Monsanto

95-338-01P

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December 14, 1995

Mr. Michael A. Lidsky, Esq.
Deputy Director, BBEP, APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Subject: Amendment to Petition for Determination of Nonregulated Status for Potatoes Producing the Colorado Potato Beetle Control Protein of *Bacillus thuringiensis* subsp. *tenebrionis* . Monsanto# 95-247U

Dear Mr. Lidsky:

On December 4, 1995, the Monsanto Company submitted a petition under 7 CFR part 340.6 to request a determination from the Animal and Plant Health Inspection Service that NewLeaf™ potato plant lines SPBT02-5 and SPBT02-7, transformed with the plasmid vector PV-STBT02 no longer be considered regulated articles. At this time, we would like to amend our petition to also include NewLeaf™ potato lines ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36 lines, which were transformed with the plasmid vector PV-STBT04.

Please replace this version of the petition for that submitted on the above date and return the earlier version to us.

Thank you for your prompt attention to this matter. Please feel free to contact either Dr. Dickerson (202-783-2460) or myself (314-537-6547) if you need additional information.

Sincerely,

Terry B. Stone
Regulatory Affairs Manager

cc: Dr. C. T. Dickerson, Jr.

95-338-01p
[AMMENDED 12/15/95]

PETITION FOR DETERMINATION OF NONREGULATED STATUS

POTATOES PRODUCING

THE COLORADO POTATO BEETLE CONTROL PROTEIN

OF

Bacillus thuringiensis subsp. *tenebrionis*

Submitted by

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PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR POTATOES PRODUCING THE COLORADO POTATO BEETLE CONTROL PROTEIN OF *Bacillus thuringiensis* subsp. *tenebrionis*

SUMMARY

The Monsanto Company submits this petition under 7 CFR part 340.6 to request a determination from the Animal and Plant Health Inspection Service (APHIS) that two Colorado potato beetle resistant (NewLeaf™) potato lines of cv. Superior transformed with PV-STBT02 and five lines of cv. Atlantic transformed with PV-STBT04, should no longer be considered regulated articles under 7 CFR part 340. Potatoes transformed with either of these plasmid vectors are resistant to the Colorado potato beetle (CPB) through the production of a small amount of an insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*). This protein is identical to that found in nature and in commercial *B.t.t.* formulations registered as pesticides with the Environmental Protection Agency (EPA) since 1988. The *B.t.t.* protein is highly selective in controlling the CPB and is expressed at a consistently effective level in the potato foliage throughout the growing season. NewLeaf™ potatoes also produce neomycin phosphotransferase II (NPTII, also known as APH(3')II), which serves as a selectable marker to aid plant transformation.

This will be the second petition for a determination of nonregulated status Monsanto Co. has made to the USDA for NewLeaf™ potatoes. The first petition for seven lines of NewLeaf™ cv. Russet Burbank potatoes transformed with the plasmid vector PV-STBT02 was submitted to the USDA on September 14, 1994. The Agency granted this request on March 2, 1995, determining that the seven potato lines are no longer considered to be regulated articles under 7 CFR part 340.6.

The two NewLeaf™ potato lines of cv. Superior; SPBT02-5 and SPBT02-7, were transformed with the same plasmid vector (PV-STBT02) as the seven NewLeaf™ Russet Burbank lines described above. The PV-STBT04 plasmid vector used to transform the five lines of cv. Atlantic; ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36, employs a different promoter for the *cryIIIA* gene (ArabSSU1A) than PV-STBT02 (E35S) to express the identical *B.t.t.* protein in NewLeaf™ Russet Burbank potatoes. In all other respects, the components of these two plasmid vectors are identical.

In contrast to cv. Russet Burbank, Superior and Atlantic potato varieties are male fertile. However, data and information for the Superior and Atlantic NewLeaf™ potato lines transformed with PV-STBT02 or PV-STBT04 are provided to demonstrate that these potatoes are no more likely to become a weed than their non-modified parental variety and are unlikely to increase the weediness potential of any cultivated plant or native wild species. In addition, these lines do not exhibit plant pathogenic properties and exhibit no toxicity to non-target organisms, including those organisms that are beneficial to agriculture.

Therefore, based on the data and information enclosed in this petition, we request that APHIS determine that the NewLeaf™ Superior and Atlantic potato lines: SPBT02-5, SPBT02-7, ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36 need no longer be regulated under 7 CFR part 340.6.

CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

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ABBREVIATIONS

AT	cv. Atlantic
B.t.t.	*Bacillus thuringiensis* subsp. *tenebrionis*
bp	Base pair
CPB	Colorado potato beetle, *Leptinotarsa decemlineata* (Say)
cv.	Cultivar
CV	Coefficient of variability
cryIIIA	Class III (Coleoptera-specific) parasporal crystal protein gene from *B.t.t.*
°C	Degree Centigrade
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
°F	Degree Fahrenheit
IPM	Integrated pest management
kb	Kilobase pair
Kg, g, mg, µg, ng, pg	Kilogram, gram, milligram, microgram, nanogram, picogram
kD	Kilodalton
LB	Left border
LSD	Least significant difference
nptII	Neomycin phosphotransferase type II gene
OD	Optical density
RB	Right border
R. B.	cv. Russet Burbank

ABBREVIATIONS (Cont'd)

SE	Standard error
SU, SP	cv. Superior
T-DNA	Transferred-DNA

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I. RATIONALE FOR THE DEVELOPMENT OF COLORADO POTATO BEETLE RESISTANT POTATOES

A. Need for NewLeaf™ Potatoes

Potatoes are produced to some extent in all fifty states and the United States is currently ranked fourth among potato producing countries (National Potato Council, 1992). The Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) is the most damaging pest of the 2.3 billion dollar U.S. potato crop (Casagrande, 1987; National Potato Council, 1992) and approximately one-third of the 2.8 million pounds of chemical insecticides annually applied to potatoes are targeted for its control (USDA, 1993). CPB damage is particularly severe in the eastern and north central potato production areas and is becoming an increasing problem in the northwest. Both larval and adult stages feed on potato foliage and, if not controlled, can undergo population growth rates exceeding 40 fold per generation (two and potentially three generations per year are possible in many areas) and a potential overwintering survival rate of more than 60% (Groden and Casagrande, 1986; Harcourt, 1971). If poorly managed, the CPB is capable of completely defoliating potato plants, resulting in yield reductions of as much as 85%, which is sufficient to prevent potato production in some areas (Roush, 1993; Hare, 1980; Ferro *et al.*, 1983; Shields and Wyman, 1984). Loss of revenue due to the CPB in Michigan alone was estimated at more than 15 million dollars in a state where total potato production in 1991 was valued at 70 million dollars (Potato Growers of Michigan, Inc. and the Michigan Potato Industry Commission, 1992; Olkowski *et al.*, 1992).

Current control of CPB relies heavily upon the use of chemical insecticides that are variably effective due to environmental factors or insect sensitivity. These insecticides are also expensive with costs that can exceed $200 per acre per season (Ferro and Boiteau, 1992). Additional management options for CPB include, crop rotation, vacuum suction (Boiteau *et al.*, 1992), propane flaming (Moyer, 1992; Moyer *et al.*, 1991), polyethylene-lined trenches (Roush, 1993; Wyman, 1993) and trap plots (Roush, 1993; Roush and Tingey, 1992). These options are not often practical, effective, economical nor easily implemented throughout the season (Roush, 1993; Wyman, 1993).

Microbial *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) formulations containing the insecticidal proteins have been commercially available for CPB control since the late 1980's (Zehnder and Gelernter, 1989). These formulations are variably effective due to poor spray timing, inadequate plant coverage, short residual activity, and an inability to control large larvae and adults (Ferro and Lyon, 1991; Ferro and Gelernter, 1989; Zehnder and Gelernter, 1989). In contrast, NewLeaf™ potatoes produce the *B.t.t.* protein throughout the potato foliage and at a level high enough to control all CPB life stages throughout the growing season (Perlak, *et al.*, 1993; Appendix 1). Such consistently sustained control of CPB is not possible with currently available microbial, chemical, or physical control methods. In addition, the specificity of the *B.t.t.* protein to CPB permits the populations of predaceous and parasitic insects to increase unhindered by the application of broad spectrum chemical insecticides as no additional applications to control CPB are required (Appendix 1). These beneficial insects can then aid in the

control of non-target potato insect pests such as aphids and leafhoppers and the diseases they transmit. The combination of NewLeaf™ potatoes and beneficial insects provides a safe and an environmentally compatible foundation for the implementation of other potato pest management practices.

B. Risk Reduction Due to the Introduction of NewLeaf™ Potatoes

Reducing the amount of chemical insecticides applied to NewLeaf™ potatoes will not only enhance potato pest management but will also reduce the potential for farm worker and environmental exposure. A wide range of chemistry has been employed for CPB control with the organophosphates, carbamates and pyrethroids currently predominating (Casagrande, 1987; Tette and Heinmiller, 1992). When applied according to the label, these insecticides are not expected to pose any unacceptable risks to workers and the environment. However, the potential for accidental release or exposure during shipping, storage, mixing and loading, application and container disposal does exist. NewLeaf™ potatoes would reduce chemical pesticide use in potato production and the associated risks of accidental exposure.

The integrity of groundwater resources is of critical concern in potato production since potatoes are frequently grown on irrigated, coarse textured soils which are highly vulnerable to pesticide leaching (Wyman, 1993). In the 1970's and 1980's systemic carbamate insecticides were widely used by potato growers because of their effectiveness in CPB control. As a result, contamination of groundwater resulted in several areas (Zaki *et al.*, 1982; Rothschild *et al.*, 1982) and carbamate systemics were withdrawn from use and replaced with intensive foliar spray programs. NewLeaf™ potatoes will offer growers a selective, long lasting control alternative to systemic chemical insecticides without risk of groundwater contamination and will provide an alternative to intensive foliar spray programs.

C. Economic Factors Due to the Introduction of NewLeaf™ Potatoes

The costs associated with potato production are considerable. The USDA (1988) estimates that as much as $1,000 per acre is spent for production with as much as 35% utilized to control the CPB (Wyman, 1993). This financial burden could be significantly alleviated by the planting of NewLeaf™ potatoes, which would eliminate the need for additional CPB pesticide applications. While the cost of NewLeaf™ seed potatoes has not yet been determined, growers can expect a savings when the cost of these potatoes is compared to their present CPB insecticide costs. Just as important, however, will be the reduction in yield losses resulting from the superior CPB control provided by these genetically modified plants. Even the best currently available CPB management programs result in some yield loss due to CPB defoliation (Guenthner, 1993). NewLeaf™ potatoes are essentially immune to CPB feeding, consequently, the full yield potential of potatoes without CPB damage can be realized. This decrease in yield loss will result in increased grower profits. In addition, NewLeaf™ potatoes will benefit equally both large and small growers. The technology will be equally accessible and available to all growers, as no additional labor, planning, or machinery will be required for implementation (Guenthner, 1993).

For the consumer, more potatoes produced at less cost may result in lower prices for potato products (Guenthner, 1993; Hill and Florkowski, 1991). The ability to successfully control CPB will also positively impact the potato processing industry. Potato processing facilities are located primarily in areas of extensive potato production. In several of these areas potato production is seriously threatened by the increasing inability to manage CPB (Hare, 1980; Ferro *et al.*, 1983). The ability of NewLeaf™ potatoes to successfully control this serious pest could contribute to the stability of the potato industry in these areas.

NewLeaf™ potatoes utilizing the *B.t.t.* CPB control protein will have a more positive impact on the environment than the use of chemical insecticides to control CPB. The *B.t.t.* protein produced by these potatoes breaks down rapidly in the soil, cannot volatilize or drift, and is safe to nontarget organisms such as fish, birds, humans and other mammals. The superior CPB control offered by these potatoes will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop. As a result, they will be able to utilize a host of IPM practices that cannot be currently implemented because of their current dependence on chemical insecticides to control this pest. An increase in the biological and cultural control of non-target potato pests and a more judicious use of chemical insecticides will also result in safer work conditions for farm employees and reduce the potential for pesticidal drift, groundwater contamination and accidental spills. In addition, the costs associated with the use of insecticide handling, storage and disposal will also decrease.

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II. THE POTATO FAMILY

The Potential for Gene Escape from Cultivated Transgenic Potatoes Within the U.S.

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A. Summary

Potato genetic engineering has caused concern that exotic genes will escape into wild relatives of potato and develop the potential for ecological disruption. In some situations this could happen. Over nine hundred species of *Solanum* have been identified, most near the centers of origin in Central and South America and many cross freely with the cultivated potato (*S. tuberosum*). However, within the borders of the U.S., only two species of tuberizing *Solanum*, *S. fendleri* and *S. jamesii*, have been confirmed to exist. Neither species hybridizes with *S. tuberosum* due to differences in ploidy level, differences in endosperm balance number (EBN) or a combination of the two. Both species are found in high elevation, arid climates and are seldom geographically adjacent to potato production areas. Several species of *Solanum* are considered weeds in cultivated fields, including several species of nightshade. None of these species are closely related and none will hybridize with potatoes. The lack of compatible wild species and the clonal propagation system used in potatoes leads to the conclusion that within the borders of the U.S. no opportunity exists for the escape of introduced genes from cultivated types to wild relatives of potato.

B. History and Geography of Potato Production and Use

The potato (*Solanum tuberosum*) is native to the western hemisphere and occurs in abundance from the tropical highlands of Mexico, southward throughout western South America. Around 1570, South American cultivated potatoes were introduced into Europe. Descendants of these early European potatoes were permanently introduced into the U.S. in 1719 by Irish immigrants when they established a colony in New Hampshire (Stevenson, 1951). As Europeans settled in North America, potato production spread throughout the geographical area currently controlled by the U.S.

Potatoes are currently produced to some extent in all fifty states and the U.S. ranks fourth in world production (National Potato Council, 1992). The major commercial production areas are located in the northernmost states of the continental United States and include Maine, New York, Michigan, Wisconsin, Idaho, Oregon and Washington. Exceptions are substantial potato acreages in California and Florida, and minor, but significant acreage in Alabama, Arizona, Louisiana, Nevada, New Mexico, North Carolina, Texas and Virginia (National Potato Council, 1992).

Per capita consumption of potatoes in the United States is ca. 130 pounds or more than one 150 g potato each per day (USDA, 1991). In 1990, 85% of the crop was used for

human consumption (either processed or as tablestock), 6% was planted as seed, and less than 1% was used for animal feed. The feed use is limited by region, by season and is confined to a few integrated potato grower/processors or individual farmers. Shrinkage, loss, and home use represented the remaining 8% (National Potato Council, 1992).

C. Modern Potato Production and Potato Life Cycle

Cultivated potatoes are a clonally propagated crop, grown as an annual, with tubers from the previous year's crop serving as propagules. In the U.S., potato acreage is rotated with other crops on a cycle of two to five years. In most potato growing regions of the U.S., winters are severe enough to freeze and destroy tubers left after the harvest season, eliminating the possibility of escapes. However, in areas with heavy snow cover or mild winters, clonally generated volunteer potatoes are common and may persist for several years. The number of volunteers is reduced, but not eliminated by cultivation and herbicide usage in subsequent crops. Small grains are a common rotation crop and herbicides used in small grains are effective for reducing the number of volunteers. The rate of decline for volunteers has not been well documented but is highly dependent on the severity of the environment. Volunteers from true seed following berry production by fertile varieties will germinate for up to eleven years following seed production with a 40-50% reduction in emergence each year (Lawson, 1983). However, in the long term, potatoes are not competitive with other cultivated crop species and are even less competitive in noncultivated areas. There has been no documented case of cultivated potatoes (*S. tuberosum*) becoming a persistent weed outside of cultivated areas.

In wild species, the predominant method of propagation is also clonal (Hawkes, 1978). Sexual reproduction occurs readily, but is not obligatory and only occasionally results in viable hybrids populations. Nearly all potato species are at least partial outcrossers and require insects, in particular bumblebees, for pollination. Insects rarely visit flowers of cultivated species because they lack nectaries (Pavek, pers. comm.). This results in very limited pollen dissemination. In the only definitive study completed to date, Tynan *et al.* (1990) found that dispersal of pollen from transgenic plants did not occur outside a range of five meters.

D. Taxonomy of Genus *Solanum*

Potatoes belong to the family Solanaceae and the genus *Solanum*. This family comprises 2000 species and includes tomatoes, peppers, eggplant, tobacco, petunia and several forms of the weed commonly called nightshade (Benson, 1959). The genus *Solanum* contains more than 900 species (Correll, 1962; Hawkes, 1990). All potatoes cultivated in the U.S. belong to a single species, *Solanum tuberosum*. Native cultivated potatoes in South America are taxonomically divided among several species including *S. ajanhuiri*, *S. curtilobum*, *S. goniocalyx*, *S. x chaucha*, *S. phureja*, *S. tuberosum*, *S. stenotomum*, and *S. juzepczukii* (Bavyko, 1978). Most can be hybridized with *S. tuberosum*. Native cultivated types are found in Peru, Columbia, Ecuador, Bolivia, and Argentina with *S. tuberosum* ssp. *tuberosum* limited to Chile (Hanneman and Bamberg, 1986).

Only two close relatives of potato, *S. fendleri* and *S. jamesii*, occur naturally within the

borders of the U.S. (Hawkes, 1990). They are considered close relatives because both are tuber bearing *Solanum* (section petota) with at least some possibility of producing hybrids with *S. tuberosum*. *S. fendleri* belongs to the series longipedicellata, is tetraploid, and has been found in Arizona, Colorado, New Mexico and Texas. It resides in dry forests at altitudes of 5,000 to 10,000 feet. *S. jamesii* belongs to the series pinnatisecta, is a diploid, and has been found in Arizona, Colorado, New Mexico, Texas and Utah. It resides in environments similar to those where *S. fendleri* is found.

Several other *Solanum* species are either native or introduced weeds in the U.S. including bitter nightshade (*S. dulcamara*), silverleaf nightshade (*S. elaeagnifolium*), black nightshade (*S. nigrum*), hairy nightshade (*S. sarrachoides*), cutleaf nightshade (*S. triflorum*), buffalobur (*S. rostratum*), and turkeyberry (*S. torvum*) (Whitson *et al.*, 1991). All of these are non-tuber bearing and will not hybridize with tuberizing *Solanum* species.

E. Genetics of Potato

The genetic structure, and crossability of potato species are important considerations in understanding the flow of genes from cultivated to wild species. A brief description follows.

1. Genetic Structure:

A basic chromosome number of 12 was established by Smith (1927) for the genus *Solanum*. Polyploidy is common in both wild and cultivated potatoes. Most species are diploid (73%), or tetraploid (15%), but triploids (4%), pentaploids (2%) and hexaploids (6%) have also been documented (Hawkes, 1990).

The production of numerically unreduced gametes is common in many diploid cultivated and wild species (Camadro and Peloquin, 1980; Yerk and Peloquin, 1990). The result is a production of tetraploid progeny from diploid x tetraploid, tetraploid x diploid, or diploid x diploid crosses with a resultant transfer of genes from the diploid into the tetraploid population. Triploid potatoes are occasionally partially female fertile, producing a limited number of both n and 2n eggs. Triploids may also be crossed as pollen parents with cultivated tetraploids (Brown, 1988; Brown and Adiwilaga, 1990). These may act as 'triploid bridges', serving to allow gene flow in both directions (Jackson *et al.*, 1978). In nature, this is probably a rare event. Crosses of either tetraploids or 2n egg producing diploids with hexaploid species are usually easily made.

2. Crossability:

Three major factors influence the crossability of species. The ploidy level, the endosperm balance number (EBN), and cross incompatibility. The ploidy level, as has been discussed above, restricts the frequency of interspecies hybrids and the direction of gene flow, but by and large, does not prevent such events.

EBN is a term given to the ratio of maternal to paternal genomes in the endosperm of a species. Crosses of species with unequal EBN's result in a nonviable endosperm, causing the embryo to abort. The result is a very effective hybridization barrier between many

Solanum species. Most South American diploid species and nearly all tetraploid species, including *S. fendleri*, have an EBN of 2. *Solanum tuberosum*, a tetraploid, is an exception with an EBN of 4. Most Mexican diploids have an EBN of 1, including *S. jamesii* (Hanneman and Bamberg, 1986). The production of 2n gametes in 2 EBN diploids effectively doubles the EBN, allowing hybridization with *S. tuberosum* to occur. EBN is an important guideline for determination of crossability, however, many exceptions have been noted.

Most diploid species are self-incompatible due to the presence of S-alleles (Howard, 1970). Many closely related species are also cross incompatible because they share identical S-alleles. For reasons not completely understood, cultivated tetraploids and tetraploids derived from self-incompatible diploids show a weakened effect of the S-alleles and are usually self-compatible.

Hawkes (1990) cites evidence from a number of studies that hybrids between wild and cultivated, or between two wild species occur frequently in nature. However, the adaptability of the hybrids is poor and they rarely survive more than one or two seasons. Crosses of *S. tuberosum* with intrageneric species outside the section petota, such as with many types of nightshade, have been attempted, but no fertile progeny have been recovered (Dale *et al.*, 1992 and Rick, 1979).

F. Hybridization of Potato With Wild Relatives

Within the borders of the 50 United States, no opportunity exists for gene flow from cultivated potatoes to wild species. None of the solanaceous weedy species growing in and around potato fields will hybridize with cultivated potatoes. *S. jamesii* and *S. fendleri* are the only closely related species that are endemic to the U.S. Both are very difficult to hybridize with *S. tuberosum* due to incompatible EBN's. The only documented hybrids have been created under carefully controlled conditions in a laboratory situation (Adiwilaga and Brown, 1991; Novy and Hanneman, 1991). *S. fendleri* is the most likely of the two to produce hybrids with *S. tuberosum* because the development of a 2n gamete will produce a compatible EBN. However, no 2n gametes have been reported for *S. fendleri*. Any resulting progeny would be hexaploid with an EBN of 4 and would not be compatible parents for further hybridization with *S. fendleri*.

In addition to genetic incompatibility, the possibility of outcrossing is diminished due to geographical separation. Both *S. fendleri* and *S. jamesii* are found in high elevation, dry forest environments, isolated from all potato production areas. In the event an unlikely hybridization event does occur, the progeny probably will not be adapted to either environment and will not survive.

G. Hybridization of Potato With Other Cultivated Varieties

Other than the common occurrence of sterility, there is no genetic mechanism to prevent the hybridization of two cultivated varieties within the U.S. However, due to production methods, it is unlikely that gene transfer will occur in this manner. Pollen transfer occurs infrequently and over short distances. Tynan and his coworkers (1990) demonstrated no pollen dispersal in a field interplanted with genetically engineered and control potatoes beyond 4 - 5 meters and Dale *et al.* (1992) in a similar study, reported

no pollen transfer beyond 10 meters. Hybrid seed that does occur is not used for further propagation and will remain in the field. If this seed germinates, long term propagation and survival of the resulting seedlings is not expected due to standard cultivation practices, and in fact has never been documented. In the event of self-pollination within a fertile variety containing the *B.t.t.* gene, germination of the resulting seed will present no more concern than clonal volunteers (J. Pavek, pers. comm.).

H. Escape of Transgenic Plant Materials

Escape of plant materials will take the form of lost tubers. Other plant parts are not suitable for propagation. Once in commerce, tubers containing the *B.t.t.* gene can and will be lost during all phases of the growing and marketing operations. The major recipient locations of lost tubers will be fields where the crop is grown, roadsides, and areas around buildings where the potatoes are stored and shipped. Given the non-competitive nature of potatoes in these locations, escape will be inconsistent and temporary. No unusual steps need be taken to control escape through vegetative plant parts.

I. Ecological Impact of Gene Escape

If the *B.t.t.* gene escapes into the environment in a persistent manner it is most likely to do so in Central or South American where appropriate wild species are present. Even there, gene movement into a diploid wild species is unlikely due to the infrequent flow of genes from tetraploids into diploids via triploid bridges, an event never documented in nature. Hybrids are more likely with tetraploid and hexaploid species, but in a native situation will likely be noncompetitive. If hybrids do survive, the predominance of clonal propagation will limit spread. In the event the gene does become established in a wild population, it will provide no competitive advantage because Colorado potato beetle resistance already occurs frequently among wild species (Hanneman and Bamberg, 1986). Also, the Colorado potato beetle is either not present or is not a pest problem in Central and South America, (Bill Cantelo, personal communication; C.A.B. International, 1991) and will not provide the selection pressure that may create an ecological advantage. The *B.t.t.* insecticidal protein does not have known activity against non-target insects and is non-toxic to other animals (Herrnstadt *et al.*, 1986; MacIntosh *et al.*, 1990). It is environmentally safe and should have no ecological consequences.

J. Conclusion

Potato production in the United States provides a closed system for the production of transgenic varieties. No likely avenue exists for uncontrolled introduction of the *B.t.t.* gene into the environment either through loss of plant material or gene flow to related species. In the unlikely event the gene does escape, it will probably provide no competitive advantage and is nontoxic to other insect and animal systems.

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III. DESCRIPTION OF TRANSFORMATION SYSTEM AND PLASMID UTILIZED

The binary double border plant expression vectors, PV-STBT02 and PV-STBT04, used to transform the NewLeaf™ Atlantic and Superior lines for which this determination is requested, contain the *cryIIIA* gene from *Bacillus thuringiensis* subsp. *tenebrionis.* The *cryIIIA* gene gene encodes the Colorado potato beetle active protein and the *nptII* gene from the prokaryotic transposon Tn5 which codes for the enzyme neomycin phosphotransferase II. These genes were stably transferred into the genome of potato plants using *Agrobacterium tumefaciens* mediated transformation (Perlak *et al.*, 1993).

A. *Agrobacterium tumefaciens* Transformation System

The *Agrobacterium tumefaciens* transformation method has been reviewed by Klee and Rogers (1989). The transformation vector contains well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the T-DNA into plant cells. The plant expression vector was assembled in *Escherichia coli* and mated into the ABI *Agrobacterium* strain. The ABI strain contains the disarmed pTi58 plasmid pMP90RK which does not carry the T-DNA phytohormone genes (Koncz and Schell, 1986). Therefore, the *Agrobacterium* is unable to cause crown gall disease and is no longer considered a threat as a plant pest (Huttner *et al.*, 1992). The pMP90RK plasmid was engineered to provide the *trfA* gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. The ABI *Agrobacterium* strain containing the binary vector was added to potato stem sections (Newell *et al.*, 1991) in tissue culture dishes. The T-DNA, which includes the *cryIIIA* and *nptII* genes, was transferred into the genome of individual potato cells thereby allowing selection in kanamycin. After a few days, the residual *Agrobacterium* cells were killed using different antibiotics. Subsequently, the potato tissues were treated to stimulate regeneration of transgenic cells into shoots and ultimately plantlets were grown in soil and assayed for Colorado potato beetle resistance.

B. Properties of the Non-transformed Cultivars Atlantic and Superior

1. Atlantic

a) Parentage: The Atlantic variety was developed from a cross between the variety Wauseon and B5141-6. The USDA, Florida, Virginia, New Jersey and Maine Agricultural Experiment Stations took part in the breeding and evaluation of this variety which was released in 1976 (Barkley and Schrage, 1993).

b) Description: Atlantic tubers are oval to round with shallow eyes. Their skin is buff in color with light to heavy netting while the flesh is white. Atlantic plants are upright and medium to large in size with large leaves and lavender flowers.

c) Characteristics: Atlantic is a male fertile, high yielding, high specific gravity potato with a mid-season maturity. It is resistant to late blight (race O), golden nematode (race A), and bacterial pink eye disease and is highly resistant to PVX and tuber net necrosis. It is highly susceptible to heat necrosis when grown on hot, sandy soils. Atlantic tubers, due to high specific gravity under dry conditions,

may accumulate starch in the cortex region as a result of bruising. Atlantic plants produce a good set of tubers of uniform size and shape (Barkley and Schrage, 1993).

2. Superior

a) Parentage: The Superior variety was developed by the University of Wisconsin from a cross between the varieties B96-56 and M59.44 and was released in 1961 (Barkley and Schrage, 1993).

b) Description: Superior tubers are round with medium shallow eyes. Their skin is buff in color with light flaky russetting and the flesh is white. Superior plants are upright and medium in size with small leaflets and pale lilac flowers with white tips.

c) Characteristics: Superior is a male fertile, medium yielding, medium specific gravity potato with an early to mid-season maturity. It is moderately resistant to common scab but highly susceptible to late blight, PVY and verticillium wilt (Barkley and Schrage, 1993).

C. Construction of the Plasmid Vectors, PV-STBT02 and PV-STBT04, Utilized for Transformation

The plasmid vectors, PV-STBT02 and PV-STBT04, are double border binary transformation vectors which differ only in the nontranslated promoter region of the *cryIIIA* gene. Both plasmid vectors contain well-characterized DNA segments required for selection and replication of the plasmid in bacteria as well as right and left borders for delineating the region of DNA (T-DNA) designated for transfer into the plant genomic DNA. The host for all DNA cloning and vector construction was the *E. coli* MM-294, a derivative of the common laboratory *E. coli* K-12 strain. The vectors are composed of the following genetic elements. The first segment is the 0.45 kb fragment from the pTi15955 octopine Ti plasmid (a *Cla*I to *Dra*I restriction fragment) which contains the T-DNA left border region (Barker *et al.*, 1983). This is joined to the 1.3 kb fragment which contain the origin of replication (*oriV*) region derived from the broad-host range plasmid RK2 (Stalker *et al.*, 1981). The next segment is a 1.8 kb from pBR322 which provides the origin of replication for maintenance in *E. coli* (*ori322*), the replication of primer (*rop*) and the *bom* region for the conjugational transfer into the *Agrobacterium tumafaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978). This is fused to the 0.93 kb fragment isolated from transposon Tn7 which encodes the 0.79 kb *aad* gene that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985) which is fused to a 0.36 kb *Pvu*I to *Bcl*I restriction fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Depicker *et al.*, 1982).

Two chimeric genes, with signals for plant expression, were introduced between the right and left border regions of the vectors PV-STBT02 and PV-STBT04. Both plasmid vectors contain a chimeric gene (35S/*nptII*/NOS 3') for selection on kanamycin which consists of the cauliflower mosaic virus 35S promoter, the neomycin phosphotrans-

ferase type II (*nptII*) gene and the nontranslated region of the 3' region of the nopaline synthase gene referred to as NOS 3' (Rogers *et al.*, 1985). The second chimeric gene is responsible for the efficacious control of Colorado potato beetle. In the case of plasmid vector PV-STBT02, the second chimeric gene (E35S/*cryIIIA*/E9 3') consists of the enhanced 35S promoter (Kay *et al.*, 1987; Odell *et al.*, 1985), the *cryIIIA* gene which encodes the *B.t.t.* protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993) and the nontranslated region of the small subunit of ribulose-1,5-bisphosphate carboxylase (RUBISCO) referred to as E9 3' (Coruzzi *et al.*, 1984). In the case of plasmid vector PV-STBT04, the second chimeric gene (ArabSSU1A/*cryIIIA*/E9 3') consists of the the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit *ats*1A promoter referred to as ArabSSU1A (Almeida *et al.*, 1989; Wong *et al.*, 1992), the *cryIIIA* gene which encodes the *B.t.t.* protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993) and the nontranslated region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (RUBISCO) referred to as E9 3' (Coruzzi *et al.*, 1984).

The plasmid maps of PV-STBT02 and PV-STBT04 are shown in Figure III.1 and III.2, respectively. A summary of the specific DNA components of vectors PV-STBT02 and PV-STBT04 are listed in Table III.1.

D. Genetic Elements Transferred to NewLeaf™ Atlantic and Superior Potato Lines.

In general, for plant tranformation systems utilizing a double border vectors, the DNA transferred and integrated into the plant genome is confined to the DNA within the border regions, the T-DNA (Zambryski, 1992). The T-DNA is integrated in an irreversible manner (Huttner *et al.*, 1992). The border sequences themselves are partially transferred during the process of insertion of the T-DNA into the plant genome (Bakkeren *et al.*, 1989); consequently, the inserted DNA is no longer a functional T-DNA; *i.e.*, once integrated, it cannot be remobilized into the genome of another plant even if acted on again by *vir* genes (genes involved in T-DNA excision and transfer). In the case of plamids PV-STBT02 or PV-STBT04, the *cryIIIA* and the *nptII* genes are located within the borders and are expected to transfer to the potato genome. The *aad* gene which allows for bacterial selection in spectinomycin and streptomycin and the bacterial origins of replication, *oriV* and *ori322* regions, for replication of the plasmid in bacterial hosts, are located outside the borders. These regions are not expected to transfer to the potato genome.

Occasionally, insertion of truncated T-DNA regions and/or insertion of DNA beyond the classically defined T-DNA region is known to occur in plants transformed with double border vectors (Deroles and Gardner, 1988; Martineau *et al.*, 1994). In the case of potato plants transformed with plasmids PV-STBT02 or PV-STBT04, these less common transformation events have yielded plants containing the *oriV*, *ori322* and/or *aad* genetic elements. The genetic elements present in the NewLeaf™ Atlantic and Superior lines are listed in Table III.2. Details of the genetic analysis are discussed in Section V.

Table III.1 Summary of DNA Components in PV-STBT02 and PV-STBT04.

Genetic Element	Size,[1] kb	Function and Source
RB	0.36	A restriction fragment from the pTiT37 plasmid containing the 24 bp nopaline-type T-DNA right border used to initiate the T-DNA transfer from *Agrobacterium tumefaciens* to the plant genome (Depicker *et al.*, 1982).
E35S[2]	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
ArabSSU1A[3]	1.7	The *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit *ats*1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992).
cryIIIA	1.8	The gene which confers resistance to CPB. The gene encodes an amino acid sequence identical to the CPB control protein (referred to as the *B.t.t.* Band 3 protein) found in *B.t.t.* as described by Perlak *et al.* (1993).
E9 3'	0.63	A 3' nontranslated region of the pea ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) E9 gene (Coruzzi *et al.*, 1984), which functions to terminate transcription and direct polyadenylation of the *cryIIIA* mRNA.
35S	0.32	The 35S promoter region of the cauliflower mosaic virus (CaMV) (Gardner *et al.*, 1981; Sanders *et al.*, 1987).
nptII	0.79	The gene isolated from Tn5 (Beck *et al.*, 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley *et al.*, 1983).
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which functions to terminate transcription and direct polyadenylation of the *nptII* mRNA (Depicker *et al.*, 1982; Bevan *et al.*, 1983).
ori V	1.3	Origin of replication segment for ABI *Agrobacterium* derived from the broad-host range plasmid RK2 (Stalker *et al.*, 1981).
ori-322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-STBT02 plasmid in *E. coli*, the replication of primer (*rop*) region and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978).
aad	0.93	A fragment isolated from transposon Tn7 containing a 0.79 kb gene which encodes for the enzyme streptomycin adenylyltransferase that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985).
LB	0.45	A restriction fragment from the octopine Ti plasmid, pTi15955, containing the 24 bp T-DNA left border used to terminate the transfer of the T-DNA from *Agrobacterium tumefaciens* to the plant genome (Barker *et al.*, 1983).

1. Sizes are approximations. 2. Present only in PV-STBT02. 3. Present only in PV-STBT04.

Table III.2. Genetic Elements Transferred to NewLeaf™ Atlantic and Superior Potatoes

Line No.	Genetic Elements[1]				
	cryIIIA	*nptII*	*oriV*	*ori322*	*aad*
SPBT02-5	√		√	√	
SPBT02-7	√	√			
ATBT04-6	√	√			
ATBT04-27	√	√			√
ATBT04-30	√	√			
ATBT04-31	√	√			
ATBT04-36	√	√	√	√	√

1. √ indicates presence of specified genetic element.

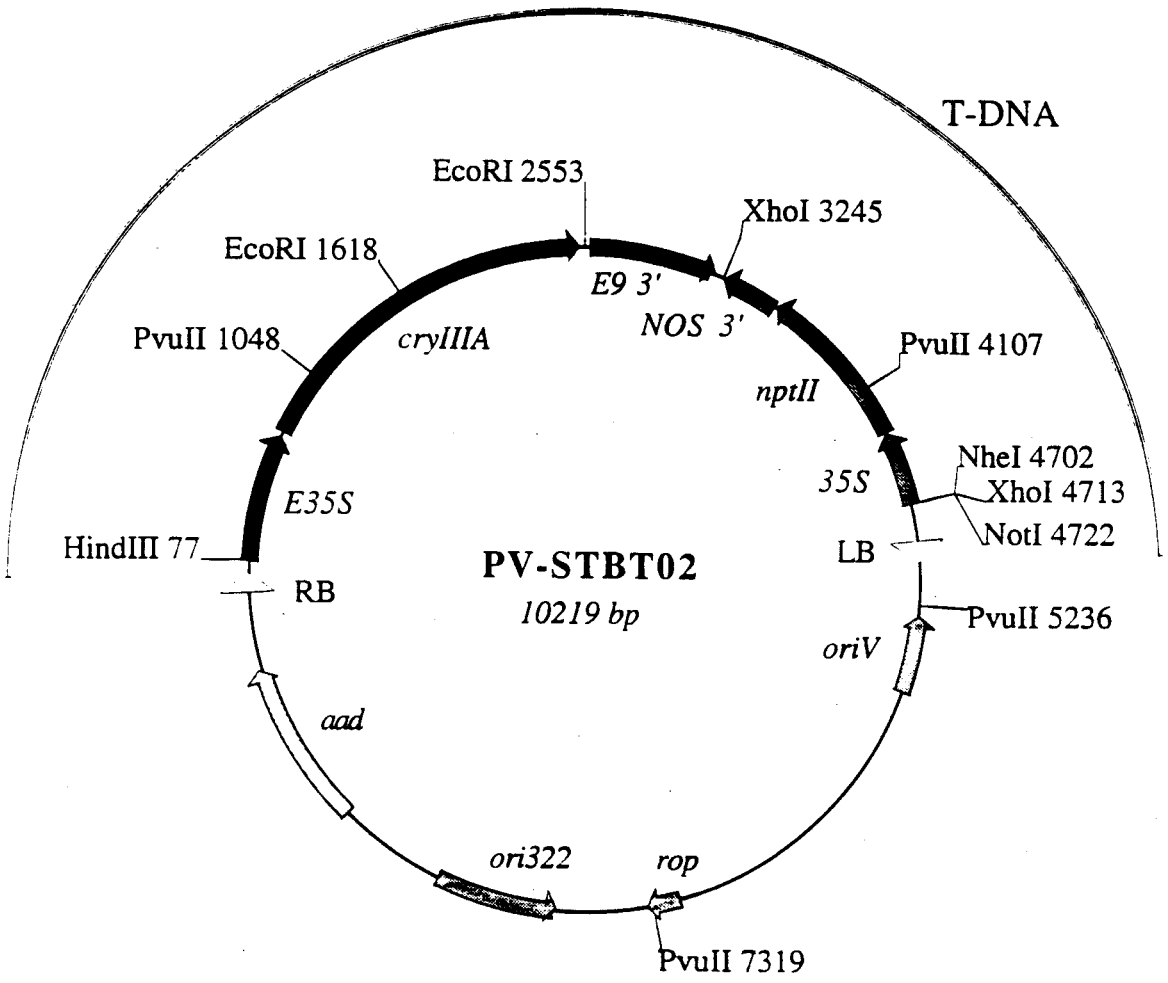

Figure III.1 Plasmid map of the binary vector PV-STBT02. A detailed description of all vector components may be found in Table III.1. Restriction sites of endonucleases utilized in the Southern analysis are shown. Location of the restriction sites are in base pairs (bp). The location of the right and left border (RB and LB, respectively) regions is indicated by the open arrows. The T-DNA, delineated by the right and left borders, is indicated by the semicircle outside the plasmid.

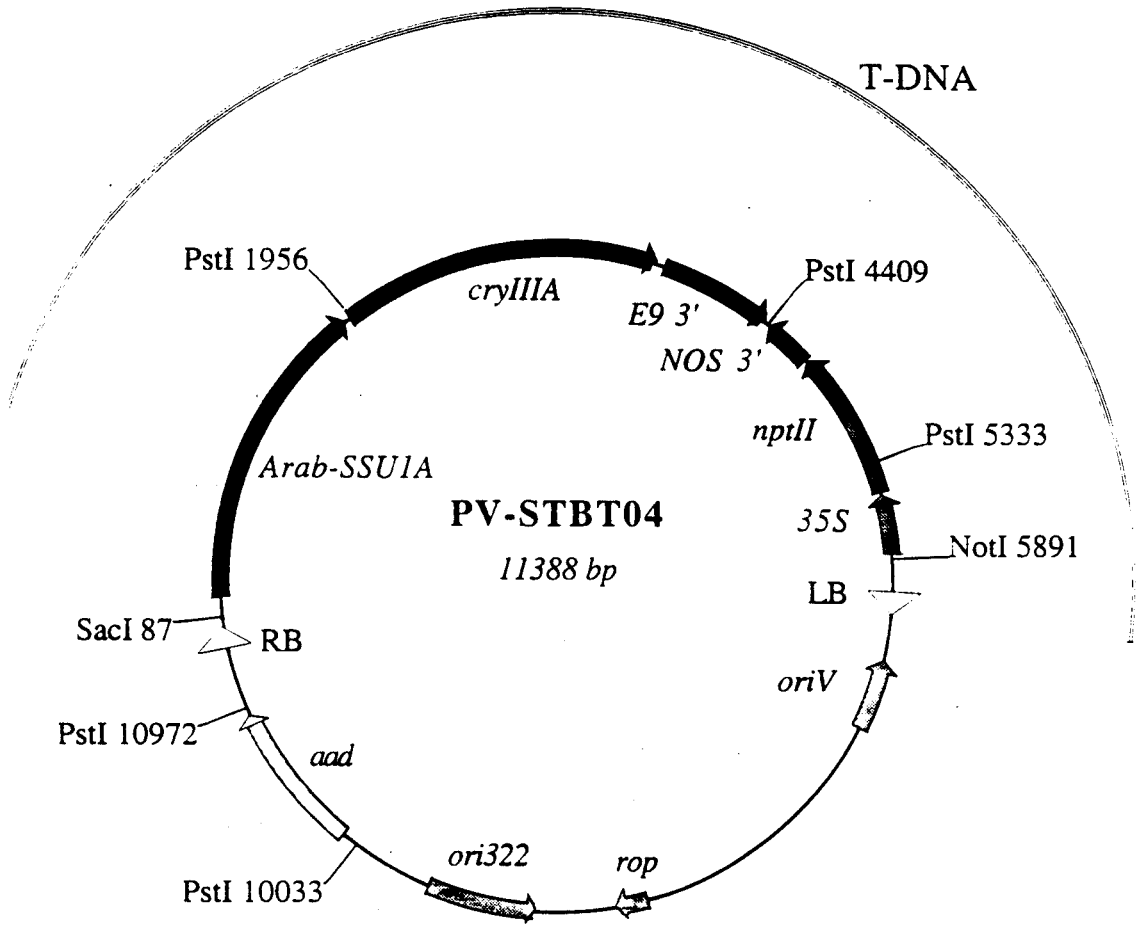

Figure III.2 Plasmid map of binary vector PV-STBT04. A detailed description of all vector components may be found in Table III.1. Restriction sites of endonucleases utilized in the Southern analysis are shown. Location of the restriction sites are in bp. The location of the right and left border (RB and LB, respectively) regions is indicated by the open arrows. The T-DNA, delineated by the right and left borders, is indicated by the semicircle outside the plasmid.

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IV. DONOR GENES

A. *cryIIIA* Gene

The gene used to produce the five lines of NewLeaf™ Atlantic and two lines of NewLeaf™ Superior potato plants, designated *cryIIIA* (Höfte and Whitely, 1989), was isolated from the DNA of *B. thuringiensis* subsp. *tenebrionis*, strain BI 256-82 (Krieg *et al.*, 1983). A full length clone and the complete nucleotide sequence has been reported for this gene (McPherson *et al.*, 1988; Perlak *et al.* 1993). The *cryIIIA* gene encodes a protein of 644 amino acids with a molecular weight of 73 kD which is produced by the bacterium during sporulation. This protein has insecticidal properties with selective activity against a narrow spectrum of Coleoptera (MacIntosh *et al.*, 1990). Upon ingestion by susceptible species, feeding is inhibited with disruption of the gut epithelium, which results in the eventual death of the insect (Slaney *et al.*, 1992). In addition to the full-length protein, the *B.t.t.* bacterium also produces a smaller form of this protein called *B.t.t.* band 3. The *B.t.t.* band 3 protein has a molecular weight of 68 kD (597 amino acids) which results from an internal translational initiation event within the same gene starting at amino acid 48 (McPherson *et al.*, 1988; Perlak *et al.* 1993). This protein has been shown to possess the same insecticidal potency and selectivity to CPB larvae as the full-length protein (McPherson *et al.*, 1988). The gene encoding the *B.t.t.* band 3 protein, modified with plant preferred codons for increased plant expression, was introduced into potato plants. The modification changed 399 out of 1791 nucleotides within the gene which codes for the *B.t.t.* band 3 protein, without altering any of the encoded amino acids. This modified gene encodes the nature identical amino acid sequence of band 3 protein as produced by the *B. thuringiensis* subsp. *tenebrionis* microbe (Perlak *et al.*, 1993).

B. *nptII* Gene

This gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). The NPTII enzyme uses ATP to phosphorylate neomycin and the related kanamycin, thereby inactivating these aminoglycoside antibiotics and preventing them from killing the cells producing NPTII. The coding sequence for the *nptII* gene is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). The sole purpose of inserting the *nptII* gene into potato cells with the *cryIIIA* gene is to have an effective method of selecting cells that contain the insecticidal gene. In general, the frequency of cells that are transformed is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et. al.*, 1984). Therefore, to facilitate this process, a selectable marker gene, *nptII*, and selective agent, kanamycin, is used. Consequently, cells selected for plant generation contain the *nptII* and *cryIIIA* genes.

C. References

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V. GENETIC ANALYSIS, AGRONOMIC PERFORMANCE, AND COMPOSITIONAL ANALYSIS OF THE NEWLEAF™ ATLANTIC AND SUPERIOR LINES.

A. Genetic Analysis

As described in Section III, NewLeaf™ potato plant lines were generated by *Agrobacterium tumefaciens* mediated transformation with either plasmid PV-STBT02 or PV-STBT04. DNA analyses were performed on the two Superior lines transformed with plasmid PV-STBT02 and five Atlantic lines transformed with plasmid PV-STBT04. The characterizations were performed by Southern blot analyses (Southern, 1975) on isolated genomic DNA treated with restriction endonucleases. All Southern blots were hybridized with ^{32}P-labelled PV-STBT02 or PV-STBT04 and the genetic constituents of the plasmids. The analyses detemined the genetic elements which were transferred to the potato genome and yielded information on the T-DNA in terms of insert number (number of genetic loci), copy number (number of T-DNA copies at a particular genetic locus) and insert integrity (deletions and/or rearrangements). Description of the analyses are summarized below.

1. NewLeaf™ Superior Transformed with Plasmid PV-STBT02

a. Insert and Copy Number

To confirm the presence of the *cryIIIA* gene and obtain information on the number of T-DNA copies transferred into the potato genome, the isolated DNA was cut with the endonuclease *Eco*RI (refer to Figure III.1 for location of restriction sites). The plasmid PV-STBT02 contains two *Eco*RI restriction sites within the T-DNA. Therefore, Southern analysis with DNA digested with *Eco*RI can yield information on the number of T-DNA copies transferred into the potato. Transformants containing a single copy/single insertion are expected to yield three DNA fragments: a 935 bp *cryIIIA* gene fragment and two fragments (>1.54 kb and >2.16 kb) containing T-DNA joined to the plant genomic DNA (referred to as border fragments). Southern blots of DNA digested with *Eco*RI were probed with ^{32}P-labelled PV-STBT02 plasmid, *oriV* and *aad*. Using the whole plasmid as a probe, background fragments were observed at approximately 5.8, 4.6, 3.0 and 2.5 kb. Figure V.1 shows the results of the Southern analyses using these probes.

Superior Line No. SPBT02-7. The whole plasmid probe showed hybridization with a single internal fragment which is part of the *cryIIIA* gene (935 bp) and two border fragments (approximately 11 and 7.5 kb) containing T-DNA joined to the plant genomic DNA (Figure V.1A, Lane 5). A weak hybridization with a fragment of approximately 8.5 kb was also detected. The presence of this fragment was attributed to incomplete digestion (results with other restriction enzymes confirmed this conclusion, see below). Two probes for regions outside the double borders (*oriV* and *aad*) did not hybridize with any DNA from Line No. SPBT02-7 (Figure V.1B, Lane 5 and Figure V.1C, Lane 5). These results, in conjunction with the intensity of the 935 bp band, indicate that this line contains a single copy of the *cryIIIA* gene and that only the region within the right and left borders of the T-DNA was transferred into the genome.

Superior Line No. SPBT02-5. The whole plasmid probe showed strong hybridization with the internal 935 bp fragment and two border fragments of approximately 10 and 3

kb (Figure V.1A, Lane 4). The intensity of the band at 935 bp suggested insertion of a single copy of the *cryIIIA* gene. Probing with *oriV* showed hybridization with the 10 kb fragment (Figure V.1B, Lane 4). No hybridization with the *aad* probe was detected (Figure V.1C, Lane 4). These results indicate that for Line No. SPBT02-5, the integration included a single copy of the *cryIIIA* gene and genetic elements beyond the left border including the *oriV* but not the *aad* gene.

b. Insert Integrity

Information on the insert integrity and confirmation on copy/insert number was obtained by digesting DNA from each of the two NewLeaf™ Superior lines and the control line with a combination of *Hin*dIII and *Xho*I and a combination of *Hin*dIII and *Not*I endonucleases. DNA from NewLeaf™ Superior Line No. 5 was also analyzed with *Pvu*II endonuclease.

i. *Hin*dIII and *Xho*I digestions:

The plasmid PV-STBT02 contains one *Hin*dIII restriction site and two *Xho*I restriction sites (refer to Figure III.1 for location of restriction sites). Therefore, for transformation events which insert a single copy of the T-DNA at a single site, this combination of endonucleases is expected to release two internal fragments of predicted sizes 3168 bp and 1468 bp. The Southern blot was probed with plasmid (PV-STBT02) and the genetic elements: *aad*, *oriV* and *ori*322. Using the whole plasmid as a probe, a background fragment was observed at approximately 5.8 kb. Figure V.2 shows the results of these analyses.

Superior Line No. SPBT02-7. The whole plasmid probe hybridized to the two predicted fragments of approximately 3100 and 1400 bp (Figure V.2A, Lane 5). The *oriV* (Figure V.2B, Lane 5), *ori322* (Figure V.2C, Lane 5) and *aad* (data not shown) probes showed no hybridization with DNA from this line. These results indicate that the entire inserted T-DNA is intact and, confirms the results observed with the *Eco*RI digest, that only the region within the right and left T-DNA borders was transferred into the genome of this line.

Superior Line No. SPBT02-5. The whole plasmid probe hybridized to a large fragment of approximately 13 kb and a second band of approximately 5.8 kb (Figure V.2A, Lane 4). Since a 5.8 kb fragment was also detected with control DNA, this fragment was attributed to background hybridization. The 13 kb fragment indicated missing restriction site(s) and/or irregular border function. This fragment did not hybridize with the *aad* probe (data not shown), but did hybridize with the *oriV* (Figure V.2B, Lane 4) and *ori322* (Figure V.2C, Lane 4) probes. These results indicate that for this line the integration included the T-DNA and vector elements beyond borders which included the *oriV* and *ori322* genetic elements but not the *aad* gene. The hybridization to a single fragment indicates linkage between the *cryIIIA*, *oriV* and *ori322* regions and also suggests that the plasmid DNA was inserted at a single site.

ii. *Hin*dIII / *Not*I digestion

Digestion with a combination of *Hin*dIII / *Not*I restriction endonucleases provided further information on insert integrity and copy number. The plasmid PV-STBT02 contains one *Hin*dIII restriction site and one *Not*I site (refer to Figure III.1 for location of restriction sites). Therefore, for transformation events which insert a single copy of the T-DNA at a single site, a combination of these enzymes is expected to release a single fragment of 4645 bp. The Southern blot was probed with the whole plasmid PV-STBT02, *cryIIIA* and *nptII*. No background hybridization was detected with this digest. The results of these analyses are shown in Figure V.3.

Superior Line No. SPBT02-7. The whole plasmid probe hybridized to a single fragment of approximately 4600 bp (Figure V.3A, Lane 5). This same fragment hybridized with the *cryIIIA* and the *nptII* probes (Figures V.3B, Lane 5 and Figure V.3C, Lane 5, respectively). These results indicate linkage of the *cryIIIA* and *nptII* genes and confirm the integrity and insertion of the T-DNA at a single site.

Superior Line No. SPBT02-5. The whole plasmid probe hybridized to a single large fragment of approximately 14 kb (Figure V.3A, Lane 4). The large fragment size obtained with this digest suggested missing restriction site(s) and/or irregular border function. The *cryIIIA* probe also hybridized to the 14 kb fragment (Figure V.3B, Lane 4). However, no hybridization was observed with the *nptII* probe (Figure V.3C, Lane 4) indicating absence of the *nptII* marker gene in this line.

iii. *Pvu*II digestion

Since Southern blots with DNA of NewLeaf™ Superior Line No. SPBT02-5 digested with *Eco*RI, *Hin*dIII / *Xho*I and *Hin*dIII / *Not*I, described above, indicated presence of plasmid elements outside the double borders as well as absence of the selectable marker gene, an additional digest with *Pvu*II was done to confirm these results.

The plasmid PV-STBT02 contains four *Pvu*II restriction sites, two of which are outside the T-DNA double border region (refer to Figure III.1 for location of restriction sites). Based on the Southern blot results described above, two *Pvu*II sites were expected to be absent in the transferred DNA: the site within the *nptII* gene and the site adjacent to the left border region. The Southern blot was probed with the genetic elements: *cryIIIA, nptII, oriV* and *ori322*. Using the *cryIIIA* and *ori322* as a probe, background fragments were observed at approximately 5.2 and 3.0 kb. Using the *oriV* as a probe, a background fragment was observed at approximately 3.2 kb. The results of these analyses are shown in Figure V.4.

Two fragments of approximately 3.2 and 2.6 kb were detected in the *Pvu*II digest hybridization analysis. The *cryIIIA* (Figure V.4A, Lane 4) and the *oriV* (Figure V.4B, Lane 4) probes hybridized with the 3.2 kb fragment. The *ori322* (Figure V.4C, Lane 4)probes hybridized with the 2.6 kb fragment. No hybridization was detected with the *nptII* probe (data not shown). These results confirmed the previous Southern analysis. Line No. SPBT02-5 contains the *cryIIIA, oriV* and *ori322* regions integrated at a single site within its genome. The *nptII* and the *aad* genes were not incorporated into the genome of this line.

In summary, Southern analyses indicate that a single copy of the T-DNA containing the *cryIIIA* and the *nptII* genes was inserted at a single site in the genomic DNA of Line No. SPBT02-7. Integrity of the T-DNA was maintained during the transfer process and no region of DNA outside the plasmid borders were detected for this line. In the case of Line No. SPBT02-5, Southern analyses indicated insertion of the *cryIIIA* and a region outside the borders containing the *oriV* and *ori322* genetic elements. These genes were inserted at a single site within the genome of this line. Integrity of the *cryIIIA* gene was maintained during the transfer process. The *nptII* and the *aad* genes were not transferred into the genome of Line No. SPBT02-5.

2. NewLeaf™ Atlantic Transformed with Plasmid PV-STBT04

a. Insert, Copy Number and Integrity

To determine the genetic elements of plasmid PV-STBT04 which were transferred to the potato genome and obtain information on the number of T-DNA copies transferred and integrity of the transferred genes, the isolated DNA from the five lines of NewLeaf™ Atlantic potatoes were digested with a combination of *Sac*I and *Not*I restriction endonucleases and with *Pst*I restriction endonuclease.

i. *Sac*I / *Not*I digestions:

The plasmid PV-STBT04 contains one *Sac*I restriction site located near the right border and one *Not*I restriction site located near the left border (refer to Figure III.2 for location of restriction sites). Digestion of the plasmid with a combination of these restriction endonucleases produces two fragments of 5.8 kb and 5.6 kb. Transformants containing a single copy of the T-DNA are expected to release a single fragment of approximately 5.8 kb. Therefore, digestion with a combination of these restriction endonucleases can provide information on copy number and integrity of the inserted DNA. The Southern blot was probed with the whole plasmid (PV-STBT04) and the following genetic elements: *cryIIIA*, *nptII*, *oriV*, *ori322*, and *aad*. DNA from control plants (nontransformed cv. Atlantic) digested with *SacI*/*Not*I and DNA from control plants spiked with plasmid vector PV-STBT04 DNA digested with *SacI*/*Not*I were included as controls in the Southern blots. Using the whole plasmid as probe, a faint background fragment was observed at approximately 4.3 kb. Using the *ori322* probe, background fragments were observed at approximately 5.8 kb and 4.3 kb. Using the *aad* probe, faint background fragments were observed at approximatley 5.8 kb and 2.1 kb. No background fragments were observed with the *cryIIIA*, *nptII* or *oriV* probes. Figures V.5 and V.6 show the results of these analyses.

Atlantic Line No. ATBT04-6. DNA from Line ATBT04-6 digested with a combination of *SacI*/*Not*I yielded a single fragment of 5.8 kb which hybridized with the whole plasmid, *cryIIIA* and *NPTII* probes (Figure V.5, Lane 4 in Panels A - C). There was no hybridization with the *ori322* (Figure V.6B, Lane 4) or the *aad* probes (Figure V.6C, Lane 4). A weak hybridization of the 5.8 kb fragment with the *oriV* probe was noted (Figure V.6A, Lane 4). However, based on the size of the fragment and the results obtained with *Pst*I digestion (see discussion below), this was attributed to background hybridization. These results indicate that only the region within the right and left borders of the T-DNA was transferred to the genome of this line. Hybridization of the

cryIIIA and *nptII* probes to the same fragment indicates that these genes are linked and that integrity of the T-DNA was maintained during the transfer. The intensity of the 5.8 kb fragment resulting from hybridization with the whole plasmid, *cryIIIA* and *nptII* probes (Figure V.5, Lane 4 in Panels A - C) indicates that multiple copies of the T-DNA were transferred to Line ATBT04-6.

Atlantic Line No. ATBT04-27. DNA from Line ATBT04-27 digested with a combination of *SacI*/*NotI* yielded three fragments which hybridized to the whole plasmid probe: 9.5 kb, 5.8 kb and 3.0 kb (Figure V.5A, Lane 5). The intensity of hybridization with the 5.8 kb fragment was approximately twice as strong as the intesity of the 9.5 kb and the 3.0 kb fragments. The *cryIIIA* probe hybridized to the same three fragments (Figure V.5B, Lane 5). The *nptII* probe hybridized to the 9.5 kb and 5.8 kb fragments (Figure V.5C, Lane 5). The *aad* probe hybridized to the 9.5 kb fragment (Figure V.6C, Lane 5). There was no detectable hybridization with either the *oriV* (Figure V.6A, Lane 5) or the *ori322* (Figure V.6C, Lane 5) probes. Weak hybridizations at approximately 9.5 kb, 8.0 kb and 3.0 kb were detected with the *ori322* probe. However, based on the results obtained with the *PstI* digest (see discussion below) and the weak intensity of the hybridizations, these bands were attributed to background hybridization. These results indicate that two copies of T-DNA were inserted at two loci of Line ATBT04-27. One insert contains a complete T-DNA. The second insert contains a complete T-DNA plus the *aad* element and part of the *cryIIIA* gene. Based on the size of the fragments, the *SacI* and *NotI* sites in the second insert appear to have been deleted during the insertion process.

Atlantic Line No. ATBT04-30. DNA from Line ATBT04-30 digested with a combination of *SacI*/*NotI* yielded a single fragment of approximately 30 kb which hybridized to the whole plasmid (Figure V.5A, Lane 6), the *cryIIIA* (Figure V.5B, Lane 6) and the *nptII* (Figure V.5C, Lane 6) probes. There was no hybridization with the *oriV*, *aad* or *ori322* probes (Figure V.6, Lane 6 in Panels A-C). These results indicate that Line ATBT04-30 contains a single copy of the T-DNA. The larger than expected fragment suggests that the *SacI* and/or *NotI* restriction site(s) are missing. The linkage between the *cryIIIA* and *nptII* genes indicates that integrity of the T-DNA was maintained during the transformation process. No genetic elements outside the borders were transferred to the genome of this line.

Atlantic Line No. ATBT04-31. DNA from Line ATBT04-31 digested with a combination of *SacI*/*NotI* yielded a single fragment of approximately 5.8 kb which hybridized to the whole plasmid (Figure V.5A, Lane 7), the *cryIIIA* (Figure V.5B, Lane 7) and the *nptII* (Figure V.5C, Lane 7) probes. No hybridization with the *oriV*, *aad* or *ori322* probes was detected (Figure V.6, Lane 7 in Panels A-C). These results indicate that Line ATBT04-31 contains a single copy of the T-DNA. Hybridization of the *cryIIIA* and the *nptII* probes to the same fragment indicates that these genes are linked and that integrity of the T-DNA was maintained during the transformation process. No genetic elements outside the borders were transferred to the genome of this line.

Atlantic Line No. ATBT04-36. The whole plasmid and the *cryIIIA* probes hybridized to three fragments of approximately 5.8 kb, 6.8 kb, and 15.0 kb (Figure V.5, Lane 8 in Panels A and B). The *nptII* probe hybridized to the 5.8 kb and 6.8 kb fragments (Figure V.5C, Lane 8). The *oriV* probe hybridized with light intensity to the 5.8 kb and 6.8 kb fragments (Figure V.6A, Lane 8). Both the *ori322* and the *aad* probes hybridized to the

5.8 kb fragment (Figure V.6, Lane 8 in Panels B and C). Although weak background hybridization at 5.8 kb was also observed with the *ori322* and the *aad* probes, the hybridization with this fragment is clearly of greater intensity than the background hybridization. These results indicated that Line ATBT04-36 contains three inserts. One insert consists of the whole plamid, PV-STBT04. The second insert consists of the T-DNA plus the *oriV* element. The third insert contains only the *cryIIIA* gene. All genetic elements present in plasmid PV-STBT04, including *oriV*, *ori322* and *aad*, were detected in Line ATBT04-36.

ii. *Pst*I digestions:

The plant transformation vector PV-STBT04 has three *Pst*I sites within the T-DNA and two *Pst*I sites outside the double borders (refer to Figure III.2 for location of restriction sites). Digestion of the plasmid with *Pst*I produces five fragments: 4.7 kb, 2.4 kb, 0.5 kb and two fragments of approximately 0.9 kb. For transformation events which insert a single copy of the T-DNA, digestion with *Pst*I is expected to yield four fragments: a 2.4 kb *cryIIIA* gene fragment, a 0.9 kb *nptII* gene fragment and two fragments (> 0.9 kb and >2.0 kb) containing the T-DNA joined to the plant genomic DNA (referred to as "border fragments"). DNA from five lines digested with *Pst*I were probed with whole plasmid (PV-STBT04), *cryIIIA*, *nptII*, *oriV*, *aad*, and *ori322*. DNA from control plants (nontransformed cv. Atlantic) digested with *Pst*I and DNA from control plants spiked with plasmid vector PV-STBT04 DNA digested with *Pst*I were included as controls in the Southern blots. No background fragments were observed with any of the probes. Figures V.7 and V.8 show the results of these analyses.

Atlantic Line No. ATBT04-6. The whole plasmid probe hybridized to a total of eight fragments. Five fragments of sizes 4.7 kb, 4.6 kb, 3.7 kb, 2.4 kb, 0.9 kb hybridized with heavy intensity. Three fragments of sizes 2.0 kb, 1.9 kb and 1.5 kb hybridized with light intensity (Figure V.7A, Lane 4). The *cryIIIA* probe hybridized only to the 2.4 kb fragment (Figure V.7B, Lane 4). The *nptII* probe hybridized to the 0.9 kb (heavy intensity), 1.5 kb (light intensity) and 2.0 (light intensity) fragments (Figure V.7C, Lane 4). A background hybridization fragment was also detected at approximately 2.4 kb (similar background fragment was also detected with DNA from control plants spiked with plasmid vector PV-STBT04 DNA digested with *Pst*I, Figure V.7C, Lane 3). No hybridization was detected with the *oriV*, *aad*, or *ori322* probes (Figure V.8, Lane 4 in Panels A - C). These results are consistent with Line ATBT04-6 containing three copies of T-DNA at three loci. The intensity of the 2.4 kb and 0.9 kb fragments using the whole plasmid probe, corresponding to the *cryIIIA* and *nptII* genes, indicated insertion of multiple copies of the T-DNA. The additional fragments represent the border fragments for the three inserts.

Atlantic Line No. ATBT04-27. The whole plasmid hybridized to a total of six fragments (Figure V.7A, Lane 5). Four fragments of sizes 8.6 kb, 3.7 kb, 2.4 kb and 0.9 kb hybridized with heavy intensity. Two fragments of sizes 3.2 kb and 1.5 kb hybridized with light intensity. The *cryIIIA* probe hybridized to 2.4 kb and 1.5 kb fragments (Figure V.7B, Lane 5). The *nptII* probe hybridized with light intensity to fragments of 3.0 kb, 1.3 kb and with heavy intensity to a fragment of 0.9 kb (Figure V.7C, Lane 5). The *aad* probe hybridized to a 0.9 kb fragment (Figure V.8C, Lane 5). No hybridization was detected with either the *oriV* or *ori322* probe (Figures V.8, Lane 5 in Panels A and

B). These results are consistent with the *SacI/NotI* digestion data, which indicated that Line ATBT04-27 has two complete copies of T-DNA inserted at two sites. One insert contains a complete copy of the T-DNA. The second insert contains a complete T-DNA plus the *aad* region and part of the *cryIIIA* gene. No genetic elements outside the double borders other than *aad* were detected.

Atlantic Line No. ATBT04-30. The whole plasmid probe hybridized to two fragments of sizes 2.4 kb and 0.9 kb (Figure V.7A, Lane 6). There were no detectable border fragments with the whole plasmid probe. The *cryIIIA* probe hybridized to the 2.4 kb fragment (Figure V.7B, Lane 6). The *nptII* probe hybridized to a 0.9 kb fragment (Figure V.7C, Lane 6). No hybridization was observed with the *oriV*, *aad* or *ori322* probes (Figure V.8, Lane 6 in Panels A - C). These results are consistent with the *SacI/NotI* digestion data. A single T-DNA copy containing the *cryIIIA* and *nptII* genes was inserted into the genome of Line ATBT04-30. No genetic elements outside the double borders inserted in the genome of this line. The absence of the expected border fragments >2.0 kb and >0.5 (which would result from cleavage at positions 1956 and 5333, respectively) suggests that truncation and/or deletion at the *PstI* site at position 1956. The second border fragment >0.5 kb (which would result from cleavage at position 5333) may have yielded a fragment below the limits of detection by this Southern blot analysis.

Atlantic Line No. ATBT04-31. The whole plasmid probe hybridized to three fragments: 2.8 kb, 2.4 kb, and 0.9 kb (Figure V.7A, Lane 7). The *cryIIIA* probe hybridized to the 2.4 kb fragment (Figure V.7B, Lane 7). The *nptII* probe hybridized to the fragment of approximately 0.9 kb (Figure V.7C, Lane 7). There was no detectable hybridization with *oriV*, *aad*, or *ori322* probes (Figure V.8, Lane 7 in Panels A - C). These results confirmed the *SacI / NotI* digest results which indicate that Line ATBT04-31 contains a single copy of the T-DNA. The absence of a second border fragment of size >0.5 kb (which would result from cleavage at position 5333) was attributed to fragmentation below the limits of detection by this Southern blot analysis. No genetic elements outside the borders were transferred to the genome of this line.

Atlantic Line No. ATBT04-36. The whole plasmid probe hybridized to a total of five bands of the following approximate sizes: a 2.4 kb fragment with heavy intensity and four fragments of sizes 0.9 kb, 4.8 kb, 18.0 kb and 20.0 kb with light intensity (Figure V.7A, Lane 8). The *cryIIIA* probe hybridized to fragments with approximate sizes of 18.0 kb and 2.4 kb (Figure V.7B, Lane 8). The *nptII* probe hybridized to a fragment of the 0.9 kb (Figure V.7C, Lane 8). The *oriV* probe hybridized with light intensity to fragments of 8.2 kb and 4.8 kb (Figure V.8A, Lane 8). The *ori322* probe hybridized to a 4.8 kb fragment (Figure V.8B, Lane 8). The *aad* probe hybridized to a 0.9 kb fragment (Figure V.8C, Lane 8). These analyses confirm the *SacI / NotI* digest results which indicate that Line ATBT04-36 contains inserts at three loci. One insert consists of the whole plamid, PV-STBT04. The second insert consists of the T-DNA plus the *oriV* element. The third insert contains only the *cryIIIA* gene. All genetic elements present in plasmid PV-STBT04, including *oriV*, *ori322* and *aad*, were detected in Line ATBT04-36.

In summary, Southern analyses indicate that Line ATBT04-6 contains three copies of T-DNA at three insertion sites. Lines ATBT04-30 and ATBT04-31 contain a single copy of the T-DNA. Integrity of the *cryIIIA* and *nptII* genes was maintained during the transfer

process and no genetic elements outside the plasmid borders were detected in these lines. In the case of Lines ATBT04-27 and ATBT04-36, the border regions did not funtion as expected. Southern analyses indicate that Line ATBT04-27 has two complete copies of T-DNA inserted at two sites; one insert contains a complete copy of the T-DNA, the second insert contains a complete T-DNA plus the *aad* region and part of the *cryIIIA* gene. No genetic elements outside the double borders other than *aad* were detected in Line ATBT04-27. Line ATBT04-36 contains inserts at three loci. One insert consists of the whole plamid, PV-STBT04. The second insert contains the T-DNA plus the *oriV* element. The third insert contains only the *cryIIIA* gene. All genetic elements present in plasmid PV-STBT04, including *oriV*, *ori322* and *aad*, were detected in Line ATBT04-36.

B. Justification for Lack of Mendelian Inheritance Data

Evidence gathered to date indicates that the transformation of the two NewLeaf™ Superior lines and five NewLeaf™ Atlantic lines, with plamid vector PV-STBT02 or PV-STBT04 resulted in the inserted genes being stably inserted into the chromosome of the plant. The consistent efficacy of these plant lines in controlling the CPB in field trials conducted in 1992 through 1994 further indicates the stable integration of the inserted genes. Therefore, any additional study of the Mendelian inheritance of the CPB reistant trait would likely lead to the same conclusion. Finally, since potatoes are vegetatively propagated, no genetic segregation in subsequent generations is expected.

C. Disease and Pest Characteristics

NewLeaf™ potato lines transformed with the plasmid vectors, PV-STBT02 and PV-STBT04 were tested in the United States in 1992 (USDA# 91-360-01, 92-002-01, and 92-262-02), 1993 (USDA# 92-363-05 and 93-004-01, and 93-253-06N) and 1994 (USDA# 93-357-01N, 93-357-02N, 94-056-01N, 94-056-02N, 94-067-09N, 94-067-10N, 94-074-05N, 94-084-15N and 94-249-03N) at a combined total of 30 locations. At nearly all of these locations the plant lines were evaluated in replicated trials. Detailed monitoring for the disease and insect susceptibility of these lines versus Atlantic or Superior control plants was performed one or more times per season at the sites listed in Table V.1. No differences in disease or insect infestation or severity other than CPB control were detected between the NewLeaf™ plant lines and Atlantic or Superior control plants. The USDA final reports for the trials conducted in 1992, 1993 and 1994 with these lines have been submitted to the Agency. These observations were made by private growers, university and USDA researchers and potato seed certification experts who compared the general vigor and disease and insect susceptibility of control and the NewLeaf™ plant lines. These observations are typical of those taken by potato crop consultants, agronomists, seed producers, and seed certifiers in detecting the presence and magnitude of a disease or insect infestation. On the basis of these critical evaluations, the NewLeaf™ plant lines were entered for certification in U.S. seed potato certification programs in 1993 and 1994 and were granted certification for current season and post harvest evaluation. Common diseases evaluated included, but were not limited to: early blight, late blight, *Verticillium*, potato leaf roll virus and potato virus Y. The primary insect pests monitored were aphids, potato leafhoppers, Colorado potato beetles, and cutworms.

Agronomic observations included plant vigor, growth, color, leaflet shape and flowering. As stated above, no differences in agronomic quality, disease or insect susceptibility, other than to the CPB, were detected between Atlantic and Superior control plants and NewLeaf™ plant lines of the same varieties tested between 1992 and 1994 and 1993 and 1994, respectively.

D. Yield and Quality Characteristics

The two NewLeaf™ Superior and five NewLeaf™ Atlantic plant lines transformed with the plasmid vectors PV-STBT02 or PV-STBT04, are still in development. These lines have exhibited quality and yield characteristics comparable to control Superior and Atlantic variety potatoes. Only those lines with commercially acceptable yield and quality characteristics will enter the marketplace.

E. Expression Levels of the *B.t.t.* and NPTII Proteins

The levels of *B.t.t.* and NPTII proteins expressed in two NewLeaf™ Superior lines and five NewLeaf™ Atlantic lines were determined in the leaf and tuber by validated enzyme linked immunosorbent assay (ELISA). A summary of the methods employed and the descriptive features of the ELISAs developed to measure the *B.t.t.* and NPTII protein levels in the various potato tissues are summarized in Appendix 2.

1. Expression in NewLeaf™ Superior Lines Transformed with Plasmid PV-STBT02

Expression in leaves of NewLeaf™ Superior potato were determined on tissue collected at approximately six weeks post planting from four replicate field trial carried out at Homestead, FL, during the winter of 1994. Expression in tuber tissue were determined in tubers harvested from three replicated plots of a field trial carried out at Island Falls, ME, during the summer of 1994.

The leaf and tuber expression results for *B.t.t.* protein in the NewLeaf™ Superior lines is shown in Tables V.2. The expression results for NPTII protein is shown in Table V.3.

The mean *B.t.t.* protein expression level in leaves of both lines was found to be 11.5 µg/g tissue fresh weight. The mean tuber *B.t.t.* protein expression level was approximately 1.0 µg/g tissue fresh weight. The *B.t.t.* protein expression levels correspond to 0.07% of total foliage protein and 0.005% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

NPTII expression was detected only in tissue from NewLeaf™ Superior Line No. SPBT02-07. The mean NPTII expression level in leaves of CPB resistant Line No. SPBT02-07 was found to be 3.82 µg/g tissue fresh weight, while overall mean tuber expression was 0.97 µg/g tissue fresh weight. The NPTII expression levels correspond to 0.02% of total foliage protein and 0.005% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

2. Expression in NewLeaf™ Atlantic Lines Transformed with Plasmid PV-STBT04

Expression in leaves of NewLeaf™ Atlantic potato were determined on tissue collected at approximately six weeks post planting from six replicate field trial carried out at Aberdeen, ID, during the summer of 1993 and a four replicate field trial carried out at Homestead, FL, during the winter of 1994. Expression in tuber tissue were determined in tubers harvested from four replicated plots of field trials carried out at four sites during the summer of 1995: Painter, VA; Stanton, MI; Coloma, WI; and New Denmark, New Brunswick, Canada.

The composite expression, results across all sites, for leaf and tuber *B.t.t.* protein in the NewLeaf™ Atlantic lines are shown in Tables V.4. The expression results for NPTII protein are shown in Table V.5.

The *B.t.t.* protein expression in leaves of all five lines across two sites was found to be in the range of 15.7 to 59.3 µg/g tissue fresh weight. The mean tuber *B.t.t.* protein expression level across four sites was found to be in the range of 0.09 to 0.53 µg/g tissue fresh weight. The *B.t.t.* protein expression levels correspond to 0.10 to 0.37% of total foliage protein and 0.0004 to 0.003% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

The NPTII protein expression in leaves of all five lines across two sites was found to be in the range of 4.4 to 36.6 µg/g tissue fresh weight. The mean tuber *B.t.t.* protein expression level across four sites was found to be in the range of 0.50 to 2.9 µg/g tissue fresh weight. The *B.t.t.* protein expression levels correspond to 0.028 to 0.23% of total foliage protein and 0.0025 to 0.014% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

F. Compositional Analyses of NewLeaf™ Potatoes

Monsanto Co. is in consultation with the FDA following their Policy "Foods Derived from New Plant Varieties" on the food safety of the NewLeaf™ Atlantic and Superior potatoes. Studies were carried out to compare the nutritional constituents of potato tubers of these plant lines with tubers obtained from control plants grown, stored, processed and analyzed under the same conditions. The study demonstrated that the tubers produced by the NewLeaf™ Atlantic and Superior plant lines were substantially equivalent to tubers produced by nontransformed Atlantic and Superior plants in the production of total solids, protein, sugars (dextrose and sucrose) vitamin C and glykoalkaloids.

Table V.1 Disease and Insect Susceptibility of NewLeaf™ Atlantic and Superior Plant Lines in Comparison to Control Plants.

Difference in Susceptibility Versus Control Variety			
Year/Site	**Vector**	**Disease[1]**	**Insect[2]**
1992			
Homestead, FL	PV-STBT04	No	No
Aberdeen, ID	PV-STBT04	No	No
Hancock, WI	PV-STBT04	No	No
1993			
Homestead, FL	PV-STBT04	No	No
Aberdeen, ID	PV-STBT04	No	No
Island Falls, ME	PV-STBT02	No	No
Island Falls, ME	PV-STBT04	No	No
Presque Isle, ME	PV-STBT04	No	No
Lakeview, MI	PV-STBT04	No	No
Freeville, NY	PV-STBT02	No	No
Rock Springs, PA	PV-STBT04	No	No
Hancock, WI	PV-STBT02	No	No
Hancock, WI	PV-STBT04	No	No
1994			
Beltsville, MD	PV-STBT04	No	No
Galena, MD	PV-STBT04	No	No
Homestead, FL	PV-STBT04	No	No
Homestead, FL	PV-STBT02	No	No
Island Falls, ME	PV-STBT02	No	No
Island Falls, ME	PV-STBT04	No	No
Presque Isle, ME	PV-STBT04	No	No
Presque Isle, ME	PV-STBT02	No	No
St. Agatha, ME	PV-STBT04	No	No
Freeville, NY	PV-STBT04	No	No
Lake Placid, NY	PV-STBT04	No	No
Clayton, NC	PV-STBT04	No	No
Wooster, OH	PV-STBT04	No	No
McClean,VA	PV-STBT04	No	No
Antigo, WI	PV-STBT04	No	No
Cassian, WI	PV-STBT04	No	No
Coloma, WI	PV-STBT04	No	No
Marathon, WI	PV-STBT04	No	No

1. Susceptibility to late blight, early blight, *Verticillium*, potato leafroll virus, potato virus Y, spindle tuber mosaic virus, bacterial ring rot, root knot nematode, and others.
2. Susceptibility to insects other than the CPB eg. aphids, potato leafhoppers, flea beetles, etc.

Table V.2. *B.t.t.* Protein Expression in Tissue of NewLeaf™ Superior Lines.

Line	Tissue	Number of Sites (reps.)	Expression Level, µg *B.t.t.* protein/g fresh weight: Least Square Mean	Standard Error	Range[3]
SPBT02-05	Leaf[1]	1 (4)	11.542	1.402	8.90 - 15.49
	Tuber[2]	1 (3)	1.146	0.078	1.00 - 1.29
SPBT02-07	Leaf[1]	1 (4)	11.474	0.170	11.11 - 11.82
	Tuber[2]	1 (3)	0.910	0.078	0.81 - 1.06
SP-Control	Leaf[1]	1 (4)	0.038[4]	0.005	0.00 - 0.01
	Tuber[2]	1 (3)	0.042[4]	0.004	0.03 - 0.05

1. Leaf tissue collected six weeks post planting from 1994 Winter field trial at Homestead, FL. The field trial arrangement consisted of four replicates per line.
2. Tubers obtained from 1994 Summer field trial at Island Falls, ME. The field trial arrangement consisted of four replicates per line, expression level assays were limited to three replicates.
3. "Range" denotes the highest and lowest individual assay for each line.
4. Value within the baackground noise level of the assay.

Table V.3. NPTII Protein Expression in Tissue of NewLeaf™ Superior Lines.

Line	Tissue	Number of Sites (reps.)	Expression Level, µg NPTII protein/g fresh weight: Least Square Mean	Standard Error	Range[3]
SPBT02-05	Leaf[1]	1 (4)	0.003	0.003	0.00 - 0.01
	Tuber[2]	1 (3)	0.016	0.005	0.01 - 0.03
SPBT02-07	Leaf[1]	1 (4)	3.817	0.215	3.20 - 4.17
	Tuber[2]	1 (3)	0.969	0.042	0.89 - 1.04
SP-Control	Leaf[1]	1 (4)	0.004[4]	0.002	0.00 - 0.01
	Tuber[2]	1 (3)	0.034[4]	0.005	0.03 - 0.04

1. Leaf tissue collected six weeks post planting from 1994 Winter field trial at Homestead, FL. The field trial arrangement consisted of four replicates per line.
2. Tubers obtained at harvest from 1994 Summer field trial at Island Falls, ME. The field trial arrangement consisted of four replicates per line, expression level assays were limited to three replicates.
3. "Range" denotes the highest and lowest individual assay for each line.
4. Value within the background noise level of the assay.

Table V.4. *B.t.t.* Protein Expression in Tissue of NewLeaf™ Atlantic Lines.

Line	Tissue	Number of Sites (reps.[3])	Expression Level, µg *B.t.t.* protein/g fresh weight: Least Square Mean[4]	Standard Error[5]	Range[6]
ATBT04-06	Leaf[1]	2 (10)	59.336	3.826	29.48 - 88.67
	Tuber[2]	4 (16)	0.528	0.024	0.26 - 0.71
ATBT04-27	Leaf[1]	2 (10)	32.027	3.826	26.10 - 36.17
	Tuber[2]	4 (16)	0.239	0.024	0.15 - 0.36
ATBT04-30	Leaf[1]	2 (10)	25.188	3.826	8.16 - 50.15
	Tuber[2]	4 (16)	0.090	0.024	0.04 - 0.25
ATBT04-31	Leaf[1]	2 (9)	15.694	4.033	8.23 - 19.81
	Tuber[2]	4 (16)	0.140	0.024	0.07 - 0.26
ATBT04-36	Leaf[1]	2 (10)	20.278	3.826	8.90 - 32.22
	Tuber[2]	4 (16)	0.126	0.024	0.05 - 0.27
AT-Control	Leaf[1]	2 (10)	0.050[7]	0.033	0.01 - 0.13
	Tuber[2]	4 (16)	0.029[7]	0.009	0.01 - 0.07

1. Leaf tissue collected six weeks post planting from 1993 Summer field trial at Aberdeen, ID and 1994 Winter field trial at Homestead, FL. The field trial at Aberdeen, ID consisted of six replicates per line, the field trial at Homestead, FL consisted of four replicates per line.
2. Tubers obtained from 1995 Summer field trial at Painter, VA; Stanton, MI; Coloma, WI; and New Denmark, New Brunswick, Canada. These field trials consisted of 12 to 15 replicates per line. Expression level assays were limited to four replicates.
3. The total number of plots across sites.
4. Population marginal or "least squares" means obtained from the MIXED procedure in SAS. In some unbalanced cases these means will not be equal to simple means calculated as it the data are unstructured.
5. Standard errors of the mean obtained from a mixed model containing error terms for block and site when relevant. All lines except the control were used in the mixed model. Control standard errors were determined separately. These standard errors reflect the **total** precision of the mean and can **not** be used to compare lines.
6. "Range" denotes the highest and lowest individual assay for each line.
7. Value within the background noise level of the assay.

Table V.5. NPTII Protein Expression in Tissue of NewLeaf™ Atlantic Lines.

Line	Tissue	Number of Sites (reps.[3])	Expression Level, µg NPTII protein/g fresh weight: Least Square Mean[4]	Standard Error[5]	Range[6]
ATBT04-06	Leaf[1]	2 (10)	36.564	2.666	21.54 - 47.63
	Tuber[2]	4 (16)	2.864	0.110	2.06 - 3.82
ATBT04-27	Leaf[1]	2 (10)	4.366	2.666	2.74 - 6.84
	Tuber[2]	4 (16)	0.975	0.110	0.29 - 1.43
ATBT04-30	Leaf[1]	2 (10)	13.163	2.666	2.27 - 28.95
	Tuber[2]	4 (16)	0.498	0.110	0.22 - 1.75
ATBT04-31	Leaf[1]	2 (9)	4.994	2.800	3.23 - 5.59
	Tuber[2]	4 (16)	0.726	0.110	0.27 - 1.45
ATBT04-36	Leaf[1]	2 (10)	12.156	2.666	5.46 - 23.02
	Tuber[2]	4 (16)	0.583	0.110	0.29 - 0.80
AT-Control	Leaf[1]	2 (10)	0.093[7]	0.070	0.01 - 0.41
	Tuber[2]	4 (16)	0.035[7]	0.019	0.00 - 0.18

1. Leaf tissue collected six weeks post planting from 1993 Summer field trial at Aberdeen, ID and 1994 Winter field trial at Homestead, FL. The field trial at Aberdeen, ID consisted of six replicates per line, the field trial at Homestead, FL consisted of four replicates per line.
2. Tubers obtained from 1995 Summer field trial at Painter, VA; Stanton, MI; Coloma, WI; and New Denmark, New Brunswick, Canada. These field trials consisted of 12 to 15 replicates per line. Expression level assays were limited to four replicates.
3. The total number of plots across sites.
4. Population marginal or "least squares" means obtained from the MIXED procedure in SAS. In some unbalanced cases these means will not be equal to simple means calculated as it the data are unstructured.
5. Standard errors of the mean obtained from a mixed model containing error terms for block and site when relevant. All lines except the control were used in the mixed model. Control standard errors were determined separately. These standard errors reflect the **total** precision of the mean and can **not** be used to compare lines.
6. "Range" denotes the highest and lowest individual assay for each line.
7. Value within the background noise level of the assay.

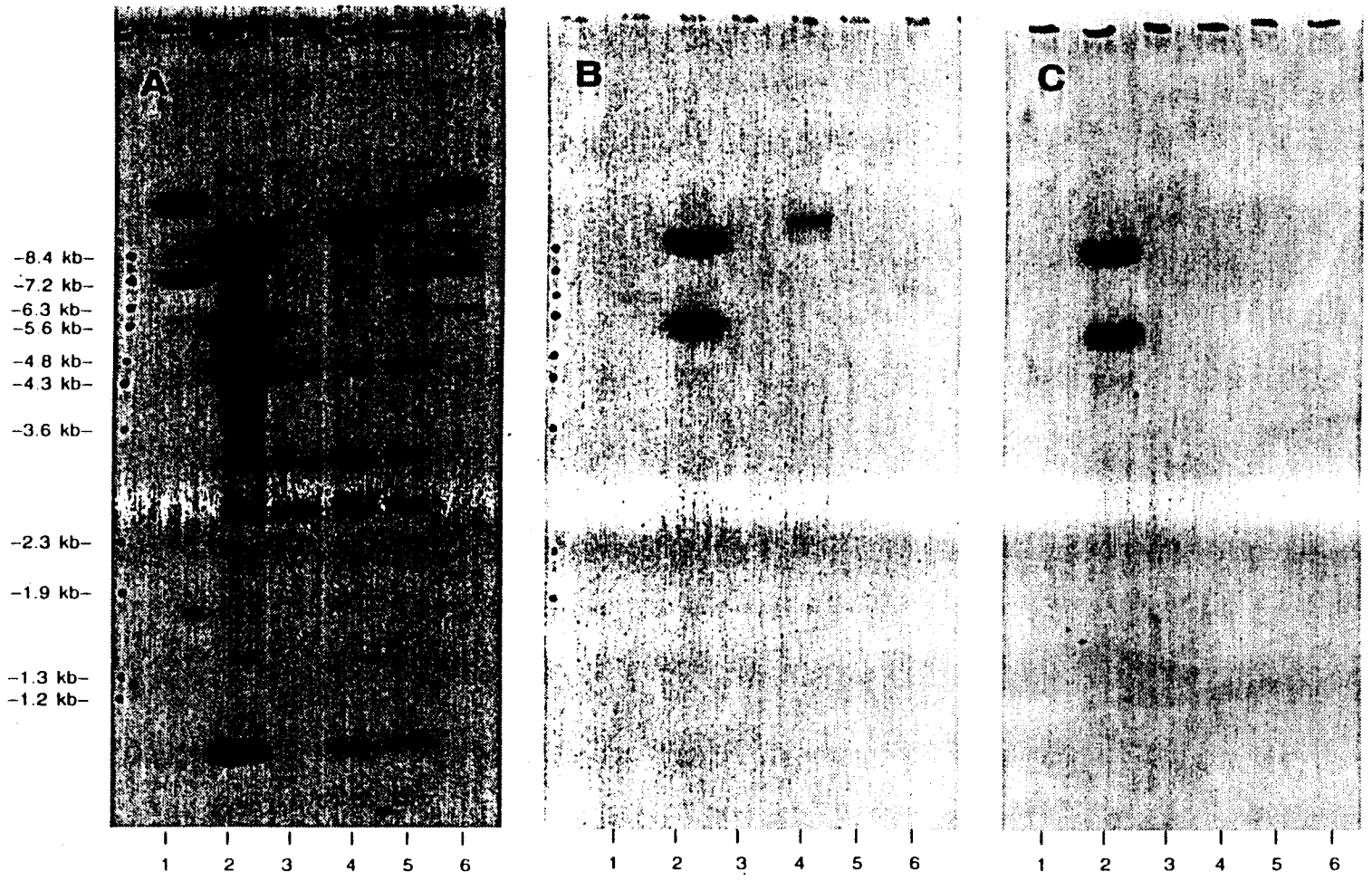

Figure V.1. Southern blot analysis of Superior lines using *Eco*RI. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with entire PV-STBT02 plasmid. Panel B was probed with *oriV*. Panel C was probed with the *aad* gene. Lane 1 and 6 contain molecular markers; Lane 2 contains plasmid, PV-STBT02, DNA digested with *Eco*RI mixed with plasmid DNA digested with a combination of *Hin*dIII/*Nhe*I and with DNA from control plant; Lane 3 contains DNA from control plant; Lane 4 contains DNA from Line SPBT02-5; and Lane 5 contains DNA from Line SPBT02-7.

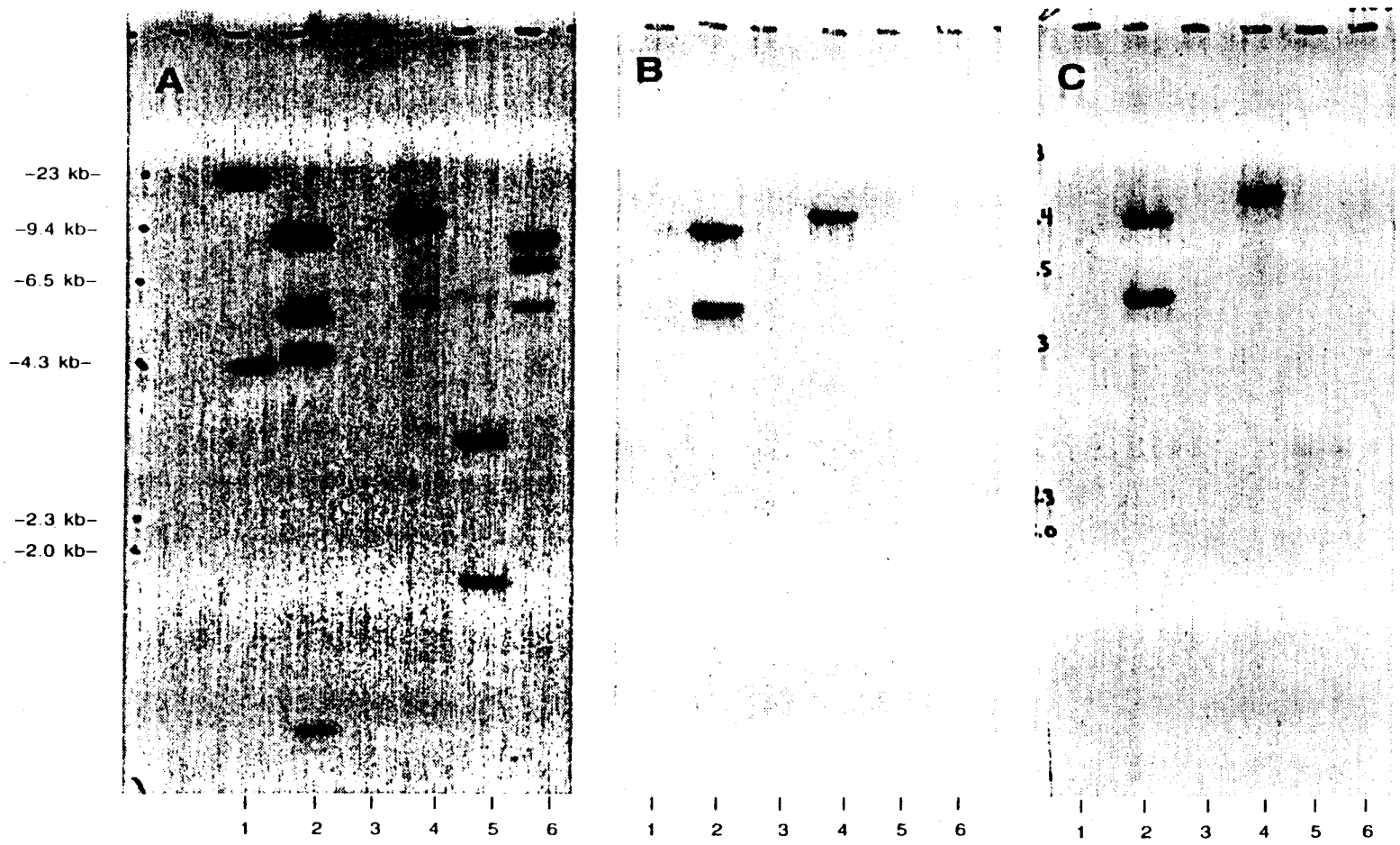

Figure V.2. Southern blot analysis of Superior lines using a combination of *Hin*dIII and *Xho*I. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with entire PV-STBT02 plasmid. Panel B was probed with *oriV* region. Panel C was probed with the *ori322* region. Lane 1 and 6 contain molecular markers; Lane 2 contains plasmid, PV-STBT02, DNA digested with *Eco*RI mixed with plasmid DNA digested with a combination of *Hin*dIII/*Nhe*I and with DNA from control plant; Lane 3 contains DNA from control plant; Lane 4 contains DNA from Line SPBT02-5; and Lane 5 contains DNA from Line SPBT02-7.

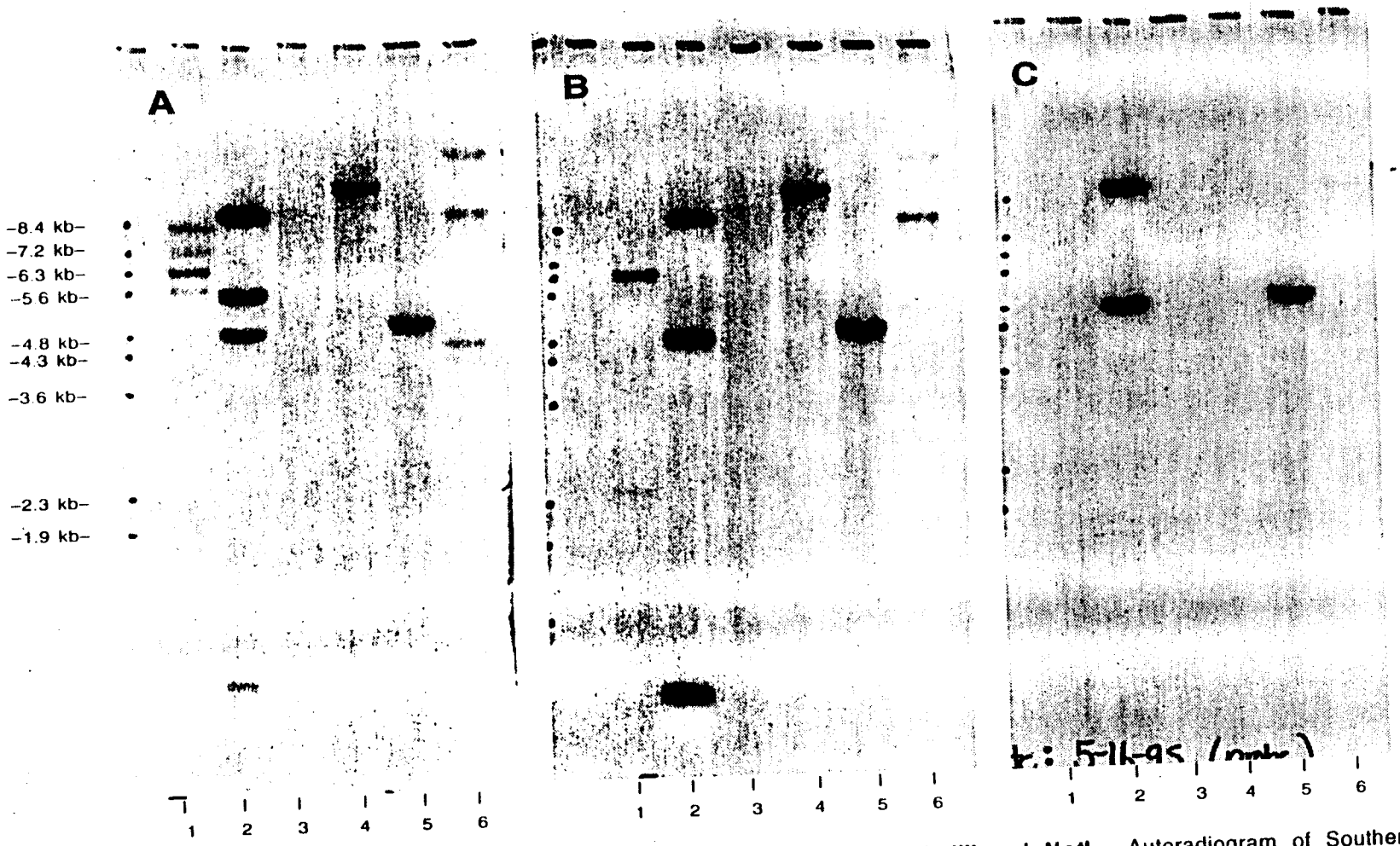

Figure V.3. Southern blot analysis of Superior lines using a combination of *Hin*dIII and *Not*I. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with entire PV-STBT02 plasmid. Panel B was probed with *cryIIIA* gene. Panel C was probed with the *nptII* gene. Lane 1 and 6 contain molecular markers; Lane 2 contains plasmid, PV-STBT02, DNA digested with *Eco*RI mixed with plasmid DNA digested with a combination of *Hin*dIII/*Nhe*I and with DNA from control plant; Lane 3 contains DNA from control plant; Lane 4 contains DNA from Line SPBT02-5; and Lane 5 contains DNA from Line SPBT02-7.

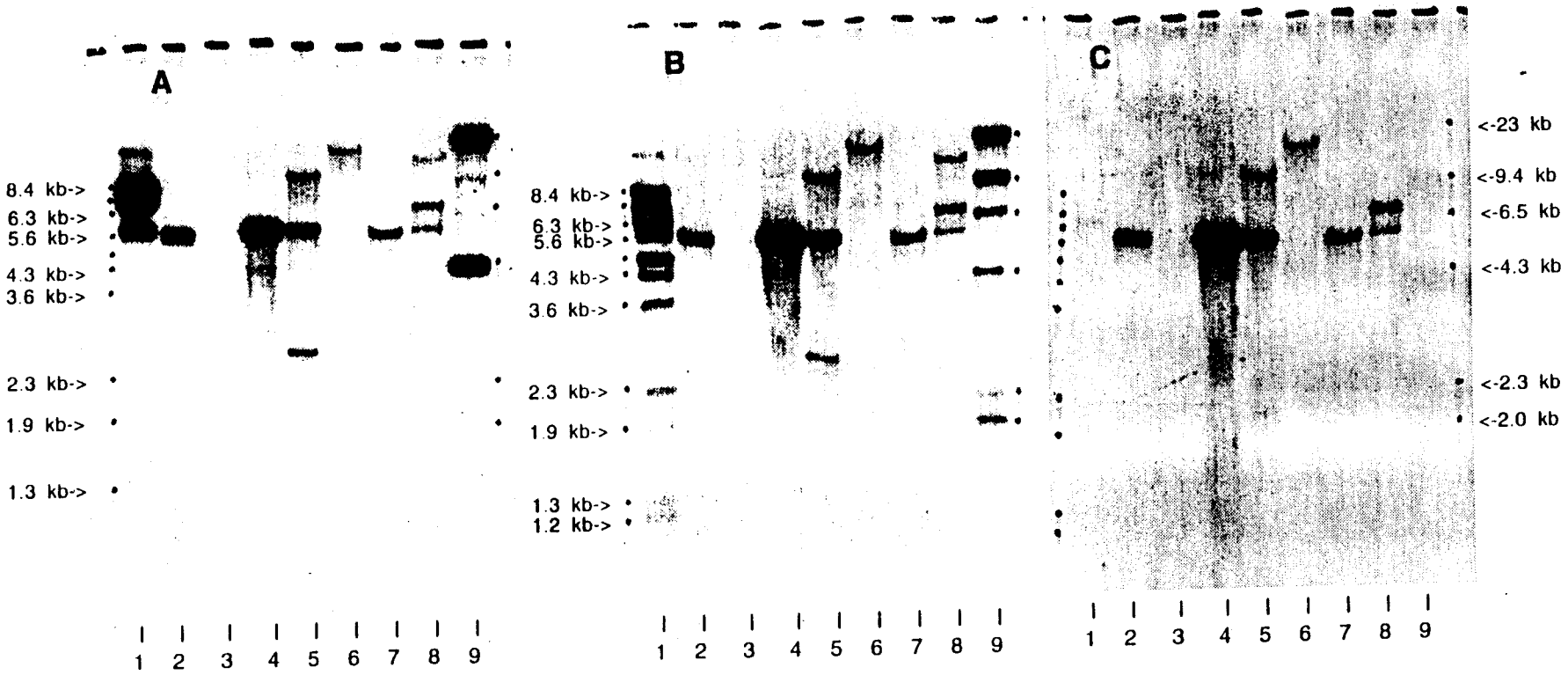

Figure V.5. Southern blot analysis of Atlantic lines using a combination of *SacI* and *NotI*. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with the entire PV-STBT04 plasmid. Panel B was probed with the *cryIIIA* gene. Panel C was probed with the *nptII* gene. Lanes 1 and 9 contain molecular markers. Lane 2 contains plasmid PV-STBT04 DNA and DNA from control plant digested with *SacI / NotI*. Lane 3 contains DNA from control plant digested with *SacI / NotI*. Lane 4 contains DNA from Line ATBT04-6. Lane 5 contains DNA from Line ATBT04-27. Lane 6 contains DNA from Line ATBT04-30. Lane 7 contains DNA from Line ATBT04-31. Lanes 8 contains DNA from Line ATBT04-36.

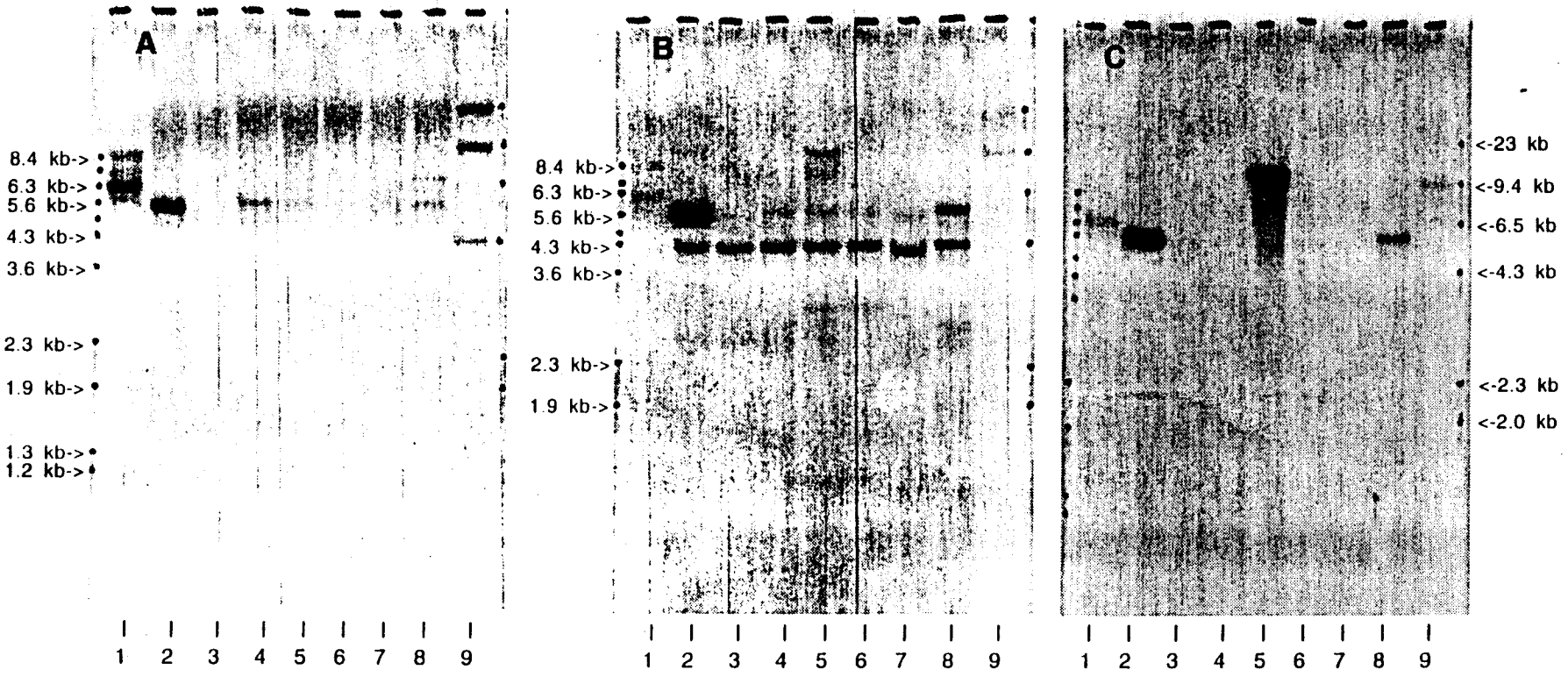

Figure V.6. Southern blot analysis of Atlantic lines using a combination of *SacI* and *NotI*. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants.
Panel A was probed with the *oriV* region. Panel B was probed with the *ori322* region. Panel C was probed with the *aad* gene. Lanes 1 and 9 contain molecular markers. Lane 2 contains plasmid PV-STBT04 DNA and DNA from control plant digested with *SacI* / *NotI*. Lane 3 contains DNA from control plant digested with *SacI* / *NotI*. Lane 4 contains DNA from Line ATBT04-6. Lane 5 contains DNA from Line ATBT04-27. Lane 6 contains DNA from Line ATBT04-30. Lane 7 contains DNA from Line ATBT04-31. Lanes 8 contains DNA from Line ATBT04-36.

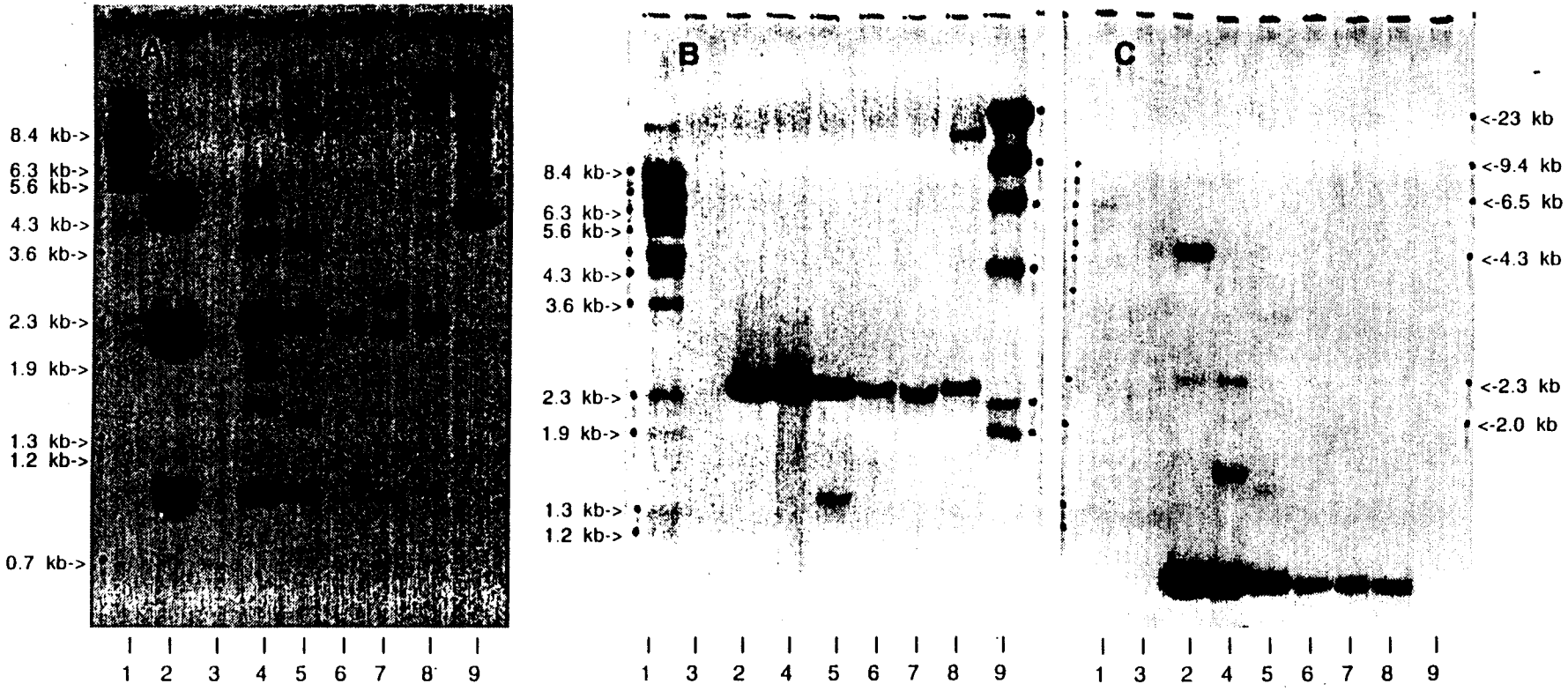

Figure V.7. Southern blot analysis of Atlantic lines using *PstI*. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with the entire PV-STBT04 plasmid. Panel B was probed with the *cryIIIA* gene. Panel C was probed with the *nptII* gene. Lanes 1 and 9 contain molecular markers. Lane 2 contains plasmid PV-STBT04 DNA and DNA from control plant digested with *PstI*. Lane 3 contains DNA from control plant digested with *PstI*. Lane 4 contains DNA from Line ATBT04-6. Lane 5 contains DNA from Line ATBT04-27. Lane 6 contains DNA from Line ATBT04-30. Lane 7 contains DNA from Line ATBT04-31. Lanes 8 contains DNA from Line ATBT04-36. Note: Loading of control DNA preparations (Lanes 2 and 3) in Panels B and C were reversed.

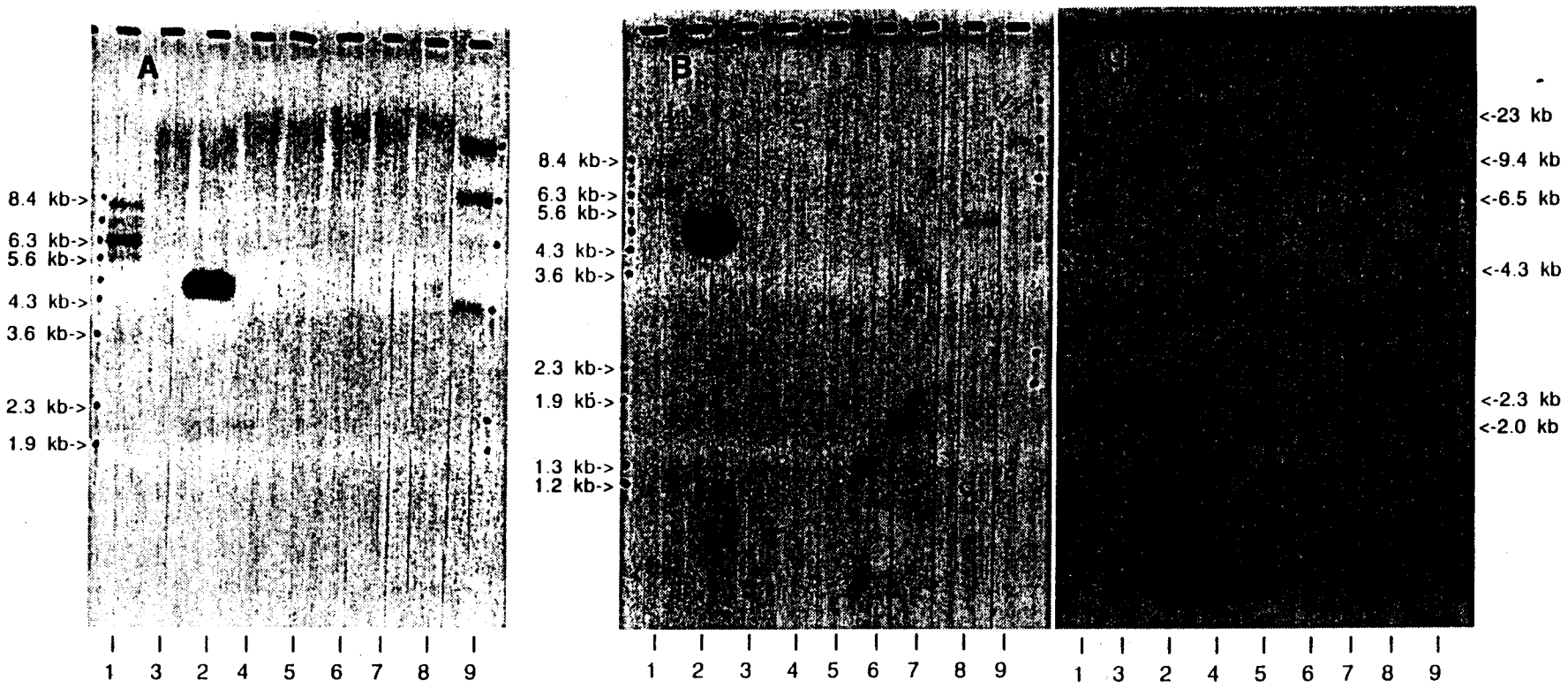

Figure V.8. Southern blot analysis of Atlantic lines using *PstI*. Autoradiogram of Southern blot analysis of approximately 10 µg of DNA isolated from young leaves of CPB resistant and control plants. Panel A was probed with the *oriV* region. Panel B was probed with the *ori322* region. Panel C was probed with the *aad* gene. Lanes 1 and 9 contain molecular markers. Lane 2 contains plasmid PV-STBT04 DNA and DNA from control plant digested with *PstI*. Lane 3 contains DNA from control plant digested with *PstI*. Lane 4 contains DNA from Line ATBT04-6. Lane 5 contains DNA from Line ATBT04-27. Lane 6 contains DNA from Line ATBT04-30. Lane 7 contains DNA from Line ATBT04-31. Lanes 8 contains DNA from Line ATBT04-36. Note: Loading of control DNA preparations (Lanes 2 and 3) in Panels A and C were reversed.

F. References

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Southern, E. M. 1975. Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. J. Mol. Biol. 98:503-517.

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCING NEWLEAF™ POTATO LINES PRODUCED UTILIZING THE PLASMID VECTORS PV-STBT02 OR PV-STBT04

The potential environmental consequences of introducing NewLeaf™ Atlantic and Superior potato lines transformed with the plasmid vectors, PV-STBT02 or PV-STBT04 have been evaluated. The use of these potatoes will have a positive impact on the environment by promoting integrated pest management practices and reducing reliance on traditional chemical insecticides. Extensive field test results, safety studies and independent scientific research establishes that the commercial use of these potatoes will not result in any adverse effects to the environment. These studies include assessing the toxicity to non-target organisms of the *B.t.t.* and NPTII proteins, environmental fate of the *B.t.t.* protein, transfer of the introduced genes to other plant species, the potential for these potato plants to become weeds, and the impact of these plants on potato pest management. These potential effects are discussed below.

A. *B.t.t.* Protein

The EPA and other regulatory agencies worldwide have determined that use of registered *B.t.t.* products offer no significant risks to human health or non-target organisms (EPA, 1988; EPA, 1991). Based on full product registration packages and other scientific information, the Agency found no evidence of any human or environmental safety concerns related to current uses of *B.t.t.* (EPA, 1988). In published reviews and the EPA documents, studies are referenced where the maximum hazard dose (5000 mg/kg) of *B.t.t.* microbial preparations was administered as single or multiple doses to different laboratory animals, with no adverse effects (EPA, 1991). Avian and aquatic organisms have also been fed *B.t.t.* microbial preparations, with no adverse effects. The preparations which were administered contained varying amounts of crystalline proteins from *B.t.t.*, either as a mixture with spores or encapsulated in killed *Pseudomonas fluorescens* cells (EPA, 1991). While target insects are susceptible to oral doses of *B.t.t.* proteins, there was no evidence of any toxic effects observed in non-target laboratory mammals, fish or birds given the equivalent of up to 10^6 µg of protein per gram of body weight. Since the *B.t.t.* protein expressed by NewLeaf™ potato plants is identical to one of the proteins present in commercial *B.t.t.* microbial preparations, the data support the safety of the *B.t.t.* protein produced in these NewLeaf™ potato lines.

Data was submitted to the EPA on September 10, 1993 (EPA Number 524-UTU) to support an exemption from the requirement of a tolerance and the registration of the *B.t.t.* protein as a plant pesticide. These requests were granted on April 25, 1995 and May 5, 1995, respectively. Studies included within the EPA submission demonstrated the non-target and environmental safety of this protein. These studies confirmed the Coleopteran selectivity of the *B.t.t.* protein expressed in NewLeaf™ potatoes (Tables VI.1 and VI.2). In addition, MacIntosh *et al.* (1990), observed no deleterious effects on non-target insects at concentrations of over 300-700 fold higher than those needed to control CPB.

An *in vitro* digestion study was conducted, which demonstrated the rapid degradation of the *B.t.t.* protein in simulated mammalian digestive fluid (Keck *et al.*, 1993). Even if insecticidal proteins could survive in the mammalian digestive tract, no *B.t.t.* receptors are expected on the surface of gastrointestinal tissues to permit binding of the protein to the cell surface. Data submitted to the EPA from a mouse acute gavage study with a large dose of *B.t.t.* protein (5000 mg/kg body weight) support this conclusion (Naylor, 1992). These results are fully consistent with the history of safe use of *B. thuringiensis* preparations.

B. Neomycin Phosphotransferase II

The NPTII protein, which has no insecticidal effect, is ubiquitous in the environment and is found in microbes present on food and within the human digestive system (Flavell *et al.* 1992; Calgene, Inc., 1993). This protein has also been used as a selectable marker for animal and human cell transformation and for human gene therapy experiments (Culver *et al.*, 1991; Brenner *et. al.*, 1993). The safety of NPTII and other selectable markers were addressed in recent reviews by Fuchs *et al.* (1993a and 1993b), Flavell *et al.* (1992) and Nap *et al.* (1992). Data were submitted to the EPA on November 25, 1993, to support the exemption from the requirement of a tolerance for this protein as a pesticidal inert ingredient (EPA Pesticide Petition #4E4301). The EPA approved this request on September 28, 1994 (EPA, 1994). The U.S. Food and Drug Administration (FDA) also approved the use of this protein as a processing aid food additive in several crops, as requested by Calgene, Inc. (FDA, 1994). All data support the safety of NPTII protein for use as a selectable marker in crops grown for human and animal consumption. This conclusion was also supported by a document published by the World Health Organization (WHO, 1993).

C. Effects of NewLeaf™ Potatoes on Non-target Organisms

NewLeaf™ potato plant lines transformed with the plasmid vectors PV-STBT02 or PV-STBT04 have been field tested at numerous sites across the U.S. since 1992. Plants of these lines show no deleterious effects towards non-target insects, birds, or other species that frequent potato fields (Appendix 1). The results of Monsanto sponsored tests on the safety of the *B.t.t.* protein expressed in NewLeaf™ potatoes on non-target beneficial insects (larval and adult honeybee, ladybird beetle, green lacewing and parasitic wasp) were reviewed by the EPA. The results of these studies are consistent with published literature showing that the biological activity of *B.t.t.* protein is highly specific to target insects only (EPA, 1988; EPA, 1991). In addition, as the protein expressed in NewLeaf™ potato plants is identical to that found in nature and in commercial *B.t.t.* formulations (Rogan and Lavrik, 1993), these results confirm that there are no differences in the selective toxicity of the protein expressed in the plant compared to the naturally occurring *B.t.t.* proteins. Therefore, no adverse effects are expected to non-target species from the use of the *B.t.t.* protein as expressed in Atlantic and Superior variety NewLeaf™ potato plants transformed with the plasmid vector PV-STBT02 or PV-STBT04.

D. Uncontrolled Movement of NewLeaf™ Potatoes into the Environment

1. Pollen Transfer

The potato, *Solanum tuberosum*, is the only tuberous *Solanum* species cultivated within the United States. Only two other tuberizing species of *Solanum* have been confirmed to exist; however, both of these species are found in high elevation arid climates, geographically distinct from cultivated potato production areas. Neither of these species can hybridize with *S. tuberosum.* Many other species of *Solanum* exist that are considered weeds in cultivated fields. However, none of these species are closely related and can hybridize with *S. tuberosum.* The article by Dr. Steven Love, Associate Professor of Potato Variety Development, College of Agriculture, University of Idaho, (Section II of this document) addresses in more detail the potential for gene escape from NewLeaf™ potatoes. In this article and in Love (1994), the conclusion is reached that outcrossing of NewLeaf™ potatoes with other *Solanum* species is not possible.

2. Hybridization With Other Cultivated Varieties

In contrast to Russet Burbank variety potatoes, most other varieties, including Atlantic and Superior are male fertile. Consequently, there is no genetic mechanism to prevent the hybridization of NewLeaf™ potatoes with other cultivated varieties within the U.S. Hybridization of NewLeaf™ potato plants with nontransformed cultivars would be expected to segregate in a normal Mendelian fashion, as the *B.t.t.* gene is stably integrated into the chromosome of the plant. However, due to production methods, it is unlikely that gene transfer will occur. Potato is bee pollinated, not wind pollinated, and flowers of cultivated potatoes are not attractive to bees because they lack nectar (Pavek, pers. comm.). In addition, pollen transfer occurs infrequently and over short distances. Tynan and his coworkers (1990) demonstrated no pollen dispersal in a field interplanted with genetically engineered and control potatoes beyond 4 - 5 meters and Dale *et al.* (1992) in a similar study, reported no pollen transfer beyond 10 meters. Hybrid seed that does occur is not used for further propagation and will remain in the field. If this seed germinates, long term propagation and survival of the resulting seedlings is not expected due to standard cultivation practices, and in fact has not been documented. Finally, the USDA (1995) concluded that "multiple barriers insure that gene introgression from NewLeaf™ potatoes into wild or cultivated sexually compatible plants is extremely unlikely, and such rare events should not increase the weediness potential of resulting progeny or have an adverse effect on biodiversity."

3. Weediness Potential

There is no indication that NewLeaf™ potatoes are more likely to become a weed than the non-modified parental variety or that they will increase the weediness potential of any other cultivated potato plant or wild species. At the 30 field locations at which the Superior and Atlantic lines transformed with PV-STBT02 and PV-STBT04, respectively and the 34 field locations at which the seven Russet Burbank NewLeaf potato lines (Monsanto, 1994) were evaluated, no difference in the number of volunteers of NewLeaf™ or control potatoes has been observed. No difference has also been noted with respect to the germination, disease and insect susceptibility, other than to CPB, of the transformed lines. In addition, the long term survival of volunteers would not be

expected due to standard cultivation practices or herbicide application. The USDA (1995) also concluded that "NewLeaf™ potatoes are no more likely to become a weed than CPB resistant potatoes, which could potentially be developed by traditional breeding techniques. Potato is not a serious, principal or common weed pest in the U.S., and there is no reason to believe that resistance to CPB would lead potatoes expressing this phenotype to become weed pests."

E. Impact of NewLeaf™ Potatoes on Potato Pest Management

It is apparent from the data developed by Monsanto, NatureMark and our cooperators and the experience of potato growers producing NewLeaf™ potatoes, that additional insecticide applications will not be required to control the CPB. Controlling CPB populations by planting NewLeaf™ potatoes will enable growers to significantly reduce the amount of chemical insecticides now applied to their crop. As a result, growers will be able to utilize a host of IPM practices that cannot be implemented now because of the current dependence on broad-spectrum chemical insecticides to control this pest. An increase in the biological and cultural control of non-target potato pests and a more judicious use of chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public.

F. Development of Pest and Resistance Management Strategies for CPB Resistant Potatoes

To achieve the numerous benefits, previously discussed, it is important that NewLeaf™ potatoes be implemented and managed properly. In this respect, these plants are no different than any other crop protection product that has been used over the last century. It is clear from the knowledge gained over that time, that to successfully maximize the long-term use of these potatoes, two interconnected management components are required. First, is the development of integrated pest management techniques that allow the farmer to optimize the utility of these plants for potato pest control. In essence, this is the development of a total insect management package that will be centered around NewLeaf™ potatoes. Second, to maximize the durability of these potatoes, is the development and implementation of strategies targeted to prevent the development of insect resistance to the *B.t.t.* protein produced by the plants.

For the last several years, extensive consultations have been held with the leading potato pest and resistance management researchers to develop a program to maximize the use and durability of NewLeaf™ potatoes. Laboratory and field studies designed in collaboration with these experts from academia and extension services are in progress and are providing the data needed to develop this management program. These studies are examining the impact of NewLeaf™ potatoes on populations of beneficial and pest insects endemic to the crop, the impact on the use of conventional insecticides for controlling non-target pests, the establishment of the baseline susceptibility of our insect targets to *B.t.t.*, and the impact of mixtures of resistant and non-resistant plants on yield loss.

Monsanto scientists have worked for several years on laboratory and field studies of insect resistance, and with outside collaborators nearly every suggestion made for

resistance management in NewLeaf™ potatoes is being examined. These strategies, developed in consultation with an expert advisory panel, take into account existing research and an understanding of potato production and agronomic practices. They include:

1) High dose expression of the *B.t.t.* protein in potatoes to control CPB heterozygous for resistance alleles.

2) Refugia as hosts for sensitive insects provided through non-NewLeaf™ potatoes.

3) Monitoring of insect populations for susceptibility to the *B.t.t.* protein.

4) Agronomic practices that minimize insect exposure to the *B.t.t.* protein.

5) Development of novel CPB control proteins with a distinct mode of action from the *B.t.t.* protein.

Those pest and resistance management strategies best suited for use in potato production and with the potential for delaying or preventing the development of resistance will be recommended. In addition, a program is in development to educate potato growers to the most effective ways to integrate these potatoes within their current production practices. This cooperative effort between growers, academia, extension services and Monsanto/NatureMark will help ensure that the benefits of NewLeaf™ potatoes are fully realized and sustained.

Table VI.1 Responses of Ten Insect Species to Ingestion of *B.t.t.* Protein.
Assays were performed at a concentration of 50 µg/ml in test diet.

Species	Reps	Insects Treated, No./Rep	% Survivor (Number of Insects) *B.t.t.* Protein	% Survivor (Number of Insects) Control	Physiological Effects on *B.t.t.* Protein Treated Insects
Anthonomus grandis	3	24	90.3 (65)	90.3 (65)	none
Diabrotica undecimpunctata	3	24	94.4 (68)	93.1 (67)	none
Leptinotarsa decemlineata	3	24	0.0 (68)	93.1 (67)	all larvae dead
Ostrinia nubilalis	31	24	87.5 (63)	97.2 (70)	none
Ostrinia nubilalis	3	24	97.2 (70)	97.2 (70)	none
Manduca sexta	3	24	97.2 (70)	95.8 (69)	none
Helicoverpa zea	3	24	98.6 (71)	97.2 (70)	none
Heliothis virescens	3	24	94.4 (68)	94.4 (68)	none
Aedes aegypti	5	10	100.0 (50)	100.0 (50)	none
Blatella germanica	2	15	93.3 (28)	96.7 (29)	none
Myzus persicae	16	12	83.3 (150)	100.0 (192)	honeydew reduction[2]
Myzus persicae	16[3]	12	69.8 (134)	76.6 (147)	honeydew reduction[2]

1. Test repeated due to a significant treatment related effect resulting in the first assay. Survivorship of treatment and control *O. nubilalis* in the second assay was identical. Analysis of the combined data set (6 reps, 2 assay dates) indicated that neither the day-related variation ($F = 4.55$, $df = 2$, $p > 0.06$) nor the treatment related variation ($F = 4.55$, $df = 2$, $p > 0.06$) was significant.
2. Slight reduction in honeydew production. A rating scale of 0 to 3 was used to estimate relative levels of *M. persicae* feeding based on honeydew reduction. 0 = no honeydew reduction (aphid feeding apparently normal), 1 = slight reduction, 2 = moderate reduction and 3 = no honeydew production (little or no aphid feeding). Using this scale, the mean ratings for bioassay 1 were: *B.t.t.* protein = 0.40, control = 0.19, and the ratings for bioassay 2 were: *B.t.t.* protein = 0.56, control = 0.06.
3. Test repeated due to a significant treatment related effect resulting in the first assay. Two-way analysis of variance was performed on the combined data set. Analysis of the combined data set from both assays (31 reps *B.t.t.* protein, 32 reps control, 2 assay dates) indicates that the day-related variation ($F = 10.57$, $p > 0.002$) explains much of the difference between the treatment and control groups.

Table VI.2 Sensitivity of Selected Beneficial Insects to the *B.t.t.* Protein.
Assays were performed using a concentration at least 100 times the estimated LC_{50} (LC_{50} = ca. 1.0ppm) of *B.t.t.* protein in the diet of Colorado potato beetle.

Taxonomic Order	Species	Reps.	Insects Treated, No./Rep	% Mortality *Btt* Protein	% Mortality Control	LC_{50} (ppm)
Coleoptera	*Leptinotarsa decemlineata*	3	16	81	03	*ca.* 1.0[1]
Coleoptera	*Hippodamia convergens*	6	25	29	22	>100[2]
Hymenoptera	*Nasonia vitripennis*	2	25	28	30	>100[3]
Hymenoptera	*Apis mellifera* (larvae)	4	50	14	18	>100[4]
Hymenoptera	*Apis mellifera* (adults)	3	39-64	25	30	>100[5]
Neuroptera	*Chrysopa carnea*	1	30	10	23	>100[6]

1. The activity of *B.t.t.* protein was assessed by incorporation of the test material in an artificial diet at five concentrations which ranged from 9.0 to 0.11 µg *B.t.t.* protein per gram diet. The assay employed first instart larvae. The reported values represent the percent mortatlity at 1.0 µg *B.t.t.* protein per gram diet
2. Insects were exposed to one maximum test concentration and observed daily for mortality and signs of toxicity. The reported percent mortality represent the average cummulative number of dead insects per number exposed during a ten day assay period.
3. Insects were exposed to one maximum test concentration and observed daily for mortality and signs of toxicity. The reported percent mortality represent the average cummulative number of dead insects per number exposed during a nine day assay period.
4. Larvae were exposed to one maximum dose of test substance by placing 5 µl of an aqueous solution of the test substatnce into each larval cell. Reported values represent the mean larval mortality from 1st - 2nd instar stage through adult emergence.
5. Tests were conducted using paper carton cages containing approximately 40 bees per replicate. Adults were exposed to test or control substances by adding the material to a 50:50 mixture of honey:water to achieve on a maximum concentration. The test material was introduced through the cage bottom using a glass vial and a cotton wick. Adults were observed daily for mortality and signs of toxicity until the control group mortality exceeded 20% . The percent mortality are the mean cumulative mortality during the three days assay period.
6. Larvae were exposed to one maximum test concentration and then observed daily for mortality and signs of toxicity. The percent mortality are the average cummulative number of dead insects per number exposed during a nine day assay period.

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VII. STATEMENT OF GROUNDS UNFAVORABLE

We know of no unfavorable grounds associated with NewLeaf™ Superior potato lines SPBT02-5 and SPBT02-7 and NewLeaf™ Atlantic potato lines ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36 developed using the plasmid vectors, PV-STBT02 or PV-STBT04. Therefore, on the basis of the substantial potential benefits to the grower, the environment, and the significantly lower potential risk to public health, Monsanto Company requests that these potato lines no longer be regulated under 7 CFR part 340.6.

APPENDIX 1

GENETICALLY MODIFIED COLORADO POTATO BEETLE RESISTANT POTATO PLANTS, FOLIAR-APPLIED MICROBIAL *B.t.t.*, AND CONVENTIONAL INSECTICIDES: COMPARATIVE IMPACTS ON NON-TARGET ARTHROPODS

Genetically Modified Colorado Potato Beetle Resistant Potato Plants, Foliar-applied Microbial *B.t.t.*, and Conventional Insecticides: Comparative Impacts on Non-target Arthropods

by

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ABSTRACT

Replicated large plot studies were conducted in 1992 at three North American locations to evaluate the impact of several insect management regimes on non-target arthropods. Genetically modified Colorado potato beetle resistant potatoes containing an insecticidal protein from *Bacillus thuringiensis* subsp. *tenebrionis* provided complete control of all Colorado potato beetle life stages at all locations. Beneficial arthropods were significantly more abundant in genetically modified Colorado potato beetle resistant potato plots than in those treated with conventional chemical insecticides. Commercially acceptable aphid control was achieved in these plots solely through predation by natural enemies. Colorado potato beetle resistant potatoes represent an effective and environmentally compatible addition to the existing methods of managing potato insect pests.

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most destructive foliar pest of potatoes in North America. Insecticide resistance has made chemical control of the Colorado potato beetle increasingly difficult in recent years (Forgash 1985). In the east and midwestern U.S., where resistance is most troublesome, insecticide control of Colorado potato beetle costs upward of $200 to $300 per acre (Wyman, unpubl. data). Insecticide resistance is now recognized as a serious threat in all potato producing areas, and alternative management strategies which utilize a combination of control factors for the Colorado potato beetle are needed.

Bacillus thuringiensis is a common soil-borne bacterium (Martin and Travers 1989). In its spore forming stage, *B. thuringiensis* produces an insecticidal protein which is non-toxic to humans, other mammals, or beneficial organisms (EPA 1988). *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) possesses specific activity against coleopteran insects (MacIntosh, et al. 1990), and has been widely adopted by organic producers for control of the Colorado potato beetle (OCIA Materials List). Despite its safety and environmental advantages, its use in conventional potato pest management programs has been limited due to high costs and the rather unpredictable results achieved with foliar-applied microbial sprays. Although microbial *B.t.t.* formulations

can be highly effective when properly applied, their success may be reduced by a number of factors including poor spray timing, inadequate coverage, and inclement weather (Ferro and Lyon 1991).

Plant expression of *B.t.t.* protein through genetic modification represents an alternative delivery system through which researchers have achieved complete control of the Colorado potato beetle (Perlak et al. 1993). This technology will facilitate a shift toward more biologically-based pest management programs and will allow growers to reduce pesticide inputs without suffering crop losses from the Colorado potato beetle. As reliance on broad spectrum insecticides diminishes, populations of beneficial non-target organisms such as predators and parasites are expected to increase in the cropping system. These natural enemies will contribute to the control of Colorado potato beetles and other potato pests including aphids, leafhoppers, and plant bugs.

The following data from studies conducted in 1992 at three North American locations represents preliminary results in a multi-year research project investigating the effect of several Colorado potato beetle control strategies on non-target pest and beneficial arthropods. The experimental insect control practices included conventional systemic or foliar insecticides, foliar-applied microbial *B.t.t.*, and genetically modified Colorado potato beetle resistant plants. The long term goal of this continuing research is to develop crop recommendations which incorporate Colorado potato beetle resistant plants and other selective insect controls for the integrated management of potato insect pests.

MATERIALS & METHODS

Research was conducted at three locations representing different potato production regions and their respective pest/beneficial complexes. The research sites included the Oregon State University-Hermiston Agricultural Research & Extension Center, (northcentral Oregon), the University of Wisconsin-Hancock Experiment Station (central Wisconsin), and the Agriculture Canada Charlottetown Experiment Station (Prince Edward Island). With the exception of insect control treatment factors, which were specific to each trial location, all crop production procedures reflected those used locally with conventional fertilizer, herbicide, and fungicide applications. The experimental treatment regimes were designed to compare conventional, broad-spectrum chemical controls with narrow-spectrum, selective alternatives.

Plot Design

In all trials, individual plots consisted of sixteen 54 ft rows, which were bordered on all sides by 6 feet of unprotected Russet Burbank buffer. Potato rows were 34 to 36 inches apart with tuber seed placed 9 to 14 inches apart in the row. Pest and beneficial arthropod populations were visually sampled once each week from an 18 ft by 6 row area in the center of each plot, designated the experimental unit. An unplanted single-row alleyway was maintained around this area to facilitate worker mobility. All sampling that required disruption of the foliage was performed in the potato rows immediately adjacent to, and outside of, the experimental unit.

Sampling Methods

The following methods were used in some or all locations to measure the densities of all foliar and soil-surface dwelling arthropods and their impact on the cropping system:

Beat cloth samples were taken by placing a 27 to 30 inch square cloth under the canopy and beating the foliage with a stick to dislodge resident insects. The primary insect pests that were sampled using this technique were aphids (family: Aphidae). Beneficial arthropods sampled in this manner included damsel bugs (family: Nabidae), big-eyed bugs (family: Geocoridae), lady beetles (family: Coccinellidae), green lacewings (family: Chrysopidae), brown lacewings (family: Hemerobiidae), minute pirate bugs (family: Anthocoridae), flower flies (family: Syrphidae), stink bugs (family: Pentatomidae), and spiders.

Sweep samples were taken by passing the 15 inch diameter sweep net through the canopy 25 times. This method was used to sample adult potato leafhoppers (*Empoasca fabae* (Harris)).

Visual plant counts were taken on the perimeter of the experimental unit. Potato flea beetle (*Epitrix cucumeris* (Harris)) adults, Colorado potato beetle adults, small larvae (instars 1 and 2), large larvae (instars 3 and 4), and egg masses were counted on whole plants.

Leaf samples were taken to evaluate populations of aphids and potato leafhopper nymphs, and damage (feeding holes) from adult flea beetles.

D-Vac samples were taken from foliage by moving the vacuum head through the canopy for 60 seconds/plot. This method was used to sample all foliage-dwelling insects.

Pitfall traps constructed from 16 oz plastic beer cups were placed in two locations/plot in the potato rows immediately adjacent to the experimental unit. Pitfall traps were used to estimate population densities of ground beetles (family: Carabidae), rove beetles (family: Staphylinidae), and other soil-dwelling fauna.

Yellow and green water pan traps were placed in each plot to monitor alate aphid populations throughout the season.

Defoliation estimates were made weekly to evaluate Colorado potato beetle damage.

Tubers were visually examined at harvest for wireworm damage.

All data for individual insects were subjected to multiple analyses of variance for each sampling date, and for seasonal population averages.

Regional Pest Problems and Conventional Practices

Oregon:

The most damaging pests of potatoes in Oregon are green peach aphids (*Myzus persicae* (Sulzer)), potato aphids (*Macrosiphum euphorbiae* (Thomas)), Colorado potato beetles, and wireworms. In northcentral Oregon, many growers utilize soil-applied systemic insecticides early in the season, followed by soil or foliar-applied insecticides when efficacy begins to decline (Pacific Northwest Insect Control Handbook 1992).

Green peach aphids, which are efficient vectors of potato leafroll virus (Klostermeyer 1953), are the most serious aphid pest of commercial (non-seed) potatoes. Green peach aphids overwinter on peach and other *Prunus* species in the Columbia basin. Winged adults typically infest potato fields in early spring as the weather warms (Tamaki and Olsen 1979). If these aphids have previously fed on virus-infected volunteer potatoes or other non-crop hosts, they may create points of infection within the potato field. During the season, potato leafroll virus may spread as wingless aphid populations build.

The majority of insecticide applications in the northwestern U.S. are targeted at aphids. While the Colorado potato beetle can be a serious pest, control is usually achieved as a result of aphid management. Most available insecticides provide good control of potato beetle larvae and adults since insecticide resistance has not yet been detected in this region.

Wireworms can be a severe problem in infested soils. In fields that are not fumigated prior to planting, non-systemic soil insecticides are applied to protect tubers from attack.

Wisconsin:

The primary pests of potatoes are Colorado potato beetles, potato leafhoppers, and green peach and potato aphids. Both Colorado potato beetles and potato leafhoppers can cause crop devastation if not controlled in a timely fashion, while aphids are primarily a concern due to their ability to vector potato leafroll virus. Potato flea beetles are sporadic pests which rarely require targeted insecticide applications.

Potato leafhoppers migrate into Wisconsin each spring on southerly winds and typically build to damaging levels over a very short period. As a result, they do not lend themselves to biological control since most beneficial insects have not become established in the cropping system so early in the season. Potato leafhoppers are susceptible to a variety of insecticides and can be controlled by timing applications to established economic thresholds.

Since green peach and potato aphids do not overwinter in Wisconsin, infestations usually does not occur until mid-summer. A range of 10 to 40 green peach aphids per 50 leaves is employed as a general treatment threshold for processing potatoes where potato leafroll virus is of concern, while a threshold of 5 green peach aphids per 50

leaves is recommended for seed potato production.

In the last ten years, following detection of pesticide residues in the ground water (Rothschild et al. 1982), growers in Wisconsin have shifted away from a reliance on soil-applied systemic insecticides. Instead, insect control is achieved with well timed applications of broad spectrum foliar insecticides (Chemical Recommendations for Commercial Potato Production, 1992). Insecticides specifically targeted at aphids are frequently required in mid to late season.

Prince Edward Island (PEI):

The primary insect pests in PEI which require control annually are Colorado potato beetles, potato flea beetles, and green peach and potato aphids. Insect control is commonly achieved with soil-applied systemic insecticides early in the season, followed by foliar-applied insecticides when systemics are no longer effective. Since most potatoes produced in PEI are grown as potential seed potatoes, aphid treatment thresholds are low in order to limit infection from all vectored viruses. Aphids do not overwinter in Canada, so infestation usually does not occur until mid-summer.

Because of the relatively short growing season experienced in eastern Canada, Colorado potato beetles are usually limited to one generation per year. Controls are targeted primarily at the larvae, although the adult progeny of these larvae can be a threat in some years. Insecticide resistance is a growing problem in Maine and northeastern Canada, but chemical control is still achieved without difficulty in PEI.

Colorado potato beetles and potato flea beetles are taxonomically related (family: Chrysomelidae), and share a similar life history. Flea beetle adults overwinter in the soil, larvae complete one discrete generation per year, and all stages of the insect feed on the same host plant species. Potato flea beetle larvae, which feed on potato roots, are effectively controlled with soil-applied systemic insecticides. The adult is the most damaging stage, however, as it makes numerous small holes in the foliage. Control of larvae early in the season does not always eliminate the need for late-season insecticides to control potato flea beetle adults.

Site Specific Treatments

Oregon

1. Russet Burbank potatoes with foliar Colorado potato beetle control.

   Permethrin (Pounce® 3.2 EC, .2 lbs ai/A) was applied every two weeks beginning June 23, with five applications total.

2. Russet Burbank potatoes with systemic insecticides for Colorado potato beetle and aphid control.

   Phorate (Thimet® 15G, 2.17 lbs ai/A) was applied on June 6, followed by foliar application of disulfoton (Di-Syston-8®, 3.36 lbs ai/A) on July 9 when aphid and Colorado potato beetle control began to decline.

3. Genetically modified Colorado potato beetle resistant Russet Burbank potatoes with systemic insecticides for aphid control.

   Phorate (Thimet® 15G, 2.17 lbs ai/A) was applied on June 6, followed by foliar application of disulfoton (Di-Syston-8®, 3.36 lbs ai/A) on July 9 when aphid control began to decline.

4. Genetically modified Colorado potato beetle resistant Russet Burbank potatoes with no additional insect control.

5. Russet Burbank potatoes with foliar-applied microbial *B.t.t.* treatment for Colorado potato beetle control.

   Microbial *B.t.t.* (M-Trak®, 0.75 qt/A) was applied weekly beginning June 23, with a total of nine applications.

6. Russet Burbank potatoes with no insect control.

All treatments were replicated six times and arranged in a Latin Square design.

Wisconsin

1. Russet Burbank potatoes with conventional foliar control of Colorado potato beetle and other pests.

   Esfenvalerate (Asana® 1.9 EC, 0.05 lbs ai/A) was applied on June 30 for control of first generation Colorado potato beetle larvae. Endosulfan (Thiodan® 50 WP, 1.0 lbs ai/A) was applied on July 15 for control of Colorado potato beetle summer adults, and methamidophos (Monitor 4® 0.75 lbs ai/A) was applied for potato leafhopper and aphid control on August 24.

2. Genetically modified Colorado potato beetle resistant Russet Burbank potatoes with selective potato leafhopper control.

   Malathion was applied at half the recommended rate (Malathion® 0.45 lbs ai/A) on July 2 when potato leafhoppers exceeded the treatment threshold of two per sweep. A second application of malathion was made on August 24.

3. Russet Burbank potatoes with foliar applied microbial *B.t.t.* and selective potato leafhopper control.

   Microbial *B.t.t.* (M Trak®, 0.75 qt/A) was applied on June 25 and July 15 for Colorado potato beetle larval control. Malathion was applied at half the recommended rate (Malathion® 0.45 lbs ai/A) on July 2 when potato leafhoppers exceeded the treatment threshold of two per sweep. As microbial *B.t.t.* has little effect on Colorado potato beetle adults, esfenvalerate (Asana® 1.9 EC, 0.05 lbs ai/A) was applied on August 24 to control potato leafhoppers and to rescue the plots from defoliation due to the Colorado potato beetle.

4. Russet Burbank potatoes with no Colorado potato beetle control and selective potato leafhopper control (designated "untreated control").

   Malathion (Malathion® 0.45 lbs ai/A) was applied at half the recommended rate on July 2 when potato leafhoppers exceeded the treatment threshold of two per sweep. A second application of malathion was made on August 24.

All treatments were replicated four times and arranged in a Latin Square design.

PEI

1. Russet Burbank potatoes with systemic insecticide for Colorado potato beetle, potato flea beetle, and aphid control.

   Phorate (Thimet® 15G, 2.17 lbs ai/A) was applied at planting.

2. Genetically modified Colorado potato beetle resistant Russet Burbank potatoes with systemic insecticide for potato flea beetle and aphid control.

   Phorate (Thimet® 15G, 2.17 lbs ai/A) was applied at planting.

3. Genetically modified Colorado potato beetle resistant Russet Burbank potatoes with no additional insect control.

4, 5, and 6. Russet Burbank potatoes with no insect control.

All treatments were replicated four times and arranged in a randomized complete block design.

RESULTS

Oregon:

Colorado potato beetle:

Genetically modified potatoes, microbial *B.t.t.*, permethrin, and the systemic insecticides all provided commercially acceptable control of Colorado potato beetles. Defoliation reached 100 percent in the untreated controls on July 17, while all other treatments incurred less than 10 percent defoliation during this time period. Widespread infection of late blight (*Phytophthora infestans*) made damage from Colorado potato beetles difficult to measure after mid-July, and defoliation estimates were discontinued as a result.

Genetically modified potatoes had fewer adults and larvae than did any other insecticide treatment (Fig. 1a, b), and egg laying was significantly reduced relative to all treatments (Fig. 1c). Weekly applications of microbial *B.t.t.* provided good Colorado

potato beetle control, and the seasonal average of larvae was lower than that in systemic insecticide-treated plots. However, while no larvae survived past the first instar in genetically modified potatoes, some late instar larvae were found in microbial *B.t.t.* plots.

Green peach aphid:

Alate green peach aphids were first detected in the Hermiston area on May 28 in yellow water pan traps. Alate aphids moved into the plots as the plants began to emerge on June 19 but did not increase substantially until after June 29, when a larger flight deposited alate aphids in large numbers throughout the field. Apterous progeny from these aphids began to build up in the plots after this point, whereupon treatment differences became evident.

Apterous aphid populations were lowest in systemic treated plots, but remained at commercially acceptable levels all season in every treatment except permethrin (Fig. 2a). In late-July to mid-August, apterous aphid populations in permethrin plots exhibited exponential-like growth, approaching 4000 per beat sample on August 13. Permethrin provided excellent control of Colorado potato beetles but was ineffective against aphids. Although a viable management option for Colorado potato beetle control, permethrin is toxic to a broad range of beneficial predators and parasites. The type of aphid population increase observed in permethrin-treated plots in this study resulted from the elimination of natural enemies as a regulating influence. This same "population bloom" did not occur in plots which received no supplemental insecticides for aphid control. Apterous green peach aphid populations increased to only 35 per beat sample in the genetically modified potato plots during this same period.

The late season population of alate aphids reflected apterous population trends, increasing significantly in permethrin-treated plots in late August (Fig. 2b). Alate populations remained below treatment threshold levels in all other treatments, where no significant differences were detected. Aphids typically mature into winged forms as a mechanism of dispersal in response to overcrowding, as was observed in permethrin plots. Since alates are capable of acquiring and transporting viruses to new locations, they may be responsible for initiating new disease outbreaks in neighboring fields. Persistent viruses such as potato leafroll virus are commonly spread between fields in this manner.

Wireworms:

Substantial wireworm populations were found in all plots. Infestation ranged from 67% of tubers in genetically modified potato plots with no insecticides, to 80% of tubers in systemic insecticide-treated potatoes. No significant differences in incidence or extent of tuber damage were detected between any treatment regimes.

Beneficial arthropod complex:

The primary predators found in the plots were generalist hemipterans and spiders. Big-eyed bugs, spiders, damsel bugs, and minute pirate bugs comprised over 97% of the predators observed in 1992, while lady beetles, brown lacewings, flower flies, and

stink bugs were present at a much lower frequency. Predacious arthropods were most abundant in genetically modified potato and microbial *B.t.t.* plots (Fig. 3). Broad spectrum insecticides were not applied in these treatments, and resident plant and detritus-feeding insect populations were high enough to maintain predator population growth.

Big eyed bugs:

Big eyed bugs were the most common predators found in the plots. Adult populations were highest during late June, giving rise to a nymphal population that increased after mid-July. Nymphs were significantly more abundant in genetically modified potato and microbial *B.t.t.* plots from July 20 to August 24 (Fig. 4 and 5). Adult populations, though greater in these treatments, were not significantly different.

Systemic insecticides generally have less impact on beneficials than do foliar insecticides, such as permethrin, because exposure is limited to those insects feeding on the plants. However, big eyed bug nymphs feed on sap during the first two instars which may account for the lower numbers observed in plots treated with phorate and disulfoton.

Spiders:

Spiders were prevalent in the experimental plots and appeared to be an important element of the natural enemy complex. Genetically modified potato and microbial *B.t.t.* plots had significantly more spiders than all other treatments from July 13 to August 3, and had more than permethrin-treated plots all season (Fig. 6).

Spider populations dropped significantly in the systemic and genetically modified potato/systemic plots after foliar application of disulfoton on July 9. Reentry of spiders into disulfoton-treated plots occurred as the material decreased in activity and pests reinfested the plots. Permethrin appeared to be toxic to spiders, as stable populations never developed in these plots despite the high pest (aphid) populations.

Damsel bugs:

Damsel bugs were most numerous in the genetically modified potato and microbial *B.t.t.* plots (Fig. 7). However, significant differences between these and the systemic treated plots only occurred for a three week period following application of disulfoton on July 9. Permethrin was detrimental to damsel bug populations, and treated plots had very few nymphs season-long.

Minute pirate bugs:

Adult minute pirate bugs, which are highly mobile, were most abundant in permethrin plots (Fig. 8) as they continually reinfested in response to large prey (aphid) populations. However, their numbers dropped sharply following each permethrin application, and very few nymphs were observed in these plots during the season. Minute pirate bug populations in general were variable throughout the season, and no significant differences between treatments were detected.

Summary:

A commercially acceptable level of Colorado potato beetle control was obtained in all plots with experimental insect management regimes. Egg laying in genetically modified potato plots was significantly lower than in any other treatments, with no larvae surviving past the first instar. Late instar larvae, which can potentially develop into destructive summer adults, were detected in all other plots.

Aphid control was achieved in all experimental treatments except permethrin. Permethrin, which is broad spectrum in activity, prevented the establishment of many beneficial arthropods such as big eyed bugs, damsel bugs, minute pirate bugs, and spiders, but provided no control of green peach aphids. As a result, aphid populations increased in an exponential-like fashion in these plots.

Both apterous and alate aphid populations were kept in check throughout the season in genetically modified potato and microbial *B.t.t.* plots, without application of chemical insecticides. In these plots, the selective control of Colorado potato beetles did not adversely affect beneficial predators and parasites, which were present in sufficient numbers to regulate aphid populations. The results of this study demonstrate the potential of *B.t.t.* to control Colorado potato beetles and enhance the potential for biological control of aphids.

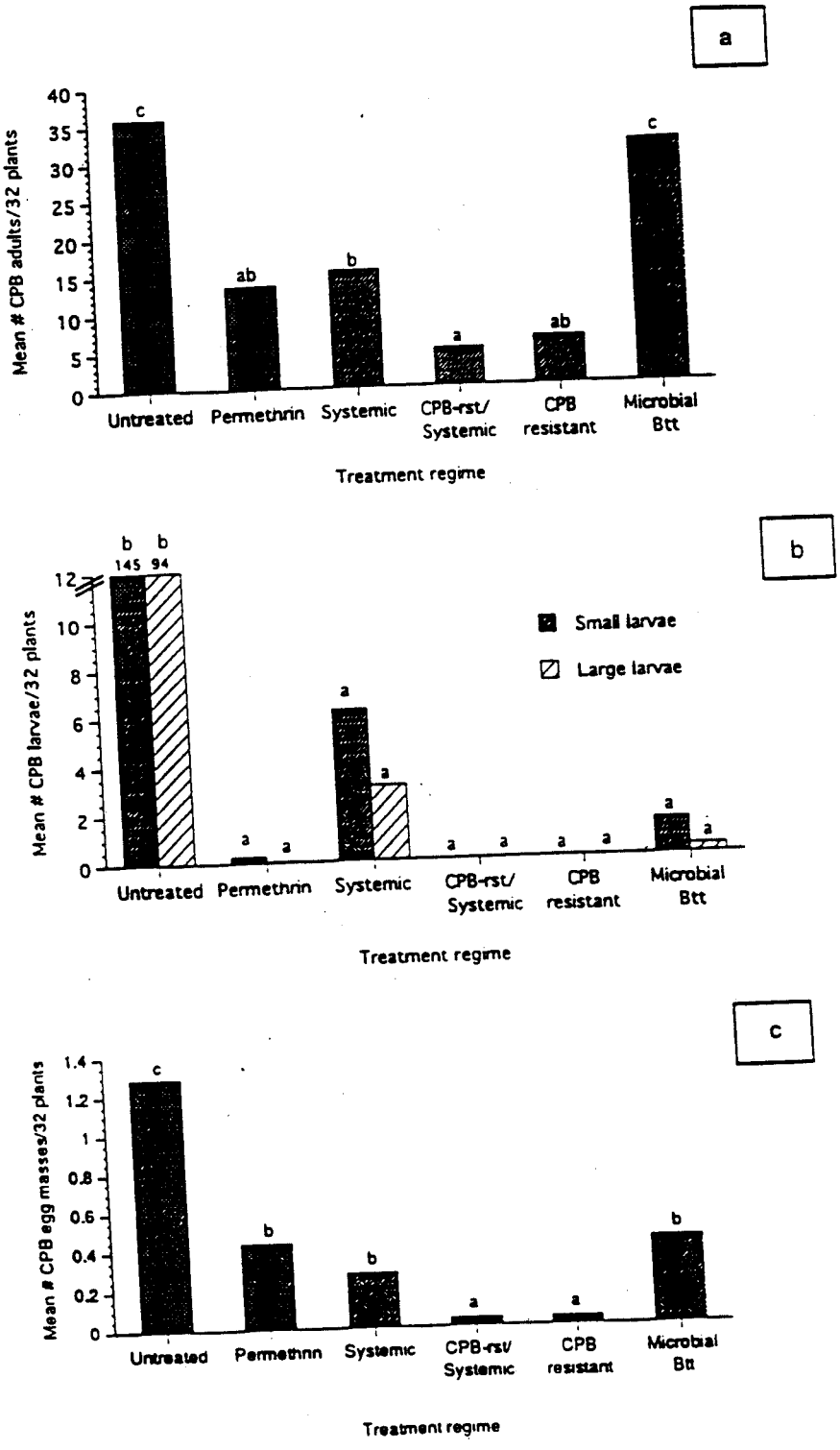

Figure 1. Colorado potato beetle (a) adults, (b) larvae, and (c) egg masses found on 32 plants in potato plots with experimental pest management regimes (average of 6 reps), Hermiston, Oregon, 1992. Means for each stage with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

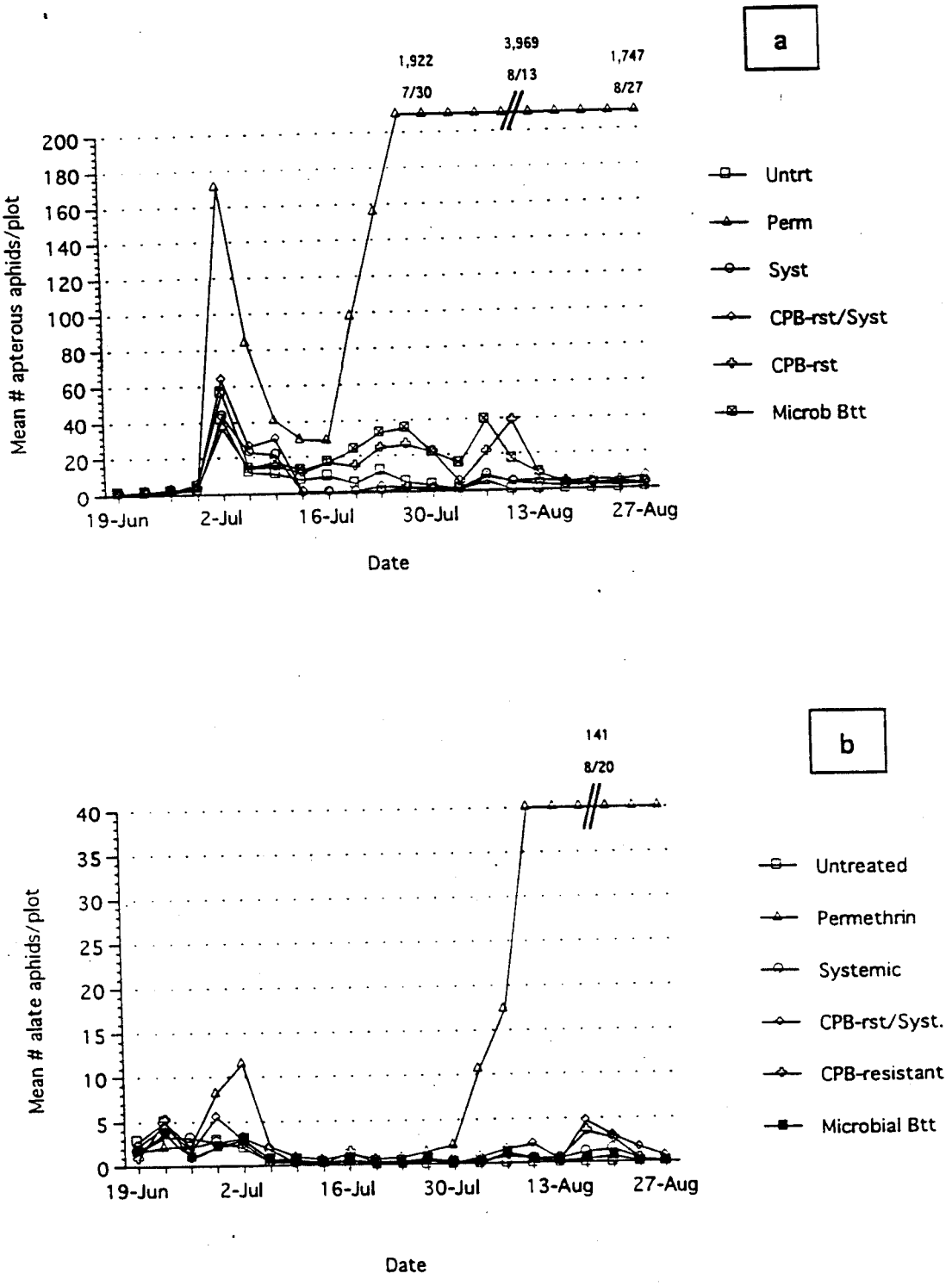

Figure 2. Seasonal distribution of (a) apterous and (b) alate green peach aphids taken from beat cloth samples in potato plots with experimental pest management regimes, Hermiston, Oregon, 1992

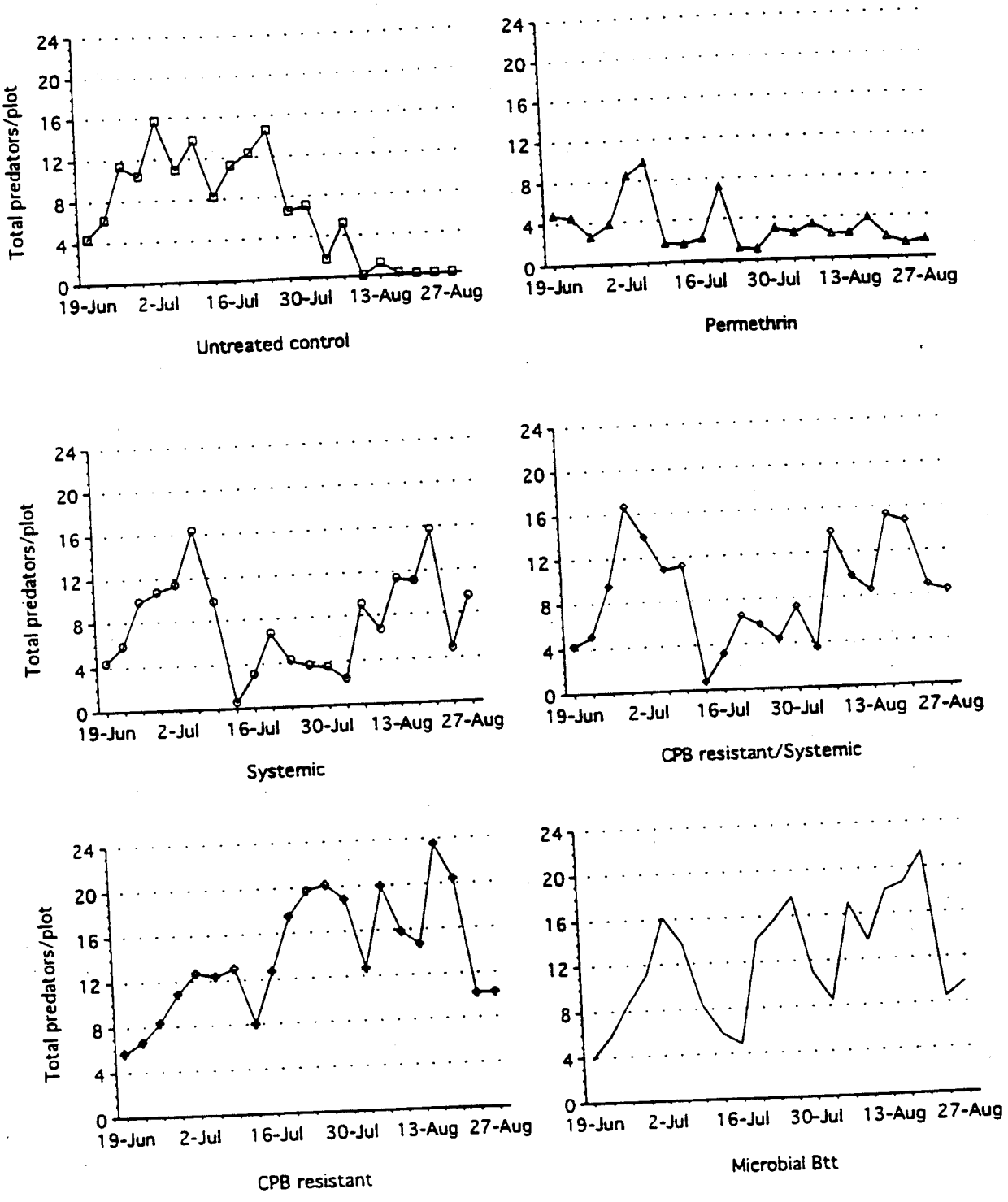

Figure 3. Seasonal distribution of generalist predators collected in beat cloth samples from potato plots with experimental pest management regimes, Hermiston, Oregon, 1992.

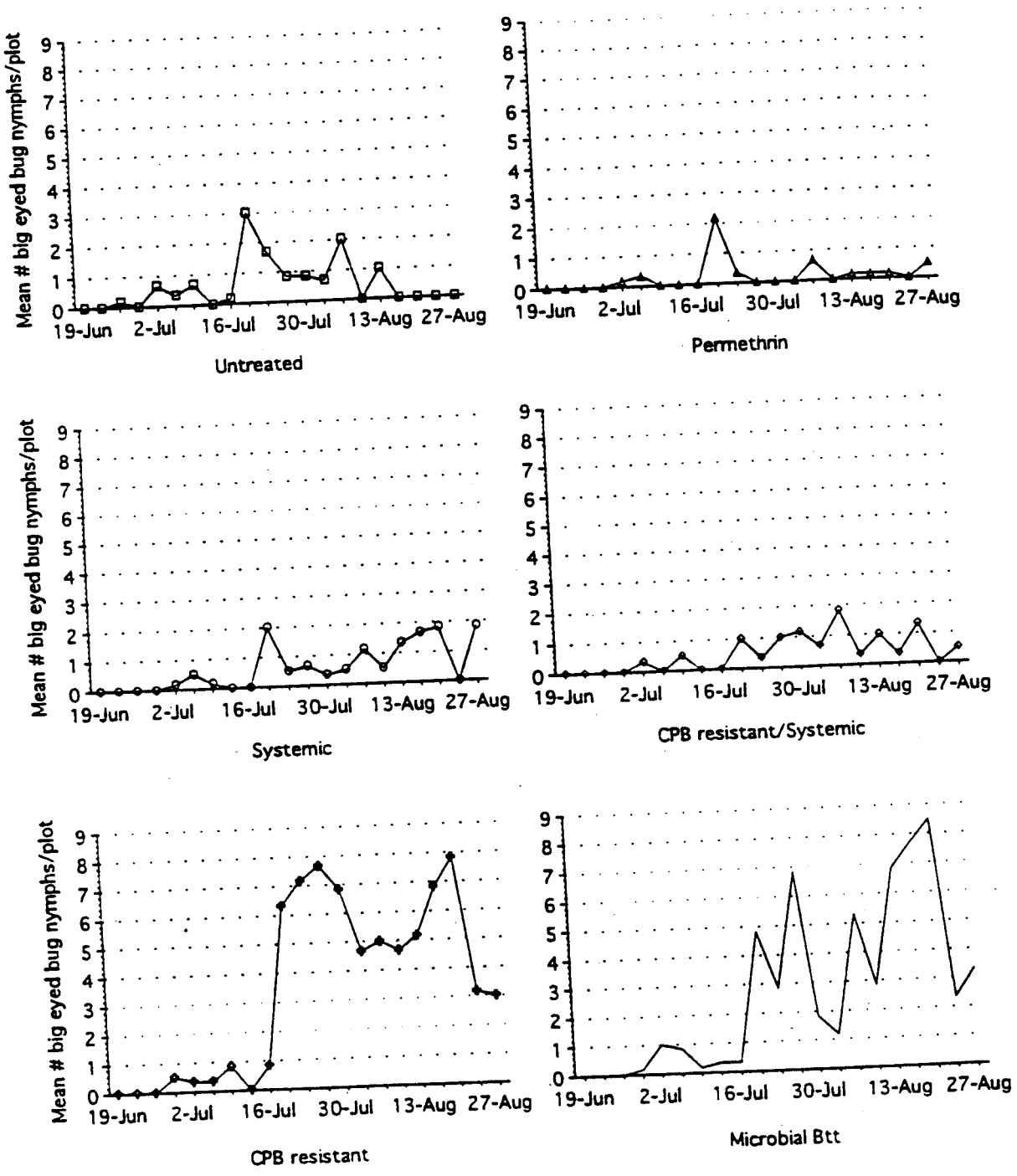

Figure 4. Seasonal distribution of big eyed bug nymphs in beat cloth samples from potato plots with experimental pest management regimes, Hermiston, Oregon, 1992.

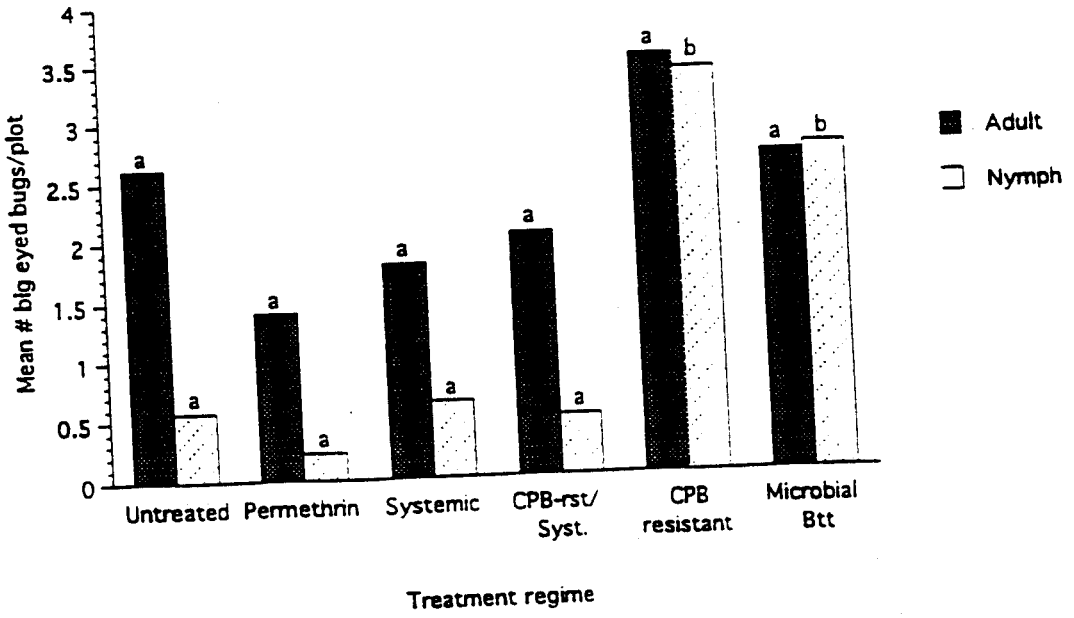

Figure 5. Seasonal average (mean of 6 reps) of big eyed bug adults and nymphs taken from beat cloth samples in potato plots with experimental pest management regimes, Hermiston, Oregon, 1992. Means for each stage with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

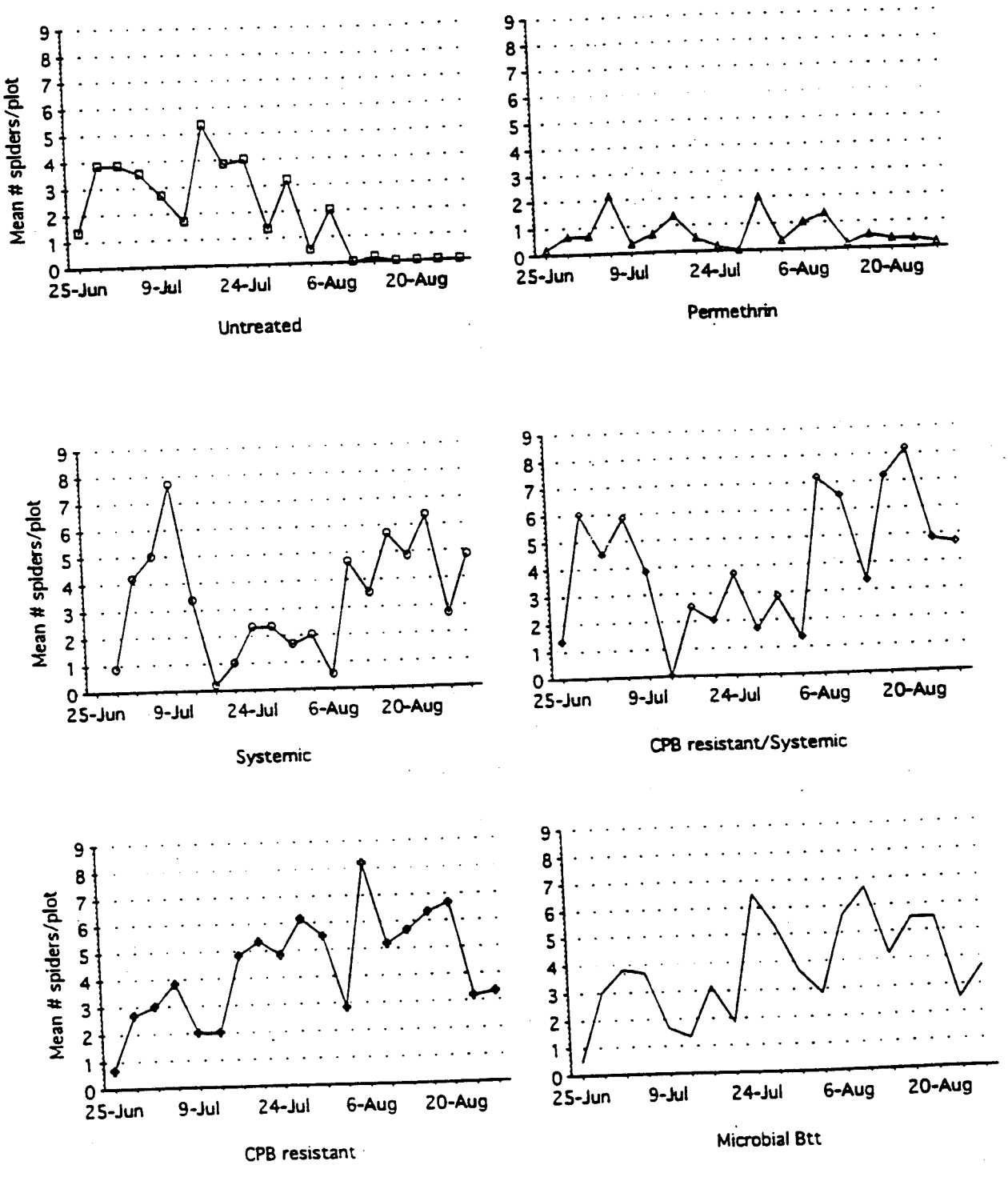

Figure 6. Seasonal distribution of spiders taken from beat cloth samples in potato plots with experimental pest management regimes, Hermiston, Oregon, 1992.

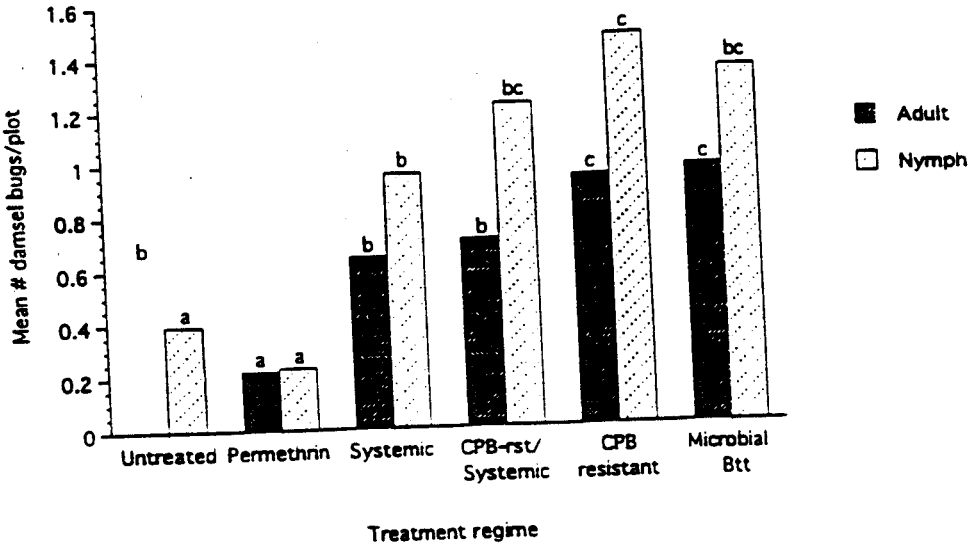

Figure 7. Seasonal average of damsel bug adults and nymphs collected in beat cloth samples from potato plots with experimental pest management regimes (average of 6 reps), Hermiston, Oregon, 1992. Means for each stage with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

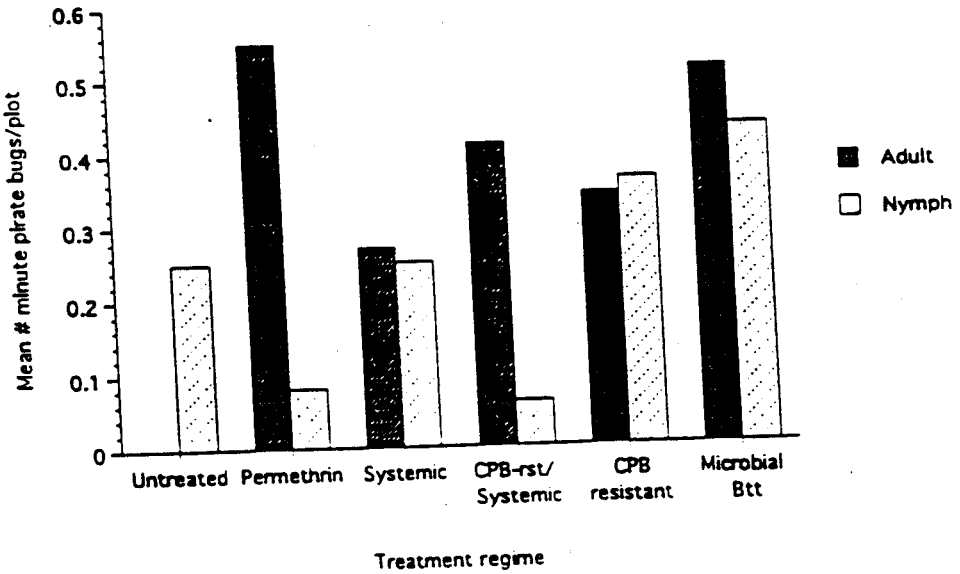

Figure 8. Seasonal average of minute pirate bug adults and nymphs taken from beat cloth samples in potato plots with experimental pest management regimes (mean of 6 reps), Hermiston, Oregon, 1992. (No significant differences)

Wisconsin:

Colorado potato beetle:

Early season adult populations were low with means of 5 adults per 20 plants in untreated plots on June 11 (Fig. 9). Overwintered adults gave rise to significantly fewer eggs in the genetically modified potato plots, while no differences in egg deposition were found between other treatment regimes (Fig. 10).

Cool weather resulted in a single larval generation which caused 50% defoliation of the untreated control by mid-July (Fig. 11). Defoliation of genetically modified potato, microbial *B.t.t.*, and conventional insecticide plots was limited to 10%. However, unlike the conventional insecticide and microbial *B.t.t.* treatments, very few small larvae (Fig. 12a) and no large larvae (Fig. 12b) were found on genetically modified potato plants throughout the season. Those small larvae that were found on these plants were probably neonates which had not yet fed on the foliage, as none developed to later instars.

Summer adults, which began to emerge on July 20, completely defoliated the untreated controls by August 13. Adult populations remained low in the genetically modified potato plots all season without additional insecticide applications. In contrast, adults began to increase in microbial *B.t.t.* plots in late August, necessitating treatment with esfenvalerate to rescue the plots from defoliation.

Potato leafhopper:

Potato leafhopper populations were unusually high, exceeding the treatment threshold of 2 per sweep on June 26 (Table 1), before natural enemy populations had a chance to establish. Malathion provided excellent control of potato leafhoppers with no apparent effect on Colorado potato beetle larvae, as seen in the untreated control (Fig. 12). After the July 2 treatment, potato leafhopper populations in genetically modified potato, microbial *B.t.t.*, and untreated plots remained below threshold until late-August, when plots were re-treated.

Aphids:

Aphid populations were extremely low throughout the season and never approached the seed treatment threshold of 5 aphids per 50 leaves. Combined counts of green peach and potato aphids in untreated controls (prior to defoliation) averaged 4.8 per 50 leaves. Although populations were low overall, differences between treatment regimes were detected on several dates using both leaf and beat cloth sampling methods (Tables 2 and 3).

The conventional insecticide treatment utilized for Colorado potato beetle control (esfenvalerate/endosulfan) effectively eliminated apterous aphids, maintaining the population at less than 1 per 50 leaves from late June through mid August. Aphid populations in genetically modified and microbial *B.t.t.* plots were significantly lower than in the untreated control from July 8 to 24 (leaf counts, $p < .05$). Similar results were obtained in beat samples, but the differences were not significant. The higher aphid populations observed in the control plots may have resulted from plant stress and

early senescence which was caused by insect feeding.

Potato flea beetles:

The potato flea beetle summer adult population peaked in late August, after untreated control plots were totally defoliated by Colorado potato beetles. Significantly fewer potato flea beetles were recovered from D-Vac samples on August 20 in genetically modified potato and conventional control plots than from microbial *B.t.t.* plots (Figure 13). Untreated control plots were not sampled at this time because of the lack of foliage.

Beneficial arthropod complex:

Predator populations were generally low and reflected the limited availability of prey. The greatest numbers of predators were collected from beat samples prior to defoliation in the controls, between July 10 and July 31 (Fig. 14). The most predators were found in the untreated controls where prey (Colorado potato beetles, potato leafhoppers, aphids, and others) were most abundant. Conversely, predator populations were lowest in the conventional control treatment, where broad spectrum insecticides were used and prey insects were few. Intermediate predator populations were found in the genetically modified and microbial *B.t.t.* treatments. Although overall numbers of individual predator species were generally low and differences were not significant, these data suggest that pest management programs which utilize selective insect controls, such as *B.t.t.*, may foster higher predator populations than do conventional insecticide programs.

Predaceous species recovered in pitfall traps were comprised primarily of ground beetles and rove beetles. Several other species normally associated with foliage were also found in the traps (e.g. spiders, minute pirate bugs, lady beetles, and lacewings). Predator numbers did not differ significantly between treatments but as was observed in beat samples, the highest numbers were detected in untreated controls and the lowest in conventionally treated plots.

The D-Vac was the most effective technique for sampling a wide variety of natural enemy species. No samples were taken in control plots due to a lack of foliage, but significantly more minute pirate bugs, lady beetles, and spiders were present in the genetically modified potato and microbial *B.t.t.* plots than were found in the conventionally treated plots (Fig. 15). Although, the number of predatory species was significantly greater in the microbial *B.t.t.* plots than the genetically modified potatoes, the profile of these species was similar, indicating that the method of *B.t.t.* delivery has no effect on non-target organisms.

Hymenopteran species, including those which are important aphid parasites, were most effectively sampled with the D-Vac. Although species determination has not been conducted, significantly more hymenopterans were recovered from genetically modified potatoes than from conventional control plots (Fig. 16). Hymenopterous species were also abundant in microbial *B.t.t.* plots, but not significantly more so than in the control.

An assessment of the total insect fauna in the potato plots was made using the insects recovered from the D-Vac sample (Fig. 17). Over 1000 insects/60 seconds were

recovered in both the microbial *B.t.t.* and genetically modified potato plots. In comparison, the conventional insecticide treatment reduced the insect fauna by over 50%.

Summary:

Genetically modified potato plants and conventional insecticides provided season long control of all Colorado potato beetle life stages. Foliar-applied microbial *B.t.t.* prevented defoliation from larvae but did not protect the plants from summer adult feeding, which necessitated application with a conventional insecticide to rescue the plots from crop loss. Aphid populations were unusually low, and commercially acceptable control was achieved in plots with all treatment regimes.

Malathion provided excellent control of potato leafhoppers when applied at half the recommended rate, and did not appear to negatively impact other insect species. Since potato leafhopper control is typically required in the early season before beneficials are established, this tactic may provide a safe control option for an integrated management system.

Natural enemy populations were generally low, and no differences were detected in beat samples or pitfall traps. However, significantly more predators (minute pirate bugs, lady beetles, and spiders) and hymenopteran parasitoid species were recovered in D-Vac samples from genetically modified potato and microbial *B.t.t.* treated plots than from conventional insecticide plots.

The total number of insects recovered from D-Vac samples was significantly reduced in potato plots treated with broad spectrum insecticides. No difference in the insect fauna was detected between foliar-applied *B.t.t.* and genetically modified potato plots. Since untreated control plots were completely defoliated by the August 20 sample date, a comparison with this treatment could not be performed.

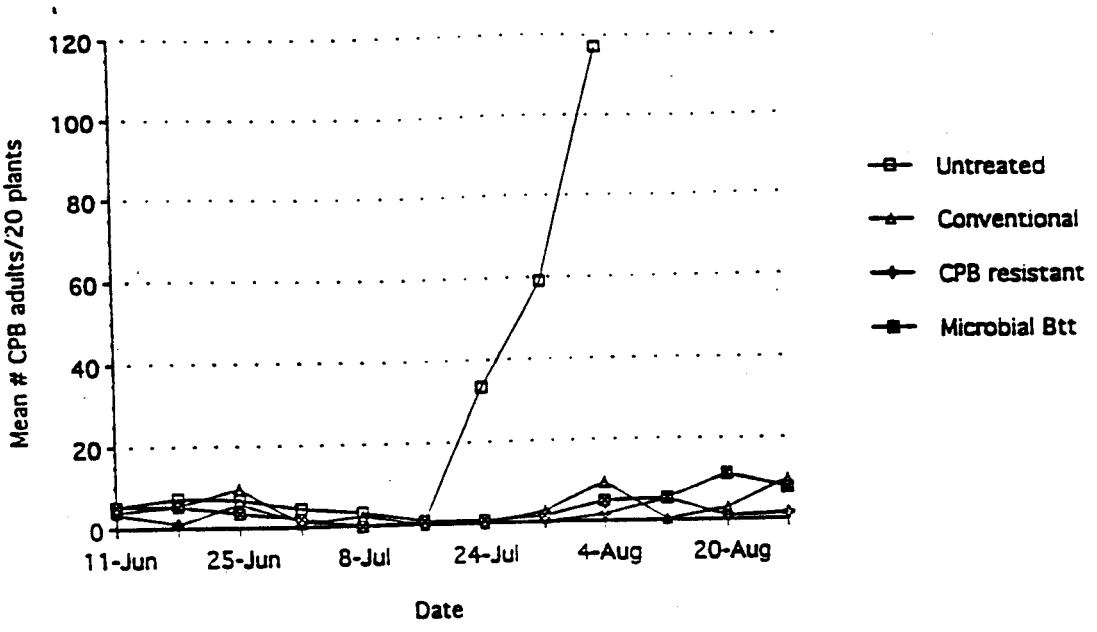

Figure 9. Seasonal distribution of adult Colorado potato beetles in potato plots with experimental pest management regimes, Hancock, Wisconsin, 1992. (Insect counts in untreated plots discontinued after August 4 due to lack of foliage.)

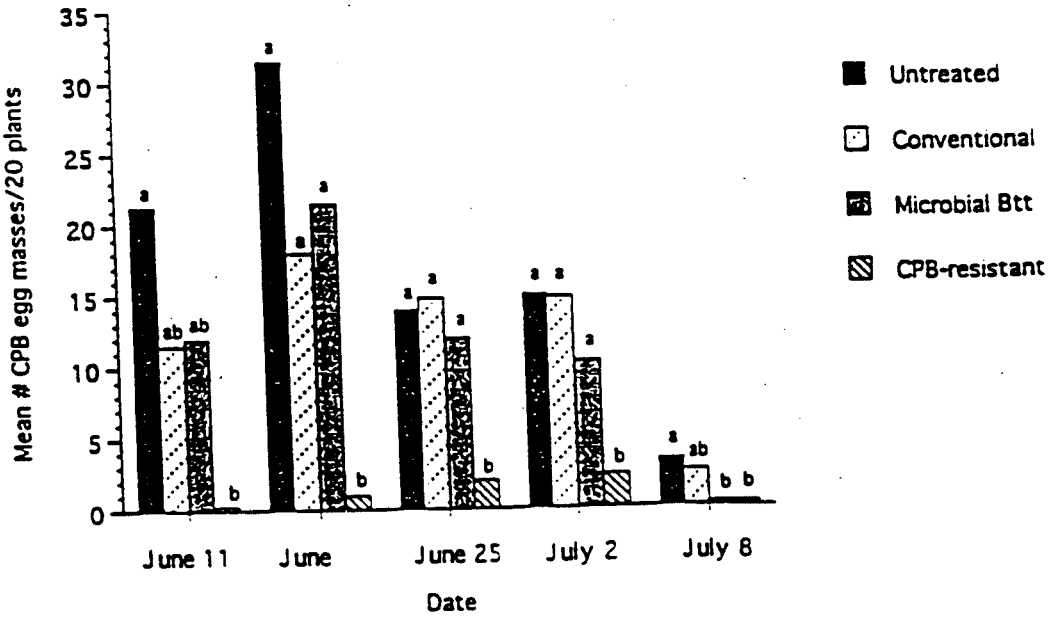

Figure 10. Colorado potato beetle egg masses (mean of 4 reps) on 20 plants in potato plots utilizing experimental pest management regimes, Hancock, Wisconsin, 1992. Means for each date with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

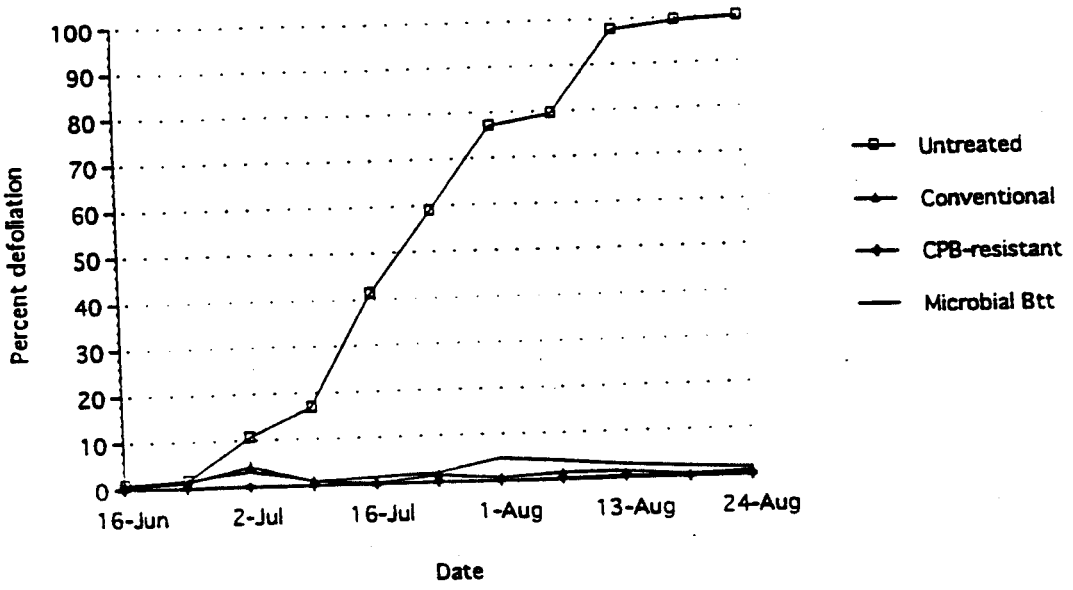

Figure 11. Defoliation caused by Colorado potato beetle feeding in potato plots with experimental pest management regimes, Hancock, Wisconsin, 1992.

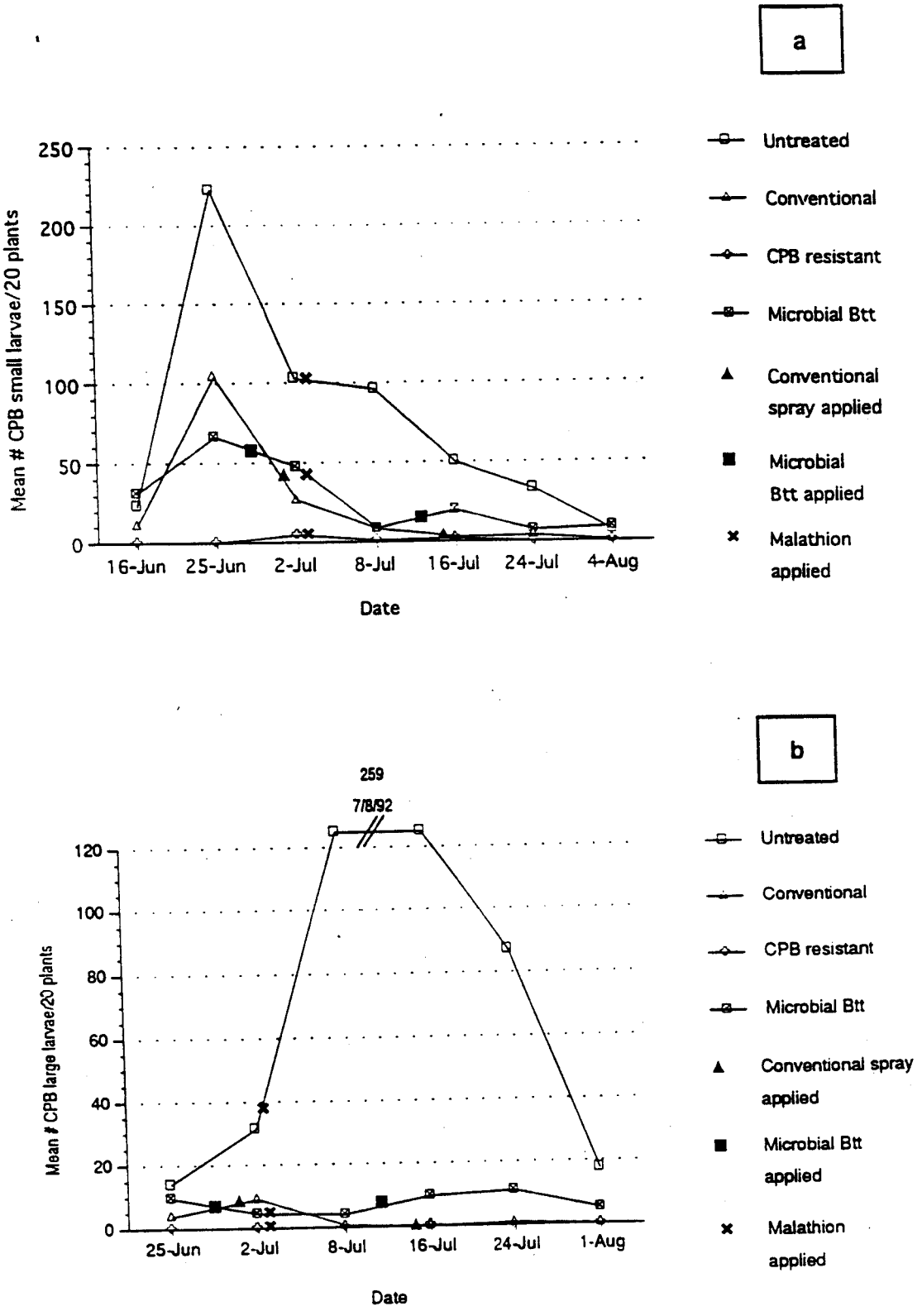

Figure 12. Seasonal distribution of CPB (a) first and second instar larvae and (b) third and fourth instar larvae in potato plots with experimental pest management regimes, Hancock, Wisconsin, 1992

Table 1. Potato leafhopper adult populations in potato plots with expenmental treatment regimes, Hancock, WI 1992

Treatment	Potato leafhoppers/50 sweeps								
	6/26	6/30	7/8	7/17	7/24	8/1	8/5	8/13	8/20
Untreated	113 a [1]	184 a *	19 a	19 a	28 a	59 a	33 b	26 bc	NA
Conventional	116 a *	17 b	1 b *	7 b	7 c	19 b	32 b *	.5 c	1 b
CPB resistant	82 a	164 a *	2 b	18 a	17 b	49 a	68 a	94 a	110 a
Microbial Btt	75 a	217 a *	2 b	11 ab	18 ab	47 ab	62 a	63 ab	41 b

[1] Means followed by the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

* = spray applied

Table 2. Potato and green peach aphids counted in leaf samples in potato plots utilizing experimental pest management regimes, Hancock, Wisconsin 1992

Treatment	Aphids/50 leaves						
	6/30	7/8	7/17	7/24	8/1	8/5	8/13
Untreated	2 a [1]	3 a	10 a	4 a	5 a	2 ab	3 a
Conventional	3 a	1 b	0 b	0 b	1 b	1 b	1 a
CPB resistant	2 a	1 b	1 b	1 b	4 ab	7 a	1 a
Microbial Btt	1 a	0 b	0 b	1 ab	5 a	2 ab	3 a

[1] Means followed by the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

Table 3. Potato and green peach aphids counted in beat cloth samples in potato plots utilizing experimental pest management regimes, Hancock, Wisconsin 1992

Treatment	Aphids/beat sample						
	7/10	7/17	7/24	7/31	8/6	8/13	8/21
Untreated	43 a [1]	27 a	15 a	5 a	NA	NA	NA
Conventional	1 b	2 b	3 a	0 b	6 a	2 b	1 a
CPB resistant	18 ab	11 ab	9 a	1 b	16 a	8 a	4 a
Microbial Btt	27 ab	20 ab	2 a	2 b	14 a	4 ab	4 a

[1] Means followed by the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

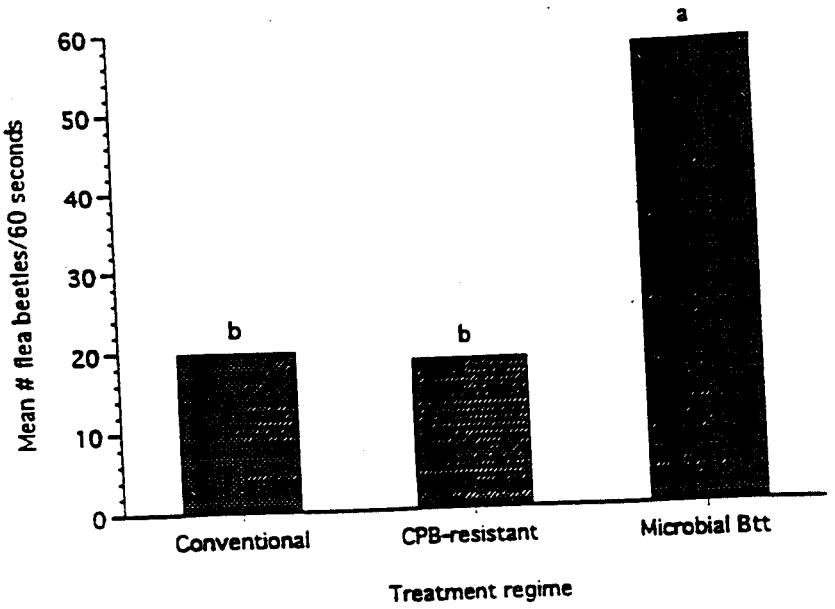

Figure 13. Potato flea beetles (mean of 4 reps) collected in 60 second D-Vac samples from potato plots utilizing experimental pest management regimes, Hancock, Wisconsin, 1992. Means with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

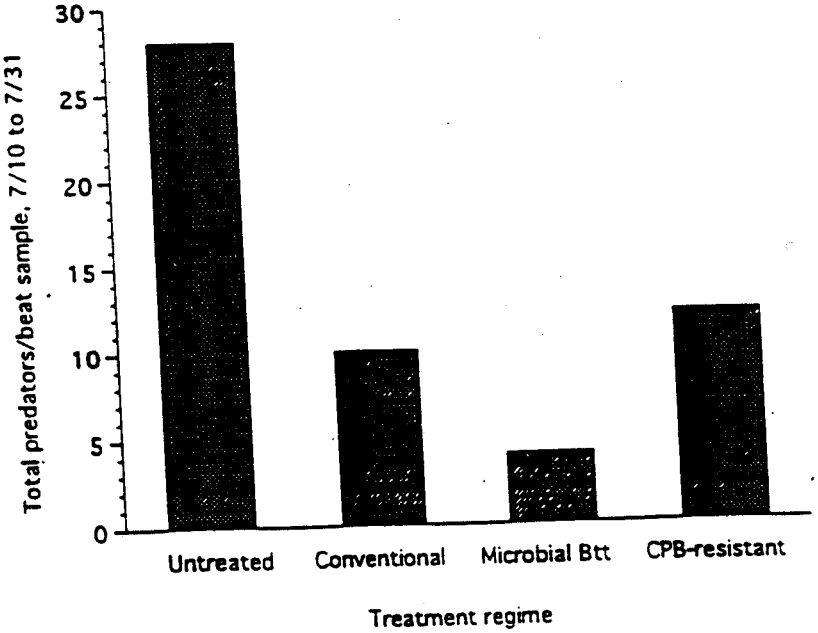

Figure 14. Total predators (mean of 4 reps) collected in beat samples between July 10 and July 31 in potato plots with experimental pest management regimes, Hancock, Wisconsin, 1992. (No significant differences)

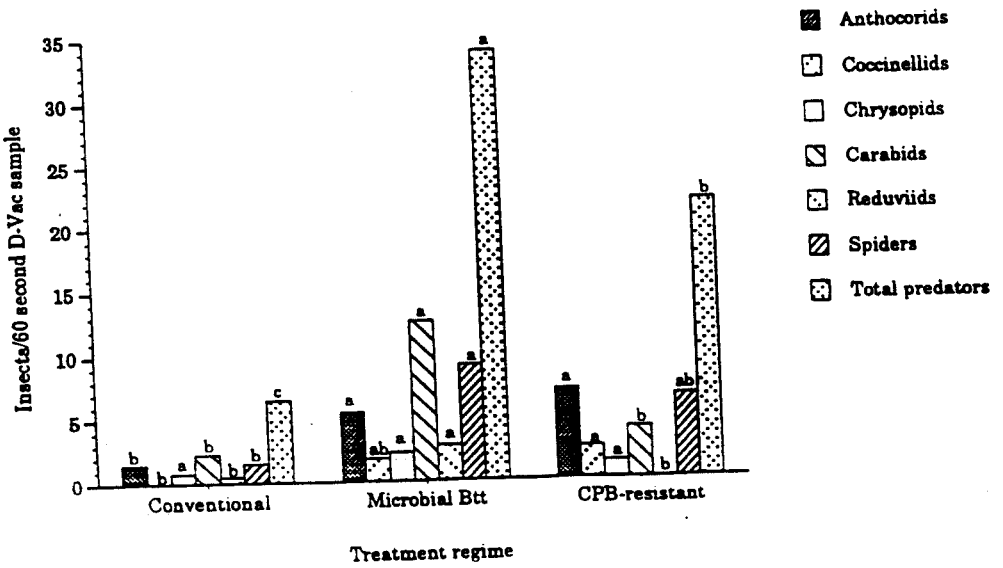

Figure 15. Individual and total predators (mean of 4 reps) collected in 60 second D-Vac samples in potato plots utilizing experimental pest management regimes, Hancock, Wisconsin, on August 20, 1992. Means for each predator with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

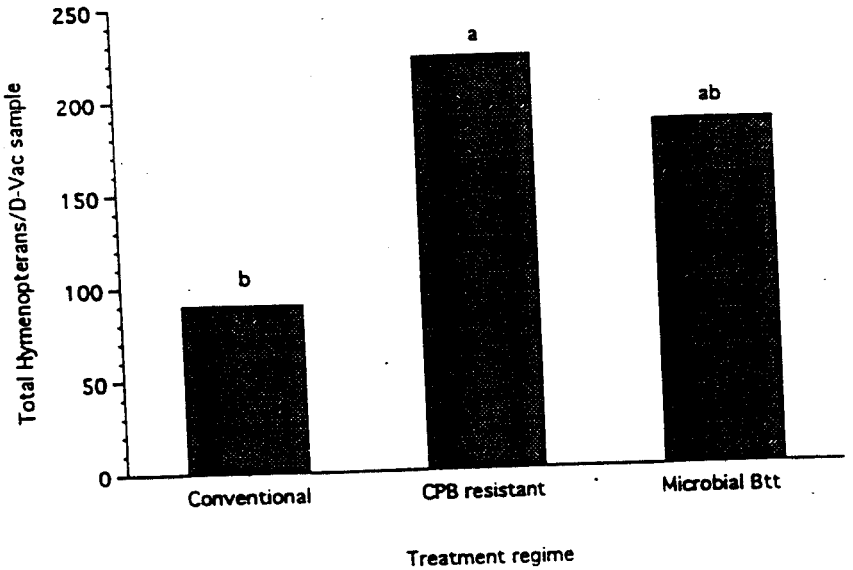

Figure 16. Total Hymenopterous insects (mean of 4 reps) collected in 60 second D-Vac samples in potato plots utilizing experimental pest management regimes, Hancock, Wisconsin, on August 20, 1992. Means with the same letter are not significantly different at the .05 level, Fisher's (1935) protected LSD.

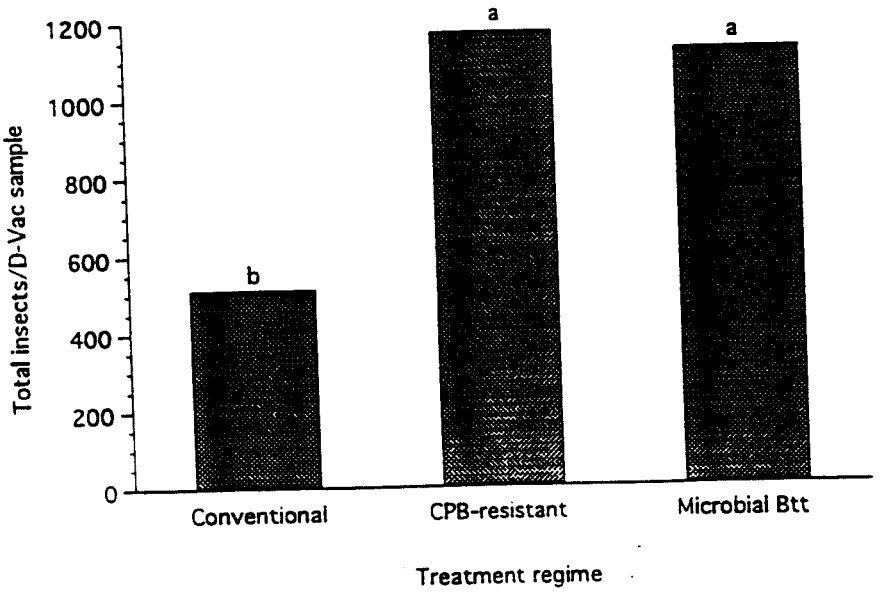

Figure 17. Total insects (mean of 4 reps) collected in 60 second D-Vac samples from potato plots utilizing experimental, pest management regimes, Hancock Wisconsin, on August 20, 1992. Means with the same letters are not significantly different at the .05 level, Fisher's (1935) protected LSD.

PEI

It was intended that the treatments in this study mirror those in the Hermiston, Oregon experiment. However, due to extreme winter weather conditions in 1991-92, insect populations in all plots were suppressed and treatment thresholds for foliar insecticide applications were never reached. With the exception of at-planting systemics, no insecticides were applied to the plots.

Colorado potato beetle:

Although Colorado potato beetle populations were atypically low in all plots, significantly fewer adults and larvae were found in genetically modified potato plots than in all others, including those treated with phorate (Fig. 18a and b). Early season adult Colorado potato beetles gave rise to one larval generation that peaked in the untreated controls and the non-resistant phorate treatment between August 4 and 25. Virtually no larvae were found in any genetically modified potato plot throughout the season.

Aphids:

Potato aphid populations were significantly lower in systemic insecticide treated plots than in untreated plots between July 27 and August 10 (Fig. 19). By the time the population peaked from August 20 to August 25, residues had begun to decline such that no differences between treatments were detected. Green peach aphids were extremely scarce all season, reaching a maximum of 3.25 per beat sample and 12.5 per 50 leaves in the untreated control. Aphid populations did not differ between genetically modified and unmodified potato plots within each treatment regime.

Potato flea beetles:

Adult potato flea beetle populations and the corresponding leaf damage were lower in phorate treated than in untreated plots. Flea beetle feeding damage was also reduced in genetically modified potato plots with no insecticide. While differences in feeding damage were not evident early in the season, these plants had significantly fewer leaf feeding holes than all three untreated controls on July 27, August 20, September 10, 15, and 21 (Fig. 20). Adult flea beetles were also less abundant in the genetically modified plots, but these differences were not statistically significant.

Beneficial arthropods:

Predator and parasite populations reflected the low pest populations. No significant differences in lady beetles, damsel bugs, flower flies, soldier beetles, or ground beetles were detected between plots.

Summary:

Insect populations were generally low, and treatment thresholds for Colorado potato beetles and aphids were not reached. Potato flea beetle summer adult feeding was significantly reduced in genetically modified potato plots.

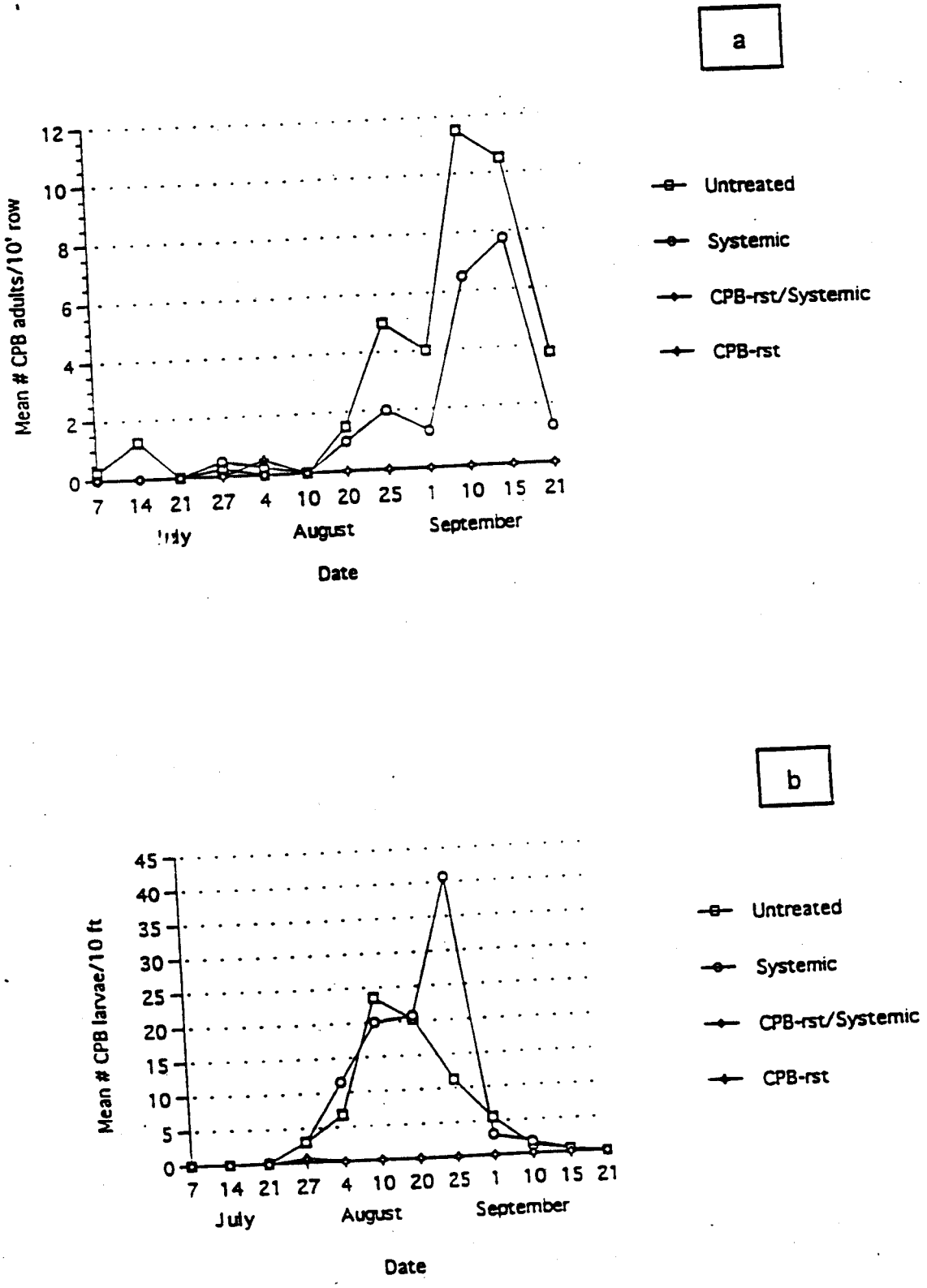

Figure 18. Seasonal distribution of Colorado potato beetle (a) adults and (b) larvae in potato plots utilizing experimental pest management regimes, Charlottetown, PEI, 1992.

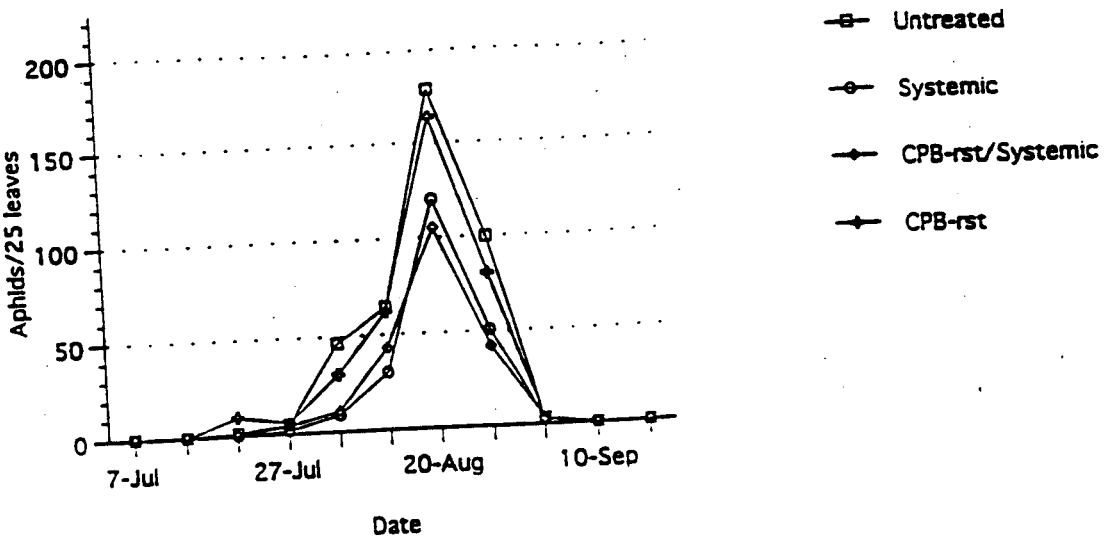

Figure 19. Seasonal distribution of potato aphids per 25 leaves (mean of 4 reps) in potato plots utilizing experimental pest management regimes, Charlottetown, PEI, 1992.

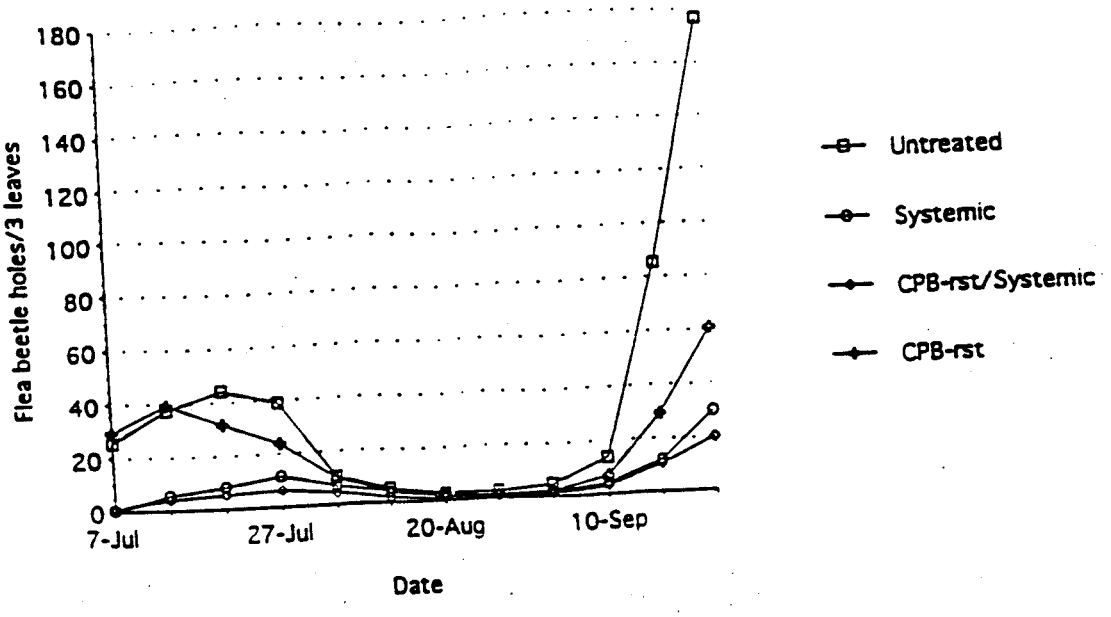

Figure 20. Seasonal distribution of potato flea beetle leaf feeding holes (mean of 4 reps) in potato plots utilizing experimental pest management regimes, Charlottetown, PEI, 1992.

CONCLUSIONS

Genetically modified Colorado potato beetle resistant potato plants provided season long control of Colorado potato beetles at all locations. No larvae were found to survive past the first instar, and the numbers of adults and egg masses on these plants were lower than in all other treatments. Microbial *B.t.t.* effectively protected the plots from Colorado potato beetle feeding damage, but allowed some larvae to survive to later instars. It is expected that such larval "escapes" will successfully pupate and emerge as summer adults. These insects are difficult to control with either microbial *B.t.t.* or chemical insecticides, and can cause substantial defoliation in a short period. Summer adult Colorado potato beetles will also overwinter to establish the succeeding year's population in potatoes.

Significantly more predators and parasites were found in the genetically modified potato and foliar-applied microbial *B.t.t.*-treated plots than in conventional insecticide treatments in both Wisconsin and Oregon, demonstrating the safety of the *B.t.t.* protein to non-target insects. As a result of elevated predator populations, aphids were maintained at commercially acceptable levels in these plots without supplemental insecticides. In contrast, aphid populations in Oregon rose exponentially in esfenvalerate-treated plots, where beneficial arthropods were eliminated and no chemical aphid control was achieved. This population response demonstrates the tremendous reproductive potential of aphids and the importance of natural enemies in their population regulation.

Data from PEI and Wisconsin suggest that plant expression of *B.t.t.* has some activity against potato flea beetles. Although summer generation adult populations were not significantly lower in genetically modified plots than in untreated controls in PEI, feeding damage was reduced. Significantly fewer potato flea beetles were recovered from Colorado potato beetle resistant plots in Wisconsin than from microbial *B.t.t.* plots. Since potato flea beetles and Colorado potato beetles are both in the family Chrysomelidae, it is possible that potato flea beetles are susceptible to *B.t.t.* Further studies specifically investigating the effect of plant expressed *B.t.t.* on potato flea beetle adult and larval feeding and development will be conducted in 1993 and 1994.

Results from this multi-year research program will be used to develop crop recommendations which incorporate genetically modified Colorado potato beetle resistant potatoes and other selective controls for the integrated management of potato insect pests. Data from 1992, which will be confirmed in subsequent studies, clearly demonstrates that genetically modified potatoes provide superior season-long control of all life stages of the Colorado potato beetle. The safety of the *B.t.t.* protein to non-target arthropods enables natural enemy populations to develop without disruption by chemical insecticides. Beneficial arthropods can significantly reduce the populations of non-target potato pests such as aphids. Upon their commercialization, these potatoes will represent an effective and environmentally compatible addition to the existing methods of potato pest management.

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APPENDIX 2

SUMMARY OF EXPERIMENTAL METHODS

APPENDIX 2. SUMMARY OF EXPERIMENTAL METHODS

Determination of *B.t.t.* and NPTII Protein Expression Levels:

The following is a summary of the methods utilized in the determination of the *B.t.t.* and NPTII proteins expression levels reported in Tables V.2 through V.5 of this petition.

Tissue Collection

Expression in leaves of NewLeaf™ Superior potato were determined on tissue collected at approximately six weeks post planting from a four replicate field trial carried out at Homestead, FL, during the winter of 1994. Expression in tuber tissue were determined in tubers harvested from three replicated plots of a field trial carried out at Island Falls, ME, during the summer of 1994.

Expression in leaves of NewLeaf™ Atlantic potato were determined on tissue collected at approximately six weeks post planting from 1993 Summer field trial at Aberdeen, ID and 1994 Winter field trial at Homestead, FL. The field trial at Aberdeen, ID consisted of six replicates per line, the field trial at Homestead, FL consisted of four replicates per line. Expression in tuber tissue were determined in tubers harvested from 1995 Summer field trial at Painter, VA; Stanton, MI; Coloma, WI; and New Denmark, New Brunswick, Canada. These field trials consisted of 12 to 15 replicates per line. Expression level assays were limited to four replicates.

Leaf tissue. Leaf tissue was collected at approximately six weeks post planting. One of the youngest leafs (1/2 to 1 inch in diameter) were collected from each of four randomly chosen plants from each of the plots. The leafs from each of the three plants per plot were combined in an appropriately labelled plastic sample bag, immediately frozen on dry ice and shipped on dry ice to Monsanto Co., Chesterfield, MO., where the tissue was stored frozen at approximately -80°C before being processed for ELISA analysis.

Tuber tissue. Tubers were harvested from the field and placed in commercial seed potato storage condition (i.e., 4-6ºC and 80-90% relative humidity) from September 25, 1994 to April 11, 1995 at Island Falls, ME. Ten tubers were non-systematically selected from each plot for ELISA analysis. Tuber samples were shipped at ambient temperatures to Monsanto Co, chesterfield, MO. At Monsanto, tubers were stored at approximately 4-8°C, 80-100% relative humidity prior to processing for ELISA.

Tissue Extraction

Leaves. Frozen leaf tissue samples were crushed to a fine powder in the collection plastic sample bags. *B.t.t.* and NPTII proteins were extracted from tissues using an aqueous extraction buffer (8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 0.14 M NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.2 % Tween 20®, pH 7.4) which had been optimized to provide for maximum extraction of both proteins. Approximately one gram of each sample was extracted in 15 ml of aqueous extraction buffer.

Tubers. The *B.t.t.* and NPTII protein expression levels in tubers were determined in lyophilized tuber powders. For preparation of lyophilized tuber samples, five tubers/plot were non-systematically selected from the ten tubers that were collected from the field. These tubers were diced into approximately 20 g pieces. One 20 g piece from each of the five tubers was sliced into thin pieces, and placed inside of an appropriately labeled paper bag and approximately 100 g of dry ice added to freeze the tuber pieces. The tuber pieces were then lyophilized at approximately -4°C for approximately seven days, and then ground into a powder in a Waring blender. The fresh weight equivalent of approximately one gram of each sample was extracted in 15 ml of extraction buffer (8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 0.14 M NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.2 % Tween 20®, pH 7.4). The fresh weight of the tissue was obtained by determining the amount of water removed from the tissues during the lyophilization process.

Expression Level Assays

ELISA Assays. The expression levels in extracts of the *B.t.t.* and NPTII proteins were determined using validated ELISA's. Descriptive features of the *B.t.t.* protein and NPTII ELISAs are included below. All extracts were analyzed in triplicate. Samples were repeated if the % coefficient of variation was greater than 10.5% (based on absorbance) or if any of the assays did not pass the established accept/reject criteria as described in the ELISA specifications. The expression level of each protein was converted to the amount of protein expressed in each tissue on the basis of the fresh weight of the tissue used.

ELISA Data Reduction. All data reductions were done using Microplate Manager™ from BioRad (Richmond, CA). Microsoft Excel™ was used to transform ELISA data for the statistical evaluation of the expression levels.

ELISA Specifications:

B.t.t. Protein ELISA

Range:

Least detectable dose:	0.020 ng/well 0.0012 µg/g tissue
Standard curve range:	0.032 to 2.0 ng/well

Accuracy:

Extraction efficiency:	63% (cv. Superior Leaf Tissue) 98% (cv. Superior Tuber Tissue) 66% (cv. Atlantic Leaf Tissue) 95% (cv. Atlantic Tuber Tissue)

Precision:

Intraplate variability:	7.2% C.V.
Interassay variability:	11.5% C.V.

Accept/Reject Criteria:

Buffer blank:	≤ 0.100 O.D. at 450 nm
Standard curve:	Correlation Coefficient (R^2) ≥ 0.95
Variability in replicates:	≤ 10.5% C.V.
Interassay control:	< 2 standard deviations from established mean of quality control sample
Absorbance in 2 ng/well standard:	> 0.500 absorbance units

B.t.t. Protein Stability:

In tuber extract:	No degradation after 3 months storage at -80°C
In leaf extract:	No degradation after 3 months storage at -80°C

NPTII Protein ELISA

Range:

Least detectable dose:	0.006 ng/well 0.0003 µg/g tissue
Standard curve range:	0.02 to 2.0 ng/well

Accuracy:

Extraction efficiency:	68% (cv. Superior Leaf Tissue) 94% (cv. Superior Tuber Tissue) 77% (cv. Atlantic Leaf Tissue) 86% (cv. Atlantic Tuber Tissue)

Precision:

Intraplate variability:	7.3% C.V.
Interassay variability:	18.7% C.V.

Accept/Reject Criteria:

Buffer blank:	≤ 0.100 O.D. at 450 nm
Standard curve:	Correlation Coefficient (R^2) ≥ 0.95
Variability in replicates:	≤ 10.5% C.V
Interassay control:	< 2 standard deviations from established mean of quality control sample
Absorbance in 2 ng/well standard:	> 0.500 absorbance units

NPTII Protein Stability:

In tuber extract:	No degradation after 3 months storage at -80°C
In leaf extract:	No degradation after 3 months storage at -80°C

APPENDIX 3

LETTERS OF SUPPORT

THE OHIO STATE UNIVERSITY
OARDC

Ohio Agricultural Research
and Development Center

Department of Entomology
1680 Madison Avenue
Wooster, OH 44691-4096
Phone 216-263-3725
Fax 216-263-3686

10 January 1994

Public Response and Program Resources Branch
Field Operations Division (7506C)
Office of Pesticide Programs
Environmental Protection Agency
401 M St. SW
Washington D.C. 20460

Comments regarding document control number OPP-30355, File symbol 524-UTU:

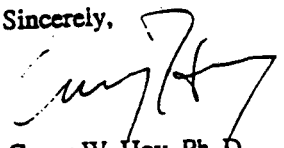
I am writing to offer my opinion in support of the use of transgenic potato plants that produce the *Bacillus thuringiensis* (Bt) d-endotoxin in agricultural production, as proposed by the Monsanto Company. In my opinion, plants containing this protein pose no more risk to producers, consumers, and the environment than do foliar sprays of Bt suspensions, which have been used in many different crops for more than 20 years. In fact, if the plant itself produces the endotoxin, then farmers can avoid damage to their soil through compaction, high foliar application costs (labor, equipment, and fossil fuels), worker exposure to sprays, and non-target contamination through drift and runoff.

Few pests are controlled exclusively with Bt foliar sprays, because they are highly selective, difficult to time properly, and have a short period of residual activity. This is particularly true for control of Colorado potato beetle with foliar Bt. We have attempted to optimize the use of foliar Bt's in Ohio potato integrated pest management programs, but have never been successful in controlling this pest entirely with Bt. The alternatives are broad spectrum insecticides that carry more environmental contamination, health and safety risks than do Bt sprays. Because the endotoxin in transgenic plants is always present in the foliage, timing and residual activity problems are avoided completely. In research conducted at the Ohio Agricultural Research and Development Center during 1993, Colorado potato beetle control was very thorough in plots containing transgenic plants. Furthermore, because no broad spectrum insecticides were used in these plots after a single early application for potato leafhopper control, aphids were successfully controlled by natural enemies. Biological control of aphids did not occur in plots where broad spectrum insecticides were used for Colorado potato beetle control. Based on last summers research results and spray records of Ohio potato growers, I estimate that use of the transgenic potato plants could reduce the number of insecticide applications, most of them broad spectrum insecticide applications, from an average of approximately 6-7 to an average of 1 application per year in our state. I believe that society as a whole would approve of that result.

The sole concern that I have with respect to the use of transgenic potatoes is the development of resistance to the endotoxin in Colorado potato beetle populations. I have the same concern, however, with respect to foliar Bt use or the use of any other insecticide. Colorado potato beetle is notoriously good at developing resistance to anything with which we try to kill it. Foliar Bt sprays have already resulted in resistance to the endotoxin in diamondback moth populations in the field, and in a Colorado potato beetle population in the laboratory. Monsanto Company has demonstrated ample concern

for this issue and has actively enlisted the assistance of professional entomologists to help devise the best strategy for avoiding resistance. In my opinion, the use of transgenic potato plants does not result in greater risk of resistance than regular use of foliar Bt sprays. The risk of resistance should not prevent the use of transgenic plants in potato production. The best way to avoid resistance is to have a large arsenal of different control measures that are used strategically in combination. Transgenic potato plants should be allowed to be part of that arsenal.

Sincerely,



Casey W. Hoy, Ph. D.
Associate Professor
Research Entomologist

SIDDOWAY, INC.

Famous Idaho Potatoes
134 E. Main #205
Rexburg, Idaho 83440

Watts 800-234-9399
In Idaho 208-356-9399

August 19, 1993

Terry Stone
Senior Regulatory Specialist
Monsanto Agricultural Group
700 Chesterfield Parkway North
St. Louis, MO 63198

Dear Terry,

I am a Broker/Dealer of potato seed in Idaho with the bulk of my sales in the Northwest. I have been following your research on the seed which is resistant to the Colorado Potato Beetle. I have talked with many of my customers concerning the potential of this new product and have found a great deal of interest in the future availability of the seed. The CPB has been a very time consuming and expensive pest considering conventional insecticides, applications and the damage the beetle does. A product of this nature which may add to the bottom line of the farmer and reduce his time and expense in handling the current chemicals, naturally draws his immediate attention.

I recently inspected a potato field in the Rexburg area of Southeastern Idaho. The field was bordered on two sides by a grain field that was full of volunteer potatoes. As you may know volunteer potatoes in grain are the perfect host for the CPB. With an abundance of food and nothing to stop them, thousands are hatched uninhibited. Several days ago these beetles started moving into the potato field. By the time the farmer hired an airplane and had them sprayed they had totally defoliated 15 feet into the field. Luckily his timing was good and his losses were minimal but the potential for loss was explosive. This is only one situation in many where a CPB resistant potato would have never allowed the propagation of the beetles in either the volunteer potatoes or those planted this spring. It would have eliminated the need to handle the insecticide and the expense of the chemical both at pre-emergence and again this summer.

For these reasons and many others the potato industry needs this product. For the benefit of my customers and my own business I would encourage any effort toward making this seed available. If there is anything I can do to help promote or market the seed to the commercial growers please contact me. Thank you.

Thane Siddoway

NATIONAL POTATO COUNCIL
9085 E. Mineral Circle, Suite 155
Englewood, CO 80112
Phone: (303) 790-1141
Fax: (303) 790-1142

August 23, 1993

Mr. Terry Stone
Senior Regulatory Specialist
Monsanto Agricultural Group
700 Chesterfield Parkway North
St. Louis, MO 63198

Dear Mr. Stone:

I am writing this letter in support of Monsanto's submission to the Environmental Protection Agency for registration of a Colorado potato beetle (CPB)-resistant potato seed.

I speak on behalf of the National Potato Council as vice president of the NPC's Environmental Affairs Committee. The NPC is the only trade association representing 10,500 commercial potato growers in 50 states. Our growers produce both seed potatoes and potatoes for consumption in a variety of forms. Approximately 132 pounds of potatoes are consumed per person per year. Annual production in 1991 was 417,762,000 cwt with a farm value of $2,045 billion.

The Environmental Affairs Committee, as well as the entire potato industry, recognizes the CPB as one of the most damaging potato pests throughout the world. Its resistance to many insecticides is well documented.

Although devastation caused by the CPB is not apparent in every growing area, several states are severely attacked by this pest. A 1991 study showed that in Michigan alone potato growers suffered average estimated crop losses of 12.2 percent from CPB representing $4.3 million in lost revenue on 42.3 percent of the potato acreage. Average cost of CPB control on surveyed acreage in 1991 was $124.55 per acre; this was up 51.4 percent from 1989 costs of control. Michigan growers spent approximately $2.475 million on insecticides for CPB control in 1991 on the 42.3 percent of Michigan potato acreage surveyed. I am confident that these same kinds of losses and control costs occur in many northeastern potato-growing states.

I grow potatoes in the Columbia Basin in Washington State. I spray at least once and in some fields twice to protect against CPB. For the last two years I have seen a resistance to the insecticide I have been using. The cost of using and changing insecticides in my operation alone amounts to several thousand dollars annually. It would be of great commercial and productional benefit to be able to plant a potato seed that is resistant to CPB.

I appreciate the opportunity to comment on your submission to the EPA for registration of a Colorado potato beetle-resistant potato seed, Mr. Stone. Please let me know if the NPC can provide additional information.

Sincerely,

Lynn J. Olsen
Vice President

Mecox Road
Bridgehampton,N.Y. 11932
April 14, 1993

Ms. Jennifer Feldman
HybriTech Seed International, Inc.
1503 Tyrell Lane
Boise, Idaho 83706

Dear Jennifer:

The presentation on transgenic potatoes which you gave at the L.I. Agricultural Forum in Riverhead, N.Y. this spring was of great interest to me. I operate a 200 acre farm here in Bridgehampton, along with my brother and father. Our farm has been in continuous operation by our family for five generations. I have been farming for 21 years since graduating from college. The farm currently supports three families, plus one worker and his family. Our primary crop is potatoes, but we also sell some grain, rye and oats, which we use for crop rotation.

The Colorado potato beetle is, without a doubt, the most expensive pest we have to contend with. Over half of our total pesticide bill is due to this single insect. At present, the best method we have of control for CPB is application of two materials, Kryocide and Novodor.

I heartily support your company's efforts in the development of transgenic CPB control for the following reasons:

1) It would likely eliminate most, if not all, CPB sprays

2) It would reduce overwintering adult populations of CPB, resulting in better crop emergence the following spring

3) It would likely break the cycle of pesticide resistance

4) It would reduce worker exposure to pesticides

5) It may reduce scouting costs

All of these things would be a benefit to our farming operation from both an economic and safety point of view. I look forward to the successful development of transgenic CPB control.

Good luck!

Sincerely yours,

Harry S. Ludlow

HSL/bal

April 27, 1993

Stephen R. Diercks
Coloma Farms Inc.
136 S. Scott St.
Coloma, WI 54930

Mr. John Cudnohufsky
HybriTech Seed International
1503 Tyrell Ln.
Boise, ID 8706

Dear Mr. Cudnohufsky,

I am a third generation potato grower in the state of Wisconsin. Our family has been growing potatoes since the 1930's, first in the Antigo area and since the mid 60's, we have been farming in Coloma, the Central Sands Area of Wisconsin. I am a 1970 graduate from the University of Wisconsin with a major in Agricultural Economics and this year I have a son graduating from the same institution with an Agricultural Engineering Degree, who will be joining my father and I on the family farm.

Our farm consists of 2200 acres of irrigated land. We grow approximately 750 acres of potatoes each year and we grow field corn, soybeans, sweet corn, peas and alfalfa in rotation with our potato crops. Our potatoes are all on a three year rotation and the crops grown in rotation are based on which crop will best suit our needs for that year. We employ 5 full time people and up to 20 people during harvest season. We purchase the majority of our inputs, approximately $500,000, from local suppliers within a 30 mile radius of the farm.

Our farm has participated in numerous research projects with the University of Wisconsin. We participated in the early development of the PCM, Potato Crop Management, and WISP, Wisconsin Irrigation Scheduling Program. We have used IPM for many years and hire scouting services to regularly scout our crops.

Even with the use of all the new Best Management Practices that we are using, we are still having problems. The publics concern over the use of pesticides, the environmental problems with some crop protectants and the probable loss of many pesticides, has led us to believe that we must find better way to control pests in our potato crop. The use of transgenic material is the next logical step.

Colorado Potato Beetles (CBP) control has become one the biggest pest problems we have on the farm. The pressure in the area is very high and if we do not have control, it is possible to lose the crop. With the excellent recommendations from Dr. Jeff Wyman, we have been able to maintain good control and little resistance build up. We rotate chemical classes and use PCM to determine when the correct time to make an application is. This is quite a change from 10 years ago when we applied Temik one time and had control for most of the season. Temik and other systemics have gone by the wayside because of ground water contamination and toxicity problems. Today we are still controlling CPB but we are using more sprays, introducing more pesticides into the environment and exposing workers to more pesticides because of more applications.

The use of transgenic material to control CPB is an exciting step forward. By integrating the use of this new material into our existing IPM program we should be able to better control

CPB and have less of an impact on the environment and be safer for my employees. While the transgenics are an exciting step forward, they cannot be expected to solve all our problems. We must learn how and when to use these materials. The plot work which we are doing is a beginning to see how to use these materials will be used and we are sure the data from this kind of plots will help us all.

I am looking forward to working with your people this coming growing season and am eagerly looking forward to the release of this new material in the coming years.

Sincerely,

Stephen R. Diercks
V.P. Coloma Farms Inc.

June 23, 1993

Mr. John Cudnohufsky
Manager, Customer Relations
HybriTech Seed International
300 E. Mallard Drive, Suite 220
Boise, ID 83706

Dear John:

I am writing in support of your company's research and development efforts with genetically improved potatoes. I have been a potato grower for several decades, concentrating on seed and table potatoes with production ranging from 150-200 acres/year. During the last 20 years I have observed that some pests (Colorado Potato Beetle, aphids) and diseases (Early Blight, scab) have impacted my potato crops more, while I have had less pesticides and techniques available to control the insects and diseases. Ir the case of Colorado Potato Beetle insecticides have lost effectiveness in a very short time and little has become available to replace them. Pesticide costs on my farm are in the $200-300/acre range, resulting in a significant expenditure each year. I find the reduced number of effective pesticides a reason for concern as we look at the future of potato production in North America. The approach of your company to develop potatoes that defend themselves without frequent and repeated pesticide applications may provide an attractive and effective alternative.

During my term as Chairman of the Potato Promotion Board I had opportunities to visit many agricultural areas in the U.S. as well as some as distant and distinct as China. Those visits reinforced my conviction that American agriculture is highly productive and successful because individual segments or areas are quick to recognize improvements and adopt new technology to be more efficient. I have also noticed that farmers are becoming more protective of their resources and environment, knowing full well that failing to do so will have serious consequences, both in the long and short term.

I see the genetically improved potatoes that your company is developing and planning to market as a major step forward in allowing a potato grower to produce a crop that continues to be safe to eat, causes less stress on the environment and require fewer inputs in terms of energy, labor and pesticides. These potatoes will also allow growers to be at the forefront of technology, a situation that will help them continue to provide high quality, inexpensive food to a rapidly expanding population.

I plan to closely follow your progress in Maine in both research and seed potato production. If your potatoes will allow me to farm smarter and better, then I will want to use them. Good luck.

Sincerely,

G. Arnold Roach

G.Arnold Roach
Forest Home Farms
P O Box 179
Smyrna Mills, Maine 04780

95-338-01p

REC'D 10 FEB 2000
DSH

MONSANTO
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February 8, 2000

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Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service,
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Attention: Dr. James White **FAX: 301/ 734-8669**

Dear Dr. White,

Subject: RE: Approved petition 95-338-01p. NewLeaf Atlantic line ABTBO4-6 research results. Provided for your information.

Monsanto is supplying the enclosed information on the nematode resistance of NewLeaf Atlantic line ABTB04-6 to the USDA. We believe that this new information should not impact the deregulation decision for this product. The information is to be presented at a public meeting of a New York State Technical Committee as indicated below.

This past year, Dr. Bill B. Brodie , a nematologist at Cornell University, conducted standard greenhouse screens for Golden Nematode (GN) resistance on three lines of NewLeaf Atlantic (ABTB04-6, ABTBO4-36, ABTB04-31, the three lines currently available to commercial growers); the parental Atlantic line; a New York Atlantic line; a known resistant line (Kanona); and a known susceptible line (Katahdin). Dr. Brodie first conducted an initial screen of ABTB04-6 as part of his standard screening program for varieties and lines available from various breeding programs. NatureMark/Monsanto supplied additional material at his request to conduct the attached study as verification of the initial screen. Resistance to GN is not a normal part of the testing conducted in variety development at NatureMark as NewLeaf products are not targeted for marketing in APHIS quarantined areas. To Monsanto's knowledge (from our database of grower licensing agreements), no ABTB04-6 has ever been planted in the APHIS quarantine areas.

NatureMark/Monsanto received a copy of the results of the verification study on January 26. A copy of the results table supplied by Dr. Brodie is included in the Attachment (page 3), along with a brief description of the testing method, and some general information on GN. The results of Dr. Brodie's work show that line ABTB04-6 is not resistant to GN. Lines ABTB04-36 and ABTB04-31, like the parental Atlantic line, are resistant. Dr. Brodie will report these results at the annual meeting of the Golden Nematode Technical Committee which is being held February 9 in conjunction with the Empire State Vegetable Growers Annual Meeting in the Holiday Inn at the Syracuse, NY Airport.

2/11/2000
Left VM

In response to this new information, NatureMark/Monsanto contacted the New York State extension agent responsible for grower communication on GN issues, Dr. Don Halseth. NatureMark is following his advice to provide recommendations relative to GN resistance to seed growers and New York extension agents and to include these recommendations in product leaflets that will be provided to prospective growers in the next few weeks. Growers in areas quarantined for GN (Long Island and parts of upstate New York) need to know this for making planting decisions. The information does not impact growers in other areas.

The attachment does not contain Confidential Business Information.

If you need further information in regards to this finding, please contact me at phone 636/ 737-5721 or via email at elizabeth.d.owens@monsanto.com.

Sincerely,
Monsanto Company

Elizabeth D. Owens, Ph.D.
Manager, Regulatory Affairs
Team Lead, Potatoes

Attachment: Results of Screening Studies Conducted by Dr. Bill B. Brodie, Cornell University, for Golden Nematode Resistance in Potatoes: NewLeaf Atlantic Lines ABTB04-6, -31, -36

cc: Russ Schneider, Monsanto
Bob Ingratta, Monsanto

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Petition 95-338-01p

Date:

February 8, 2000

Provided to:

Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
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U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Provided by:

Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO 63098

Bioassay for Resistance to Golden Nematode (*Globodera rostochiensis*):
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Individual tubers are planted into pots inoculated with enough golden nematode cysts to provide ca 5000 viable eggs/pot. Eight weeks after emergence, the roots are examined for cyst development (data provided under "Roots"). A plant is considered resistant if five cysts or less develop on the roots. If additional information is desired, the plants are allowed to grow another four weeks then the soil is processed to determine the number of cysts /pot (data provided under "Soil") and the cysts are examined for the number of viable eggs/cyst (no data taken).

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The presence of the H_1 gene, a gene associated with GN resistance, can now be determined by testing for a marker associated with the gene on the chromosome. Using the method outlined below, line ABTBO4-6 was demonstrated to have the marker present although it is not a GN resistant line. Dr. Brodie provided this summary of the method developed to detect the gene marker.

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Collection of plant material
DNA extraction
Digestion of DNA
Southern blotting
Pre-hybridization
Random Hexamer Labeling
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Expose film to radioactive membrane
Develop film

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Atlantic NewLeaf/NY -6	12	1,394	Sus.
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* Average of 10 replications

** Carries the H_1 gene for GN resistance

***NT = non transformed. Atlantic also carries the H_1 gene for GN resistance.

FACT Sheet
PPQ -- made available through the CAPS program
March 23, 1993

FACTS-08

GOLDEN NEMATODE (GLOBODERA rostochiensis)

This nematode was first noticed in 1881 during Germany's campaign against beet nematodes where it was recorded as a curiosity, a possible subrace of Heterodera schachtii. It was not until 1913 in Scotland and in Germany in 1914 that this "oddity" was proved to be the cause of "soil sickness of potatoes".

The pest was discovered in England in 1917, and in Sweden and Ireland in 1922. In 1923 it was described as a separate species from the nematode that affected sugar beets, but it took several more years before this pest was generally recognized as a separate species from H. schachtii, The scientific name Heterodera rostochiensis was assigned because the samples used for the description came from Rostock, Germany.

Infested seed had been spread over the world long before the nematode was discovered and named, and as a result, has had a spectacular impact on the potato industry. Infestations were discovered in such widespread places as Peru in 1952, Iceland in 1943, India in 1961, and Panama in 1967. For many years it was thought that the pest originated in Europe. The discovery of the infestation in Peru showed that the nematode was endemic to this same area from where the potato itself had originated. Interestingly, it was USDA Plant Quarantine Inspectors who put this revelation in motion by finding infested potatoes on a ship from Peru that arrived in Seattle in 1951. The discovery was made after tracing back this find to the origin of the infested potatoes.

Potato tubers had been distributed to Europe from South America by the early 1600's, but under primitive conditions, build up of populations was slow. Only much later, after the potato had become a vital component of the basic food supply in Ireland, Scotland, Germany and other western European nations, did the nematode gain a foothold in European potato fields. This was brought on by the intense production of new varieties and the widespread distribution of seed potatoes for the establishment of commercial potato production between 1856 and 1876.

The nematode was discovered in the United States near Hicksville, Long Island, New York, in 1934 when a farmer noticed a few isolated spots where the vines were stunted and off color. These spots became worse and began to have a serious impact on potato production. After repeated requests by the farmer, the field was examined and the nematodes were found on the roots of the crop, and identified as Heterodera (now Globodera) rostochiensis. Crop losses were at 70 percent in the affected field. From all indications, the entire Long Island infestation can be traced to this 40 acre field. Spread resulted from reuse of burlap bags for harvesting potatoes and the movement of contaminated farm machinery by renters from one area to another. Exactly how the nematode gained entry no one knows, but speculation blames the return of equipment from Europe after World War I. The nematode had probably been present 20 years in the original field before it was identified.

The life cycle takes 38 to 48 days to complete. Eggs hatch within the dead, swollen, flask-shaped bodies of fertilized females smaller than a pinhead, called cysts. Each cyst, which is the protective covering for the eggs, may contain up to 500 eggs. These cysts are resistant to chemicals, drying, and some soil organisms. In the spring, at about 60 degrees F., the larvae hatch in response to a chemical given off by potato or tomato roots. They leave the cyst and migrate to the host plant where they enter the roots and feed. Below 55 degrees F. there is little activity.

In the roots the nematodes take up a position near the plant's vascular system, the females swell and break through the roots, remaining attached by a thin neck. The males remain thin and eel-like and mate with the females. The fertilized female produces several hundred eggs and dies. The cyst, visible to the unaided eye, is at first white, then a golden color (thus the name "golden nematode") and finally brown. Cysts become detached and remain in the soil after the crop has been harvested.

Commercial plants infected by this nematode are potato, tomato, and eggplant. Wild plants known to be infected all belong to 90 species of SOLANUM, several of which are found in this country, but most are found in South America, the indigenous range of the nematode.

The first infestation outside of Long Island was discovered in Steuben County, New York, December 1967. Additional infestations have been discovered since in upper New York State in additional counties. An infestation was allegedly found in New Castle County, Delaware, February 1969. This infestation was either eradicated, or was not a valid find to begin with, depending on one's point of view.

The Federal Golden Nematode Quarantine was invoked in 1969 with the states of New York and Delaware quarantined. After intensive survey, Delaware was removed from quarantine in 1970. Regulations presently stay in effect for the two agricultural counties of Long Island and seven upstate New York counties.

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FACT SHEETS, PLANT PROTECTION AND QUARANTINE PROGRAMS
Calendar Year 1977

CODE OF FEDERAL REGULATIONS Subpart--Golden Nematode (301.85) 1992

CBI DELETED

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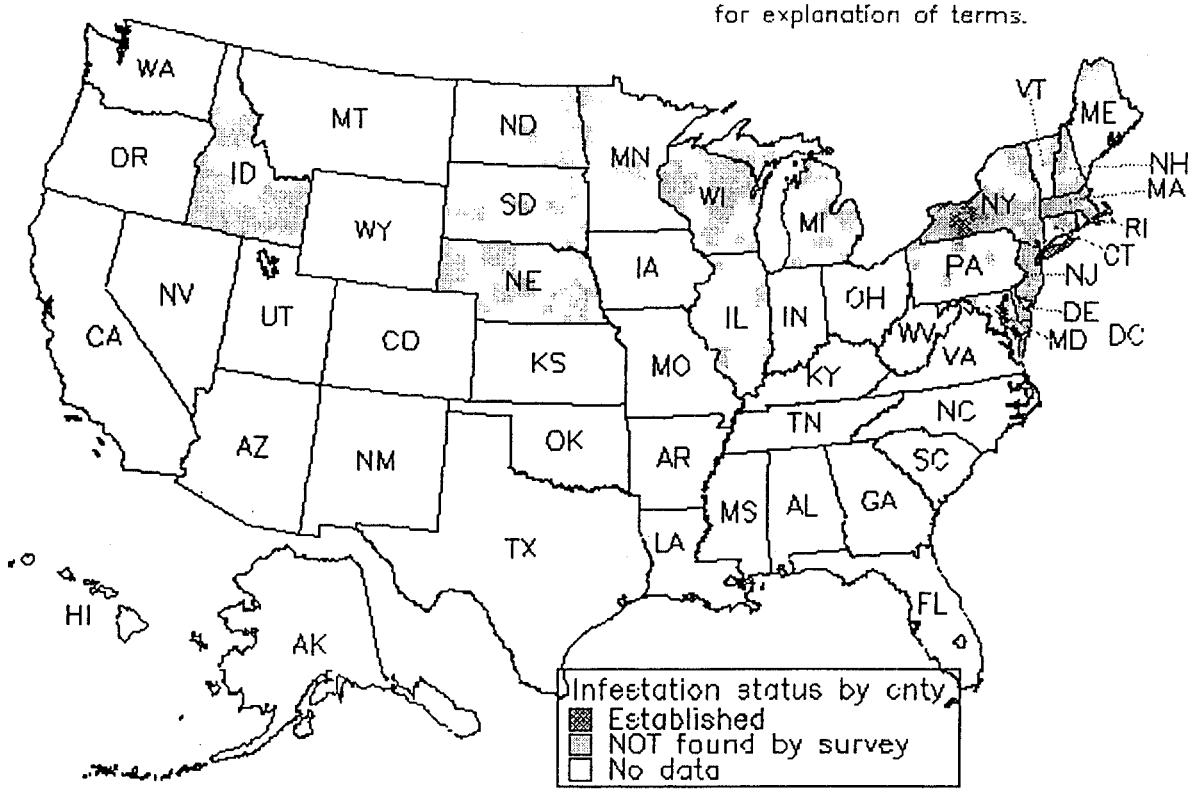
CLICK on legend area
for explanation of terms.



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Petition 95-338-01p

Date:

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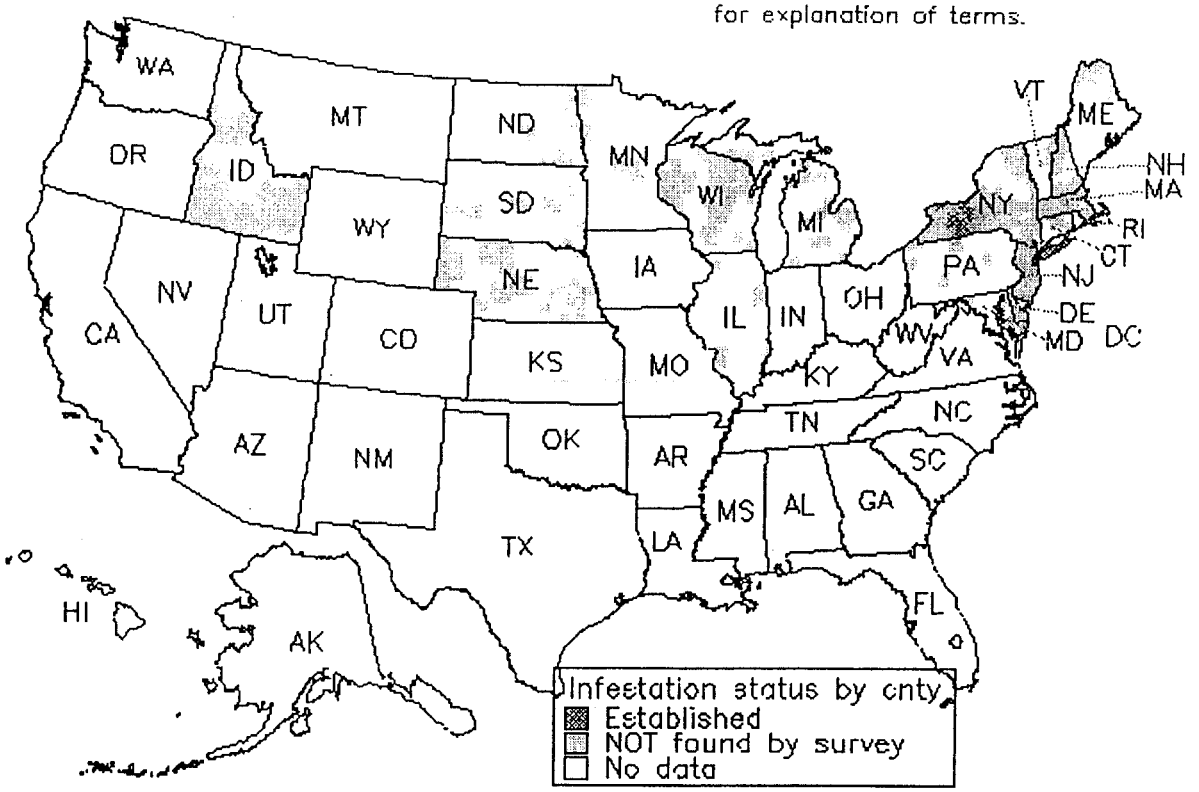

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February 22, 2000

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Attention: Dr. David Heron

Dear Dr. Heron,

Subject: Approved petition 95-338-01p. NewLeaf Atlantic line ABTBO4-6 . Responses to your questions of February 16.

Attached are responses to your questions of February 16 with supporting documents. There are three copies of the response, but is just one copy of the original extension bulletin is provided.

The attachment does not contain Confidential Business Information.

If you need further information in regards to this finding, please contact me at phone 636/ 737-5721 or via email at elizabeth.d.owens@monsanto.com.

Sincerely,
Monsanto Company

Elizabeth D. Owens, Ph.D.
Manager, Regulatory Affairs
Team Lead, Potatoes

Attachment: Responses to questions of February 16, 2000.

cc: Russ Schneider, Monsanto
Bob Ingratta, Monsanto

RESPONSES TO QUESTIONS ON Btt POTATOES POSED FEBRUARY 16 BY DR. DAVID S. HERON OF USDA/APHIS

Submitted by Elizabeth D. Owens, Ph.D., Monsanto, with the assistance of David F. Hammond, Ph.D., Michael K. Thornton, Ph.D. and James C. Zalewski, Ph.D.

Note: Original email from Dr. Heron included as Appendix 1.

1. Has ABTB04-6 been evaluated for susceptibility to other nematodes which infect potatoes ?

Monsanto/NatureMark follows the standard selection practices for breeders developing new potato varieties. Most breeding programs do not screen for nematode resistance. USDA/ARS scientist Dr. Bill Brodie screens potato lines for golden nematode resistance that come from the Cornell University potato breeding and variety evaluation program. This program has the development of golden nematode resistant varieties for New York state as one of its primary objectives.

Resistance of NatureMark lines to common nematode pests is evaluated by natural exposure during the variety development process (Appendix 2). Potato tubers are examined for defects of any kind and nematode tuber damage is easily recognized (Appendix 3). Since most areas where the field trials are conducted would have natural infestations of the common potato nematodes, including root knot, damage would be noted as part of the normal agronomic screening practice. No unusual or more severe symptoms of root knot nematode have ever been observed on any of our transgenic lines since 1993 when they were first grown in research trials and in seed increase plots that were certified and monitored by US and Canadian seed inspection agencies.

Susceptibility of a new variety to pests and diseases, whether it is produced by traditional breeding or by biotechnology is always a possibility. Observation of unusual disease or pest symptoms during agronomic screening is a simple and effective method of detecting such changes if the disease or pest normally occurs in the region(s) in which the variety is being tested.

The process of observing unusual disease symptoms or pests during variety selection trials and during early commercial production is followed not only by potato breeding programs but by conventional breeding programs in all types of crops world-wide. There are so many diseases and pests of most crops that specific screening trials designed to evaluate potential susceptibility to every known disease or pest would be economically and practically impossible. Breeding programs focus on the major pests and diseases that have the greatest economic impact in the target market. Nematode resistance is not one these primary focus diseases in the potato industry of the United States and Canada.

2. From your comments, I understand that potatoes are not usually evaluated for such resistance. That surprised me, since the GRIN database lists resistance to eight different nematodes which attack potato. Can you clarify this for me?

New potato varieties are screened for all commonly occurring pests and diseases, including nematodes, by natural exposure in the numerous agronomic trials conducted during the development of new variety (Appendix 2).

The GRIN database lists sources for resistance in potato plant breeding material. It is not a listing of nematode pests for which new potato varieties are being screened in the United States. Dr. Saad Hafez, Univerisity of Idaho potato nematologist located in Parma, Idaho, said that four of the listed nematodes, blackroot, horsenettle, Osborne cyst, and white potato cyst nematode, do not exist in North America.

Of the nematodes listed, only 2, 4, and 5 have significant presence in the United States and all produce readily observable symptoms on tubers. The golden nematode, 2, is highly localized in quarantined areas in New York state and Canada. Commercial varieties currently produced are not considered resistant to northern and columbia root knot nematodes (4, 5) so growers use long rotation cycles and chemical treatments where necessary in infested areas (Appendix 3) .

3. One control strategy used to stabilize the development of resistant races is to alternate seasons of resistant and susceptible potato varieties. How will the results for ABTB04-6 influence these practices?

These golden nematode screen results are only important in the quarantined areas where the golden nematode is present. We have already notified extension agents and seed growers supplying those areas that ABTB04-6 is not resistant to the golden nematode and that lines ABTB04-31 and ABTB04-36 are recommended for use in these areas instead. Growers in quarantined areas are only allowed to plant non-resistant potato varieties once in a four-year rotation cycle. The information on lack of golden nematode resistance has no impact on grower practices in areas where this nematode is not present.

4. When did Monsanto receive the preliminary results from Dr. Brodie that ABTB04-6 appeared to be susceptible to golden nematode?

Dr. Brodie first contacted Dr. David Hammond of Monsanto/NatureMark in the spring of 1999 after Dr. Brodie had completed some preliminary studies which indicated that the line of NewLeaf that Cornell had in house was not resistant to golden nematode. At that time, Dr. Brodie was not sure of the line identification and therefore requested material that was of known stock. NatureMark supplied the additional material, which included all three NewLeaf Atlantic lines and the parental Atlantic line, with the understanding that Dr. Brodie would supply the results of this final study when it was complete. The results were received January 26th and subsequently supplied to Dr. James L. White at the USDA on February 8th.

5. Where and how extensively has ABTB04-6 been grown in the U.S. each year since the determination of nonregulated status?

ABTB04-6 was first planted by a commercial grower in 1998 (1 location, 19 acres). Prior to then, only seed growers planted ABTB04-6 on limited acreage. It wasn't until 1999 that seed potatoes became widely available to commercial growers who then planted ABTB04-6 on approximately 2500 acres, mostly in the states of Michigan, North Dakota, and Wisconsin. These three states accounted for approximately 60% of the acres planted to this line. There were only 11 acres of ABTB04-6 planted commercially in New York in 1999, none of which were in quarantined areas.

6. Have there been any reports of nematode diseases on ATBT04-6 ?

Any of the common potato nematodes present in the soils of North America would produce above ground and/or tuber symptoms in a susceptible variety that would be observed during the production of such a variety on infested soil.

In addition to not observing nematode damage differences in pre-commercial field trials, there have been no reports from seed or commercial growers of unexpected nematode damage to ABTB04-6 or any of the other NewLeaf lines. Most growing regions have soils infested at some level with nematodes of one species or another so some damage is expected and normal. Most growers, particularly seed growers, notify NatureMark if tuber or plant damage from any disease or pest is greater than that normally experienced with conventional varieties.

APPENDIX 1

FEBRUARY 16 QUESTIONS

From: David S Heron [David.S.Heron@usda.gov]
Sent: Wednesday, February 16, 2000 1:35 PM
To: ELIZABETH.D.OWENS@monsanto.com
Cc: James.L.White@usda.gov
Subject: Potato line ABTB04--6

Sensitivity: Personal

Elizabeth,

Thank you for the information on the telephone today regarding your recent correspondence about NewLeaf potato line ABTB04-6. Here are some of the things we talked about that will help me put a clearer picture together.

1. Has ABTB04-6 been evaluated for susceptibility to other nematodes which infect potatoes ? From your comments, I understand that potatoes are not usually evaluated for such resistance. That surprised me, since the GRIN database lists resistance to eight different nematodes which attack potato. Can you clarify this for me?

From the GRIN database
http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?73
Category: NEMATODE

1.Blackroot nematode (BNEM)
Resistance to Blackroot Nematode (Diplotheca rhizophila)
Code values.

2.Golden cyst nematode (GPCNEM)
Resistance to Golden Potato Cyst Nematode (Heterodera- Globodera rostochiensis).
Code values.

3.Horsenettle cyst nematode (HCNEM)
Resistance to Horsenettle Cyst Nemat. (Globodera virginiae)
Code values.

4.Meloidogyne Hapla Nematode (MHNEM)
Resistance to Meloidogyne hapla Nematode
Code values.

5.Meloidogyne chitwoodi Nematode (MCNEM)
Resistance to Meloidogyne chitwoodi Nematode
Code values.

6.Osbornes cyst nematode (OSNEM)
Resistance to Osbornes Cyst Nemat. (Globodera solanacearum)
Code values.

7.Root knot nematode (RKNEM)
Resistance to Root Knot Nematode (Meloidogyne spp.).
Code values.

8.White-potato cyst nematode (WPCNEM)
Resistance to White-Potato Cyst Nemat. (Heterodera/Globodera pallida)
Code values.

2. One control strategy used to stabilize the development of resistant races is to alternate seasons of resistant and susceptible potato varieties. How will the results for ABTB04-6 influence these practices?

3. When did Monsanto receive the preliminary results from Dr. Brodie that ABTB04-6 appeared to be susceptible to golden nematode?

4. Where and how extensively has ABTB04-6 been grown in the U.S. each year since the determination of nonregulated status?

5. Have there been any reports of nematode diseases on ATBT04-6 ?

Thanks,
David Heron

APPENDIX 2

PROTOCOLS FOR FIELD EVALUATIONS OF POTATO LINES

MONSANTO/NATUREMARK

FIELD TRIAL MONITORING PROTOCOL AND MONITORING FORMS

Although not specifically indicated in the forms/protocols, tubers are also examined for any disease symptoms, including nematodes, during the harvest, grading, and internal evaluations that are conducted on all lines.

Appendix II

Pre-Harvest Data Collection

1) Emergence Date

   a) Date of 50% emergence recorded as Julian date provided at the front of each field book.

2) Stand Count

   a) Record number of plants emerged. Adjust if count changes due to rogueing, mechanical damage, etc.

3) Vigor Rating

   a) Conducted approximately two months after planting, (i.e., recordings should be taken when tops have reached maximum size and before there are visible signs of maturity). Rate on scale from 1-5 on the basis of top size (vigor). One being poor vigor and 5 excellent vigor. Half-points may be used when necessary. Check cultivars should be observed before and during rating, but rate absolute not relative values. Record as 10 to 50 in data books, i.e. no decimal point.

   b) Checks normally read as follows:

      2 - Norland
      3 - Norchip
      4 - Russet Burbank

4) Maturity Rating

   a) Conducted in late August when a range of maturities are readily apparent. Early varieties should show yellowing and be slumped into interrow area, mid-season varieties should be showing some signs of maturity, and late varieties little sign of maturation. Check cultivars should be observed before and during the rating, but rate absolute not relative values. Rate on a scale of 1-5 (1 being early and 5 being late). Half-points may be used when necessary. Record as 10 to 50 in data books, i.e., no decimal point.

   b) Checks normally read as follows:

      2 - Norland
      3 - Norchip
      4 - Russet Burbank

Appendix III

Post-Harvest Data Collection

1) Yield

All yield measurements should be weighed to closest 100 g. Report in kg per plot.

a) Total.--All harvested tubers. Count total tuber numbers.

b) <48 mm yield. --All tubers less than 48 mm diameter. Count tuber number.

c) >48 mm yield.--All tubers greater than 48 mm in diameter but less than 88mm. Count tuber number.

d) >88 mm yield.--All tubers greater than 88 mm in diameter. Count tuber number.

e) Marketable.--For replicated trials only. All tubers conforming to a Canada No. 1 grade (see attached grading regulations) or better in all aspects besides oversize (i.e., marketable yield includes oversize yield). Count tuber number. No deformities of any description.

f) Oversize.--For replicated trials only. All tubers conforming to a Canada No. 1 grade in all respects besides oversize (>88 mm). Count tuber number.

2) Tuber characteristics of >48 mm or marketable yield rated as follows:

a) Shape (***Use attached chart for rating.)

1 - Round
2 - Oval
3 - Oblong
4 - Long

b) Unusual and unwanted shapes.--Only record if 10% or more of the >48 yield is affected (including >88 mm). (***Use attached chart for rating.)

0 - Absent
1 - Flattened (when the length of a transverse section at any point of the tuber is more than three times longer than its breadth).
2 - Ovate
3 - Compressed
4 - Clavate
5 - Reniform
6 - Fusiform (spindle shaped, tapering gradually at both ends).

c) Uniformity of tuber shape

1 - Very variable
2 - Variable
3 - Acceptable
4 - Uniform
5 - Very uniform

d) Uniformity of tuber size

1 - Very variable
2 - Variable
3 - Acceptable
4 - Uniform
5 - Very uniform

e) Skin color.—Include depth of color, flake skin, etc. in comments column.

1 - White
2 - Light Russet
3 - Heavy Russet
4 - Red
5 - Deep Red
6 - Purple

f) Eye depth

1 - Deep (e.g., Warba)
2 - Undesirable
3 - Acceptable (e.g., Russet Burbank)
4 - Shallow
5 - Very shallow

g) Overall appearance

1 - Very poor
2 - Poor
3 - Acceptable
4 - Good
5 - Outstanding

h) Flesh color

1 - White
2 - Off-white
3 - Yellow
4 - Deep yellow

i) Deformity.--Growth cracks and second growth only. Number and weight of tubers affected in the >48 mm category and >88 mm category.

j) Hollow heart and brown center (one combined reading).--Assessed on the basis of cutting 25 marketable-size tubers (>48 mm) per plot, but less than < 88 mm in the three adaptation trials. In the replicated trials assess Hollow Heart in two categories # >48 mm and # >88 mm. Cut all tubers across the stem end to apical end.

k) Internal necrosis.--Assessed on the basis of cutting 25 marketable size tubers (>48 mm) per plot. Necrotic flecks throughout the tuber tissue, mainfry from vascular ring inward. Flecks range in color from rust to brown and black.

3) Disposal 1,2,3

1 - Discard
2 - Unsure
3 - Save

4) Comments

Appendix IV

Quality Data Collection

At harvest, an 6-kg sample of marketable tubers should be collected from each plot in each replication. This sample will be used for assessment of quality variables. Tubers to be used for the chipping test six weeks after harvest should be placed in a well humidified 10°C storage. The remainder of the sample should be stored at 6.5°C in a well humidified storage.

1) Specific Gravity (SG)

Use the above sample (6 kg) for this measurement (i.e., separate SG reading for each replication).

Method

a) Weigh tubers in air and water (accurate to 1 gm).
b) Water temperature should be approximately 18°C.
c) Sample should be clean so as not to influence the density of the water.
d) Calculate specific gravity as follows:

$$\text{Specific Gravity} = \frac{\text{weight in air}}{\text{weight in air minus weight in water}}$$

a) Calculation should be taken to 3 decimal places.

b) An automatic device for the calculation of SG is available from Engineering Research Services, Agriculture Canada, Ottawa (Young's Specific Gravity Calculator).

2) Chip Color

Use tubers gathered randomly for above 6 kg plot sample, (Note: Separate samples for each replication in the replicated advanced yield trails; composite sample made up of 2 tubers from each replication for adaptation and advanced adaptation trials).

Method

a) Sample should consist of 5-8 average sized marketable tubers (>48 mm to >88 mm) per replication plot.

b) Chip slices are 1.0 mm thickness (thickness of a dime). Tubers peeled. Slices from five tubers mixed.

c) Chip slices washed (2x) with lukewarm water to remove excess starch and then blotted with paper towelling to remove excess moisture.

d) 400 g (max.) of slices weighed.

IN-SEASON FIELD MONITORING

CBI DELETED

Complete one form per notification and RETURN THIS FORM WITHIN 14 DAYS AFTER HARVEST to your Compliance Specialist (name and address on the bottom of this page).
USDA Regulation 7CFR 340.3(d)(4) require that a final report must be submitted within six months after harvest of a field trial established under a USDA Notification. The information provided on this form is part of the process in compiling data to enable Monsanto to meet this requirement.

USDA Ref. #	Monsanto Ref. #

Site Information

Crop _____

Project/Study # _____

Site Name/Company _____

Location (including county) _____

Contact/Phone _____

Instructions for recording observations:

Following are guidelines for consideration while making observations at least once every 4 weeks during the planting season. This list is in no way inclusive of what may be observed. If multiple lines are planted under the same notification, please identify the specific lines affected. If no control is available in the plot, compare to other plants within the growing area. If something unusual is noted during the field trial that is not covered by any of the categories listed, please record the observation on the reverse side of the form or attach additional sheets as necessary.

Disease Susceptibility

Do transgenic plants have a higher incidence of disease than non-transgenic plants? What diseases were observed? Indicate if this occurred field trial-wide or in the transgenic plants only.

Insect Susceptibility

Do transgenic plants have a higher incidence of non-target insect species than non-transgenic plants? Are the transgenic plants more susceptible to insect feeding than non-transgenic plants? What insects were observed? Indicate if this was a field trial-wide observation or in transgenic plants only.

Plant Growth (Morphology)

Is there a difference in the general appearance, growth, flowering, and/or seed production of the transgenic and non-transgenic plants? Please describe the differences.

Weediness Characteristics

Is the germination of transgenic plants in any way different from non-transgenic plants? If yes, describe differences and potential causes.

Plant Stand

Is the final stand of the transgenic plants any different than the non-transgenic plants?

RETURN THIS COMPLETED FORM TO YOUR COMPLIANCE SPECIALIST:

Use a new line for each observation date recorded. Attach additional sheets if more room is needed to record observations.

Disease Susceptibility

Date	Yes	No	% of Plants Affected Trans.	Non-Trans.	Diseases Identified/Comments

Insect Susceptibility

Date	Yes	No	% of Plants Affected Trans.	Non-Trans.	Insects Identified/Comments

Plant Growth Characteristics

Date	Yes	No	% of Plants Affected Trans.	Non-Trans.	Observed Char./Comments (any adverse effect?)

Weediness Characteristics

Date	Yes	No	% of Plants Affected Trans.	Non-Trans.	Observed Char./Comments

RETURN THIS COMPLETED FORM TO YOUR COMPLIANCE SPECIALIST:

Plant Stand

Date	Standcount Yes	No	Trans.	Non-Trans.	Observed Char./Comments

Lines/Events Planted	
Plant Date	
Harvest Date	
Destruct Date and Method	
Disposition of Seeds	

General Results of Field Trial

SIGNATURE _____ DATE _____

RETURN THIS COMPLETED FORM TO YOUR COMPLIANCE SPECIALIST:

WESTERN REGIONAL
POTATO VARIETY TRIAL REPORT
DISEASE RESISTANCE EVALUATION

These regional trials are conducted in Washington, Oregon and Idaho. These are the standard trials used for potato line evaluation for materials released from all regional breeding programs. Monsanto/NatureMark submits material to these trials and utilizes the same rating system for their own field evaluations during line selection.

1998
WESTERN REGIONAL
POTATO VARIETY TRIAL REPORT

State Experiment Stations and
USDA-ARS Cooperating

California
Colorado
Idaho
New Mexico
Oregon
Texas
Washington

1998 WESTERN REGIONAL POTATO VARIETY TRIAL REPORT

Compiled by Brian Schneider

Monsanto
February 22, 2000

Revision - 1/22/99

TABLE 21: 1998 Western Regional Potato Variety Trial - DISEASE EVALUATION AND METRIBUZIN REACTION

No	Clone	Verticillium Wilt AB[1]	Verticillium Wilt HRM[2]	Early Blight AB[1]	Common Scab AB[1]	Late Blight Foliar AUDPC Mt. Vernon[3]	Late Blight Tuber % by Wt. Mt. Vernon[3]	PVY Foliar Symptom HRM[2]	Fusarium Dry Rot AB[1]	Erwinia Soft Rot AB[1]	Metribuzin Reaction AB[4]
1	R. BURBANK	S	S	S	VR	4592	0.1	MS	S	S	R
2	RANGER R.	MR	S	MS	S	5178	0.1	VR	MS	S	R
3	R. NORKOTAH	VS	VS	VS	R	4839	2.8	MR	MR	S	MR
4	SHEPODY	S	-	VS	S	4575	0.0	-	MS	S	VS
5	AVALANCHE	MR	MR	S	MR	3960	0.0	VR	S	S	VS
6	A88338-1	R	R	MR	VR	3990	0.0	R	S	MR	MR
7	AC87084-3	MR	R	MS	R	3611	0.0	MR	S	MS	VS
8	AC88042-1	S	MS	VS	R	4970	0.0	R	S	S	MS
9	AC88165-3	S	S	S	R	5041	7.3	MR	S	S	MR
10	AO87277-6	S	MS	S	MS	4977	0.4	R	S	VS	MS
11	AO89128-4	R	MS	MS	MR	5404	0.5	MS	S	S	VR
12	CORN-3	S	MS	S	VR	3402	0.2	MS	MR	S	R
13	CORN-8	S	S	VS	VR	4253	0.2	MS	MR	S	R
14	NDD840-1	MR	-	S	VR	4134	0.0	-	S	S	VR
15	TX1385-12RU	S	MS	VS	R	4973	1.0	MS	S	S	VR
16	TXNS112	S	S	VS	R	4678	0.0	MR	MR	S	R
17	TXNS223	S	S	VS	R	4541	0.0	MR	MR	S	R
18	TXNS278	S	S	VS	VR	4612	0.0	MS	MR	S	R

1 Evaluations made at Aberdeen, Idaho by Dennis Corsini.
2 Evaluations made at Hermiston, Oregon by Dan Hane.
3 Evaluations made at Mt. Vernon, Washington by Debbie Inglis.
4 Evaluations made at Aberdeen, Idaho by Steve Love.

APPENDIX 3

EXTENSION BULLETIN ON POTATO NEMATODES

University of Idaho
College of Agriculture
Cooperative Extension System
Agricultural Experiment Station

Current Information Series No. 925

Potato nematodes and their control

S. L. Hafez and M. K. Thornton

Nematodes are microscopic, wormlike animals that inhabit the soil and often attack plant roots and tubers. Potato plants can be parasitized and severely damaged by several nematode species. Damage by these nematodes results not only in yield losses but in inferior-quality tubers.

Nematodes parasitic on potatoes in Idaho

Root-knot nematodes (*Meloidogyne* spp.)

Root-knot nematodes are the most damaging nematodes occurring in Idaho. Of the several species of root-knot nematodes, two occur in many commercial potato-growing areas of Idaho. They are known as northern (*Meloidogyne hapla*) and columbia (*M. chitwoodi*) root-knot nematodes. These nematodes mainly affect potato quality. A wide host range (Table 1) makes their control by crop rotation difficult. Although the first generation usually attacks the roots, economic damage occurs as reduced yield and tuber quality.

Symptoms of nematode infestation are galls of various sizes and shapes on roots (Fig. 1) and wartlike bumps on the surface of tubers (Fig. 2). *M. hapla* tends to cause less distinct tuber symptoms than *M. chitwoodi*.

Carefully peeling thin layers off an infected tuber will reveal small brown spots, mostly within the outer one-fourth of an inch (Fig. 3). Tuber symptoms are rarely seen before harvest. They are most conspicuous during storage at temperatures above 45°F. Some reports indicate a possible interaction of root-knot nematodes with Verticillium wilt in increasing the symptoms of early dying.

Root lesion nematodes (*Pratylenchus* spp.)

These nematodes affect potato yield directly by reducing the size and weight of tubers. They reduce yield

Table 1. Some hosts of the major species of root-knot nematode that infest potatoes.

	Columbia *M. chitwoodi*	Northern *M. hapla*
Crops		
Alfalfa	+[a]	+
Bean	+	+
Carrot	+	+
Cole crops	+	+
Corn	+	–
Cotton	–	–
Eggplant	–	+
Grains	+	–
Grape	–	+
Hops	–	–
Lettuce	0	+
Mint	–	+
Melon	–	+
Pea	+	+
Pepper	–	+
Strawberry	–	–
Sudangrass	–	–
Sugarbeet	+	+
Tomato	+	+
Weeds		
Barnyardgrass	–	–
Bindweeds	0	+
Canada thistle	+	+
Foxtails	–[b]	–
Kochia	0	+
Lambsquarters	–	+
Mallows	0	+
Mustards	–	+
Nightshades	–	+
Nutsedges	0	0
Pigweeds	–	–
Russian thistle	–	0
Sowthistles	+	+

Source: Flint, M. L., director and technical editor. 1986. Integrated pest management for potatoes in the western United States. University of California, Division of Agriculture and Natural Resources Publication 3316 and WREP 3316.

Note: +, good host; –, poor or nonhost; 0, unknown.

[a]Alfalfa is a host for race 2 of *M. chitwoodi*.

[b]Green foxtail is a moderate host; yellow foxtail and meadow foxtail are poor or nonhosts.

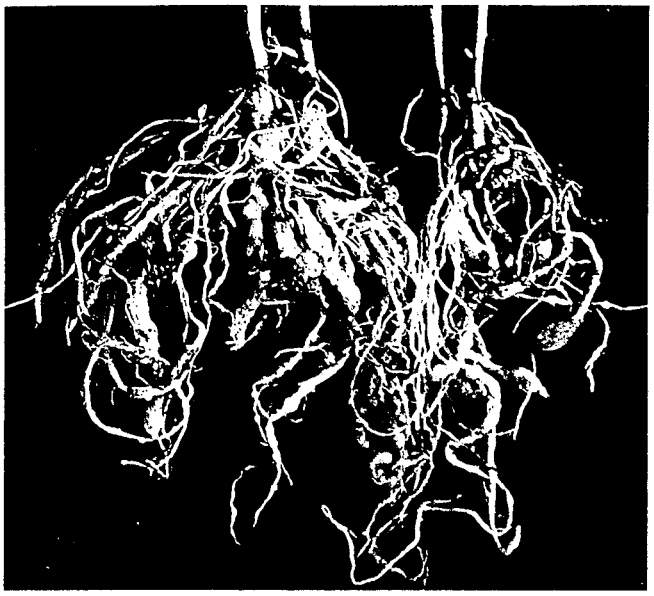

Fig. 1. Root-knot nematodes cause swellings on potato roots.

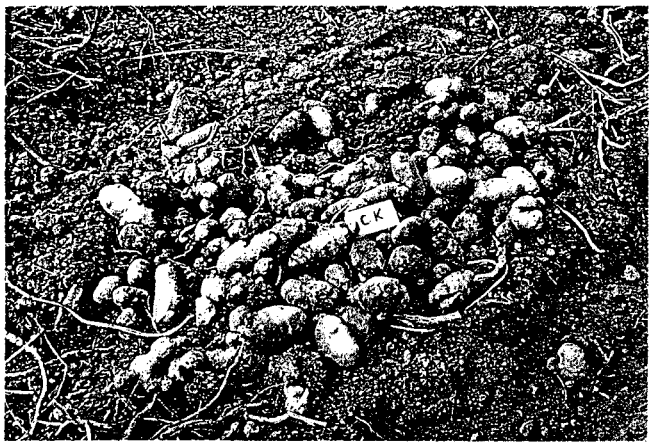

Fig. 2. Root-knot nematodes cause bumps and warts on the tuber surface.

Fig. 3. Root-knot nematode damage. The tuber at left is uninfected; the tuber at right has a severe, advanced infection of columbia root-knot nematode; and the tuber at center shows brown spots caused by nematode egg masses.

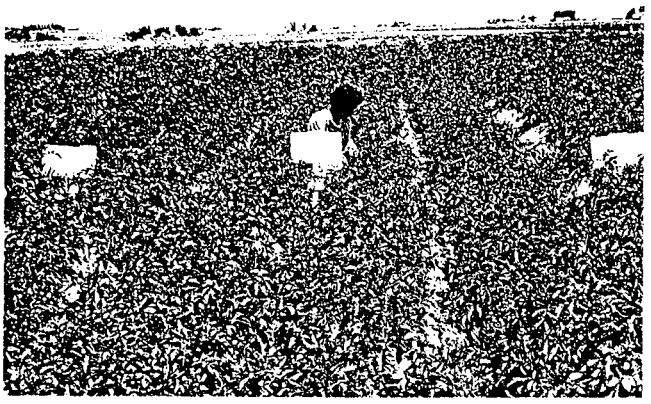

Fig. 4. A potato field infested with root-knot and root lesion nematodes shows poor growth in the untreated four rows (center) and stronger growth in the treated rows on either side.

indirectly by weakening and increasing stress on the plants and by making them more susceptible to fungal and bacterial diseases. In some cases, a strong relationship has emerged between the amount of Verticillium wilt and populations of certain root lesion nematode species. Aboveground symptoms caused by high nematode populations resemble general plant stress. Patchy areas of poor growth and stunted, yellowing plants indicate nematode infestation (Fig. 4). The root symptom is sunken lesions.

Although most root lesion nematodes are primarily root parasites, some species are also known to damage potato tubers, causing a severe reduction in tuber quality. The most common root lesion nematode present in Idaho potato fields, *P. neglectus*, causes little damage. However, *P. coffeae* and *P. penetrans*, which have been found recently in a few areas, can cause severe yield and quality losses.

Stubby root nematodes (*Trichodorus* spp.)

Stubby root nematodes are important parasites of potatoes, not so much for the direct damage they cause but for the tobacco rattle virus (TRV) they transmit to potatoes. This virus causes a disease of potato tubers called corky ringspot. TRV sometimes causes a stem mottle consisting of yellowish rings and line patterns together with malformed leaves. Tubers infected with TRV may become irregularly shaped during the early stage of growth. The skin tissue cracks into arc-shaped lesions, and brown, concentric rings develop on the surface of many tubers. Rusty-brown, irregularly shaped lesions that have a corky texture may appear in the flesh of the tuber (Fig. 5). At harvest, tubers may have deep cracks and shallow, corky depressions on their surfaces, rendering them unmarketable.

Fig. 5. Corky ringspot in Russet Burbank tubers may appear as an area of concentric rings of brown tissue.

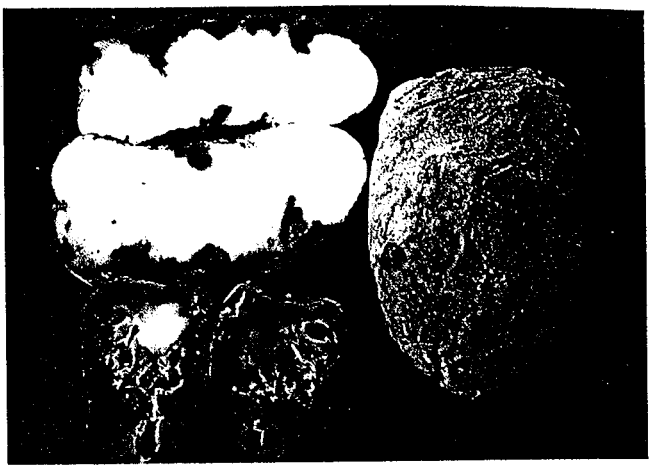

Fig. 6. Internal (left) and external (right) symptoms of potato rot nematodes.

Potato rot nematode (*Ditylenchus destructor*)

This nematode damages tubers, causing a serious problem in stored potatoes. Symptoms of the disease are not usually observable in the foliage except in cases of severe infection, which can reduce growth and deform leaves.

Initial tuber symptoms are small white spots just beneath the skin with holes in their centers. Infected areas become soft and are more readily detected by touch. Later, the tissues under the skin turn grayish brown and form slight depressions. As the disease progresses, the skin above the spots thins and frequently splits, exposing an inner, dry crumbled mass (Fig. 6). A cross section of a lesion will often reveal many nematodes along the border between diseased and healthy tissue.

Control

Nonchemical control

Before the discovery of chemical nematicides, cultural practices and land management were the most common means used to control nematode problems. Such practices include prevention, crop rotation, clean fallow, early harvest, and organic manures.

Prevention — Prevention is generally more effective and less expensive than any other control measure. There are many ways to prevent new nematode infestations and to prevent recontamination after applying nematicides:

- Use clean, certified, nematode-free seed.
- Do not return tare dirt to any cultivated land.
- Avoid moving farm machinery from infested fields to clean fields.
- Avoid using contaminated water for irrigation.
- Avoid using nondecomposed manure.
- Use a clean, disinfected storage to store potato seed.
- Don't use return-flow water from other farm operations.

Crop rotation — Crop rotation to reduce nematode populations is the most effective and most widely used land management practice. Successful practices include the following:

- Planting crops unfavorable for nematodes in the rotation
- Using resistant varieties of the rotation crop, if available
- Using a systemic nematicide on the rotation crops preceding the potato crop (if chemicals are labeled)
- Practicing good weed control in the rotation crops

Fallowing — Fallowing is the practice of keeping land dry and free of all vegetation for various periods of time depending on the target nematodes. Fallowing can be accomplished through frequent tilling of the soil by disking, plowing, or harrowing or by applying herbicides to prevent plant growth. Repeated cultivation reduces nematode populations in the upper layer of soil by exposing them to heat and air.

Plant-parasitic nematodes depend on living plant tissues for the food they need to develop to maturity and to reproduce. Some weeds can act as hosts for nematodes; therefore, it is important to keep the field weed free. During fallow, most active stages of nematodes die by starvation and desiccation.

Early harvest — Planting varieties that mature early is a good practice for reducing root-knot nematode damage. A short growing period reduces the time available for nematodes to infect tubers and cause significant symptom development. Delaying harvest will make nematode symptoms more severe and noticeable.

Organic manures — Use of aged organic manure or decomposed crop residues (green manure) affect nematodes in two ways: (1) by producing toxic fumes

and chemicals that kill nematodes and (2) by increasing the activity of naturally occurring biological control agents in the soil.

Chemical control

Two types of chemicals are in use today as nematicides: fumigants and nonfumigants. Fumigants are volatile compounds that produce toxic fumes when injected into the soil. Soil fumigation is the most cost-effective chemical method for root-knot nematode control.

Nonfumigants are nonvolatile compounds that kill nematodes by direct contact. Nonfumigant systemic nematicides are the most cost-effective chemicals for control of root lesion and stubby root nematodes.

Nematicides are generally used as a preventive measure. By the time nematode damage becomes apparent, the infested crops are usually damaged so severely that control measures are ineffective. Therefore, nematicides are normally applied preplant (mostly fumigant and contact compounds) or at planting (nonfumigant contact or systemic compounds). Because of the high cost of soil fumigation, it is most cost effective if it is used to control the quality-damaging root-knot nematodes or severe infestations of stubby root nematodes. Other nematodes can be controlled effectively by the use of nonfumigant nematicides.

Reasons for inadequate nematode control

Reinfestation — Reinfestation can occur for one or more of the following reasons:

- Missing strips
- Leaving field ends untreated
- Using contaminated water for irrigation
- Bringing contaminated equipment into a treated field
- Planting infected seed in a treated field
- Practicing poor weed control and allowing volunteer plants in the field

The wrong chemical or wrong rate — Certain nonfumigants control *M. hapla* but not *M. chitwoodi*. All chemicals are most effective when used at the rate specified on the label.

Poor timing of fumigant application — Early fall application of fumigants is recommended. Fumigants can be applied in the spring, but it is more difficult to obtain the proper soil temperature, moisture, and soil conditions.

Improper soil conditions before and during the application — Nematicides should be applied only under conditions specified on the label. Soil tilth, moisture, and temperature influence the effectiveness of chemical treatments.

Inadequate waiting period after fumigation — The soil must be left undisturbed for a period of time after application. Exposure time depends on soil condition and type and rate of fumigant.

Improper placement — Placement of nematicides is critical for proper control. Nonfumigants should be incorporated into the zone where potato roots and tubers develop. Fumigants are generally injected or watered in to a depth of 18 inches or more.

Weather favoring nematode survival and reproduction — Warm winter and spring temperatures increase nematode survival and reproduction. Even low initial populations of root-knot nematodes can lead to tuber damage when conditions are favorable for their rapid increase.

Soil sampling for nematodes

Most control practices require knowing the types and populations of nematodes present in the fields. Keep records of field locations where tuber symptoms were observed in previous potato crops. Sample soil in the fall while the crop previous to potatoes is still in the field. Take samples in the row because nematodes tend to concentrate within the root zone. Take soil samples when soils are not too dry, excessively wet, or frozen. It is a good practice to sample soils after fumigation if the field had a high nematode population or a history of infected crops.

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For further information

CIS 868, *The Potato Rot Nematode* (25 cents)
CIS 914, *Corky Ringspot of Potatoes* (50 cents)
PNW 190, *Root-Knot Nematodes of the Pacific Northwest* (25 cents)

To order publications, contact the Extension agricultural agent in your county or write to Agricultural Publications, Idaho Street, University of Idaho, Moscow, ID 83843-4196 or call (208) 885-7982. Idaho residents add 5 percent sales tax.

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