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Maize spermosphere bacterial endophytes and their biotic and abiotic stress tolerance traits

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Abstract
In the present study, 12 bacterial endophytes (C1 to C12) were isolated from three different genotypes of maize seed (COH6). Seed endophytes are considered as pioneers of plant microbiome and enhance the plant defense response at an early stage of crop growth and later stages of seedling development. The seed-associated endophytes exhibit the antagonistic property against the fungal pathogen (*Fusarium oxysporum* and *Macrophomina phaseolina*) of maize. Among 12 isolates, C11 suppressed the mycelial growth of both the tested pathogens. Furthermore, the selected isolates were subjected to *in vitro* abiotic stress tolerance such as salt, drought and temperature stress. The isolates C3, C5, C7, and C12 showed increased tolerance to drought, salt and temperature stress and might assist the host plant in alleviating the ill-effects of abiotic stress on maize. On a whole, microbial isolates associated with the seeds could be a better candidate in biotic and abiotic stress tolerance and would develop those isolates as consortia for obtaining desirable growth in maize.

Keywords: Biochemical characteristics, maize, seed endophytes, stress tolerance

1. Introduction
Stress is one of the major agricultural constraints that limit crop yield, health, and productivity [1]. Biotic and abiotic stress upon plants is mainly responsible for the disruption of plant equilibrium having deleterious effects on plant health and functioning. The predominance of biotic stress such as pathogen, pest, and abiotic stress such as salt, drought, and high temperature negatively impact crop growth and functioning. Microbial inoculants have been recognized as beneficial and eco-friendly ones that can enhance host response to withstand biotic and abiotic stress.

The bacterial endophytes are symbionts present everywhere in plant species without harming the host, known to improve plant growth and development. They exhibit ecological benefits to plant fitness which can be used for sustainable agricultural production. The seeds serve as a vehicle for the transmission of endophytes passed on to successive generations (vertical transmission). Though, seed endophytes are unexploited source now it is emerging as a potential source of microbial inoculants [2]. From a native pool of microorganisms, beneficial microbes are likely to colonize the seeds, establishes an initial microflora and support the host plant in every phase of seedling development.

Maize is an important cereal crop and is called as Queen of cereals, widely cultivated throughout the world. Maize is one of the major energy-providing crops for more than half of the population after rice and wheat [3]. It is being cultivated throughout the world and contributes around one-fourth of global cereal grain production. However, the production and yield of maize were influenced by both biotic and abiotic stress. To mitigate these factors microbial-based remediation is to be taken which enhances the survival ability of plants and helps in sustaining the crop yield. The endophytes isolated from the maize seeds would be an efficient inoculum that can act as an initial colonizer and recruits the indigenous microbes and participates in stress alleviation [4].

Therefore, the present study aims in identifying the bacterial endophytes associated with seeds isolated from three different genotypes (Male, female, and hybrid) of maize and screening of isolates for their potential to withstand the abiotic stress such as drought, temperature, and salt and also for their antagonistic property against the seed-borne pathogen.

2. Materials and Methods

2.1 Seed sample collection and isolation of endophytes

The fresh seeds of three different genotypes (male, female, and hybrid) of maize (COH6) were obtained from the Department of Millets, TNAU, Coimbatore. For isolation of seed bacterial endophytes, the collected seeds were surface sterilized using 4% sodium hypochlorite for 2 min, followed by 70% ethanol for 2-3 min and finally rinsed with sterile double distilled water to eliminate the epiphytes and other undesirable microorganisms under aseptic condition. The surface-sterilized seeds were macerated using phosphate buffer saline in a sterile pestle and mortar. From the macerated wash, 1mL was used to make serial dilutions up to (10^{-4}) plated on six different media such as Tryptic soy agar (TSA), Nutrient agar (NA), Luria Bertani (LB), Soil extract agar (SEA), Reasoner's 2A agar (R2A) and Starch casein agar (SCA). The plates were incubated at 28±2°C for 24-48 h. At the end of the incubation period, the plates were observed for the appearance of bacterial colonies [5]. The morphologically different colonies were picked and purified by repeated streaking onto a respected media and the purified isolates were stored in 60% glycerol and kept at -80°C.

2.2 Morphological identification of bacteria

Bacterial colonies formed on each media were characterized morphologically by observing the traits such as shape, size, elevation, form, color, edge, and appearance. According to the morphological observations, 12 different bacterial colonies were picked and screened for biochemical and biotic & abiotic stress tolerance.

2.3 Biochemical characterization of selected bacterial endophytes

The bacterial endophytes were screened for biochemical characterization namely indole production test, catalase test as per the protocol [6], methyl red and Voges Proskauer test [7], citrate utilization test, KOH test [8], Gram staining [9], Cellulase, Pectinase, and Amylase test [10-12].

2.4 Screening of endophytes for antagonistic activity

The antagonistic activity of selected twelve bacterial endophytes was assessed against major fungal plant pathogens of maize namely *Macrophomina phaseolina* and *Fusarium oxysporum* by dual culture method [13]. 5 mm of a fungal disc of actively grown *Macrophomina phaseolina* and *Fusarium oxysporum* were placed on PDA plates. The test bacterial isolates were cross-streaked at the other edge of the plate 3 cm away from the fungal disc. The fungal disc without bacterial streak was maintained as a control plate and the plates were sealed with parafilm and incubated at 27 ± 2°C for 5 days until the fungal growth covers the edge of the control. The mycelial growth inhibition by the bacterial isolates shows the antagonistic activity and percent inhibition was calculated.

$$Percentage\ of\ inhibition = \frac{C-T}{C}$$

C – Radial mycelial growth of the pathogen in the control plate

T – Radial mycelial growth of the pathogen in bacterial isolates-streaked plate

2.5 Screening of bacterial endophytes for abiotic stress tolerance

The twelve selected bacterial endophytes were tested for their different abiotic stress tolerance. The bacterial endophytes were grown under different abiotic stresses such as salt (1%, 3% and 5% NaCl), moisture stress (-1MPa, -2MPa and -3MPa) PEG concentration and temperature (4°C and 50°C) [14]. The growth of each bacterial endophyte was recorded at different time intervals and their absorbance was measured at 600nm.

2.6 Salt tolerance

0.1 mL of each bacterial endophyte was inoculated into different test tubes containing 10 mL of tryptic soy broth (TSB) supplemented with different salt concentrations (1%, 3%, and 5% (w/v)). The test tubes were incubated at 28°C and the growth was recorded. The bacterial cell growth was measured using a spectrophotometer at an optical density of OD 600nm.

2.7 Moisture stress tolerance

To determine the moisture stress tolerance by the bacterial isolates, 0.1 mL of bacterial suspension was poured into TSB broth amended with different concentrations of PEG 6000 (-1MPa, -2MPa, and -3MPa). The uninoculated were maintained as control. The inoculated tubes were incubated at 28°C and the growth was recorded. The bacterial cell growth was measured using a spectrophotometer at an optical density of OD 600nm.

2.8 Temperature tolerance

The temperature tolerance of selected bacterial isolates was tested at 4°C (low temperature) and 50°C (high temperature). 10 mL of tryptic soy broth dispensed with 0.1 mL of bacterial suspension and the tubes were incubated at 4°C and 50°C and the growth of cells were recorded. The bacterial cell growth was measured using a spectrophotometer at an optical density of OD 600nm.

3. Statistical Analysis

All the experiments were carried out with triplicates by applying a completely randomized block design and analyzed by standard analysis of variance (one-way ANOVA). The treatment mean was compared by Duncan multiple range test (DMRT) at $p<0.05$ using SPSS 16.0 software.

4. Result and Discussion

4.1 Isolation of bacteria and total viable count

The fresh seeds of three different maize seeds were collected and surface sterilized using 4% sodium hypochlorite and 70% ethanol as mentioned in materials and methods. The seeds were macerated, serially diluted up to 10^4, and plated on six different media, and colonies were purified by repeated streaking on respective medium. The endophytic bacterial population of maize seeds was shown in Table 1. Among the six different media used, the highest bacterial count of 5.5 $x10^5$ cfu's/g of seed on NA followed by 2.3 $x10^5$ on LB, 7.0 $x10^5$ on TSA was observed and the count was gradually reduced in SEA, R2A, and SCA. The viable count of male seed was recorded the highest on NA with a value of 4.0 $x10^4$ followed by 3.3 $x10^4$ on LB and 3.1 $x10^4$ on TSA. On accounting for the population of female seeds of maize, the highest population count of 1.4 $x10^4$ was observed on TSA followed by 1.2 $x10^4$ on LB while on the SEA and SCA, no growth was observed for both male and female seeds.

Plants living in a natural habitat have to confront diverse stress factors. Many scientific reports have revealed the benefits of endophytic microbes in enhancing the growth potential of crops under various stress conditions. On a morphological basis, 12 isolates were selected, purified,

screened for their biotic and abiotic stress tolerance, and pure isolates were maintained in TSA slants for future works (Table 2) [15]. Reported that most of the endophytes isolated from the maize seeds show- antagonistic activity against phytopathogens and could withstand inanimate stress conditions.

4.2 Characterization of selected endophytes

The biochemical characteristics and carbohydrate utilization test of twelve selected bacterial endophytes of maize seed are shown in Table 3. The twelve endophytes were characterized morphologically and biochemically. Among 12 endophytes, 4 endophytes were found to be gram-negative and the remaining 8 endophytes were gram-positive. None of the isolates showed positive for indole production whereas 7 isolates showed positive for methyl red production and 5 isolates showed positive for Voges-Proskauer test. Almost all the 12 isolates utilized all the sugars (Table 3). Out of 12, only 5 isolates showed positive for citrate utilization, 4 isolates showed positive for amylase production and 8 isolates showed positive for cellulase production.

4.3 Antagonistic assay

To screen the antagonistic activity of bacterial endophytes s, *in vitro* dual culture plate assay was used. Out of 12 bacterial endophytes, 9 isolates (C1, C2, C3, C4, C6, C7, C9, C11, and C12) were able to inhibit the growth of *Fusarium oxysporum* whereas the *Macrophomina phaseolina* growth was arrested by only one isolate C11 (Table 5). C11 was able to inhibit the growth of both *Fusarium oxysporum* and *Macrophomina phaseolina* as shown in figure 4 [2]. Reported that seed-associated bacterial endophytes of cultivated cucurbits have significant antagonistic activity against major fungal plant pathogens (*Fusarium graminearum, Rhizoctonia solani*, *Pythium aphanidermatum*, and *Phytophthora capsici*).

4.4 Salinity tolerance

The seed bacterial endophytes are known to be subjected to different abiotic stresses. As they could sustain in such environments, it could complement the plants in stress alleviation [14]. The endophytes enhance the adaptation ability of plants and protect the crops from stresses such as high temperature, drought, salinity, and other harsh environmental conditions [16]. Many workers [15] reported the maize seed-associated endophytic bacterial isolates could tolerate the different concentrations of salinity up to 10%.

The salinity tolerance of all the bacterial isolates was evaluated using TSB with different NaCl concentrations (1%, 3%, and 5%) and the results are shown in Table 4 & Figure 1. At high salt concentration, C12 showed maximum growth followed by C8, C5, and C1, which showed prominent growth. Almost all the bacterial isolates showed increased growth in 1% NaCl and the growth of all the endophytes reduced gradually in 3% and 5% NaCl. The endophytes of peanuts have also been reported to alleviate the salinity stress and enhance the plant yield [17].

4.5 Moisture stress tolerance

A total of 12 isolates were subjected to different PEG 6000 concentrations (-1MPa, -2MPa, and -3MPa) and the absorbance was measured at 600nm. The results revealed that all the 12 endophytes could tolerate moisture stress of -1.0MPa and thereafter the growth of endophytes declined gradually with an increase in PEG 6000 concentration (Table 5 and Figure 2). Out of 12 isolates, C7 showed prominent growth under all 3-water potential [18]. Reported that *Kosakonia cowanii* endophytic bacteria isolated from a xerophytic invasive plant, *Lactuca serriola* possess the drought-tolerant ability and promoted the growth of *Arabidopsis thaliana*.

4.6 Temperature tolerance

Temperature is one of the prominent factors which is evident for the growth of bacterial communities under extreme environmental niches [19]. Based on the temperature limits, the endophytes were tested for their growth at 4°C (lower temperature) and high temperature (40°C and 50°C) at different time intervals (0, 24, 48 h), respectively (Table 3). All the 12 isolates showed enhanced growth at 40°C, while a decrease in growth was observed at 50°C, and growth of the isolates was negligible at 4°C (Figure 3). Out of 12 isolates, C2, C3, and C8 showed increased growth at 50°C among those, C2 was found to be better stress-tolerant. On the other hand, C5 showed better growth at 4°C. To ameliorate the impact of heat stress, endophytic bacteria with plant growth-promoting activity is an effective and eco-friendly approach that could be used for economically significant crops worldwide. Many of the reports suggest that the use of the plant endophytic bacteria aids in improving the stress tolerance and protect the plants from adverse environmental conditions.

Table 1: Endophytic bacterial flora of Maize hybrid COH6, parental line (Male), parental line (Female)

Name of the Sample	Medium used	Colony morphology				Population (cfu/g of seed)
		Color	Elevation & Edge	Size	Form	
Maize COH6 (Hybrid seed)	NA	White	Translucent, flat	Small	Irregular	5.5×10^5
	LB	Pale white	Convex, entire	Small	Irregular	2.3×10^5
	TSA	White	Entire, Opaque	Small	Circular	7.0×10^4
	SEA	White	Flat, entire	Small	Circular	10.0×10^3
	R2A	White	Convex, entire	Small	Circular	2.0×10^3
	SCA	White	Convex, flat, Pinpointed	Small	Circular	2.0×10^3
Maize UMI-1230 (Male seed)	NA	Pale white	Raised, entire	Small	Circular	4.0×10^4
	LB	White	Raised, entire	Medium	Circular	3.3×10^4
	TSA	Pale white	Raised, opaque	Small	Irregular	3.1×10^4
	R2A	Creamy white	Raised, opaque	Large	Circular	9.0×10^3
Maize UMI-1200 (Female seed)	NA	Pale white	Flat, misty, entire	Small	Circular	12.0×10^3
	LB	Pale white	Raised, entire	Small	Circular	5.0×10^3
	TSA	White	Raised, entire	Medium	Irregular	14.0×10^3
	R2A	Pale white	Raised, Opaque	Small	Circular	11.0×10^3

Table 2: Dominant and unique endophytic bacterial flora of male, female and hybrid of Maize COH6

Isolate	Source	Morphological characteristics	Gram's reaction characteristics
C1	Male-LB	Thick, golden pinpoints, raised colonies	-
C2	Male-LB	Thin, slight creamy yellow with white pinpoints	+
C3	Male-LB	Yellow color, thin, slightly raised colonies	+
C4	Female-TSA	Thick, creamy yellow, polysaccharides producing colonies	+
C5	Female-TSA	Thick, exopolysaccharide producing, slight light yellow creamy	+
C6	Female-TSA	Dry, moderate thickness, slight reddish tinge color colonies	+
C7	Male-TSA	Dry, thick, white creamy, slightly raised colonies	+
C8	Female-NA	Moderate thick, white creamy, exopolysaccharide producing colonies	+
C9	Female-NA	Moderate thick, white colony at edge, slight yellow color, exopolysaccharide producing colonies	+
C10	Male-NA	Thin, small, pale white, creamy, exopolysaccharide producing	-
C11	Hybrid-R_2A	Large, pale white yellow creamy color colonies	-
C12	Hybrid-R_2A	Thin. Slight yellow creamy colony with cloudy appearance	-

Note: + and – sign indicates the positive and negative for gram staining

Table 3: Biochemical characteristics of selected endophytes

Isolates	IP test	MR test	VP test	CUT test	KOH test	Catalase test	Amylase test	CMC test	Pectinase test
C1	-	-	+	+	+	-	-	-	-
C2	-	-	+	-	-	-	+	+	-
C3	-	-	+	-	-	-	+	+	+
C4	-	+	-	-	-	-	-	-	-
C5	-	-	+	+	-	-	-	-	-
C6	-	+	-	-	-	-	-	+	+
C7	-	+	-	-	-	-	+	+	+
C8	-	-	+	+	-	-	-	-	-
C9	-	+	-	-	-	-	+	+	-
C10	-	+	-	-	+	-	-	+	-
C11	-	+	-	+	+	+	-	+	+
C12	-	+	-	+	+	+	-	+	+

Note: + and – negative sign indicates the positive and negative results for biochemical test
IP- Indole Production, MR- Methyl red, VP – Vogus-Proskauer, CUT- Citrate Utilization test

Table 4: Growth of the bacterial isolates under different abiotic stress measured in terms of absorbance 600nm

Isolates	Non-Stressed	Salt stress (NaCl)			Moisture stress (PEG)			Temperature stress (°C)		
		1%	3%	5%	-1 MPa	-2 MPa	-3 Mpa	4°C	40°C	50°C
C1	1.025^{h}	1.618^{c}	1.499^{a}	0.916^{c}	0.622^{ab}	0.13^{fg}	0.061^{f}	0.178^{b}	2.201^{a}	0.113^{g}
C2	1.043^{gh}	0.726^{f}	0.567^{e}	0.557^{e}	0.647^{a}	0.128^{fg}	0.057^{f}	0.071^{h}	1.876^{c}	0.917^{a}
C3	1.149^{ef}	0.621^{g}	0.350^{f}	0.242^{g}	0.439^{fg}	0.471^{b}	0.164^{c}	0.073^{h}	0.694^{h}	0.719^{b}
C4	1.177^{def}	0.527^{gh}	0.297^{f}	0.254^{g}	0.546^{cd}	0.209^{e}	0.08^{e}	0.106^{ef}	1.026^{g}	0.309^{d}
C5	1.275^{bcd}	1.440^{d}	1.048^{c}	0.898^{c}	0.533^{de}	0.112^{gh}	0.041^{g}	0.386^{a}	2.118^{ab}	0.117^{fg}
C6	1.128^{fg}	0.315^{i}	0.29^{f}	0.162^{h}	0.506^{de}	0.085^{h}	0.039^{g}	0.086^{gh}	1.494^{e}	0.164^{ef}
C7	1.793^{a}	0.345^{i}	0.339^{f}	0.334^{f}	0.427^{g}	0.616^{a}	0.519^{a}	0.118^{de}	1.744^{d}	0.272^{d}
C8	1.369^{b}	1.885^{a}	1.373^{b}	1.011^{b}	0.487^{ef}	0.248^{d}	0.062^{f}	0.156^{c}	1.489^{e}	0.549^{c}
C9	1.002^{h}	0.502^{h}	0.192^{g}	0.095^{i}	$0.589b^{c}$	0.114^{gh}	0.106^{d}	0.127^{d}	1.321^{f}	0.093^{g}
C10	1.343^{b}	0.900^{e}	0.516^{e}	0.238^{g}	0.525^{de}	0.407^{c}	0.212^{b}	0.106^{ef}	2.056^{b}	0.181^{e}
C11	1.286^{bc}	1.814^{ab}	0.826^{d}	0.778^{d}	0.645^{a}	0.105^{gh}	0.071^{ef}	0.09^{fg}	0.786^{h}	0.091^{g}
C12	1.240^{cde}	1.784^{b}	1.460^{a}	1.162^{a}	0.401^{g}	0.15^{f}	0.059^{f}	0.0182^{i}	1.514^{e}	0.104^{g}

Note: The same letter in the superscripts indicates no significant difference between the observation

Table 5: Percentage inhibition of Maize seed-borne pathogen *Macrophomina phaseolina* and *Fusarium oxysporum* by bacterial seed endophytes

Isolates	Percent inhibition of Fungal pathogen (%)	
	Macrophomina phaseolina	***Fusarium oxysporum***
C1	0	24.64
C2	0	18.84
C3	0	31.88
C4	0	2.9
C5	0	0
C6	0	23.19
C7	0	8.7
C8	0	0
C9	0	2.9
C10	0	0
C11	14.86	23.19
C12	0	2.89

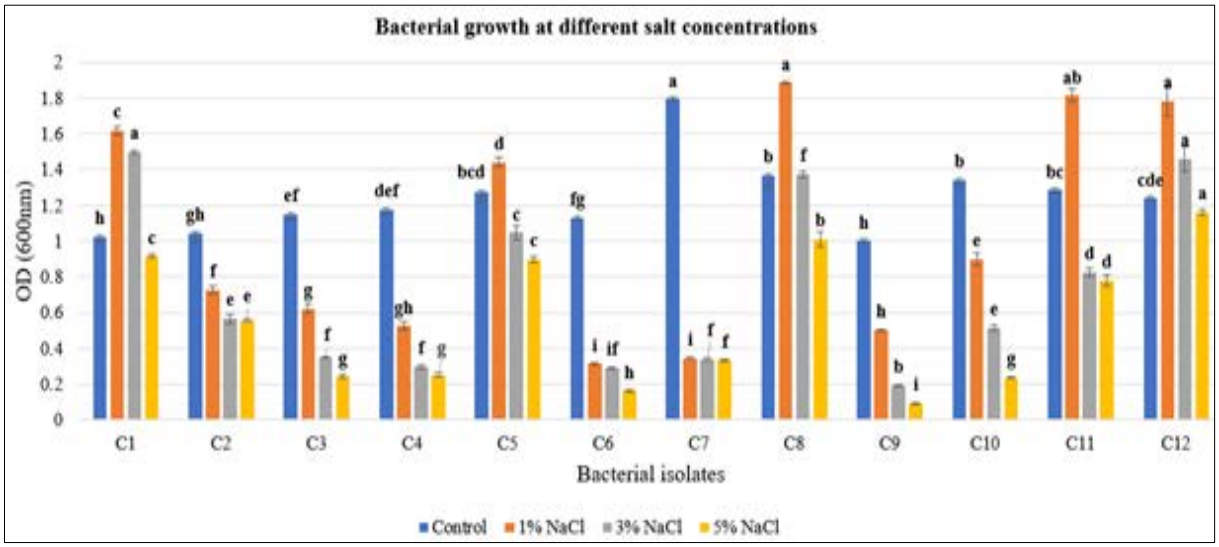

Fig 1: Salt tolerance by seed bacterial endophytes

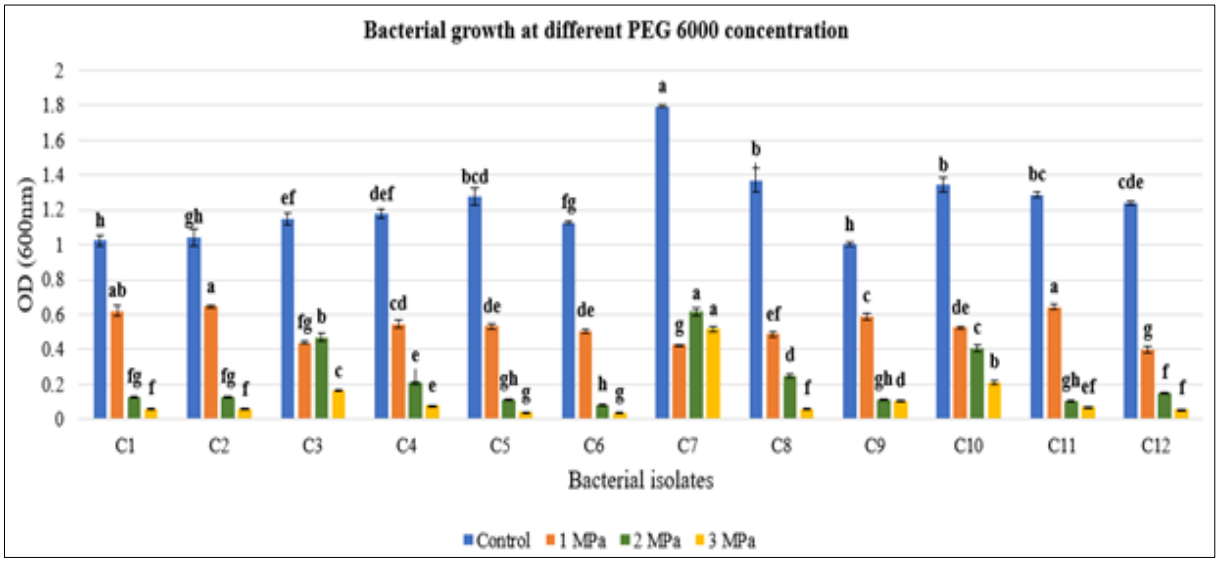

Fig 2: Moisture stress tolerance by seed bacterial endophytes

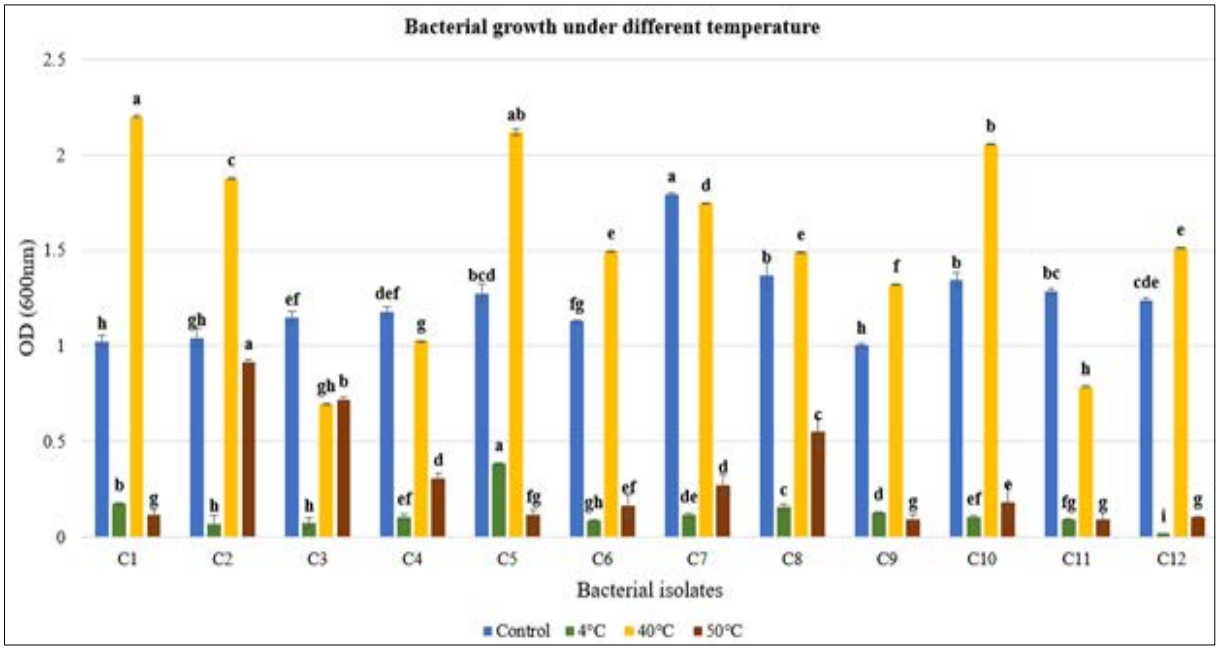

Fig 3: Temperature tolerance by seed bacterial endophytes

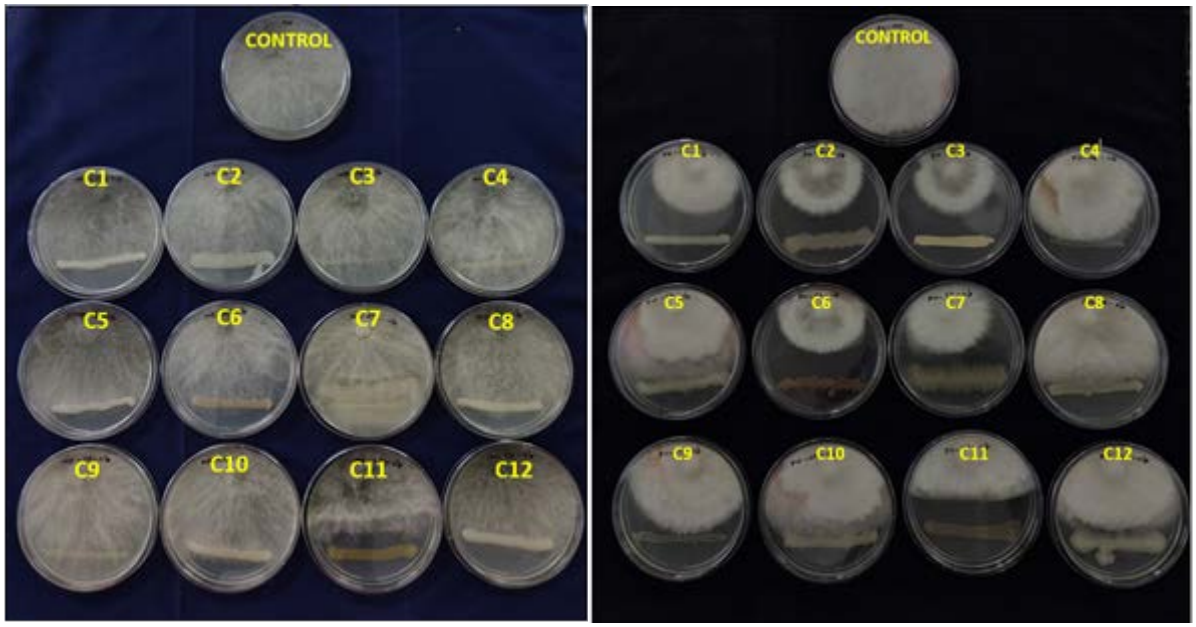

Fig 4: *In vitro* antagonistic activity of bacterial isolates against fungal pathogen *Macrophomina phaseolina* and *Fusarium oxysporum*

5. Conclusion

From the present study, it is confirmed that the seed-associated bacterial endophytes of maize successfully tolerate environmental stresses. The results suggest that the isolates C3, C7, C11, and C12 could be utilized as consortia to mitigate the stress condition and these isolates could assist the crop in addressing the problem of biotic and abiotic stresses and act as an effective strategy for improving the sustainability in agriculture. The microorganisms from the seed source favor the plants for the effective colonization of beneficial microorganisms in the seedling establishment stage and assisting plant growth.

6. Authors Contribution

The authors of the manuscript have an equal contribution

7. Acknowledgements

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8. Conflict of interest

The authors declare that there is no conflict of interest.

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