# Isolation and characterization of bacterial endophytes from maize genotypes varying in resistance against *Macrophomina phaseolina*


## Abstract

The present work aims to isolate and characterize the bacterial endophytes associated with different maize genotypes varying in resistance to disease reaction against *Macrophomina phaseolina*. A total of 50 endophytic bacteria were isolated from root and stem of healthy maize plants at 30 DAS, 60 DAS by using two different media like TSA, NA medium and maximum number of endophytic bacterial population were recovered from root followed by stem in all the genotypes and TSA medium was found to be the most suitable medium for deciphering maximum endophytic bacterial diversity. The isolated bacterial endophytes were characterized on the basis of morphological parameters *viz.*, size, shape, colour, margin and texture, elevation, gram staining reaction and it was observed that Gram positive bacteria (68.0 %) formed the dominant group. The colony characterization revealed that circular forms (38.0 %) were dominated. Among the colony features the colonies with entire margins (66.0 %) and convex elevation (44.0 %) were found to be dominating.



**Key words:** Endophytes, genotypes, maize, morphological characteristics.




## 1. Introduction

Endophytes are a significant group of common and diverse plant symbionts that exist asymptomatically and occasionally systemically within plant tissues without manifesting disease symptoms. (Promputtha *et al*., 2005; Porras-Alfaro and Bayman, 2011) [13,11]. Due to their capacity to colonise plant internal tissues and their capacity to foster plant development and prevent plant diseases, endophytes can be utilised in agriculture as a technique to enhance crop performance (Yuan *et al*., 2009) [20]. Many facts about the variety and function of endophytes in agriculture have been gathered during the past two decades (Yuan *et al*., 2009) [20].

The endophytic bacteria mainly have been researched for their beneficial activities in terms of nutrients availability, plant growth hormones and control of soil borne and systemic pathogens. (Marag *et al.*, 2018) [6]. Some of the successful plant associated endophytes like *Gluconacetobacter diazotrophicus, Herbaspirillum, Burkholderia, Azospirillum, Ba*

*cillus* spp. of crop plants have drawn the attention of the scientific world to unravel more properties of these natural associations of the plant with the microbial world (Compant *et al*., 2005) [3].

Maize, a crop cultivated worldwide, was investigated for colonization of endophytic bacteria. The characterization of endophytic bacteria associated with the maize crop may provide valuable information to our still poor knowledge of the plant–bacteria interaction with different genotypes in addition to the possibility of recognizing strategies that may benefit maize management and production (Ikeda *et al.*, 2013) [4]. Early studies have shown that the genotypes of plants can impact the endophytic communities coexisting with the plants (Zhang *et al.*, 2019) [21]. So, the aim of this study was to isolate and to characterize the endophytic bacteria obtained from roots and stems of different maize genotypes varying in resistance to *M. phaseolina.*

## 2. Materials and methods

### 2.1 Collection of maize genotype samples

Fresh samples were collected from different maize genotypes at 30 DAS, 60 DAS from fields of Maize research centre, during Rabi, 2022. The plants were uprooted and placed in sterile polythene bags and transferred to laboratory for further analysis. Endophytes were isolated from both stem and root by following direct plate impression of tissues and serial dilution technique.

### 2.2 Surface sterilisation of maize root and stem bits

Surface sterilization was carried out by following modified protocol (Qin *et al*., 2009; Sarangthem and Momota, 2012) [14,16]. The freshly collected samples were washed thoroughly under running tap water to remove adhering soil along with associated unwanted particles and soaked for 10 min in distilled water containing a few drops of tween 80. Stems and roots were cut into appropriate segments and washed twice with sterile distilled water before proceeding for surface sterilization by using 80 % ethanol for 2 min (stem) and 3 min (root) depending on plant parts. The samples were treated with 4 % sodium hypochlorite, rinsed with sterile distilled water and treated with 70 % alcohol for 1 min, followed by 3-4 successive rinses with sterile distilled water and dried in a sterile condition for 5-10min.

### 2.3 Isolation of maize endophytes

The indigenous methods followed were (i) direct plate impression of sterilized tissues: The samples were carefully made into thin slice, removing the outer cover and placed on Nutrient agar (NA), Tryptic soy agar (TSA). Further, the plates were incubated at $30^0$ C ± $2^0$ C for 2 days until the observation of bacterial growth. (ii) Spread and pour plate technique: Surface-disinfected tissue was aseptically macerated using mortar and pestle. The sample extract was directly plated on the media (50 µl) in one set and in another set 1 ml of the sample suspended in 9 ml solution until to get $10^{-7}$ dilutions and isolations were done through the spread plate and pour plate techniques from $10^{-5}$ to $10^{-7}$. The plates were incubated at $30^0$ C ± $2^0$ C for 24-48 hrs for the observation of bacterial colonies.

## 2.4 Validation of surface sterilisation method

Surface sterilized tissues and aliquot from the final rinse was tested as a sterility check measure (Schulz *et al.*, 1998) [18]. The success of the surface sterilization method was confirmed by the absence of microbial growth on the media plates impregnated with 50 µl aliquots of the final rinse water.

### 2.5 Mass multiplication of pathogen on toothpicks for inoculation into genotypes

Sterilized toothpicks were inoculated with the culture of the pathogen aseptically. Fungal growth covers the toothpicks and inoculum is ready for use in about 10 days. Inoculation was done on 55 days old plants just before flowering. The lower internode (second/third) of the plant just above soil level is opened with a jabber and the toothpick with fungal growth was inserted into the hole and symptoms were recorded after 30 days of inoculation.

## 2.6 Scoring of disease reaction for different maize genotypes

The disease severity of PFSR was recorded at harvesting stage by following 1-9 rating scale of Payak and Sharma (1985) [10] with slight edition as given below.



**Table 1:** Disease rating scale for toothpick method

| In vitro lesion Length (cm) | Intensity and extent of severity (%) | PDI% | Disease reaction |
|---|---|---|---|
| 1.0 | 25 per cent of the inoculated internode discoloured | < 11.11 | Resistant (R) (Score: < 3.0) (PDI: < 33.33) |
| 2.0 | 26-50 per cent of the inoculated internode discoloured | 22.22 | |
| 3.0 | 51-75 per cent of the inoculated internode discoloured | 33.33 | |
| 4.0 | 76-100 per cent of the inoculated internode discoloured | 44.44 | Moderately resistant (MR) (Score: 3.1–5.0) (PDI: 33.33-55.5) |
| 5.0 | Discolouration of less than 50 per cent of adjacent internode | 55.55 | |
| 6.0 | Discolouration of less than 50 per cent of adjacent internode | 66.66 | Moderately susceptible (MS) (Score: 5.1–7.0) (PDI:55.56- 77.77) |
| 7.0 | Discolouration of three internodes | 77.77 | |
| 8.0 | Discolouration of four internodes | 88.88 | Susceptible(S) (Score: > 7.0) (PDI: >77.7) |
| 9.0 | Discolouration of five or more internodes and premature death of plant | 99.99 | |



**Table 2:** List of different bacterial and fungal endophytes isolated along with the maize genotype disease reaction against *Macrophomina phaseolina*

| S.NO | Name of maize genotype | Disease score of genotype | Part of the plant used for isolation | Isolate name allotted |
|---|---|---|---|---|
| 1 | MGC 157 | **3.5** | Stem | ESB1, ESB19 |
| | | | Root | ERB2, ERB9, ERB13 |
| 2 | 11001 | **4.8** | Stem | ESB2, ESB3 |
| | | | Root | ERB4, ERB10, ERB21 |
| 3 | 11011 | **4.5** | Stem | ESB5, ESB10, ESB22 |
| | | | Root | ERB5, ERB11, |
| 4 | PFSR 104 | **2.9** | Stem | ESB6 |
| | | | Root | ERB16 |
| 5 | PFSR 151 | **2.9** | Stem | ESB8, ESB9, ESB20, ESB23 |
| | | | Root | ERB7, ERB20 |
| 6 | PFSR 30 | **3.0** | Stem | ESB11 |
| | | | Root | ERB1, ERB19 |
| 7 | YA10-230 | **4.0** | Stem | ESB13, ESB14, ESB15 |
| | | | Root | ERB6 |
| 8 | 12016 | **7.0** | Stem | ESB21, ESB24 |
| | | | Root | ERB3, ERB23 |
| 9 | KNR-47 | **3.0** | Stem | ESB7, ESB17 |
| 10 | YA10-162 | **4.0** | Stem | ESB4, ESB12, ESB16 |
| 11 | KNR-40 | **2.0** | Stem | ESB18 |
| 12 | YA10-235 | **5.0** | Root | ERB8 |
| 13 | 11040 | **6.5** | Root | ERB12, ERB18 |
| 14 | KNR-1 | **5.5** | Root | ERB14, ERB24 |
| 15 | 11004 | **2.8** | Root | ERB15 |
| 16 | 11041 | **3.1** | Root | ERB17 |
| 17 | BML 14 | **5.0** | Root | ERB22, ERB26 |
| 18 | YA10-46 | **3.0** | Root | ERB25 |

(ESB: Endophytic Stem Bacteria, ERB: Endophytic Root Bacteria)

## 2.7 Morphological characterization of endophytic bacterial isolates

Morphological characterization of the endophytic bacterial isolates was carried out by growing each isolate in 3 replications on Nutrient agar (NA) to study their growth characteristics such as Colony colour, Shape, Size, Elevation, Margin, Texture, Cell shape and Gram staining reaction. All isolates were purified by quadrant streaking on NA medium plates. Purified bacterial isolates were preserved in NA slants at 4 °C as working culture and as 25% glycerol stock at −20 °C for future use.



**Fig 1:** Endophytic Root Bacteria (ERB) isolated from roots of different maize genotypes

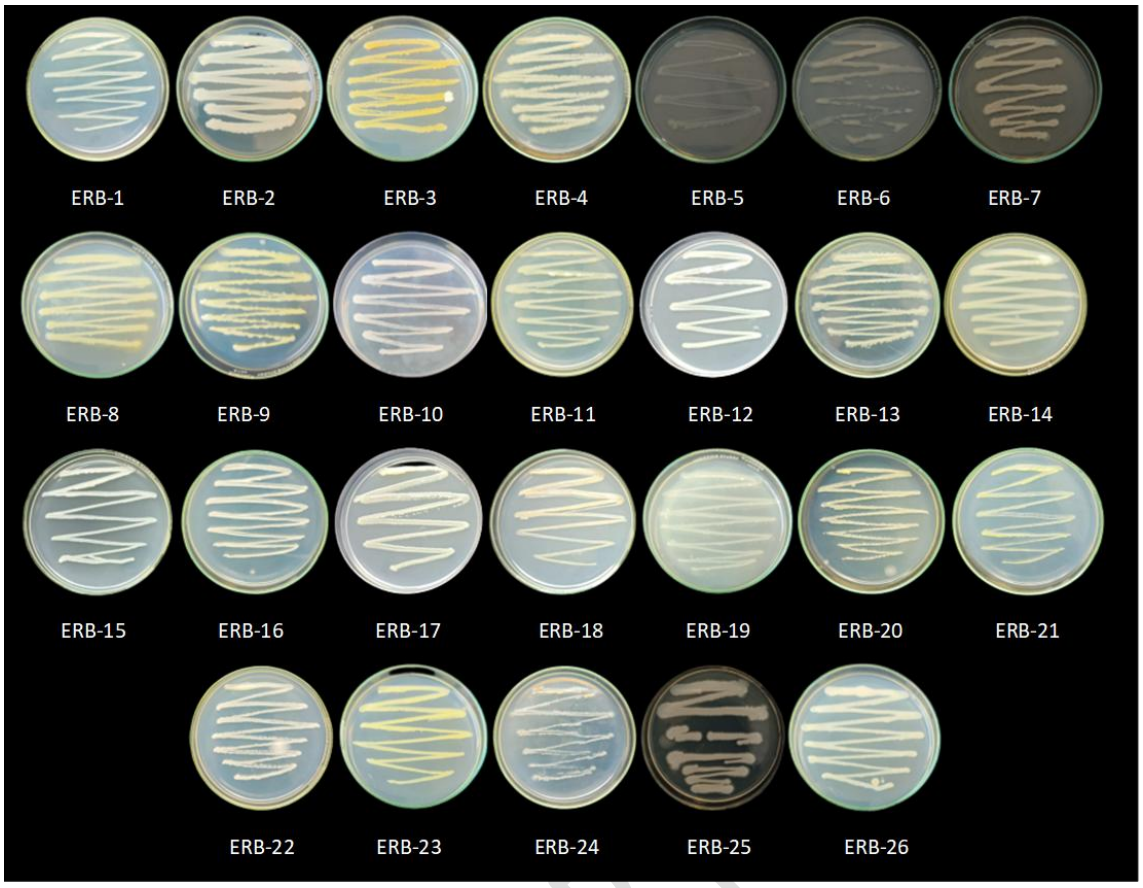


**Fig 2:** Endophytic Stem Bacteria (ESB) isolated from stems of different maize genotypes

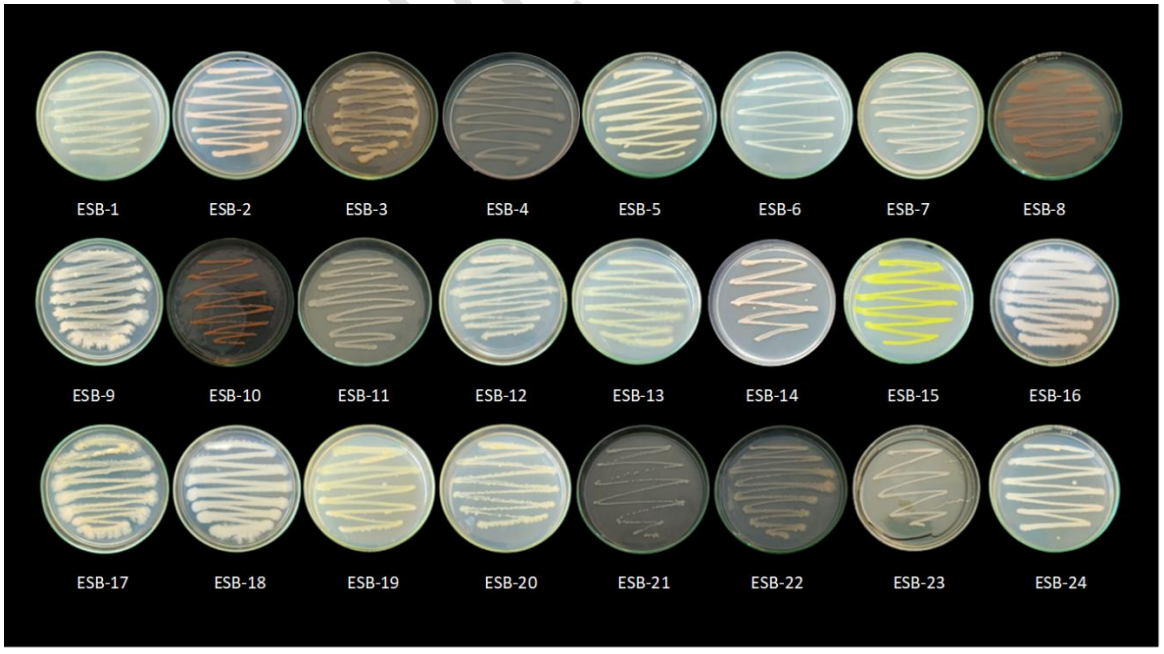

**Table 3:** Morphological and colony characters of endophytic bacterial isolates isolated from different maize genotypes

| S.NO | Isolate ID | Colony colour | Colony shape | Size | Optical property | Elevation | Margin | Texture | Cell shape | Gram staining reaction |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ESB1 | Creamy White | Filamentous | Large | Opaque | Raised | Filiform | Mucoid | Rod | + |
| 2 | ESB2 | Light peach | Circular | Medium | Opaque | Convex | Entire | Mucoid | Coccus | - |
| 3 | ESB3 | Cream | Irregular | Large | Opaque | Flat | Lobate | Mucoid | Rod | + |
| 4 | ESB4 | Cream | Circular | Medium | Opaque | Convex | Entire | Butyrous | Coccus | _ |
| 5 | ESB5 | Cream | Punctiform | Small | Opaque | Convex | Entire | Mucoid | Coccus | + |
| 6 | ESB6 | White | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 7 | ESB7 | Light cream | Filamentous | Large | Opaque | Raised | Lobate | Mucoid | Coccus | _ |
| 8 | ESB8 | Light orange | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | - |
| 9 | ESB9 | Creamy White | Irregular | Large | Opaque | Flat | Lobate | Mucoid | Rod | + |
| 10 | ESB10 | Light orange | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | - |
| 11 | ESB11 | Cream | Circular | Large | Opaque | Flat | Entire | Mucoid | Rod | + |
| 12 | ESB12 | White | Irregular | Medium | Opaque | Flat | Undulate | Dry | Rod | + |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | ESB13 | Dull white | Circular | Small | Transparent | Convex | Entire | Butyrous | Rod | + |
| 14 | ESB14 | Light peach | Circular | Small | Opaque | Convex | Entire | Mucoid | Coccus | _ |
| 15 | ESB15 | Yellow | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Coccus | - |
| 16 | ESB16 | White | Filamentous | Medium | Opaque | Flat | Filiform | Dry | Rod | + |
| 17 | ESB17 | Creamy White | Filamentous | Large | Opaque | Raised | Filiform | Mucoid | Rod | - |

| **S.NO** | **Isolate ID** | **Colony colour** | **Colony shape** | **Size** | **Optical property** | **Elevation** | **Margin** | **Texture** | **Cell shape** | **Gram staining reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| 18 | ESB18 | White | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 19 | ESB19 | Creamy white | Irregular | Medium | Opaque | Raised | Undulate | Mucoid | Rod | + |
| 20 | ESB20 | Cream | Irregular | Large | Opaque | Convex | Undulate | Mucoid | Coccus | + |
| 21 | ESB21 | Light White | Irregular | Medium | Opaque | Flat | Undulate | Dry | Rod | + |
| 22 | ESB22 | Cream | Irregular | Medium | Opaque | Raised | Undulate | Mucoid | Coccus | + |
| 23 | ESB23 | Creamy white | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 24 | ESB24 | White | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 25 | ERB1 | Creamy white | Circular | Large | Opaque | Convex | Entire | Butyrous | Rod | + |
| 26 | ERB2 | Cream | Irregular | Large | Opaque | Flat | Lobate | Mucoid | Coccus | + |
| 27 | ERB3 | Orange | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 28 | ERB4 | Creamy white | Circular | Large | Opaque | Convex | Entire | Butyrous | Rod | + |
| 29 | ERB5 | Light White | Irregular | Medium | Transparent | Flat | Lobate | Mucoid | Rod | + |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 30 | ERB6 | Creamy white | Circular | Large | Transparent | Convex | Entire | Butyrous | Coccus | _ |
| 31 | ERB7 | Creamy White | Irregular | Large | Opaque | Flat | Undulate | Mucoid | Rod | + |
| 32 | ERB8 | Creamy white | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 33 | ERB9 | Creamy white | Circular | Large | Transparent | Convex | Entire | Butyrous | Coccus | _ |
| 34 | ERB10 | Creamy white | Circular | Large | Opaque | Convex | Entire | Mucoid | Coccus | _ |
| 35 | ERB11 | Creamy white | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 36 | ERB12 | Dull white | Irregular | Small | Opaque | Raised | Lobate | Mucoid | Rod | + |

| **S.NO** | **Isolate ID** | **Colony colour** | **Colony shape** | **Size** | **Optical property** | **Elevation** | **Margin** | **Texture** | **Cell shape** | **Gram staining reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| 37 | ERB13 | Creamy white | Irregular | Large | Opaque | Raised | Lobate | Mucoid | Rod | + |
| 38 | ERB14 | Creamy white | Irregular | Medium | Opaque | Raised | Lobate | Mucoid | Rod | + |
| 39 | ERB15 | White | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 40 | ERB16 | White | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 41 | ERB17 | Creamy white | Circular | Large | Opaque | Convex | Entire | Butyrous | Coccus | _ |
| 42 | ERB18 | Light peach | Circular | Small | Opaque | Convex | Entire | Mucoid | Rod | + |
| 43 | ERB19 | Light cream | Irregular | Large | Opaque | Raised | Entire | Mucoid | Coccus | _ |
| 44 | ERB20 | Light cream | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 45 | ERB21 | Light yellow | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 46 | ERB22 | Cream | Irregular | Large | Opaque | Convex | Entire | Mucoid | Coccus | + |
| 47 | ERB23 | Light yellow | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Coccus | + |
| 48 | ERB24 | Dull white | Punctiform | Small | Transparent | Convex | Entire | Mucoid | Coccus | _ |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 49 | ERB25 | White | Punctiform | Small | Opaque | Raised | Entire | Mucoid | Rod | + |
| 50 | ERB26 | Creamy white | Circular | Large | Transparent | Convex | Entire | Butyrous | Rod | _ |

## 3. Results and discussion

Maize genotypes were screened for disease resistance by following artificial toothpick method of inoculation (Table 1). The resistant and susceptible reactions of genotypes ranged from 2.0 in genotype KNR 40 to 7.0 in genotype No. 12016 on 1-9 scale. A total of 50 endophytic bacteria were isolated from different maize genotypes varying in disease reaction. The endophytes were isolated both from root (26 no.) and stem (24 no.). it was noticed that more number of endophytes were recovered from root as compared to stem indicated in Table 2, Fig 1, Fig 2. Out of these 50 endophytic bacteria, 28 of them were isolated from TSA medium and 22 on NA medium. All the endophytes were studied for morphological & colony characters. It was observed that all endophytes varied in colony colour, shape, size, elevation, margin, texture, cell shape and gram staining reaction. Among all 34 endophytic bacteria shown gram positive reaction and 16 were given gram negative reaction. Colony forms among isolates varied from punctiform, circular, irregular to filamentous and the colony margins varied from entire, undulate, lobate to filiform. The colony elevation varied from flat, raised to convex with different colors like light yellow, white, creamy white, dull white, light peach, light orange, cream, light cream, yellow, light white, orange. All the isolates were opaque except 6 isolates *i.e* ESB 13, ERB 5, ERB 6, ERB 9, ERB 24, ERB 26 which were transparent. Colony texture of all isolates were mucoid except 8 isolates (ESB4, ESB13, ERB1 ERB4, ERB6, ERB9, ERB17, ERB26) were butyrous and 3 were dry (ESB12, ESB16, ESB21). Among 50 isolates 33 exhibited rod shape, 17 were coccus in shape (Table 3).

The results are in conformation with the earlier work done by Roncato-Maccari *et al.,* 2003 [15] who reported that bacterial load was high in root tissues followed by stem and leaves in both hybrid and composite of maize. Similarly, Liu *et al.* (2006) [5] also reported colonization by *Bacillus megaterium* in maize plant was declining from roots to stems and leaves. Marag *et al.* (2018) [6] also said that roots harboured more endophytes followed by stem. The roots were the preferential site of colonization, independent of cultivar type and growth stage.

In another study Michiels *et al.* (1989) [7] found that the genotype of plant controls the composition and quantity of root exudates and this in turn correlates with quantity of bacteria colonizing the rhizosphere. Likewise, Neal *et al.* (1973) [8] also studied microbes found in rhizosphere of different wheat genotypes and confirmed the mutants contained more endophytes than wild types. so, he suggested that genotype of

the plants determined the species of microorganisms colonizing the rhizosphere. Similarly, Andreote *et al.* (2010) [1] reported that the plant genotype influences colonization by bacteria and also the difference in endophytic colonization has been observed in both root and stem.

Sawarkar *et al.* (2021) [17] isolated different endophytic bacteria from *Tamarindus indica* and studied their growth characteristics on King's B media and revealed that the colonies were either irregular or circular in shape, either flat or raised, their margins were either undulated or entire and the surface of the growth was opaque and white in colour and all the endophytic bacterial isolates were gram positive rods. Our results also in accordance with Padder *et al.* (2017) [9], who reported that out of 81 endophytic bacteria isolated circular forms were dominated. Among the colony features the colonies with entire margins and convex elevation were dominated. Sgroy *et al.* (2009) [19] also isolated both Gram-positive bacteria (68.9 %) and Gram-negative bacteria (31.1 %) from the roots of *Prosopis strombulifera*.

## 4. Conclusion

The results presented in this study revealed that a total of 50 bacterial endophytes were isolated from 22 maize genotypes varying in resistance to *M. phaseolina*. The endophytes which vary in colony colour, shape, size, optical property, texture, elevation, margin, cell shape, gram staining reaction. The different genotypes with different level of resistance varied in collecting endophytes both from root and stem as well as on different media.

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