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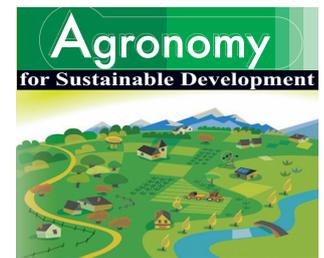
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Research article

Occurrence of *Bacillus thuringiensis* harboring insecticidal *cryI* genes in a corn field in Northern Italy

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Abstract – *Bacillus thuringiensis* (Bt) is a ubiquitous bacteria widely used as a biopesticide to control a number of important insect pests. Since the mid-1990s, genetically modified (GM) plants expressing Bt genes have been used as an effective tool to control a wide range of insect pests. In recent years, a wide number of articles addressing the environmental impact of genetically modified plants have been published. However, only a few have addressed the occurrence and distribution of the indigenous Bt population in agricultural systems. Here, culturing and molecular methods were used to study the occurrence of Bt harboring insecticidal *cryI* genes in a corn field. Samples of corn leaves and soil were collected in July and August 2007 from a 10 ha corn field in Northern Italy. The results showed that the highest Bt density was in leaves located near the soil surface. The incidence of Bt isolates harboring antilepidopteran *cryI* genes was 42% of the total tested isolates. Approximately 20% of the Bt isolates harbored the *cryIAb* and *cryIAc* genes. Similarly to Bt density, the highest abundance of isolates harboring *cryI* genes was found in leaves collected near the plant collar. Less than 9% of the Bt isolated from soil harbored *cryI* genes. Density of Bt was reduced by application of the insecticide chlorpyrifos. This effect appeared to be due to simply washing off effects of the insecticide treatment to the corn phyllosphere. This study showed that Bt was fairly abundant in the corn agroecosystem and that the high incidence of isolates harboring antilepidopteran *cryI* genes could have a role in preserving the sustainability of the agroecosystem.

Bacillus thuringiensis / corn phyllosphere / epiphytic bacteria / chlorpyrifos

1. INTRODUCTION

In recent years, growing interest in sustainable agriculture has led to emphasizing the role of biodiversity in agroecosystems (Clergue et al., 2005). Research has demonstrated that, among other factors, preservation of biodiversity is a critical component in the achievement of sustainability (Ho and Ulanowicz, 2005). Nowadays, this aspect is highly considered for setting up crop management strategies, especially in the era of genetically modified (GM) crops. One of the practical implications of this view is that modern pest control strategies, including integrated and biological pest control, are based on the concept of preserving biological diversity (Bignal and McCraeken, 2000). During the past decade, the role of biodiversity in agroecosystems has been the subject of a number of field studies. Investigations dealing with the biodiversity of agroecosystems have been mainly focused on occurrence and spatial or temporal variability of beneficial insects in cultivated fields (Burel et al., 2004). In contrast, only a limited number

of studies have dealt with microorganisms (Abbas et al., 2004; Mulder et al., 2006; Walter et al., 2007).

The Gram-positive, spore-forming bacterium *Bacillus thuringiensis* (Bt) is widely distributed in the environment and has been isolated from several habitats, including soil, insect cadavers, stored grain and the phylloplane (Schnepf et al., 1998). During sporulation, Bt cells produce parasporal crystal inclusions comprised of a mixture of crystal (Cry) proteins, which are lethal to a variety of insect larvae upon ingestion. Classification of Cry proteins is currently based on amino acid sequence identity and is roughly correlated with the taxonomic order of susceptible insect species. Bt-based formulations, consisting of a formulated mixture of spores and crystals, have been used for more than four decades in pest control of agricultural crops and forestry (Crickmore, 2006). Due to high selectivity, and low toxicity to mammals and to non-target insects, Bt-based insecticides are compatible with integrated pest management and organic farming programs (Clark et al., 2005). Concerns over insecticide resistance have resulted in a continuous search for novel Bt strains with specificity for a much broader range of target insects (Cerón et al., 1995; Bravo

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et al., 1998). Apart from the search for novel Bt strains, these investigations have increased the knowledge on occurrence and distribution of Bt and *cry* genes in the environment (Chak et al., 1994). Although some aspects of the ecology of this bacterium remain unclear, Bt is commonly described as an opportunistic insect pathogen (de Maagd et al., 2001; Broderick et al., 2006). In addition, some authors have assumed that Bt may be present in the phyllosphere in order to protect plants from insect herbivores and that some plants would encourage the growth of this bacterium (Smith and Couche, 1991; Elliot et al., 2000). Available molecular techniques have permitted the cloning and sequencing of a number of *cry* genes. In recent years, gene(s) encoding for truncated Cry toxins have been engineered into agricultural plants, including corn and cotton (Carozzi et al., 1991). Apart from the development of Bt-protected plants, molecular methods and the availability of selective medium have facilitated the study of Bt distribution in the environment. Although information concerning the distribution of Bt and genes encoding for Cry toxins (*cry* genes) in natural or undisturbed environments is available in the literature, only a few studies have focused on agroecosystems (Accinelli et al., 2006; Jara et al., 2006; Marchetti et al., 2007; Accinelli et al., 2008). Therefore, the main objectives of this study were to investigate the occurrence of Bt harboring insecticidal *cryI* genes in a corn field in Northern Italy and to estimate whether or not insecticide treatment against the European corn borer would affect its distribution.

2. MATERIALS AND METHODS

2.1. Site description and sample collection

This study was conducted in a 10 ha (approximately $10^3 \text{ m} \times 10^2 \text{ m}$) corn field located in the Po Valley (Portomaggiore, Italy; $44^\circ 41' 39'' \text{ N}$, $11^\circ 51' 23'' \text{ E}$). The corn variety DKC 6818 (Dekalb Italia S.p.A., Venezia-Mestre, Italy) was grown from April 21st to September 6th, 2007, adopting recommended agricultural practices for the region (Accinelli et al., 2001). The selected field and surrounding area were not treated with Bt-based insecticides for at least the previous ten years. The field was divided into 10 randomly selected 4 m^2 areas (A-L). Five areas (A-E) were randomly selected and treated with insecticide directed at 2nd-generation larvae of the European corn borer (*Ostrinia nubilalis* Hübner; ECB); the remaining areas (F-L) served as untreated controls. Treatments were applied on 27 July by spraying $1.0 \text{ kg a.i. ha}^{-1}$ of chlorpyrifos (Dursban, Dow AgroScience Italia s.r.l., Bologna, Italy) with an over-top sprayer (Whirlwind B612, Martignani s.r.l., Ravenna, Italy). Sampling was conducted on July 25th and August 3rd by taking samples from a 4 m^2 area located approximately in the center of each plot. For each area, three plants were randomly selected and three mature leaves were collected for each plant at the following heights from the ground: 2.5 m (11–12th leaf collar; top leaves), 1.5 m (4–5th leaf collar; middle leaves) and 0.5 m (1–2nd leaf collar; bottom leaves). Three samples of surface soil (0–5 cm depth) for single areas were also collected. Leaf and soil samples were

placed in sterile plastic bags, transported to the laboratory in a cooler, and processed within 48 hours.

2.2. Bt isolation from corn leaves and soil

Single leaves were aseptically cut into ten 4.9 cm^2 disks and placed into 50 mL centrifuge tubes with 20 mL of sterile phosphate buffer saline (PBS; pH 7.2) and 5 g glass beads. Tubes were shaken on a horizontal shaker for 20 min at 200 rpm. After centrifugation, the resulting suspensions were decimally diluted in PBS and 100 μL aliquots were spread-plated onto *Bacillus cereus* (Bc) selective agar (BcSA; Oxoid Ltd, Basingstoke, UK) supplemented with polymyxin B (100 IU mL^{-1}) and egg yolk. Soil samples were passed through a 4 mm sieve and 10 g aliquots were placed in a 250 mL bottle containing 95 mL of PBS and 10 g glass beads. Bottles were shaken for 20 min at 200 rpm and centrifuged at $5000 \times g$ for 10 min. The supernatant was processed as described for leaf samples. Plates inoculated with leaf or soil extracts were incubated at 30°C for 24 hours and colonies with Bt/Bc morphology were counted. A random selection of Bt/Bc-like colonies were streaked onto T3 agar (Travers et al., 1987) and incubated at 30°C for 48–72 hours. Isolates were examined for the presence of parasporal crystals by phase-contrast microscopy. Isolates containing parasporal crystals were counted as Bt and stored in 50% glycerol at -70°C for polymerase chain reaction (PCR) analysis. In order to deactivate vegetative cells, a limited number of leaf samples (20 random samples from bottom leaves) and soil extracts (10 samples) were incubated at 80°C for 10 min (heat-shock treatment), serially diluted in PBS and spread-plated onto T3 agar.

2.3. Polymerase chain reaction analysis

Bt isolates were grown overnight at 30°C on Luria-Bertani (LB) agar. Bt DNA was isolated from liquid cultures using the UltraClean Microbial DNA Kit (MoBio Laboratories Inc., Solana Beach, CA), according to the manufacturer's instructions. Isolated DNA was resuspended in $1 \times \text{TE}$ buffer, stored at 4°C and used as a DNA template for PCR analysis. The PCR mixture contained 5 μL of DNA extract, 2.5 μM of each primer, 20 μL of a $2.5 \times$ Master Mix (Eppendorf AG, Hamburg, Germany) and molecular biology grade water to the final volume of 50 μL . Amplification of the highly conserved region of *cryI* genes was performed with the universal primers Un1f/Un1r described in Ben-Dov et al. (1997). The specific primers used to amplify *cryIAa*, *cryIAb* and *cryIAc* genes are described in Cerón et al. (1995). All primers were provided by MBI Fermentas (St. Leon-Rot, Germany). Amplification was done in a T3 DNA thermal cycler (Biometra GmbH, Göttinger, Germany) with the step cycle program set for 30 cycles, each consisting of a 1 min denaturation step at 95°C , a 1 min annealing step at 48°C , and a 1 min elongation step at 72°C . An extra step of extension at 72°C for 10 min was then performed. PCR products were separated on a 2% agarose gel and

stained with SYBR Green I (Sigma-Aldrich Corp., St. Louis, MO).

DNA from thirty randomly picked Bt isolates was subjected to amplified ribosomal DNA restriction analysis (ARDRA). Amplification of 16S rDNA was carried out using the primers 63f (5'-CAGGCCTAACACATGCAAGTC) and 1387r (5'-GGGCGGWGTGTACAAGGC) (Marchesi et al., 1998). Ten microliters of 16S rDNA products were separately digested with 10 U of *AluI* and *AluI/EcoRI* in a total volume of 40 μ L at 37 °C for 2 hours. Digested products were separated by vertical non-denaturing 8% polyacrylamide gel electrophoresis and visualized by SYBR Green I staining.

2.4. Influence of chlorpyrifos on Bt growth and *cry* gene expression

The potential effect of the insecticide chlorpyrifos on Bt growth and *cryI* gene expression was estimated using the *B. thuringiensis* subspecies *kurstaki* strain HD-1 obtained from the Bacillus Genetic Stock Center (Columbus, Ohio). Erlenmeyer flasks containing 50 mL of LB broth were inoculated with a 1 mL aliquot of an overnight Bt culture. The optical density at 600 nm of inocula was adjusted to within 0.1 units. Viable bacteria in adjusted inocula were enumerated by plating serial tenfold dilutions onto LB agar. Filter-sterilized chlorpyrifos was added to give final concentrations ranging from 0.01 to 10 mg L⁻¹. Flasks were incubated for 48 h at 30 °C in an orbital shaker. The experiment was conducted in triplicate. Untreated flasks and uninoculated flasks were included to serve as the control and blank for spectrophotometric determination, respectively. Growth was monitored by measuring the optical density (OD₆₀₀) using a Jenway 6305 spectrophotometer (Jenway Ltd., Dunmow, UK). The ratio of average OD₆₀₀ values obtained from treated flasks to that of control flasks was used to calculate the percent of growth and finally, to estimate the percent of inhibition.

The influence of chlorpyrifos on gene expression of Bt genes was estimated by using the reverse-transcriptase polymerase chain reaction (RT-PCR) approach. total soil rna was isolated using the ultraclean microbial rna kit (mobio Laboratories Inc., Solana Beach, CA), according to the manufacturer's instructions. Contaminant DNA was removed by incubating subsamples (4 μ L) at 37 °C for 30 min in the presence of 5 U of RQ1 RNase-free DNase (Promega Co., Madison, WI), 1 μ L 10 \times Reaction Buffer and nuclease-free water to a final volume of 10 μ L. After DNase digestion, RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI). Approximately 1 μ g RNA was incubated at 70 °C for 5 min in the presence of 0.5 μ g Oligo(dT)15 primer in a total volume of 20 μ L. After cooling on ice, 5 μ L of the primer/template mix was added to 15 μ L of a reaction mixture containing 4 mM MgCl₂, 0.5 mM of each dNTP, 4 μ L of the 5 \times buffer, 20 U of recombinant RNasin ribonuclease inhibitor and nuclease-free water. Reaction was performed by incubating samples at 25 °C (5 min.), 42 °C (60 min.) and 70 °C (15 min.). Aliquots of cDNA (approximately 5–10 ng) were amplified using the same three

primer couples (*cryIAa*, *cryIAb* and *cryIAC*) and conditions described above. RT-PCR products were separated by agarose gel electrophoresis as described above. The efficiency of the RT-PCR methodology was tested by including amplification of the 16S rDNA using the primers PRBA338f/PRUN518r (Muyzer et al., 1993).

2.5. Statistical analysis

Data of microbial propagule density were analyzed by analysis of variance. Means were separated by Fishers' least significant difference (LSD) and significant differences were detected at the $P = 0.05$ level.

3. RESULTS AND DISCUSSION

3.1. Bt occurrence and distribution

Of a total of 90 leaf samples collected during the first survey (July 25th), 32 (36%) had detectable Bt/Bc bacteria (Fig. 1). The highest abundance of positive samples was obtained from leaves located at 0.5 m above the ground (bottom leaves). Samples from top (2.5 m) and middle leaves (1.5 m) showed similar results. The average incidence of Bt on the total Bt/Bc-like colonies was 16, 24 and 39% in the top, middle and bottom leaves, respectively (Fig. 1). As indicated in Figure 2, Bt density ranged from 38 to 123 colony-forming units (CFUs) cm⁻² of leaf surface. More Bt isolates were recovered from bottom than top and middle leaves. Information on distribution and density of Bt in the phyllosphere of agricultural crops is scarce. In a survey conducted in Colombia, Bt was recovered from 56 and 46% of the total analyzed corn and bean leaves, respectively. Density of Bt was 0.46 and 1.5 CFUs cm⁻² of corn and bean leaf surface, respectively (Jara et al., 2006). Apart from difficulties in comparing data obtained from regions with different climates, the values reported by Jara et al. (2006) are lower than those we report here. As indicated by these authors, counted Bt colonies did not include vegetative cells. Most of the surveys concerning distribution and enumeration of Bt in environmental samples have been conducted using methodologies relying on heat-shock treatment (Collier et al., 2005). Even though the ecology of Bt remains unclear, several studies have demonstrated that soil and stored products are the principal reservoirs of natural Bt strains (Bernhard et al., 1997; de Maagd et al., 2001). Bt survives in soil mainly as spores and only limited evidence supports its ability to grow in soil (West et al., 1985; Hendriksen and Hansen, 2002). Consequently, methods relying upon heat-shock treatment give an acceptable estimation of abundance of Bt in soil samples. In contrast to soil, the presence of metabolically active Bt cells in the phyllosphere has recently been confirmed (Collier et al., 2005). In this latter case, the heat-shock step is expected to affect the estimation of the size of Bt populations. More recently, alternative methods and highly selective media have been proposed for efficient isolation of Bt cells and spores

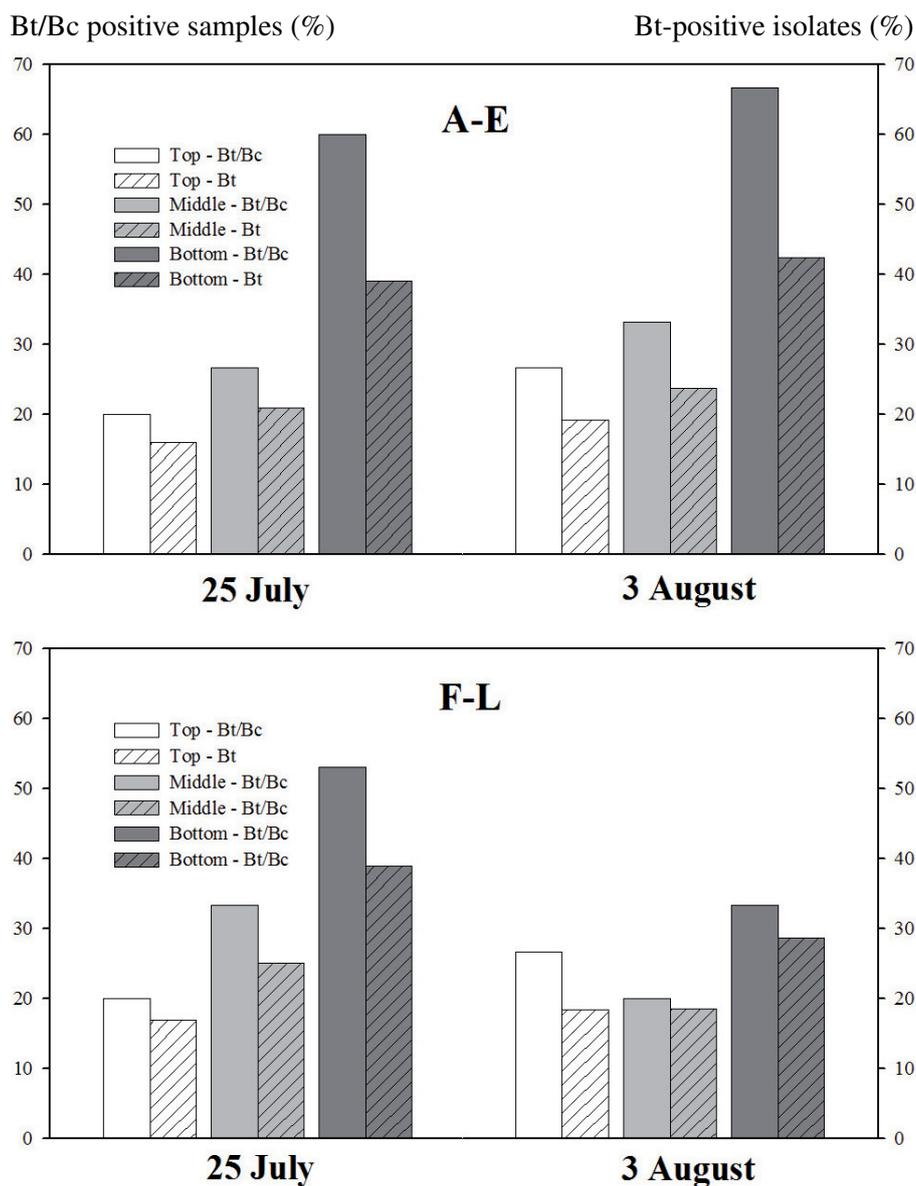


Figure 1. Relative abundance of *Bacillus thuringiensis* (Bt) and *B. cereus* (Bc), and frequency of Bt-positive corn leaf samples collected before and after the insecticide treatment. A–E and F–L are the untreated and treated areas, respectively.

from environmental samples. Using *Bacillus cereus* (Bc) selective agar (BcSA) media supplemented with polymyxin B and egg yolk, Collier et al. (2005) investigated the distribution of Bt/Bc in the phyllosphere of broad-leaved docks and surrounding habitats, confirming the presence of metabolically active cells on plant leaves, with Bc/Bt density $> 1.5 \log \text{CFUs g}^{-1}$ leaf. Similarly to the research described here, these authors isolated more Bt/Bc bacteria from senescent than mature or young leaves. In the present experiment, 23 (77%) of the soil samples collected during the first survey were positive for Bt/Bc (Fig. 3). These findings further confirmed that Bt/Bc bacteria are more likely associated with soil than the phyllosphere. Sixty percent of the colonies isolated from soil samples yielded parasporal crystals (Fig. 3). The estimated

density of Bt in soil samples was $> 3.7 \log \text{CFUs g}^{-1}$ soil (Fig. 4). The heat-shock did not reduce the number of counted Bt isolates from soil (data not shown). Consequently, our results seem to confirm the absence or undetectable presence of Bt vegetative cells in soil. In contrast to soil samples, the heat-shock treatment led to a 10% decrease in Bt isolates from leaf samples, with no differences in leaf heights (data not shown). Considering the moderate incidence of Bt vegetative cells, differences between our density data and those reported by Jara et al. (2006) cannot be explained by differences in methodologies, but are more likely due to differences in climate and agro-environmental conditions.

The results from the second survey (August 3rd) which was conducted shortly after insecticide treatment are shown in

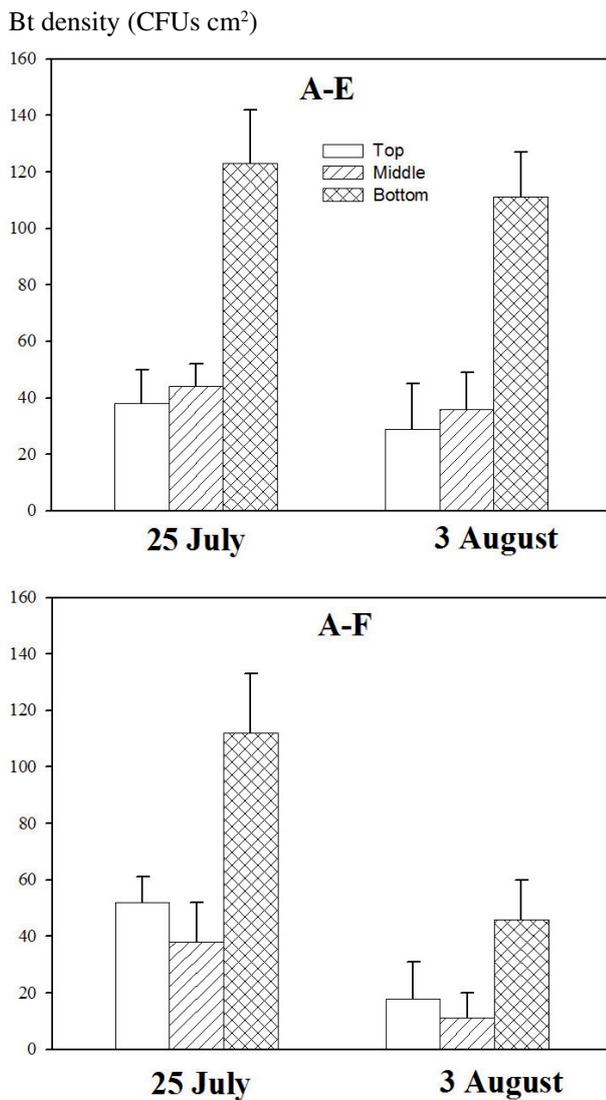


Figure 2. Density of *Bacillus thuringiensis* (Bt) on corn leaves collected at different heights (top: 2.5 m; middle: 1.5 m; bottom: 0.5 m).

Figures 1–4. In the five areas receiving insecticide treatment (areas F–L), a decrease in the number of Bt/Bc positive samples and Bt density on leaf surfaces with respect to the untreated areas (A–E) was observed (Figs. 1–2). The highest decrease in Bt density was observed in top leaves. Middle and bottom leaves showed similar values. In addition, conversely to samples from the untreated areas, the distribution of Bt in the three treated areas (E, F and G) was found to not approximate the log-normal distribution. No differences in the number of Bt isolates between non-heat-treated and heat-treated leaf extracts were observed. This is consistent with a lower abundance of vegetative cells with respect to the untreated areas (A–E). A number of studies have demonstrated that epiphytic bacterial population are log-normally distributed in natural habitats. Even though Bt cannot be correctly defined as epiphytic bacteria and a corn field is not an undisturbed habitat, our results suggest that insecticide treatment has the potential

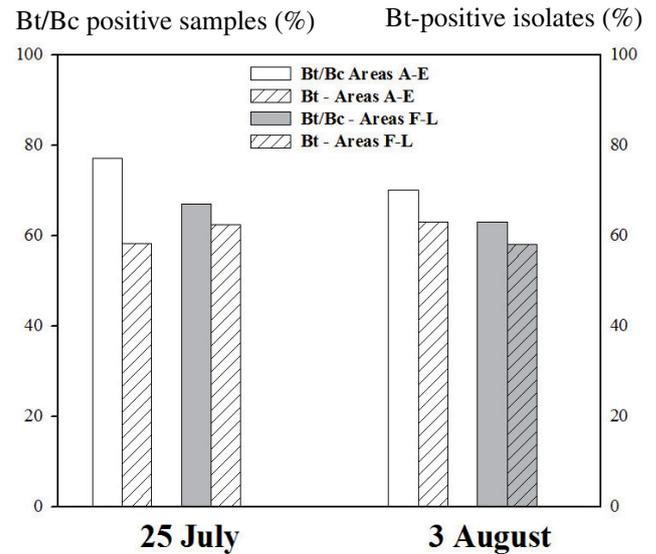


Figure 3. Relative abundance of *Bacillus thuringiensis* (Bt) and *B. cereus* (Bc), and frequency of Bt-positive soil samples collected before and after the insecticide treatment. A–E and F–L are the untreated and treated areas, respectively.

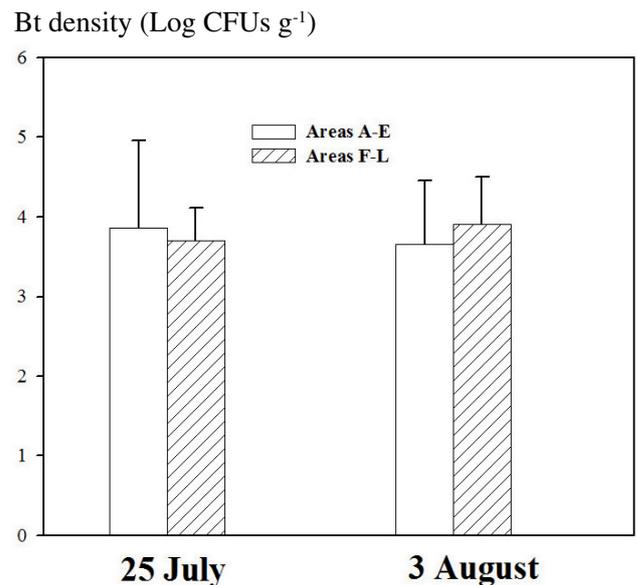


Figure 4. Density of *Bacillus thuringiensis* (Bt) in soil samples on corn leaves collected before and after the insecticide treatment. A–E and F–L are the untreated and treated areas, respectively.

to affect its distribution. As expected, insecticide treatment did not affect distribution or size of Bt populations of soil samples (Fig. 4).

3.2. Occurrence of *cry* genes

Among 300 tested Bt isolates from leaf samples collected during the first survey, the DNA of 126 (42%) were positive for the *cryI* gene (Figs. 5, 6). Approximately 20% of Bt isolates

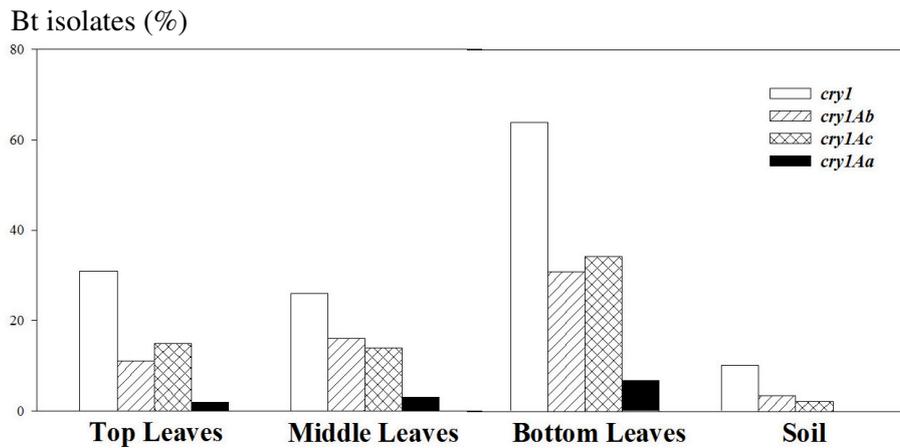


Figure 5. Frequency of *cryIAa*, *cryIAb* and *cryIAc* in leaf samples collected in the 10 areas (A-L) before and shortly after insecticide treatment.

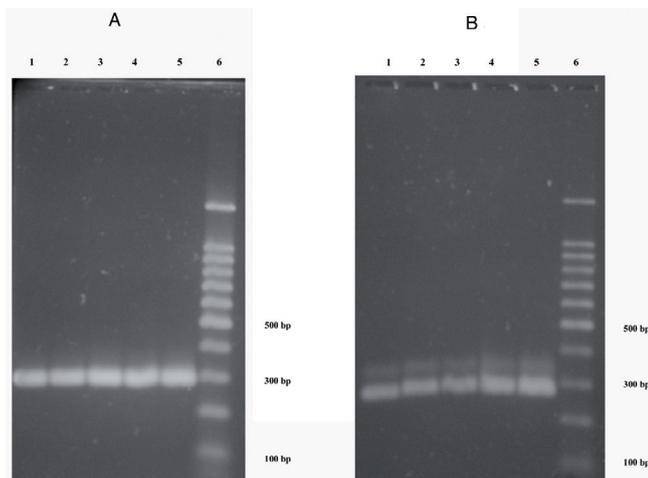


Figure 6. Representative polymerase chain reaction (PCR) detection of *cryI* (A) and *cryIAb* (B) from 5 randomly selected phyllosphere Bt isolates. Lanes 1–5: *cryI*, lane 6: DNA ladder.

were positive for both *cryIAb* and *cryIAc*. Only 4% of the isolates harbored the *cryIAa* gene. Percentages of isolates harboring *cryI* genes were higher in bottom than in middle and top leaves. Comparable results have been found in other surveys conducted in natural and agricultural habitats (Theunis et al., 1998; Martínez and Caballero, 2002; Bizzarri and Bishop, 2007). Although less Bt isolates were recovered shortly after insecticide treatment, the relative incidence of isolates that harbored *cryI* genes did not significantly change. Frequency data were consequently pooled together (Fig. 5). Less than 9% of the Bt isolates from soil harbored *cryI* genes. The low observed frequency of *cryI* genes in soil samples suggests that the Bt population of soil is more complex than that of the corn phyllosphere (Fig. 5).

In the present experiment, PCR analysis was conducted using DNA from Bt isolates selected on the basis of presence of parasporal crystals. According to Bravo et al. (1998), isolates that harbor *cryI* genes commonly produce bipyramidal

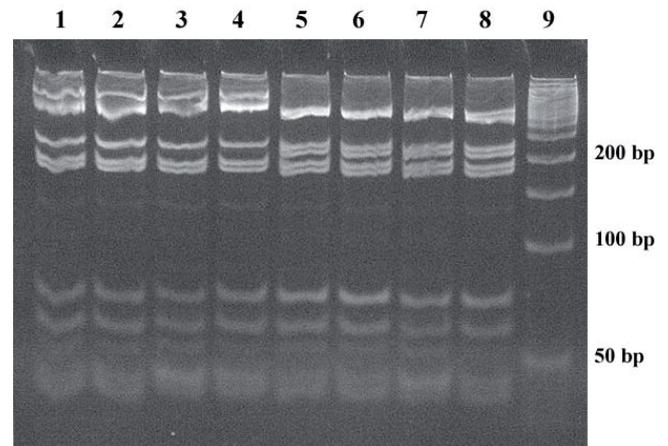


Figure 7. Analysis of 16S rDNA fragments from randomly selected phyllosphere Bt isolates digested with *AluI* (lanes 1–4) and with *AluI* plus *EcoRI* (lanes 5–8). The reference Bt strain HD1 is in lane 1 and lane 5; lane 9: DNA ladder.

crystals which are more easily distinguishable than isolates with rhomboid, oval or pointed crystal types. In this study, we were interested in obtaining basic information on the distribution of indigenous Bt isolates harboring the three most representative antilepidopteran *cryI* genes, namely, *cryIAa*, *cryIAb* and *cryIAc*. Obviously, the presence of existing or unknown *cryI*-type genes encoding for different antilepidopteran toxins cannot be excluded. The results from ARDRA analysis showed the high similarity of our Bt isolates from the phyllosphere with the well-known antilepidopteran Bt strain HD1 (Fig. 7). Even considering these limitations, our study confirmed the occurrence of Bt strains harboring *cryI* genes which are associated with antilepidopteran toxins. More recently, some authors have assumed that the occurrence and the genetic profile of Bt populations is to some extent related to the types of insects feeding on the foliage (Damgaard et al., 1998; Martínez and Caballero, 2002). The lepidopteran European Corn Borer (ECB; *Ostrinia nubilalis* Hübner) is the major pest

Table I. Effect of chlorpyrifos on *Bacillus thuringiensis* growth. Test was conducted at 30 °C.

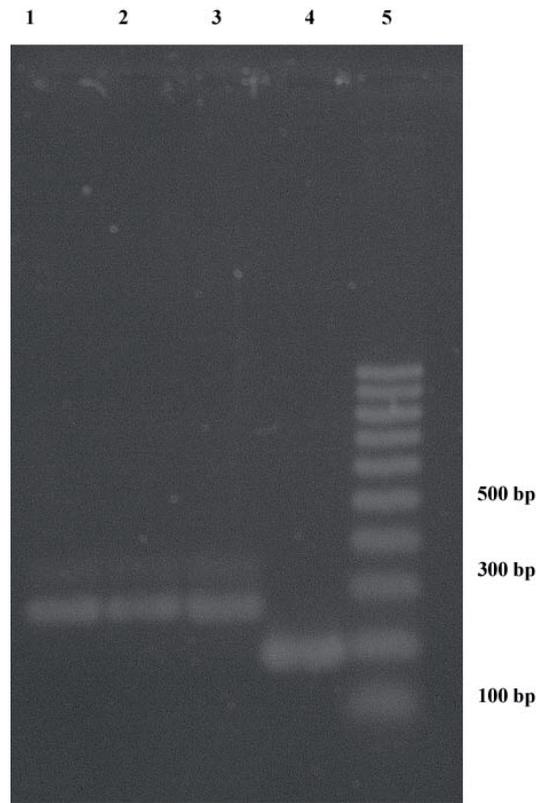
Chlorpyrifos concentration (mg L ⁻¹)	Specific growth rate μ (h ⁻¹)	Growth rate as percentage of control
0.01	0.81*	95
0.1	0.85	100
1.0	0.79	93
10	0.83	98

* Numbers are means of three replicates.

of corn in Italy. The ECB infests nearly all of the corn acreage every year with yield losses of 7–15%. Until the last five years, only 5% of the total corn acreage of Italy was chemically treated against the ECB, mostly using synthetic pyrethroids. Increased direct and indirect damage (i.e. secondary infections of fungi associated with ECB feeding) caused by the ECB has led to changes in strategies adopted by farmers to control ECB infestations. Most of the corn fields are now treated with the insecticide chlorpyrifos. Research has shown that this broad-spectrum insecticide can persist longer in the environment and side-effects on non-target organisms have been demonstrated (Wiktelius et al., 1999). The observed differences in numbers of Bt isolates and distribution of *cry* genes between treated and untreated areas may be likely due to the reduction of ECB larvae of treated corn plants. Although more research is needed to support these assumptions, this survey indicated detectable levels of antilepidoteran Bt isolates even in an altered ecosystem, such as a corn field, and that Bt population of corn leaves seemed to be not related to that of the soil.

3.3. Potential effect of the insecticide chlorpyrifos on Bt growth and *cry* gene expression

Increasing concentrations of the insecticide chlorpyrifos did not affect the growth of the Bt strain HD-1 (Tab. I). RT-PCR analysis showed that expression of the *cryIAb* gene was not affected by the insecticide chlorpyrifos (Fig. 8). As discussed above, we found differences in Bt distribution in the sampling area receiving insecticide treatment with respect to untreated areas. Even though a more complex study would be necessary to investigate the effect of insecticide treatment on the density and distribution of microorganisms in the phyllosphere, our results seemed to exclude direct potential effects of chlorpyrifos on Bt growth and Cry toxin synthesis. Under some circumstances, the detrimental effect of pesticide treatment on microorganisms is mainly caused by substances included in commercial formulation (i.e. surfactants, etc.) rather than active ingredients per se. Other factors (i.e. environmental conditions, different exposition to UV rays or simply wash-off of bacterial cells during insecticide application) would not be excluded. As described above, some authors have assumed that the presence of Bt in the phyllosphere is to some extent related to the type of insect feeding on the foliage. The observed reduction of Bt isolates in sampling areas treated with chlor-

**Figure 8.** Expression profiles of Bt genes. Lane 1: *cryIAb*, lane 2: *cryIAc*, lane 3: 16S rDNA, lane 4: 100 bp DNA ladder.

pyrifos may be likely due to a dramatic decrease in ECB larvae feeding on foliage.

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