Co-infection and Disease Severity of Ohio *Maize dwarf mosaic virus* and *Maize chlorotic dwarf virus* Strains

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Abstract

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Two major maize viruses have been reported in the United States: *Maize dwarf mosaic virus* (MDMV) and *Maize chlorotic dwarf virus* (MCDV). These viruses co-occur in regions where maize is grown, such that co-infections are likely. Co-infection of different strains of MCDV is also observed, and a synergistic enhancement of symptoms in co-infected plants was previously reported. Here, we examined the impact of co-infections of two strains of MCDV (MCDV-S and MCDV-M1, severe and mild, respectively), and co-infections of MCDV and MDMV in the sweet corn hybrid 'Spirit' in greenhouse experiments. Quantitative plant growth and development parameters were measured and virus accumulation was measured by reverse-transcriptase quantitative polymerase chain reaction. Virus symptoms were enhanced and plants showed no recovery over time in co-infections of MDMV-OH and MCDV-S but virus titers and quantitative growth parameters did not indicate synergy in co-infected plants. MCDV-M1 co-infections with either MDMV-OH or MCDV-S did not show symptom enhancement or evidence of synergism.

Maize dwarf mosaic virus (MDMV) and *Maize chlorotic dwarf virus* (MCDV) are both common maize-infecting viruses in the United States (18). MDMV is an aphid-transmitted member of the genus *Potyvirus*, family *Potyviridae*, and was originally described and isolated in Southern Ohio (13,33). In infected plants, it and related viruses in the sugarcane mosaic virus subgroup of the genus *Potyvirus* cause Maize dwarf mosaic disease (18,33). MCDV, transmitted by the blackfaced leafhopper, *Graminella nigrifrons*, was also originally isolated in southern Ohio (27). MCDV is a member of the genus *Waikavirus* in the family *Secoviridae* (29). As the name indicates, it causes stunting and chlorosis in infected plants, with characteristic veinal chlorosis.

Both MDMV and MCDV were first described in the southeastern and Midwest corn-growing regions of the United States, emerging as a significant disease complex in the 1960s to 1970s (8,9,13,20,27). Control measures have been successful in reducing the impact of disease in subsequent decades but both viruses are still present, and some varieties of cultivated sweet corn in the United States remain susceptible, at least to MDMV (40; *unpublished data*). Given the co-occurrence of the viruses, we examined whether they had potentially agriculturally significant interaction in co-infections. Despite the overlapping distribution of the viruses and some anecdotal reports of more severe disease pressure with both viruses, controlled studies testing disease outcomes of co-infections have never been reported to our knowledge (8).

Virus co-infections are not unusual in cropping systems and in natural ecosystems. Several possible outcomes of virus co-infections have been described and reviewed elsewhere (26,36), and can be categorized as neutral (no interaction between co-infecting viruses), antagonistic, or synergistic. Antagonistic interactions include competition or superinfection exclusion of co-infecting viruses and can be an important agronomic outcome with utility for controlling virus diseases such as by cross-protection (36). Synergistic interactions, in which transmission or virulence of one or both viruses is increased in a more than additive manner in co-infections (41), are especially important to agriculture because they result in exacerbated disease. Synergy has been measured differently in different studies, usually using multiple parameters. A synergistic interaction may result in higher titers of one, both, or neither virus but, by definition, results in more than additive disease symptoms or enhanced transmission of at least one virus (41). Synergistic virus interactions cause several important viral diseases worldwide, including rice tungro disease (10,11), maize lethal necrosis (7,30), and sweet potato virus disease (15).

MDMV is prevalent in southern Ohio, and infection of maize with MDMV has also been shown to increase susceptibility to other nonviral diseases (22). A prior study indicated that co-infection of two different strains of MCDV, one mild and one severe, resulted in synergistic infection with very severe symptoms (6). The relative rarity of synergy resulting from co-infections of different strains or isolates of the same virus (3,23), and findings that MCDV-M1 is prevalent in southern Ohio (L. R. Stewart, R. Teplier, J. C. Todd, M. W. Jones, B. J. Cassone, S. Wijeratne, A. Wijeratne, and M. G. Redinbaugh, *in press, Phytopathology*), as well as the absence of information about virus titer during co-infection, prompted our reexamination of interactions between co-infecting MCDV strains as well as MDMV/MCDV co-infection.

Materials and Methods

Treatments. MCDV-S/MDMV-OH, MCDV-M1/MDMV-OH, and MCDV-M1/MCDV-S co-infections were tested in three sepa-

rate sets of experiments, each with four treatments: single infection of each virus, co-infection of two viruses, and healthy control. For MCDV-S/MDMV-OH co-infection experiments, 30 plants of sweet corn hybrid 'Spirit' were tested per treatment; whereas, for MCDV-M1/MDMV-OH and MCDV-M1/MCDV-S co-infection experiments, 20 plants were tested per treatment. Each experiment set was repeated three times.

Virus isolates. MCDV-M1, MCDV-S, and MDMV-OH were used. Isolates were originally collected near Portsmouth, OH (12,17) and complete sequences have been reported (4,35; R. E. Gingery, R. Chaouch-Hamada, M. G. Redinbaugh, and S. A. Hogenhout, *unpublished*).

Planting. Single seed were planted into 21-by-3.8-cm "Conetainers" (Stuewe and Sons Inc.) containing greenhouse soil (Potting Mix; Lawn Products Inc.) and one tablespoon of Scotts Osmocote Classic Controlled Release Fertilizer.

Inoculation with MCDV. Insects were used for inoculation with MCDV, using methods similar to those previously described (19,37). Five days after planting, uniform seedlings were selected and distributed among four 30.5-by-30.5-cm racks (Stuewe and Sons Inc.), each holding 49 seedlings. Then, 400 viruliferous *G. nigrifrons* were introduced to seedlings in two dacron cages for inoculation with MCDV-S or MCDV-M1. For postinoculation with MDMV-OH and healthy control, 400 nonviruliferous *G. nigrifrons* were introduced in two cages of plants. To maximize infection, insects were removed and a fresh batch of viruliferous insects was added every other day for a total of three inoculations. At day 11 post-planting, plants were fumigated and transferred to greenhouses for symptom development. MCDV-infected plants were selected by symptoms (MCDV-S) or reverse-transcriptase polymerase chain reaction (RT-PCR) (MCDV-M1). For MCDV-M1/MCDV-S co-infection, three inoculations with 400 viruliferous leafhoppers were done for each virus.

Inoculation with MDMV-OH. Twelve-day-old plants were inoculated with MDMV-OH after leafhopper inoculations. MDMV-OH-infected maize leaves were ground 1:10 (wt/vol) in 0.01 M potassium phosphate buffer, pH 7.0, and inoculated to test plants with carborundum. Rub inoculation was performed three times every other day on all leaves of the plants, as previously described (14).

Symptom evaluation. Symptoms were evaluated after the final day of inoculation with MDMV. At this time, the MCDV/MDMV-OH co-infected plants were 7 days post first inoculation with MDMV-OH and 14 days post first inoculation with MCDV. For simplicity, this time point will be referred to as T1. Symptoms were evaluated four times (T1, T2, T3, and T4) at 7-day intervals. Length of longest leaf (centimeters), length of youngest leaf (centimeters), height of highest node (centimeters), and fresh plant weight (pounds) were the quantitative growth properties measured; vein banding, chlorosis, and mosaic were the qualitative symptoms. The two uppermost leaves of individual plants were scored for each symptom using a three-point scale, where 0 = no symptoms, 1 = mild or limited symptoms, and 2 = intense symptoms.

Primer design. Primer3 v. 0.4.0 (28) was used for primer design and MacVector v. 12.6 was used for multiple sequence alignments of published virus genome sequences. RT-quantitative (q)PCR primers were MCDV-S 4148f 5′-TTGGGTGTGTGCCATTCT CCC-3′ Tm 57.9°C and MCDV-S 4275r 5′-TGCCGCAGGTTG ACTGTCTAAG-3′ Tm 59.4°C, yielding a 127-bp product; and MDMV-OH 4272f 5′-GGATGTTGACGTTGTTGTCG-3′ Tm 54.3°C and MDMV-OH 4413r 5′-TGGTTTTGTCCTCCC AACTC-3′ Tm 55.0°C, yielding a 171-bp product. Normalization 18S primers based on GenBank sequence U42796.1 were designed and provided by Dr. Bryan Cassone (Ohio State University): 18S-f 5′-GATTCCGGTCCTATTGTGTTG-3′ Tm 53.4°C and 18S-r 5′-TTTCGCAGTTGTTCGTCTTT-3′ Tm 52.9°C, yielding a 125-bp product. Primers were tested for amplification of a single product of expected size from template cDNA and tested for cycle threshold (Ct) values and primer efficiency. Standard curve amplifications were performed using RT-PCR products (amplified

using Access RT-PCR System; Promega Corp.) cloned into pGEM-T Easy Vector (Promega Corp.), and expected insertions were verified by sequencing and plasmid digestion with *Eco*RI HF enzyme (New England Biolabs). Plasmids were used for dilutions for standard curves to validate primer pairs for RT-qPCR. Reactions were performed in a CFX96 Touch Real-Time PCR Detection System, using Bio-Rad's EvaGreen Fast SSO supermix and Hard-Shell 96-Well 480 PCR Plates, using a cycle of 95°C for 30 s; followed by 95°C for 3 s, 57°C for 5s, and plate read repeated 30 times. Melting curve analysis was performed at 65 to 95°C, in increments of 0.5°C per 5 s followed by plate reading.

SYBR Green RT-qPCR. Total RNA was extracted from –80°C frozen leaves using Direct-zol RNA MiniPrep kit (Zymo Research Corporation). RNA was treated with DNase I (Invitrogen) according to the manufacturer's instructions. Total RNA (1.0 µg) was used for each cDNA synthesis reaction. cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad) using oligo(dT) and random primers. RT-qPCR was performed using EvaGreen Fast SSO supermix according to the manufacturer's instructions. Primers were used at 0.25 µM concentrations with 1:10 diluted cDNA (absolute cDNA concentrations were not measured). Five samples per treatment per experiment were used to measure virus [RNA]. RT-qPCR reactions were performed with three pairs of primers and each sample was analyzed in duplicate. Fold changes were calculated using ΔΔCt values.

Data analysis. Calculations for analysis of variance (ANOVA), least significant difference (LSD), and Dunnett tests were performed using SAS (SAS Institute, Inc.). For RT-qPCR analyses, ANOVA and LSD tests were performed using PROC GLM. Samples in which virus was detected or not detected did not match the treatment set and samples in which the values were outside of the mean ± two standard deviations of the sample set were discarded. At least three biological replicates were used for RT-qPCR analyses per treatment and time point.

Results

MCDV-S/MDMV-OH co-infection. For MCDV-S/MDMV-OH co-infected plants, we observed no quantitative growth parameters that were significantly different from at least one of the single virus-infected plant sets, except for area under the disease progress curve (AUDPC). Infected treatments were different from healthy plants for all parameters except length of longest leaf, in which MDMV-OH-infected plants were not significantly different from healthy plants. There were significant differences in longest leaf length between experiments, which were carried out from summer to fall (*data not shown*). Mean wet weights of MCDV-S-infected plants (1.7 lb [0.77 kg]) and co-infected plants (1.5 lb [0.68 kg]) were significantly were significantly lower than that of MDMV-OH-infected plants (3.0 lb [1.4 kg]), which was significantly lower than that of healthy plants (4.0 lb [1.8 kg]) (Table 1). AUDPC from T1 to T4 was significantly higher for co-infected plants than either single infection set, reflecting persistence of strong symptoms in co-infections but not single infections after T2 (*data not shown*). Chlorosis, mosaic, and vein-banding symptoms were stronger in co-infected plants compared with single-infected plants at T3 and T4 (*data not shown*).

The amount of MCDV-S RNA detected by RT-qPCR in the co-infection treatment was always greater than the amount present in the single-infection treatment but the difference was only significant in the first experiment. The amount of MDMV-OH RNA detected was significantly higher in co-infected plants than single-infected plants only in the third experiment (Table 2).

MCDV-M1/MDMV-OH co-infection. MCDV-M1/MDMV-OH co-infection and single infections had similar longest-leaf lengths and highest-node heights (Table 3), differing only from healthy plants. The youngest leaf height was not significantly different between any treatments (Table 3). The fresh weight was lowest in plants singly or co-infected with MCDV-M1 (Table 3). Chlorosis symptoms were limited, and virus symptoms decreased from T1 to T4 in both MDMV-OH and co-infected plants (*data not shown*).

MCDV-M1 symptom AUDPC was indistinguishable from healthy plants, and co-infected plants had symptom AUDPC similar to that of MDMV-OH single-infected plants (Table 3). Virus levels were not measured by RT-qPCR for MCDV-M1/MDMV-OH co-infection experiments.

MCDV-M1/MCDV-S co-infection. There was no significant variation between MCDV-M1/MCDV-S co-infection experiments, and no significant differences between co-infected plants and MCDV-S-infected plants for longest leaf length, highest node height, or wet weight (Table 4). No significant differences between healthy and MCDV-M1 single-infected plants were observed for any measurement except wet weight (Table 4). Symptom AUDPC of co-infected plants was intermediate between each single infection set (Table 4). Typical vein-banding symptoms induced by MCDV-S alone were strongest at T2 and T3 but limited by T4 (*data not shown*). Although MCDV-M1 was asymptomatic in most plants at the time points scored, infection was confirmed in ran-

domly selected plants by RT-PCR. Some co-infected plants showed very severe symptoms before T1, with strong twisting and tearing most notable. However, these symptoms subsided by T1 and later time points, and twist and tear was negligible at these time points and, therefore, omitted from analyses (Table 4; *data not shown*).

Discussion

Natural virus co-infections in plants are common, and can impact disease potential. Because two major U.S. maize viruses, MDMV and MCDV, overlap in distribution, we compared single and co-infections for disease symptoms and quantitative plant growth parameters. Co-infection of MCDV-S and MDMV-OH resulted in enhanced persistence of disease symptoms but not reduced growth over 4 weeks after co-infection. Measurements indicated that neither MCDV-S nor MDMV-OH titer consistently increased in co-infected plants. The mild strain of MCDV (MCDV-M1) showed no interaction with MDMV-OH in co-infections and

Table 1. Maize growth and symptoms in *Maize dwarf mosaic virus* (MDMV)-OH, *Maize chlorotic dwarf virus* (MCDV)-S, and co-infected compared with healthy plants

	Phenotype score[a]									
Treatment[b]	Longest leaf (cm)	LSD	Youngest leaf (cm)	LSD	Node height (cm)	LSD	Wet weight (kg)	LSD	AUDPC	LSD
HC	91.7	A	69.9	A	60.4	A	1.8	A	0.0	A
MDMV-OH	77.6	AB	58.5	BC	46.5	C	1.4	BC	80.5	B
MCDV-S	62.1	BC	56	C	48.7	BC	0.77	D	72.3	B
S + OH	52.3	C	47.3	DC	42.4	C	0.68	D	156.3	C
Prob F Treat[c]	0.0049	…	0.0012	…	0.0138	…	0.0012	…	<0.0001	…
Prob F Exp[c]	0.0006	…	0.0039	…	0.0358	…	0.0113	…	0.9842	…

[a] Except for area under the disease progress curve (AUDPC), data presented are the means of T4 measurements from three experiments with 30 plants each. Quantitative scores: length of longest leaf, length of youngest leaf, height of highest node, fresh plant weight (lb), and AUDPC from T0 to T4 were calculated from summed scores for chlorosis, vein-banding, and mosaic.
[b] HC = healthy control, OH = Ohio isolate, S = severe strain, and S + OH = co-infected.
[c] Probability value from analysis of variance for differences between treatments and experiments.

Table 2. Amount of viral RNA detected by reverse-transcriptase quantitative polymerase chain reaction in *Maize dwarf mosaic virus* (MDMV)-OH, *Maize chlorotic dwarf virus* (MCDV)-S, and co-infected plants compared with healthy control maize

	Relative amount of virus[a]											
	Experiment 1				Experiment 2				Experiment 3			
Treatment[b]	S	LSD	OH	LSD	S	LSD	OH	LSD	S	LSD	OH	LSD
HC	0.86	C	0.9	B	0.98	B	2	B	1	B	1	C
MDMV-OH	1.05	C	1,097	A	1.28	B	20,139	A	2	B	20,835	B
MCDV-S	128	B	1	B	318	A	2	B	1,199	A	1	C
S + OH	740	A	1,315	A	388	A	16,898	A	1,606	A	38,416	A
Prob F Treat[c]	<0.0001	…	<0.0001	…	<0.0001	…	<0.0001	…	<0.0001	…	<0.0001	…
Prob F Exp[c]	0.7825	…	0.931	…	0.556	…	0.3421	…	0.2774	…	0.5326	…

[a] Amount of virus relative to one healthy control sample calculated with ΔΔ cycle threshold fold change. ANOVA was performed on fold changes. Three to five samples per experiment per treatment were tested. Columns: S and OH indicate amount of virus detected using MCDV-S and MDMV-OH primers, respectively. Treatments with the same letters for Fisher least square difference (LSD) do not statistically differ.
[b] HC = healthy control, OH = Ohio isolate, S = severe strain, and S + OH = co-infected.
[c] Probability value from analysis of variance for treatment and experiment.

Table 3. Maize growth in *Maize dwarf mosaic virus* (MDMV)-OH, *Maize chlorotic dwarf virus* (MCDV)-M1, and co-infected compared with healthy plants

	Phenotype score[a]									
Treatment[b]	Longest leaf (cm)	LSD	Youngest leaf (cm)	LSD	Node height (cm)	LSD	Wet weight (kg)	LSD	AUDPC	LSD
HC	111.8	A	80.4	A	68	A	1.5	A	0.0	A
MDMV-OH	98.2	B	75.2	A	57	B	1.1	B	67.0	B
MCDV-M1	95.2	B	60	A	48.2	B	1.0	BC	4.7	A
M1 + OH	92.3	B	68.6	A	52.2	B	0.86	C	65.6	B
Prob F Treat[c]	0.0003	…	0.004	…	<0.0001	…	0.0103	…	<0.0001	…
Prob F Exp[c]	0.012	…	0.2033	…	0.0106	…	0.002	…	0.3423	…

[a] Except for area under the disease progress curve (AUDPC), data presented are the means of T4 measurements from three experiments with 20 plants each. Quantitative scores: length of longest leaf, length of youngest leaf, height of highest node, fresh plant weight, and AUDPC from T0 to T4 were calculated from summed scores for chlorosis, vein-banding, and mosaic.
[b] HC = healthy control, OH = Ohio isolate, M1 = mild strain, and M1 + OH = co-infected.
[c] Probability value from analysis of variance for differences between treatments and experiments.

Table 4. Maize growth in *Maize dwarf mosaic virus* (MDMV)-M1, *Maize chlorotic dwarf virus* (MCDV)-S, and co-infected compared with healthy plants

Treatment[b]	Phenotype score[a]							
	Longest leaf (cm)	LSD	Node height (cm)	LSD	Wet weight (kg)	LSD	AUDPC	LSD
HC	115.6	A	38.9	A	1.8	A	0.0	A
MCDV-M1	105	A	34.1	AB	1.4	B	0.0	A
MCDV-S	75	B	25.5	C	0.64	C	62.5	C
S + M1	79.1	B	27.7	BC	0.59	C	39.7	B
Prob F Treat[c]	0.0006	…	0.0237	…	<0.0001	…	<0.0001	…
Prob F Exp[c]	0.1853	…	0.7366	…	0.9114	…	0.2795	…

[a] Except for area under the disease progress curve (AUDPC), data presented are the means of T4 measurements from three experiments with 20 plants each. Quantitative scores: length of longest leaf, length of youngest leaf, height of highest node, fresh plant weight, and AUDPC from T0 to T4 were calculated from summed scores for chlorosis, vein-banding, and mosaic.

[b] HC = healthy control, M1 = mild strain, S = severe strain, and S + M1 = co-infected.

[c] Probability value from analysis of variance for differences between treatments and experiments.

had no effect on symptom development or duration. Co-infection of two of the MCDV strains, MCDV-S and MCDV-M1, resulted in plant growth reduction similar to MCDV-S single infection and visible virus symptoms intermediate between S and M1.

Co-infection of MDMV-OH and MCDV-S might be considered mildly synergistic because of persistence of stronger symptoms, according to a limited definition of virus synergism in which co-infection symptoms are more severe than additive effects of each individual virus (41). However, other parameters did not support characterization of the co-infection as synergistic. In several reported cases of virus synergy, a potyvirus enhances accumulation of another virus (1,24–26,39). However, MDMV-OH did not enhance titer of MCDV-S, nor did MDMV-OH titer rise. Similarly, Scott et al (31) reported an absence of synergism when evaluating the effects of MCDV and MDMV in several F1 crosses between resistant and susceptible maize lines. However, they relied on natural MCDV infections and obtained relatively low infection rates (31 and 65% infection), confounding results with missed infections.

We did observe significant differences between replicated experiments in MDMV-OH/MCDV-S co-infection experiments. Plants were grown in the same greenhouses but experiments were a month apart (August, September, and October for experiments 1, 2, and 3, respectively); therefore, ambient light, day lengths, and temperatures were different. Temperature has been reported to affect MDMV titer over time (38), and other environmental factors may also be important.

Despite the previous report indicating synergy of co-infecting mild and severe MCDV (6), co-infection of MCDV-M1 and MCDV-S was not synergistic in our experiments. There are few reports of co-infection by closely related viruses causing synergy (2,16). More often, cross-protection or superinfection exclusion are observed in co-infections of closely related viruses (5,32). In the case of MCDV, strains MCDV-M1 and MCDV-S are considered the same species but their sequences are quite divergent, sharing only 57% nucleotide sequence identity and 59% amino acid identity (34). We observed symptoms intermediate between the two viruses in co-infected plants, which might be explained by competitive interaction of the related viruses. Interestingly, however, wet weight in co-infected plants was as low as in MCDV-S-infected plants, rather than intermediate. Some of the experimental differences between our study and the previous report could be important, such as use of different maize cultivars or scoring over different time periods. Because MCDV strains used in the prior report of synergy were not sequenced at the time (4,21), we cannot exclude the possibility of sequence differences in the viruses used.

We examined disease and plant growth outcomes of co-infections of major U.S. maize viruses with overlapping distributions: MDMV and MCDV. Despite extensive studies of these U.S. maize viruses because of their historic disease impact, the interactions of viruses in co-infected plants had not been the subject of careful examination. Although we did not observe strong synergism in the MCDV-S/MDMV-OH co-infection, as is seen in disease complexes such as maize lethal necrosis (30), the persistence of stronger

symptoms in co-infected plants may have important implications for disease in susceptible maize where these viruses overlap.

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