Plant Archives
Journal home page: www.plantarchives.org
DOI Url: https://doi.org/10.51470/PLANTARCHIVES.2021.v21.no1.062

ANTAGONISTIC POTENTIAL OF SALT TOLERANT BACTERIA AND OPTIMIZATION OF THEIR CULTURE CONDITIONS FOR ENHANCEMENT OF THE ACTIVITY

Pooja Verma[1,2], Priyanka Chandra[1]*, Kailash Prajapat[1], Awtar Singh[1], Parul Sundha[1] and Baljeet Singh Saharan[2]*

[1]ICAR-Central Soil Salinity Research Institute, Karnal - 132001, Haryana, India
[2]Department of Microbiology, Kurukshetra University, Kurukshetra-136119, Haryana, India
*E-mail: priyanka.chandra921@gmail.com

(Date of Receiving-09-11-2020; Date of Acceptance-22-01-2021)

ABSTRACT

The antagonistic potential of bacteria is being applied to biocontrol the infectious diseases caused by pathogenic fungi in plants that are one of the major threats to the growth and productivity of crop plants. In the present study, bacterial strains were isolated from soil samples collected from the rhizosphere of Sorghum (*Sorghum bicolor*) and Wheat (*Triticum aestivum*). Microscopic analysis revealed that all three bacterial isolates were Gram-positive, rod-shaped and spore-forming. The isolates Bacillus subtilis BP171 and *Bacillus amyloliquefaciens* BP124 demonstrated salt tolerance up to 12% while Bacillus subtilis BP67 tolerated up to 10% of NaCl. All the three strains were screened against seven test pathogenic fungi like Bipolaris sorokiniana, Fusarium oxysporum, *Aspergillus sp., Penicillium sp., Rhizoctonia solani, Aspergillus niger,* and *Fusarium sp*. for their antagonistic activity. BP124 was found to be the most potent in comparison to BP67 and BP171. *Bacillus amyloliquefaciens* BP124 demonstrated significantly highest ($p<.0001$) inhibition percentage against *Fusarium sp*., (61%) and *Fusarium oxysporum* (60%). The optimization of various parameters like pH, temperature, inoculum size, agitation, carbon sources, and nitrogen sources was carried out to enhance the antagonistic potential of bacterial isolates. The results revealed that the bacterial isolates were able to demonstrate significantly highest ($p<.0001$) antagonistic potential when inoculum size required for the growth was 1ml, agitation rate at 150 rpm, while the medium of pH at 7.0 and 30°C incubation temperature. Starch as carbon source and peptone as nitrogen source supported significantly highest ($p<.0001$) antagonistic activity against all the fungal pathogens for all the bacterial isolates. Therefore, the study showed that appropriate and optimum fermentation conditions can be of great importance in enhancing the antagonistic potential of bacterial isolates.

Keywords: *Bacillus*, Biological control, Optimization, Salt-tolerance, Rhizosphere

INTRODUCTION

Plant diseases caused by the fungal pathogens are causing remarkable losses in yield and economy. Chemical fungicides are widely used for controlling such diseases but their excessive use leads to adverse and toxic effects on soil, crops, and the environment. However, continuous uses of chemical agents are leading to environmental pollution, resistant-plant pathogen outbreaks, and toxicity in humans (Wu *et al.,* 2016). The application of antagonistic bacteria as biological control agents is an alternative approach for controlling these fungal pathogens. Many bacterial genera show potential to control the growth of fungal pathogens through several mechanisms such as lysis of pathogenic fungal cells through production of hydrolytic enzymes such as chitinases, glucanases, proteases, and lipases), also compete with the pathogens at the root surface for nutrients and colonization, producing antifungal metabolites such as bacteriocins, siderophores, and antibiotics.

Bacillus species are aerobic or facultatively anaerobic, gram-positive, rod-shaped endospore-forming bacteria widely spread in nature (Graumann 2007; Al-janabi 2006). *Bacillus* species display a broad range of physiological qualities that allow the organism to flourish in all environmental conditions. these species form endospores that are stable to heat, cold, radiation, desiccation, and disinfection and helps to compete favorably with other organisms in vicinity., They also, produce secondary metabolites which have an antagonistic effect on different microorganisms (Kuta *et al.,* 2009). *Bacillus* species producing antibiotics have been used as biocontrol agents against pathogenic fungi and bacteria (Pederson, Reddy 1997; Yilmaz *et al.,* 2005). Bacillus-based biological agents are being widely accepted and their production at a commercial level in the form of the product is required. An appropriate medium for bacterial growth and production of antimicrobial metabolites is a critical step and to achieve this, modifications in the composition of the medium along with different carbon and nitrogen sources have been reported for effective production of antibiotics by microorganisms. The physiochemical parameters such as inoculum size, pH, incubation time, and temperature, *etc.* are essential for the cultivation of bacteria and the productionof important bioactive compounds (Bundale *et al.,* 2015). The alteration of an economic culture medium is required to obtain a huge quantity of biomass as well as secondary metabolites. The components used for a medium must fulfill the basic requirements for the production of cell biomass and metabolites. Since, physiochemical and nutritional conditions greatly influence the growth, as well as the metabolic activities of the microorganisms and optimization of such parameters, is an important step for

	Characteristics	BP67	BP124	BP171
1.	Gram reaction	Positive	Positive	Positive
2.	Cell morphology	Rod shaped	Rod shaped	Rod shaped
3.	Colony morphology	Flat, irregular, lobate margins	Raised, irregular, lobate margins	Flat, irregular, wavy margins
4.	Colony colour	Cream	Cream	White
5.	NaCl tolerance (%)	0-10	0-12	0-12
6.	Endospore staining	+	+	+
7.	Catalase test	+	+	+
8.	Lactose	-	+	-
9.	Xylose	-	+	-
10.	Maltose	+	-	-
11.	Fructose	+	-	-
12.	Dextrose	-	-	-
13.	Galactose	-	-	-
14.	Raffinose	-	-	-
15.	Trehalose	-	-	-
16.	Melibiose	-	-	-
17.	Sucrose	-	+	-
18.	L-Arabinose	+	-	+
19.	Mannose	+	+	-
20.	Inulin	-	-	+
21.	Sodium gluconate	-	-	-
22.	Glycerol	+	-	+
23.	Salicin	-	-	+
24.	Dulcitol	-	-	-
25.	Inositol	-	-	+
26.	Sorbitol	+	-	+
27.	Mannitol	+	-	+
28.	Adonitol	-	-	+
29.	Arabitol	-	-	-
30.	Erythritol	-	-	-
31.	α-Methyl-D-glucoside	-	-	-
32.	Rhamnose	-	-	-
33.	Cellobiose	-	-	-
34.	Melezitose	-	-	-
35.	α-Methyl-D-mannoside	-	-	-
36.	Xylitol	-	-	-
37.	ONPG	+	-	+
38.	Esculin hydrolysis	+	+	+
39.	D-Arabinose	-	-	-
40.	Citrate utilization	+	+	+
41.	Malonate utilization	+	-	-
42.	Sorbose	-	+	-

the enhancement of activity. The present study was planned to screen *Bacillus* strains for their antagonistic potential and evaluate their growth conditions to define the most effective parameters for their enhanced biocontrol activity.

MATERIALS AND METHODS

Microbial strains

The fungal plant pathogens used for the study were *Bipolaris sorokiniana*, *Fusarium oxysporum, Fusarium* sp., *Aspergillus* sp., *Aspergillus niger, Penicillium* sp. and *Rhizoctonia solani.* The cultures were maintained by regular sub-culturing on Potato Dextrose agar at 25°C for 5 days and stored in potato dextrose agar slants at 4°C for further study.

Table 2 : Effect of inoculum size on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
Inoculum size	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**
250 µl	40.8B	28.8BC	52.2B	17.8D	38.33A	40.00A	33.33A	22.50D	31.11BC	64.44C	18.89D	21.67C	28.33C	30.00BC	43.33C	28.89CD	52.22B	22.22D	43.33	46.67B	33.33B
500 µl	48.3A	31.1B	55.5AB	44.4B	36.67A	46.67A	36.11A	48.33B	32.22B	68.89B	41.11B	33.33B	38.33B	38.33B	50.83B	32.22BC	54.44B	50.00B	41.67	48.33AB	36.11B
1 ml	54.1A	60.0A	61.1A	56.6A	45.00A	43.33A	40.00A	70.83A	64.44A	78.89A	55.56A	48.33A	50.00A	51.67A	56.67A	65.56A	64.44A	64.44A	50.00	51.67A	43.33A
1.5 ml	38.3B	32.2B	31.1C	25.5C	36.67A	38.33A	36.67A	46.67B	34.44B	32.22D	27.78C	31.67B	38.33B	35.00BC	45.83C	35.56B	36.67C	31.11C	45.00	45.00B	36.67B
2 ml	25.0C	24.4C	24.4D	17.7D	26.67B	26.67B	25.00B	38.33C	27.78C	24.44E	17.78D	28.33BC	21.67D	25.00C	28.33D	25.56D	32.22C	24.44D	35.00	30.00C	25.00C
p-Value	<.0001	<.0001	<.0001	<.0001	0.0134	0.0209	0.017	<.0001	<.0001	<.0001	<.0001	0.0005	<.0001	0.0033	<.0001	<.0001	<.0001	<.0001	0.0617	<.0001	0.0005
CV(%)	8.04	9.59	7.24	8.70	12.32	14.52	11.90	4.67	5.99	4.16	4.63	12.34	6.59	15.32	5.02	7.43	6.15	8.52	11.72	5.45	7.87

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp*.

Table 3: Effect of agitation rate on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
Inoculum size	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**
120 RPM	33.33B	28.89B	30.30B	34.44C	33.33B	38.33C	21.33B	22.50B	32.22B	32.32B	31.11B	42.67B	38.33B	28.00B	39.17B	32.22B	32.32B	42.22B	37.33B	45.00B	25.33B
150 RPM	59.17A	62.22A	64.65A	58.89A	49.33A	48.33A	42.67A	71.67A	68.89A	72.73A	57.78A	54.67A	51.67A	53.33A	61.67A	66.67A	67.68A	65.56A	54.67A	53.33A	46.67A
Static	31.67B	20.00C	24.24C	51.11B	26.67C	38.33B	6.67C	20.83B	18.89C	23.23C	17.78C	34.67C	21.67C	13.33C	38.33B	30.00B	25.25C	41.11B	29.33C	41.67B	14.67C
p-Value	<.0001	0.0001	<.0001	0.0018	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	0.0011	0.0004	0.0001	<.0001	<.0001	0.0001	0.0035	0.0008	0.0178	0.0018
CV(%)	4.03	7.94	6.73	6.73	3.66	0.00	11.32	8.42	4.81	6.25	5.41	5.25	7.08	8.45	1.80	4.09	7.05	8.53	6.59	6.19	14.60

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp*.

Table 4: Effect of pH on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
Inoculum size	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**	**F.S.**	**F.O.**	**B.S.**	**A.S.**	**P.S.**	**R.S.**	**A.N.**
5 pH	29.17B	40.00BC	45.05B	28.89AB	22.22AB	25.00	24.44B	29.17C	41.90B	37.84BC	28.89BC	27.78CD	23.33	17.78C	36.67B	41.90C	44.14B	32.22B	26.67B	43.33AB	27.78BC
6 pH	31.37B	45.56B	40.86B	27.78BC	24.36A	28.57	20.51B	39.22B	48.89A	37.63C	22.22CD	35.90B	26.19	28.21B	37.25B	48.89B	34.41C	38.89AB	21.79B	26.19D	19.23CD
7 pH	42.67A	54.17A	53.33A	35.00A	26.98A	36.11	33.33A	46.67A	54.17A	58.67A	40.00A	46.03A	38.89	39.68A	48.00A	56.94A	58.67A	43.33A	47.62A	47.22A	39.68A
8 pH	35.00B	40.00BC	41.67B	31.37AB	15.56BC	23.33	20.37B	38.33B	40.00B	46.67B	33.33AB	31.11BC	26.67	20.37BC	36.67B	40.00C	45.00B	31.37B	17.78B	36.67BC	16.67D
9 pH	35.19B	33.33C	22.22C	22.22C	13.89C	33.33	13.33C	22.22D	33.33C	26.67D	19.44D	22.22D	33.33	13.33C	37.04B	29.17D	31.11C	22.22C	19.44B	33.33CD	33.33AB
p-Value	0.0095	0.0029	<.0001	0.0205	0.0105	0.2559	0.0017	0.0001	0.0004	0.0004	0.0013	0.0002	0.2862	0.0004	0.0066	<.0001	<.0001	0.0024	0.0012	0.0013	0.0018
CV(%)	9.61	9.78	6.67	12.06	18.12	25.04	16.15	9.16	7.31	11.50	13.77	9.97	29.98	17.48	7.75	6.46	6.03	12.47	21.54	10.54	17.53

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp*.

Table 5: Effect of temperature on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.
25oC	40.74B	29.49C	44.85B	25.72C	13.33C	26.67B	17.56B	37.78C	35.43C	44.52C	27.24C	21.33C	28.33B	16.89B	45.81AB	33.00B	44.00C	30.00C	18.67C	31.67B	24.33B
30oC	48.33A	57.47A	59.27A	54.60A	43.33A	42.00A	38.67A	69.17A	63.33A	70.00A	54.00A	46.00A	48.33A	46.67A	49.17A	59.67A	62.33A	58.33A	48.33A	51.33A	42.67A
37oC	37.73B	50.67B	50.67B	44.70B	33.33B	16.67B	20.00B	47.67B	46.67B	56.00B	45.67B	36.67B	36.67B	40.00A	42.33B	54.67A	53.33B	49.33B	38.33B	23.33B	25.00B
p-Value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
CV(%)	5.75	5.12	5.59	6.74	10.21	16.83	15.20	5.84	5.50	6.45	6.75	9.73	13.17	11.88	4.06	7.12	4.37	7.75	8.73	13.79	12.18
p-Value	<.0001	<.0001	<.0001	<.0001	0.0134	0.0209	0.017	<.0001	<.0001	<.0001	<.0001	0.0005	<.0001	0.0033	<.0001	<.0001	<.0001	<.0001	0.0617	<.0001	0.0005
CV(%)	8.04	9.59	7.24	8.70	12.32	14.52	11.90	4.67	5.99	4.16	4.63	12.34	6.59	15.32	5.02	7.43	6.15	8.52	11.72	5.45	7.87

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp.*

Table 6 : Effect of different carbon sources on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.
Glucose	49.17B	39.05B	48.33C	34.44B	35.42B	42.67B	20.51B	35.00B	43.81B	51.67B	32.22B	42.71B	37.33B	41.03B	51.67B	41.90B	49.17B	36.67B	38.54B	45.33B	25.64B
Sucrose	21.67D	27.08C	33.33D	17.33C	17.78C	20.29C	11.59C	21.67C	29.17C	30.48C	17.33C	21.11C	10.14C	18.84C	25.00D	30.21C	36.19C	21.33C	20.00C	27.54D	14.49C
Dextrose	41.11C	38.67B	52.22B	33.33B	34.67B	39.22B	18.52BC	32.22B	40.00B	47.78B	28.99B	41.33B	33.33B	38.89B	43.33C	41.33B	54.44B	37.68B	37.33B	41.18C	24.07B
Starch	60.95A	63.81A	69.17A	60.95A	52.38A	52.56A	48.89A	72.38A	70.48A	74.17A	61.90A	55.24A	55.13A	54.44A	63.81A	68.57A	72.50A	67.62A	55.24A	60.26A	57.78A
p-Value	<.0001	<.0001	<.0001	<.0001	0.0004	0.0003	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
CV(%)	8.03	8.15	3.09	3.22	12.10	9.86	14.19	7.66	5.85	8.79	8.99	6.22	6.59	10.57	8.00	3.69	5.48	4.37	6.92	4.43	11.76

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp.*

Table 7: Effect of nitrogen source on the antagonistic potential of bacterial isolates

	BP67							BP124							BP171						
	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.	F.S.	F.O.	B.S.	A.S.	P.S.	R.S.	A.N.
NH4NO3	50.00B	47.00B	56.00B	52.67B	46.67B	41.33B	48.67B	53.33B	52.00B	56.33B	40.33B	46.67B	42.67B	47.67B	53.33B	50.20B	59.33B	55.00B	50.67B	45.33B	51.33B
Peptone	63.33A	64.00A	71.33A	64.67A	55.00A	53.33A	52.67A	73.33A	72.67A	75.00A	63.33A	56.00A	58.67A	57.33A	65.83A	69.00A	73.83A	67.67A	61.00A	61.33A	59.00A
Casein	16.00D	14.00D	15.33D	18.67D	13.33D	13.33D	18.00D	15.67D	12.33D	14.67D	6.67D	11.67D	11.67D	12.00D	19.00D	17.67D	18.67D	22.67D	16.67D	18.33D	22.33D
Yeast extract	35.00C	25.00C	31.33C	24.67C	32.67C	32.00C	27.33C	35.00C	27.67C	29.00C	26.33C	32.00C	31.33C	29.67C	37.50C	27.00C	32.33C	29.33C	34.67C	40.67C	31.00C
p-Value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
CV(%)	7.78	4.42	5.67	6.27	5.83	10.33	3.49	4.98	4.99	11.40	12.21	9.21	6.20	8.61	6.27	7.00	4.15	6.86	6.01	5.22	5.15
CV(%)	9.61	9.78	6.67	12.06	18.12	25.04	16.15	9.16	7.31	11.50	13.77	9.97	29.98	17.48	7.75	6.46	6.03	12.47	21.54	10.54	17.53

B.S. - *Bipolaris sorokiniana*, F.O. - *Fusarium oxysporum*, A.S. - *Aspergillus sp*., P.S. - *Penicillium sp*., R.S. - *Rhizoctonia solani*, A.N. - *Aspergillus niger*, F.S. - *Fusarium sp.*

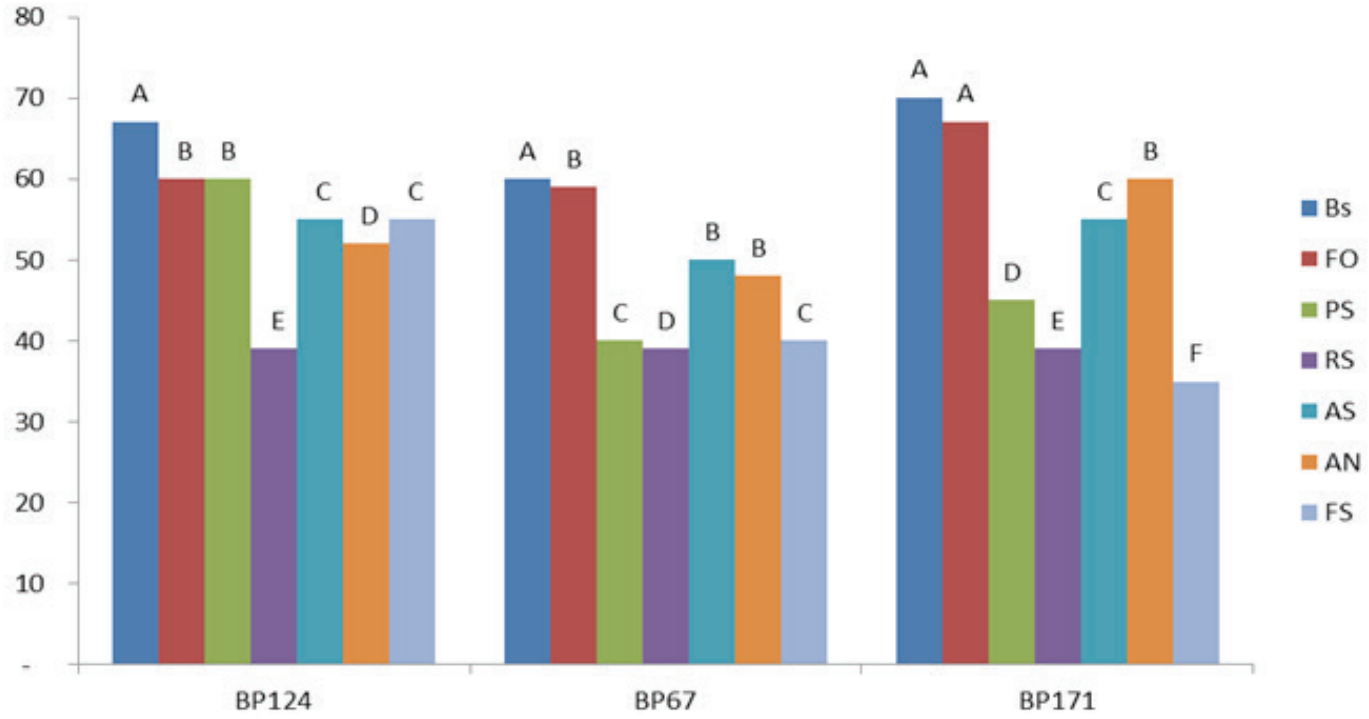

Figure 1: Antagonistic potential of all the three bacterial isolates on different phytopathogens (BS - *Bipolaris sorokiniana*, FO - *Fusarium oxysporum*, PS - *Penicillium sp*., RS - *Rhizoctonia solani*, AS - *Aspergillus sp*., AN - *Aspergillus niger* and FS - *Fusarium sp*.)

The bacterial isolates, two strains of *Bacillus subtilis* BP67 (NCBI accession number MT448859.1) and BP171 (NCBI accession number MT448856.1) and *Bacillus amyloliquefaciens* BP124 (NCBI accession number MT448858.1) were isolated from the rhizosphere of Sorghum (*Sorghum bicolor*) and Wheat (*Triticum aestivum*). The isolation was carried out by the serial dilution method on the nutrient agar medium. The bacterial isolates were sub-cultured regularly on fresh nutrient agar medium, incubated at 30°C for 24 h, and stored at 4°C.

The growth and morphological characteristics of bacterial cultures were checked. The isolates were characterized by Gram staining and biochemical tests. Salt tolerance capability of bacterial isolates was tested by inoculating fresh culture on sterile nutrient agar plates supplemented with various levels of NaCl (0.5, 1, 2, 4, 6, 8, 10, and 12%) using pour plate technique. The plates were incubated at 30°C for two days. Sterile Petri plates having nutrient agar supplemented with 0.5%-12% NaCl (w/v) without inoculation of the bacteria served as a control. The results were observed for growth and recorded after two days of incubation.

Antagonistic activity

The antagonistic potential of the isolates was screened by the dual-culture method. The bacterial isolates were grown in nutrient broth at 25°C whereas fungal pathogens were grown on potato dextrose (PD) medium. Five-day-old fungal mycelial disc (5 mm) of each pathogen was then placed in the center of sterile PD medium plates and bacterial culture was streaked 2 cm juxtaposed from the fungal disc. The plates were incubated at 28°C for 3–7 days.

The percentage of growth inhibition (I) was calculated by measuring the distance between the edges of the bacterial and fungal colonies by using the following formula:

$$I\% = [(C-T) / (C_0-C)] \times 100$$

Where C refers to the radial growth of fungus in control and T refers to the radial growth of fungus in dual culture plate (Aeron *et al.,* 2011). C_0 is the diameter of the test fungus agar discs (5 mm).

Optimization of culture conditions

To investigate the consequence of different cultural conditions and nutrients on the bacterial isolates for enhanced antagonistic potential, various parameters of physio-chemical growth had been studied such as different pH, various temperatures, different inoculum size, and different levels of agitation, different carbon sources and nitrogen sources.

Effect of inoculum size: Bacterial isolates were inoculated into the sterile nutrient broth at different concentrations of inoculum i.e. 250µl, 500µl, and 1ml were used. The flasks were incubated at 30°C for 48 hrs. After incubation, the antagonistic potential was checked.

Effect of agitation rate: The bacterial isolates were inoculated into the sterile nutrient broth in 50ml conical flasks and were kept at different agitation rates of 120 rpm, 150 rpm, and one set at static for 48 hrs at 30°C. The antagonistic potential was checked by the agar well diffusion method.

Effect of pH: The effect of pH on the antagonistic potential was determined by preparing nutrient broth of different pH

(5, 6, 7, 8, and 9) in a 50ml Erlenmeyer flask. The pH of media was retained with 1N HCl and 1N NaOH by using a pH meter. The loopful of fresh culture was inoculated in each flask under aseptic conditions. The flasks were incubated at 30°C for 48hrs. And further, the antifungal activity of bacterial isolates was checked by the agar well diffusion method on potato dextrose agar plates.

Effect of different temperatures: Different ranges of temperature (25°C, 30°C, and 37°C) were tested for the enhanced antagonistic potential of bacterial isolates. The pH of the medium used was adjusted with 1N HCl and 1N NaOH by using a pH meter. The antifungal activity was checked by the agar well diffusion method.

Effect of different carbon sources: The bacterial isolates were evaluated for their antagonistic activity at different concentrations of carbon sources under optimized pH conditions. During this experiment, glucose, sucrose, dextrose, and starch were tested as alternate carbon sources. Carbon source of basal medium (glucose-20g/l, yeast extract-5g/l, K_2HPO_4-6g/l, NaH_2PO_4-7g/l, NH_4Cl-0.7g/l, $MgSO_4$-0.5g/l) was substituted with one of these sources. Autoclaved Erlenmeyer flasks containing medium were inoculated with the selected isolates. The pH of the various media was adjusted to 7 (before autoclaving) and the flasks were incubated at 30°C for 48 h on a rotary shaker (150 rpm). 1 ml of the culture filtrate was then used aseptically to check the antifungal activity and growth was measured by the inhibition zone method.

Effect of different nitrogen sources: In this experiment, ammonium nitrate, casein, peptone, and yeast extract were tested as substitute nitrogenous sources. Nitrogenous source of basal medium (glucose-20g/l, yeast extract-5g/l, K_2HPO_4-6g/l, NaH_2PO_4-7g/l, NH_4Cl-0.7g/l, $MgSO_4$-0.5g/l) was substituted with one of the mentioned sources. Erlenmeyer flasks containing medium were inoculated with the selected isolates. The initial pH of the different media was adjusted to 7, before sterilization and the flasks were further incubated at 30°C for 2 days on a rotary shaker (150 rpm). 1 ml of the culture filtrate was then taken aseptically and the antagonistic potential was evaluated by the inhibition zone method.

RESULTS AND DISCUSSIONS

Morphological and biochemical characteristics

Microscopic analysis revealed that all the three bacterial isolates were Gram-positive, spore-forming, and rod-shaped. Colony color and characteristics are described in Table 1. They also gave a positive test for catalase. The test organism *B. subtilis* BP67 showed the ability to utilize various carbon sources such as maltose, fructose, dextrose, L-arabinose, mannose, glycerol, sorbitol, mannitol, ONPG, citrate, malonate and able to hydrolyze esculin while the other strain *B. subtilis* BP 171 utilizes various carbon sources such as L-arabinose, inulin, glycerol, salicin, inositol, sorbitol, mannitol, adonitol, ONPG, citrate, and malonate while also able to hydrolyze esculin. However, *B. amyloliquefaciens* BP124 utilized different carbon sources such as lactose, xylose, sucrose, mannose, sorbose, citrate, and was able to hydrolyze esculin. The carbohydrate utilization pattern was *Bacillus subtilis* BP67, *Bacillus subtilis* BP171 and *Bacillus amyloliquefaciens* BP124 was similar to the *Bacillus licheniformis* which was reported by Salkinoja-Salonen *et al.,* (1999).

Salt tolerance

The bacterial isolates *Bacillus subtilis* BP171 and *Bacillus amyloliquefaciens* BP124 demonstrated salt tolerance up to 12% while *Bacillus subtilis* BP67 could able to tolerate up to 10% of NaCl (Table 1). However, the density of growth declined with the increase of salt concentration. Hence, these results suggest that bacterial isolates are halophiles and have high salt tolerance properties. In earlier studies, isolates belonging to *Bacillus* genera demonstrated salt tolerance up to 12% of NaCl (Tomohiko *et al.,* 2003). Bokhari *et al.,* (2019) reported *B. subtilis*, *B. tequilensis* and *B. circulans*, demonstrated salt tolerance. Baindara *et al*., (2013) observed that *B. ssubtilis* isolated from a rhizosphere soil can tolerate salt up to 14% of NaCl. Hence, it can be inferred that salt tolerance varies in different bacterial isolates and also depends on the environment from which they are isolated.

Antagonistic potential of bacterial isolates

All three bacterial isolates demonstrated antagonistic potential against different phytopathogens *viz. Bipolaris sorokiniana*, *Fusarium oxysporum, Fusarium* sp., *Aspergillus* sp., *Aspergillus niger, Penicillium* sp. and *Rhizoctonia solani.* Based on the results, *Bacillus amyloliquefaciens* BP124 was found to be most potent in comparison to *B. subtilis* BP67 and BP171 (Figure 1). *Bacillus amyloliquefaciens* BP124 demonstrated significantly highest ($p<.0001$) inhibition percentage against *Fusarium* sp., (61%) and *Fusarium oxysporum* (60%), following *Penicillium* sp. (51%) and *Rhizoctonia solani* (49.67%) then *Bipolaris sorokiniana* (41%) and *Aspergillus niger* (41%) while least activity was found against *Aspergillus* sp. (39%). A similar pattern was followed by *B. subtilis* BP 171. However, *B. subtilis* BP67 demonstrated the highest ($p<.0001$) inhibition percentage against *Fusarium sp.,* (67%) which was followed by *Fusarium oxysporum* (60.33%) and *Bipolaris sorokiniana* (61%), following *Penicillium sp.* (54.67%) and *Aspergillus niger* (54%). The antagonistic potential of *B. subtilis* BP67 was minimum against *Rhizoctonia solani* (51.67%) *and Aspergillus sp.* (40.67%). Among the Bacillus species, *B subtilis* is most studied for its antagonistic activity and occasionally *B megaterium*, *B.cereus*, *B.pumilus,* and *B.polymyxa*. Shahzad *et al*., (2017) studied plant growth-promoting endophytic *Bacillus amyloliquefaciens* which displayed antifungal activity against pathogenic *Fusarium oxysporum* f. sp. *lycopersici*.

Effect of inoculum size

The bacterial isolates were able to demonstrate significantly highest (p<.0001) antagonistic potential when inoculum size required for the growth was 1ml followed by 500 µl while activity decreased when inoculum size was increased or decreased (Table 2). Similar Results were found for all the bacterial isolates. Secondary metabolites produced at lag phases are dependent on inoculum size plays a crucial role in such activities of bacteria (Maier, 2009)

Effect of agitation

The bacterial isolates grown at the agitation rate of 150 rpm demonstrated significantly highest (p<.0001) antagonistic potential (Table 3). However, antagonistic potential reduced at an agitation rate of 120 rpm while the activity was found to be minimum at static condition. Li *et al.,* (2009) reported that 150 rpm was the ideal shaking condition for the production of antifungal protein from *Bacillus subtilis* strain B29. The higher level of agitation could lead to damage of cells and causes inactivation of enzymes as well as metabolites production (Shioya *et al.,* 1999). Agitation plays an important role in mixing and shearing resulting in improved oxygen transfer for higher biomass production continuous stirring and shaking maintain the homogeneity of chemical and physical conditions are in the medium. Bacterial growth was less at static conditions in comparison to shaking.

Effect of different pH

The significantly highest (p<.0001) antagonistic potential was recorded at pH at 7.0 (Table 4). The results were similar for all the isolates while antagonistic potential decreased pH 5, 6, 8, and 9. However, higher pH showed adverse effects on both growth and the production of the antifungal metabolites. Microorganisms release acidic or alkaline metabolites that changes the pH of the culture medium and affects the growth and production of the antibiotic produced. A change in the external medium alters the ionization of nutrient molecules and thus its availability to the microorganisms is reduced. The importance of pH in the production of antifungal compounds by *Streptomyces* was reported by several investigators and the optimum pH for antibiotic production range between 7.0 and 7.5 (Locci, 1989).

Effect of different temperatures

The bacterial isolates demonstrated significantly highest (p<.0001) antagonistic potential at 30°C followed by 37°C and 25°C (Table 5). Similar results was found for all the microbial isolates. Singh *et al.,* (2017) reported that beyond the optimum temperature, the growth and antifungal metabolite production was decreased. Also, higher temperatures showed an adverse effect on both growth and bioactive compound production. Johnson (1974) reported that the optimum temperature for *Bacillus cereus* ranged between 30 and 37°C, however some strains could grow at temperature as low as 45°C and up to 55°C on the higher side. Afrin and Bhuiyan (2019) revealed that an adequate level of growth and zone of inhibition was observed at 30°C to 45°C and pH 6.0 to 7.5 by *Bacillus amyloliquefaciens subsp. amyloliquefaciens.* Anjhana and Sasikala (2017) found 35°C to be ideal for the growth of *B. subtilis*.

Effect of different carbon sources

Starch as carbon source supported significantly highest (p<.0001) antagonistic activity against all the fungal pathogens for all the bacterial isolates followed by glucose (Table 6). As carbon substrate has a two-fold role in biosynthesis and energy generation, complex carbohydrates such as starch are being more suitable for microbial fermentation and production of secondary metabolites. Several researchers observed that starch and lactose are the ideal carbon sources for biocontrol activity (Pathak, 2011; Usama *et al.,* 2003). Singh *et al.,* (2017) reported that starch is considered to be an important medium component for the production of antifungal compounds from microorganisms, maximum growth, as well as antibiotic production, when starch is used as the solitary source of carbon. However, significantly least (p<.0001) antagonistic activity was found when bacterial isolates were grown on sucrose.

Effect of different nitrogen sources

The best nitrogen source for all the bacterial isolates was found to be peptone as it demonstrated the highest (p<.0001) antagonistic activity, followed by ammonium nitrate and yeast extract (Table 7). The bacterial isolates showed the least antagonistic potential against fungal pathogens in a medium containing casein as a nitrogen source. The results obtained in this study demonstrated that organic nitrogen sources such as peptone and yeast extract had supported the rapid growth and high production of the biocontrol agent. It has been suggested that peptone and yeast extract are good substrates for many microorganisms (Jackson *et al.,* 1998; Costa *et al.,* 2002) because of the amino acids and peptides, water-soluble vitamins, and carbohydrates. However, inorganic salts such as ammonium nitrate are also effective and can be used as nitrogen sources for the production of biocontrol agents that can take in ammonium and reduce nitrate. Durairaj *et al.,* (2017) also demonstrated that peptone, ammonium nitrate, and ammonium chloride effectively increased the zone of inhibition against various fungal pathogens while Joshi *et al.,* (2016) found that ammonium nitrate is a good nitrogen source in minimal salt media for enhanced biocontrol activity and production of the antagonistic compound, lichenysin in *Bacillus licheniformis.*

CONCLUSION

From the present study, it can be concluded that the selected salt-tolerant *Bacillus* strains isolated from rhizospheric soil showed the highest antagonistic potential against phytopathogenic fungi. These strains could be used for controlling harmful diseases caused by fungal

pathogens. The study also showed that appropriate and optimum fermentation conditions including inoculum size, agitation, pH, temperature, carbon, and nitrogen sources, could play an important role in the enhancement of antagonistic potential of bacterial isolates. The bacterial strains were also able to tolerate high salt concentration i.e., 10-12% NaCl. Hence, these salt-tolerant bacterial cultures (*Bacillus subtilis* BP67, *Bacillus subtilis* BP171 and *Bacillus amyloliquefaciens* BP124) are ideal subsitutes for the promotion of crop growth as well as biocontrol agents, and also, for coordinated use in disease and nutrient management strategies under salt-stressed conditions.

ACKNOWLEDGEMENT

The authors express their gratitude towards the Respective Director, ICAR- Central Soil Salinity Research Institute, Karnal, Haryana, for providing necessary support for conducting the research work.

REFERENCES

Aeron, A., S. Kumar, P. Pandey and D. K. Maheshwari (2011). Emerging role of plant growth promoting rhizobacteria in agrobiology, in *Bacteria in Agrobiology: Crop Ecosystems* ed. Maheshwari D.K. (Berlin: Springer ;), 1–36.

Afrin, S. and M.N.I. Bhuiyan (2019). Antagonistic activity of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* against multidrug resistant *Serratia rubidaea. bioRxiv. Corpus ID: 208581683*

Al-janabi, A.A.S. (2006). Identification of Bacitracin produced by local isolate of *Bacillus licheniformis. Afr. J. Biotech.* 5 (18):1600-1601.

Anjhana, V.R. and S.L. Sasikala (2017). Isolation, screening and growth optimization of antagonistic *Bacillus subtilis* MS21 from Thengapattanam estuary against plant fungal pathogens. *Int. J. Adv. Res. Biol.Sci.,* 4(12): 15-26.

Baindara, P., S.M. Mandal, N. Chawla, P.K. Singh, A.K. Pinnaka and S. Korpole (2013). Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK.DU.4 isolated from a rhizosphere soil sample. *AMB Express,* 3:2.

Bokhari, A., M. Essack, F. Feras, C. Andres-Barrao, R. Jalal, S. Alamoudi, R. Razali, H. Alzubaidy, K.H. Shah, S. Siddique, B.V. Bajic, H. Hirt, and M.M. Saad (2019). Bioprospecting desert plant Bacillus endophytic strains for their potential to enhance plant stress tolerance. *Sci. reports*, 9:18154.

Bundale, S., B. Deovrat, N. Nashikkar, T. Kadam and A. Upadhyay (2015). Optimization of Culture Conditions for Production of Bioactive Metabolites by *Streptomyces* spp. Isolated from Soil. *Adv. Microbiol.,* 5: 441-451.

Costa, E., N. Teixidó, J. Usall, E. Atarés and I. Viñas (2002). The effect of nitrogen and carbon sources on growth of the biocontrol agent *Pantoea agglomerans* strain CPA-2. *Lett. Appl. Microbiol.,* 35(2):117-20.

Durairaj, K., P. Velmurugan, J.H. Park, W.S. Chang, Y.J. Park, P. Senthilkumar, K.M. Choi, J.H. Lee and Byung-Taek (2017). Potential for plant biocontrol activity of isolated *Pseudomonas aeruginosa* and *Bacillus stratosphericus* strains against bacterial pathogens acting through both induced plant resistance and direct antagonism. *FEMS Microbiol. Lett.,* 364: 225.

Graumann, P (2007). *Bacillus: Cellula. Mol. Biol.* (1st Ed.): Caister Academic.

Jackson, M.A., J.S. Frymier, B.J. Wilkinson, P. Zorner and S. Evans (1998). Growth requirements for production of stable cells of the bioherbicidal bacterium *Xanthomonas camprestris*. *J. Ind. Microbiol. Biotechnol.,* 21: 237–241.

Johnson, U. and B.G. Snygg (1974). Lipase production and activity as a function of incubation, time, pH and temperature of four lipolytic organisms. *J. Appl. Bacteriol.,* 37: 571-581.

Joshi, S.J., Y.M. Al-Wahaibi, S.N. Al-Bahry, A.E. Elshafifie, A.S. Al-Bemani, A. Al-Bahri and M.S. Al-Mandhari (2016). Production, Characterization, and Application of Bacillus licheniformis W16 Biosurfactant in Enhancing Oil Recovery. *Front. Microbiol.,* 7:1853.

Kuta, F.A., Nimzing L., Yahemba P. (2009). Screening of *Bacillus* Species with Potentials of Antibiotics Production. *Appl. Med. Info*, 24(1-2): 42-46.

Locci, R. (1989). Streptomyces and related Genera. *In: Bergey's Manual of Systematic Bacteriology*, 4: 2451–2508.

Li, J., Q. Yang, L.H. Zhao, S.M. Zhang, Y.X. Wang and X.Y. Zhao (2009). Purification and characterization of a novel antifungal protein from *Bacillus subtilis* strain B29. *J. Zhejiang Uni. Sci.,* 10(4): 264-272.

Maier, R.M. (2009). Bacterial growth. In Environmental Microbiology ed. Maier, R.M., Pepper, I.L. and Gerba, C.P., pp. 37–54. Burlington, MA: Elsevier/Academic Press.

Pathak, K.V. (2011). Purification and characterization of antifungal compounds produced by banyan endophytic Bacilli, Ph.D., Thesis, Dept. of Microbiology, BRD School of Biosciences, Sardar Patel University, India.

Pederson, E.A., M.S. Reddy (1997). A potential biological control agent for damping-off and root rot diseases on multiple crops. *Can. J. Plant Path.* 19: 114-115.

Salkinoja-Salonen, M.S., R. Vuorio, M.A. Andersson, P. Kämpfer, M.C. Andersson, T. Honkanen-Buzalski and A.C. Scoging (1999). Toxigenic strains of Bacillus licheniformis related to food poisoning. *Appl. Environ. Microbiol.,* 65(10):4637-45.

Shahzad, R., A.L. Khan, S. Bilal, S. Asaf and In-Jung Lee (2017). Plant growth-promoting endophytic bacteria versus pathogenic infections: an example of Bacillus

amyloliquefaciens RWL-1 and *Fusarium oxysporum* f. sp. lycopersici in tomato. *PeerJ,* 5: e3107

Shioya, S., M. Morikawa, Y. Kajihara and H. Shimizu (1999). Optimization of agitation and aeration conditions for maximum virginiamycin production. *Appl. Microbiol. Biotechnol.,* 51: 164-9.

Singh, C., R.S. Parmar, P. Jadon and A. Kumar (2017). Optimization of Cultural Conditions for Production of Antifungal Bioactive Metabolites by *Streptomyces spp.* Isolated from Soil. *Int. J. Curr. Microbiol. Appl. Sci., 6*(2): 386-396.

Tomohiko, I., I. Akio, T. Masayuki and N. Shinichi (2003). Induction of salt tolerance in *Bacillus subtilis* IFO 3025. *J. bios. bioengin.,* 96:184-6.

Usama, B., E.E. Hesham, I.M.K. Ismail, M. Hassan, W. Ewa and A.E.G. Sawsan (2003). β-glucanase production from genetically modified recombinant Escherichia coli: Effect of growth substrates and development of a culture medium in shake flask and stirred tank bioreactor. *Process Biochem.,* 39: 307- 313.

Wu, L., H. Shang, Q. Wang, H. Gu, G. Liu and S. Yang (2016). Isolation and characterization of antagonistic endophytes from *Dendrobium candidum* Wall ex Lindl., and the biofertilizing potential of a novel *Pseudomonas saponiphila* strain. *Appl. Soil Ecol.,* 105: 101–108.

Yilmaz, M., H. Soran and Y. Beyatli (2005). Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiol. Res.* 161: 127-131.