Comments Regarding Polyoxin D Zinc Salt
for the April 10, 2013 NOSB Public Hearing

Submitted by
Kaken Pharmaceutical Co., Ltd.
c/o Cynthia Ann Smith
Vice President
Conn & Smith, Inc.
6713 Catskill Road
Lorton, VA 22079 USA

March 6, 2013

CONTENTS

Page

EXECUTIVE SUMMARY

1. NOT AN ANTIBIOTIC

Neither "antibiotic" nor "antibiotic drug" are defined:

- In the Federal Insecticide Fungicide and Rodenticide Act (FIFRA);
- By the U.S. Department of Agriculture; or
- By the National Organic Program.

The decision to include or exclude polyoxin D zinc salt from 7 CFR §205.601 is a regulatory decision. Therefore, only a regulatory definition of "antibiotic" or "antibiotic drug" should be used in NOSB's and NOP's decision making. Otherwise, the regulatory decision would be arbitrary and capricious.

The Federal Food Drug and Cosmetic Act (FFDCA) defines an "antibiotic drug" and requires intended use in humans or animals. Section 201 of 21 U.S.C. 321 states:

> "(jj) *The term "antibiotic drug" means any drug (except drugs for use in animals other than humans) composed wholly or partly of any kind of penicillin, streptomycin, chlortetracycline, chloramphenicol, bacitracin, or any other drug intended for human use containing any quantity of any chemical substance which is produced by a micro-organism and which has the capacity to inhibit or destroy micro-organisms in dilute solution (including a chemically synthesized equivalent of any such substance) or any derivative thereof.*" [Emphasis added.]

Polyoxin D zinc salt has always been marketed exclusively as a plant protectant. Polyoxin D zinc salt is not and has never been intended for use in humans or animals. Therefore, polyoxin D zinc salt is not an antibiotic as defined by the FFDCA.

2. POTENTIALLY NON-SYNTHETIC, BUT PROPOSED AS SYNTHETIC

Polyoxin D is produced via a fermentation process and is believed to be non-synthetic. Kaken buys the zinc source to convert polyoxin D to polyoxin D zinc salt. Kaken does not control the production process for the zinc source and cannot assure that it is mined. Therefore, polyoxin D zinc salt is proposed as a synthetic material.

3. UNIQUE, NON-TOXIC MODE OF ACTION

Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal. This makes polyoxin D zinc salt an excellent tool for integrated pest management (IPM).

Polyoxin D zinc salt is the only registered pesticide with this mode of action. This makes polyoxin D zinc salt an excellent tool for resistance management.

4. POLYOXIN D ZINC SALT IS PRACTICALLY NON-TOXIC TO HONEYBEES

The 96-hr LD_{50} of polyoxin D zinc salt to honeybees was determined to be 28.774 µg/bee. Using EPA's classification criteria, polyoxin D zinc salt is practically non-toxic to honeybees.

EPA Bee Hazard Category [1]	96-hr LD_{50} (µg/bee)
Highly toxic	< 2
Moderately toxic	2-11
Practically non-toxic	> 11

1. http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm

5. POLYOXIN D ZINC SALT HAS NO ADVERSE EFFECTS ON OTHER BENEFICIAL INSECTS

Polyoxin D zinc salt has no adverse effects on silkworm, marmalade hoverfly, and green lacewing.

Beneficial Insect	End-point	Observations
Silkworm (Kinshu x Showa)	LC_{50} > 2100 mg/L	No adverse effects observed.
Marmalade hoverfly	10-day LC_{50} > 2100 mg/L	No adverse effects observed.
Green lacewing	14-day LC_{50} > 2100 mg/L	No deaths.

6. RAPID DEGRADATION UNDER NORMAL ENVIRONMENTAL CONDITIONS

Polyoxin D zinc salt rapidly degrades in the presence of water and sunlight. In sterile natural water, polyoxin D degraded by:

- 50% in 0.4 days (9.6 hours); and
- 90% in 1.2 days (less than 29 hours).

7. NEGLIGIBLE EXPOSURE AND RISK TO FISH AND AQUATIC INVERTEBRATES

Aquatic exposure and aquatic risk are negligible because:

- Polyoxin D zinc salt formulations are for terrestrial use only;
- Application rates are low; and
- Polyoxin D zinc salt degrades rapidly under normal environmental conditions.

8. SAFETY TO HUMANS

The polyoxin D zinc salt formulation developed for the organic market (EPA Reg. No. 68173-4) has such low toxicity that EPA does not require a first aid statement. Also, polyoxin D zinc salt has been determined by EPA to not cause DNA damage or long-term health effects.

On September 12, 2012, EPA established an exemption from the requirement of a tolerance for the residues of polyoxin D zinc salt in or on all food commodities when applied as a fungicide and used in accordance with good agricultural practices (40 CFR § 180.1285). This exemption includes pre-harvest and post-harvest uses.

9. EFFICACY

Polyoxin D zinc salt provides curative control for most diseases; the alternatives generally provide only preventative control.

Polyoxin D zinc salt provides curative control for three crop/disease combinations with no OMRI listed alternatives:

- Cucurbits/Southern blight (*Sclerotium rolfsii*);
- Ginseng/Cylindrocarpon root rot (*Cylinderocarpon destructans*); and
- Pome fruit/Leaf blotch (*Diplocarpon mali*).

Polyoxin D zinc salt is not phytotoxic and does not cause russeting (cosmetic effect with crop value reduction) of apples.

10. LARGE NUMBER OF EPA AND CALIFORNIA REGISTERED USES

There are 73 EPA registered crop/disease combination uses of polyoxin D zinc salt, many of which are for entire crop groups. Most of the uses are also registered in California. New uses are in development.

11. REQUESTED SUPPORT FOR INCLUSION IN 7 CFR §205.601

For the reasons stated above, Kaken Pharmaceutical Co. Ltd. requests the support of the National Organic Standards Board and National Organic Program for the inclusion of polyoxin D zinc salt in 7 CFR §205.601 to permit the use of polyoxin D zinc salt in organic crop production.

DETAILED COMMENTS

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
1. Are there adverse effects on environment from manufacture, use, or disposal? [§205.600 b.2]			X		Not adverse.
2. Is there environmental contamination during manufacture, use, misuse, or disposal? [§6518 m.3]	X			The TR (lines 190-195) states that the EPA considers polyoxin D zinc salt a low environmental risk, listing several reasons for this rationale. Also, included in the supplemental information submitted by the petitioner on October 2, 2012 as part of an EPA posting to the Federal Register on September 12, 2012.	Not adverse.
				The TR does mention (line 194) that failure to follow the product label could result in death of fish and aquatic organisms. In the TR (lines 197-204) states that biopesticides generally pose lower risks than chemically produced pesticides.	Aquatic exposure and aquatic risk are very low because: • Polyoxin D zinc salt formulations are for terrestrial use only; • Application rates are low; • Polyoxin D zinc salt degrades rapidly under normal environmental conditions. Please see pages 19-20 of http://tinyurl.com/C-Smith-1-23-2013 . The September 12, 2012 published final rule for polyoxin D zinc salt states on page 56131 of the Federal Register: *"2. Drinking water exposure. As stated in the previous tolerance exemption (73 FR 69562), there is a small potential for trace amounts of polyoxin D zinc salt to enter drinking water sources after a significant rainfall, via surface water runoff, and/or via incidental spray drift. The petitioner submitted a photodegradation in water study (MRID 48653305) to support this tolerance exemption. The results of the study show that polyoxin D zinc salt has a net photolytic half-life of 0.4 days in sterile natural water (See Ref.). Even if residues of polyoxin D zinc salt enter water sources, residues are expected to degrade and be so diluted as to be negligible. The data and information demonstrate a lack of aggregate dietary risk via drinking water and is sufficient to support this expansion of the tolerance exemption."*

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				The manufacturing process is CBI, but the TR states the process would be similar to other antibiotics produced from *Streptomyces.* (TR July 11, 2102)	Please see page 5 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D is produced via an aerobic fermentation process. Polyoxin D is converted to polyoxin D zinc salt using an aqueous process. No organic solvent impurities are present in Polyoxin D Zinc Salt Technical. Zinc is a mined mineral. Please see http://www.zinc.org/basics/zinc_production. Zinc is also recycled. Please see http://www.zinc.org/basics/zinc_recycling. Kaken is not the producer of the zinc source used in the production of polyoxin D zinc salt and does not know if the zinc is "virgin" zinc from a mine or recycled zinc.
				The TR states (lines 190-204) that polyoxin D could get into water if misused by not following the label.	Aquatic exposure and aquatic risk are very low because: • Polyoxin D zinc salt formulations are for terrestrial use only; • Application rates are low; • Polyoxin D zinc salt degrades rapidly under normal environmental conditions. Please see pages 19-20 of http://tinyurl.com/C-Smith-1-23-2013 : The September 12, 2012 published final rule for polyoxin D zinc salt states on page 56131 of the Federal Register: *"2. Drinking water exposure. As stated in the previous tolerance exemption (73 FR 69562), there is a small potential for trace amounts of polyoxin D zinc salt to enter drinking water sources after a significant rainfall, via surface water runoff, and/or via incidental spray drift. The petitioner submitted a photodegradation in water study (MRID 48653305) to support this tolerance exemption. The results of the study show that polyoxin D zinc salt has a net photolytic half-life of 0.4 days in sterile natural water (See Ref.). Even if residues of polyoxin D zinc salt enter water sources, residues are expected to degrade and be so diluted as to be negligible. The data and information demonstrate a lack of aggregate dietary risk via drinking water and is sufficient to support this expansion of the tolerance exemption."*

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				Waste may be disposed of on site or at an approved waste facility, but not disposed of in waste water. (TR July 11, 2012)	The Environmental Hazard statement for products containing polyoxin D zinc salt includes: "Do not contaminate water when disposing of equipment wash water or rinsate." This is a standard statement included in the Environmental Hazards section of EPA registered pesticide labels, including OMRI alternative products, *e.g.*, • *Bacillus subtilis* strain QST 713 (Seranade Max; EPA Reg. No. 264-1151); • *Reynoutria sachalinensis* (Regalia Max; EPA Reg. No. 84059-6): • *Streptomyces lydicus* WYEC 108 (Actinovate SP; EPA Reg. No. 73314-1); and • *Bacillus amyloliquefaciens* strain D747 (Double Nickel; EPA Reg. No. 70051-108).

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
3. Is the substance harmful to the environment and biodiversity? [§6517c(1)(A)(I); 6517(c)(2)(A)I]	X	X		Polyoxin D zinc salt is moderately toxic to fish and aquatic invertebrates and should not be discharged into water. (TR lines 279-280). If label instructions followed, those concerns would be mitigated (EPA, 2001)(TR lines 290-291).	Aquatic exposure and aquatic risk are very low because: • Polyoxin D zinc salt formulations are for terrestrial use only; • Application rates are low; • Polyoxin D zinc salt degrades rapidly under normal environmental conditions. Aquatic exposure and aquatic risk are very low. Please see pages 19-20 of http://tinyurl.com/C-Smith-1-23-2013 : The September 12, 2012 published final rule for polyoxin D zinc salt states on page 56131 of the Federal Register: *"2. Drinking water exposure. As stated in the previous tolerance exemption (73 FR 69562), there is a small potential for trace amounts of polyoxin D zinc salt to enter drinking water sources after a significant rainfall, via surface water runoff, and/or via incidental spray drift. The petitioner submitted a photodegradation in water study (MRID 48653305) to support this tolerance exemption. The results of the study show that polyoxin D zinc salt has a net photolytic half-life of 0.4 days in sterile natural water (See Ref.). Even if residues of polyoxin D zinc salt enter water sources, residues are expected to degrade and be so diluted as to be negligible. The data and information demonstrate a lack of aggregate dietary risk via drinking water and is sufficient to support this expansion of the tolerance exemption."*
				Should be considered toxic to various soil fungi and bacteria (TR lines 234-235). However, the TR (lines 241-251) does state that alternative fungicides, such as copper or sulfur, may have similar or more severe effects. No documented studies to verify the effects by comparison to other fungicides.	**Polyoxin D zinc salt is NOT toxic to fungi, including soil fungi.** Please see pages 6-7 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal. **Polyoxin D zinc salt is NOT toxic to bacteria, including soil bacteria.** Please see pages 57-62 of http://tinyurl.com/C-Smith-1-23-2013 for the Maximum Inhibitory Concentration data. Polyoxin D zinc salt is not efficacious for use as an antibiotic to kill bacteria.

<table>
<tr><th colspan="5">Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?</th></tr>
<tr><th colspan="4">Crops Subcommittee Petitioned Material Proposal (January 29, 2013)</th><th rowspan="2">Kaken's Comments</th></tr>
<tr><th>Question</th><th>Yes</th><th>No</th><th>N/A</th><th>Documentation
(TAP; petition; regulatory agency; other)</th></tr>
<tr><td></td><td></td><td></td><td></td><td>In the TR it mentions (TR line 54) Action of Substance: Inhibits cell wall chitin synthesis (Misato, 1977, O'Neill, 2006).</td><td>EPA's May 11, 2012 science review of polyoxin D zinc salt states on page 3,
"[The mode of action of] Polyoxin D and its zinc salt is the inhibition of chitin synthesis in the cell walls of fungi, some of which are pathogenic to plants. This inhibition of chitin synthesis is limited to chitin in fungal cell walls. Polyoxin D and its zinc salt do not inhibit the synthesis of chitin in animals that contain chitin, such as for insects and crustaceans that contain chitin in their exoskeletons. Polyoxin D Zinc Salt does not affect mammals because mammalian cells have plasma membranes that do not contain chitin."</td></tr>
<tr><td></td><td></td><td></td><td></td><td>It further states (TR lines 257-262) it has been shown to inhibit chitin synthetase in cockroaches, and may therefore affect beneficial insects.</td><td>Please see pages 25-26 of http://tinyurl.com/C-Smith-1-23-2013 for data regarding non-target insects. Polyoxin D zinc salt:
• Is practically non-toxic to honeybees using EPA's criteria (EPA's least hazardous classification); and
• Has no adverse effects on:
  • Silkworm;
  • Marmalade hoverfly;
  • Green lacewing; and
  • Wolf spider.
The article by Leighton et al. (1981) referenced in the TR reports research using insect organ cultures, not whole insects. The article makes no statement about any effects of polyoxin D zinc salt or polyoxin D on whole insects as suggested in the TR. A copy of the article is provided as APPENDIX 1.</td></tr>
<tr><td></td><td></td><td></td><td></td><td>EPA: Toxic to Honey Bees. [1]</td><td>Please see page 26 of http://tinyurl.com/C-Smith-1-23-2013 .
The 96-hr LD_{50} of polyoxin D zinc salt to honeybees was determined to be 28.774 µg/bee. Using EPA's classification criteria, polyoxin D zinc salt is practically non-toxic to honeybees (EPA's least hazardous classification).
http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm
<table>
<tr><th>EPA Bee Hazard Category</th><th>96-hr LD_{50} (µg/bee)</th></tr>
<tr><td>Highly toxic</td><td>< 2</td></tr>
<tr><td>Moderately toxic</td><td>2-11</td></tr>
<tr><td>Practically non-toxic</td><td>> 11</td></tr>
</table>
If polyoxin D zinc salt were toxic to bees, there would be a bee hazard statement in the Environmental Hazards section of the label. None of the polyoxin D zinc salt product labels have a bee hazard statement on the label.</td></tr>
</table>

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				Kaken cites EPA.[2] "Polyoxin D and its zinc salt do not inhibit the synthesis of chitin in animals that contain chitin, such as for insects and crustaceans that contain chitin in their exoskeletons."	Not adverse.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
4. Does the substance contain List 1, 2 or 3 inerts? [§6517 c (1)(B)(ii); 205.601(m)2]		?		The TR states that Polyoxin D Zinc Salt is formulated with undisclosed inert ingredients. TR line 58 (TR July 11, 2012) The TR further states that the preferred surfactants used in the dry flowable form are formalin sodium naphthalenesulfonate (inert list 4B) or non-ionic polyoxyethylene alkyl ethers (inert list 4B) (Tokumura, et al., 2001). Formulation process is CBI.	The formulation that has been developed for the organic market is VEGGIETURBO 5SC Suspension Concentrate Fungicide (EPA Reg. No. 68173-4). None of the ingredients in this formulation are on EPA's Inert Ingredient List 1, 2, or 3.
5. Is there potential for detrimental chemical interaction with other materials used? [§6518 m.1]	X	X		Because of its activity as a fungicide, it may have a negative impact on beneficial fungi. Polyoxin D inhibits the germination of *Trichoderma viride* (Benitez, et al., 1976). *T. viride* is closely related to *T.harzianum,* which is used in organic farming under the brand name Root Shield (OMRI, 2012). There are a couple of other fungi used as biological controls in organic farming. (TR lines 216-222).	Polyoxin D zinc salt: • Does not kill fungi; it prevents its growth. • Degrades rapidly under normal environmental conditions. In 1.2 days, 90% degradation has occurred. Any adverse impacts on beneficial fungi in the soil will be only temporarily. Polyoxin D zinc salt can be used in the same field that is treated with live fungal active ingredients. If it were tank mixed with products with live fungal active ingredients, polyoxin D zinc salt would not kill the other active ingredient but instead would delay its action.
				However, it has also been shown to promote the biocontrol of Bacillus subtilis, with a strong synergistic effect on Alternaria mali suppression. (TR lines 225-226) (TR July 11, 2012)	Not adverse.
				Also, in the TR (TR lines 220-224) it lists *Gliocladium virens, Paecilomyces fumosoroseus,* and *Streptomyces griseoviridis* as other fungi used as biological control agents in organic agriculture. *G virens* is marketed as SoilGard, *P. fumosoroseus* is the active ingredient in PFR-97 and *S.griseoviridis* is sold as Mycostop (OMRI, 2012).	See above.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				(TR line 223) states that polyoxin D zinc salt was found to reduce the efficacy of the virus used to control the black cutwork (sic)(*Agrotis ipsilon)* (Bixby-Brosi and Potter, 2012)	Please see page 22-23 of http://tinyurl.com/C-Smith-1-23-2013 . Bixby-Brosi and Potter (2012) concludes that polyoxin D is compatible with *Agip*MNPV. The abstract for Bixby-Brosi and Potter (2012) includes: *"This study tested whether applying the virus [AgipMNPV] together with such a fungicide [polyoxin D] can synergize AgipMNPV activity against A. ipsolon in turfgrass."* *"RESULTS: The addition of chitin synthesis inhibitor failed to increase AgipMNPV infectivity to A. ipsolon in the field. Rather, delayed and slightly reduced mortality from viral infection was seen when larvae fed on fungicide/virus treated grasses as opposed to virus-only treatment. Choice tests revealed fungicide residues to be a mild feeding deterrent."* *"CONCLUSION: Because polyoxin-d does not inactivate AgipMNPV, the two substances are compatible. However, combination applications of polyoxin-d and AgipMNPV on turfgrass might interfere with the larval ingestion of a lethal virus dose, resulting in prolonged larval feeding in the field."* [Emphasis added.] A copy of Bixby-Brosi and Potter (2012) is provided as APPENDIX 2.
				In the soil tests, the half-lives were 15.9 days for aerobic soils and 59.2 days for anaerobic soils. (EPA science review, p12). However, in the document provided by the petitioner (January 18, 2013 section 5.2) it states that in the presence of sunlight polyoxin D zinc salt degrades by 50% within 0.4 days (9.6 hours) "in sterile natural water, pH 5.0, pH 7.0, and pH 9.0 buffers, respectively."	Degradation rates are driven by the fastest available route. Please see page 21 of http://tinyurl.com/C-Smith-1-23-2013 . Polyoxin D zinc salt degrades rapidly under normal environmental conditions. The values reported are half-lives ($T_{1/2}$). For example, in the presence of sunlight and water, polyoxin D zinc salt: • Degrades by 50% within 0.4 days (9.6 hours); • Further degrades another 50% within another 0.4 days (another 9.6 hours); and • Further degrades another 50% within another 0.4 days (another 9.6 hours), etc. In 1.2 days (less than 29 hours), 90% degradation has occurred. "In the soil tests, the half-lives were 15.9 days for aerobic soils and 59.2 days for anaerobic soils." These studies were done in the dark (absence of sunlight).

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				The petitioner says that it inhibits fungi growth but does not kill it, maintain that it would not be a detriment to organic products such as Root Shield, currently used in organic farming (same doc. Pg 24 section 5.5).	Not adverse. See above.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
6. Are there adverse biological and chemical interactions in agro-ecosystem? [§6518 m.5]	X	X		TR 233-237: "As a broad-spectrum antibiotic and fungicide, polyoxin D Zinc Salt is toxic to soil fungi.	**Polyoxin D zinc salt is NOT an antibiotic.** Please see pages 7-14 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D zinc salt: • Is and always has marketed in the United States and elsewhere in the world exclusively as a plant protection product. • Has never been marketed for use as a pharmaceutical for use in human or animal health. • Is not efficacious for use as an antibiotic to kill bacteria. • Is not an antibiotic as defined by the Federal Food Drug and Cosmetic Act (FFDCA). (USDA, NOP, and EPA do not have a definition of "antibiotic.") Polyoxins have been repeatedly described in the literature as antibiotics based upon an arbitrary definition used in a Gottlieb and Shaw (1970). This arbitrary definition would be arbitrary and capricious if used for regulatory decision making. **Polyoxin D zinc salt is NOT toxic to fungi, including soil fungi.** Please see pages 6-7 of http://tinyurl.com/C-Smith-1-23-2013 . Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal.
				Polyoxins and other antibiotics were found to increase melanins in *Alternaria kikuchiana* (Kohno, et al., 1983; Butler and Day, 1998). The ecological functions of melanins are still unknown, but they are believed to enhance the phytotoxic and pathogenic properties of plant pathogens (Butler and Day, 1998). Earthworms were shown to have a preference for melanized fungi (Marfenina and Ischenko, 1997; Butler and Day, 1998)."	Please see pages 23-24 of http://tinyurl.com/C-Smith-1-23-2013 . • Both Kohno, *et al.* (1983) and Butler and Day (1998) are not relevant to the NOP petition for polyoxin D zinc salt. • Kohno, *et al.* (1983) describes experiments that used exclusively polyoxin B. Neither polyoxin D nor polyoxin D zinc salt were used in the study. • Butler and Day (1998) is a review article regarding fungal melanins that references Kohno, *et al.* (1983) without specifying that the findings of Kohno, *et al.* (1983) are limited to polyoxin B.
				There is some concern that polyoxin D used on turf to have a moderate risk of resistance. (Vincelli and Williams 2012)(TR lines 253-261)	Please see pages 27 of http://tinyurl.com/C-Smith-1-23-2013 . Polyoxin D zinc salt has been used for over 40 years as a crop protectant without a single observation of pest resistance.
				Again alternative materials may have similar or worse effects. (TR lines 246-248) (TR July 11, 2012)	Not adverse.
				In the Jan. 18, 2013 (pages 20 -26) document provided by the petitioner it does not actually kill fungi, just inhibits growth.	Not adverse.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				Also is not harmful to beneficial insects.	Not adverse.
				Same report (pages 27-28) also that polyoxin D zinc salt is a FRAC 19 class (Kaken 2008) (EPA Reg. No. 68173-1) of fungicide. It has a unique mode of action that would aid in resistance management as part of an IPM disease control program. Only class 19 fungicide currently listed.	Not adverse.
7. Are there detrimental physiological effects on soil organisms, crops, or livestock? [§6518 m.5]	X			The TR states that there may be adverse effects to beneficial soil organisms when exposed to polyoxin D. TR lines 241-242. It goes on to state that alternative fungicides may have similar or even greater effects on soil ecology, but that no studies could be found that compare the impacts between polyoxin D and other fungicides in organic production, specifically. TR lines 246-251. (TR July 11, 2012)	**Polyoxin D zinc salt is NOT toxic to fungi, including soil fungi.** Please see pages 6-7 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal. **Polyoxin D zinc salt is NOT toxic to bacteria, including soil bacteria.** Please see pages 57-62 of http://tinyurl.com/C-Smith-1-23-2013 for the Maximum Inhibitory Concentration data. Polyoxin D zinc salt is not efficacious for use as an antibiotic to kill bacteria. **Polyoxin D zinc salt degrades rapidly.** Please see page 21 of http://tinyurl.com/C-Smith-1-23-2013 . Polyoxin D zinc salt degrades rapidly under normal environmental conditions. In the presence of sunlight and moisture, Polyoxin D zinc salt: • Degrades by 50% within 0.4 days (9.6 hours); and • Degrades by 90% within 1.2 days.
				Is not labeled for use on livestock or pastures.	Not adverse.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
8. Is there a toxic or other adverse action of the material or its breakdown products? [§6518 m.2]		X		The following refers to polyoxin D zinc's use as an antibiotic: Polyoxin D has been shown to be effective as a drug to treat human and animal pathogens *Candida albicans* and *Cryptococcus neoformans* (Becker, et al., 1983: Hilenski, et al., 1986). Polyoxin D also shows some efficacy in the reduction of the protozoan parasite *Encephalitiozoon cuniculi* infecting immune-compromised AIDS patients (Sobottka, et al., 2002). All three of the above mentioned studies were *in vitro* experiments and not substantiated by any *in vivo* claims or studies. Polyoxin D zinc salt in currently not listed for use in human or veterinary medicine.	Please see #7 above. Please see page 11-12 of http://tinyurl.com/C-Smith-1-23-2013 for additional details.

				Moderate acute dermal toxicity; moderate toxicity primary eye irritation. (TR Table 2.)	Please see Table 2 on page 16 of http://tinyurl.com/C-Smith-1-23-2013 :
					VEGGIETURBO 5SC Suspension Concentrate Fungicide (EPA Reg. No. 68173-4) is the polyoxin D zinc salt formulation developed for the organic market. This formulation:
					• Is practically non-toxic via dermal exposure.
					  • Category IV; EPA's least hazardous category
					  • $LD_{50} \geq$ 5050 mg/kg (males, females, and combined); and
					• Is non-irritating to eyes
					  • Category IV; EPA's least hazardous category.
					  • No irritation was observed in any eyes 24 hours after treatment.
					The toxicity of the formulation is so low that EPA does not require a First Aid statement on the label.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
9. Is there undesirable persistence or concentration of the material or breakdown products in environment? [§6518 m.2]		X		The EPA's risk assessment of polyoxin D Zinc Salt to carry a low environmental risk due to its specific mode of action, low toxicity, rapid degradation and low application rate (EPA 2008) TR lines 190-191. "The EPA waived environmental fate and ground water data due to the use pattern, application methods, and mitigation of non-target aquatic organism toxicity with appropriate precautionary label statements under Environmental Hazards."	Not adverse.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				Failure to follow the label instructions may result in the death of fish and ~~194~~ aquatic organisms (EPA, 2001, 2008)." (TR 191-195)	Aquatic exposure and aquatic risk are very low because: • Polyoxin D zinc salt formulations are for terrestrial use only; • Application rates are low; • Polyoxin D zinc salt degrades rapidly under normal environmental conditions. EPA's Biopesticide Registration Action Document (BRAD) was included in the petition as Appendix 8. EPA states on page 122 of the petition (page 3 of the BRAD): "Potential exposure to freshwater invertebrates and fish, via runoff after application, will be minimized by mitigating Environmental Hazards label text." The Environmental Hazards section for the EPA stamped accepted labels state: "For terrestrial use. This pesticide is moderately toxic to aquatic invertebrates and fish. Do not apply directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water when disposing of equipment wash water or rinsate. Do not allow runoff into lakes, streams, ponds or public waterways. Drift and runoff may be hazardous to aquatic organisms in water adjacent to treated areas. Observe the most restrictive labeling limitations and precautions of all products used in mixtures." Please see pages 19-20 of http://tinyurl.com/C-Smith-1-23-2013 : The September 12, 2012 published final rule for polyoxin D zinc salt states on page 56131 of the Federal Register: *"2. Drinking water exposure. As stated in the previous tolerance exemption (73 FR 69562), there is a small potential for trace amounts of polyoxin D zinc salt to enter drinking water sources after a significant rainfall, via surface water runoff, and/or via incidental spray drift. The petitioner submitted a photodegradation in water study (MRID 48653305) to support this tolerance exemption. The results of the study show that polyoxin D zinc salt has a net photolytic half-life of 0.4 days in sterile natural water (See Ref.). Even if residues of polyoxin D zinc salt enter water sources, residues are expected to degrade and be so diluted as to be negligible. The data and information demonstrate a lack of aggregate dietary risk via drinking water and is sufficient to support this expansion of the tolerance exemption."* "194" is a line number from the TR. Inclusion of "194" in the Crops Subcommittee statement is a word processing error.

<table>
<tr><th colspan="6">Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?</th></tr>
<tr><th colspan="5">Crops Subcommittee Petitioned Material Proposal (January 29, 2013)</th><th rowspan="2">Kaken's Comments</th></tr>
<tr><th>Question</th><th>Yes</th><th>No</th><th>N/A</th><th>Documentation
(TAP; petition; regulatory agency; other)</th></tr>
<tr><td></td><td></td><td></td><td></td><td>Soil half-life from aerobic microbial metabolism is reported to be 15.9 days. Degradation in water and sunlight is reported to be approximately 2.3 days (Smith, 2012). (TR line 153)(July 11, 2012)</td><td>The fastest route of degradation drives the overall degradation rate.
Study: Aerobic soil metabolism — Observed DT_{50}: 15.9 days
Study: Aqueous photolysis: Sterile natural water — Observed DT_{50}: 0.4 days
Study: Sterile water, pH 7 buffer — Observed DT_{50}: 2.3 days</td></tr>
<tr><td>10. Is there any harmful effect on human health?
[§6517 c (1)(A)(i); 6517 c(2)(A)i; §6518 m.4]</td><td>X</td><td>X</td><td></td><td>All polyoxins have shown to have low mammalian toxicity. (Copping and Duke, 2007)(TR lines 305-309)).</td><td>Not adverse.</td></tr>
<tr><td></td><td></td><td></td><td></td><td>Could case slight skin irritation.</td><td>Please see Table 2 on page 16 of http://tinyurl.com/C-Smith-1-23-2013 :
VEGGIETURBO 5SC Suspension Concentrate Fungicide (EPA Reg. No. 68173-4) is the polyoxin D zinc salt formulation developed for the organic market.
• This formulation has Category IV skin irritation (EPA's least hazardous category).
• At 72 hours, the primary irritation index was 0.3.
The toxicity of the formulation is so low that EPA does not require a First Aid statement on the label.</td></tr>
<tr><td></td><td></td><td></td><td></td><td>Positive benefits for human and animal pathogens Candida albicans and Cryptococcus neoformans (Becker, et al. 1983: Hilenski, et al., 1986) (TR lines 311-314) Polyoxin D Zinc Salt is currently not listed for use for human or veterinary medicinal uses.</td><td>Please see page 11 of http://tinyurl.com/C-Smith-1-23-2013 .
Both Becker, et al. (1983) and Hilenski, et al. (1986) describe in vitro (outside a living organism) experiments only and makes no claim for in vivo (within a living organism) efficacy in humans or other animals.</td></tr>
<tr><td></td><td></td><td></td><td></td><td>Also has be shown to have an effect on the protozoan parasite Encephalitozoon cuniculi infecting the immune system in AIDS patients (Sobottka, et al., 2002) (TR lines 311-314) This was the result of one in vitro experiment. (TR July 11, 2012)</td><td>Please see pages 11-12 of http://tinyurl.com/C-Smith-1-23-2013 .
Sobottka, et al. (2002) provides no data to support the suggestion in the September 23, 2012 technical evaluation report that polyoxin D is an effective drug for the treatment of Encephalitozoon infections in AIDS patients.</td></tr>
</table>

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				EPA: results of the mutagenicity studies indicated Polyoxin D Zinc Salt Technical was weakly mutagenic in an Ames Assay (MRID# 433230-01) and not mutagenic in a host mediated assay (MRID # 432618-36). If a food/feed use is ever sought, the test results will require a review of the mutagenicity data base to determine the need for additional studies.[3] Mammalian chromosome aberration studies with hamster cells showed highly significant increases in chromosomal aberrations over solvent control.[4] However, in view of other studies submitted by the petitioner, EPA decided that the studies indicate that polyoxin D zinc salt is not mutagenic or clastogenic.	Not adverse. Please note that food/feed use was sought and additional data were submitted to EPA. As noted, EPA concluded that polyoxin D zinc salt is not mutagenic or clastogenic, *i.e.*, polyoxin D zinc salt does not have an adverse effect on DNA.

Category 1. Adverse Impacts on Humans or the Environment of Polyoxin D Zinc Salt?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
11. Is there an adverse effect on human health as defined by applicable Federal regulations? [205.600 b.3]			X		Not adverse.
12. Is the substance GRAS when used according to FDA's good manufacturing practices? [§205.600 b.5]			X		Not adverse.
13. Does the substance contain residues of heavy metals or other contaminants in excess of FDA tolerances? [§205.600 b.5]			X		Not adverse.

1. EPA, May 11, 2012, Science Review of Product Chemistry, Residue Chemistry, Non-Target Organism, and Toxicity Data in Support of Label Amendment for Polyoxin D Zinc Salt. (Included with supplemental petition).
2. EPA, May 11, 2012, Science Review of Product Chemistry, Residue Chemistry, Non-Target Organism, and Toxicity Data in Support of Label Amendment for Polyoxin D Zinc Salt. (Included with supplemental petition).
3. EPA. Consideration of Eligibility for Registration of the New Pesticide Active Ingredient Polyoxin D Zinc Salt – DECISION MEMORANDUM, p 15. (1997)
4. EPA, May 11, 2012. Science Review of Product Chemistry, Residue Chemistry, Non-Target Organism, and Toxicity Data in Support of Label Amendment for Polyoxin D Zinc Salt. (Included with supplemental petition.

http://tinyurl.com/C-Smith-1-23-2013
= http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5102221
= "Polyoxin D Zinc Salt: Reply to and Comments Regarding the National Organic Program Technical Evaluation Report Dated September 23, 2012" submitted January 18, 2013 and revised January 23, 2013.

Category 2. Is Polyoxin D Zinc Salt Essential for Organic Production?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
1. Is the substance formulated or manufactured by a chemical process? [6502 (21)]	X	X		Included in a new document received on January 18, 2013 from the petitioner it states on page 5 section 1.1, that, polyoxin D is made from an aerobic fermentation process, thus a natural process. However, they do state that they do not know whether the zinc salt is from a mined or from a recycled zinc source. The TR states that the manufacturing process has at least one step that would be similar to other *Streptomyces* products that are classified as synthetic on section 205.601 of the National List: streptomycin and tetracycline (terramycin). Similarly, polyoxin D Zinc Salt may also be classified as a synthetic. TR lines 146-148. It would appear that polyoxin D may be non-synthetic, but it would be assumed that the zinc salt would be synthetic, due to the lack of being able to properly verify its source.	Kaken agrees. Kaken believes that the fermentation product, polyoxin D, is non-synthetic. However, because Kaken: • Does not control the production of the source of zinc used in the production of polyoxin D zinc salt; and • Cannot provide details of the production of the source of zinc used in the production of polyoxin D zinc salt. Kaken agrees that polyoxin D zinc salt should be classified as a synthetic material under these circumstances. If in the future Kaken secures a certified organic source of the zinc starting material, Kaken may seek a non-synthetic classification of polyoxin D zinc salt.
2. Is the substance formulated or manufactured by a process that chemically changes a substance extracted from naturally occurring plant, animal, or mineral, sources? [6502 (21)]	X	X		Refer to the above answer in Category 2, Question 1.	See above.
3. Is the substance created by naturally occurring biological processes? [6502 (21)]		X		It is produced from a natural occurring soil microorganism Streptomyces cacaoi by a controlled fermentation process, according to the TR lines 119 – 120. (TR July 11, 21012) The petition states that polyoxin D Zinc Salt is isolated from a broth (extraction media) and then dried. Actual process is part of their CBI information. One part of the TR states that a review of all the structural forms of polyoxin does not include the Zinc Salt as a natural product (Worthington, 1988). TR lines 141-142. Also, refer to the answers as stated in Category 2, Question 1 & 2.	See above.
4. Is there a natural source of the substance? [§205.600 b.1]		X	X		

Category 2. Is Polyoxin D Zinc Salt Essential for Organic Production?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
5. Is there an organic substitute? [§205.600 b.1]			X		
6. Is the substance essential for handling of organically produced agricultural products? [§205.600 b.6]			X		
7. Is there a wholly natural substitute product? [§6517 c (1)(A)(ii)]	X	X		There is a natural occurring quinone plumbagin, isolated as a botanical that is comparable to polyoxin D (Dekeyser and Downer 1994), but it is not commercially available in the US at this time.	Polyoxin D zinc salt is not comparable to quinone plumbagin. • Dekeyser and Downer (1994) is a review article about the development of miticides. • Polyoxin D zinc salt is not registered for use for control of mites. Based upon efficacy testing, polyoxin D zinc salt does not provide control of mites. Polyoxin D zinc salt is registered for control of crop fungal diseases only.
				There are coppers and sulfur materials currently allowed for use. TR 321-328. (TR July 11, 2012)	The following are listed in 7 CFR §205.601(I) as synthetic materials allowed for use in organic crop production for plant disease control: "(2) Coppers, fixed - copper hydroxide, copper oxide, copper oxychloride, including products exempted from EPA tolerance, *Provided*, That, copper-based materials must be used in a manner that minimizes accumulation in the soil and shall not be used as" herbicides; "(3) Copper sulfate - Substance must be used in a manner that minimizes accumulation of copper in the soil"; "(6) Lime sulfur"; and "(10) Elemental sulfur".
8. Is the substance used in handling, not synthetic, but not organically produced? [§6517 c (1)(B)(iii)]			X		

Category 2. Is Polyoxin D Zinc Salt Essential for Organic Production?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	

9. Are there any alternative substances? [§6518 m.6]	X			There are other alternative substances available. The TR lists several that are currently allowed: JMS Stylet Oil, Dow's M-Pede, Regalia, Sonata, and Kaligreen to name just a few. See TR July 12, 2012 table: Comparison of the Endorse WDG label with Alternative Pesticides., located between lines 355-356. The efficacy of each of these materials is not listed.	Please see http://tinyurl.com/C-Smith-1-23-2013 :
					• Pages 43-46 discuss crop/disease combinations with no OMRI listed alternative:
					  • Cucurbits/Southern blight (*Sclerotium rolfsii*);
					  • Gingeng/Cylindrocarpon root rot (*Cylinderocarpon destructans*); and
					  • Pome fruit/Leaf blotch (*Diplocarpon mali*).
					• Pages 63-128 of for a comparison of polyoxin D zinc salt to registered alternatives on a crop/disease basis. Polyoxin D zinc salt provides curative control for most diseases; the alternatives generally provide only preventative control.
					• Pages 6-7 and 37-38 describe the unique, non-toxic mode of action that make polyoxin D zinc salt an important tool in resistance management and integrated pest management.

10. Is there another practice that would make the substance unnecessary? [§6518 m.6]	X	X		(TR lines 376-391) The TR lists several possible practices that could be used possibly in place of polyoxin D Zinc Salt. Antibiosis – using the live organisms rather than their extracts. This seems to be more consistent with organic farming principles. (Milner, et al. 1997)	Please see pages 63-128 of http://tinyurl.com/C-Smith-1-23-2013 . There is only one noted OMRI listed alternative for which the active ingredient is a live organism: • Actinovate Soluble; EPA Reg. No. 73314-1; *Streptomyces lydicus* WYEC 108. • This product is registered for use on only 41 of the 73 crop/disease combinations for which polyoxin D zinc salt is registered.
				Also beneficial antagonistic Streptomyces spp – but commercial development is slow in coming. (Liu, et al., 1997) (TR July 11, 2012)	Please see pages 63-128 of http://tinyurl.com/C-Smith-1-23-2013 for comparisons current to OMRI listed alternative products. Comparisons to possible future products is not a realistic or productive exercise.
				Also, crop rotation, crop nutrient management practices, sanitation to remove disease vectors, selection of resistant species and varieties (where applicable) beneficial antagonistic bacteria, monitoring. TR 367-382	These practices, even when employed judiciously, do not always prevent infection. Polyoxin D zinc salt provides curative control when these preventative measures did not successfully prevent infection. Polyoxin D zinc salt can be an important tool for preventing crop loss on organic farms.

http://tinyurl.com/C-Smith-1-23-2013

= http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5102221

= "Polyoxin D Zinc Salt: Reply to and Comments Regarding the National Organic Program Technical Evaluation Report Dated September 23, 2012" submitted January 18, 2013 and revised January 23, 2013.

<table>
<tr><th colspan="6">Category 3. Is Polyoxin D Zinc Salt Compatible with Organic Production Practices?</th></tr>
<tr><th colspan="5">Crops Subcommittee Petitioned Material Proposal (January 29, 2013)</th><th rowspan="2">Kaken's Comments</th></tr>
<tr><th>Question</th><th>Yes</th><th>No</th><th>N/A</th><th>Documentation
(TAP; petition; regulatory agency; other)</th></tr>
<tr><td>1. Is the substance compatible with organic handling? [§205.600 b.2]</td><td></td><td></td><td>X</td><td></td><td></td></tr>
<tr><td rowspan="2">2. Is the substance consistent with organic farming and handling? [§6517 c (1)(A)(iii); 6517 c (2)(A)(ii)]</td><td rowspan="2">X</td><td rowspan="2">X</td><td rowspan="2"></td><td>There are concerns with the possible impact on beneficial soil organisms.</td><td>Please see pages 21 of http://tinyurl.com/C-Smith-1-23-2013 :
• Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt does not kill beneficial soil fungi, but instead prevents the growth of fungi. Polyoxin D zinc salt is fungistatic, not truly fungicidal.
• Polyoxin D zinc salt degrades rapidly under environmental conditions. The May 11, 2012 EPA science review regarding the expanded tolerance exemption for polyoxin D zinc salt states on page 12:
"The net photolytic half-lives of [^{14}C]Polyoxin D were calculated to be 0.4 days, 4 days, 2.4 days, and 1.6 days in sterile natural water, pH 5.0, pH 7.0, and pH 9.0 buffers, respectively."
Please note that a half-life is the time during which a material degrades by 50%. Also, the rate of degradation is determined by the fastest route of degradation. In the presence of sunlight, polyoxin D zinc salt degrades by 50% in 0.4 days (9.6 hours). In 1.2 days, 90% degradation has occurred.
• Because polyoxin D zinc salt (1) does not kill fungi and (2) degrades rapidly under environmental conditions, any adverse effects on beneficial fungi would be only temporary.</td></tr>
<tr><td>Toxic to bees. (TR lines 305-309)</td><td>Please see page 26 of http://tinyurl.com/C-Smith-1-23-2013 .
The 96-hr LD_{50} of polyoxin D zinc salt to honeybees was determined to be 28.774 µg/bee. Using EPA's classification criteria, polyoxin D zinc salt is practically non-toxic to honeybees (EPA's least hazardous classification).
http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm
<table>
<tr><th>EPA Bee Hazard Category</th><th>96-hr LD_{50} (µg/bee)</th></tr>
<tr><td>Highly toxic</td><td>< 2</td></tr>
<tr><td>Moderately toxic</td><td>2-11</td></tr>
<tr><td>Practically non-toxic</td><td>> 11</td></tr>
</table>
If polyoxin D zinc salt were toxic to bees, there would be a bee hazard statement in the Environmental Hazards section of the label. None of the polyoxin D zinc salt product labels have a bee hazard statement on the label.</td></tr>
</table>

Category 3. Is Polyoxin D Zinc Salt Compatible with Organic Production Practices?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
				EPA exempts it from tolerance (40 CFR 180.1285) Also in a petition Addendum dated October 2,2012 the EPA has granted the petitioner an expanded exemption of tolerance to "all food commodities" and given expanded uses for all food and feed crops pre-harvest and post-harvest.	Polyoxin D zinc salt is registered for use on 73 crop/disease combinations. The expanded exemption of tolerance significantly reduces the time needed to commercialize new uses. New uses are in development but are not ready for discussion in a public forum.

3. Is the substance compatible with a system of sustainable agriculture? [§6518 m.7]	X	X		No, because it is not a unnecessary synthetic input.	Please see http://tinyurl.com/C-Smith-1-23-2013 :
					• Pages 43-46 discuss crop/disease combinations with no OMRI listed alternative:
					  • Cucurbits/Southern blight (*Sclerotium rolfsii*);
					  • Gingeng/Cylindrocarpon root rot (*Cylinderocarpon destructans*); and
					  • Pome fruit/Leaf blotch (*Diplocarpon mali*).
					• Pages 63-128 for a comparison of polyoxin D zinc salt to registered alternatives on a crop/disease basis. Polyoxin D zinc salt provides curative control for most diseases; the alternatives generally provide only preventative control.
					• Pages 6-7 and 37-38 describe the unique, non-toxic mode of action that make polyoxin D zinc salt an important tool in resistance management and integrated pest management.

				Also, because it does show toxicity to fungi and bees.	Fungi: Polyoxin D zinc salt is NOT toxic to fungi, including soil fungi. Please see pages 6-7 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal.
					Bees: Please see page 26 of http://tinyurl.com/C-Smith-1-23-2013 for a summary of the honeybee data. Using EPA toxicity descriptors, polyoxin D zinc salt is practically non-toxic to honeybees (EPA's least hazardous classification). If polyoxin D zinc salt were toxic to bees, there would be a bee hazard statement in the Environmental Hazards section of the label. None of the polyoxin D zinc salt product labels have a bee hazard statement on the label. See above.
				However, some felt it was a useful tool as part of a rotational disease control program.	Kaken agrees.

Category 3. Is Polyoxin D Zinc Salt Compatible with Organic Production Practices?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
4. Is the nutritional quality of the food maintained with the substance? [§205.600 b.3]			X		
5. Is the primary use as a preservative? [§205.600 b.4]			X		
6. Is the primary use to recreate or improve flavors, colors, textures, or nutritive values lost in processing (except when required by law, e.g., vitamin D in milk)? [205.600 b.4]			X		
7. Is the substance used in production, and does it contain an active synthetic ingredient in the following categories: a. copper and sulfur compounds;		X			
b. toxins derived from bacteria;	X			According to the TR (TR line 110) polyoxin D is a toxin derived from a bacteria (Streptomyces cacaoi var. asoensis) (TR July 11, 2012)	Polyoxin D zinc salt is NOT a toxin. Please see pages 6-7 of http://tinyurl.com/C-Smith-1-23-2013 : Polyoxin D zinc salt has a non-toxic mode of action. Polyoxin D zinc salt inhibits the chitin synthetase found in fungi. This prevents the growth of fungi without killing the fungi. As such, polyoxin D zinc salt is truly *fungistatic* rather than fungicidal.
c. pheromones, soaps, horticultural oils, fish emulsions, treated seed, vitamins and minerals?		X			
d. livestock parasiticides and medicines?		X			
e. production aids including netting, tree wraps and seals, insect traps, sticky barriers, row covers, and equipment cleaners?		X			

http://tinyurl.com/C-Smith-1-23-2013

= http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5102221

= "Polyoxin D Zinc Salt: Reply to and Comments Regarding the National Organic Program Technical Evaluation Report Dated September 23, 2012" submitted January 18, 2013 and revised January 23, 2013.

All questions from §205.600(b) are not applicable. Polyoxin D zinc salt is proposed for use in crop production.

Category 4. Is the Commercial Supply of an Agricultural Substance as Organic, Fragile or Potentially Unavailable?					
Crops Subcommittee Petitioned Material Proposal (January 29, 2013)					Kaken's Comments
Question	Yes	No	N/A	Documentation (TAP; petition; regulatory agency; other)	
1. Is the comparative description provided as to why the non-organic form of the material /substance is necessary for use in organic handling?			X		
2. Does the current and historical industry information, research, or evidence provided explain how or why the material /substance cannot be obtained organically in the appropriate **form** to fulfill an essential function in a system of organic handling?			X		
3. Does the current and historical industry information, research, or evidence provided explain how or why the material /substance cannot be obtained organically in the appropriate **quality** to fulfill an essential function in a system of organic handling?			X		
4. Does the current and historical industry information, research, or evidence provided explain how or why the material /substance cannot be obtained organically in the appropriate **quantity** to fulfill an essential function in a system of organic handling?			X		
5. Does the industry information provided on material / substance non-availability as organic, include (but not limited to) the following: a. Regions of production (including factors such as climate and number of regions);			X		
b. Number of suppliers and amount produced;			X		
c. Current and historical supplies related to weather events such as hurricanes, floods, and droughts that may temporarily halt production or destroy crops or supplies;			X		
d. Trade-related issues such as evidence of hoarding, war, trade barriers, or civil unrest that may temporarily restrict supplies; or			X		
e. Are there other issues which may present a challenge to a consistent supply?			X		

All questions from §205.600(b) are not applicable. Polyoxin D zinc salt is proposed for use in crop production.

APPENDIX 1. Leighton *et al.* (1981)

general, the average R of neural and liver tissues was somewhat lower than that of fibroblasts.

The mobility of a single surface molecule, the neural cell adhesion molecule (N-CAM), was also measured on chick brain and retina cells (Table 1, experiments 6 and 7). This cell surface molecule has been identified with the use of highly specific antiserums, and its role in cell-cell adhesion and development of neural tissue has been intensively studied in our laboratory (*13*). Despite its role in cell-cell adhesion, the mobility of this specific receptor was similar to that of the more general population of receptors measured using polyspecific anti-brain membrane serum.

Our results indicate that the average D's of a wide variety of surface receptors (but not necessarily all) fall within a narrow range, varying less than twofold under different conditions of cell growth and interaction. This variation is much less than the sixfold decrease in D seen on lectin-induced anchorage modulation (*4*). We conclude that if reversible modulation of receptor mobility is a significant mechanism for signaling cell-cell interactions, it must take place by the specific modulation of a small set of particular individual receptors rather than by general modulation of surface properties.

The differences in the fraction of mobile receptors observed between fibroblasts and the other cells suggest that the distribution of individual receptors in the population between the anchored and free mobility states may be characteristic of differentiation states, cell types, or morphologies. Consistent with this suggestion is the observation that about half of the cells measured in liver tissue showed no apparent recovery. Also, it has been shown that half of human lymphocytes labeled uniformly with a fluorescent monoclonal antibody against HLA antigens show no detectable redistribution of fluorescence after photobleaching, while the other half show redistribution with D of 6.9×10^{-10} cm^2/sec (*14*).

In summary, while Con A binding decreases receptor mobility in a variety of cells, the presence of cells in tissues does not appear to mimic this kind of modulation. However, receptors on about half of the cells in liver tissue labeled with polyspecific antibodies recognizing at least 15 different surface antigens, they were essentially immobile ($D < 5 \times 10^{-12}$ cm^2/sec). In contrast, the same receptors on dissociated liver cells showed values for D and R comparable to other cells. This suggests that naturally occurring modulation may take place by an "all or none" change (or greater than 100-fold decrease) in the mobility of specific receptors, rather than by a sixfold decrease as induced by lectins. Further experiments with monoclonal antibodies or other antibodies of very restricted specificity will be required to test this hypothesis.

W. EINAR GALL
GERALD M. EDELMAN
Rockefeller University, New York 10021

References and Notes

1. I. Yahara and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 608 (1972); *Nature (London)* **246**, 152 (1973).
2. G. M. Edelman, *Science* **192**, 218 (1976), and references therein.
3. I. Yahara and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1579 (1975).
4. J. Schlessinger, E. L. Elson, W. W. Webb, I. Yahara, U. Rutishauser, G. M. Edelman, *ibid.* **74**, 1110 (1977).
5. D. Axelrod, D. E. Koppel, J. Schlessinger, E. L. Elson, W. W. Webb, *Biophys. J.* **16**, 1055 (1976); M. Edidin, Y. Zagyansky, T. J. Lardner, *Science* **191**, 466 (1976); K. Jacobson, E.-S. Wu, G. Poste, *Biochim. Biophys. Acta* **433**, 215 (1976).
6. See, for example, J. Schlessinger, D. Axelrod, D. E. Koppel, W. W. Webb, E. L. Elson, *Science* **195**, 307 (1977); B. A. Woda, J. Yguerabide, J. D. Feldman, *Exp. Cell Res.* **126**, 327 (1980); M. Johnson and M. Edidin, *Nature (London)* **272**, 448 (1978); see also R. J. Cherry, *Biochim. Biophys. Acta* **559**, 289 (1979).
7. Measurements were made with an apparatus similar to that described by D. E. Koppel, D. Axelrod, J. Schlessinger, E. L. Elson, W. W Webb [*Biophys. J.* **16**, 1315 (1976)], connected to a DEC PDP-12 computer for on-line data collection, shutter control, and data processing. Intensity measurements were made with a beam power (at 568.2 nm) of 15 watt/cm^2 in the specimen plane; pulses about 200 msec long with intensities from 1×10^4 to 6×10^4 watt/cm^2 were used for photobleaching. The fluorescence was monitored every 200 msec during the initial recovery, then every second for the remainder of the measurement. Bleaching by the measuring beam was less than 5 percent. These conditions are in the range devoid of detectable photoinduced artifacts [D. E. Wolf, M. Edidin, P. R. Dragsten, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2043 (1980)]. The time constant of recovery and the fluorescence intensities at the start of recovery and at infinite recovery time (used to estimate the mobile fraction) were obtained from a nonlinear least-squares fit of the observed data to the theoretical equation for recovery. Control measurements of the diffusion of rhodamine-labeled Fab' fragments in glycerol solutions gave the expected coefficients (*6*). In a typical experiment, cells or tissue slices were incubated with rhodamine-labeled monovalent Fab' fragments of rabbit antibodies to cell surface antigens (50 μg/ml) for 15 minutes at room temperature in Hanks balanced salt solution (lacking phenol red) containing 50 m*M* *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (*p*H 7.3) and crystalline bovine serum albumin (1 mg/ml). The cells were then washed in the same buffer, and measured at room temperature (20° to 23°C), usually with a ×40 water-immersion objective (numerical aperture, 0.75), giving a spot radius ($1/e^2$) of 1.2 μm. The relative accuracy of the D's is not affected by errors in the estimation of the spot radius.
8. J. Schlessinger, L. S. Barak, G. G. Hammes, K. M. Yamada, I. Pastan, W. W. Webb, E. L. Elson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2909 (1977).
9. R. Dulbecco and M. G. P. Stoker, *ibid.* **66**, 204 (1970).
10. D. A. McClain and G. M. Edelman, *ibid.* **77**, 2748 (1980).
11. S. W. de Laat, P. T. van der Saag, E. L. Elson, J. Schlessinger, *ibid.*, p. 1526.
12. P. J. Sims, A. S. Waggoner, C. H. Wang, J. F. Hoffman, *Biochemistry* **13**, 3315 (1974).
13. J.-P. Thiery, R. Brackenbury, U. Rutishauser, G. M. Edelman, *J. Biol. Chem.* **252**, 6841 (1977); U. Rutishauser, J.-P. Thiery, R. Brackenbury, G. M. Edelman, *J. Cell Biol.* **79**, 371 (1978); U. Rutishauser, W. E. Gall, G. M. Edelman, *ibid.*, p. 382.
14. H. R. Petty, L. M. Smith, D. T. Fearon, H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6587 (1980).
15. Supported by NIH grants AM-04256, AI-11378, AI-09273, and RR 07065. We thank A. Waggoner for the lipid probe and M. C. Coogan for technical assistance.

18 March 1981; revised 22 May 1981

Pesticides: Insecticides and Fungicides Are Chitin Synthesis Inhibitors

Abstract. *Several important groups of fungicides and insecticides are specific inhibitors of chitin synthesis in a* Phycomyces *enzyme system and in insect organ cultures. The recently discovered benzoylphenylurea insecticides, which prevent chitin synthesis in insect tissues, are apparently not direct-acting chitin synthetase inhibitors. These insecticides may prevent insect chitin synthesis by interfering with the proteolytic activation of the chitin synthetase zymogen.*

The biosynthesis of chitin skeletal structures is a promising molecular target for pesticide action, since chitin is restricted in its biological distribution (*1*). The effects of fungicides and insecticides on chitin synthesis have received increasing attention following reports (*2*, *3*) that several agricultural chemicals affect insect chitin synthesis [also see review (*4*)].

We have developed biochemical and tissue culture methodologies that allow us to examine the mode of action of compounds that specifically interfere with chitin biosynthesis in insects and fungi. Table 1 lists compounds that are specific inhibitors of a cell-free preparation of chitin synthetase derived from the fungus *Phycomyces*. Specificity is defined as resistance of I_{50} level inhibition to the addition of excess protein (ovalbumin) (I_{50} is the concentration in moles of the compound that produces 50 percent inhibition of control chitin synthetase activity). This test establishes that the specific compounds do not react nonselectively with polypeptide functional groups. Compounds active in the *Phycomyces* system include chlorinated hydrocarbons, triazines, nitrophenols, organophosphates, sulfenimides, and thiolanes. Many of these compounds were not pre-

0036-8075/81/0821-0905$01.00/0

Table 1. Compounds that are chitin synthesis inhibitors in the *Phycomyces* and cockroach systems. *Phycomyces* germlings were harvested at mid-log growth phase from a defined minimal medium (*22, 23*) by vacuum filtration and were washed with breakage buffer (0.1*M* tris, *p*H 8.0; 0.02*M* $MgCl_2$; 10 percent glycerol; 0.001*M* EDTA, and 0.001*M* dithiothreitol). The washed germlings were resuspended in breakage buffer and disrupted by grinding with glass beads on a chilled mortar and pestle. The homogenate was centrifuged at 10,000*g* for 10 minutes. The pellet fraction was resuspended in breakage buffer and centrifuged again at 10,000*g* for 10 minutes. The resulting supernatant fraction was discarded, and the washed pellet fraction was resuspended in breakage buffer and used as a cell-free chitin synthetase preparation (*24*). The utilization of the cockroach leg regenerate system for assessing chitin synthesis inhibitors has been described in detail (*4*). The cytotoxicity of compounds was assessed by observing explant cultures in Rose chambers in the presence of an I_{90} to I_{99} concentration of the test material. If no interference with cuticle deposition was observed, the compounds were considered nontoxic.

Compound class	Compounds	I_{50} (mole/liter)	
		Phycomyces	Cockroach
Chloro and fluoro hydrocarbons	Chlordane	3.1×10^{-5}	
	p,p'-DDT	3.1×10^{-5}	7.3×10^{-6}
	Endosulfan	3.0×10^{-5}	
	Dieldrin	1.6×10^{-4}	
	2,2'-Methylenebis[4-chlorophenol]	4.9×10^{-5}	4.0×10^{-6}
Nucleoside and base analogs	Polyoxin D	2.9×10^{-4}	3.4×10^{-9}
	2,4-Dichloro-6-methylpyrimidine	1.6×10^{-4}	
Triazines	Azidotriazine	1.5×10^{-3}	4.0×10^{-9}
Sulfenimides	Captan	3.9×10^{-4}	See (*3*)
Dithiazoliums	Dithiazolium iodide	1.1×10^{-3}	7.7×10^{-8}
Organophosphates	Kitazin-P	4.7×10^{-4}	See (*3*)
Dinitrophenols and benzoylphenylureas	Dinocap	5.0×10^{-5}	
	Dimilin	$> 10^{-2}$	9.4×10^{-11}
	SIR8514	$> 10^{-2}$	2.1×10^{-11}
	SIR6874	$> 10^{-2}$	6.0×10^{-11}
	Penfluron	$> 10^{-2}$	1.6×10^{-11}
Thiolanes	Isoprothiolane	6.6×10^{-5}	1.3×10^{-7}

viously suspected to be chitin synthetase inhibitors. Various chemical structures can block chitin synthetase; most of these compounds are reversible inhibitors and in general do not compete with the substrate for access to the active site of the enzyme. The fungicide polyoxin D is a competitive, substrate-analog type of chitin synthetase inhibitor (*5*). A number of chlorinated hydrocarbons, carbamates, and other compounds demonstrate nonspecific inhibition in the *Phycomyces* system (data not shown).

Benzoylphenylureas (60-40, 60-38, DU119111, SIR8514, SIR6874, and Penfluron), herbicides (2,4-dichlorophenoxyacetic acid and simazine), Dichloran, and 5-fluorouracil have no effect on *Phycomyces* chitin synthetase when tested at their aqueous solubility limits or at a concentration of at least $10^{-2}M$. Chymostatin ($1.1 \times 10^{-5}M$), soybean trypsin-chymotrypsin inhibitor ($1.4 \times 10^{-4}M$), and lima bean trypsin-chymotrypsin inhibitor ($1.2 \times 10^{-4}M$) also have no effect on chitin synthetase activity.

A number of compounds that inhibit chitin synthesis in the *Phycomyces* system also inhibit chitin synthesis in cultured insect tissues (Table 1). The organophosphates kitazin-P and parathion, and the sulfenimide captan are inhibitory in this insect system (*3*). Since none of these compounds produce cytotoxic effects in cockroach organ cultures, they selectively prevent chitin synthesis without affecting the biosynthesis of other cuticular components.

The benzoylphenylureas do not inhibit cell-free preparations of either fungal or insect chitin synthetase (*6, 7*) but are highly active in insect systems. Their potency might therefore be explained by assuming that these compounds do not interact with the large amount of active chitin synthetase present in insect cells but rather affect a cascade event involved in enzyme biosynthesis—namely, the proteolytic activation of the chitin synthetase zymogen. The existence of zymogen forms of chitin synthetase in fungi has been reported (*8*).

Table 2. Inhibition of chymotrypsin activity by 60-40 and 60-38. The activity of chymotrypsin was assayed as described by Leighton *et al.* (*9*). In typical protease assays, 6 to 10 pmole of enzyme in 1 ml of reaction mixture was incubated for 18 hours at 37°C. Experiments (30 to 50 pmole of enzyme in 0.2 ml of 0.05*M* tris, *p*H 8.0, and 0.01*M* $CaCl_2$) included appropriate solvent and enzyme controls. All molar ratio circulations were based on the aqueous solubility limits of the inhibitors.

Molar ratio of inhibitor to enzyme	Time before incubation (hours)	Percent inhibition
	Compound 60-40	
20:1	1	36
20:1	2	57
20:1	4	72
20:1	6	87
10:1	3	43
	Compound 60-38	
60:1	1	3
60:1	2	18
60:1	4	20
60:1	6	32
30:1	3	17

Model experiments show that the benzoylphenylurea 60-40 (Dimilin) and the less effective insecticide 60-38 (*10, 11*) are direct-acting serine protease inhibitors (Table 2). These compounds have a slight preference for chymotrypsin-like proteases (data now shown). Several known chymotrypsin inhibitors (*12–14*) prevent chitin synthesis in the cockroach system (I_{50} values of active inhibitors are chymostatin, $2.3 \times 10^{-7}M$; 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate, $4.0 \times 10^{-6}M$; lima bean trypsin-chymotrypsin inhibitor, $1.2 \times 10^{-6}M$; and soybean trypsin-chymotrypsin inhibitor, $1.9 \times 10^{-6}M$). Leupeptin, antipain, and pepstatin A—inhibitors of trypsin, plasmin, pepsin, renin, and a variety of proteolytic enzymes other than chymotrypsin (*12, 15*)—show no effect in this system at $10^{-5}M$. None of these inhibitors produce cytotoxic effects in cockroach organ cultures.

An unexpected finding is that many currently used insecticides and fungicides are specific chitin synthetase inhibitors (Table 1). Surprisingly, neurotoxins (*16, 17*) and oxidative phosphorylation or respiratory chain inhibitors (*18*) affect the process of chitin biosynthesis. Whether the targets of these inhibitors share common receptor sites, or whether these molecules can disturb membrane structure in a manner that affects the activity of a few cognate polypeptides, is not known. Since many of the inhibitory activities we observe are stereochemically constrained, further quantitative structure-activity studies should aid in the design of more selective and effective chitin synthetase inhibitors. Isoprothiolane is a chitin synthesis inhibitor in both the *Phycomyces* and cockroach systems. It also inhibits carbohydrate uptake in fungal cells (*19*). Its low mammalian toxicity makes it an attractive new compound class for the development of antimycotic and anti-insect agents.

The benzoylphenylureas, which control insect populations at extremely low doses, appear to selectively derange the synthesis of insect chitin-containing

structural elements (*2*, *3*, *10*). The data presently available indicate that benzoylphenylureas are not direct-acting chitin synthetase inhibitors, but rather that they are direct-acting serine protease inhibitors that block the conversion of chitin synthetase zymogen into active enzyme. Thus a variety of specific chymotrypsin inhibitors, which are not directed against other serine and nonserine active site proteases, are capable of selectively blocking insect chitin synthesis. These data provide evidence for the critical involvement of a chymotrypsin-like protease in insect chitin biosynthesis. Recently, Strauss *et al.* (*20*) implicated a chymotrypsin-like protease in the processing of the pre-segment of human secretory proteins. Furthermore, Green and Ryan (*21*) observed that injury to plant leaf surfaces elicits a hormonally mediated response resulting in the production of large quantities of polypeptide trypsin and chymotrypsin inhibitors. This plant defense system may have the same mode of action as the benzoylphenylureas.

TERRANCE LEIGHTON
Department of Microbiology and Immunology, University of California, Berkeley 94720

EDWIN MARKS
Metabolism and Radiation Research Laboratory, SEA-USDA, Fargo, North Dakota 58102

FRANCES LEIGHTON
Department of Microbiology and Immunology, University of California

References and Notes

1. R. A. Muzzarelli, *Chitin* (Pergamon, Oxford, 1977).
2. L. C. Post, B. J. DeJong, W. R. Vincent, *Pestic. Biochem. Physiol.* **4**, 473 (1974).
3. E. P. Marks and B. A. Sowa, *The Insect Integument* (Elsevier, New York, 1976), pp. 339–357.
4. T. Misato and T. Kakiki, *Antifungal Compounds* (Dekker, New York, 1977), vol. 2, pp. 277–300.
5. A. Endo, K. Kakiki, T. Misato, *J. Bacteriol.* **104**, 189 (1970).
6. R. Mayer, A. Chen, J. DeLoach, *Insect Biochem.*, in press.
7. E. Cohen and J. Casida, *Pestic. Biochem. Physiol.* **13**, 129 (1980).
8. R. E. Ulane and E. Cabib, *J. Biol. Chem.* **249**, 3418 (1974).
9. T. J. Leighton, R. H. Doi, R. A. J. Warren, R. A. Kelln, *J. Mol. Biol.* **76**, 103 (1973).
10. A. Verloop and C. D. Farrell, *Pesticide Chemistry in the 20th Century* (American Chemical Society, Washington, D.C., 1977), pp. 237–270.
11. E. P. Marks, unpublished data.
12. H. Umezawa, *Methods Enzymol.* **45**, 678 (1976).
13. B. F. Erlanger and F. Edel, *Biochemistry* **3**, 345 (1964).
14. Y. Birk, *Methods Enzymol.* **45**, 695 (1976).
15. T. Aoyagi, *Bioactive Peptides Produced by Micro-organisms* (Wiley, New York, 1978), pp. 129–151.
16. R. D. O'Brien, B. Hetnarski, R. K. Tripathi, G. J. Hart, *Mechanism of Pesticide Action* (American Chemical Society, Washington, D.C., 1974), pp. 1–13.
17. F. Matsumura, *Toxicology of Insecticides* (Plenum, New York, 1975), pp. 105–164.
18. G. W. Ware, *The Pesticide Book* (Freeman, San Francisco, 1978), pp. 97–107.
19. T. Kakiki and T. Misato, *J. Pestic. Sci.* **4**, 129 (1979).
20. A. W. Strauss, M. Zimmerman, I. Boime, B. Ashe, C. A. Munford, A. W. Alberts, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4225 (1979).
21. T. R. Green and C. A. Ryan, *Plant Physiol.* **51**, 19 (1973).
22. Y. N. Jan, *J. Biol. Chem.* **249**, 1973 (1974).
23. R. P. Sutter, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 127 (1975).
24. The chitin synthetase reaction mixture contained 0.025*M* tris, pH 8.0; 0.05*M* $MgCl_2$; 0.001*M* UDP-*N*-acetylglucosamine (UDP, uridine diphosphate); 0.05*M* *N*-acetylglucosamine; 0.075 μCi of [^{3}H]UDP-*N*-acetylglucosamine ([6-^{3}H]glucosamine, 6.6 Ci/mmole); 80 μg of pellet fraction protein; and H_2O to make a total volume of 150 μl. The reaction mixture was incubated for 90 minutes at 28°C, and chitin synthesis was terminated by the addition of 5 percent trichloroacetic acid (4°C). The reaction mixture was filtered onto a glass fiber filter, washed with trichloroacetic acid and 95 percent ethanol, dried, and counted in a liquid scintillation spectrometer. The protein specificity of an inhibitory molecule was assessed by adding 500 μg of ovalbumin to a reaction mixture containing an amount of the test compound which would result in a 50 percent inhibition of enzyme activity. If the ovalbumin addition caused less than 10 percent deviation from the expected I_{50} value, the compound was classified as a specific chitin synthetase inhibitor. The I_{50} values were determined by graphical analysis of dose-response data; the standard error of these data is in the range of ± 10 percent. In substrate competition experiments, UDP-N-acetylglucosamine concentrations were 1 m*M* to 0.5 μ*M*. The specific activity of the ^{3}H-labeled substrate was increased fivefold in these experiments. The reversible or irreversible nature of inhibition was determined by incubating (90 minutes at 28°C) 25 μl of pellet material in the presence of the test compound at a concentration in that volume known to produce 50 percent inhibition under usual assay conditions. This preliminary incubation was terminated, and the test compound was diluted sevenfold by the addition of the remainder of the assay components. The level of inhibition after a 90-minute assay was observed. A decrement greater than 10 percent from the expected I_{50} value was interpreted as reversible inhibition.
25. We thank A. Glazer for helpful discussions, J. Stock and M. Delbrück for stimulating our interest in fungal biochemistry, and A. B. Borkovec for providing samples of dithiazolium iodide and Penfluron.

29 August 1980; revised 15 December 1980

Disease Resistance: Incorporation into Sexually Incompatible Somatic Hybrids of the Genus *Nicotiana*

Abstract. *Somatic hybrid plants of* Nicotiana nesophila *and* N. stocktonii *with* N. tabacum *(cultivated tobacco) were produced by protoplast fusion. These combinations cannot be achieved with conventional sexual hybridization, yet are important in that the wild* Nicotiana *species are resistant to numerous diseases. Hybridity was verified by chromosome number, isoenzyme analysis, morphological characteristics, and genetic behavior. Local lesion-type resistance to tobacco mosaic virus has been observed in leaves of these somatic hybrid plants.*

Wild species of *Nicotiana* have been used to incorporate disease resistance into cultivated tobacco (*1*). The three *Nicotiana* species of the section *Repandae* (*N. nesophila*, *N. repanda*, and *N. stocktonii*) are among those species resistant to the most diseases of cultivated tobacco. Attempts to crossbreed these wild species with cultivated tobacco by conventional breeding techniques have been unsuccessful (*2*, *3*). Two of these species, *N. nesophila* and *N. stocktonii*, have been crossed with *N. tabacum* (*4*) by means of ovule culture in vitro. How-

Table 1. Comparison of morphological characteristics of *N. tabacum* + *N. nesophila* (NN + Su/Su) somatic hybrid plants with the two parental species.

Plant	Flower		Leaf (cm)		Pollen viability (%)
	Color	Length* (cm)	Length of blade*	Maximum width*	
N. tabacum (Su/su)	Dark pink	5.28 ± 0.07	23.00 ± 2.48	7.25 ± 1.23	97.5
NN + Su/Su (somatic hybrids)	Light pink	5.09 ± 0.08	18.25 ± 1.31	8.13 ± 0.32	55.3
N. nesophila (NN)	White	4.95 ± 0.07	13.14 ± 0.46	9.29 ± 0.48	96.5

*Measurement expressed as mean ± standard error with differences significant, with P less than .05, for each group of plants.

Table 2. Segregation of the Su locus controlling leaf pigmentation in sexual progeny of *N. tabacum* + *N. nesophila* (NN + Su/Su) somatic hybrid plants.

Sexual cross	Dark green	Light green	Albino
(NN + Su/Su) × *N. tabacum* (su/su)	58	36	0
(NN + Su/Su) × *N. nesophila*	11	14	0
N. tabacum (su/su) × (NN + Su/Su)	3	2	0
(NN + Su/Su) × self	11	35	1

0036-8075/81/0821-0907$01.00/0

APPENDIX 2. Bixby-Brosi and Potter (2012)

Research Article

SCI

Received: 30 March 2011 Revised: 11 June 2011 Accepted article published: 13 June 2011 Published online in Wiley Online Library: 4 August 2011

(wileyonlinelibrary.com) DOI 10.1002/ps.2252

Can a chitin-synthesis-inhibiting turfgrass fungicide enhance black cutworm susceptibility to a baculovirus?

Andrea J Bixby-Brosi and Daniel A Potter*

Abstract

BACKGROUND: Developmental resistance, i.e. reduced virulence and speed of kill of late instars, is a limiting factor in the use of baculoviruses for caterpillar control. *Agrotis ipsilon* multicapsid nucleopolyhedrovirus (*Agip*MNPV) is highly infective to young black cutworms, *Agrotis ipsilon*, but too slow-acting against late instars for effective curative control on golf courses or sports fields. Chitin-synthesis-inhibiting fungicides containing the active ingredient polyoxin-d are used to control fungal diseases in turfgrass, and similar compounds have been shown in the laboratory to synergize baculoviruses by disrupting peritrophic membrane function. This study tested whether applying the virus together with such a fungicide can synergize *Agip*MNPV activity against *A. ipsilon* in turfgrass.

RESULTS: The addition of a chitin synthesis inhibitor failed to increase *Agip*MNPV infectivity to *A. ipsilon* in the field. Rather, delayed and slightly reduced mortality from viral infection was seen when larvae fed on fungicide/virus-treated grasses as opposed to virus-only treatments. Choice tests revealed the fungicide residues to be a mild feeding deterrent.

CONCLUSION: Because polyoxin-d does not deactivate *Agip*MNPV, the two substances are compatible. However, combination applications of polyoxin-d and *Agip*MNPV on turfgrass might interfere with larval ingestion of a lethal virus dose, resulting in prolonged larval feeding in the field.

Keywords: baculovirus; *Agip*MNPV; fungicide; polyoxin-d; chitin synthesis inhibitor; *Agrotis ipsilon*; turfgrass

1 INTRODUCTION

Baculoviruses (family Baculoviridae, genus Nucleopolyhedrovirus), present a seemingly good alternative to broad-spectrum insecticides because of their efficacy, specificity and safety to humans and other non-target organisms. They have been used worldwide to manage pests in various cropping systems and forests.[1–5] It is striking, however, given that >400 insect species, mostly members of the orders Lepidoptera and Hymenoptera, have been reported as hosts for baculoviruses, how infrequently they are successfully used in integrated pest management programs.[3,6,7]

One of the limitations of baculovirus-based insecticides is their relatively slow speed of kill, especially of late instars.[3–5,8] As larvae mature, they typically become less susceptible to virus infection and may continue to feed for several days after ingesting a lethal dose, so targeting early instars is necessary to avoid economic damage to plants.[9–11] Most research to enhance the usefulness of baculoviruses has focused on using optical brighteners to protect them from degradation by ultraviolet light.[12–14] Another approach is to increase the virulence of the virus itself. For an insect to become infected, it must first ingest virus occlusion bodies (OBs) while feeding. After ingestion, the OBs release virions in the host midgut, which then must pass through the peritrophic membrane to initiate virus infection in the midgut.[10] This chitinous membrane is the insect's first line of defense against a virus, so a compound that disrupts its function may help facilitate infection and increase speed of kill. Additives such as optical brighteners may work this way,[12] but chitin synthesis inhibitors, too, have been shown to synergize baculoviruses and dramatically increase their activity by disrupting peritrophic membrane function.[15,16]

Polyoxins are *Streptomyces*-derived antibiotics that inhibit fungal and insect chitin syntheses.[17–19] Polyoxin-d strongly affected peritrophic membranes *in vitro* in adult blowflies, *Calliphora erythrocephala*, by inhibiting chitin synthesis and by changing the fine structure of the membrane.[20] Nucleopolydrovirus (NPV) susceptibility was increased in larvae of the silkworm, *Bombyx mori*, when commercially available polyoxin fungicidal agents were incorporated into the insect's artificial diet.[15,16] Enhanced biological activity of *Spodoptera litura* NPV by a chitin-synthesis-inhibiting compound was attributed to obvious ruptures on the outer surfaces of the peritrophic membrane, which potentially facilitated the passage of virions through the peritrophic membrane.[21] These compounds have been validated as synergists in laboratory experiments but have not been tested in the field.

The black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae), is nearly a worldwide pest of golf-course putting greens and tees, as

* *Correspondence to: Daniel A Potter, Department of Entomology, S-225 Agriculture Science Bldg N., University of Kentucky, Lexington, KY 40546-0091, USA. E-mail: dapotter@uky.edu*

Department of Entomology, University of Kentucky, Lexington, KY, USA

well as sports fields and various garden crops.[22,23] In turf, the night-active larvae chew down the grass surrounding their burrows, causing brown pock marks that reduce smoothness and uniformity of playing surfaces.[23] Prater *et al.*[11] documented a natural epizootic of *Agrotis ipsilon* multicapsid nucleopolyhedrovirus (*Agip*MNPV) decimating black cutworm populations on Kentucky golf courses, established dose–mortality relationships and demonstrated that a sprayed viral suspension can provide short-term control in the field.[11] When sprayed suspensions of *Agip*MNPV were evaluated for season-long control of black cutworm on creeping bentgrass (*Agrostis stolonifera* L.) golf-course tees under actual maintenance and play, one-week-old virus residues reduced larval populations resulting from introduced eggs by 76–82%. Residual control, however, lasted no more than a few weeks.[24] *Agip*MNPV quickly controls young larvae, but larger late instars require higher dosages and continue to feed for several days before being killed.[11,24] Combinations of *Agip*MNPV with adjuvants, such as optical brightener and lignin, failed to accelerate or extend efficacy of the virus against *A. ipsilon* in the field.[24] Even if they had worked, such adjuvants likely would be too expensive to use in synergizing virus applications targeting grass-feeding caterpillars on golf courses or sports fields.

If baculovirus efficacy could be enhanced by something already being used in the turf or crop system, land managers would incur no additional cost. For example, fungicides containing the active ingredient polyoxin-d are already being used, sometimes several times per season, to control turfgrass diseases such as brown patch, *Rhizoctonia* spp. An overlapping application of polyoxin-d fungicide and baculovirus would be a practical combination in golf-course settings, because fungal diseases and cutworm infestations often occur on the same tees, greens and other highly maintained sites. The purpose of this study was to determine whether the combined use of a chitin-synthesis-inhibiting substance, polyoxin-d, could enhance or synergize *Agip*MNPV activity against *A. ipsilon* in turfgrass.

2 MATERIALS AND METHODS

2.1 Insects, virus and fungicide

Agrotis ipsilon eggs and larvae were obtained from a commercial insectary (Benzon, Carlisle, PA) where they had been maintained on soybean-based diet. They were shipped in cups of diet by overnight mail and transferred to the present assays within a few hours of arrival. The *Agip*MNPV isolate used in all experiments was originally obtained from naturally infected late-instar *A. ipsilon* from central Kentucky golf courses.[11] Frozen infected caterpillars were macerated in 0.1% sodium dodecyl sulfate (SDS) for 10 min and filtered through five layers of cheesecloth. Virus OBs were then centrifuged at 900 × *g* for 10 min. The pellet was resuspended in 0.5% SDS and centrifuged again. Resuspension and centrifugation were repeated with 0.5 M NaCl with the final suspension in distilled water. Sodium azide was added at 0.02% concentration to prevent bacterial growth. This purified OB suspension was stored at 4 °C. OB concentrations were determined using a phase contrast microscope and a Neubauer bright-line hemocytometer (Fisher, Pittsburgh, PA).

The polyoxin formulation evaluated as a synergist for *Agip*MNPV was Endorse® wettable powder fungicide (Arysta LifeScience, Cary, NC), containing 2.5% active ingredient polyoxin-d zinc salt (equivalent to 2.2% polyoxorim and 0.3% metallic zinc), zinc 5-{[2-amino-5-*O*-(aminocarbonyl)-2-deoxy-L-xylonoyl]amino}-1-[5-carboxy-3,4-dihydro-2,4-dioxo-1(2*H*)-pyrimidinyl]-1,5-dideoxy-β-D-allofuranuronate. Endorse® is a group-19 fungicide and is labeled for controlling fungal diseases on golf courses, residential lawns, parks and commercial and institutional grounds. The wettable powder was dissolved in distilled water for all applications.

2.2 Evaluating virus/fungicide combinations in small field plots

An experiment initiated in July 2010 tested whether increased activity is provided to *Agip*MNPV residues by the fungicide. The trial was conducted in a stand of 'Penncross' creeping bentgrass on a Maury silt loam (fine, mixed, mesic typic Paleudalf; pH = 6) at the University of Kentucky's Turfgrass Research Center (UKTRC), Spindletop Farm, near Lexington, Kentucky. The turfgrass, representative of a golf-course fairway, was mowed at 1.6 cm 3 times per week, irrigated as necessary to prevent drought stress and fertilized in September, October and November at 0.48 kg actual N per 100 m^2 per application from urea (46-0-0). Fungicides (non-polyoxin) had been applied curatively, as needed, for control of fungal diseases, but were not used for at least 4 weeks before the present trials.

Individual plots were 0.5 m^2, with 1 m^2 buffers, and arranged in a randomized complete block with six replicates of each treatment. Virus suspensions were prepared as described above. Treatments included high, medium and low rates of virus (5×10^8, 5×10^7 and 5×10^6 OB m^{-2}) with and without fungicide, fungicide alone and an untreated control. Fungicide treatments were at a high label rate for golf-course fairways [1.2 g (product) m^{-2}]; virus rates were based on previous field experiments.[11,24] Larvae were confined in circular metal enclosures (39.0 cm diameter, 10.2 cm height) which were twisted and pressed to seat their lower edge about 1 cm into the ground. Each solution was dissolved in 50 mL of water and applied using a hand-pump sprayer inserted into a 50 mL plastic vial. The area inside each enclosure (0.12 m^2) was treated, and larvae were introduced as soon as the residues had dried.

Twenty third-instar *A. ipsilon* were introduced into each of the metal enclosures, which were then covered with 0.64 cm mesh wire hardware cloth to prevent bird predation. Grass was not mowed while cutworms and enclosures were in the plots. Surviving larvae were recovered after 4 days by using a soap flush consisting of 1.3 mL of lemon-scented dishwashing detergent (Joy®; Proctor & Gamble, Cincinnati, OH) per liter of water.[23] Larvae were rinsed with distilled water as soon as they surfaced, placed in individual capped 30 mL cups with soybean-based noctuid diet,[25] held at 25 °C and monitored until death or pupation. Death due to viral infection was verified by examining blood for viral OBs by using a phase contrast microscope at 400× magnification.

2.3 Testing for direct insecticidal effects of fungicide

The soap drench brought up relatively few cutworms from fungicide-treated plots in the above experiment, suggesting that there had been a disproportionately high number of escapes from those enclosures, or mortality from the fungicide itself. Therefore, a follow-up trial was conducted at the same field site to determine whether the fungicide alone reduced cutworm survival. Treatments included high and low rates of fungicide (1.2 and 0.6 g m^{-2}) and an untreated control. Plots were again 0.5 m^2 with a 1 m^2 buffer, and set up in a randomized complete block design with six replicates of each treatment. The experiment was carried out as described above; however, the metal enclosures were driven more deeply (3 cm) into the turf to ensure that larvae could not escape by burrowing beneath their edges.

2.4 Testing fungicide/virus synergism; greenhouse trials

In August 2010, creeping bentgrass cores (15.2 cm diameter, 6.5 cm deep) were harvested with an oversized golf-course cup cutter from the aforementioned creeping bentgrass stand. Grass cores were placed in pots with a small amount of potting mix below and around them to help maintain moisture. The potting mix consisted of 3 : 1 Pro-Mix BX (Premier Horticulture, Quakertown, PA) and autoclaved topsoil. Plants were watered as needed. The turfgrass was maintained in a glasshouse under a 14 h photoperiod with supplemental lighting from 1000 W sodium vapor bulbs unless ambient light was $\geq$450 L mol m^{-2} s^{-1}, and watered as needed to maintain vigor. Day and night temperatures were set at 22 and 18 °C respectively.

The treatments (virus/fungicide combinations) included high, medium and low rates of virus (5×10^9, 5×10^6 and 5×10^3 OB m^{-2}) with or without fungicide at high, medium and low rates (2.1, 0.21 and 0.012 g m^{-2}), plus an untreated control. Each solution was dissolved in 50 mL of water and applied using a separate hand-pump sprayer inserted into a disposable tube containing the treatment combination. Six replicates of each treatment were arranged on greenhouse benches in a randomized complete block design. Treatments dried for 20 min before third-instar *A. ipsilon* (12 per pot) were introduced into pots. Larvae were allowed to feed on treated grasses for 24 h. Cutworms were recovered by removing the grass plugs from their containers and examining the soil, roots, thatch and grass. The grass plug was then placed back into the pot, and those few remaining larvae were extracted using a soap disclosing solution and immediately rinsed with fresh water to remove soap as soon as they surfaced. All larvae were placed individually in 30 mL rearing cups with artificial diet and monitored until death or pupation, as above. Days until death were recorded. Death due to viral infection was verified by examining blood for OBs using a phase contrast microscope.

The above experiment was repeated to determine how varying the duration of exposure by feeding cutworms might affect virus synergism by the fungicide. Two virus rates (1×10^8 and 5×10^8 OB m^{-2}) and one fungicide rate (2.1 g m^{-2}) were applied alone and in combination, plus an untreated control. Cohorts of five replicates per treatment were set up in a randomized complete block design to be sampled at three different times (after 1, 2 and 4 days of feeding and exposure).

2.5 Fungicide effects on consumption of treated grass

Feeding preference of neonates and third instars was compared between fungicide-treated and untreated grass to try to reconcile results from the field and greenhouse experiments. More specifically, the hypothesis was tested that reduced consumption of fungicide-treated grass might interfere with cutworm ingestion of a lethal virus dose, thus resulting in lower infection rates. Creeping bentgrass cores were collected from the UKTRC on 13 September and maintained in a glasshouse as described above. Grass clippings were cut into 2.5 cm sections. The clippings were treated with the label rate of fungicide (2.1 g m^{-2}) by dipping them into the mixed fungicide solution for 5 s and then allowing the residues to air dry. Three treated and three untreated clippings were placed in an alternating, spoke-like arrangement on a moistened filter paper in the bottom of a polystyrene petri dish (90 mm × 15 mm). Ten neonates were placed in the center of each dish before replacing the lid. For the no-choice tests, one treated or untreated grass blade and one neonate were placed in each arena. There were 20 replicates for each test. Larvae were left to feed in the dark for 17 h at room temperature (about 22 °C). The total area of leaf tissue consumed in each treatment was visually estimated to the nearest 10% by two independent observers whose ratings were averaged, and the number of larvae actively feeding was also scored for each dish and treatment.

The trials were repeated with third instars, using larger arenas (styrofoam bowls, 115 mm × 50 mm). Grass blades were held in place on moistened filter paper using insect pins to prevent them from being scattered by the larvae. A single larva was added to each bowl; bowls were then capped with plastic wrap, covered with another styrofoam bowl and placed in a dark growth chamber (27 °C). The percentage of each grass blade that had been consumed was visually estimated, as above, at 1, 4 and 18 h.

2.6 Statistical analysis

Larval recovery and weights, percentage mortality from virus and other variables were analyzed by a 2 × 4 (small-plot field experiment) or a 4 × 4 (greenhouse experiment) factorial analysis of variance (ANOVA) for main effects and interaction of fungicide and virus rate (weighted ANOVA was used for field experiment percentages). The effect of virus rate was analyzed by polynomial contrasts for significance of linear or quadratic trends. A three-way repeated-measures ANOVA also was conducted on cumulative percentage mortality for greenhouse experiments. Fixed factors were fungicide rate and virus rate (between-subject factors) and time after exposure to treatments (within-subject factor), with repeated measure (mortalities) on the time factor, as mortalities were recorded on groups of larvae within the same replicates over time. Dunnett's tests were performed to compare virus mortalities in control groups (virus alone) to fungicide/virus combinations. The percentage of fungicide-treated or untreated leaf tissue consumed in the choice and no-choice tests was compared by Wilcoxon signed-rank tests or two sample *t*-tests for no-choice tests respectively. Replicates were omitted from analysis if there was no feeding on either treatment. Chi-square tests also were used to compare total proportions of treated or untreated blades with some feeding damage. Statistix 8[26] was used for all statistical analyses except for weighted ANOVAs, for which SAS[27] was used. Percentage data were normalized by arcsine square root transformation for analysis. All data are reported as original (non-transformed) means ± SE.

3 RESULTS

3.1 Evaluating virus/fungicide combinations in small field plots

Few larvae were recovered from fungicide-treated plots, regardless of whether or not virus was included in the treatment (Table 1). The percentage of recovered larvae that ultimately died from viral infection increased at higher virus rates with a significant linear trend for rate. Lower rates of virus infection occurred in combination treatments than with virus alone, resulting in a significant fungicide by virus interaction; however, there was no main effect of fungicide on percentage mortality (Table 1).

3.2 Testing for direct insecticidal effects of fungicide

Unlike the first experiment, in which the relatively small number of larvae recovered from fungicide-treated plots had suggested mortality from the fungicide itself, or proportionately more escapes from those enclosures, similar numbers of larvae were recovered from fungicide-treated and untreated plots (control = 15.3 ± 0.6;

Table 1. Numbers of *A. ipsilon* recovered from small-plot field experiments, and percentage that died from virus infection

Rate (OB m^{-2})	Fungicide	Larvae recovered	% mortality[a]
0	−	11.3 ± 2.3	1.5 ± 1.5
	+	3.3 ± 1.7	4.8 ± 4.8
5×10^6	−	4.1 ± 2.0	8.5 ± 4.7
	+	2.5 ± 1.3	0 ± 0
5×10^7	−	8 ± 3.2	24.3 ± 8.9
	+	3.3 ± 1.9	7.8 ± 5.1
5×10^8	−	6.5 ± 1.9	61.7 ± 14.3
	+	1.0 ± 1.0	47.7 ± 21.4
ANOVA (*F*-values)[b]			
Fungicide		11.7**	0.2
Virus rate		1.6	18.4**
F × V		0.8	6.1*

[a] Weighted ANOVA.
[b] df = 1, 3, 3 and 35 for fungicide, virus rate, interaction and error respectively.
* and ** denote significance at $P \leq 0.05$ and 0.01 respectively.

Table 2. Numbers of larvae recovered from virus/fungicide-treated pots after 24 h of feeding, and percentage infected by virus in greenhouse trials

Rate (OB m^{-2})	Fungicide	Larvae recovered	% mortality[a]
0	High	10.3 ± 0.8	1.5 ± 1.5
	Medium	9.8 ± 0.9	4.8 ± 4.8
	Low	10.0 ± 0.4	4.8 ± 3.4
	0	11.2 ± 0.3	0 ± 0
5×10^3	High	10.7 ± 1.0	17.1 ± 4.7
	Medium	8.8 ± 0.5	1.3 ± 1.3
	Low	7.1 ± 0.8	17.5 ± 9.2
	0	10.1 ± 0.5	4.8 ± 3.1
5×10^6	High	7.8 ± 1.3	6.5 ± 3.1
	Medium	9.7 ± 0.7	4.5 ± 2.9
	Low	9.3 ± 1.0	5.3 ± 2.5
	0	9.1 ± 1.0	16.3 ± 6.2
5×10^9	High	12.0 ± 0.8	70.3 ± 4.9
	Medium	7.1 ± 1.5	78.5 ± 9.5
	Low	9.7 ± 1.5	87.5 ± 5.6
	0	9.7 ± 1.4	77.1 ± 4.3
ANOVA (*F*-values)[b]			
Fungicide		1.8	1.2
Virus rate		1.4	126.5**
F × V		1.9	2.1*

[a] ANOVA.
[b] df = 3, 3, 9 and 75 for fungicide, virus rate, interaction and error respectively.
* and ** denote significance at $P \leq 0.05$ and 0.01 respectively.

high fungicide rate = 18.0 ± 0.9; low fungicide rate = 15.7 ± 1.5; $F_{2,10} = 2.9$; $P = 0.1$). This indicated that the fungicide itself did not have direct adverse effects on larval survival.

3.3 Testing fungicide/virus synergism; greenhouse trials

When larvae were exposed to treated turfgrass in the greenhouse for 24 h, the number recovered from the pots was similar for all treatments. Percentage mortality from virus infection increased as virus rate increased, but was similar for the two lowest virus rates. There was no significant main effect of fungicide (Table 2). A virus × fungicide interaction was seen; however, when fungicide/virus combinations were compared with comparable rates of virus alone, the percentage mortality from virus was similar regardless of whether or not fungicide was included.

When larvae were exposed to treated grasses for 1, 2 and 4 days, the number recovered from the pots again was similar for all treatments. Longer duration of feeding on treated grasses and exposure to the higher virus rates corresponded to greater mortality from virus infection ($F_{2,67} = 4$; $P = 0.02$ and $F_{2,67} = 115$; $P \leq 0.01$ respectively) for all exposure durations (Table 3). Within rates, larvae exposed to the low rate of virus alone experienced significantly higher mortality (41.8 ± 4.7 versus 22.1 ± 5.5; $F_{1,24} = 10.1$; $P \leq 0.01$) and died more quickly compared with larvae feeding on grasses treated with the low-virus/fungicide combination for all exposure times (Fig. 1 and Table 3). Larvae also died more quickly at the high virus rate compared with high-virus/fungicide combinations when exposed for 2 and 4 days; however, the rate of death was similar when exposed for only 1 day (Fig. 1 and Table 3).

Table 3. Analysis of variance for main effects and interaction of virus rate, fungicide and days of exposure on percentage of black cutworms that died from viral infection

		ANOVA (*F*-values) for cohorts exposed for		
		1 day[a]	2 days[b]	4 days[b]
Main effects	Virus	34.1**	3.9	37.3**
	Fungicide	0.1	16.3*	0.4
Interactions	Virus × fungicide	3.5*	2.7	1.6
	Virus × time	37.9**	0.9	19.5**
	Fungicide × time	0.9	2.6**	1.17
Contrasts[c]	VL versus VLF	17.11**	3.14**	19.17**
	VH versus VHF	0.47	9.24**	65.7**

[a] df = 2, 1, 2, 10, 5 and 120 for virus rate, fungicide, interactions and error respectively.
[b] df = 2, 1, 2, 8, 4 and 96 for virus rate, fungicide, interactions and error respectively.
[c] Preplanned single-degree-of-freedom contrasts.
* and ** denote significance at $P \leq 0.05$ and 0.01 respectively.

3.4 Fungicide effects on consumption of treated grass

In choice tests with neonates, the total number of grass blades with some damage caused by cutworm feeding was similar for the treated and the non-treated grasses (52 versus 58; $\chi^2 = 0.33$; $P = 0.56$). Larvae consumed proportionately less of the treated than of the non-treated grass tissue (17.1 ± 1.8% versus 24.7 ± 1.7% respectively; Wilcoxon signed-rank test, $P \leq 0.01$). The numbers of larvae feeding on treated versus untreated grass blades were similar at the time of assessment, however. In no-choice tests with neonates, the percentage feeding damage on treated grass blades (8.5 ± 2.6%) was significantly lower than for non-treated blades (22 ± 3.9%; $t_9 = -2.76$; $P = 0.01$), but the number of blades with some cutworm damage was similar regardless of treatment. Third

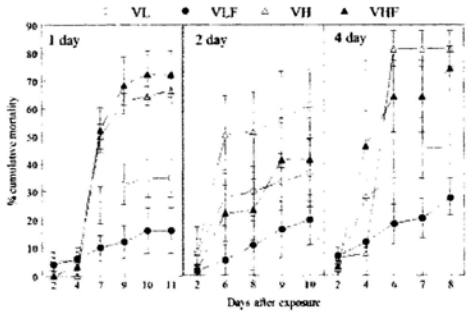

Figure 1. Cumulative lethal virus infection for *A. ipsilon* fed on bentgrass cores treated with two rates of *Agip*MNPV (VL = low virus, 1×10^8; VH = high virus, 5×10^8 OB m^{-2}) and one fungicide rate (F = 2.1 g m^{-2} of formulated product), applied alone and in combination. Larvae were exposed to treated grasses for 1, 2 and 4 days. Data are means (± SE). Delayed and slightly reduced mortality from *Agip*MNPV occurred when larvae fed on fungicide/virus-treated grasses as opposed to virus-only treatments for all cases, except those in which larvae were exposed to the high virus rate for 1 day.

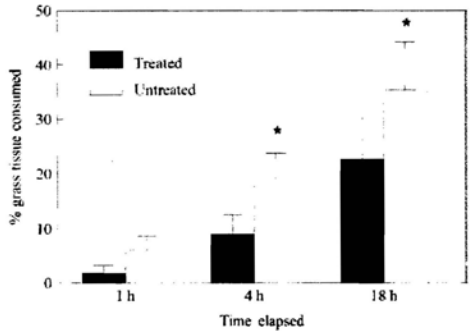

Figure 2. Mean (± SE) percentage of fungicide-treated versus non-treated grass leaf tissue consumed by third-instar *A. ipsilon* in choice tests. Asterisks denote significant feeding preference for untreated grass (Wilcoxon signed-rank tests, $P < 0.05$). The trend after 1 h was significant at $P = 0.08$.

instars showed significant preference for non-treated grass blades in choice tests (Fig. 2).

4 DISCUSSION

Combined or overlapping applications of a labeled polyoxin-d fungicide and *Agip*MNPV would be practical in turfgrass settings, so it was hoped that the combination would enhance infectivity of the virus against the black cutworm, an important golf-course pest, compared with levels of control provided by virus alone. That hypothesis is reasonable given previous laboratory studies with other insect species in which chitin-synthesis-inhibiting agents facilitated passage of virions through the chitinous peritrophic membrane, enhancing viral infection.[15,16,20,21] However, no synergism by the chitin-synthesis-inhibiting fungicide was seen; instead, there was delayed and slightly reduced mortality from *Agip*MNPV when larvae fed on fungicide/virus-treated grasses compared with virus-only treatments.

Poor recovery of larvae from fungicide-treated plots in the first field experiment initially suggested that polyoxin-d might have an insecticidal effect on cutworms. However, in a second experiment, when metal enclosures were driven deeper into the turf, similar numbers of larvae were recovered from fungicide- and non-fungicide-treated plots, revealing that the fungicide does not kill the cutworms. In choice tests, cutworms avoided feeding on polyoxin-d-treated grass. This suggests that larvae disproportionately escaped from the fungicide-treated turf by crawling beneath the shallow-driven enclosures used in the first field experiment. Because polyoxin-d does not deactivate *Agip*MNPV, and high virus rates can knock down and overwhelm cutworm populations in the short term,[24] the two substances are compatible and can be used together in the field. However, polyoxin-d residues on treated grass might interfere with larval ingestion of a lethal virus dose by inhibiting feeding or repelling larvae from putting greens, tees or other treated sites.

Previous studies examining the insecticidal effects of chitin synthesis inhibitors have all been done in laboratory settings and involved direct injection of the compound into the insect or incorporating it into artificial diet.[28–32] To the present authors' knowledge, this is the first study to examine the use of a chitin synthesis inhibitor as a synergist to an entomopathogen on living plants in greenhouse or field settings. Adjuvants such as stilbene optical brighteners, which have been shown to protect baculoviruses from UV degradation, enhance their longevity or act as synergists to virus infection in laboratory studies, may or may not provide the same benefits in the field.[33,34] The optical brightener M2R, for example, reduced the LD_{50} value of *Agip*MNPV to *A. ipsilon* in the laboratory but failed to enhance its efficacy against the same pest in greenhouse- or field-grown corn (*Zea mays* L.),[35] and also failed to accelerate or extend the efficacy of *Agip*MNPV against *A. ipsilon* in turfgrass field plots.[24] Optical brighteners can also deter feeding, and therefore results from a laboratory experiment may not translate to field settings where insects can disperse away from treated plant material.[36] Possibly, polyoxin chitin synthesis inhibitors consumed on plant tissue are less disruptive to caterpillar peritrophic membranes than when ingested in artificial diet. Plant secondary chemicals can alter the susceptibility of insects to naturally encountered pathogens as well as to microbial insecticides applied for biological control.[37] Caterpillar mortality, for example, can differ by as much as 50-fold, depending on the species of host plant upon which baculoviruses are consumed.[38–40]

The authors are still optimistic that *Agip*MNPV has potential as a microbial insecticide for managing black cutworms on golf courses, sports fields and in garden crops. Selecting for more virulent strains, or formulating the virus with adjuvants that enhance its persistence in field settings, could be productive. Testing *Agip*MNPV in combination with other chitin-synthesis-inhibiting fungicides suited for golf-course use is warranted, because some may be more disruptive to peritrophic membranes without discouraging feeding on treated grass as occurred with polyoxin-d. Another approach might be to combine a high dose of virus with a short-lived natural feeding stimulant[41,42] so that targeted larvae more rapidly ingest a lethal dose. The commercial success of *Agip*MNPV, like most entomopathogens, largely depends on future development of *in vitro* production methodology allowing the virus to be produced more economically and in greater amounts.[2,43]

ACKNOWLEDGEMENTS

The authors thank E Dobbs, C Redmond, D Williams, and L Williams for technical assistance, D Houseworth (Arysta Life Sciences) for donating the fungicide for experiments and C Keathley and S Vanek for critically reviewing an earlier draft of this article. This research was partially supported by grants from the United States Golf Association Turfgrass and Environmental Research Program, and from the OJ Noer Research Foundation. This is paper 11-08-034 of the Kentucky Agricultural Experiment Station.

REFERENCES

1 Cunningham JC, Baculoviruses as microbial insecticides, in *Novel Approaches to Integrated Pest Management*, ed. by Reuveni R. Lewis, Boca Raton, FL, pp. 261–92 (1995).
2 Black BC, Brennan LA, Dierks PM and Gard IE, Commercialization of baculoviral insecticides, in *The Baculoviruses*, ed. by Miller LK. Plenum, New York, NY, pp. 341–387 (1997).
3 Moscardi F, Assessment of the application of baculoviruses for control of Lepidoptera. *Annu Rev Entomol* **44**:257–289 (1999).
4 Szewczyk B, Hoyos-Carvajal L, Paluszek M, Skrzecz I and Lobo de Souza M, Baculoviruses – re-emerging biopesticides. *Biotechnol Adv* **24**:143–160 (2006).
5 Erlandson M, Insect pest control by viruses. *Encycl Virol* **3**:125–133 (2008).
6 Tanda Y and Kaya HK, *Insect Pathology*. Academic, New York, NY (1993).
7 Lacey LA, Frutos R, Kaya HK and Vail P, Insect pathogens as biological control agents: do they have a future? *Biol Control* **21**:230–248 (2001).
8 Bonning BC and Hammock BD, Development of recombinant baculoviruses for insect control. *Annu Rev Entomol* **41**:191–210 (1996).
9 Hoover K, Washburn JO and Volkman LE, Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. *J Insect Physiol* **46**:999–1007 (2000).
10 Cory JS and Myers JH, The ecology and evolution of insect baculoviruses. *Ann Rev Ecol Syst* **34**:239–272 (2003).
11 Prater CA, Redmond CT, Barney W, Bonning B and Potter DA, Microbial control of the black cutworm (Lepidoptera: Noctuidae) in turfgrass using *Agrotis ipsilon* multiple nucleopolyhedrovirus. *J Econ Entomol* **99**:1129–1137 (2006).
12 Wang P and Granados RR, Calcofluor disrupts the midgut defense system in insects. *Insect Biochem Mol Biol* **30**:135–143 (2000).
13 Mukawa S, Nakai M, Okuno S, Takatsuka J and Kunimi Y, Nucleopolyhedrovirus enhancement by a fluorescent brightener in *Mythimna separata* (Lepidoptera: Noctuidae). *Appl Entomol Zool* **38**:87–96 (2003).
14 Okuno S, Takatsuka J, Nakai M, Ototake S, Masui A and Kunimi Y, Viral-enhancing activity of various stilbene-derived brighteners for a *Spodoptera litura* nucleopolyhedrovirus. *Biol Control* **26**:146–156 (2003).
15 Arakawa T, Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), by flufenoxuron. *Appl Entomol Zool* **37**:7–11 (2002).
16 Arakawa T, Chitin synthesis inhibiting antifungal agents promote nucleopolyhedrovirus infection in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), larvae. *J Invert Pathol* **83**:261–263 (2003).
17 Hori M, Kakiki K, Suzuki S and Misato T, Studies on the mode of action of polyoxins. Part III. Relation of polyoxin structure to chitin synthetase inhibition. *Agric Biol Sci* **35**:1280–1291 (1971).
18 Cohen E, Chitin biochemistry: synthesis and inhibition. *Ann Rev Entomol* **32**:71–93 (1987).
19 Decker H, Zähner H, Heitsch H, König WA and Fiedler HP, Structure–activity relationships of nikkomycins. *J Gen Microbiol* **137**:1805–1813 (1991).
20 Becker B, Effects of Polyoxin-D on *in vitro* synthesis of peritrophic membranes in *Calliphora erythrocephala*. *Insect Biochem* **10**:101–106 (1980).
21 Hui-Fang G, Ji-Chao F, Bao-Sheng L, Jie-Ping W, Wan-Fang Z and Fang-Hao W, Enhancement of the biological activity of nucleopolyhedrovirus through disruption of the peritrophic matrix of insect larvae by chlorfluazuron. *Pest Manag Sci* **63**:68–74 (2007).
22 Williamson RC and Potter DA, Oviposition of black cutworm (Lepidoptera: Noctuidae) on creeping bentgrass putting greens and removal of eggs by mowing. *J Econ Entomol* **90**:590–594 (1997).
23 Potter DA, *Destructive Turfgrass Insects: Biology, Diagnosis, and Control*. Wiley, New York, NY (1998).
24 Bixby-Brosi AJ and Potter DA, Evaluating a naturally occurring baculovirus for extended biological control of the black cutworm (Lepidoptera: Noctuidae) in golf course habitats. *J Econ Entomol* **103**:1555–1563 (2010).
25 Blanco CA, Portilla M, Abel CA, Winters H, Ford R and Streett D, Soybean flour and wheat germ proportions in artificial diet and their effect on the growth rates of the tobacco budworm, *Heliothis virescens*. *J Insect Sci* **9**:59 (2009).
26 *Statistix User's Manual*. Analytical Software, Tallahassee, FL (2008).
27 *SAS User's Guide*. SAS Institute, Cary, NC (2005).
28 Gijswijt MJ, Deul DH and De Jong BJ, Inhibition of chitin synthesis by benzoyl- phenylurea insecticides, III. Similarity in action in *Pieris brassicae* (L.) with polyoxin D. *Pestic Biochem Physiol* **12**:87–94 (1979).
29 Nishioka T, Fujita T and Nakajima M, Effect of chitin synthesis inhibitors on cuticle formation of the cultured integument of *Chilo suppressalis*. *J Pestic Sci* **4**:367–374 (1979).
30 Cohen E and Casida JE, Properties and inhibition of insect integumental chitin synthesis. *Pestic Biochem Physiol* **17**:301–306 (1982).
31 Gelman DB and Borkovec A, The pharate adult clasper as a tool for measuring chitin synthesis and for identifying new chitin. *Comp Biochem Physiol* **85C**:193–197 (1986).
32 Arakawa T, Yukuhiro F and Noda H, Insecticidal effect of a fungicide containing polyoxin B on the larvae of *Bombyx mori* (Lepidoptera: Bombycidae), *Mamestra brassicae*, *Mythimna separate*, and *Spodoptera litura* (Lepidoptera: Noctuidae). *Appl Entomol Zool* **43**:173–181 (2007).
33 Ignoffo CM and Batzer OF, Microencapsulation of ultraviolet protectants to increase sunlight stability of an insect virus. *J Econ Entomol* **64**:850–853 (1971).
34 Shapiro M, Use of optical brighteners as radiation protectants for gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *J Econ Entomol* **85**:1682–1686 (1992).
35 Boughton AJ, Lewis LC and Bonning BC, Potential of *Agrotis ipsilon* nucleopolyhedrovirus for suppression of the black cutworm (Lepidoptera: Noctuidae) and effect of an optical brightener on virus efficacy. *J Econ Entomol* **94**:1045–1052 (2001).
36 Farrar RR, Jr, Ridgway RL, Cook SP, Thorpe KW and Webb RE, Nuclear polyhedrosis virus of the gypsy moth (Lepidoptera: Lymantriidae): potency and effects of selected adjuvants on feeding behavior. *J Entomol Sci* **30**:417–428.
37 Cory JS and Hoover K, Plant-mediated effects in insect–pathogen interactions. *Trends Ecol Evol* **21**:278–286 (2006).
38 Keating ST, Yendol WG and Schultz JC, Relationship between susceptibility of gypsy moth larvae to a baculovirus and host plant foliage constituents. *Environ Entomol* **17**:952–958 (1988).
39 Hoover K, Yee JL, Schultz CM, Rocke DM, Hammock BD and Duffey SS, Effects of plant identity and chemical constituents on the efficacy of a baculovirus against *Heliothis virescens*. *J Chem Ecol* **24**:221–252 (1998).
40 Ali MI, Felton GW, Meade T and Young SY, Influence of interspecific and intraspecific host plant variation on the susceptibility of heliothines to a baculovirus. *Biol Control* **12**:42–49 (1998).
41 Farrar RR, Jr, Shapiro M and Shepherd BM, Enhanced activity of the nucleopolyhedrovirus of the fall armyworm (Lepidoptera: Noctuidae) on *Bt*-transgenic and non-transgenic sweet corn with a fluorescence brightener and a feeding stimulant. *Environ Entomol* **34**:525–532 (2005).
42 Lasa R, Willams T and Caballero P, The attractiveness of phagostimulant formulations of a nucleopolyhedrovirus-based insecticide depends on prior insect diet. *J Pest Sci* **82**:247–250 (2009).
43 Kompier R, Tramper J and Vlak JM, A continuous process for the production of baculovirus using insect-cell cultures. *Biotechnol Lett* **10**:849–854 (1988).

