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# Development of *Bt-cry5* Insect-resistant Potato Lines 'Spunta-G2' and 'Spunta-G3'

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***Abstract.*** **The potato tuber moth (*Phthorimaea operculella* Zeller) is the primary insect pest of cultivated potato (*Solanum tuberosum* L.) in tropical and subtropical regions, causing both foliar and tuber damage. In contrast, the Colorado potato beetle (*Leptinotarsa decemlineata* Say) is the most important insect pest in the northern potato production latitudes. The codon-modified *Bacillus thuringiensis Bt-cry5* gene (revised nomenclature *cry1IaI*), specifically toxic to Lepidoptera and Coleoptera, was transformed into cultivar Spunta using an *Agrobacterium* vector to provide resistance to both potato tuber moth and Colorado potato beetle. The *Bt-cry5* gene was placed downstream from the constitutive CaMV35S promoter. Two transgenic 'Spunta' clones, G2 and G3, produced high levels of mortality in first instars of potato tuber moth in detached-leaf bioassays (80% to 83% mortality), laboratory tuber tests (100% mortality), and field trials in Egypt (99% to 100% undamaged tubers). Reduced feeding by Colorado potato beetle first instars was also observed in detached-leaf bioassays (80% to 90% reduction). Field trials in the United States demonstrated that the horticultural performance of the two transgenic lines was comparable to 'Spunta'. These *Bt-cry5* transgenic potato plants with high potato tuber moth resistance have value in integrated pest management programs.**

Insect damage to tubers is often a serious problem in cultivated potato; one such destructive insect in the subtropic and tropical regions is the potato tuber moth. The larvae mine the foliage, stems, and tubers in the field and tubers in storage. Tuber infection by various other insects and secondary diseases that subsequently attack damaged tubers can cause dramatic losses. Annual losses in storage alone range from 30% to 70% in India and similar losses occur in the Middle East, North Africa, and South America (Raman and Palacios, 1982). Currently, available means to control the potato tuber moth and avoid major crop losses have left farmers with no alternatives to chemical control and, in some cases, the pesticides are applied directly to the potatoes in storage (Raman, 1988). The general utility of insecticides, however, is limited by high cost, persistence of residue in tubers and the environment, and development of insecticide-resistant pests.

The Colorado potato beetle is the most important pest of potato production in northern latitudes. This insect has shown the ability to adapt to many insecticides over the past half-century (Casagrande, 1987). Currently, it is reported to be resistant to 37 insecticides worldwide, including organophosphates, carbamates, organochlorines, pyrethroids, and hydrogen cyanide (Georgiou and Lagunes-Tejeda, 1991). Thus, if the Colorado potato beetle is to be properly managed, host plant resistance needs to be combined with integrated pest management practices.

The *Bt-cry5* toxin gene (designated *cry1Ia1* under the revised nomenclature as stated by Crickmore et al., 1998) from *B. thuringiensis* exhibits activity against both Lepidoptera and Coleoptera (Tailor et al., 1992), and was codon-modified to increase its expression level in plants. Douches et al. (1998) transformed this gene into potato and, subsequently, Mohammed et al. (2000) identified high *Bt-cry5* expressing potato lines using tuber bioassays.

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This paper describes the development of the *Bt-cry5* transgenic lines 'Spunta-G2' and 'Spunta-G3', which were subjected to potato tuber moth bioassays (foliar and tuber), Colorado potato beetle detached-leaf bioassays, agronomic field studies, and field and storage evaluations of tuber damage from potato tuber moth.

## Materials and Methods

'Spunta' is a long, white, tablestock cultivar bred in the Netherlands and grown widely in subtropical regions, such as North Africa and South America. 'Spunta' was transformed via *Agrobacterium tumefaciens* LBA4404 with a *Bt-cry5* construct (Fig. 1) according to Douches et al. (1998). The vector was the binary pBI121 (Clontech, Palo Alto, Calif.) with the codon-modified *Bt-cry5* gene (Tailor et al., 1992) supplied by Zeneca (Berkshire, U.K.). The pBI121 vector was digested with *SmaI* and *EcoICRI,* and self-ligated. The resulting plasmid, pBIML4, was then cleaved with *BamHI* and ligated with the *Bt-cry5* fragment that was cut from a Bluescript SK$^+$ based plasmid (Stratagene, La Jolla, Calif.), resulting in the plasmid pBIML5. Two *Bt-cry5*-

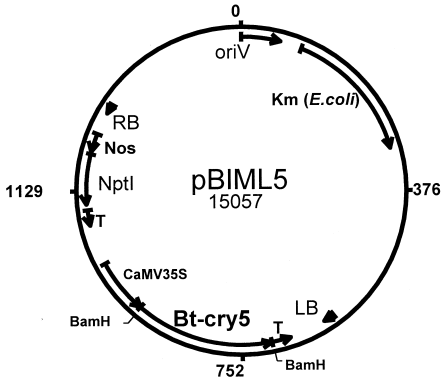


Fig. 1. pBIML5, plant transformation vector for *Bt-cry5*. Important functional region of the vector originates from plasmid pBI121, containing a chimeric neomycin phosphotransferase II gene and CaMV 35S promoter.

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Spunta lines, G2 and G3, with high insecticidal activity were selected from more than 20 lines for advanced testing because of their performance in preliminary detached-leaf bioassays.

*Insect bioassays.* Young, fully expanded leaves from pesticide-free, greenhouse-grown plants were collected for potato tuber moth and Colorado potato beetle detached-leaf bioassays. The petiole with leaf was removed from the plant using a new single-edged razor blade. A petiole leaf was inserted through a pre-moistened sponge (1 $cm^2$) into each 3.5-mL glass vial full of water. The single detached leaf was then placed horizontally in a petri dish (15 cm × 2 cm) containing a 15-cm-diameter 3MM Whatman paper. One leaf was used per replication and the bioassay was replicated four times per potato line. Ten neonate larvae were placed near the mid-rib of each leaf. The petri dish was covered with the lid and placed at 25 ± 2 °C and 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent light. Mortality of the larvae was determined after 72 h. For Colorado potato beetle leaf bioassays, leaf defoliation as a percentage of feeding of the susceptible cultivar Russet Burbank was also estimated. RBN15 and RBN20 (*Bt-cry3A*-transgenic lines from John Kemp, New Mexico State Univ.) were used as positive controls. 'Russet Burbank' and the 'Spunta' lines were negative controls. NYL235-4, a line with glandular trichome-based insect resistance (Plaisted et al., 1992), was also tested.

For tuber bioassays, a greenhouse-grown tuber (5–40 g) from 'Spunta-G2' or 'Spunta-G3' was placed in a Phytatray II box (Sigma, St. Louis). Holes were punched through the top for aeration. Five potato tuber moth neonates were placed on each tuber and kept at 23 ± 2 °C in the dark. The number of pupae, adults, and larvae in each box was counted after 4 weeks and percent mortality calculated. The tuber bioassay included four replications for 'Spunta-G2' or 'Spunta-G3'.

Field experiments were conducted at the International Potato Center (CIP) field station, Kafr-el-Zyat, Egypt, and AGERI, Giza, Egypt, to evaluate resistance to tuber moth for 'Spunta', 'Spunta-G2', 'Spunta-G3', and the glandular trichome-based insect resistant line, NYL235-4 (Plaisted et al., 1992) (tested in 1999 only). Seed tubers used in the field trials were obtained from seed plots at the Lake City Experiment Station, Lake City, Mich. In each trial, 10-plant (85.4-cm spacing) one-row plots were replicated four times using a randomized complete-block design. A row of a susceptible cultivar was planted between each plot. The trials were planted 24 and 25 Jan. and harvested 7 and 8 June 1999; in 2000 the CIP Egypt trial was planted 20 Feb. and harvested June 5. Normal agronomic practices were followed, except that no insecticides were applied. At harvest each tuber was visually examined for potato tuber moth damage and the percent tubers free from tuber moth damage was calculated.

To evaluate storage damage of the tubers, at least 100 undamaged tubers from each line from the CIP field trials in 1999 were placed in a traditional, ambient temperature potato storage building (nawalla). The tubers were examined monthly for tuber moth damage for 3 months.

A field trial was conducted at the Montcalm Research Farm, Entrican, Mich., in 2000 to measure total and marketable yields, determine tuber size distribution and specific gravity, and to evaluate tuber appearance, and incidence of external and internal defects. 'Spunta-G2', 'Spunta-G3', 'Spunta', 'Atlantic', and NYL235-4 were planted in a randomized complete-block design with four replications. The plots were 7 m long with 31-cm spacing between plants in-row. Between-row spacing was 86 cm. Normal agronomic practices were followed and supplemental irrigation was applied as needed.

All leaf and tuber bioassay data and potato tuber moth damage in the Egypt field experiments and the Michigan agronomic performance trial were analyzed by analysis of variance (ANOVA) as a randomized complete-block design using SAS general linear models procedure (SAS 1998). All percentage data were arcsine transformed before statistical analysis and untransformed data were presented. Mean comparisons were done using Fisher's least significant differences ($\alpha = 0.05$).

Table 1. Results from tuber and leaf bioassays of 'Spunta-G2' and 'Spunta-G3' transgenic lines and Spunta by feeding with potato tuber moth first instars.

| | % Mortality[z] | | Mining[y] |
|---|---|---|---|
| Potato line | Leaf | Tuber | (72h) |
| Spunta-G2 | 83 b | 100 b | N |
| Spunta-G3 | 80 b | 100 b | N |
| Spunta[x] | 8 a | 25 a | Y |

[z]Means in a column with different letters are significantly different, $P < 0.05$ (Fisher's Protected LSD).
[y]N: Leaf mining stopped within 72 h; Y: mining continued after 72 h.
[x]Non-transgenic control.

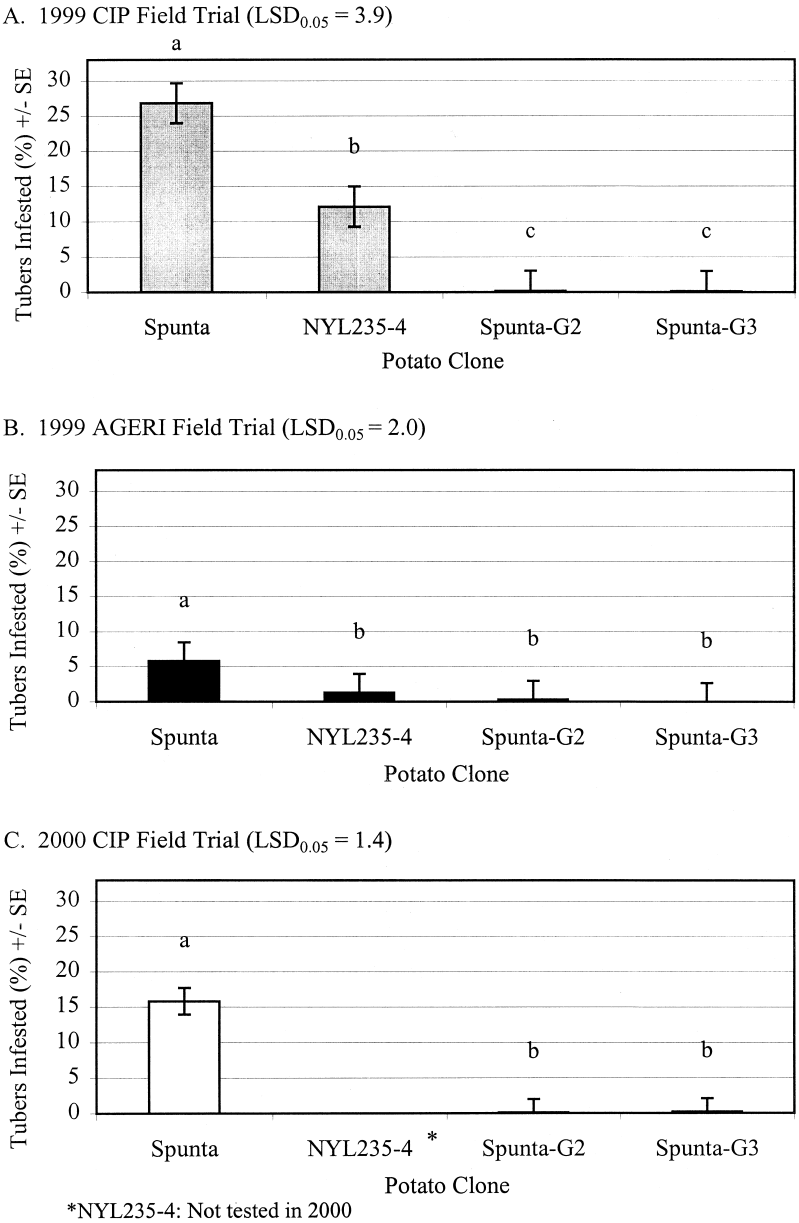


Fig. 2. Potato tuber infestation by potato tuber moth in insecticide-free field trials. Within each year/location, means with the same letter designation are not statistically different (Fisher's LSD, $\alpha = 0.05$).

## Results

Mortality of potato tuber moth larvae in detached-leaf bioassays on the transgenic plants was 80% to 83% after 72 h of feeding, compared to 8% on the non-transgenic 'Spunta' (Table 1). Although mortality was not 100%, growth of the surviving larvae on the transgenic plants was severely restricted during the first 72-h assay, and all larvae were dead after 5 to 7 d of feeding (data not shown). Very little feeding damage or mining of the leaves was observed on the transgenic plants. The leaves of non-transformed 'Spunta' were seriously damaged after 72 h of feeding and larvae grew rapidly (data not shown). In the tuber bioassays, adults emerged from 'Spunta' non-transgenic tubers after 4 weeks. Mortality was 25% on 'Spunta'. In contrast, the 'Spunta-G2' and "Spunta-G3' lines caused 100% tuber moth mortality with no adults emerging; no feeding damage was visible because the newly hatched larvae died before significant mining occurred in the tuber (Table 1).

In the 1999 field experiment at CIP Egypt, tuber moths damaged 27% of the 'Spunta' tubers. NYL235-4, 'Spunta-G2', and 'Spunta-G3' had significantly less tuber damage (Fig. 2). NYL235-4 had 12% damaged tubers, while feeding on the two *Bt-cry5*-Spunta lines was negligible. In 2000, the incidence of potato tuber moths was lower. 'Spunta' had 16% damaged tubers, while the 'Spunta-G2' and 'Spunta-G3' tubers were less than 1% damaged by tuber moths (Fig. 2). In the 1999 field experiment at AGERI Egypt, tuber moth damage was low. 'Spunta' had 6% damaged tubers, while NYL235-4, 'Spunta-G2' and 'Spunta-G3' were significantly better, with only 0% to 2% damaged tubers (Fig. 2).

During the 3-month storage trial conducted at CIP Egypt, 'Spunta' progressed from 0% to 97% tuber damage and NYL235-4 reached 93% damaged tubers (Fig. 3). 'Spunta-G3' and 'Spunta-G2' tubers were much less damaged, with 17% and 10% damaged tubers, respectively. Also, the severity of damage and invasion by rotting organisms was much less in 'Spunta-G2' and 'Spunta-G3' than in 'Spunta' (data not shown).

In the detached-leaf bioassays with first instar Colorado potato beetles, defoliation of 'Spunta' leaves was the same as for 'Russet Burbank' (100%). Likewise, feeding on NYL235-4 was not different from 'Spunta'; however, 'Spunta-G2' and 'Spunta-G3' had only 22% and 10% of the degree of feeding on 'Russet Burbank', respectively. In comparison, RBN15 and RBN20, two *Bt-cry3A*-transgenic lines, had negligible feeding (Fig 4a). Mortality of the larvae generally inversely paralleled the defoliation data. Larvae mortality on NYL235-4 and 'Spunta' was 5% or less, while larvae mortality on 'Spunta-G2' and 'Spunta-G3' lines was significantly greater (18 and 37%, respectively) (Fig. 4b). In comparison, *Bt-cry3A*-lines RBN15 and RBN20 had 67% to 70% larvae mortality.

In the Michigan agronomic trial, the yield of 'Spunta' was similar to 'Atlantic' (Table 2). The agronomic performance of the two *Bt-*

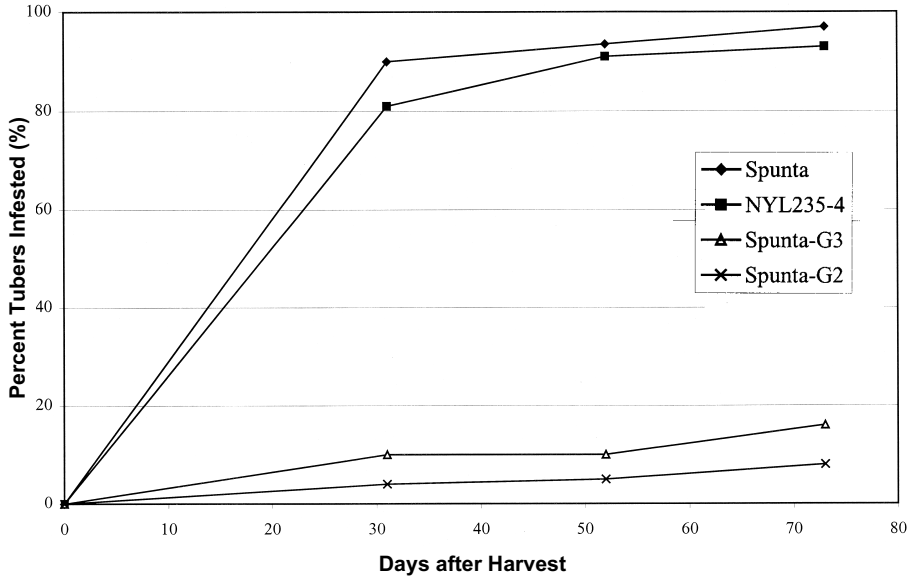


Fig. 3. Potato tuber moth infestation of insecticide-free tubers in nawhalla storage trials conducted at CIP, Egypt (1999).

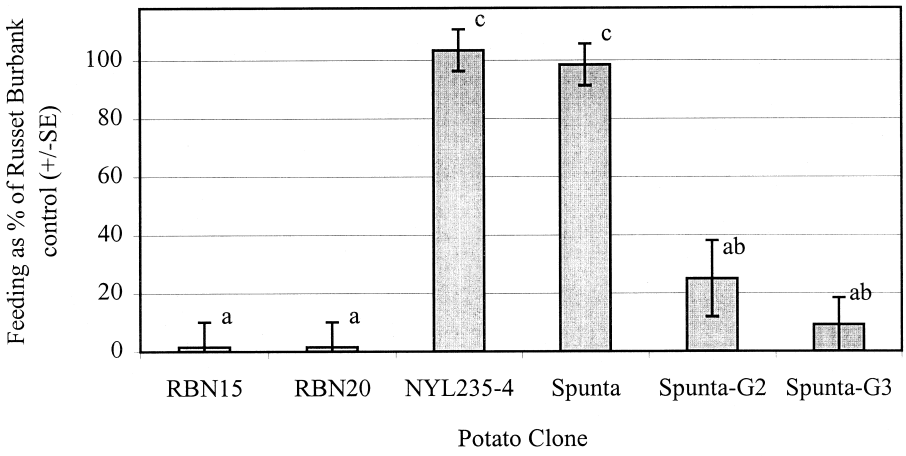


Fig. 4A. Feeding of Colorado potato beetle larvae in detached-leaf bioassays. Means with the same letter designation are not statistically different (Fishers's LSD, $\alpha = 0.05$).

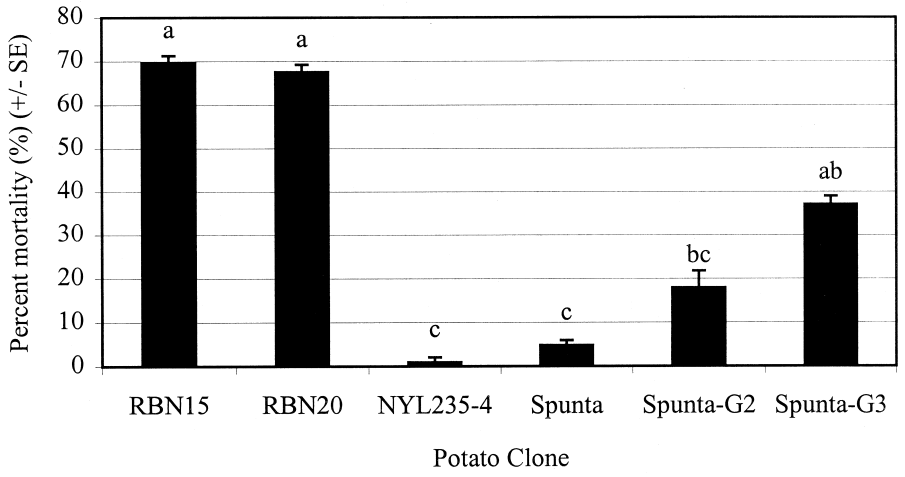


Fig. 4B. Mortality of Colorado potato beetle larvae in detached-leaf bioassays. Means with the same letter designation are not statistically different (Fishers's LSD, $\alpha = 0.05$).

*cry5*-Spunta lines was similar to 'Spunta'. There were no differences in total or US#1 yield, or tuber specific gravity between 'Spunta-G2', 'Spunta-G3', and 'Spunta'. Similar tuber size distributions and tuber quality were also observed between the two transgenic lines and 'Spunta'. NYL235-4 was the highest yielding of all lines.

Table 2. Field trial yield and tuber quality characteristics of potato lines and comparison cultivars, Montcalm Research Farm, Entrican, Mich.

| | Yield (t·ha$^{-1}$) | | Percent of total[z] | | | | | | Tuber quality[y] | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Line | US#1 | Total | US#1 | Bs | As | OV | PO | SP GR | HH | VD | IBS | BC | Cut |
| NYL235-4 | 61.1 | 67.7 | 90 | 9 | 80 | 11 | 0 | 1.084 | 0 | 0 | 0 | 0 | 40 |
| Spunta-G2 | 54.2 | 62.7 | 86 | 11 | 61 | 25 | 3 | 1.063 | 0 | 0 | 0 | 0 | 40 |
| Spunta | 51.4 | 60.5 | 85 | 10 | 62 | 23 | 5 | 1.065 | 1 | 0 | 0 | 0 | 40 |
| Spunta-G3 | 47.9 | 58.2 | 82 | 14 | 64 | 18 | 4 | 1.062 | 1 | 0 | 2 | 1 | 40 |
| Atlanitc | 47.0 | 51.1 | 92 | 7 | 72 | 20 | 0 | 1.090 | 16 | 0 | 0 | 1 | 30 |
| Mean | 52.3 | 60.1 | | | | | | 1.073 | | | | | |
| $LSD_{(0.05)}$ | 10.3 | 10.4 | | | | | | 0.005 | | | | | |

[z]Size distribution: B: < 5.1 cm; A: 5.1–8.3 cm; OV: > 8.3 cm; PO: Pickouts.
[y]Tuber quality: HH = Hollow Heart; VD = Vascular Discoloration; IBS = Internal Brown Spot; BC = Brown Center. Planted 4 May 2000; harvested 5 Sept. 2000 (124 d).

## Discussion

Insect pests are a primary problem facing potato farmers in developing countries. To implement an integrated pest management (IPM) program for potato tuber moth, host plant resistance would be a valuable first line of defense. The 'Spunta-G2' and 'Spunta-G3' potatoes would easily fit into an integrated pest management program to manage potato tuber moth. The two lines we have developed offer both protection from foliar and tuber mining. Combining the Bt-based resistance with natural resistance mechanisms may provide a more durable host plant resistance (Douches et al., 1998).

Earlier reports indicated that expression of *cry* genes was weak in transgenic plants compared to many other heterologous genes (Barton et al., 1987; Fischhoff et al., 1987). The insufficient production of toxic protein in situ led to poor host plant resistance. Higher A-T content was found in native δ-endotoxin coding regions than in plant DNA sequences, reducing the stability of mRNA (Fujimoto et al., 1993). In recent years, Bt gene modifications focused on the truncation of A-T rich regions and codon-modification in native *cry* protein genes (e.g., *cry1* and *cry3*), resulting in a distinct increase in Bt gene expression in certain crops (Adang et al., 1993; Koziel et al., 1993; Perlak et al., 1990; Wünn et al., 1996). The *Bt-cry5* gene used in this study was codon-modified to optimize expression in plants (Zeneca, personal communication). The CaMV35S promoter was employed for *Bt-cry* gene expression in transgenic potatoes (Douches et al., 1998; Peferoen et al., 1990; Perlak et al., 1990), since this promoter confers high constitutive gene expression in a wide variety of tissues during most stages of development (Odell et al., 1985). In our evaluations, both foliage and tuber tissue were tested, and high insect mortality was found in both tissues. In the field, tuber resistance to the tuber moth larvae resulted in little or no tuber damage.

Constitutive expression of the Bt gene in plants in the field can lead to insect resistance management issues. The use of tissue-specific promoters, such as the granule bound starch synthase (GBSS) promoter for tuber-specific expression (Visser et al., 1991) and the ST-LS1 promoter for leaf/stem-specific expression (Eckes et al., 1986), may help circumvent continuous exposure of potato tuber moths to Bt.

Egypt was chosen for field testing because of its high natural populations of *P. operculella* (Mohammed et al., 2000). CIP Egypt is located in the Nile River Delta potato production region of Egypt and was a more ideal site than AGERI Egypt, which is located in Giza. To further enhance insect pressure during tuber development, the trials were planted 1 month later than other commercial plantings. Natural infection levels varied between the 2 years; however, the infection levels were sufficient each year to discriminate 'Spunta-G2' and 'Spunta-G3' from the non-transgenic 'Spunta' (Fig. 2). The CIP Egypt location also supported nawalla storage experiments that relied on natural tuber moth pressure. Even starting with tubers with no visible damage or infestation, the tuber moth infestation was sufficiently high to reach near 100% tuber infection levels in 'Spunta' tubers following 2–3 months of storage (Fig. 3).

The experiments reported here have used one potato tuber moth species; other species of tuber moths occur in developing countries, including *Scrobipalpula absoluta* (Meyrick), *Symmetrischema plaeseosema* (Turner), and *Scrobipalpopsis solanivora* (Povolny). Lagnaoui et al. (2000) tested 'Spunta-G2' and 'Spunta-G3' with *Symmetrischema tangolias* (Gyen) in detached-leaf bioassays and observed similar mortalities to our data with *P. operculella.*

The *Bt-cry5* gene has both lepidopteran and coleopteran activity (Tailor et al., 1992). We tested 'Spunta-G2' and 'Spunta-G3' against Colorado potato beetle first instars in detached-leaf bioassays. Defoliation levels with 'Spunta-G2' and 'Spunta-G3' were not significantly different from the *Bt-cry3A*-transgenic lines (RBN15 and RBN20); however, the differences in mortality were significant (Fig. 4 a and b). A "high dose" expression level of Bt toxin has been proposed for Bt potatoes (Whalon and Ferro, 1998) for the purpose of resistance management. Our *Bt-cry5*-Spunta lines do not fit this requirement for managing Colorado potato beetle. However, the partial host plant resistance to the beetle may be of value in an IPM program as another control strategy option.

## Availability

Due to private ownership of the *Bt-cry5* gene and the CaMV 35S promoter, virus-free tissue culture plantlets or small amounts of seed of the 'Spunta-G2' and 'Spunta-G3' lines are available for research purposes only at this time. These lines can be obtained from Dr. Dave Douches at Michigan State Univ. (douchesd@msu.edu).

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