*International Research Journal of Environment Sciences*_____ ISSN 2319–1414
Vol. **3(9),** 27-31, September **(2014)** *Int. Res. J. Environment Sci.*

# *Bacillus Thuringiensis* as Endophyte of Medicinal Plants: Auxin Producing Biopesticide

**Minu M. Ali and Dipak Vora**
Dept. of Microbiology, Ramnarain Ruia College, Matunga, Mumbai 400019, INDIA

**Available online at: www.isca.in, www.isca.me**
Received 8th July 2014, revised 11th August 2014, accepted 12th September 2014

## Abstract

*Occurrence of Bacillus thuringiensis, a biopesticide living as endophytes in the leaves of medicinal plant and their ability to produce Indole-3-acetic acid was studied. Phenotypic characterization identified the endophytes as Bacillus sp. The presence of parasporal crystalline inclusion was observed using phase contrast microscopy for all the isolated endophytic Bacilli. Coomassie brilliant blue (CBB) staining further established the presence of endospore and parasporal body. Appearance of insecticidal proteins (ICP) in the sporulation phase observed as a dark stained blue structure confirmed the endophytes as B. thuringiensis. Genotypic variation was studied by 16S rRNA gene amplification and sequencing. All isolated endophytic B. thuringiensis were observed to have the ability to produce phytohormone Indole-3-acetic acid. This study shows the presence and association of endophytic biopesticide with its host medicinal plant and its potential to produce an important plant growth hormone. This unique finding of such naturally occurring association is of great value to research as well as agricultural industry.*

**Keywords:** *B. thuringiensis,* parasporal crystals, genetic sequencing, agricultural tool, phytohormone, endophytic association

## Introduction

Biological pesticides are being used extensively to control agricultural insects and diseases in crop plants. *Bacillus thuringiensis* is the most widely used of these microbial pesticides. Insecticidal activity in *B. thuringiensis* is controlled by the *cry* genes encoded parasporal crystals that are host specific. Their toxicity depends on the insect type with all reported 200 strains of *B. thuringiensis* existing as different insecticides[1]. Synthetic insecticides are effective chemical pesticides that have been used for ages to control the agricultural pests. Although these chemical insecticides are effective and efficient but their use has been limited due to their adverse effect on environment, animals and human health[2]. Since first successfully cloned crystal toxin gene in 1981, researchers have created *B. thuringiensis* with highly specific and efficient target spectra and extremely toxic strains for agricultural employment. These naturally occurring biopesticides have now slowly replaced the synthetic insecticides universally. 'Formulated and sporulated cultures of *B. thuringiensis* are widely used as foliar sprays as a part of integrated pest management strategies against insect pests of agricultural crops'[3-5].

*B. thuringiensis* based biopesticides are environment friendly and are not harmful to non-target organisms including humans and have been used successfully as pesticides for more than fifty years[5-8]. The present study aims to isolate native *B. thuringiensis* strains living as endophytes in medicinal plant tissue, to understand their environmental distribution and their potential and relation to the plant. The isolated endophytic *B. thuringiensis* were also evaluated for production of phytohormone, auxin. Degradable and safe insecticidal *B. thuringiensis* existing as plant associated endophytes with plant growth hormone producing added ability is a blessing to the ecosystem and agriculture.

## Material and Methods

**Plant Material:** Leaves of *Withania somnifera* plants authenticated from St. Xaviers Blatter herbarium, Mumbai were screened for this study.

**Isolation of endophytic bacteria:** The plant tissue was collected at the flowering stage. Seven healthy plants of *Withania somnifera* were carefully removed. From each plant, four (4) mature symptomless leaves were obtained. Leaves were processed in the laboratory within 48 hours. The leaf tissue was cleaned and surface sterilized using 70% ethanol and 0.1% HgCl2 before isolation[9].

**Efficiency of sterilization and negative controls:** A 100µl sample of the water from the third rinse of the leaf tissue was surface spread on sterile nutrient agar (NA) to verify the efficacy of sterilization. Negative controls for each leaf were carried by pressing leaf tissue using sterile forcep on nutrient agar to confirm bacteria being recovered were endophytes.

**Bacterial strains and Identification / phenotypic characterization:** Pure cultures were classified according to

their physical appearance in culture. Gram staining, capsule staining and endospore staining were performed. All the isolated endophytes were plated on selective/ differential media and further characterized using tests including IMViC, oxidase, urease, catalase, pigment production, starch hydrolysis and motility as per the established methods. Motility was confirmed by both hanging drop technique as well as agar diffusion method.

**Identification and Confirmation of *B. thuringiensis* : Phase contrast microscopy:** All the colonies that were Gram positive bacilli were examined for the presence of parasporal inclusion by phase contrast microscopy. 50h and 90h wet mount slides were prepared from the inoculated cultures to visualize the vegetative phase morphology and endospore and the parasporal bodies characteristics of *B. thuringiensis*[4].

**Coomassie brilliant blue (CBB) staining:** The CBB stain allows high throughput evaluation and enhanced resolution of the bacterial colonies for the presence of crystals[10]. Characterization using CBB stain was done at two phases, the sporulation phase and at the autolysis phase. These selected isolates were inoculated into Nutrient Broth and incubated in an orbital shaker (250 rpm) for 90h (sporulation stage) and 110 h (autolysis stage) at room temperature.

An aliquot of colony was stained using 0.133% Coomassie Blue stain in 50% acetic acid[11]. The dried slides were then observed under phase contrast microscope to visualize the formation of toxin-containing parasporal bodies and the spores. The morphological characters of the crystals were also examined, whether the shapes of the crystals were bipyramidal, cuboidal, oval or irregularly shaped.

**16S rRNA gene amplification and sequencing:** The identification of the bacterial endophytes was done by DNA sequencing of the 16S rRNA gene. DNA Sequencing of all the isolates was done by Microbial culture collection, *National Centre for Cell Science* (*NCCS*), Pune, India. Sequencing of pure isolate(s) was done using multiple PCR primers in ABI 3730XL sequencing machine. DNA isolation (PCR Template preparation) was done by Phenol-Chloroform method and PCR purification by PEG-NaCl method[12,13]. Nucleotide sequence similarities were determined by using BLAST, version 2.2.28 (National Center for Biotechnology Information databases).

**IAA production:** IAA production was estimated by Gordon and Weber spectrophotometric method (1951)[14].

**Extraction and purification of IAA:** Ethyl acetate extracts of endophytic isolates were prepared[6,15]. The extracts were stored at -20 ºC till estimation.

**Thin layer chromatography:** Thin-layer chromatography (TLC) of IAA was performed using silica gel TLC plates (Silica gel G f254, thickness 0.25 mm) and developed in ethyl acetate: chloroform: formic acid (55:35:10)[16]. Rf values of observed spots were compared with Rf value of authentic IAA spots.

**High pressure liquid chromatography:** The ethyl acetate extract of the isolates were analyzed by HPLC. HPLC chromatograms were produced by injecting 20 µl of the filtered extract onto a Cosmosil C-18 (150 mm x 4.6 mm, internal diameter) in a chromatograph equipped with an ultraviolet detector absorbing at 233 nm. The solvent system used to separate IAA was 0.1 M Potassium dihydrogen orthophosphate Buffer: methanol 65:35 (v/v), flow rate was 1 ml/min. The sample temperature was maintained at 4ºC.

HPLC results achieved of the extracts of isolates were compared and contrast with standard IAA[17].

## Results and Discussion

Selected leaves from the medicinal plant of *Withania somnifera* were surface sterilized and screened for endophytes. The isolated colonies were confirmed to be endophytic as the efficiency of sterilization was achieved to be 100%. As observed by the colony as well as gram's characteristics and using various biochemical tests, endophytes were characterized and were confirmed to belong to *Bacillus* sp. (figure 1)[18].

All isolated bacilli were selected and subjected to phase contrast microscopy to screen for the presence of endospore and parasporal body in order to distinguish *B. thuringiensis* from other *Bacillus* groups (figure 3). Phase contrast microscopy of slide of 50h old inoculate shows the presence of rod-shaped vegetative cells. Parasporal bodies were observed in 90h slides. The colonies were microscopically examined and all the isolates were observed to have visible parasporal inclusions and were thus classified as *B. thuringiensis* [19] and selected for further characterization.

All the isolates were further stained with Coomassie Brilliant Blue and viewed under Phase Contrast Microscopy. These isolates were confirmed as *B. thuringiensis* with prominently apparent parasporal bodies as dark stained blue objects (figures 2,3). Nucleotide sequence similarities of observed DNA sequences as determined by BLAST, version 2.2.28 (National Center for Biotechnology Information databases) further confirmed the presence of *B. thuringiensis* in the medicinal plant *Withania somnifera*.

These endophytic *B. thuringiensis* were screened for production of Indole-3-acetic acid. Preliminary screening of these *B. thuringiensis* shows their ability to produce more than 50µg/ml of Indole-3-acetic acid. HPLC of ethyl acetate extracts confirmed their ability to produce IAA. Thus all of the isolated endophytic *B. thuringiensis* had the ability to produce a major auxin, Indole-3-acetic acid.

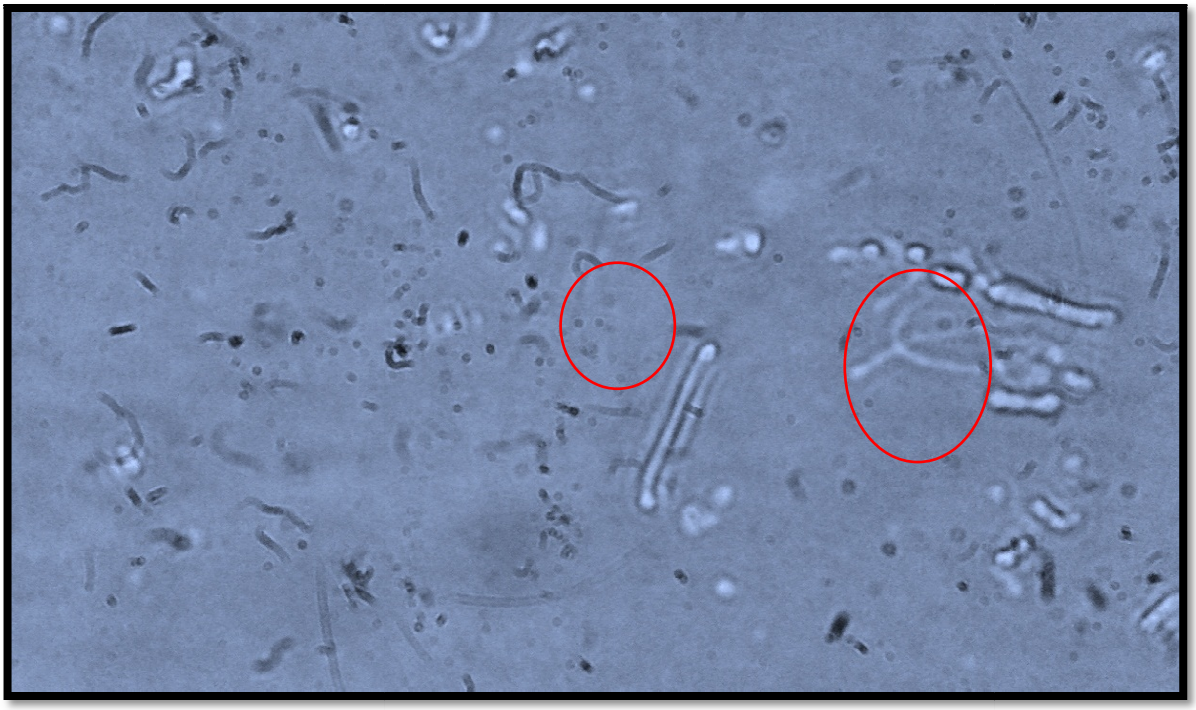

**Figure-1**
**The cells in the dark red circle represent the vegetative form of Bacillus cells at 1000 X magnification**

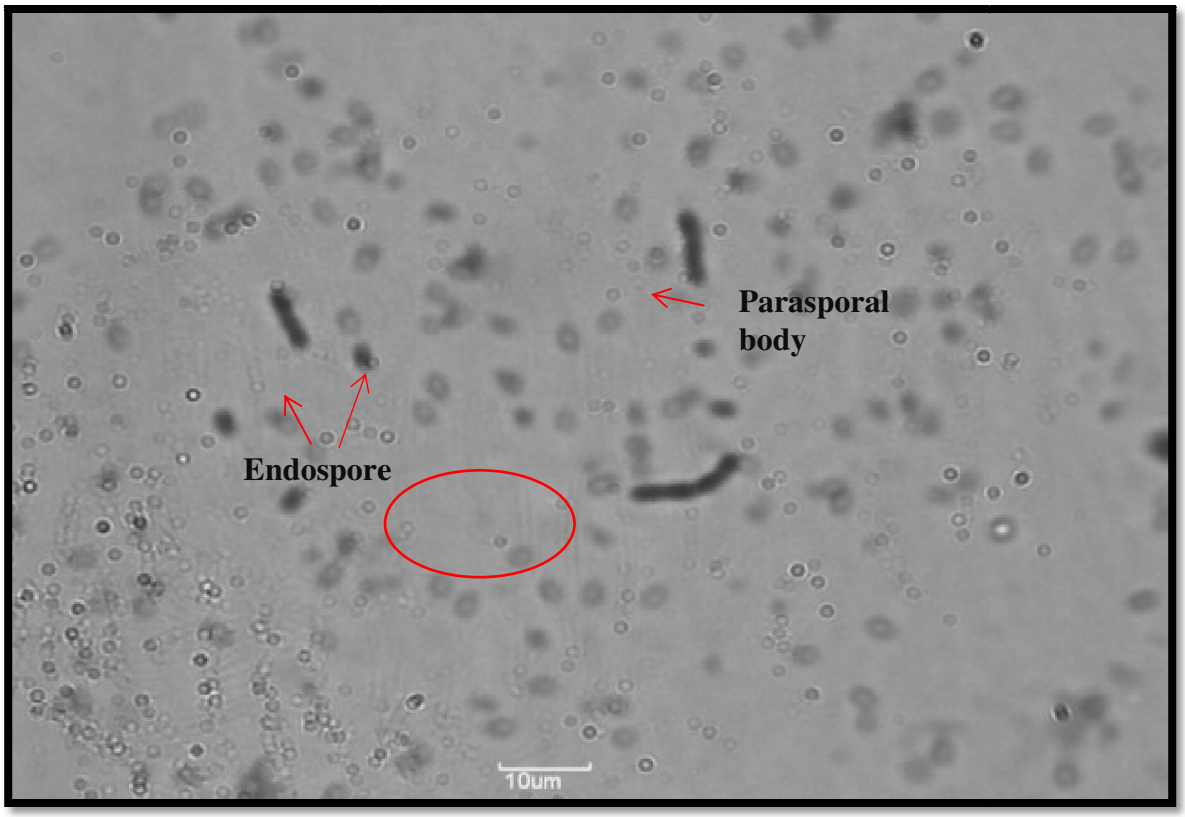


**Figure-2**
**The sporulated forms of *Bacillus* were shown in the dark red circle. The clear and bright portion is the spore whereas the parasporal body appears as dark blue, when observed under 1000X magnification**

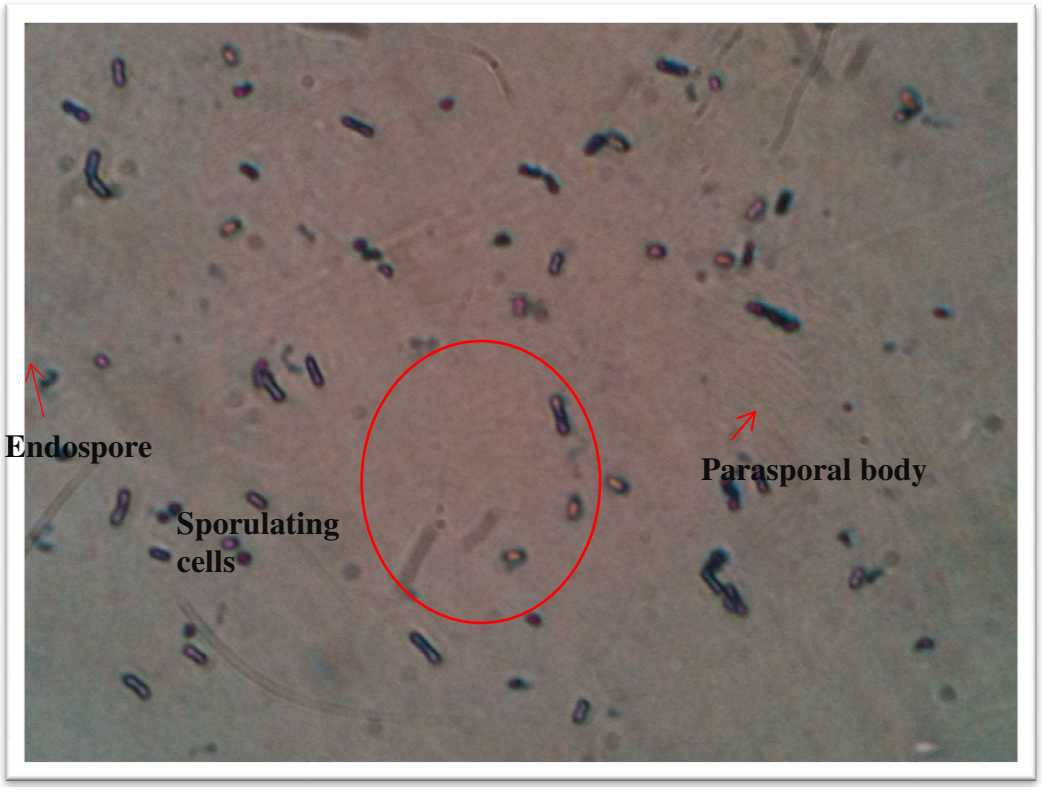


**Figure-3**
**Phase contrast microscopy of sporulated cells (in dark red circle), endospore formation and dark blue stained parasporal bodies (1000 x magnification)**

## Conclusion

This study demonstrates the occurrence and application of culturable endophytes. Presence of the most conventional biopesticide of *B. thuringiensis* in the leaf tissue of important medicinal plants, living as endophytes establishes a definite relation between the two. This novel plant-bacterial association has given new insight with the host medicinal plant having added advantages of inherent biocontrol agent benefitting the plant growth as well.

Medicinal plants with such novel bacteria can be explored for future research and application. The remarkable property of these naturally occurring endophytic microbial pesticides to produce phytohormone increases their interest as agricultural and biotechnological tool. This present research intrigues the interest to further investigate the molecular interdependence of *B. thuringiensis* and the host plants.

## Acknowledgement

We thank Ramnarain Ruia College, Matunga, Mumbai 400019, India for the facility provided. We are grateful to Microbial culture collection, National Centre for Cell Science *(*NCCS*),* Pune, India for all the help.

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