J. Farm Sci., 35(2): (223-227) 2022

RESEARCH PAPER

Biochemical and molecular characterization of few endophytes isolated from cotton

POOJA S. GHANTI, M. S. L. RAO, VENKATESH R. KULKARNI AND JONES NIRMALNATH

Department of Plant Pathology, College of Agriculture, Dharwad
University of Agricultural Sciences, Dharwad - 580 005 Karnataka, India
E-mail: poojaghanti1997@gmail.com

(Received: January, 2022 ; Accepted: May, 2022)

Abstract: The endophytic bacteria reside in plant tissues have often been known to promote plant growth. Endophytic bacteria were isolated from seeds and roots of cotton. A total of 35 bacterial endophytes from Bt cotton (12 from seed, 7 from root) and non-Bt cotton (5 from seed, 11 from root) were obtained from apparently healthy cotton plant samples. Morphological, biochemical and molecular identification were done to identify the bacteria isolated. Morphological characters of bacterial endophytes on nutrient agar medium varied with respect to colony colour, appearance (form). Colony colour varied from creamish white to bright yellow colour, colony form varied from regular to irregular. Among the 35 bacterial endophytes five endophytes (BRR-1, BRS-2, JAS-3, BRS-5 and AJS-1) were further tested for biochemical and molecular studies. Biochemical tests revealed that, the isolate BRS-5 showed positive reaction to methyl red, oxidase, starch, casein hydrolysis and gelatin liquefaction and isolate Jas-3 showed negative reaction to all tests conducted . Based on 16S rRNA the five endophytes were identified as *Streptomyces sampsonii* (BRR-1), *Pantoeadispersa* (BRS-2), *Pseudoclavibacterhelvolus* (JAS-3), *Bacillus cereus* (BRS-5) and *Bacilluspumilis* (AJS-1).

Key words: Biochemical, Cotton, Endophytic bacteria, Molecular characterization

Introduction

Cotton is one of the most important commercial crop of the world as well as India which belongs to the botanical family "Malvaceae". Cotton is referred to as "King of Fibers" and is also popularly known as "white Gold". India is the only country throughout the world where all the four cultivated *Gossypium* species *viz.*, *G. hirsutum* (American upland cotton), *G. arboreum, G. herbaceum* (Asian cotton) and *G. barbadense* (Egyptian cotton) besides hybrid cotton are cultivated on commercial scale.

Many biotic and abiotic factors are responsible for reduction in yield and deterioration of cotton in India, Diseases occupy a vital place. Cotton crop in India is known to suffer from fungal, bacterial, viral and nematode diseases. Among them, the economically most important ones are Alternaria leaf spot, bacterial blight, grey mildew, rust and vascular wilts which occur throughout the world (Kotasthane and Agarwal, 1970).

Now a days in India, majority of the farmers cultivate Bt cotton instead of non-Bt cotton. This has created tremendous competitiveness in hybrid seed production as the production area for hybrid cotton has remained stagnant.Conventionally, the farmers use agrochemicals to protect the crop and increase the crop yield. The tremendous use of agrochemicals leads to development pest resistance, kill beneficial microflora of soil and leads to pollution of soil to a great extent. Use of biocontrol agents, such as *Trichoderma*, *Psuedomonas*and *Bacillus* are recommended but use of endophytes as biocontrol agents is becoming new area of research in plant protection in recent decades because of many advantages with them.

Plant growth promoting bacterial endophytes have the ability to colonize a plant's interior and to establish a special type of relationship where both partners may derive benefits from this interaction (Reiter and Sessitsch, 2006). Bacterial endophytes have been reported to promote plant growth by a number of different mechanisms. These mechanisms include phosphate solubilization activity, production of phytohormones, nitrogen fixation, siderophore biosynthesis and supplying essential nutrients to the host plant.

Seed endophytes are known to protect the incoming seed borne pathogens through various mechanisms. Although endophytes may be isolated from all parts of plants, endophytes of seeds have developmental and evolutionary significance because seeds include the next generation of plant, and it is logical that seeds would be invested with all resources (including endophytes) needed to grow and compete (Verma *et al.*, 2019). Considering the benefits of endophytic bacteria, the present investigation was undertaken.

Material and methods

Seed and root samples of both Bt and non-Bt cotton were collected from AICRP research blocks on cottonHebballi farm Dharwad, private companies.The laboratory study was conducted in the Department of Plant Pathology, College of Agriculture Dharwad, University of Agricultural Sciences, Dharwad during the year 2019-20.

Isolation of bacterial endophytes

Root and seeds of healthy cotton plants were collected. Selected plants were uprooted, collected in sealed sterile plastic bags and transported aseptically to the laboratory. Initially plants were washed in running tap water to remove adhering soil particles. Root sections of 1 to 2 cm length were excised using flame sterilized scalpel.

Selected roots and seeds were thoroughly washed with distilled water and samples were blotted dry with filter paper

and then weighed to have final sample weight of 1 g. After this, surface sterilization of seeds and roots was done with ethanol (70%) for a minute followed by sodium hypochlorite (1%) for three minutes, with three rinses in sterilized distilled water. The sterilized sample was rinsed with 0.02M potassium phosphate buffer. An aliquot of 1ml of the final buffer wash was transferred to sterile petri plate to which nutrient agar (NA) was added and it served as sterile check. One gram of plant parts (seed and roots) were macerated with 9 ml of potassium phosphate buffer in pestle and mortar. Serial dilutions were made up to 10^{-3} dilutions. Dilution of 10^{-2} and 10^{-3} were plated on nutrient agar (NA) medium. The plates were incubated at 28 ± 2 °C for 48-72 h for observing colonies developed on it and isolated colonies were picked up and streaked on fresh nutrient agar plates and incubated. Final pure cultures were transferred on to NA slants and stored for further studies in refrigerator at 4°C.

Coding of endophytes

Bacterial endophytes were coded by using three letters. First two letters indicated the genotype of cotton (ex. Ajeet-155: AJ, Dr. Brent: BR), third letter indicated the part of plant (S: Seed, R: Root).

Morphological, biochemical and molecular identification of endophytic bacterial isolates

Morphological, biochemical and molecular identification were studied to identify the bacteria. The morphological characters of each bacterium was carried out by streaking the 72 h old culture on Nutrient agar (NA) media. Characters of the bacteria were recorded with respect to colony colour and form.

Biochemical characterization of bacterial endophytes

After carrying out the preliminary morphological characterization, biochemical analysis was carried out to so as to authenticate the identification of bacteria. Among the 35 endophytic bacteria isolated some of the bacteria were similar with respect to colony colour and form, the five bacterial endophytes which are having different colony colour and form were selected for further studies. The biochemical tests that were carried out under present investigation included: methyl red test, starch hydrolysis test, gelatin liquifaction, casein hydrolysis test and oxidase test. Control tubes were placed in each of the biochemical test which are not inoculated with the test bacteria whereas the rest others were inoculated with test bacteria.

The biochemical characterization of selected bacterial endophytes was essentially done as per the procedures outlined by Cappuccino and Sherman (1992).

Gram staining

Bacterial culture of 24 hour old was used for Gram staining. Later it was observed in oil immersion microscope or light microscope (Bartholomew and Finkelstein, 1958).

Methyl red test

One day old endophytic bacterial culture was inoculated in 10 ml of nutrient broth and incubated at 28°C for 48-72 hours. After 72 hours of incubation, 5 drops of methyl red solution was added to culture and observed for the change in color of the medium. Bright red color indicates the positive interaction and yellow/orange indicates negative interaction.

Starch hydrolysis

Endophytic bacterial cultures of 24 hour old culture was spotted on starch agar medium and incubated at 28±2 °C for 24 to 48 hours. After incubation, the plates were flooded with Lugol s iodine solution of 5ml. Formation of clear zone around the bacterial growth or colony was taken as positive for the test (Eckford, 1927).

Gelatin liquifaction

To the pre-sterilized nutrient gelatin deep tubes, the test endophytic bacterial cultures were inoculated and tubes were incubated at 28±2 °C for 24 h. the tubes were later kept in a refrigerator at 4 f C for 30 minutes. The tubes with cultures that remained liquified were taken as positive and those that solidified on refrigeration were taken as negative for the test (Blazevic and Ederer, 1975).

Casein hydrolysis

Endophytic bacterial cultures of 24 hour old were spotted on skimmed milk agar plates and incubated at 28±2 °C for 24 h. The production of clear halo around the colony was taken as positive for the test (Seeley and Vandemark, 1970).

Oxidase test

Endophytic bacterial cultures of 24 hour old were spotted on oxidase disc and after few minutes change in the color of disc from white to blue colour indicates positive reaction for test. White colour of the disc shows negative reaction for test.

Molecular identification of bacterial endophytes

After carrying out the biochemical test, molecular analysis was carried out to identify the bacteria. The five endophytic bacterial cultures were sent to National Collection of Industrial Microorganisms (NCIM), CSIR- National Chemical Laboratory (NCL) Pune, India. PCR amplification was conducted by using bacterial 16S rRNA gene. Purified amplicons were sequenced by Sanger method and were further analyzed using Basic Local Alignment Search Tool (BLAST) with closest culture sequence retrieved from the National Center for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences. The one which has the highest similarity was taken as information to be interpreted. Decoding was done to identify bacteria to highlight the results.

Results and discussion

Isolation of bacterial endophytes

A total of 35 bacterial endophytes from Bt cotton (12 from seed, 7 from root) and non-Bt cotton (5 from seed, 11 from root) were obtained from apparently healthy cotton plant samples. The isolates of endophytic bacteria were named as BRS-1, BRS-2, BRS-3, BRS-4, BRS-5, AJS-1, AJS-2, AJS-3, AJS-4, MRS-1, MRS-2, KCS-1, AJR-1, AJR-2, BRR-1, BRR-2, BRR-3, MRR-1, MRR-2, JAS-1, JAS-2, JAS-3, ABS-1, ABS-2, JAR-1, JAR-2, JAR-3, JAR-4, ABR-1, ABR-2, SAR-1, SAR-2, DHR-1, DHR-2

and DHR-3. The results obtained are presented in the Table 1 and Plate 1. The results are in agreement with the work of Okunishi *et al.* (2005) who isolated endophytic bacteria from rice seeds and based on 16S rRNA sequence identified as *Bacillus* and *Pantoea*. Inderiati *et al.*(2008) who isolated endophytic Actinomycetes from tomato plants and were identified as *Streptomyces* based on 16S rRNA.

Table 1. List of endophytic bacteria isolated from seed and roots of Bt and Non-Bt cotton

Sample	Genotype	Source	Bacterial endophytes isolated
Bt cotton	Dr. Brent	Seed	BRS-1, BRS-2, BRS-3, BRS-4, BRS-5
	Ajeet-155	Seed	AJS-1, AJS-2, AJS-3, AJS-4
	MRC 7373	Seed	MRS-1, MRS-2
	KCH-100	Seed	KCS-1
	Ajeet-155	Root	AJR-1, AJR-2
	Dr. Brent	Root	BRR-1, BRR-2, BRR-3
	MRC 7373	Root	MRR-1, MRR-2
Non-Bt Cotton	Jayadhar	Seed	JAS-1, JAS-2, JAS-3
	Abhadita	Seed	ABS-1, ABS-2
	Jayadhar	Root	JAR-1, JAR-2, JAR-3, JAR-4
	Abhadita	Root	ABR-1, ABR-2
	Sahana	Root	SAR-1, SAR-2
	DHH 263	Root	DHR-1, DHR-2, DHR-3

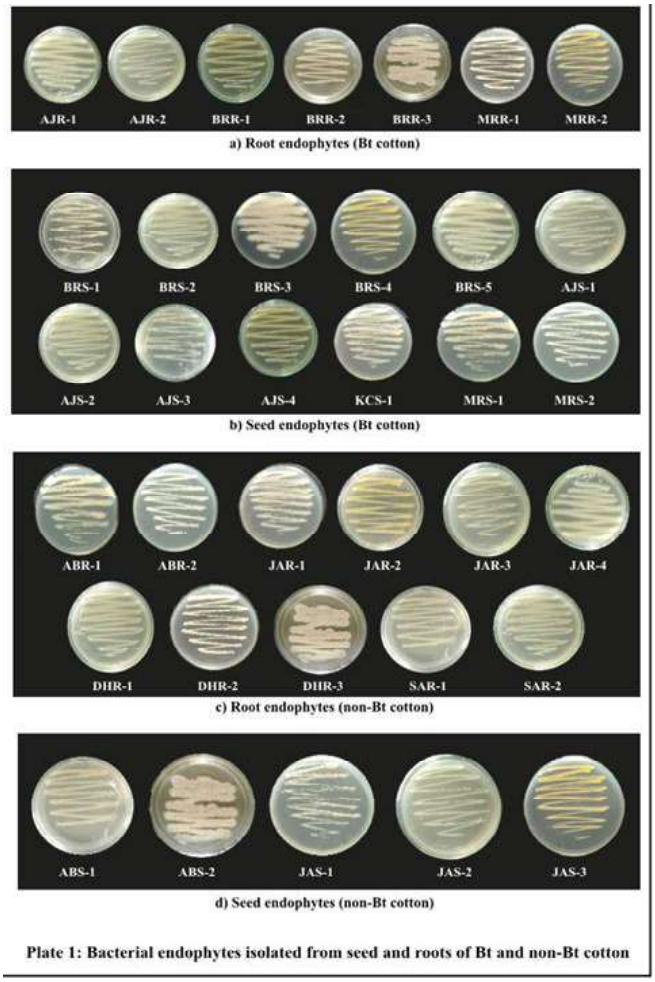

Plate 1: Bacterial endophytes isolated from seed and roots of Bt and non-Bt cotton

Morphological characters of bacterial endophytes

Morphological characters of bacterial endophytes on nutrient agar medium varied with respect to colony colour, appearance (form). Colony colour varied from creamish white to bright yellow colour, colony form varied from regular to irregular. The results are presented in Table 2.

Among 35 bacterial isolates, in Bt cotton 10 isolates were white, 6 isolates were creamish white, 2 isolates were bright yellow and 1 isolate was light yellow colour. In non-Bt cotton 6 isolates were white, 6 isolates were creamish white in colour, 2 isolates were bright yellow in colour and 2 isolates were light yellow in colour. Totally 15 isolates had circular form and remaining 4 had irregular form in Bt cotton and in non-Bt cotton 12 isolates were regular form while remaining 4 were of irregular form.

Biochemical characterization of bacterial endophytes

The results on biochemical tests for characterization of the effective bacterial endophytes revealed that, out of five endophytic isolates three isolates (BRR-1, BRS-5 and AJS-1) were gram positive and the remaining two isolates (BRS-2 and JAS-3) were gram negative. For methyl red test two isolates (BRS-2 and BRS-5) showed positive reaction and remaining three isolates (BRR-1, JAS-3 and AJS-1) showed negative reaction. For oxidase test four isolates (BRR-1, BRS-2, BRS-5 and AJS-1) showed positive reaction except for the isolate JAS-3. For starch hydrolysis two isolates (BRR-1 and BRS-5) showed positive reaction and three isolates (BRS-2, JAS-3 and AJS-1) showed negative reaction. For casein hydrolysis three isolates (BRR-1, BRS-5 and AJS-1) showed positive reaction whereas two isolates (BRS-2 and JAS-3) showed negative reaction. Gelatin liquifaction test showed positive reaction for three isolates (BRR-1, BRS-2 and BRS-5) whereas negative reaction for two isolates (JAS-3 and AJS-1). The results obtained were presented in Table 3 and Plate 2a, 2b.Jain *et al.* (2007) isolated *Streptomyces* strain which was gram positive and hydrolyze starch, casein and gelatin where as tested negative for methyl red test.

Molecular characterization of the bacterial endophytes

The five endophytic bacterial cultures were sent to NCIM, CSIR-NCL Pune. Purified amplicons were sequenced by Sanger method and were further analyzed by BLAST with closest culture sequence retrieved from the NCBI database that finds regions of local similarity between sequences. Based on sequence comparison, the bacterial endophytes were identified as *Streptomyces sampsonii* (BRR-1), *Pantoea dispersa* (BRS-2), *Pseudoclavibacterhelvolus* (JAS-3), *Bacillus cereus* (BRS-5), *Bacillus pumilis* (AJS-1) Table 4.

The results were in agreement with the work of Adeleke *et al.* (2021) isolated bacterial endophytes from the sunflower roots,identified by morphological, biochemical and molecular study. The strain was gram positive and exhibited positive reaction to oxidase, casein and starch hydrolysis. Based on 16S rRNA sequence it was identified as *Bacillus cereus* T_4S.

Table 2. Morphological characterization of bacterial endophytes from cotton

Sample	Source	Isolate code	Colony colour	Colony form	Gram reaction
Bt cotton	Seed	BRS-1	White	Regular	+
		BRS-2	Light yellow	Regular	-
		BRS-3	Creamish white	Irregular	+
		BRS-4	Bright yellow	regular	-
		BRS-5	White	Irregular	+
		AJS-1	White	Regular	+
		AJS-2	Creamish white	Regular	+
		AJS-3	White	Regular	+
		AJS-4	Creamish white	Regular	+
		MRS-1	White	Irregular	+
		MRS-2	White	Regular	+
		KCS-1	White	Regular	+
	Root	AJR-1	Creamish white	Regular	+
		AJR-2	White	Regular	+
		BRR-1	Creamish white	Regular	+
		BRR-2	White	Regular	+
		BRR-3	Creamish white	Irregular	+
		MRR-1	White	Regular	+
		MRR-2	Bright yellow	Regular	-
Non-Bt cotton	Seed	JAS-1	White	Regular	+
		JAS-2	White	Regular	+
		JAS-3	Bright yellow	Regular	-
		ABS-1	White	Regular	+
		ABS-2	Creamish white	Irregular	+
	Root	JAR-1	Creamish white	Regular	+
		JAR-2	Bright yellow	Regular	-
		JAR-3	White	Regular	+
		JAR-4	Creamish white	Irregular	+
		ABR-1	Creamish white	Irregular	+
		ABR-2	White	Regular	+
		SAR-1	Creamish white	Regular	+
		SAR-2	Light yellow	Regular	-
		DHR-1	Light yellow	Regular	-
		DHR-2	White	Regular	+
		DHR-3	Creamish white	Irregular	+

+ : Gram positive - : Gram negative

Table 3. Biochemical characterization of endophytic bacteria

Isolates	Methyl red test	Oxidase test	Starch hydrolysis	Casein hydrolysis	Gelatinliquifaction
BRR-1	-	+	+	+	+
BRS-2	+	+	-	-	+
JAS-3	-	-	-	-	-
BRS-5	+	+	+	+	+
AJS-1	-	+	-	+	-

+ : Positive reaction
- : Negative reaction

Table 4. Molecular identification of the bacterial endophytes by 16S rRNA gene sequences

Isolates	Similarity	Closest Gene Bank Match	Strain Identified	Accession No.
BRR-1	99.00%	*Streptomyces sampsonii* strain ATCC 25495 16S ribosomal RNA complete sequence	*Streptomyces sampsonii*	NR_025870.2
BRS-2	99.76%	*Pantoea dispersa* gene for 16S ribosomal RNA, partial sequence, strain: DSM 30073	*Pantoea dispersa*	AB907780.1
JAS-3	99.64%	*Pseudoclavibacter helvolus* strain DSM 20419 16S ribosomal RNA, partial sequence	*Pseudoclavibacter helvolus*	NR_029264.1
BRS-5	100.00%	*Bacillus cereus* strain CCM 2010 16S ribosomal RNA gene, partial sequence	*Bacillus cereus*	$MT_4$21928.1
AJS-1	100.00%	*Bacillus pumilis* strain NCTC10337 genome assembly, chromosome:1	*Bacillus pumilis*	$LT_9$06438.

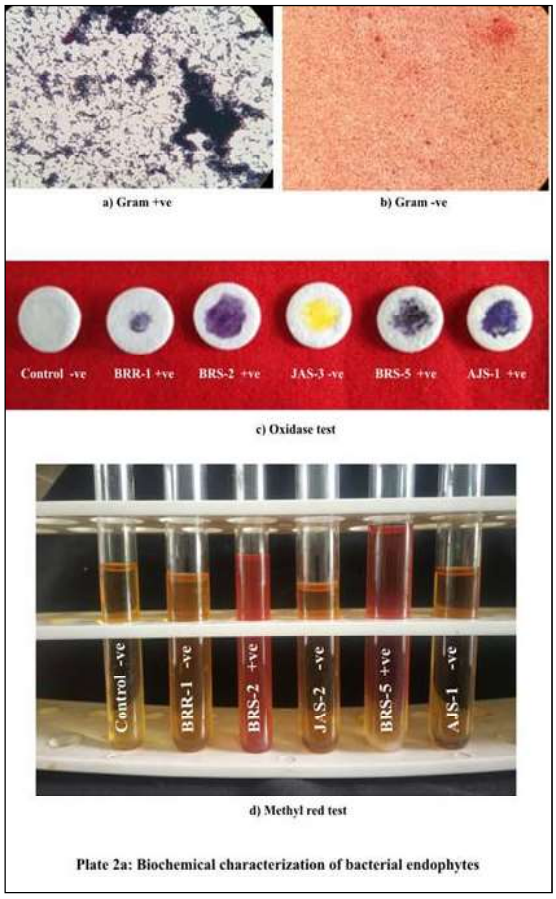

Plate 2a: Biochemical characterization of bacterial endophytes

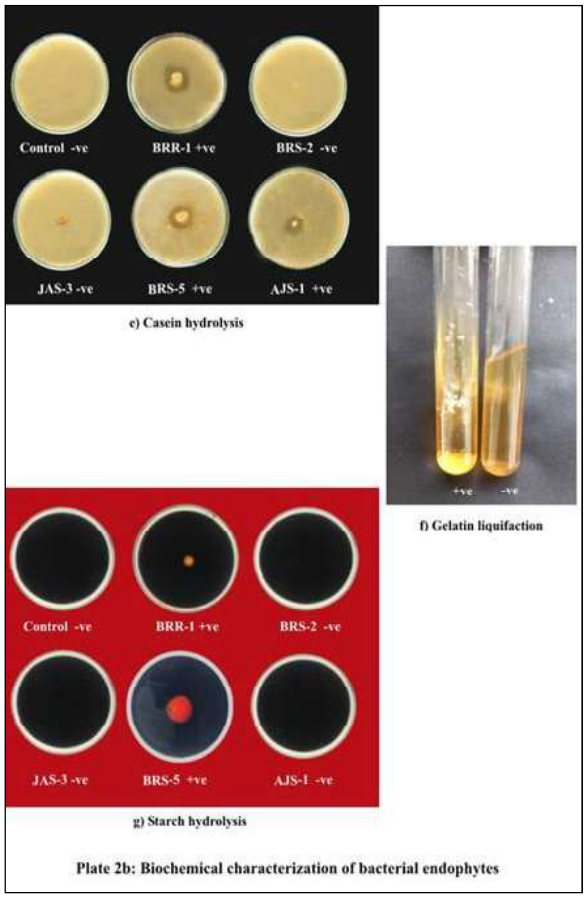

Plate 2b: Biochemical characterization of bacterial endophytes

Conclusion

Total of 35 bacterial endophytes were isolated from both Bt and non-Bt cotton. Among them five endophytes were identified based on biochemical and molecular studies. Bacterial endophytes were identified as *Streptomyces sampsonii* (BRR-1) *Pantoea dispersa* (BRS-2), *Pseudoclavibacter helvolus* (JAS-3), *Bacillus cereus* (BRS-5) and *Bcaillus pumilis* (AJS-1). Bioefficacy of promising endophytes tested under current investigation may be further confirmed by large scale field trials and demonstrations Bioefficacy of fungal endophytes may also be tested against seedling diseases of cotton.

References

Adeleke B S, Ayangbenro A S and Babalola O O, 2021, Genomic analysis of endophytic *Bacillus cereus* T_4S and its plant growth-promoting traits. *Plants*, 10(9): 1776.

Bartholomew J W and Finkelstein H, 1958, Relationship of cell wall staining to Gram differentiation. *Jouranl of Bacteriol*ogy, 75(1): 77-84.

Blazevic D J and Ederer G M, 1975, *Principles of Biochemical Tests in Diagnostic Microbiology*, Wiley and Company, New York, pp. 13-45.

Cappuccino J G and Sherman N, 1992, *Microbiology: A Laboratory Manual*. The Bejamin/Cummings Publishing Company, Inc., California.

Eckford M O, 1927, Thermophillic bacteria in milk. *American Journal of Hygiene*, 7: 201-202.

Inderiati S and Franco M M Christopher, 2008, Isolation and identification of endophytic actinomycetes and their antifungal activity.*Journal of Biotechnology Research in Tropical Region,* ISSN: 1979-9756.

Jain P K and Jain P C, 2007, Isolation, characterization and antifungal activity of *Streptomyces sampsonii* GS 1322. *Indian Journal of Experimental Biol*ogy, 45: 203-206.

Kotastane S R and Agarwal S C, 1970, Efficacy of four seed protectants for the control of bacterial blight of cotton. *Pest Articles and News Summaries*,16: 334-335.

Okunishi S, Sako K, Mano H, Imamura A and Morisaki H, 2005, Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*). *Microbes and Environments,* 20(3): 168-177.

Reiter B and Sessitsch A, 2006, Bacterial endophytes of the wildflower *Crocus albiforus* analyzed by characterization of isolates and by cultivation-independent approach. *Cannadian Journal of Microbiology,* 52: 140-149.

Seeley H W and Vandemark P J, 1970, *Microbe in Action, A Laboratory Manual of Microbiology*, D. B. Taraporvala Sons and Company Pvt. Ltd., Mumbai, pp. 86-95.

Verma S K, Kharwar R N and White J F, 2019, The role of seed-vectored endophytes in seedling development and establishment. *Symbiosis*, 78(2): 107-113.