Combining genetic engineering and traditional breeding to provide elevated resistance in potatoes to Colorado potato beetle

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Abstract

The sustainable deployment of resistant crop varieties is a critical issue for the implementation of biotechnology in crop pest management. Feeding, biomass accumulation, and mortality were evaluated for susceptible, insecticide-resistant, and *Bacillus thuringiensis* (Bt) Cry 3A-selected Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera, Chrysomelidae) larvae fed on: cultivated potato, a *Solanum chacoense* line expressing leptine glycoalkaloids, a transformed line expressing Bt toxin, or the leptine line transformed to express Bt toxin. Larvae selected for resistance to Bt-Cry3A performed better on Bt foliage, but not as well on the leptine foliage, compared to susceptible or insecticide-resistant larvae. Neither leptine nor Bt toxin completely inhibited the feeding and growth of 3rd and 4th instars of all three strains of Colorado potato beetle. However, for all three strains of Colorado potato beetle on leptine + Bt foliage, feeding was almost zero, growth was zero or negative, and mortality was near 100%.

Introduction

The deployment of genetically engineered insect-resistant crop varieties is a critical issue for the implementation of biotechnology in crop pest management (Gould, 1998). Mostly single host plant resistance factors are available commercially (*Bacillus thuringiensis* Berliner toxins), so current discussion emphasizes a high dose/refugia model for managing the adaptation of insect pests to resistant crop varieties (Whalon & Norris, 1999). According to Shelton et al. (2002), the high dose/refugia model is the 'only strategy currently available'. Regulations in place for the deployment of Bt transgenic crops in the USA are based on this model (Anonymous, 2001), but there are serious concerns about the level of compliance (Jaffe, 2003). A model using both genetically engineered and traditionally bred host plant resistance has the potential to be more durable.

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The major biological concerns surrounding crops engineered to produce *B. thuringiensis* (Bt) toxins are sustainability and management. Continual exposure to Bt toxins from biopesticides, transgenic crops, and laboratory selection has led to the development of resistance in several species (Tabashnik, 1994; Ferre & Van Rie, 2002). The rate of a pest's resistance development is positively correlated with increasing selection pressure (Tabashnik et al., 1990). Transgenic crops can increase selection pressure compared to foliar sprays of the pesticide toxin by: (1) increasing the level of toxin exposure to the pest, (2) producing toxin over a long period in all plant parts, and (3) increasing the acreage of crops expressing Bt (Gould, 1998; Hilder & Boulter, 1999; Whalon & Norris, 1999).

The higher dose of transgenic plants, along with the rising acreage of Bt plants using the same or similar toxin can increase selection pressures on an insect pest (Hilder & Boulter, 1999). From 1996 to 2003, Bt cotton acreage has increased from 12% to 75% in the USA (Anonymous, 2003a). From 1996 to 2003, Bt corn acreage has also increased from 1% to 40% in the USA (Carpenter & Gianessi, 2001; NASS, 2002; Anonymous, 2003a). With the acreage of Bt crops

increasing, it is important to evaluate methods which preserve Bt and other host plant resistance mechanisms.

Host plant resistance management methods typically fall into one of three categories: (1) maintaining a susceptible insect population through seed mixtures, refuges, and crop rotation, (2) using trap crops to attract pests away from more economically important crops, and (3) combining different toxins, assuming the insect is less likely to develop resistance to more than one toxin simultaneously (Neppl, 2000). These tactics can delay resistance by orders of magnitude (Neppl, 2000). Combined toxins can be employed by combining insecticides with host plant resistance factors, or by 'stacking' host plant resistance factors into plants (Mani, 1985; Roush, 1998). We have focused on combining multiple host plant resistance factors into a single plant using both classical breeding and genetic engineering.

The Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera, Chrysomelidae) feeds on plants in the Solanaceae family, including potato (*Solanum tuberosum* ssp. *tuberosum* L.), tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), and nightshade (*Solanum nigrum* L.) (Solanales, Solanaceae) (Jacques, 1988). It is highly adaptable, and is a model organism for studying resistance development. The Colorado potato beetle has consistently demonstrated an ability to adapt to insecticides: it has developed resistance to every insecticide used to control it, and is presently resistant to 37 insecticides (Bishop & Grafius, 1996; Whalon et al., 2000). The beetle's ability to become resistant to insecticides may be related to its ability to tolerate the glycoalkaloids produced by potatoes and other Solanaceae. Gylcoalkaloids are cholinesterase inhibitors, functioning much like organophosphate and carbamate insecticides (Lawson et al., 1993; Rangarajan et al., 2000). Colorado potato beetle has also adapted to Bt-Cry3A in the laboratory, suggesting it may have the ability to do so in the field (Whalon et al., 1993). This underscores the importance of evaluating deployment strategies for varieties resistant to Colorado potato beetle. We also believe the potato/Colorado potato beetle system will be a vigorous model for the deployment of other pest resistant crops.

The cultivated potato naturally produces glycoalkaloid compounds, which are associated with deterring Colorado potato beetle feeding (Sinden et al., 1980; Sinden et al., 1986). While high glycoalkaloid levels could be useful host plant resistance factors, at high concentrations they impart a bitter taste in the tuber, and can induce nausea and vomiting in mammals (Sinden & Webb, 1972; Van Gelder, 1990). Most glycoalkaloids are distributed throughout the potato plant tuber and foliage. However, *Solanum chacoense* Bitter, a wild relative of the potato, produces a novel leptine glycoalkaloid that is only expressed in the foliage (Lorenzen et al., 2001). Although leptines have not been introgressed into any current commercial cultivars, leptines could potentially provide protection from foliar pests and alleviate the human health concern associated with high glycoalkaloid content in the tuber (Sinden et al., 1986).

Bacillus thuringiensis (Bt) is a Gram-positive soil-borne bacterium that produces insecticidal crystalline (Cry) proteins during sporulation (Sharma et al., 2000). Cry proteins are highly specific; often they are only toxic to individual insect orders, and often only a few species within an order. The Cry proteins are produced by single genes, many of which have been isolated, codon-modified, and cloned. The *cry3A* gene from *B. thuringiensis* ssp. *tenebrionis* has been cloned and expressed in potato to target Colorado potato beetle (Adang et al., 1993; Perlak et al., 1993). Recently, Coombs et al. (2002) expressed the *Bt-cry3A* gene in both a susceptible commercial cultivar cv. Yukon Gold and a high leptine-expressing accession from *S. chacoense*. In detached leaf bioassays, the combined resistance of Bt-Cry3A and leptine provided a greater control of Colorado potato beetle larvae than either leptine or Bt-cry3A alone (Coombs et al., 2002).

We have developed combined host plant resistance in potato and have Colorado potato beetle strains in our laboratory that differ in levels of resistance to insecticides and Bt-Cry3A. This puts us in a unique position to study the effectiveness of the single vs. combined-host plant resistant factor strategy on insects expressing resistance to Bt-Cry3A and insecticides. We hypothesized that: (1) the susceptible strain would be vulnerable to both resistant factors, (2) insecticide-resistant larvae would be tolerant to plants expressing leptine, (3) Bt-selected Colorado potato beetle larvae would be tolerant to the plants expressing Bt, (4) neither host plant resistant factor alone would completely inhibit larvae of all three strains of Colorado potato beetle, and (5) the combined resistance would be more effective than either host plant resistance factor alone.

The objective of this study was to determine the effects of combining two host plant resistance factors, leptine and Bt-cry3A, on susceptible, insecticide-resistant, and Bt-selected Colorado potato beetle larvae. We measured consumption, biomass accumulation, and mortality for each larval instar. The results may have implications for the deployment of genetically engineered potatoes and other pest resistant crops.

Materials and methods

Plant material

The potato lines used include single and multiple resistance factors (Table 1). YGc3A.12 is a line of a cv. Yukon Gold, engineered with the *Bt-cry3A* gene under control of the constitutive $(ocs)_3$ mas promoter. 80c3A.01 is a USDA

Table 1 Potato lines

Potato line	Designation	Source	Host plant resistance factor(s)
Untransformed	cv. Yukon Gold	–	None
Leptine	USDA 838001	*Solanum chacoense*, USDA Beltsville, MD (Sinden et al., 1986)	Leptine
Bt	YGc3A.12	Yukon Gold, transformed at Michigan State University (Coombs et al., 2002)	Bt-Cry3A
Leptine + Bt	80c3A.01	USDA 8380-1, transformed at Michigan State University (Coombs et al., 2002)	Leptine + Bt-Cry3A

line, USDA8380-1, which expresses leptine, engineered with the same Bt gene construct; it has both foliar leptine and *Bt-cry3A* expression (Coombs et al., 2002).

All the potato lines were maintained in tissue culture as previously described (Coombs et al., 2002). When tissue culture plants reached about 60 mm in height, they were transferred to soil in seedling trays (50 cells per tray, 3 cm diameter) in the greenhouse. After a month, the seedlings were transferred into plastic pots (3.78 l). When the plants reached between 45 and 60 cm in height in the greenhouse, the young leaves from each potato line were harvested for glycoalkaloid and Bt-Cry3A analyses and detached leaf bioassays.

Glycoalkaloid level quantification

The concentrations of glycoalkaloids in potato foliage are positively correlated with light intensity (Deahl et al., 1991; Lafta & Lorenzen, 2000). To determine the level of glycoalkaloids at different times during our experiments, young leaf tissue was collected from the four potato lines (susceptible, leptine, Bt-cry3A, and Bt-cry3A + leptine) simultaneously for the detached leaf bioassay and glycoalkaloid analysis from August 2000 to March 2002. The leaf tissue was kept at −80 °C. The tissue was freeze-dried and sent to Dr Ken Deahl (USDA/ARS PSI VEG LAB, Beltsville, MD) for glycoalkaloid analysis by high performance liquid chromatography (Sinden et al., 1980). The data were analyzed using a mixed linear model with repeated measures. Means were separated using least-squares means with a pair-wise t-test (SAS Institute, 2000).

Cry3A expression level quantification

To determine whether the Cry3A levels were consistent throughout the year, leaf tissue from the Bt potato lines (Bt and leptine + Bt) was collected and tested from August 2000 to March 2002. The tissue was kept at −80 °C until the Bt-Cry3A analysis. The level of Bt-Cry3A protein in the tissue samples was quantified with a DAS-ELISA test system for Bt-Cry3A endotoxins (Agdia Inc., Elkhart, IN). The manufacturer's protocol was followed. Leaf tissue (75 mg) from the greenhouse grown plants was macerated in 750 ml of extraction buffer in disposable extraction pouches. Twenty microlitres of the extract solution was diluted to 1000 µl, resulting in a final dilution of 1 : 500 (w/v). A negative control and concentrations of 64, 32, 16, 8, 4, 2, and 1 ng ml^{-1} of positive control were dispensed individually into the first eight wells of an ELISA plate in order to generate a standard curve to quantify Cry3A protein levels in the leaf tissue. The standard curves had an R^2 value of 0.95 or greater. The plate was incubated for 1.5 h with the enzyme conjugate. Absorbance was detected at 405 nm on an Automated Microplate Reader (EL311S, Bio-tek Instruments Inc., Winooski, VT) at 15, 30, 45, and 60 min after incubation with p-nitro phenyl phosphate (PNP) substrate buffer, prepared from the PNP substrate tablet. A standard curve was generated for each reading and the standard curve with the highest R^2 value was used to quantify the Cry3A protein levels in the tissue. Data were analyzed using a mixed linear model using repeated measures. Means were separated using least-squares means with a pair-wise t-test (SAS Institute, 2000).

Beetle strains and selection

Three strains of Colorado potato beetles were used in this study: an insecticide-susceptible strain, an insecticide-resistant strain, and a Bt-selected strain (Table 2). All strains were reared on greenhouse grown cv. Superior potato at 25 ± 2 °C under a photoperiod of L16:D8.

Table 2 Colorado potato beetle strains

Strain	Source	Characterization
Susceptible	New Jersey Department of Agriculture – in culture since 1983	A susceptible strain, raised in laboratory culture without exposure to insecticides
Insecticide-resistant	Long Island, NY – in culture at Michigan State University – in culture since 1997	From a field population resistant to carbamate, organophophate, and neonicotinoid insecticides
Bt-selected	Michigan State University – in culture since 1987 (Whalon et al., 1993)	Selected initially using foliar Bt insecticide in field, then select in the laboratory by exposing 2nd instars to Bt-Cry3A plants

The susceptible strain was obtained as egg masses from the New Jersey Department of Agriculture's Phillip Alampi Beneficial Insect Rearing Laboratory, West Trenton, NJ. It was originally collected in 1983 from potato and eggplant fields in New Jersey, and has been continuously reared without exposure to insecticides. After the egg masses were received, the larvae were reared on cv. Superior potato foliage until the instar needed for the assay was reached.

The insecticide-resistant strain was collected in 1997 from Suffolk County, Long Island, NY, by Dale Moyer, Cornell Cooperative Extension, Riverhead, NY. Colorado potato beetles from Long Island, NY, are highly resistant to all groups of chemical insecticides (Gauthier et al., 1981; Bishop & Grafius, 1996). This strain was originally collected and studied for its resistance to imidacloprid (Zhao et al., 2000a), a neonicotinoid insecticide similar in structure and activity to the plant chemical nicotine, but this strain is also resistant to carbamates, organophosphates, and pyrethroids (Bishop & Grafius, 1996). The egg masses were collected from the $F_{13} - F_{15}$ insecticide-resistant strain to use in this study. The insecticide-resistant strain does not undergo selection at each generation, but the resistance level is monitored. In 2003, the LD_{50} for this strain on imidacloprid and thiamethoxam was 2.196 μg/beetle and 0.204 μg/beetle, respectively (Bishop et al., 2003). We hypothesized that this strain might be tolerant of leptine because organophosphate and carbamate insecticides are acetyl cholinesterase inhibitors like the potato glycoalkaloids (Bushway et al., 1987; Lawson et al., 1993; Rangarajan et al., 2000).

The Cry3A-sel strain was selected for resistance to Bt, as previously described by Whalon et al. (1993). The F_{34} generation of this strain was over 700-fold resistant to Cry3A-Bt compared to the susceptible strain (Trisyono & Whalon, 1997). Resistance in the current culture has declined in subsequent generations for unknown reasons. At the higher resistance level, the newly molted 2nd instars (4-day-old) were exposed to cv. Atlantic Newleaf (Monsanto Company, St Louis, MO), a commercial Bt-cry3A line, for 5 days. With the loss of resistance within strain, the selection was reduced to a 3 day exposure on cv. Russet Burbank Newleaf (Monsanto Company, St Louis, MO), a commercial Bt-cry3A line. Mortality from selection is generally 50–70%. Surviving larvae are transferred to non-transformed cv. Superior plants until pupation. The individual Bt-selected larvae used for this study were not selected prior to the assay.

Detached leaf bioassays (consumption, biomass accumulation, and mortality)

Fully expanded leaves were collected from each of the four potato lines: non-transformed, leptine, Bt, and leptine + Bt. Each petiole was immersed in a water-filled vial (1 dram) sealed with parafilm and placed in a Petri dish (125 mm diameter) lined with Whatman no. 2 filter paper. A group of 10 1st instars of the same strain (susceptible, insecticide-resistant, or Cry3A-sel) was weighed on a Mettler balance (Model PB 153) and placed on each detached leaf (non-transformed, leptine, Bt, or leptine + Bt) for 5 days in a no-choice test. The assays were performed in groups over time with each potato line represented in each group to compensate for any variance in plant quality between replications. This procedure was repeated separately for all four instars of the three Colorado potato beetle strains. Larvae often molted during the 5-day period; the final larval stage was noted at the end of the assay. The detached leaf bioassay was replicated 12–16-fold (120–160 larvae × 4 instars × 3 strains × 4 plant lines), depending on the availability of larvae. The assays were observed daily to check for mortality and leaf degradation. If leaf quality

had degraded significantly, or a large area of leaf had been consumed, the leaf was replaced and consumption was visually estimated with mm^2 grid paper and recorded for each group of larvae (Coombs et al., 2002).

We attempted various methods of ascertaining the area of consumption, such as scanning the leaves, using a fresh/dry leaf mass ratio, and weighing them. Scanning or weighing leaves did not appear to reduce the variability associated with this measurement, and were much more time consuming than using mm^2 grid paper (Coombs et al., 2002). After 5 days, the estimated area of consumption, mass of the larvae, and mortality were measured. Biomass accumulation was estimated by the change in mass during the assay. Larvae were considered dead if no movement was observed after being lightly touched with a small paintbrush.

The data sets were unbalanced because replications varied from 12 to 16. Mortality was transformed to an arcsine of the square root to homogenize variance. The data sets were analyzed using the SAS Least-squares means model procedure for a two-factorial design analysis of variance, with the factors of potato line and Colorado potato beetle strain, used to analyze consumption, biomass accumulation, and arcsine square root mortality. The interactions between potato line and Colorado potato beetle strain were significant for all data sets except 1st instar growth; 1st instar growth data was analyzed using SAS general linear model for analysis of variance. Means of the 1st instar biomass accumulation were compared using Fisher's LSD (SAS Institute, 2000).

Results

Expression and analysis of glycoalkaloids

Time of year was a significant factor in the level of glycoalkaloids in the leaf tissue ($F = 20.50$, d.f. = 3, $P<0.001$). The total glycoalkaloid content in the leptine (26.86 ± 1.28 mg g^{-1}) and leptine + Bt (20.31 ± 1.28 mg g^{-1}) lines were approximately five- and threefold higher than in the untransformed (5.38 ± 1.28 mg g^{-1}) and Bt (6.66 ± 1.28 mg g^{-1}) line, respectively. The untransformed and Bt line glycoalkaloid levels did not significantly differ from each other over time ($P = 0.5735$). The leptine + Bt glycoalkaloid level was significantly higher than both the untransformed and Bt line ($P< 0.0001$). The leptine glycoalkaloid level was significantly higher than untransformed, Bt, and Bt + leptine line ($P<0.0001$).

Expression and analysis of Bt-Cry3A proteins

The time of year did not significantly effect the Cry3A levels of the Bt or leptine + Bt line ($F = 0.20$, d.f. = 3, $P = 0.8174$). The Cry3A level did not significantly differ between the Bt (5.71 ± 1.06 ng mg^{-1}) or leptine + Bt (3.04 ± 1.06 ng mg^{-1}) ($F = 3.18$, d.f. = 1, $P = 0.1000$).

Bioassays

Consumption. Consumption by susceptible and insecticide-resistant strains was similar in the four potato lines over the four instars (Figure 1a–d). For 1st and 2nd instars of the two beetle strains, feeding was generally highest on the

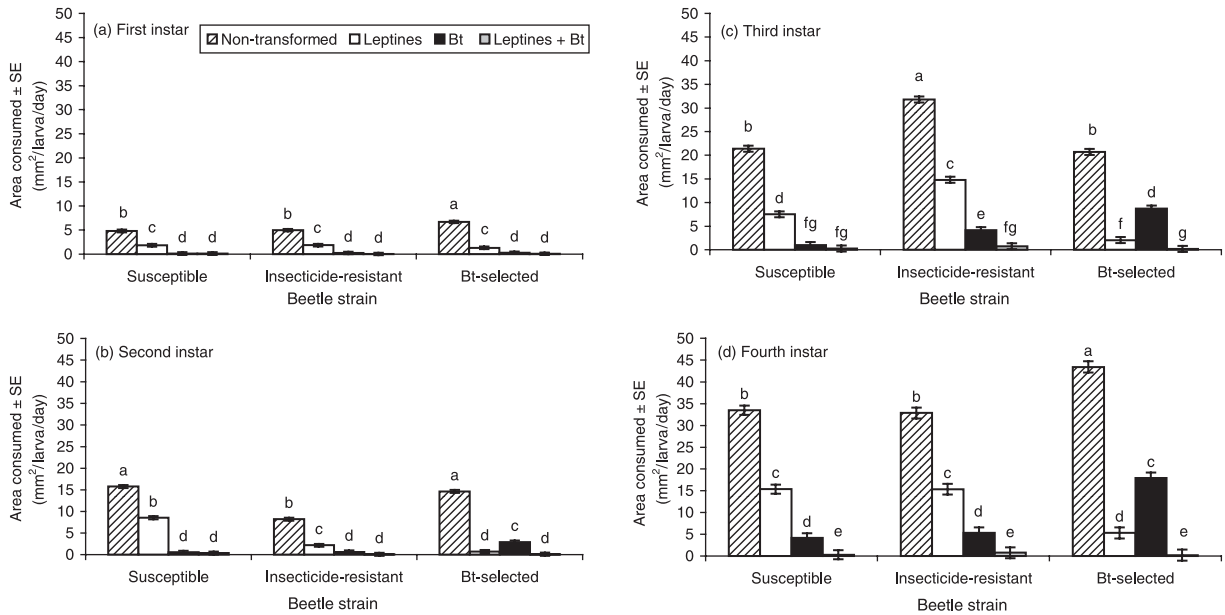

Figure 1 Mean consumption of foliage from four potato lines (untransformed, leptine, Bt, and leptine + Bt) by susceptible, insecticide-resistant, or Bt-selected Colorado potato beetle for 1st–4th instars (a–d) in a 5-day no-choice detached leaf bioassay. Means within an instar followed by different letters are significantly different at the 0.05 level according to a two-factorial design ANOVA and LS means procedures.

untransformed foliage, significantly lower on the leptine line, and significantly lower still on the Bt and leptine + Bt lines. These observations are similar to results of a previous study (Coombs et al., 2002). The 1st instars are particularly susceptible to insecticidal compounds compared with later larval stages; the more potent toxin (Bt) may mask the effects of the lesser toxin. Moreover, insecticide resistance may not be expressed until later larval stages or perhaps 1st instars lack the nutritional resources to cope as well with toxins as later instars (Wierenga et al., 1996; Hilton et al., 1998). For the 3rd instars of the insecticide-resistant strain and the 4th instars of both strains, feeding was significantly lower on the leptine + Bt foliage than on the Bt foliage. In later instars, which have large nutritional reserves, the combined resistance of leptine + Bt deters feeding more than either single resistance factor.

Consumption by Bt-selected 1st instars was similar to consumption by susceptible and insecticide-resistant 1st instars (Figure 1). In 3rd and 4th instars, the Bt-selected larvae consumed significantly more Bt foliage than either the leptine or leptine + Bt foliage (Figure 1b–c). A previous study found the Bt-selected 2nd instars much less vulnerable to Cry3A than 1st instars (Wierenga et al., 1996). In our study, the Bt-selected strain appears to be more sensitive to leptine from the 2nd instar onward than the other strains. Bt-selected 3rd and 4th instars fed significantly less on the leptine + Bt foliage than on either leptine foliage or Bt foliage, again suggesting the combined resistance may be more effective than either alone.

Biomass accumulation. The susceptible and insecticide-resistant larvae showed some significant differences in biomass accumulation over the 5-day period; the biomass accumulation of susceptible 1st–3rd instars was significantly higher than insecticide-resistant 1st–3rd instars on untransformed foliage and leptine foliage (Figure 2a–d). Otherwise, the two strains performed similarly on the four potato lines over all four instars. Susceptible and insecticide-resistant biomass accumulation was significantly inhibited on leptine foliage compared to the untransformed potato line over all four instars. This was consistent with the inhibition of feeding on the leptine line, compared to feeding on the non-transformed line (Figure 1a–d). These results correlate with previous studies suggesting that leptine glycoalkaloids possess strong anti-feedant properties which delay development (Lorenzen et al., 2001). The Bt and leptine + Bt foliage inhibited the biomass accumulation of the susceptible and insecticide-resistant larvae significantly more than untransformed foliage or leptine foliage, but there were no significant differences between

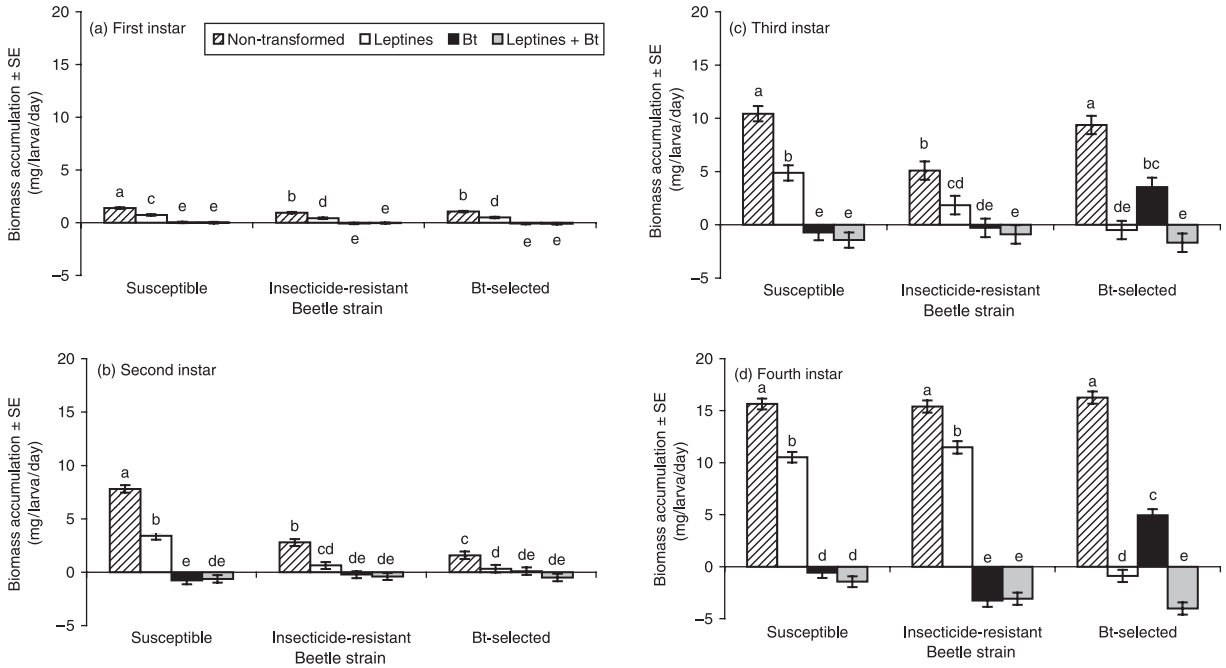

Figure 2 Mean biomass accumulation of 1st–4th instars (a–d) of susceptible, insecticide-resistant, or Bt-selected Colorado potato beetle for on four potato lines (untransformed, leptine, Bt, and leptine + Bt) in a 5-day no-choice detached leaf bioassay. Means within an instar followed by different letters are significantly different at the 0.05 level according to a two-factorial design ANOVA and LS means procedures.

the biomass accumulation of larvae on the Bt or leptine + Bt. Biomass accumulation for these two strains on Bt or leptine + Bt was mostly negative, largely due to little or no growth of survivors and to the desiccation of dead individuals. Survivors on Bt or leptine + Bt foliage were visibly smaller than their original size, extremely weak, and would probably have died in a longer bioassay.

The biomass accumulation of the Bt-selected 1st and 2nd instars on all four potato lines showed a similar pattern to the susceptible and insecticide-resistant strains (Figure 2a,b). In the 2nd instar, Bt-selected larvae biomass accumulation was minor, even on the untransformed foliage. Previous studies reported that the Bt-selected strain has a longer development time on untransformed potato plants (Alyokhin & Ferro, 1999). Perhaps the slow growth in the 2nd instar may assist the strain in overcoming Bt-toxicity.

In the 3rd and 4th instars, Bt-selected biomass accumulation was significantly higher on the Bt foliage than on leptine or leptine + Bt foliage (Figure 2c,d). For 3rd instars, the biomasss accumulation of Bt-selected was positive on Bt foliage, but negative on leptine foliage. Bt-selected 4th instars had negative biomass accumulation on both leptine and leptine + Bt foliage, but the biomass accumulation was significantly less on leptine + Bt foliage than on leptine foliage; combined resistance was more effective at inhibiting biomass accumulation than either leptine or Bt alone.

Mortality. The mortality of the susceptible and insecticide-resistant larvae closely followed trends seen for consumption; mortality of susceptible and insecticide-resistant larvae was similar for respective instars (Figures 1a–d and 3a–d). While consumption was significantly lower on the leptine foliage than on the untransformed foliage for susceptible and insecticide-resistant larvae, leptine foliage did not cause a significantly higher mortality than the untransformed foliage. Leptine may act as a feeding deterrent rather than a toxin (Sinden et al., 1986). Bt foliage and the leptine + Bt foliage killed significantly more 1st and 2nd instar susceptible larvae than either the leptine or the untransformed foliage. Mortality on leptine + Bt foliage was significantly higher than mortality on the Bt foliage for the insecticide-resistant larvae. For the susceptible 3rd and 4th instars, and all insecticide-resistant instars, the combined resistance, leptine + Bt, was significantly more effective than either leptine or Bt alone.

Although the leptine foliage did not cause significantly higher mortality than the untransformed foliage in the susceptible and insecticide-resistant strains, the leptine foliage caused much higher mortality than untransformed foliage in the Bt-selected strain. The mortality of Bt-selected 1st instars was nearly 100% for Bt and leptine + Bt foliage. Previous studies have demonstrated that the Bt-selected 1st instars are more susceptible to Cry3A than later instars (Wierenga et al., 1996). Leptine foliage caused significantly more mortality for the Bt-selected 2nd, 3rd, and 4th instars

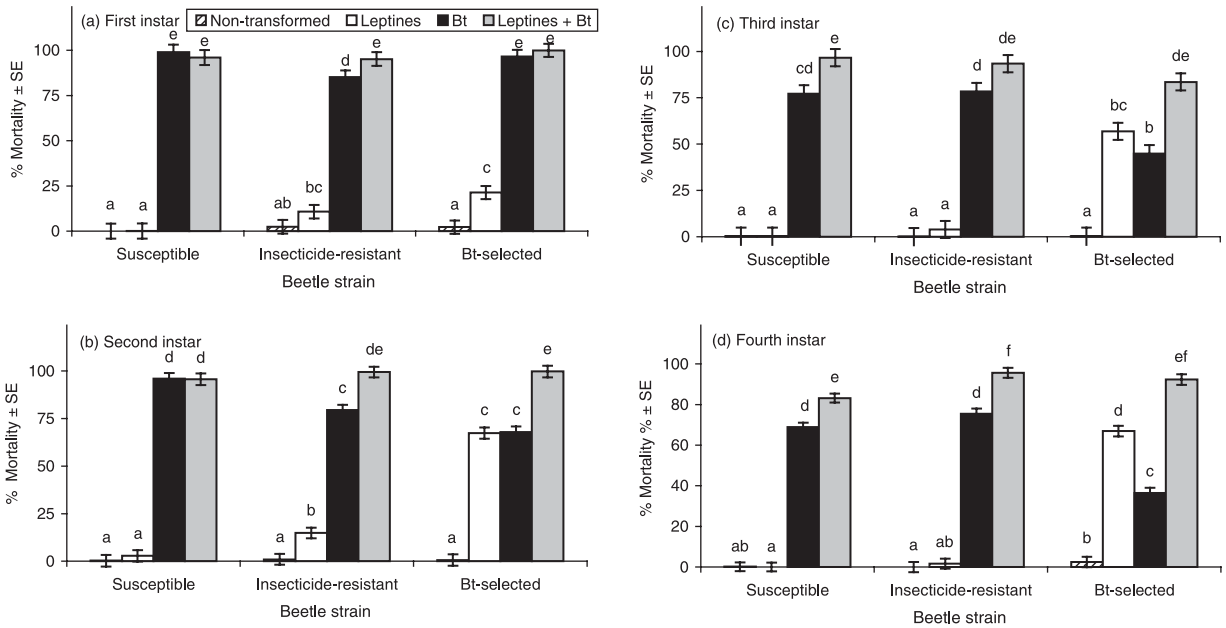

Figure 3 Mean per cent mortality of 1st–4th instars (a–d) of susceptible, insecticide-resistant and Bt-selected Colorado potato beetle for on four potato lines (untransformed, leptine, Bt, and leptine + Bt) in a 5-day no-choice detached leaf bioassay. Means within an instar followed by different letters are significantly different at the 0.05 level according to a two-factorial design ANOVA and LS means procedures.

than for the other two beetle strains. The mortality of Bt-selected 4th instars was higher on leptine foliage than on Bt foliage. The combined resistance of leptine + Bt caused significantly higher mortality than either leptine or Bt foliage.

Insecticide-resistant strain. The insecticide-resistant strain may be insensitive to acetyl cholinesterase or have elevated detoxification systems for insecticides. The strain does not appear to be resistant to leptine glycoalkaloids, suggesting that leptine may differ in its mode of action or mode of detoxification from organophosphates or carbamates. Similarities in feeding, growth, and mortality suggest that the susceptible and insecticide-resistant strains have a similar tolerance to leptine.

Bt-selected strain. Although the increased sensitivity of the Bt-selected strain to leptine was unexpected, previous studies have described a diminished fitness of this strain in the absence of Bt-Cry3A (Alyokhin & Ferro, 1999). The reduced fitness may be an artifact of the Bt-Cry3a selection resulting in reduced vigor, the results of genetic drift from a relatively small initial population, and the instability of number of individuals in the colony, or perhaps the leptine glycoalkaloid sensitivity may be inversely linked with Cry3A resistance. Further studies will be needed to determine the cause of the leptine glycoalkaloid sensitivity within the Bt-selected strain.

Discussion

The combined resistance was more effective across a range of Colorado potato beetle strains with varying resistance backgrounds, even though the glycoalkaloid levels were significantly lower in the leptine + Bt than in the leptine line. In early instars the elevated resistance may be masked due to the inherent sensitivity of smaller larvae. In the later instars, the combined resistance was significantly more effective than either single resistance factor. Although durability was not specifically addressed in this study, the susceptibility of the Bt-selected Colorado potato beetle strain to the leptine line demonstrates an instance of individuals resistant to one toxin being controlled by the other. This concept is fundamental to the pyramiding management strategy.

Bacillus thuringiensis toxins are the only biotechnology-based insect resistance factors available commercially (Anonymous, 2003b). Cry1Ab, Cry1Ac, Cry1F, and Cry3Bb1 proteins are available in commercial varieties of corn and cotton. Cry3A was available commercially in potatoes resistant to Colorado potato beetle from 1996 to 2001 (NewLeaf, Monsanto Corp., St Louis MO) but those varieties were taken off the market in 2001 due to low sales and buyer concerns about genetically modified organisms.

Colorado potato beetle has proven itself to be one of the most adaptable of all crop pests. It has developed resistance to all chemical insecticides used for its control (Bishop & Grafius, 1996; Whalon et al., 2000), and a laboratory population has also been selected with resistance to *Bacillus thuringiensis* Cry3A (Whalon et al., 1993). Diamondback moth, *Plutella xylostella* L., is another pest being targeted with genetic engineering for control. However, field populations of diamondback moth already show resistance to foliar applications of Bt, and a laboratory population with more than 60 000-fold resistance has been selected on Bt transgenic broccoli (Zhao et al., 2000b). The tobacco budworm, *Helicoverpa virescens* (Fabricius), one of the major pests of cotton, has developed resistance to 37 insecticides (Whalon et al., 2000). No single form of resistance, either genetically engineered or classically bred, is likely to provide long-term control to such highly adaptable insects, particularly if other mortality factors, such as crop rotation or biological control, are not included in the management system. More durable strategies than single factor Bt-based host plant resistance must be developed.

Host plant resistance management combining genetically engineered resistance with traditionally bred host plant resistance has the potential to be much more sustainable and easily implemented than a high dose / refuge strategy. Combining host plant resistance factors as a resistance management strategy does not require grower cooperation or regulatory monitoring or enforcement. Potential pest resistance mechanisms to the genetically engineered and traditionally bred resistance factors will likely be completely different, as is probably the case with leptine and Bt-based resistance for management of Colorado potato beetle. Incorporation of host plant resistance into an integrated pest management system involving multiple biological, cultural, and chemical controls will further increase the sustainability of a pest management system.

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