www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(2): 01-05

www.thepharmajournal.com
Received: 01-12-2021
Accepted: 03-01-2022

S Archana Devi
Department of Entomology, S.V. Agricultural College, Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

K Devaki
IFT, Regional Agricultural Research Station, Tirupati, Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

M Rajasri
Department of Entomology, S.V. Agricultural College, Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

MK Jyothsna
Krishi Vigyan Kendra, Kalikiri, Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

Corresponding Author
S Archana Devi
Department of Entomology, S.V. Agricultural College, Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

Collection and isolation of *Bacillus thuringiensis* Berliner from the soils of cruciferous vegetable growing areas in Andhra Pradesh

S Archana Devi, K Devaki, M Rajasri and MK Jyothsna

Abstract
In the present study *B. thuringiensis* was isolated from the soil samples of cruciferous vegetable growing areas in Andhra Pradesh, using sodium acetate selection method. The soil samples were buffered with 0.5M sodium acetate followed by heat shock at 80 ^{o}C and serial diluted up to 10^{-5} for eliminating non-spore forming bacteria and other microbes in the culture and then grown on LBA medium. After 24 hr of incubation, the colonies which were round, creamish with fried-egg appearance were produced. A total of 38 colonies from 50 soil samples were tested for Gram staining and observed 37 Gram positive bacteria with rod shaped structure. These 37 Gram positive cultures resulted in 22 endospore producing cultures and finally 15 crystal producing *B. thuringiensis* cultures were identified. Only bipyramidal types of crystalline inclusions were observed in the 15 new isolates of *B. thuringiensis.*

Keywords: *B. thuringiensis*, collection, isolation, soil samples

Introduction
Biological control is an important component of integrated pest management (IPM) in which microbial biocontrol plays a key role in the control of insect pests. Over 100 bacteria identified as insect pathogens, *Bacillus thuringiensis* Berliner (*Bt*) has got maximum importance as a microbial biocontrol agent (Muhammad *et al*., 2016) [19].
B. thuringiensis is a Gram positive, rod shaped, facultative aerobic, spore-forming saprophytic soil bacteria which constitutes 95% of all commercial bio-insecticides, due to its high specificity, safety and effectiveness in the control of wide spectrum of human disease vectors and agriculture-pests. Spore-formation enables *B. thuringiensis* to survive in harsh environments resulting in a ubiquitous distribution (Schunemann *et al*., 2014) [23]. Besides spore formation, insecticidal properties of *B. thuringiensis* are mainly attributed to the synthesis of insecticidal crystal proteins (ICPs) and vegetative insecticidal proteins (VIP), which are synthesized during sporulation and vegetative growth, respectively. Bravo *et al.*, 1998 [3] reported that, the crystal proteins toxic to lepidopteran insects belongs to the *cry*1, *cry*9, and *cry*2 groups, toxins active against coleopteran insects are *cry*3, *cry*7, and *cry*8 proteins, *cry*1B and *cry*1I proteins have dual activity. Among the Vip proteins, Vip1 and Vip2 are effective against Coleoptera and Hemiptera. The third group Vip3 is suitable for Lepidoptera which is having mode of action similar to Cry proteins in terms of proteolytic activation. The mode of action of Vip 4 is still unknown and the target group is also unidentified. *B. thuringiensis* strains have been isolated worldwide from many habitats, including soil, aquatic environments, dead insects and their breeding sites, herbivore faeces, stored-products, dust and deciduous and coniferous leaves (Cavado *et al*., 2001) [5]. A typical method of isolation involves heat treatment to select for spores, sometimes with an acetate enrichment step (Travers *et al*., 1987) [25], antibiotic selection (Yoo *et al*., 1996) [28] or non selective agar media (Chilcott and Wigley, 1993) [6].

Materials and Methods
The present study was carried out at the Department of Entomology, Institute of Frontier Technology, RARS, Tirupati

Collection of soil samples
A total of fifty soil samples were collected from five cruciferous crop growing districts *viz.*, Visakhapatnam, Vizianagaram, Krishna, Kurnool and Chittoor of Andhra Pradesh, of which

'11'samples were from Visakhapatnam, '13' samples from Vizianagaram, '10' samples from Krishna, '5' samples from Kurnool and '11' samples from Chittoor. These samples were used for the isolation of *B. thuringiensis.*

Sampling method

Surface soil was scraped off to avoid surface contamination and about 10 g of soil sample was taken from a depth of 5–10 cm with sterile spatula and subsequently transferred into sterile plastic bags and brought to the laboratory for further processing. These samples were stored at 4ºC until processed for isolation of *B. thuringiensis*.

Isolation of *B. thuringiensis* from Soil Samples

Modified Sodium acetate selection method given by Travers *et al*., 1987 [25] was followed for isolating *B. thuringiensis* from soil samples. Half a gram of soil sample was added to 10 ml of Luria Bertani broth (LB) in a 50 ml of conical flask. The Luria Bertani broth was buffered with 0.5 M sodium acetate. The mixture was kept on a shaker at 250 rpm for 4 h at 28°C. The sample was taken and subjected to heat shock at 80°C for 15 min. Each sample was then subjected to five ten-fold dilutions (from 10^{-1} to 10^{-5} dilutions). One ml of heat shocked culture broth was taken and mixed with 9 ml of sterile distilled water to get 10^{-1} dilution. From 10^{-1} dilution, one ml was taken and mixed with 9 ml of sterile distilled water to get 10^{-2} dilution. This is repeated up to 10^{-5} dilutions. One hundred micro litre of 10^{-5} dilution was spread on Luria Bertani (LB) agar media petriplates with 'L' rod for bacterial growth. Then the plates were incubated at 37°C for overnight.

Staining and Microscopic observations of strains for identification of *B. thuringiensis*

After incubation, bacterial colonies were selected based on the typical morphological characteristics of *B. thuringiensis*, creamish coloured colonies having appearance of fried egg (Figure 1). These selected colonies were then sub-cultured onto new Nutrient Agar plates by repeated four way streaking (Merdan *et al*., 2010) [17] and incubated at 37 ºC.

Smears of bacterial cultures were prepared and subjected to Gram staining, endospore staining and crystal staining for identification of *B. thuringiensis*. Gram staining of bacteria was done by following Hucker's method as described by Cappuccino and Sherman (1992) [4]. Gram positive cells appear violet colour under microscope. Gram positive cultures were streaked on T_3 medium for sporulation. After 48 hr of incubation, crystal protein staining was performed according to the protocol given by Sharif and Alaeddinoglu (1988) [24] and they appear as dark blue coloured crystals under microscope. Endospore staining was done by using Schaeffer-Fulton method. Under the microscope, the endospores appear as green and the vegetative cells will appear red or pink (Lalitha, 2012) [15].

Results and Discussion

Out of 50 soil samples, 38 samples have shown creamy white, flat bacterial colonies with fried egg appearance, of which 10 were from Visakhapatnam, 11 from Vizianagaram, 7 from Krishna, 4 from Kurnool and 6 from Chittoor.

Out of 37 samples produced rod shaped gram positive bacteria, 10 (90.9%), 11 (84.61%), 6(60.0%) 4 (80.0%) and 6 (54.54%) were positive in Gram staining with rod shaped bacterial cells after 18 hours of incubation from the soil samples of Visakhapatnam, Vizianagaram, Krishna, Kurnool and Chittoor, respectively (Table1 and figure 2). The present results are in line with earlier findings of Lalitha, (2012) [15]; Devaki, (2017) [8], Zhenxiang *et al.* (2018) [29], Kesini, (2020) [14] who reported that the bacterial colonies having colony morphology of creamy white or gray-white, round, opaque, flat, drying, medium-sized are positive in Gram staining.

Among the 37 gram positive cultures tested for endospore production, a total of 22 cultures were able to produce endospores from Visakhapatnam, Vizianagaram, Krishna, Kurnool and Chittoor respectively. The gram positive samples obtained from Kurnool district produced highest per cent endospores (80.00%), followed by 54.54, 45.45, 30.76 and 30.00 per cent in Chittoor (6), Visakhapatnam (5), Vizianagaram (4) and Krishna (4) districts respectively (Table 2 and figure 3). Similarly, Cetinkaya (2002) observed 136 *B. thuringiensis* colonies with subterminal endospores out of 359 isolates.

In total, 15 samples which were identified with parasporal bodies had stained dark blue colour. Among the 15 *B. thuringiensis* crystal positive strains tested for the presence of parasporal inclusions or crystal proteins soil samples from Visakhapatnam harboured high frequency of 45.45 per cent crystal positive *B. thuringiensis* strains followed by , 40 per cent from Krishna, 30.76 per cent from Vizianagaram and 18.18 per cent from Chitoor (Table3 and figure 4). The microscopic observation of the crystals revealed the presence of small and medium bipyramidal crystals. Out of 22 endospore positive strains, 15 strains were able to produce bipyramidal crystals of which 5 from Visakhapatnam, 4 from Krishna, 4 from Vizianagaram and 2 from Chitoor.

There was no variation among the 15 *B. thuringiensis* crystal positive strains regarding crystal morphology in the present study. All 15 crystal positive strains showed the presence of bipyramidal crystals. Similar to the results of present studies, Wangondu *et al.* (2003) [26] and Harpal Singh, (2005) [12] obtained only bipyramidal inclusions. Although the relationship between the type of crystal morphology and the level of insecticidal activity is not clear, it has been reported that the strains with bipyramidal crystals are more toxic to lepidopteran larvae (Obeidal *et al.*, 2004, Asokan and Puttaswamy, 2007, Monnerat *et al*., 2007 and Opondo *et al*., 2010) [20, 2, 18, 22].

The overall recovery of *B. thuringiensis* strains from soil samples in this study was similar to Liang *et al*. (2011) [16] who reported a 30 per cent *B. thuringiensis* positive strains in their collection, followed by 23.11 per cent (Devaki, 2017) [8], 19.7 per cent (Johnson and Bishop, 1996) [13], 12.5 per cent (Ammouneh *et al*.. 2011) [1], 10 per cent (Ohba and Aizawa (1986) [21] and Ejiofor and Johnson (2002) [11], 2.1 per cent (Meadows *et al.*, 1992 and Dias *et al*., 1999) [9] and 1.0 per cent (Donovan *et al.*, 1988) [10], 0.5 per cent (Delucca *et al.*, 1981) [7], but it was lesser than Yadav *et al*. (2007) [27] who reported 82.60 per cent and 32 per cent (Kesini, 2020) [14].

Table 1: List of Gram-positive bacterial cultures isolated from the soils of cruciferous vegetables growing areas in Andhra Pradesh

S. No	District	Isolate code	Total No of soil Samples	No of Isolates	Gram positive Samples (%)
1	Visakhapatnam	VP1,VP2,VP3,VP4,VP5,VP6,VP7,VP8,VP9,VP10	11	10	90.90
2	Vizianagaram	VZ12,VZ13,VZ14,VZ15,VZ16,VZ17,VZ18,VZ19,VZ20,VZ22,VZ23	13	11	84.61
3	Krishna	KR30,KR31,KR33,KR34,KR35,KR37	10	06	60.00
4	Kurnool	KN38, KN40, KN41, KN42	05	04	80.00
5	Chittoor	CT43, CT44, CT45, CT46, CT47, CT49	11	06	54.54
	Total		50	37	74.00

Table 2: List of Endospore positive bacterial cultures isolated from the soils of cruciferous vegetables growing areas in Andhra Pradesh

S. No	District	Isolate code	Total No of Soil Samples	No. of Endospore Producing samples	Per cent Endospore Positive strains
1	Visakhapatnam	VP1, VP3, VP4, VP5, VP7	11	05	45.45
2	Vizianagaram	VZ12, VZ14, VZ19, VZ22	13	04	30.76
3	Krishna	KR30, KR33, KR34	10	03	30.00
4	Kurnool	KN38, KN40, KN41, KN42	05	04	80.00
5	Chittoor	CT43, CT44, CT45, CT46, CT47, CT49	11	06	54.54
	Total		50	22	44.00

Table 3: List of Crystal positive bacterial strains isolated from the soils of cruciferous vegetables growing areas in Andhra Pradesh

S. No	District	Isolate code	Total No of Samples	Crystal positive strains	Per cent crystal positive strains
1	Visakhapatnam	VP1, VP3, VP4, VP5, VP7	11	05	45.45
2	Vizianagaram	VZ12, VZ14, VZ19, VZ22	13	04	30.76
3	Krishna	KR30, KR33, KR34, KR37	10	04	40.00
4	Kurnool	-	05	00	0.00
5	Chittoor	CT45, CT47	11	02	18.18
	Total		50	15	30.00

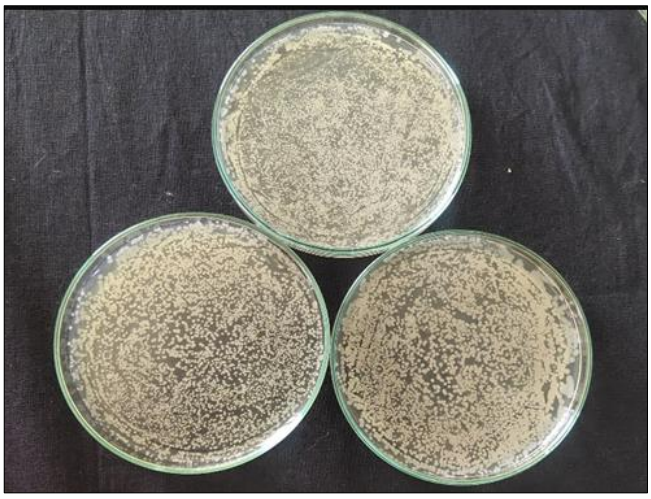

Fig 1: Colony morphology of *Bacillus thuringiensis*

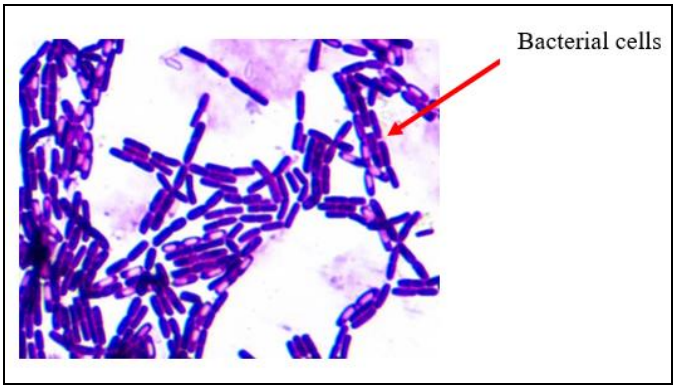

Fig 2: Microscopic observations of Gram stained cultures (Magnification X 1000)

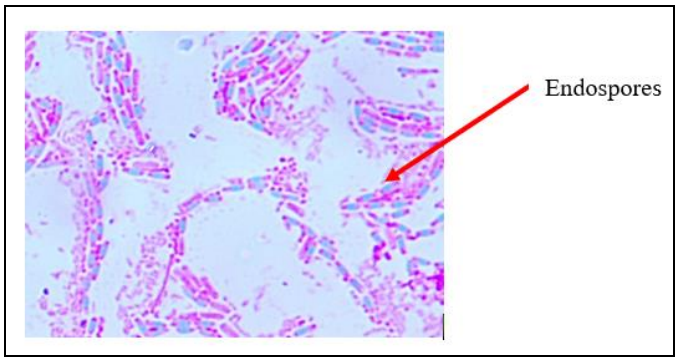

Fig 3: Microscopic observations of endospore stained cultures (Magnification X 1000)

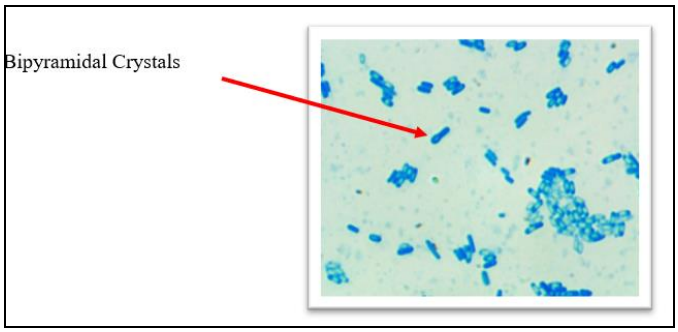

Fig 4: Microscopic observations of crystal stained cultures (Magnification X 1000)

Conclusion

Fifteen *B. thuringiensis* strains were isolated and identified through Gram staining, endospore staining and crystal staining from 50 soil samples collected from cruciferous vegetables growing areas in Andhra Pradesh. All the fifteen *B. thuringiensis* strains were identified with bipyramidal

crystals based on microscopic observations. Soil samples From Visakhapatnam, harboured high frequency of 45.45 per cent crystal positive *B. thuringiensis* strains followed by, 40 per cent from Krishna, 30.76 per cent from Vizianagaram and 18.18 per cent from Chitoor. A future study can be conducted on the efficiency of new *B. thuringiensis* strains against other major insect pests *viz.*, *Spodoptera litura*, *S. frugiperda*, *H. armigera* and *Tuta absoluta.*

References

1. Ammouneh, Hassan, Muhanad Harba, Emad Idris, Hayat Makee. Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. Turkish Journal of Agriculture and Forestry. 2011;35(4-9):1007-1117.
2. Asokan, Puttaswamy R. Toxicity of different isolates of *Bacillus thuringiensis* to the larvae of diamondback moth, *Plutella xylostella* (L). Journal of Biological Control. 2007;21:305-308.
3. Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, *et al*. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Applied Environmental Microbiology. 1998;64:4965-4972.
4. Cappuccino JG, Sherman N. Microscopy a laboratory manual. Third Edition. The Benjamin publishing company, Inc. New York, USA, 1992, 521.
5. Cavado CFG, Fonseca RN, Chaves JQ, Rabinovitch L, Araujo-Coutinho C JPC. Identification of entomopathogenic *Bacillus* isolated from Simulium (Diptera, Simulidae) larvae and adults. Memories of the Oswaldo Cruz Institute. 20019;6:1017–1021.
6. Chilcott CN, Wigley PJ. Isolation and toxicity of *Bacillus thuringiensis* from soil and insect habitats in New Zealand. Journal of invertebrate pathology. 1998;61:244-247.
7. Delucca AJ, Simonson ZG, Larson AD. *Bacillus thuringiensis* distribution in soils of the US states. Canadian Journal of Microbiology. 1981;27:865-870.
8. Devaki K. Molecular Characterization of *Bacillus thuringiensis* cry Genes with insecticidal activity against *Spodoptera litura* in groundnut. Ph.D. Thesis. Acharya N G Ranga Agricultural University, Guntur, 2017.
9. Dias SC, Sagardoy MA, Silva SF, Dias JM. Characterization and pathogenic evaluation of *Bacillus thuringiensis* and *Bacillus sphaericus* isolates from Argentinean soils. Biocontrol. 1999;44:59-71.
10. Donovan WP, Gonzalez JM Jr, Gilbert MP, Dankocsik C. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. Molecular Genetics and Genomics. 1988;214:365-372.
11. Ejiofor AO, Johnson T. Physiological and Molecular Detection of Crystalliferous *Bacillus thuringiensis* Strains from Habitats in the South Central United States. Journal of Industrial Microbiology and Biotechnology. 2002;28:284-290.
12. Harpal Singh. Bioefficacy of native isolates of *Bacillus thuringiensis* Berliner against rice leaf folder, *Cnaphalocrocis medinalis* (Guenee). Ph.D. Thesis. Punjab Agricultural University, Ludhiana, Punjab, 2005.
13. Johnson C, Bishop AH. A technique for the effective enrichment and isolation of *Bacillus thuringiensis*. FEMS Microbiology Letters. 1996;142:173-177.
14. Kesini B. Isolation and molecular characterization of *Bacillus thuringiensis* virulent to *Spodoptera Frugiperda* (J.E. Smith) From Tirupati. M.Sc. thesis. Acharya N G Ranga Agricultural University, Guntur, 2020.
15. Lalitha C. Isolation and identification of novel *Bacillus thuringiensis* cry genes with insecticidal activity against *Spodoptera litura* and *Helicoverpa armigera* through molecular approaches. Ph.D. thesis. ANGRAU, Hyderabad, 2012.
16. Liang H, Liu Y, Zhu J, Guan P, Li S, Wang S, Zheng A, Liu H, Li P. Characterization of *cry*2-type genes of *Bacillus thuringiensis* strains from soil- isolated of Sichuan basin. Brazilian Journal of Microbiology. 2011;42:140-146.
17. Merdan A, Salama HS, Labib E, Ragaei, Mand AEG. *Bacillus thuringiensis* isolates from soil and diseased insects in Egyptian cotton fields and their activity against lepidopteran insects. Archives of phytopathology and plant protection. 2010;43(12):1165-1176.
18. Monnerat RG, Batista AC, Medeiros PT, Martins ES, Melatti VM, Prac LB, *et al*. Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis.* Biological Control. 2007;41:291-295.
19. Muhammad N, Juma IM, Hongxia H. Current status and advancement of biopesticides: Microbial and botanical pesticides. Journal of Entomology and Zoology Studies. 2016;4(2):241-246.
20. Obeidal M, Al-Momani F, Saadoun I. Diversity of *Bacillus thuringiensis* in different habitats of northern Jordan. Journal of Basic Microbiology. 2004;40:385-388.
21. Ohba M, Aizawa K. Insect toxicity of *Bacillus thuringiensis* isolated from soils of Japan. Journal of Invertebrate Pathology. 1986;47:12-20.
22. Opondo SO, Ngure RM, Nguu EK, Chanda JO, Ogoyi DO. Molecular characterization of *Bacillus thuringiensis* strains with differential toxicity to the spotted stalk borer, *Chilo partellus.* Journal of Applied Bioscience. 2010;31:1878-1886.
23. Schünemann R, Knaak N, Fiuza LM. Mode of action and specificity of *Bacillus thuringiensis* toxins in the control of caterpillars and stink bugs in soybean culture. ISRN Microbiology, 2014, 1-12.
24. Sharif FA, Alaeddinoglu NG. A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. Journal of Industrial Microbiology. 1988;3:227-229.
25. Travers RS, Martin PAW, Reicnel Dorfer CF. Selective process for efficient isolation of soil *Bacillus* sps. Applied and Environmental Microbiology. 1987;53:1263-1265.
26. Wangondu VM, Kahin Di JHP, Olembo NK, Ochanda J. Isolation and characterization of *Bacillus thuringiensis* strains from soil samples from kakamega and Machakos districts in Kenya. Journal of Tropical Microbiology. 2003;2(1):3-10.
27. Yadav BP. Molecular characterization of cry / vip genes and efficacy of native *Bacillus thuringiensis* isolates. M.Sc. Thesis, University of Agricultural Sciences, Dharwad, India, 2007.
28. Yoo KH, Kim SY, Kang MH, Cho MH, Lee HH. Characterization of *Bacillus thuringiensis* isolates from soil in Wonju Area. Journal of Microbiology. 1996;34:370-37.

29. Zhenxiang LU, Weina GU, Chang LU. Isolation, identification and characterization of novel *Bacillus subtilis*. The Journal of Veterinary Medical Science. 2018;80(3):427-433.