J. Amer. Soc. Hort. Sci. 127(1):62–68. 2002.

Combining Engineered (*Bt-cry3A*) and Natural Resistance Mechanisms in Potato for Control of Colorado Potato Beetle

Joseph J. Coombs and David S. Douches[1]
Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824

Wenbin Li
Department of Plant Agriculture, University of Guelph, Biotechnology Division, Guelph, Ontario, Canada N1G 2W1

Edward J. Grafius and Walter L. Pett
Department of Entomology, Michigan State University, East Lansing, MI 48824

Additional index words. *Solanum tuberosum*, *Leptinotarsa decemlineata*, host plant resistance, *Bacillus thuringiensis*, leptine glycoalkaloids, glandular trichomes

Abstract. The Colorado potato beetle [*Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)] is a destructive pest of the cultivated potato (*Solanum tuberosum* L.) in northern latitudes. Combining resistance mechanisms of leptine glycoalkaloids and glandular trichomes with the synthetic *Bacillus thuringiensis* Berliner (*Bt*) *cry3A* gene in potato may be an effective strategy for controlling the Colorado potato beetle. *Bt-cry3A* transgenic plants were developed for three potato lines with differing levels of resistance to Colorado potato beetle ['Yukon Gold' (susceptible control), USDA8380-1 (leptine glycoalkaloids), and NYL235-4 (glandular trichomes)]. Polymerase chain reaction, and Southern and northern blot analyses confirmed integration and transcription of the *cry3A* gene in the transgenic lines. Detached-leaf bioassays of the *cry3A* engineered transgenic lines demonstrated that resistance effectively controlled feeding by first instar Colorado potato beetles. The susceptible 'Yukon Gold' control suffered 32.3% defoliation, the nontransformed high foliar leptine line (USDA8380-1) had 3.0% defoliation, and the nontransformed glandular trichome line (NYL235-4) had 32.9% defoliation. Mean percentage defoliation for all transgenic lines ranged between 0.1% and 1.9%. Mean mortality ranged from 0.0% to 98.9% among the *Bt-cry3A* transgenic lines, compared to 20% for the susceptible 'Yukon Gold' control, 32.2% for USDA8380-1, and 16.4% for NYL235-4. Results indicate that genetic engineering and the availability of natural resistance mechanisms of potato provide the ability to readily combine host plant resistance factors with different mechanisms in potato.

The potato (*Solanum tuberosum*) has many pests and pathogens that can reduce yields and overall plant vigor. The Colorado potato beetle (*Leptinotarsa decemlineata*) is a highly destructive pest of potato in north central and eastern North America (Wyman et al., 1994), Europe, and Asia (Hare, 1990). Defoliation by adults and larvae can reduce yields and even result in total tuber loss (Hare, 1980). Despite breeding efforts, no potato cultivars with demonstrated resistance to Colorado potato beetle have been released commercially (Yencho and Tingey, 1994).

The Colorado potato beetle is a unique insect pest of potatoes because it has shown the ability to adapt to every chemical insecticide ever deployed for its control (Forgash, 1985). The combination of the insect's genetic diversity, its ability to detoxify toxic plant compounds, and strong insecticide selection pressure, have contributed to the Colorado potato beetle's adaptation to insecticides (Grafius and Bishop, 1996; Weber and Ferro, 1994). It can be anticipated that the Colorado potato beetle will develop resistance to future insecticides used for its control (Hare, 1990; Kennedy and French, 1994). As chemical control costs increase and pesticide efficacy decreases, successful, long-term control measures for Colorado potato beetle will need to incorporate host plant resistance (Spooner and Bamberg, 1994) and integrated crop management techniques.

Received for publication 29 Mar. 2001. Accepted for publication 28 Aug. 2001. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
[1]Corresponding author; e-mail: douchesd@msu.edu.

Host plant resistance has been suggested as the only practical long-term solution for controlling the Colorado potato beetle (Spooner and Bamberg, 1994). Leptine glycoalkaloids and glandular trichomes are two natural insect host plant resistance mechanisms available in potato. Leptines, a type of steroid glycoalkaloid, are effective natural resistance mechanisms of potato against Colorado potato beetle (Sinden et al., 1980, 1986a, 1986b; Stürckow and Löw, 1961; Tingey, 1984; Tingey et al., 1984), but are found in only a few accessions of *S. chacoense* Bitter (Sinden et al., 1986b). The glycoalkaloids chaconine and solanine are found commonly in all plant organs of cultivated potato. Leptines I and II are the acetylated forms of solanine and chaconine, however, they are only synthesized in the leaves and not in the tubers (Sinden et al., 1986b). This is an important advantage for breeders who are concerned with selecting against potentially toxic levels of total glycoalkaloids in the tubers. The high leptine-producing accession of *S. chacoense*, USDA8380-1 (PI 458310), is a diploid selection containing approximately 300 mg per 100 g leaf fresh weight leptines (constituting 90% of the total glycoalkaloids) with strong antifeedant properties against Colorado potato beetle (Deahl et al., 1991; Sinden et al., 1986a).

Glandular trichomes of the wild Bolivian potato, *S. berthaultii* Hawkes, confer resistance to at least 10 major insect pest groups or species, including the potato tuber moth (*Phthorimaea operculella* Zeller), green peach aphid (*Myzus persicae* Sulzer), potato leaf hopper (*Empoasca fabae* Harris), potato flea beetle (*Epitrix cucumeris* Harris), and the Colorado potato beetle (Tingey, 1991). The presence of type A and B trichomes leads to entrap-

ment and death of small-bodied insects (Gregory et al., 1986) and reduces developmental time, survival, and oviposition of Colorado potato beetle (Casagrande, 1982; Wright et al., 1985; Yencho and Tingey, 1994). The trichomes of *S. berthaultii* are associated with insect responses including host avoidance, restlessness, reduced feeding, reduced development, reduced adult weight, reduced reproductive performance, decreased longevity, and increased mortality (Tingey, 1991). Plaisted et al. (1992) released a glandular trichome germplasm line NYL235-4. This line caused a 40% to 70% reduction in Colorado potato beetle population densities of first generation larvae and up to 90% reduction in first generation adults and second generation larvae, compared with commercial controls (Plaisted et al., 1992).

The bacterium, *Bacillus thuringiensis* (*Bt*) ssp. *tenebrionis* Berliner, produces a Cry3A protein that has toxic effects on Coleoptera, including Colorado potato beetle (Krieg et al., 1983). The choice of a *Bt* gene for engineering host plant resistance has multiple advantages. *Bt* proteins have very specific modes of action, such that a protein with specific toxicity towards Coleoptera would not be toxic to other orders of insects; and *Bt* crystal proteins have not shown any toxicity towards humans, other mammals, or birds (Lavrik et al., 1995). Synthetic *Bt-cry3A* genes have been constructed specifically to optimize expression in plants (Adang et al., 1993; Perlak et al., 1993; Sutton et al., 1992). The ability to transform and express *Bt* in plants provides a high-dose strategy of deploying *Bt* toxins for specific insect control.

Current integrated crop management strategies of potato include various combinations of cultural practices and chemical controls (Reiners et al., 2000). Additionally, engineered host plant resistant cultivars are also available (Sherman, 2000). However, insecticide applications continue to be the primary means used by growers to control Colorado potato beetle. New strategies must be developed to offer other options for use in integrated pest management programs. Development of combined natural and engineered host plant resistance mechanisms in potato will provide alternatives for control of Colorado potato beetle in an integrated pest management program.

The overall objective of this research was to generate and test combined engineered and natural resistance mechanisms in potato for effective management of Colorado potato beetle. The specific objectives were to: 1) transform a high leptine-expressing diploid line, a glandular trichome line, and a susceptible cultivar with a synthetic *Bt-cry3A* gene, 2) characterize the insertion and transcription of the *Bt-cry3A* in these transgenic lines, and 3) evaluate resistance of the combined natural and engineered host plant resistance mechanism lines to Colorado potato beetle in no-choice, detached-leaf bioassays.

Materials and Methods

Plant material. Plant materials used for transformation were 'Yukon Gold' (2n = 4x = 48), USDA8380-1 (2n = 2x = 24), and NYL235-4 (2n = 4x = 48). 'Yukon Gold' is a yellow-flesh tablestock cultivar (Johnston and Rowberry 1981) that is susceptible to the Colorado potato beetle. The high leptine-producing accession of *S. chacoense,* USDA8380-1 (PI 458310), is a diploid selection containing ≈300 mg per 100 g leaf fresh weight leptines (constituting 90% of the total glycoalkaloids) with strong antifeedant properties against Colorado potato beetle (Deahl et al., 1991; Sinden et al., 1986a). The germplasm line NYL235-4 is a result of a 2x x 4x cross between *S. tuberosum* 'Hudson' and *S. berthaultii* (PI 310925) and six generations of backcrossing, and has resistance to Colorado potato beetle via glandular trichomes (Plaisted et al., 1992). The *Bt-cry3A* transgenic line RBN15, obtained from John Kemp (New Mexico State University, Las Cruces), is a transgenic 'Russet Burbank' line with the *Bt-cry3A* gene under control of a CaMV35S promoter. RBN15 was selected from more than 20 different *Bt-cry3A* transgenic 'Russet Burbank' lines for strong resistance to Colorado potato beetle and was used as a positive control.

All potato lines were maintained in tissue culture by nodal propagation in 25 × 150-mm culture tubes or GA-7 Magenta vessels (Magenta Corp., Chicago, Ill.) in modified Murashige and Skoog (MS) (1962) medium (MS salts at 4.3 $g \cdot L^{-1}$, 3% sucrose, 1.4 mM sodium phosphate, 1.1 mM thiamine, 0.55 mM myo-inositol, pH 6.0, and Bactoagar at 8 $g \cdot L^{-1}$ (Difco, Detroit, Mich.)). All culture tubes, Magenta vessels, and petri dishes were sealed with surgical tape (Micropore; 3M Co., St. Paul, Minn.). Cultures were maintained at 25 ± 2 °C with a 16-h photoperiod of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ produced by cool-white fluorescent lamps, as measured at 25 cm from the light source.

Transformation. The gene used in this study was supplied by John Kemp at New Mexico State University and included a synthetic *Bt-cry3A* gene optimized for expression in plants (Sutton et al., 1992). The *cry3A* gene was subcloned into vector pE1120 resulting in a plasmid (pSPUD8) that included the constitutive $(ocs)_3$mas promoter and the selectable marker neomycin phosphotransferase (*nptII*) under the control of its own nopaline synthase promoter. The resulting plasmid, pSPUD8, was introduced into *Agrobacterium tumefaciens* Smith and Townsend strain LBA4404 (Clontech, Palo Alto, Calif.) by triparental mating (Bevan, 1984).

Bt-cry3A transgenic potato lines were generated using *A. tumefaciens*-mediated transformation (Li et al., 1999). The plants were prepared for transformation by removing leaf tip and petiole ends from tissue culture plantlet leaves; stem pieces were also used, carefully avoiding any meristematic tissue by cutting between nodes.

When callus nodules produced shoots 5 to 7 mm in length, the shoots were excised and placed in rooting medium (modified MS medium with the addition of kanamycin at 50 $mg \cdot L^{-1}$) in 25 × 150 mm culture tubes. Only a single shoot was removed from each callus to ensure selection from independent transformation events. Rooted transformants, expressing resistance to kanamycin were maintained in tissue culture by micropropagation and were transplanted to 3.8-L pots in the greenhouse for further analyses. Unless otherwise stated, all chemicals used were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Molecular characterization: polymerase chain reaction (PCR). DNA was isolated by the quick DNA method (Wang et al., 1993) from one 8-mm-diameter leaf disc of a young (4 to 5 weeks old), greenhouse-grown, tissue culture transplant. For PCR, 1 mL of the resulting DNA was used directly. PCR components for 40 mL reactions were used following Gibco BRL instructions (Gaithersburg, Md.) (1× PCR buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 1.0 mM of each primer, 100 ng template DNA, and 2.5 U Taq DNA polymerase).

Optimal primers for amplifying a region of the *cry3A* gene were selected using DNASTAR (DNASTAR, Inc., Madison, Wis.). Two 25-base primers were chosen to amplify a 439 base pair (bp) length DNA fragment between bases 253 and 692 of the synthetic *cry3A* gene. The primer sequence complimentary to the transcribed strand was 5'-GAG CTG CAA GGC CTT CAA AAC

AAT-3'. The second primer was complimentary to the nontranscribed strand and had a sequence of 5'-TCT AGC ACG GTA AGG GTC ATC TCT-3'.

PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and primer extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The reactions were held at 4 °C before being analyzed. Reaction products were electrophoresed on a 1% (w/v) agarose gel (Boeringer Mannheim, Indianapolis, Ind.) containing ethidium bromide at 0.5 mg·mL^{-1} in 1× Tris-acetate/EDTA (pH 8.0) buffer at 80 mV for 1.5 to 2 h (Sambrook et al., 1989) and viewed under ultraviolet light (254 nm).

Southern analysis. Total plant genomic DNA was extracted from the fresh leaf tissue (2 g) of greenhouse-grown tissue culture transplants using the cetyltrimethylammonium bromide (CTAB) extraction protocol (Saghai-Maroof et al., 1984), modified by adding 2% beta-mercaptoethanol to the extraction buffer. The DNA was quantified using a miniflourometer (model TKO100; Hoeffer Scientific, San Fernado, Calif.).

To confirm *Bt-cry3A* insertion, DNA (20 mg) was digested with *EcoRI* and *BamHI* restriction enzymes (Gibco BRL). To determine the number of *Bt-cry3A* gene insertion events, the DNA was digested with *XbaI*. Agarose-gel electrophoresis, Southern blotting, membrane hybridization, and detection was performed as per Li et al. (1999), with the exception of the *cry3A* RNA probe, which was made by in vitro SP6 RNA polymerase transcription of the *cry3A* gene cut from pSP73 with *BamHI* as per manufacturer's instructions (Boeringer Mannheim).

Northern analysis. The 100 mg leaf samples from greenhouse grown transplants were placed in 1.5-mL Eppendorf tubes, frozen in liquid nitrogen, and stored at –80 °C until total plant RNA was extracted using the Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, Calif.). RNA quantification and purity were determined by measuring the absorbance of the sample at 260 and 280 nm. Total RNA was electrophoresed, blotted, hybridized, and detected as per Li et al. (1999).

Double-antibody sandwich enzyme-linked immunosorbant assay (DAS-ELISA). *Bt-cry3A* protein was quantified in the transgenic lines using a DAS-ELISA test system (Agdia Inc, Elkhart, Ind.). The manufacturers' instructions were used with the following specifications. Leaf tissue (75 mg) from field-grown plants was ground in 750 mL of extraction buffer in an Eppendorf tube using a disposable pestle attached to a mounted drill before being adjusted to a final dilution of 1:500 (w/v). The extracts were incubated with the enzyme conjugate for 1.25 h. Absorbance was detected at 405 nm after 60 min incubation with the PNP substrate using an automated microplate reader (model EL311S; Bio-tek Instruments, Inc., Winooski, Vt.).

Detached-leaf bioassays. A Colorado potato beetle strain was collected originally from Long Island, N.Y. and has been maintained in culture for use in laboratory bioassays. All Colorado potato beetle stages were fed nontransgenic potato foliar tissue from various commercial cultivars grown in the greenhouse under a 16-h photoperiod. The beetle colony was maintained with a 16-h photoperiod at 25 ± 2 °C using 25 μmol·m^{-2}·s^{-1} provided by cool-white fluorescent lamps or high-sodium greenhouse lights, as measured at 70 cm from the light source.

No-choice, detached-leaf bioassays of the plants confirmed previously to contain the *Bt-cry3A* gene were conducted to determine biological activity of the transgene. Young, fully expanded leaves of similar age and size were removed from greenhouse transplants. The petiole of each leaf was wrapped in a small piece of cotton and placed into a 3.5 mL vial of distilled water. This detached-leaf was placed on 90 mm Whatman filter paper in a covered 20 × 100 mm polystyrene petri dish. Egg masses designated for bioassay use were maintained at 10 °C for 0 to 5 d until enough were collected for a single round of bioassays. Only first instars that hatched on the same day and had no prior feeding were used for detached-leaf bioassays. Ten first instars from a compiled randomized sample were gently transferred to each leaf using a fine-tipped paintbrush that was moistened with water. Detached-leaf bioassays were maintained at 25 ± 2 °C with constant light of 25 μmol·m^{-2}·s^{-1} provided by cool-white fluorescent lamps. The detached-leaf bioassays were conducted as a completely randomized design consisting of two replications.

A visual estimation of percentage defoliation, insect mortality, and the developmental stages (instars) of the larvae were recorded after 4 d. Percentage defoliation was determined by visually estimating the percentage of the entire leaf area that was consumed by the larvae. For lines that had very little feeding, the following criterion were used: no signs of any feeding = 0.0%; very little sampling of leaf tissue, feeding not progressing entirely through the leaf = 0.5%; sampling of leaf tissue resulting in pinhole feeding that did not progress beyond 1 mm feeding diameter = 1.0% defoliation. Percentage mortality was calculated as the number of dead or absent larvae divided by 10 and multiplied by 100%. Larvae were considered dead if no movement was observed after being gently touched with a fine-tipped paint brush.

Percentage defoliation and percentage mortality data were arcsine transformed before analysis of variance was performed. Mean comparisons were accomplished using Fisher's protected least significant difference (LSD, $P = 0.05$) in the general linear models procedure of SAS (SAS Inst., Inc., 1998). Reported means were retransformed into percentages.

Results and Discussion

Transformation. *Agrobacterium*-mediated transformation of the three lines used in this study was effective in producing *Bt-cry3A* transgenic potato lines. The number of shoots emerging from any one callus ranged from 0 to 30, although only a single shoot was removed from each callus to select for independent transformation events.

Successful shoot regeneration appeared to be genotype dependent. Between 103 and 143 d after cocultivation, 27 shoots were removed from 46 'Yukon Gold' explants and 96.3% of the shoots rooted in the presence of kanamycin at 50 mg·L^{-1}. The USDA8380-1 line produced 28 independent shoots from 37 explants between 93 and 125 d post co-cultivation with Agrobacterium. Of these 28 shoots, 89.3% rooted in MS medium with kanamycin at 50 mg·L^{-1}. NYL235-4 produced the fewest shoots (eight) from 53 explants between 93 and 105 d after exposure to Agrobacterium, and only three (37.5%) rooted in the presence of kanamycin. Transformation of USDA8380-1 and NYL235-4 has been reported previously (Douches et al., 1998), and similar difficulties in transforming and regenerating shoots from NYL235-4 with a low frequency (33%) of recovered shoots rooting in kanamycin medium were observed.

Molecular characterization. Nearly all (88%) of the rooted 'Yukon Gold', 56% of the rooted USDA8380-1, and all of the rooted NYL235-4 lines were PCR positive following DNA amplification of the 439 bp *Bt-cry3A* fragment. Ten PCR-positive, phenotypically normal, *Bt-cry3A* transgenic lines selected from both USDA8380-1 and 'Yukon Gold', as well as all three

NYL235-4 transgenics were analyzed via Southern and northern blotting and detached-leaf bioassays.

All selected lines were confirmed via PCR to contain the *Bt-cry3A* gene (exemplary PCR results shown for USDA8380-1 transgenic lines in Fig. 1A, data not illustrated for 'Yukon Gold' or NYL235-4 transgenic lines). Southern blotting showed that the number of inserted gene copies (copy number) per transgenic potato line varied between one and three (exemplary Southern analysis shown for USDA8380-1 transgenic lines in Fig. 1B, data not illustrated for 'Yukon Gold' or NYL235-4 transgenic lines). Transformation of similar potato lines with a *Bt-cry5* gene resulted in plants with copy numbers ranging from one to three as well (Douches et al., 1998). Jelenkovic et al. (1998), using the same synthetic *Bt-cry3A* gene, reported that most transgenic *Bt-cry3A* eggplant (*Solanum melongena* L.) lines regenerated had one or two gene inserts, although as many as five copies were noted in a single plant.

In general, the *Bt-cry3A* transgenic lines transcribed similar levels of *Bt-cry3A* mRNA, as determined by comparing the relative amounts of 18S rRNA (exemplary northern analysis shown for USDA8380-1 transgenic lines in Fig. 2, data not illustrated for 'Yukon Gold' or NYL235-4 transgenic lines). One exception was YGc3.15, which had a similar amount of 18S rRNA compared to the other transgenic lines, however, lower *Bt-cry3A* mRNA (data not illustrated). YGc3.15 was not significantly different from the other 'Yukon Gold' *Bt-cry3A* transgenic lines for percentage defoliation or mortality in Colorado potato beetle detached-leaf bioassays (Table 1). The *Bt-cry5* mRNA levels of transgenic potato lines developed by Douches et al. (1998) were similar to each other for all but one transgenic line, which also caused the lowest level of mortality in potato tuber moth bioassays. Adang et al. (1993) observed relative differences among the *Bt-cry3A* transgenic lines for mRNA amounts and this correlated to the relative differences they observed for protein toxin levels and Colorado potato beetle larval weight bioassay results. They also found that Cry3A protein levels were a good indicator of relative efficacy, and mRNA levels were a good (but not absolute) indicator of relative protein amount. A positive correlation between resistance to Colorado potato beetle and *Bt-cry3A* mRNA expression has also been reported in eggplant (Jelenkovic et al., 1998).

Cry3A protein content in the transgenic lines ranged from 0.4 to 1.0 $\mu g \cdot mg^{-1}$ fresh leaf tissue as determined by DAS-ELISA. All transgenic lines were significantly less than RBN15, with 1.5 $\mu g \cdot mg^{-1}$ fresh leaf tissue. However, resistance of these lines was not significantly different from RBN15.

We chose the $(ocs)_3$mas promoter because the transient *gus* expression was reported to be 156-fold greater than the Cauliflower Mosaic Virus 35S (CaMV35S) promoter (Ni et al., 1995).

It was unexpected to discover lower Cry3A protein levels in our *Bt-cry3A* transgenic lines under the control of the $(ocs)_3$mas promoter compared to the levels of Cry3A protein produced by the same gene under control of the CaMV35S promoter found in RBN15. However, RBN15 was selected from more than 20 different *Bt-cry3A* transgenic 'Russet Burbank' lines for strong resistance to Colorado potato beetle.

Detached-leaf bioassays. The *Bt-cry3A* transgenic potato lines showed 0.1% to 1.9% defoliation by first instar Colorado potato beetles in no-choice, detached-leaf bioassays, regardless of the source potato line (Table 1). The nontransformed 'Yukon Gold' control suffered 32.3% defoliation. The 'Yukon Gold' transgenic lines had 0.9% to 1.9% defoliation. The nontransformed USDA8380-1 had only 3.0% defoliation, further demonstrating natural host plant resistance of leptines against Colorado potato beetle. The USDA8380-1 *Bt-cry3A* transgenic lines had only 0.1% to 0.7% mean percentage defoliation, and all but two had significantly lower defoliation than the nontransformed USDA8380-1. The nontransformed NYL235-4 had 32.9% defoliation compared to the *Bt-cry3A* transgenic lines from NYL235-4 with between 0.6% and 1.1% defoliation. The positive control RBN15 had 1.0% defoliation.

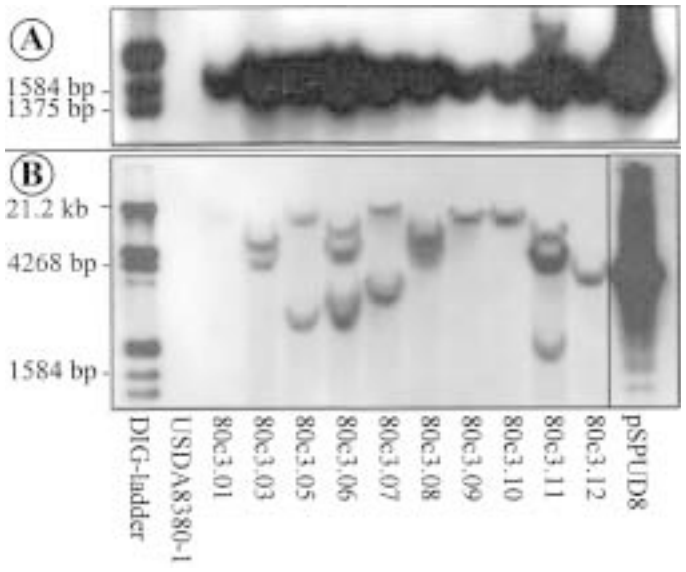

Fig. 1. (**A**) Southern analysis of total plant DNA from *Bt-cry3A* transgenic lines (designated c3.xx) digested with *EcoRI* and *BamHI* and hybridized with a *cry3A* RNA probe (the resulting digested *cry3A* DNA fragment is a 1540 bp fragment of the 1790 bp gene) and (**B**) Southern analysis of total plant DNA from *Bt-cry3A* transgenic lines digested with *XbaI* and hybridized with a *cry3A* RNA probe. USDA8380-1 is a high leptine diploid. The *cry3A* plasmid, pSPUD8, was also digested. Exemplary Southern analysis is shown for USDA8380-1 transgenic lines. However, data are not illustrated for 'Yukon Gold' or NYL235-4 transgenic lines.

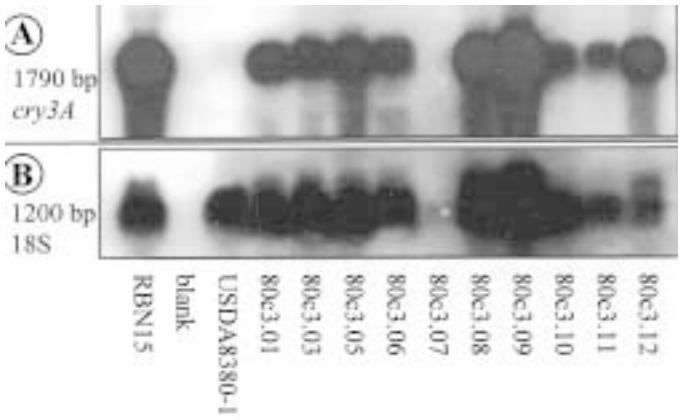

Fig. 2. (**A**) Northern analysis of total plant RNA from *Bt-cry3A* transgenic lines (designated c3.xx) hybridized with a *cry3A* RNA probe and (**B**) the same membrane rehybridized with an 18S ribosomal RNA probe. USDA8380-1 is a high leptine diploid. RBN15 is a *Bt-cry3A*-positive line. Exemplary northern analysis is shown for USDA8380-1 transgenic lines. However, data are not illustrated for 'Yukon Gold' or NYL235-4 transgenic lines. Amounts of RNA loaded in the gel varied, as noted by the differences in the amount of 18S RNA; in this gel 80c3.07 had very little RNA loaded, accounting for lack of the *cry3A* band presence.

The NYL235-4 no-choice, detached-leaf bioassay results differed from earlier findings of Colorado potato beetle resistance for this line in the field (Plaisted et al., 1992). Percentage defoliation was relatively high (32.9%) and percentage mortality was low (16.4%) for NYL235-4 and neither was significantly different from the susceptible cultivar 'Yukon Gold'. Plaisted et al. (1992) reported field results for NYL235-4 demonstrating that Colorado potato beetle resistance was due to reduced egg laying and an increase in first and second instar mortality. In our no-choice detached-leaf bioassays, the egg laying preference was not tested and host plant properties did not reduce defoliation or increase mortality of first instars compared to nontrichome lines.

The observed lack of resistance of NYL235-4 in our detached-leaf bioassays may have resulted from differences between the Plaisted et al. (1992) study and our detached-leaf bioassays in trichome density and other related resistance factors. França and Tingey (1994b) showed that lower irradiances reduced type A and B trichome densities, volume of trichome exudate, and type A trichome phenolic oxidation activity in *S. berthaultii*. The plants used for bioassays in the present study were grown in the greenhouse, with lower irradiances than in the field. Furthermore, the larvae used in the detached-leaf bioassays were placed on the upper side of the leaf (lower trichome densities than the lower leaf surface), which differs from where eggs are usually laid on the lower leaf surface of potato plants in the field. However, neonates feed on both sides of the leaf, eating entirely through the leaf, regardless of where they begin. Lack of resistance of NYL235-4 to Colorado potato beetle has also been observed in field studies in Michigan (Coombs et al., 1999).

Mean mortality after 4 d was much more variable, ranging from 0.0% to 98.9% between the *Bt-cry3A* transgenic lines (Table 1). Percentage mortality of the 'Yukon Gold' transgenics had the most variable response, 0.0% to 94.7% mortality, compared to 20% for the nontransformed 'Yukon Gold' control, while mortality for RBN15 was 97.4%. USDA8380-1 *Bt-cry3A* transgenic lines resulted in 50.0% to 97.4% mortality, compared with 32.2% mortality on the nontransformed control. *Bt-cry3A* NYL235-4 lines had significantly higher mortality (83.3% to 98.9%) than the NYL235-4 control (16.4%).

Results for percentage defoliation were much less variable than for percentage mortality. Although leaves sampled from plants were chosen to be of similar size and age, and larvae from a single hatch date were chosen randomly, environmental differences in both the plants and the insects could contribute to the variability seen in the mortality results. Some of this variability was due likely to the strict criterion for determining mortality; larvae were considered dead only if no movement occurred. Mortality from *Bt* can take several days and some larvae judged

Table 1. Mean percentage defoliation of detached-leaves and mean percentage mortality of first instar Colorado potato beetles feeding on detached-leaves obtained from combined natural and engineered insect resistant potato clones.

Potato clone	Defoliation (%)	Mortality (%)	Host plant resistance mechanism
USDA8380-1[z]	3.0 b[y]	32.2 defg	Leptine glycoalkaloids
80c3A.01	0.7 bcd	70.5 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.03	0.1 d	50.0 bcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.05	0.5 cd	94.7 abc	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.06	0.7 bcd	55.0 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.07	0.5 cd	76.7 abcde	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.08	0.1 d	65.1 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.09	0.1 d	65.1 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.10	0.1 d	65.1 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.11	0.1 d	55.0 abcdef	Leptine glycoalkaloids + *Bt-cry3A*
80c3A.12	0.1 d	97.4 ab	Leptine glycoalkaloids + *Bt-cry3A*
NYL235-4[x]	32.9 a	16.4 fg	Glandular trichomes
L235c3A.2	1.1 bcd	83.3 abcd	Glandular trichomes + *Bt-cry3A*
L235c3A.3	0.6 bcd	97.6 a	Glandular trichomes + *Bt-cry3A*
L235c3A.5	0.8 bcd	98.9 a	Glandular trichomes + *Bt-cry3A*
Yukon Gold[w]	32.3 a	20.0 efg	Susceptible control
YGc3A.01	1.9 bc	29.5 defg	*Bt-cry3A*
YGc3A.03	1.0 bcd	18.4 efg	*Bt-cry3A*
YGc3A.04	1.7 bcd	50.0 bcdef	*Bt-cry3A*
YGc3A.06	1.7 bcd	0.0 g	*Bt-cry3A*
YGc3A.12	0.9 bcd	72.4 abcdef	*Bt-cry3A*
YGc3A.13	1.0 bcd	94.7 abc	*Bt-cry3A*
YGc3A.15	1.5 bcd	90.0 abc	*Bt-cry3A*
YGc3A.17	1.0 bcd	94.7 abc	*Bt-cry3A*
YGc3A.19	1.5 bcd	85.4 abcd	*Bt-cry3A*
YGc3A.21	1.0 bcd	44.8 cdef	*Bt-cry3A*
RBN15	1.0 bcd	97.4 ab	*Bt-cry3A*
$LSD_{0.05}$	1.0	34.6	

[z]The high leptine glycoalkaloid potato clone USDA8380-1 and *Bt-cry3A* transgenics (80c3A.xx).
[y]Mean separation within columns by Fisher's protected LSD, $P = 0.05$.
[x]The glandular trichome potato clone NYL235-4 and *Bt-cry3A* transgenics (L235c3A.x).
[w]The potato cultivar 'Yukon Gold' and *Bt-cry3A* transgenics (YGc3A.xx).

to be 'alive' in this study may have died later. Extended bioassay periods would likely better differentiate mortality and insect developmental differences among the different transgenic lines.

Combining host plant resistance mechanisms. Use of combined resistance mechanisms in a single deployable unit has been suggested as a strategy for delaying development of insect adaptation to host plant resistance mechanisms, especially *Bt*-based resistance (Gould, 1986, 1988, 1998; Gould et al., 1994; McGaughey and Whalon, 1992; McGaughey et al., 1998; and Tabashnik, 1994). Our strategy was to combine natural (leptine glycoalkaloids and glandular trichomes) and engineered (*Bt-cry3A*) host plant resistance mechanisms.

The strong antifeedant properties of leptine glycoalkaloids found in a few accessions of *S. chacoense* confer natural host plant resistance against Colorado potato beetle (Sinden et al., 1980, 1986a, 1986b; Stürckow and Löw, 1961; Tingey, 1984; Tingey et al., 1984). Although the mode of action of leptines is not understood thoroughly, solanaceous glycoalkaloids and alkaloids inhibit acetyl cholinesterase (Bushway et al., 1987), and disrupt membranes by lysis of sterol-containing liposomes (reviewed in Lawson et al., 1993). The Colorado potato beetle adapts readily to chemical insecticides with similar modes of action to the proposed glycoalkaloid function (Forgash, 1985). Additionally, DDT-resistant Colorado potato beetles were more resistant to leptines than susceptible beetles (Stürckow and Löw, 1961). These issues should be evaluated before leptine-based resistant potato lines are deployed.

The percentage defoliation results from the detached-leaf bioassays for the combination of *Bt* + leptines suggest that the amount of feeding by first instar Colorado potato beetles is reduced significantly in some transgenic lines compared to leptines alone (Table 1). However, these differences were not evident in the mortality results, comparing leptines with *Bt* + leptines. Further experiments may indicate whether host plant resistance combination is more effective than either alone, and more importantly, whether the combination is more durable.

Early reports by Groden and Casagrande (1986) suggested that the Colorado potato beetle could overcome the resistance of *Solanum berthaultii* within two to three generations. More recent studies (França et al., 1994; Pelletier and Smilowitz, 1991) report that adaptation of Colorado potato beetle to *S. berthaultii* is possible, but it would most likely occur much more slowly than suggested previously. França et al. (1994) also noted that it is important to consider the regional genetic dynamics of different Colorado potato beetle populations and the underlying differences in resistance mechanisms of different *S. berthaultii* clones. The complexity of the interactions between glandular trichomes and the responses of Colorado potato beetle suggest that for adaptation to occur, Colorado potato beetles may have to overcome a variety of behavioral, developmental, and metabolic factors elicited by *S. berthaultii* against both Colorado potato beetle adults and larvae (França and Tingey, 1994a).

Glandular trichomes combined with *Bt* in a single cultivar may be an important host plant resistance strategy for broad-spectrum insect control in potato production. Glandular trichomes may not be as effective in full scale commercial plots for controlling Colorado potato beetle; however, in the combined *Bt* + glandular trichome resistance potato cultivar, *Bt* may provide effective control of the Colorado potato beetle and the glandular trichomes would confer resistance to other small-bodied insects such as aphids and leaf-hoppers.

The present study is the first report of combining natural and engineered resistance mechanisms in potato for control of Colorado potato beetle. Whalon et al. (1993) has shown that Colorado potato beetle can adapt to *Bt* in the laboratory, and the severe selection pressure applied by use of *Bt* potatoes in the field will likely eventually lead to adaptation by the Colorado potato beetle to *Bt*. Genetic engineering and the availability of natural resistance mechanisms of potato provide the ability to readily pyramid host plant resistance factors with different mechanisms in potato.

Literature Cited

Adang, M.J., M.S. Brody, G. Cardineau, N. Eagan, R.T. Roush, C.K. Shewmaker, A. Jones, J.V. Oakes, and K.E. McBride. 1993. The reconstruction and expression of a *Bacillus thuringiensis cry3A* gene in protoplasts and potato plants. Plant Mol. Biol. 21:1131–1145.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711–8721.

Bushway, R.J., S.A. Savage, and B.S. Ferguson. 1987. Inhibition of acetyl cholinesterase by solanaceous glycoalkaloids and alkaloids. Amer. Potato J. 64:409–413.

Casagrande, R.A. 1982. Colorado potato beetle resistance in a wild potato, *Solanum berthaultii*. J. Economic Entomol. 75:368–372.

Coombs, J., D. Douches, W. Li, E. Grafius, and W. Pett. 1999. Field evaluation of potato resistance mechanisms for control of Colorado potato beetle. Amer. J. Potato Res. 76:366–367 (abstr.).

Deahl, K.L., W.W. Cantelo, S.L. Sinden, and L.L. Sanford. 1991. The effect of light intensity on Colorado potato beetle resistance and foliar glycoalkaloid concentration of four *Solanum chacoense* clones. Amer. Potato J. 68:659–666.

Douches, D.S., A.L. Westedt, K. Zarka, B. Schroeter, and E.J. Grafius. 1998. Potato transformation to combine natural and engineered resistance for controlling tuber moth. HortScience 33:1053–1056.

Forgash, A.J. 1985. Insecticide resistance in the Colorado potato beetle, p. 33–52. In: D.N. Ferro and R.H. Voss (eds.). Proc. Symp. of the Colorado potato beetle. XVIIth Intl. Congr. Entomol. Mass. Agr. Expt. Sta. Bul. 704.

França, F.H., R.L. Plaisted, R.T. Roush, S. Via, and W.M. Tingey. 1994. Selection response of the Colorado potato beetle for adaptation to the resistant potato, *Solanum berthaultii*. Entmologia Experimentalis et Applicata 73:101–109.

França, F.H. and W.M. Tingey. 1994a. *Solanum berthaultii* Hawkes affects the digestive system, fat body and ovaries of the Colorado potato beetle. Amer. Potato J. 71:405–410.

França, F.H. and W.M. Tingey. 1994b. Influence of light level on performance of the Colorado potato beetle on *Solanum tuberosum* L. and on resistance expression in *Solanum berthaultii* Hawkes. J. Amer. Soc. Hort. Sci. 119:915–919.

Gould, F. 1986. Simulation models for predicting durability of insect-resistant germ plasm: A deterministic diploid, two-locus model. Environ. Entomol. 15:1–10.

Gould, F. 1988. Evolutionary biology and genetically engineered crops. BioScience 1:26–33.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: Integrated pest genetics and ecology. Annu. Rev. Entomol. 43:701–726.

Gould, F., P. Follett, B. Nault, and G.G. Kennedy. 1994. Resistance management strategies for transgenic potato plants, p. 255–277. In: G.W. Zehnder, M.L. Powelson, R.K. Jansson, and K.V. Raman (eds.). Advances in potato pest biology and management. APS Press, St. Paul, Minn.

Grafius, E.J. and B.A. Bishop. 1996. Resistance to imidacloprid in Colorado potato beetles from Michigan. Resistant Pest Mgt. 8:21–25.

Gregory, P., W.M. Tingey, D.A. Ave, and P.Y. Bouthyette. 1986. Potato glandular trichomes: A physicochemical defense mechanism against insects, p. 160–167. In: M.B. Green and P.A. Hedin (eds.). Natural resistance of plant pests. Amer. Chem. Soc. Symp. Ser. 296. ACS Books, Wash., D.C.

Groden, E. and R.A. Casagrande. 1986. Population dynamics of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera:

Chrysomelidae) on *Solanum berthaultii*. J. Economic Entomol. 79:91–97.
Hare, J.D. 1980. Impact of defoliation by the Colorado potato beetle on potato yields. J. Economic Entomol. 73:369–373.
Hare, J.D. 1990. Ecology and management of the Colorado potato beetle. Annu. Rev. Entomol. 35:81–100.
Jelenkovic, G., S. Billings, Q. Chen, J. Lashomb, G. Hamilton, and G. Ghidiu. 1998. Transformation of eggplant with synthetic *cryIIIA* gene produces a high level of resistance to the Colorado potato beetle. J. Amer. Soc. Hort. Sci. 123:19–25.
Johnston, G.R. and R.G. Rowberry. 1981. 'Yukon Gold'; a new yellow-fleshed, medium-early, high quality table and French-fry cultivar. Amer. Potato J. 58:241–244.
Kennedy, G.G. and N.M. French. 1994. Monitoring resistance in Colorado potato beetle populations, p. 278–293. In: G.W. Zehnder, M.L. Powelson, R.K. Jansson, and K.V. Raman (eds.). Advances in potato pest biology and management. APS Press, St. Paul, Minn.
Krieg, A., A.M. Huger, G.A. Langenbruch, and W. Schnetter. 1983. *Bacillus thuringiensis* var. *tenebrionis*, a new pathotype effective against larvae of Coleoptera. Z. Angew. Entomol. 96:500–508.
Lavrik, P.B., D.E. Bartnicki, J. Feldman, B.G. Hammond, P.J. Keck, S.L. Love, M.W. Naylor, G.J. Rogan, S.R. Sims, and R.L. Fuchs. 1995. Safety assessment of potatoes resistant to Colorado potato beetle. Amer. Chem. Soc. Symp. Ser. 605:148–158.
Lawson, D.R., R.E. Veilleux, and A.R. Miller. 1993. Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. Current Topics in Bot. Res. 1:335–352.
Li, W., K.A. Zarka, D.S. Douches, J.J. Coombs, W.L. Pett, and E.J. Grafius. 1999. Coexpression of potato PVY^{o} coat protein and *cryV-Bt* genes in potato. J. Amer. Soc. Hort. Sci. 124:218–223.
McGaughey, W.H., F. Gould, and W. Gelenter. 1998. Bt resistance management. Nature Biotechnol. 16:144–146.
McGaughey, W.H. and M.E. Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science 285:1451–1454.
Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
Ni, M., D. Cui, J. Einstein, S. Narasimhulu, C.E. Vergara, and S.B. Gelvin. 1995. Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. Plant J. 7:661–676.
Pelletier, Y. and Z. Smilowitz. 1991. Biological and genetic study on the utilization of *Solanum berthaultii* Hawkes by the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Can. J. Zool. 69:1280–1288.
Perlak, F.J., T.B. Stone, Y.M. Muskopf, L.J. Petersen, G.B. Parker, S.A. McPherson, J. Wyman, S. Love, G. Reed, D. Biever, and D.A. Fischhoff. 1993. Genetically improved potatoes: Protection from damage by Colorado potato beetles. Plant Mol. Biol. 22:313–321.
Plaisted, R.L., W.M. Tingey, and J.C. Steffens. 1992. The germplasm release of NYL235-4, a clone with resistance to the Colorado potato beetle. Amer. Potato J. 69:843–846.
Reiners, S., C.H. Petzoldt, and M.P. Hoffmann (eds.). 2000. The 2000 integrated crop and pest management recommendations website. http://www.nysaes.cornell.edu/recommends/note.html
Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard. 1984. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014–8018.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Lab. Press, Cold Spring Harbor, N.Y.
SAS Inst., Inc. 1998. The SAS system for windows. Software release 6.12. SAS Inst., Inc. Cary, N.C.
Sherman, A. 2000. National potato council 2000–2001 potato statistical yearbook. Natl. Potato Council, Englewood, Colo.
Sinden, S.L., L.L. Sanford, W.W. Cantelo, and K.L. Deahl. 1986a. Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense*. Environ. Entomol. 15:1057–1062.
Sinden, S.L., L.L. Sanford, and K.L. Deahl. 1986b. Segregation of leptine glycoalkaloids *in Solanum chacoense* Bitter. J. Agr. Food Chem. 34:372–377.
Sinden, S.L., L.L. Sanford, and S.F. Osman. 1980. Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. Amer. Potato J. 57:331–343.
Spooner, D.M. and J.B. Bamberg. 1994. Potato genetic resources: Sources of resistance and systematics. Amer. Potato J. 71:325–337.
Stürckow, B. and I. Löw. 1961. Die wirkung einger *Solanum*-alkaloidglykoside auf den kartoffelkafer, *Leptinotarsa decemlineata* Say. Entomol. Expt. Applicata 4:133–142.
Sutton, D.W., P.K. Havstad, and J.D. Kemp. 1992. Synthetic *cry3A* gene from *Bacillus thuringiensis* improved for high expression in plants. Transgenic Res. 1:228–236.
Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39:47–79.
Tingey, W.M. 1984. Glycoalkaloids as pest resistance factors. Amer. Potato J. 61:157–167.
Tingey, W.M. 1991. Potato glandular trichomes: Defense activity against insect attack, p. 126–135. In: P.A. Hedin (ed.). Naturally occurring pest bioregulators. Amer. Chem. Soc. Symp. Ser. 449. ACS Books, Wash., D.C.
Tingey, W.M., P. Gregory, R.L. Plaisted, and M.J. Tauber. 1984. Research progress: Potato glandular trichomes and steroid glycoalkaloids, p. 125–131. In: Rpt. XXII Planning Conf. on Integrated Pest Management, Intl. Potato Center, Lima, Peru.
Wang, H., M. Qi, and A.J. Cutler. 1993. A simple method of preparing plant samples for PCR. Nucleic Acids Res. 21:4153–4154.
Weber, D.C. and D.N. Ferro. 1994. Colorado potato beetle: Diverse life history poses challenge to management, p. 54–70. In: G.W. Zehnder, M.L. Powelson, R.K. Jansson, and K.V. Raman (eds.). Advances in potato pest biology and management. APS Press, St. Paul, Minn.
Whalon, M.E., D.L. Miller, R.M. Hollingworth, E.J. Grafius, and J.R. Miller. 1993. Selection of a Colorado potato beetle (Coleoptera: Chrysomelidae) strain resistant to *Bacillus thuringiensis*. J. Econ. Entomol. 86:226–233.
Wright, R.J., M.B. Dimock, W.M. Tingey, and R.L. Plaisted. 1985. Colorado potato beetle (Coleoptera: Chrysomelidae): Expression of resistance in *Solanum berthaultii* and interspecific potato hybrids. J. Econ. Entomol. 78:576–582.
Wyman, J.A., J. Feldman, and S.K. Kung. 1994. Cultural control of Colorado potato beetle: Off-crop management, p. 376–385. In: G.W. Zehnder, M.L. Powelson, R.K. Jansson, and K.V. Raman (eds.). Advances in potato pest piology and management. APS Press, St. Paul, Minn.
Yencho, C.G. and W.M. Tingey. 1994. Glandular trichomes of *Solanum berthaultii* alter host preference of the Colorado potato beetle, *Leptinotarsa decemlineata*. Entomol. Expt. Applicata 70:217–225