Genetic background and development stage of maize influence disease severity and yield loss due to maize lethal necrosis (MLN) and Maize chlorotic mottle virus (MCMV)

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Abstract

Nine inbred lines and nine hybrids of maize (*Zea mays*) were tested in an insect-free net-house in Naivasha, Kenya, by inoculating them with either *maize chlorotic mottle virus* (MCMV) alone or together with *sugarcane mosaic virus* (SCMV), a combination that causes maize lethal necrosis, and the impact was assessed in terms of disease severity and grain yields. The effect of the combination was much more in plants inoculated while at the early growth stage: the disease severity scores were higher, the area under disease progress curve (AUDPC) was greater, and titres of MCMV were higher. Seeds from all the inoculated plants were contaminated with MCMV irrespective of the stage at which they had been inoculated and irrespective of their level of resistance. The study highlights the need to take proper prophylactic measures before sowing, such as using disease-free seeds or treated seeds to eliminate any possible contamination with MCMV at early growth stages, to lower the adverse impact of maize lethal necrosis in countries in which the disease is endemic. Also, genotypes susceptible to the disease need to be replaced with those resistant to it.

Introduction

Maize is one of the major staple food crops in sub-Saharan Africa, providing food security to millions of people [21]. Maize lethal necrosis (MLN) emerged as a serious disease of maize in Kenya in 2011 and consequently in several countries across eastern Africa, with significant economic impact [5, 15]. The estimated annual economic impact associated with MLN on smallholder farmers in eastern Africa is about US$291–US$339 million [14]. Maize lethal necrosis is caused by a synergistic co-infection by *maize chlorotic mottle virus* (MCMV) [12, 19] and any one of the viruses of the Potyviridae family, especially *wheat streak mosaic virus* (WSMV), *maize dwarf mosaic virus* (MDMV), or *sugarcane mosaic virus* (SCMV). MCMV was first reported in Peru in 1974 [3], followed by several countries worldwide [21], whereas SCMV has been endemic in Africa since several decades and has a world wide distribution, it has a moderate effect on maize; however, when SCMV and MCMV co-infect maize plants causing MLN, it has a severe impact [14].

Plants have evolved sophisticated mechanisms to resist pathogens, including viruses that can cause heavy crop losses [18]. These resistance mechanisms are often associated with the host plant recognizing the specific pathogen, the recognition triggering the defence mechanism, and the plant then showing resistance. Resistance to a pathogen shown by a tolerant or resistant genotype can come about by the host becoming less susceptible, which hampers the infectivity of the pathogen [18, 20]. Breeding for resistance against key diseases is an effective and environment-friendly strategy. The International Maize and Wheat Improvement Center (CIMMYT) is engaged in an intensive breeding programme to develop and deploy high-yielding and multiple-stress-tolerant maize varieties in sub-Saharan Africa, including resistance to MLN, through conventional and molecular breeding [2, 9, 21].

The stage of development at which a host plant is infected may determine its response to the infection [28], and this phenomenon, namely the change in resistance as a function of the developmental stage at the time of infection, has been variously labelled as age-related resistance, developmental resistance, ontogenic resistance, and so on (Kus et al., 2002; [28]. For example, yield losses in maize plants inoculated with MDMV were higher when they were infected during the early stage of growth than in plants infected during the later stages of growth [24,(.13] examined the relationship between injury and yield loss due to viral diseases and reported that the relationship was non-linear because the spatial distribution of the injury in a crop stand was not uniform, nor was the date of disease onset. Prasanna et al. [21] reported maize yield losses due to MLN among three groups of hybrids – susceptible, tolerant, and resistant –with or without MLN artificial inoculation. The mean grain yield of the susceptible hybrids was 0.7 t/ha with a yield penalty of 77.7% under artificial inoculation; the tolerant and resistant hybrids recorded 3.4 t/ha and 4.6 t/ha, with a yield penalty of 22.9% and 3.1%, respectively. In a 2-year field experiment, Batchelor et al. [1] evaluated 17 maize hybrids, either inoculated or uninoculated, with different levels of tolerance to MLN at the MLN screening facility in Naivasha, Kenya. Disease progress scores were recorded over time, and translated into as daily damage, including leaf necrosis and death, as inputs in the crop model. The model resulted showed a good correlation between the yield predicted by the model and actual yield for the calibration data set (R^2 of 0.97) and for the evaluation data set (R^2 of 0.92), indicating that the model could predict the impact of MLN on grain yield reliably.

Understanding the effect of developmental stages at the time of MLN infection, and the consequent effects, especially in terms of yield loss, is important for devising effective disease management strategies. The present study focused on analysing the influence of (1) provenance (hybrids or inbred lines), (2) degree of resistance (resistant, tolerant, or susceptible), (3) the stage of development

at which maize plants were inoculated (4) either with MCMV alone or together with SCMV, under controlled conditions, on the severity of MLN, grain yield, virus titre, and the extent of seed contamination.

Materials And Methods

Plant materials and experimental design

A set of 18 maize inbred lines and 9 hybrids (Table 1) developed by CIMMYT with different levels of tolerance to MLN and MCMV were tested. Of these 27 entries, a set of 9 inbred lines and another of 9 hybrids were used for experiments involving two types of inoculum, one consisting of MCMV alone and another consisting of MCMV and SCMV, because it takes both to cause MLN (referred to simply as the combination from now on). In the resistant entries, the growth and development of a specified pathogen were severely restricted under given normal disease pressure when compared to the pathogen growth and development in susceptible varieties. Also, under a given level of disease pressure, resistant entries produce economic yield close to their genetic potential, whereas susceptible entries produce little yield, with tolerant lines intermediate between the resistant and the susceptible lines: on the one hand, tolerant lines restrict the growth and development of the pathogen; on the other, symptom development in such lines is more pronounced than that in resistant lines. Yield from tolerant lines is also intermediate, higher than that from susceptible lines but lower than that from resistant lines at a given disease pressure.

Table 1
List of inbred lines and hybrids used for MLN and MCMV inoculation experiments.

Inbred lines for MCMV experiment	Inbred lines for MLN experiment	Hybrids for MLN and MCMV experiments
Resistant	*Resistant*	*Resistant*
CML494	CKDHL120918	CKHMLN150077
KS23-6	CKDHL120275	CKHMLN150078
CML550	CKLMLN146285	CKHMLN150080
	CKDHL163577	WE5135
Tolerant	*Tolerant*	*Tolerant*
CKDHL120312	CKDHL120312	CKH12603, CKH12602
CLRCY034-B	CLRCY034-B	CKH12600, CKH10086
(CML494*/OFP1)-B-1-1-1-B-B-B; (CML494/CML550)PHDH68-B*	CLWN270	CKH12613, WE6110
Susceptible	*Susceptible*	*Susceptible (Commercial Checks)*
CML442	CML442	DK8031
CML395	CML395	DUMA 43
CML543	CML543	PHB3253

Note: Inbred line (CML494/OFP1)-B-1-1-1-B-B-B was replaced by (CML494/CML550) PHDH68-B in the second year of evaluation due to seed shortage of the former.

Seeds of the inbred lines and hybrids used in the experiments were obtained from plants grown in MLN-free environment at the KALRO (Kenya Agricultural and Livestock Research Organization) Research Station, Kiboko, Kenya. The plants were inoculated and grown in screen houses at the MLN Screening Facility in Naivasha, Kenya (0°43'00" S, 36°26'09" E; elevation 1884 m) in two different cropping seasons, namely, April–September 2018 and January–June 2019. Each screenhouse was tightly covered with insect-proof nylon mesh (40 µm) to prevent the entry of insect vectors.

The experiment was laid out in an alpha lattice design with two replications each of the inbred lines and three replications each of the hybrids. Each plot in the screenhouse was 2.5 m long and 0.75 m wide, with the seeds sown in rows 0.75 m apart and at a

spacing of 0.25 m within each row. Each plot thus comprised 11 plants, of which the last two in each row at either end were excluded while collecting leaf samples for enzyme-linked immunosorbent assay (ELISA). Two seeds were sown at each hill and the seedlings thinned 3 weeks after emergence to ensure one plant per hill. All the standard agronomic practices were followed in each trial.

Disease severity was recorded following the protocols explained by Clark and Adams [4], and virus titres were determined using ELISA at specified intervals following inoculation. At the time of harvest, grain weight and grain moisture content were recorded, and grain yield was calculated after adjusting for a moisture content of 12.5%.

[Table 1 here]

Preparation of inoculum and artificial inoculation

Mother cultures of SCMV and MCMV were maintained at the MLN Screening Facility in Naivasha on susceptible maize plants, MCMV on hybrid H614 and SCMV on hybrid PHB30G19, in separate greenhouses with strict phytosanitary measures. The plants to be used as the source of inoculum were tested for purity with reference to MCMV and SCMV at regular intervals using ELISA. The inocula were prepared following an optimized protocol [8, 14; https://mln.cimmyt.org]. Healthy maize plants of these hybrids were raised in the respective greenhouses and inoculated when at the 4- to 5-leaf stage. The leaves of these plants were collected at the 12-leaf stage (V12) as sources of pure inoculum for MCMV or SCMV. These leaves were weighed, chopped into small pieces using a paper cutter, and homogenized in 0.1 M potassium phosphate buffer (pH 7.0) using a ratio of 1:10 (v/v). The suspension was filtered through a nylon mesh, and carborundum was added to the filtrate at a rate of 0.02 g/mL. The test entries were inoculated at each of the five developmental stages by rubbing the mixture onto the leaves, twice at an interval of one week after 1st post inoculation, at the each of the five developmental stages to ensure uniform inoculation.

Inoculum for the combination (MLN) was prepared the same way, with MCMV and SCMV mixed in optimal proportions (1:4, w/v). The five developmental stages mentioned above were as follows (V denotes the vegetative stage and R denotes the reproductive stage): 5-leaf (V5), 10-leaf (V10), 14-leaf (V14), tasselling (VT), and grain-filling (R1). The experiment therefore comprised ten treatments (five growth stages and two types of inoculum). Separate sets of inbred lines and hybrids were maintained for each of the developmental stages for MCMV and for the combination: thus, a total of 10 sets were assigned to MCMV and another 10 sets to the combination (five stages and two sets, one of hybrids and one of inbred lines). Across all the treatments and at each stage, the plants were observed carefully before inoculation. At every stage, the presence of MCMV in MCMV-inoculated plants as well as that of MCMV and SCMV in those inoculated with the combination was independently confirmed by ELISA two weeks following inoculation.

Determination of disease severity and grain yield

Disease severity was assessed visually by scoring each plot on a scale of 1 (highly resistant, with no disease symptoms) to 9 (highly susceptible, leading to necrosis and death), beginning 21 days after inoculation, and further observations were recorded at 14-day intervals on hybrids and at 7-day intervals on inbred lines. For plants inoculated at V5, V10, and V14, the scores were recorded on three occasions whereas for those inoculated at VT and R1, the scores were recorded on two occasions. The area under the disease progress curve (AUDPC) calculated for each plot served as the measure of the progression of severity over time. Grain yield and moisture level were recorded at physiological maturity. The harvested grains were sampled according to the International Seed Testing Association (ISTA) specifications and tested for the presence or absence of MCMV or SCMV using double antibody sandwich (DAS) ELISA, for which we used Bioreba antiserum for detecting the two viruses [SCMV IgG Art. No. 140412; SCMV IgG Conjugated W/AP, Art No. 14022; MCMV AgG Art. No. 140712; MCMV IgG Conjugate W/AP Art. No. 140722].

Estimating virus titre

Five plants were selected from each of the test entries such that every other plant in the row was chosen to make up the sample for the leaf DAS-ELISA test. The leaves were placed in self-sealing polythene bags, labelled, and stored at − 20°C. The plants were sampled every two weeks for hybrids and every ten days for inbred lines to coincide with the days on which the plants were also scored for disease severity. MCMV antibody and its enzyme conjugate were supplied by Bioreba Company, and DAS-ELISA was carried out following the protocol provided by the manufacturer. MCMV IgG of the Bioreba antibody, diluted to 1:1000 in carbonate coating buffer, was added to each well of the microplate, 200 µL of the solution to a well. Each microplate, which was a polystyrene,

U-bottom, clear, Microlon®, medium-binding (Greiner Bio-one, Kremsmünster, Austria) plate, consisted of 96 wells and was incubated for 4 h at 30°C. The unbound antibody was washed off using PBS-Tween-20. The prepared leaf samples (0.1 g added to 5 mL of extraction buffer) were loaded, 200 µL to each well, in duplicates and incubated overnight at 4°C. Unbound antigens in the sample were washed off several times using PBS-Tween 20, and excess fluid was removed by blotting with a paper towel. Next, 200 µL of MCMV IgG AP (Bioreba) diluted to 1:1000 with ECI buffer was added to each well and incubated for 5 h at 30°C, and the plate was washed as described above. Finally, 200 µL of para-nitrophenyl phosphate tablets dissolved in 1 × PNP buffer pH 9.8 (1 mg/mL) was loaded into each well and the plate was incubated for 30 min to allow for colour development. Absorbance was measured at 405 nm using a microplate photometer (Multiskan FC, ThermoFisher Scientific). A sample was considered positive if its absorbance was at least two times higher than that of the negative control.

The leaves and seed samples from plants inoculated with the combination were tested for MCMV and SCMV, whereas those inoculated with MCMV alone were tested only for MCMV. The virus titre of the leaf tissue was recorded for all entries including resistant, tolerant, and susceptible hybrids and inbred lines that had been inoculated at various growth stages. Seeds harvested from the plants under various treatments were also tested for MCMV and SCMV: for both the viruses in the plants inoculated with the combination and for MCMV alone in the plants inoculated only with MCMV. Lastly, seedlings grown from the seeds harvested from the various experiments were also tested for the two viruses.

Seed contamination with MCMV

To determine the level of seed contamination by MCMV in all the different treatments, the experiments were conducted in protected screenhouses to avoid any external contamination by means of insect transmission. Seeds were sampled according to the ISTA guidelines. The sampled seeds were sown in 20-litre plastic pots filled with a mixture of sterilized soil, manure, and peat moss. The experiment was set up in a randomized complete block design with three replications for each entry and five seeds in each pot in each replication. The plants were strictly quarantined and observed for five weeks from sowing. From each pot, one young growing leaf was collected from an asymptomatic plant and another from a symptomatic plant, the leaves were placed separately in a self-sealing polythene bags, stored at − 20°C, and further tested for the viruses using DAS-ELISA.

Detection of viruses in maize seeds

Seeds harvested from the test entries and sampled according to the ISTA sampling guidelines were analyzed for the presence or absence of the viruses by testing the ‘soakate’, namely the liquid (a general extraction buffer) in which the seeds had been soaked overnight. For another experiment, the soakate was in the form of a supernatant collected after crushing the soaked seeds and was referred to as the ‘seed crush solution’. Twenty seeds were put into each of 50 mL Falcon tubes and soaked overnight in 10 mL general extraction buffer at 4°C: 200 µL of the supernatant was used in testing for the viruses using DAS-ELISA. In the second experiment, 20 seeds were crushed using an IKAR Tube-mill 100 (Deutschland, Germany) in a grinding chamber at 5000 rpm for 3 min, and 5 g batches of the pulverized mass were transferred to a series of 15 mL Falcon tubes containing 5 mL of the buffer. From each tube, 200 µL of the supernatant was used for testing with DAS-ELISA.

Statistical analysis

Grain yield, disease severity, and ELISA test data were analysed using a linear mixed model for each treatment. Data from the two crop seasons were analysed together, and season effect was included in the model as a random effect. The statistical model considered the design effects of replicate nested in season [rep(season)$_{ji}$], and a random effect of blocks nested in replicates and season [block/rep*season$_{kji}$]. Because not all the genotypes used in the two seasons were the same, and because the most important effect was the type of genotype (resistant, tolerant, or susceptible), type effect [type$_{l}$] and type by season interaction [type*season$_{li}$] effects were included in the model, whereas the genotype [genotype(type)$_{ml}$] effect was nested in type as well as the interaction between genotype and season [genotype*season(type)$_{mil}$]. All the interaction effects which involved the season effect were considered as random effects with normal distribution, zero mean, and homogeneous variance, pairwise independent and of the experimental error. Adjusted means of type were compared using the least significant difference *t*-test at 0.05 level of significance to consider the means as statistically different. In all cases, the degree of freedom was approximated by the Kenward-Roger 2 method available in SAS. All analyses were performed in SAS ver. 9.4 with the capabilities of STAT/GLIMMIX. Variance components were estimated using REML, according to the following model, with a normal distribution assumed for the residuals:

Y_{ijklm} = media + $season_i$ + $rep(season)_{ji}$ + $block(rep*season)_{kji}$ + $type_l$ + $genotype(type)_{ml}$ + $type*season_{li}$ + $genotype*season(type)_{mil}$ + $error_{ijklm}$

where Y_{ijklm} is the response variable in the i^{th} season, j^{th} replicate, k^{th} block, l^{th} type, and m^{th} genotype, either grain yield, AUDPC, or ELISA test.

Results

Disease severity and AUDPC values in the MCMV- and MLN-inoculated experiments

Disease severity scores were recorded four times in plants that had been inoculated at growth stages V5 and V10; three times, in those at stages V14 and VT; and twice, in those at stage R1. To compare the treatments in terms of disease severity, we used the score recorded 40 days after the inoculation, which corresponded to the scores recorded for the third time for V5, V10, and V14 and that for the second score for VT and R1.

[Table 2 here.]

Table 2
P-values to test type of genotype effect on disease severity scores for hybrids and inbred lines artificially inoculated with MLN or MCMV viruses.

Experiment	Data	Hybrids					Inbred lines				
		V5	V10	V14	VT	R1	V5	V10	V14	VT	R1
MLN	AUDPC	0.0889	< .0001	< .0001	0.0613	0.5855	0.0014	0.1586	0.0002	0.6253	0.0235
	Grain yield	0.0002	< .0001	0.3560	0.4782	0.1879	0.0331	0.6456	0.4941	0.1648	0.0237
	MLN1	< .0001	0.0260	0.6927	0.1004	0.5537	< .0001	0.2192	0.0992	0.5822	0.3287
	MLN2	< .0001	0.2196	0.2342	0.1121	0.5903	0.0091	0.2096	0.0010	0.6323	0.0754
	MLN3	0.1217	0.1523	0.2405	.	.	0.0011	0.2056	0.0003	.	.
	MLN4	0.3911	0.1092	.	.	.	0.0017	0.0013	.	.	.
MCMV	AUDPC	0.0104	0.1574	0.4593	0.5860	0.9839	< .0001	< .0001	0.4680	0.7334	0.1119
	Grain yield	0.0010	0.1236	0.0976	0.6368	0.2225	0.0192	< .0001	< .0001	0.1978	0.1227
	MCMV1	0.0130	0.9953	0.3975	0.9549	1.0000	0.0571	0.2528	0.5180	0.8471	0.4078
	MCMV2	< .0001	0.2428	0.5729	0.6319	0.9496	< .0001	< .0001	0.5297	0.7302	0.0751
	MCMV3	< .0001	0.1364	0.2520	0.2923	.	0.0667	< .0001	0.3525	0.6906	.
	MCMV4	0.0321	0.0948	.	.	.	< .0001	< .0001	.	.	.

AUDPC: Area under disease pressure curve

Stages of disease severity scoring:

MLN1*: 1st MLN disease severity score after 21st days after inoculation

MLN2*: 2nd MLN disease severity score after 28th days after inoculation

MLN3*: 3rd MLN disease severity score after 35th days after inoculation.

MLN4*: 4th MLN disease severity score after 42nd days after inoculation.

MCMV1*: 1st MCMV disease scoring after 21st days after inoculation

MCMV2*: 2nd MCMV disease severity score 28th days after inoculation

MCMV3*: 3rd MCMV disease severity score 35th days after inoculation.

MCMV4*: 4th MCMV disease severity score 42nd days after inoculation.

Table 2 shows the *P* values for comparing the three types of materials, namely resistant, tolerant, and susceptible. All three showed a common pattern: entries inoculated at the early stages showed significantly greater variation, in terms of disease severity and AUDPC, than that shown by the entries inoculated at the later stages. The hybrids were less sensitive than the inbred lines were to MCMV or to the combination. More significant effects were detected for inbred lines than for hybrids, especially at the later stages (V10 to R1). The hybrids showed significant differences at V5 in all the traits following inoculation with MCMV whereas following inoculation with the combination, the hybrids differed only in terms of grain yield and in the set of observations designated as MLN1 and MLN2; the effects were significant in plants that had been inoculated at V14, VT, or RT was MLN AUDPC at V14. The inbred lines inoculated at V5 showed significant effects at all four scoring stages (MLN1 to MLN4) following MLN inoculation and at three scoring stages (MCMV2 to MCMV4) following inoculation with MCMV, with a slightly non-significant effect ($P = 0.0571$ and 0.00667) for the first (MCMV1) and the third (MCMV3) observations. Inbred lines also presented significant effects when inoculated with MCMV alone at V10 and with the combination (MLN) at V14 in the second (MLN2) and third (MLN3) round of observations, and even at R1 in terms of AUDPC. Grain yield showed significant differences between different types of hybrids inoculated at V5

and V10, and at V5 for MLN and MCMV inoculation, respectively. The inbred lines differed significantly at V5 and R1 when inoculated with the combination and at V5, V10, and V14 when with MCMV alone (Table 2).

The adjusted means showed the expected pattern for all the significant effects: resistant entries showed the lowest disease severity and AUDPC values whereas susceptible entries showed higher values, and the tolerant entries showed intermediate values, closer (statistically) at times to the values shown by the resistant entries and at other times to those shown by the susceptible entries (Table S1).

The hybrids showed a clear pattern in terms of the adjusted means of disease severity, with susceptible entries showing higher values than resistant and tolerant entries. None of the entries differed significantly at VT and R1, and the AUDPC values were also very similar, possibly due to a large decrease in disease severity at VT and R1 (Table S1; Figs. 1 and 2).

In the case of the inbred lines, the susceptible entries differed significantly from the resistant and tolerant entries at V5, V14, and R1, but not at V10 and VT. Values of AUDPC were higher in plants inoculated at the early stages because we used four measurements for those stages but only two measurements at stages VT and R1. However, the average across genotypes and early stages was nearly four times (152.0) the average for stages VT and R1 (38.1) for (MLN) the combination whereas for MCMV alone, it was only 2.1 times (Table S1 and Fig. 2). Therefore, the progress of MLN was higher in plants that had been inoculated with the combination at the early stages than in those inoculated with it at the later stages (VT and R1) whereas in plants that had been inoculated only with MCMV, the stage at which they had been inoculated made no difference to disease severity. The values of AUDPC in the case of the combination were similar among all the three categories – resistant, tolerant, and susceptible – in both hybrids and inbred lines, except that at when inoculated at R1, the resistant types showed statistically significant differences from those shown by the tolerant and susceptible types (Table S1 and Fig. 2).

The results of inoculation with MCMV alone showed a pattern similar to that seen in the plants inoculated with the combination. The adjusted means of disease severity for the hybrids showed significant differences among the resistant, tolerant, and susceptible entries inoculated at V5, but not among those inoculated at V10, V14, VT, and R1. The values of AUDPC for the resistant and the tolerant entries were much lower than those for the susceptible entries inoculated at V5, V10, and V14, whereas in the case of plants inoculated at VT and R1, the values were lower than in plants inoculated at the early stages (Table S1 and Fig. 3).

In the case of inbred lines, the tolerant entries were statistically different from the resistant and the susceptible entries. In plants inoculated at V5 or V10, the resistant entries showed disease scores that were statistically different from those shown by the susceptible entries, but in plants inoculated at V14, VT, and R1 the differences were not significant; however, the values of AUDPC in plants inoculated at these stages were very similar to those seen in the hybrids inoculated at the same stages (Table S1 and Fig. 3). For all the significant effects, the adjusted means showed the expected pattern: disease severity in the sets of hybrid and inbred lines was the lowest in resistant entries, intermediate in tolerant entries, and high in susceptible entries irrespective of the type of inoculation (that is, MCMV alone or a combination of MCNV and SCMV).

[Fig. 1 here.]

[Fig. 2 here.]

[Fig. 3 here.]

[Fig. 4 here.]

Grain yield

In the hybrids inoculated with the combination at V5 and V10, the adjusted means showed statistically significant differences in grain yield between resistant and tolerant entries and also between resistant or tolerant entries and susceptible entries. Grain yield showed no statistically significant differences among plants inoculated with the combination at V14, VT, and R1 stages. In inbred entries inoculated with the combination at V5 and R1, the adjusted means showed statistical differences. In plants inoculated with the combination at V5, grain yield from *resistant* entries was significantly different from grain yield from the tolerant and

susceptible entries, whereas in plants inoculated with the combination at R1, grain yield from *susceptible* entries was significantly different from grain yield from resistant and tolerant entries (Table S1 and Fig. 5).

In the hybrids inoculated with MCMV alone at V5, the adjusted means were significantly different; however, the means were not significantly different in plants similarly inoculated but at V10, V14, VT, and R1. Among plants inoculated at V5, the resistant and tolerant entries differed significantly between themselves and also from the susceptible entries. In inbred lines, the adjusted means for grain yield were significantly different among plants inoculated at V5, V10, and V14, but not so between plants inoculated at V14 and R1. Among plants inoculated at V5, V10, and V14, the resistant entries differed significantly from the tolerant and susceptible entries (Table S1, Fig. 6).

In both types of inoculations, grain yield was higher in the resistant entries than in the susceptible entries. In both hybrid and inbred entries inoculated with the combination, the resistant ones showed a significant effect when inoculated at the early stages – V5 and V10 – on grain yield; however, when inoculated at the later stages, the differences due to provenance (hybrids and inbred entries) were smaller. Among the plants inoculated with MCMV alone, the resistant entries recorded higher grain yield than the susceptible entries did irrespective of the stage at which they had been inoculated except the VT stage; however, the average yields of both resistant hybrids and inbred lines inoculated with MCMV alone were higher than those inoculated with the combination (Table S1, Fig. 7).

[Fig. 5 here.]

[Fig. 6 here.]

[Fig. 7 here.]

The hybrids were vulnerable to MLN up to V10, whereas the inbred lines were vulnerable up to V14 (Fig. 7): inoculation at VT or R1 led to smaller differences in grain yield (lower than 50%) among the hybrids, whereas the difference among the inbred lines were marked even for inoculations up to R1. These results indicate that vulnerability is significant at the early stages and relatively lower at the late stages.

ELISA virus titre values in plants inoculated only with MCMV

Differences between resistant, tolerant, and susceptible entries for the ELISA titres were significant in almost all the combinations of inoculation and sampling stages for both hybrids and inbred lines, except for hybrids inoculated at stage V5 and sampled at V14, VT, and R1 and those inoculated at V10 and sampled at R1. ELISA titres from seeds (whether intact (SW) or crushed (SC)) obtained from inoculated plants were significantly different irrespective of the stage of inoculation. However, plants grown from such seeds (grow out tests; GOT1 and GOT2) showed no differences in ELISA titres among genotypes that differed in the degree of resistance or among plants inoculated at different stages, whether hybrids or inbred lines.

In both hybrids and inbred lines inoculated either with MCMV alone or with a combination of MCMV and SCMV, titre values in leaves sampled at various sampling intervals from V5 to V10 were higher than those in leaves sampled at V14, VT, and R1. Titre values in plants inoculated with the combination were significantly higher than those in plants inoculated with MCMV alone irrespective of the stage of inoculation (Table S2).

Comparison of mean titre values (Supplementary Table 3) showed that resistant and susceptible hybrids and inbred lines differed significantly, with the susceptible types showing much higher values than those shown by the resistant entries. In some cases, the tolerant entries showed the highest values among all the three types (Supplementary Figs. 1 and 2).

With crushed seeds, the resistant and susceptible types presented similar ELISA titre values, which were lower than those seen in the tolerant types of hybrids inoculated at V5 or V14 and of inbred lines inoculated at V5, V10, V14, and R1. With intact seeds (SW), the same pattern was found in inbred lines inoculated at stages V5, V10, and V14. Other sampling stages with a similar pattern for inbred lines were R1 when inoculated at V5 and V10, and VT, R1, and SW when inoculated at V10 (Table S3, Supplementary Figs. 1 and 2).

Supplementary Figs. 1 and 2 show the mean ELISA titre patterns for plants inoculated at different stages and sampled at different stages for hybrids and inbred lines, respectively. In general, ELISA titre values were higher in plants inoculated at early stages. As a method of measuring virus titres, the supernatant from crushed seeds (SC) was more sensitive than that from intact seeds (SW).

ELISA virus titre values in plants inoculated with a combination of MCMV and SCMV

Supplementary Table 4 shows the p-values for the comparison of mean ELISA titre values. Only in a few cases, especially inbred lines, were the mean differences statistically different. SCMV was significant only in one case, namely inbred lines inoculated at V5 and evaluated at R1 (Supplementary Table 4). As in inoculations with MCMV alone, ELISA titres in plants raised from seeds produced by inoculated plants (GOT) did not differ significantly in all cases (Table S4).

ELISA titres from plants inoculated with MCMV alone showed greater statistical differences between genotypes than did those inoculated with the combination. Significant effects were found in plants inoculated at V5, V10, V14 and sampled at R1 and always between inbred lines. Inoculation at early stages, V5 and V10, showed more significant differences than inoculations at late stages. ELISA titres of plants inoculated at V5 and sampled at R1, and titres from crushed (SC) or intact seeds (SW), differed between genotypes, while after inoculation at stage V10, we found significant effects at stages V14, VT, R1, and for measurements taken after inoculation at both V5 and V10, differences were found between genotype types in seed, seed crushing (SC) and seed washing (SW) (Table S4 and S6).

The mean MCMV titre values in case of plants, both hybrids and inbred lines, inoculated with the combination showed a pattern similar to that seen in plants inoculated with MCMV alone, but most of the effects were not significant (Supplementary Tables 2 and 3, Figs. 1 to 4). Early-stage inoculation showed the largest values of ELISA titre, and ELISA titre values for SCMV were lower than those for MCMV. When we compared the ELISA titre values for MCMV in the two types of inoculation, those in the combination were higher.

Discussion

The study sought to evaluate the response of maize hybrids and inbred lines, categorized as resistant, tolerant, or susceptible to MLN, inoculated with either MCMV alone or in combination with SCMV, at different stages of crop growth. The impact of inoculation was assessed in terms of disease severity, grain yield, and the extent of contamination observed in seeds.

In both the plants inoculated with (a combination of MCMV and SCMV) MLN- and MCMV-inoculated experiments, the disease severity scores and AUDPC values for the hybrids and inbred lines were much higher at the early inoculated growth stages (V5 and V10) than at the later growth stages.

In an earlier study [29], in which maize plants were co-infected with the same combination as used in the present study (MCMV and SCMV) at the 3rd-leaf stage, severe chlorotic symptoms appeared on leaves 9 days post-inoculation (dpi) and developed necrotic areas at 10 days dpi, respectively. It was also observed that in the mixed infected leaf, the accumulated level of MCMV genomic RNAi was much higher and there was a significantly decreased level of SCMV RNA. The same study also reported that the expression levels of MCMV genomic RNAs and coat protein (CP) were higher in maize plants inoculated with the combination than in those inoculated with MCMV alone. It was also demonstrated in two earlier studies [22, 26] that HC-Pro, the silence suppressor encoded by potyviruses, could enhance the pathogenicity and accumulation of heterologous viruses.

The titres of MCMV were also much higher in plants inoculated with the combination than in those inoculated with MCMV alone, irrespective of their genetic background or the stage at which they had been inoculated, probably because of the synergistic effect of the combination, especially during the early stages of growth. Greater disease severity during the early growth stages led to greater yield losses in the susceptible entries than in either resistant or tolerant entries. However, in plants inoculated with the combination at their later growth stages, the titres of MCMV and SCMV as well as disease severity were much lower, with little impact on yield.

Understanding the host–pathogen–vector interaction is important especially in diseases caused by multiple viruses. Such interactions may be neutral or synergistic or antagonistic [16]. In neutral interactions, accumulation of viruses or disease dynamics

show little difference between joint infections and those by individual viruses, nor does the phenotype change significantly, whereas in synergistic interactions, joint infections result in higher titres and greater disease severity. Such synergy has been attributed to various factors including greater replication of the pathogens, enhanced ability to invade new plant tissue, or greater interference with the plant's defences [16]. Maize lethal necrosis is a typical example of a synergistic interaction involving MCMV and a potyvirus such as SCMV [21].

Disease severity scores, AUDPC values, and MCMV titres (ELISA) were much lower in the hybrids than in the inbred lines irrespective of the type of inoculum (single or combination). The hybrids certainly offered greater resistance than the inbred lines did to MCMV or SCMV or both and the resistance to the combination was greater (V14 to R1). In plants inoculated early, the higher titres of both MCMV and SCMV clearly showed the synergism. Early inoculation resulted in greater necrosis and little yield in the susceptible hybrids, whereas the resistant and the tolerant entries showed less chlorosis and no significant reduction in yield. Host–pathogen interaction changed depending on the stage of inoculation, the provenance (hybrids or inbred lines), and the inoculum (MCMV alone or together with SCMV), changes that may also be attributed to the host's defence mechanism or to the combined effect of the interaction and the defences.

In MLN, titres of MCMV increased with infection with anyone of the potyviruses and resulted in greater chlorosis and greater economic losses with, symptoms of MCMV showing more chlorosis with increased at higher temperatures [25]. However, interference with the plant's defence mechanism by viral proteins may facilitate replication, intracellular movement, and spread within the plant of other viruses as well [6]. Increased virulence can be predicted as the result of the competitive advantage of a virulent parasite because mixed infections can alter the host's immunity and bring about phenotypic changes in the host. Thus, higher virulence in a mixed infection can be the consequence of competition for resources .

In hybrids, the titres of SCMV following inoculation with the combination were higher in plants inoculated early than in those inoculated later, and titres of SCMV were much lower than those of MCMV, indicating that the impact of infection by SCMV on hybrids and inbred lines is much lower in the presence of MCMV. This finding is consistent with the findings of earlier studies, which showed clearly that the expression level of MCMV genomic RNA and of CP was higher in maize plants infected by both SCMV and MCMV than in those infected by MCMV alone [29]. The synergistic effect of the combined infection by SCMV and MCMV was especially apparent in plants inoculated early: MLN was more severe in those plants than in those inoculated at later. It is likely that in maize plants inoculated with a mix of MCMV and SCMV at later growth stages, the interaction tends to be closer to being neutral, as evident in the lower titres of SCMV, whereas in plants inoculated early the interaction tends to be closer to being synergistic. In another study, in maize plants co-infected with MCMV and either WSMV, MDMV, or SCMV, the titre of MCMV was 1.6- to 11-fold higher than that in plants infected with MCMV alone [7, 25; 29Xia et al., 2016). In the present study (Supplementary Table 5), titres of MCMV (as expressed in terms of OD values) were much higher (1.676–3.635) than those of SCMV (1.246–2.2425).

Little increase in the titres of MCMV was observed when those of SCMV (ELISA) were negative or below the threshold; the titres of MCMV increased when the OD value of SCMV ELISA was at a certain threshold, because the multiplication of SCMV was minimal or negative; MCMV alone cannot multiply exponentially unless SCMV is present at or beyond a certain minimum threshold. This also correlates with the percentage yield loss. Either SCMV may favour faster multiplication of MCMV or SCMV may make plants more susceptible when they are inoculated at an early stage. Earlier studies on co-infection by MCMV and SCMV showed increased accumulation of MCMV and virus-derived, small interfering RNAs (vsi RNAs) from MCMV [29].

In an earlier investigation on ultrastructural changes in infected maize leaf cells, it was observed that starch grains present in the chloroplasts in the cells co-infected by MCMV and SCMV were much smaller than those in MCMV-infected cells (Wang et al., 2017). It was suggested that photosynthesis in these cells was significantly affected. It was also found in the study that the mitochondria in the co-infected leaf cells were severely damaged much earlier than in the leaf cells infected by MCMV alone. Thus, systemic necrosis in MLN-affected plants was mainly due to disruption of chloroplast photosynthesis and mitochondrial respiration. In another study, it was noticed that in cells co-infected with MCMV and SCMV, the concentration of MCMV increased more than fivefold, whereas there was no difference in concentration of SCMV between co-infected and single-infected inoculated plants [7]. From these observations in the present studies, we can deduce that MCMV infection alone can cause mild symptoms and that in cells co-infected with MCMV and SCMV, both the viruses accumulate in greater amounts.

Grain yield of MLN-resistant hybrids inoculated with the combination at early growth stages (V5 or V10) was negatively correlated with disease severity scores and AUDPC values, whereas the susceptible hybrids showed no such correlation. When inoculated later, the yields of resistant, tolerant, and susceptible hybrids were negatively correlated to disease severity and AUDPC values. Beyene et al. [2] observed during their genetic analysis of various tropical inbred lines for resistance to MLN that the combining ability estimates indicate the prevalence of additive gene action rather than of non-additive gene action. The grain yield of hybrids inoculated at later stages could be possibly protected by the defence mechanism. In another study of several lines in Hawaii, it was observed that the level of resistance varied widely, and it was suggested that MCMV resistance in maize is a quantitative trait [17]. Host factors might decrease viral multiplication; however, this needs to be confirmed. In plants inoculated at later growth stages by the combination, symptoms were less severe and MCMV titres were lower than in those that had been inoculated early. Susceptible hybrids inoculated early recorded higher disease severity, greater AUDPC values, and much lower yield.

As expected, grain yield in resistant hybrids and inbred lines was higher than that in susceptible ones irrespective of the type of inoculation. The differences between resistant and susceptible types were more pronounced in plants inoculated at the vegetative stages (up to V10) than in those inoculated at V14, VT, or the reproductive stage (R1). This result is different from that seen in a study involving maize dwarf mosaic virus (MDMV), which reported higher losses in grain yield in plants inoculated at an advanced growth stage [10]. Predicting the performance of hybrids from the performance of their parental inbred lines has been unsuccessful due to the masking of dominance effects

Seeds of both hybrids and inbred lines were contaminated with MCMV irrespective of the stage at which they were inoculated, irrespective of their level of resistance, and irrespective of the type of inoculum. Thus, contamination with MCMV appears to be unrelated to either the level of resistance or the growth stage.

In conclusion, the experiments in the present study further our understanding of the effect of genetic background and development stage on disease severity, grain yield, and seed contamination. The study also confirmed the synergistic effect of SCMV and MCMV co-infection on disease severity, titre values, and grain yield, because these parameters were affected more in plants inoculated with the combination than in those inoculated with MCMV alone. Inoculation at an early growth stage resulted in higher levels of both SCMV and MCMV, the result of greater synergy. The synergistic interaction of MCMV and SCMV affected the susceptible genotypes more severely, with higher chlorosis and necrosis and total loss of yield. As expected, the resistant hybrids and inbred lines showed were better able to withstand the pressure of the disease and recorded much lower yield loss. Inoculation at the early stages accentuated the differences between the hybrids and the inbred lines and also resulted in the highest disease severity and AUDPC values; this observation is important in evaluating the impact of MLN in the field. We found that disease severity, especially virus titre (content,) was not necessarily correlated to loss in grain yield, except when resistant and susceptible genotypes were compared; however, in tolerant genotypes, the differentiation could be more complicated. Thus, the use of MLN-resistant hybrids is a better option for protecting maize from the devastating effect of MLN on grain yield, as these hybrids can withstand disease pressure following infection even at early stages. The study also showed that MCMV titres were not affected either by the genotype or by the growth stage at which the plants had been inoculated. Therefore, there is a need to develop a seed treatment strategy to combat seed contamination of MCMV, particularly when maize is grown under conditions conducive to the virus.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

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Figures

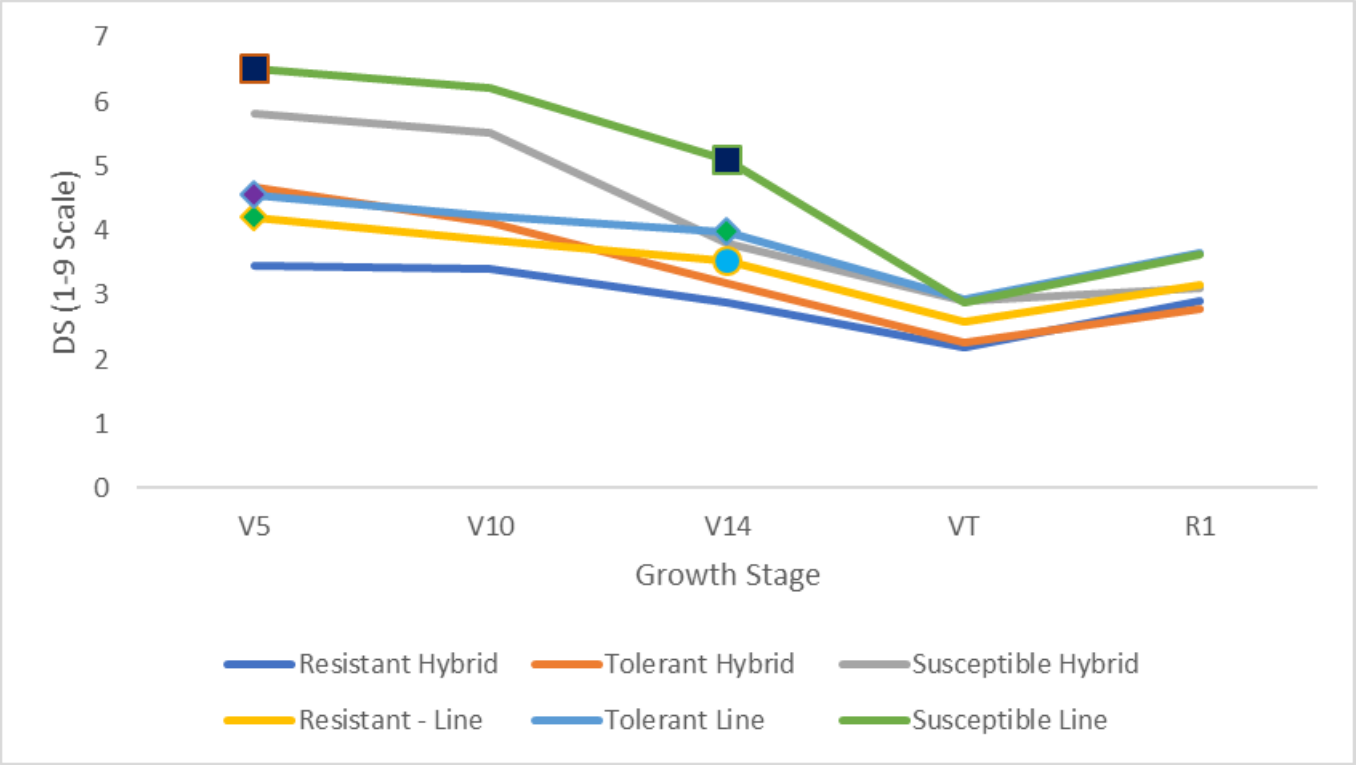

Figure 1

Disease severity in the test entries 40 days after inoculation with MLN under protected net-house conditions. Symbols in the figure indicate statistically significant means within each group (hybrids or inbred lines) by developmental stage. For V5, V10 and V14 stages, the MLN score corresponds to MLN3, while for VT and R1 the score corresponds to MLN2.

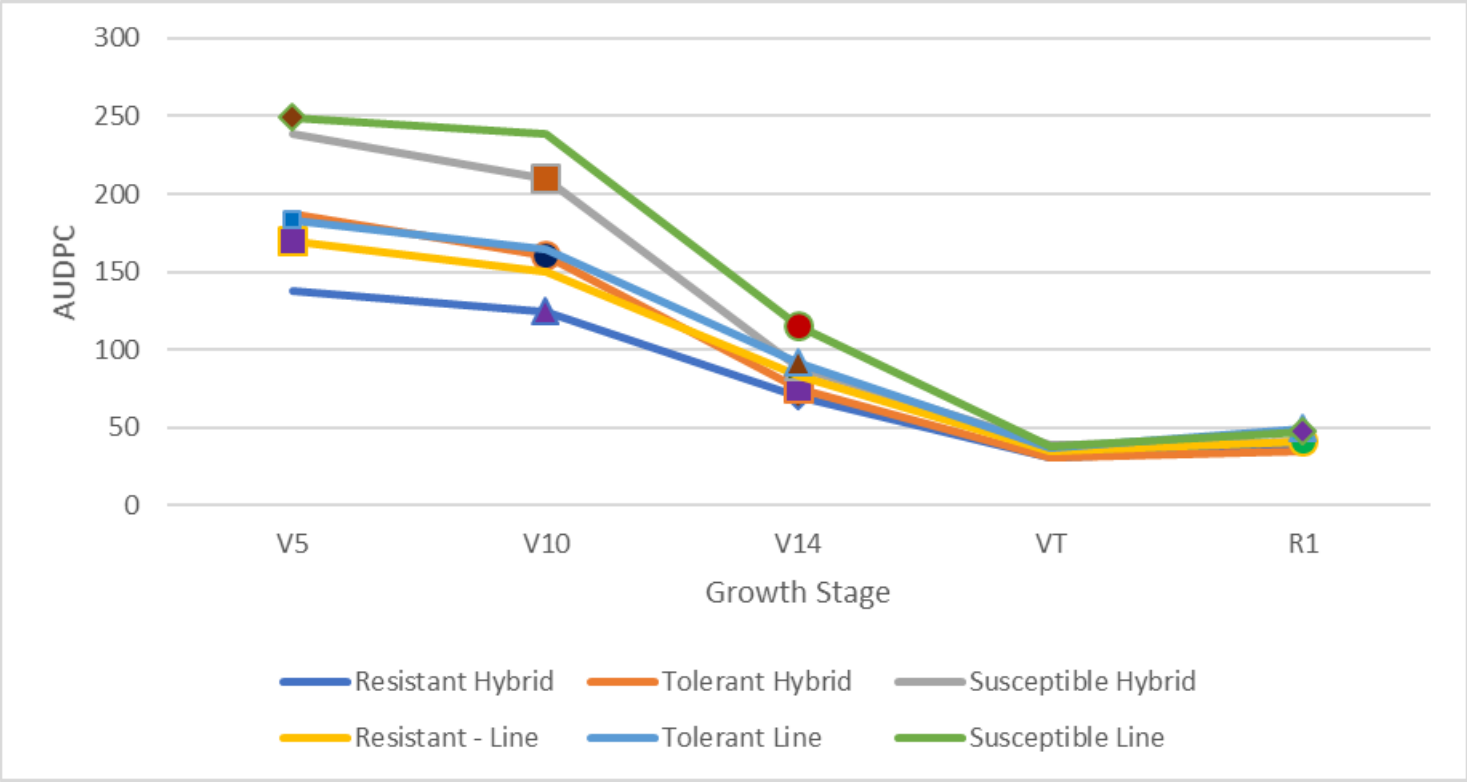

Figure 2

AUDPC of MLN in test entries inoculated at various growth stages under protected net house conditions. Symbols in the figure indicate statistically significant means within each group (hybrids or inbred lines) by developmental stage. AUDPC was calculated with four points for V5 and V10, three points for V14, and two points for VT and R1.

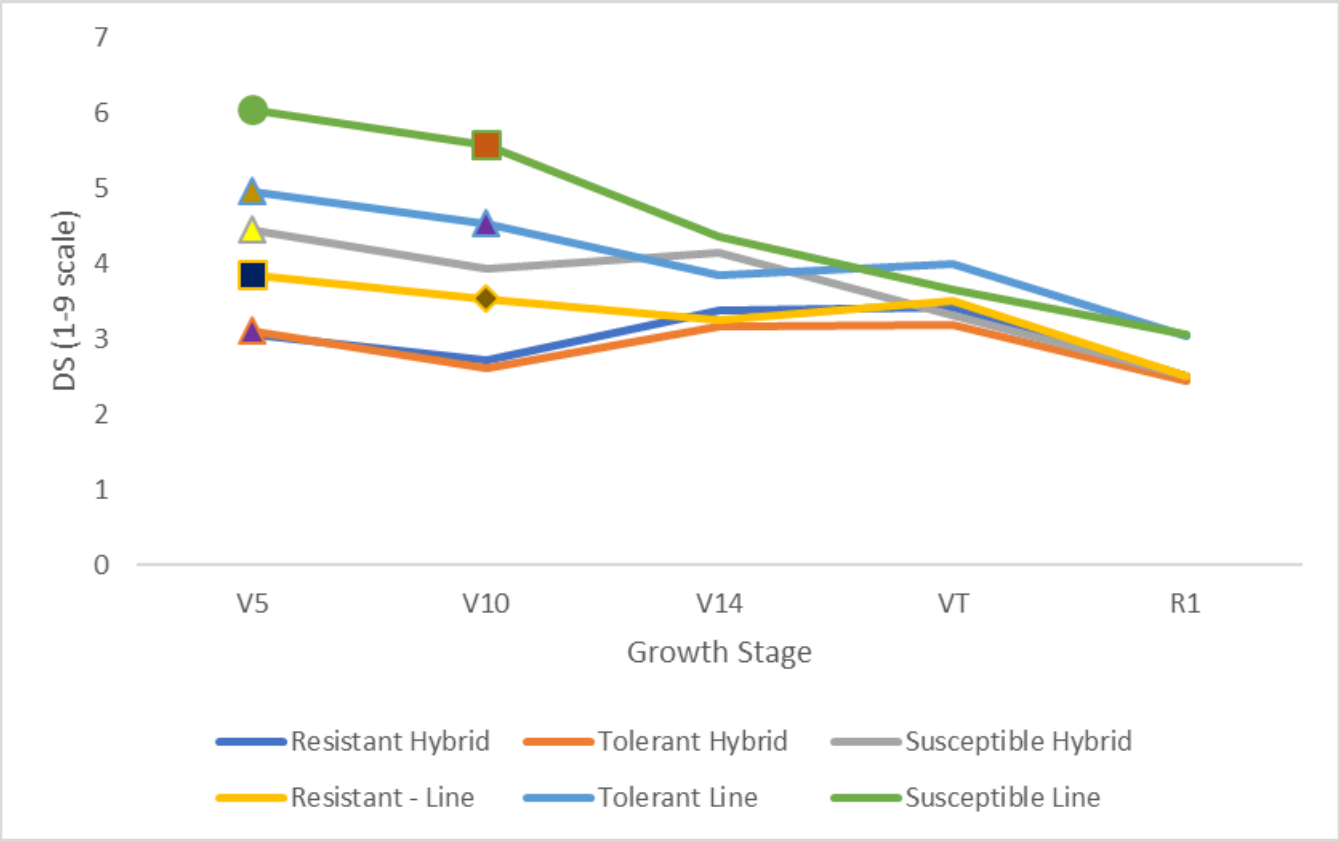

Figure 3

Disease severity in the test entries 40 days after artificial inoculation with MCMV at different developmental stages under protected nethouse conditions. Symbols in the figure indicate those statistically significant means within each group (hybrids or inbred lines). For V5, V10 and V14 stages, the MCMV score corresponds to MCMV3, while for VT and R1 the score corresponds to MCMV2.

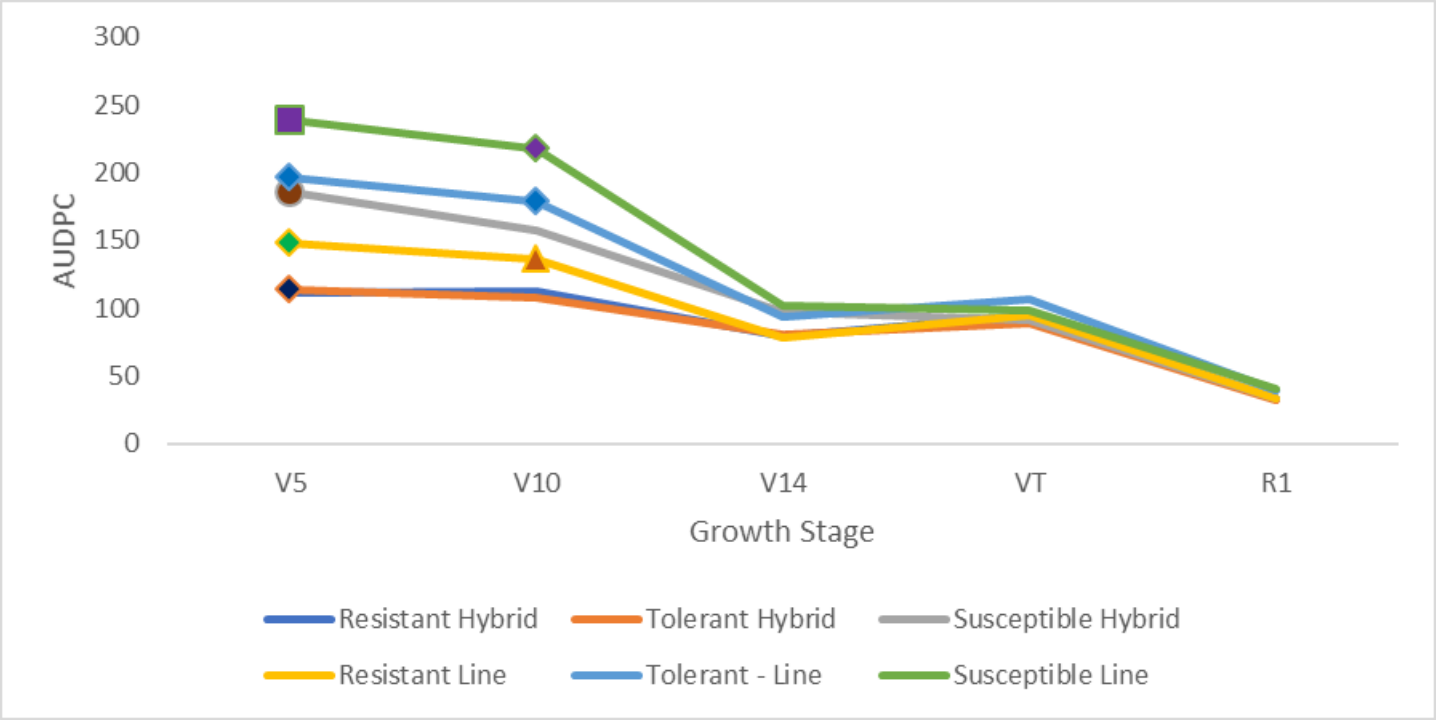

Figure 4

MCMV disease severity in the test entries inoculated at various growth stages under protected nethouse conditions. Symbols in the figure indicate statistically significant means within each group (hybrids or inbred lines). AUDPC was calculated with four points for V5 and V10, three points for V14, and two points for VT and R1.

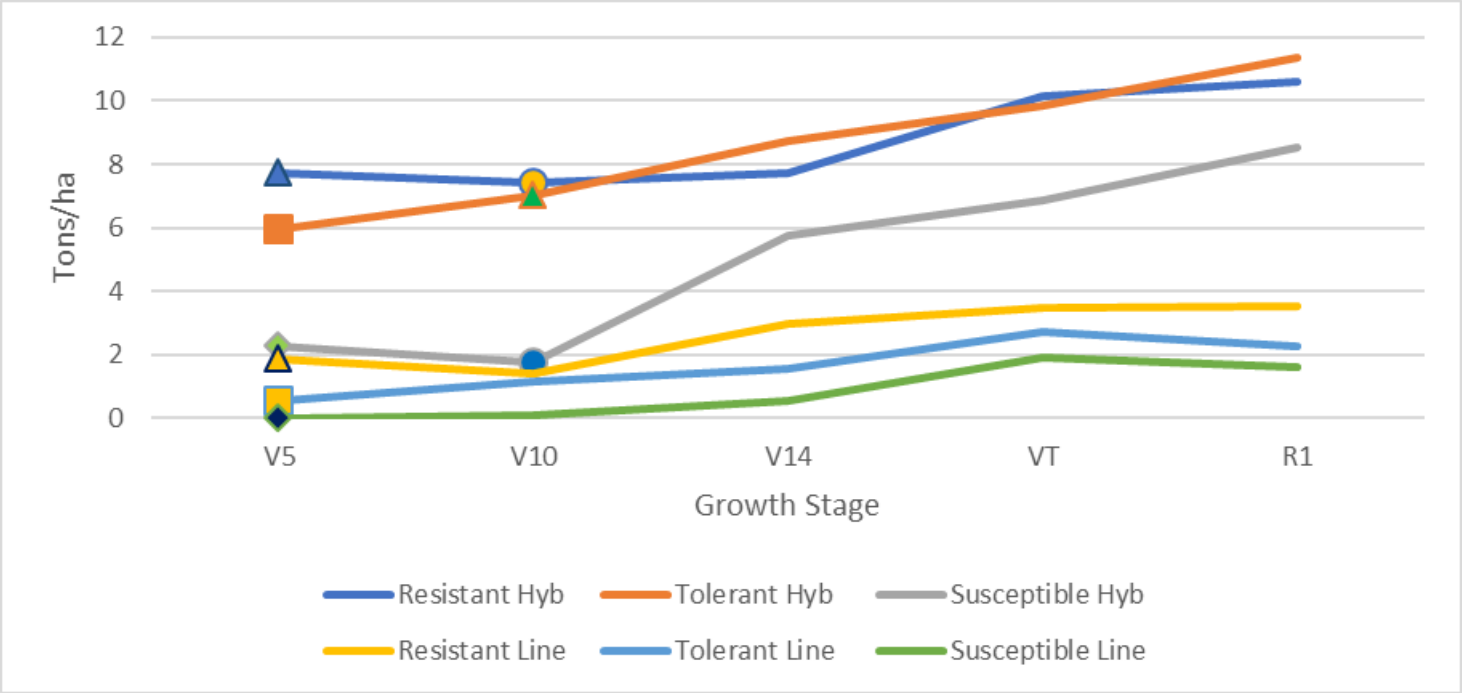

Figure 5

Grain yield of the test entries inoculated with MLN at various growth stages under protected nethouse conditions. Symbols in the figure indicate statistically significant means within each group, (hybrids or inbred lines) by developmental stage.

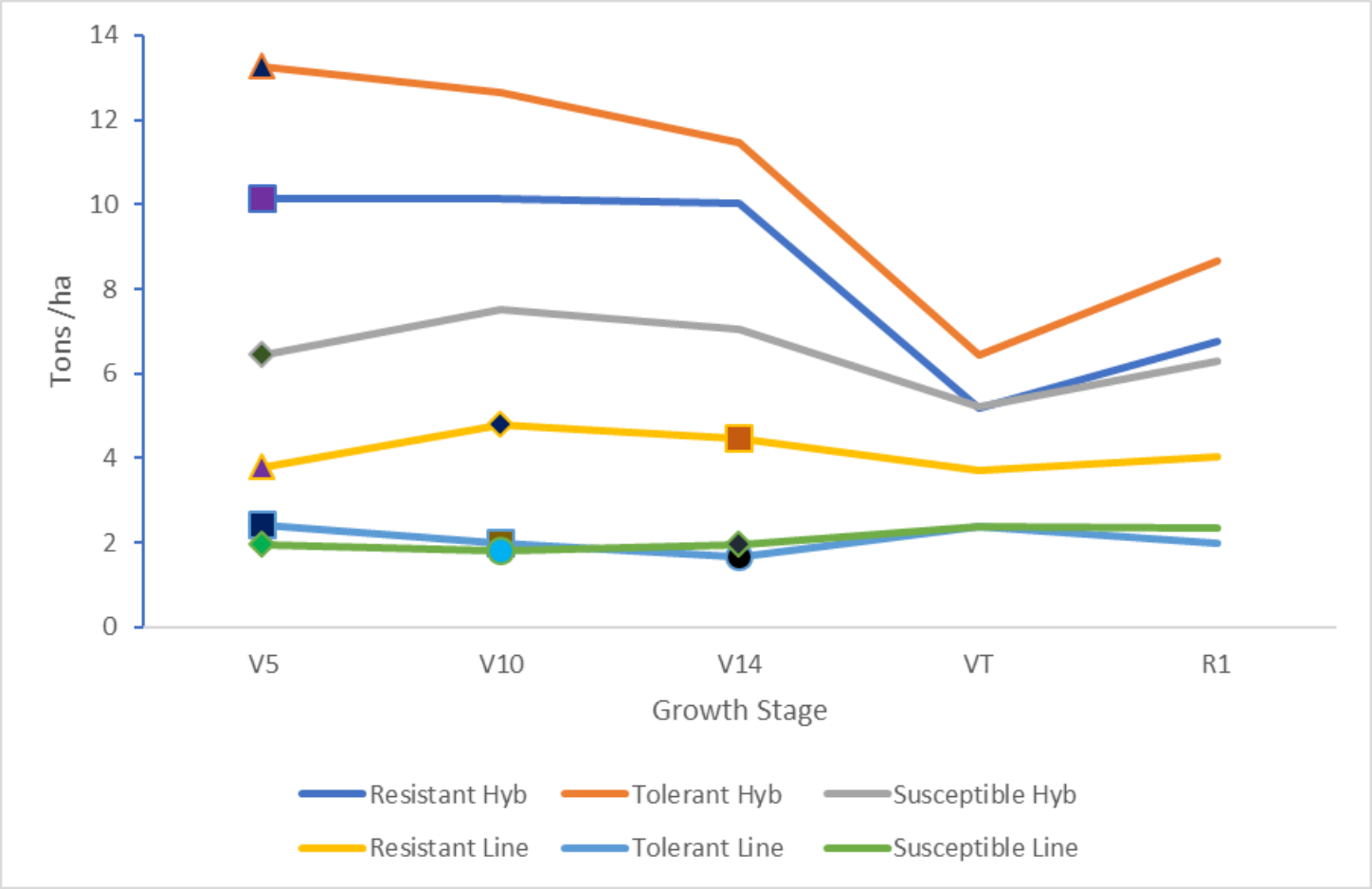

Figure 6

Grain yield of test entries inoculated with MCMV at various growth stages under protected nethouse conditions. Symbols in the figure indicate the statistically significant means within each group (hybrids or inbred lines) by developmental stage.

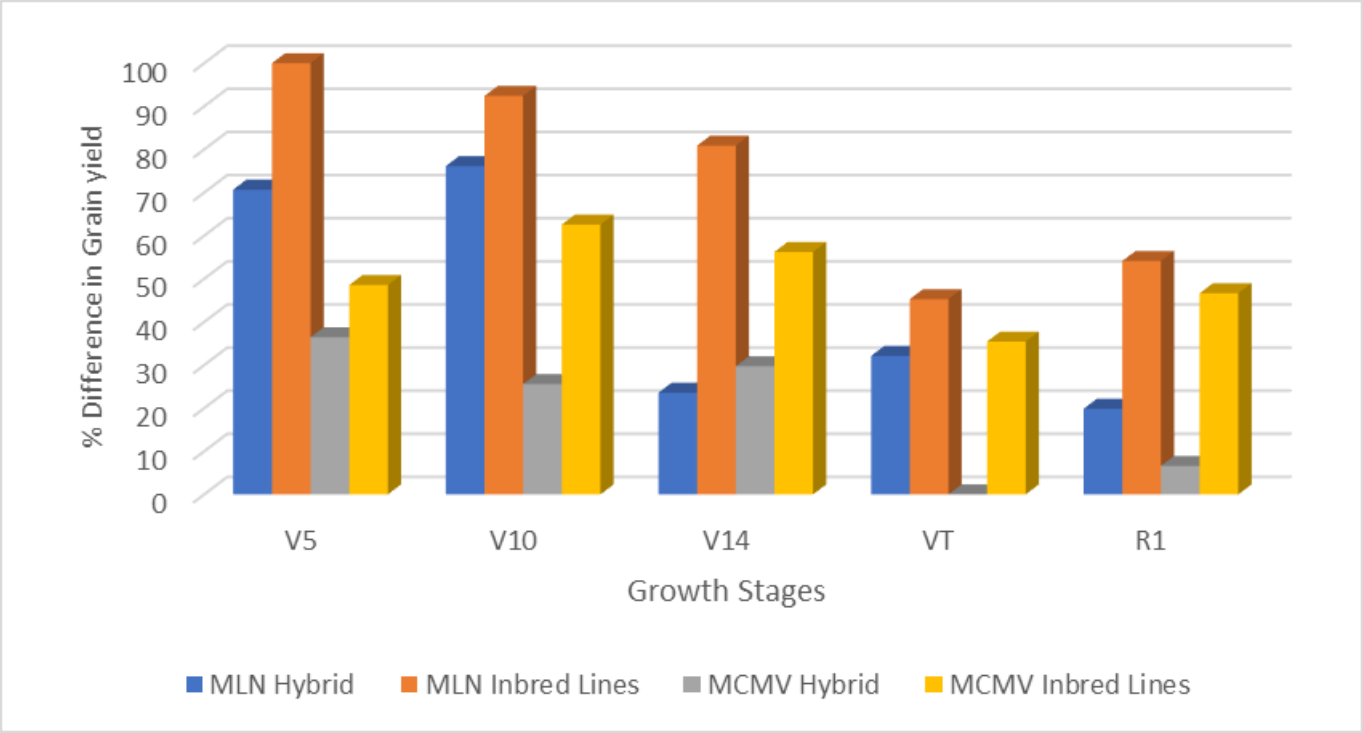

Figure 7

Percentage grain yield differences between resistant and susceptible entries at various growth stages under MLN and MCMV infection at different stages of inoculation.

Supplementary Files

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