Journal of Biological Control, 26 (2): 138-143, 2012

Research Article

Development of recombinant Cry3A expressing *Bacillus cereus* isolated from the rhizosphere of brinjal (*Solanum melongena* L.) in the management of ash weevil (*Myllocerus subfasciatus* Guerin)

MANAMOHAN MALIGEPPAGOL*, R. ASOKAN and SUKHADA MOHANDAS

Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta Lake PO., Bangalore - 560 089, Karnataka, India.
** Corresponding author E-mail:* manamohan.s.m@gmailcom

ABSTRACT: A predominant *Bacillus cereus* (BR145) was engineered to express Cry3A protein by electroporation from *B. thuringiensis tenebrionis* (*Btt*.) The rhizospheric survival of these *Bacillus* spp., was compared where survival of the former was better in the rhizosphere of brinjal than the latter. The efficacy of Cry3A protein obtained from the lysed cells of rBR145 and *Btt* were evaluated for toxicity against grubs and adult *M. subfasciatus* in pot and detached leaf bioassays, respectively. The toxicity of Cry3A protein produced from both the bacilli was on par.

KEY WORDS: Cry3A, *Myllocerus subfasciatus*, *Bacillus cereus*, *Bacillus thuringiensis* ssp. *tenebrionis,* rhizosphere.

(Article chronicle: Received:26-3-2012 Revised:12-6-2012 Accepted:28-6-2012)

INTRODUCTION

Cry3A proteins produced by *Bacillus thuringiensis tenebrionis* are known to be toxic to coleopteran insects. Ash weevil, *Myllocerus subfasciatus* Guerin has become a severe pest on brinjal (Gowda and Veeresh, 1984). Here, we have employed the coleopteran active Cry3A protein producing *Bacillus* species to manage the ash weevil infesting brinjal. The ability to colonize the rhizosphere (competence) is an essential criterion for bacteria to function as biological control agents. Rhizospheric competence of soil microbes has been known to be strain specific rather than the species or the genus (Milus and Rothrock, 1993). Preparations based on *B. thuringiensis* (*Bt)* strains producing insecticidal crystal proteins have been commercially available and are used as environmentally acceptable insecticides as they are toxic to the specific target insect, but are essentially harmless to most other non-target organisms. This specificity makes the use of *Bt* in the pest management very attractive. Since, the commercialization of transgenic plants expressing Cry toxins is an elaborate and time consuming process requiring extensive bio safety trials and regulatory approvals (Sudha, *et al.,* 1999), use of microbial formulations is a viable option. However, *Bt* spores and crystal proteins are available commercially and are used extensively to combat the crop pests. Most of the *Bt* preparations were targeted towards the lepidopteran pests, except for a few preparations which are successfully used against the coleopteran pests, *viz.,* Novodor, has been used to control the important pests such as Colorado potato beetle (*Leptinotarsa decemlineata* Say) and carrot weevil (*Listronatus oregonensis* Le Conte) (Saade *et al*., 1996). However, the effectiveness of the application depends upon the stability of the cry toxin and its concentration in the insect feeding zone and its replenishment by multiplication and sporulation of the bacterium. The half-life of the *Bt* crystal protein in the rhizopshere is short (4-20 days) and it is rendered ineffective by the proteolytic attack and by adsorption to the clay particles (Palm *et al.,* 1996). This puts severe limitations on the use of the spore and crystal protein formulations in controlling soil dwelling pests. In the recent years, *Bt* insecticide research has focussed on i) the development of strains possessing increased virulence, potency, selectivity, and environmental longevity through selection and genetic engineering and the ii) the introduction of *B. thuringiensis* ä-endotoxin genes in to a wide variety of prokaryotic and eukaryotic hosts (Selinger *et al.,* 1998). Compared to *B. cereus*, *B. thuringiensis* has low rhizospheric competence and is sparsely present and its survivability in the rhizosphere is also low (Weller,

1988). In view of this, in the present study, Cry3A toxin was expressed in a rhizospheric *B. cereus* having higher natural survivability than the *Btt* by gene transfer and its toxicity against *M. subfasciatus* infesting brinjal was evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B.t tenebrionis was obtained from the Institut de Pasture, France and grown on nutrient agar (NA) at 30°C that harbours mega plasmids containing the *cry3*A gene. A predominant *Bacillus* isolate (>10^5 cfu/g soil) from the rhizosphere of brinjal was isolated and identified as *B. cereus*, using the API CHB 50 kit, API NE 20 kit, from Biomuerieux (France) following the instructions of the manufacturer and also by employing 16S rRNA gene sequence analysis, this isolate was designated as BR145 and grown on NA at 30°C.

Survival studies in the brinjal rhizosphere were carried out on the 6-week old brinjal plants grown in pots of 30 cm diameter containing about 12 kg of steam sterilised potting mix. Two hundred ml of NA was inoculated with *Btt* and rBR145 and incubated for 2 days to an OD_{600} of 0.5. Cells were harvested by centrifugation and washed twice with sterile distilled water and re-suspended in sterile peptone (0.1%) to give a final cell density of 10^7cfu/ml. Five ml of cell suspension was added to the potted brinjal plants. Bacterial counts were made at seven days intervals for four weeks by serial dilution and colony counting (cfu/g). The stability of the recombinant cry3A plasmid in rBR145 was confirmed by random selection and light microscopy.

Transformation of *Bacillus cereus*

Transformation of BR145 with plasmid isolated from *Btt.* Plasmid preparations were made by modified alkaline lysis method (Bimboim and Doly, 1979) with inclusion of lysozyme in the lysis buffer and 5u of proteinase K. Plasmids were resolved on 0.8% agarose gel by electrophoresis and quantified using a spectrophotometer. Competent cells of BR145 were prepared by pelleting the cells and washing thrice with ice cold sterile water, the pellet was suspended in 1 ml of 30% ice cold polyethylene glycol-1000 and cells were kept on ice until electroporation. An aliquot of 45 ì l of cells was mixed with 5 ì l of the plasmid DNA, electroporation was performed in a BTX Electro cell manipulator 600 at 14.4 KVolts/cm, with a pulse length of 3.02 milliseconds in a 1 mm gap sterile cuvette. After electroporation 1 ml of twice the concentration of LB (TLB) was added and incubated for 90 minutes at 30°C. A 100 ì l of aliquot was spread plated on the NA plates and incubated for 3 days and individual colonies were observed in a microscope after staining the smear with crystal violet for 20 seconds and the transformants were selected based on the presence of bi-pyramidal crystals by microscopic observations.

PCR confirmation

Presence of *cry*3A gene in rBR145 was confirmed by PCR gene specific primers, CJIII20 (5'TTAACCGTTTT CGCAGAGA) and CJIII21 (5'TCCGCACTTCTATG TGTCCAAG) which yield a PCR product of about 700 bp (Ceron *et al.,* 1995). The crystal toxin was partially purified by washing the spore and crystal mixture with 2% NaCl for three times most of the spores were removed as frothy suspension after each washing, the pellet was washed with cold sterile, distilled water and dissolved in carbonate buffer pH 10.5. An aliquot of 200 ì l was used for estimating the protein concentration by Bicinchinoninic (BCA) method.

Bioassay

The partially purified toxin was used for the bioassay, against grubs and adult weevils. Brinjal leaves were dipped for 2 minutes in spore crystal mixture in sterile water with 0.1% Tween 20 as surfactant, the leaves were air dried in a laminar air flow chamber for 10 minutes. Three leaves were placed in each Petri-dish (15 x 2 cm), and 10 adult weevils were introduced and replicated four times. Observation on the mortality was recorded at 24 hours interval for two weeks.

Bioassay using grubs was performed on the potted brinjal plants in to which 30 newly hatched grubs were released. Five ml of the bacterial culture of both *Btt* and rBR145 was poured in to the rhizosphere of brinjal. After three weeks, the brinjal plants were carefully uprooted and checked for the grub mortality. Total number of grubs surviving in the rhizosphere were counted and averaged for each of the treatments including controls.

RESULTS AND DISCUSSION

It was observed that after 5 days the *Btt* count came down to 5 x 10^3 cfu per g soil, from the initial count of about 10^7 cfu and at the end of 3 weeks to about 0.5% of the initial count. Whereas, rBR145 count decreased to 5.9 x 10^5, after 3 weeks the counts were about 3.5% of the initial count of about 10^7.

Introduction and expression of *cry3*A gene in BR145, the selection of the transformants was based on the presence of the crystal proteins by microscopy to further confirm the gene transfer *cry3*A gene was amplified by PCR using specific primers, PCR products were resolved on 1% agarose gels and scored for the presence of the expected band size.

Initially the LD_{50} for the purified CRY3A protein against *M. subfasciatus* grubs was 0.947µg/ml. In bio-assay experiment using *M. subfasciatus* grubs, of the 30 grubs introduced in to the potted brinjal plants, the treatment with rBR145 and *Btt*, only a mean numbers of 4.6 and 5 grubs survived whereas in control 25.6 grubs survived. More than 70% of the adult *M. subfasciatus* were dead within 4 days in rBR145 and *Btt* treated leaves. In control treatments weevils survived for more than two weeks.

M. subfasciatus is a serious pest in brinjal causing huge crop losses, Use of Cry toxins is a highly targeted and ecologically sound approach in pest management. In view of this a recombinant *B. cereus,* (rBR145) expressing Cry3A crystal protein has been developed to manage the ash weevil infesting brinjal. The major damaging stage is grub, which feeds on the roots while the adults feed on the foliage resulting in the characteristic notching of the leaf edges. The damage by grubs is more severe in young seedlings, where they girdle the tap root resulting in the death of the seedling.

B. thuringiensis tenebrionis has been shown to control this pest, however, the survivability in the rhizopshere is low, thus a predominant rhizospheric *Bacillus* species was isolated and identified as *B. cereus.* Since, many *Bacillus* species, mainly members of the *B. cereus* group, possess the enterotoxin genes and they may be pathogenic (Granum *et al.,* 1999), we tested the presence of enterotoxic gene using *hblA* PCR primers and was found negative. This isolate was transformed with *cry3*A genes by using the plasmid of *Btt* and the expression of toxin protein was stable. *B thuringiensis* has been known to reproduce poorly in the soil (Raymond *et al.,* 2010). Comparison of rhizospheric survival of *Btt* and rBR145 have shown that the latter can survive for longer periods, at the end of 3 weeks rBR145 population was approximately 7 times higher than that of *Btt.* Since, the BR145 was the predominant one in the brinjal rhizosphere, its competence also was naturally higher compared to the introduced *Btt.* This could be attributed to the specific bacterial lipopolysaccharides, antibiotics, the root exudates and also the ability to attach itself to the roots of the plants etc. It is documented that the *B. cereus* strains produce and secrete a number of antibiotics such as cerecin (Oscariz *et al.,* 1999; Vijayalakshmi and Suseela, 2010).

The bioassay results on adult weevils and grubs have demonstrated the effectiveness of the Cry3A toxin. These results also showed that the Cry3A proteins produced by rBR145 was equally toxic to both stages of *Myllocerus* weevil as that of *Btt.* The mortality rate of *Myllocerus* grubs released on to the potted brinjal plants receiving soil application of rBR145 and *Btt* cultures had exhibited about 85% mortality within 3 weeks of application as compared to the control, indicating that recombinant *B. cereus* produced Cry3A protein with similar toxicity to the target pest as that of *Btt*.

The transferred plasmids were highly stable in the rBR145 host cells for a number of generations in the antibiotic free medium by microscopic observations of the randomly selected colonies of host cells. A number of reports support the highly stable replication and maintenance of the *Bt* toxin producing plasmids in the recombinant hosts. Wang *et al.* (2006) developed an engineered wild type *B. thuringiensis* subsp. *aizawai* G03 which naturally contains Lepidopteran active cry toxins with a Coleopteran active cry toxin cry3Aa7 and monitored the inheritance of the plasmids in the recombinant cells and observed that the plasmid was maintained in bacteria cultured for 180 generations in the culture media containing no antibiotics. Similarly, Casique-Arroyo *et al.* (2007) a recombinant plasmid in *B. thuringiensis* subsp. *kurstaki* HD-73 showed high segregation stability in the antibiotic free medium.

Many studies have shown that recombinant heterologous Cry protein production was effective in pest management as these can be engineered by domain swapping or by constructing chimeric toxins with an objective to enhance the toxicity, to widen host range and also to manage or delay resistance development in the pest (Knight *et al.,* 2004). Thus, we have developed a recombinant *B. cereus* having significantly higher rhizospheric survival than the natural host of *cry*3A genes, *Btt* by the heterologous production of Cry3A protein that is toxic to the *M. subfasciatus* weevil infesting brinjal. Bioassay using adult weevils has indicated that both the transformed *B. cereus* and *Btt* have led to a mortality of over 70% post four days of treatment, in control the weevils have survived for more than two weeks. With this approach many beneficial soil microbes can be used for pest control with reduced number of sprayings. Thus, by employing the recombinant microbes it is possible to tailor the microbes for multiple uses,

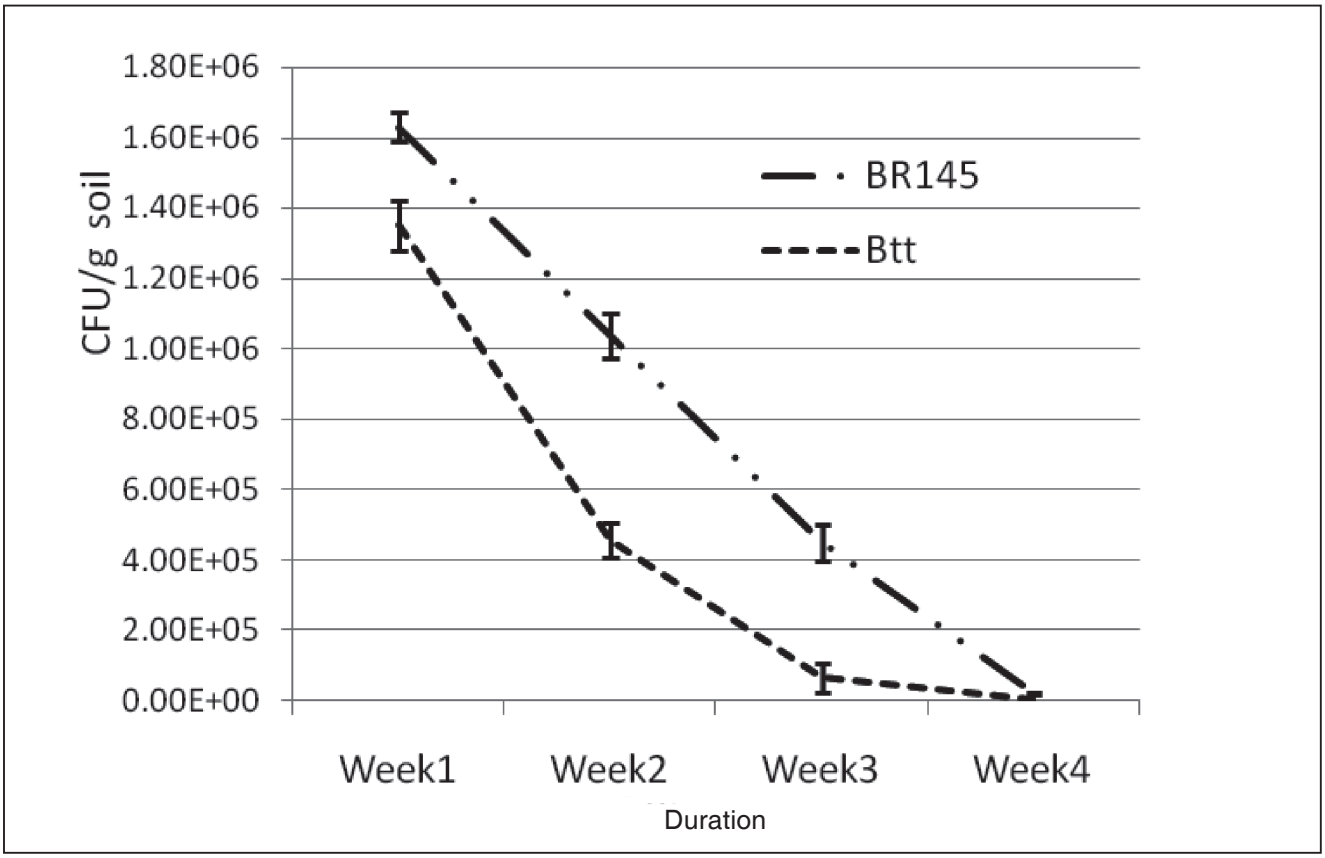

Fig 1 Survival of the inoculated bacterial cells in the rhizosphere of brinjal. Error bars represent standard error of mean of three replications

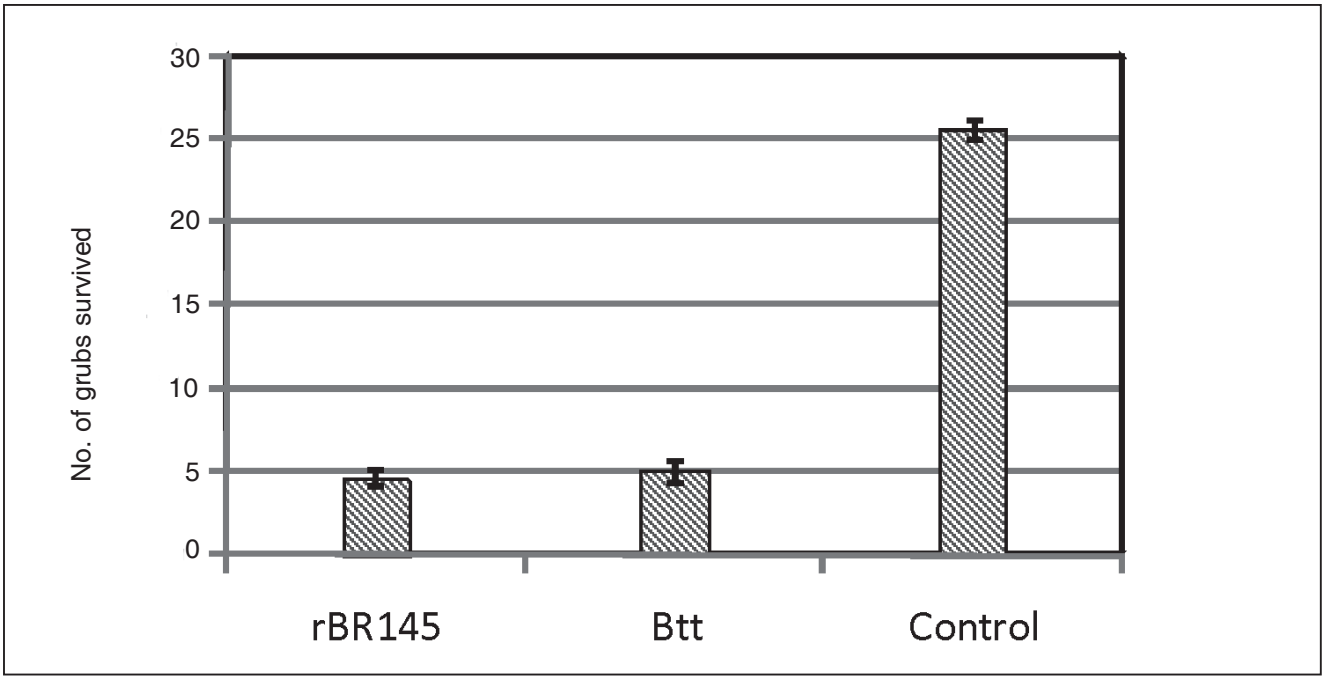

Fig 2 Effect of Cry3a producing *B. cereus* and *B.t.t.* on the rhizospheric survival of *Myllocerus subfasciatus* Guerin grubs. Error bars represent standard error of mean at 95% confidence interval

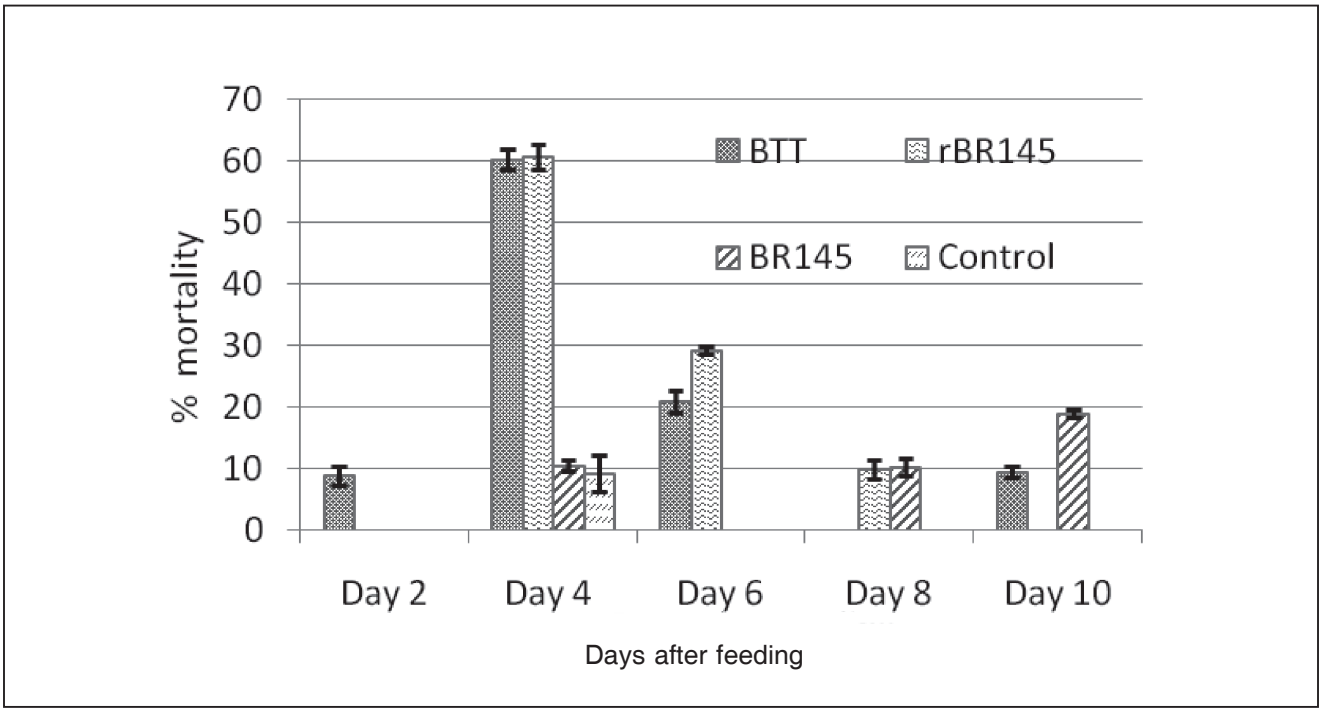

Fig 3 Percent mortality of the adult *Myllocerus subfaciatus* Guerin fed with leaves treated with Cry toxin. The error bars represent standard error of mean at 95% confidence level

such as biofertilizers, biopesticides and microbes inhibiting plant pathogens.

ACKNOWLEDGEMENT

The authors thank the Director, IIHR, for encouragement and providing required facilities to conduct this experiment. The help rendered by the technical staff is gratefully acknowledged.

REFERENCES

Bimboim HC, Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513–1523.

Casique-Arroyo G, Bideshi D, Salcedo-Herna´ndez R, Barboza-Corona JE. 2007. Development of a recombinant strain of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 that produces the endochitinase *ChiA74*. *Antonie van Leeuwenhoek,* **92**: 1–9.

Cerón J, Ortíz A, Quintero R, Güereca L, Bravo A. 1995. Specific PCR primers directed to identify cryI and cryIII genes within a *Bacillus thuringiensis* strain collection. *App Env Microbiol.* **61**: 3826–3831.

Gowda KS, Veeresh GK. 1984. Assessment of crop loss in brinjal (*Solanum melongena* L.) due to as weevil *Myllocerus subfasciatus* Guerin. (Curculionidae : Coleoptera). *J Soil Biol Ecol.* **4**: 41–52.

Granum PE, O'Sullivan K, Lund T. 1999. The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiol Letters* **177**: 225–229.

Knight JS, Broadwella AH, Grant WN, Shoemaker CB. 2004. A strategy for shuffling numerous *Bacillus thuringiensis* crystal protein domains. *J Econ Ent.* **97**: 1805–1813.

Milus EA, Rothrock CS. 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. *Canadian J Microbiol.* **39**: 335–341.

Oscariz JC, Lasa I, Pisabarro AG. 1999. Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. *FEMS Microbiol Letters,* **178**: 337–341.

Palm CJ, Schaller DL, Donegan KK, Seidler RJ. 1996. Persistence in soil of transgenic plant-produced *Bacillus thuringiensis* var. *kurstaki* ä-endotoxin. *Canadian J Microbiol.* **42**: 1258–1262.

Raymond B, Wyres KL, Sheppard SK, Ellis RJ, Bonsall MB. 2010. Environmental factors determining the

epidemiology and population genetic structure of the *Bacillus cereus* group in the field. *PLoS Pathogens,* **6**: e1000905. doi:10.1371/journal.ppat.1000905

Saade FE, Dunphy GB, Berlier RL. 1996. Response of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Cucrulionidae), to strains of *Bacillus thuringiensis*. *Biol Control* **7**: 293–298.

Selinger, LB, Kachatourians GG, Byers JR, Hynes MF. 1998. Expression of a *Bacillus thuringiensis* ä-endotoxin gene by *Bacillus pumillus*. *Canadian J Microbiol.* **44**: 259–269.

Sudha SN, Jayakumar R, Vaithilingam S. 1999. Introduction and expression of the *cry1Ac* gene of *Bacillus thuringiensis* in a cereal-associated bacterium, *Bacillus polymyxa. Curr Microbiol.* **38**: 163–167.

Vijayalakshmi K, Suseela R. 2010. Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: An application of statistical optimization technique. *African J Microbiol Res.* **4**: 2388–2396.

Wang G, Zhang J, Song FWJ, Feng S, Huang D. 2006. Engineered *Bacillus thuringiensis* GO33A with broad insecticidal activity against lepidopteran and coleopteran pests. *Appl Microbiol Biotechnol*. **72**: 924–930.

Weller DM. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annl Rev Phytopathol.* **26**: 379–407.