

Original Research Article

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 asymptotically 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*, *Bacillus* 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 $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 days until the observation of bacterial growth. (ii) Spread and pour plate technique: The sterile tissues after aseptically processing and removing the outer edged portion was homogenized using sterile 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 $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ 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
	25 per cent of the inoculated		

1.0	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	Disease score of	Part of the plant used for	Isolate name allotted
------	---------------	------------------	----------------------------	-----------------------

	genotype	genotype	isolation	
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.

UNDER PEER REVIEW

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

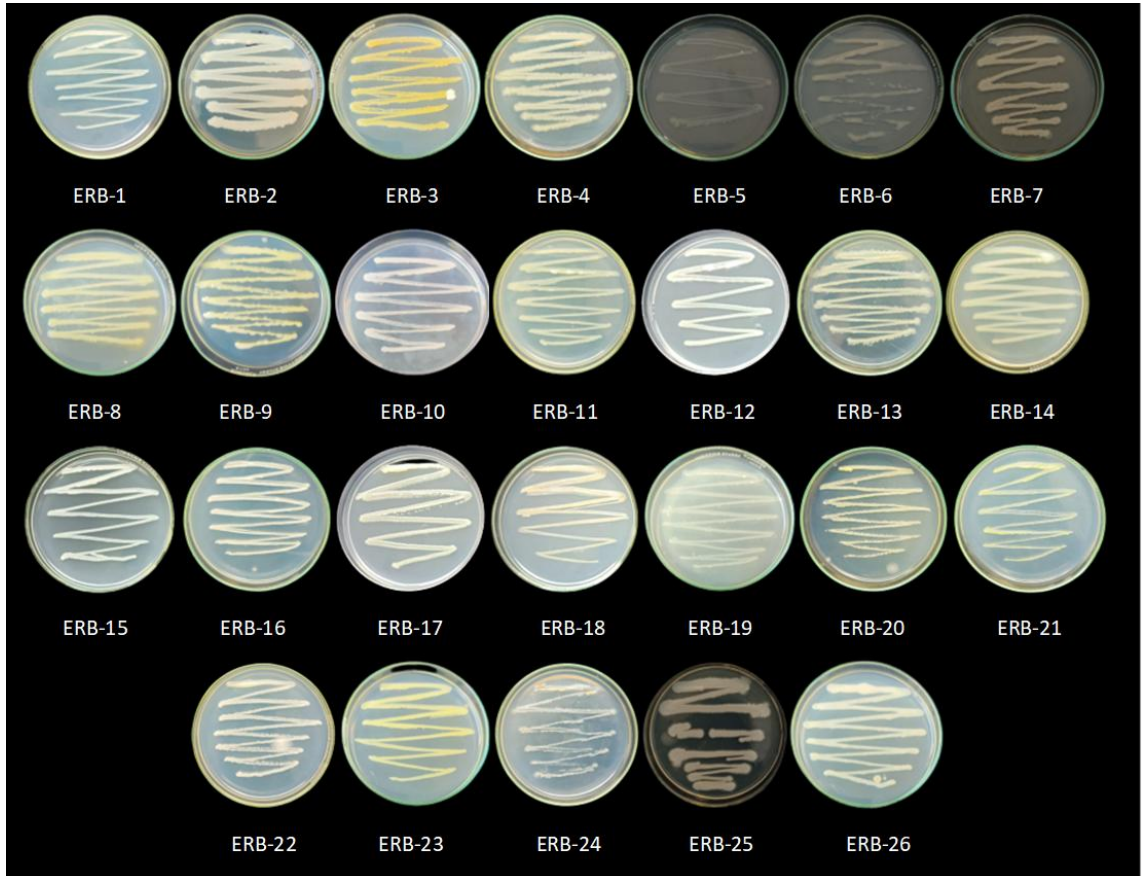


Fig 2: Endophytic Stem Bacteria (ESB) isolated from stems of different maize genotypes

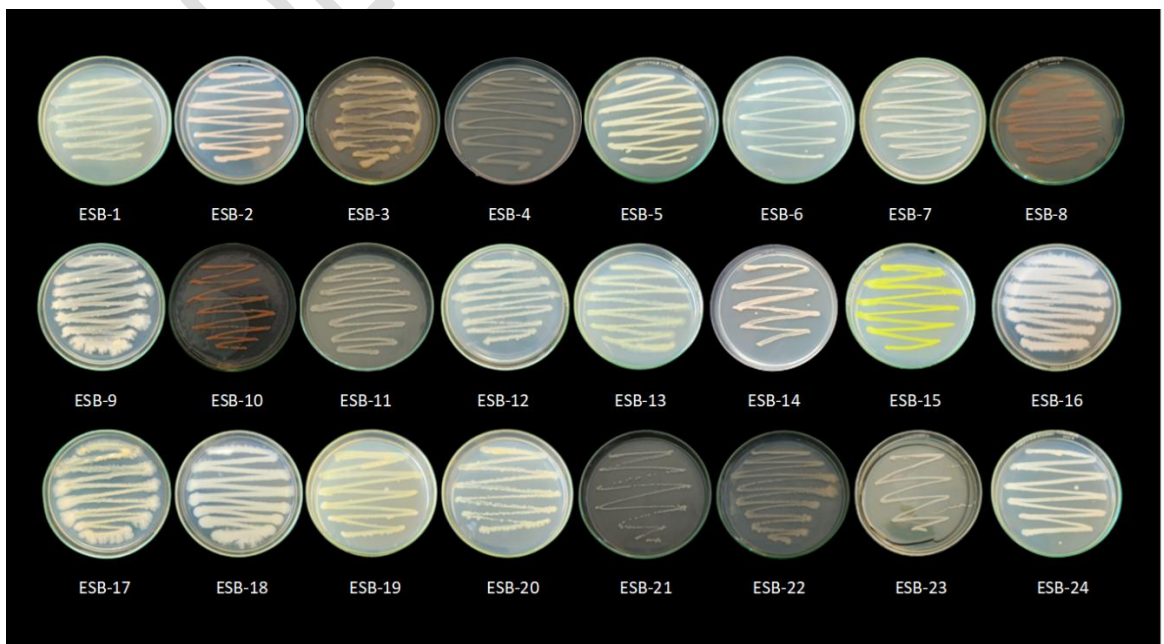


Table 3: Morphological and colony characters of endophytic bacterial isolates isolated from different maize genotypes

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	-

UNDER PEER REVIEW

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. 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. Sgroby *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.

6. References

- [1] Andreote FD, Rocha UND, Araújo WL, Azevedo JL and van Overbeek LS. Effect of bacterial inoculation, plant genotype and developmental stage on root-associated and endophytic bacterial communities in potato (*Solanum tuberosum*). *Antonie Van Leeuwenhoek*. 2010;97: 389-399.
- [2] Chiranjeevi N, Kumar MR, Padmodaya B, Venkateswarlu NC, Sudhakar P and Devi RSJ. Phenotypic, bio-chemical and molecular characterization of potential endophytic bacterial isolates and evaluation of endophytic bacterial formulations and extracted antibiotic substances under glass house conditions. *The Pharma Innovation Journal*. 2021;10(5): 923-936.

- [3] Compant S, Duffy B, Nowak J, Clément C and Barka EA. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*. 2005; 71(9): 4951-4959.
- [4] Ikeda AC, Bassani LL, Adamoski D, Stringari D, Cordeiro VK, Glienke C *et al.* Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes. *Microbial ecology*. 2013;65: 154-160.
- [5] Liu X, Zhao H and Chen S. Colonization of maize and rice plants by strain *Bacillus megaterium* C4. *Current Microbiology*. 2006;52: 186-190.
- [6] Marag PS and Suman A. Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (*Zea mays* L.). *Microbiological Research*. 2018;214: 101-113.
- [7] Michiels K, Vanderleyden J and Van Gool A. *Azospirillum*—plant root associations: A review. *Biology and Fertility of Soils*. 1989;8: 356-368.
- [8] Neal JL, Larson RI and Atkinson TG. Changes in rhizosphere populations of selected physiological groups of bacteria related to substitution of specific pairs of chromosomes in spring wheat. *Plant and Soil*. 1973;39: 209-212.
- [9] Padder SA, Dar GH, Bhat ZA, Verma K, Wani AB. Morphological metabolic and biochemical characterization of bacterial root endophytes associated with brown sarson (*Brassica rapa* L.). *Journal of Pharmacognosy and Phytochemistry*. 2017;6(2): 226-232.
- [10] Payak MM, Sharma RC. Maize diseases and approaches to their management in India. *Tropical Pest Management*. 1985;31: 302-310.
- [11] Porrás-Alfaro A and Bayman P. Hidden fungi, emergent properties: endophytes and microbiomes. *Annual review of phytopathology*. 2011; 49: 291–315.
- [12] Potshangbam M, Devi SI, Sahoo D, Strobel GA. Functional characterization of endophytic fungal community associated with *Oryza sativa* L. and *Zea mays* L. *Frontiers Microbiology*. 2017;8: 325.
- [13] Promputtha I, Jeewon R, Lumyong S, McKenzie EHC and Hyde KD. Ribosomal DNA fingerprinting in the identification of non-sporulating endophytes from *Magnolia lilifera* (Magnoliaceae). *Fungal Diversity*. 2005;20: 167–186.

- [14] Qin S, Li J, Chen HH, Zhao GZ, Zhu WY, Jiang CL *et al.* Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Applied Environmental Microbiology*. 2009;75: 6176–6186.
- [15] Roncato-Maccari LD, Ramos HJ, Pedrosa FO, Alquini Y, Chubatsu LS, Yates MG, *et al.* Endophytic *Herbaspirillum seropedicae* expresses nif genes in gramineous plants. *FEMS microbiology ecology*. 2003;45(1): 39-47.
- [16] Sarangthem ID and Momota P. Isolation and characterization of Endophytic microbiome from indigenous maize (*Zea mays*) variety of Manipur and its impact on biological control. *International Journal of Human Genetics Medical Biotechnology & Microbiological Studies*. 2012;1: 11–17.
- [17] Sawarkar A, Sharma RK, Gautam V, Shraman K and Jain S. Antibacterial activity of endophytic bacteria from leaves of *Tamarindus indica*. *Pharma Innovations Journal*. 2021;10(2): 472-475.
- [18] Schulz B, Guske S, Dammann U and Boyle C. Endophyte-host interactions II. Defining symbiosis of the endophyte-host interaction. *Symbiosis*. 1998;25: 213–227.
- [19] Sgroy V, Cassan F, Masciarelli O, Papa MF, Lagares A, Luna V. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. *Applied Microbiology and Biotechnology*. 2009;85(2): 371-381.
- [20] Yuan Z, Zhang C, Lin F, Kubicek CP. Identity, diversity, and molecular phylogeny of the endophytic mycobiota in the roots of rare wild rice (*Oryza granulate*) from a nature reserve in Yunnan, China. *Applied Environmental Microbiology*. 2009;76(5): 1642-1652.
- [21] Zhang J, Zhang C, Yang J, Zhang R, Gao J, Zhao X *et al.* Insights into endophytic bacterial community structures of seeds among various *Oryza sativa* L. rice genotypes. *Journal of Plant Growth Regulation*. 2019;38: 93-102.