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MVP® Bioinsecticide, A Novel *Bacillus thuringiensis* Delta-endotoxin-Based Insecticide for the Control of Grape berry moth, *Lobesia botrana*

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Abstract

MVP® Bioinsecticide is the first in a new class of biopesticide products based on Mycogen Corporation's novel CellCap® bioencapsulation technology. MVP contains a selected ∂-endotoxin of *Bacillus thuringiensis* variety *kurstaki* (B.t.k.) that is highly active against the grape berry moth species, *Lobesia botrana* and *Eupoecilia ambiguella*, as well as the diamondback moth, *Plutella xylostella* (L.), *Helicoverpa* spp, and a number of other important agricultural pest species. In the CellCap system, the gene for this toxin was first introduced into the genome of a free-living *Pseudomonas fluorescens* (P.f.) strain. In the manufacturing process this *P. fluorescens* strain is cultured in large fermentors, where the toxin is produced as a large intracellular crystal, much as it is produced in *B. thuringiensis*. However, unlike *B. thuringiensis*, the P.f. cells do not lyse or break apart at maturity. When the fermentation is completed, these intact P.f. cells are killed and fixed in the fermentor using a patented process that encapsulates the toxin crystals within the stabilized cytoplasm and cell walls of the killed bacterial cells.

The effect of the CellCap bioencapsulation process in protecting the ∂-endotoxin from environmental degradation was evaluated in small plot trials on cabbage and broccoli compared to two standard conventional *B. thuringiensis* products to small plots of cabbage and broccoli. Residual activity was estimated using a leaf disc bioassay. These tests demonstrated that MVP provided residual activity that was two to three times greater than conventional B.t. products containing unprotected toxin crystals. Similar trials were conducted on grape vines in Italy with a similar increase in residual activity being noted.

Efficacy of MVP was evaluated in small plot tests on grape vines in Italy, France, Spain, Germany, and Switzerland. In these tests MVP provided excellent levels of control of the grape berry moth species, *Lobesia botrana* and *Eupoecilia ambiguella*. Performance was superior to that seen with conventional *B. thuringiensis* products and generally similar to that achieved with standard chemical insecticides.

Bioinsetticida MVP®, Un Nuovo Insetticida a Base di Delta-endotossina del *Bacillus thuringiensis* per la Lotta contro la *Lobesia botrana* della Vite

Riassunto

MVP® è il primo in una nuova classe di prodotti biopesticidi basato sulla nuova tecnologia di bioincapsulazione CellCap® di Mycogen Corporation. MVP contiene una δδ-tossina di *Bacillus thuringiensis* varietà *kurstaki* (B.t.k.) che è altamente attivo contro le specie di mosca dell'uva, *Lobesia botrana* e *Eupoecilia ambiguella*, come anche la *Plutella xylostella (L.), Helicoverpa spp*, ed un numero di altre importanti specie agricole di parassiti. L'efficacia di MVP è stata valutata in prove parcellari su vigneti in Italia, Francia, Spagna, Germania e Svizzera. Nel sistema CellCap, il gene di questa tossina è stato prima introdotto nel genoma di un ceppo di *Pseudomonas fluorescens* (P.f.). Nel processo di lavorazione questo ceppo di *P. fluorescens* viene messo in coltura in grandi fermentatori, dove la tossina è prodotta come un grande cristallo intracellulare, più grande di quello prodotto nel *B. thuringiensis*. Tuttavia, diversamente dal *B. thuringiensis* le cellule P.f. non si dissolvono o spezzano a maturazione. Quando la fermentazione è completa, le cellule di P.f. intatte vengono uccise e fissate nel fermentatore usando un processo brevettato che incapsula i cristalli di tossina tra il citoplasma stabilizzato e le pareti cellulari delle cellule batteriche devitalizzate.

L'effetto del processo di bioincapsulazione CellCap nella protezione della δ-tossina dal degradamento ambientale è stato valutato in prove parcellari su cavoli e broccoli e confrontato con due prodotti standard di *B. thuringiensis*. L'attività residua è stata stimata usando un test biologico su un disco fogliare. Queste prove hanno dimostrato che MVP fornisce attività residua due o tre volte maggiore dei prodotti B.t. convenzionali contenenti i cristalli di tossina non protetti. Prove simili sono state condotte su vigneti in Italia ottenenedo un incremento simile nell'attività residua.

Introduction

The demand for alternative insecticide products and pest management strategies for managing pest populations has grown as growers, policy makers, and the general public in many countries have become more aware of the problems associated with the use of broad spectrum chemical insecticides in crop protection. Environmental contamination, increasing pest resistance, worker safety concerns, and the potential hazards associated with pesticide residues on crops are all problems that have become increasingly associated with the dependence upon broad spectrum chemical insecticides. In addition to insecticide resistance, the effect of these compounds on beneficial insect populations frequently results in secondary pest outbreaks and pest resurgence, and is increasingly recognized as adversely impacting our ability to manage pest populations in general.

The need for alternative strategies to mitigate these side effects of dependence on broad spectrum insecticides, has been noted many times and led to the concept of integrated pest management (IPM) (Smith and Reynolds 1966, Smith and van den Bosch, 1967). The development of economic injury levels and action thresholds and at least the first stages of progress toward implementing IPM programs has now occurred in many different crop situations.

However, the development of efficacious, selective, alternative control methods and products to use in IPM programs is essential for these programs to be successful. Among the most promising of these alternatives are products based on insect pathogens and naturally occurring insect toxins. These bioinsecticides or microbial insecticides, are effective and specific for target pests, offering a great deal of promise in resolving some of the problems commonly associated with dependence on broad spectrum insecticides. In addition, by offering a completely different mode of action from conventional neurotoxic chemical insecticides they can be very useful in managing resistance to these chemicals.

The most successful of the bioinsecticide products developed to date are those based on *Bacillus thuringiensis* (B.t.), a spore-forming bacteria that produces a selectively toxic protein in the form of an inclusion or crystal within the cell. This protein inclusion is the active component in B.t. products and consists of the protoxin form of one or more *δ*(delta)-endotoxins. When this inclusion is ingested by a susceptible insect hose, it is solubilized and the protoxin(s) processed to the active *δ*-endotoxin form. This active toxin then binds to and destroys the midgut epithelium, causing a rapid gut paralysis and cessation of feeding in less than an hour after ingestion in the most sensitive species. Death generally occurs within one to three days.

Effective commercial B.t. products for the control of caterpillars have been available for over twenty years and have been used against caterpillar pests of grapevines, vegetables, forests and fruit for many years. However, conventional B.t. products have some serious limitations. One of the most significant of these is the short residual activity under field conditions.

During the fermentation of B.t. cells, spores and toxin crystals are produced and released into the medium when cell walls lyse at the conclusion of the fermentation cycle. It is these spores and crystals which constitute the active ingredient in conventional B.t. products. When a grower applies a conventional B.t. insecticide to his crop, he is applying these spores and naked crystals of *Bacillus thuringiensis*. It is these unprotected toxin crystals that are so susceptible to degradation (Figure 1A). Ultraviolet radiation (U.V.) has been shown to rapidly degrade the activity of B.t. products (Ignoffo et al. 1977, Morris 1983; Sneh and Schuster 1981). In a given crop, the bulk of activity can be lost within two to three days. The

insecticidal half-life of B.t. products exposed to direct sunlight has been estimated to be 1.5 to 2 days on cotton foliage (Beegle et al. 1981) and two days on white spruce (Morris and Moore, 1975). This means a lethal dose is present for only a relatively short time, decreasing efficacy and making frequent applications necessary. This lack of foliar persistence is a key factor in the inconsistent performance of conventional B.t. insecticides. Mycogen has developed the patented CellCap encapsulation system to address this limitation.

Mycogen's CellCap Biological Encapsulation System

The CellCap bioencapsulation and delivery system is Mycogen's proprietary technology for enhancing the field persistence and efficacy of B.t. toxins.

The development of a new biopesticide product based on this CellCap process actually begins when a B.t. isolate with the desired host range and level of potency is identified in a focused screening and bioassay effort against a particular target pest.
The gene(s) coding for the desired ∂-endotoxin(s) is then isolated and transferred into a *Pseudomonas fluorescens* (P.f.) host isolated from tomato foliage. In the manufacture of a CellCap product the transformed P.f. cells are cultured in large-scale fermentors. Unlike B.t. cells which lyse at the end of the fermentation cycle, the P.f. cell walls remain intact. The P.f. cells are then killed in the fermentor before harvest using a proprietary physical and chemical process. This process also fixes the cell wall and cytoplasm, creating a stable, dead cell "biocapsule" which encapsulates and protects the toxin crystal. Thus, the active component of any CellCap product contains no living cells, but rather consists of a selected toxin (or toxins) encapsulated within a dead cell "biocapsule" (Figure 1B).

MVP Bioinsecticide is the first of Mycogen's Cell Cap products, and has been developed for the control of pest lepidopterous larvae. A ∂-endotoxin from B.t. variety *kurstaki* with high activity against DBM and a number of other key caterpillar pests in vegetable crops, was selected for this product. The studies reported here were conducted to evaluate MVP and assess the effect of the CellCap biological encapsulation technology on the efficacy and foliar persistence of B.t. toxins.

Materials and Methods

1. MVP® Bioinsecticide Foliar Persistence Studies

Cabbage and broccoli - Foliar persistence of MVP on cabbage and broccoli was evaluated in small plot tests in 1988 and 1989 in the United States in the states of Florida, California, Wisconsin, and North Carolina. A total of 20 studies were conducted over these two years. These experiments were conducted in small plots of broccoli or cabbage treated with MVP and two representative registered B.t. products, coded as Check "A" and Check "B". Applications were made at label rates of each product. Test materials were applied either with a CO_2 backpack sprayer or tractor mounted boom sprayer, using one overhead and two drop hollow cone nozzles (spraying Systems TXUS8). Leaf disc samples (200 discs per treatments) were collected at several post-treatment intervals beginning with the day of treatment and ending at 12 days. These leaf discs were brought back to the lab and activity assayed by placing a single third instar DBM larvae on each disc and evaluating mortality four days after infestation. The method developed for these tests has proven to be a highly sensitive technique for quantifying residual B.t. activity.

Grapevines - Foliar persistence was also evaluated on grapevines in Italy by Ioriatti (1992) at the Istituto San Michele all'Adige. In these tests, field treated grapes were sampled at intervals after application with MVP and three conventional B.t. products. Sampled grape berries were brought back to the laboratory and residual activity determined using a biological assay with *L. botrana* larvae.

2. MVP Bioinsecticide Field Efficacy Evaluations

Small plot tests - Between 1989 and 1993, MVP was tested in Italy, Spain, France, Germany, and Switzerland in cooperation with Shell Italia, Shell España, Agrishell, Shell Agrar and Agroplant respectively, against the grape berry moths, *L. botrana*, and *E. ambiguella* in small plot trials on grapes. These trials included competitive B.t. products, as well as at least one standard chemical treatment generally utilized by growers in the particular region. One or two treatments were made for control of second and third generation. Some trials on first generation were also conducted. The first application was made when the

first eggs began to hatch based on direct field observations. Pheromone traps were also used as a guide in determining when oviposition might begin. In a limited number of trials, a second treatment was made seven to 14 days later. Either hydraulic or mist blower backpack sprayers were used to apply the test materials.

Label rates of registered products were applied in these trials. MVP was applied at one to four liters/ha, with most treatments being made at two to four liters/ha. Grape bunches were generally sampled 30 days post-treatment and the number of *L. botrana* larvae and the number of superficial and deep penetrations of berries estimated. Several trials evaluated the efficacy of MVP on *E. ambiguella*.

The use of sugar (sucrose) has been shown to enhance the efficacy of B.t. products (Charmillot, et. al., 1991) and several trials were conducted to evaluate the effect of adding sugar to the spray tank (0.5% or 1%) on the efficacy of MVP.

Results

1. MVP Bioinsecticide Persistence Studies

Foliar persistence test in vegetables - The CellCap system was found to consistently enhance foliar persistence of B.t. ∂-endotoxin on cabbage and broccoli when compared to conventional B.t. products. Generally, within the first two days after application, insecticidal activity of the conventional B.t. products tested had dropped well below 70% of the original activity. In contrast with MVP, insecticidal activity remained above 70% of the original level even after four days. By seven days, residual activity of MVP was generally above 40%, while both Check "A" and Check "B" had dropped below 20%. In 20 studies conducted in four different geographic regions, MVP consistently provided high levels of residual activity two to three times greater than those recorded for the conventional products seven days after treatments. Figure 2 provides an example of the data collected during the two years of foliar persistence studies. In this study, residual activity of MVP averaged 35% after seven days versus 14% for Check "A".

Foliar persistence studies in grapevines - Studies in grapevines on the persistence of MVP, demonstrated a two -fold enhancement of residual activity compared to three conventional dry B.t. products, two of which were new products and one an older established product (Ioriatti, 1992). Activity of the three traditional B.t. products after seven days had fallen to 54, 36, and 33% mortality from 78,66 and 90% initial activity, while MVP was still at 79% mortality as compared to 90% mortality initially. The conventional products were reapplied at eight days. MVP was not reapplied, but at 21 days after treatment still showed 40% mortality as compared to 40, 35, and 15% for the second treatment of the other products. Additional trials were conducted by in 1992 and 1993 with similar results showing the enhanced persistence of MVP and are being reported at this meeting by Dr. Ioriatti.

2. MVP Bioinsecticide Field Efficacy

Small Plot Trials - MVP provided excellent control of *L. botrana* for both second and third generations in small plot tests conducted between 1989 and 1993 in Italy, Spain, France, and Germany. Typically MVP provided performance that was similar to that achieved with the chemical standards and was superior to that observed with other B.t. products. Sample data from these trials are presented in Figures 3 as well as Table 1 and show that levels of control well in excess of 95% were achievable with MVP.

The optimum rate for control of *L. botrana* appears to be three liters per hectare, although a number of trials such as that presented in Figure 3, showed that doses of less than three liters could provide good efficacy. Timing of application was shown to be important, with best results being achieved when applications were made at initiation of egg hatch. Good spray coverage of the grapes was likewise very important.

The addition of sugar (sucrose) a 0.5 or 1 kg. to the spray tank gave variable results in trials on second generation. In Italy, sugar appeared to enhance the activity of MVP slightly from 93% to 99% efficacy (Table 1). In France, the results were similar with little or no improvement being noted when sugar was used (Figure 3). Tests in Germany at lower product rates, however, showed that sugar could substantially improve activity. In one test, for example, a 1 liter/ha rate plus 1 kg sugar produced 50% control, while 3 liters/ha without sugar showed only 30% control. The three liter rate with sugar, produced 73% control, more than twice the control with the product alone.

Charmillot and his co-workers (1992) in Switzerland found MVP to have excellent field efficacy on both *L. botrana* and *E. ambiguella*. In their trials in 1991 with *L. botrana*, they found that the cumulative efficacy (of MVP over 21 days to be 93% while the B.t. standard, provided 86% efficacy. The other two B.t. products had cumulative control levels of 88 and 73%. In these trials, the efficacy of the chemical standard, parathion, at 91% was below that of MVP.

Small plot trials in Germany and Switzerland also demonstrated good efficacy of MVP on *E. ambiguella*. In the Swiss trials, for example, the cumulative efficacy of MVP over 17 days was found to be 86% while the B.t. standard was second at 80%. The other two B.t. products tested had efficacy below this at 76 and 63% (Charmillot et al 1992).

Discussion

The results obtained in the studies reported here have demonstrated that the CellCap bioencapsulation and delivery system can be used to produce very efficacious, longer residual bioinsecticides. The increased residual activity of MVP, coupled with its selected toxin with high activity against *L. botrana* and *E. ambiguella* is proving to be a highly effective combination for control of these species in grapevines in Italy and the rest of Europe. MVP performance against these key pests of grapes has surpassed conventional B.t. products, and has proven to be comparable to that of the standard synthetic chemical treatments.

In addition to the benefits of a high potency toxin and longer residual activity in the field, the CellCap system also offers superior shelf life stability in a liquid formulation, something difficult to achieve in spore-containing formulations.

MVP also has benefits it shares with other B.t.-based products. The *B. thuringiensis* variety *kurstaki* ∂ -endotoxins have been extensively tested on mammals and other non-target organisms as part of the regulatory requirements for the registration of the various commercial B.t. products now on the market in many countries. In these test, the B.t. ∂-endotoxins examined have been found to be highly selective, showing a consistent lack of toxicity to mammals, birds, fish, beneficial insects, key aquatic invertebrates, and plants. Acute toxicology tests conducted with MVP have likewise shown no toxic effects on any of these non-target species. Because of their very high degree of safety, B.t. ∂-endotoxins are exempted from

Figure 1. Transmission electron micrograph of the CellCap® active ingredient in MVP ® Bioinsecticide, showing the *Bacillus thuringiensis* var. *kurstaki* ®∂-endotoxin protein crystal encapsulated within killed and stabilized *Pseudomonas fluorescens* cells.

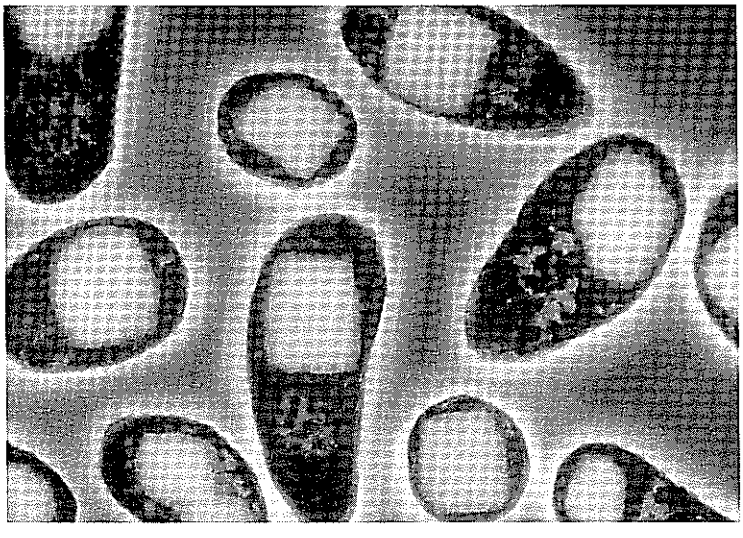
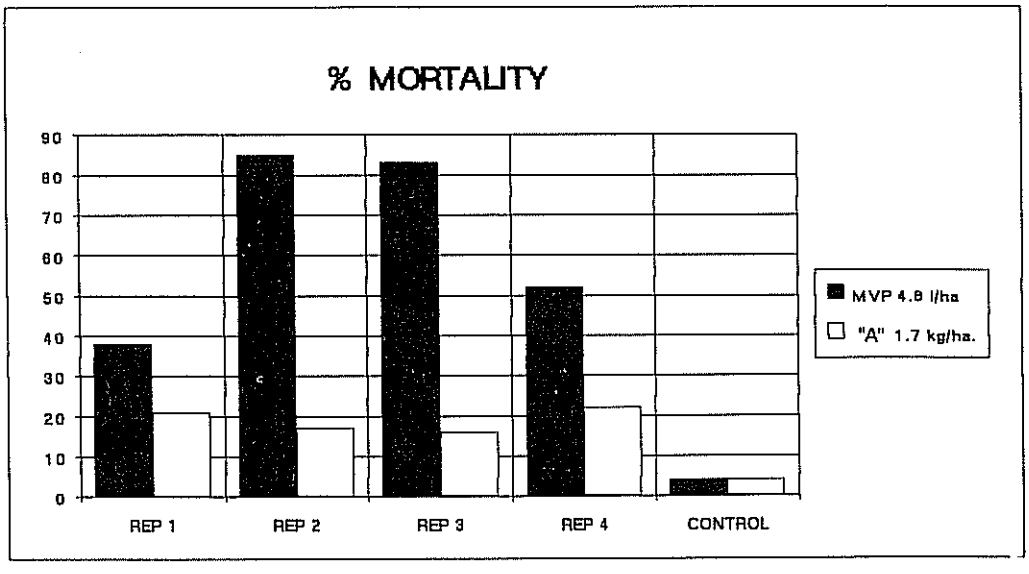


Figure 2. Comparison of persistence of MVP® Bioinsecticide aqueous flowable at 4.6 l/ha and a conventional *Bacillus thuringiensis* var. *kurstaki* product (water dispersible granule) coded as check "A", applied at 1.7 kg/ha, on broccoli, as determined by laboratory biological assays (*Plutella xylostella* third instar larvae) of leaf disc samples of field treated broccoli plants from plots in Yuma, Arizona, U.S.A..

A) Bar graph showing residual activity found in each of 4 replicate samples (50 leaf discs per replicate) taken 4 days after treatment.



B) Residual activity degradation curve for first seven days after treatment (error bars show standard deviation above and below the mean)

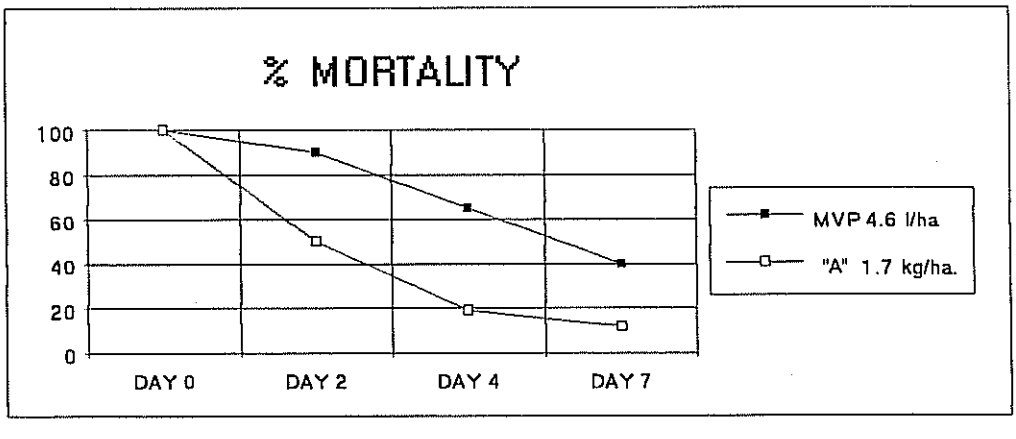


Figure 3. Efficacy of MVP® Bioinsecticide on second generation Grape Berry Moth, *Lobesia botrana*, on Grapevines in southern France (Provence) (Control: 4 larv/bunch)

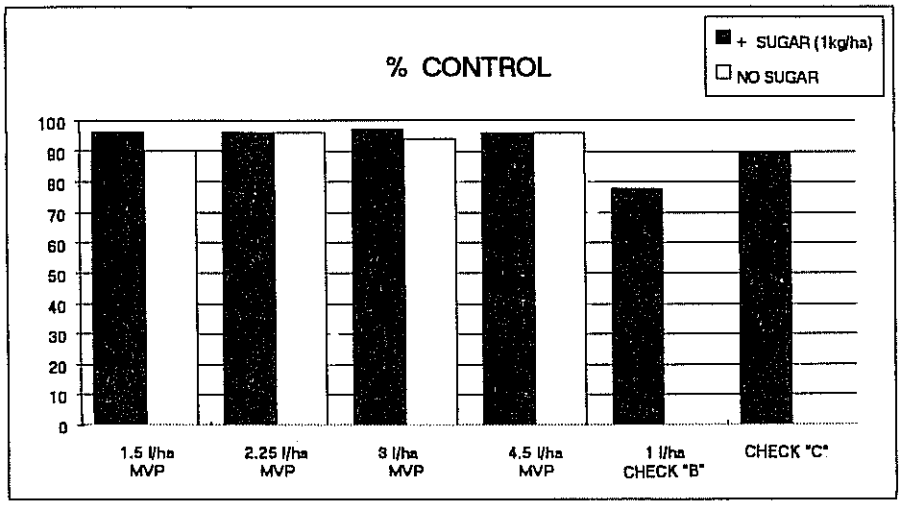


Table 1. Efficacy of MVP® Bioinsecticide on the second generation Grape Berry Moth, *Lobesia botrana*, in Northern Italy, in comparison with a conventional B.t. product and chemical standard

	Treatment	Dose Rate per ha.	No. of Applications	Sugar added/ha.	No. larvae/ 100 bunches	% Control
1	MVP Bioinsecticide	4 liter	1	500 gm.	3	99
2	Check "D"	1 kg.	1	500 gm.	22	92
3	MVP Bioinsecticide	4 liter	2	0	9	97
4	MVP Bioinsecticide	2 liter	2	0	10	96
5	MVP Bioinsecticide	4 liter	1	0	10	96
6	Check "E"	40 gm.	1	0	5	98
7	Untreated check				275	0

tolerance in the US and other countries and an be applied up to the day of harvest with no re-entry period or pre-harvest restrictions. This provides growers with important flexibility of use.

The selectivity of action is a key advantage offered by MVP and other B.t. products, particularly when these products are used integrated pest management (IPM) programs. This specificity means that B.t. products can be used to regulate pest populations without direct toxicity to the natural enemies of the target pest and other phytophagous insects in the crop. The result of this complementary action is that secondary pest outbreaks and pest resurgence problems can be avoided.

The concept of IPM stresses the need to understand and build on existing ecological processes and key interspecific relationships that are present in particular agro-ecosystems and utilizing these to maximum advantage in regulating and managing pest populations. This construction of pest management systems from the bottom up, building on this foundation of existing ecological factors is still a long way from being the primary approach in pest management and inmost crop systems we are at best still in the very earliest stages of IPM development and implementation. It is clear that as these more sophisticated and presumably more successful IPM systems are developed, the availability and use of selective control tactics will become increasingly important as management tools that are effective and yet also compatible with existing natural enemies and other ecological factors that contribute to regulation of pest populations. B.t. δ-endotoxin-based products like MVP are among the most effective of the selective insecticides that are currently available and among the least disruptive to the existing natural enemy complex, therefore minimizing the potential for pest resurgence and secondary pest outbreaks. In this regard, they can fill a key niche in many IPM programs by offering effective yet selective action, a benefit which is not offered by many other products. The novel CellCap technology gives MVP Bioinsecticide some unique features that can offer even greater benefits to growers in the use of these toxins in crop protection in general and IPM in particular.

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