Salvatore Arpaia

TRANSGENIC RESISTANCE OF EGGPLANTS TO THE COLORADO POTATO BEETLE

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TRANSGENIC RESISTANCE OF EGGPLANTS TO THE COLORADO POTATO BEETLE

PROPOSITIONS

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1. Prolonged ingestion of Cry3B toxin almost completely inhibits reproduction of female Colorado potato beetles. This is due to a specific lack of vitellogenin, preventing normal egg maturation. Further studies on the impact of Bt-derived toxin on adult insects should take this type of effects into account

   Zehnder *et al.* (1989) J. Econ. Entomol. 82:756-761; Johnson *et al.* (1993) J. Econ. Entomol. 86:330-333; Perlak *et al.* (1993) Plant Mol. Biol. 22: 313-321; Arpaia *et al.*, Entomol. Exper. Appl., in press).

2. Unlike other insects, Colorado potato beetles do not avoid either Cry3 toxins or Cry3-expressing plants. The obvious effect of continuous exposure to food containing these toxins is a perturbation of beetle metabolism. The lack of antifeedant effects of Bt on *L. decemlineata* should be considered when planning a strategy of resistance management

   Perlak *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:3324-3328; Ferro & Lyon (1991) J. Econ. Entomol. 84:806-809; Arpaia & Ricchiuto (1993) Environ. Entomol. 22: 334-338

3. The ecologically based approach to pest control, as conceived in IPM programs, is fundamental in the management of transgenic resistance. Studies by insect ecologists in transgenic agroecosystems will be conclusive in providing basic data for more realistic predictions obtained with computer simulation models

4. Herbivory by the wooly adelgid (Homoptera:Adelgidae) on firs, is dramatically changing the floral composition of the forests on the Appalachian Mountains

5. A commitment of the scientific community to rapid and extensive communication of scientific information and progress achieved in the field of transgenic resistance to policy makers* is essential to support the fundamental role of scientific information as a basis for policy making

6. The continuous exploitation of useful genes for transgenic expression in crops may contribute to the increase of the economic gap between developing and developed countries

7. The world is much bigger than a double strand of DNA

8. Mr. Louis van Gaal's predictions have been falsified by reality, as Juventus has reached the final game of the Champions League for the fourth year in a row

   v. Gaal, L. (1996), interview published by 'La Gazzetta dello Sport' on May 23, 1996

Propositions with the thesis "Transgenic resistance of eggplants to the Colorado potato beetle" by S. Arpaia.

Wageningen, April 16, 1999

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To my wife Lella, who's always been at my side.
No matter what.

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Arpaia, S., Chiriatti, K. and Giorio, G. 1998. Predicting the Adaptation of Colorado Potato Beetle to Transgenic Eggplants Expressing cry3 Toxin: the Role of Gene Dominance, Migration and Fitness Costs. Journal of Economic Entomology 91(1): 21-29

Rotino, G.L., Perri, E., Acciarri, N., Sunseri, F. and Arpaia, S. 1997. Development of Eggplant Varietal Resistance to Insects and Diseases via Plant Breeding. Advances in Horticultural Science 11:193-201

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Preface

I went to the Netherlands for the first time in 1990. I was absolutely fascinated by Prof. Schoonhoven's scientific work and I tried, almost hopelessly, to be appointed as a visiting scientist with his group just by writing a letter with my wishes, and mentioning only my research grant and my CV. Quite unexpectedly, his daughter Madeleine phoned me one day in the lab speaking with a perfect Italian accent, and inviting me to the Netherlands. It took me several minutes to realize it was not just a joke!

After only a few weeks there, I took a fancy to Holland, in spite of the cow smell, wet weather, and that infinite sense of envy due to my being unable to ride two bicycles at once on a rainy day.

The idea of starting a PhD program at Wageningen got me a couple of years later, when I was back in Italy and had already been given a permanent position as a researcher at Metapontum Agrobios. To complete this program I have had to travel back and forth from The Netherlands several times ever since (but I love flying, anyway). In spite of the difficulties and also the cost of this project for my own bank account, I never felt as if I were about to give up and I really wanted to complete this work. Thank God, I've met many people who have helped me and spurred me on without whom this book would never have been completed.

First and foremost, I wish to thank my promoter Prof. Louis Schoonhoven, who so warmly accepted the proposal of my being one of his students (even in a very particular way), in spite of the fact that the topic was not really very close to his research. By the way, he and his family sort of adopted me by putting me and my wife up whenever we needed to stay in Wageningen. Talking about entomology, science or just life with Louis was always a great pleasure. Something to be proud of.

Dr. Joop van Loon, my co-promoter, and his family carried on the wonderful tradition of Dutch friendliness hospitality towards me, quite free of charge. Moreover, José van Loon gave me a fundamental requirement for an 'external' Wageningen student: a recipe for poffertjes. Thanks to her, I'm pretty sure I've become the best 'poffertjes-maker' in southern Italy. Dr. Joop van Loon has been a really attentive careful scientific guide for my thesis, by continuously improving, making useful suggestions and thinking about my scientific work; occasionally when he was unable to do this because of the distance, the e-mail cut the mileage and everything ran pretty smoothly.

Special thanks is due to Dr. Fred Gould at the Department of Entomology, North Carolina State University. I spent six months in his lab, which was most certainly the best place to go for my studies. The people over there proved to be a really great group of scientists and also extremely nice people to live with. I never felt as if I were a newcomer to Method Road and they even made me feel appreciated for my small contribution to the research group. I came back from Raleigh with what I consider to be one of the nicest chapters regarding this thesis, yet much more than that. I do apologise for not writing a complete list, since I wish to show a sense of my appreciation to everyone by quoting them now: George Kennedy, Toni Riggin-Bucci, Angelika Hilbeck, Tracy Johnson, Bradley Klepetka, Amy Shack, Doug

Sumerford, Scott Costa, and all the Method Road staff.

I also most warmly thank Prof. James Lashomb at Department of Entomology, Rutgers State University of New Jersey, my first Colorado potato beetle teacher. He knows a lot about this beetle, and he's always open both to doing and learning more. He has mainly contributed to my *Leptinotarsa*-mania and has shared a lot of his thoughts with me about Colorado potato beetle, entomology, and our world today.

Finally, I'm most indebted to the 'Milinciana Group', a link between the two research units of Metapontum Agrobios and the Istituto Sperimentale per l'Orticoltura, who have worked together with the highest enthusiasm and professionality for 6 years so far, on two research projects "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici " and "Progetto finalizzato orticoltura" sponsored by the Italian Ministry of Agricultural Politics which funded most of the activity reported in this thesis. Namely, I thank the project leader, Giuseppe Rotino, the soul of the group (and the real 'eggplant-man' of Italian horticulture), Giuseppe Mennella, Vincenzo Onofaro, Domenico Perrone, Francesco Sunseri, Nazareno Acciarri and Rina Iannacone, the 'gene maker'.

The list of references presented at the end of the book was most kindly and carefully reviewed by Grazia Maria Di Leo.

Summary

The subject of this thesis is the use of transgenic plant resistance as a method to control the Colorado potato beetle, *Leptinotarsa decemlineata* Say in eggplant. The gene conferring resistance is coding for a Cry3B toxin and it is a synthetic version of a wild-type gene originally obtained from the soil bacterium *Bacillus thuringiensis* Berl.

Eggplant cultivations are constantly attacked by a number of serious pests (e.g. the fruit and shoot borer, the Colorado potato beetle, soil-borne fungi). In spite of the heavy losses they may cause, breeding for resistance on this crop has been very limited because of the lack of desirable traits in the eggplant genome or sexual incompatibility with the resistant wild related species. The first chapter reviews Colorado potato beetle biology and its control, with a special emphasis on beetle-eggplant relationships. *L. decemlineata* has become a major problem for eggplant cultivation and sometimes its control on this crop is even more problematic compared to potato cultivation. The longer life cycle of the plant and the intensive regime under which horticultural crops are cultivated have contributed to the increasing importance of this pest.

In the second chapter, a review of the source of resistant genes available in both the eggplant gene pool and wild *Solanum* relatives is presented. Considering the genetic basis of resistant traits, the possible strategies for eggplant breeding are discussed with emphasis on approaches based on the integration of classical breeding methods (crosses and selection) with biotechnological ones (anther culture, genetic transformation, protoplast fusion and marker-assisted selection).

In the third chapter, the results of the study in which protein extracts of *Escherichia coli* expressing the toxin gene from *B. thuringiensis* were tested for effect on the behavior and development of *L. decemlineata* larvae using in vitro bioassays are presented. Protein extracts proved to be very toxic against neonate *L. decemlineata*; no antifeedant effect due to Cry3B toxin was found, even in concentrations which caused mortality or severely inhibited larval growth.

In the fourth chapter, the production of transgenic eggplants and their evaluation is presented. A modified Bt gene of *Bacillus thuringiensis* var. *tolworthi,* encoding a coleopteran insect-specific Cry3B toxin, was transferred via *Agrobacterium tumefaciens* to the female parent of the commercial F1 hybrid 'Rimina' eggplant. A large number of transgenic plants were regenerated and tested by PCR and NPTII expression assays. The presence of the Cry3B toxin in leaf extracts was demonstrated by the DAS-ELISA test in 57 (62.3%) transgenic plants which contained a 74 kDa protein cross-reacting with the serum anti-Cry3B toxin. Twenty-three out of 44 *S. melongena* plants tested by an insect bioassay showed a significant insecticidial activity on neonate larvae of Colorado potato beetle (CPB). The Bt transgene and the toxic effect on CPB larvae were transmitted to progenies derived by selfing. Thus, transgenic Bt eggplants may represent a very effective means of CPB pest control.

Transgenic potato clones expressing a Cry3B endotoxin were used to study the trophic interactions between newly emerged Colorado potato beetle adults and these resistant clones (Chapter 5). Adult longevity and fitness were studied for the first 3 weeks after emergence. The reproductive biology of the beetle on highly resistant clones, partly resistant clones and

control potato plants was monitored by dissecting females after 7-15 days of feeding and by analyzing the haemolymph's protein content after 3 days of feeding. Feeding behavior on either highly toxin expressing or control plants was monitored individually for 36 beetles feeding on leaf-discs. Beetles feeding on transgenic or control clones as the sole source of food had similar longevity. However egg production was completely inhibited on transgenic plants. Dissection studies indicated that adult males living on transgenic plants were still able to mate and produce mobile sperms, but the females were impaired in their reproductive ability since ovarioles were not normally developed. An exam of the haemolymph revealed the protein concentration in females living on transgenic plants to be dramatically reduced (about 50% compared to the control).

The feeding behavior of Colorado potato beetle adults was not affected by the different food plants. This shows that transgenic potato plants were readily accepted as host plants by the beetles. The implications of these findings for the use of transgenic plants as a means of *L. decemlineata* control are discussed.

In the sixth chapter the most relevant results of the first year of a field experiment with the transgenic eggplant lines are presented. Two of the 3 transgenic lines used, showed a high level of resistance in two separate trials, as indicated by the analyses of *L. decemlineata* population levels and crop yield. Fruit production was almost doubled in the resistant lines compared to a DR2 untransformed control. Only one transgenic line showed an intermediate level of resistance, giving results more similar to the control under heavy CPB attack, whereas it gave comparable results to the other transgenic lines where natural infestation was milder. No detrimental effects on non-target arthropods (including the chrysomelid beetle *Altica* spp.) were apparent.

Field observations confirmed that Bt-expressing transgenic plants might be able to successfully control Colorado potato beetle infestations in eggplant cultivations, representing a potentially effective and environmentally safe means of pest control.

In chapter 7, the relationships between *L. decemlineata* egg density and *Coleomegilla maculata* DeGeer predatory behavior is presented. Despite aggregation in areas of the highest prey density by *C. maculata*, egg consumption was inversely related to egg mass density at the smallest and the largest spatial scales tested. The experimental data on predation rates in high and low density field treatments, were included in a mathematical model to simulate the impact of natural enemies on the rate of *L. decemlineata* adaptation to Bt-toxin-expressing transgenic potato plants when Bt-expressing plants are mixed at the plot-to-plot level with normal potato plants. Results showed that *C. maculata* predatory behavior could decrease the rate at which *L. decemlineata* adapted to Bt-toxins if plot-to-plot mixed plantings were used.

Finally, a simulation model to predict the possible adaptation of *L. decemlineata* to the Cry3 toxin expressed in transgenic eggplant is presented (Chapter 8). The use of mixed fields of transgenic and susceptible isolines at a 90:10 ratio has been simulated. Beetle movement, which is a fundamental parameter when studying plant mixtures, has been addressed with a 'two-stage' hypothesis. The biological and genetic characteristics of the beetles have been set to specifically address their possible interactions with resistant eggplant. The role of gene dominance, migration, and fitness costs associated with the resistant genotype have been exa-

mined. Using the hypothesis of partial dominance of the resistant gene, only a high level of migration (yet, very likely in most agricultural areas) or a considerable reduction of the fitness of resistant beetles, associated with the change in their genome, can guarantee a long-lasting efficacy of the germ plasm. The simulations clearly indicate that the effect of resistance in transgenic clones expressing *Bacillus thuringiensis*-derived toxins can be optimized only in accordance with opportune agricultural practices.

Riassunto

L'argomento di questa tesi di dottorato è l'uso della resistenza transgenica in piante di melanzana per il controllo della dorifora, *Leptinotarsa decemlineata* Say. Il gene che conferisce la resistenza è un gene sintetico ottenuto per mutagenesi di un gene 'wild-type' originario di *Bacillus thuringiensis* Berl. e che codifica per l'espressione in pianta della tossina Cry3B.

La coltura della melanzana è interessata da un buon numero di avversità piuttosto serie (es. il nottuide *Leucinodes orbonalis* Guenee, la dorifora, alcuni funghi terricoli). Nonostante le gravi perdite causate da tali avversità, il breeding per le resistenze agli stress biotici in questa coltura è stato molto limitato a causa della mancanza di tratti utili nel genoma di melanzana o della incompatibilità sessuale con specie selvatiche affini.

Il primo capitolo descrive la biologia della dorifora e le sue possibilità di controllo, con particolare riferimento alle sue relazioni con la melanzana quale pianta ospite. Su tale coltura infatti, la dorifora rappresenta spesso un problema ancora più complesso rispetto alla patata. La maggiore lunghezza del ciclo colturale ed il regime colturale intensivo cui la melanzana viene sottoposta (colture in ambiente protetto, alto uso di fertilizzanti, ecc.), hanno contribuito a rendere tale agroecosistema particolarmente consono al fitofago.

Nel secondo capitolo viene presentata una review sulle possibili fonti di geni di resistenza nel genoma di melanzana o di specie di *Solanum* selvatiche compatibili. Considerando il determinismo genetico dei tratti di resistanza, vengono discusse le possibili strategie per il breeding della melanzana con particolare attenzione per gli approacci basati sull'integrazione fra i metodi di breeding classico (incroci e selezione) con quelli biotecnologici (cultura di antere, trasformazione genetica, fusione dei protoplasti e selezione assistita).

Nel terzo capitolo, vengono presentati i risultati di uno studio in cui gli estratti proteici del batterio *Escherichia coli* trasformato per l'espressione del gene 'wild type' derivato da *B. thuringiensis*, sono stati testati per valutare gli effetti sul comportamento e lo sviluppo di *L. decemlineata* tramite biosaggi in vitro. L'estratto proteico si è rivelato molto tossico per le larve neonate di dorifora; non sono stati rilevati effetti fagodeterrenti della tossina Cry3B, anche a concentrazioni che hanno causato mortalità o severamente inibito lo sviluppo delle larve neonate.

Nel quarto capitolo, viene descritto l'ottenimento e la valutazione biologica delle piante di melanzana transgeniche. Un gene mutagenizzato di *Bacillus thuringiensis* var. *tolworthi* che consente la produzione della tossina Cry3B specifica per i coleotteri è stato trasferito via *Agrobacterium tumefaciens* al genitore femminile dell'ibrido commerciale F1 'Rimina'. Un gran numero di piante transgeniche sono state rigenerate e testate con PCR e saggio NPTII. La presenza della tossina Cry3B negli estratti fogliari è stata dimostrata tramite DAS-ELISA test in 57 (62.3%) piante transgeniche che contenevano una proteina da 74 kDa che reagiva con il siero anti-Cry3B. Ventitre piante di *S. melongena* su 44 testate con biosaggi con insetti hanno mostrato una attività tossica su larve neonate di dorifora. Il gene esogeno Bt e gli effetti tossici a carico delle larve di dorifora sono stati altresì rilevati nella progenie ottenuta tramite autofecondazione. Le piante di melanzana esprimenti la tossina Bt rappresentano quindi un potenziale mezzo di controllo della dorifora.

Riassunto

Cloni transgenici di patata esprimenti la endotossina Cry3B sono stati utlizzati per studiare le interazioni trofiche fra adulti neosfarfallati di dorifora e tali piante autoprotette (Capitolo 5). La longevità degli adulti e la loro fitness sono state valutate per le prime tre settimane dopo l'emergenza. Gli effetti sulla biologia riproduttiva del coleottero alimentato su cloni altamente resistenti, parzialmente resistenti e cloni di patata di controllo sono stati valutati tramite dissezione di femmine dopo 7-15 giorni di permanenza sulle piante test ed analizzando il contenuto proteico dell'emolinfa dopo 3 giorni di alimentazione. Il comportamento alimentare su cloni fortemente resistenti o su cloni controllo è stato valutato per mezzo di singole osservazioni di 36 individui alimentati su leaf-disc. Le dorifore alimentate sui cloni transgenici hanno mostrato un TL_{50} comparabile a quello degli insetti viventi sul controllo. La loro longevità tendeva però a diminuire sensibilmente in caso di allevamento in condizioni ambientali non ottimali. La loro produzione di uova è risultata completamente inibita. Gli studi di dissezione hanno indicato che i maschi adulti che hanno vissuto su piante transgeniche sono stati in grado di accoppiarsi normalmente e di produrre spermi mobili che sono stati rinvenuti all'interno dell'apparato genitale femminile. Le femmine invece sono state completamente inibite nella loro capacità riproduttiva poiché gli ovari non si sono sviluppati normalmente. L'analisi dell'emolinfa ha rivelato che la concentrazione proteica dell'emolinfa di femmine sviluppatesi su piante transgeniche era notevolmente ridotta (circa il 50% rispetto agli animali alimentati su piante controllo). Il comportamento alimentare degli adulti di dorifora non è stato condizionato dai diversi cloni di patata con cui sono stati nutriti. Ciò indica che le piante transgeniche sono state prontamente riconosciute quali piante ospiti da parte del coleottero. Le implicazioni derivanti da queste evidenze sull'uso delle piante transgeniche come mezzo di controllo di *L. decemlineata* sono discusse nel capitolo.

Nel sesto capitolo sono riportate le osservazioni relative al primo anno di prove in campo effettuato con le linee di melanzana transgeniche ottenute. Progenie autofecondate di 3 linee transgeniche sono state utilizzate in due prove di campo per valutarne le performance agronomiche con particolare riferimento ai caratteri di resistenza alla dorifora in condizioni di infestazione naturale.

I livelli di popolazione del fitofago rilevati nelle due località sono stati sensibilmente diversi. Due delle linee transgeniche hanno mostrato alta resistenza verso il fitofago in entrambi i campi sperimentali come rilevato dall'analisi sulla presenza di dorifora e sui dati produttivi delle diverse linee. La produzione delle linee transgeniche è stata quasi doppia rispetto al controllo DR2 non trasformato. Soltanto una delle linee transgeniche ha mostrato livelli intermedi di resistenza, fornendo risultati più simili al controllo in condizioni di forte attacco, mentre ha dato risultati comparabili alle altre linee transgeniche quando l'infestazione naturale è risultata più lieve. Non si sono riscontrati effetti negativi a carico di insetti non bersaglio (incluso il crisomelide *Altica* spp.).

Le osservazioni di campo hanno confermato che le piante transgeniche esprimenti la tossina Bt sono in grado di controllare infestazioni di dorifora in coltivazioni di melanzana, e rappresentano un potenziale efficace mezzo di controllo con limitato impatto ambientale.

Nel capitolo 7 viene affrontato l'argomento delle relazioni fra la densità di ovature di dorifora ed il comportamento di un suo nemico naturale, il coccinellide predatore

Coleomegilla maculata DeGeer. Nonostante l'osservata aggregazione di *C. maculata* in aree con più alta densità di preda, il consumo di uova è risultato inversamente correlato alla densità di ovature alla diverse scale di grandezza dell'esperimento realizzato. I dati sperimentali sui tassi di predazione rilevati in campi ad alta e bassa densità di prede, sono stati poi inclusi in un modello matematico per simulare l'impatto dei nemici naturali sulla possibilità di adattamento di *L. decemlineata* alle piante di patata transgeniche esprimenti la tossina Bt, quando queste sono utilizzate in combinazione con piante di patata normali. I risultati hanno indicato che il comportamento del predatore può diminuire la velocità con cui la dorifora si adatta alle piante transgeniche esprimenti la tossina Cry3 in condizioni di utilizzo in campi misti.

Infine, nel Capitolo 8 viene illustrato un modello di simulazione in grado di prevedere il possibile adattamento di *L. decemlineata* alla tossina Cry3 espressa in piante di melanzana transgeniche. E' stato ipotizzato l'uso di campi misti formati da isolinee transgeniche e suscettibili ad un rapporto di 90:10. Il movimento dell'insetto, una caratteristica fondamentale nello studio dei campi misti, è stato valutato con una ipotesi a 'due stadi'.

Le caratteristiche biologiche della dorifora sono state fissate in modo da simulare specificamente il loro sviluppo a carico della melanzana. Sono stati esaminati il ruolo della dominanza del gene, della migrazione e i costi in termini di fitness associati con il genotipo resistente. Usando l'ipotesi della parziale dominanza del gene, solo un alto livello di migrazione (peraltro molto probabile nella maggior parte delle situazioni colturali) o una sensibile riduzione della fitness degli individui resistenti, associata con il cambio nel loro genoma, può garantire una efficacia di lunga durata del germoplasma transgenico. Le simulazioni indicano chiaramente che il mantenimento della resistenza in piante transgeniche che esprimono le tossine derivate da *Bacillus thuringiensis*, può essere ottimizzato solo in combinazione con le opportune pratiche agronomiche.

Chapter 1

The Biology of *Leptinotarsa decemlineata* Say and its Control in Eggplant Crops

Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), is the most important insect pest of potatoes in North America and Europe; it causes serious damage to eggplant and tomato as well. This beetle is oligophagous and accepts only a few species of the family *Solanaceae* as host plants. It was originally linked to wild species of the genus *Solanum,* and *S. rostratum* is considered to be its ancestral host (Tower, 1906). The insect's area of origin is Central America, and from there it successively shifted from its original wild hosts to potato cultivations in southwestern North America. The first major crop losses due to CPB infestation were reported in the state of Nebraska in 1859. Ever since then, it has spread throughout the rest of the continent and eventuallly moved to Europe (ca. 1875) and Asia. Currently, its distribution covers about 8 million km^2 in North America (Hsiao, 1985) and about 6 million km^2 in Europe and Asia (Jolivet, 1991).

The beetle's pest status reached a very important level, due mainly to the following reasons: i) high feeding rates (Ferro *et al.*, 1985; Arpaia *et al.*, 1996); ii) high fecundity, with one female laying on average 300-800 eggs (Harcourt, 1971) and even up to 4000 eggs/female have been recorded under laboratory conditions (Brown *et al.*, 1980), iii) its 'bet-hedging' reproductive strategy that allows the species to minimize risks of severe losses (Voss and Ferro, 1990); and iv) an impressive ability to quickly develop resistance to virtually every insecticide that has been used for its control (Forgash, 1985).

Life history

The Colorado potato beetle's life history varies over its geographic range. The beetles overwinter in the soil as adults, with the majority aggregating in the areas adjacent to fields where they have spent the previous summer (Weber and Ferro, 1993; French *et al.*, 1993). These results partly disagree with previous observations made by Lashomb *et al.* (1984) who found that an economically important number of beetles overwinter within the field.

The emergence of post-diapause beetles depends on the latitude, and may start as early as April (Follett *et al.*, 1993), but more commonly this happens in late May.

If fields are not rotated, they are colonized by overwintered adults that walk to the field from their overwintering sites or emerge from the soil within the field. If fields are rotated, the beetles can fly up to several kilometers to find a new host habitat (Ferro *et al.*, 1985), with the unfed females being the most successful in doing so (Ferro *et al.*, 1991); their colonization pattern seems to be well explained with a linear decrease with the distance (Weisz *et al.*, 1996).

Once overwintered beetles have colonized the field, they first feed and then oviposit within 5-6 days depending on the temperature. Newly emerged adults are unable to fly as the flight muscles have not yet developed (de Kort, 1969) and in this case walking is the only means of dispersal in host finding.

Eggplant cultivations may be the primary host in a new growing season, thus being attacked at a rather early stage (even immediately after transplanting), then the beetles complete two or three yearly generations on this crop. In other cases eggplants represent a secondary host plant for adults moving from harvested potato fields; in the latter case, beetles may spend one or two generations on potato and move on to eggplant fields.

Studies of spatial dispersion of CPB in eggplant fields, indicated an aggregated distribution for both eggmasses and combined mobile stages, however the degree of aggregation may be different between eggplant and potato (Hamilton *et al.*, 1997a).

Consumption of eggplant foliage by Colorado potato beetle is influenced by temperature. Figure 1 shows the average consumption for second through fourth instars at different temperatures (Arpaia *et al.*, 1996). Second instar consumption ranges from 17 to 25.9 % that of fourth instars, third instar consumption ranges from 33 to 66.3 % that of fourth instars and adult consumption ranges from 38 to 81.4 % that of fourth instars (Arpaia *et al.*, 1996).

Colorado potato beetle larvae develop at different rates when fed on eggplant rather than potato. Developmental times from second instar until pupation ranged, at different temperatures (between 20 and 35°C), from 7.29 to 11.38 days (Arpaia *et al.*, 1996), both of which are longer than beetles fed on potato at the same general range of temperatures (Ferro *et al.*, 1985). The slower development may reflect that eggplant is not as suitable as potato for development. This consideration seems to be confirmed by studies on beetle fitness conducted by Jansson *et al.* (1989) who found that fecundity of *L. decemlineata* on eggplant is substantially lower than on potato. Adult longevity though, is not affected by different host plants (Jansson *et al.*, 1989).

The differences in developmental biology on the two host plants will produce significant differences in the population dynamics in the

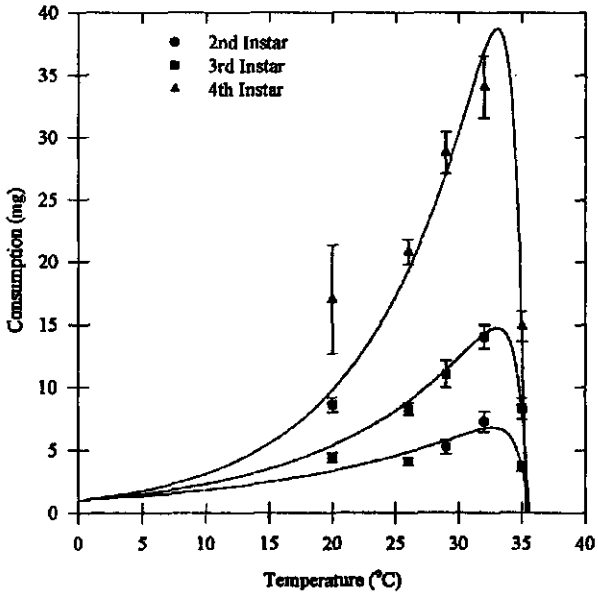

Fig.1 - Average consumption (mg) of eggplant by dry weight for Colorado potato beetle larvae relative to temperature (2nd instar - $y=e^{0.084x}$ $(1-(1.85 \times 10^{-15} * e^{0.953x}))$, $r^2=0.73$, $P\leq0.01$; 3rd instar - $y=e^{0.084x} * (1-(4.697 \times 10^{-16} * e^{0.992x}))$, $r^2=0.56$, $P\leq0.01$; 4th instar - $y=e^{0.114x} * (1-(3.712 \times 10\text{-}16 * e1.006x))$, $r^2=0.75$, $p\leq0.01$). (from Arpaia *et al.*, 1996)

field. I ran a simulation using the logistic population growth curve (Populus software version 3.4, University of Minnesota) to analyze comparative growth trends on the two different host plants. To initialize the simulation model, the requested imputs were set as follows:

- carrying capacity on the two crops at 500 larvae per square meter in potato (Ferro, 1993), and 3000 larvae per square meter in eggplant (S. A., unpublished data);

- two hypothetical r values at 0.36 in potato and 0.18 in eggplant (original calculations, partly based on observations presented in Harcourt, 1971).

The output of the simulation is presented in figure 2.

Migration capacity and flexible diapause response are additional biological features of the Colorado potato beetle which render the species capable of minimizing risks by balancing their offspring production between different years and locations in response to adverse climatic and nutritional conditions (Voss and Ferro, 1990).

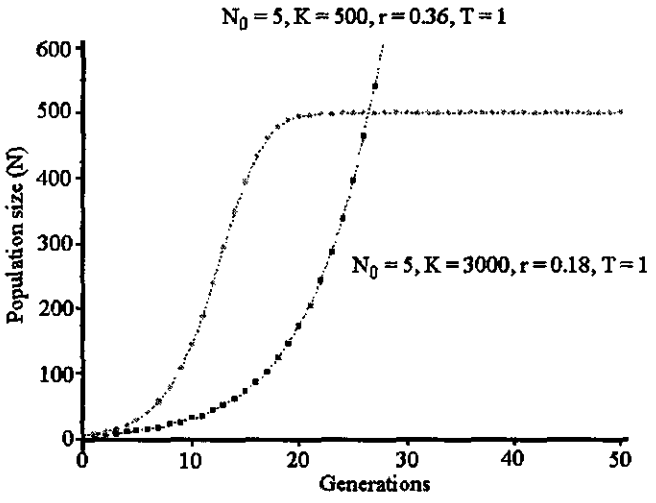

Fig.2 - Hypotetical growth curves of Colorado potato beetle population development on two different host plants; dots = potato, squares = eggplant.

As already mentioned, eggplant fields are regularly infested by migrating *L. decemlineata* adults coming from harvesting potato fields. This phenomenon also plays an important role in the possible adaptation of the beetle to resistant germoplasm (see chapter 8).

Beetles that emerge under short-day photoperiod do not develop their reproductive system and flight muscles that season. They feed actively for several weeks and then either walk to overwintering sites nearby or burrow into the soil directly in the field (Voss, 1989). Photoperiod is the primary factor triggering diapause, food quality and temperature are also important in this respects (Hare, 1990; Weber and Ferro, 1994). Diapause is usually preceded by an intense period of feeding (de Kort, 1990).

Beetle control

Colorado potato beetle is an active leaf feeder whose infestations may completely defoliate fields. Nevertheless, both potato and eggplant may withstand the effects of feeding by quite a large number of individuals without suffering economic losses. An accurate determination of economic injury levels (E.I.L.) is then important and may avoid unnecessary resort to

spray. Thresholds are very variable if different varieties of potato and eggplant are considered; for instance Senanayake and Holliday (1990) determined that on 'Norland' potatoes in Canada, typical values for the economic injury levels ranged from 0.14-0.82 larvae per plant and their values are considerably lower when compared to other studies (e.g. Martel *et al.*, 1986 gave an E.I.L. of 20 larvae; Wright *et al.*, 1987 indicate an E.I.L. of 1.5 large larvae per stalk). Zehnder *et al.* (1995) proposed an action threshold based on defoliation levels, and they defined for the cv. 'Superior' in Virginia the following limits: 20% from emergence to early bloom, 30% early bloom to late bloom, and 60% from late bloom to harvest.

For eggplants, the study performed by Cotty and Lashomb (1982) found an E.I.L. of 8 large larvae per plant for the hybrid 'Harris Special'. Arpaia *et al.* (1996), based on consumption studies, correlated the food consumption of each single instar to the temperature and formulated E.I.L.s in terms of feeding equivalents at different temperatures. In general, thresholds of 8 fourth instars, 12 adults, 19 third instars and 44 second instars can be generally utilized for highly vigorous hybrids. In the thermal range of 29-32°C the maximum consumption was recorded (Fig. 1), and in this case E.I.L.s should be consequently reduced.

Chemical Control

The control of *L. decemlineata* both in the field and greenhouses has been continuously hampered by the well known ability of the insect to adapt to many different chemicals. The first report of CPB resistance to synthetic pesticides was in 1952 for DDT (Quinton, 1955). Presently, the beetle has acquired resistance to a wide range of insecticides, including arsenicals, organochlorines, carbamates, organophosphates, and pyrethroids. In some cases, a new insecticide failed even during the first year of use (e. g. oxamyl, Forgash, 1985). Resistance mechanisms are highly variable even within the same geographical area (Ioannidis *et al.*, 1991). Furthermore, the beetles show cross-resistance to organophosphates and carbamates, and multiple resistance to organophosphates, carbamates, and pyrethroids (Ioannidis *et al.*, 1991). Laboratory experiments showed that under strong selective pressure, the beetle may be able to adapt to the delta-endotoxin produced by *Bacillus thuringiensis* subsp. *Tenebrionis* (Whalon *et al.*, 1993; Rahardja and Whalon, 1995). This extreme capacity for adaptation forces growers to repeated sprays in a single season, up to 12-15 on eggplants. There is a number of synthetic insecticides currently available for CPB control, the efficacy of the treatments, however, may vary from area to area. In Table 1 the most used active molecules are indicated.

Among the newest active molecules commercially available for CPB control, very good results have been obtained with the use of imidacloprid (e.g. Hoy and Dunlap, 1995; Boiteau *et al.*, 1997), whose systemic properties guarantee a longlasting protection of the crop. Its mode of action shows some similarities with that of carbamates, organophosphates and pyrethroids because the molecule blocks neural transmission by inhibiting the acetylcoline esterase activity. Unlike the above mentioned insecticides, imidacloprid mimics the acetylcoline molecule and specifically binds to receptors on the membrane of neural cells. This mechanism should avoid a simple onset of cross-resistance in insect strains resistant to other active molecules. So far, no onset of resistance to imidacloprid has been observed in any *L. decemlineata* population.

Table 1. Availability of insecticides to control Colorado potato beetle on different crops (only products allowed for use on these crops in Kentucky are reported; from Bessin, 1994).

Insecticide Class	Product Name	Potato	Eggplant	Tomato
Organophosphates	Guthion	+	+	
	Diazinon*	+		
	Imidan*	+		
	Di-Syston	+	+	
Carbamates	Sevin*	+	+	+
	Furadan	+		
Pyrethroids	Ambush	+	+	
	Asana XL	+	+	+
	New Spectracide	+		
	Warrior		+	
Chlorinated	Methoxychlor*	+	+	
Hydrocarbon	Thiodan*	+	+	+
B. thuringiensis	M-One*	+	+	+
Chloronicotinyl	Admire 2F	+		
	Provado 1.6F	+		
IGR	Align*	+	+	+
Other	Kryocide	+		

Control by microbial preparations

Microbial insecticides based on *Bacillus thuringiensis* (Bt) crystals and spores are becoming a very popular means of pest control, since they are environmentally safe, very specific in their action and harmless to the herbivore's natural enemies and humans. Several Bt strains have been proven to be toxic to coleopterans and the Colorado potato beetle has probably been the main target insect of these microbial formulations (e.g. Cantwell and Cantelo, 1981; Zehnder and Gelernter, 1989; Maini *et al.*, 1990b; Ferro and Lyon, 1991; Johnson *et al.*, 1993). Beetle susceptibility decreases with its development (Zehnder and Gelernter, 1989), thus spray should be directed against young larvae. A proper timing of field application is then a fundamental requirement for a successful control. A method for correlating spraying with the occurrence of peak egg hatching was proposed by Zehnder *et al.* (1992).

The weak point of Bt-based insecticides is represented by their sensitivity to sunlight and a generally short persistence in the field. Several techniques have then been proposed to prolonge its activity over time. The proposed methods vary quite a lot: the incapsulation of the bioinsecticide with starch (Dunkle and Shasha, 1988), clay (Tapp and Stotzky, 1995), casein based formulation (Behle *et al.*, 1996), the preparation of a flour matrix (Ridgway *et al.*, 1996) and more diverse sprayable granular formulations (e.g. Tamez-Guerra *et al.*, 1996).

A recent technology has enabled the achievement of higher expression levels in *Bacillus thuringiensis* strains by utilizing some natural genetic processes, such as 'plasmid curing' and 'bacterial conjugation'. The first process allows the selection of special *B. thuringiensis* strains which have lost some extrachromosomial DNA (plasmids) and consequently compensate this loss with the production of an higher level of insecticidal proteins. By utilizing bacterial conjugation, plasmids producing highly active proteins can be transferred into less active strains, thus more effectively targeting the bioinsecticide.

Alternatives, other than spraying with conventional or biological insecticides, are available for CPB control:

Cultural control practices

The use of common cultural practices such as crop rotation, a change in planting time, use of mulches and trap crops can reduce CPB populations.

In a rotated field, the maximum density of egg masses could be less than 10% of that in the non-rotated field (Lashomb and Ng, 1984). Wright (1984) reported that when potatoes were planted following a non-host crop, early season CPB adult densities were reduced by 95.8%. Follett *et al.* (1996) suggest that to reduce the beetle population, effective field rotation from year to year will require a distance > 500 m.

Late and early planting is aimed at the suppression of the second generation larval populations. On late-planted crops, summer-generation adults emerging under short-day photoperiod are stimulated to enter reproductive diapause, thus largely eliminating the second-generation larvae. Early planting also eliminates the second generation larvae, because the crop is already being removed at the time of their emergence (Weber and Ferro, 1995).

Trap cropping may be effective in intercepting overwintered beetles colonizing a field in the spring (Weber and Ferro, 1993).

Larval populations of the beetle were also significantly reduced in strawmulched plots of potato (Stoner, 1993) and eggplant (Stoner, 1997). A peak of 1st and 2nd instars was observed 1 - 2 weeks later on the mulched potato fields than on the unmulched ones (Stoner, 1993). Furthermore, the mulch may increase the time required by the beetles to find potatoes (Ng and Lashomb, 1983), and increase the predation on eggs and larvae (Brust, 1994). Overall, a 6- 10 cm layer of wheat straw produced 2.5-5 fold decrease in potato defoliation (Zehnder and Hough-Goldstein, 1990; Brust, 1994).

Another cultural practice that affects CPB populations is vine killing. This practice, originally adopted to facilitate mechanical harvesting, proved to be useful in beetle control because it did not permit their reproduction on the crop (Casagrande, 1987).

Unfortunately, changes in grower practices in the past decades in the use of rotations, vine killers and fertilizers, have all helped to render the agroecosystem more favourable to Colorado potato beetle populations. This has led to a larger request for insecticides, contributed to the resistance problem and caused enormous CPB populations where insecticides failed.

Finally, Casagrande (1987) suggests that, among others, the two cultural practices which can have a major effect on beetle control are: crop rotation and shortening the growing season. Alas!, the latter is not feasible in eggplant crops.

Biological control

There are a number of arthropods attacking the Colorado potato beetle, and some of them show a good potential as biocontrol agents (Hough-Goldstein *et al.,* 1993). The coccinellid *Coleomegilla maculata* DeGeer (Coleoptera:Coccinellidae), for instance, is an active predator of *L. decemlineata* eggs and first instars. Field studies in North Carolina potato fields, found that egg mortality due to predation ranges from 17-34% in the early season, with peaks as high as 100% during the latter part of the season (Hilbeck, 1994). The parasitic wasp *Edovum puttleri* Grissel was found to parasitize 71-91% of Colorado potato beetle egg masses on eggplant, killing 67-79% of the eggs per mass (Lashomb *et al.,* 1987). *E. puttleri* has been used successfully for the implementation of biological control in eggplant (Lashomb, 1989).

Unfortunately, the use of natural enemies does have limitations. First of all, because many of the CPB antagonists are of sub-tropical origin, and so often have difficulty surviving the winter in temperate regions, as it is the case of *E. puttleri* which requires an annual reinoculation. This may not always be feasible, as the inundative release technique proved to be too expensive for CPB control when the stinkbug *Perillus bioculatus* (F.) was released (Hough-Goldstein and Whalem 1993). The tachinid *Myiopharus doryphorae* (Riley) though, is native to the USA and has frequently been reported in field populations of CPB, but its action is inversely density dependent (Harcourt, 1971) and normally it is not able to compete with increasing beetle populations (e.g. Tamaki *et al.,* 1983). Another possible drawback is due to the fact that population dynamics of some predators, such as *C. maculata,* are driven by outside factors that cannot be controlled (e.g. their strong orientation towards corn, their overwintering behavior in masses in or at trees, the presence of alternative preys, etc.).

Unpublished observations by J.H. Lashomb show that combined effects of the predator guilds is significant at low host density, and in unsprayed fields natural enemy guilds arrive

quickly. Nevertheless, the high fecundity of the Colorado potato beetle may also cause its population to rise quickly above economic treshold levels before it can be controlled by natural enemies (Hough-Goldstein *et al.*, 1993), thus these autors suggest the use of natural enemies in conjunction with other compatible control methods.

Genetic engineering

The newest approach for controlling *L. decemlineata* infestations, is represented by the introduction of transgenic plants expressing the Cry3 proteins derived from the soil bacterium *Bacillus thuringiensis* Berliner, strains *tenebrionis*, *san Diego* and *tolworthii*. The first reports of the successful transformation of tobacco and tomato plants with *B. thuringiensis*—derived genes (e.g. Vaeck *et al.*, 1987; Fischhoff *et al.*, 1987, Barton *et al.*, 1987) was followed by an enormous flow of research aimed at the production of engineered plants highly resistant to insects, namely Coleoptera and Lepidoptera. Ten years later, transgenic plants resistant to insects have been allowed for commercial cultivations in North America, Argentina and Australia (Table 2) and transgenic corn resistant to the European corn borer is presently being sold in the European Community.

Table 2. Surfaces cultivated with insect resistant transgenic crops worldwide during 1997 (source: Commissione Interministeriale per le Biotecnologie. Rome, june 1998).

Crop	Country	Surface (Ha)
Maize resistant to European corn borer	USA	3,200,000
	Argentina	120,000
	Canada	20,000
	TOTAL	**3,340,000**
Cotton resistant to Lepidoptera	USA	1,000,000
	Australia	170,000
	TOTAL	**1,170,000**
Potato resistant to Colorado potato beetle	USA - Canada	40,000
GLOBAL SURFACE		**4,550,000**

Several major steps have been made towards the production of effective insect—resistant clones. Transformation methods have been optimized and the current registered varieties

have been obtained either via *Agrobacterium*—mediated transformation (e.g. tomato, potato, cotton, rice) or by using the particle gun (corn).

'Wild type' Bt genes however, were poorly expressed in higher plants (e.g. Murray *et al.*, 1989; Murray *et al.*, 1991; Perlak *et al.*, 1991). One of the first explanations suggested for this phenomenon was the different codon usage between eukaryotes and prokaryotes.

In our attempts to reach a high expression level of Cry3 protein in transgenic eggplant lines, we tested more than one hundred plants (eggplant and the related wild species *Solanum integrifolium*, used as a model plant) transformed with different versions of the 'wild type' Cry3B gene and we could not detect insecticidal activity for any of these plants (Table 3). Subsequently the modification of the coding sequence was achieved by changing polyadenylation sequences, splicing sites, ATTTA sequences (Iannacone *et al.*, 1997).

Table 3. Transgenic plants transformed with the 'wild type' Bt gene and tested for toxicity to neonate *L. decemlineata* larvae (from Iannacone *et al.*, 1995, partly modified).

Vector constructs	no. of different transformations	total no. of plants tested
Solanum integrifolium 35S-cry3b-OCS	24	34
S. integrifolium 35SΩ-cry3b-OCS	8	20
S. integrifolium RbcΩ-cry3b-OCS	7	20
S. integrifolium 35Sturbo-cry3b-OCS	8	21
S. melongena 35S-cry3b-OCS pD09	17	20
TOTAL	**64**	**125**

Field experiments with Bt—expressing transgenic plants are being carried on at an increasing frequency (e.g. Delannay *et al.*, 1989; Hoffmann *et al.*, 1992; Koziel *et al.*, 1993; Benedict *et al.*, 1993; Hamilton *et al.*, 1997b) and are confirming the effectiveness of insect resistance characters. In the U.S.A. alone, since 1987 the USDA has either approved or acknowledged 3,176 field trials at 13,518 field sites (I.S.B. News Report, January 1998).

Two major concerns are presently being carefully considered by researchers in this area: i) the possible onset of resistance to the Cry toxins expressed in transgenic plants in insect populations and ii) the possibly detrimental effects derived from the mass release of genetically engineered plants in the environment.

The first point is discussed in several parts of this thesis and in particular, chapter 7 considers the possible effect of a predator on the rate of Colorado potato beetle adaptation to the Cry3 toxin expressed in eggplant in different field conditions.

The study of the possible effects of transgenic plants on the environment is widespread and complex and would need the cooperative effort of experts active in different disciplines. Preliminary studies have been conducted using the transgenic materials mentioned in the present thesis, and the first available data may be found in Arpaia (1996), Arpaia and Sunseri (1996), Noteborn and Arpaia (in preparation).

Induced resistance against *L. decemlineata* by means of genetic engineering is the main topic of this thesis and the subject is thoroughly discussed in the following chapters.

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Chapter 2

Development of Eggplant Varietal Resistance to Insects and Diseases via Plant Breeding

Abstract

Eggplant cultivations are constantly attacked by a number of harmful pests (e.g. the fruit and shoot borer, the Colorado potato beetle, soil-borne fungi). In spite of the heavy losses they may cause, breeding for resistance on this crop has been very limited because of the lack of desirable traits into eggplant genome or sexual incompatibility with resistant wild related species.

The present paper reviews the source of resistant genes available in both the eggplant gene pool and wild *Solanum* relatives. Considering the genetic determinism of resistant traits, the possible strategies for eggplant breeding are discussed with an emphasis made on approaches based on the integration of classical breeding methods (crosses and selection) with biotechnological ones (anther culture, genetic transformation, protoplast fusion and marker-assisted selection).

Introduction

Solanum melongena L. (2n = 24) is also known as eggplant, aubergine, brinjal or Guinea squash. It is mainly cultivated in the tropical Asian and Mediterranean countries.

The annual worldwide production of eggplants was about 17.5 million metric tons in 1997 and this has increased in the last ten years by about 10 million metric tons (FAO, 1998).

The largest producer is China with 50% of total world production followed by India, then Turkey, Japan, and Mediterranean countries such as Egypt, Italy, Spain, etc. The eggplant is an important and popular vegetable in the food habits of these countries inhabitants.

Most of the produce is freshly marketed, but the use of frozen pre-cooked eggplants is spreading, mostly in the developed countries. The fruits and leaves have other medicinal uses. Eggplant is a slow-growing, perennial, solanaceous crop in the tropical countries, while in the temperate zones it behaves as an annual. However, its growing season can be extended under protected cultivation. The plant grows to a height of 50 to 150 cm and bears fruits of very different sizes, shapes and skin colour. It is a day-neutral plant and starts flowering at the 6th to 10th- leaf stage lasting for a long period. It is considered an autogamous species, however, the frequency of natural cross-pollination is estimated to vary from 0.2 to 48%.

Eggplant breeding is mainly focused on F1 hybrid cultivars, which almost replaced the open pollinated varieties, particularly in the intensive growing areas. The major objectives of breeding are the development of high-quality and pest resistant varieties. In the countries where intensive and successive cropping is practised, the main goal of breeding programs is to

develop varieties resistant to soil-borne diseases (*Verticillium* and *Fusarium* wilt, bacterial wilt and nematode infections). Eggplant cultivations are severely damaged also by insects (e.g. *Leucinodes orbonalis* Guenee, *Leptinotarsa decemlineata* Say, *Trialeurodes vaporariorum* - Westw-), mites and fruit rot. Since the eggplant is highly responsive to *in vitro* manipulation (Hinata, 1986), biotechnological approaches as *in vitro* doubled-haploids production (Rotino, 1996), culture and fusion of protoplasts (Sihachakr *et al.*,1994), and genetic transformation (Rotino and Gleddie, 1990), may well help to solve several of its agronomic problems.

Breeding for resistance and crop improvement

Resistance source within eggplant germplasm. The breeding procedures usually followed are: pedigree, backcross, bulk methods, recurrent selection and single seed descent (Kalloo, 1993).

In spite of the vast morphological variability, there is a lack of resistance traits in the *Solanum melongena* gene pool; thus, the release of eggplant cultivars resistant or tolerant to the main diseases and pests has been very limited. A certain degree of genetic variability related to resistance to several pests has been found and germplasm was reported as a suitable source (Table 1). Neverthless most of this genetic material gave unsatisfactory or contrasting results when employed in breeding programs. Several reasons, such as: resistance restricted to a specific pathogen strain or isolate, poligenic and complex resistance traits and unreliable tests for assessing the resistance may explain the relatively poor results obtaineded so far. In spite of this, the efforts made have permitted the improvement of the tolerance to certain diseases (e.g. even in the absence of an effective resistance trait, lines showing a reasonable field tolerance to *Verticillium spp.* have been selected).

With regard to resistance to insects, studies have been carried out by Indian scientists to exploit genetic variation, aimed at the selection of genotypes with improved tolerance or resistance to the fruit and shoot borer, *L. orbonalis* (Dahnkhar and Sharma, 1986), which is the most destructive pest in Asia. Many field screenings of different genotypes infested by this insect consented the correlation of some plant characters with improved tolerance to this pest.

Morphological traits which have been associated with increased tolerance to insect attack are: tightly arranged seeds in the mesocarp (Lal, 1991); more lignified and compact hypodermal sclerenchyma, broader and more compact vascular bundles (Isahaque, 1984). Plant chemicals potentially involved in tolerance to this pyralid were also identified: 1) lower sugar and protein content (Isahaque, 1984), 2) a higher level of peroxidase and poliphenoloxidase and a higher level of glicoalkaloids (Bajaj *et al.*, 1989). The latter are well known compounds involved also in resistance to *L. decemlineata* in several solanaceae (Flanders *et al.*, 1992).

However, Tewari and Moorthy (1985) reported that the variation in tolerance under field conditions among different genotypes, was lost in artificial infestation with a high pest population density and all genotypes were equally susceptible. The tolerance seemed to be inherited as a poligenic trait with a high additive effect, supported by more than one recessive

gene (Dahnkhar *et al.*, 1977; 1980).

A partial resistance to *T. vaporariorum*, based on antibiosis and antixenosis, was also noted among seven eggplant genotypes in greenhouse and laboratory tests and the differences in hairiness and colour among genotypes had no relation whatsoever with the resistance (Malausa *et al.*, 1988).

In germoplasm field screenings, variation was noted also in relation to the response to natural infestations of jassid (*Amrasca biguttula* - Ishida -) (Khaire and Lawande, 1986). Unlike the previous case, the presence of trichomes was considered to be associated with increased levels of resistance (Schreiner 1990), while other morphological characters such as leaf lamina and midrib tickness were positively correlated with the insect infestation (Subbaratnam *et al.*, 1983).

Trichomes are also involved in resistance to insects in potato and other wild *Solanum* species. The combined action of mechanical obstruction and the production of phenolic compounds (Avé and Tingey, 1986) and sucrose esters of carboxylic acids (Neal *et al.*, 1990) considerably reduce attack by aphids, leafhoppers, flea beetles and Colorado potato beetle (Flanders *et al.*, 1992).

A certain degree of variation in susceptibility to the Coleopteran *L. decemlineata* (Fiume, 1987) and *Epilachna vigintioctopunctata* F. (Sambadan *et al.*, 1976; Raju *et al.*, 1987) was reported for some eggplant accessions.

Partial resistance to *Tetranychus cinnabarinus* (Boisduval) based on antibiosis, was found to be positively correlated with the density of leaf hairs (Misra *et al.*, 1990) or else to an antixenosis mechanism (Shalk *et al.*, 1975).

Resistance source in wild species and distant hybridization. Many attempts have been made to introgress in eggplant, resistance genes possessed by wild *Solanum* species by means of sexual hybridization (Kalloo, 1993). The first step in a program aiming at the introgression of useful traits from wild relatives into the eggplant gene pool is their evaluation for disease and pest resistance. The second step is the selecting and fixing of useful resistance levels in segregating progenies. In addition, different accessions of the wild species may give different results with respect to resistance to the same pathogen.

Several solanaceous species have been identified as possible sources of resistance to the main pests of the eggplant (Table 2); however the genetics of the resistance is not completely known. Source of resistance to the most serious soil-borne diseases (*Verticillium*, *Fusarium* and nematodes) have been identified in *S. sisymbrifolium* and *S. torvum*. *S. khasianum* was found resistant to the shoot and fruit borer (*L. orbonalis*).

In spite of numerous studies and the enormous amount of work undertaken by some research groups, the contribution of wild relatives to eggplant breeding has been very limited so far. The main reasons for the unsatisfactory results obtained may be concerned with a certain confusion regarding the taxonomic classification within the *Solanum* genus which makes very difficult for breeders to make a reasonable prediction of the suitable wild species to be employed for crossing (Daunay and Lester, 1988; Daunay *et al.*, 1995).

Successful distant hybridization between *S. melongena* and wild relatives are reported

in Table 3; most of the interspecific hybrids obtained were sterile or not crossable with eggplant, thus this material resulted useless for starting a breeding program. Nishio *et al.* (1984) classified 11 *Solanum spp.* into 3 groups on the basis of their interspecific compatibility: 1. *S. melongena, S. incanum, S. macrocarpon;* 2. *S. integrifolium, S. gilo, S. nodiflorum;* 3. *S. indicum, S. mammosum, S. torvum, S. sisymbrifolium, S. toxicarium.* Crosses are only possible between and within the first and second groups.

The INRA in Monfavet (France) started a project to evaluate the crossability of a collection of wild relatives in order to guide breeders towards the use of the genetic variability within the species crossable with eggplant. The strategy of this kind of work is reversed when compared to the previous approaches about distant hybridization in eggplant. In fact, the evaluation for resistance to pathogens is restricted to the wild species crossable with the eggplant. By using this procedure, 15 interspecific hybrids have been obtained among the 21 *Solanum* so far tested (Daunay *et al.*, 1995).

In Japan, where grafting is a normal practice for most of eggplant cultivation, an intensive resistance breeding is carried out to confer multiple resistance in the rootstocks (Yamakawa, 1982). Wild relatives (*S. integrifolium, S. torvum,* etc.), or selected fertile sexual or somatic interspecific hybrids are employed as rootstock. Grafting on suitable rootstock is also developing in the Mediterranean countries under protected cultivation.

Biotechnological approaches

Incorporation of doubled-haploid in breeding for disease resistance. The eggplant anther culture technique is currently incorporated in commercial breeding programs in France, Italy and other countries. Compared to successive selfing, the main advantage of the anther culture is that it saves time in obtaining pure lines. Two years after anther culture, it is possible to include eggplant doubled-haploid (DH) lines in field trials, which represents less than half the time required by sexual reproduction. Anther-derived DH lines may be released as self-pollinated cultivars or else used as parents of F_1 hybrids. For breeding purposes, a large number of homozygous plants is needed and it is important that DH lines be a representative sample of the genetic variation obtained from the sexual recombination of the donor plant. Other factors which must be taken into account are the heterozygosity level of the anther donor (F_1, F_2 or advanced selected progenies) and the genetic inheritance of the desirable traits. Genetic variation has been observed among DH lines derived from both inbred cultivars and heterozygous donors (Rotino, 1996).

Although good recombinant DH lines may be recovered from F_1 hybrids, it should be considered that most of the DH lines do not present useful characters for practical breeding. Therefore, it is advisable to apply the anther culture technique to a segregating plant population previously selected for disease resistance and other agronomic traits. In these plants, there is a higher probability of finding favourable gene combinations, since the parental chromosomes have already undergone at least two recombination cycles.

The production of DH lines can be applied effectively when a relatively small number

Table 1. Eggplant germplasm reported as resistant to its main pests.		
Pest	**Source**	**Reference**
Insects		
Jassid *(Amrasca biguttula)*	S488-2; S34; S258 'Manjari Gota'	Pawar *et al.*, 1987
Aphis gossypii	Green-fruited local populations AC 49A	Schreiner, 1990
Glasshouse whitefly *(Trialeurodes vaporariorum)*	'Shinkuro'	Malausa *et al.*, 1988
Shoot and fruit borer *(Leucinodes orbonalis)*	F_3 progenies *S. melongena* x *S. incanum* 'Pusa Purple Cluster' AM62 SM17-4	Rao, 1981; Nathani 1983; Singh and Sidhu, 1988
Diseases		
F. oxysporum and *Phomopsis vexans*	F_4 plants *S. melongena* x *S. indicum*	Rao and Kumar, 1980
Fusarium wilt	K61, K7, Ghana Local	Abdullaeva and Shifman, 1988
Verticillum dahliae	PI 1649, PI 174362	Lockwood and Markarian, 1961
Verticillum albo-atrum	'Florida Market', 'Hanis Hybrid 7763'	O'Brien, 1983
Cercospora solani	UdipiGulla, GO-3	Madalageri *et al.*, 1988
Pseudomonas solanacearum	SM6-1, PPC, ARU2C, SM 6-1, SP, SM 6-7, SP	Sheela *et al.*, 1984; Ushamani and Peter, 1987
Nematodes		
M. incognita race 1 & 2	'Gulla'	Ravichandra *et al.*, 1988

Table 2. Wild *Solanum* species reported as resistant to the main pests of eggplant.		
Pest	**Source**	**Reference**
Insects		
Shoot and fruit borer *(Leucinodes orbonalis)*	*S. integrifolium*, *S. sisymbrifolium*, *S. xantocarpon*, *S. khasianum*, *S. hispidum*	Chelliah and Srinivasan, 1983 Khan *et al.*, 1978 Sharma *et al.*, 1980
Glasshouse whithefly *(T. vaporariorum)*	*S. macrocarpon*	Malausa *et al.*, 1988
Aphis gossypii	*S. sisymbrifolium*, *S. mammosum*	Sambandam and Chelliah, 1983
Colorado potato beetle *(L. decemlineata)*	*S. pinnatisectum*, *S. polyadenium*, *S. jamesii*, *S. trifidum*, *S. capsici-baccatum*, *S. tarijense*, *S. chacoense*, *S. berthaultii*, *S. chomatophilum*	Flanders *et al.*, 1992
Mites		
Two-spotted spider mite *(Tetranychus urticae)*	*S. macrocarpon* *S. integrifolium*	Schaff *et al.*, 1982 Dikii and Voronina, 1985
Carmine spidermite *(T. cinnabarinus)*	*S. mammosum* *S. pseudocapsicum* *S. sisymbrifolium*	Shalk *et al.*, 1975
Diseas		
Fusarium wilt	*S. indicum*, *S. integrifolium*, *S. incanum* *S. sisymbrifolium*	Yamakawa and Mochizuki, 1979 Cappelli *et al.*, 1995
Verticillium dahliae *V. albo-atrum*	*S. sisymbrifolium*	Fassuliotis and Dukes, 1972
Phomopsis fruit rot *(P. vexans)*	*S. gilo*, *S. integrifolium*	Ahmad, 1987
Cercospora solani	*S. macrocarpon*	Madalageri, 1988

Table2. (continued)

Pest	Source	Reference
Verticillium dahliae	*S. torvum, S. caripense,* *S. persicum, S. scabrum,* *S. sodomaeum* *S. sisymbrifolium,* *S. torvum*	Sakata *et al.*, 1989 Anonymous, 1979 Alconero *et al.*, 1989
Bacterial wilt *(Pseudomonas solanacearum)*	*S. integrifolium,* *S. torvum,* *S. integrifolium*	Yamakawa, 1982 Sheela *et al.*, 1984
Nematodes		
Meloidogyne incognita	*S. sisymbrifolium* *S. khasianum, S. torvarum* *S. toxicarium* *S. sisymbrifolium* *S torvarum*	Fassuliotis, 1973 Ali *et al.*, 1992 Di vito *et al.*, 1992
Meloidogyne incognita *M. arenaria*	*S. torvum,* *S. sisymbrifolium*	Daunay and Dalmasso, 1985
Meloidogyne incognita *M. javanica*	*S. sisymbrifolium*	Fassiulotis and Dukes, 1972
Meloidogyne spp.	*S. torvum, S. aethiopicum*	Hébert, 1985
M. javanica	*S. torvum*	Di Vito *et al.*, 1992
M. hapla	*S. sisymbrifolium*	Di Vito *et al.*, 1992
Phytoplasma		
Little leaf	*S. hispidum,* *S. integrifolium*	Rao, 1980 Khan *et al.*, 1978
Virus		
Eggplant mosaic virus	*S. hispidum*	Rao, 1980

of genes are involved in the resistance trait or when the desirable alleles are recessive and not closely linked. A very useful application of the anther culture technique is to extract DH lines from advanced cycles of a recurrent selection scheme. Resistance to insects seems to be partial and its inheritance polygenic and the use of anther culture could thus significantly improve the selection efficacy, because it may facilitate the fixing of favourable gene combinations at the homozygous level.

Somatic hybridization. Protoplast fusion and somatic hybrids regeneration has been attempted to overcome sexual barriers between the eggplant and its wild relatives. From the first successful production of somatic hybrids between the eggplant and *Solanum sisymbrifolium* (Gleddie *et al.*, 1986), several other somatic interspecific hybrids have been obtained. Out of 13 successful protoplast fusion experiments, somatic hybrid plants were regenerated in eleven cases, but fertile hybrid plants were obtained only in combination with 3 wild species (Table 4). The useful resistance traits to *Pseudomonas*, spider mites, *Fusarium* and *Verticillium* derived from wild species were maintained in the regenerated somatic hybrids. By using a somatic fusion hybrid, Balbyshev and Lorenzen (1997) obtained a clone that responded to egg-masses of the Colorado potato beetle with a hypersensitive necrotic zone which subsequently disintegrated around the border and became detached from the leaf. In general, information about backcrosses with the eggplant are limited.

This technique consents the obtainment of somatic hybrids, in which recombination of both the nuclear and cytoplasmic DNA occurs and thus represents a powerful tool for enlarging the genetic variability in eggplant. An improvement in somatic hybrids regeneration efficiency and the use of better selection schemes may allow the regeneration of a large number of both symmetric and asymmetric somatic hybrids, which should give a stronger probability of finding backcrossable plants. In addition, the anther culture of somatic hybrids may be a suitable tool to bring the amphidiploid hybrids or their backcrosses with eggplant back to the diploid status.

Genetic transformation. Protocols for introducing foreign, agronomically useful genes into the eggplant genome via *Agrobacterium tumefaciens* are available (Rotino and Gleddie, 1990). *Bacillus thuringiensis* wild type genes, active against *L. decemlineata* were obtained, but the low expression level of the transgene did not permit a satisfactory insect control (Rotino *et al.*, 1992; Chen *et al.*, 1995). Recently, transgenic eggplants resistant to Colorado potato beetle have been obtained by using mutagenized Bt *cry3* genes (Arpaia *et al.*, 1997b; Hamilton *et al.*, 1997b). Anti-lepidopteran Bt genes *cry1* and *cry2* could then be used to verify the sensitivity of the fruit and shoot borer to the toxin and its possible control in the field.

Other primary gene products (e.g. proteinase inhibitors, lectins), which are toxic to insects, may be employed alone or in various combinations.

The availability of engineered resistant eggplants, based on a single dominant gene, will pose the problem of the durability of resistance obtained by genetic manipulation. Resistance to the Cry3A toxin in a laboratory strain of *L. decemlineata* was inducted by conti-

Table 3. Sexual hybrids between eggplant and wild *Solanum* spp.		
S. melongena x S. indicum	F_4 progeny obtained	Rao and Kumar, 1980
S. sodomeum x S. melongena	Fertile F_1	Tudor and Tomescu, 1995
S. melongena x S. macrocarpon	Partially fertile F_{1s} Sterile F_1	Schaff *et al.* 1982 Rajasekaran, 1961
S. melongena x S. khasianum	F_1 obtained by embryo rescue F_2 obtained	Sharma *et al.*, 1980
S. aethiopicum x S. melongena	F_1 obtained by embryo culture	Ano *et al.*, 1991
S. melongena x S. insanum	F_1 obtained	Ali and Fujieda, 1990
S. melongena x S. gilo	F_1 obtained Sterile F_1	Ali and Fujieda, 1990 Nashrallah and Hopp, 1963
S. integrifolium x S. melongena	Sterile F_{1S} F_1 obtained	Kirti and Rao, 1982 Ali and Fujieda, 1990
S. gilo x S. melongena	Sterile F_{1S}	Omidiji, 1981 Rao and Baksh, 1981
S. melongena x S. hispidum	Sterile F_{1S}	Rao, 1980
S. melongena x S. torvum	Sterile F_{1S}	McCammon and Honma, 1983
S. melongena x S. insanum/S. incanum/S. integrifolium/ S. gilo	Functional seeds	Rao, 1979

nuous exposure to this toxin (Whalon *et al.*, 1993), and the presence of resistance alleles in relatively high frequencies for some beetle populations in the field has been reported (Whalon and Rahardja, pers. comm.).

Resistance management is an effort aimed at preventing or delaying adaptation in insects and should therefore be considered as the management of a genetic resource represented by insect susceptibility genes or alleles. Computer simulations have been widely used to investigate the possible outcome of an insect-host coevolution under different levels of selection pressure (e.g. Gould, 1986; Mallet and Porter, 1992; Alstad and Andow, 1995). The results

are also different depending on host and insect ecology and genetics. A specific model is also available to investigate the most relevant features of the Colorado potato beetle-eggplant interactions (Arpaia *et al.*, 1998). Indications retrieved by the latter model indicate that a mixed planting of transgenic and non transgenic clones could facilitate the preservation of a longlasting efficacy of the germplasm.

Therefore, methods of resistance preservation should be incorporated in the philosophy of Integrated Pest Management (IPM). These methods fit in well with the IPM goal of implementing strategies to hold pest population below a density which could cause economic injury by using natural, biological and cultural tools as the first level for the control of pests and diseases. Field observations have also indicated that in some cases a synergistic action of Bt-transgenic plants and CPB natural enemies is possible and will enable the maintaining of a longlasting efficacy of the resistant lines (Arpaia *et al.*, 1997a).

Within the IPM context, the strategies proposed for a better management of plant resistance genes are: i) diversification of mortality source; ii) reduction of selection pressure for each mortality mechanism; iii) refuges or immigration to supply susceptible individuals; iv) estimation and/or prediction of progress made toward insect resistance (McGaughey and Whalon, 1992). Extensive field studies are most certainly needed, to support the indications retrieved by simulation models in order to target the field deployment of insect resistant transgenic eggplants better.

Engineering insect resistance, based on secondary compounds involved in the resistance (e.g. glicoalkaloids), will be much more difficult because a more complex biochemical pathway should be known and then altered.

Marker assisted selection. Molecular markers can be used to facilitate the localization of genomic traits which show continuous variations in expression, and are more complex than simple mendelian characters in inheritance (quantitative traits loci or QTLs). Unfortunately, the development in eggplant of marker assisted selection is far from becoming a reality since, at the present time, the species lacks of a genetic map. Allozyme and Random Amplified Polymorphic DNA (RAPD) variations in eggplants was reported recently (Karihaloo and Gottlieb, 1995; Karihaloo *et al.*, 1995). RAPD and allozyme analyses were performed on 52 accessions, comprising 27 cultivars of *Solanum melongena* and 25 lines of the related weedy form *"insanum"*. The results show a very high degree of similarity between the accessions tested (I = 0.947 by RAPD analysis); overall, the *"insanum"* accessions were more diverse than those of *S. melongena* (Karihaloo *et al.*, 1995). These preliminary results indicate a low degree of polymorphism in eggplant by using the above mentioned markers. In the near future, considering the advantage gained from the synthenic relationship with the well studied solanaceous species, the potato and the tomato, it might be possibile to obtain genetic information on segregating (F_2, recombinant inbred lines or DH) populations by using markers such as RFLP, AFLP plus microsatellites.

Molecular markers were recently used to locate genes for resistance to *L. decemlineata* in hybrid *Solanum tuberosum* x *Solanum berthaultii* potato progenies (Yencho *et al.*, 1996). Two and three QTLs influencing resistance were identified in mutual backcrosses with

Table 4. Result of somatic hybridization between eggplant and *Solanum* spp.

Fusion partners	Results of fusion	Hybrid characteristics	Reference
S. melongena cv. Imperial Black Beauty + *S. sisymbrifolium*	26 somatic hybrid plants; Aneuploids close to 48	Sterile. Mites and rootknot nematode resistant	Gleddie *et al.*, 1986
S. melongena cv. Dourga + *S. khasianum*	83 somatic hybrid plants; Most tetraploids (48) and few aneuploids (46-48)	Sterile	Sihachakr *et al.*, 1989a
S. melongena cv. Black Beauty + *S. torvum*	10 somatic hybrid plants; Tetraploids and aneuploids (46-48)	Sterile. *Verticillium* Resistant, Spider mites partially resistant	Guri and Sink, 1988a
S. melongena cv. Black Beauty + *S. nigrum*	2 somatic hybrid plants	Sterile. Atrazine resistant	Guri and Sink, 1988b
S. melongena cv. Dourga + *S. torvum*	10 somatic hybrid plants; Most Tetraploids (46-48)	Sterile. *Verticillium* filtrate and nematode resistant	Sihachakr *et al.*, 1989a
S. melongena cv. Dourga + *S. nigrum*	1 somatic hybrid plant Aneuploid	Sterile. Atrazine resistant	Sihachakr *et al.*, 1989b
S. melongena cv. Shironasu + *S. integrifolium*	16 somatic hybrid plants; Tetraploid	Fertile. Offsprings *Pseudomonas* resistant	Kameya *et al.*, 1990
S. melongena cv. Shironasu + *Nicotiana tabacum* (chlorophyll-deficient, streptomycin-resistant	Green shoots from 2 somatic hybrid colonies		Toki *et al.*, 1990

Table 4. (continue)

Fusion partners	Results of fusion	Hybrid characteristics	Reference
S. melongena cv. Black Beauty + (sexual hybrid tomato x *Lycopersicon pennellii*)	2 hybrid calli with leaf-like primordia		Guri *et al.*, 1991
S. melongena cv. Dourga + *S. aethiopicum*	35 somatic hybrid plants; 32 tetraploids, 1 hexaploid, 2 mixoploids	Fertile. *Pseudomonas* and *Fusarium* resistant	Daunay *et al.*, 1993
S. melongena cv. Senryou + *S. sanitwongsei*	1 somatic hybrid plant Tetraploid	Fertile. *Pseudomonas* resistant	Asao *et al.*, 1994
S. melongena cv. Black Beauty + sexual cross *L. esculentum* and *L. pennelli*	4 somatic hybrid plants; 45-60 chromosomes	Sterile.	Liu *et al.*, 1995
S. melongena cv. Dourga + *S. nigrum*	1 somatic hybrid plant Aneuploid	Sterile. Atrazine resistant	Sihachakr *et al.*, 1989b
S. melongena breeding lines + *S. integrifolium*	More then 100 hybrid plants; Mostly tetraploids	Fertile. Backcrossed. *Fusarium* resistant	Rotino *et al.*, 1995

S. berthaultii and potato respectively. These QTLs generally coincided with the loci associated with glandular trichomes, confirming their role in the mechanism of resistance. However a constant association of QTL on chromosome 1 not linked with trichomes was noted, suggesting that other factors were contributing to insect resistance in these progenies.

Perspectives

Breeding for resistance in eggplant has received limited research efforts, considering the heavy losses pests may cause to the cultivation of this crop. Moreover, the possibility of pest adaptation to resistance genes should lead to a re-orientation of breeding for pest resistance.

The crucial point for the development of a durable crop protection strategy is the interdisciplinary cooperation between breeders, entomologists, plant pathologists and agronomists. The field evaluation of breeding materials should be carried out taking into consideration the entire agroecosystem, trying to reduce chemical interference to a minimum.

Other important points are represented by:

Exploitation of resistance genes. Searching for partial resistance within the eggplant gene pool may be particularly important, as evidenced by the genetic variation observed, which is involved with the response to the major pests. In addition, established biotechnological techniques, mainly based on tissue culture and gene transfer, can enlarge the availability of resistance genes from wild relatives or unrelated organisms.

The availability of a genetic map to start marker assisted selection also on eggplant would be an important effort towards identifying QTLs which confer tolerance/resistance to the major diseases (*Verticillium* and *Fusarium* wilt, bacterial wilt and nematode infections).

Finally, the improvement of techniques to obtain interspecific, fertile, somatic hybrids, should facilitate the use of genes derived from wild species (and maintained in the regenerated somatic hybrids), and increase the quota of recovery in backcross populations.

Development of efficient and reliable test procedures to assess resistance. Plant resistance to insects is often expressed in terms of its negative effects on individual development and/or population biology. If a first screening is to be performed, for simplicity and economy, on a single criterion (e.g. mortality), more comprehensive surveys are needed to assess the cumulative impact on insect populations. Parameters such as fertility and fecundity, or adverse impact on insect behavior should be included in the investigations. Collection of all the data necessary for life tables or population growth curves may be justified as well. Investigations should also include the effects of infestations on plants on a progressively larger scale (plant tissues, organs, single plants, and greenhouse and field trials). The ultimate goal of insect resistance is obviously crop yield and quality, which should then represent the final characters to be looked at in field experiments.

Collect field data to validate simulation models and predict insect population growth. This will be a helpful instrument both during the selection of the most effective genotypes in specific plant-insect interaction and in field trials to address the study by looking at the entire agroecosystem in such a way as to eventually optimize the germ plasm resources and fit them into an appropriate IPM perspective.

Chapter 3

Effects of *Bacillus thuringiensis* Berl. Toxin Extracts on Feeding Behavior and Development of Colorado Potato Beetle (Coleoptera: Chrysomelidae) Larvae

Abstract

Protein extracts of *Escherichia coli* expressing the toxin gene from *Bacillus thuringiensis* Berliner were tested for effects on the behavior and development of *Leptinotarsa decemlineata* Say using in vitro bioassays. Potato leaf-discs coated with protein extracts were used as a diet. Toxicant properties and sublethal effects were scored with no-choice tests, and the relationships between dose of protein extract and larval growth and mortality was defined using regression analysis. Feeding behavior of young larvae was studied with choice tests. No antifeedant effects of *B. thuringiensis* toxins were found, even at concentrations that caused mortality or severely inhibited larval growth. The lack of feeding inhibition may limit strategies for producing transgenic potato plants with a low selection pressure against the target insect in the field.

Introduction

In the past few years, the attention given to different *Bacillus thuringiensis* Berl. strains and their toxicological properties has greatly increased. *B. thuringiensis* crystal proteins are environmentally compatible and can be synthesized in genetically engineered higher plants. In anticipation of a wider use of *B. thuringiensis* toxins in transgenic plants, studies are now in progress on the ecological impact of large increases in the presence of the toxin in the field.

Field populations of some lepidopteran species with resistance to *B. thuringiensis*-based insecticides have been reported (Kirsch and Schmutterer, 1988; Tabashnik *et al.*, 1990). Van Rie *et al.* (1990) and Ferré *et al.* (1991) suggest that the mechanism of resistance involves midgut membrane receptors. A desirable goal is the attainment of transgenic plants with a reduced selection pressure towards target pests, as with Integrated Pest Management approaches.

Gould (1988) suggested the use of different strategies to reduce possible insect adaptation to the toxins: 1) mixtures of plants, 2) partially resistant plants, 3) tissue-specific resistance in plants, and 4) inducible resistance in plants via specific promoters.

Plant mixtures or tissue-specific resistance would be more effective if *B. thuringiensis* toxins were active as feeding deterrents. However, feeding deterrent effects are likely to be variable in different situations (Dulmage *et al.*, 1978; Mohd-Salleh and Lewis, 1982; Herbert

and Harper, 1985; Hornby and Gardner, 1987; Gould and Anderson, 1991; Gould *et al.*, 1991a; Schwartz *et al.*, 1991). The aim of the present study was to assess the effect of *B. thuringiensis* protein extracts on the biology and behavior of *Leptinotarsa decemlineata* Say, to predict efficacy for potato-pest management.

Materials and Methods

Insects. The Colorado potato beetle larvae were laboratory strains, reared on potted potato plants (cv. Desirée) at the Metapontum Agrobios Research Center since 1990. The colony is maintained every year with adults collected from the field.

Bacillus thuringiensis Toxins. The gene which encodes the protein was isolated at Ecogen (Langhorne, PA), using the radiolabeled Cry3A gene from the *Bacillus thuringiensis* strain EG2158. The full length gene (Cry3B, 1.96 kb) which encodes for a 74.228 kd protein was cloned in the bacterial expression vector pDG1, derived from pGEM3Zf(+) (Promega). The resulting plasmid was named pDG1Cry3B13s. Cells of *Escherichia coli* (strain DH5 alfa) harboring the plasmid were grown for 16 h in 100 ml Lauria-Bertani medium supplemented with 100 mg/l ampicillin. Bacterial cells were harvested by centrifugation, washed three times with extraction buffer (50 mM tris pH 8.7) and resuspended in 2 ml of the same buffer. The total protein extract was obtained by sonicating *E. coli* cells for 3 min (Vibra Cell, Sonics and Material, Danbury, CT).

Experimental Design. *No-choice tests.* Potato leaf-discs (26 mm diameter) were cut from fully expanded 'Desirée' leaves and placed in petri plates (50 mm diameter) lined with a water-saturated filter paper, one disc per plate. Each disc was coated with 30 µl of toxin extract on each side, evenly distributed with a small camel hair brush. Twelve protein concentrations (six in each of two experiments) were used, with five replications set up for each dose. There were two different controls; in the first, leaf-discs were coated with distilled water and in the second, protein extracts from *E. coli* transformed with a plasmid (pUC19) carrying a reporter gene were used. When the discs were completely dry, four neonate *L. decemlineata* larvae were placed on each. All plates were placed in a growth chamber at 26°C, RH 70% and a 16:8 (L:D) h photoperiod. The discs were replaced daily, and larval mortality was recorded. In the second experiment, larval fresh weight (the survived larvae in each plate were pooled and the average weight was determined) and larval instar were scored at the end of the trial (72 h).

Choice tests. Two leaf-discs were placed 1 cm apart in 90-mm petri plates lined with a water-saturated filter paper. One disc on the right side was treated with protein extract as in the no-choice tests and the other treated with distilled water. In the control, both discs were treated with water. In a first experiment, there were four treatments (0, 5, 10, 20 µg of protein extract). In a second experiment, the highest dose (20 µg) was eliminated to avoid high mortality according to previous observations, and, as suggested by Gould *et al.*, (1991a), to eliminate a possible incapacitation effect at high doses on young larvae. A control of *E. coli* extract carrying the pUC19 plasmid was used to assess possible effects on larval feeding because of other proteins. Five neonate *L. decemlineata* larvae were placed between the discs in the midd-

le of each plate. Each treatment was replicated five times, and plates were incubated as previously described. The leaf-discs were changed every day, and the residual area measured on the removed discs with an image analyzer (Delta T devices - Burwell, Cambridge, UK). Each leaf was measured twice and the average value recorded as actual area.

The larval position was monitored twice per day and scored as on the control disc (a), on the treated disc (b) or elsewhere (c). Larval mortality, fresh weight, and instar were recorded at the end of the bioassay (72 h) as in the no-choice tests.

Data Analyses. *No-choice tests.* Statistical significance of differences among mortalities obtained at different doses was established by a completely randomized design analysis of variance (ANOVA). In spite of the fact that only crude protein extracts had been used (we did not assess the actual amount of toxin in the extracts), a probit analysis was carried out, and LC_{50}s were determined. The main effect of the treatment on larval weight was studied using regression analysis. The separation of treatment effects was realized by the ANOVA (Ryan's Q test p=0.01). Log-transformed data of larval fresh weight were used in the analyses.

Choice tests. A repeated measures ANOVA design was adopted to analyze larval choice; time was the within-subject factor and concentration of toxin was the between-subject factor.

The measure of larval choice used was the ratio LC/TL, where LC is the number of larvae on the untreated disc and TL is the total number of larvae on food. For the statistical analysis the arcsine transformation of these proportions was used. The percentage of larvae found away from the food was always negligible (< 5%).

The same experimental design was set up to determine whether differences between leaf-disc areas, after larval feeding, indicated any preference. The difference between residual untreated and treated leaf areas was the parameter chosen for the analysis.

Results

No-choice tests. The toxicological properties of the *B. thuringiensis* toxin expressed in *E. coli*, and the harmlessness of the pUC19 plasmid, previously assessed in several preliminary tests, were also confirmed in these experiments by ANOVA analysis. The actual *B. thuringiensis* delta endotoxin content in crude bacterial extracts was not determined; based on previous experience, the delta endotoxin content in crude protein extracts was approximately 1% wt/vol. Significant differences in larval mortality were obtained at doses as low as 12.5 µg per disc in the first experiment, and at 20.0 µg per disc in the second experiment (Table 1). Sublethal effects were clear at lower doses (2.5 µg), in terms of a slowing-down in weight increase. Using data from the first no-choice experiment, a probit analysis gave a significant (p=0.05) slope value (Table 2). The regression equation is $y = 2.23 + 2.60x$. The regression line obtained with data from the second experiment is $y = 2.27 + 2.17x$. Since these results were obtained with crude protein extracts, their significance is only indicative, and should not be considered as a prediction for toxic activity in plants.

The relationships between the amount of toxin extract and larval fresh weight can be

Table 1. Effects of protein extracts on survival and growth of *L. decemlineata* larvae in no-choice tests

Experiment no.	Treatment	Mean[a] mortality per plate	% Corrected mortality[b]	Mean larval wt[c]
1	Control	0.0a	0.0	-
	pUC19 50 µg	0.0a	0.0	-
	1.6 µg Bt	0.2a	5.0	-
	3.1 µg Bt	0.2a	5.0	-
	6.2 µg Bt	0.8ab	20.0	-
	12.5 µg Bt	2.0bc	50.0	-
	25.0 µg Bt	3.6c	90.0	-
	50.0 µg Bt	3.6c	90.0	-
2	Control	0.2a	-	0.0039a
	pUC19 40 µg	0.2a	0.0	0.0038a
	1.2 µg Bt	0.0a	-5.2	0.0035a
	2.5 µg Bt	0.0a	-5.2	0.0020b
	5.0 µg Bt	0.4ab	5.2	0.0014bc
	10.0 µg Bt	1.6abc	36.8	0.0008c
	20.0 µg Bt	2.2bc	52.6	0.0008c
	40.0 µg Bt	3.2c	78.9	0.0007c

[a] ANOVA Ryan's Q test ($p<0.01$). Means followed by the same letter are not significantly different. First experiment $F=20$, df=6, 28; second experiment $F=11.9$, df=6, 28.
[b] Abbot's modified formula.
[c] ANOVA Ryan's Q test ($p<0.01$) $F=38.7$, df=6, 24.

adequately explained with a nonlinear regression model (Fig. 1) ($r^2 = 0.91$; df = 2, 5 $p<0.05$) The equation is $y = -1.98/x + 7.12$ (residual mean-square error = 0.0289). None of the larvae that were fed > 10 µg was able to reach the second instar within 72 h. (Fig. 2).

Choice tests. In both bioassays, statistical analysis on LC/TL ratios gave no evidence of significant differences due to the treatments. Tests of hypotheses using the type III MS for

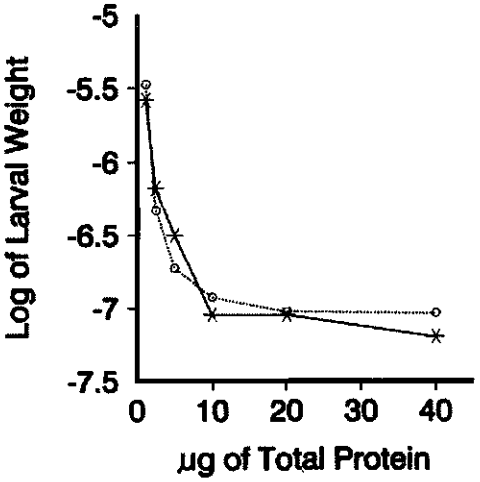


Fig.1 - Relationship between dose of protein and larval fresh weight (second no-choice experiment).
*, predicted values; °, actual values.

'replications x treatment' as an error term gave in the first experiment F = 0.29 df =3, 12 p = ns, and in the second experiment F = 0.21 df = 3, 12 p = ns. The interaction time x treatment had no significance whatsoever in either of the experiments. Feeding behavior was variable between replications, thus the fraction of larvae found on the control discs did not vary with treatment (Table 3). Leaf-area analysis showed no larval preference in daily leaf consumption (Table 3).

Table 2. Probit analysis relative to the first no-choice experiment

n	Slope±SE	LC_{50}	95% CL
6	2.60 ± 0.51	11.6	8.5-16.1

Tests of hypotheses using type III MS for replications x treatment as an error term gave in the first experiment F = 0.93 df = 3, 12 p = ns and F = 2.71 df = 3, 12 p = ns in the second experiment. The leaf-area difference between treated and control discs in each treatment after 24 h feeding, showed in all cases that untreated leaves, on average, had been eaten more than the treated ones, however differences were usually small, with a great deal of variation among replications. Mortality and larval weight were affected differently in the two experiments (Table 3). This is not surprising, considering the variation in time spent feeding on treated leaves. The data on larval weight show that the presence of an untreated disc in the choice test reduced the negative effects of the toxin on insect growth (Tables 1 and 3). As antifeedant molecules may need time to exert an effect on herbivorous insects (Arpaia and van Loon, 1993), constancy in feeding behavior over time may assume a primary role. Fig. 3A and B show variations in feeding behavior over time. The trends in both experiments show a homogeneous division between the two leaf discs, thus

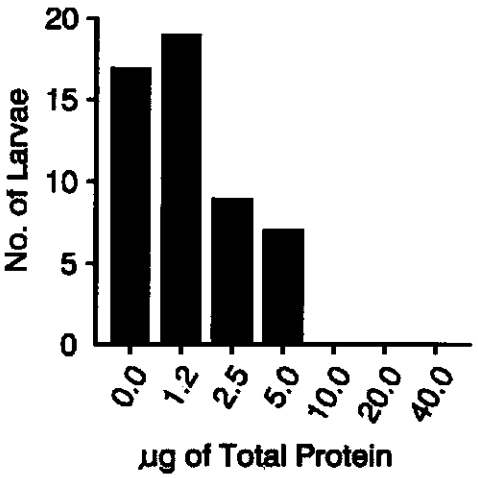


Fig.2 - Relationship between dose of protein and larval development at 72 h (second no-choice experiment). Bars indicate the number of second instars recovered at the end of the test.

Table 3. Effects of protein extracts on survival, growth and behavior of *L. decemlineata* larvae in choice tests

Exp. no.	Treatment	Mean[a] mortality per plate	% Mortality	Mean[b] larval wt	% larvae on control ± SE	Mean[c] LAD; ± SE
1	Control	0.0a	0.0	0.0037a	43.5±8.3	52.8±44.7
1	5 µg Bt	1.0ab	20.0	0.0025a	47.6±7.2	7.1±48.5
1	10 µg Bt	1.2ab	24.0	0.0025a	56.6±7.9	66.9±28.2
1	20 µg Bt	3.4b	68.0	0.0023a	35.3±7.9	5.4±24.3
2	Control	0.0a	0.0	0.0058a	54.0±5.3	-38.3±25.7
2	pUC19 10 µg	0.2a	4.0	0.0064a	50.3±9.8	75.7±39.9
2	5 µg Bt	0.2a	4.0	0.0050a	52.7±4.3	58.8±15.9
2	10 µg Bt	0.4a	8.0	0.0029a	45.7±3.6	111.1±37.3

a ANOVA Ryan's Q test ($p<0.01$). Means followed by the same letter are not significantly different. First experiment $F=7.47$ df=3, 16; second experiment $F=0.76$ df=3, 16.
b ANOVA Ryan's Q test ($p<0.01$). First experiment $F=1.95$ df=3, 15; second experiment $F=4.62$ df=3, 16.
c Leaf Area Difference (mm^2) between treated disks (right side of the plate) and control disks (left side) after 24-h feeding.

strengthening confidence in the statistical analyses.

Discussion

Transgenic plants expressing *B. thuringiensis* toxins will most likely be widely employed in the field in the near future. In this perspective, knowledge of transgenic plant-insect interactions is a fundamental requirement, along with the enhancement of the plant's insecticidal properties.

In this study, the toxicological properties of *Bacillus thuringiensis* endotoxin Cry3B were evaluated after their expression in *Escherichia coli* via a bacterial promoter. Our data confirm the effect of low doses of *B. thuringiensis* toxins obtained from engineered organisms on the survival of *L. decemlineata* larvae. Sublethal amounts of toxin in the insect diet dramatically affected larval development in no-choice situations, thus suggesting a further possibility of reducing crop damage using molecular techniques. Toxic effects were attenuated when insects were given the possibility of choosing between different diets. Neither behavioral

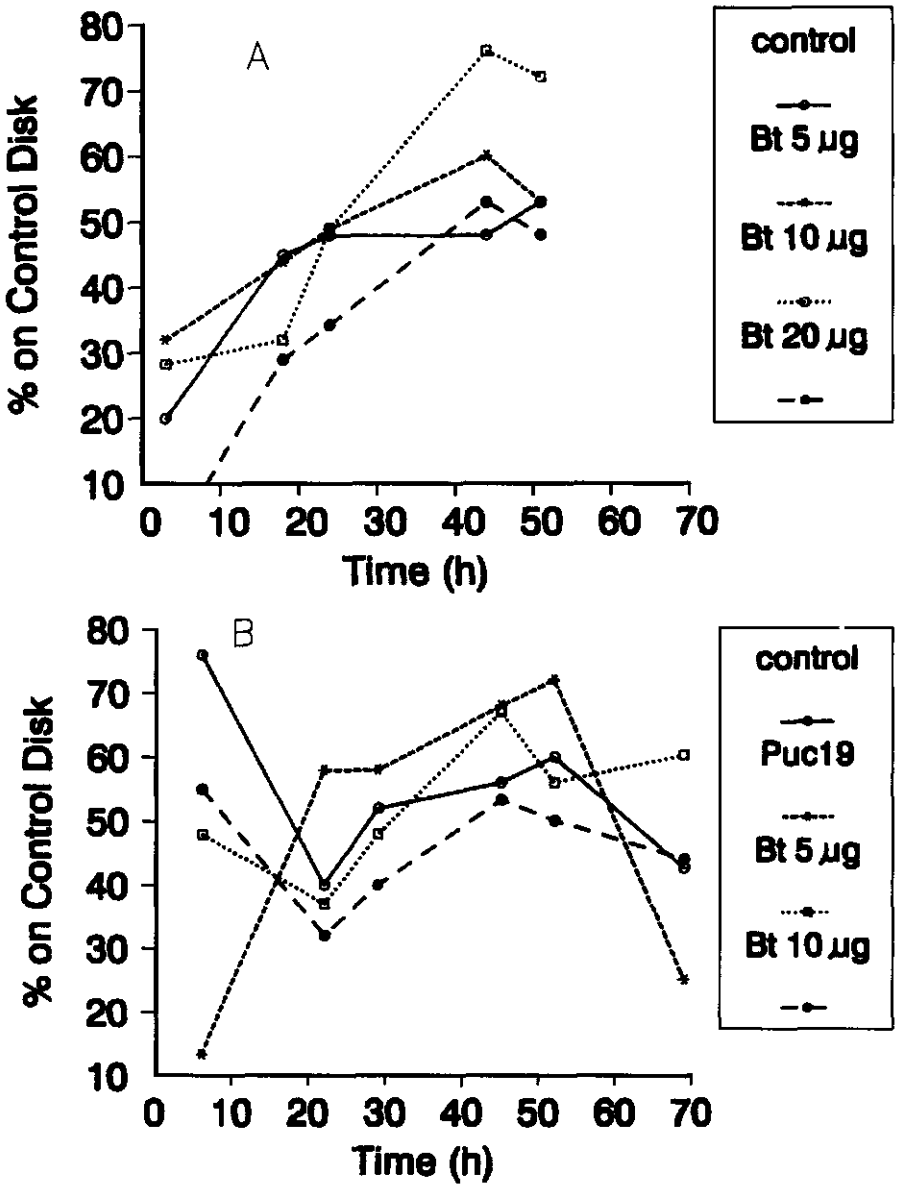


Fig. 3 - Percentage of larvae found on control disks over time. (A) First choice experiment. (B) Second choice experiment.

observations nor food consumption analysis indicated any preference between the treated and control diets.

It may therefore be concluded that there was no evidence of an antifeedant effect of *B. thuringiensis* toxins on the behavior of young Colorado potato beetle larvae under our conditions. In the light of these results, great care should be taken in the choice of the strategies to be adopted in developing transgenic potato plants resistant to *L. decemlineata.* If the insects do not avoid the plants, the effectiveness in reducing selection pressure by strategies such as tissue-specific resistance can be lessened. Three alternative strategies for potato-pest management are a) inducible resistance, obtainable using time-specific promoters or inducible promoters; b) using mixtures of plants, some of which do not produce toxins, or c) the expression of sublethal doses of toxins in the plants. The latter could effectively contain Colorado potato beetle problems in early potatoes in the Mediterranean area by slowing-down the insect life cycle.

To confirm a complete lack of antifeedant effects, electrophysiological studies are necessary to verify behavioral observations, and antifeedant properties must be investigated in whole plants before any definite conclusion can be drawn. It should be noted however that findings cannot be extrapolated to different insect-host plant systems.

For instance the results reported in this article are in concordance with the findings of Schwartz *et al.* (1991) regarding *Plutella xylostella* L., but not with the findings of Gould et al. (1991a) in *Heliothis virescens* (F.). It would be advantageoud to examine each single case for effects on both fitness and behavior before choosing strategies for biotechnology projects.

Acknowledgment

We thank R. Iannacone (Metapontum Agrobios) for supplying protein extracts. We also thank A. Tranfaglia (Universita' della Basilicata), A. Eaglesham and M. Antonelli (Metapontum Agrobios) for a critical revue of the manuscript, and D. Palazzo (Metapontum Agrobios) for help with statistical analyses

Chapter 4

Production of Transgenic Eggplant (*Solanum melongena* L.) Resistant to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say)

Abstract

A modified gene of *Bacillus thuringiensis* var. *tolworthi* (Bt), encoding a coleopteran insect-specific Cry3B toxin, was transferred via *Agrobacterium tumefaciens* to the female parent of the eggplant commercial F1 hybrid 'Rimina'. One-hundred and fifty eight transgenic plants were regenerated and tested by PCR and NPTII expression assays. The presence of the Cry3B toxin in leaf extracts was demonstrated in 57 out of 93 transgenic plants tested by DAS-ELISA assay. High Bt-expressing plants contained a 74 kDa protein cross-reacting with serum anti-Cry3B toxin. Seventy-five out of 131 *S. melongena* plants tested by insect bioassay showed significant insecticidial activity on neonate larvae of Colorado potato beetle (CPB). The Bt transgene and the toxic effect on CPB larvae were transmitted to progenies derived by selfing. Thus, transgenic Bt eggplants represent a very effective means of CPB pest control.

Introduction

Bacillus thuringiensis (Bt)-derived Cry genes have been widely used to generate transgenic plants resistant to insects (Fischhoff, 1996). The level of toxin expression in Bt-transgenic plants may differ depending on plant species, age, tissues and organs (Koziel *et al.*, 1993). This can affect the survival of target insects. Thus, insect-plant relationships need to be investigated in each single case to evaluate the potential of any field release of Bt-transgenic crops (Boulter, 1993). The Colorado potato beetle *(Leptinotarsa decemlineata* Say) (CPB) represents the target insect of transgenic potatoes transformed with the Cry3A gene (Perlak *et al.*, 1993). CPB also feeds on eggplants and is the most important insect pest of this crop in Europe and America. When not properly controlled, this insect causes heavy economic losses (Cotty and Lashomb, 1982; Maini *et al.*, 1990a; Arpaia *et al.*, 1995). Eggplants transgenic for wild-type Bt genes have shown that the low expression of the transgene did not allow successful insect control (Rotino *et al.*, 1992; Chen *et al.*, 1995). In the present paper we report that transformation of an eggplant commercial F1 hybrid parent with a mutagenized *Bacillus thuringiensis* Berl. var. *tolworthi* gene (Cry3B) results in transgene expression levels sufficient for CPB control.

Materials and Methods

Plant material. The female parent of the F_1 eggplant hybrid 'Rimina' (hereafter indicated as *Solanum melongena*), released by Istituto Sperimentale per l'Orticoltura Sezione di Monsampolo del Tronto, was used for genetic engineering.

Vector. The Bt Cry3B gene employed was a mutant version of the modified Bt gene reported in Iannacone *et al.* (1995). The binary plasmid pBinCry3B was obtained by subcloning the 35S-WTMV-Cry3B-OCS cassette into the pBin19 plasmid (Bevan, 1984), which also contains the selectable marker gene NOS-NPTII-NOS. The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) carrying the plasmid pBinCry3B was employed in the transformation experiments.

Plant transformation. The procedure for eggplant transformation was essentially the one described in Rotino and Gleddie (1990) and Rotino *et al.* (1992) with modifications. Leaf, cotyledon and hypocotyl explants were pre-cultured for two days in MS macro- and micronutrients (Murashige and Skoog, 1962), Gamborg vitamins (Gamborg *et al.*, 1968), 0.5 gl^{-1} of MES, 20 µM acetosyringone supplemented with the growth regulators (mgl^{-1}) 0.5 ZEA, 0.3 BAP, 0.2 KIN and 0.1 NAA, media were solidified with 2 gl^{-1} of phytagel (Sigma), pH 5.8. For explant infection, an overnight *Agrobacterium tumefaciens* liquid culture was centrifugated and the pellet re-suspended at an 0.1 OD600 density in MS basal medium, 2% glucose and 200 mM acetosyringone pH 5.5. The cut edges of the hypocotyls were cut again and all the explants were infected by being dipped in the bacterial suspension for 5 min, blotted dry onto sterile filter paper and then put back in the same plates. After 48 h the explants were transferred to selective medium (described above) without acetosyringone and supplemented with 30 mgl^{-1} kanamycin and 500 mgl^{-1} cefotaxime. Shoot-bud differentiation and shoot elongation were achieved by transferring calli with compact green nodules to the same selective medium without NAA. Shoots were rooted and propagated in V3 medium (Chambonnet, 1985) without antibiotics. Regenerated plants were labelled according to the original callus (first number) and shoot (second number). Transgenic plantlets were grown in the greenhouse, flower buds were covered with paper bags for self-pollination.

Re-callusing and kanamycin-spraying assays. Leaf-discs from putative transformants were cultured on regeneration medium containing 30 mgl^{-1} of kanamycin so as to verify their ability to produce a callus. Expression of the NPTII marker gene was also monitored just after plantlet acclimatation by spraying a 300 mgl^{-1} kanamycin solution according to Sunseri *et al.* (1993).

Polymerase chain reaction. The plant DNA was isolated from young leaves according to Doyle and Doyle (1990). PCR analysis was performed using the primers 5'ATGATTGAACAAGATGGATTGCACGCAGG3' and 5'GAAGAACTCGTCAAGAAGGCGATA3', which amplified a 839-bp fragment of the NPTII coding region, and the primers 5'AAGTTCGAAGTTCTGTTCCTTCCA3' and 5'TAGTCTACAGATCTATGGGTCC3', which amplified a 1000-bp fragment of the Bt Cry3B coding region. PCR reactions were performed using 100 ng of template DNA in 50 ml of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatine, 200 µM dNTPs, 50 pM of each primer and 1 U AmpliTaq polymerase

(Perkin Elmer). Amplifications were carried out in a thermocycler (Perkin Elmer) programmed for one cycle of 5 min at 95°C; 35 cycles of 15 sec at 95°C, 1 min at 60°C, 3 min at 72°C, and one final cycle of 10 min at 72°C. PCR products were subjected to electrophoresis in a 1% (w/v) agarose gel containing 0.1 μgml-1 of ethidium bromide.

DAS-ELISA and Western blotting. Toxin extraction was carried out by grinding a young leaf in 50 mM of Na_2CO_3, pH 9.5, 100 mM of NaCl, 0.05% Tween 20, 1 mM of phenylmethylsulfonyl fluoride and 1 μM of leupeptin. The ratio w/v was 1:10 for double- antibody ELISA (DAS-ELISA) tests and 1:3 for Western blots. For DAS-ELISA analyses we employed a monoclonal antibody (1:1000 dilution) against Cry3B toxin (Grassi *et al.* 1995) for the direct coating of ELISA plates, a rabbit anti-Cry3B toxin serum (1:6000 dilution) and a peroxidase-conjugate anti-rabbit immunoglobulin G (1:15000 dilution). In each specific experiment, a transgenic plant was considered positive when its OD492 nm value was more than twice that of control plants. The approximate Cry3B toxin concentration in plant extracts was determined by a standard (Cry3B toxin produced by *E. coli*) according to the procedure described in Mennella *et al.* (1995). The total protein content was measured according to Bradford (1976). For Western-blot analyses, the samples were further diluted 1:1.5 in the above mentioned extraction buffer containing 2.5% SDS and 5% β-mercaptoethanol. After centrifugation, the supernatants were boiled for 5 min and 4 μl was analysed by PAGE. Proteins were blotted onto a nitrocellulose filter and incubated with rabbit anti-Cry3B toxin serum (1:2000 dilution) overnight at room temperature. The subsequent steps were as described by the manufacturer (ECL-Amersham). By using these procedures, 93 *S. melongena* plants representing 22 different transformation events were analyzed at least twice for Cry3B toxin presence by DAS-ELISA; ten plants were also analyzed by Western blotting.

Insects. Colorado potato beetle larvae came from a laboratory colony, reared on potted potato plants at Metapontum Agrobios from 1990 onwards. This colony is maintained every year with new individuals obtained from potato and eggplant fields.

Leaf-disc bioassays. To assess the toxicity of transformed plants, 131 transgenic plants were tested in 20 different experiments. Leaf-discs were used for in vitro bioassays according to the protocol described in Iannacone *et al.* (1995). The effect of the treatments was established by an ANOVA completely randomized design. Differences between control and transgenic plants were investigated by using ANOVA-Dunnett's test.

Progeny analysis. A genetic analysis of four transformed T_2 progenies, derived by selfing, were carried out by spraying with 300 mgl^{-1} of kanamycin solution according to Sunseri *et al.* (1993). All plants were scored for kanamycin resistance/sensitivity by observing the absence/presence of bleaching sectors in the sprayed leaves. Data were analyzed using chi-square for evaluating segregation ratios of the active NPTII gene. Two resistant and two sensitive randomly chosen plants of the segregating progenies were transferred to pots and bioassayed.

Results

Plant transformation. A high percentage of calli (51%) were obtained from 639 leaf and cotyledon explants compared to the 14% kanamycin-resistant calli produced from 432 hypocotyl segments. However, since hypocotyl-derived calli showed a better morphogenetic response, 70% of the 158 regenerated plants were produced from hypocotyls. Transgenic plants appeared phenotypically normal and set seeds upon controlled self-fertilization.

Re-callusing, spraying, Bt and NPTII PCR assays. Seventy-five plantlets obtained from 31 independent putative transgenic calli were analyzed. Agreement was observed among the four kinds of assays employed regarding the presence of transgenes and the expression of kanamycin resistance (data not shown). Successful transformation was evident, based on PCR analysis, in 64 plantlets derived from 27 different calli. In a few cases, both transformed and untrasformed plantlets were regenerated from the same callus. Moreover, five plantlets derived from two calli were positive to the PCR test for the NPTII gene, and yet no amplification product for the Bt gene was detected (examples in Fig. 1, lanes 9 and 10).

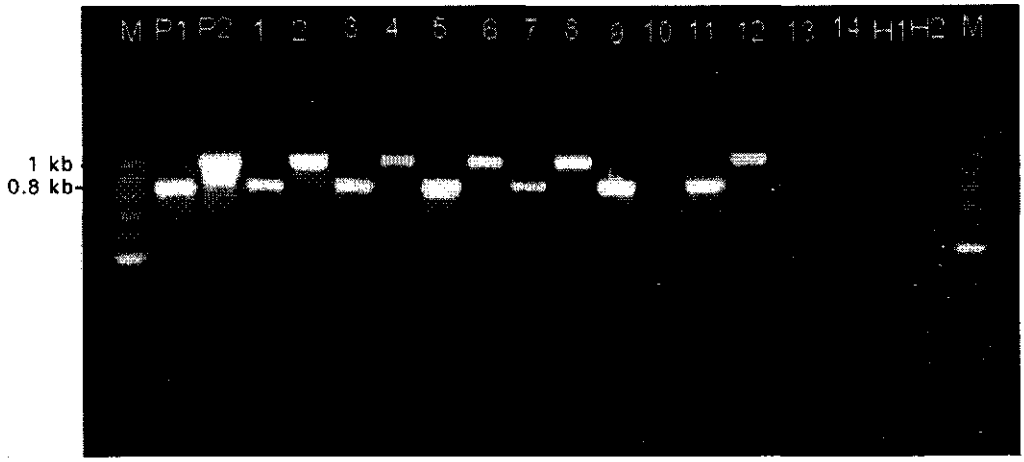


Fig.1 - PCR analysis of DNA from transgenic *S. melongena. Odd lanes*, amplification with NPTII gene primers; *even lanes*, amplification with BT gene primers; *lanes:* 1-2, plant # 7-3; 3,4 plant # 6-1; 5-6, plant # 1-2; 7-8, plant # 3-1; 9-10, plant # 41-1; 11-12, plant # 1-1, 13-14, untransformated control plant. *P1 and P2* positive control plasmids containing the NPTII and Bt CryIIIB gene. *H1 and H2* negative control NPTII and Bt CryIIIB gene amplifications. M1 molecular-weight marker (100-bp DNA Ladder).

DAS-ELISA and Western blotting. Fifty-seven (61.3%) *S. melongena* plants were proved positive with the DAS-ELISA test. On the basis of OD_{492} values, compared to results from the *E. coli*-produced Cry3B toxin, we estimate that approximately 320 ng/ml of toxin were present in extracts of high expressing transgenic plants. The level of Cry3B toxin in leaves correlates significantly (r^2=0.906) with the result of the insect bioassays, indicating that the plants with a high level of the Cry3B toxin were more toxic to CPB larvae (Table 1). In protein extracts of high Bt-expressing transgenic plants a specific immunoreactive polypeptide

of approximately 74000 Da co-migrates with the standard Cry3B toxin generated in *E. coli* (Fig. 2). Some additional bands of lower mobility were also noted. Tissues from the untransformed control plant did not contain the 74000 Da polypeptide, although other cross-reacting polypeptides were detected.

Insect bioassays. Most of the larvae feeding on transgenic leaf-discs died within 72 h. The few survivors had always a significantly lower body weight compared to the control larvae and they rarely reached the second instar. Fifty-seven percent of the transgenic plants were toxic to neonate CPB larvae. Pooled data from the first ten experiments are shown in Table 1. Sublethal effects were evident when the larval stadium of the survivors is considered. Regular moults were severely hampered in larvae feeding on Bt-expressing leaves. Weight reduction was a second evident effect, but was also occasionally observed in some susceptible plants. For example, larvae feeding on *S. melongena* # 1-3 and # 3-10 showed a strong weight difference compared to the control larvae, but reached the second instar as quickly as the control larvae. It is concluded that data of larval fresh weight should only be considered in association with other parameters. Two plants showed sublethal effects on larvae (larval growth and moult were severely reduced compared to the control), but did not cause a significantly different mortality with respect to control plants. These partially resistant plants may be of some interest in obtaining transgenic lines exerting low selection pressure against the target insect, with the aim of delaying its possible adaptation to the resistant plants.

Comparison of detection methods. The data of six different tests available for 39 plants provided an evaluation of NPTII and the Cry3B gene presence (PCR analyses) and activity (leaf disc, spraying, ELISA and insect assays). Twenty-six plants gave a constant response (positive or negative) to all the different tests. Six plants were successfully transformed as evi-

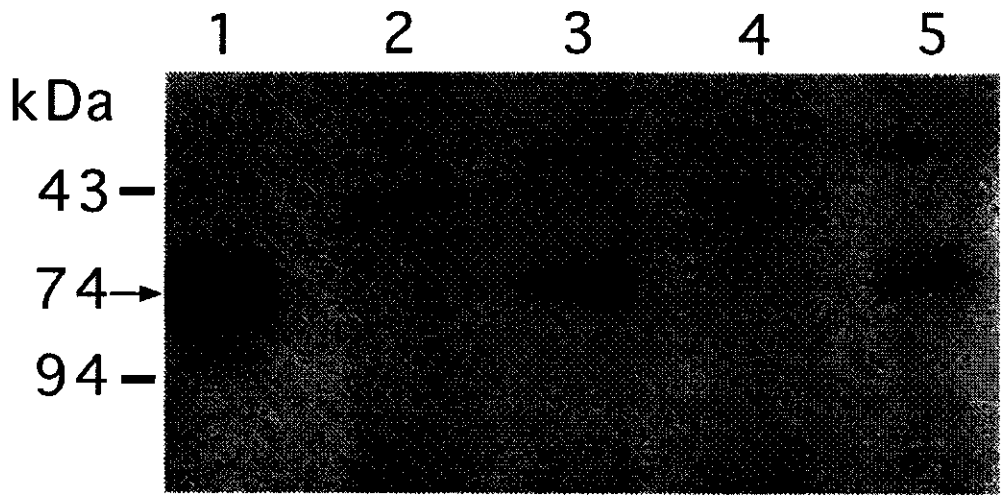


Fig. 2 - Western blot analysis of *S. melongena* plant and *E. coli* extracts. Gel electrophoresis and blotting onto nitrocellulose membrane was performed by a Phast System apparatus (Pharmacia). *Lane 1*, standard Bt-toxin generated in *E. coli*; *lane 2*, plant # 3-1; *lane 3*, plant # 6-2; *lane 4*, untransformated control plant; *lane 5*, plant # 9-2.

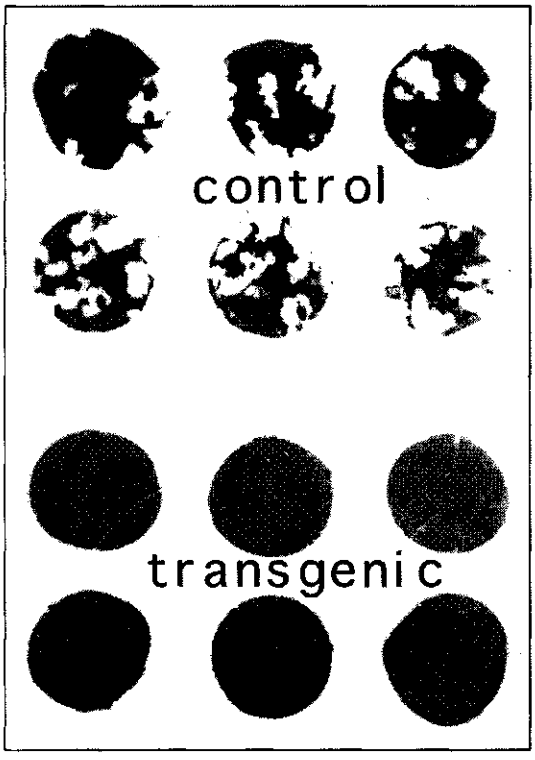
Table 1. Results of insect bioassays and DAS-ELISA immunoassays. Asterisks indicate a significant difference (ANOVA Dunnett p=0.05) from the control in the specific insect bioassay experiment. The OD ratio represents the ratio between the adsorbance value at 492 nm of the transgenic plant and that of the untrasformed control in the specific DAS-ELISA experiment; nt, not tested.

Plant #	Insect bioassay[a]			Toxin concentration DAS-ELISA OD ratio (transformed vs control)
	Mortality[b]	Larval weight (% of control)	Larvae reaching the second instars (% of control)	
3-9	100*	-	-	5.0
6-2	100*	-	-	4.7
6-6	100*	-	-	5.6
7-1	100*	-	-	5.3
7-2	100*	-	-	4.4
7-3	100*	-	-	3.7
9-1	100*	-	-	5.4
9-2	100*	-	-	5.6
9-4	100*	-	-	5.6
19-1	100*	-	-	3.6
6-3	95*	21.05*	0*	5.4
8-2	94.74*	8.97*	0*	3.6
9-3	94.74*	14*	0*	4.7
14-2	94.12*	20*	0*	6.3
4-1	90*	26.32*	0*	4.2
6-1	88.23*	15.38*	0*	5.6
6-4	88.23*	30*	0*	5.8
9-5	76.47*	36*	0*	6.5
3-2	64.71*	17.95*	0*	3.9
6-5	50*	28.95*	11.11*	3.8
28-2	16.66	31.15*	38.89*	2.1
1-1	11.76	61.54	50	1.6

Table 1. (continued)

Plant #	Insect bioassay[a]			Toxin concentration DAS-ELISA OD ratio (transformed vs control)
	Mortality[b]	Larval weight (% of control)	Larvae reaching the second instar (% of control)	
1-7	11.76	27.94*	47.06*	2.9
3-4	10.53	>100	89.47*	1.3
3-6	10	91.7	89.47	1.2
3-5	5.88	>100	100	1.1
1-5	5.26	72*	66.32*	0.7
15-7	5	>100	100	nt
1-2	0	81.97	100	2.2
3-1	0	67.8	100	1.1
3-7	0	96.15	100	1.3
3-11	0	63.24	>100	1.0
3-12	0	61.76	>100	1.0
5-1	0	74.58	>100	2.1
5-2	0	>100	94.74	3.2
5-9	0	>100	>100	2.1
41-1	0	73.53	>100	1.1
2-1	-5.26	>100	>100	1.0
1-3	-5.80	57.35	100	2.1
13-1	-5.88	97.44	100	2.4
2-6	-11.76	89.71	>100	1.0
3-3	-11.76	80.88	>100	1.0
3-8	-11.76	>100	>100	1.2
3-10	-11.76	54.41	100	1.0
34-1	-11.76	75	>100	1.0

[a] Data collected at 72h
[b] Expressed according to the Abbot's formula



denced by positive PCR for Bt and NPTII genes but did not produce enough protein to be detected in the Western-blot analysis or in the bioassays (e.g. plant # 3-1 in Fig. 1, lanes 7-8 and Fig. 2, lane 2; Table 1). Six other plants produced Cry3B protein as evidenced by DAS-ELISA tests, but the amount of toxin was not sufficient to show a significant effect on CPB neonate larvae. The higher sensitivity of the DAS-ELISA test, when compared to the CPB larvae bioassay, had already been proved in previous laboratory experiments with Cry3B toxin expressed in *E. coli* (Mennella *et al.*, 1995). In one case only the NPTII gene was present in the plant DNA, but the Bt gene was not.

Fig. 3 - Insect bioassay carried out on progenies derived by self-pollination of plant # 9-3. The two upper rows show Petri plates containing the larvae eating on leaf-discs cut from plants which showed chlorotic symptoms due to the kanamycin spray treatment. Larvae of the two lower rows were fed with leaf-discs taken from non-chlorotic plants.

Table 2. Segregation ratios for kanamycin resistance in four T_2 progenies obtained by selfing (KmR, kanamycin-resistant: KmS, kanamycin-sensitive)

Progeny #	Tested plants	Phenotype		Ratio (KmR/KmS)	χ^2 value	P value
		KmR	KmS			
9-3	114	89	24	3:1	0.582	0.40-0.50
5-1	92	88	4	15:1	0.713	0.30-0.40
5-5	100	92	8	15:1	0.709	0.30-0.40
6-2	119	119	0	-	-	-
Control	114	0	114	-	-	-

Progeny analysis. Among the T2 progenies sprayed with kanamycin, the chi-square test showed a 3KmR:1KmS ratio, as expected for a monogenic dominant trait in the progeny derived from plant # 9-3. Two other plants segregated as if two independent loci were involved in kanamycin resistance. All the selfed seed-derived plantlets of *S. melongena* # 6-2 were resistant to kanamycin, suggesting the integration of several T-DNA copies in different chromosomes and/or independent integration in a close allelic position into two homologous chromosomes (Table 2). Insect bioassays conducted on two chlorotic and two symptomless plants, chosen among the plants of the selfed segregating progenies, showed that in all cases kanamycin resistance co-segregated with the insect resistance trait (Fig. 3).

Discussion

Bt-expressing transgenic plants of plant species of worldwide importance such as rice, potato and corn have already reached the market. Valuable vegetable crop species, cultivated on a smaller scale, are also of interest particularly if their transformation and the level of Bt gene expression can be optimized. This article reports the engineering of transgenic eggplants bearing a mutagenized Bt gene which allows the production of a level of Cry3B protein sufficient to control Colorado potato beetle. Transformed plants of *S. melongena* showed complete protection from CPB larval attack. Indeed, the transgenic plants were so toxic that individuals surviving after three days of feeding on transgenic leaves were rarely visible.

Among the types of explants tested, hypocotyl segments showed a better capability for regenerating shoots. Since this process is extremely fast, a second cutting was done to expose freshly-wounded less-differenziated cells to agrobacteria and so reduce the frequency of escapes. The presence of transgenic and non-transgenic plants from the same callus could be due to a chimeric origin of some selected calli. An incomplete integration of the Bt gene might explain the fact that, in a few cases, the presence and expression of the selectable NPTII gene was noted, while both the expected Bt gene fragment (lacking at least one of the annealing sites) and the Cry3B protein were not detected.

The results indicate that DAS-ELISA represents a sensitive test which has a remarkable specificity for detecting a very low amount of the Cry3B toxin expressed in transgenic plants. In the most actively expressing plants, Cry3B toxin levels ranged from 800 to 1400 ng per g fresh weight and these levels were sufficient to demonstrate that extracts of plants contain a polypeptide of the same size as the Cry3B toxin. Low amounts of the toxin, still detectable with immunochemical methods, did not prevent CPB larvae from damaging plants. Nevertheless, the high degree of accordance between the DAS-ELISA test and the insect bioassay allows a rapid screening of the transgenic eggplants. The identification of eggplant lines with a high, medium or low toxic effect on CPB will amplify the possibility of testing different methods (McGaughey and Whalon, 1992) for an effective management of transformed CPB-resistant eggplants.

Gene transmission to the progeny occurred according to the expected mendelian ratio, except for one case in which all the progeny was constituted by resistant plants. The correla-

tion between the absence of bleaching (resistance to kanamycin) in the leaves and the presence of an active Bt gene has been demonstrated, based on the insect bioassays.

Experimental field trials with our transgenic eggplant lines are now planned in order to follow protein expression over time and in different tissues.

Acknowledgements

We wish to thank Prof. Angelo Spena for a critical reading of the manuscript and Bruno Ricchiuto and Maria Grazia Tacconi for their valuable technical help. This research was supported by the Italian "Ministero delle Risorse Agricole Alimentari e Forestali" in the framework of the project "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici".

Chapter 5

Trophic Interactions Between *Leptinotarsa decemlineata* Say Adults and Bt—expressing Transgenic Potatoes: Effects on Beetle Feeding Behavior and Reproductive Biology

Abstract

Transgenic potato clones expressing a Cry3B endotoxin were used to study the trophic interactions between newly emerged Colorado potato beetle *(Leptinotarsa decemlineata* Say) adults and these resistant clones. Adult longevity and fitness were studied for the first 3 weeks after emergence. Beetle reproductive biology on highly resistant clones, intermediary resistant clones and control potato plants was monitored by dissecting females after 7-15 days of feeding and also by analyzing haemolymph protein content after 3 days of feeding. Feeding behavior on either transgenic plants expressing high toxin concentrations or control plants was monitored individually for 36 newly emerged beetles feeding on leaf-discs during the first two meals. Lethal Time 50 for beetles feeding on transgenic clones as the sole source of food was not significantly shorter than for beetles on control clones reared in growth chamber. Differences tended to be higher under the more stressing situation of a greenhouse environment with a less optimal temperature range. Female egg production on transgenic plants appeared instead almost totally inhibited. Dissection studies indicated that adult males living on high-level Bt-expressing transgenic potatoes were still able to mate and produce mobile sperms, but the females were impaired in their reproductive ability since their ovaries were in general not normally developed. An examination of the haemolymph revealed the protein concentration in females living on transgenic plants to be dramatically reduced (≡ 50%), and electrophoresis showed specific changes in the protein pattern.

Feeding behavior of adult Colorado potato beetles was not affected by the different food plants; this indicates that transgenic potato plants were readily accepted as suitable host plants by beetles. The effects of these findings on the use of transgenic plants as a means of *L. decemlineata* control are discussed.

Introduction

Transgenic potato clones resistant to *Leptinotarsa decemlineata* Say have been obtained and presently are commercially available (James, 1997). While immature *L. decemlineata* are rapidly killed after feeding on *Bacillus thuringiensis*—expressing transgenic potatoes (Perlak *et al.*, 1993; Whalon and Wierenga, 1994), adults are less susceptible, thus a certain amount of feeding may occur and even a few eggs might be laid (Perlak *et al.*, 1993). Initial infestation of potato fields is normally performed by post-diapausing adults. Consequently, up to three gene-

rations per season follow depending on the availability of host plants and climatic conditions (e.g. Cappaert *et al.*, 1991). Food conversion by adult beetles feeding on *B. thuringiensis*—expressing transgenic clones will subsequently determine egg production and consequently population levels of the beetle in the field and eventually the final yield of the crop.

Sensory detection of Cry toxin by target insects could enable them to avoid transgenic plants, thus leading to escape of the selective pressure exerted by resistant clones on the herbivores. While some lepidopteran larvae showed avoidance of *B. thuringiensis*—based diet (e.g. Gould *et al.*, 1991), *L. decemlineata* larvae did not avoid *B. thuringiensis*—treated potato leaf discs (Ferro and Lyon, 1991; Arpaia and Ricchiuto, 1993).

We have conducted this study with the aim of assessing how continuous feeding on transgenic potatoes affects the physiology and behavior of adult Colorado potato beetle. In particular, we wished to clarify: 1) whether beetles accept transgenic plants as a host or whether they tend to avoid these plants, 2) whether adults can withstand the effects of Cry3 toxin expressed in transgenic plants and still live and reproduce, thus giving rise to a new generation.

Answers to these questions will contribute to elucidate the mechanism of insect resistance in Cry3-expressing transgenic plants to the adult phase of the insect and furnish more accurate indications on the effectiveness and possible durability of this pest control strategy.

Materials and methods

Insects. All the beetles used in these experiments were newly emerged adults collected daily from a laboratory colony maintained at Metapontum Agrobios on potted potato plants (cv. Desirée) since 1990. Beetles were kept starved until experiments started.

Plants. Three transgenic potato clones (H1, H2 and L) transformed with the synthetic gene BtI belonging to the Cry3 class (Iannacone *et al.*, 1997), together with a control clone of the same cultivar (Desirée), were used during these experiments. The first two were clones expressing high levels of resistance (i.e. 100% of first instar mortality after three days of feeding; in the following indicated as H1- and H2-clones respectively), while the third one showed intermediate levels of resistance (i.e. 50% of first instar mortality after three days of feeding; hereafter referred to as L-clone).

Study of fitness and survival. Three 3 week-old plants were used for each potato clone, isolated in metal mesh-screened cages (90x65x65 cm). Three cages per clone were prepared and kept either in growth chambers at 26±1°C (experiment no. 1) or in a greenhouse at temperatures ranging between 12 and 31°C (experiment no. 2). Three pairs of beetles were confined to each plant. The number of eggs laid and adult mortality were scored daily. Newly laid eggs were immediately removed from plants and kept in a growth chamber at 26±1°C until hatching. Number of hatched larvae was recorded. Clones H1 and L were used for these experiments together with an untransformed control.

Study of female reproductive apparatus. Two transgenic plants (clone H2) and two control plants in each experiment were placed in cages as described above and kept in a

greenhouse (experiment no. 3) or in growth chambers at 26±1° C (experiments no. 4-5). Mortality was recorded every other day. Three *L. decemlineata* females and three males were put onto each plant. After 7 days and/or 15 days, the females were collected from each plant, anaesthetized and dissected for an examination of the reproductive apparatus. In particular, the size and development of the ovarioles, the consistency and colour of the spermatheca and the presence of sperms in the spermatheca were observed.

Analysis of the haemolymph. Two more plants for each of the H2 and control clones were put into cages and maintained in a growth chamber as described above. Only three females were put onto each plant. After three days all the beetles were collected, and their haemolymph was extracted using a microsyringe after a cut made on both elytrae. The haemolymph from individuals feeding on each type of clone was pooled (≅ 10 ml were obtained) and collected in Eppendorf vials with a few grains of glutathione. In order to remove the haemocytes the solution was centrifuged at 10000 rpm for 4 minutes and diluted with extraction buffer. Protein concentration was determined by use of BIO-Rad protein assay (Bradford method) using bovine serum albumin protein as standard. The experiment was repeated five times.

Behavioral observations. Beetles were monitored individually, by using a table magnifying glass (5x), while feeding on potato leaf discs in petri dishes. For each feeding trial, two leaf discs (42 mm diam.) were cut from the same young leaf of either the H2 or the control clone. Leaf discs were put individually in petri dishes (55 mm diam.) lined with a water saturated filter paper and maintained in a growth chamber at 25±1 °C. The mean fresh weight of leaf-discs obtained from transgenic and control plants was not statistically different (Control = 0.137 g ± 0.018, H2 = 0.144 g ± 0.018). One adult *L. decemlineata* was introduced into one of the two dishes, and its behavior visually monitored until the second meal ended. To define the difference between intra and intermeal we referred to the timing indicated by Mitchell and Low (1994). Behavioral recording was carried out on 18 beetles for each treatment, by using a portable data logger. We recorded the behavioral categories as follows (cf. Mitchell and Low, 1994):

- *feed:* feeding, such as noticeable portions of the leaf are removed and as long as mouth parts are engaged in movement;
- *rest:* no change in positions and often no visible movements at all;
- *groom*: grooming using legs;
- *local:* moving with very little change in position, the beetle remains on a small part of the leaf disc;
- *walk :* walking, such as the beetle moves around the whole leaf disc circumference;
- *defecate:* defecation, scored as a frequency event, no duration measured.

At the end of each observation, the two leaf discs were photocopied. The area eaten was then determined by using an image analyser (Delta T Devices - Burwell, Cambridge, UK) as the difference between the areas of the two leaf-discs (uneaten leaf disc and partly fed leaf disc).

Statistical analyses. Data were analysed by using the LIFETEST, GLM and NPAR1WAY procedures of SAS version 6.12 for windows (SAS, 1989). Survivorship data

were right censored at day 21, and we calculated the non-parametric estimate Wilcoxon test. Behavioral data were treated with a non parametric ANOVA. Differences in leaf consumption were analyzed with the T test of analysis of variance.

Results

Study of fitness and survival. In the growth chamber experiment, adult longevity was not affected by clone type. Individuals feeding on plants of the H1-clone lived on average 10.27 days, on the L-clone 11.5 days and adults on untransformed control plants lived 14.1 days. No significant differences in longevity between clones were found (Wilcoxon test: Chi-square 0.2398, Pr> Chi square 0.8870). In the greenhouse experiment differences between transgenic clones were more pronounced as revealed by lifetest analysis (Wilcoxon test: Chi-square 3.2376, Pr> Chi square 0.1981), yet the results are not statistically significant. Survival in greenhouse experiments was lower for all the treatments: on the H1-clone beetles lived on average 9.52 days, on the L-clone they averaged 10.45 days, and adults on the control plants lived on average 13.86 days. LT50s calculated for each of the two experiments are shown in Table 1. Survival function estimates are represented in Fig. 1. It is obvious that in greenhouse conditions (panel A) the curves are better separated, while more censored data appear in growth chamber survival estimates (panel B).

We found no eggs either on L or on H1 potato clones. Females on control plants started laying eggs at day 9 in the greenhouse and at day 4 in the growth chamber. In the first three weeks of adult life, they produced an average number of 326 eggs per female in the greenhouse experiment and 920 eggs per female in the growth chamber. The peak of egg production was recorded toward the end of the second week (days 11-14). The average percentage of eggs that hatched was 95.16 %.

Study of female reproductive apparatus. Virgin females feeding on transgenic potato clones were affected negatively by this diet. Their ovarioles were completely inhibited in their development after feeding on Bt-plants; sometimes also the spermatheca was not properly developed and appeared not normally sclerotized. Feeding on transgenic plants did not hinder

Table 1. LT_{50} and 95% confidence intervals calculated for the different clones.

Clone	Greenhouse				Growth chamber			
	LT_{50}	upper	lower	%21[1]	LT_{50}	upper	lower	%21[1]
H1	7.00	15.00	4.00	10.52	n.c.	n.c.	3.00	66.66
L	7.00	17.00	7.00	9.09	11.00	n.c.	9.00	33.33
Control	14.00	n.c.	10.00	33.33	13.00	n.c.	9.00	31.25

[1]indicates the % of individuals surviving at day 21 (censored observation).

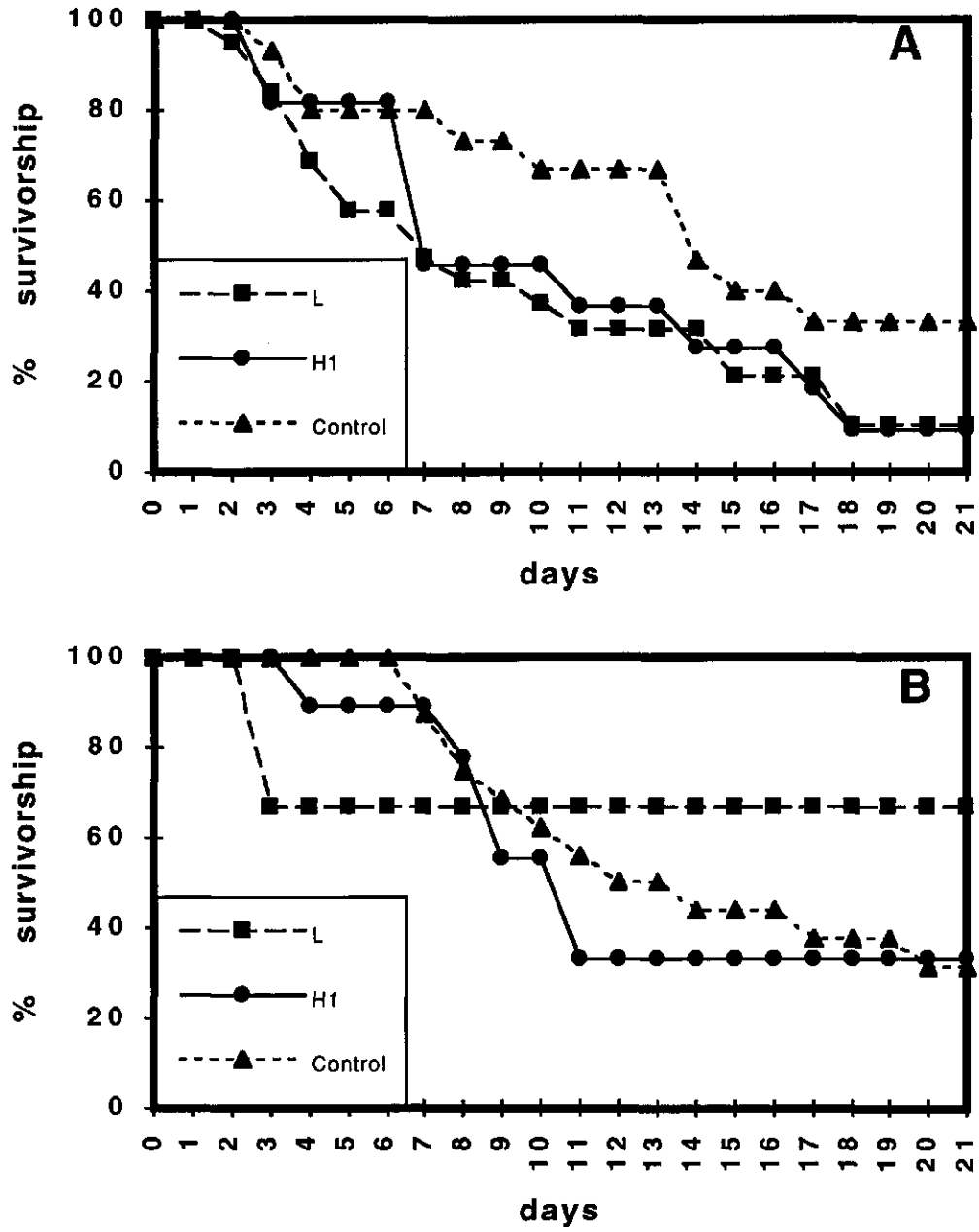


Fig.1 - Cumulative survivorship of adult beetles on different potato clones. A) Greenhouse experiment, B) Growth chamber experiment.

normal mating as revealed by the observation of mobile sperms in almost all the spermathecae examined, even in those cases when the organ was not normally sclerotized. In one case only, we found regularly developed ovaries. In Table 2, the results of dissection of 15 females (fed transgenic potatoes) are summarized. The 15 females fed on control plants generally showed a good ovary development and at day 15 a fairly large number of mature eggs were found inside the ovaries. In three cases though, not normally developed ovaries were found too.

During experiment no. 5, egg laying occurred. On control plants, eggs started to appear at day 11 and female fecundity averaged 122 and 301 eggs per female in two different cages. Also in one of the H2-plants we found one egg mass at day 15 which contained 11 eggs resulting in an average number of eggs laid per female of 3.66.

Analysis of the haemolymph. The analysis of the haemolymph revealed about two-fold reduction of the protein concentration in the females fed with the H2 clone compared to the

Table 2. Results of examination of reproductive apparatus of female beetles fed on transgenic clone H2.

Exp.	no. Beetles	Day of dissection	Atrophic ovaries	Partly developed ovaries	Completely developed ovaries	Developed spermatheca	Presence of mobile sperms
3	5	15	5	0	0	4	4
4	4	7	4	0	0	4	4
5	4	7	0	3	1	4	1
5	2	15	2	0	0	2	2

control (Table 3). Preliminary observations of the haemolymph protein composition with gel electrophoresis indicated the lack of a few specific proteins in the specimen fed on transgenic clones.

Behavioral observations. In Table 4, data referring to the principal categories of behavior are reported for beetles feeding on either clone type. No significant differences were found by analysis of variance when data on meal duration (Kruskal-Wallis: Chisquare =0.464, p=0.496, DF=1), pre-ingestive sampling (Kruskal-Wallis: Chisquare =0.402, p=0.526, DF=1)

Table 3. Total protein content (mg/ml) of the haemolymph of 3-day old *L. decemlineata* females observed on five different experiments.

Clone						Means (±SD)
H2	11.25	11.08	15.97	4.70	8.00	10.20 (±2.05)
Control	24.23	22.23	24.58	10.64	13.63	19.06 (±2.54)

Table 4. Mean length (in seconds) of the principal components of beetle feeding behavior.

Clone	Duration (±SD)	Interruptions (±SD)	Intermeal (±SD)	Examination (±SD)	Fedratio[1]	Areafed
H2	1237.0 (±598.44)	1.0294 (±0.922)	1566.6 (±505.0)	17.33 (±14.16)	0.000415	0.7689*
Control	1148.3 (±639.7)	0.7895 (±0.799)	1570.4 (±844.1)	37.37 (±87.69)	0.000419	0.4574

Asterisk indicates statistically significant difference p=0.01
[1]mm^2/sec

or number of interruptions during feeding (Kruskal-Wallis: Chisquare =0.511, p=0.475, DF=1) were analysed. The leaf area removed was even higher for beetles feeding on transgenic leaf-discs (ANOVA F=5.92 p=0.0205 df=1, 33) whereas the feeding rate expressed in mm^2/sec was very similar.

Discussion

Post-diapause adult Colorado potato beetles that move from their overwintering sites cause the spring infestation in potato fields each year. In transgenic potato fields beetles are faced with a crop that expresses the Cry3B toxin; the impact of these plants on the incoming beetles will obviously affect the infestation levels caused by subsequent generations.

Previous reports on Colorado potato beetle feeding behavior indicated that feeding on less preferred hosts is accompanied by a change in feeding behavior, such as reduced meal size, increased pre-ingestive sampling and frequent interruptions during each meal (Harrison, 1987). We conducted about 50 hours of visual observation on 36 newly emerged beetles feeding either on transgenic or control leaf-discs, and no food-induced change of any of these behaviors was noted. Instead, the area removed from the transgenic leaf-discs was even higher and the intermeal intervals tended to be shorter while feeding on plants of the H-clone, probably because of their lower nutritional value to which the beetle responded with compensatory feeding. These results demonstrate that *L. decemlineata* adults readily accept Bt—transgenic potato plants.

Experiments on whole plants indicated that under optimal environmental conditions beetles' longevity is not significantly affected by feeding on transgenic clones as compared with control potatoes. A small difference in LT50 appeared under more variable temperature conditions, this may indicate that stressed beetles are less able to withstand Cry3B toxin effects under those conditions. Observations on whole plants showed that the beetles feed for some days on resistant clones causing limited damage, but in general no offspring is produced. The effects of Cry3B toxin on adult beetles after feeding on transgenic plants appeared to be

Chapter 6

Field Performance of Transgenic Eggplant Lines Resistant To Colorado Potato Beetle

Abstract

Selfed progenies of transgenic eggplants (DR2 line, female parent of the hybrid 'Rimina') expressing a mutagenized *Bacillus thuringiensis* Berl. gene coding for the Cry3B toxin, were used in a field trial to assess their level of insect resistance. Three different transgenic lines were evaluated in field trials at two different locations according to a randomized block experimental design. Sampling was carried out twice per week to assess the density of *Leptinotarsa decemlineata* Say populations and the presence of other insects on the canopy. Only natural infestation by Colorado potato beetle (CPB) was considered in order to evaluate the resistance of the germ plasm. At harvest, total yield and fruit appearence was recorded on a plot base.

The density of CPB populations, was quite different at the two locations. Two of the transgenic lines showed high level of resistance at both locations, as revealed by the analyses of *L. decemlineata* abundance and crop yield. Fruit production was almost double in the resistant lines when compared to a DR2 untransformed control. The third transgenic line showed an intermediate level of resistance, giving results more similar to the control under heavy CPB attack whereas it gave comparable results to the other transgenic lines when the level of natural infestation was lower. No detrimental effects due to their presence in transgenic plots were evidenced on nontarget arthropods (including the chrysomelid *Altica spp.*). The examination of eggplant fruits revealed that on control plants significantly higher percentage of fruits was damaged by *Phtorimaea operculella* Zell. compared to all the transgenic lines.

These field observations confirmed laboratory data suggesting that the use of Bt-toxin expressing transgenic plants control Colorado potato beetle infestations below the economic threshold in eggplant cultivations, thus representing a potentially effective and environmentally safe means of pest control.

Introduction

Crop protection is a critical aspect of eggplant cultivation due to the heavy damage caused by pathogens and insects, especially the soil borne fungi *Verticillium spp.* and the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say. In Italy, early potatoes are being cultivated on increasing acreages, so that the growing season of potatoes (the preferred host plant of CPB) has become partially asynchronous with the beetle's life cycle and this phenomenon has contributed to the augmentation of *L. decemlineata* damage to eggplant cultivations (Arpaia *et*

al., 1995). Transgenic plants expressing Bt-derived toxins have proven to be effective in controlling insect populations during several field trials (e.g. Benedict *et al.*, 1996; Hamilton *et al.*, 1997b; Jansen *et al.*, 1997). The use of transgenic plants engineered for insect resistance involved 23.9% of all the requests for field release of genetically modified organisms approved or acknowledged by USDA until January 1998, and represent the second most common exogenous trait inserted into cultivated plants (BSS Biotechnology Newsletter, 2/1998) . Obviously, the most widespread crops (corn, potato, rice, cotton) have been the first to be transformed for insect control. Now that the technology is quite well established crops cultivated on smaller acreages could be likewise used. Horticultural crops are grown under labour intensive regimes with quite an abundant use of both fertilizers and pesticides. The alternative use of transgenic resistant varieties for these high value crops could be an important means of pest control which should be considered thoroughly. The use of transgenic eggplants resistant to CPB by expression of the Cry3 toxins (Arpaia *et al.*, 1997b; Hamilton *et al.*, 1997b; Iannacone *et al.*, 1997; Jelenkovic *et al.*, 1998) might represent a new and most effective means of pest control. Resistant lines are supposed to be non toxic for non-target organisms (including mammals), environmentally safe, and are also easy to use.

In this paper, we report the results of field trials, performed at two locations, of transgenic eggplant lines expressing a modified *Bacillus thuringiensis* gene, under natural CPB infestation, to evaluate their agronomic performance with particular attention to insect resistance.

Materials and methods

Plants. A parental line, named DR2, of the "Rimina" hybrid released from the Istituto Sperimentale Orticoltura, was used for genetic transformation (Rotino *et al.*, 1992). Transgenic eggplant lines obtained by selfing of primary transformant plants (Arpaia *et al.*, 1997b) were used for field experiments. With the aim of choosing only those plants bearing the transgene, segregating progenies were screened by means of *in vivo* spray with kanamycin solution (Sunseri *et al.*, 1993).

Experimental fields. Fields were prepared according to a randomized block design with 4 replications at two different locations: Metaponto and Monsampolo del Tronto, Italy (approval of the Italian Ministry of Public Health No. B/IT/97- 05A). This field experiment was setup to evalutate 3 transgenic eggplant lines (labeled as 3-2, 6-1 and 9-8), together with a control (DR2 untransformed plants) deployed as pure stands of transgenics. Only natural infestation was used to evaluate insect resistance characters. The plants were manually transferred into the fields on May, 25 1997 at Monsampolo and on June, 23 1997 at Metaponto. Eggplants were mulched, planted in coupled rows with a density of 2,22 plants per square meter and cultivated following the traditional cultural practices of each location. Each plot measured 4.5x9.6 m and contained 96 plants.

Field observations. Three weeks after transplanting, the sampling program started and was carried out for two times per week. Ten percent of the plants, randomly chosen, were visually inspected and the presence of insects on the plants recorded. Surveys continued for

about 8 weeks. Eggplants were periodically harvested and the fruit number and weight were then recorded. Seven harvests were considered for data collections (july-september 1997).

Cry3B toxin quantification. DAS-ELISA tests were performed on fruit tissues according to the protocol described in chapter 4.

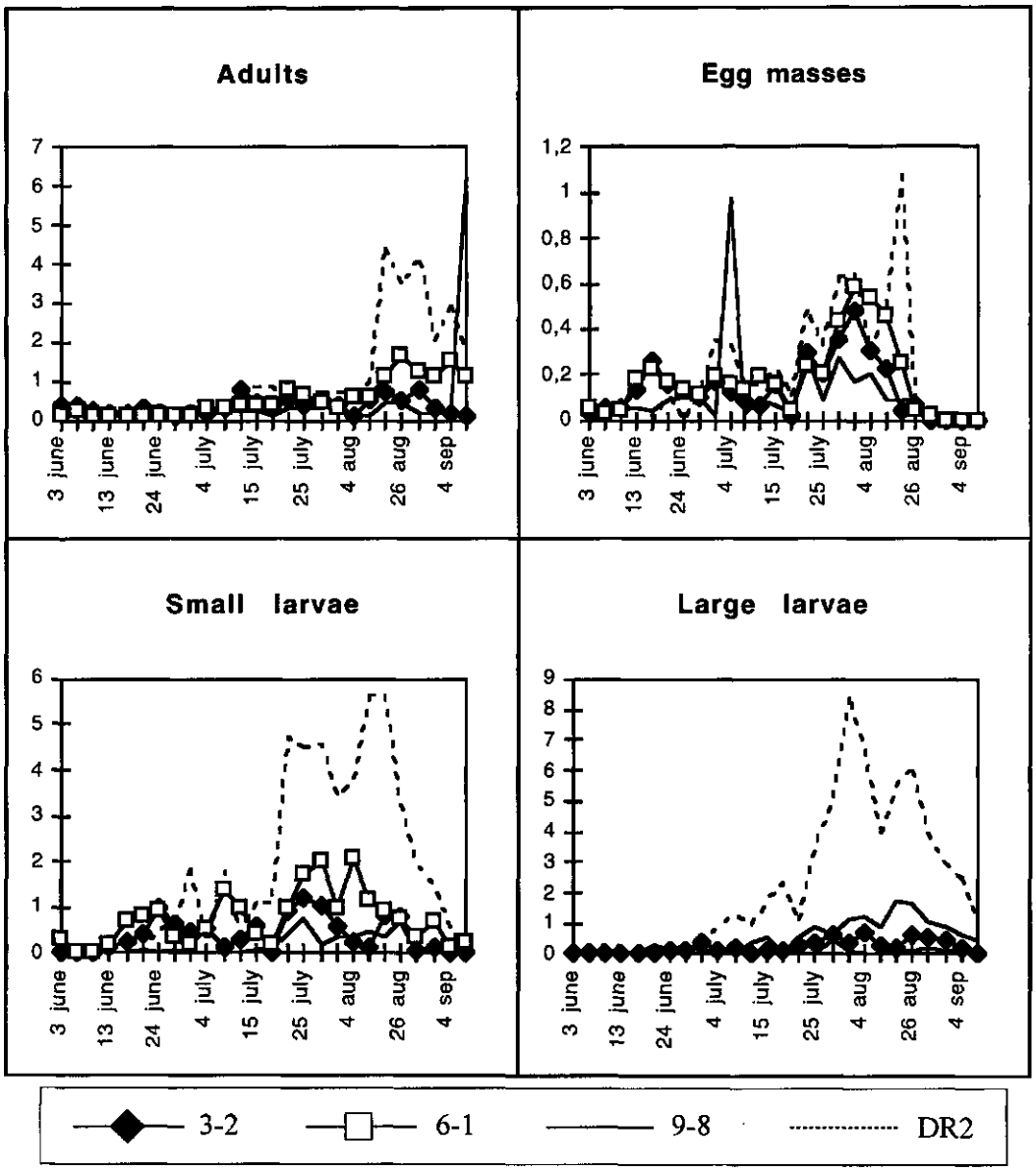
Statistical analyses. Data on insect abundance and crop yield were analyzed according to a randomized block design analysis of variance with repeated measures. Separations between means were obtained by the Least Significant Difference. All the analyses were performed by using SAS software, version 6.12 for Windows (SAS, 1989).

Results

Colorado potato beetle infestation course. Monsampolo. In this field trial the size of the beetle population was considerable. In Fig. 1 it can be seen how the beetles apparently completed two generations. Initially their population was most likely made up of overwintered beetles and later on the population peaked two times (early July and second half of August) as it is especially visible for insects in control plots (Fig.1). While incoming adult beetles spread quite evenly over all plots, small and large larvae were mainly found on the DR2 (control) and the 6-1 line and this led to a higher number of adults on the same eggplant lines toward the end of the season (Fig. 1). The analysis of variance confirmed that the beetle population (expressed in terms of egg masses and larvae per plant) was significantly higher on control plants than on transgenic ones (Tab. 1). Among the transgenic lines, the 3-2 and 6-1 lines bore a comparable number of egg masses, but only a small percentage of them were normally developping as larvae. On the 9-8 line, the smallest number of larvae was found throughout the whole growing season. Line 6-1 gave results intermediate between the untrasformed control and the 9-8 and 3-2 lines.

Metaponto. The attack by *L. decemlineata* started from one edge of the field near which a potato crop had been harvested three weeks before. As a result of this, the first block was severely damaged compared to the other blocks (data not shown). Due to the late planting date, incoming beetles in this case were mostly first generation adults. Infestation level was not very high throughout the season and the adults appeared to be evenly distributed among the different eggplant lines. Lines 6-1 and DR2 (control) initially bore a higher number of egg masses, whereas toward the end of the growing season only the egg mass number on control plants was still higher (Fig. 2). Pooled numbers of small and large larvae were used to represent the following infestation which only towards the end of the growing season reached an average level of two individuals per plant in the control plots. The analysis of variance showed that the presence of both egg masses and larvae was statistically higher on DR2 as compared to all other lines (table 2).

Presence of non-target organisms. In table 3, the presence of the most common insects found in Metaponto field trial is summarized. The only natural enemy of CPB regularly found on the canopy was the lacewing, *Chrysoperla carnea* Stephens. During our samplings, immature stages were only sporadically present until early August, then increased when plants



F ig. 1 - Colorado potato beetle infestation course at Monsampolo field trial.

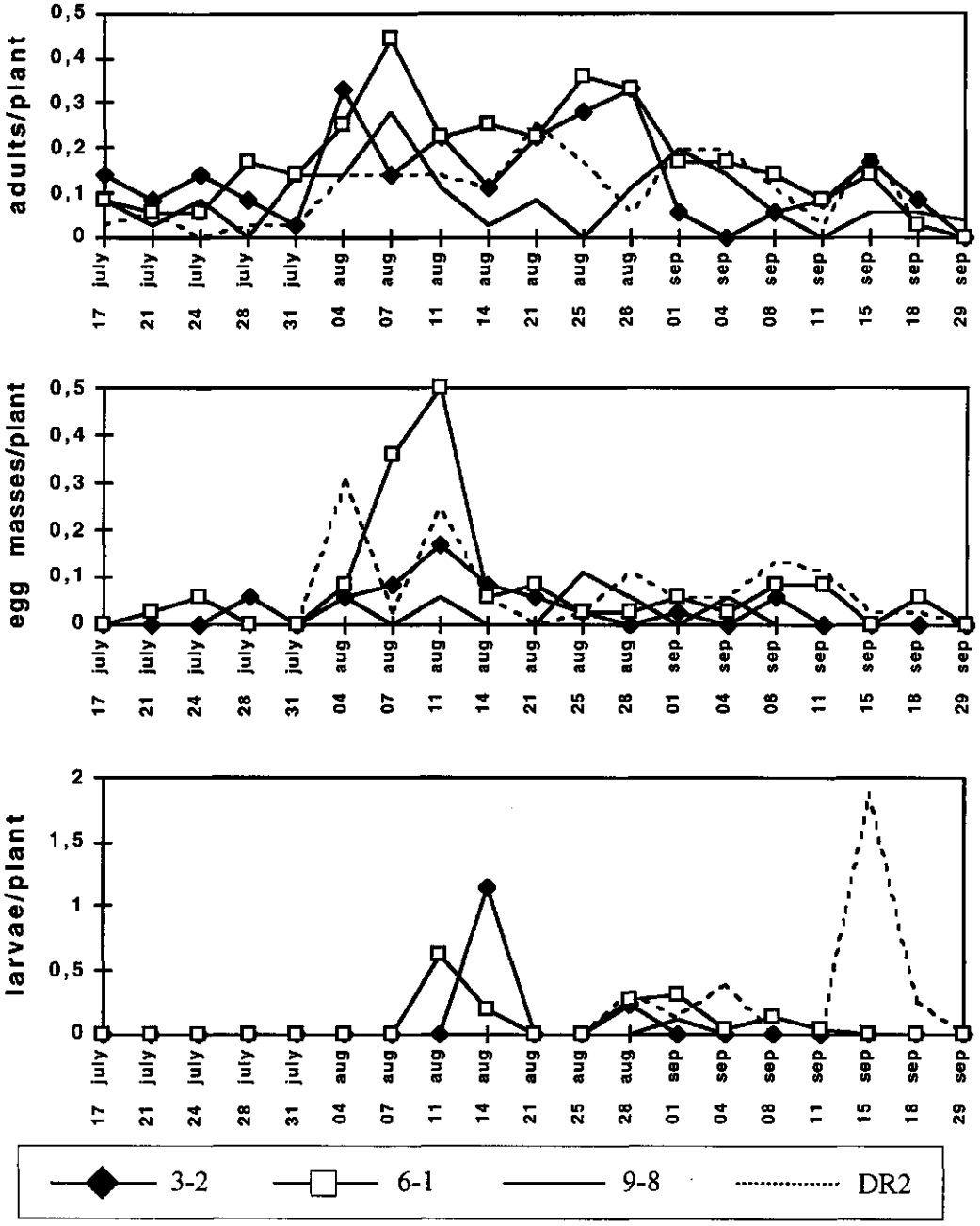


Fig. 2 - Colorado potato beetle infestation course at Metaponto field trial. Data on small and large larvae have been pooled.

Table 1. Statistical analyses of the field data collected at Monsampolo.

Line	Egg masses/plant	Larvae/plant	Total production[1]	no. fruits[2]
3-2	0.132 B	0.209 C	1.298 A	6.232 A
6-1	0.168 B	0.449 B	0.584 BC	3.477 B
9-8	0.076 C	0.071 C	0.978 AB	6.176 A
DR2	0.214 A	1.910 A	0.400 C	2.752 B

[1]Kg/plant
[2]Cumulative per plant

were fully grown. Their presence was not numerically relevant and no difference between lines was detected (ANOVA DF=3, 54 F=0.03 Pr> F 0.993).

Among non-target insect pests, the most common herbivores were the green peach aphid *Myzus persicae* Sulz., the flea beetle *Altica spp.*, and the potato tuber moth *Phtorimaea operculella* Zell. Aphid colonies were found spread all over the field in all the different plots and no difference in the average number of colonies per plot was evidenced (table 3). Extremely interesting observations were obtained regarding flea beetles and potato tuber moth. The chrysomelid beetle *Altica spp.* was apparently not affected by the presence of Cry3B toxin in the eggplants. The number of leaves damaged by the beetle increased over time, until we noticed up to 80% of damaged leaves in most of the plots. The mean number of damaged leaves was even higher for transgenic lines (table 3) but this might be due to the better vegetative status achieved by transgenic lines withstanding CPB's attack.

Table 2. Statistical analyses of the field data collected at Metaponto.

Line	Egg masses/plant	Larvae/plant	Total production[1]	no fruits[2]
3-2	0.035 B	0.050 B	0.938 A	8.466 A
6-1	0.067 A	0.077 B	1.015 A	9.341 A
9-8	0.016 B	0.034 B	1.064 A	10.063 A
DR2	0.039 A	0.210 A	0.434 B	4.178 B

[1]Kg/plant
[2]Cumulative per plant

When harvested fruits were checked, we noticed quite relevant damage caused by *P. operculella* larvae. Surprisingly enough, a statistically significant difference due to the eggplant lines was found (ANOVA DF=3, 9 F=11.61 Pr> F 0.019). The control line suffered significantly more damage compared to the three transgenic lines (Table 3).

Eggplant production. The data collected upon harvesting showed a significantly higher production for all the transgenic lines when compared to controls (Table 1-2). Fruit production for transgenic lines was approximately doubled compared to that of DR2 control line

Table 3. Summary of field data relatives to the presence of the main non-target insects collected at Metaponto.

Line	no. of *C. carnea*/plant[1]	Aphid colonies/plant	Leaves damaged by *Altica spp.*[2]	Fruits damaged by *P. operculella*[3]
DR2	0.016 A	0.009 A	5.23 B	7.72 A
3-2	0.015 A	0.009 A	5.65 AB	4.47 B
6-1	0.016 A	0.028 A	6.06 A	4.98 B
9-8	0.015 A	0.015 A	6.19 A	5.17 B

[1]Larvae+eggs
[2]Average of three samplings during the month of August
[3]Average of all harvests

and differences were even more pronounced at Monsampolo where the CPB infestation level was considerably higher. A higher number of fruits produced per plant accounts for the differences recorded (Table 1-2). The line 6-1 gave intermediate results in terms of yield level and in the case of mild infestation its production tended to be similar to the other transgenic lines, whereas in case of high pressure by *L. decemlineata* its production was closer to DR2 than other transgenic lines.

Discussion

The widespread availability of insect resistant transgenic cultivars will bring new possibilities for a sustainable pest control; to prove that these varieties will be safe and effective in this respect, sound insights must be obtained from repeated field studies. Assessing insect resistance is obviously the main subject of such studies, but the impact of the resistant germ plasm on the whole entomofauna should also be examined to better evaluate its possible effect when used on a large scale. Several recent studies have addressed this topic by considering specific aspects such as the effects on secondary pests (e.g. Hardee and Bryan, 1997; Pilcher *et al.*, 1997a; 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) or the evaluation of possibly detrimental effects on insect predators feeding on transgenic pollen (Pilcher *et al.*, 1997b).

In the present field study we have begun to look at the 'eggplant agroecosystem' in order to obtain a prediction of the success of introduction of Bt-expressing transgenic lines. Our experiments clearly indicate that resistance to the Colorado potato beetle in eggplants expressing the Cry3B protein, already demonstrated in laboratory bioassays, was confirmed under field conditions. In transgenic plots, a constantly lower abundance of all CPB stages

during the season was found. Transgenic lines furnished a higher production also in the case of strong infestation levels. Line 6-1 showed levels of resistance intermediate between the two other transgenic lines on the one hand and the untransformed control line on the other.

The evaluation of field observations made on the main non-target insects led to some interesting conclusions. According to the adopted rating of plant damage, flea beetles, which are taxonomically closely related to the Colorado potato beetle, were apparently not impaired in their development on transgenic eggplants. This suggests the Cry3B gene affects one chrysomelid species but not the other. However, more accurate observations on *Altica* biology, along with the rating of field damage, should then be conducted to reliably compare the sensitivity of the two species. The extent of fruit damage inflicted by *P. operculella* seems to indicate an effect of Cry3B toxin on this lepidopteran; if confirmed this observation would imply a cross-resistance for these eggplant lines. We did assess Cry3B presence in eggplant fruits with DAS-ELISA test. The OD492 ratio between transgenic and control plants indicated a high expression for the 9-8 and 3-2 transgenic lines (averege OD ratio values of 6.8 and 4.13 respectively) and a slightly positive value of 2.2 for the 6-1 line. Also in this case, gathering field observations over several years is of paramount importance for integral germ plasm evaluation.

No evidence of detrimental effects on other arthropods (e.g. aphids, lacewings) was collected.

Preliminary observations in a mixed field, made by 80% transgenic plants and 20% control plants, showed that even in this case the transgenic lines could withstand natural infestation by *L. decemlineata* and yet provide appreciably higher production.

The two most effective transgenic lines have now been used to obtain eggplant hybrids which could more likely be a final product of the research. Hybrids are currently under evaluation in field trials at different locations in order to gather more accurate information on their possible role for commercial use.

Acknowledgments

I wish to thank N. Acciarri, G.M. Di Leo, G. Mennella, G. Sabino, F. Sunseri and G.L. Rotino who partecipated in the field experiments and who are co-authors of a shorter version of this manuscript, published in the Proceedings of the Xth EUCARPIA Meeting on Capsicum and eggplant held in Avignon, France, September 1998.

Dalla Basilicata una melanzana che resiste agli insetti dannosi

Yes, now the beetles do not eat my eggplants anymore, but they do prefer my ham sandwich .

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Chapter 7

Potential impact of *Coleomegilla maculata* DeGeer (Coleoptera:Coccinellidae) predation on Colorado Potato Beetle (Coleoptera: Chrysomelidae) adaptation to Bt-transgenic plants.

Abstract

The relationships between *Leptinotarsa decemlineata* Say egg density and *Coleomegilla maculata* DeGeer predatory behavior was investigated at different spatial scales (plant-to-plant and plot-to-plot). Both adult *C. maculata* location and daily egg consumption rates were monitored over time in greenhouse and field tests. Despite aggregation in areas of highest prey density by *C. maculata*, egg consumption was inversely related to egg mass density at the smallest and the largest spatial scales tested. The experimental data on predation rates in high and low density field treatments were included in a mathematical model to simulate the impact of natural enemies on the rate of *L. decemlineata* adaptation to Bt-toxin-expressing transgenic potato plants when Bt-expressing plants are mixed with normal potato plants at the plot-to-plot level. Results showed that *C. maculata* predatory behavior could decrease the rate at which *L. decemlineata* adapted to Bt-toxins if plot-to-plot mixed plantings were used.

Introduction

It has often been assumed that the effects of natural enemies and crops with host plant resistance (HPR) were so distinct that predators were not considered to be one of the factors which could affect the durability of a HPR trait (for example, Bergman and Tingey 1979). Gould *et al.* (1991a), used computer simulations to demonstrate that the rate of predation by natural enemies could indeed affect the rate of pest adaptation to partially resistant host plants deployed in homogeneous plots. More recent simulations (Gould, 1994) indicate that natural enemies could also affect the rate of pest adaptation to plants with high levels of HPR if these plants are grown near other plants which are susceptible to the pest insect. Simulations indicate that if natural enemies prey on the pest in a density-dependent fashion, this could lead to faster adaptation by the pest to the toxin. In contrast, inverse density-dependent predation is expected to slow down the rate of adaptation.

Transgenic potatoes resistant to *Leptinotarsa decemlineata* Say based upon expression of a toxic protein derived from *Bacillus thuringiensis*, will be among the first transgenic crops deployed commercially. Several deployment strategies have been suggested to reduce the rate at which target insects may adapt to the Bt toxins in these potatoes (Arpaia and Ricchiuto,

1993; Gould *et al.*, 1994). One of these strategies, planting a mixture of transgenic and susceptible potato isolines, could retard adaptation by providing refugia where susceptible beetles could survive. If, however, predation occurred in a density-dependent manner, a higher proportion of the beetles and their eggs in the Bt refugia would be killed, relative to the few resistant individuals in the areas of Bt expression. This would have a negative impact on the utility of the refuges.

The coccinellid *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) is an active predator of *L. decemlineata* eggs and first instars. Field studies in North Carolina found egg mortality due to predation ranges from 17-34% in the early season, with peaks as high as 100% during the latter part of the season (Hilbeck, 1994). Hilbeck indicated that *C. maculata* was the most important predaceous species in the area. Giroux *et al.* (1994) found no toxic effects of Bt-based insecticides to *C. maculata* in laboratory experiments.

The relationship between CPB egg density and egg consumption by *C. maculata* was determined in laboratory experiments by Hazzard and Ferro (1991). They found a Type II functional response to egg density. While these petri dish results are interesting, we do feel that determining the impact of *C. maculata* on the rate of *L. decemlineata* adaptation to Bt toxins requires experiments conducted in more realistic environments. Here we report the results of an investigation on *C. maculata* 's response to various *L. decemlineata* egg mass densities in arenas that mimicked different spatial scales of mixed plantings ranging from plant-to-plant to plot-to-plot mixtures of transgenic and susceptible plants. We used data from these experiments to set the parameters of a simulation model which predicts rates of pest adaptation to resistant host plants.

Materials and Methods

Insects. *L. decemlineata.* Egg masses were obtained from a colony maintained at the Department of Entomology, North Carolina State University, and from a culture maintained at the Philip Alampi laboratory, Beneficial Insects Rearing, New Jersey Department of Agriculture. For experiments requiring up to 70 egg masses per day, we always used fresh egg masses. However, in preparing for large scale experiments, egg masses were sealed in petri dishes and frozen at -20°C. Egg masses were then thawed by leaving petri dishes at 4°C overnight. Preliminary bioassays showed that thawed egg masses are readily accepted by *C. maculata* and do not deteriorate for over five days after thawing (data not shown).

C. maculata. Adults (15-25 days old) were received from the USDA-APHIS-PPQ Mission Biological Control laboratory. Prior to experiments, insects were kept overnight without food. Previous research has demonstrated that females are more responsive than males to food availability and will eat more (Hazzard and Ferro, 1991). Therefore, we used only female *C. maculata* when this was feasible. When only females were to be used in an experiment, we ensured accurate sexing by selecting only females from mating pairs.

Experimental protocols. *Small cage experiments.* Metal mesh screened cages 42x42x42 cm were used in all of the small-cage experiments. Two 3-week-old potted potato

plants were placed in these cages with limited contact between the leaves of different plants. Eight female *C. maculata* were released into each cage. Plants were infested by pinning egg masses on top of single potato leaflets. All the experiments were conducted in a greenhouse with controlled temperature between 20 and 38 °C.

Presence-absence treatments. Four different egg mass densities were tested, each in a different cage: 1, 3, 10 and 20 egg masses per plant. Newly laid egg masses were pinned on only one of the two plants. Four *C. maculata* adult females were placed on each of the two plants. The number of *C. maculata* on each plant was recorded at 2, 4, 6, 20, 22, 24, 28, 30, 44, 46, 48, 52, 54, 68, 70 and 72h after the initial placement. The number of eggs eaten was determined daily for three days. Egg masses were examined by removing them from the plants and inspecting them for predation under a dissecting stereomicroscope. Undamaged egg masses were replaced in the same spot from which they were taken. Attacked egg masses were replaced with new ones. Two replicates were set up for each egg mass density. The experiment was repeated four times.

Low versus high density treatments. The protocol was the same as in 'presence-absence treatments', except that one of each pair of plants always had one single egg mass instead of no egg masses (i.e. low density plants). The other plants had 1, 3, 10 or 20 egg masses and are referred to as the 'high density plant'. Egg masses on both plants were scored for percent of eggs eaten. The experiment was repeated three times.

Large cage experiments. Four benches, 265x120 cm, were constructed in a greenhouse in a rectangular arrangement such that each bench was 50 cm from the neighboring benches on all sides. Forty, 3-week-old potted potato plants were placed on each bench (8 rows of 5 plants each). A large fabric net, supported by 4 m high poles, was placed over all four benches to prevent the escape of the *C. maculata* adults. Two of the plots (on diagonally positioned benches) were infested with three egg masses per plant, while the plants on the other two benches received an egg mass density of 0.1 per plant (i.e. 1 for every 10 plants). Forty *C. maculata* females were released in each plot (1 per plant). The number of *C. maculata* in each plot was counted twice per day and egg predation was scored daily. All the egg masses in the low density plots were scored for predation, but in the high density plots, a total of 40 egg masses per plot (1 per plant) were examined. Attacked egg masses were not replaced in this experiment. Each of the three replicates of the experiment lasted for five days (frozen egg masses were used to prevent hatching). The placement of the high and low egg mass density treatments was alternated between the benches following each replicate.

Field experiments. Seed pieces of transgenic potatoes which expressed Bt toxins were provided by Monsanto Agriculture Company (St. Louis, MO). We chose to use a potato line that expressed Bt-toxin to prevent natural *L. decemlineata* infestations. Four plots were demarcated within a potato field of approximately 0.1 Ha in a completely randomized block design. Plot size was 8.8x5.0 m, each plot consisted of 12 rows of 17 plants each, but only 10 rows were used for the observations (the two border rows were excluded). Soil type was sandy loam. Preplant fertilizer (12-6-24) was applied at a rate of 600 lbs per acre and a post-planting top dressing of fertilizer (15-0-4) was applied at 200 lbs per acre. When potatoes were in the early bloom stage, plots were artificially infested with previously frozen Colorado potato bee-

tle egg masses by pinning egg masses to the upper side of the potato leaves. Thereafter, approximately 5000 adults of *C. maculata* were released throughout the plots. The same egg mass densities used in the large cage experiments were chosen to assess *C. maculata* predation rates and movement in the field experiments, because these two densities (0.1 and 3 egg masses per plant) represent potential field situations in North Carolina (Kennedy, unpublished data). Egg predation was scored every day using a stereomicroscope. All egg masses were counted in the low density treatments, but only 170 egg masses (1 per plant) were monitored in each high density plot. The number of *C. maculata* per plot was scored daily by examining 50 randomly chosen plants per plot. The experiment was repeated twice with each replicate lasting four days. The plots used for the high and low density treatments were switched between replicates of the experiment. The first replicate was initiated on June 7, 1994 and the second one on June 16, 1994. During the time of these experiments, the plots were devoid of significant numbers of alternate *C. maculata* prey, such as aphids.

Statistical analyses. *Small cage experiments. Presence-absence treatments.* To evaluate the functional response of *C. maculata* to egg masses, data from the presence-absence experiments were fitted to the Holling disc equation (Holling, 1959) by using a nonlinear regression (SAS, 1989). A reciprocal linearization of the disc equation (Livdahl and Stiven, 1983) was used to obtain the initial estimates of a and Th, as required in the SAS NLIN procedure. The percentage of beetles on the 'treatment' plant was used to investigate the effect of egg mass density on beetle movement. A Wilcoxon signed rank test was used to determine if, overall, plant with egg masses had more beetles on them than plants without egg masses. A repeated measures ANOVA was adopted to determine if there were differences among 1, 3, 10 and 20 egg masses regarding the extent of beetle preference (as measured by beetle position). The repeated measure design was also used to analyze effects of time during the course of an experiment and the effect of experimental block on the degree of beetle preference. Density treatments used in the experiment were analyzed using a random effect statistical model. Data were arcsin square root transformed before performing ANOVA.

Low versus high density treatments. Beetle preference for the high and low density plant was determined as described in 'presence-absence tests'. A repeated measure ANOVA was used to test whether the proportion of eggs attacked on the high density versus low density plants was differentially affected by the four egg mass densities. The ratio of percent predation on low versus high density plants was the dependent variable. Egg mass density and experimental block were considered random effects.

Large cage experiments and field experiments. The same general type of analyses used in the 'small cage' low versus high density tests were used for the large cage and field tests. The most important difference in the analyses was that in the small cage densities of egg masses were considered random effects, whereas in the large cage and field tests the two densities were chosen specifically to reflect expected field densities and were therefore considered fixed effects. Predator preference in the field was assessed by analyzing the percentage of beetles in the high and low density plots, over time.

Genetic model. A deterministic population genetic model described by Gould (1986) was used to see how the movement and predatory behavior of *C. maculata* might influence the

rate of *L. decemlineata* adaptation to Bt-expressing potatoes that were planted as a mixture of plots, one half containing 100% Bt-expressing plants and one half containing 100% non-Bt-plants. The inheritance of resistance was assumed to be controlled by a partially recessive allele with an initial frequency of 0.02. In the absence of *C. maculata*, the fitness of *L. decemlineata* RR, RS and SS genotypes on Bt-expressing plants was set at 1.00, 0.005 and 0.001, respectively. On non Bt plants, all genotypes were assumed to have a fitness of 1.00 in the absence of *C. maculata*. The impact of *C. maculata* on the fitness of beetles in the Bt and non-Bt plots was determined based on the results of four days of predation in the field experiments. A second simulation was run, assuming that there was equal predation on beetle eggs in both Bt and non-Bt plots.

Results

Small cage experiments. *Presence-absence treatments.* The relationship between *L. decemlineata* egg density and the number of eggs eaten by *C. maculata* can be explained well by the Holling disc equation (Williams and Juliano, 1985):

$$Na/TP=aN/(1+aThN),$$

where a = area of discovery, Na = number of prey attacked, T = total time, P = number of predators, and Th = handling time. Estimated parameters (mean ± confidence interval) were: a = 0.47481 ± 0.2772, and Th = 0.5232 ± 0.1330 with an R^2 = 0.933. An approximate curve is plotted in Fig. 1. Mean daily consumption at different densities is shown in Tab.1.

The percentage of females *C. maculata* on different plants over time is presented in Fig. 2. It was clear that predator abundance was greater on plants with *L. decemlineata* egg masses relative to control plants (mean difference 27.95, Wilcoxon signed rank test S = 12939 $p < 0.0001$). Different prey densities induced a significantly different *C. maculata* behavioral response as measured by the location of the predators (ANOVA F= 4.09, df=3, 9; p=0.043, see Tab. 2). There was also significant effect of time (ANOVA F= 2.76, df=15, 40; p=0.005).

Low versus high density treatments. Fig. 3 presents the percentage of *C. maculata* on plants in the different treatments over time. Most of the predators were found on the plant with the higher egg mass density (mean difference = 18.42 Wilcoxon signed rank test S= 17889, $p < 0.0001$). There was a significant positive relationship between the difference in egg mass densities on the high and low density plant (ANOVA F= 9.53, df=3, 9; p=0.011. See Table 2). None of the other factors or interactions were significant. These results match those obtained in the presence-absence treatments, indicating that prey density does affect *C. maculata* position at a plant-to-plant spatial scale.

Analysis of variance of the ratio of the percent of the eggs eaten on high and low density plants indicated the relationship between the difference in egg mass densities on the high and low density plants and this ratio (F=7.83, df=1, 6, p= 0.031 linear contrast). However, this relationship was in contrast with the effect of density on plant preference by adults. A higher proportion of eggs was preyed upon in the low density treatment than in high density treatments and the significant linear contrast indicated that the higher the egg mass density on the

Table 1. Mean daily consumption (± standard errors) of *Leptinotarsa decemlineata* eggs per *Coleomegilla maculata* female at different prey densities. In the presence-absence test, one plant had no egg masses, while a second plant had either 1, 3, 10, or 20 egg masses. In the low versus high density experiment, one plant always had a single egg mass, while a second treatment plant had 1, 3, 10, or 20 egg masses.

Experiment	Day	Number of egg masses			
		1	3	10	20
Presence-absence	1	2.98(±0.82)	4.95(±1.79)	10.49(±2.38)	10.72(±1.70)
	2	2.88(±0.83)	6.72(±1.77)	10.38(±2.12)	11.56(±1.56)
	3	2.63(±0.83)	7.44(±0.81)	13.52(±1.88)	14.68(±1.55)
Low versus high density	1	4.00(±0.50)	7.77(±0.72)	15.33(±0.95)	18.60(±0.93)
	2	4.15(±0.59)	8.46(±1.82)	12.69(±1.39)	17.59(±1.13)
	3	4.13(±0.78)	8.02(±1.04)	15.55(±2.65)	20.07(±1.75)

'high density plant', the bigger the difference in percent predation on the high and low density plants (Table 3). This logically indicates an inverse density dependence in *C. maculata* predation on *L. decemlineata*. The treatment*experiment interaction was not significant (ANOVA F=1.74, df=6, 36 p=0.139). Mean daily egg consumption was generally higher in the low versus high density experiment compared to the egg consumption in the presence-absence tests (Table 1). This indicates that the *C. maculata* used in the choice tests were more active that those used in the presence-absence tests.

Large cage experiments. Over the five day period of the experiment, an average of more than 40% of the *C. maculata* adult females released in the three replicates were detected in our daily samplings, but the mean number of individuals declined almost constantly over time (day 1 = 80 beetles, day 2 =55 beetles, day 3= 45 beetles, day 4= 37 beetles, day 5= 27.5 beetles). Predator location over time is shown in Fig. 4A. More *C. maculata* were found in the high density plots than in the low density plots (ANOVA F=107.53, df=1, 26; p=0.0001). The treatment*experiment interaction (ANOVA F=35.69, df=2, 26; p=0.0001) and the treatment*experiment*time interaction (ANOVA F=3.17, df=6, 26; p=0.0181) were also significant.

The percentage predation per day was higher at low egg mass density than at high egg mass density (Table 4), but statistically this difference was only marginally significant.

Field experiments. The percentage of predators in the high and low density plots is presented in Fig. 4B. To quantify the pattern of coccinellid aggregation in the field situation, it

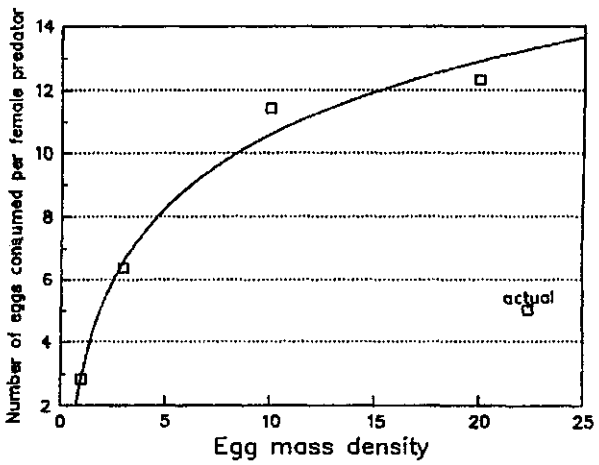

Fig.1 - Relationship between daily egg consumption per *C. maculata* female and egg mass density. $R^2 = 0.933$ in the regression of experimental data against the data expected based on the Holling equation. The curve in this figure is a nonlinear fit to the data points.

was necessary to consider the natural decrease of *C. maculata* numbers over time (Fig. 5). We therefore estimated the average time *C. maculata* individuals remained in each plot, using the method of Ives *et al.* (1993). This was determined by multiplying the fraction of predators remaining in each plot on a given day by the number of days since the release. The sum over all days was then calculated. Even after one single day, the number of *C. maculata* in the low density plots was substantially lower than those in the high density plots. Results obtained with this method are consistent with what we observed in cage experiments where *C. maculata* movement was more restricted (Fig. 4A, B). Analysis of variance, indicated that significantly more *C. maculata* were found in high density plots than in low density plots (F= 48.71, df=1, 16, p=0.0001). These results indicate that at the larger spatial scale predators aggregated in response to food availability. *C. maculata* individuals of both sexes were released in field plots. We did not assess whether males or females responded more strongly to egg mass density in the field experiments.

The scoring of eggs within egg masses for evidence of *C. maculata* feeding (Tab. 4) indicated that predation was significantly greater in the low density plots than in the high density plots (ANOVA F= 5.42, df=1, 16; p=0.031). This result is similar to that of the greenhouse experiments, confirming the existence of inversely density-dependent predation.

At least some eggs in almost all egg masses were fed upon during the four-day field experiments. The average percent of egg masses found without even one egg attacked in the high density plots was 0.53% and in the low density plots all the egg masses were attacked. After four days, 86.54% of the eggs in the low density plots were killed by predators, while only 66.28% were killed in the high density plots.

Predictions of the genetic model. By assessing the percentage of eggs in the high and the low density field treatments which were not killed by the end of the experiment, we were able to arrive at an expectation for the difference in fitness caused by predation in Bt and non-Bt potato plots. In the low density plots (i.e. mimick of Bt potatoes) the overall egg survival in face of predation was 13.46% (i.e. 100%-86.54%). In the high density plots (*i. e.* mimick of non-Bt potatoes), 33.72% survived. We used these values to set genotype-specific relative fitnesses in hypothetical Bt and non-Bt fields. *L. decemlineata* with RR genotypes were assigned a fitness of 0.3372 in non-Bt fields and were assigned a fitness of 0.1346 in Bt

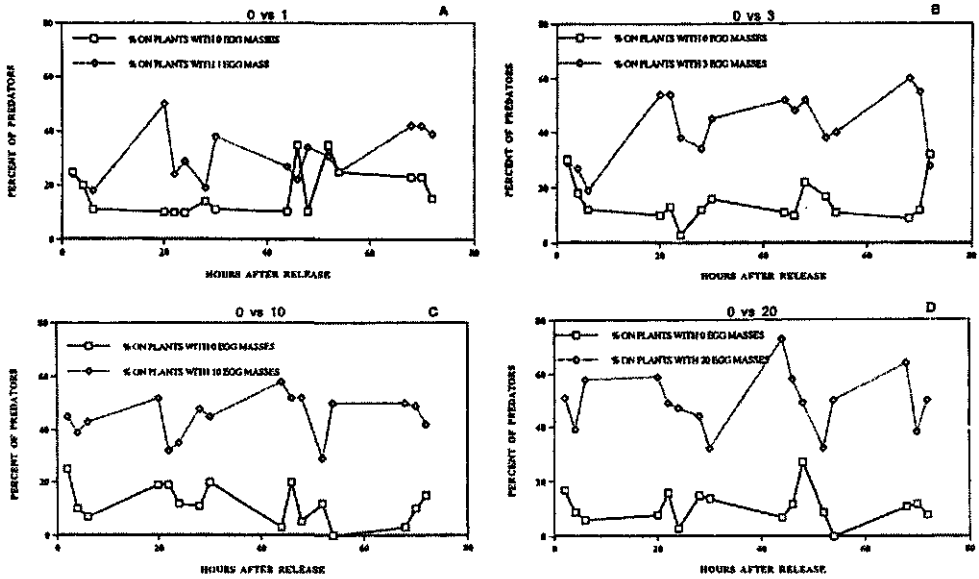

Fig. 2 - Predator position over time in small cage, presence-absence experiments.

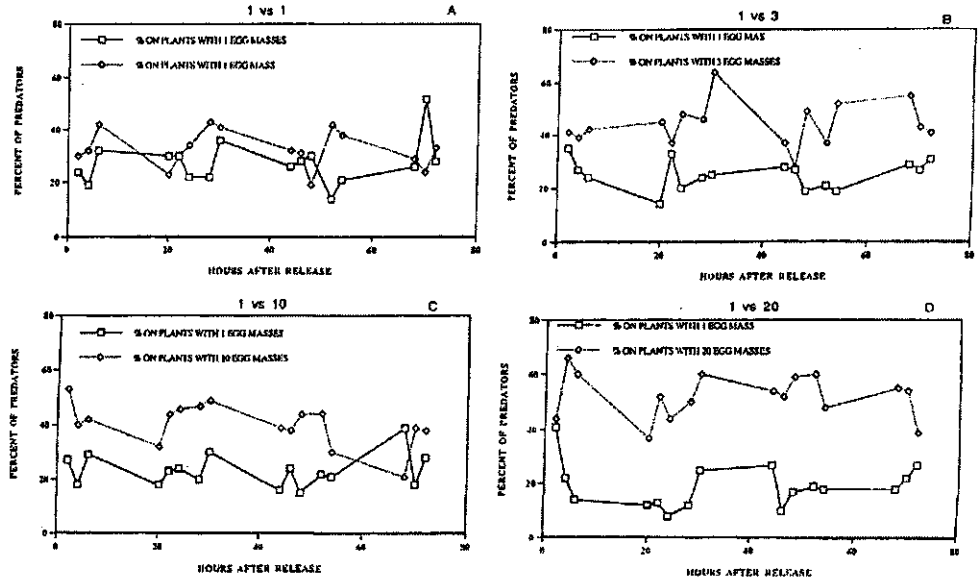

Fig. 3 - Predator position over time in small cage, low versus high density experiment.

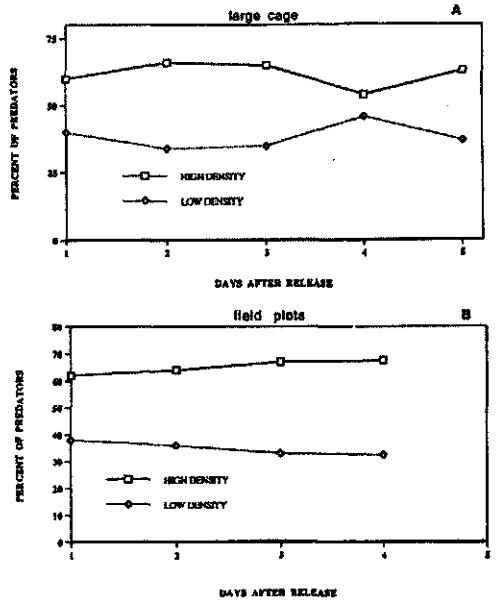

Fig. 4 - Predator position over time. A = large cage experiments; B = field experiments. In both A and B, one-half of the plots had three egg masses per plant, while in the other plots, there were 0.1 egg masses per plant.

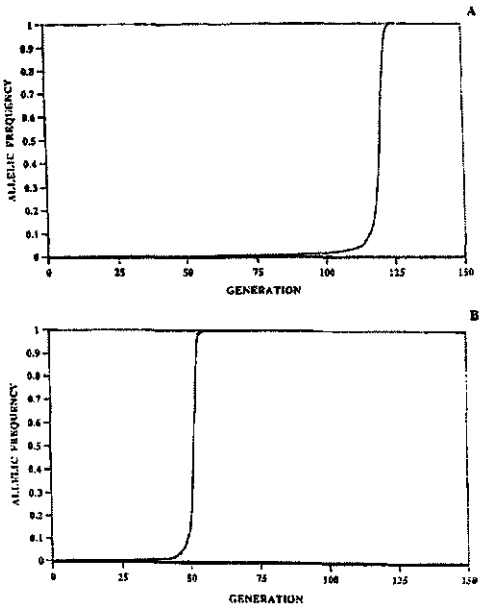

Fig. 6 - Results of single locus genetic model with fitness values set based on A) data from the field experiment (Table 4), or B) the assumption of equal predation in high *L. decemlineata* density, non-Bt plots, and in low density Bt plots.

fields because they were not affected by the Bt, but suffered mortality from predation. The RS and SS genotypes had a fitness of 0.1346 in the non-Bt plots just like the RR genotypes. In Bt plots, however, the fitness of the RS genotypes were estimated to be (0.005)X(0.1346) = 0.000673, and the fitness of the SS genotypes was estimated to be (0.001)X(0.1346) = 0.000135 because of high toxin induced mortality. Assuming that a beetle population lives in an area with one half Bt plots and one half non-Bt plots, the average relative fitness of the RR, RS and SS genotypes would be 0.2359, 0.1689, and 0.01687 respectively. When these values were used to initialize the computer model, it took 118 generations for the the resistance allele to reach a frequency of 0.5 (Fig. 6A). When

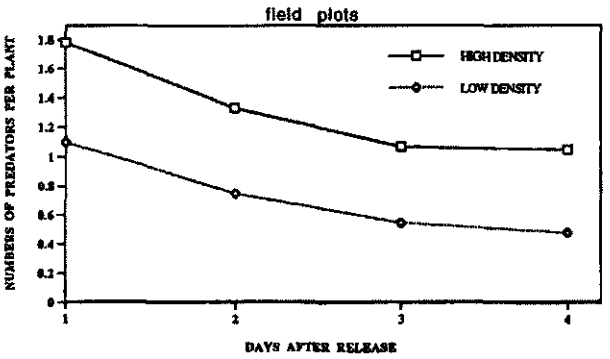

Fig. 5 - Natural decrease of *C. maculata* population over time in experimental fields. This decrease could have been due to dispersal and/or mortality of the beetles.

Table 2. Mean percentage of *Coleomegilla maculata* found on the higher density plant at different egg mass densities in the small cage experiments. The percentage on the control plant is equal to 100% minus the percent on the treatment plant because predators that were on neither plant were excluded from the analysis.

Presence-absence test		**Low versus high density**	
No. egg masses	Percent *C. maculata*	No. egg masses	Percent *C. maculata*
20	81.40	20	73.78
10	78.29	10	63.36
3	71.85	3	61.89
1	65.19	1	53.70

Table 3. Daily percent egg consumption by *Coleomegilla maculata* when given a choice of one plant with low egg mass density and one plant with higher egg mass density in small cages.

Egg mass densities	**Mean consumpion (%) (± standard error)**		**Ratio between percent predation on egg masses on high and low density plants****
	Low density	High density	
1 versus 20	33.62(±4.73)	19.46(±0.94)	1.90
1 versus 10	49.56(±6.54)	28.02(±2.21)	1.76
1 versus 3	49.46(±6.61)	52.68(±4.99)	1.12
1 versus 1*	65.43(±5.61)	74.97(±3.92)	0.94

* In the 1 versus 1 treatment, the 'high density plant' was arbitrarily assigned.
** Ratios were computed for each experimental replicate and then the mean was determined. Therefore, simply dividing the mean percent consumption of egg on the low density plants by the mean percent consumption of the high density plants will not give the values in this column.

Table 4. Percentage (±S.E.) of eggs eaten per day in low (0.1 egg masses/plant) and high density (3 egg masses/plant) plots in tests conducted in a greenhouse and in the field

Greenhouse experiments[a]			Field experiments[b]	
	Treatment		Treatment	
Day	Low density	High density	Low density	High density
1	22.70(±6.17)	14.61(±3.20)	30.54(±5.57)	20.04(±5.58)
2	5.59(±6.17)	7.88(±3.36)	31.71(±4.22)	17.61(±2.31)
3	10.26(±5.32)	14.44(±7.91)	50.17(±6.45)	27.86(±2.18)
4	10.97(±8.98)	11.53(±6.96)	44.81(±13.73)	24.84(±6.69)
5	26.07(±12.42)	11.88(±4.21)		

[a]Average of three experiments.
[b]Average of two experiments.

the same genetic model was run under the assumption of equal mortality due to predation in Bt and non-Bt fields (50% in both) the model indicated that it would take only 52 generations for the resistance allele to reach a frequency of 0.5 (Fig. 6B).

Discussion

Changes in feeding rate and movement are fundamental responses of natural enemies to changes in prey density. These responses determine a natural enemy's effectiveness in controlling insect pest populations (e. g. Huffaker and Messenger, 1976; Hassel, 1978). To properly assess these characteristics in specific predators, experiments must be conducted over a range of spatial scales (Heads and Lawton, 1983; Freeman and Smith, 1990). Hodek (1973) pointed out different feeding responses of the same coccinellid species when tested in laboratory experiments and also in a field situation. Ives *et al.* (1993) highlighted the importance of examining different spatial scales to examine *C. maculata* movement appropriately.

In order to collect adequate information on *C. maculata* interactions with *L. decemlineata* egg masses when these egg masses were the only source of food, both feeding response and predator movement were studied at different spatial scale (i.e. plant-to-plant and plot-to-plot prey density variation). Our results indicate that *C. maculata* distribution at a plant-to-plant spatial scale, as well as in potato fields, is driven by *L. decemlineata* egg mass density, but in spite of this aggregation our experiments indicate that a significant number of beetles occur in areas where food density is low. The proportion of beetles in our high density treatments, relative to our low density treatments, did not reflect the difference in prey density in a one-to-one relationship (i.e. when the high prey density was 30x the low prey density, the den-

sity of beetles in the high prey density treatments was < 30x higher than in low prey density treatments). Therefore, an inversely density dependent pattern of egg consumption was found. Our results have significant implications for biological control projects which use augmentation of *C. maculata.* While *C. maculata* will aggregate in high pest density areas, the extent of this aggregation is not sufficient to cause spatially density-dependent predation (see Huffaker and Messenger, 1976 and Hassel, 1978).

Our results are also useful for understanding the genetics of tritrophic interactions in a crop system which includes transgenic plants resistant to *L. decemlineata.* When our data were used in a genetic model to assess how *C. maculata* would affect the rate at which *L. decemlineata* adapted to Bt toxin in potatoes, the results indicated that the inverse density dependence would slow down the rate of adaptation significantly if Bt potatoes were mixed with non-Bt potatoes at a plot-to-plot spatial scale. This result makes sense at an intuitive level. In a mixed deployment of transgenic and non transgenic potatoes in the field, a much lower density of egg masses on transgenic plants is expected. If predators prey in an inversely density-dependent fashion, the likelihood of a Bt resistant *L. decemlineata* individual reaching adulthood on transgenic plants will be lowered, thus slowing down the rate at which the resistance alleles increase in frequency. Our field experiment only examined a single set of relative prey densities, and our simulation model made a number of assumptions about genetic resistance. Therefore, the quantitative impact on resistance development predicted from our work should not be considered as a general prediction. The qualitative outcome that *C. maculata* could slow down the development of resistance in plot-to-plot mixtures is somewhat more solid. We studied a simple system in which there was only one predator and one prey species. In most field situations, generalist predators such as *C. maculata* have a choice of more than one prey species. In the case of *C. maculata* in potatoes, aphids often serve as a source of prey. It will be important to know whether the results that we obtained in our one prey/one predator system would hold good in a more species-rich field environment. Our experiments and our analyses are also limited, because they did not examine how the density dependent spraying of broad and narrow spectrum pesticides would be expected to interact with the genetics of our tritrophic system. Further experiments are most certainly needed before any final conclusions can be made about how natural enemies will affect the rate at which pests adapt to engineered plants. However, our results clearly point out that adaptation to plant defenses in agricultural and natural settings does not occur in isolation and can be significantly influenced by the ecological setting in which the target plant and herbivore occur.

Acknowledgments

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Chapter 8

Predicting the Adaptation of Colorado Potato Beetle (Coleoptera: Chrysomelidae) to Transgenic Eggplants Expressing Cry3 Toxin: the Role of Gene Dominance, Migration and Fitness Costs.

Abstract

A simulation model was developed to predict the possible adaptation of *Leptinotarsa decemlineata* Say to Cry3 toxin expressed in transgenic eggplants. The use of mixed fields of transgenic and susceptible isolines at a 90:10 ratio has been simulated. Beetle movement, a fundamental feature when studying plant mixtures, has been addressed with a 'two-stage' hypothesis. Biological and genetic characteristics of the beetles have been set to specifically address their possible interactions with resistant eggplant. The role of gene dominance, migration, and fitness costs associated with the resistant genotype have been examined. Using the hypothesis of partial dominance of the resistant gene, only a high level of migration (very likely, in most agricultural areas), or a sensible reduction of the fitness of resistant beetles associated with the change in their genome can guarantee a long-lasting efficacy of the germ plasm. The simulations clearly indicate that the effect of resistance in transgenic clones expressing *Bacillus thuringiensis*-derived toxins can be optimized only in accordance with opportune agricultural practices.

Introduction

Population genetics models have often been used to choose the best strategies for deploying insect resistant germ plasm (e.g. Gould, 1986, 1994; Gould *et al.*, 1991b; Ferro, 1993; Alstad and Andow, 1995). The outcome of these simulations is different depending on the ecology and genetics of host plants and herbivores.

Given their high level of toxin production and its presence all season long, transgenic plants expressing *Bacillus thuringiensis* Berliner Cry toxins may exert a high selection pressure against insect pests, which may enable some of them to give rise to resistant strains. An indiscriminate use of transgenic plants could lead to a quick loss of effectiveness of this resistant germ plasm and affect the efficacy of *B. thuringiensis*-based insecticides as well.

The Colorado potato beetle *Leptinotarsa decemlineata* (Say), is a likely candidate to becoming resistant to Cry3 toxin, because it has shown ability to adapt to a large variety of chemicals (Forgash, 1985). Moreover Colorado potato beetle strains have already been selected for resistance to the Cry3 toxin in the laboratory (Whalon *et al.*, 1993). Although several studies have been published on *L. decemlineata*—transgenic potato interactions (Ferro, 1993;

Perlak *et al.*, 1993; Gould *et al.*, 1994; Arpaia *et al.*, 1997a), similar information on eggplant—Colorado potato beetle interactions are lacking.

In this study, a simulation model (PACE) was implemented, to predict the spread of *B. thuringiensis*-resistance genes in Colorado potato beetle populations in transgenic eggplant fields. In doing so, we used as a baseline the Mallet and Porter (1992) model which includes the effect of movement. Using the same model, Tabashnik (1994) clarified, the effect of plant mixing in delaying the pest adaptation to transgenic plants, if compared with the use of pure stands. We studied the effects of a few other important parameters (gene dominance, insect migration, and fitness costs) in the attempt to more accurately fit a general model to the *B. thuringiensis*-transgenic eggplant—Colorado potato beetle system.

Materials and Methods

Model Development. PACE (Predicting Adaptation of the Colorado potato beetle to Eggplant) is a deterministic model based on the following standard genetical assumptions about the insect population: (1) large population size, (2) random mating, (3) a single diallelic autosomal locus A (A_R for resistant and A_S for susceptible alleles) and (4) very low mutation rate, compared with the initial resistant allele frequency.

A mixed field made up by 90% transgenic and 10% nontransgenic plants is used as a general condition, no crop rotation is considered.

PACE is different from the general model of Mallet and Porter (1992) because it can allow for some phenomena more specific to the *B. thuringiensis*-transgenic eggplants—Colorado potato beetle system.

We specifically addressed the effect of movement by considering Colorado potato beetle behavior. In nature, although food is abundant, the movement of larvae is minimal and adult movement consists primarily of short localized flights (Voss and Ferro, 1989). In field experiments, Gould *et al.* (1994) observed that a considerable percentage of beetles can move between potato plants in the same row and that their movement is not influenced by the presence of Cry3 toxin on plants. The movement between rows was negligible. We considered the 'two stage model' of Mallet and Porter (1992): stages 1 (larva) and 2 (adult). For simplicity's sake, we considered only the movement of the adults at the moment in which they emerge from the soil after the pupal stage. The probability that an adult coming from the soil moves to a plant different from that where it lived as a larva is M, and (1-M) represents the probability that the adult returns to the same plant. The proportion of toxin-free plants in seed-mixture is defined as V. Following the movement, the newly emerged beetle can end up on a toxic plant or on a toxin-free plant with probabilities (1-V) and V, respectively.

Secondly, the PACE model can consider the immigration of beetles from harvested potato fields in the surroundings into the transgenic eggplant field. Immigration has been incorporated in the model, defining m as the coefficient of migration. It was considered useful to include this variable because a strong increase in beetle density in eggplant fields, following the harvest of potato fields in the surrounding area, has been observed (J.H. Lashomb, personal

communication; S. Maini and S.A., unpublished data). Unfortunately, the extent of this migration has not been specifically estimated; therefore in the simulations, we used two hypothetical and extreme values of *m*: 0.5 and 0.99. When *m* is set to 0.5 it means that after immigration the mixed population is composed of 50% individuals which survived in transgenic fields and 50% immigrants. In the second case, immigrants constitute the great majority of the population. The latter seems to be the most likely situation because the expected insect survival in a transgenic fields, regardless of the proportions of different genotypes, may be considered very low in most situations.

In Mediterranean areas, overwintering Colorado potato beetle adults usually resume their activities during the end of April—beginning of May. Normally eggplants are not transplanted in the fields until the second half of May. Beetles then start feeding on potato or wild host plants and thereupon invade eggplants. They complete three generations per year on eggplant and the third generation is active during the latter part of the growing season when potato fields have already been harvested. Movement from potato to eggplant is a continuous common phenomenon for beetles, but the largest migration coincides with the potato harvest. In the model then, the effect of migration is added every three generations of selection.

Table 1. Probabilities for beetles developing on either susceptible (S) or transgenic plant (T) in 1st and 2nd stage in a mixed field

Event	Probability
SS	$V\ M\ V + V\ (1 - M)$
ST	$V\ M + (1 - V)$
TS	$(1 - V)\ V\ M$
TT	$M\ (1 - V)^2 + (1 - V)\ (1 - M)$

V, percentage of susceptible plants in the mixture;
M, probability of moving to a different plant during the 2nd stage.

Immigrant individuals might never have been faced with Cry toxins before. In this case the same initial gene frequency is hypothesized for both immigrant and resident Colorado potato beetle individuals. In other cases, immigrant individuals may have already been under selection pressure because of exposure to sprays with *B. thuringiensis*-based insecticides or previous feeding in *B. thuringiensis*-transgenic fields in the surrounding area. To consider the second possibility, different gene frequencies (referred to as *z* in the model) for the immigrant population have been simulated.

Finally, the model can allow for the possible action of a specific selection against the resistant gene A_R when considering insects feeding on toxin-free plants. Selection against resistant allele in toxin-free plant systems could be realistically hypothesized because Ferro (1993) has reported that a Colorado potato beetle strain resistant to *B. thuringiensis* has shown reduced fertility compared with nonselected strains. The phenomenon of fitness costs associated with resistant traits was also found in other Colorado potato beetle strains (Argentine *et al.*, 1989), hence we assume that these traits can be metabolically costly for the species.

Having considered the resistance to the Cry toxin as controlled by a single locus A with 2 alleles (Ar and As), 3 genotypes are possible: AsAs (susceptible), ArAs (heterozygous), and

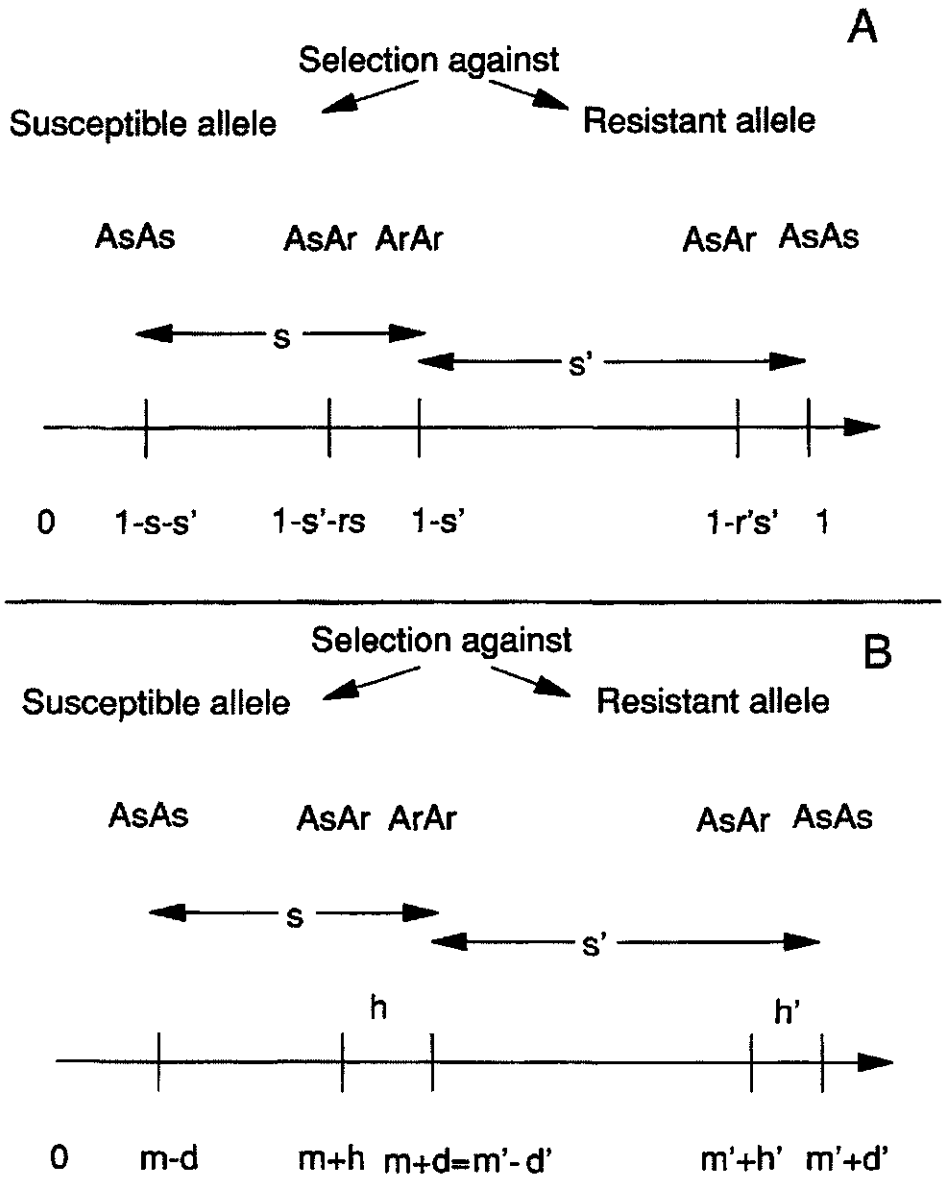

Fig. 1 - Fitness of the three possible genotypes in case of resistance against either the susceptible or the resistant allele. (A) Genotypic scale. (B) Phenotypic scale.

ArAr (resistant). Using the same probability tree of Mallet and Porter (1992), it is possible to calculate for an insect its chance of spending: the larval stage on a toxic plant and the adult stage on a toxin-free plant (TS); the larval stages on a non-toxic plant and the adult stage on a toxic plant (ST); all stages on toxic plants (TT) or on toxin-free plants (SS).

These probabilities are found in terms of V and M. The four possibilities and the relative probabilities are reported in Table 1. When selection acts against the susceptible allele, the relative fitnesses are 1 for ArAr, 1-s for AsAs, and 1-rs for AsAr. The selection coefficient *s* can assume two different values for larvae and adults, because of their different sensitivity to the toxin (Johnson *et al.*, 1993) and it will be indicated as s_l and s_a for larvae and adults, respectively.

The relative fitnesses of the 3 genotypes when the selection acts against the resistant allele are 1 for AsAs, 1-s' for ArAr and 1-r's' for AsAr. These selective values only apply to insects feeding on toxin-free plants. Now if we assume, firstly, that the fitness of the AsAs genotype is the highest possible in absence of selection (i.e., absence of toxin), and, secondly, that the fitness of the ArAr genotype is always the same regardless of the kind of selection acting (i.e., type of plant it is feeding on), it is possible to derive the fitness of the 3 genotypes for the two selection situations expressed relatively to the maximum value of fitness. The fitness of AsAs when selection is against Ar gene will be 1 and the fitness of ArAr will always be 1-s', regardless of the direction of selection acting. The postulated model is described in Fig. 1A.

The coefficient of selection s applies when the AsAs is the unfavoured genotype, and s' is the proportionate reduction in the gametic contribution of ArAr compared with AsAs when selection acts against Ar. The fitness of the heterozygotes in the two situations can be derived in terms of r , r', s and s'. The r and r' can be defined as the proportionate reduction of the strength of selection (s and s') for the heterozygotes in the two cases of selection, or as the degrees of dominance of fitness. As stated by Falconer (1989), 'dominance, in this context, means dominance with respect to the fitness, and this is not necessarily the same as the dominance with respect to the main visible effects of the gene'. However, in this specific case, the resistance of a genotype to the toxin can be measured as its ability to survive and reproduce in the presence of the toxin, or, in other words, the resistance is an indirect measure of fitness. We can consider then, that the dominance shown by the gene with respect to the fitness is the same as the dominance shown by the gene with respect to its visible effects (e.g., the resistance to the toxin measured as LC_{50}). The coefficients r and r' can be derived in terms of the corresponding coefficients of dominance of gene. As defined by Mather and Jinks (1982), the phenotypic difference between AsAs and ArAr and the departure of AsAr phenotype from the midpoint between the two homozygotes (m) can be represented by the two parameters d and h. Thus, the phenotypes of the two homozygotes are m+d and m-d, whereas that of the heterozygote is m+h. In all cases, d is positive, whereas the sign of h will depend on the degree of dominance; if there is no dominance h = 0, if dominance is positive h > 0, if dominance is negative h < 0.

The degree of dominance is h/d = D. It is quite simple to show that s = 2 d and that rs = d - (h). If we now write r = (d-h)/2d; r = 1/2(1-h/d); r = 0.5(1-D). The coefficient r can be

expressed in terms of the coefficient of dominance of the gene, D. Thus, if dominance is absent (D = 0) it follows that r = 0.5 and the fitness of AsAr is 1-0.5s.

However, in this specific case, the phenotypes of the three genotypes (the mortality, the LC_{50}, or the number of eggs laid) change in relation to the presence or the absence of the toxin. Therefore, for the same locus A we assume two different parameters d and h. In a situation like this, the genetical analysis of the character would be better carried out by introducing two more parameters, one accounting for the environmental factors and the other for the interaction between genetical and environmental components. However, we made a simplification of the model and considered for the same locus two sets of parameters. So that, in the case of selection against the susceptible alleles the phenotypes of the three genotypes, AsAs AsAr and ArAr, would be m-d, m+h and m+d, whereas in the case of selection against the Ar, the phenotypes of the 3 genotypes would be m'+d', m'+h', and m'-d'.

The relative fitness can be used to derive the cumulative fitness of the three genotypes for each of the four situations an insect can face (SS, ST, TS, TT) (Table 2). The overall relative fitness for each of the three genotypes is calculated by multiplying the fitnesses of each genotype in any specific situation by the probabilities of each situation occurring and then summing up the four.

Table 2. Stage specific fitnesses for the 3 genotypes

Event	AsAs	AsAr	ArAr
SS	1	$(1-r's')^2$	$(1-s')^2$
ST	$(1-s_a-s')$	$(1-r's')\ (1-s-rs_a)$	$(1-s')^2$
TS	$(1-s_l-s')$	$(1-s'-rs_l)\ (1-r's')$	$(1-s')^2$
TT	$(1-s_a-s')\ (1-s_l-s')$	$(1-s'-rs_a)\ (1-s'-rs_l)$	$(1-s')^2$

s_a coefficient of selection against susceptible adults;
s_l, coefficient of selection against susceptible larvae;
s' coefficient of selection against resistant beetles;
r, proportionate reduction of strength of selection s;
r', proportionate reduction of strength of selection s'.

Using the same notation of Mallet and Porter (1992), the overall relative fitness of AsAs, AsAr, and ArAr insects across the two selection situation, WSS, WSR and WRR respectively, are then

$WSS = VMV + V(1-M) + V M (1-V)(1-s_a-s') + V M (1-V)(1-s_l-s') + [M(1-V)^2 + (1-V)(1-+M)][(1-s_a-s')(1-s_l-s')]$;

$WSR = [VMV + V(1-M)](1-r's')^2 + V M (1-V)(1-r's')(1-s'-rs_a) + V M (1-V)(1-r's')(1-s'-+rs_l) + [M(1-V)2 + (1-V)(1-M)](1-s'-rs_a)(1-s'-rs_l)$;

$WRR = \{[VMV + V(1-M)] + 2[V M (1-V)] + [M(1-V)_2 + (1-V)(1-M)]\}(1-s')^2$.

Changes in the Ar gene frequency were calculated by using the following expression:

$qi = [q^2i\text{-}1\ WRR + qi\text{-}1\ (1-qi\text{-}1)\ WSR]/[q^2i\text{-}1\ WRR + 2\ qi\text{-}1\ (1-qi\text{-}1)\ WSR + (1-qi\text{-}1)^2\ WSS]$.

The initial frequency of resistance allele was set at 0.0001.

Computer Program. The PACE model was implemented as a C++ program for DOS. The requested inputs are the susceptible plant percentage (V), to differentiate between a completely transgenic eggplant field and a mixed field; M to define the probability of insect movement at the second stage; s_a, s_l , and s' to choose the selection pressure acting against As and

Ar; r and r' value to define the fitness of heterozygotes; m to consider the effect of migration and z to establish the initial allele frequency in the migrant individuals.

The output of the model is a plot of allelic frequency variation over the Colorado potato beetle generations.

Results

Figure 2 shows the different adaptation pattern under the hypothesis of partial dominance [*L. decemlineata*, Rahardja and Whalon, 1995], partial recessiveness [*Plutella xylostella* L., Tabashnik *et al.*, 1992], and codominance [*Heliothis virescens* (F.), Gould *et al.*, 1992] of the Ar allele. The difference on the effect of adaptation is obvious and if the hypothesis of partial dominance is correct, the beetles may adapt very quickly.

If beetle migration is taken into account, the adaptation is slowed down with a periodical dropping of the resistance allele frequency in the population every three generations (Figs.

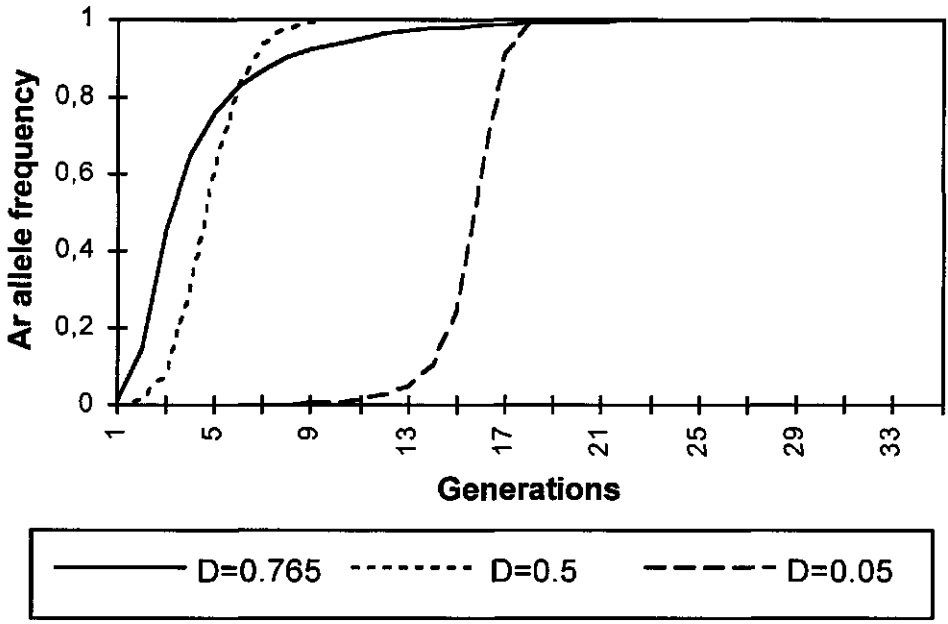

Fig. 2 - Change in Ar allele frequency over time in case of different levels of dominance of the resistance gene in an adapted beetle strain. A mixed field of 90% transgenic and 10% susceptible eggplants is hypothesized. D, dominance (see text).

3—4). The extent of the phenomenon is also affected by the degree of dominance of the gene. In the case of a partially recessive trait, the adaptation may be delayed for ≡12 generations as is seen in the case of low levels of z (Fig. 3A). A higher level of migration will keep the allele

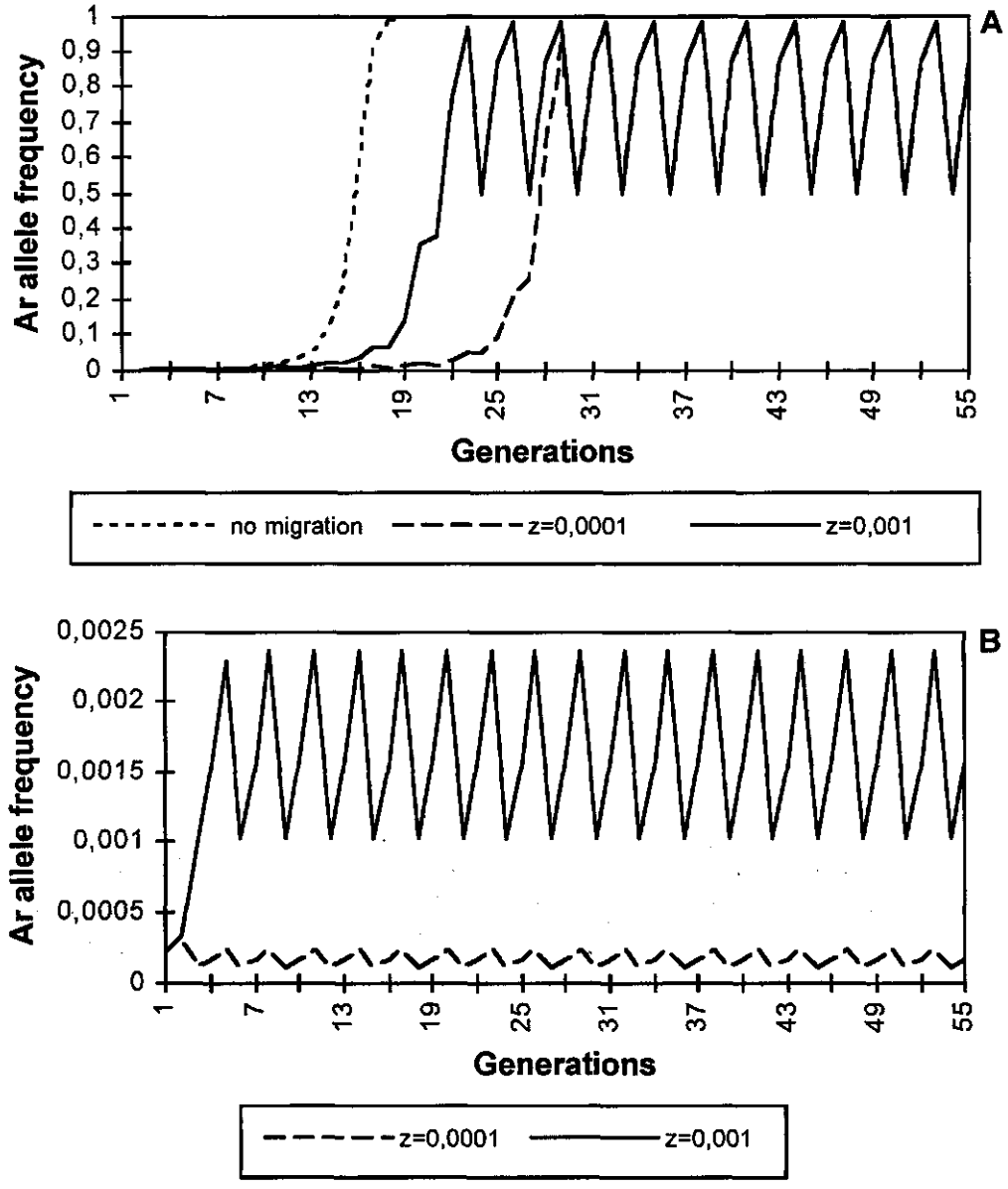

Fig. 3 - Change in Ar allele frequency over time in case of partial recessiveness of the resistance gene (D=0.05) in an adapted beetle strain with the effect of immigrant beetles considered every three generations. z, Ar allele frequency in the immigrant beetles. (A) m=0.5 (B) m=0.99.

frequencies below 0.002 independently of the allele frequency of migrants (Fig. 3B).

In the case of partial dominance, at a migration level of 0.5, the beetles will adapt to the toxin in a time lag comparable with what hypothesized in absence of migration (Fig. 4A). The level of migration of 0.99 will instead always keep the allele frequency below 0.5 (Fig. 4B). If the hypothesis of partial dominance holds, the allele frequencies of migrant beetles will not be critical, because their contribution is hidden by the stronger effects of migration and dominance.

When the possibility of fitness costs is added to this scenario, even more possibilities exist. Also, in the case of low levels of migration, a fitness cost as high as 90% will keep the Ar allele frequency very low and will avoid adaptation (Fig. 5).

The combined effect of fitness costs and a high level of migration are illustrated in Fig. 6, which shows the most favorable conditions.

Discussion

Transgenic plants resistant to insects may become a valuable means of pest management. Great care should be given to their deployment in the environment if we are to utilize their potential properly. Moreover, only a long-lasting commercial life can compensate for the expensive research and development required to obtain such new varieties. The expression of resistance in transgenic plants can be generally assimilated to vertical resistance, hence one of the main concerns is a possible onset of resistance to the toxin in the insect population. The spread of resistance genes in the pest population is affected by the ecology and genetics of insect pests and also the agricultural practices involved. Therefore, each single case needs to be specifically investigated.

In a previous study (Ferro, 1993) a fast adaptation by the Colorado potato beetle to *B. thuringiensis*—transgenic potatoes was predicted, raising serious doubts about the reliability of this pest management strategy. Yet by contrast, quite different results were hypothesized by Gould *et al.* (1994).

The Colorado potato beetle—eggplant interactions are characterized by specific phenomena that make the possible evolution of resistance very different from the case of Colorado potato beetle-potato interactions. First of all, in areas in which both potato and eggplant are available, late in the season eggplant fields are regularly affected by the immigration of Colorado potato beetle adults which come from harvesting potato fields. Immigration from neighboring areas is a variable phenomenon, because this is regulated by many different parameters, such as the nutritional status of migrating individuals, distance from the field and weather conditions (e.g. Weber *et al.*, 1995). Nevertheless, in agricultural areas such as the Mediterranean zone, the ratio between surfaces cultivated with potatoes and with eggplants varies between 6:1 and 50:1; consequently, even though part of the beetles will enter diapause after the harvest of potatoes, the beetles moving toward eggplant fields usually outnumber the resident population in eggplant fields greatly.

Secondly, the eggplant has a fairly high economic injury level (Cotty and Lashomb, 1982; Arpaia *et al.,* 1995) which could prevent from spraying in nontransgenic refuge zones set in the fields, allowing a large mixing between the two sub-populations.

We think that Colorado potato beetle movement in mixed transgenic fields can be well studied by using a two stage model (Mallet and Porter, 1992). However, this model does not match the biology of the Colorado potato beetles in the way the authors have indicated. Because larval movement between plants is an unusual phenomenon under normal conditions, we set the adult as the 'second stage' in the model. The adults may move more than once, but for the sake of simplicity this further complication was not studied during our simulations.

Different simulation models have clearly shown that deployment of resistant clones in pure stands can induce a fast adaptation of the pest to the toxin (e.g., Gould, 1986; Tabashnik, 1994). However, it is not that simple. Whalon and Wierenga (1994) demonstrated that first instars from a laboratory strain of *L. decemlineata* showing 400-fold resistance to the Cry3A toxin, could not survive on transgenic potatoes. Thus, toxin expression in transgenic plants may determine a different effect on the beetle populations.

We ran simulations with the PACE model to assess the effectiveness of the mixed field strategy in delaying the adaptation by Colorado potato beetle to *B. thuringiensis*—expressing transgenic eggplants. In a mixed field, a low percentage of susceptible plants was indeed able to avoid the adaptation of the Colorado potato beetle to resistant clones in the several situations analyzed. This indicates that the mixing approach may be used to render resistance genes functionally recessive and also in the case of a medium level of toxin expression. The extent of the migration, together with low survival in transgenic areas, causes a considerable reduction of the allele frequency in the population every 3 generations.

Among the other biological features of the Colorado potato beetle included in our simulations, the fitness costs associated with the presence of the resistance gene have a fundamental role in this interaction and determine the lack of adaptation at even lower migration levels.

In some cases, the assumption of random mating cannot be correct because of siblings mating or the asynchrony of emergence between resistant adults from toxic plants and susceptible adults from toxin-free plants (Tabashnik, 1994). If such an event is ascertained, the model can be easily modified to account for the partial inbreeding in the population. With considerable deviations from a completely random mating system, this beetle may adapt to transgenic plants at an even faster rate. However, biological observations (e.g. Williams, 1988; French *et al.,* 1993) seem to confirm that the assumption of random mating for *L. decemlineata* is a reasonable one.

Our hypotheses necessarily need to be confirmed by extensive field observation. When more of this becomes available from studies in transgenic fields, more accurate indications can be retrieved by PACE simulations.

The results obtained in the current study indicate that the use of *B. thuringiensis*-expressing eggplant clones are fruitful and confirm that benefits will be maximized only in case of rational use of transgenic clones to hamper the selection of resistant beetles. As reported by Tabashnik (1994), the evolution of resistance can also be delayed by using temporal

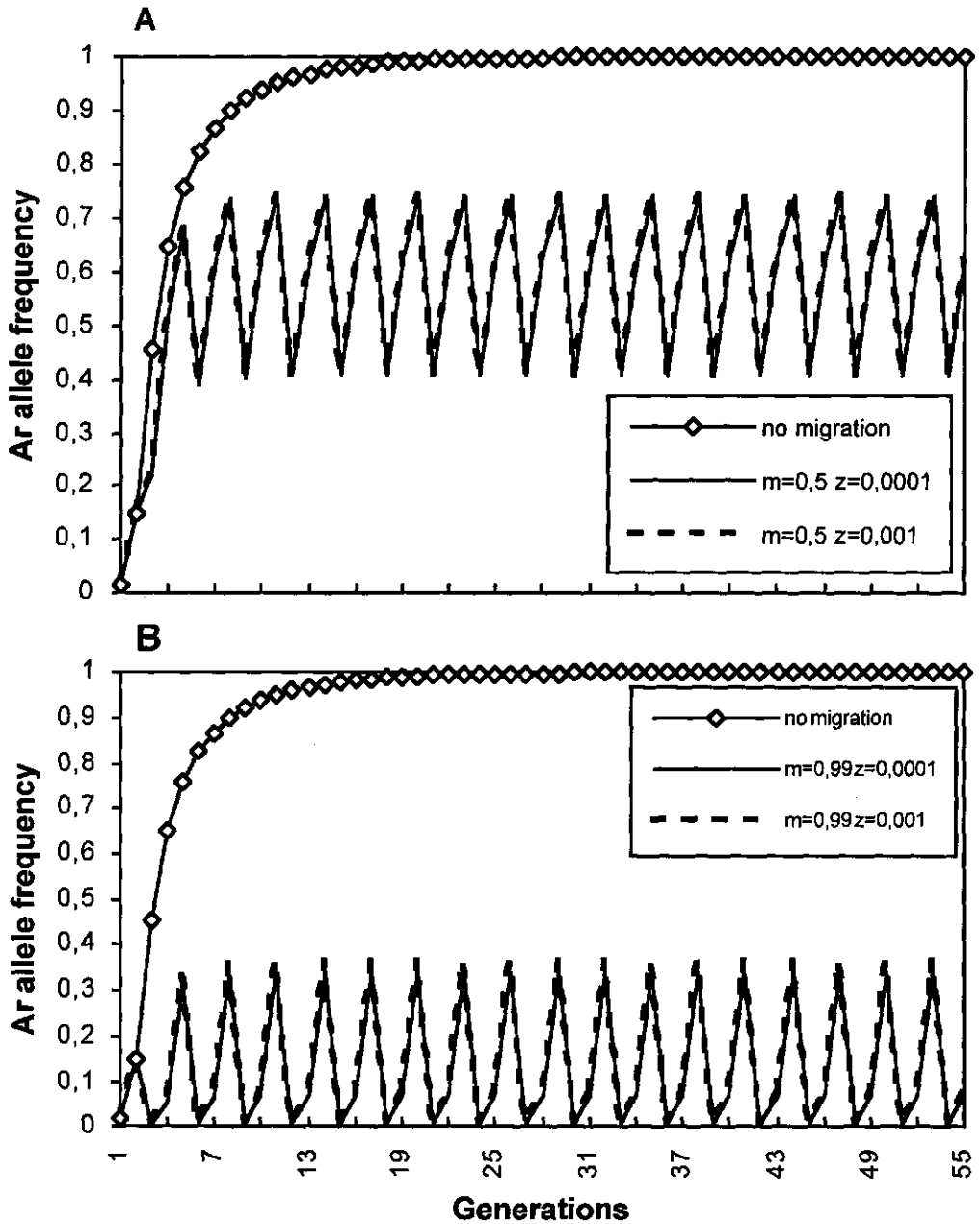

Fig. 4 - Change in Ar allele frequency over time in case of partial dominance of the resistance gene (D=0.765) in an adapted beetle strain at different levels of migration. z, Ar allele frequency in the immigrant beetles. (A) m=0.5. (B) m=0.99. Lines 2 and 3 exactly overlap.

refuges for susceptible insects. These refuges can be implemented in several ways, either through rotation with toxin-free varieties or else with different crops, or more technologically, through the use of transgenic plants with temporal expression control. In addition to this, our results seem to indicate that in regions where an alternative host for the target insect is cultivated on much larger areas (e.g. potato) the use of transgenic plants would not be risky. In such a situation, potato fields would provide an unlimited source of susceptible alleles, thus reducing the chance of the resistant allele to become fixed.

Similar studies on each specific agro-ecosystem are needed to furnish consistent guidelines for both growers and companies, using transgenic plants resistant to insects.

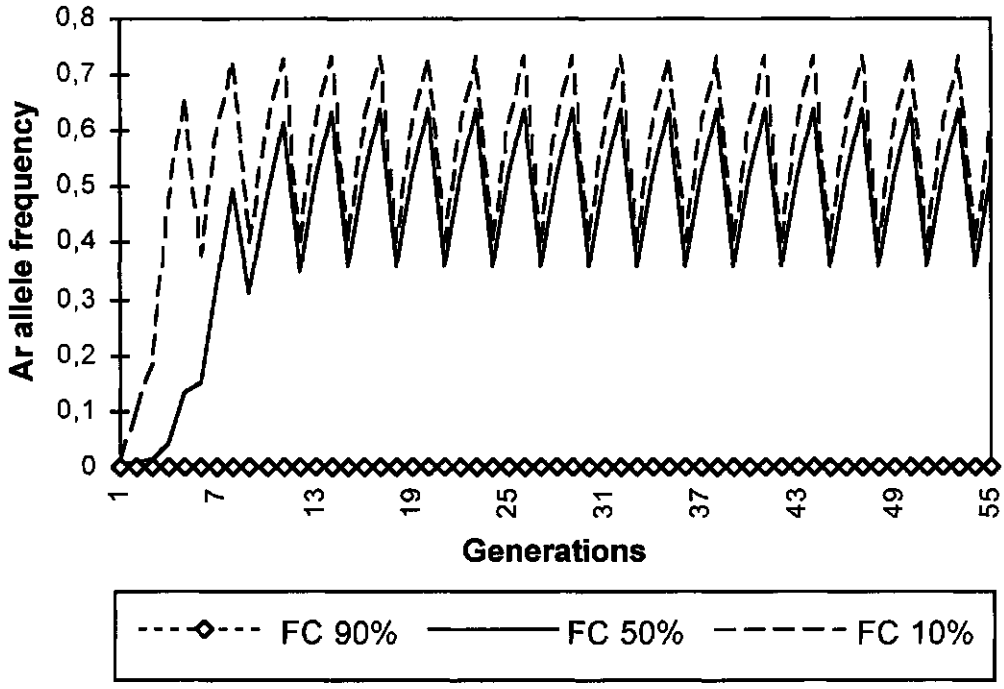

Fig. 5 - Change in Ar allele frequency over time in case of partial dominance of the resistance gene (D=0.765) and m=0.5, if fitness costs linked to the resistant trait are hypothesized. FC, fitness costs.

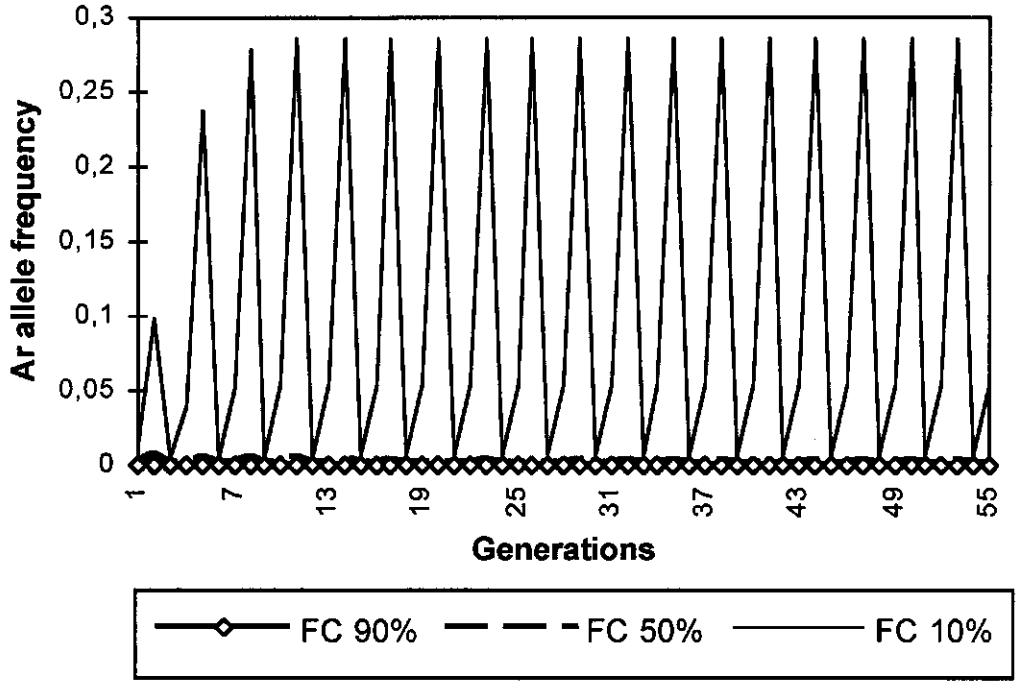

Fig. 6 - Change in Ar allele frequency over time in case of partial dominance of the resistance gene (D=0.765) and m=0.99, if fitness costs linked to the resistant trait are hypothesized. FC, fitness costs.

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Chapter 9

Conclusions

Eggplant is an intensive, high value horticultural crop. It is widely distributed in the Mediterranean area and in Italy it is cultivated both in open air and in greenhouse, especially grown in southern regions. Due to the large contribution of greenhouse cultivations, eggplants have an almost year round availability. The crop acreage in Italy is about 11.000 Ha, with an average yield of ≅ 29 MT/Ha. Several pathogens and insects can cause serious damage to the crop, then a fairly large amount of pesticides is often used. In a recent survey (Osservatorio per le malattie delle piante di Acireale, 1997), conducted on eggplant fruits obtained from greenhouse cultivations in Sicily, the 15.9 % of samples contained pesticide residues in amounts exceeding the maximum level legally allowed.

The aim of this doctoral thesis was to explore the possibility of an effective control of Colorado potato beetle, namely on eggplant, by means of transgenic plants expressing a *Bacillus thuringiensis*-Cry3B toxin, obtained via *Agrobacterium*-mediated transformation. I have followed a step-by-step research, from the laboratory determination of toxic activity in row protein extracts, to the field assessment of resistance characters.

The main conclusion is that transgenic *Solanum melongena* plants have proven to be effective in controlling *Leptinotarsa decemlineata* infestations, while keeping all the agronomic characters of the original eggplant accessions.

Some aspects of the toxic activity of the Cry3B toxin on the Colorado potato beetle have been pointed out. No antifeedant effect due to the *B. thuringiensis* Cry3B toxin was found on larvae, even at concentrations which caused mortality or severely inhibited larval growth. The same conclusion was reached when adult feeding behavior on transgenic leaves was observed.

While adult Colorado potato beetles do not suffer of acute poisoning as neonate do, they are affected by feeding on Bt-expressing plants and this effect is largely dependent on the sex of the beetle. Beetles feed for some days on resistant clones causing limited damage, but usually no offspring are produced. After feeding on transgenic plants, males are still able to mate and produce mobile sperms. The different effect suffered by the two sexes can be explained by the lowered capacity of accumulating protein in the haemolymph which hinder females from normally developping their ovaries.

According to the present study, two major points are fundamental for the correct use of resistant lines.

1. Adaptation to the toxin.

One of the major concerns linked to the widespread use of transgenic plants expressing

Bacillus thuringiensis toxins is the onset of resistance to the toxin in the populations of the target insect. In the case of *L. decemlineata*, the lack of feeding inhibition by Bt-expressing plants may limit strategies for producing transgenic plants with a low selection pressure against the target insect in the field.

In Chapter 8, I have described the implementation of PACE, a mathematical model aimed at the study of the possible adaptation by *Leptinotarsa decemlineata* to Bt-expressing eggplants. It was clearly highlighted that a wise use of transgenic plants can keep their resistance longlasting and effective, because the resistance allele frequency can be maintained at low levels by means of plant mixing and due to beetle biology. Preliminary field observations with our transgenic eggplant lines seem to indicate that an intraplot mixture, made of 80% transgenic plants and 20% control plants, is effective in controlling beetles and may even lead to comparable yields with plots made of pure stands of transgenic plants. Similar results were obtained by Riggin-Bucci and Gould (1997), when an intraplot mixture of *Bacillus thuringiensis* treated and untreated collard plants was prepared, in order to study its effect on *Plutella xylostella* L. population dynamics. Beetle biology, and especially migration and fitness costs linked to the resistant allele, will have a major role in spreading the resistant allele in the beetle population. Further versions of PACE will incorporate a sex-dependent effect on fitness (see Chapter 5) and more recent observations on fitness costs in resistant *Leptinotarsa decemlineata* populations (Trisyono and Whalon, 1997) and also movement in the field. Unlike the case of the Colorado potato beetle, Tang *et al.* (1997) found a lack of fitness costs in *Plutella xylostella* populations resistant to the cryI toxin.

Therefore, the study of the possible adaptation by a target insect to Bt-expressing transgenic plants need to be faced case by case, so as to define the best strategy according to the insect's biology and its relationships with the host plant.

2. Integration of transgenic plants with other means of pest control.

Transgenic plants resistant to *L. decemlineata* may be successfully employed in combination with other means of control. As reported in chapter 7, the effect of feeding activity by the predator *Coleomegilla maculata* on *L. decemlineata* eggmasses in conditions of mixed plantings may delay the adaptation of the target insect to the toxin expressed in transgenic clones. Moreover, the field study presented in chapter 6 revealed an even distribution of lacewings which represents the only relevant Colorado potato beetle predator in the area, in both transgenic and control plots. While no specific attempt has been made to isolate the contribution of these predators to the control of the beetle (due to the general low level of their population), the regular presence of eggs, larvae and adults in transgenic areas allowed me to assume an active presence in transgenic fields. These observations seem to indicate that transgenic eggplants might be compatible with the use of beneficial arthropods. A relevant flow of literature is recently being published confirming that in several cases this interaction between resistant plants and natural enemies can be fruitful (e.g. Johnson, 1997; Mascarenhas and Luttrell, 1997; Orr and Landis, 1997; Pilcher *et al.*, 1997b). Other reports in contrast, furnished

different indications about the compatibility of Bt-transgenic plants with herbivore's natural enemies. For example, Hilbeck *et al.* (1998) found a significantly higher mortality of *Chrysoperla carnea* Stephens larvae reared on Bt-affected lepidopterous larvae than when raised on Bt-free prey.

One clear indication of these studies however, is that the management of each whole agroecosystem needs to be taken into consideration, to determine the best indications for field deployment more accurately. Transgenic germ plasm should be thoroughly handled by companies, growers, extensionists, etc., to preserve a longlasting field efficacy of the resistance characters and also prevent the loss of effectiveness for all the Bt-based insecticides.

In 1996 for the very first time, several new agricultural biotechnology products developed in the United States entered the marketplace nationally and internationally, both as produce and as bulk commodities. Ongoing bilateral environmental consultations with the Commission of the European Union continue, to discuss policy developments on the commercialization of, and trade in, new agricultural commodities. A project on the commercialization of agricultural products derived through modern biotechnology was also started. The aims of this project, which was initiated in 1994, are to assist member countries in their oversight of these organisms, and more specifically in their efforts to ensure safety measures to make oversight policies more transparent and facilitate trade. A key work area has been the development of scientific consensus documents on specific topics relevant to the environmental biosafety of transgenic plants, such as the biology of particular crop plants and issues associated with the introduction of particular traits in plants.

The biosafety of transgenic plants and products represents the main scientific issue to be faced nowadays before a widespread use of these plants may be truly desirable. I have conducted preliminary studies with the Cry3B toxin expressed in transgenic plants. These observations have shown that the presence of transgenic plants expressing this toxin is compatible with the activity of pollinators, such as the honeybee *Apis mellifera* L. (Arpaia, 1996). It has also been shown that the toxin does not have binding sites in the gut tissues of mammals (Arpaia and Noteborn, 1998). The continuation of these studies however, goes far beyond the aims of this thesis.

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