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Evaluations of Transgenic Potatoes for Resistance to Potato Tuberworm in the Laboratory and Field

David S. Douches · Joseph J. Coombs · Lawrence A. Lacey · Kimberly J. Felcher · Walter L. Pett

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Abstract The potato variety 'Spunta' was previously transformed to constitutively express the *cry1Ia1* gene from *Bacillus thuringiensis* from which three transgenic lines (SpuntaG2, SpuntaG3 and Spunta6a3) were chosen to evaluate for resistance to potato tuberworm (*Phthorimaea operculella* Zeller). Because potato tuberworm is becoming a serious pest in the Pacific Northwest of the United States, 'SpuntaG2', 'SpuntaG3' and 'Spunta6a3' were evaluated in Washington State through laboratory and field experiments. In the laboratory, both choice and no-choice experiments demonstrated that the transgenic 'Spunta' lines were completely resistant (100% mortality) to potato tuberworm. Potato tuberworm resistance was further supported by choice, field-cage studies in which the transgenic 'Spunta' lines harbored no potato tuberworm larvae at any sampling date while the controls were heavily infested (averaging 6.4 to as many 17.0 larvae per stem). These results indicate that the *cry1Ia1* gene could be an effective component of potato tuberworm management in the Pacific Northwest as well as the international venues where it has already been proven effective.

Resumen Se transformó previamente a la variedad de papa "Spunta" para que expresara constitutivamente el gen *cry1Ia1* de *Bacillus thuringiensis*, de la cual se seleccionaron tres líneas transgénicas (SpuntaG2, SpuntaG3, y Spunta6a3) para evaluarles su resistencia a la palomilla de la papa (*Phthorimaea operculella* Zeller). Considerando que la palomilla de la papa se está convirtiendo en una plaga seria en el Pacífico Noroeste de los Estados Unidos, "SpuntaG2", "SpuntaG3", y "Spunta6a3" se evaluaron en el Estado de Washington en experimentos de laboratorio y campo. En el laboratorio, ambos, experimentos seleccionados y no seleccionados, demostraron que las líneas transgénicas de Spunta fueron completamente resistentes (100% de mortalidad) a la palomilla de la papa. La resistencia fue posteriormente respaldada por estudios de selección, en jaulas de campo, en donde las líneas transgénicas de Spunta no mantuvieron a las larvas de la palomilla en ninguna de las fechas de muestreo, mientras que los testigos estuvieron fuertemente infestados (promediando de 6.4 a tanto como 17.0 larvas por tallo). Estos resultados indican que el gen *cry1Ia1* pudiera ser un componente efectivo en el manejo de la palomilla de la papa en el Pacífico Noroeste, así como a nivel internacional donde ya se ha probado que es efectivo.

Keywords SpuntaG2 · *Phthorimaea operculella* · Host plant resistance · Integrated pest management · *Bacillus thuringiensis*

D. S. Douches (✉) · J. J. Coombs · K. J. Felcher
Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824, USA
e-mail: douchesd@msu.edu

L. A. Lacey
USDA/ARS, Yakima Agricultural Research Laboratory, Wapato, WA 98951-9651, USA

W. L. Pett
Department of Entomology, Michigan State University, East Lansing, MI 48824, USA

Introduction

Potato tuberworm, *Phthorimaea operculella* (Zeller), is the most serious insect pest of potatoes worldwide (Radcliffe 1982; Kroschel and Lacey 2009). It is of greatest importance in tropical and subtropical latitudes, including the southern and southwestern US, but has recently become

established in the Pacific Northwest (De Bano et al. 2010). Damage caused by potato tuberworm can tremendously reduce the potato yield and quality because it attacks both the foliage and the tuber (Capinera 2001). In warmer climates, the quantity and quality losses in unrefrigerated storages can be as high as 100% (Lagnaoui et al. 2000). Potato tuberworms spend their larval stages in the foliage and/or in the tubers. In the foliage, the larvae mine the leaves and the petiole and the resulting damage can be extensive enough to cause severe plant health issues or even plant death. Eggs that are oviposited on exposed tubers hatch and the larvae enter the tubers through the "eyes". Once inside the tubers, the larvae create narrow tunnels throughout the tuber and introduce bacterial infestation. Insecticide use is the most common means of potato tuberworm control in both the field and in storage. In sub-tropical locations, 12–20 insecticide applications are commonly used to control potato tuberworm during the growing season (Madkour 1999).

Integrated pest management is the use of complementary strategies to control pest damage and limit any potential hazards to people or the environment. One of the key components of an integrated pest management program to control potato tuberworm is host plant resistance. At present there is no potato material produced from host plant resistance breeding that has appreciable levels of resistance to the potato tuberworm (Lagnaoui et al. 2000). The introduction of the codon-modified *Bacillus thuringiensis* (*Bt*) *cry1Ia1* gene via genetic engineering offers a form of plant resistance against potato tuberworm. The expression of Bt in crop plants offers an ecologically sound means to control specific crop insect pests. Naturally occurring Bt strains possess genes which code for a number of distinct toxic proteins (δ-endotoxins) with different spectra of activity against insects (Gould et al. 1994).

Since 1992, the Michigan State University potato breeding and genetics program has been involved in a USAID-funded international project to develop potato tuberworm-resistant potatoes using *Bt* technology (Douches et al. 1998; Westedt et al. 1998; Li et al. 1999; Lagnaoui et al. 2000; Mohammed et al. 2000). The long yellow variety, 'Spunta', has been transformed with the *cry1Ia1* Bt gene (Douches et al. 2002). Several *cry1Ia1*-Spunta lines have been characterized at the molecular level (Zarka et al. 2010), tested for food safety (Quemada et al. 2010), and field tested in Egypt, South Africa and Michigan for efficacy against potato tuberworm and for agronomic traits (Mohammed et al. 2000; Douches et al. 2010). This research has led to germplasm that has commercial potential in the U.S. and abroad (Egypt, South Africa, South America, Mexico and Indonesia) (Mohammed et al. 2000; Douches et al. 2002). Because potato tuberworm has become a problem in the Pacific Northwest of the United States, the transgenic lines 'SpuntaG2', 'SpuntaG3' and 'Spunta6a3' were evaluated in Washington State in both laboratory and field experiments with both choice and non-choice experimental designs.

Materials and Methods

Plant Material The potato variety 'Spunta' was transformed using vector constructs designed to constitutively express the *cry1Ia1* gene as described in Li et al. (1999) and Zarka et al. (2010). Tubers of these transformed Spunta lines ('SpuntaG2', 'SpuntaG3' and 'Spunta6a3') were produced under field conditions by the Michigan State University Potato Breeding Program and held at 3°C prior to use in bioassays.

Potato Tuberworm Choice, No-Choice Bioassays A 4 L Food Quality Storage Container (17.7 cm top diam.× 21.3 cm in height×14.0 cm bottom diam.) was set up with a ventilated lid using organdy material (6 cm diameter opening) and a tuber/s to be studied (either non-transgenic 'Spunta', or 3 transgenic lines: 'SpuntaG2', 'SpuntaG3' or 'Spunta6a3') of about 100 g. No-choice tests consisted of a single test tuber in the container, while choice tests used a combination of a transgenic tuber and a control tuber ('Spunta'/'SpuntaG2', 'Spunta'/'SpuntaG3', and 'Spunta'/'Spunta6a3'). The potato tuberworms used in this study were originally collected from an infected field in Oregon and the colony was maintained as indicated by Lacey et al. (2010). For no-choice, indirect infestation, 30 newly hatched potato tuberworm larvae were taken from the colony and placed on the side of the container within 5 cm from the bottom. The assay was conducted on six separate dates, with four replicate tubers tested at each date, resulting in 24 tubers tested in total. For the no-choice, direct infestation bioassays, 30 potato tuberworm neonates were placed directly on the tuber. The assay was conducted on three separate dates, with four tubers tested at each date, resulting in 12 tubers tested in total. Choice tests were conducted in a similar manner with the following adjustments: both a 'Spunta' control tuber and a transgenic tuber were included in each container, 15 neonates were applied for both direct and indirect infestation and only three containers were set up at each testing date. Therefore, 18 tubers of each transgenic line were tested in indirect infestation assays and nine tubers of each were tested in direct infestation assays. Following infestation, test containers were labeled and stored at 27°C for a total of 15 days. Three to five days after infestation, the tubers were carefully lifted and 1 cm of dry, sterile, fine-grit sand was placed at the bottom of the tub to allow for pupation of emerging potato tuberworm larvae. On the 15th day

following infestation, the tubers were removed from the containers, bisected and examined under a dissecting microscope to confirm that the exposed mines were caused by potato tuberworm larvae. Damage to the tubers was assessed on a scale of 0 (no damage), 0.5 (superficial damage with no evidence of tuberworms) and 1.0 (one or more verifiable entry/entries of tuberworm larvae) (Fig. 1). The remaining sand in the containers was sifted and potato tuberworm pupae were counted.

Field Cage Testing The field trial was planted (May 13, 2005). The 1 m^3 cages consisted of PVC pipe frames covered on the top and sides with mesh fabric (mesh size=38×44) with a 61 cm^2 door on one end, attached with Velcro. The frames were staked down with rebar-hooks on two opposite sides and the fabric was placed over the frame and the ends buried. The experimental design was a randomized complete block with four replicate plots. Each plot consisted of four rows, with a different treatment in each row ('Spunta', 'Spunta6a3', 'SpuntaG3', and 'SpuntaG2').

At 32 days after planting, a cage was placed over three potato plants in each row of every plot for a total of 16 cages. Caged plants were selected for their general health and cleaned of any insects and egg masses. Thirty-five potato tuberworm pupae were introduced into each cage along with containers of 15% honey water at 48 days after planting. The cages were checked periodically in an attempt to circumvent any unwanted insect population growth inside the cage (e.g. Colorado potato beetle). The potato tuberworm population was given 27 days to develop inside the cages, at which time (75 days after planting) three major stems from each cage were sampled and brought back to the lab to determine the number of larvae per stem. Plants were sampled again in the same manner, 90 and 97 days after planting. At 171 days after planting, 25 tubers were harvested from each cage and evaluated for damage, as previously described.

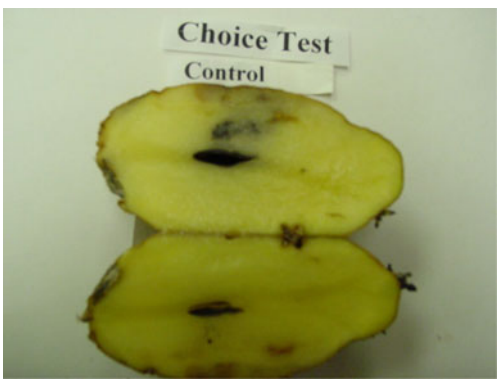

Fig. 1 Damage to Spunta-control (damage rating 1)

Data were analyzed using analysis of variance and Fisher's protected least significant difference for means separation (α=0.05). All analyses of variance were performed using the SAS general linear model procedure at α=0.05 (release 8.20; SAS Institute, Cary, NC).

Results

In the laboratory no-choice potato tuberworm assays with indirect application of neonates, the three transgenic lines ('SpuntaG2', 'SpuntaG3' and 'Spunta6a3') had 0 pupae recovered, 0 emerged adults. 'SpuntaG2' was given a 0 damage rating at all evaluation points and in all replications whereas; 'SpuntaG3' and 'Spunta6a3' were given a mean damage rating of 0.02. However, the observed damage in these two transgenic lines was probably not due to potato tuberworm as the damage was superficial and without any sign of frass. Dissection of the damaged area confirmed this conclusion. In contrast, the 'Spunta' controls had an average of 4.4 pupae/tuber, an average of 4.2 emerged adults/tuber and an average damage rating of 1.0. When neonates were applied directly to the tubers the results were similar (Table 1). In laboratory choice potato tuberworm assays, results were similar for both direct and indirect neonate application (Table 2). No tuberworm pupae, adults or damage were observed in the transgenic potato lines ('SpuntaG2', 'SpuntaG3' and 'Spunta6a3'). However, in the 'Spunta' controls the damage rating was always 1.0, the average number of pupae/tuber ranged from 3.1 to 5.2 and the average number of adults/tuber ranged from 3.1 to 4.7.

In the field cage studies, the transgenic varieties harbored no potato tuberworm larvae at any of the sampling dates and the plants remained green and vigorous throughout the study. However, the 'Spunta' control foliage was heavily infested with averages of 6.4±3.1, 17.0±5.6 and 6.2±2.7 potato tuberworm larvae per stem at 75, 90 and 97 days after planting respectively. Furthermore, at the third and final sampling date, vines in one of the control cages were so wilted and senescent that they were not sampled (i.e. n=3 cages). Shortly thereafter, 'Spunta' control vines in the other three cages were in similar condition.

When the tubers were harvested from the field-cage trials, the 'Spunta' controls had 5.3% damaged tubers, whereas, there was no damage to any of the transgenic tubers (data not shown). It was noted that there were no cracks in the hills prior to harvest and that there were still adult tuberworms within the cages at harvest. The lack of cracking in the soil would prevent or limit tuber access by adults which may account for the low level of tuber damage in the controls.

Table 1 Effect of 'Spunta' and *cry1Ia1*-Spunta potato varieties on the survival of potato tuberworm larvae and on tuber damage in no-choice assays with direct and indirect infestation

Test tuber	Indirect infestation (n=24 tubers) (mean/tuber)[a]			Direct infestation (n=12 tubers) (mean/tuber)[a]		
	Damage[b]	Pupae	Adults	Damage[b]	Pupae	Adults
Spunta	1.0	4.4	4.2	1.0	5.3	5.3
SpuntaG2	0	0	0	0	0	0
SpuntaG3	0.02[c]	0	0	0	0	0
Spunta6a3	0.02[c]	0	0	0	0	0
$LSD_{0.05}$		0.1	0.1		0.3	0.4

[a] For indirect infestation, neonates were placed on the side of the container 5 cm from the bottom; for direct infestation, neonates were placed directly onto the tubers

[b] Damage ratings: 0=no damage, 0.5=superficial damage, no evidence of tuberworm larvae, 1.0=one or more verifiable entry/entries of tuberworm larvae

[c] Damage was superficial, exhibited no frass and therefore was not likely to be caused by potato tuberworm

Discussion

Chemical control of potato tuberworm can be difficult therefore the development of host plant resistance is vital to the management of this insect pest. The use of *Bt* genes in agricultural crops to control insect pests is well documented including commercialized examples in cotton and corn. In 2010, *Bt* cotton accounted for 73% of the cotton acreage in the U.S. and *Bt* corn accounted for 63% of the U.S. corn acreage (Fernandez-Cornejo 2010). In the U.S. the use of pesticides decreased by 2.5 million pounds in the 10 years following the adoption of *Bt* crops in 1996 (Fernandez-Cornejo and Caswell 2006) indicating that *Bt* genes are an important and effective component of pest management strategies. The results of our study corroborate this evidence. All three transgenic 'Spunta' lines gave complete control of potato tuberworm in both the laboratory and in the field. This is supported by multiple years of trials in South Africa and Michigan in which 'SpuntaG2' gave complete resistance to potato tuberworm infestation in both the field and in storage (Douches et al. 2010). Agronomic trials conducted over multiple years and locations demonstrated that 'SpuntaG2' does not differ from non-transgenic 'Spunta' with respect to yield, other important agronomic properties, tuber quality traits and processing traits (Douches et al. 2010). Furthermore, previous studies have characterized 'SpuntaG2' at the molecular level (Zarka et al. 2010) and demonstrated that it is safe for human consumption (Quemada et al. 2010). The results of our study clearly indicate that the *cry1Ia1* gene could be an effective component of potato tuberworm management in the Pacific Northwest in addition to the international venues where it has already been proven effective.

Table 2 Effect of 'Spunta' and *cry1Ia1*-Spunta potato varieties on the survival of potato tuberworm larvae and on tuber damage in choice assays with direct and indirect infestation

	Indirect infestation (n=36 tubers) (mean/tuber)[ab]			Direct infestation (n=18 tubers) (mean/tuber)[ab]		
	Damage[cd]	Pupae	Adults	Damage[cd]	Pupae	Adults
Spunta/SpuntaG2	1.0/0	4.7	4.6	1.0/0	4.6	4.3
Spunta/SpuntaG3	1.0/0	3.5	3.4	1.0/0	3.1	3.1
Spunta/Spunta6a3	1.0/0	3.2	3.1	1.0/0	5.2	4.7
$LSD_{0.05}$		0.3	0.3		1.3	1.1

[a] For indirect infestation, neonates were placed on the side of the container 5 cm from the bottom; for direct infestation, neonates were placed directly onto the tubers

[b] Only 'Spunta' control tubers (n=18 for indirect infestation and n=9 for direct infestation) were used to calculate the mean/tuber for pupae and adults, as all the transgenic tubers were undamaged

[c] Damage ratings: 0=no damage, 0.5=superficial damage, no evidence of tuberworm larvae, 1.0=one or more verifiable entry/entries of tuberworm larvae

[d] First damage rating refers to 'Spunta' control tuber; second damage rating refers to the transgenic variety

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