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# Translocation of *Bacillus thuringiensis kurstaki* strain HD-1in brinjal seedling by different inoculation methods

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**ABSTRACT:** In the present study, *Btk* strain HD1 was inoculated at a concentration of $10^8$ cfu/ml to brinjal in three methods under pot culture studies. The methods include seed treatment, soil application and foliar spray. *Btk* strain HD1 from leaves of seedlings was recovered in all the application methods. In seed treatment,*Btk* HD1 was detected in leaves after 30$^{th}$ day after inoculation while in soil application and foliar spray the *Btk* HD1 was detected upto 10$^{th}$ DAI. Further the colonies recovered from leaf tissues collected from three inoculation methods were examined for morphological, spore crystal formation and*cry*1 gene amplification. This is the first kind of report regarding the endophytic ability of *Btk* strain HD1 in brinjal by which the approach could be a possible way for the development of insect resistant crop for sustainable agriculture.

**Keywords:** *Bacillus thuringiensis*, endophytic ability, brinjal

## INTRODUCTION

Brinjal or Eggplant (*Solanum melongena* L.) often called as king of vegetables belongs to family Solanaceae and is originated in India. India holds second rank next to china in brinjal production. Brinjal shoot and fruit borer, *Leucinodes orbonalis* is the serious pest and the incidence is still in alar`ming stage despite the continuous use of many synthetic chemicals. As an alternative to synthetic chemicals, microbial pesticides based on fungi, bacteria, viruses and nematodes have been used against various major field pests due to their target specificity and safety to non-target organisms and environment. *Bacillus thuringiensis* (*Bt*) is a well known commercial entomopathogen with more than 240 holotypes of cry toxins that are active against Lepidoptera, Diptera, Coleoptera and Hymenoptera. *Bacillus thuringiensis* with its specific crystal tox in production has been occupied 90% share in biopesticide market. Furthermore, the efficacy of *B. thuringiensis* strains against insects from Lepidoptera, Coleoptera, Diptera and Hemiptera onvarious crops in India have already been documented (Dharavath *et al.*, 2016; Kumar *et al.*, 2019; Rajashekhar *et al.*, 2018a & 2018b and Tripathi *et al.*, 2016). However, the beneficial features of *Bt* are not limited to its role as an insecticide; it is also used as plant growth promoter by encompassingin nutrient uptake (Azizoglu, 2019; Lee *et al.*, 2009; Qi *et al.*, 2016 and Raddadi *et al.*, 2007).Certain entomopathogenic *Bt* strains were reportedto colonize various host plants keeping their efficacy against target pests either by toxin production or altering the plant physiology besides showing plant growth promotion and extra cellular enzymes production. Additionally, *B. thuringiensis* which is a well-known entomopathogen has been reported as an endophytic bacterium in plants such as cotton, soybean, maize, sugarcane, and cabbage (Subrahmanyan *et al.*, 1983; Suzuki *et al.*, 2008; Praca *et al.*, 2012).

The ability of *B.thuringiensis* to colonize endophytically has also been identified as an important feature to actas a biological control agent against pests. Several authors have reported the different delivery methods of endophytic *Bacillus* spp. to host plants (Algam *et al.*, 2005; Bressan and Borges, 2004; Jahanian *et al.*, 2012; Siddiqui and Shaukat, 2002 and Zhang *et al.*, 2009 ) as well as colonization of *Bacillus* spp. in the target host plants (Fan *et al.*, 2011; Nair *et al.*, 2002; Liu *et al.*, 2006 and Yan *et al.*, 2003) and biocontrol of pests (Disi *et al.*, 2018; Gadhave and Gange,2016 and Zehnder *et al.*,1997).Additionally, tracking the presence of inoculated bacterial strain in the interior plant parts (root,stem and leaf ) among the overcrowded microbes by a selective detection methods such as reportergene technology which is found to be an important approach that is used to monitor micro organisms in various environmental samples (Ramos *et al.*, 2000).Hence, with this background the present study on endophytic ability of *Btk* strain HD1 brinjal was investigated.

## MATERIALS AND METHODS

### Preparation of *Bacillus thuringiensiskurstaki* strain HD-1

For seed treatment and other inoculation methods, bacterial inoculum was prepared with a 24h old bacterial

culture grown on LB agar. The fresh actively grown cell culture then transferred to sterile falcon tubes and centrifuged 8000 rpm for 10 min at 30°C by using sterile oak ridge tubes, pellets were prepared and stored at 0°C for future use. Subsequently, the supernatant was discarded and the pellet was re suspended in 10ml sterile distilled water. The amount of inoculum used for the experiment was OD 600 = 0.8 ($10^8$ cfu /ml).The surface sterilized seeds were allowed to soak in the bacterial suspension ($10^8$ cfu/ml) for 3h and dried on sterilized tissue paper before sowing in the potted soil. For control, seeds were soaked in sdw and sown in pots. In soil application method, *Btk* HD1 @ 5ml ($10^8$cfu /ml) was given near the root zone of each seedling of one month old seedlings. In case of control, sdw @ 5ml was given to each seedling. One month old seedlings were given foliar spray at a concentration of 1x$10^8$cfu /ml until the leaves become wet.

**Lay out of Pot Experiment**

The potting mixture was prepared with soil, vermiculite and coco peat in 1:1:1 ratio and autoclaved for three times at 24 h interval. Pots were filled with material. Seeds of brinjal (variety: Bhagyamati) were washed first with tap water thrice, followed by 70% ethanol for 5 min and 2% sodium hypochlorite for 5min and then 3 times washing with sterile distilled water. To ensure that the seed samples had been completely surface disinfected, a volume of 100 µl of last wash water was plated on LBAgar plate and incubated at 30 °C for 48 h; if colonies were found the samples were discarded. Seeds were sown in the 15cm x 10cm size pots and two seedlings were maintained per pot during the experiment. The seedlings were maintained in plant growth chamber under 12 h dark +12 h light period with 30°C temperature and 65-70% relative humidity and watered regularly.

**Reisolation and morphological confirmation of *B. thuringiensis* colonies recovered from leaves**

Leaves were collected randomly from brinjal seedlings on 3DAI and 10th DAI in soil application and foliar spray treatments while in seed treatment the leaves from seedlings were collected on 15th DAI and 30th DAI. Leaves were macerated and diluted solutions were plated on selective antibiotic plates. Colonies that grew on selective LBA plates similar to the colonies on the reference plates were examined for colony morphology (Algam *et al.*, 2005 Bressan and Borges, 2004 and Tanuja *et al.*, 2013). After confirmation on the plates the recovered colonies of each *Bt* strain were further inoculated into LB broth with selective antibiotic markers and kept for incubation at 30 °C for 48h to 72h and examined vegetative cells as well as crystal and spores in cells under phase contrast microscope in order to confirm the reisolated colonies were similar to inoculated *Btk* strain HD1.

**Molecular confirmation of *Btk*HD-1 colonies recovered from leaves**

Confirmation of recovered *Bt* colony identity by PCR method was done as per Algam *et al.*, 2005 and Maduell *et al.*, 2007 with little modification. To ensure the confirmation of the recovered *Btk* strain HD1 corresponded to the inoculated *Btk* strain HD1, *cry*1 gene primers (FP-CATGATTCATGCGGCAGATAAAC;RP-TTGTGACACTTCTGCTTCCCATT) were used. A loopful cells was transferred to 100 µl of sterile distilled water, and the mixture was frozen for 20 min at -80°C and then transferred to boiling water for 10 min to lyse the cells. The resulting cell lysate was centrifuged at 10,000 rpm for 30 s and supernatant was used as a DNA template in the PCR. DNA template (10 µl) was mixed with reaction mixture of 4.3 µl consisting of Taq assay buffer (10x) with $MgCl_2$ (15 mM),1µl dNTPs (10 mM), 1 µl of each primer (10 pM), 0.2 µl Taq DNA polymerase (5 U/µl) and 7.5 µl nuclease free water. The reactions were placed in a thermocycler (Flexigene Techne, UK) programmed as initial denaturation at 94 °C for 5 m followed by 35 cycles of denaturation at 94 °C for 1 m, annealing at 50 °C for 1 m, extension at 72 °C for 2 min and a final extension at 72°C for 5 m. After amplification, 2 µl of loading buffer (0.5% bromophenol blue in glycerol 50%) was added to 5µl of each amplified PCR product and were electrophoresed (at 70 volts for 10 min, followed by 90 volts for 45 min) on a 1x Tris-acetate-EDTA (TAE with ethidium bromide) buffer in 1.2% agarose gel. Gels were visualized in a gel documentation system (AlphaimagerTM) and analyzed with Alpha EaseFC.

**RESULTS AND DISCUSSION**

**Morphological confirmation of *Btk*-HD1 colonies recovered from leaves**

The colonies observed on antibiotic amended nutrient agar plates were found to be similar to that of original colonies of *Btk* strain on the basis of colour, elevation and margin of the colony. *Btk*-HD1 colonies were confirmed by white, small and rough margins.No *B.thuringiensis* colonies were appeared on control plates. Further confirmation of colony as *B. thuringiensis* was done by examination of cells, spores and crystal inclusions for *B. thuringiensis* strain under phase contrast microscope (Plate 1). Recovery of *B. thuringiensis* from leaf tissues of brinjal seedlings from seed treatment, soil application and foliar spray confirmed that *Btk*-HD1 was able to colonize leaf tissues of brinjal seedling.

**Confirmation of *cry* gene in colonies of *Btk*-HD1 recovered from leaves through PCR**

PCR studies showed that the *Btk*-HD1 reisolated from leaves collected from plants inoculated by seed treatment on 15th day after inoculation (DAI) soil application on 3rdDAI and foliar spray on 3rdDAIamplified expected amplicon size of 276 bp of *cry*1 gene. Thus, proved that the *Bt* strain *Btk* -HD1 was successfully colonized the seedlings of brinjal and translocated to leaves from seed, soil and phylloplane of leaves. Further, *Bt* strain reisolated on 30th DAI of seed treatment, 10th DAI of soil application and foliar spray also amplified 276 bp band of *cry* 1 gene (Fig.1) indicated that the *Bt* strains could persist in the leaves in all the inoculation methods.

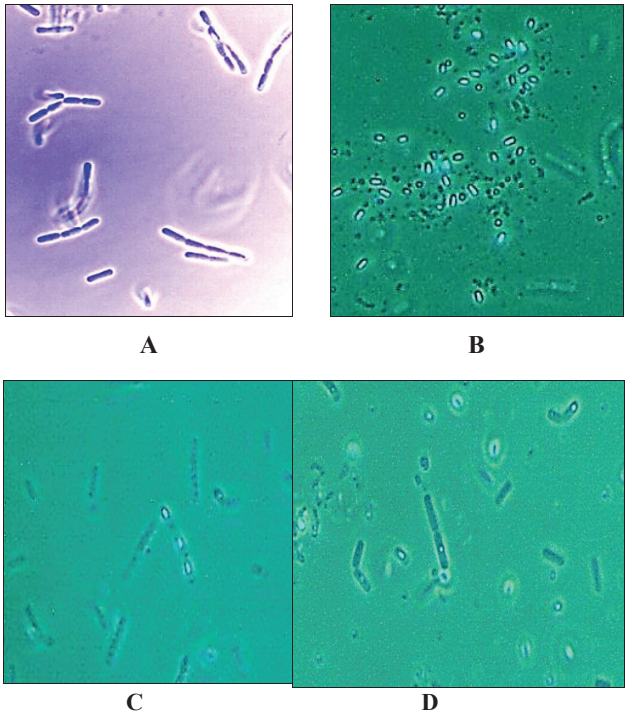


**Plate 1. Phase contrast microscopic images of cells and spore crystals of *Bacillus thuringiensis* sub species *kurstaki* strain HD-1recovered from leaves after seed treatment (B), soil application(C) and foliar spray (D). Original culture of *Btk*-HD1 (A)**

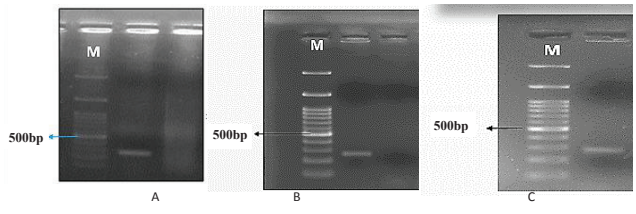


**Fig. 1 Amplification of *cry1* gene (276 bp) in colonies of *Bacillus thuringiensis* re isolated from leaf samples of brinjal seedlings from seed (A&B) and soil treatment (C); Lane M: Marker**

Only a few studies are reported so far related to colonization of potential entomopathogenic bacteria (*B.thuringiensis*) as endophytes. In our study the main emphasis has been given on detection of *Bt* inside the brinjal leaf tissue rather than root and stem tissues as the neonates of brinjal shoot and fruit borer tend to migrate towards leaf veins and more specifically leaf midribs immediately after hatching (Hanur *et al.*, 2014).Based on the results it can be understood that the *Btk*-HD1 find some favorable site inside the seed tissues by which proliferation could be possible. Earlier, similar findings have been reported by Tanuja *et al.* (2013), showed that *Bt* strains were able to colonize different host plants such as ricebean, soybean, gahat and lentil seedlings and were recovered from leaf sections after 45 days of sowing. In another report, three *Bt* strains possessing high activity against lepidopteran pests exhibited endophytic ability in cabbage seedlings after seed treatment (Praca *et al.*, 2012). In soil application *Btk*-HD1 was detected in leaf tissues of brinjal seedlings and it has been evident that the *Bt* might have entered into root system initially from soil followed by ascending migration into aerial parts probably via xylem vessels. *Bt* strains may get entry into root tissue via germinating radicles (Huang, 1986) or natural wounds due to root growth or through root hairs and at epidermalconjunctions.Hallmann *et al.*, 1997; McCully 2001 reported that bacteria may get entry into leaf tissues during foliar spray due to the possible entry via stomata, wounds and even by means of ability of production of hydrolytic enzymes.

The present investigation clearly demonstrated that *Btk* strain HD1 has the potential to be associated with brinjal as an endophyte as it gain access into the interior of brinjal root, stem and leaf. It was evident that *Btk* strain HD1 showed colonization inside the brinjal seedling as it migrated endophytically upward into stem and leaves of the plants. The new findings indicated that thorough attention need to be paid on beneficial plant bacterium interactions and its potential application for the sustainable management of major pest brinjal shoot and fruit borer, *L.orbonalis*.

## REFERENCES

Algam, S, A., Guan-lin X. and Coosemans, J. 2005. Delivery methods for introducing endophytic Bacillus into tomato and their effect on growth promotion and suppression of tomato wilt. *Plant Pathology Journal,* **4**(1): 69-74.

Azizoglu, U. 2019. *Bacillus thuringiensis* as a biofertilizer and biostimulator: a mini-review of the little-known plant growth-promoting properties of *Bt. Current microbiology* 1-7.

Bressan, W. and Borges, M, T. 2004. Delivery methods for introducing endophytic bacteria into maize. *BioControl,* **49**(3): 315-322.

Dharavath, V., Mittal, A., Rajashekhar, M. and Kalia, V. K. 2016. Potential of Native *Bacillus* Strains against *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Progressive Research– an International Journal,* **11**: 2861-2865.

Disi, J. O., Kloepper, J, W. and Fadamiro, H, Y. 2018. Seed treatment of maize with *Bacillus pumilus* strain INR-7 affects host location and feeding by Western corn rootworm, *Diabrotica virgifera*. *Journal of Pest Science,* **91**(2): 515-522.

Fan, B., Chen, X. H., Budiharjo, A., Bleiss W., Vater J. and Borriss, R. 2011. Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. *Journal of Biotechnology,* **151**(4): 303-311.

Gadhave, K, R. and Gange, A. C. 2016. Plant-associated *Bacillus* spp. alter life-history traits of the specialist insect *Brevicoryne brassicae* L. *Agricultural and Forest Entomology,* **18**: 35–42.

Hallman, J., Quadt-Hallmann, A., Rodriguez-Kabana, R. and Kloepper, J. W. 1997. Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. *Soil Biology and Biochemistry,* **23**: 117–131.

Hanur, V. S., Boopal, K., Arya, V. V., Srividya, K, N. and Saraswathi, M, S. 2014. Why is management of brinjal shoot and fruit borer, *Leucinodes orbonalis* Guenee, difficult? An examination into the pest's unique feeding behavioral biology. *Journal of Entomology and Zoology Studies,* **2**(6): 257-260.

Horticultural Statistics at a Glance. 2018-19. Department of Agriculture, Co-operation and Farmers Welfare [online]. Available from www.agricoop.nic.in.

Huang, J. S. 1986. Ultrastructure of bacterial penetration in plants. *Annual Review of Phytopatology,* **24**: 141–157.

Jahanian, A., Chaichi, M. R., Rezaei, K., Rezayazdi, K. and Khavazi, K. 2012. The effect of plant growth promoting rhizobacteria (PGPR) on germination and primary growth of artichoke (Cynarascolymus). *International Journal of Agriculture and Crop Sciences,* (IJACS), **4**(14): 923-929.

Kumar, K. K., Sridhar, J., Murali-Baskaran, R. K., Senthil-Nathan, S., Kaushal, P., Dara, S. K. and Arthurs, S. 2019. Microbial biopesticides for insect pest management in India: Current status and future prospects. *Journal of Invertebrate Pathology,* **165**: 74-81.

Lee, H., Churey, J. J. and Worobo, R. W. 2009. Biosynthesis and transcriptional analysis of thurincin H, a tandem repeated bacteriocin genetic locus, produced by *Bacillus thuringiensis* SF361. *FEMS Microbiology Letters,* **299** (2): 205-213.

Liu, X., Zhao, H. and Chen, S. 2006. Colonization of maize and rice plants by strain *Bacillus megaterium* C4. *Current Microbiology,* **52**: 186–190.

Maduell P, Armengo G, Llagostera M, Lindow S, Orduz S. 2007. Immigration of Bacillus thuringiensis to bean leaves from soil inoculum or distal plant parts. Journal of *Applied Microbiology,***103** (6): 2593-2600.

McCully, K. S. 2001. The biomedical significance of homocysteine. *Journal of Scientific Exploration,* **15** (1): 5-20.

Nair, J. R., Singh, G. and Sekar, V. 2002. Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. *Journal of Applied Microbiology,* **93** (5): 772-780.

Praca, L. B., Gomes, A, C, M, M., Cabral, G., Martins, E, S., Sujii, E. R. and Monnerat, R. G. 2012. Endophytic colonization by Brazilian strains of *Bacillus thuringiensis* on cabbage seedlings grown in vitro. *Bt Research* 3: 11–19.

Qi, J., Aiuchi, D., Tani, M., Asano, S. I. and Koike, M. 2016. Potential of entomopathogenic *Bacillus thuringiensis* as plant growth promoting rhizobacteria and biological control agents for tomato Fusarium wilt. *International journal of environmental* and agriculture *research,* **2** (6): 55-63.

Raddadi, N., Cherif, A., Ouzari, H., Marzorati, M., Brusetti, L., Boudabous, A. and Daffonchio D. 2007. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. *Annals of Microbiology,* **57**(4): 481-494.

Rajashekhar, M., Mittal, A., Dharavath, V. and Kalia, V. K. 2018a. Potential of Native *Bacillus thuringiensis* Strains against Cotton Aphid, *Aphis gossypii* Glover. *Pesticide Research Journal,* **30**(1): 24-30.

Rajashekhar, M., Mittal, A., Dharavath, V. and Kalia, V. K. 2018b. Characterization of potential native *Bacillus thuringiensis* strains isolated from insect cadavers against cotton aphid Aphis gossypii Glover (Hemiptera: Aphididae). *Indian Journal of Entomology,* **80**(2): 177-184.

Ramos C, Molbak L, Molin S. 2000. Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents and synthesis rates. Applied and Environmental Microbiology 66(2): 801-809.

Subrahmanyan, P., Reddy, M, N. and Rao A S. 1983. Exudation of certain organic compounds from seeds of groundnut. *Seed Science and Technology,* 11: 267–272.

Suzuki, M. T., Hernandez-Rodriguez, C. S., Araujo, W. L. and Ferre, J. 2008. Characterization of an endophytic *Bacillus thuringiensis* strain isolated from sugar cane. In Proceedings of 41st Annual Meeting of the Society for Invertebrate Pathology and 9th International Conference on *Bacillus thuringiensis* ed. Chandler D, Winstanley D. Coventry, UK: *Society for Invertebrate Pathology*, pp. 25.

Siddiqui, I. A. and Shaukat S, S. 2002. Rhizobacteria mediated induction of systemic resistance (ISR) in tomato against *Meloidogyne javanica*. *Journal of Phyto pathology,* **150** (89): 469-473.

Tanuja., Bisht, S. C. and Mishra P K. 2013. Ascending migration of endophytic *Bacillus thuringiensis* and assessment of benefits to different legumes of N.W. *Himalayas European Journal of Soil Biology,* 56.

Tripathi, M., Kumar, A., Kalia, V. K., Saxena, A. K. and Gujar, G. 2016. Isolation and characterization of lepidopteran specific *Bacillus thuringiensis* strains predominantly from northeastern states of India. *Journal of Experimental Biology,* **54**: 431-452.

Yan, Z., Reddy, M. S. and Kloepper, J. W. 2003. Survival and colonization of rhizobacteria in a tomato transplant system. *Canadian Journal of Microbiology,* **49**(6): 383-389.

Zhang, S., Cheng, H., Gao, Y., Wang, G., Liang, G. and Wu, K. 2009. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis*. Cry1Ac toxin. *Insect Biochemistry and Molecular Biology*, **39**(7): 421-429.

Zehnder, G, W., Kloepper, J, W., Tuzun, S., Yao, C., Wei, G., Chambliss, O. and Shelby, R. 1997. Insect feeding on cucumber mediated by rhizobacteria-induced plant resistance. *Entomologia Experimentalis et Applicata,* **83**:81-85

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