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# Molecular Characterization of *Bacillus thuringiensis* Strains from Native Soils in Southern Zone of Andhra Pradesh, India

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***Abstract*:** In search of novel insecticidal *Bacillus thuringiensis* strains effective against *Spodoptera litura,* a total of 44 cultures were isolated from 205 soil samples collected in Nellore district, Andhra Pradesh, India and evaluated against third instar *Spodoptera litura*. The isolates exhibiting more than 50 per cent larval mortality were chosen for molecular characterization with 14 *cry* gene primers specific to Lepidoptera. The bioassay studies indicated that, larval mortality of 86.67 per cent was observed with N30 at 168h after treatment, followed by N3 with 83.33 per cent which were comparable with standard check HD-1 (96.67%). The Other promising isolates, N44, N48 (%), N58 %), N93 %), N115 (%) and N141 which were statistically on par with each other. Molecular characterization of virulent *B.thuringiensis* strains with *cry* gene specific primers indicated that *cry*1I gene was predominant in present *B. thuringiensis* collection and it was reported from seven (N20, N23, N30, N63, N93, N141, N143) strains. The most effective strain, N30 harboured a combination of three *cry* genes *i.e.*, *cry*1, *cry*2 and *cry*9. N3 contained only one *cry* gene (*cry*1C). Among the 19 strains characterized, N93 possessed the highest diversity with five *cry* genes (*cry*1Aa, *cry*1C, *cry*1I, *cry*2A(a)1, *cry*9Ca1). The study identified potential *B. thuringiensis* strains with diverse *cry* gene profiles encoding insecticidal crystal proteins. The presence of multiple *cry* genes (*cry*1, *cry*2 and *cry*9) in N30 likely contributed to its superior efficacy against test insect, *S. litura*. This strain shows promise for development into a cost effective *B. thuringiensis* formulation for use as a biopesticide and may also serve as a valuable resource for developing *Bt* transgenic crop plants.

***Keywords*:** *Bacillus thuringiensis*, *Cry* genes, Environmental isolates, *Spodoptera litura*

*Bacillus thuringiensis* (Berliner) is an agriculturally important microbe having key role in biocontrol of insect pests. The bacterium produces specific crystalline protein inclusions (δ-endotoxin) during sporulation, which are toxic to insect pests belong to the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga (Alsaedi et al., 2017). This unique insecticidal property has made *B. thuringiensis* the most widely used microbial biopesticide in the world market. In addition to δ-endotoxins, *B. thuringiensis* also synthesizes insecticidal proteins during its vegetative growth phase, which are subsequently secreted into the culture medium. These proteins are commonly known as vegetative insecticidal proteins (Vips) and exhibit potent insecticidal activity against several lepidopteran, coleopteran and some homopteran pests.

*Bacillus thuringiensis* is a rod shaped, Gram-positive, filiform, spore forming bacterium, that is ubiquitous in nature. This organism has been isolated worldwide from a great diversified environments including soil, water, dead insects, dust from silos, leaves from deciduous trees, diverse conifers, and insectivorous mammals, and even from human tissues with severe necrosis (Palma et al., 2014). Among the various sources for *B. thuringiensis* isolation, soil is the most suitable media for collecting diverse isolates. There are several *B. thuringiensis* collections across the world, which are being tested against a number of insect pests. Li et al. (2007) reported that, *B. thuringiensis* isolates are distributed worldwide, and more than 60,000 have been collected by various industries in an effort to obtain novel crystal proteins. Each *B. thuringiensis* isolate produces different types of crystal toxins either alone or in combination and exhibits specific activity against one or a few related species of insects. Therefore, large-scale screening programmes have been conducted worldwide, leading to important collections of isolates from different environments and characterized to evaluate their toxic potential against various insect orders (Quesada and Valverde 2004, Ramalakshmi and Udayasuriyan 2010). The studies were conducted at Regional Agricultural Research Station, Tirupati to isolate and characterize the environmental *B. thuringiensis* strains from soil samples collected in Nellore district of Andhra Pradesh, India for their potential to control *Spodoptera litura* (Fabricius).

## MATERIAL AND METHODS

**Collection, isolation and identification of *B. thuringiensis* from soil samples:** During the study, 205 rhizosphere soil samples were collected from various locations in Nellore district of Andhra Pradesh covering crop fields, waste lands, virgin soils and other habitats. Samples were collected aseptically in sterile polythene bags. Sodium acetate selection method was adopted for isolating *B. thuringiensis* with slight modifications (Santana et al., 2008, Devaki et al., 2020). Half a gram of soil sample was added to 10 ml of Luria Bertani (LB) broth in a 100 ml conical flask

along with 0.5 M sodium acetate. The mixture was kept on a shaker for 4h at 250 rpm at room temperature and subjected to heat shock at 80°C for 15 min and plated at $10^{-5}$ dilution on LB agar (LBA) medium. Cream coloured colonies with a characteristic 'fried egg' appearance were picked up after 18h of incubation on LB agar media, followed by Gram's staining. The Gram positive cultures grown on T3 media for 72 h for crystal and endospore production were further characterized for the presence or absence of crystal proteins and endospores using standard protocols (Jisha and Benjamin 2014). The Gram positive culture which were confirmed for crystal and endospore production were further purified by repeated four-way streaking (Merdan et al., 2010) on pre-pasteurized LB agar plates. The cultures which were observed positive in Gram's staining, crystal and endospore staining were further used in bioassay studies.

**Bioassay of *B. thuringiensis* against *S. litura:*** Fourty four *B. thuringiensis* strains were assayed to ascertain larvicidal activity against third instar larvae of *S. litura*. The standard *B. thuringiensis* (HD1) strain obtained from National Beaureau of Agricultural Insect Resources (NBAIR), Bengaluru was used as a reference strain. Individual *B. thuringiensis* culture was streaked on plain LB agar plates and incubated overnight at 37°C. One loop of the overnight culture was inoculated in LB broth and kept for sporulation under shaking conditions (250 rpm) at 28°C for 72 h. The culture containing spore crystal mixture was pelletized in Eppendorf centrifuge at 10000 rpm for 10 min. The resultant pellet was diluted in sterile water, plated on LBA and colony counts were taken for fixing the doses.

The bioassay was conducted by leaf dip method at a concentration of 5 -$10^8$ CFU/ml (Devaki et al., 2017). Groundnut compound leaves containing four leaflets were dipped into culture broth containing 0.2 per cent Triton X-100 for 10 minutes and air-dried. After drying, leaf petiole was swabbed with wet cotton to maintain leaf turgidity and dip the leaf in *B. thuringiensis* isolates with different dilutions. One treated groundnut leaf for one replication was placed in a petriplate. Ten third instar larvae were released per replication. HD-1 served as a reference strain. The leaves dipped in distilled water served as control. The larval mortality was assessed after 72 h at regular intervals till pupation and $LC_{50}$ values were determined using probit analysis. Isolates with more than 75 per cent mortality (N3, N30, N44, N48, N58, N93, N115) along with HD1 were tested for determining lethal concentrations following the same leaf dip procedure. Five concentrations viz., $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ CFU/ml were used for carrying probit analysis and $LC_{50}$ determination.

**DNA extraction and PCR amplification:** Genomic DNA was isolated from 18 *B. thuringiensis* strains with ≥ 75% larval mortality of III instar *S. litura* by adopting the protocol of Sambrook and Russell (1998). The resultant DNA was verified for its quality and purity through agarose gel electrophoresis as well as nanodrop spectrophotometer. A total of 14 cry gene-specific primers (*cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1Da1, *cry*1Ea1, *cry*1F, *cry*1Fa1, *cry1I*, *cry*2, *cry*2A(a)1, *cry*8, *cry*9Aa1 and *cry*9Ca1) were used in the study. The primers were synthesized with the known sequences available in the literature (Table 1). The conditions like initial denaturation, annealing temperature, elongation and final extension were standardized by using gradient PCR. Composition of the reaction mixture was also standardized and a final proportions of PCR reagents and buffers were as follows; Taq assay buffer (10X) 2.5 µl, dNTP's (2 mM) 1.5 µl, 2.5 µl of each forward and primer, $MgCl_2$ (2.0 mM) 1.5 µl, Taq DNA polymerase (3 U /µl) 0.5 µl, Template DNA (100 ng) 2.5 µl and made to a final volume of 25 µl. Thermal cycling was fixed for each primer in Biorad thermal cycler and resultant PCR product was ran through electrophoresis on 1% agarose gel. The particulars of primer sequencing, annealing temperature, and PCR recipe are listed in the Table 1, 2 and 3.

## RESULTS AND DISCUSSION

**Isolation and screening of *B. thuringiensis* from soil samples:** Out of 205 samples collected from diverse soil environments screened for bacterial cultures, a total of 68 samples were identified as Gram positive organisms. Among these, 48 isolates were capable of producing endospores, of which 44 were confirmed as crystal positive *B. thuringiensis* strains. Based on crystal morphology, 16 produced spherical crystals, 11 irregular, 7 bipyramidal, 6 cuboidal and 4 showed a combination of bipyramidal & cuboidal forms. All these 44 crysal producing strains were used in the laboratory bioassays against third instar larvae of *S. litura*.

**Bioassay of *B. thuringiensis* isolates:** In the bioassay, larval mortality was recorded from 72 h to 168 h after treatment and the mortality was ranged from 0.0 to 50.0, 0.0 to 36.67, 0.0 to 36.67, 0.0 to 26.67 and 0.0 to 33.33 per cent at 72, 96, 120, 144 and 168 hours after treatment, respectively (Table 4). At 72 hours after treatment, among the 44 isolates, N115 recorded the highest larval mortality of 40 per cent which was statistically on par with standard check HD-1. The next best treatments were N30, N48, (N58 %), N69 , N100 , N118 and N93 and all statistically at par with each other. Whereas, at 96 hours after treatment, isolates N3, N45, N58, N100, N189 each recorded 23.33 per cent mortality and were d significantly superior over the other treatment. At 120 hours after treatment, isolates N173, N141, N93, N3, N115

were effective and on par with each other. Subsequently, N44 (26.67%) and N34 (33.33%) recorded higher larval mortality at 144 and 168 hours after treatment, respectively.

Cumulative mortality data revealed that isolate N30 caused the highest larval mortality (86.67%), followed by N3 which were comparable with standard check HD-1 (96.67%). The other treatments in the order of efficacy was N44, N48, N58, N93, N115, and N141, which were statistically similar in their efficacy. The rest isolates were no way superior to untreated check (Table 3). Probit analysis revealed that the lowest $LC_{50}$ was observed for N30 (1.90-$10^5$CFU/ml) followed by N3 (2.18- $10^5$ CFU/ml), which recorded quicker lethal time to kill 50 per cent population ($LT_{50}$ = 95.70 h). In standard check HD1 recorded the lowest $LC_{50}$ and $LC_{90}$ values with $LT_{50}$ and $LT_{90}$ of 61.99 h and 121.64 h, respectively (Table 4a).

**Molecular characterization of *B. thuringiensis* isolates:** Molecular characterization of 19 *B. thuringiensis* strains native to Nellore district soil samples, revealed distinct *cry*

**Table 2.** PCR reaction mixture for amplification of native *B. thuringiensis* strains with specific *cry* primers

| Reagents | Volume/reaction |
|---|---|
| Taq assay buffer (10X) | 2.5 µl |
| dNTP's (2 mM) | 1.5 µl |
| Forward primer (5 pmole) | 2.5 µl |
| Reverse primer (5 pmole) | 2.5 µl |
| $MgCl_2$ (2.0 mM) | 1.5 µl |
| Taq DNA polymerase (3 U /µl) | 0.5 µl |
| Template DNA (100 ng) | 2.5 µl |
| Sterile distilled water | 11.5 µl |
| Total | 25.0 µl |

**Table 1.** Details of *cry* primers used in the study

| Primer name | Forwardion | Primer sequence | Reference |
|---|---|---|---|
| *cry*1Aa | Forward | ATTATCATATTGATCAAGTTC | Salek et al., 2012 |
| | Reverse | CATAAGGAACCCGTACCTGG | |
| *cry*1Ab | Forward | GGACCAGGATTTACAGGAGG | -do- |
| | Reverse | GTTCTCCTACTAATGGTTTCC | |
| *cry*1Ac | Forward | CTCAATGGGACGCATTTCTT | -do- |
| | Reverse | CGGTTGTAAGGGCACTGTTC | |
| *cry*1C | Forward | AAAGATCTGGAACACCTTT | Ceron et al., 1994 |
| | Reverse | CAAACTCTAAATCCTTTCAC | |
| *cry*1Da | Forward | GTAGCAGACATTTCATTAGG | Pooja et al., 2013 |
| | Reverse | ACATGAATAAGGCTAGTCAG | |
| *cry*1Ea1 | Forward | ATATAGAAGTAGGGGGACAG | -do- |
| | Reverse | TAGCCCTAGTTGATTTGTAG | |
| *cry*1Fa1 | Forward | GATTTGCTAATACAGACGAC | -do- |
| | Reverse | CGTGAACTCACTAAGTGTCC | |
| *cry*1F | Forward | TGTAGAAGAGGAAGTCTATCCA | Ceron et al., 1994 |
| | Reverse | TATCGGTTTCTGGGAGTA | |
| *cry*1I | Forward | AGCTATGGCCTAAGGGGAAA | Nariman, 2007 |
| | Reverse | TTCCAACCCAACTTTCAA | |
| *cry*2 | Forward | GTTATTCTTAATGCAGATGAATGGG | Ben-Dov et al., 1997 |
| | Reverse | CGGATAAAATAATCTGGGAAATAGT | |
| *cry*2A(a) | Forward | AAGGAGGAATTTTATATGAA | Ogunjimi et al., 2002 |
| | Reverse | CATTTAGTTCCGTCAATATG | |
| *cry*8 | Forward | ATGAGTCCAAATAATCTAAATG | Bravo et al., 1998 |
| | Reverse | TTTCATTAATGAGTTCTTCCACTCG | |
| *cry*9Aa1 | Forward | ATCGTAGAGAGTGACATTG | Pooja et al., 2013 |
| | Reverse | TGTTGTCCAGAGATTAGTTC | |
| *cry*9Ca1 | Forward | GGATCTAAATGCAAGTGTAG | -do- |
| | Reverse | ACCATTTACATCGTAGTCAC | |

gene profiles. Among these, seven (N20, N23, N30, N63, N93, N141, N143,) *B. thuringiensis* strains harboured *cry*1I genes, six strains (N45, N69, N93, N130, N141, N143) contained *cry*1Aa and four strains (N3, N93, N100, N115) possessed *cry*1C. Three strains were positive for *cry*1Da1 (N44, N69, N96) and *cry*1Fa1 (N141, N143, N158). Only one strain N69 harboured *cry*1Ea1 gene (Tables 5 , 6). Among the *cry*2 group positive isolates, one strain (N20) harboured *cry*2 and five strains (N23, N30, N58, N93, N100) harboured both *cry2* and *cry*2A(a)1 genes. Four strains (N23, N30, N93, N141) possessed *cry*9Ca1 gene. None of the isolates in the present study tested positive for *cry*1Ab, *cry*1Ac*, cry*1F, *cry*9Aa1, *cry*8 genes. There was no definite relationship between the isolate efficacy and number of crystal proteins encoding *cry* genes. However, isolates harbouring multiple *cry* genes tended to exhibit higher larvicidal activity. One representative strain N115 was sequenced using 16S rRNA primer, the gene sequence was deposited in NCBI GenBank with accession Number MF487791. The highest mortality of 86.67 and 83.33 per cent mortality was observed in *B. thuringiensis* isolates, N30 and N3, respectively which were statistically on par with the HD1. The present studies are in line with the earlier studies made by Meihiar et al. (2015) who reported that, forest, beach and cultivated soils had more *B. thuringiensis* strains than uncultivated and interior arid soils. The frequency of *B. thuringiensis* occurrence was partially dependant on organic matter and pH content of the soil, with about 65 per cent of the isolates found toxic to *Galleria mellonella*. The most toxic isolate of *B. thuringiensis* was obtained from cultivated area and produced bipyramidal, cuboidal and rectangular inclusions.

**Table 3.** PCR conditions for each set of *cry* primers used in the analysis

| Step | | Temperature (°C) | Duration (min) | No. of cycles |
|---|---|---|---|---|
| Initial denaturation | | 94.0 | 10 | 1 |
| Denaturation | | 94.0 | ½ | 35 |
| Annealing | *cry*1Aa | 48.9 | 1 | 35 |
| | *cry*1Ab | 53.4 | | |
| | *cry*1Ac | 54.4 | | |
| | *cry*1C | 52.0 | | |
| | *cry*1Da 1 | 51.3 | | |
| | *cry*1Ea1 | 51.3 | | |
| | *cry*1Fa1 | 50.3 | | |
| | *cry*1F | 52.0 | | |
| | *cry*1I | 47.3 | | |
| | *cry2* | 57.2 | | |
| | *cry*2Aa | 46.2 | | |
| | *cry*8 | 49.0 | | |
| | *cry*9Aa1 | 51.0 | | |
| | *cry*9Ca1 | 52.3 | | |
| | 16s rRNA | 49.0 | | |
| Extension | | 72.0 | 2 | 35 |
| Final extension | | 72.0 | 10 | 1 |
| Hold | | 4.0 | ∞ | - |

Similarly, Sharma (2000) reported a mortality of 66.66 to 100 per cent with five *B. thuringiensis* formulations against *S. litura* and *Spilarctia obliqua* under controlled conditions at 26±1°C and 75 per cent relative humidity which were on par with endosulfan. Further, Nariman et al. (2009) reported a higher mortality of 100 per cent and 90 per cent of second instar *S.littoralis* with two *B. thuringiensis* isolates Ts-5 and As-3, respectively collected from seven governorates of Egypt. Lalitha et al. (2012) also reported a mortality of 16.67 to 94.44 per cent with *B. thuringiensis* isolates against second instar larvae of *H. armigera* where *B. thuringiensis* isolates 122 and 22 recorded 83.33 per cent mortality and was statistically on par with HD-1. At the same time Al-Otaibi (2013) reported a higher positive efficiency of *B. thuringiensis* isolates in spore+ crystal mixtures @ $10^9$ CFU/ml at 168h after treatment against second instar *S. littoralis* larvae. Ricardo et al. (2000) also reported a mortality of 100 and 80.4 per cent in second instar larvae of *S. frugiperda* with the suspensions of *Bt aizawai* HD 68 and *Bt thuringiensis* 4412, respectively, containing 3- $10^8$ cells/ml. Azzouz et al. (2014) also confirmed a higher toxicity of *B. thuringiensis* against *S. littoralis* while Pooja et al. (2013) reported a cumulative mortality of 83.33 per cent with the *B. thuringiensis* isolate DBT153 against *Plutella xylostella* collected from hill ecosystems in Coorg district. Devaki et al. (2017) reported a cumulative mortality of 83.33 per cent with *B. thuringiensis* strains viz., C79, C97, C134 and C212 against third instar larvae of *S. litura* at 5 x $10^5$ CFU/ml. As against this, Mohan et al. (2014) reported a low toxicity of *B.thuringiensis* HD-1 strain against *S. litura* and non-toxic effects of HD-73 strain against *S. litura* and *S. oblique*. Liao et al. (2002) also observed that *H. armigera* strain from Australia was poorly susceptible to *cry*9Ca1 toxin produced by *Bt tolworthi*.

The present study also aligns with Nazarian et al. (2009) and Konecka et al. (2012) with high frequency of *cry*1I gene from *B. thuringiensis* isolates. Similarly, Salama et al. (2015) revealed that, the *cry*1 gene (83.33%) was the most abundant in the soil samples of Egypt. Yılmaz (2010) reported that the frequency of *cry*1Ab or *cry*1Ac was highest (47.72%) of all the *cry* genes studied in isolates obtained from Adana region of Turkey. Further, Devaki et al (2020) reported

**Table 4.** Bioassay of *B. thuringiensis* isolates collected from Nellore district against third instar *S. litura* larvae under laboratory conditions

| Isolate | Per cent mortality | | | | | |
|---|---|---|---|---|---|---|
| | 72h | 96h | 120h | 144h | 168h | Cumulative |
| N2 | 10.00 (15.00)$^{a-g}$ | 10.00 (15.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{a}$ | 23.33 (28.78)$^{cde}$ |
| N3 | 0.00 (0.00)$^{a}$ | 23.33 (28.78)$^{de}$ | 23.33 (28.29)$^{defgh}$ | 16.67 (23.86)$^{de}$ | 20.00 (26.57)$^{gh}$ | 83.33 (70.78)$^{klm}$ |
| N7 | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 16.67 (23.86)$^{fgh}$ | 16.67 (23.86)$^{cd}$ |
| N9 | 30.00 (33.00)$^{h-n}$ | 6.67 (12.29)$^{abc}$ | 6.67 (8.86)$^{abc}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{ab}$ | 50.00 (45.08)$^{def-j}$ |
| N11 | 10.00 (18.43$^{efgh}$ | 3.33 (6.14)$^{ab}$ | 10.00 (15.00)$^{a-e}$ | 0.00 (0.00)$^{a}$ | 10.00 (11.07)$^{a-f}$ | 33.33 (34.63)$^{cdefg}$ |
| N15 | 13.33 (21.14)$^{fghi}$ | 6.67 (12.29)$^{abc}$ | 20.00 (21.14)$^{b-h}$ | 3.33 (6.15)$^{ab}$ | 6.67 (8.86)$^{a-e}$ | 50.00 (45.79)$^{ef-j}$ |
| N20 | 10.00 (15.00)$^{a-g}$ | 10.00 (15.00)$^{abcd}$ | 10.00 (15.00)$^{a-e}$ | 0.00 (0.00)$^{a}$ | 30.00 (33.00)$^{h}$ | 60.00 (51.14)$^{f-jk}$ |
| N23 | 10.00 (15.00)$^{a-g}$ | 16.67 (23.86)$^{cde}$ | 10.00 (15.00)$^{a-e}$ | 0.00 (0.00)$^{a}$ | 30.00 (33.00)$^{h}$ | 66.67 (55.37)$^{ghijkl}$ |
| N30 | 36.67 (37.14)$^{jklmn}$ | 13.33 (21.14)$^{bcd}$ | 13.33 (17.71)$^{bcdef}$ | 13.33 (21.15)$^{cde}$ | 10.00 (15.00)$^{a-g}$ | 86.67 (72.78)$^{lm}$ |
| N32 | 0.00 (0.00)$^{a}$ | 10.00 (18.43)$^{bcd}$ | 3.33 (6.14)$^{ab}$ | 6.67 (12.29)$^{abcd}$ | 6.67 (12.29)$^{a-f}$ | 26.67 (31.00)$^{cdef}$ |
| N34 | 3.33 (6.14)$^{a-e}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 6.67 (8.86)$^{abc}$ | 33.33 (35.01)$^{h}$ | 43.33 (41.15)$^{defghi}$ |
| N37 | 23.33 (28.29)$^{g-m}$ | 10.00 (18.43)$^{bcd}$ | 3.33 (6.14)$^{ab}$ | 16.67 (23.86)$^{de}$ | 6.67 (12.29)$^{a-f}$ | 60.00 (50.94)$^{f-jk}$ |
| N44 | 26.67 (31.00)$^{h-n}$ | 3.33 (6.14)$^{ab}$ | 20.00 (26.57)$^{defgh}$ | 26.67 (30.79)$^{e}$ | 0.00 (0.00)$^{abc}$ | 76.67 (61.22)$^{hijkl}$ |
| N45 | 3.33 (6.14)$^{a-e}$ | 23.33 (28.78)$^{de}$ | 20.00 (26.07)$^{cdefgh}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{abc}$ | 53.33 (46.92)$^{ef-j}$ |
| N48 | 33.33 (35.22)$^{i-n}$ | 16.67 (23.86)$^{cde}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 20.00 (21.93)$^{efgh}$ | 76.67 (61.92)$^{ijkl}$ |
| N58 | 33.33 (34.93)$^{i-n}$ | 23.33 (28.78)$^{de}$ | 13.33 (21.14)$^{b-h}$ | 0.00 (0.00)$^{a}$ | 6.67 (12.29)$^{a-f}$ | 76.67 (61.92)$^{ijkl}$ |
| N61 | 0.00 (0.00)$^{a}$ | 6.67 (12.29)$^{abc}$ | 0.00 (0.00)$^{a}$ | 10.00 (18.44)$^{bcde}$ | 0.00 (0.00)$^{abc}$ | 16.67 (23.86)$^{cd}$ |
| N63 | 20.00 (22.14)$^{f-j}$ | 16.67 (19.93)$^{bcd}$ | 13.33 (21.14)$^{b-h}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 50.00 (44.21)$^{def-j}$ |
| N64 | 0.00 (0.00)$^{a}$ | 10.00 (18.43)$^{bcd}$ | 6.67 (12.29)$^{abcd}$ | 10.00 (18.44)$^{bcde}$ | 0.00 (0.00)$^{abc}$ | 26.67 (31.00)$^{cdef}$ |
| N65 | 0.00 (0.00)$^{a}$ | 10.00 (18.43)$^{bcd}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 16.67 (23.86)$^{cd}$ |
| N69 | 33.33 (35.22)$^{i-n}$ | 16.67 (23.86)$^{cde}$ | 6.67 (12.29)$^{abcd}$ | 10.00 (15.00)$^{bcd}$ | 0.00 (0.00)$^{abc}$ | 66.67 (55.07)$^{ghijkl}$ |
| N93 | 23.33 (24.15)$^{f-jk}$ | 10.00 (18.43)$^{bcd}$ | 30.00 (33.00)$^{fgh}$ | 3.33 (6.15)$^{ab}$ | 10.00 (18.44)$^{defg}$ | 76.67 (65.85)$^{jklm}$ |
| N100 | 33.33 (35.22)$^{i-n}$ | 23.33 (28.78)$^{de}$ | 0.00 (0.00)$^{a}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 60.00 (50.85)$^{f-jk}$ |
| N105 | 0.00 (0.00)$^{a}$ | 16.67 (23.86)$^{cde}$ | 3.33 (6.14)$^{ab}$ | 10.00 (18.44)$^{bcde}$ | 3.33 (6.15)$^{abcd}$ | 33.33 (35.22)$^{cdefg}$ |
| N106 | 10.00 (18.43)$^{efgh}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 10.00 (18.43)$^{ac}$ |
| N107 | 6.67 (12.29)$^{a-f}$ | 6.67 (12.29)$^{abc}$ | 0.00 (0.00)$^{a}$ | 13.33 (21.15)$^{cde}$ | 6.67 (12.29)$^{a-f}$ | 33.33 (35.01)$^{cdefg}$ |
| N108 | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 6.67 (8.86)$^{abc}$ | 10.00 (18.44)$^{defg}$ | 16.67 (23.36)$^{cd}$ |
| N115 | 40.00 (39.15)$^{kmn}$ | 10.00 (18.43)$^{bcd}$ | 23.33 (28.78)$^{defgh}$ | 0.00 (0.00)$^{a}$ | 3.33 (6.15)$^{abcd}$ | 76.67 (65.85)$^{jklm}$ |

**Cont..**

**Table 4.** Bioassay of *B. thuringiensis* isolates collected from Nellore district against third instar *S. litura* larvae under laboratory conditions

| Isolate | Per cent mortality | | | | | |
|---|---|---|---|---|---|---|
| | 72h | 96h | 120h | 144h | 168h | Cumulative |
| N117 | 0.00 (0.00)$^{ab}$ | 30.00 (28.08)$^{cde}$ | 10.00 (18.43)$^{b-g}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 43.33 (39.99)$^{cdefgh}$ |
| N118 | 33.33 (35.22)$^{i-n}$ | 20.00 (26.07)$^{cde}$ | 3.33 (6.14)$^{ab}$ | 0.00 (0.00)$^{a}$ | 3.33 (6.15)$^{abcd}$ | 60.00 (51.14)$^{f-jk}$ |
| N122 | 0.00 (0.00)$^{abc}$ | 10.00 (18.43)$^{bcd}$ | 3.33 (6.14)$^{ab}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 16.67 (23.86)$^{cd}$ |
| N130 | 23.33 (28.78)$^{g-m}$ | 20.00 (26.07)$^{cde}$ | 3.33 (6.14)$^{ab}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 50.00 (45.08)$^{def-j}$ |
| N131 | 16.67 (23.86)$^{f-j}$ | 10.00 (18.43)$^{bcd}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 33.33 (35.22)$^{cdefg}$ |
| N138 | 10.00 (18.43)$^{efgh}$ | 3.33 (6.14)$^{ab}$ | 10.00 (18.43)$^{b-g}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 26.67 (30.79)$^{cdef}$ |
| N141 | 20.00 (26.07)$^{f-jklm}$ | 20.00 (26.07)$^{cde}$ | 33.33 (35.22)$^{gh}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 73.33 (59.71)$^{hijkl}$ |
| N143 | 16.67 (23.86)$^{f-j}$ | 10.00 (18.43)$^{bcd}$ | 26.67 (31.00)$^{efgh}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 53.33 (46.92)$^{ef-j}$ |
| N148 | 16.67 (23.86)$^{f-j}$ | 20.00 (26.07)$^{cde}$ | 13.33 (21.14)$^{b-h}$ | 10.00 (15.00)$^{bcd}$ | 0.00 (0.00)$^{abc}$ | 60.00 (51.14)$^{f-jk}$ |
| N158 | 23.33 (24.15)$^{f-l}$ | 10.00 (18.43)$^{bcd}$ | 13.33 (21.14)$^{b-h}$ | 3.33 (6.15)$^{ab}$ | 0.00 (0.00)$^{abc}$ | 50.00 (45.00)$^{def-j}$ |
| N173 | 0.00 (0.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 36.67 (37.14)$^{h}$ | 3.33 (6.15)$^{ab}$ | 3.33 (6.15)$^{abcd}$ | 43.33 (41.07)$^{defghi}$ |
| N188 | 0.00 (0.00)$^{abcd}$ | 10.00 (15.00)$^{abcd}$ | 10.00 (15.00)$^{a-e}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{abc}$ | 26.67 (30.29)$^{cdef}$ |
| N189 | 0.00 (0.00)$^{abcd}$ | 23.33 (24.15)$^{cde}$ | 10.00 (18.43)$^{b-g}$ | 0.00 (0.00)$^{a}$ | 10.00 (15.00)$^{abdefg}$ | 43.33 (40.86)$^{defghi}$ |
| N190 | 0.00 (0.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 16.67 (23.86)$^{cdefgh}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 16.67 (23.86)$^{cd}$ |
| N192 | 0.00 (0.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 0.00 (0.00)$^{a}$ |
| N193 | 0.00 (0.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 0.00 (0.00)$^{a}$ |
| HD1 | 50.00 (45.00)$^{n}$ | 36.67 (37.22)$^{e}$ | 3.33 (6.14)$^{ab}$ | 6.67 (12.29)$^{abcd}$ | 0.00 (0.00)$^{abc}$ | 96.67 (83.86)$^{m}$ |
| Control | 0.00 (0.00)$^{abcd}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{abc}$ | 0.00 (0.00)$^{ab}$ |
| SEd | 6.27 | 6.50 | 7.06 | 5.95 | 5.99 | 8.88 |
| LSD | 12.46 | 12.92 | 14.03 | 11.81 | 11.88 | 17.65 |

Figures in parentheses are arcsine transformed values
Alphabets indicating Duncan Multiple Range Test (DMRT)

**Table 4a.** Lethal dose calculation for effective *B. thuringiensis* strains

| *Bt* isolates | Regression equation | $LC_{50}$ values CFU/ ml | $LC_{90}$ values CFU/ ml | $LT_{50}$ (h) | $LT_{90}$ (h) |
|---|---|---|---|---|---|
| N3 | Y =-2.267 +0.425X | $2.18 \times 10^{5}$ | $2.27 \times 10^{8}$ | 129.25 | 175.33 |
| N30 | Y =-2.616+0.496X | $1.90 \times 10^{5}$ | $7.33 \times 10^{7}$ | 95.70 | 180.74 |
| N44 | Y =-1.891 +0.315X | $9.93 \times 10^{5}$ | $1.15 \times 10^{7}$ | 116.47 | 193.62 |
| N48 | Y =-2.256 +0.365X | $1.50 \times 10^{6}$ | $4.85 \times 10^{9}$ | 107.79 | 233.62 |
| N58 | Y =-2.687 +0.453X | $8.42 \times 10^{5}$ | $5.65 \times 10^{8}$ | 92.78 | 206.34 |
| N93 | Y =-2.398 +0.387X | $1.59 \times 10^{6}$ | $3.27 \times 10^{9}$ | 115.31 | 196.39 |
| N115 | Y =-2.689+0.434X | $1.57 \times 10^{6}$ | $1.40 \times 10^{9}$ | 88.68 | 205.28 |
| HD-1 | Y =-2.252+0.452X | $9.59 \times 10^{4}$ | $6.56 \times 10^{7}$ | 61.99 | 121.64 |

**Table 5.** Origin of native *B. thuringiensis* isolates with ≥ 50 per cent mortality against *S. litura* collected from Nellore district

| Isolate | Mortality (%) | Place of collection | Crop/ location |
|---|---|---|---|
| N3 | 83.33 | Mambattu | Groundnut |
| N9 | 50.00 | Mamillapadu | Cultivated Fallow |
| N15 | 50.00 | Vaddipalem | Grass |
| N20 | 60.00 | Chamellapadu | Fodder |
| N23 | 66.67 | Venambakkam | Cultivated Fallow |
| N30 | 86.67 | Chembedupalem | Grass |
| N37 | 60.00 | Sullurpet | Sugarcane |
| N44 | 76.67 | Venkatagiri | Jack fruit |
| N45 | 53.33 | Petlur | Fallow |
| N48 | 76.67 | Petlur | Neem |
| N58 | 76.67 | Venkatagiri | Cultivated Fallow |
| N63 | 50.00 | Peddayachasamudram | Grass |
| N69 | 66.67 | Marlagunta | Teak |
| N93 | 76.67 | Chaaganam | Hill |
| N100 | 60.00 | Saidapuram | Mango |
| N115 | 76.67 | Kasumur | Eucalyptus |
| N118 | 60.00 | Gurivindapudi | Neem |
| N130 | 50.00 | Anuppallipadu | Grass |
| N141 | 73.33 | Narasareddypalli | Sugarcane |
| N143 | 53.33 | Narasareddypalli | Fodder |
| N148 | 60.00 | Gudur | Cultivated Fallow |
| N158 | 50.00 | Naidupet | Brinjal |

that the *cry*1I was the predominant gene among *B. thuringiensis* collections from soil samples in undisturbed environments of Andhra Pradesh, India. Some of the strains in present *B. thuringiensis* collections harbored *cry*2 genes. Similar findings were reported by Liang et al. (2011), who studied the diversity of *cry*2 genes in Sichuan basin, western China. Among the 791 *Bt* strains isolated from 2650 soil samples in different ecological regions, it was found that, 322 *B. thuringiensis* strains harboured *cry*2-type genes with four different RFLP patterns. The combination of *cry*2Aa*/cry*2Ab gene was the most frequent (90.4%), followed by *cry*2Aa (6.8%) and *cry*2Ab alone (2.5%).

## CONCLUSIONS

The soil samples from Nellore district of Andhra Pradesh, India harboured good number of *B. thuringiensis* isolates. Out of 205 soil samples screened, 44 cultures were identified and evaluated against third instar larvae of *S. litura*. The highest per cent mortality was observed with N30, followed N3 both of which were statistically superior over the other strains and on par with standard strain HD1**.** Molecular characterization of the virulent *B. thuringiensis* strains with *cry* gene specific primers indicated that *cry*1I gene was predominant in seven *B. thuringiensis* strains, which was reported to confer specific toxicity against lepidopteran insects.

**Table 6.** *Cry* genes in effective native *B. thuringiensis* strains effective against *S. litura* collected from southern zone of Andhra Pradesh

| Isolate | Mortality (%) | *cry* gene(s) observed | No. of *cry* genes |
|---|---|---|---|
| N3 | 83.33 | *cry*1C | 1 |
| N20 | 60.00 | *cry*1I, *cry*2 | 2 |
| N23 | 66.67 | *cry*1I, *cry*2A(a)1, *cry*9Ca1 | 3 |
| N30 | 86.67 | *cry*1I, *cry*2A(a)1, *cry*9Ca1 | 3 |
| N44 | 76.67 | *cry*1Da1 | 1 |
| N45 | 53.33 | *cry*1Aa | 1 |
| N48 | 76.67 | - | - |
| N58 | 76.67 | *cry*2A(a)1 | 1 |
| N63 | 50.00 | *cry*1I | 1 |
| N69 | 66.67 | *cry*1Aa, *cry*1Da1, *cry*1Ea1 | 3 |
| N93 | 76.67 | *cry*1Aa, *cry*1C , *cry*1I, *cry*2A(a)1, *cry*9Ca1 | 5 |
| N100 | 60.00 | *cry*1C , *cry*2A(a)1 | 2 |
| N115 | 76.67 | *cry*1C | 1 |
| N118 | 60.00 | - | 0 |
| N130 | 50.00 | *cry*1Aa | 1 |
| N141 | 73.33 | *cry*1Aa, *cry*1Fa1, *cry*1I | 3 |
| N143 | 53.33 | *cry*1Aa, *cry*1Fa1, *cry*1I | 3 |
| N148 | 60.00 | - | 0 |
| N158 | 50.00 | *cry*1Fa1 | 1 |

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