Polyoxin D and Other Biopesticides Reduce Gummy Stem Blight But Not Anthracnose on Melon Seedlings

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ABSTRACT

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Biopesticides may be useful to prevent fungal foliar diseases on cucurbit seedlings in greenhouses because of the short re-entry intervals, normally 4 h. *Bacillus subtilis* QST 713 (Serenade ASO), root extract of *Reynoutria sachalinensis* (Regalia), hydrogen dioxide (Oxideate), paraffinic oil (JMS Stylet Oil), polyoxin D (Oso), and 92% edible fish oil + 5% sesame oil (Organocide) were evaluated against gummy stem blight on muskmelon seedlings and anthracnose on

watermelon seedlings. Polyoxin D applied alone, *B. subtilis* QST 713 followed by polyoxin D, and *R. sachalinensis* extract followed by *B. subtilis* QST 713 reduced the severity of gummy stem blight and were as effective as mancozeb. None of the biopesticides applied alone or sequentially were effective against anthracnose. Polyoxin D could be used to prevent outbreaks of gummy stem blight on watermelon and muskmelon seedlings grown for use as transplants.

INTRODUCTION

Gummy stem blight of cucurbits is caused by the fungus previously named *Didymella bryoniae* (Auersw.) Rehm, which recently was shown to be a complex of three morphologically identical, genetically distinct species: *Stagonosporopsis citrulli* M.T. Brewer & J.E. Stewart; *S. cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley; and *S. caricae* (Sydow & P. Sydow) Aveskamp (Stewart et al. 2015). Gummy stem blight can occur on watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*) and muskmelon (*Cucumis melo* L.) seedlings grown for use as transplants and on greenhouse-grown cucumber (*C. sativus* L.) and melon crops (Boughalleb et al. 2007; Brown et al. 1970; Cedeño et al. 2000; Crinò et al. 2007; Hendricks and Roberts 2015; Keinath 2009, 2011, 2012; Koike 1997; Latin 1993; Stevenson et al. 2004; van Steekelenburg 1983). Infected seedlings can be a source of inoculum for field crops (Boughalleb et al. 2007; Keinath 1996). Seeds contaminated with *Stagonosporopsis* spp. produce diseased seedlings, and the fungus spreads rapidly from these initial foci to surrounding seedlings (Brown et al. 1970; Keinath 1996, 2013; Lee et al. 1984). Airborne ascospores of *Stagonosporopsis* spp. produced on debris remaining from diseased, previous crops are an additional source of inoculum in greenhouses (van Steekelenburg 1983).

Anthracnose, caused by the fungus *Colletotrichum orbiculare* (Berk. & Mont.) Arx, is another cucurbit seedling disease found in greenhouses. It was found on greenhouse-grown watermelon seedlings in Indiana and California in 1991 (Koike et al. 1991; Latin 1993). Cucumber seed contaminated with *C. orbiculare* produced cucumber seedlings with anthracnose symptoms (Horn and Wilson 1955); however, reports of transmission of *Colletotrichum* on cucurbit seed are less common than reports of

Stagonosporopsis. When watermelon seed was inoculated with either pathogen, 67% of the cotyledons and embryos were infected by *Stagonosporopsis*, but <2% were infected by *C. orbiculare* (Rankin 1954). Hybrid watermelon is susceptible to *C. orbiculare* race 2, whereas muskmelon is susceptible to races 1, 2, and 2B (Keinath 2015; Wasilwa et al. 1996). Anthracnose is the only disease mentioned in the USDA (2006) standards for grades of watermelon fruit; therefore, to maintain fruit quality it is critical to eliminate diseased seedlings as a source of inoculum.

Biopesticides, as defined by the U.S. Environmental Protection Agency, include microorganisms and their products and other biochemical substances “that have a nontoxic mode of action” (Leahy et al. 2014). Biopesticides would be very useful for managing gummy stem blight and anthracnose in greenhouse operations that produce melon transplants. The short re-entry intervals for biopesticides, generally 4 h, make it easier to schedule applications around hand watering times and other tasks. Mancozeb and cyprodinil-fludioxonil are the only fungicides effective against gummy stem blight that are registered for greenhouse use on cucurbits, but re-entry intervals are 24 and 12 h, respectively (Keinath 2013b). In addition, growers of organic transplants need effective biopesticides or synthetic substances allowed by the National Organic Program (NOP). Although applications of fixed copper and sulfur are allowed in organic agriculture, copper is ineffective against gummy stem blight (Keinath et al. 2000). In general, sulfur products are registered only against powdery mildew on cucurbits. Another advantage of using biopesticides in place of conventional fungicides is that they could prevent the occurrence of resistance to fungicides in *Stagonosporopsis* spp. Isolates of this pathogen resistant to quinone-outside-inhibitor fungicides, such as azoxystrobin, and to succinate-dehydrogenase-inhibitor fungicides, such as boscalid, have been found in commercial transplant production facilities in the southeastern United States (Hendricks and Roberts 2015; Keinath 2009, 2012; Stevenson et al. 2004).

A number of biopesticides are registered for use on cucurbits in the greenhouse and are marketed to control gummy stem blight

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and anthracnose. These biopesticides include potassium bicarbonate (Armicarb), *Bacillus subtilis* (Serenade ASO), root extract of *Reynoutria sachalinensis* (Regalia), hydrogen dioxide (Oxidate), and polyoxin D zinc salt (Oso). In addition, paraffinic oil (JMS Stylet Oil) may be used in the greenhouse on cucurbits against gummy stem blight. In a previous field experiment, polyoxin D reduced severity of gummy stem blight (Keinath et al. 2011). Potassium bicarbonate reduced severity of gummy stem blight in the greenhouse but not in the field (McGovern 1993; Ziv and Zitter 1992). Because paraffinic oil was effective in a previous greenhouse experiment, another biopesticide oil, a product of 92% edible fish oil + 5% sesame oil (Organocide), also was included in the present study (Ziv and Zitter 1992).

SCREENING BIOPESTICIDES AGAINST GUMMY STEM BLIGHT

The experimental design was a randomized complete block with four replications. Each replication was one pot with three seedlings of muskmelon ‘Athena.’ Seedlings with two true leaves were sprayed with the maximum-labeled rates of biopesticides dissolved or suspended in the equivalent of 100 gpa (Table 1). The following day, treated seedlings were inoculated by spraying with a suspension of 10⁶ conidia/ml of *S. citrulli* isolate W771. Inoculated seedlings were immediately placed in a humidity chamber at 100% relative humidity. Gummy stem blight incidence (percentage of plants with any symptoms) and severity (percentage leaf area diseased) were rated 3 days after inoculation. The experiment was done twice. Data were transformed with arcsine of the square root before analysis with SAS PROC GLM for mixed model analysis of variance (SAS 9.3, SAS Institute Inc., Cary, NC). Model fits were evaluated with

TABLE 1 Efficacy of biopesticides against gummy stem blight on muskmelon seedlings.				
Treatment	Product	Rate per 100 gal	Incidence (%) ^x	Severity (%) ^x
Water (inoculated control)		NA	100.0 a	21.5 a
Paraffinic oil 97%	JMS Stylet Oil	6 qt	100.0 a	19.7 a
Hydrogen dioxide 27%	Oxidate	1 gal	99.4 ab	14.0 a
Edible fish oil 92% + sesame oil 5%	Organocide	2 gal	100.0 a	6.1 b
<i>Bacillus subtilis</i> QST713 1.34%	Serenade ASO	6 qt	85.9 bc	2.7 bc
<i>Reynoutria sachalinensis</i> extract 5%	Regalia	1 gal	73.0 c	2.7 bc
Polyoxin D zinc salt 5%SC	Oso	14 fl oz	23.3 d	0.7 cd
Mancozeb 75DG	Manzate Pro-Stick	2-3 lb ^y	11.8 d	0.1 d
Water (non-inoculated control)		NA	0.0 e	0.0 d

^x Means were calculated from two experiments with four replications per experiment. Means within columns followed by the same letter are not significantly different, Waller-Duncan k-ratio t tests, *P* = 0.01.
^y Mancozeb was applied at 3 lb/100 gal in the first trial and 2 lb/100 gal in the second trial.

plots of residuals. Data from the two trials (repetitions of the experiment) were combined for analysis, as there were no differences between the trials and no interactions between trial and treatment.

All nontreated, inoculated plants had a least one leaf spot of gummy stem blight (an incidence of 100%) (Fig. 1) (Table 1). Biopesticides varied in their performance against gummy stem blight from completely ineffective to very effective (Table 1). Polyoxin D reduced the number of seedlings with symptoms (incidence) and limited the size of leaf spots (severity). It was the only product as effective as mancozeb, and it resulted in a significantly lower incidence than all other biopesticides. Severity on seedlings treated with mancozeb or polyoxin D did not differ from severity on the non-inoculated control seedlings, which were completely free of gummy stem blight. A reduction in incidence is important because *Stagonosporopsis* spp. can spread rapidly from infected seedlings to surrounding seedlings once spores are produced (Keinath 1996, 2011, 2013a). *B. subtilis* QST 713 and *R. sachalinensis* extract also reduced incidence compared to the nontreated control but not to the extent that polyoxin D did. Although *B. subtilis* QST 713 and *R. sachalinensis* extract reduced severity as well as Polyoxin D, severity with these two biopesticides was greater than on the non-inoculated seedlings. JMS Stylet Oil, Oxidate, and Organocide did not reduce incidence, and, of these three, only Organocide reduced severity.

Potassium bicarbonate 85% (Armicarb 100) was tested at 5 lb/100 gal in the first trial, but it caused phytotoxicity on all treated seedlings. Organocide also caused phytotoxicity on treated seedlings in the first trial but not in the second trial (*data not shown*). Because of the phytotoxicity observed, a separate experiment to test phytotoxicity was done. Seedlings were treated with biopesticides as described above, not inoculated, and left on the greenhouse bench. Seedlings were rated for severity of phytotoxicity at 1, 3, and 7 days after treatment. Phytotoxicity was observed with Armicarb applied at 5 and 2.5 lb/100 gal but not with any other treatments.

FIGURE 1
 Symptoms of gummy stem blight on muskmelon seedlings 3 days after inoculation. The seedlings in the four pots in the foreground were inoculated with *Stagonosporopsis* sp., and the seedlings in the rear pot were sprayed with water.

COMBINING BIOPESTICIDES AGAINST GUMMY STEM BLIGHT

R. sachalinensis extract, *B. subtilis* QST 713, and Polyoxin D were applied alone or in sequence with each other to determine if applications of two or three biopesticides were more effective than single biopesticides (Table 2). The sequential experiment used the same basic procedures as the screening experiment. In order to standardize comparisons among treatments, *R. sachalinensis* extract, *B. subtilis* QST 713, and Polyoxin D were applied 4, 2, and 0 days before inoculation, respectively, in the first trial, and 4, 2, and 1 day before inoculation in the second trial in all sequences (Table 2). For the final application in the first trial, plants were treated in the morning and inoculated in the afternoon to ensure that leaves had dried. Treated seedlings were inoculated with *S. citrulli* isolate W732 and incubated, and severity was rated and analyzed, as described previously. Data from the two trials were combined for analysis, as there were no interactions between trial and treatment.

Gummy stem blight was somewhat more severe in this experiment than in the screening experiment, as mean severity on inoculated control seedlings was 37% compared to 21%, respectively. Using a different isolate of *Stagonosporopsis* may have increased severity. The more severe disease may partially explain why *R. sachalinensis* extract and *B. subtilis* QST 713 applied alone were not effective in this experiment (compared to the water control), whereas they were in the previous experiment (Table 2). Disease was less severe with all other biopesticide treatments and with mancozeb than with the water control ($P =$

0.01). No disease developed in the noninoculated control. The level of disease that developed on seedlings treated with mancozeb or Polyoxin D ($\leq 3\%$) was not significantly greater than on the noninoculated control seedlings. *B. subtilis* QST 713 followed by polyoxin D and *R. sachalinensis* extract followed by *B. subtilis* QST 713 did not differ from mancozeb.

In general, polyoxin D was the most effective biopesticide against gummy stem blight, which verified the results of the screening experiment. In the sequential experiment, seedlings treated with polyoxin D had less severe disease than seedlings treated with *R. sachalinensis* extract or *B. subtilis* QST 713 (Table 2). In addition, applying polyoxin D after *R. sachalinensis* extract or *B. subtilis* QST 713 improved control compared to *R. sachalinensis* extract or *B. subtilis* QST 713 used alone. *R. sachalinensis* extract was the least effective of the three biopesticides. Seedlings sprayed with *R. sachalinensis* extract followed by *B. subtilis* QST 713 (10.4%) or *R. sachalinensis* extract followed by polyoxin D (14.7%) had less severe gummy stem blight than seedlings treated with only *R. sachalinensis* extract (32.6%). Severity on seedlings treated with *R. sachalinensis* extract followed by *B. subtilis* QST 713 (10.4%) did not differ from severity on seedlings treated with *B. subtilis* QST 713 (22.6%).

All combinations of two products were as effective against gummy stem blight as the treatment with three biopesticides (Table 2). In addition, the treatment with three biopesticides was less effective than mancozeb. Therefore, there is no advantage to using *R. sachalinensis* extract, *B. subtilis* QST 713, and polyoxin D on the same crop of seedlings.

In only one case was a combination of two biopesticides less effective against gummy stem blight than one of the components. Seedlings treated with *R. sachalinensis* extract followed by polyoxin D had more severe disease (14.7%) than seedlings treated only with polyoxin D (2.8%). This interaction was confirmed in a separate analysis of variance done with the eight combinations involving the three biopesticides, in which there

TABLE 2
Efficacy of three biopesticides applied alone or in combination against gummy stem blight on muskmelon seedlings.^x

Treatment 1	Treatment 2	Treatment 3	Severity (%) ^y
Water (inoculated control)	Water	Water	37.0 a
<i>Reynoutria sachalinensis</i> extract 5%	Water	Water	32.6 a
Water	<i>Bacillus subtilis</i> QST713 1.34%	Water	22.6 ab
<i>Reynoutria sachalinensis</i> extract 5%	Water	Polyoxin D zinc salt 5%	14.7 bc
<i>Reynoutria sachalinensis</i> extract 5%	<i>Bacillus subtilis</i> QST713 1.34%	Polyoxin D zinc salt 5%	13.1 bcd
<i>Reynoutria sachalinensis</i> extract 5%	<i>Bacillus subtilis</i> QST713 1.34%	Water	10.4 bcde
Water	<i>Bacillus subtilis</i> QST713 1.34%	Polyoxin D zinc salt 5%	4.7 cde
Water	Water	Polyoxin D zinc salt 5%	2.8 def
Water	Water	Mancozeb 75%	1.9 ef
Water (non-inoculated control)	Water	Water	0.0 f

^x Biopesticides were applied at the rates given in Table 1.

^y Means were calculated from two experiments with four replications per experiment. Means within columns followed by the same letter are not significantly different, Waller-Duncan k-ratio t tests, $P = 0.01$.

FIGURE 2

Symptoms of anthracnose at the hypocotyl-cotyledon juncture on watermelon seedlings.

was a significant interaction between treatments that included *R. sachalinensis* extract and polyoxin D ($P = 0.0009$). All other combinations of two or three biopesticides were more effective than, or equivalent to, the components.

In the sequential experiments, water was applied on days when no biopesticide was applied to certain treatments, so that leaves of all seedlings were wet the same number of times. Thus, water was applied twice to seedlings sprayed with *R. sachalinensis* extract and once to seedlings sprayed with *B. subtilis* QST 713 after treatment, but no water was applied after seedlings were treated with polyoxin D. If water removed some biopesticide residues, then this could be an explanation why *R. sachalinensis* extract and *B. subtilis* QST 713 were less effective than polyoxin D. The order in which the biopesticides were applied was determined based on their effects on the host plant. *R. sachalinensis* extract, a resistance inducer, was applied first, so there would be a short period after treatment before the seedlings were challenged with the pathogen (Fungicide Resistance Action Committee 2016). *B. subtilis* QST 713 was applied second, because *B. subtilis* QST 713 produces one or more lipopeptide antibiotics, and other strains of *B. subtilis* have been reported to induce resistance (Fungicide Resistance Action Committee 2016; Joshi and McSpadden Gardener 2006; Kloepper et al. 2004). Polyoxin D was applied last, since it is recommended to be used preventatively (Highland et al. 2014). Further experiments are needed to determine if the application sequence affects the efficacy of biopesticides.

COMBINING BIOPESTICIDES AGAINST ANTHRACNOSE

The same experimental design was used to screen *R. sachalinensis* extract, *B. subtilis* QST 713, and polyoxin D at the same rates and in the same sequences against *C. orbiculare* race 2 isolate WMF2 with an inoculation concentration of 10^5 conidia/ml (Keinath 2015). Seedless watermelon ‘Tri-X 313’ seedlings with two true leaves were sprayed with the same rates of biopesticides at 3, 2, and 1 day before inoculation. Seedlings were incubated in a humidity chamber for 24 h at 100% relative humidity, returned to the greenhouse, and rated 7 days after inoculation (Keinath 2015). The experiment was done twice. Data were analyzed with SAS PROC MIXED.

B. subtilis QST 713, *R. sachalinensis* extract, polyoxin D, and all combinations were ineffective against anthracnose on watermelon seedlings. With both incidence (*data not shown*) and severity ratings (Table 3), mancozeb reduced anthracnose in trial

one but not in trial two. There was a significant interaction between trial and treatment, because *R. sachalinensis* extract applied alone differed in efficacy between the two trials ($P = 0.01$) (Table 3). Incidence of anthracnose was high, $\geq 65\%$, in both trials. Approximately 23% of all seedlings had anthracnose lesions at the base of the hypocotyl or at the point on the hypocotyl where the cotyledons were attached, as observed previously (Fig. 2) (Keinath 2015; Koike et al. 1991; Latin 1993). Between 70 and 50% of the seedlings with hypocotyl lesions collapsed in trials one and two, respectively. Leaves or cotyledons on 39% of the remaining seedlings with hypocotyl lesions wilted in the second trial. Wilted portions of seedlings were included in the severity ratings, even if no leaf spots were present. Thus, hypocotyl lesions increased the severity of anthracnose in this experiment. Biopesticides were not effective enough to prevent hypocotyl lesions; mancozeb, with an anthracnose incidence of 98% in the second trial, also was ineffective. In the field, *B. subtilis* QST 713, *R. sachalinensis* extract, and polyoxin D were ineffective against anthracnose on watermelon, when biopesticides were applied weekly in the same sequences tested in this experiment (Keinath and DuBose 2014a).

CONCLUSIONS

Polyoxin D can be used on greenhouse cucurbit seedlings, as it protects against both gummy stem blight and powdery mildew. In Arizona, polyoxin D reduced severity of powdery mildew on muskmelon in the field (Matheron and Porchas 2008). In a previous greenhouse study, *B. subtilis* QST 713 and *R. sachalinensis* extract were ineffective against powdery mildew on cucurbit seedlings (polyoxin D was not tested) (Keinath and DuBose 2012). However, because polyoxin is not approved by the NOP, it cannot be used in certified organic production. Polyoxin inhibits chitin synthase, the Fungicide Resistance Action Committee Code 19 mode of action class, and is considered to have a medium risk of resistance development (Fungicide Resistance Action Committee 2016; Highland et al. 2014). Therefore, polyoxin D should be applied only once to each planting of cucurbit seedlings in a greenhouse. If additional applications are needed to manage gummy stem blight, mancozeb or cyprodinil-fludioxonil should be used (Keinath 2013b). Because no biopesticides tested in this study controlled anthracnose, mancozeb is likely the best fungicide to use in the greenhouse against this cucurbit seedling disease (Keinath and DuBose 2014b).

TABLE 3
Efficacy of three biopesticides applied alone or in combination against anthracnose on watermelon seedlings.^x

Treatment 1	Treatment 2	Treatment 3	Severity (%) ^y	
			Trial 1	Trial 2
Water (inoculated control)	Water	Water	35.2 ab	32.1 ab
Water	<i>Bacillus subtilis</i> QST713 1.34%	Water	30.7 ab	28.1 ab
<i>Reynoutria sachalinensis</i> extract 5%	Water	Water	62.0 a ^z	6.1 bc ^z
Water	Water	Polyoxin D zinc salt 5%	27.1 ab	12.2 abc
<i>Reynoutria sachalinensis</i> extract 5%	<i>Bacillus subtilis</i> QST713 1.34%	Water	41.8 ab	9.5 abc
Water	<i>Bacillus subtilis</i> QST713 1.34%	Polyoxin D zinc salt 5%	12.4 bc	18.6 ab
<i>Reynoutria sachalinensis</i> extract 5%	Water	Polyoxin D zinc salt 5%	13.6 bc	36.3 a
<i>Reynoutria sachalinensis</i> extract 5%	<i>Bacillus subtilis</i> QST713 1.34%	Polyoxin D zinc salt 5%	12.9 bc	27.5 abc
Water	Water	Mancozeb 75%	0.0 c	12.2 abc
Water (non-inoculated control)	Water	Water	0.0 c	0.0 c

^x Biopesticides were applied at the rates given in Table 1.

^y Means within columns followed by the same letter are not significantly different, Fisher's Protected LSD, $P \leq 0.01$ for trial 1 and $P \leq 0.05$ for trial 2.

^z Treatment means for *Reynoutria sachalinensis* extract differed between the two trials ($P = 0.0113$).

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