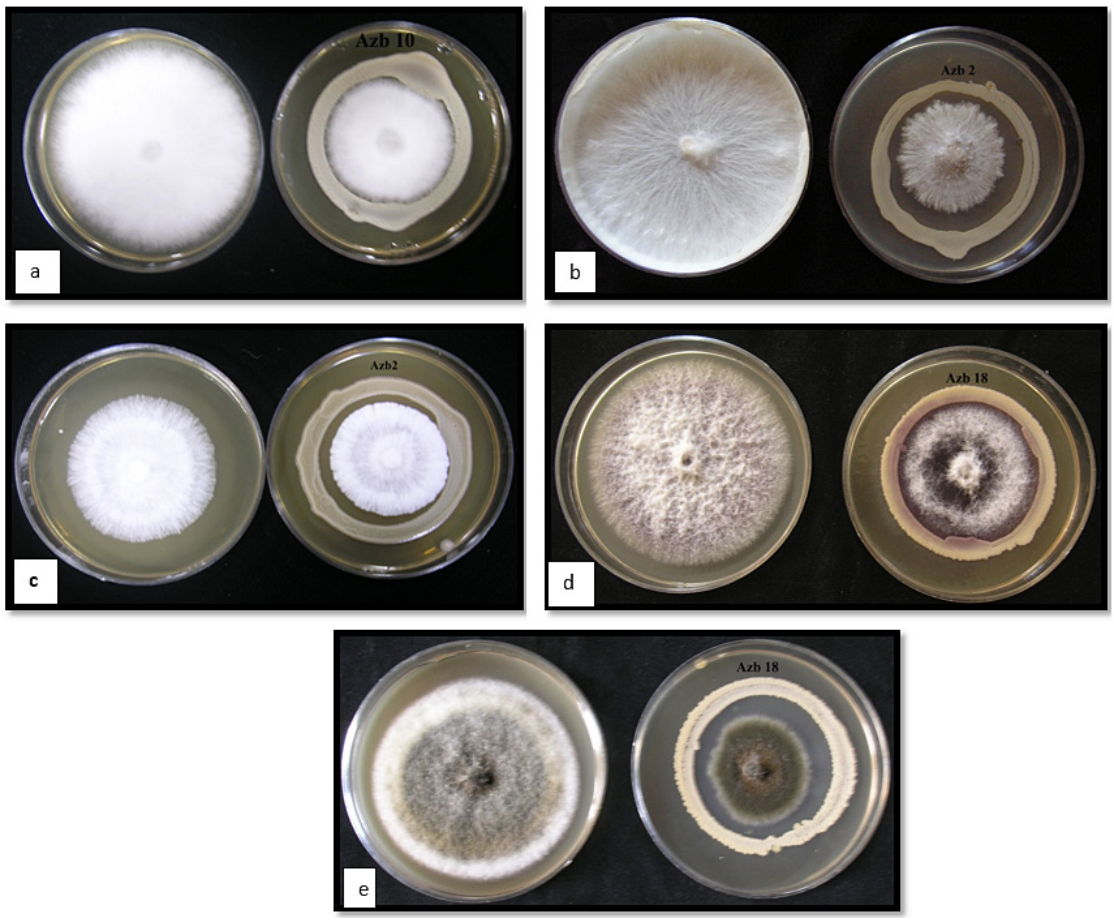

Fig. 4: *In vitro* antagonistic activity of *Azotobacter* strains against a) *M. Phaseolina* b) *S.rolfsii* c) *R.solani* d) *F. oxysporum* and e) *A.tenuissima* (dual culture assay)

and salinity during crop growing season. Such conditions also affect growth and survival of microorganisms. Change in climate can alter the environmental conditions drastically as a result of which plant-microbe associations are affected. Screening and isolation techniques have been developed for isolation of efficient stress tolerant microbial inoculants for improved farming in rainfed agriculture. Hence, an attempt was made to screen and identify promising *Azotobacter* and *Azospirillum* isolates with abiotic stress tolerance in addition to PGP traits. Eight isolates of *Azotobacter* and seven isolates of *Azospirillum* were able to grow at 50 °C. The cyst formation protects from desiccation

process. It is generally accompanied by the production of a thick coat or capsule.[3,27] Similarly, five isolates of *Azotobacter* and four isolates of *Azospirillum* were found to possess salinity tolerance. All the nine isolates survived even at 1.8M NaCl. In *Azospirillum* spp. there is accumulation of compatible solutes such as glutamate, proline, glycine betaine and trehalose in response to salinity and osmolarity reported by Tripathi.[25] Proline plays a major role in osmo-adaptation and with increase in osmotic stress, a shift of the dominant osmolyte from glutamate to proline has been observed. Therefore, it could be observed from the current results that some of the salt tolerant isolates may have good saprophytic and competitive abilities to perform well in the rhizosphere. Six isolates of *Azotobacter* and three isolates of *Azospirillum* could grow under osmotic stress. Inoculation with *Azotobacter* was effective for qualitative and quantitative yield of wheat. Inoculation with *Azotobacter* promoted early flowering, a long grain filling period, late maturity period, a high number of grains per spikelet and short spike length for increasing yield under drought conditions as reported by.[8] Trehalose accumulation in *Azospirillum* brasilense improved drought tolerance and biomass in maize plants.[6] The capacity to form cysts and to produce metabolites like proline, trehalose protects *Azospirillum* and *Azotobacter* isolates from environmental stresses.

The phytopathogenic fungi are one of the leading causes of loss in agricultural productivity. Out of 38 *Azotobacter* and 41 *Azospirillum*, 5 isolates of *Azotobacter* and 6 isolates of *Azospirillum* showed significant inhibition of the mycelium development of major soil-borne phytopathogens. *Azotobacter*

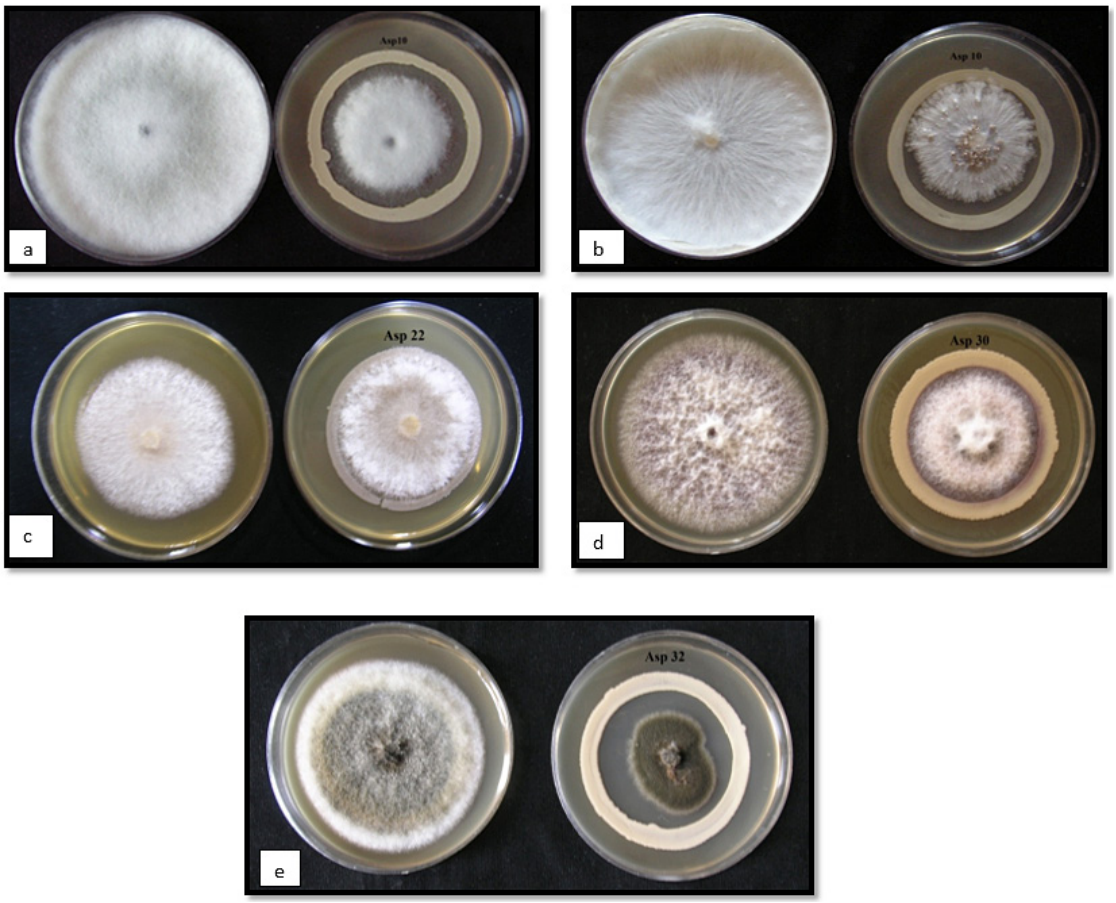

Fig. 5: *In vitro* antagonistic activity of *Azospirillum* strains against a) *M. Phaseolina* b) *S.rolfsii* c) *R.solani* d) *F. oxysporum* and e) *A.tenuissima*

Azb 2, 6, 10, 16, 18 and *Azospirillum*, Asp2, 10, 22, 30, 32 and 39 inhibited the growth of all fungal pathogens (Table 2 and Table 3). These results suggest that antibacterial activities of *Azospirillum* and *Azotobacter* could offer additional protection to crop plants when used as plant growth promoters by suppressing phytopathogens also. Various mechanisms have been attributed to antagonistic activity of *Azotobacter* strains namely, production of hydrolytic enzymes, antibiotics, siderophores and volatile compounds like HCN, tetra amine polyphosphates, etc. *Azospirillum* antibacterial activities could be related to its already known ability to produce bacteriocins and siderophores.[19, 25, 27] In addition, *Azospirillum* was recently reported to synthesize phenylacetic acid (PAA), an auxin-like molecule with antimicrobial activity Sandhya *et al*. (2010). The major issue in production of biofertilizers using *Azotobacter* and *Azospirillum* is the search for the efficient strains possessing an array of beneficial characteristics viz. high rate of dinitrogen fixation, ability to produce growth promoting substances and broad-spectrum antifungal activity against phytopathogens. In the present study, *Azotobacter* (Azb18) inhibited the growth of all five pathogenic fungi and in turn was tolerant to temperature and salinity stress. In case of *Azospirillum*, Asp32 inhibited the growth of all fungal pathogens except *Sclerotium rolfsii* and in turn high temperature and salinity tolerant and drought tolerant. This feature of possessing both characters makes the selection an ideal one for their possible better performance under field conditions.

Conclusion

Microbial bioinoculants with the characteristics described above are good candidate strains to promote plant yield under stressful environmental conditions. An alternative promising strategy of chemical pesticides to control plant pests has been the implementation of biological control. The present *in vitro* study shows that *Azotobacter* and *Azospirillum* have antagonistic activities against fungal phytopathogens. The successful exploitation of these isolates replacing chemical fertilizers will be beneficial, especially in rainfed agriculture.

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Conflict of Interest

The authors do not have any conflict of interest.

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