5.06 Studies on biodegradation of Cry1Ac protein by rizospheric bacteria from Bt cotton soil

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Bacillus thuringiensis, Bt, the most used bacteria for insect control, can also be genetically engineered into crops in order to protect them against insects. The development of such insect-resistant crops has some benefits and some potential risks. The release of Cry proteins (originated by Bt *cry* gene inserted) to the soil (pollen deposition, root exudation, cell senescence, residues left in the field after harvesting, among others) is one of the potential risks foreseen and this may occur during the plant lifespan.

Root exudates influence rizosphere microorganism and in this way biosafety studies were developed. A few or non-toxic effects of Cry proteins on woodlice, collembolans, mites, earthworms, nematodes, protozoa, and on the activity of various enzymes in soil are reported, but the question concerning the influence on microbial communities still remains incomplete.

The literature shows that at least part of the Cry proteins could remain adsorbed to mineral or organic-mineral soil particles, remaining protected against degradation and inactivation, and could retain much of its biological activity for a period that will depend on soil characteristics. In addition the structure of the bacterial community around Bt cotton plant is less affected by transgenic Bt-traits than by other environmental factors like plant age, soil characteristics, climate and cotton variety. There are studies on dissipation of the Bt proteins exudating from roots of transgenic plants, but few on degradation of the protein. One study conducted in a Dirradiated sterilized soil indicated that the decline in extractable Cry protein concentration could be due to biotic degradation rather than to physical adsorption by the soil particles.

Bt cotton (Bollgard™ event 531) has been approved for commercial release in Brazil by the National Biosafety Technical Committee (CTNBio) in 2005 and since then the cultivation area is increasing annually. There are some concerns in the country about risks to non target species including soil microbiota. Brazilian Agricultural Research Corporation - Embrapa - leads the research on Bt cotton biosafety issues in Brazil, and Embrapa Environment, one of Embrapa Research Centers, develops studies on the impacts on soil microorganisms. This work was done under the umbrella of Embrapa's Biosafety Research Network - BioSeg -looking for impacts of the approved Bt cotton on soil microorganisms. The objective was to identify and characterize the degradation of Cry protein by soil microorganisms using the microbial purified and activated version of the Cry1Ac protein (66 kDa) supplied by Marianne Pusztai-Carey from Case Western Reserve University. This protein is the one synthesized by the approved Bt cotton in Brazil.

Soil from crop land around Campinas (São Paulo State, Brazil) was collected and used to fill pots where Bt cotton (Bollgard™ event 531) would be cultivated. At a defined period of plant development freshly rizospheric soil was collected from these pots and added to defined culture medium (mentioned here as MMCry, meaning mineral salts medium [MM] plus Cry1Ac protein as the sole carbon and nitrogen source) for bacterial isolation. For comparative purposes the bacterial growth was also observed on the same MM medium amended with glucose and ammonium nitrate instead of Cry1Ac (mentioned here as MMGA). Bacterial growth was undertaken through sussessive culture on MMCry. Each 48 hours an aliquot was transferred to a fresh MMGA or MMCry. This subculture was repeated six times. At the final harvest time it was serially diluted and spread on MMGA or MMCry agar plates, respectively. Twenty four colonies bacterial-like were selected based on observation of its growth fitness and among them the best three were used in growth kinetic studies.

The three bacteria were identified by gas chromatography of cellular fatty acids (FAME) as *Gordonia rubripertincta*, *Bacillus megaterium* and *Bacillus pumilus*. For growth kinetic studies a suspension of young bacteria cells was used as inoculum for fresh MMCry liquid medium at a final concentration of 10^3-10^4 cells/mL. Cultures were grown at 28°C under rotary agitation at 250 rpm. Samples were taken at pre-established intervals, serially diluted and plated for CFU determination (colony forming units) on MMCry agar for 96 hours at 28°C. For verification of Cry protein degradation the three bacteria were grown for 96 hours in MMCry liquid medium, followed by precipitation of bacterial cells and supernate electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Brilliant Blue R250 was used as dye solution and MMCry liquid medium was run in a separate lane as pattern.

The three bacteria selected belong to distinct genera and species but they showed similar behavior regarding the utilization of Cry protein for their development. Statistical analysis of the growth rate (Tukey's test) showed that Cry protein allows bacterial growth, and the growth rate for MMGA and MMCry are similar.

The electrophoresis experiments showed a single band on the reference lane containing MMCry, corresponding to a molecular mass of 66 kDa. For each of the three different bacteria several bands of smaller molecular mass were detected corresponding to Cry1Ac degradation products. Further studies about the kinetic of such biodegradation are under development at Embrapa Environment. Data of growth kinetic and electrophoresis run will be presented at the 10 ISBGMO.