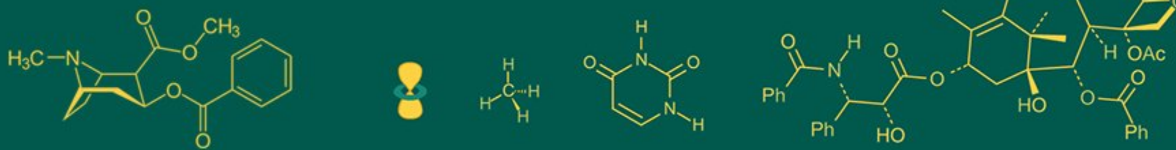


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# Efficacy of *Bacillus* spp., against *Fusarium sacchari* causing Pokkah boeng disease of sugarcane under *in vitro* condition

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**Abstract**
Pokkah boeng, once a minor disease is becoming major disease of sugarcane a total of 14 bacterial isolates were obtained from sugarcane phylloplane using HiCrome Bacillus Agar and characterized culturally for colony colour, shape and number of colonies per plate. Grams' test differentiated the Gram-positive bacteria from Gram negative bacteria which was done using 3% KOH test. All these isolates were evaluated for their antagonism against *Fusarium sacchari* through dual culture technique. Out of 14 isolates, ScANKP-9 and ScANKP-13 isolates were found to be the most effective with maximum antagonistic potential by significantly restricting the growth of *F. sacchari* to 4.47 and 4.80 cm, in comparison to monocultured *F. sacchari* (9 cm) with 50.33 per cent and 46.67 per cent inhibition in radial growth. Molecular characterization of effective isolates using modified CTAB method following PCR amplification of DNA with FGPS-63 and FGPL-132-38 primers, BLAST and phylogenetic analysis confirmed the isolates, ScANKP-9 and ScANKP-13 as *Bacillus amyloliquefaciens* and *Pseudomonas fluorescence*, respectively.

**Keywords:** *Bacillus* spp., *Fusarium sacchari*, *Saccharum officinale* L., DNA

## Introduction
Sugarcane (*Saccharum officinale* L.) is one of the most important cash crops in the world for sugar production. Globally, India is the second largest producer of sugarcane after Brazil and largest consumer of sugar. In India, sugarcane is grown in an area of 4.55 million hectare with an annual production of 353.84 million tonnes and productivity of 77.75 tonnes per hectare. Major sugarcane growing areas in India are Uttar Pradesh which is accounting for 178.42 million tonnes production followed by Maharashtra, Karnataka, Bihar, Tamil Nadu, Panjab, Haryana, Gujarat and Andhra Pradesh. In Andhra Pradesh, sugarcane occupies an area of 0.99 lakh hectare, giving a total production of 65.5 lakh tonnes and productivity of 76.14 tonnes per hectare (www.indiaagristat.com, 2019-20). It grows well in deep, well-drained soils of medium fertility of sandy loam soil textures with a pH range from 6.0 to 7.7. It plays a major role in the economy of sugarcane-growing areas.
The crop suffers from various biotic and abiotic factors leading to severe economic losses. Among the diseases, pokkah boeng is emerging as a major disease associated with *Fusarium* species complex. The pathogen is transmitted by air currents and airborne conidia colonize the leaves, flowers and stems of the plant.
Pokkah boeng disease, considered as a minor foliar disease earlier, is now emerging as a major disease, causing substantial losses in cane weight, length, girth, total juice and total sugars (Singh *et al.*, 2006) [10]. In India, *Fusarium sacchari*, *F. proliferatum* and *F. moniliforme* var. *subglutinans* were found associated with Pokkah boeng disease (Arya *et al.*, 2017) [2].
Management practices for this disease include use of resistant varieties, spraying of fungicides and use of biocontrol agents. Spraying of fungicides like carbendazim, copper oxychloride, mancozeb, propiconazole and difenoconazole on first appearance of disease was found effective for disease management (Vishwakarma *et al.*, 2013; Sharma and Arun, 2015) [23, 9]. Owing to ill-effects of chemical pesticides ecologically sustainable approaches towards tackling diseases are gaining importance.

One among them is biological control through application of fungal and bacterial antagonists that has wide range applicability against plant diseases. *Burkholderia* species, *Pseudomonas fluorescens*, *Bacillus* spp., *Trichoderma harzianum*, *T. hamatum*, *T. viride*, *Aspergillus flavus* and *A. Niger* were tested against *F. moniliforme* under *in vitro* conditions by dual culture technique (Vishwakarma *et al*., 2013; Choudhury *et al*., 2019) [12, 4]. Therefore, the current study is aimed to determine the antagonistic nature of *Bacillus* spp., against *Fusarium sacchari* and to identify at species level using molecular approaches.

## Materials and Methods

The experiment was conducted during 2021 at the Department of Plant Pathology, Agricultural College, Bapatla. The study was designed with fourteen treatments and three replications with completely randomized design (CRD).

**Isolation of bacteria from sugarcane phylloplane:** Leaf samples were collected from ten genotypes of sugarcane from 3rd or 4th top leaf of the plant whorl. Sugarcane genotypes used for the above experiment were 81A 99, 87A 298, 93A 145, 97A 85, 2000A 56, 2007A 63, 2003V 46, 2003A 255, 2009A 107 and 2005A 128.

Leaves were cut into small pieces and 10g of weighed sample was taken in conical flask containing 100 ml distilled sterilized water which is considered as $10^{-1}$ concentration. Conical flask was shaken thoroughly for few minutes. Then serial dilution was done to bring down the bacterial population. This was done by transferring 1 ml solution from the $10^{-1}$ conc. to 9 ml water blank resulting in $10^{-2}$ conc. and repeated for several times until $10^{-7}$ concentration was obtained.

The inoculum of 1 ml volume from $10^{-6}$ and $10^{-7}$ dilution was taken through sterilized pipette and poured on the HiCrome Bacillus Agar media plates. Sterilized spreader is used to spread over the media and kept for incubation at 27±2 °C (AICRP on sugarcane, Technical Report-Plant Pathology (2020-21), SBI, Coimbatore).

**Identification and maintenance of phylloplane bacteria:** One day old culture plates previously inoculated with serially diluted phylloplane bacteria were observed for different coloured colonies and noted the colour, shape of each distinguishable colony and counted number of colonies from each plate.

Single colony was taken with sterilized inoculation loop and streaked on HiCrome Bacillus Agar plates to get pure culture of the same. One day after incubation single colony was taken from those plates and streaked on LB media containing plates.

**Slant preparation:** Luria Bertanis Agar medium was filled to the ¼th volume of test tube, autoclaved (121 °C, 15 lbs) and kept in slant position for further use. Slants were streaked with single bacterial colonies with sterilized inoculation loop aseptically.

**Testing for gram reaction of isolated bacteria:** A drop of 3% KOH was placed on clean and dried slide. Loopful of bacteria was taken and mixed in the drop of KOH solution. In the presence of KOH, gram negative cell walls were broken down. Disintegration of cell wall of gram-negative bacteria show stickiness when touched due to the release of viscid chromosomal material, indicating the respective bacteria as gram negative otherwise it is gram positive.

**Storage of bacterial cultures:** Bacteria in its pure form were stored in glycerol at -20 °C.

**Evaluation of phylloplane bacteria against *Fusarium sacchari*:** Isolates of bacteria obtained from sugarcane phylloplane were evaluated *in vitro* for their antagonism against *F. sacchari* using dual culture technique.

An agar block (5 mm diameter) of 5-day-old culture of *F. sacchari* was placed in the centre of Petri plates (90 mm diameter) containing Potato Dextrose Agar (PDA) and loopful of one-day old culture of bacterial test antagonist was streak inoculated on either side of *F. sacchari* disc at a distance of 2 cm apart.

The pathogen culture inoculated centrally on PDA plates without bacterial streak served as control. Each treatment was replicated thrice and the inoculated plates were incubated at 28 ± 2 °C for 5 days and per cent inhibition was calculated (Dennis and Webster, 1971) [14] using the formula given,

$$I = \frac{C - T}{C} X\ 100$$

Where,
I = per cent inhibition,
C = growth of the fungus in control plate, and
T = growth of the fungus in dual culture plate.

**Molecular identification of potential phylloplane bacteria**

Two potential bacterial isolates were grown in Nutrient Broth for 24 hr. to 48 hr. and genomic DNA was extracted by modified CTAB method. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in molecular grade water and stored at 4 ºC. Primers for 16S rRNA genes were selected from standard scientific literature. Sequences of the primers used to amplify and detect 16S rRNA genes are "FGPS6-63-GGAGAGTTAGATCTTGGCTCAG and FGPL-132-38 CCCGGTT TCCCCATTCGG" (Normand *et al.*, 1992) [7].

**Bacterial DNA isolation protocol:** Respective bacterial isolates grown in NA broth were used for DNA isolation. The culture solution was shaken for few seconds and 1.5 ml of the bacterial suspension was pipetted out in appropriate centrifuge tube. The sample was centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. The pellet was re-suspended in lysozyme buffer of 590 µl volume, mixed well and incubated at 37 ºC for 30 min. After incubation, 40 µl of 10% SDS solution and 4 µl of proteinase K (20mg/ml) was added in the solution and incubated at 56 ºC for 1-3 h. Now 100 µl of 5 M NaCl and 100 µl of CTAB/NaCl were added, mixed well and incubated at 65 ºC for 10 min.

Then, 0.5 ml of chloroform: isoamyl alcohol solution (24:1) was added and mixed well in vortex. This mixture was centrifuged at max speed for 10 min at room temperature. Now, the upper phase was carefully transferred into a fresh tube and 0.5 ml of Phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at max speed for 10 min at room temperature. Again the supernatant obtained was transferred into a fresh tube and

mixed with 0.5 ml of chloroform: isoamyl alcohol (24:1) properly and centrifuged at max speed for 10 min at room temperature. The upper aqueous phase containing thick material was transferred to a fresh tube and added with 0.6 ml volume of isopropanol and incubated at -20 ºC for 2 h and centrifuged at max speed for 15 min at 4 ºC.
Now, the supernatant was discarded and 500 µl of 70% alcohol was added and mixed properly. The solution was spinned at max speed for 5 min. The ethanol was discarded and the pellet was air dried at room temp and the obtained pellet was dissolved in 20 µl of molecular grade water and checked in 0.8% agarose gel. The DNA concentration was checked in Nanodrop system further.

| | |
|---|---|
| 10X PCR buffer (Thermo Scientific): | 2.5 µl |
| 25 mM MgCl2 (Thermo Scientific): | 2 µl |
| 10 mM dNTPs (Thermo Scientific): | 1 µl |
| Molecular grade water: | 15 µl |
| 5U Taq polymerase (Thermo Scientific): | 0.5 µl |
| 10 µM Primer F: | 1 µl |
| 10 µM Primer R: | 1 µl |
| Template DNA: | 2 µl |

**PCR amplification using universal primers:** The composition of the reaction mixture (25 µl) used in polymerase chain reaction (PCR) for DNA amplification of respective bacterial isolate is as follows:

**Programme for PCR amplification of bacterial DNA**

| Steps in PCR | Temperature |
|---|---|
| Initial denaturation | 95 ºC for 3 min |
| Denaturation | 95 ºC for 1 min |
| Annealing | 55 ºC for 1 min |
| Primer extension | 72 ºC for 2 min |
| No. of cycles | 35 |
| Final extension | 72 ºC for 3 min |
| Storage | Infinite hold at 4 ºC |

The aliquots were checked for the amplification of the expected size (approximately 1500 bp) on 1.2% agarose gel and visualized in gel documentation system.
The amplicons produced after PCR were separated by gel electrophoresis.

**Preparation of gel cast and loading of DNA samples:** Gel solution was prepared by adding 1.2 g of agarose to 100 ml of 1X TAE buffer in a conical flask and micro waved until a clear solution was obtained. Five µl of ethidium bromide was added to luke warm solution and poured in to casting tray after placing the Teflon comb. When the gel was polymerised, the comb was gently removed and the gel cast was placed in horizontal electrophoresis unit.
Five micro liter of genomic DNA of was mixed with loading dye (2.5 µl) (Novagen, U.S.A) and was loaded in agarose gel wells. The gel was run in horizontal electrophoresis unit (Genaxy, India) at 60 V and 60 mA for 1 h.

**DNA sequencing:** The amplified samples were chromatographed by gel electrophoresis on 1.2% agarose. The gel was photographed using gel documentation system. Amplicons of 500 to 600 bp were selected for sequencing the ITS region. For size selection a co-resolved 100 bp ladder was used. The amplified products were sequenced by Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad.

**Molecular identification:** Molecular identification of the fungal isolates was done using partial amplified nucleotide sequences of ITS region and *TEF-alpha* region through NCBI, BLAST programme and checked for homology. The generated sequences were deposited in NCBI Gene Bank database to obtain the accession numbers.

**Phylogeny tree construction:** Using maximum parsimony method in MEGA software (Ver. 10.0.5), a phylogenetic tree was constructed by considering most relevant sequences from the NCBI database along with sequence data of test isolates.

**Results and Discussion**

Fourteen sugarcane bacterial isolates were obtained from phylloplane using HiCrome Bacillus agar and numbered them as ScANKP-1 to 14. Of them, thirteen isolates were found to be Gram positive and one isolate as gram negative when 3% KOH test was done. These isolates were used for dual culture to test their antagonistic potential against *F. sacchari* using streak plate method. The results obtained in dual culture were presented here with Table 1 and Plate 1.
Among the fourteen bacterial isolates obtained through phylloplane isolation, isolate ScANKP-9 followed by ScANKP-13 were found to be the most effective with maximum antagonistic potential by significantly restricting the growth of *F. sacchari* to 4.47 and 4.8 cm respectively, in comparison to monocultured *F. sacchari* (9 cm) with 50.33 per cent and 46.67 per cent inhibition in radial growth respectively. A clear zone of inhibition was also observed in these isolates measuring 0.53 and 0.20 cm respectively. Though other isolates showed little inhibition in *F. sacchari* radial growth, upon prolonged incubation, these isolates couldn't stop invading *F. sacchari* resulting in over growth of *F. sacchari* on these bacterial isolates. Hence, ScANKP-9 and ScANKP-13 bacterial isolates were confirmed as the best among all the isolates used in dual cultural technique.
Reports were also published on the efficacy of bacterial and fungal species antagonists to *F. sacchari.* Pal *et al.* (2001) [8] with *Pseudomonas* sp., Cavaglieri (2005) [3] with *Bacillus subtilis*, Mostafa *et al.* (2009) [5] with *Pseudomonas* sp. and Arya *et al.* (2017) [2] with *Trichoderma* sp.
The most effective bacterial bioagent against *F. sacchari i.e.*, ScANKP-9 and ScANKP-13 were further identified by molecular methods.

**Molecular characterization of effective antagonistic bacterial isolates**

Bacterial DNA was extracted by modified CTAB method (Murray and Thompson, 1980) [6] and obtained good concentration of genomic DNA (132.56 gm/µl) with purity value 2.03. The 16S rRNA gene of this isolate was amplified using universal primers, FGPS-63-GGAGAGTTAGATCTTGGCTCAG and FGPL-132-38-CCCGGTTTCCCCATTCGG (Normand *et al.*, 1992) [17] in PCR. Amplified products were checked for amplification by gel running with the help of gel electrophoresis unit and obtained band near 500bp. This amplicon was outsourced for partial sequencing. Similarity of 16 S rRNA gene sequence was aligned using BLAST programme of GenBank database (NCBI).
BLAST search for similarities using NCBI database showed the percentage similarity of the isolate ScANKP-9 (GenBank accession No. OK576714) ranged from 95.98-

99.55% with *B. amyloliquefaciens*, *B. siamensis*, *B. subtilis*, *B. velezensis*, *B. tequilensis*, *B. atrophaeus* and *B. aerius* but highest similarity ranging from 99.33-99.55% reported with *Bacillus amyloliquefaciens*. Whereas, ScANKP-13 (GenBank accession No. OL628761) isolate showed 98.96% similarity with *Pseudomonas fluorescence* in Blast search with NCBI reference sequence *P. fluorescence* (GenBank accession No. KP119846). Therefore, identity of the ScANKP-9 and ScANKP-13 isolates were confirmed as *Bacillus amyloliquefaciens* and *P. fluorescence* respectively, based on NCBI-BLAST search. Phylogenetic analysis revealed distant evolutionary relationship between the two isolates by forming two separate clades where ScANKP-9 isolate joined with *B. amyloliquefaciens* and ScANKP-13 isolate with *P. fluorescence* (Fig 1).

Varma *et al.* (2017) [11] isolated a phylloplane bacterium on sugarcane antagonistic to *F. sacchari* which was identified as *B. amyloliquefaciens* using molecular tools.

**Plate 1:** Antagonistic potential of sugarcane phylloplane bacterial isolates against *F. sacchari*

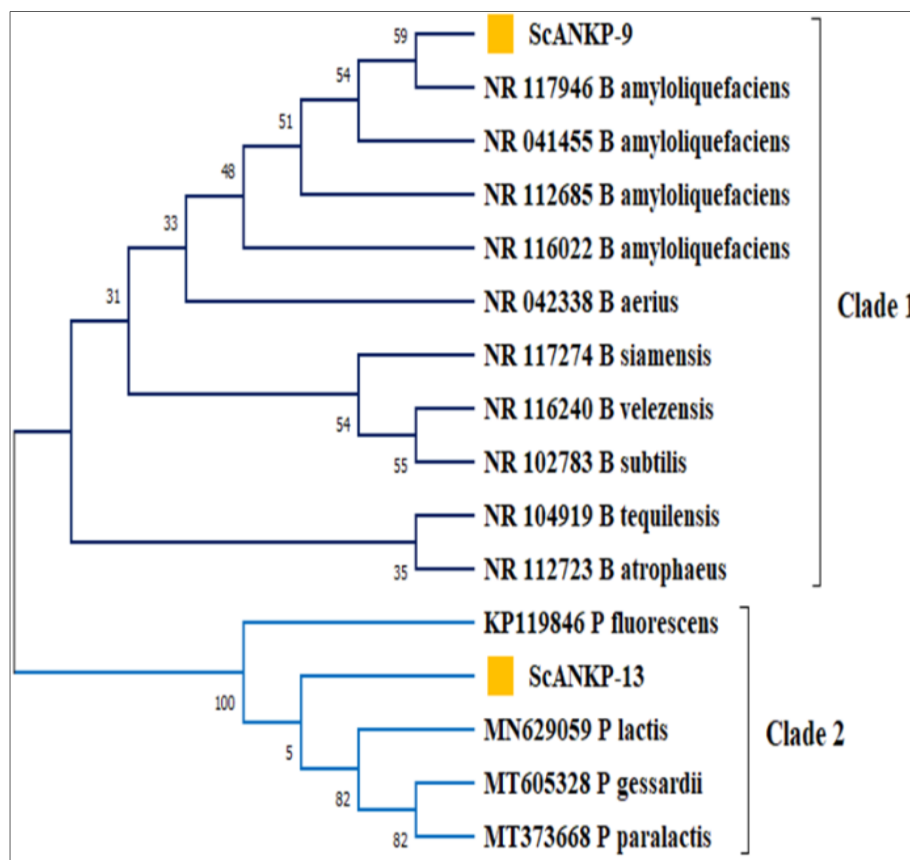


**Fig 1:** Neighbour-joining tree showing the relationship of ScANKP-9 and ScANKP-13 isolates with other species of Bacillus and Pseudomonas, inferred using p-distance method. The bootstrap values (1000 replicates) higher than 50% are shown next to the branches

**Table 1:** Efficacy of sugarcane phylloplane bacterial isolates against *Fusarium sacchari* radial growth *in vitro*

| S. No. | Isolates | Radial Growth of (cm) | Inhibition over control (%) | Inhibition zone (cm) |
|---|---|---|---|---|
| 1 | ScANKP-1 | 7.8 $^{g}$ | 13.33 | - |
| 2 | ScANKP-2 | 8.77 $^{abcd}$ | 2.56 | - |
| 3 | ScANKP-3 | 7.77 $^{g}$ | 13.33 | - |
| 4 | ScANKP-4 | 8.4 $^{e}$ | 6.67 | - |
| 5 | ScANKP-5 | 8.07 $^{f}$ | 10.33 | - |
| 6 | ScANKP-6 | 7.77 $^{g}$ | 13.67 | - |
| 7 | ScANKP-7 | 8.77 $^{abcd}$ | 2.56 | - |
| 8 | ScANKP-8 | 8.87 $^{ab}$ | 1.44 | - |
| 9 | ScANKP-9 | 4.47 $^{i}$ | 50.33 | 0.53 |
| 10 | ScANKP-10 | 8.93 $^{a}$ | 0.78 | - |
| 11 | ScANKP-11 | 8.8 $^{abc}$ | 2.22 | - |
| 12 | ScANKP-12 | 8.8 $^{abc}$ | 1.89 | - |
| 13 | ScANKP-13 | 4.8 $^{h}$ | 46.67 | 0.20 |
| 14 | ScANKP-14 | 8.87 $^{ab}$ | 1.44 | - |
| Control | | 9 | | |
| SEM | | 0.012 | | |
| CD | | 0.036 | | |
| CV | | 0.758262 | | |

## Conclusion

Among fourteen bacterial isolates obtained through phylloplane isolation, ScANKP-9 isolate was found to be significantly superior as compared to other bio-agents and inhibited maximum mycelial growth (50.33% inhibition) of *F. sacchari* which was followed by ScANKP-13 isolate (46.67% inhibition). These isolates were identified as *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* respectively, with molecular characterization and phylogenetic analysis. This information provides a choice to the sugarcane growers to trust and choose bioagents against the pokkah boeng disease and the researchers to consider these for field evaluation against the pathogen.

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