Transgenic Resistance to the Colorado Potato Beetle in Bt-expressing Eggplant Fields

Nazareno Acciarri and Gabriele Vitelli
Istituto Sperimentale Orticoltura, I-63030 Monsampolo del Tronto (AP), Italy

Salvatore Arpaia
Metapontum Agrobios, S.S. 106 Km. 448.2 I-75010 Metaponto (MT), Italy

Giuseppe Mennella
Istituto Sperimentale Orticoltura, P.O. BOX 48 I-84098 Pontecagnano (SA), Italy

Francesco Sunseri[1]
Dipartimento Biologia, Difesa, Biotecnologie AgroForestali, Università della Basilicata I-85100 Potenza, Italy

Giuseppe L. Rotino
Istituto Sperimentale Orticoltura, Via Paullese 28 I-26836 Montanaso L. (LO), Italy

Abstract. **Colorado potato beetle (CPB; *Leptinotarsa decemlineata*-Say) is a serious pest because it has developed resistance against insecticides. Three transgenic eggplant (*Solanum melongena* L.) lines bearing a mutagenized *Bacillus thuringiensis* Berl. gene coding for the Cry3B toxin, and the nontransformed control DR2-line were tested in field trials to assess their insect resistance. The transgenic lines 3-2, 6-1, and 9-8 were tested at two different locations in a randomized complete-block design. Samples were taken biweekly to assess the level of CPB and the presence of other insects. At harvest, total yield and fruit number per plot were recorded. Two transgenic lines showed high levels of resistance at both locations, as measured by CPB abundance and yield. Fruit production was almost twice as great in the highly resistant lines (3-2 and 9-8) as in the nontransformed control. The 6-1 transgenic line showed an intermediate level of resistance; it was similar to the control under heavy CPB pressure and was comparable to the other transgenic lines under milder infestations. Analysis by double antibody sandwich–enzyme linked immunosorbent assay (DAS–ELISA), performed on different tissues, revealed a lower amount of Cry3B protein in the 6-1 transgenic line than in lines 3-2 and 9-8. No detrimental effects on nontarget arthropods (including the chrysomelid *Altica*) were evident. Field observations confirmed that Bt may be able to control CPB infestation in eggplant, representing a potential effective and environmentally safe means of pest control.**

Resistance in eggplant to Colorado potato beetle (CPB) is critical for production in America and Europe due to heavy damage (Arpaia et al., 1995; Hamilton et al., 1997). In Italy, cultivation of early potato (the preferred host plant of CPB) allows the beetle's life cycle to overlap with cultivation of eggplant (Arpaia et al., 1995).

The availability of insect-resistant transgenic cultivars provides new possibilities for a sustainable pest control. Assessing insect resistance and yield performance are obviously the main subject of field studies, but the impact of the resistant germplasm on the whole entomofauna should also be examined to better understand its possible effect on a large scale. Several recent studies have addressed the effects on secondary pests (e.g., Hardee and Bryan, 1997; Pilcher et al., 1997b; Wagner et al., 1996) and their natural enemies (Dogan et al., 1996), the combined effects of transgenic plants and natural enemies on the target herbivores (Johnson, 1997; Orr and Landis, 1997), and the evaluation of possible detrimental effects on insect predators feeding on transgenic pollen (Pilcher et al., 1997a).

Transgenic crops expressing Bt-derived toxins are effective in controlling insect populations (e.g., Benedict et al., 1996; Hamilton et al., 1997; Jansen et al., 1997). The use of transgenic plants engineered for insect resistance were the topic of 24% of all requests for field release of genetically modified organisms approved or acknowledged by the U.S. Dept. of Agriculture (USDA) through Jan. 1998, and represents the second most common transgene.

The use of transgenic resistant cultivars of horticultural crops could be an important alternative means of pest control. Transgenic eggplants resistant to CPB by expression of Bt Cry3 genes (Arpaia et al., 1997; Hamilton et al., 1997; Iannacone et al., 1997; Jelenkovic et al., 1998) may represent a new and effective means of pest control. Resistant lines should be nontoxic to nontarget organisms (including mammals) and environmentally safe. The objective of this study was to evaluate transgenic eggplant lines expressing a modified Bt gene under natural CPB infestation for their yield performance and insect resistance.

Materials and Methods

Plants. Transgenic eggplant lines were obtained by selfing parental transformed plants from line DR2 (Arpaia et al., 1997) and were used for field experiments. Kanamycin-resistant plants were self-pollinated and screened by spraying with a kanamycin solution (Sunseri et al., 1993). Segregation analysis, chi square, and *P* values on selfed progenies of the transgenic lines and the nontransformed control were obtained, indicating the number of nonlinked transgenes. Kanamycin-resistant plants were used in field experiments.

Experimental fields. Fields were prepared in a randomized complete-block design with four replications, at two different locations: Metaponto (MT-Italy) and Monsampolo del Tronto (AP-Italy) with the approval of the Italian Ministry of Public Health (No. B/IT/97-05A). Transgenic eggplant lines 3-2, 6-1, and 9-8 and the nontransformed control DR2 were planted and natural infestation was used to evaluate resistance. The plants were transplanted on 25 May 1997 at Monsampolo and on 23 June 1997 at Metaponto. Eggplants were mulched, planted in double rows at a density of 2.2 plants/m², and cultivated following the traditional cultural practices of each location (e.g., La Malfa, 1990; Panero, 1981). Each plot measured 4.5 × 9.6 m and contained 96 plants.

Field observations. Data were collected twice a week beginning 3 weeks after transplanting. Ten percent of the plants in each replication were randomly chosen for visual inspection and the numbers of CPB adults, egg masses, small and large larvae on each plant were recorded. At Metaponto, field data on the presence of other insects in the canopy were also collected. Surveys were carried out for 7 and 10 weeks at Metaponto and

Monsampolo fields, respectively. At each harvest, fruit number and weight were recorded. Plants were harvested seven times between July and Sept. 1997.

Cry3B toxin quantification. In the field trial at Monsampolo, during the growing season, Cry3B protein levels were quantified by double antibody sandwich–enzyme linked immunosorbent assay (DAS–ELISA) analyses (Arpaia et al., 1997) using different tissues of transgenic eggplants; young and old leaf, fruit, and flower. The dilution ratio for fruit and flower samples was 1:3 (w/v) in extraction buffer. Four plants were randomly sampled from each replication in July and Sept. 1997.

Statistical analyses. Insect abundance and yields were analyzed as a randomized complete-block design with repeated measures. DAS–ELISA values were analyzed as a completely randomized design of analysis of variance (ANOVA). Means were compared by least significant difference (LSD). All the analyses were performed by using SAS software, version 6.12 for Windows (SAS Inst., 1989).

Results and Discussion

The selection of the offspring bearing the transgene either in homozygous or heterozygous state were performed as described in Sunseri et al. (1993). The data, collected after in vivo spray test and analyzed by chi-square test, suggested the presence and the expression of one (lines 3-2 and 9-8) or two copies (line 6-1) of the transgene (Table 1). More-over, since the transgenic line 6-1 showed an intermediate level of insect resistance, both in insect bioassays and DAS–ELISA tests, a gene silencing phenomenon may be present (Dieguez et al., 1998; Stam et al., 1997).

At Monsampolo, the beetle population was sizeable and apparently completed two generations. Initially, the population was probably from overwintering beetles. At the beginning of July and in the second half of August, the number of egg masses peaked, especially in the 9-8 line (July) and the DR2 control line (August). While incoming beetles spread quite evenly on all plots, small and large larvae were found mainly on lines DR2 (control) and 6-1, and this trend led to a higher number of adults on the same eggplant lines toward the end of the season (Figs. 1–2). Beetle populations (expressed as number of egg masses and larvae per plant) were significantly higher on the control line DR2 than on the transgenic lines (Table 2). Among the latter, 9-8 showed the lightest attack; the other two transgenic lines had comparable numbers of egg masses, but a smaller per-centage of eggs developed into larvae. The average number of eggs per egg mass on eggplant has been reported to be from 26 to 30 (Jansson et al., 1989). Survival values of 10% to 11.5% and 13.7% to 15.8% were observed on the lines 3-2 and 6-1, respectively, vs. 29% to 34.1% on DR2 and 5.1% to 5.9% on 9-8. Line 6-1 was intermediate between the nontransformed control and the other two transgenic lines for average number of larvae per plant.

In the field trial at Metaponto, the attack by CPB started from one edge of the field near where a potato crop had been harvested 3 weeks before. The first block was more severely damaged than the other blocks (data not shown). Because of the late planting date, incoming beetles were mostly first generation adults. The infestation level was not high throughout the season. Therefore, pooled numbers of small and large larvae represented the level of infestation. Adults appeared evenly distributed among different eggplant lines and only toward the end of the growing season did the average number per

Table 1. Segregation ratios, chi-square values and *P* values for kanamycin resistance in S_1 families transgenic eggplant progenies.

Family	No. plants tested	KmR[z]	KmS[y]	Ratio (KmR/KmS)	χ^2 value	*P* value
3-2[x]	945	675	270	3:1	6.430	0.01
6-1[x]	1140	1078	62	15:1	1.281	0.26
9-8[x]	965	730	235	3:1	0.214	0.64
DR2	970	0	970	---	---	---

[z]KmR, kanamycin-resistant.
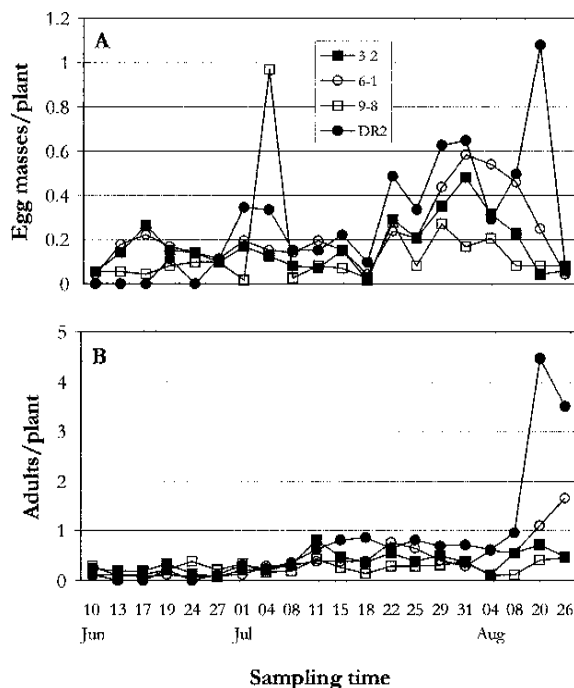[y]KmS, kanamycin-sensitive.
[x]Self-pollinated.

Fig. 1. Colorado potato beetle infestation course at Monsampolo field trial in 1997: egg masses and adults per plant.
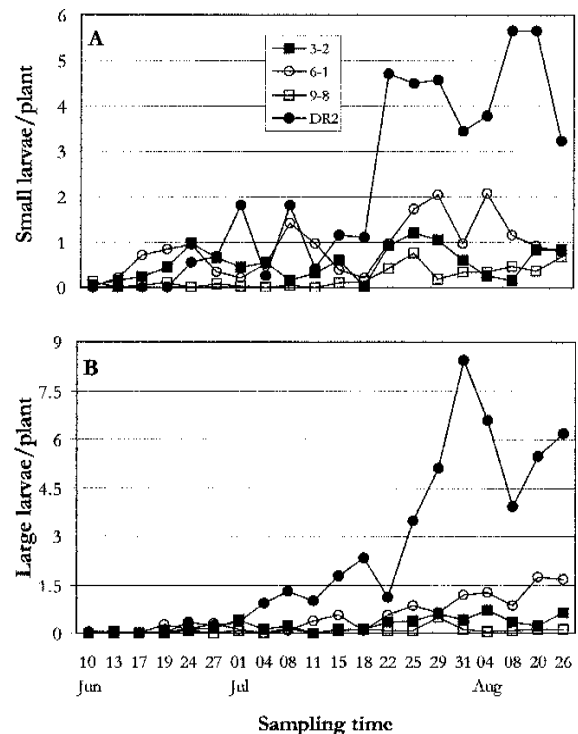

Fig. 2. Colorado potato beetle infestation course at Monsampolo field trial in 1997: small and large larvae per plant.

plant reach two in the control plots (Fig. 3). Lines 6-1 and DR2 initially had higher numbers of egg masses, while toward the end of the growing season the numbers were higher on control plants (Fig. 3). Considering the egg masses laid, the percentage of small larvae surviving were 8.3% ± 1.3%, 3.6% ± 0.9%, 0.7% ± 0.1%, and 8.9% ± 1.7% on the lines 3-1, 6-1, 9-8, and DR2, respectively. Nevertheless, the numbers of both egg masses and larvae were significantly higher on the nontransformed DR2 line (Table 2).

The numbers of the insects most commonly found in the Metaponto field trial are summarized in Table 3. The only natural enemy of CPB regularly found on the canopy was the lacewing, *Chrysoperla carnea* Stephens. The number of eggs and larvae were not numerically relevant and no difference among lines was detected ($P > 0.9934$). Among nontarget insect pests, the most common herbivores were the green peach aphid (*Myzus persicae* Sulz.), the flea beetle (*Altica* sp.), and the potato tuber moth (*Phthorimaea operculella* Zell.). Aphid colonies occurred throughout the field and no significant difference in the average number of colonies per plot was observed (Table 3). The chrysomelid

beetle (*Altica* sp.) was apparently not affected by the presence of Cry3B toxin. The maximum number of leaves damaged by the beetle was 80% in most of the plants, but was higher in transgenic lines (Table 3), perhaps because of the better vegetative growth achieved by transgenic clones, allowing them to withstand CPB attack. *Phthorimaea operculella* larvae caused damage on berries. Surprisingly, the control line suffered significantly more damage ($P > 0.019$) than did the transgenic lines (Table 3).

ELISA showed that the transgenic line 6-1 had a lower amount of Bt toxin than did lines 3-2 and 9-8 (Table 4). In particular, the leaf and fruit tissues of line 6-1 contained significantly lower levels of Bt toxin than did corresponding tissues of lines 3-2 and 9-8 (young leaf: $P > 0.0001$; old leaf: $P > 0.0073$; fruit: $P > 0.0002$). Bt toxin content in lines 3-2 and 9-8 did not differ significantly.

Fruit production was about twice as great in transgenic lines than in the DR2 control (Table 2), largely because of the higher number of fruits produced per plant. Differences were more pronounced at Monsampolo, where CPB infestation was greater. Yields of line 6-1 were intermediate and, under mild infestation, tended to be similar to those of the other transgenic lines; however, under high pressure by *L. decemlineata*, production did not differ significantly from that of the DR2 control line. These results are in agreement with the surveys of CPB larvae in the lines tested, whose feeding activity drastically reduced the canopy in the nontransformed line. Although the Bt gene was in the heterozygous or homozygous state, no visual differences in the response to CPB was noted among plants within each transgenic line.

These preliminary field experiments clearly indicate that the resistance to CPB in eggplant expressing the Cry3B protein is expressed under field conditions. Moreover, transgenic plots had lower populations of all CPB stages and higher yields, even at strong infestation levels. The resistance of line 6-1 was intermediate between the other two transgenic lines and the nontransformed control.

The development of flea beetles, which are taxonomically close to CPB, was apparently not impaired on transgenic eggplants, indicating a specific effect of resistance. Further studies should be conducted to confirm the different sensitivities of two chrysomelids to the Cry3B protein. The extent of fruit damage by *P. operculella* seems to indicate an activity of Cry3B toxin on Lepidoptera. This implies that a larger range of insect resistance may exist for these eggplant lines. Also, in this case, field observations over several years are of paramount importance for germplasm evaluation. No evidence of detrimental effects on other arthropods [e.g., aphids (*Myzus percicae* Sulz.), lacewings (*Chrysoperla carnea* Stephens)] were found.

The two most effective transgenic lines have been used to produce eggplant hybrids that are now in field trials to gather more accurate information on their possible commercial use.

Table 2. Mean number of Colorado potato beetle egg masses, larvae per plant, and eggplant fruit production in field experiments conducted at Monsampolo and Metaponto, Italy, in 1997.

Line	No. egg masses/plant	No. larvae/plant	Yield per plant Kg	Yield per plant No. fruit
		Monsampolo		
3-2	0.13 b[z]	0.21 c	1.30 a	6.23 a
6-1	0.17 b	0.45 b	0.58 bc	3.48 b
9-8	0.08 c	0.07 c	0.98 ab	6.18 a
DR2	0.21 a	1.91 a	0.40 c	2.75 a
		Metaponto		
3-2	0.03 b	0.05 b	0.94 a	8.47 a
6-1	0.07 a	0.08 b	1.01 a	9.34 a
9-8	0.02 b	0.03 b	1.06 a	10.06 a
DR2	0.09 a	0.21 a	0.43 b	4.18 b

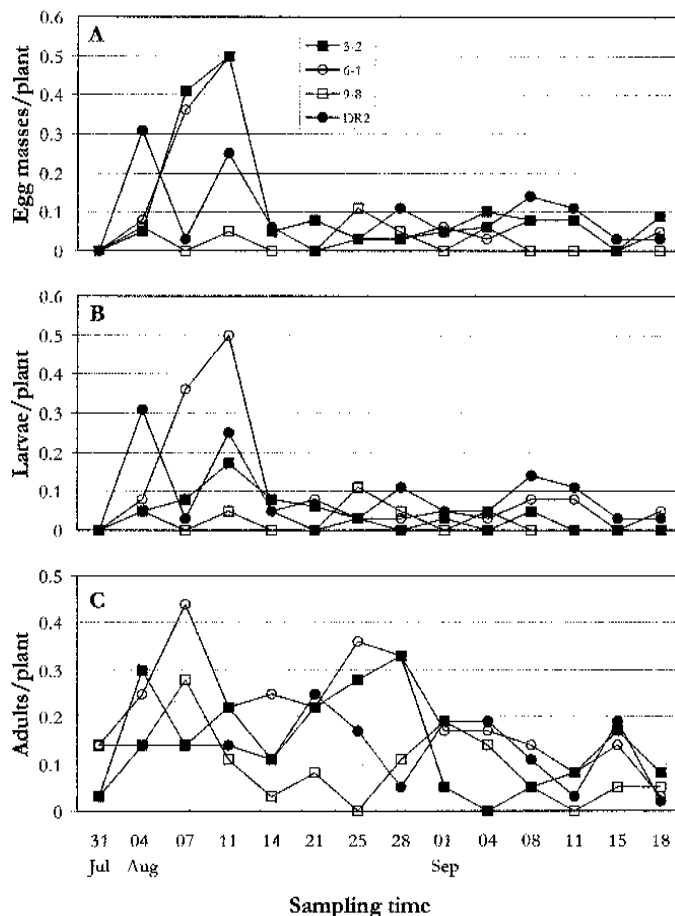[z]Mean separation within columns and parameters by LSD, $P = 0.05$.

Fig. 3. Colorado potato beetle infestation course at Metaponto field trial in 1997. Data relative to small and large larvae have been pooled.

Table 3. Mean numbers of nontarget insects collected at Metaponto, Italy, in 1997.

Line	No. $C.\ carnea$/plant[z]	No. aphid colonies/plant	No. leaves damaged by $Altica$ sp./plant[y]	No. fruits damaged by $P.\ operculella$/plant[x]
DR2	0.02 a[w]	0.01 a	5.23 b	7.72 a
# 3-2	0.01 a	0.01 a	5.65 ab	4.47 b
# 6-1	0.02 a	0.03 a	6.06 a	4.98 b
# 9-8	0.01 a	0.01 a	6.19 a	5.17 b

[z]Larvae + eggs.

[y]Average of three samplings during the month of August.

[x]Average of all harvests.

[w]Mean separation within columns and parameters by LSD, $P \leq 0.05$.

Table 4. Mean DAS–ELISA (OD_{ratio})[z] results for eggplant lines carrying the Cry3B protein.

Line	Mean	Young leaf	Old leaf	Flower	Fruit
9-8	6.81 a[y]	6.87 a	6.47 a	5.38 a	8.52 a
3-2	6.19 a	6.60 a	6.10 a	5.65 a	6.40 a
6-1	3.42 b	2.70 b	4.10 b	5.20 a	1.67 b

[z]OD_{ratio} represents the ratio of absorbance values of transformed plants to those of on transformed plants at 492 nm.

[y]Mean separation within columns and parameters by LSD, $P \leq 0.05$.

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