

EFFICACY OF TRANSGENIC CORN EXPRESSING THE *BACILLUS THURINGIENSIS* *CRYIA(b)* GENE AGAINST ASIATIC CORN BORER, *OSTRINIA FURNACALIS* (GUENEE)¹

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ABSTRACT

Transgenic corn plants (MON 810) expressing the *Bacillus thuringiensis cryIA(b)* gene were evaluated for efficacy against repeated artificial infestations with Asiatic corn borer *Ostrinia furnacalis* (Guenee) at the CL4 confinement facility of IRRI. The transgenic plants were highly resistant to foliar damage by the borer throughout the three infestation periods compared to an equivalent non-Bt hybrid and a local check variety, Supersweet corn. The number and length of borer tunnels in the stalk and the length of tunnels in the ear shank of the transgenic plants were also significantly reduced.

KEY WORDS: Transgenic corn, *Ostrinia furnacalis*, Asiatic corn borer, *Bacillus thuringiensis* var. *kurstaki*, *cryIA(b)*, *CryIA(b)*

INTRODUCTION

Advances in genetic engineering has made possible the insertion of various *Bacillus thuringiensis* insecticidal crystal protein (Cry) gene into crop plants. Plants expressing a Bt Cry gene produce small quantities of a protein which has been demonstrated to be highly effective in specifically controlling certain insect pests. Transgenic tomato plants expressing the *B. thuringiensis* var. *kurstaki* HD-1 [*cryIA(b)*] genes (Delannay *et al.*, 1989) cotton plants expressing the HD-1 and HD73 [*cryIA(c)*] genes (Perlak *et al.*, 1990), tobacco with HD-73 or CpTI genes (Hoffman *et al.*, 1992) and potato containing [*cryIA(c)*] gene (Ebora *et al.*, 1994) have all been shown to be effective against major insect pests.

Extensive field experiments with Bt corn, conducted by Pioneer Hi-Bred International, Inc. in the US since 1993, showed that certain transformation events including MON 810, resulted in very effective control of the European corn borer (*Ostrinia nubilalis* Hubner), an important insect pest of corn in North America and Europe. A number of U.S. seed companies, including Pioneer Hi-Bred, have commercialized transgenic Bt corn hybrids containing the *cryIA(b)* gene, after completing a rigorous field testing regime and undergoing safety reviews by three different U.S. regulatory agencies.

In the Philippines, the most important insect pest of corn is the Asiatic corn

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borer (ACB), *Ostrinia furnacalis* (Guenee). This species is also distributed over a large part of Asia. Since this insect is very closely related to *O. nubilalis*, both taxonomically and biologically, there is a strong possibility that Bt corn will also resist ACB. It has been demonstrated in laboratory experiments conducted locally that the purified *CryIA(c)* protein from *Bacillus thuringiensis* var. *kurstaki* is lethal to ACB (personal communication with Dr. Cesar Demayo). Incorporation of a Bt *cry* gene active against lepidopteran larvae into local corn germplasm would improve control of ACB, thereby, increasing the productivity of corn farmers and enhancing the Philippines' competitiveness in the corn world market.

The objective of this trial was to determine the efficacy of a transgenic Bt corn hybrid expressing the *cryIA(b)* gene against ACB, under conditions of artificial infestation, in a containment facility.

MATERIALS AND METHODS

Planting Materials: Forty seeds each of MON810, a Bt-transformed HI-2 hybrid (Bt corn) and untransformed HI-2 plants (non-Bt corn) were provided by Pioneer Hi-Bred International, Inc., Johnston, Iowa, USA through Pioneer Overseas Corporation (Phil.), Los Baños, Laguna.

The hybrid material derived from transformation event designated as MON 810, was supplied to Pioneer under license by Monsanto Company, St. Louis, Missouri U.S.A. Line MON 810 was obtained by transforming the corn line "HI-2" (a cross between public inbreds B73 and A188) by the particle acceleration method or microprojectile bombardment. The introduced genetic material comprised a 3.6 Kb full length synthetic gene encoding the *B. thuringiensis* var. *kurstaki* insect control protein *CryIA(b)* under control of the cauliflower mosaic virus 35S promoter (CaMV 35S), in a pUC-Kan based delivery plasmid vector (Croon *et al.*, 1996). Expression of the introduced genes has been monitored through many generations. The *cryIA(b)* gene is stably inherited in a normal Mendelian fashion with no signs of instability.

Test Site and Experiment Design. Thirty (30) size 10 clay pots were filled with soil medium composed of 1 garden soil: 1/4 coir dust: 1/4 decomposed hog manure. The potted soil were autoclaved for 2 hrs and then placed inside Bay 5 of the CL4 containment facility of the International Rice Research Institute (IRRI). The containment facility satisfies the requirements set by the National Committee on Biosafety of the Philippines (NCBP) for greenhouse testing of transgenic plants. It is equipped with proper environmental controls to assure proper growth of the corn plants to maturity and at the same time effectively prevents the dissemination of plant reproductive structures (i.e. pollen or seeds).

Planting was done on August 9, 1996, immediately upon receipt of the test materials. Four (4) kernels each of Bt corn, non-Bt corn and the local susceptible check variety, Supersweet corn, were planted in the center of each pot. Water was applied immediately after planting. Recommended agronomic practices were followed to ensure healthy growth of the plants.

A complete randomized design (CRD) was used with 10 replications. Each pot served as a replicate. Treatments consisted of the three corn genotypes described above, which were subjected to three separate artificial infestations 25, 41 and 56 days after planting (DAP).

Infestations. Asiatic corn borer (ACB) larvae were reared at the Entomology Laboratory of the Institute of Plant Breeding, UPLB and were transported to the CL4 facility of IRRI immediately prior to infestation. Infestation was done using neonate ACB larvae placed in the whorl of the plant with the use of a soft camel hair brush (Figure 1). This was done late in the afternoon to promote larval establishment since light intensity was lower then, and prevented dessication of the young larvae. The first infestation was conducted on September 2, 1996, twenty-five (25) days after planting (DAP), with 40 larvae per plant. Nine (9) days later, each plant was assessed for leaf feeding damage using the 1 to 9 rating scale of Guthrie *et al.* (1960). After the first rating, the plants in each pot were thinned to a single plant that showed the least amount of leaf feeding damage. On 18 September 1997 (41 DAP), the second infestation was done by placing 50 neonate larvae on the whorl of each plant. Seven (7) days later the leaf feeding damage was assessed. After the second rating, the plants were enclosed in a white mosquito net to prevent the escape of emerging adults.

On September 30, 1996, (53 DAP) the Bt corn plants were detasselled prior to full tassel emergence. Removed tassels were autoclaved immediately. Only the tassels from the non-Bt and Supersweet corn plants were allowed to emerge and used to pollinate the ears of all the plants. On 2 October 1996 (56 DAP), final infestation was done by placing 50 neonate larvae on each plant. Final whole plant damage assessment was done on 6 November 1996 (90 DAP). Ear shank damage, number of borer tunnels and stalk damage were recorded. Ear damage was also noted.

The ear shank and stalk damage was determined by splitting the shank and the stalk with a knife and measuring the tunnel length. Measurements were taken from the base, middle and upper portion of the stalk. The number of tunnel holes was also counted. After the data were collected, all plants were chopped, placed in polyethylene bags and autoclaved immediately. Used pots were also autoclaved prior to soil disposal.

Data Analysis. The first rating of leaf feeding damage is a mean of 3 to 4 plants per replicate. Likewise, the length of borer tunnel is a mean of three sampling sites in the stalk. Except for the length of borer tunnels, the data were subjected to square root transformation $(X + 0.5)^{1/2}$ and then to Analysis of Variance (ANOVA), with the three genotypes as treatments. Duncan Multiple Range Test (DMRT) was used to compare the means (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Leaf Feeding Damage. Analysis of variance of the leaf feeding damage by the ACB taken 7 to 9 days after each infestation showed highly significant difference ($P < 0.01$) between the Bt corn and the non-Bt corn or the local check Supersweet corn (Table 1 and Figure 2). The non-Bt and Supersweet corn did not, however, differ significantly with each other in susceptibility. Throughout the evaluation periods at 34, 48, and 90 DAP, the Bt corn remained highly resistant to leaf feeding by the borer with mean ratings of 1.6, 2.1 and 2.2, respectively.

Koziel *et al.* (1993) previously showed that transgenic corn plants with the synthetic *cryIA(b)* gene produce high levels of insecticidal protein and exhibited excellent protection against repeated heavy infestations with European Corn Borer

Table 1. Mean leaf feeding damage rating at 3 stages of evaluation after three infestations with Asiatic corn borer larvae, *O. furnacalis* (Guenee).¹

TREATMENT	LEAF FEEDING DAMAGE ²		
	1st Rating (34 DAP)	2nd Rating (48 DAP)	3rd Rating (90 DAP)
Bt corn	1.6 ^a	2.1 ^a	2.2 ^a
Non-Bt: Isogenic line	6.8 ^b	9.0 ^b	9.0 ^b
Supersweet corn (Local check)	7.5 ^b	8.0 ^b	8.5 ^b

^{1/} Rating scale based from Guthrie *et al.* (1960) where:

- 1 - No visible leaf injury or small amount of pin or fine shothole type on few leaves.
- 5 - Several leaves with elongated lesions.
- 9 - Most of the leaves with long lesions.

^{2/} Data represent average of ten replications with one (1) plant per replicate except in the first rating where 4 plants were used per replication.

Means within a column followed by the same letter are not significantly different ($p < 0.01$; DMRT).

Figure 2. Degree of leaf feeding damage by Asiatic corn borer larvae during the vegetative stage on the Bt corn, non-Bt plants and Supersweet (local check) (a) and final plant and ear damage 90 days after planting (b).

Table I. Mean leaf feeding damage rating at 3 stages of evaluation after three infestations with Asiatic corn borer larvae, *O. furnacalis* (Guenee).

LEAF FEEDING DAMAGE °	TREATMENT		
	1st Rating (24 DAP)	2nd Rating (48 DAP)	3rd Rating (90 DAP)
Bt corn	1.6*	2.1*	2.3*



Means within a column followed by the same letter are not significantly different ($p < 0.01$; DMRT).

Figure 2. Degree of leaf feeding damage by Asiatic corn borer larvae during the vegetative stage on the Bt corn, non-Bt plants and Supersweet (local check) (a) and final plant and ear damage 90 days after planting (b).

(ECB). The resistance of the Bt corn to insect damage is due to the failure of the larvae to establish in the plant. Most of the neonate ACB larvae died after initial feeding on the transgenic Bt plant (Figure 3a), demonstrating the efficacy of the Bt toxin against this insect pest. Our results showed that Line MON 810, expressing the *cryIA(b)* gene in also highly resistant to ACB. Three (3) successive infestations of neonate ACB (a total of 140 larvae per plant) failed to cause significant leaf feeding damage. Resistance to this level of infestation is more than adequate compared to the level of ACB resistance available in other non-transgenic corn genotypes.

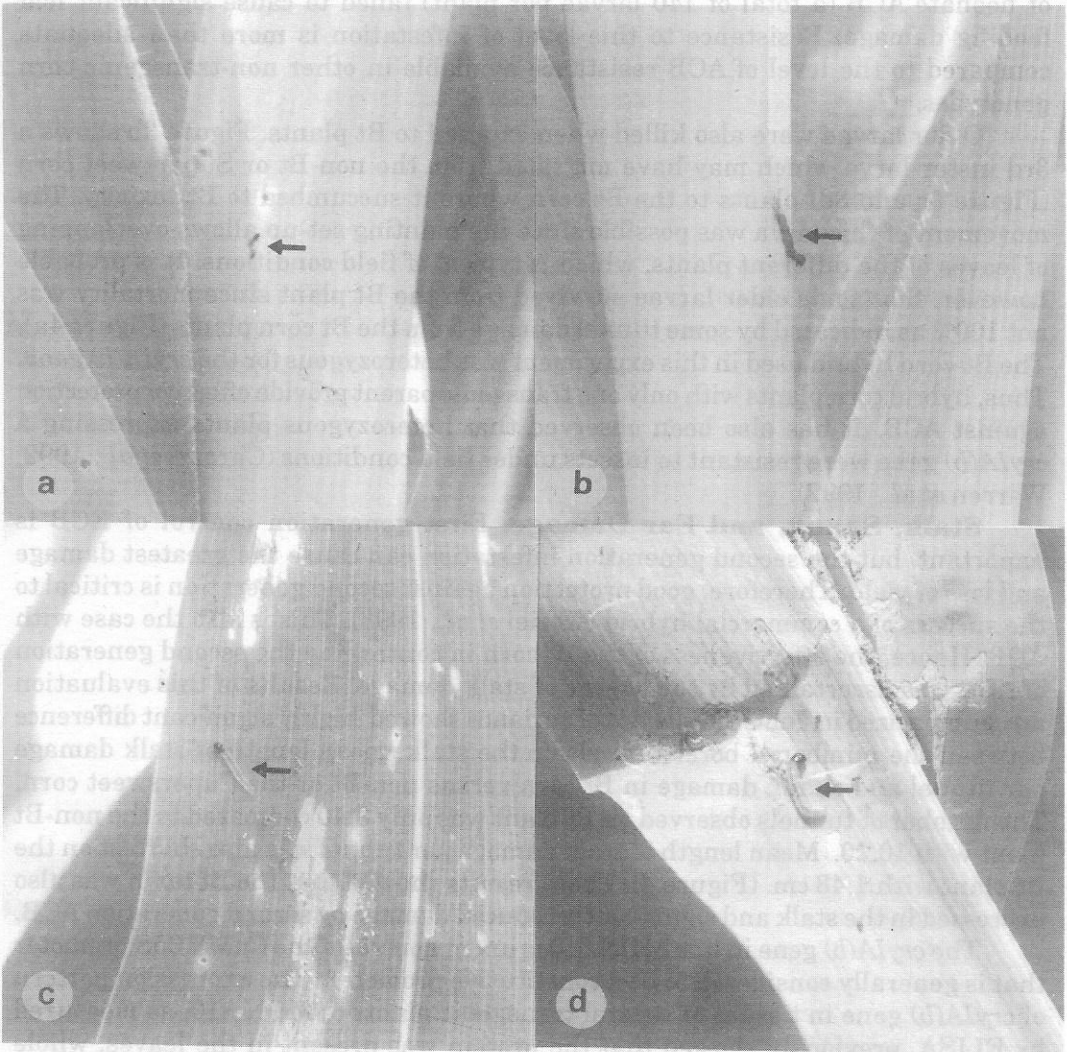
Older larvae were also killed when exposed to Bt plants. Figure 3b shows a 3rd instar larva which may have migrated from the non-Bt or Supersweet corn (Figure 3c and 3d) plants to the Bt corn where it succumbed to Bt toxicity. The movement of this larva was possible since the planting set-up allows overlapping of leaves of the different plants, which is typical of field conditions. It is probable however, that some older larvae survived from the Bt plant since mortality was not 100% as indicated by some tunnel damage from the Bt corn plants (Figure 4a). The Bt-corn hybrid used in this experiment was heterozygous for the *cryIA(b)* gene. Thus, hybrid corn plants with only one transgenic parent provide effective protection against ACB. It has also been observed that heterozygous plants expressing a *cryIA(b)* gene were resistant to insects under field conditions (Carozzi *et al.*, 1992; Warren *et al.*, 1992).

Stalk, Shank, and Ear Damage. First generation control of ECB is important, but the second generation infestation can cause the greatest damage and loss of yield. Therefore, good protection against second generation is critical to the success of a commercial hybrid (Koziel *et al.*, 1993). This is also the case with ACB. Hence, the effectiveness of the Bt corn in controlling the second generation of ACB was ascertained by the degree of stalk damage. Results of this evaluation are summarized in Table 2. Analysis of variance showed highly significant difference between the number of borer tunnels on the stalk, mean length of stalk damage per tunnel and shank damage in Bt corn versus non-Bt or the Supersweet corn. The number of tunnels observed on Bt plant was only 3.40 compared to the non-Bt plant with 10.20. Mean length of stalk damage per tunnel was also shortest on the Bt plant with 1.48 cm. (Figure 4). These results showed that the Bt toxin was also expressed in the stalk and significantly reduced damage by second generation ACB.

The *cryIA(b)* gene in line MON 810 is under control of the CaMV 35S promoter that is generally considered to be a constitutive promoter. The expression pattern of *cryIA(b)* gene in tissues of several transgenic plants of MON 810, as measured by ELISA, previously showed that the protein was present in the leaves, whole plant, grain and very little in the pollen (Croon *et al.*, 1996). The transgenic hybrid evaluated in this experiment apparently has high *CryIA(b)* protein in the pith as well as in the shank as indicated by the high level of protection exhibited by the plants. Similarly, Koziel *et al.* (1993) earlier showed that transgenic plants having high levels of *CryIA(b)* protein in leaf, root, pith, pollen/anther and kernels had little or no tunneling damage.

Kernel damage was not included in the statistical analyses of the data derived from the current study. While most of the Bt plants showed no ear feeding damage, ears of the non-Bt and Supersweet corn were mostly completely damaged as early as ear formation stage and thus the data could not be compared statistically. The

(Bt). The resistance of the Bt corn to insect damage is due to the failure of the larvae to establish in the plant. Most of the neonate ACh larvae died after initial feeding on the transgenic Bt plant (Figure 3a), demonstrating the efficacy of the Bt toxin against this insect pest. Our results showed that Line MON 810, expressing the *cryIA(b)* gene in also highly resistant to ACh. Three (3) successive instars of neonate ACh (total of 140 larvae) failed to cause significant damage to the Bt plant. This resistance to the ACh larvae is due to the presence of the *cryIA(b)* gene in the Bt plant. The non-Bt corn plants were highly susceptible to ACh larvae. The neonate ACh larvae fed on the non-Bt corn plants and caused significant damage to the plant. The neonate ACh larvae fed on the non-Bt corn plants and caused significant damage to the plant. The neonate ACh larvae fed on the non-Bt corn plants and caused significant damage to the plant.



plant grain and very little in the pollen (Cron et al., 1996). The transgenic hybrid evaluated in this experiment apparently has high *CryIA(b)* protein in the pit as well as in the stink as indicated by the high level of protection exhibited by the plants. Similarly, Kester et al. (1993) earlier showed that transgenic plants having high levels of *CryIA(b)* protein in leaf, root, tith, pollen/another and kernels had little or no tunneling damage.

Figure 3. Dead first instar larvae (a) and 3rd instar larvae (b) showing typical effect of Bt toxin observed on the Bt plants compared to the healthy 3rd instar larvae on non-Bt plant (c) and Supersweet corn (d). Also note the larger feeding damage in the untransformed corn plants.

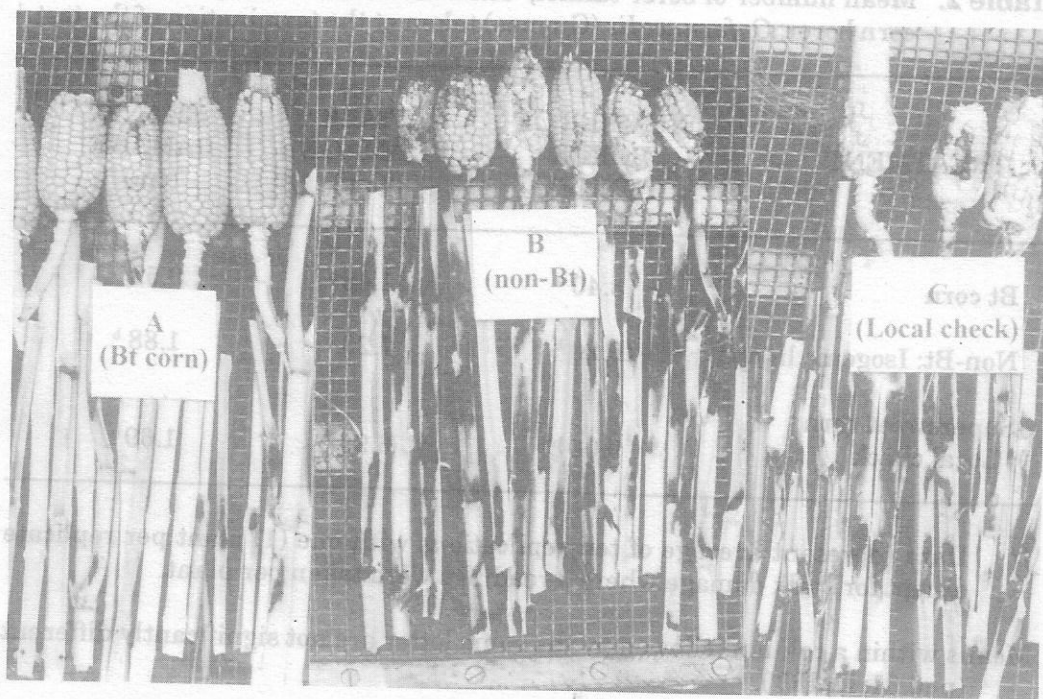


Figure 4. Degree of stalk and ear damage on the Bt (A), non-Bt (B) and Supersweet corn (C) by the Asiatic corn borer.

Table 2. Mean number of borer tunnel, stalk and shank damage by the Asiatic corn borer, *O. furnacalis* (Guenee) taken at the termination of the test.¹

TREATMENT	NUMBER OF BORER TUNNEL	STALK DAMAGE (cm)	SHANK DAMAGE (cm)
Bt corn	3.40 ^a	1.48 ^a	0.52 ^a
Non-Bt: Isogenic line	10.20 ^b	7.23 ^b	1.88 ^b
Supersweet corn: Local check	9.00 ^b	6.96 ^b	1.69 ^b

1/ Data represent average of ten replications with one (1) plant per replicate except for stalk damage where 3 readings were taken per plant.

Means within a column followed by the same letter are not significantly different ($p < 0.01$; DMRT).

high degree of ear damage observed in the non-Bt and Supersweet corn was due to the greater number of surviving larvae feeding on these plants. In the Bt corn, the toxin present in the different plant parts either eliminated the larvae or greatly weakened the survivors that attempted to enter into the ears. The Bt plants had good ear yield and less kernel damage compare with non-Bt or Supersweet corn (Figure 4). According to Koziel *et al.* (1993), some developers have used tissue specific regulating elements like phosphoenol pyruvate carboxylase (PEPC) and pollen specific promoters, that express high levels of *CryIA(b)* protein in green tissue and pollen and low levels in kernel. This combination is particularly suited for producing a pattern of expression effective for controlling ECB while minimizing expression of *CryIA(b)* protein in the grain. However, this leaves the grain vulnerable to attack by second generation ECB and other lepidopteran pests that feed in the ear.

SUMMARY/RECOMMENDATION

The work presented here marks the first insect resistance evaluation of a transgenic crop, other than rice, in the Philippines. Although the test was conducted in a confinement facility, the results demonstrated that a commercial transgenic Bt corn hybrid providing effective protection against ECB is also effective against the ACB. The control was effective against both first and second generations of this very serious local pest and therefore has potential for use in an integrated pest management program.

The protection afforded by the hybrid derived from transgenic corn line MON 810 expressing the *cryIA(b)* gene, as observed in this trial, is significantly better than the level of resistance currently available in tropical genotypes developed using conventional breeding strategies. However, the *cryIA(b)* gene will have to be introgressed into tropical corn populations if it has to be of value to the efforts of improving the level of ACB resistance in tropical corn. Research is currently limited to evaluation in contained facilities. It will be important to determine how this transgenic corn line will perform under field conditions in a tropical environment like the Philippines.

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