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ORIGINAL ARTICLE

Virulence assay and role of *Bacillus thuringiensis* TS110 as biocontrol agent against the larval stages of rice leaffolder *Cnaphalocrocis medinalis*

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Abstract The bacterial isolate *Bacillus thuringiensis* TS110 was isolated from the rice field soil of Burdwan district, West Bengal, India. Bioassay test of the bacteria TS110 against 3rd, 4th and 5th instar larvae of *Cnaphalocrocis medinalis* was carried out. Cut leaf assay, potted plant assay and field assay were done. During filed assay, it has been observed that the LC_{50} ($\times 10^7$) values of TS110 against 3rd, 4th and 5th instar larvae of *C. medinalis* were 3.77, 5.29, 4.83 and 4.93, 4.42, 4.72 in dry and wet season, respectively. The morphological, biochemical and phylogenetic analysis of the isolate TS110 were done. TS110 was positive for catalase, nitrate reductase, methyl red, voges-proskauer, oxidase, urease, indole, citrate utilization, arginine dihydrolase test, starch, lipid, gelatin, casein, and lecithin hydrolysis test. TS110 showed fermentation test positive for glucose, fructose, mannose, arabinose and trehalose in nutrient broth medium. The antibiotic sensitivity test showed that the bacterial isolate was sensitive to kanamycin (30 µg/disc), nalidixic acid (30 µg/disc), rifampicin (5 µg/disc), doxycycline (30 µg/disc), gatifloxacin (10 µg/disc), vancomycin (30 µg/disc), gentamycin (10 µg/disc), ampicillin (10 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc), gentamycin (10 µg/disc). The phylogenetic analysis revealed that TS110 was closely related to different species of *B. thuringiensis* submitted to the GenBank. On the basis of morpho-physiological and molecular characterization, the bacterial isolate was identified as *B. thuringiensis.*

Keywords *Cnaphalocrocis medinalis* · LC_{50} · Antibiotic sensitivity · Phylogenetic analysis · Fermentation test · *Bacillus thuringiensis*

Introduction

Insects of different orders constitute the majority of pests infesting different plants. Almost 128 species of insects have been reported to cause damages of rice crop and out of these, 15–20 species are most important in relation to economic loss (Kalode et al. 1995). Insect pests caused extensive damages in rice crop at different developmental stages and among them leaf feeding pests are the important ones due to their ability of decomposing chlorophyll or defoliating that led to yield loss (Kaur et al. 2008). The lepidopteran insect rice leaffolder (*Cnaphalocrocis medinalis)* is one of the major pests of rice plants accounting great economic loss in rice cultivating countries (Riley et al. 1995). In India, the infestation of rice leaffolder has been increased after late 80's and became the major cause of economic loss in rice cultivation (Nanda and Bisoi 1990). Chemical insecticides applied against this rice pest caused environmental hazards and the insects also developed resistance against them (Shahid et al. 2003). In view of these, use of Bt as pesticide became the need of the day. *Bacillus thuringiensis* (Bt) is a gram-positive, rod-shaped, spore-forming bacterium producing different crystal proteins due to which it is widely used in biocontrol of different insect pests (Shishir et al. 2012). They are eco-

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friendly, do not have a negative effect on nontarget animals, including vertebrates (Joung and Cote 2000). *B. thuringiensis* produce Cry and Cyt proteins that are effective against different insect pest (Ibrahim et al. 2010). Till now 68 groups of Cry toxin and 3 groups of Cyt toxins have been isolated from different subspecies of Bt (Crickmore et al. 1995). Proteins coded by genes cry1, cry2, cry7, cry8, cry9, cry15, cry22, cry51,and cyt1 are effective against Lepidopteran pests (Frankenhuyzen 2009). Several Bt strains have been used against different lepidopteran insects all over the world; and wild strains of *B. thuringiensis* isolated from different environmental samples can show higher activity against different insect pests in comparison to already available *B. thuringiensis* formulated as pesticides (Konecka et al. 2012). On the view of this, the aim of the present study was to isolate and characterize novel Bt strain from the rice field soils of Burdwan district, West Bengal, India and evaluate its pathogenicity against rice leaffolder *C. medinalis*.

Materials and methods

The soil samples were collected from rice fields of different village areas of Burdwan district, West Bengal. The top most soil (1 cm) was scrapped off and then about 100 g soils (pH 6.9–7.1) from each area were collected in sterile polythene bags, sealed with rubber bands.

Bacteria isolation

Soil samples were collected from the rice fields of Burdwan and taken to the laboratory for further analysis. The soil samples were blotted and diluted serially up to 10^{-3} level. A 100 µl suspension was mixed with 100 ml Nutrient Agar (NA) (g/l: peptone 5, beef extract 3, agar 2, pH7), distributed in five plates and incubated at 30 ± 0.1 °C in the BOD incubator for 24 h. The colonies were checked under a phase-contrast microscope and those having spores were purified on NA plates and the pure cultures were maintained at 4 ± 0.1 °C on NA slants.

Bio assay of the bacterial isolates against rice leaffolder

Cut leaf assay

Bioassay was carried out on 3rd, 4th and 5th instar larvae of *C. medinalis* against the bacterial isolates. Rice leaf pieces were surface sterilized and soaked separately in each bacterial suspension (of different concentrations; 10^4, 10^5, 10^6, $10^{7,}$ 10^8 cfu/ml) for 15–20 min. Then different larval instars of rice leaffolders (10 larae/test tube) were placed in test tubes and covered with an insect proof cloth. The mortality of the larvae were recorded up to 72 h.

Potted plant assay

Pots containing freshly cultivated 30-day old rice plants (5 tillers/pot) of insect's favourable variety was infested with *C. medinalis* larvae (10 larvae/pot) before 2 weeks of spraying. Then the infested potted plants with folded leaves were treated separately with different concentrations (10^4, 10^5,10^6, 10^7, 10^8 cfu/ml) of the bacterial isolates. The control plants were sprayed with water.

Characterization of bacterial isolate

The bacterial isolate giving best mortality of rice leaffolder was then characterized on the basis of morphological, physiological and biochemical and molecular aspects (Collee and Miles 1989; Smibert and Krieg 1994; Lacey 1997; Logan and de Vos 2009; Janssen 1994; Thompson et al. 1994). Scanning electron micrographs of the isolates were taken to study the morphology of the bacterial isolates. Antibiotic sensitivity was tested using different antibiotic discs viz. amoxycilin (10 µg/disc), kanamycin (30 µg/disc), nalidixic acid (30 µg/disc), rifampicin (5 µg/disc), doxycycline (30 µg/disc), gatifloxacin (10 µg/disc), vancomycin (30 µg/disc), gentamycin (10 µg/disc), ampicillin (10 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc), penicillin G (10 U/disc) and gentamycin (10 µg/disc) following Brown (2004). Phylogenetic tree of the potential bacterial isolate TS 110 was constructed following Tamura et al. (2007).

Field assay of spore crystal formers

The isolate TS110 was grown on Nutrient Broth (NB) for 10 d on a rotary shaker (200 rpm, 30 ± 0.1 °C). After that the culture was centrifuged at 10,000 rpm for 10 min and washed three times with distilled water by centrifugation and then preserved at −20 °C. Suspension of the isolate was made in distilled water containing 0.001 % APSA (commercial sticker, 1 µl/100 ml) and diluted logarithmically to obtain 0–10^8 bacteria/ml and APSA sprayed in the LF infected rice field twice a year i.e. in dry and wet season, respectively, from 2012 to 2014. The mortality was recorded daily.

Result

The bacterial isolate TS110 formed filamentous, off-white, elevated colony ranging 2.45–3.87 mm in diameter. The isolate was rod shaped having spores and crystals (Fig. 1).

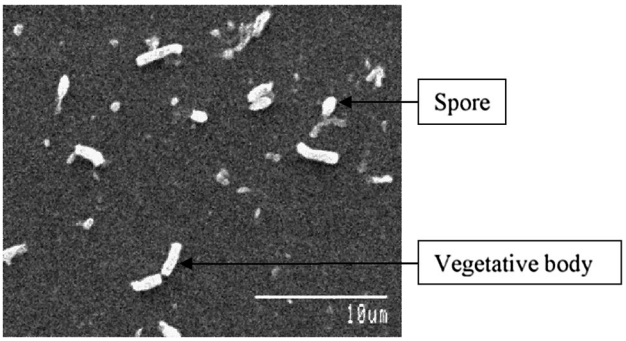

Fig. 1 Scanning electron micrograph of the bacterial isoalte TS110 (KT725786)

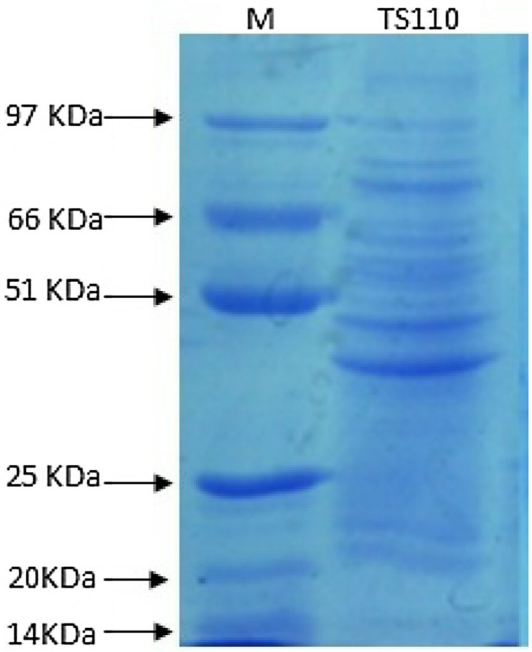

Fig. 2 SDS-PAGE analysis of the cellular protein of TS110 (KT725786)

TS110 was found to be positive for catalase, oxidase, citrate, nitrate, urease, methyl red, voges-proskauer and arginine dihydrolase test. TS110 had the ability to hydrolyse starch, gelatin, casein, lipid and lecithin. The isolate could ferment glucose, fructose, mannose, arabinose and trehalose but could not ferment sucrose, xylose and mannitol present in the medium. TS110 showed growth at pH 5.7–6.8 and 4 –50 °C temperature; this isolate could tolerate up to 10 % NaCl in the Nutrient Broth (NB) medium (Table 1). The total DNA and protein content of the isolate were 7.6 µg/ml and 7.8 µg/ml respectively. SDS-PAGE protein profiling showed that TS110 contained 18 bands of protein having molecular weight of 85.101 to 11.00 kDa (Fig. 2). Antibiotic sensitivity test showed that the isolate was sensitive to kanamycin (30 µg/disc), nalidixic acid (30 µg/disc), rifampicin (5 µg/disc), doxycycline (30 µg/disc), gatifloxacin (10 µg/disc), vancomycin (30 µg/disc), gentamycin (10 µg/disc), ampicillin (10 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc), gentamycin (10 µg/disc) but resistant to amoxycilin (10 µg/disc) and penicillin G (10 U/disc) (Table 1). The Neighbour joining tree of TS110 revealed that the isolate *Bacillus thuringiensis* TS110 (KT725786) branched with the cluster containing other species of *Bacillus thuringiensis, Bacillus cereus and Bacillus sp.* with 33 % bootstrap value (Fig. 3).

Table 1 Characterization of TS110 isolated from the ricefields of Burdwan district, West Bengal, India

Characters	TS110	
Colony	Filamentous, offwhite, elevated colony ranging 2.45–3.87 mm in diameter	
Vegetative body	Bacterium rod shaped, Gram (+)ve, motile	
Spore	Spore elliptical	
Crystal	Present	
Growth characteristics	Growth on nutrient media, NaCl (up to 10 %), 4–50 °C temperature, pH 5.7–6.8	
Biochemical tests	Positive	Negative
	Catalase, nitrate reductase, MR, VP, oxidase, urease, Indole, citrate, arginine dihydrolaase	–
Enzymatic activity	Starch, lipid, gelatin, casein, lecithin hydrolysis	–
Fermentation	Glucose, fructose, mannose, arabinose, trehalose	Sucrose, xylose, mannitol
Antibiotic sensitivity test	Sensitive	Resistant
	Kanamycin (30 µg/disc), nalidixic acid (30 µg/disc), rifampicin (5 µg/disc), doxycycline (30 µg/disc), gatifloxacin (10 µg/disc), vancomycin (30 µg/disc), gentamycin (10 µg/disc), ampicillin (10 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc), gentamycin (10 µg/disc)	Amoxycilin (10 µg/disc) penicillin G (10 U/disc)

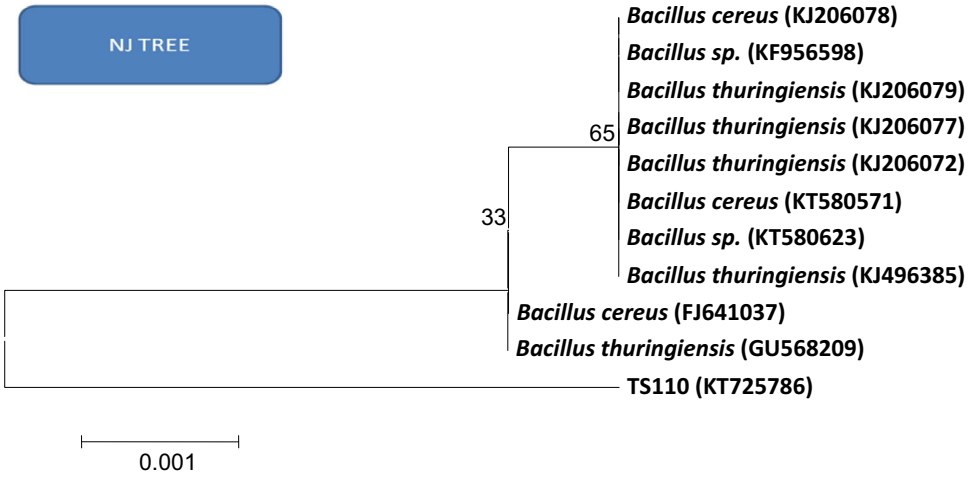

Fig. 3 Phylogenetic tree constructed based on partial 16S rRNA genes sequences of TS110 strain along with the other 16S rRNA gene sequences retrieved from NCBI and RDP

For cut leaf assay the LC_{50} ($\times 10^7$cfu/ml) values of the *B. thuringiensis* strain TS 110 were against 3rd, 4th and 5th instar larvae of *C. medinalis* in dry and wet season was 2.36, 2.37, 4.34 and 2.41,2.87, 5.25 respectively. The potted plant assay showed the LC_{50} ($\times 10^7$cfu/ml) of TS110 in dry and wet season as 4.43, 3.94, 4.89 and 4.00, 3.21, 3.98 against 3rd, 4th and 5th instar larvae of *C. medinalis* respectively. The field assay of the bacterial pathogen BtTS110 against 3rd, 4th and 5th instar larvae of *C. medinalis* showed the LC_{50} ($\times 10^7$cfu/ml) values of 3.77, 5.29, 4.83 and 4.93, 4.42, 4.72 in dry and wet season respectively (Fig. 4).

Discussion

The morphological, biochemical and phylogenetic analysis of TS110 characterized it as *Bacillus thuringiensis.* Phylogenetic analysis revealed very close similarity between the bacterial isolate TS110 and other *B. thuringiensis* sequences present in the Gen Bank. The range of LC_{50} in cut leaf assay was less than that of potted plant assay. This is because cut leaf assay was done in the laboratory condition which was more controlled than the net house environment where potted plant assay was performed. No such differences have been found in the effect of *Bacillus thuringiensis* TS110 against larvae of *C. medinalis* in different seasons but the LC_{50} value of TS110 against *C. medinalis* was instar dependent($p = 0.029$) which was found to be similar the findings of Tabashnik and Carrie‘ re (2004) who also observed the same phenomenon.

The mean LC_{50} value (cfu/ml) of TS110 against larvae of *C. medinalis* were lower than the findings of Ramamourti et al. 2012 who recorded the LC_{50} as 0.6×10^8–22.5×10^8 of indigenous Bt isolates against rice LF. The comparatively lower LC_{50} value in the present study was due to the biotoxicity of the native isolate TS110.

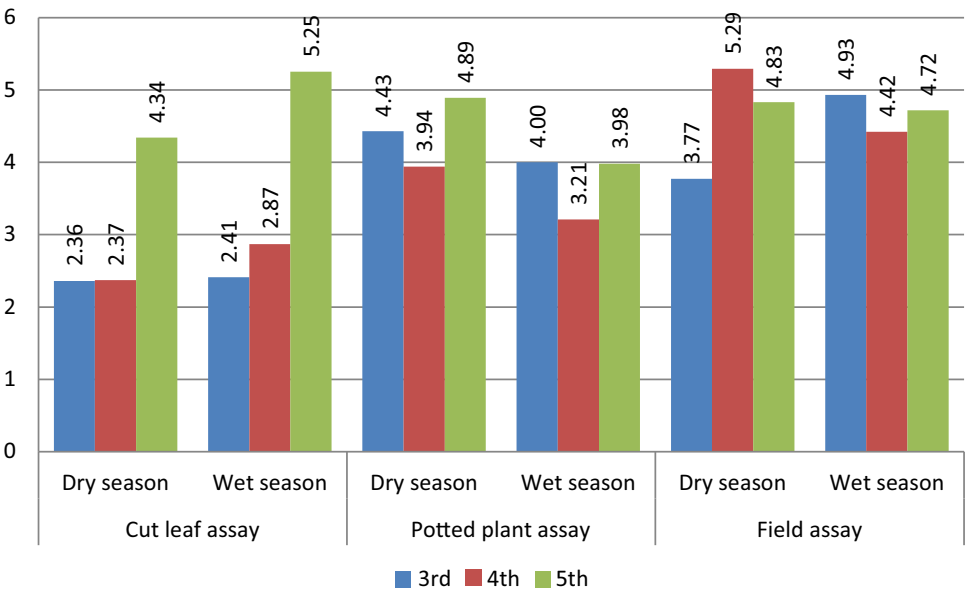

Fig. 4 LC_{50} values of TS110 against *C. medinalis* in cut leaf, potted plant and field assay

Effectiveness of the bacterial pathogen varied with concentration and the period of exposure (Savitri and Murali Mohan 2003). Application of higher concentration of *Bacillus thuringiensis* reduced the food consumption of *C. medinalis* and enhanced larval mortality (Singh et al. 2000). The insect mid gut is the main target area for Bt to infect the larvae. The crystal proteins of Bt get solubilized and activated in the mid gut at high pH and produce delta-endotoxins that showed the toxicity against the larvae (Knowles 1994). The delta-endotoxin firstly binds with the membrane glycoprotein of the midgut epithelium and disrupts it leading to cell lysis and killing the larvae ultimately (English and Slatin 1992). A wide variation in the effectiveness of *B. thuringiensis* isolates was found according to different target insects (Yaradoni, 1999). Environmental parameters viz; temperature, pH and light affected the pathogenicity of *B. thuringiensis* (Bt) in field (Somasekhar and Krishnayya 2004).

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Compliance with ethical standards

Conflict of interest Declared none.

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