Short Communication

CHALLENGES IN ASSESSING EFFICACY OF POLYOXIN-D ZINC SALT AGAINST *COLLETOTRICHUM* SPECIES

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SUMMARY

Polyoxin-D zinc salt was recently made available for disease control of small fruit pathogens in the United States, but very little is known about its efficacy against diseases other than gray mold. In this study, the inhibitory activity of polyoxin-D zinc salt against *Colletotrichum* species from peach, the causal agent of peach fruit anthracnose, was assessed based on mycelial growth and germ tube elongation assays on four growth media, and in detached fruit assays. EC_{50} values between isolates of different *Colletotrichum* species varied within and between media for both mycelial growth and germ tube elongation tests. Occasionally, variability in sensitivity to polyoxin-D zinc salt was also found between isolates within the same species on the same media. Polyoxin-D zinc salt applied on detached fruit at recommended label rates inhibited mycelial growth of *C. nymphaeae* (100%), *C. fructicola* (88.50%), *C. siamense* (78.97%), *C. fioriniae* subgroup 2 (75.47%), *C. fioriniae*-subgroup 1 (73.55%), and *C. truncatum* (58.90%). In our assays, polyoxin-D zinc salt was equal or superior in efficacy compared to penthiopyrad. The results indicate that the EC_{50} values determined for *Colletotrichum* species are strongly dependent on the medium used and that none of the media accurately reflected observations on detached fruit assays.

Keywords: fungicide sensitivity, Ph-D, peach anthracnose.

Polyoxin-D, the active portion of the polyoxin-D zinc salt molecule, is a peptidyl-pyrimidine nucleoside antibiotic isolated from *Streptomyces cacaoi* var. *asoensis* (Isono *et al*., 1969). It inhibits chitin synthesis and consequently the biosynthesis of chitin in the fungal cell wall (Endo *et al*., 1970; Ohta *et al*., 1970; Cabib, 1991). The low toxicity profile makes polyoxin-D an attractive candidate for agricultural use. It was developed in the 1980s for the control of black spot of pear and apple in Japan caused by *Alternaria kikuchiana* and *A. mali* (Maria and Sullia, 1986). It has also been found highly effective in controlling fungal diseases of a variety of plants (Tewari and Skoropad, 1979; Yamaguchi, 1998; Adaskaveg *et al*., 2014; Adaskaveg and Förster, 2014). It is not toxic to vertebrates and there is no detectable toxicity in mammalian systems (Becker *et al*., 1983). Polyoxin-D has no residual effects after the compound has degraded or after it is washed off from plant tissue surfaces. The fungicide has recently been registered in the United States of America for disease management in several crops, including the control of gray mold of small fruits. The product labels indicate efficacy against anthracnose fruit rot caused by *Colletotrichum* spp., but little evidence is available in the literature about the efficacy of polyoxin-D on members of this genus. Species affecting peach include *C. fructicola* and *C. siamense* of the *C. gloeosporioides* complex, *C. nymphaeae* and *C. fioriniae* of the *C. acutatum* species complex, and *C. truncatum* (Hu *et al*., 2015b; Grabke *et al*., 2015; Chen *et al*., 2016).

Members of the methyl benzimidazole carbamates (MBC) and the quinone outside inhibitors (QoI) are effective fungicides against *Colletotrichum* spp. (Chen *et al*., 2016; Horton *et al*., 2016; Pansera *et al*., 2016), but their efficacy is threatened by resistance development. Resistance to MBC and QoI fungicides has already been detected in *C. siamense* isolates collected from multiple peach orchards in South Carolina (Hu *et al*., 2015a). Furthermore, some members of the *C. acutatum* complex are inherently resistant to MBC fungicides. Fungicide resistance based on selection or inherent resistance to MBC and QoI fungicides justifies the search for other effective products, such as polyoxin-D, which does not have cross-resistance with other chemical classes such as MBC and QoI fungicides. It therefore could be used in resistance management programs. The objective of this study was to assess *in vitro* sensitivity of *Colletotrichum* spp. to polyoxin-D on commonly-used culture media in comparison to *in vivo* efficacy in form of detached fruit assays.

All *Colletotrichum* spp. isolates used in this study were collected from peach fruit and were identified to the species level in our previous studies (Hu *et al*., 2015b; Grabke

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Table 1. *Colletotrichum* spp. isolates used in the study.

Species	Isolates	Host	Origin (county, state)	Year of isolation
C. fructicola	Cg_RR12_1	Peach	Chesterfield, SC	2013
	Cg_RR12_3	Peach	Spartanburg, SC	2012
	Cg_SE12_1	Peach	Spartanburg, SC	2012
C. siamense	Cg_Ey12_1	Peach	Saluda, SC	2012
	Cg_OD13_2	Peach	Saluda, SC	2012
	Cg_Ey12_10	Peach	Aiken, SC	2012
C. truncatum	Ct_RR13_1	Peach	Saluda, SC	2013
	Ct_RR13_2	Peach	Saluda, SC	2013
C. nymphaeae	CaCO4_35	Peach	Unknown, SC	2004
	CaPH40	Peach	Spartanburg, SC	2004
	CaPH44	Peach	Spartanburg, SC	2004
C. fioriniae-subgroup 1	CaEY12_1	Peach	Spartanburg, SC	2004
C. fioriniae-subgroup 2	C.2.2.2	Peach	Unknown, GA	2015
	C.2.3.2	Peach	Unknown, GA	2015
	C.2.4.2	Peach	Unknown, GA	2015

et al., 2015; Chen *et al*., 2016). A total of 15 *Colletotrichum* spp. isolates, including three *C. fructicola*, three *C. siamense*, two *C. truncatum*, three *C. nymphaeae*, and four *C. fioriniae* isolates were recovered for this study from filter paper stocks (Table 1). Filter paper with dried mycelium was placed on Potato Dextrose Agar (PDA) plates and incubated for 5 days in darkness at 25°C. Polyoxin-D zinc salt was obtained as formulated compound Ph-D WDG (Arysta LifeScience North America, Cary, NC). Four different media were used to determine polyoxin-D zinc salt EC_{50} values of 15 *Colletotrichum* isolates: PDA, Malt Extract Agar (MEA; 10 g malt extract, 15 g agar, 1000 ml water), V8 agar (Tewari and Skopopad, 1979; 200 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 800 ml water, adjust pH to 7.2), and Czapek-Dox agar (CzA; Yourman and Jeffers, 1999; 3 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g Fe_2SO_4, 1 g K_2HPO_4, 1 g KH_2PO_4, 0.005 g $CuSO_4{\cdot}5H_2O$, 10 g glucose, 15 g agar, 1000 ml water, pH 6.6). Final concentrations of 0, 3, 10, 30, and 100 µg/ml active ingredient were used for mycelial growth tests. Mycelial plugs (6 mm) were removed from the margins of 7-day-old colonies and transferred to the centers of 9-cm plastic Petri dishes containing fungicide-amended or non-amended medium. Experiments were conducted in triplicates for each isolate and fungicide concentration. The colony diameters of each replicate were measured in two perpendicular directions, with the original mycelial plug diameter (6 mm) subtracted from this measurement, after 5 days of incubation at 30°C for *C. siamense*, *C. fructicola* isolates, and 7 days of incubation at 25°C for *C. truncatum*, *C. nymphaeae* and *C. fioriniae* isolates in the dark. EC_{50} values were calculated by regressing the relative inhibition of growth against the $\log_{10}$ fungicide concentration.

In order to determine the sensitivity of spore germ tube elongation, spore suspensions (10^5 spores ml^{-1}) were prepared in sterile distilled water from 7-day-old cultures. Four different media mentioned above were supplemented with a range of fungicide concentrations (0, 3, 10, 30, 100 µg/ml active ingredient, respectively) and poured into 9-cm plastic Petri dishes. 0.1 ml of the spore suspension was spread on the surface of the plates and incubated at 30°C (*C. siamense*, *C. fructicola*, and *C. truncatum*) or 25°C (*C. nymphaeae* and *C. fioriniae*) for 7 to 10 h in darkness. Subsequently, the length of germ tubes (about 120 spores for each treatment) was measured with a stage micrometer using a microscope at 400× magnification. Three replicates were used for each isolate and fungicide concentration.

For *in vivo* assays, mature but still firm peach fruit were washed with soap and water to remove fungicide residue, then surface-disinfected for 10 min in 1% sodium hypochlorite, rinsed with sterile, distilled water, and allowed for air dry at room temperature. Penthiopyrad (E.I du Pont de Nemours and Company, Wilmington, DE, USA) was used as positive control. The effect of each fungicide on lesion development in detached fruit was assessed by mixing an aqueous solution of each fungicide with an equal amount of conidial suspension. The final inoculum concentration in mixtures was kept at 5×10^5 conidia/ml. For negative control treatments, conidial suspension was mixed with an equal amount of water. Each fruit was pricked at 3 sites (each about 2 cm apart) to a depth of 2 mm wound using a syringe needle. Each site was inoculated with 20 µl of each aqueous mixture. Fruit were placed in plastic boxes containing wet filter paper, covered to maintain high humidity and held at 28°C for 6-8 days. Lesion diameter of decay produced at each inoculation site was measured. Three fruit were used for each isolate/fungicide combination, and the experiment was conducted twice independently.

Results of *in vitro* assays based on mycelial growth showed *C. fructicola* isolates were insensitive to polyoxin-D on each of the media tested with EC_{50} values greater than 100 µg/ml for all four media. More than half of the isolates (i.e. *C. fructicola, C. siamense, C. truncatum*, and *C. fioriniae*-subgroup 1 isolates) tested had EC_{50} values higher than 100 µg/ml. Furthermore, *C. fructicola, C. nymphaeae*, and *C. fiorinieae*-subgroup 1 isolates were insensitive to polyoxin-D on MEA. The lowest EC_{50} values across the

Table 2. Sensitivity of *Colletotrichum* spp. mycelial growth to polyoxin-D zinc salt.

		EC_{50} values for polyoxin-D (µg/ml)			
Species	Isolate	PDA	MEA	V8 agar	CzA
C. fructicola	Cg_RR12_1	>100	>100	>100	>100
	Cg_RR12_3	>100	>100	>100	>100
	Cg_SE12_1	>100	>100	>100	>100
C. siamense	Cg_Ey12_1	>100	19.41	10.14	4.71
	Cg_OD13_2	>100	>100	28.35	55.95
	Cg_Ey12_10	>100	13.45	16.85	4.95
C. truncatum	Ct_RR13_1	>100	10.53	24.28	9.19
	Ct_RR13_2	>100	13.68	19.71	15.98
C. nymphaeae	CaCO4_35	32.63	>100	6.59	35.25
	CaPH40	31.62	>100	9.73	39.98
	CaPH44	32.43	>100	3.14	6.80
C.fioriniae-subgroup 1	CaEY12_1	>100	>100	1.19	22.52
C.fioriniae-subgroup 2	C.2.2.2	18.31	2.52	8.43	4.64
	C.2.3.2	17.92	7.00	26.19	10.78
	C.2.4.2	20.64	7.17	35.71	14.11

Table 3. Sensitivity of *Colletotrichum* spp. germ tube elongation to polyoxin-D zinc salt.

		EC_{50} values for isolates (µg/ml)			
Species	Isolate	PDA	MEA	V8 agar	CzA
C. fructicola	Cg_SE12_1	>100	30.19	65.90	>100
C. siamense	Cg_Ey12_1	>100	>100	96.28	>100
C. truncatum	Ct_RR13_2	>100	42.90	22.88	39.24
C. nymphaeae	CaPH44	>100	1.46	4.59	>100
C. fioriniae-subgroup 1	CaEY12_1	>100	>100	16.99	>100
C.fioriniae-subgroup 2	C.2.4.2	>100	>100	20.82	>100

Table 4. Suppressive activity of polyoxin-D and penthiopyrad against *Colletotrichum* spp. isolates on detached peach fruit.

Species	Isolate	Growth inhibition rate (%)	
		Polyoxin-D	Penthiopyrad
C. fructicola	Cg_SE12_1	88.50	55.23
C. siamense	Cg_Ey12_1	78.97	36.33
C. truncatum	Ct_RR13_2	58.90	60.06
C. nymphaeae	CaPH44	100.00	78.67
C. fioriniae-subgroup 1	CaEY12_1	73.55	36.82
C. fioriniae-subgroup 2	C.2.4.2	75.47	63.53

species were obtained on V8 and CzA medium. With the exception of *C. siamense* isolates on MEA and *C. fioriniae* subgroup 1 (for which we only had one isolate to test), the EC_{50} values for isolates of the same species revealed consistent results. For example, all three *C. nymphaeae* isolates were insensitive to polyoxin-D on MEA, highly sensitive on V8, and moderately sensitive on PDA and CzA media (Table 2). Again, EC_{50} values of the 15 *Colletotrichum* spp. isolates varied between media in germ tube elongation assays (Table 3). The least sensitivity of germtube elongation to polyoxin-D was detected on PDA and CzA media. Only the *C. truncatum* isolate was moderately sensitive on CzA medium. Three of the six isolates tested were insensitve to polyoxin-D on MEA, whereas isolates tested on V8 revealed highest sensitivity. The most unaffected species were *C. fioriniae* subgroups 1 and 2 as well as *C. siamense* (Table 3). With respect to detached fruit assays, the results showed polyoxin-D at highest label rate provided disease suppression for each species and controlled completely lesion development of *C. nymphaeae*. Disease suppression ranged from 58.9 % to 100% (Table 4). Similarly, penthiopyrad provided disease suppression as well and growth inhibition rates ranged from 36.3 to 78.67%. None of the isolates were inhibited completely (Table 4).

Evaluation of polyoxin-D sensitivity of *Colletotrichum* species appears to be a challenging task. None of the media amended with 100 µg/ml of polyoxin-D completely inhibited mycelial growth or germ tube elongation of *Colletotrichum* species tested (data not shown). Similarly, Maria and Sullia (1986) found that *Alternaria solani* and *Sclerotium rolfsii* isolates were capable of growing on carrot agar amended with 200 µg/ml polyoxin-D. However, EC_{50} values for *Botrytis cinerea* isolates were less than 10 µg/ml based on mycelial growth test (Mamiev *et al*., 2013). Dowling *et al.* (2016) showed that malt extract medium amended with 5 µg/ml can distinguish *B. cinerea* isolates sensitive or reduced sensitive to polyoxin-D. Variability between mycelial growth and germ tube elongation assays were also found for polyoxin-B, another cell-wall chitin biosynthesis inhibitor. Reuveni and Sheglov (2002) showed EC_{50} value of *Alternaria alternata* for polyoxin-B was 0.008 µg/ml based on conidial germination test on PDA, whereas the EC_{50} of the same isolate was 120 µg/ml based on mycelial growth test. Hwang and Yun (1986) reported that ED_{50} values of polyoxin-B sensitive and resistant *A. mali* isolates ranged from 2.2 to 2.7 µg/ml and 2.2 to 9.3 µg/ml, respectively, based on conidial germination test on potato-sucrose agar, but the ED_{50} ranged from 15.7 to 25.3 µg/ml and 191.7 to 323.3 µg/ml based on mycelial growth test on the same medium. The higher sensitivity of conidia of *Alternaria* species to polyoxin-D was then attributed to the inhibitory action by polyoxin-B (Reuveni and Sheglov, 2002). In contrast, conidia of *Colletotrichum* species tested in this study seemed no more sensitive than the mycelium regardless of medium type.

Species-specific responses to fungicides were reported previously. Chen *et al.* (2016) determined differential sensitivity of five *Colletotrichum* species from peach to demethylation inhibitor (DMI) fungicides and similar variability in sensitivity was reported between *Colletotrichum* species to various succinate dehydrogenase inhibitor (SDHI) fungicides (Ishii *et al*., 2016). These species-specific responses to fungicides must be considered when trying to manage anthracnose of crops.

Among SDHI fungicides, penthiopyrad is one of the more active ones against *Colletotrichum* species. Ishii *et al.* (2016) found isolates of *C. gloeosporioides*, *C. acutatum*, and *C. cereale* were more sensitive to penthiopyrad compared

to boscalid and fluopyram. In addition, penthiopyrad was found to be more effective against *C. gloeosporioides* and *C. acutatum* on detached apple fruit, and *C. fructicola* and *C. siamense* on detached peach fruit compared to boscalid, fluxapyroxad, and fluopyram (Ishii *et al*., 2016). Fontelis (ai. penthiopyrad; DuPont, Wilmington, DE) is currently registered for the control of anthracnose disease on vegetables and nuts. Future studies such as field trials may better investigate the efficacy of polyoxin-D for different *Colletotrichum* species.

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