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Enhanced cell Viability with Induction of Pathogenesis Related Proteins Against *Aspergillus niger* in Maize by Endo-Rhizospheric Bacteria

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Abstract

Two endorhizospheric bacteria were isolated from the root of saline tolerant plant *Suaeda nudiflora* wild mosque by using nitrogen free selective medium and identified as *Pseudomonas aeruginosa* and *Bacillus megaterium* by the various biochemical and molecular analysis. The isolates were assayed for the production of the secondary metabolite/antibiotics and also analyzed for its infection antagonizing potential against the pathogen *Aspergillus niger*. The growth promotion potential of the isolates was analyzed on maize plant for enhanced plant growth/yield by induction of chitinase, catalase production and reduced programmed cell death (PCD) for acquiring resistance against pathogen *Aspergillus niger*. Comparative analysis of secondary metabolite production was studied in healthy and infected maize by GCMS analysis. The results showed that isolates were able to induce defense enzymes prior to infection and its activity further increased on pathogenic interaction. The isolates also induced PR proteins as well as expression of catalase like gene in the infected plant. The isolated endorhizospheric bacteria also modulate the types and level of secondary metabolites in maize plant for the better survival of maize under stress.

Keywords: Endorhizospheric, Chitinase, Catalase, Programmed cell death, Secondary metabolite, GCMS analysis, Gene expression**.**

1. Introduction

Ecological factors like biotic and abiotic ones continuously effect the growth of plant in agricultural farms. A co-evolution takes place between plants and microbes, which establish synergistic and antagonistic relations with microbes and the plant pathogen. A multilayered process is mediated by the pathogen for its establishment; at the same time, plant-derived metabolites as sugars, lipo-polysaccharides and proteins are produced by the plant to develop resistant against such stress (Boyd *et al.*, 2013). Numerous self-governing and well-elaborated mechanisms have been developed by the plant pathogens to penetrate the plant cell and retrieving the plant cell contents. Zandalinas *et al.*, (2017) reported, that during infection the penetration of pathogens usually depends on the precise time-course of the pathogen interaction with the host plant cells, which initiate various biochemical reactions, resulting in production of reactive oxygen species (ROS), secondary metabolites, and PR proteins. Sometime all these mechanism employed altogether in combination by the plant, to activate specific defense mechanism against the pathogen infection. A plant, when assessed by the pathogens, will stimulate production of several biochemical compounds like the oxidation of phenolic compounds, accumulation of secondary metabolites and many mechanisms related with defense in plants, upsurge specially during fungal infections (Zhou *et al.*, 2016). The oxidation of phenolic compounds develops a tendency for the formation of insoluble complexes with proteins, which act as enzyme inhibitors or are responsible for the oxidation of toxic elements to inhibit the pathogen infection. The most imperative protective response of plants towards the pathogenic factors is the production of pathogenesis-related proteins (PR proteins). The pathogenesis-related proteins are the group of plant encoded proteins responsible for specific forms of resistance to pathogens and related stress (Jha, 2019). The hydrolytic proteins like chitinase and catalase have some basic property and have three main functions in protecting plant from pathogens. Firstly, the hydrolysis of the particular substrates are catalyzed by such enzymes, thus releasing biologically active oligosaccharides (elicitors and suppressors) proficient of managing the immune status of plant tissues. Secondly, as the main constitution of fungal pathogen cell walls is chitin, such an enzyme has the ability to hydrolyze the cell walls of pathogenic fungi. Third, it also modulates the concentration of defense enzyme like peroxides to control the pathogen spread and initiate programmed cell death (PCD) (Park *et al.*, 2018). In the healthy plant, the PR proteins are present in insignificant amount and are induced at certain stages of growth only. Programmed cell death is accountable for the removal of undesirable damaged/dangerous cells and is an important genetically controlled process. PCD is critically for different aspects of plant life cycle, and also involved in hypersensitive response (HR) for the biotic defenses toward plant

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pathogens attack. Many bacteria, fungi, protozoa, algae like numerous microorganisms reside together in the plant rhizosphere, among them bacteria are the most abundant. Being the most abundant microorganism in the rhizosphere, bacteria directly influence the plants metabolism and physiology. There is a tough competition among the rhizospheric bacteria and pathogenic microorganism for root exudates/colonization (Barriuso *et al.,* 2008). The pathogen-induced production of PR proteins and systemic response in the plants and induction of systemic response resulted in accumulation of PR proteins not only at pathogen invasive site, but systemically induced in entire plant. Maize production in the field is constrained by both abiotic and biotic stress factors. In the present study, we investigate the efficiency of endorhizospheric bacteria to develop resistance against the fungal pathogen *Aspergillus niger* in maize through induction of PR proteins, secondary metabolites, phenolic content and gene expression. The effect of endorhizospheric bacteria has been analyzed on the degree of programmed cell death in infected/uninfected maize plant.

2. Materials and Methods

2.1. Isolation and identification of bacteria

Bacterial strains were isolated from root of plant *Suaeda nudiflora* wild mosque by serial dilution techniques as our published method (Jha and Subramanian, 2014). The selection of bacteria for further experiment was done on the basis of their ability to solubilize phosphate (Chen *et al.,* 2014), produce siderophore (Ferreira *et al.,* 2019), modulate indole-acetic acid (IAA) (Mohite, 2013), gibberellins, and utilizes 1-aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen source (Polko and Kieber, 2019). Morphology and Gram properties were determined using a light microscope (1,000X) (Zeiss, Argentina S.A). Pellicle-forming ability and aerobic N_2-dependent growth were assessed in semisolid nitrogen free semi-solid medium (NFb) (Himedia, Mumbai, India) with different carbon sources. For this purpose, malate was replaced by fructose, glucose, glycerol or sucrose. All the isolates were biochemically characterized for utilization of metabolites and enzymes such as Urease, Lysine Decarboxylase, Ornithine Decarboxylase, Esculin, Adonitol, Rhamnose, Mannitol, Sorbitol, Cellobiose, Melibiose, Glucuronate, Mannose, Maltose, Trehalose, Indole, Malonate, Phenylalanine desaminase, Sucrose, 5-Ketogluconate, Palatinose, Galacturonate, Colistin, Coumarate, Tetra thionatereductase, α-Galactosidase, Indoxylphosphate, Raffinose, o- NitroPhenyl N-Acetyl β – D Glucosaminide, P- Nitrophenylβ - D Galactopyranoside, Oxidase tests as per the standard methods (Patra *et al.,* 2020).

2.2. Molecular identification of bacterial isolates

The molecular identification of the isolates were done by 16s rDNA analysis with 16s rDNA primers 16S F: 5'AGAGTTTGATCCTGGCTCAG3' and 16S R: 5'AGGTTACCTTGTTACGACTT3' for PCR amplification of the DNA, with1kb ladder BLUE from GeneON, followed by sequencing (Bangalore, GeNei). The DNA sequences were compared with the sequence obtained from the nucleotide database. The sequences were aligned with the CLUSTAL-W program, and evolutionary distances were generated. Alignment gaps and ambiguous bases were not taken into consideration for comparison. Phylogenetic trees were constructed using the neighbor-joining method and the maximum likelihood method in PHYLIP package.

2.3. Compatibility between bacterial isolates and fungus

Bacterial isolates were tested for its compatibility with each other by streaking parallel on Yeast Extract Glucose Agar medium and incubated in incubator at 28 °C. Compatibility was tested by overgrowth or inhibition of growth after 72 hrs of incubation. Antagonism between bacteria and fungus was in-vitro determined by inoculating both organisms on the same Yeast Extract Glucose Agar plate at 28 °C in incubator for a week, and antagonistic effects were tested by inhibition of growth of fungus by bacteria.

2.4. Antibiotic production ability assay

The antibiotic production ability of the selected isolates was assessed by extracting and testing toxicity of metabolites produced by them. The strains were grown for 48 hrs in 5 ml of N broth with glucose (2% w/v). The culture was centrifuged at 10,000 rpm for 10 min and filtrate was collected. The metabolites from the filtrates were extracted with an equal volume of chloroform. The metabolites were also extracted from pellet and pooled. The upper aqueous layer was discarded and to remaining chloroform phase, a pinch of sodium sulphate was added to dry off water. It was again centrifuged at 8,000 rpm for 10 min to palettes out sodium sulphate. The clear layer was discarded, and chloroform was removed by flushing air. The residue was re-dissolved in 200 ml of acetone, and 70 µl was spotted on to TLC plate (Silica gel 60 F254, 20*20 cm, 0.2 mm thickness, Merck). The plate was chromatogrammed using chloroform: acetone (9:1) as solvent system. Later the plates were observed under UV light at 254 nm. The metabolites were eluted and re-dissolved in acetone: water (1:10) 100 µl of eluted portions were centrifuged to pellet. The silica gel clear suspension was further analyzed for toxicity against test pathogen. The percentage inhibition of individual antibiotic produced by each strain was calculated with known concentration of antibiotics Streptomycin.

2.5. Plant inoculation and treatment

Certified seeds of maize variety GM4 were obtained from the Gujarat state seed cooperation and were inoculated with endorhizospheric bacteria (Jha and Subramanian, 2015). After 4 weeks of inoculation, plants were infected with *Aspergillus niger* by spore suspension with a spore load of 10^4 conidia ml^{-1}, which caused more than 75% infection under greenhouse condition. Plants inoculated with bacteria and infected or non-infected with fungus were transplanted in pot, having five transplants per pot holding 10 kg soil and allowed to grow without additional nutrient for 5 weeks at 20 to 25 °C in the greenhouse.

2.6. Effect on total phenolic content after inoculation and infection

The total phenolic content of leaf extracts was measured using colorimetric Folin–Ciocalteu method

(Kaur and Kapoor, 2002). The leaf extract (1ml) was mixed with 5ml of distilled water and 250 µl of 1N folin-ciocalteau reagent. The mixture was covered and allowed to stand for 3 min at 25°C. In this mixture, 1ml of saturated Na_2CO_3 and 1ml of distilled water were added. The mixture was incubated for 1 hr at 25°C to develop color and measured at 725 nm using spectrophotometer. Standard graph was prepared by using different concentration of phenol crystals.

2.7. Assay of defense enzymes

Plant leaf tissues were collected after 2 weeks of inoculation and infection with pathogen. Leaf samples were homogenized with liquid nitrogen in a pre-chilled mortar and pestle. One gram of leaf sample was homogenized with 0.1 M sodium citrate buffer (pH 5.0) at 4^oC. The homogenate was centrifuged for 20 min at 10, 000 rpm. The supernatant was used as a crude enzyme extract for enzyme assay.

2.8. Estimation of chitinase

One gram of leaf tissue was homogenized in 5 ml of 0.1 $molL^{-1}$ sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 g at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared by taking 2 g of crab-shell chitin (Sigma, USA). It was slowly added to 35 ml of cold concentrated hydrochloric acid with vigorous stirring and placed at 4°C for 24 h. The mixture was filtered through glass wool into ethanol (200 ml) with rapid stirring. The resultant chitin suspension was centrifuged at 10,000 g for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. The commercial lyophilized snail gut enzyme chitinase was used as standard for colorimetric assay. The reaction mixture consist 10 ml of 1 $molL^{-1}$ sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) in an eppendorf tube. After 2 h at 37°C, the reaction was stopped by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 ml of desalted snail gut enzyme for 1 h. After 1 h the reaction mixture was brought to pH 8.9 by adding 70 ml of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice bath. After the addition of 2 ml of 3-(dimethylamino) benzoid acid (DMAB), the mixture was incubated for 20 min at 37°C and immediately thereafter the absorbance was measured at 585 nm. N-Acetyl glucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as nmolGlcNAc equivalents per min per mg of protein (Kumar *et al.,* 2018).

2.9. Estimation of Catalase (CAT) activity

CAT activity was assayed by Chioti and Zervoudakis, (2017) method by measuring the initial rate of disappearance of H_2O_2. The reaction mixture contains of 0.1 ml enzyme from plant source, 0.1 mM EDTA and 3% (v/v) H_2O_2 in 0.05 M Na-phosphate buffer (pH 7). The activity was expressed as µmol H_2O_2 consumed per min by taking optical density at 240 nm.

2.10. Analysis ofprogrammed cell death

Caspase-like activity was assayed in the maize leaves after 2 weeks of infection treatment using the method of Sueldo and van der Hoorn, (2017). The leaf (200 mg) was ground in liquid nitrogen into a fine powder and homogenized in 2 ml of assay buffer containing 100 mM Tris–HCl (pH 7.2), 5 mM $MgCI_2$,2m MEDTA, 10%(v/v) glycerol, 10 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). This mixture was centrifuged at 13,000 g for 30 min at 4 °C to obtain tissue extract, and 25 µl of the tissue extract was incubated in70 µl of assay buffer at 37 °C for 5 min, followed by addition of 10 µl of 5 mM N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) as substrate (dissolved in dimethyl sulfoxide) for caspase-like activity to a final concentration of 0.5 mM. A blank reaction was set up in which Ac-DEVDpNA was substituted with 10 µl of DMSO. These reaction mixtures were incubated at 37 °C for 60 min, within which caspase-like activity was followed by measuring absorbance at 405 nm every 20 min during the 60-min incubation period. Caspase-like activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for the p-nitroaniline.

2.11. Agarose gel analysis of DNA for programmed cell death

Hundred milligram of leaf tissue from each treatment was frozen in liquid nitrogen immediately after sampling and ground with a mortar and pestle to a fine powder. Isolation of DNA was performed using a DNeasy plant mini kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's instructions. To observe DNA fragmentation, samples (0.5 µg ml^{-1} $lane^{-1}$, final concentration) were run on 1%ethidium bromide agarose gel at a constant 50 V.

2.12. GCMS analysis for secondary metabolites production

Analysis by GC-MS was performed using a Thermo Gc–Trace Ultra Ver: 5.0, Pyrolysis auto sampler interfaced to a Perkin Elmer Turbomass Gold equipped with a fused silica capillary column (J & W; DBI; 30m length x 0.25 mm id. film thickness 0.25 µm). The fraction was pyrolysed at 610 °C and then introduced to the GC column. The transfer line was held at 280 °C and the source temperature was maintained at 180 °C and ionization energy was set at 70eV. Helium was employed as carrier gas (1 mL/m). The GC oven temperature was programmed: The column held initially at 70 °C/ m (isothermal) and then increased by at 8 °C/m to 260°C/ m min^{-1} (isothermal). Qualitative identification of the different constituents was performed by composition of the relative retention times and mass spectra with those of authentic reference compounds by retention indices (RI) and mass spectra. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns (Jha *et al.,* 2014).

2.13. RNA extraction and RT-PCR from isolated RNA

The bacterized plant material without infection was used for the isolation of total mRNA and the RT-PCR was done using a GeNei RT PCR Kit with certain modifications related with cyclic variation in time. 100 ng mRNA was incubated at $65^{\circ}C$ for 10 min at room temperature, subsequently with oligo (T) primers, 0.25 mM deoxynucleotide triphosphates (dNTP's), 5 mM 1,4-dithiothreitol (DTT), 4 mM Tris-HCl pH 7.5, 20 mM KCl,

0.02 mM EDTA, 0.1% (v/v) Polyoxyethylene sorbitanmonolaurate (Tween 20), 0.5 µl M-MULV reverse transcriptase and 9µl nuclease free water for the synthesis of cDNA. The mixture was incubated at 37 °C for 1 h. Temperature was then raised to 95 °C for 2 min to denature RNA-DNA hybrid. The catalase gene was amplified by using forward primer 5'-TTAATCAGCCATGGATCCt-3' and reverse primer 5'-TTAATCAGCCATGGATCCT-3' and documentation was done by Gel Documentation System (Alpha Digi Doc RT, USA). All PCR with different primer combinations were performed in duplicate.

2.14. Statistical data analysis

All the data were analyzed for significance using analysis of variance ANOVA followed by Fisher's least significant difference test (P 0.05), using SAS software (SAS Institute, Cary, NC, USA).

3. Results

Thirty-five bacterial isolates were obtained from the root of *Suaeda nudiflora* plant, collected from coastal region of Gujarat using semi-solid nitrogen-free medium (Nfb). Out of 35, two isolates were selected on the basis of their growth promotion efficiency, by analyzing their ability to solubilize phosphate, produce siderophore, indole-acetic acid (IAA), gibberellins, and utilizes ACC as sole nitrogen source. It was also analyzed for their ability to overcome or suppress infection by producing β-1, 3-glucanases and chitinases and was identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergey's Manual of Determinative Bacteriology (Table -1).

The total genomic DNA was isolated from both isolates for molecular identification and amplified by 16S rDNA specific primers. Nucleotides homology and phylogenetic analysis of the isolate was used for identification and isolate was identified as as *Pseudomonas aeruginosa* (Gene Bank Accession Number: JQ790515) and other as *Bacillus megaterium* (GeneBank Accession Number: JQ790514). The two bacterial isolates were compatible to each other as observed on nutrient plate. *B. megaterium* and *P. aeruginosa* were also tested for its compatibility with the phyto-pathogenic fungus *A. niger* , and results indicate that both isolates were remarkably suppressed the growth of fungus in its vicinity. A clear zone of inhibition was observed around the both *B. megaterium* and *P. aeruginosa* and it was larger in *P. aeruginosa* than the *B. megaterium* (Fig.1).

The ability of the isolates for the production of antibiotic showed that the *P.aeruginosa* (S1) had more variation in bands of antibiotic in compared to *B. megaterium* (R1-R4) on TLC plate. Antibiotic(s) produced by *P.aeruginosa* (S1) do not migrate on TLC plate and that fraction does not show any zone of inhibition, so it may be some other metabolite having no effect on pathogen, while antibiotic produced by *B. megaterium* (R1-R4) were different Rf value and showed significant variation in zone of inhibition. The isolates fraction of antibiotic on TLC were analyzed for their efficacy against *A. niger* and most of the antibiotics fraction showed the efficacy against *A.niger* (Table-2) by formation of zone of inhibition.

Table 1.Biochemical properties of the isolates to be used as bio-control agent.

S.No	Biochemical Tests	*B.megaterium*	*P.aeruginosa*
1	Urease	-ve	-ve
2	Lysine Decarboxylase	-ve	-ve
3	Ornithine Decarboxylase	-ve	-ve
4	Esculin	-ve	+ve
5	Arabinose	-ve	-ve
6	Adonitol	-ve	-ve
7	Rhamnose	-ve	-ve
8	Mannitol	-ve	-ve
9	Sorbitol	-ve	-ve
10	Cellobiose	-ve	-ve
11	Melibiose	+ve	-ve
12	Glucuronate	-ve	-ve
13	Mannose	-ve	+ve
14	Maltose	+ve	+ve
15	Trehalose	-ve	+ve
16	Indole	-ve	+ve
17	Malonate	-ve	+ve
18	Phenylalanine desaminase	-ve	-ve
19	Sucrose	+ve	+ve
20	5 Ketogluconate	-ve	-ve
21	Palatinose	-ve	-ve
22	Galacturonate	-ve	-ve
23	Colistin	-ve	+ve
24	Coumarate	-ve	+ve
25	Tetra thionate reductase	-ve	-ve
26	α- Galactosidase	-ve	-ve
27	Indoxylphosphate	-ve	-ve
28	Raffinose	-ve	-ve
29	O-Nitro phenyl N-Acetylβ-D Glucosaminide	-ve	-ve
30	p- Nitrophenyl β-D Galatopyranoside	-ve	+ve
31	Oxidase	-ve	-ve
32	Phosphate Solubilization	+ve	+ve
33	Siderophore production	+ve	+ve
34	Indoleacetic acid (IAA)	+ve	+ve
35	ACC Utilization	+ve	+ve
36	β-1, 3-glucanases	+ve	+ve
37	Chitinases	+ve	+ve

Note- The +ve sign indicate presence and –ve sign indicate absence of activity.

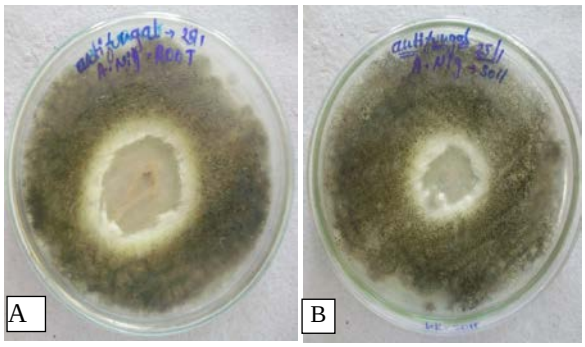

Figure 1. Endophytic bacteria showing antifungal activity against *A.niger* as the formation of Zone of clearance around the culture A= *P. aeruginosa* and B= *B. megaterium*.

The efficacy of the isolated antibiotics (from TLC) had been determined by comparing the zone of inhibition with known standard antibiotics (streptomycin). The zone of inhibition the isolated fractions of antibiotics from the TLC plate showed remarkable variation and the highest efficacy (73%) was shown by the fourth fraction of antibiotics of *P. aeruginosa*.

Plant phenols are secondary metabolites that encompass several classes of structurally diverse products arising from the shikimate–phenylpropanoid pathways. Plants use phenolic compounds for pigmentation, growth, reproduction, and resistance to pathogens. In the present study, the endorhizospheric bacteria considerably enhanced the phenolic compounds in plant under both conditions, i.e. inoculated with isolates alone and infected with the *Aspergillus niger*. The endorhizospheric bacteria alone able to induced production of sufficient amount the phenolic in inoculated plant, while when such inoculated plant get infected with fungal pathogen *Aspergillus niger*, enhanced production of the phenolic was recorded as shown in Table 2.

Table 2. Analysis of antibiotic(s) production by endophytic bacteria on TLC and their efficacy against *A. niger*.

Endophytic Strain	Antibiotic	Rf values	Zone Of Inhibition, ZOI (mm)	Antibiotic efficacy (%)
B. megaterium	S1	Absent	Absent	00
P.aeruginosa	R1	0.20	12	67%
P.aeruginosa	R2	0.515	8	54%
P.aeruginosa	R3	0.656	Absent	00
P.aeruginosa	R4	0.781	15	73%

In the present study, the protective enzymes considerably enhanced after inoculation with endorhizospheric bacteria in the plant and infection by *Aspergillus niger*, further changed in its activity. A simultaneous increase was recorded in chintase and catalase activity in the plant inoculate with endorhizospheric bacteria alone or in mixture and infected with fungal pathogen *Aspergillus niger*. Chitinase belong to the glycosyl hydrolase family, which catalyzes the hydrolysis of glycosidic bonds of chitin.

However, the CAT activity was significantly decreased in bacterized plant up to (22.2 nmol/min/g) at normal condition, but after infection with pathogen its concentration further increased upto (38.2nmol/min/g) (Table 3). So, the CAT activity remarkably increased after fungal infection in non-inoculated plants compared to plants inoculated with endophytic bacteria.

Table 3: Effect of endophytic bacteria on the phenolic, catalase

Treatment	Phenolic (mgg^{-1} of the gallic acid equivalent)	CAT (nmol $min^{-1}g^{-1}$)	Chitinase (nmol of NAG $min^{-1}g^{-1}$)
	Normal		
	m mm		
Control	0.93^{cd}	0.34^{d}	0.21^{d}
Control+*B. megaterium*	1.61^{bc}	0.47^{bc}	0.28^{c}
Control + *P.aeruginosa*	1.88^{ab}	052^{b}	0.27^{b}
Control+ *B.megaterium*+ *P.aeruginosa*	2.11^{a}	0.61^{a}	0.34^{a}
Infected			
Control	1.21^{cd}	0.83^{d}	0.29^{d}
Control+*B. megaterium*	1.87^{c}	0.53^{ab}	0.37^{b}
Control + *P.aeruginosa*	2.14^{b}	0.51^{a}	0.41
Control +*B.megaterium*+ *P. aeruginosa*	2.45^{a}	0.41^{c}	0.51^{bc}
Signification	**	*	ns

and chitinase activity in maize after infection (n=5).

$^{ns}P > 0.05$ (not significant), $*P < 0.05$ (significant) and $**P < 0.05$ (highly significant), m- mean. For each Parameter, values in columns followed by the same letter are not significantly different at ($P \leq 0.05$).

GCMS analysis of leaf extract of inoculated and non-inoculated plants after infection showed significant difference in the types and number of secondary metabolites and the results revealed the presence of different phytocompounds in the plant inoculated with selected bacteria. The healthy-inoculated plant showed 8 secondary metabolites (Table 4), while plant inoculated and infected showed 12 different secondary metabolites (Table 5) belong to phenols and terpenoids group. The compounds like androstane-3,17-dione, methyl dihydroisosteviol, isosteviol methyl ester in healthy and androstan-17-one, 3-ethyl-3-hydroxy-, (5.alpha.), 1h-3a,7-methanoazulene, octahydro-1,4,9,9-tetramethyl, aromadendrene oxide-(2) belong to phenols and terpenoids group in infected plant.

Fungal infection can trigger activation of caspase-like activity in the infected leaves. On this basis, we investigated the level of caspase-like enzymatic activity differed in maize plant inoculated with endorhizospheric bacteria and infected with fungal pathogen *Aspergillusniger*. Cell caspase-like enzymatic activity increased for both the inoculated plant with endophytes and infected with pathogen and more in non-inoculated infected plants (Fig. 2).

Table 4. Different secondary metabolites in endophytes inoculated maize leaves extract identified in GC–MS analysis.

Hit	Rev	For	Compound Name	MW	Formula	CAS
1	812	565	2,4-DIBROMOETIOCHOLA-3,17-DIONE	444	C19H26O2Br2	900251-89-1
2	787	576	ANDROSTANE-3,17-DIONE	288	C19H28O2	5982-99-0
3	750	512	ISOSTEVIOL METHYL ESTER	332	C21H32O3	900256-08-8
4	741	521	1,3,6-HEPTATRIENE, 2,5,5-TRIMETHYL	136	C10H16	29548-02-5
5	724	489	METHYL ISOLITHOCHOLATE	390	C25H42O3	5405-42-5
6	720	509	METHYL DIHYDROISOSTEVIOL	334	C21H34O3	202577-02-4
7	715	538	ALPHA.-FARNESENE	204	C15H24	502-61-4
8	714	478	TRILOSTANE	329	C20H27O3N	13647-35-3

Where hit means attempt Number, Rev Reverse match of peak, For Forward match of Peak, M.W. Molecular weight of compound, CAS Chemical Abstract service.

Table 5. Different secondary metabolites in endophytes inoculated maize leaves extract identified in GCMS analysis after fungal infection.

Hit	Rev	For	Compound Name	M.W	Formula	CAS
1	798	601	3-KETO-ISOSTEVIOL	332	C20H28O4	900255-38-4
2	779	561	3-HYDROXY-12-KETOBISNORCHOLANIC ACID	362	C22H34O4	900252-01-9
3	749	553	3-HYDROXY-11-KETOCHOLANIC ACID	390	C24H38O4	910-28-1
4	738	543	1-PROPENE, 2-NITRO-3-(1-CYCLOOCTENYL)	195	C11H17O2N	80255-21-6
5	722	531	PSEDUOSARSASAPOGENIN-5,20-DIEN	414	C27H42O3	900214-84-5
6	715	538	ALPHA.-FARNESENE	204	C15H24	502-61-4
7	714	478	TRILOSTANE	329	C20H27O3N	13647-35-3
8	713	425	ANDROSTAN-17-ONE, 3-ETHYL-3-HYDROXY-, (5.ALPHA.)-	318	C21H34O2	57344-99-7
9	703	506	1-NAPHTHALENECARBOXYLIC ACID, DECAHYDRO-1,4A-DIMETHYL-6-METHYLENE-	316	C21H32O2	10178-35-5
10	703	522	1H-3A,7-METHANOAZULENE, OCTAHYDRO-1,4,9,9-TETRAMETHYL	206	C15H26	25491-20-7
11	702	501	AROMADENDRENE OXIDE-(2)	220	C15H24O	900151-98-6
12	699	537	GAMMA.-ELEMENE	. 204	C15H24	339154-91-5

Where hit means attempt Number, Rev Reverse match of peak, For Forward match of Peak, M.W. Molecular weight of compound, CAS Chemical Abstract service.

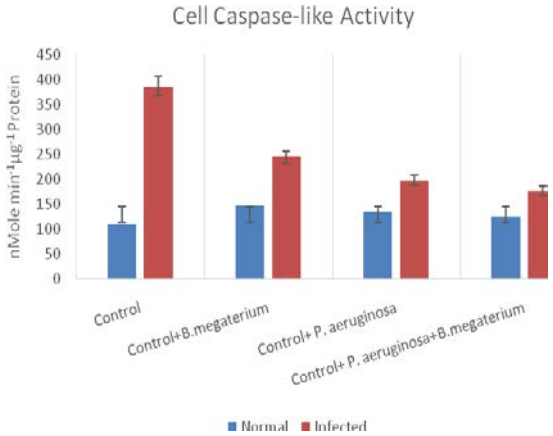

Figure 2. Effect of inoculation with *B. megaterium, P. aeruginosa* alone and in combination on cell caspase-like activity in maize in normal and infected condition (n=5).

Genomic DNA was isolated from leaves of all different treatment and separated in agarose gel electrophoresis to analyze programmed cell death (Fig. 3). Fungal infection showed extensive DNA fragmentation as detected in both the inoculated or non-inoculated maize. Less fragmentation of DNA was observed in the plants inoculated with endophytes only. To characterize the induction of catalase gene in maize inoculated with endorhizospheric bacteria, the RT-PCR-amplified product was observed as1.8 kb bands on agarose gel electrophoresis (Fig. 4) uniformly by inoculated with either single isolate or in combination.

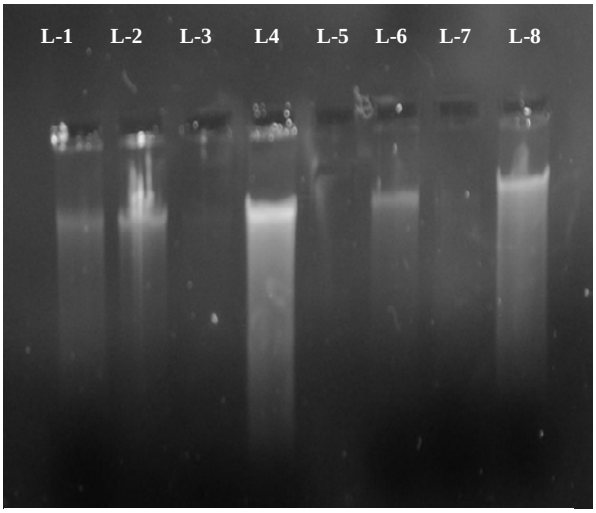

Figure 3: Effect of *B. megaterium, P. aeruginosa* alone and in combination on programmed cell death in in maize in healthy and infected condition (n=5). Where L1-infected inoculated with both the isolates, L2- infected inoculated with *B. megaterium*, L3-infected inoculated with *P. aeruginosa*, L4- Infected Control, L5-Pure control, L6- inoculated with both the isolates, L7-inoculated with *B. megaterium*, L8- inoculated with *P. aeruginosa*.

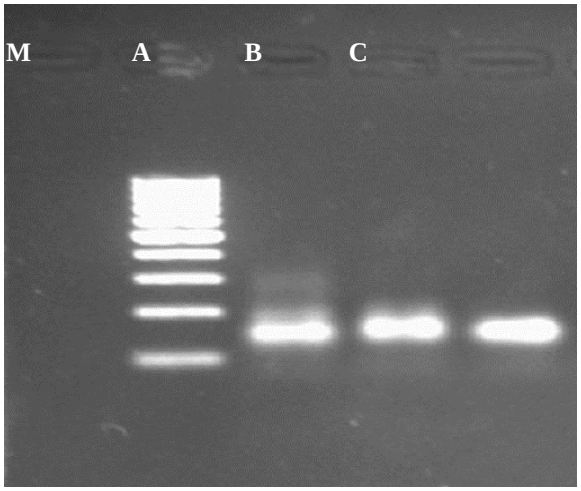

Figure 4. Agarose gel showing induction of gene catalase. Lane M=1kb *ladder* BLUE from GeneON Bioscience is composed of 13 individual *DNA* fragments , Lane A= plant inoculated with *B. megaterium*, and Lane B= plant inoculated with *P. aeruginosa*, Lane C= plant inoculated with *B. megaterium* and *P. aeruginosa*.

4. Discussion

Many interactions of microbes with plants takes place in the endosphere, due to the occurrence of rich and diverse microbial food source, so it is the most active ecological niches, (Jha and Subramanian, 2016). Such interaction of plant with endorhizospheric bacteria helps in maintaining the plant health by improving nutrient status, developing resistant against phyto-pathogen and tolerance towards the environmental stress. The plant root colonized by endorhizospheric bacteria has numerous mechanisms to employ favorable effect on plant growth and development, i.e. by associative nitrogen fixation, solubilization of phosphorus and potassium, production of siderophores, and by modifying the cell permeability (Qessaoui *et al.*, 2019). Such isolates also enhance bioavailability of nutrients, modify root proton efflux, directly motivate plant ion uptake and/or plant transport system and enhance the overall biomas production. In this study, *Pseudomonas aeruginosa* and *Bacillus megaterium*, were two bacterial isolates obtained from root of *Suaeda nudiflora wild mosque* plant, and it is well reported that bacterial genera such as *Pseudomonas*, *Bacillus* and *Brevibacillus* are well known to endorse plant growth and yield in diverse groups of non-leguminous plant under normal as well as stress conditions (Karlidag *et al.*, 2007).

Antibiotics are generally low molecular weight organic compounds produced by microbes, to inhibit the growth of other microbes. Antibiosis plays an active role in the bio-control of plant disease and often acts in concert with competition and parasitism. The result of present study showed that the antibiotic produced by the isolates has the potential to suppress the growth of fungal pathogen and ultimately help in plant growth promotion. Olanrewaju *et al.*, (2017) has also been reported that *Bacillus* and *Pseudomonas* produce a variety of antibiotic metabolites which serve as antifungal, antibacterial, antihelminthic, antiviral, antimicrobial, phytotoxic, antioxidant and cytotoxic agents. The bio-control potential and induced response of these isolates is an effective tool for yield management in economically important crops.

The phenolics normally produced by the plant to strengthen the plant cell wall also have fungicidal activity. In the present study, healthy as well as maize plants infected with fungal pathogen showed better induction of phenolic compounds after being inoculated with endorhizospheric bacteria. Induction/accumulation of phenolics takes place prior to pathogens attack or pathogen infection to protect the plant from pathogens as well as to check the complete hold pathogen on the host plant (Nicaise *et al.*, 2009). In this study, production of phenolics induced in the endophytes inoculated maize plant helps the plant to overcome phytopathogenic infection. The results of the present study showed higher induction of phenolic compounds in the plants colonized with beneficial microorganisms after pathogen infection, compared to healthy controls and are responsible for inducible protection in plant, to maintain plant health for much longer time as also reported by Wallis and Galarneau, (2020).

In response to biotic stress, plants implement a vast range of mechanisms like modification of specific metabolites/up regulation of defense-related proteins to develop resistant over pathogen. Pathogenesis-related proteins include a huge family of plant proteins, which induced in response to pathogen invasion and are developmentally controlled. Pathogen induced SAR is similar to endorhizospheric bacteria mediated ISR to develop resistant towards a wide range of plant pathogens and enhance resistant to uninfected plant parts also (Choudhary *et al.*, 2008). Induction of defence enzymes chitinase and catalase take place more efficiently in maize plant inoculated with endorhizospheric bacteria, to develop resistant against the phytopathogens. Chitinase is an important hydrolytic enzyme, which has been induced frequently in plants upon infection by diverse group of pathogens. The amount of chitinase considerably increases in the maize plant inoculated with endophytes in this study and has an effective role in plant defense against fungal pathogen, as it is responsible for degradation of fungal cell wall. Daulagala and Allan- Atkins, (2015) reported, about the chitinolytic activities of endophytic bacteria isolated from symptom-free chinese cabbage leaves, which clearly indicated that the induction of defence enzyme by such bacteria alone is sufficient for the host plant protection. The main structural component of fungal cell wall is chitin, substrate for chitinase enzyme. Pathogen infection in plant can result in generation of reactive oxygen, which has direct impact as antimicrobial action and plays critical role in inducing various other defense mechanisms, like production of phyto-alexin, lignin deposition, peroxidation of lipid and hypersensitive cell death or PCD.

Catalase is an essential enzyme have major role in plant defense against pathogens. In this study, plant inoculated with endorhizospheric bacteria showed significantly enhanced catalase activity, but it reduced after infection by fungal pathogen. The reduced catalase activity may cause the suppression of H_2O_2 scavenging activity, perhaps has a major role in modulating H_2O_2 homeostasis, as accumulation of threshold levels of H_2O_2 is necessary for the activation of vigorous programmed cell death for pathogen clearance (Lapshina *et al.*, 2016). In the plant's life cycle, cell death is a crucial process, and two main

approaches for action in plants are programmed cell death (PCD) and necrosis. PCD is genetically controlled and has some common characteristic features with apoptotic cell death of the cell like cytoplasmic condensation, cell shrinkage and DNA fragmentation (Greenberg and Yao, 2004).

Pathogen induced host cell destruction observed as enhanced DNA fragmentation in infected plant and plant inoculated with endophytes showed reduced DNA fragmentation, which indicates the role of endophytic bacteria in managing cell activity. During the plant pathogen interaction, at the site of pathogen attack an oxidative burst occur with initiation of cell death at the site of pathogen attack and such localized cell death restrict the spread of the pathogen infection to the healthy plant cell/part.

In the GC-MS analysis there are eight different metabolites/compounds obtained in the leaf extracts of plant inoculated with endophytes alone and twelve different metabolites in infected plant inoculated with endophytes. Among these twenty different metabolites ten metabolites exhibit antioxidant, antibacterial and antimicrobial activity as also reported by Nivas and Boominathan, (2015). Several bioactive natural compounds having antimicrobial activity are induced by the endophytes, which help host plant to acquire resistant against pathogen. The pathogenic intrusion regulated by modulation of secondary metabolites by the endophytes, to develop resistance against pathogen in the host plants. Many such secondary metabolites having antimicrobial activity belong to diverse structural classes such as alkaloids, peptides, steroids, terpenoids, quinines, flavonoids and phenols are reported has been induced by endophytes (Yu *et al.,* 2010). In this present study, the production of diverse types and number of secondary metabolites in the endophytes inoculated plants in presence and absence of pathogen was observed during GCMS analysis.

Suppressing the fungal pathogen by initiating the plant defense mechanism by endorhizospheric bacteria is known as “Induced Systemic Resistance”. Plants immune system activates upon interaction of plant with endorhizospheric bacteria remain active throughout the life by inducing the defense gene. The endophytes alone are able to induce the defense gene like catalase in the plant as observed in the present study. Ramos Solano *et al.,* (2008) also reported about the induction of defense-related marker genes by some strains of *Chryseobacterium* and *Bacillus* in host plant. The plant growth and yield in the radish, potato, sugar beet, ground nut and mango increased by the *Pseudomonas,* in addition to disease management has been reported by Liu *et al.,* (2017). The major effects of inoculation of plant with such bacteria may be similar to treatment with pathogenic microorganisms or abiotic agents, ensued in the induction of systemic resistance for bacterially induced resistance and systemic acquired resistance for the other forms. The development of bio-agents using such bacterial strains is an emerging trend in crop protection to reduce the economic loss caused by pathogen.

5. Conclusion

The endorhizospheric bacteria having ability for the induction of phenolic production, PR protein and secondary metabolites with antimicrobial activity in the host plant, can serve as a potential biocontrol agent against wide range of phytopathogen. Such secondary metabolites belong to diverse structural classes such as alkaloids, peptides, steroids, terpenoids, quinines, flavonoids and phenols. The present study strongly supports the development of bio-control strategies on the basis of *invitro* effect of the isolates against the fungal pathogen and production of other biologically active metabolites using such endorhizospheric strain, to reduce the damage caused by plant pathogens in economically important crops.

Conflict of Interest

There is no conflict of interest to declare.

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