Evaluation of Natural and Engineered Resistance Mechanisms in Potato Against Colorado Potato Beetle in a No-Choice Field Study

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ABSTRACT The Colorado potato beetle, *Leptinotarsa decemlineata* Say, is the major insect pest of potato, *Solanum tuberosum* L., in eastern North America and is renowned for resistance development, currently resistant to >40 insecticides worldwide. Host plant resistance may assist in delaying in resistance development to insecticides. We evaluated natural host plant resistance mechanisms (glandular trichomes and *Solanum chacoense* Bitter-derived resistance) and engineered resistance mechanisms (*Bacillus thuringiensis* [Bt] Berliner *cry3A* and *cry1Ia1*) in a no-choice cage study. Six different potato lines representing four host plant resistance mechanisms were evaluated over 2 yr. Egg masses were placed in each cage (one egg mass per plant). Almost no feeding was observed in the *Bt-cry3A* lines, and only minor feeding was observed in the *Bt-cry1Ia1* lines in either year. On the *S. chacoense*-derived line, there was significantly less defoliation than on either the susceptible line or the glandular trichome line in 2003. In 2004, there was significantly higher defoliation on the *S. chacoense*-derived line than on the susceptible line or glandular trichome line. The defoliation of the *Solanum chacoense*-derived line was largely due to larvae clipping the petioles, rather than consumption of the leaves. Defoliation on the glandular trichome line did not differ significantly from the defoliation of the susceptible line, suggesting glandular trichomes may not be effective in controlling larvae and preventing defoliation. This study suggested that Bt can provide high levels of resistance, but the natural resistance mechanisms tested here are variable for control of Colorado potato beetle larvae in no-choice situations.

KEY WORDS *Bacillus thuringiensis* (Bt), *cry3A*, *cry1Ia1*, *S. chacoense*-derived resistance, glandular trichomes

The Colorado potato beetle, *Leptinotarsa decemlineata* Say, is among the most economically significant pests of potatoes, *Solanum tuberosum* L., in North America, Europe, and western Asia. As little as 12.5% defoliation caused by Colorado potato beetle can lead to economic losses (Hare 1980, Mailloux and Bostanian 1989). An outbreak of Colorado potato beetle in 1840 led to the first large-scale use of insecticides, which continues to be the chief means of crop protection against this pest (Gauthier et al. 1981, Casagrande 1987). As a result of heavy reliance on insecticides, Colorado potato beetle has developed resistance to nearly every insecticide used for its control, >40 insecticides worldwide (Whalon et al. 2003).

Compared with many other staple crops, the cultivated potato has a broad pool of genetic diversity for natural host plant resistance to pests and diseases within its wild relatives. The wild tuber-bearing species such as *Solanum berthaultii* Hawkes, *Solanum polyadenium* Greenman, and *Solanum tarijense* Hawkes are associated with insect resistance, largely due to glandular trichomes (Pelletier et al. 1999). Glandular trichomes are specialized hair-like epidermal cells that release exudates that encase the mouthparts and tarsi of small-bodied insects such as aphids and that deter feeding and oviposition of larger insects such as the Colorado potato beetle (Tingey 1991). The potato line NYL235-4 (Cornell University), evaluated in this study is a result of backcrosses from *S. berthaultii* to *S. tuberosum*, and it is reported to be resistant to Colorado potato beetle (Plaisted et al. 1992).

Glycoalkaloids are also natural toxins that are produced by many solanaceous species, including potato (Van Gelder 1990). The predominant glycoalkaloids in cultivated potato are α-solanine and α-chaconine, and they comprise almost 95% of the total glycoalkaloids in potato (Kuhn and Low 1955). Glycoalkaloids are distributed throughout most of the plant's tissues, and at high concentrations they provide insect resistance primarily via antibiosis (Kuhn and Low 1955; Sinden et al. 1980). The North Dakota State University breeding line ND5822C-7 (ND4103-2 × Dakota Pearl) was previously evaluated in our Colorado potato beetle field nursery and has shown some resistance that may be attributed to these glycoalkaloids (D.S.D., unpublished data).

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In addition to its genetic diversity, potato is amenable to genetic engineering that increases the range of resistance sources. *Bacillus thuringiensis* (Bt) Berliner is a common soilborne bacterium that produces several types of insecticidal proteins (Whalon and Wingerd 2003). These *cry* genes have been commonly exploited for genetic engineering (Shelton et al. 2002). *Bt-cry* toxins can be classified into 24 major groups and are often toxic to specific insect orders (Hilder and Boulder 1999). In this study, plants expressing *Bt-cry3A* or *Bt-cry1Ia1* were tested. *Bt-cry3A* is effective against many groups of Coleoptera, including Colorado potato beetle (Perlak et al. 1993, Chattopadhyay et al. 2004). *Bt-cry1Ia1* is highly toxic to some Lepidoptera and has some toxicity to Coleoptera as well (Douches et al. 2002).

We would expect there to be differences in the host plant resistance potato lines to Colorado potato beetle in a no-choice cage situation. Our previous research has evaluated these resistance factors in a choice setting with small field plots (10 plants per plot) where Colorado potato beetle adults could freely move between the plots (Coombs et al. 2005). Plant resistance chiefly falls into three categories: tolerance, antibiosis, and antixenosis (repellency). With antixenosis, a cultivar may seem resistant in small plot field trials, but it may fail in a no-choice setting. Insects in many instances do not have a choice of cultivar in a commercial production situation. Thus, no-choice field cages will better emulate commercial conditions and also screen more directly for antibiosis. In our study, we examined the natural resistance factors (glandular trichomes and a *S. chacoense* Bitter-derived resistance) and engineered resistance factors (*Bt-cry1Ia1*, *Bt-cry3A* [low and high expressing]) on newly emerged first-stage Colorado potato beetles.

Materials and Methods

Plant Material. Two nontransgenic lines and three transgenic lines that together represented four different host plant resistance mechanisms were evaluated in comparison with the susceptible 'Atlantic'.

Nontransgenic potato lines included 1) NYL235-4 with glandular trichomes (Plaisted et al. 1992) and 2) ND5822C-7 with nonpreference and avoidance by Colorado potato beetle under choice conditions. NYL235-4 is a breeding line developed at Cornell University that is a result of backcrosses from *S. berthaultii* to *S. tuberosum*, and it has reported resistance to Colorado potato beetle (Plaisted et al. 1992). ND5822C-7 is a breeding line developed at North Dakota State University that is a result of backcrosses from *S. chacoense* to *S. tuberosum*, and it has reported resistance to Colorado potato beetle, due to glycoalkaloids and an uncharacterized resistance from *S. chacoense* (J. Lorenzen, personal communication).

The three transgenic potato lines included Spunta-G2, NORc3.8, and 'Atlantic Newleaf'. The *Bt-cry1Ia1* (lepidopteran- and coleopteran-specific) gene used for transformation of 'Spunta' in this study was obtained from Syngenta (Basel, Switzerland). The constitutive cauliflower mosaic virus 35S promoter was used to express *Bt-cry1Ia1*. The *Bt-cry1Ia1* transgenic line Spunta-G2 was developed in our laboratory (Douches et al. 2002). The technology was not available to determine Bt-Cry1Ia1 protein expression levels.

One of the *Bt-cry3A* (coleopteran-specific) genes used for transformation of the transgenic potato lines in this study was supplied by Dr. John Kemp at New Mexico State University (Sutton et al. 1992). The constitutive ocs_3mas promoter (Ni et al. 1995) was used to express *Bt-cry3A*, and the low-expressing *Bt-cry3A* transgenic potato line NORc3.8 was generated in our laboratory by using *Agrobacterium tumefaciens*-mediated transformation (Coombs et al. 2002). The high-expressing *Bt-cry3A* transgenic potato line Atlantic Newleaf was developed by NatureMark (Monsanto, St. Louis, MO).

Bt-Cry3A expression levels were evaluated using a Bt-Cry3A enzyme-linked immunosorbent assay (ELISA) kit from Agdia, Inc. (Elkhart, IN). Leaf tissue was collected and stored at −80°C until Bt-Cry3A analysis. The level of Bt-Cry3A protein in the tissue samples was quantified with a double antibody sandwich-ELISA test system for Bt-Cry3A endotoxins (Agdia, Inc.) following the manufacturer's protocol. Leaf tissue (75 mg) from the greenhouse grown plants was macerated in 750 ml of extraction buffer in disposable extraction pouches. The extract solution (20 μl) was diluted to 1,000 μl, resulting in a final dilution of 1:500 (wt:vol). Absorbance was measured 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 substrate. Data were analyzed using SAS general linear model for analysis of variance (ANOVA) (SAS Institute 2001). Mean comparisons were conducted using Fisher least significant difference (LSD) ($\alpha = 0.05$).

No-Choice Field Cage Trials. No-choice field cage studies were conducted in 2003 and 2004 at Michigan State University (MSU), East Lansing, MI. Treatment plots were arranged in a randomized complete block design with 10 plants per cage, 25 cm between plants, 86 cm between rows, and three replications per potato line (18 cages). Screen cages (2 by 2 by 2 m) were constructed over the field-grown plants before they were infested with Colorado potato beetle egg masses.

Egg masses from first generation adults were collected from the MSU Montcalm Research Farm, Entrican, MI. One egg mass with ≈25 eggs were added to each plant (10 egg masses per cage) on 24 June 2003 and 17 June 2004. Plants were observed weekly (24 June–12 August 2003 and 17 June–13 August 2004) for a visual estimation of percentage of defoliation by Colorado potato beetles and the number of egg masses, larvae, and adults per plant. Insect data were collected by counting all individuals on whole plants.

Percentage of defoliation data were used to calculate the area under the defoliation curve (AUDC), based on calculation of the area under the disease progress curve (Shaner and Finney 1977). AUDC divided by the maximum AUDC (e.g., 4,900 if 100%

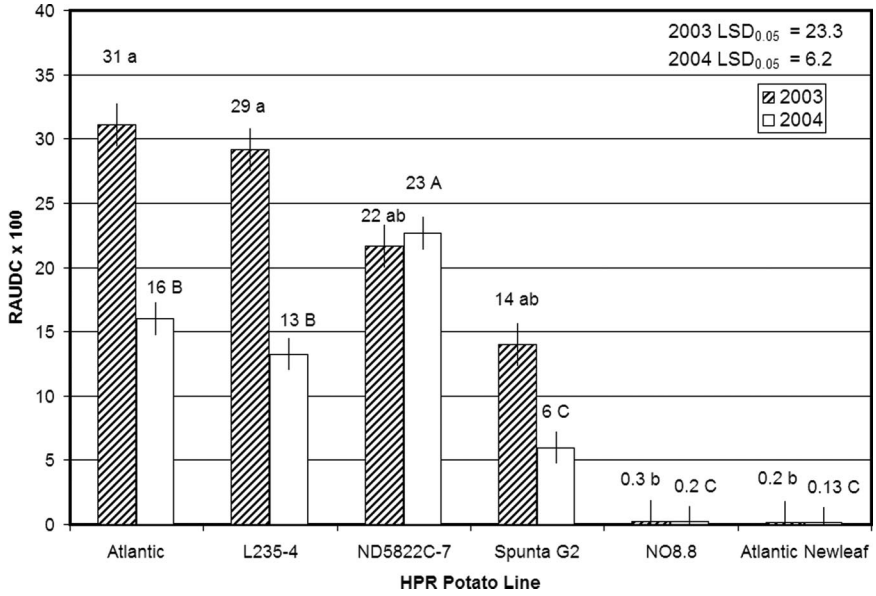

Fig. 1. Mean relative area under the defoliation curve (RAUDCx100) caused by Colorado potato beetle on six potato lines (Atlantic, NYL235-4, ND5822C-7, Spunta-G2, NORc3.8, and Atlantic Newleaf) in a no-choice cage study in 2003 and 2004. Means within a year followed by different letters are significantly different ($\alpha = 0.05$).

defoliation was present from initial observation of defoliation through 49 d), converted the value to relative AUDC (RAUDC). RAUDCx100 values are the RAUDC as a proportion × 100 that provide a season-long whole number value of defoliation over time. Seasonal mean numbers of Colorado potato beetle large larvae (third and fourth instars) per plant was calculated by dividing the number of large larvae per plant by the number of observational dates. Data were analyzed as a randomized complete block design by using SAS general linear model procedure for ANOVA (SAS Institute 2001). Mean comparisons were conducted using Fisher LSD ($\alpha = 0.05$).

Results and Discussion

Expression and Analysis of Bt-Cry3A Proteins. Atlantic Newleaf (183 ± 39 ng/mg) had significantly higher Bt-Cry3A protein levels compared with NORc3.8 (80 ± 39 ng/mg) ($F = 21.58$, df = 1, $P < 0.0001$). There was no Bt-Cry3A protein in the nontransgenic potato lines (Atlantic, ND5822C-7, and NYL235-4) or the *Bt-cry1Ia1* transgenic Spunta-G2, as expected.

No-Choice Field Cage Trial. In 2003, the RAUDCx100 for Atlantic was 31 ± 2; by the last sample date, the plants were 100% defoliated (Fig. 1). In 2004, defoliation was less severe in Atlantic (RAUDCx100 = 16 ± 1), probably because cooler weather favored potato plant growth and slowed Colorado potato beetle feeding and development. In 2004, the average temperature for June–August was 2.2°C below normal.

Although Bt-Cry3A levels were two-fold higher in Atlantic Newleaf compared with NORc3.8, the defoliation did not differ significantly between the two lines and was extremely low for both 2003 and 2004 (Fig. 1). Although Spunta-G2 (*Bt-cry1IaI*) had higher RAUDCx100 values than the *Bt-cry3A* lines in 2003 and 2004, the differences were not statistically different. *Bt-cry1Ia1* has been reported to provide partial resistance to Colorado potato beetle (Douches et al. 2002). The effectiveness of *Bt-cry1Ia1* in this study is likely because we began with neonates. In addition, neonates are more sensitive to toxins than later stages because they lack the nutritional and metabolic resources to cope with toxins (Wierenga et al. 1996). Consumption rates (milligrams of food per milligram of body weight) are highest for Colorado potato beetle neonates. *Bt-cry1Ia1* may be effective controlling neonates, but it would likely not provide strong plant protection against later stages or adults. The advantage of transforming plants with *Bt-cry1Ia1* or *Bt-cry3A* compared with foliar Bt sprays is that the toxin is uniformly present in the plant as soon as neonates begin to feed. The disadvantage is that limited commercial cultivars are available that express Bt-Cry3A.

In 2003 and 2004, RAUDCx100 values for NYL235-4 did not differ significantly from the susceptible Atlantic (Fig. 1). No-choice detached leaf bioassays in the laboratory by Coombs et al. (2002) also showed no differences in defoliation between susceptible potato varieties and NYL235-4, suggesting the majority of the resistance, if any, may be due to adult preference and differentiated egg laying. The NYL235-4 has a lower density of trichomes compared with its parent *S. berthaultii* (Plaisted et al. 1992). The resistance associated with *S. berthaultii* is attributed to type A and B trichomes. When trichomes were removed mechanically, Colorado potato beetle larvae readily consumed the leaves of *S. berthaultii* (Yencho and Tingey 1994).

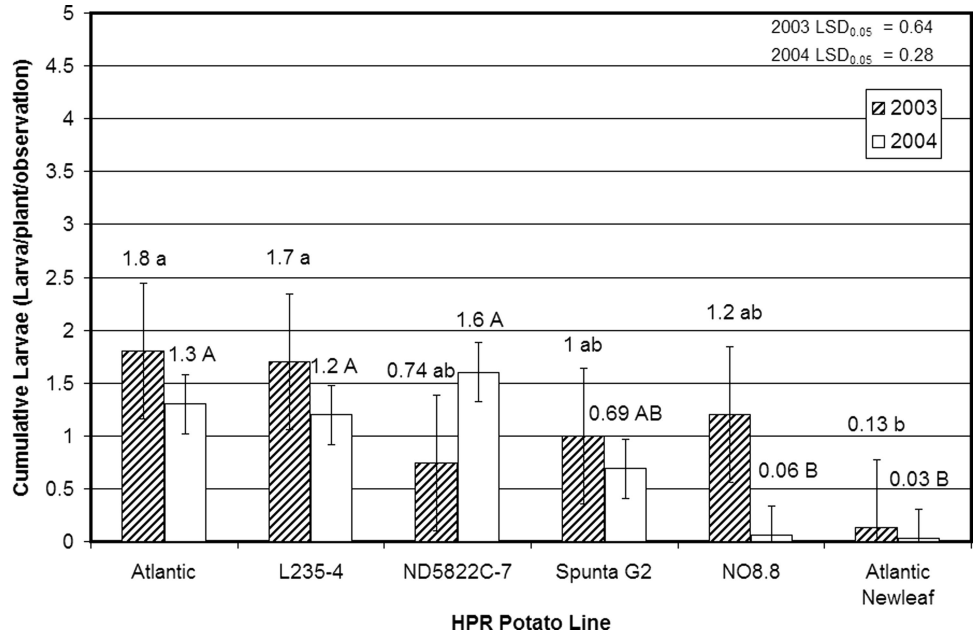

Fig. 2. Mean numbers of small Colorado potato beetle larvae (first and second stages) per plant on six potato lines (Atlantic, NYL235-4, ND5822C-7, Spunta-G2, NORc3.8, and Atlantic Newleaf) in a no-choice cage study in 2003 and 2004. Means within a year followed by different letters are significantly different ($\alpha = 0.05$).

The detached leaf assay in conjunction with our no-choice field assay suggest that glandular trichomes at lower densities, as in NYL235-4, may not be effective against Colorado potato beetle larvae in a no-choice setting (Coombs et al. 2002). However, NYL235-4 has exhibited excellent resistance at the beginning of the season in choice field studies, but it did not demonstrate resistance late in the season after susceptible potato lines had been defoliated (Coombs et al. 2005).

In 2003, RAUDCx100 values for ND5822C-7 (*S. chacoense*-derived resistance) did not significantly differ from RAUDCx100 for Atlantic (Fig. 1). In 2004, the RAUDCx100 value was significantly higher compared with Atlantic; however, the high RAUDCx100 value was not due to direct consumption by larvae; instead, larvae clipped the leaves at the petioles and fed on the stems of the plants. Although field resistance was observed in small plot choice trials, the *S. chacoense*-derived resistance in ND5822C-7 did not deter feeding in the no-choice situation (Coombs et al. 2005).

The resistance classification of ND5822C-7 was previously observed to be largely attributed to avoidance by adults (D.S.D., unpublished data). ND5822C-7 was not classified as resistant to Colorado potato beetle in this no-choice study. Large larvae clipped the petioles of the ND5822C-7, probably causing more defoliation than if they had just fed on the foliage. In 2004, the defoliation of ND5822C-7 was significantly higher defoliation than Atlantic (Fig. 1). The numbers of larvae and adults on ND5822C-7 were either not significantly different or significantly higher than the number on Atlantic. The large vigorous haulm of ND5822C-7 may limit the percentage of defoliation and the economic damage in no-choice field situations.

The numbers of small larvae on the susceptible Atlantic was 1.8 ± 0.6 and 1.3 ± 0.3 larvae per plant per observation in 2003 and 2004, respectively (Fig. 2). The numbers of small larvae were generally highly variable due in part to the difficulty of sampling for these on large plants. In addition, depending on the temperature, it requires 5–8 d to develop from neonates to third-stage larvae (Mailloux and Bostanian 1989). Data were collected every 7 d. Therefore, the number of small larvae observed was low regardless of the effects of host plant resistance on larval mortality. The numbers of small larvae were significantly lower on only Atlantic Newleaf in 2003 and 2004 and NORC3.8 in 2004 compared with the numbers of small larvae on the susceptible Atlantic. Previous studies have demonstrated that Colorado potato beetle neonates generally do not survive past 5 d on even low-expressing Bt potatoes (Cooper et al. 2004). Although larvae were observed 7 d after the egg masses were placed on NORc3.8 and Atlantic Newleaf, larvae were not observed for the remainder of the field season. In 2003 and 2004, the number of small larvae on Atlantic, NYL235-4, ND5882C-7, and Spunta-G2 did not significantly differ from each other.

In 2003, the numbers of large (third- and fourth-stage) larvae were also highly variable. Numbers of large larvae on each line did not significantly differ from each other or from the numbers of large larvae on the susceptible Atlantic line, even though no large larvae were observed on NORc3.8 or Atlantic Newleaf (Fig. 3).

In 2004, significantly more large larvae were found on ND5882C-7 than on the susceptible Atlantic line, suggesting antibiosis does not strongly contribute to its

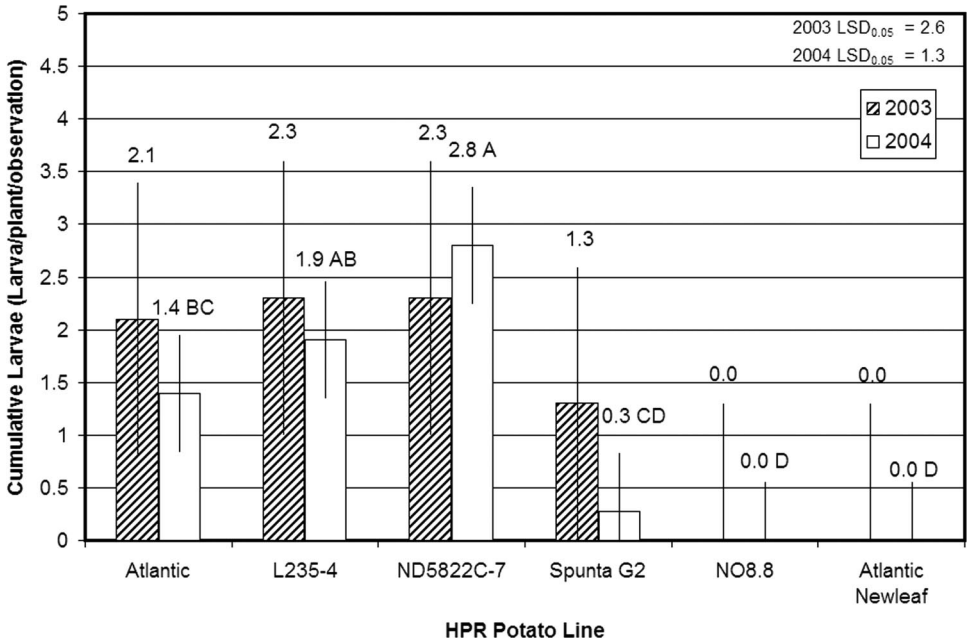

Fig. 3. Mean numbers of large Colorado potato beetle larvae (third and fourth stages) per plant on six potato lines (Atlantic, NYL235-4, ND5822C-7, Spunta-G2, NORc3.8, and Atlantic Newleaf) in a no-choice cage study 2003 and 2004. Means within a year followed by different letters are significantly different ($\alpha = 0.05$).

resistance in a no-choice situation (Fig. 3). The mean numbers of large larvae on NYL235–4 and Spunta-G2 were not significantly different from the number of large larvae on Atlantic. No large larvae were observed on either *Bt-cry3A* lines, significantly fewer than the numbers of large larvae on susceptible Atlantic, NYL235-4, or ND5822C-7.

No adults were observed on NORc3.8 or Atlantic Newleaf in either year (Fig. 4). In 2003, the number of emerging adults from larvae feeding on Atlantic,

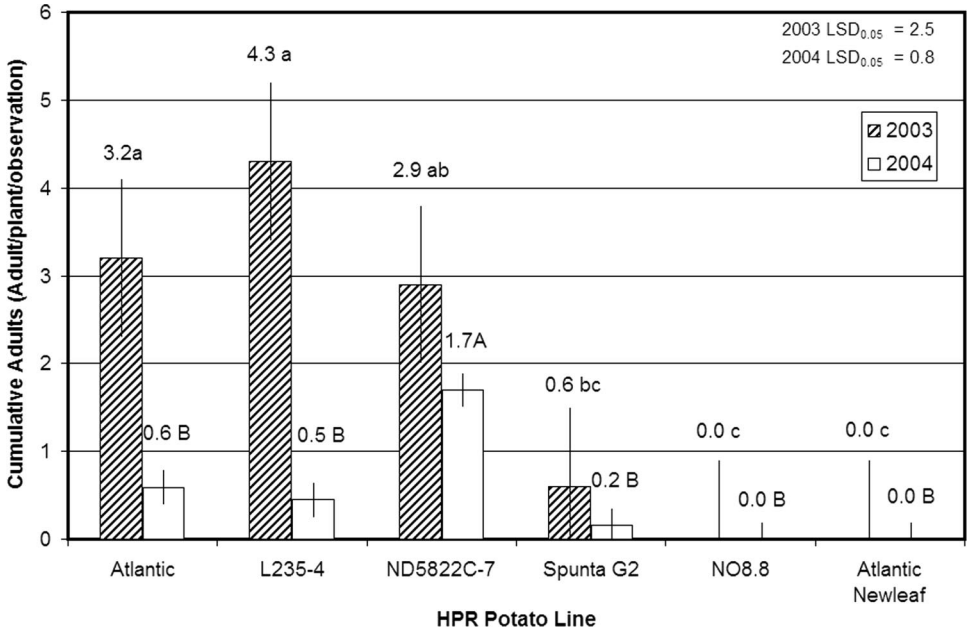

Fig. 4. Mean numbers of Colorado potato beetle adults per plant emerged on six potato lines (Atlantic, NYL235-4, ND5822C-7, Spunta-G2, NORc3.8, and Atlantic Newleaf) in a no-choice cage study in 2003 and 2004. Means within a year followed by different letters are significantly different ($\alpha = 0.05$).

NYL235-4, and ND5822C-7 did not significantly differ from each other. The number of emerging adults from larvae feeding on Spunta-G2, NORc3.8, and Atlantic Newleaf were significantly lower compared with adults on Atlantic. *Bt-cry1Ia1* targets a different receptor than coleopteran-specific *Bt-cry3A* (Griffitts and Aroian 2005). *Bt-cry1Ia1* could be combined with *Bt-cry3A* to provide a more durable resistance and also to combat multiple pests (Zhao et al. 2005).

In 2004, very few individuals from the egg masses developed into adults. The average temperature for June–August was 2.2°C below normal. The lower temperatures may have slowed the rate of development and affected survival to adult stage. Adult emergence also may have been delayed beyond the last evaluation date in 2004. The number of emerging adults on NYL235-4, Spunta-G2, NORc3.8, and Atlantic Newleaf did not differ significantly from the number of adults on Atlantic (Fig. 4). Only 0.59 ± 0.2 adults per plant per observation emerged from larvae feeding on Atlantic. The number of emerging adults from larvae feeding on ND5822C-7 was significantly higher compared with adults on all other lines.

Under the no-choice conditions in our study, only the *Bt-cry3A*- (NORc3.8 and Atlantic Newleaf)-expressing potato lines consistently and significantly reduced pest densities and injuries compared with the susceptible standard Atlantic. No large larvae or adults were detected on the plants in either year, and only minor defoliation, never exceeding 2%, was observed on the plants. The *Bt-cry1Ia1*-expressing line (Spunta-G2) significantly reduced injury 1 yr of 2 yr. The *S. chacoense*-derived resistance line (ND5822C-7) suffered significantly greater defoliation than the susceptible standard 1 yr of 2 yr. The glandular trichome line (NYL235-4) did not significantly differ from the susceptible standard for both years.

In this study, we attempted to remove Colorado potato beetle adult preference, both as food choice and oviposition site, by placing egg masses on potato lines with different resistance sources in field cages. The natural resistance, derived from glandular trichomes or *S. chacoense*-derived, did not withstand the beetle pressure under no-choice conditions. Future studies should be conducted to evaluate the behavior of adult beetles, including oviposition, on potato lines with *Bt-cry3A*, *Bt-cry1Ia1*, glandular trichome or *S. chacoense*-derived based resistance in a no-choice situation. Neither the choice field trial nor the no-choice laboratory trial closely mimics all aspects of commercial field conditions. Small plot choice field trials are critical to identify potentially resistant lines. Although the no-choice field cages are not equivalent to commercial conditions, they offer additional insight into resistance factors under these conditions. No-choice field trials are also essential to evaluate strength of resistance and to determine whether resistance may be effective in a large-scale commercial production. For example, the *Bt-cry3A*-expressing lines would be effective in large commercial field conditions because of high larval mortality. The *S. chacoense*-derived resistance and glandular trichome host plant resistance lines would not be effective in large commercial field conditions based on insect feeding and development on these lines in the no-choice cage trial.

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