Biological and Genomic Sequence Characterization of *Maize streak virus* Isolates from Wheat

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ABSTRACT

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Maize streak virus (MSV) is best known as the causal agent of maize streak disease. However, only a genetically uniform subset of the viruses within this diverse species is actually capable of producing severe symptoms in maize. Whereas these "maize-type" viruses all share greater than 95% sequence identity, MSV strains isolated from grasses may share as little as 79% sequence identity with the maize-type viruses. Here, we present the complete genome sequences and biological characterization of two MSV isolates from wheat that share ≈89% sequence identity with the maize-type viruses. Clonal populations of these two isolates, named MSV-Tas and MSV-VW, were leafhopper-transmitted to

Digitaria sanguinalis and a range of maize, wheat, and barley genotypes. Whereas the two viruses showed some differences in their pathogenicity in maize, they were both equally pathogenic in *D. sanguinalis* and the various wheat and barley genotypes tested. Phylogenetic analyses involving the genome sequences of MSV-Tas and MSV-VW, a new maize-type virus also fully sequenced in this study (MSV-VM), and all other available African streak virus sequences, indicated that MSV-Tas and MSV-VW are close relatives that together represent a distinct MSV strain. Sequence analyses revealed that MSV-VM has a recombinant genome containing MSV-Tas/VW-like sequences within its movement protein gene.

Additional keywords: *Cicadulina mbila*, host adaptation, recombination, strain classification.

The African streak viruses—a group within the genus *Mastrevirus* of the family *Geminiviridae*—are responsible for diseases of grasses throughout the African continent. The best-characterized and most economically significant member of this group is *Maize streak virus* (MSV), the causal agent of maize streak disease. Other African streak viruses identified to date include *Panicum streak virus* (PanSV) (4), *Sugarcane streak virus* (SSV) (10), *Sugarcane streak Mauritius virus* (SSMV), and *Sugarcane streak Egypt virus* (SSEV) (1), which together with MSV, are all transmitted by a number of leafhopper species in the genus *Cicadulina*.

Historically, all streak viruses isolated from graminaceous hosts in Africa, and only mildly infectious in maize, were referred to as "non-maize-adapted" MSV strains (2,25). Early studies of MSV noted different degrees of pathogenicity in maize and, on this basis, MSV strains were consequently separated into two groups. Those that were highly pathogenic in maize (i.e., maize-adapted) were designated as the "MSV-A form," whereas viruses producing milder infections in maize (i.e., non-maize-adapted) made up a group called the "MSV-B form" (16,25). Despite being distinguishable by their relative pathogenicity in maize, the A and B forms had similar host ranges that, prior to the development of molecular techniques, complicated their identification. Furthermore, different MSV-B isolates, obtained from various wild grasses, were found to produce symptoms in maize that ranged from extremely mild to moderately severe (16,25).

In the past 20 years, serological assays and DNA-based techniques have shown that a number of virus isolates originally identified as non-maize-adapted MSV-B strains are, instead, distinct species of the genus *Mastrevirus* (e.g., PanSV, SSV, SSMV,

and SSEV). Although these distinct species share between 63 to 66% genome sequence identity with MSV-A isolates, some apparently non-maize-adapted, genuine MSV-B viruses sharing between 79 to 89% sequence identity with MSV-A isolates have recently been isolated from streak-infected nonmaize hosts (21, 23,28). It has also been demonstrated that mixed MSV-A and MSV-B infections do occur (28) and that MSV-A isolates can be obtained from streak-infected wild grasses by passaging the viruses through an MSV-susceptible maize host (3). The extent to which wild grasses with mixed MSV infections might act as reservoirs for the economically important MSV-A form remains to be determined.

MSV-B isolates naturally infect wheat crops (28), but little is known about the ability of isolates to cause disease in other Graminea. Furthermore, the full genomic sequences of such wheat-infecting isolates have not been examined and their exact phylogenetic relationships to other MSV isolates remain to be determined. Therefore, the purpose of this study was to biologically characterize two MSV-B isolates from wheat (MSV-Tas and MSV-VW) and to compare their genomic sequences with those of other MSV isolates, including a new maize-infecting isolate, MSV-VM.

MATERIALS AND METHODS

Virus origins. Cloning from viral replicative form DNA, restriction mapping, and partial nucleotide sequencing of the MSV isolates MSV-Tas, MSV-VW (both isolated from wheat), and MSV-VM (isolated from maize) have been described previously (21,22). The following clones were provided by F. L. Hughes (University of Cape Town, Cape Town, South Africa): pTas100, a full-length *EcoRI* genomic clone of the isolate MSV-Tas in pBlue-script (SK) (pSK; Stratagene, La Jolla, CA); and pVM100 and pVW100, full-length *BamHI* genomic clones of the isolates MSV-

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VM and MSV-VW in pSK, respectively. An infectious clone of MSV-MatA in *Agrobacterium tumefaciens*, pMSV-AIII, has been described previously (14). Infectious clones of MSV-Kom, MSV-Ns, and MSV-R2X2 in *A. tumefaciens* were obtained from W. Schnippenkoetter (University of Cape Town), M. I. Boulton (John Innes Centre, Norwich, UK), and M. Peterschmitt (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France), respectively.

Sequencing of MSV-VW, -VM, and -Tas. Subclones of pVW100, pTas100, and pVM100 were made in pSK using conveniently positioned restriction endonuclease sites. Subclones with overlapping ends were sequenced in both directions with an ALF-Express automated sequencer (Pharmacia Corporation, Peapack, NJ). DNA sequence manipulations were performed using the Genetics Computer Group (GCG; Madison, WI) (5) and DNAMAN version 3.0 (Lynnon BioSoft, Quebec) software packages. Multiple sequence alignments and phylogenetic analyses were performed using the PILEUP function of GCG and CLUSTALW (26), respectively.

Construction of infectious clones in *A. tumefaciens* and agroinoculation. Unless otherwise stated, all DNA manipulations were performed according to Martin et al. (15). Infectious multimeric clones of MSV-Tas, -VW, and -VM were constructed by cloning partial tandem repeats of each viral genome into the binary cloning vector pBIN19. The resulting recombinant plas-

mids (pVM601, pVW203, and pTas301) were transformed into *A. tumefaciens* C58C1 [pMP90] (11) according to the freeze-thaw method of Holsters et al. (8). Agroinoculation of sweet corn (cv. Jubilee; Starke-Ayres Nursery, Cape Town, South Africa) with the MSV-B isolates, MSV-VW and MSV-Tas, and the MSV-A isolates, MSV-VM, MSV-MatA, MSV-R2X2, MSV-Ns, and MSV-Kom, was carried out according to Martin et al. (15). For each virus × host combination, a minimum of 14 seedlings were agroinoculated for each of three independent experiments.

Symptom assessment. Disease severity was measured in terms of (i) percentage of agroinoculated plants that developed symptoms, (ii) percentage of leaf areas covered by chlorotic lesions in symptomatic plants, and (iii) heights of symptomatic plants relative to uninfected control plants (15). The proportion of plants showing symptoms was determined at 3-day intervals between 5 and 14 days postinoculation. For each virus × host combination, these disease incidence measurements were integrated into an infection rate (IR) value calculated as the mean percentage of symptomatic plants observed at the four assessment times. Percentage of leaf area covered by chlorotic lesions in symptomatic plants was estimated for leaves two through six using a micro-computer-based image analysis technique described previously (13). The percentage of chlorotic areas of leaves two and three was assessed 15 days after agroinoculation; the percentage of chlorotic areas of leaves four, five, and six was assessed 22, 29, and 35 days after agroinoculation, respectively. Heights of symptomatic plants and uninfected control plants were measured 15 days after agroinoculation as the distance from the coleoptilar node to the tip of the fourth leaf. For specific virus–host combinations, a value designated S (representing stunting occurring as a result of infection) was calculated as the mean height of symptomatic plants expressed as a proportion of the mean height of uninfected control plants. The value of 1–S was used as a description of stunting.

Leafhopper transmissions. Nonviruliferous leafhoppers (*Cicadulina mbila*) were maintained on maize in gauze-covered wooden cages. For leafhopper transmission studies, symptomatic MSV-Tas or MSV-VW agroinoculated sweet corn plants (cv. Jubilee) were placed in a cage and leafhoppers were allowed to acquire the virus over a 3-day period. After a latency period of 7 days, the viruliferous leafhoppers were divided into cages (≈20 leafhoppers each) containing different host plants. Hosts included the MSV-susceptible maize genotypes popcorn, PAN6549, and PAN6363, the MSV-resistant maize genotypes PAN6552, PAN6099, PAN6364 (Pannar Ltd., Greytown, South Africa), and “Vaalharts Geel” (VHGeel; Grain Crops Institute, Potchefstroom, South Africa), *Digitaria sanguinalis*, the wheat genotypes Dias, SS66, SS44, Agent, and Marquis (Elsenberg Agricultural Development Institute [EADI], South Africa), and the barley genotypes Clipper, Adam Tas, Chokka, and Festiquay (EADI). MSV-Tas was also transmitted into the sugarcane (*Saccharum officinarum*) genotypes Uba and H44-3098 (South African Sugar Association Experimental Station, Mt. Edgecombe, South Africa). Four or five sweet corn plants (susceptible to both MSV-VW and MSV-Tas) were included in each cage as a positive control for virus transmission. A period of 1 to 2 weeks was allowed for transmission, after which the leafhoppers were culled with a carbamate insecticide. Infection symptoms were rated on a 5-point rating scale (0 = no streaks and no stunting, 4 = 80 to 100% of leaf area chlorotic with severe stunting) as described previously (23).

RESULTS

Sequence and phylogenetic analysis of MSV-Tas, MSV-VW, and MSV-VM. The assembled sequence data for MSV-Tas, -VW, and -VM (GenBank Accession Nos. AF239962, AF239960, and AF239961, respectively) indicated that their genome lengths were 2,683, 2,682, and 2,690 nucleotides, respectively (lengths that are

Fig. 1. Neighbor-joining tree illustrating the phylogenetic relationships between the full-length genomic sequences determined in this study (names in bold) and those of other African streak viruses. The tree was rooted using the full genome sequence of *Chloris striate mosaic virus* (CSMV). Accession numbers for the sequences not determined in this study: K02026 (*Maize streak virus* [MSV]-Ns), AF329881 (MSV-MatA), X01089 (MSV-Ken), AF003952 (MSV-Kom), Y00514 (MSV-SA), AJ224504 (MSV-R2X2), AF007881 (MSV-Set), M23022 (*Digitaria streak virus* [DSV]), L39638 (*Panicum streak virus* [PanSV]-Kar), X60168 (PanSV-Ken), AF039528 (*Sugarcane streak Egypt virus* [SSEV]-Asw), M82918 (*Sugarcane streak virus* [SSV]-N), AF072672 (*Sugarcane streak Mauritius virus* [SSMV]-Reu), and M20021 (CSMV). Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes. Nodes with less than 60% support have been collapsed. Whereas vertical distances are arbitrary, horizontal distances reflect sequence divergence.

within 8 nucleotides of previously sequenced MSV isolates). Positions of genes (replication associated protein [Rep], movement protein [MP], and coat protein [CP]) and intergenic regions (long and short) were inferred by analogy with previously characterized mastreviruses (20). The predicted Rep proteins of MSV-Tas, -VW, and -VM had the same number of amino acids as those of MSV-Kom, -SA, and -Set (12,23); and had 5 amino acids less than those of MSV-Ns, -Ken, and -Reu (9,17,19). All vital nucleotide sequence elements and amino acid sequence motifs (with either known or suspected functions) that have been identified in other MSV isolates were also present in the MSV-Tas, -VW, and -VM sequences (7,18).

MSV-VW and MSV-Tas share 98.1% nucleotide sequence identity with one another and $\approx 89\%$ with MSV-VM and other pre-

viously sequenced maize-type MSV isolates. Phylogenetic analysis of all available full-length MSV sequences suggested that MSV-Tas and MSV-VW represent a distinct MSV strain group (Fig. 1). The only other distinct MSV strain reported to date is MSV-Set, an isolate that shares only ≈ 78 to 80% nucleotide sequence identity with MSV-Tas, MSV-VW, and the maize-type MSV isolates. For descriptive purposes and in line with historical precedents (16), we have tentatively named these three strains MSV-A (comprising the maize-type isolates), MSV-B (MSV-Tas and MSV-VW), and MSV-C (MSV-Set).

Phylogenetic analysis of individual MSV open reading frame (ORF) and intergenic sequences (data not shown) revealed that MSV-VM is most likely a recombinant virus comprising a predominantly MSV-A-like sequence that shares $>98\%$ sequence

Fig. 2. The potentially recombinant region within the movement protein (MP) gene of *Maize streak virus* (MSV)-VM. **A**, A schematic representation of the MSV-VM genome. LIR = long intergenic region, CP = coat protein gene, SIR = short intergenic region, C2 = open reading frame (ORF) encoding C terminal portion of the replication associated protein, RepA = ORF encoding N-terminal portion of the replication-associated protein. MSV-A-like sequences are represented in white, and MSV-B-like sequences are represented in black. Sequences within the boxed region are presented in **B**. **B**, Nucleotide sequence alignment of MSV-Kom, MSV-VM, and MSV-VW sequences surrounding the potential breakpoint junctions within the MSV-VW MP. Nucleotide polymorphisms indicating an MSV-A or MSV-B origin for MSV-VM sequences are labeled below the sequences with A and B, respectively. MSV-Kom and MSV-VM MP codons that are not synonymous are indicated by * above the center nucleotide. Nucleotide sequence numbering is relative to the MSV-VM origin of (+) strand replication. **C**, Neighbor-joining trees constructed from different portions of a multiple African streak virus sequence alignment. Regions I and II are marked in **A**. Region I includes the entire genome, excluding nucleotides between positions 165 and 218 in the MSV-VM genome. Region II corresponds with nucleotides between 133 and 262 in the MSV-VM genome. Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes. Nodes with less than 70% support have been collapsed. Trees were rooted with *Chloris striate mosaic virus* (data not shown). Vertical distances are arbitrary, whereas horizontal distances reflect degrees of sequence divergence. Note the movement of MSV-VM (in bold) from the MSV-A branch of the tree constructed using region I to the MSV-B branch of the tree constructed using region II.

identity with MSV-Kom and MSV-SA, and a portion of MP sequence that is most similar to that of MSV-B isolates. Closer examination of the MP gene and surrounding sequences enabled localization of possible recombination breakpoints and a more precise phylogenetic analysis that supported the hypothesis of recombination (Fig. 2). The putatively recombinant MSV-VM MP gene would be predicted to express an MP that differs at 5 of its first 23 N-terminal amino acid positions from the MPs of MSV-Kom and MSV-SA.

Agroinoculation of MSV isolates and symptom assessment in maize. The pathogenicities of MSV-Tas, -VW, and -VM in sweet corn were compared with those of a group of previously characterized MSV isolates. After agroinoculation, MSV-Tas, -VW, and -VM produced symptomatic infections. Symptoms were relatively severe (MSV-VM), moderate (MSV-Tas), or mild (MSV-VW) (Fig. 3). Plants infected with MSV-VM had induced chlorotic areas that were similar to those induced by MSV-R2X2 and MSV-Kom (Fig. 3A). MSV-MatA- and MSV-Ns-infected plants had the most extensive chlorotic areas. Similarly, IR varied from the relatively low rate of MSV-VW (IR = $45.73 \pm 2.49\%$; 95% CI using Student's *t* test) to the relatively high rate of MSV-R2X2 (IR = $79.1 \pm 4.29\%$; 95% CI using Student's *t* test). However, the distinction among infectivity rates for viruses from maize and wheat was considerably less clear (e.g., rates of MSV-VM and MSV-Tas in Figure 3B). Plants infected with the MSV-B isolates (MSV-VW and MSV-Tas) had significantly less stunting compared with those infected with the MSV-A isolates (Fig. 3C).

Leafhopper transmissions to various hosts. The pathogenicities of MSV-VW and MSV-Tas in various maize and nonmaize genotypes were compared with those of a MSV-A isolate (MSV-Kom) and a MSV-C isolate (MSV-Set), which have been described previously (23). Viruliferous leafhoppers transmitted both MSV-Tas and MSV-VW to *D. sanguinalis* and a variety of wheat, barley, and maize genotypes (Fig. 4). Both MSV-VW and MSV-Tas produced symptomatic infections in the susceptible maize genotypes (including sweet corn, popcorn, PAN6549, and PAN6363). In most cases however, MSV-VW produced milder symptoms (Fig. 4) and infected fewer plants compared with MSV-Tas (data not shown). MSV-Tas also produced mild symptoms in PAN6364, a moderately MSV-resistant maize genotype, whereas MSV-VW did not induce symptoms in this genotype. Both MSV-Tas and MSV-VW did not cause symptomatic infections in the

MSV-resistant maize genotypes VHGeel, PAN6552, and PAN6099. It has been shown previously that all of these MSV-resistant maize genotypes were susceptible to infection by the MSV-A isolate MSV-Kom (23).

The wheat genotypes Marquis, Agent, and SS44 and the barley genotype Festiquay were relatively resistant to the MSV isolates tested here and in a previous study (23) (Fig. 4). In the previous study, only MSV-Set (MSV-C strain) produced symptoms in all of the wheat and barley genotypes used in this study (23). The barley genotypes, Clipper, Adam Tas, and Cokka, the wheat genotype Dias, and *D. sanguinalis* were most susceptible to MSV-Tas and MSV-VW infection, with both viruses inducing severe chlorosis and stunting in these hosts. Although MSV-Tas failed to produce a symptomatic infection in the sugarcane genotype H44-3098, this isolate induced symptoms when transmitted into the sugarcane genotype Uba (data not shown). Interestingly, MSV-Tas-infected Uba plants maintained in our plant growth rooms have remained symptomatic for 3 years, a finding in contrast with the transient symptomatic MSV infections of sugarcane that have been reported previously (25).

DISCUSSION

In this study, full genomic sequences and partial host ranges were determined for two MSV isolates obtained from wheat plants with streak symptoms. The genomic sequence of a third, apparently recombinant MSV-A isolate, MSV-VM, was also determined.

The MSV-VW, -Tas, and -VM genomic sequences contained all ORFs, intergenic regions, functionally significant nucleotide sequence elements, and predicted amino acid sequence motifs that have been identified in other MSV isolates (7,18). However, phylogenetic analyses indicated that MSV-VW and MSV-Tas form a distinct MSV strain grouping. Similar analyses of MSV-VM placed this isolate firmly among the other maize-type isolates by all criteria (phylogenetic analysis of individual ORF and intergenic region nucleotide sequences), except when MP nucleotide sequences were compared (Fig. 2).

Based on the genomic sequence and pathogenicity results of this and other studies, there appear to be at least three distinct MSV groups (21,23,28). Historically, MSV isolates were separated into A and B forms based on the severity of symptoms induced in maize (16,25). In consideration of the nucleotide

Fig. 3. Symptoms observed in sweet corn (cv. Jubilee) agroinoculated with a range of different *Maize streak virus* (MSV) isolates. **A**, The mean percentage of chlorotic area observed on leaves one through six of symptomatic plants agroinoculated with MSV-MatA (●), MSV-Ns (□), MSV-Kom (▲), MSV-VM (○), MSV-R2X2 (◆), MSV-Tas (■), and MSV-VW (▼). **B**, The number of plants showing symptoms between 5 and 14 days after agroinoculation, assessed at four time points, was averaged to give the infection rate (IR) for each isolate. **C**, Stunting of symptomatic plants expressed as $1 - (\text{height of infected plants})/(\text{height of uninfected control plants})$. Error bars represent 95% confidence intervals of means (using Student's *t* test).

sequence relationships between currently described MSV isolates, we have split the B group (formerly identified as those which produce mild symptoms in maize) into two groups, MSV-B and MSV-C. MSV-A comprises isolates that share approximately >95% sequence identity and induce more severe streak symptoms in maize than MSV-B and MSV-C isolates (23). MSV-B includes isolates that have been obtained from wheat and a number of wild grass species (28) and share ≈89% genome sequence identity with MSV-A isolates. We have shown that the MSV-B isolates, MSV-Tas and MSV-VW, generally produce more severe symptoms than the MSV-A isolate, MSV-Kom, in barley and wheat but are invariably milder than the MSV-A isolate in maize. MSV-C is currently represented only by MSV-Set, an isolate from a *Setaria* sp. that shares ≈80% genome sequence identity with MSV-A and MSV-B isolates. Comparing our results to that of a previous study (23), it is apparent that MSV-Set has a more narrow host range among maize genotypes than MSV-B isolates but a slightly broader host range than MSV-B isolates among wheat genotypes.

It is interesting that although the MSV-B isolates share significantly less sequence identity with MSV-C than MSV-A isolates, they are biologically more similar to the MSV-C isolate, MSV-Set, than they are to the MSV-A isolate, MSV-Kom. It is possible that, based on the ≈20% nucleotide sequence divergence between MSV-Set and other completely sequenced MSV isolates, MSV-Set should be classified as belonging to a new African streak virus species. We have felt compelled to classify MSV-Set as a MSV strain because, apart from its host range being so similar to that of MSV-B isolates, it can also be transreplicated by an MSV-A isolate (27). A mixture of biological and sequence data have been used to resolve similar classification problems with the curtovirus species *Beet curly top virus* (BCTV) and the begomovirus species *Bean golden yellow mosaic virus* (BGYMV). Although there are currently three described BCTV strains sharing between 80 and 83% sequence identity, transreplication and pathogenicity studies have suggested that each of the strains should be considered separate species (24). BGYMV and *Bean golden mosaic virus*, despite causing very similar symptoms in beans, are considered different species primarily because they share less than 80% sequence identity and are unable to form infectious pseudorecombinants (6). There are four described BGYMV strains that share between 91 and 95% sequence identity across their genomes (6). Although the

ability of the A components of isolates from each BGYMV strain to form infectious pseudorecombinants with B components of all other strains confirms that these viruses should all be considered members of the same species, they are all considered distinct strains because of their unique biological characteristics (6).

It is probable that MSV-VM is the product of a recombination event between strains A and B MSV genotypes. A 61-nucleotide region of the MSV-VM MP, 8 nucleotides downstream of the start codon, shares 100% sequence identity with MSV-VW (an MSV-B isolate) but only 87 and 85% identity with homologous sequences of the MSV-A isolates MSV-Kom and MSV-SA, respectively. The rest of the MSV-VM sequence shares >98% sequence identity with MSV-SA and MSV-Kom. It is possible that the recombination event that produced MSV-VM may have occurred somewhere within South Africa. MSV-VM, -VW, and -Kom were all collected in South Africa between 1987 and 1989 (21). MSV-VM and MSV-VW were collected in the same area during successive growing seasons, whereas MSV-Kom was collected a year later 400 km away. In addition, mixed populations of closely related isolates have been detected in maize, grasses, and wheat at the locations where these viruses were obtained (28). The importance and prevalence of recombination between distinct MSV strains is uncertain. Although MSV-VM is slightly less infectious in sweet corn than the very closely related but apparently nonrecombinant isolate, MSV-Kom, these viruses induce nearly indistinguishable stunting and chlorotic area symptoms in this host (Fig. 3). The discovery of MSV-VM does, however, suggest that MSV strains that are not directly responsible for yield losses in maize could potentially contribute to the evolution of economically significant MSV-A genotypes.

The pathogenicity of the MSV-B isolates (MSV-Tas and MSV-VW) in the wheat and barley genotypes was greater than that previously observed for MSV-Kom (an MSV-A isolate) in these hosts (23). Conversely, the MSV-B isolates were significantly less pathogenic in maize than MSV-Kom. The pathogenicity of the MSV-B isolates in *D. sanguinalis* and the barley, wheat, and maize genotypes was generally more similar to that previously observed for MSV-Set (the MSV-C isolate) (23). To properly establish whether these isolates from nonmaize hosts have similar host ranges, however, will require a more extensive study involving numerous wild grass species.

Fig. 4. Differential severity of symptoms produced by *Maize streak virus* (MSV)-A (MSV-Kom), MSV-B (MSV-Tas and MSV-VW), and MSV-C (MSV-Set) isolates in *Digitaria sanguinalis* and a range of barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and maize (*Zea mays*) genotypes. Data for MSV-Kom and MSV-Set was obtained from Schnippenkoetter et al. (23). A symptom rating of 0 implies plants were asymptomatic and a rating of 4 implies >80% chlorosis and severe stunting.

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