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Short Communication

Effect of 6-Methoxy-2-Benzoxazolinone (MBOA) on *Pythium* Species and Corn Seedling Growth and Disease

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Abstract

Corn yield reduction following a cereal rye cover crop has been attributed to, among other factors, allelochemicals released from decomposing cereal rye residue. The allelopathic effect of 6-methoxy-2-benzoxazolinone (MBOA) was evaluated on corn seedling growth, mycelial growth of seven pathogenic species of *Pythium*, and root rot of corn seedlings caused by *Pythium* spp. at 13, 16, and 22 to 23°C (room temperature) using a plate assay. Mycelial growth of all *Pythium* spp. tested was slower with MBOA at 0.25 mg/ml compared with MBOA at 0.125 and 0.0625 mg/ml and the check (4% V8 juice medium containing neomycin sulfate and chloramphenicol with 0.5% dimethyl sulfoxide). Therefore, no further tests were done with MBOA at 0.25 mg/ml. In general, MBOA reduced corn radicle length and did not cause root rot across all temperatures. However, greater root rot severity in corn was observed on corn seedlings grown in the presence of *Pythium lutarium* and *P. oopapillum* on media amended with MBOA compared with the check at all temperatures. Similarly, more root rot caused by *P. torulosum* and *P. spinosum* was observed when MBOA was present at 16°C compared with the check with no MBOA. These data suggest that corn seedling disease caused by *Pythium* spp. could be more severe when corn is planted following a cover crop of winter cereal rye due to the presence of allelochemicals that are released from the cover crop.

Keywords: 6-methoxy-2-benzoxazolinone, allelopathy, benzoxazinoids, cereal rye, MBOA, *Pythium*

Allelopathy is a complex process in which plants release chemical compounds that enable ecological adaption to biotic and abiotic stresses in addition to other possible functions such as plant defense (Field et al. 2006; Hu et al. 2018; Isah 2019). Allelochemicals are reported to affect plant–microbiome interactions (Inderjit et al. 2011). One such group of compounds are the benzoxazinoids (BXs) that are produced by numerous plant species belonging to the Poaceae family, which includes corn (*Zea mays* L.), and winter cereal rye (*Secale cereale* L.). Predominant BXs produced in cereal rye include 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA), which breaks down to benzoxazolin-2(3*H*)-one (BOA), and 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3)(4*H*)-one (DIMBOA), which breaks down to 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) (Copaja et al. 2006; Rice et al. 2005).

Levels of BX in cereal rye can vary depending on the cultivar, growth stage of the cereal rye cover crop, and growing conditions. Burgos et al. (1999) reported that levels of DIBOA and BOA ranged from 112 to 1,240 µg/g and 18 to 229 µg/g, respectively, in rye shoot tissue among nine cultivars of cereal rye harvested at boot stage. Souza et al. (2019) quantified MBOA at a concentration of 0.5 to 2.5 mg/g of dry shoot tissue based on the growth stage of cereal rye grown under monocropping or intercropping systems. In another study, total BX levels of 12.2 to 145.0 mg/kg of dry shoot weight of cereal rye were detected in the boot to early heading stage (Rice et al. 2012). In addition, the levels of BX compounds can vary between root and shoot tissues.

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Greater MBOA concentrations have been reported in the cereal rye root tissue (7.2 to 115 µg/g of dry root tissue) compared with 0.7 to 2.7 µg/g in dry rye shoot tissue (Rice et al. 2005). Finally, the persistence, availability, and activity of allelochemicals in soil is influenced by a range of biotic and abiotic factors (Inderjit and Weiner 2001; Schulz et al. 2013; Teasdale et al. 2012). Studies have shown that BOA and MBOA are relatively more stable and present at higher concentrations in soil than other BXs (Schulz et al. 2012; Teasdale et al. 2012).

There are several reports of BXs suppressing the growth of numerous small grains and other plant species (Burgos and Talbert 2000; Kato-Noguchi and Macias 2005; Rice et al. 2005), reducing mycelial growth of fungi (Martyniuk et al. 2006), increasing nematode mortality (Zasada et al. 2005), and inhibiting larval development of insects (Klun and Brindley 1966; Klun et al. 1967). Still other studies have reported that fungi, specifically *Fusarium* spp., are tolerant to MBOA and BOA (Glenn et al. 2001; Saunders and Kohn 2009). Chen et al. (2010) found that DIMBOA and MBOA affected the soil microbial community structure; in particular, fungal populations increased in the wheat rhizosphere.

In the upper Midwest, cold temperatures and short growing seasons limit the variety of cover crops that can be grown. In Iowa, cereal rye is the most frequently used cover crop because it establishes well even if planted in late fall, is winter hardy, produces adequate biomass for good ground cover, and fits well within the corn-soybean production system (Singer et al. 2007; Snapp et al. 2005). However, in some years and fields, corn yield has been reported to decrease following cereal rye cover crop (Kaspar and Bakker 2015; Miguez and Bollero 2005). Consequently, many corn producers are hesitant to use cover crops. Potential causes of this yield decline include allelopathy (Raimbault et al. 1990; Tollenaar et al. 1993), nitrogen immobilization (Karlen and Doran 1991; Kessavalou and Walters 1997), poor planter performance (Duiker and Curran 2005; Raimbault et al. 1991), and a higher risk of seedling disease (Acharya et al. 2017; Bakker et al. 2016).

Greater corn seedling disease following a cereal rye cover crop is not surprising because cereal rye and corn share some of the same root pathogens. Our previous research has shown that growing a cereal

rye cover crop before corn can serve as a green bridge for corn seedling pathogens, specifically *Pythium* spp. that belong to clade B, resulting in increased root rot and reduced plant population (Acharya et al. 2017; Bakker et al. 2016). Additionally, allelochemicals released from cereal rye plants during growth or decomposition may interact with these pathogens to increase pathogenicity. We hypothesized that cereal rye allelochemicals may affect *Pythium* pathogenicity and corn seedling disease development and, therefore, contribute to corn yield decreases observed in cereal rye cover crop–corn production systems. The goal of this research was to elucidate the effect of an allelopathic compound (MBOA) on growth of *Pythium* spp., corn seedling development, and seedling disease of corn in vitro. One of the most prevalent BXs associated with *S. cereale* is MBOA, which is formed after the degradation of DIMBOA, a dominant BX compound produced by *S. cereale* (Schulz et al. 2012).

The effect of four concentrations of MBOA was evaluated on the mycelial growth of seven *Pythium* spp. recovered from the Iowa soils, the growth of corn seedlings, and corn seedling disease development using a plate assay. The *Pythium* spp. chosen—namely, *Pythium torulosum*, *P. oopapillum*, *P. lutarium*, *P. arrhenomanes*, *P. sylvaticum*, *P. irregulare*, and *P. spinosum*—are commonly associated with diseased corn seedlings in Iowa (Matthiesen et al. 2016) (A. Robertson, personal communication). Each assay was done in an incubator set at one of three temperatures: 13°C (55°F), 16°C (61°F), or 22 to 23°C (room temperature [RT]; 72°F). In Iowa, corn is planted late April to early May, when soil temperature reaches 10°C (50°F) and continues to warm up (Abendroth et al. 2017).

MBOA (catalog number 543551-1G; Sigma-Aldrich) was dissolved in 100% dimethyl sulfoxide (DMSO) to make a stock solution and then added to a 4% V8 juice medium (DV8) containing neomycin sulfate (50 µg/ml) and chloramphenicol (10 µg/ml) (DV8++) (Schmitthenner and Bhat 1994) after autoclaving, when media temperature had cooled to 50°C, for final concentrations of MBOA at 0.25, 0.125, or 0.062 mg/ml, or no MBOA (0 mg/ml). The final concentration of DMSO was adjusted to 0.5% based on preliminary studies which indicated that 0.5% DMSO had no effect on the growth of corn or *Pythium* spp. Therefore, all plates, including the controls (treatment without the addition of MBOA), contained 0.5% DMSO. Corn seed (NK0760-3111) were sterilized with 1% sodium hypochlorite (NaOCl) for 3 min, rinsed with sterile water four times for approximately 1 min each time, placed between two sterile paper towels, and pressed to remove excess water. Five corn seeds were spaced evenly apart around the perimeter of each petri plate (100-mm diameter by 15-mm depth) containing DV8++ media amended with MBOA. For treatments that required inoculation with *Pythium* spp., discs of *Pythium* (5 mm in diameter) were cut from the leading edge of a *Pythium* culture grown on DV8++ and placed in the center of the plates. Treatments (i.e., DV8++ plates amended with different concentrations of MBOA containing either *Pythium* spp. alone, corn seed alone, or *Pythium* spp. and corn seed) were incubated at 13 or 16°C or RT for 10 to 25 days in a completely randomized design within each incubator. There were four (run 1) or five (run 2) replications per treatment. The experiment was repeated twice.

The mycelial growth of *Pythium* was recorded daily by measuring the diameter of the colony for up to 7 days at all three temperatures tested. Area under the mycelial growth curve (AUMGC) was calculated as a measurement of the growth of *Pythium* spp. over time (Kandel et al. 2012). When the mesocotyl of the corn seedlings was approximately 1 cm long in any treatment, the seedlings were destructively sampled for measurements. Therefore, because the seedlings germinated and grew more slowly at cooler temperatures, seedlings incubated at 13 and 16°C and RT were destructively sampled and measured at 24 to 25 days, 16 days, and 11 days after incubation, respectively. Corn growth parameters (mesocotyl length, radicle length, and coleoptile length) and percentage of seedling root rot of each seedling was recorded following methods described by Acharya et al. (2020).

Analysis of variance was performed for all variables using PROC GLIMMIX in SAS (version 9.4; SAS Institute Inc., Cary, NC, U.S.A.). To analyze the effect of MBOA on *Pythium* growth, run, temperature, MBOA, corn seed, and their interactions were considered as fixed-effect factors and replication within a run as a random

factor. Run–MBOA interaction was not significant ($P = 0.054$); therefore, data from both runs were combined. Because a significant temperature–MBOA interaction ($P < 0.0001$) was detected, temperatures were analyzed separately. Run–temperature–MBOA–seed interaction was not significant ($P = 0.7346$) for *Pythium* growth. Similarly, to analyze the effect of MBOA and MBOA + *Pythium* on growth of corn seedlings, run, temperature, MBOA, and MBOA + *Pythium* were considered as fixed factors and replication within a run as a random factor. Run–MBOA interaction was not significant for all corn seedling parameters (mesocotyl length, radicle length, coleoptile length, and root rot) measured ($P = 0.9848$, 0.3863, 0.0712, and 0.4146, respectively). A significant run–MBOA + *Pythium* interaction ($P = 0.049$) was detected for radicle length; however, there was a consistent trend across runs showing longer radicles in check plates (containing no MBOA) compared with plates amended with MBOA for *P. lutarium*, *P. oopapillum*, *P. torulosum*, and *P. spinosum*, while radicle length was shorter in check plates compared with those amended with MBOA for *P. sylvaticum*, *P. irregulare*, and *P. arrhenomanes*; therefore, results from both runs were combined. Temperature–MBOA and temperature–*Pythium* were significant ($P < 0.05$) for all variables measured except for mesocotyl length; therefore, data were analyzed separately for each *Pythium* sp. at each temperature. When treatment effects were detected, Fisher's protected least significant difference was used to separate treatment means at $P = 0.05$.

In general, BXs are known to have antimicrobial properties. In our study, all *Pythium* spp. that were tested were tolerant to MBOA at 0.0625 and 0.125 mg/ml across all temperatures using AUMGC as a measurement of mycelial growth (Fig. 1). We defined tolerance to MBOA as greater than 90% mycelial growth (AUMGC) on MBOA in comparison with the check plates (no MBOA). At 13°C, AUMGC of *P. torulosum*, *P. arrhenomanes*, *P. irregulare*, and *P. spinosum* were approximately 80% of the AUMGC of each check. In contrast, the highest concentration of MBOA tested (0.25 mg/ml) considerably inhibited growth of all *Pythium* spp. at all temperatures tested. Similarly, previous research (Glenn et al. 2001) assessed fungal growth on MBOA at 0.25 to 1.0 mg/ml and found that most *Fusarium* spp. were tolerant to MBOA. Because we were interested in looking at the interaction effect of MBOA and *Pythium* spp. on corn seedling disease, we did not include the highest concentration of MBOA (0.25 mg/ml) in subsequent experiments evaluating the effect of MBOA alone and MBOA + *Pythium* on corn growth and corn seedling disease development.

In this study, the 0.0625 and 0.125 mg/ml concentrations of MBOA had no effect on mycelial growth of the *Pythium* spp. tested. Previous studies have shown that BXs and their breakdown products directly inhibit mycelial growth of fungi (Martyniuk et al. 2006) or are involved in the activation of plant innate immune responses against pathogens (Ahmad et al. 2011; Makowska et al. 2015). Still other studies have reported that some fungi, specifically *Fusarium* spp., are tolerant to MBOA and BOA (Glenn et al. 2001; Saunders and Kohn 2009). Moreover, Neal et al. (2012) found that BXs attracted the bacterium *Pseudomonas putida* to the rhizosphere. Chen et al. (2010) found that DIMBOA and MBOA affected the soil microbial community structure and, in particular, increased fungal populations in the wheat rhizosphere, and may have favored some species over others. Because our tests were conducted in sterile conditions with only the target species present, we may not have detected an effect of lower concentrations of MBOA on mycelial growth of *Pythium* spp. that may otherwise occur in a field in the presence of the microbial community.

Presence of corn seed did not affect the growth of *Pythium* spp. ($P = 0.7346$). Because seed exudates stimulate the production and germination of sporangia of *Pythium* (Nelson 1990; Serrano and Robertson 2018), we expected that mycelial growth might be faster in the presence of corn seed; however, under the conditions of our study, this did not occur. Temperature, however, did affect *Pythium* spp. growth ($P < 0.0001$) (Fig. 1), as has been previously reported (Matthiesen et al. 2016; Radmer et al. 2017; Rojas et al. 2017; Serrano and Robertson 2018).

MBOA without pathogens affected root length but the effect of MBOA was not consistent for coleoptile length (Table 1).

Coleoptiles of corn seedlings grown on plates amended with MBOA at 13°C were longer than those of corn seedlings grown on check plates (no MBOA) at the same temperature. Conversely, at RT, plates amended with MBOA had shorter coleoptiles than the control. At 16°C, there was no significant effect of MBOA on coleoptile length. The radicles of corn seedlings grown on plates amended with MBOA were shorter at 13 and 16°C than the radicles of corn seedlings grown on check plates at 13 and 16°C ($P < 0.05$). At RT, there was no significant effect of MBOA on radicle length. Similarly, no effect of MBOA on mesocotyl length was detected at any temperature (Supplementary Table S1). As expected, without addition of pathogens,

there was no effect of MBOA on root rot or lesions (Table 1). There are numerous reports on the reduction of plant growth and seedling emergence due to BX compounds (Gavazzi et al. 2010; Hasegawa et al. 1992; Schulz et al. 2012; Souza et al. 2019), including corn (Burgos and Talbert 2000). Hasegawa et al. (1992) reported that MBOA was an antiauxin that inhibited shoot and root growth of timothy grass and *Amaranthus* seedlings. Furthermore, BXs were reported to interfere with auxin-binding sites through competition and, therefore, inhibit auxin-induced growth in plants (Hoshi-Sakoda et al. 1994; Venis and Watson 1978). In the current study, corn shoot and root growth inhibition

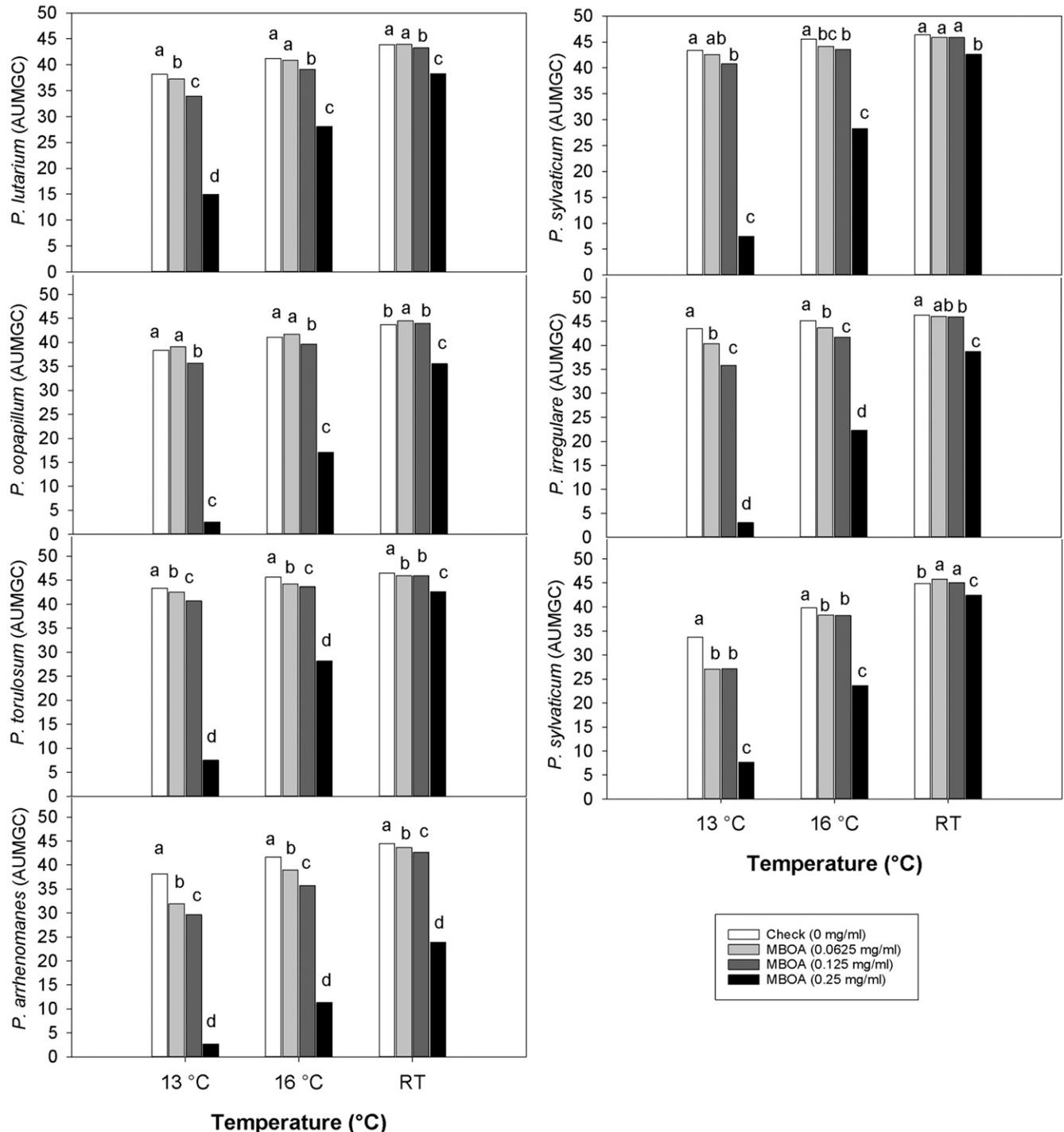**Fig. 1.** Area under the mycelial growth curve (AUMGC) of *Pythium* spp. grown on 4% V8 juice medium containing neomycin sulfate and chloramphenicol media amended with 6-methoxy-2-benzoxazolinone (MBOA) for 7 days at 13 and 16°C and room temperature (RT) in a plate assay. Bars within each temperature with the same letter are not significantly different at $P = 0.05$ compared using Fisher's least significant difference.

observed on plates amended with MBOA may have been due to an interference in auxin-induced growth from the exogenous MBOA. We suggest that the exogenous MBOA tested in this study simulates MBOA that would be released by the terminating rye cover crop.

In our study, the severity of corn seedling root rot varied with temperature and the *Pythium* spp. being evaluated. Several studies have demonstrated the effect of temperature on root rot severity of corn and soybean caused by *Pythium* spp. (Matthiesen et al. 2016; Radmer et al. 2017; Rojas et al. 2017; Wei et al. 2011). In general, root rot severity caused by *Pythium* spp. increased as the temperature decreased. Some authors (Munkvold 1999; Serrano and Robertson 2018) have speculated that lower temperature slows seed germination and seedling development, thus allowing more time for infection of the host plant by *Pythium* spp.

When corn seedlings were grown on DV8++ media amended with MBOA and inoculated with *Pythium* spp. (MBOA + *Pythium*), root rot severity increased with some of the *Pythium* spp. tested at some temperatures (Table 1; Supplementary Fig. S1). Root rot severity was greater on corn seedlings grown on DV8++ amended with MBOA and inoculated with *P. lutarium* compared with corn seedlings grown on the check across all temperatures (no MBOA + *P. lutarium*). More root rot was observed on corn seedlings grown on DV8++ amended with MBOA at 0.125 mg/ml and inoculated with *P. oopapillum* at 16°C and RT compared with the check at 16°C (no MBOA + *P. oopapillum*). Similarly, more root rot was detected on corn seedlings grown on DV8++ amended with MBOA and inoculated with *P. torulosum* or *P. spinosum* at 16°C when compared with the corresponding check. Interestingly, three of these four *Pythium* spp. i.e. (*P. lutarium*, *P. oopapillum*, and *P. torulosum*) for which an interaction with MBOA was observed (more root rot) belong to the *Pythium* clade B group. Although Bakker et al. (2016) reported that cereal rye was a host of *Pythium* spp. belonging to clades B and F, we found that *Pythium* clade B was primarily involved in seedling disease of corn following cereal rye (Acharya et al. 2017, 2020). It remains unclear why *Pythium* clade B is the dominant clade of *Pythium* associated with the system of corn grown after cereal rye is grown in Iowa.

Table 1. Effect of 6-methoxy-2-benzoxazolinone (MBOA) concentration on corn seedling growth and root rot in the absence or presence of *Pythium* spp. at three temperatures in a plate assay[x]

Species, MBOA[y]	Coleoptile length (cm)			Radicle length (cm)			Root rot (%)[z]		
	13°C	16°C	RT	13°C	16°C	RT	13°C	16°C	RT
None									
0	1.94 b	2.41	4.81 a	6.11 a	6.81 a	5.61	0.0	0.0	0.0
0.0625	4.13 a	2.11	3.75 b	3.39 b	6.0 b	5.37	0.0	0.0	0.0
0.125	3.68 a	2.27	3.26 b	2.72 b	4.56 c	5.01	0.0	0.0	0.0
P value	0.0198	0.1505	0.0063	0.0046	<0.0001	0.08	NA	NA	NA
Pythium lutarium									
0	2.93	6.51	7.73 ab	2.07	3.41 a	4.72 a	70.2 b	20.7 b	0.7 b
0.0625	2.36	6.65	8.30 a	1.94	2.88 a	3.75 b	80.6 a	32.8 ab	6.8 b
0.125	2.41	6.46	6.37 b	1.69	2.03 b	2.76 c	78.7 a	46.5 a	27.4 a
P value	0.7634	0.9729	0.0481	0.8743	0.0044	<0.0001	0.0219	0.0250	0.0004
P. oopapillum									
0	2.61	7.50 a	8.00	2.46	3.73 a	3.43	74.6	5.6 c	0.1 b
0.0625	2.18	7.01 a	8.29	1.79	2.72 b	3.38	85.5	19.7 b	2.9 b
0.125	2.26	5.0 b	8.16	1.51	2.08 c	3.32	80.3	40.7 a	7.2 a
P value	0.8252	0.0003	0.9429	0.2139	0.0001	0.9728	0.0623	<0.0001	0.0039
P. torulosum									
0	3.74	6.07 a	7.79 a	2.78	3.1471	5.17 a	42.6	7.3 b	1.7
0.0625	3.66	4.52 b	6.11 b	2.68	2.5714	3.53 b	36.5	9.9 b	4.4
0.125	3.13	3.91 b	5.61 b	2.34	2.4849	3.01 b	42.2	13.7 a	8.2
P value	0.3618	0.0142	0.0110	0.6260	0.1123	0.0119	0.3305	0.0078	0.2031
P. arrhenomanes									
0	0.31	3.21	2.92	0.68	0.48 b	0.85	98.4 a	89.6	69.7
0.0625	0.91	3.58	3.06	1.62	0.66 b	0.86	94.9 b	88.0	68.9
0.125	1.21	2.99	2.53	1.73	1.10 a	1.16	86.5 c	78.6	68.0
P value	0.1009	0.6992	0.3754	0.1681	0.0003	0.3671	0.0022	0.0769	0.9837
P. sylvaticum									
0	0.29	2.37	4.42	0.66	0.32	0.52	97.3	87.5	74.2
0.0625	0.47	1.95	3.75	0.34	0.26	0.76	98.5	91.3	75.0
0.125	1.33	2.40	3.73	0.61	0.28	0.55	94.4	89.0	75.0
P value	0.1739	0.4964	0.2636	0.6251	0.4901	0.7108	0.0570	0.7093	0.9550
P. irregulare									
0	0.01	2.41	2.65	0.09	0.22	0.28	99.0	92.1	82.7 a
0.0625	0.43	1.40	2.86	0.18	0.25	0.32	98.8	95.2	85.4 a
0.125	0.40	2.84	2.50	0.28	0.42	0.36	98.3	87.5	72.5 b
P value	0.5238	0.2625	0.7175	0.5659	0.1633	0.1235	0.8584	0.1773	0.0189
P. spinosum									
0	2.90	6.4 a	5.37	2.29	2.02 a	1.96	44.0	18.1 b	28.7
0.0625	2.81	3.9 b	4.93	2.14	1.34 b	1.74	45.4	33.3 a	34.1
0.125	2.12	4.30 b	4.66	2.07	1.66 ab	1.89	50.4	41.3 a	28.0
P value	0.3029	0.0018	0.5930	0.8086	0.0216	0.7186	0.4533	0.0010	0.2863

[x] Values followed by the same letter within a column are not significantly different at *P* value = 0.05. Abbreviations: RT = room temperature and NA = not applicable.

[y] *Pythium* spp. and concentration of MBOA (mg/ml). Check (0) = 4% V8 juice medium containing neomycin sulfate and chloramphenicol with 0.5% dimethyl sulfoxide.

[z] Root rot was calculated based on the percentage of root tissue rotted.

In general, presence of MBOA with the *Pythium* spp. increased the percentage of root rot severity in corn seedlings. Thus, we can assume that MBOA did not activate an innate immune response in the corn radicals that may have inhibited or reduced infection. A possible reason for increased corn root rot by all but one *Pythium* sp. in this study in the presence of exogenous MBOA could, again, be due to interference in the auxin signaling pathway (Hasegawa et al. 1992; Hoshi-Sakoda et al. 1994; Venis and Watson 1978). Ludwig-Müller (2015) stated that auxin regulates genes required for plant defense, and affecting the regulation of auxin-regulated genes in the auxin signaling pathway increased the susceptibility of plants to pathogens. In the current study, we speculate that the exogenous MBOA modulated auxin actions, as shown by decreased growth, and that may have increased susceptibility of the seedlings to *Pythium* spp. These data suggest that, in some situations, the presence of exogenous MBOA as a result of cereal rye cover crop decomposition may play a role in increased seedling disease observed on corn planted after a cereal rye cover crop. Further research is warranted to understand the mechanisms behind the interaction of MBOA and *Pythium* spp. to enable a thorough understanding of the negative effects that a cereal rye cover crop may have on the following corn crop.

This study provides additional insight into why increases in corn seedling disease occur in the winter rye cover crop–corn production system. Based on our previous work, we had recommended terminating a cereal rye cover crop 10 days or more before planting corn to reduce the cereal rye "green bridge" effect by allowing more time for cereal rye to die, begin decomposition, and eliminate the presence of a host for corn pathogens (Acharya et al. 2017). Based on the current study, we would speculate that terminating the cover crop 10 or more days before planting corn not only disrupts the green bridge effect but also allows more time for allelochemicals produced by the cover crop to breakdown to products that are not harmful to the corn seedling. BX compounds decomposed over a 10-day period to minimal (Krogh et al. 2006; Rice et al. 2012). Therefore, further research to understand the role of allelopathy and its interaction with corn seedling pathogens could contribute to better management of cereal rye cover crops before corn, improved corn growth and yield, and more adoption of cover crops by Iowa farmers.

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