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Journal of Invertebrate Pathology 88 (2005) 8–16
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Isolation and toxicity of *Bacillus thuringiensis* from potato-growing areas in Bolivia

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Received 20 September 2004; accepted 18 October 2004
Available online 4 January 2005

Abstract

Bacillus thuringiensis was isolated from 116 samples collected in high altitude potato-growing areas in Bolivia. In these regions, main potato pests are the potato tuberworm *Phthorimaea operculella*, and the Andean weevils *Premnotrypes latithorax* and *Rhigopsidius tucumanus*. *B. thuringiensis* was found in 60% of the samples. The main percentage of samples with *B. thuringiensis* was found in larvae of *R. tucumanus* (78%). Bioassays were performed with 112 isolates. None resulted toxic to either larvae or adults of the two Andean weevils. However, 18 isolates from this study showed more toxicity against the beet armyworm *Spodoptera exigua* than the standard strain var. *kurstaki* isolated from DELFIN. Among these isolates, three were also effective against *P. operculella*, conferring better or equal protection to the tubers than the reference strain HD-1 isolated from DIPEL. The most toxic strains against *S. exigua* and *P. operculella* were characterized in terms of serotyping, crystal morphology, protein profile, and *cry* gene content. PCR was performed with primers amplifying genes from the *cry1*, *cry2*, *cry3*, *cry4*, *cry7, 8*, and *cry9Aa* families. The toxic strains presented bipyramidal crystals, at least a band of 130 kDa in SDS–PAGE, and showed an amplification product with *cry1* family primers. One of the isolates did not amplify with any specific primer belonging to known *cry1* genes. Restriction Fragment Length Polymorphism (RFLP) confirmed the presence of a novel gene and sequence comparison showed that this gene had homology to *cry1G*.

Keywords: Screening; Ecology; Insect toxicity; Serotyping; *cry* genes

1. Introduction

The natural occurrence of the spore-forming grampositive bacterium *Bacillus thuringiensis* has been extensively studied throughout several decades. *B. thuringiensis* has the ability to produce a parasporal crystal which contains one or more Cry proteins that can be toxic for a number of insects, including important insect pests. Cry proteins are codified by *cry* genes and, up to date, more than 200 *cry* genes have been described and classified into a large number of groups and subgroups due to their amino acid sequence homology (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/toxins2.html). On the basis of their flagellar (H) antigens, *B. thuringiensis* has been classified in 69 serotypes comprising 82 serovars (Lecadet et al., 1999). Collections of isolates of *B. thuringiensis* have been established with samples collected from different ecological habitats around the world. In this respect, this bacterium has been isolated from soil samples (Chilcott and Wigley, 1993; Hossain et al., 1997; Iriarte et al., 1998; Martin and Travers, 1989); insects and their habitats (Apoloyo et al., 1995; Kaelin et al., 1994); stored products and warehouses (Hongyu et al., 2000; Meadows et al., 1992); plant materials (Bel et al., 1997; Mizuki et al., 1999); and aquatic environments (Ichimatsu et al., 2000; Martínez and Caballero, 2002), among others. Very few *B. thuringiensis* screening programs have been performed in Latin

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0022-2011/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2004.10.006

America (Bravo et al., 1998; Ibarra et al., 2003; Uribe et al., 2003), and none has included Andean tropical high valleys and the Altiplano in Bolivia.

The Andes cross the western area of Bolivia from North to South. The Andean region includes different agricultural and ecological zones ranging from the Altiplano (plateaus at altitudes above 3500 m) to inter-Andean and mesothermic valleys. The large biodiversity found in the Andean region is an extremely valuable resource of germplasm. Many peasant families in the Andes depend on potato as their staple food and as the most important source of income. The most important pests in these high altitude potato growing areas are the potato tuber moth, *Phthorimaea operculella*, and the Andean potato weevils *Premnotrypes latithorax* and *Rhigopsidius tucumanus*. These pests act as borers in potato tubers, where larvae tunnel through potatoes, filling tunnels with excrements and allowing fungi to colonize the tunnel. Some tubers are completely spoiled and other become unsightly and of little food value. Losses in potato tubers by *P. operculella* during the storage period can reach up to 50%. Losses in tuber quality by *P. latithorax* and *R. tucumanus* have been estimated as 100 and 80%, respectively. The lack of access to adequate technologies and market opportunities cause agricultural productivity of this region to be one of the lowest in the continent. Since many Andean farmers cannot afford purchasing insecticides, this major constraint to the production could be addressed best by the use of insect-resistant potatoes. *B. thuringiensis* screenings provide a source of isolates with potential toxic genes active against insect pests and useful to be introduced in transgenic crops.

In this study, a new collection of *B. thuringiensis* isolates has been developed from samples collected in potato-growing areas from the Bolivian high valleys and the Altiplano. Toxicity has been tested against the two Andean weevils, *P. latithorax* and *R. tucumanus*, and two lepidopteran pests, *Spodoptera exigua* and *P. operculella*. The most toxic isolates have been characterized in terms of serological identification, potency, crystal morphology, protein profile, and *cry* gene content, and a new *cry* gene has been found and partially characterized.

2. Materials and methods

2.1. Sample collection and B. thuringiensis isolation

Bacillus thuringiensis occurrence was examined in samples from Bolivian high valleys and the Altiplano, between 2000 and 4500 m of altitude. This area is known as "the potato zone" and includes the Departamentos of Potosí, Chuquisaca, Tarija, and Cochabamba. Soil samples were collected after removing the surface layer to avoid detrimental effects of UV radiation on *B. thuringiensis* (Addison, 1993). Dust samples were collected by scooping directly from the floor of warehouses. Potato samples were obtained from tubers that had been infested by larvae of Andean weevils; tubers were cut open along the trajectory of the gallery and the debris was collected. Dead larvae, pupae, and adults of Andean weevils found in these galleries were also collected. In all cases, samples were collected into sterile plastic tubes or bags and stored at −20 °C until processed.

Bacillus thuringiensis isolation was performed as described previously (Bel et al., 1997). Colonies with parasporal inclusions were identified as *B. thuringiensis* by phase-contrast microscopy. These isolates were plated again for single colony purification on CCY agar plates (Stewart et al., 1981) and stored in sterile liquid Nutrient Broth containing 50% glycerol at −20 °C.

2.2. Preparation of spore–crystal suspensions

A loopful of cells of each isolate grown on CCY agar medium (Stewart et al., 1981) were inoculated in 1 ml of sterile water and heated to 70 °C for 30 min to kill vegetative cells. The suspension was inoculated into 50 ml of liquid CCY medium and grown at 30 °C and 250 rpm until at least 90% of the cells had lysed and the spores and crystals released. The spores and crystals were concentrated and washed with ice-cold distilled water several times. The final pellet was re-suspended in 4 ml of 1 mM NaCl. Estimation of spore–crystal concentration was carried out in 1:100 dilutions of the final suspensions by measuring the optical density at 600 nm. Samples were stored at −20 °C until used. The spore–crystal suspension of the *B. thuringiensis* var. *kurstaki* from the commercial product DELFIN (Sandoz Agro, Switzerland) and DIPEL (Abbot Laboratories, Illinois) were used as reference standards for *S. exigua* and *P. operculella* bioassays, respectively.

2.3. Toxicity assays against adults and larvae of R. tucumanus and P. latithorax

The activity of *B. thuringiensis* isolates was tested against adults of *R. tucumanus* and *P. latithorax* by spreading on the surface of potato leaves a highly concentrated suspension of spores and crystals (OD_{600} reading of 13), to which 2% Triton X-100 was added. Each leaf was placed in a plastic container with an adult weevil. To avoid rapid desiccation of potato leaves, their stem tips were embedded in a soaked cotton. Three replicates were used for each isolate and insects were inspected after 14 days. Bioassays with larvae of *R. tucumanus* and *P. latithorax* were performed by making a hole in the tuber by removing a piece of flesh. The two surfaces (that of the hole and the piece removed) were treated with the concentrated crystal-spore suspension and, after absorption, a neonate larvae was placed into the potato hole and covered with the corresponding