Research Article

Received: 21 July 2023 Revised: 24 September 2023 Accepted article published: 25 September 2023 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.7802

Host-virus interaction between tobacco mild green mosaic virus strain U2 and tropical soda apple resulting in systemic hypersensitive necrosis and the host range, survival, spread, and molecular characterization of the virus

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Abstract

BACKGROUND: Tobacco mild green mosaic virus strain U2 (TMGMV-U2) is a registered active ingredient in a bioherbicide to control tropical soda apple (TSA), *Solanum viarum*, an invasive weed. As required for registration, we developed empirical data on the host-virus interaction and the virus's host range, survival, spread, and genomic sequence.

RESULTS: TMGMV-U2 killed TSA plants by causing systemic hypersensitive necrosis (SHN). It elicited local lesions in inoculated leaves which was followed by the plant's wilting and death. It moved from inoculated terminal leaves through the vasculature to roots and then to newly developed leaves. Phloem death was implicated in wilting and plant death. The SHN response was attenuated in plants grown at constant 32 °C. TMGMV-U2 titer in TSA was low compared to a systemically susceptible tobacco. The virus remained infective for up to 6 months in infected dead TSA tissues and in soil in which infected plants had grown. Susceptible tobacco and pepper plants grown in soil that previously had infected dead TSA or in soil amended with the virus remained asymptomatic and virus-free. A susceptible pepper crop grown in a field block following two consecutive crops of TMGMV-U2-infected susceptible tobacco grew disease-free and virus-free and without yield loss. Purified TMGMV-U2 was infective for 1 year when stored at −20 °C or 5 °C and for 1 month at room temperature. No virus spread was found in the field. Genomic analyses confirmed the registered isolate to be a U2 strain and free of satellite TMV. The TMGMV-U2-susceptible species preponderantly belonged to the Solanaceae. A few hosts that were killed belonged to this family. Several new hosts to TMGMV-U2 were found. These data enabled registration of TMGMV-U2.

CONCLUSION: TMGMV-U2 can be used safely as a bioherbicide without risks to nontarget plants and the environment.

Supporting information may be found in the online version of this article.

Keywords: bioherbicide; *tobacco mild green mosaic tobamovirus*; TMGMV-U2; host range; invasive weed; systemic necrosis; SolviNix LC

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1 INTRODUCTION

Solanum viarum, commonly called the tropical soda apple (TSA), is an invasive weed of South American origin.[1] Since the 1980s, it has been an invasive weed of pastures and surrounding natural areas in Florida, USA. It is designated as a noxious weed[2] in the United States and is reported to be invasive in nine countries.[3] In August 2010, it was found in the Upper Macleay Valley in New South Wales (NSW), Australia and subsequently in south-eastern Queensland. It is now listed as a Class 2 noxious weed under the New South Wales Noxious Weed Act of 1993.[4]

While searching for a suitable biological control agent for TSA, we discovered that it is susceptible to tobacco mild green mosaic virus strain U2 (TMGMV-U2) and is killed by a virus-elicited systemic necrosis throughout the plant. With this finding, coupled with the knowledge that the virus could be produced and purified relatively easily, we ventured to develop and register TMGMV-U2 as a bioherbicide with the U.S. Environmental Protection Agency (EPA). As required for the agency's review, we designed experiments and developed data to support our registration application. The objective was to address four areas of possible risks from the use of the virus as an herbicide which were identified by the agency: (1) Toxicology of TMGMV-U2, (2) risks to nontarget fauna (aquatic organisms, fish, bird, animals, and humans), (3) risks to nontarget flora (crops, native plants, and Threatened and Endangered [T&E] plants), and (4) the potential for environmental persistence and spread of the virus from its use as a bioherbicide. Other required data were the storage stability and survival/persistence of TMGMV-U2 in infected plant tissues, infested soil, and water. The genomic sequence of the registered TMGMV-U2 isolate was also determined. The toxicology data were waived because TMGMV-U2, a plant pathogenic virus, is not known to be toxic, infective, or pathogenic to any organism other than species of angiosperms. Presented here are the data on the host-virus interaction and symptom expression as well as the virus's plant host range, survival in dead TSA tissues, soil, and water, the potential for horizontal spread, and stability under storage.

TMGMV-U2 has been known for nearly a century as a pathogen of tobaccos (*Nicotiana* spp.), peppers (*Capsicum* spp.), and about 20 other plants.[5–7] It was first described as a mild strain of tobacco mosaic virus and was referred to by various names until its classification as a separate Tobamovirus species (*Tobacco mild green mosaic tobamovirus*; ICTV Decimal Code 71.0.1.0.011).[5–7] Three naturally occurring strains of TMGMV have been described, namely U2, U5, and J.[8–10] The TMGMV-U2 isolate that we registered as the bioherbicide was recovered from tissues of *Columnea* hybrid 'Oneidan' (*C. crassifolia* x *C. allenii*, flying goldfish plants, Gesneriaceae) that originated from a nursery in Ohio. It was purified and identified by Zettler and Nagel[11,12] who deposited it as PV-0113 at the German Collection of Microorganisms and Cell Cultures.[13] (*Note: throughout this paper, TMGMV is used to refer to the virus in general and TMGMV Strain U2 [TMGMV-U2] specifically to the virus strain studied and registered*).

Of the three strains, U2 and U5 have been described from the United States,[8,9] and they differ in the size in their 3′ untranslated regions (UTR). Strain TMGMV-U5 has a repeated sequence of 147-nucleotides in the 3′ UTR compared with TMGMV-U2. The strains elicit different biological responses in some host plants,[9] and they could cross-protect and modulate symptoms when co-inoculated. Moreover, a satellite tobacco mosaic virus (STMV), a small spherical ssRNA virus, has been reported to be associated with TMGMV-U5 recovered from *Nicotiana glauca* (tree tobacco) in southern California.[9,14,15] Since the satellite can attenuate TMGMV disease expression in plants, it was necessary to determine its presence or absence in the isolate we registered as the bioherbicide. For these reasons and because it was required for registration, we characterized this isolate by genome sequencing. A partial account of our attempt to develop TMGMV-U2 as a bioherbicide has been published previously.[16] Also, extensive field trials conducted under an Experimental Use Permit to develop application methods and tools and pesticide labels have been published.[17]

2 MATERIALS AND METHODS

2.1 Host-virus interaction

2.1.1 Reaction of TSA to inoculation with three tobamoviruses

We tested three tobamoviruses, namely tobacco mosaic virus (TMV), tobacco mild green mosaic virus strain U2 (TMGMV-U2), and tomato mosaic virus (ToMV) that were readily available to us for pathogenicity to TSA hoping to find one with the most potential as a biocontrol agent. *Nicotiana tabacum* cv. Turkish 'Samsun' *NN* (hereafter 'Samsun NN') and *N. tabacum* cv. Turkish 'Samsun' *nn* ('Samsun nn') were also inoculated to check for the viruses' infectivity and symptoms. The viruses were provided to us in freeze-dried, infected Samsun nn leaves by F.W. Zettler, University of Florida. They were multiplied from the leaves by inoculating greenhouse-grown Samsun nn plants. Symptomatic leaves were triturated at 1:10 w:v in 0.02 M sodium phosphate buffer, pH 7.2 (hereafter, the 'buffer'). The extracted sap, without dilution, was used as inoculum. In subsequent studies, the TMGMV-U2 inoculum was produced in greenhouse-grown Samsun nn plants and purified in the laboratory by known methods,[18,19] or it was industrially produced from the same host as described.[20] The latter was termed the technical grade active ingredient (TGAI) for registration purposes.

Seeds from field-collected TSA fruits and greenhouse-grown tobacco cultivars were used to raise seedlings in a commercial potting soil. At their first- to second-leaf stage, the seedlings were transplanted individually into pots. The tobacco and TSA plants were manually inoculated with virus inoculum by lightly dusting the leaves with 600-mesh Carborundum powder and gently rubbing two or three leaves with a sterile cheesecloth pad dipped in the inoculum (hereafter the 'manual inoculation' method). *Nicotiana* spp. and *Solanum* spp. that differ in their response to infection by the three tobamoviruses were used as indicator hosts to confirm infection. Table 1 summarizes the species- and cultivar-specific host symptoms used for this purpose.

For testing the viruses, approximately 60-day-old potted TSA plants in a greenhouse were used, with 10 replicates for the inoculated treatment and two for the buffer-treated controls. The experiment was repeated (minimum two trials). The inoculated TSA plants were assayed for each virus in immunodiffusion plates using antisera provided by F.W. Zettler. Randomly collected symptomatic leaves from each treatment were ground 1:1 w:v in sterile deionized water for use in the immunodiffusion plates. Positive and negative control leaf extracts were included.

TMGMV-U2 was field-tested in two trials in a TSA-infested cattle pasture in Hawthorne, FL using a completely randomized design with three virus-inoculated treatments and respective mock-inoculated controls. Plants were manually inoculated or by leaf-infiltration. The latter was done by placing the nozzle of a CO_2-propelled backpack sprayer on the adaxial leaf surface and discharging the inoculum at 138 kPa pressure. The leaves were checked visually for infiltration. Three leaves per plant were

Table 1. Differential host reactions to three tobamoviruses used in this study

Host	Host reaction(s) to the virus		
	Tobacco mild green mosaic virus strain U2 (TMGMV-U2)	Tobacco mosaic virus (TMV)	Tomato mosaic virus (ToMV)
Nicotiana tabacum cultivar 'Samsun' *NN* (Turkish/oriental tobacco)	Resistant, hypersensitive local lesions in inoculated leaves	Resistant, hypersensitive local lesions in inoculated leaves	Resistant, hypersensitive local lesions in inoculated leaves
N. tabacum cultivar 'Samsun' *nn*	Mild systemic mosaic	Systemic mosaic	Systemic mosaic
Nicotiana sylvestris (woodland tobacco)	Resistant, local lesions	Susceptible, systemic mosaic	Susceptible, systemic mosaic
Solanum lycopersicum cultivar Rutgers (tomato)	Immune, no reaction	Susceptible, systemic mosaic	Susceptible, systemic mosaic
S. viarum (tropical soda apple, TSA)	Susceptible, local lesions, systemic necrosis, plant death	Susceptible, systemic mosaic	Susceptible, systemic mosaic

inoculated, first applying the virus-free control treatments followed by the virus treatment to prevent cross-contamination. For manual inoculation, 1 g and 3 g of infected Samsun nn leaves per L were used as inoculum, respectively, in the first and second trials. For infiltration, 0.5 g and 1.0 g per L (first trial) and 1.5 g and 3 g per L (second trial) were used. One gram of carborundum (320 grit) was added per L of inoculum. There were 30 or 32 plants (replicates) per treatment. The plants were rated for first symptom appearance on the sixth day after inoculation and then at 2- to 3-day intervals until the inoculated plants died. Final results were recorded 6 weeks after inoculation.

2.1.2 Host-virus interaction and systemic hypersensitive necrosis in TSA

The *in planta* movement of TMGMV-U2 in TSA was determined from its presence in leaf, stem, and root tissues over time with the dot-blot assay along with enzyme-linked immunosorbent assay (ELISA).[21] Sixteen TSA plants, approximately 2 months old and comparably sized in 4-L pots were trimmed to a single stem with 5 to 10 leaves. The symptom expression was monitored each day following inoculation for 15 days, and one plant was harvested and blotted each day. The leaves were numbered starting from the youngest to the oldest and the stem from the middle to the soil line. Roots (the upper, middle, and lower fine roots) were also blotted. The samples were cut with a sterile razor blade and blotted onto nitrocellulose membranes in sequence from the first leaf at the top to the small roots. The blotting was done by a procedure described by Lin *et al.*[22] modified by Elliott and Zettler[23] and Siegmann *et al.*[24] Each sample was replicated in six wells. The ELISA plates were read for absorbance at 405 nm (A405) using a BioTek EL309 and/or a BioRad 680 microplate reader. Absorbance values equal to or greater than 3 times the average absorbance value of the controls were considered positive for virus presence. Positive ELISA reactions were mapped daily to follow the virus movement through the TSA plant. This experiment was done twice.

In a separate study, five 2-month-old TSA plants were manually inoculated with TMGMV-U2 and another five of the same age kept without any treatment. The plants were maintained outdoors under the same conditions and photographed on different days after inoculation to illustrate disease progression. A pair of control and inoculated plants were photographed on day 25 after inoculation and the lower third of their main stems were cut, split vertically, and photographed to record vascular discoloration of the inoculated stem compared to the healthy control stem.

2.1.3 TMGMV-U2 titer in TSA from infection to plant death

The buildup of TMGMV-U2 titer in TSA plants was quantified as part of the host-virus interaction and to provide a basis to assess its persistence in the host and soil. Twenty-one 2-mo-old, single-stemmed TSA plants approximately 41-cm tall were used. Eighteen of them were inoculated and three uninoculated plants served as healthy controls. The second, fully expanded, large, healthy leaf from the top was inoculated. Leaves were tagged to keep track of the inoculated and the abscised. The plants were grown in a shade house. Leaves from 12 individual plants of Samsun nn tobacco grown in the same shade house were used for comparing the virus titers. Three plants of TSA and Samsun nn were harvested on the following times: Time 0, the day of inoculation (control healthy plant); Time 1, 3 days after inoculation (DAI); Time 2, 10 DAI; Time 3, 17 DAI; Time 4, 21 DAI; Time 5, 28 DAI; and Time 6, 31 DAI. Samples were collected from the following locations on the plant: Location 1 = a leaf above the inoculated leaf; Location 2 = the inoculated leaf; Location 3 = one leaf below the inoculated leaf; Location 4 = developing leaves/meristem; and Location 5 = abscised, dropped leaves. In addition, stems and roots were sampled.

Tissues were stored frozen until tested for TMGMV-U2 by ELISA. The virus titer was computed by applying A405 values to a formula generated by a dilution series curve using purified TMGMV-U2. The virus titers at different locations in the infected TSA plant were compared with those from leaves of Samsun nn assayed comparably.

2.1.4 Effect of temperature on disease expression

To establish whether TMGMV-U2 would be infective in the field under Florida's subtropical temperature, the effects of three temperature regimes on disease development were studied. Six-month-old potted TSA plants were manually inoculated with TMGMV-U2 while control plants were rubbed with buffer only. The inoculated and the control plants were exposed to: (1) 18 °C continuously; (2) 32 °C continuously; and (3) 32/22 °C alternating day/night. The plants were kept in separate controlled-environment chambers under a 12-h photoperiod with

250 μE/m2/s illumination from fluorescent and incandescent lights.

2.1.5 Plant host range of TMGMV-U2

Although the host range of TMGMV-U2 was known from published accounts,[6] as required, we did an extensive host range study to identify susceptible nontarget plants that might be at risk from the use of the virus as a bioherbicide. The host range test list was comprised of: (1) representative species reported in the literature to be susceptible to TMGMV; (2) members of the Solanaceae, the family to which TMGMV is adapted;[25] (3) major crop plants grown in North America; (4) a selection of North American weeds; (5) 10 species of *Eryngium*, including *E. planum*, a previously known host to TMGMV,[6] and *E. cuneifolium*, an endangered species in Florida;[26] and (6) 12 accessions of TSA from New South Wales, Australia.

The test plants were raised from seeds purchased from commercial sources, provided by cooperators, or collected locally. *Eryngium cuneifolium* plants were collected from the wild under a permit from the State of Florida. Some tree and shrub species were purchased from nurseries and visually checked to be free of virus symptoms. One or two seedlings of dicots or higher numbers of monocots were raised per 10-cm clay pot. Seedlings in five replicate pots were manually inoculated with TMGMV-U2 and those in one pot, rubbed with buffer as the control. *Nicotiana sylvestris* plants were used to confirm the inoculum's infectivity. Inoculum was prepared from Samsun nn leaves and manually inoculated. The plants were observed and visually rated for symptoms as no symptoms (NS), hypersensitive local lesions on inoculated leaves (HS), systemic susceptibility-systemic mosaic (SS), hypersensitive reaction turning to systemic mosaic (HS_SS), or hypersensitive and systemic symptoms turning to partial or complete plant death (HS_SS_D). New leaves from each plant were used for ELISA. Samples that gave questionable ELISA results were back-inoculated onto Samsun NN or *N. silvestris* for local lesions development on inoculated leaves to confirm virus presence. ELISA results were recorded as POS, positive in ELISA (virus present) or NEG, negative in ELISA (virus not present). The host reactions based on symptoms were further reduced to Reaction Categories of Asymptomatic when there were no symptoms, but the plant was ELISA-positive in at least one of the repeated trials, Immune, Resistant, Susceptible, and Lethal-Susceptible and entered into an Excel worksheet.

Tree and shrub species were maintained over one winter season to observe for possible delayed symptom development. A total of 435 genus-species-cultivar combinations representing 183 genera and 61 families were tested. All species were tested at least twice at different times of the year and the results from the trials are presented side-by-side in the Excel file ('TMGMV-U2_HostRangeTestResults_07-20-2023' in Supporting Information, hereafter 'Excel file in Supporting Information').

2.2 TMGMV-U2 survival/persistence in dead TSA tissue, soil, water, and storage

As part of the required studies, we examined the survival of the virus in dead TSA tissue and in soil and after storage of purified virus (TGAI) at three temperatures and in three different waters. The virus was considered to have survived/persisted in soil, plant tissue, or water if symptoms developed in indicator plants inoculated with or exposed to a putatively TMGMV-U2-containing soil or plant tissue or their water extracts.

2.2.1 Persistence in potted soil

To determine whether TMGMV-U2 persisted in potting soil in an infective state under outdoor conditions, two similar studies were done. In Trial 1, 13 TSA plants, approximately 2 months old, were grown in 4-L pots containing potting soil. Twelve plants were inoculated with TMGMV-U2, and one control was rubbed with buffer only. At various times, after the inoculated plants had died from TMGMV-U2 infection, soil cores from 10- to 15-cm depth were collected, four from each pot, pooled, mixed, and stored in plastic bags at 4 °C until extracted for inoculation. Soil was collected: (1) before treatment (as control); (2) 1 month after the inoculated TSA plants had died; (3) 6 months after; and (4) 1 year after. The soil was extracted with water and the extracts tested by inoculating systemically susceptible and resistant tobacco plants. Development of TMGMV disease symptoms was indicative of virus persistence in the soil, while the lack of symptoms implied non-persistence.

Six months after inoculation with TMGMV-U2 and after the TSA plants had died, one each of a Samsun nn tobacco and *C. annuum* 'Crusader' pepper (susceptible) plants were planted in each pot. Three months later, leaf and stem samples were collected from these plants and tested by ELISA. The development of systemic mosaic symptoms in these hosts was considered proof of virus persistence in the soil. Positive ELISA readings, with or without visible symptoms, were considered confirmatory of virus persistence. Control pot was used similarly to check for symptom development and ELISA.

Soil extraction and inoculation of indicator plants were done as follows. Ten grams of each collected soil were mixed with 10 mL of the buffer in a 50-mL beaker and stirred. The beaker was covered with Parafilm and incubated overnight at 4 °C. The samples were then stirred and filtered through cheesecloth and each sample was used to inoculate a Samsun NN plant that was observed for local lesions development. Also, 300 μL of each extracted sample was placed in a Microfuge tube and frozen for ELISA. Dead TSA stem tissues collected 6 months after inoculation were also processed for back-inoculation and ELISA. For inoculation, 0.5 g of stem tissue was triturated in 5 mL of buffer and used as inoculum.

In Trial 2, done similarly to Trial 1, five *C. annuum* 'Enterprise' plants (susceptible) were raised from seed and transplanted one per pot in which the TSA plants had been previously inoculated with and killed by TMGMV-U2 6 months prior. TMGMV disease development in the pepper plants would indicate virus persistence in the soil.

2.2.2 Persistence in field soil and spread at a research farm site

The persistence of TMGMV-U2 in field soil in an infective state was determined from two studies each done from fall through summer. A 4 × 5 randomized block design with four treatments and five replicates was set up using 1 m^2 plots with 1-m alleyways between plots. Each plot had nine TSA plants raised from seed and transplanted. The treatments were (1) TSA plants inoculated with TMGMV-U2 and 1 month after they had died, were mowed to about 5 cm above the soil surface. (2) Same as treatment 1 but the plots tilled after the plants had died. (3) Non-inoculated control TSA plants that were mowed at the same time as treatment 1. (4) Non-inoculated control TSA plants that were tilled as in treatment 2. Three leaves on each TSA plant were manually Inoculated with TMGMV-U2 while the control treatments, 3 and 4, were left untreated.

Nine soil samples were collected with a soil-sampler from each plot within the root zone of the TSA plants, 10 to 15 cm below

the soil surface. The samples were pooled, mixed, and stored in plastic bags at 4 °C until extracted for inoculation and ELISA. Samples were collected three times to determine the virus presence. (1) Prior to the treatments. (2) One month after TSA plants were inoculated and had died. (3) Approximately 6 months after plants had died and the plants were mowed or tilled.

In Trial 1, two Samsun nn tobacco and two *C. annuum* 'Wizard' plants (susceptible) were planted in each plot in the same locations where the TSA plants had grown. Leaf and/or stem samples were collected from these plants for ELISA. In Trial 2, 5 months after mowing or tilling, four Samsun nn and five *C. annuum* 'Camelot' (susceptible) were planted in each plot and sampled as in Trial 1.

The soil collected 6 months after tilling was tested twice. Twenty grams of each soil were mixed with 5 mL of buffer and prepared as described above. Each filtered sample was manually inoculated onto a Samsun NN plant and observed for hypersensitive local lesions development. For ELISA, leaf samples were collected from the tobacco and pepper plants and stored frozen and assayed as described.

2.2.3 *Disease incidence in a susceptible pepper grown in TMGMV-U2-amended soil*

To establish whether a susceptible host of TMGMV-U2 raised in virus-amended soil might become infected, the cultivar *C. annuum* 'Wizard' was used as follows. Four-liter plastic pots, each containing an equal weight of a commercial potting soil, were prepared and the soil was amended with 10 different concentrations of TMGMV-U2 virions from 0 mg (water only, control) to 5 mg per pot. The concentrations were prepared in water by serial dilution from a spectrophotometrically quantified stock of purified virions (TGAI). The virions were added at the appropriate concentrations in 100-mL volume and mixed thoroughly with the soil. The pots were kept in a greenhouse at 30 ± 2 °C and sown with surface-disinfested pepper seeds, 10 per pot, and the seedlings were thinned to three per pot. There were three replicates per treatment for a total of nine plants per treatment. The plants were examined at intervals for symptoms. Eighty-four days after sowing, leaf and root samples were collected from each plant in each pot for virus detection by inoculating indicator plants and ELISA. Samples from each pot were pooled and used, providing three replicates for each virus concentration. Pepper fruits were also harvested on this day to visually examine for virus symptoms.

2.2.4 *Disease incidence in a sentinel pepper crop grown in a TMGMV-U2 production block*

The persistence of TMGMV-U2 in soil in a field block where two consecutive crops of TMGMV-U2-infected Samsun nn tobacco had been previously grown for large-scale production of the virus was studied by raising a crop of a TMGMV-U2-susceptible pepper in the same block. This same block is referred to as the 'production block' when it had tobacco, the 'production crop', or the 'sentinel block' when it had the 'sentinel crop' of pepper. The two production crops consisted of a 'summer crop' and a 'fall crop'. The former was cropped from spring to summer and the latter from summer to fall. The summer crop of approximately 13 746 tobacco plants was grown on a 5755 sq m (0.58 ha) area. The fall crop of about 6200 tobacco plants was grown on 2963 sq m (0.30 ha). After each production crop was harvested by cutting the shoots above the soil, the remnant stems and roots were disked twice into the soil and bottom-plowed. After the second crop was harvested and the plot similarly disked and plowed, the block was planted with Wren Abruzzi rye (*Secale cereale*).

In spring, the rye was mowed, disked, and bottom-plowed and the plot prepared for planting the sentinel pepper crop. This sentinel block had eight plots, each with 4 beds, 0.64 m wide and 15.24 m long, with one pepper plant at every 0.5 m (total 4266 plants). The beds were laid perpendicular to the direction of tobacco beds of the production crops. Each bed was planted with double rows of pepper *C. annuum* 'Crusader' that is susceptible to TMGMV-U2 and may be killed (Excel file in Supporting Information). The pepper seedlings were purchased ready to transplant. Presumptively, transplanting seedlings rather than direct seeding would increase the chances for virus infection through handling and root injuries during planting. The control block with eight plots of 'Crusader' pepper was laid out in a block that was not previously cropped with tobacco or other TMGMV-U2-susceptible plants. The layout and planting details were the same as in the sentinel block. However, unlike the production block that received a preemergent herbicide treatment before the production crops were planted, the control block was prepared with only cultivation. Consequently, as the study progressed, the weed pressure in this block was considerably greater than in the sentinel block, which depressed crop yield.

Pepper fruit was harvested twice during the study period and graded. Harvest 1 was on the 98th day after transplanting and Harvest 2 was 15 days later. Throughout, the plants were observed for symptoms of TMGMV-U2 infection once a week. Leaf samples were collected, four from each bed, 64 samples each from the sentinel and control blocks, and tested for TMGMV-U2 by ELISA. They were also used to inoculate TSA, *N. sylvestris*, and tomato to detect the presence of TMGMV or other tobamoviruses.

2.2.5 *Stability of TMGMV-U2 at three storage temperatures and in three water samples*

The storage stability of the TGAI of TMGMV-U2 was required information for registration. The TGAI consisted of purified virions in water as a liquid concentrate. The stability was determined from the retention of infectivity of the virions following storage under three temperatures most likely to be used for the commercial bioherbicide product. The virus was stored for 1 year in a − 20 °C freezer, a 5 °C refrigerator, and at room temperature (25 to 28 °C). At the end of this period, three vials per treatment were retrieved and tested for infectivity of the stored virus by inoculating *N. silvestris* plants.

Also, the persistence of the virus stored in sterile deionized water, nonsterile tap water, and eutrophic lake (Lake Alice, Gainesville, FL) water over 1 year was determined. One mL of TGAI at approximately 0.3 mg virions per mL, was diluted with 9 mL of sterile deionized water, tap water, or lake water. One mL of each of these dilutions was dispensed into Microfuge tubes and stored at room temperature. The stored virus samples were tested for infectivity by inoculating *N. sylvestris* plants starting at 19 days of storage and then monthly for 372 days. Three plants were manually inoculated on six leaves per plant with 0.5 mL of each stored virus. Local lesions were counted, 3 to 5 days after inoculation and averaged per leaf. Virions from one vial per treatment were tested for infectivity on different days since the start of storage. Due to time constraints, these studies were run for only 1 year before registration. We now have long-term data as mentioned in Results.

2.3 Molecular characterization of the registered TMGMV-U2 isolate

2.3.1 Genomic sequence of the isolate and the absence of a satellite in it

Although genomic sequences of TMGMV are available in the GenBank, we sequenced the TMGMV-U2 isolate registered as the bioherbicide because it was required. Standard protocols for polymerase chain reaction (PCR) amplification, cloning, and sequencing were applied in analyzing and comparing the bioherbicide isolate with known TMGMV sequences. An isolate of TMGMV-U5 + Satellite was purchased from the American Type Culture Collection-Virology, Catalog No. Pv-586, for comparison. Rabbit anti-STMV antiserum was provided by Deborah M. Matthews, University of California, Riverside.

Primers used in amplifying and sequencing TMGMV-U2 genome are as follows:

Forward (fwd) GAT GTT TTA ATA GTT TTC GAC AAC (seq 1–24)
Reverse (rev) CTG TTT TGG TTG AGC CTT GAT C (3982–3961)
fwd GCT GGA GTG ATG ACG AAG GAT TC (3839–3861)
rev TGG GCC GCT ACC GCG GTT (6355–6335)
Cloning primer w T7 fusion GTA ATA CGA CTC ACT ATA GGA TGT TTT AAT AG (1–32)
rev GCT ATC TAC TAC CTG CTT CTA CC (3407–3388)
rev TGG GCC GCT ACC GGC GGT TAG (6355–6335).

Gibb's Universal Tobamo 2 primer 5′-TTBGCYTCRAARTTCCA-3 (4572–4588) and Universal Tobamo 3 5′-CARACNATWGTBT AYCA-3 (4034–4050) were also used.[27] Letschert *et al.* universal forward primer Tob GTY GTT GAT GAG TTC RTG GA (5479 to 5498) and 3′ half reverse primer (6355-6335)TGG GCC GCT ACC GGC GGT TAG were used to compare the 3′ ends TMGV-U2 and TMGMV-U5.[28] Sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research.

3 RESULTS

3.1 Host-virus interaction

3.1.1 Reaction of TSA to inoculation with three tobamoviruses

All three tobamoviruses infected TSA (Fig. 1) while the uninoculated control plants remained symptom-free and virus-free based on immunodiffusion tests. Hypersensitive local lesions developed in 3 or 4 days in resistant Samsun NN tobacco inoculated with the viruses. In comparison, the susceptible Samsun nn tobacco developed mosaic symptoms in 9, 14, and 10 days after inoculation with TMV, ToMV, and TMGMV-U2, respectively.

In TSA, TMV and ToMV elicited systemic mosaic in new leaves without noticeably affecting plant growth. TMGMV-U2 elicited necrotic local lesions 8 days after inoculation and approximately 1 week later the infected plants started to wilt (Fig. 1). The plants died within the next 5 to 6 weeks. In two trials, all inoculated TSA plants were killed, apparently by systemic hypersensitive necrosis (SHN) while the control plants were alive and healthy. The local lesions on TMGMV-U2-inoculated TSA were irregularly shaped unlike the distinct spots seen in Samsun the NN. An immunodiffusion assay and back-inoculations to Samsun nn and TSA confirmed TMGMV-U2 as the causal agent of TSA death. TSA leaf extracts from the tobamovirus-infected plants reacted strongly with the respective virus antiserum in immunodiffusion plates. The antisera were also tested against heterologous antigens and the results ruled out cross-contamination of these viruses (data not shown).

In two greenhouse trials on 1- to 7-month-old TSA plants, the younger plants developed symptoms and died sooner than older plants (Table 2). In two field trials, 97% to 100% of the inoculated plants became symptomatic and 83% to 97% of them died irrespective of the inoculation method or the applied inoculum level (Table 3). None of the control plants became symptomatic or died, and the difference between the controls and inoculated treatments was highly significant.

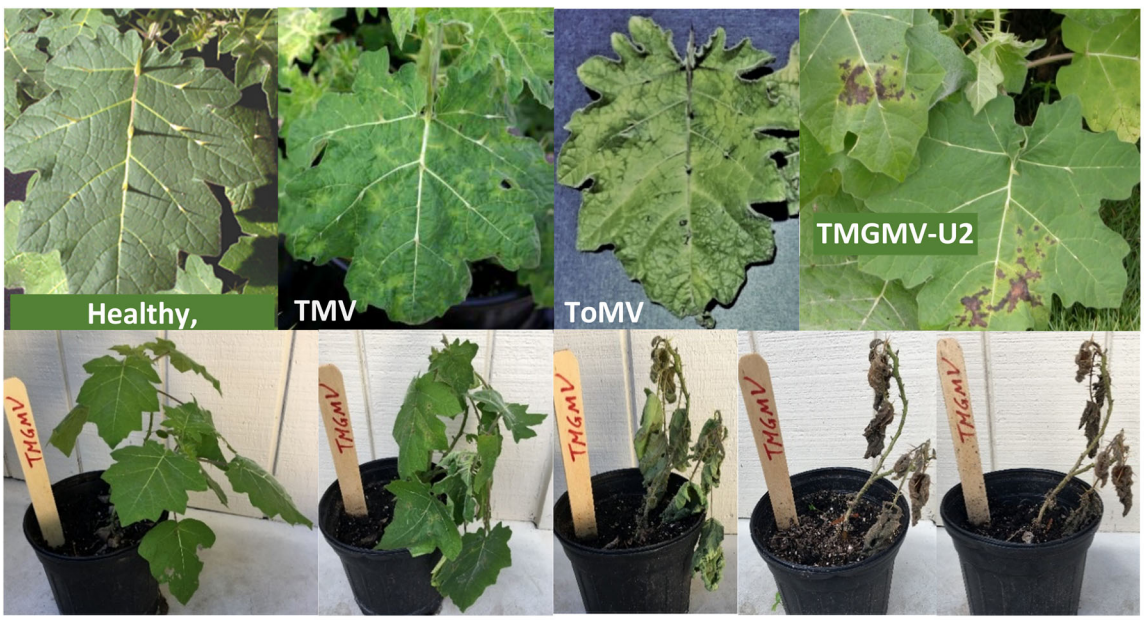

Figure 1. Top row, from left to right: TSA leaves, healthy, TMV-inoculated with systemic mosaic, ToMV-inoculated with systemic mosaic, and TMGMV-U2-inoculated with hypersensitive necrotic local lesions. Bottom row: progression of systemic hypersensitive necrosis (SHN) in a TMGMV-U2-inoculated TSA plant. Left to right: The plant on day 1 (before inoculation); day 16 after inoculation (DAI); 22 DAI, 33 DAI, and 43 DAI.

Table 2. Effect of plant age on first symptom appearance and time of death of plants inoculated with TMGMV-U2*

	First symptom appearance			Time to death
	Plant Age†	N‡	Mean§	Mean§
Trial 1	>12	6	10.0 a	21.5 a
	7	10	9.4 b	18.0 b
	3	10	5.0 c	13.0 c
	2	10	5.0 c	13.0 c
	1	9	5.0 c	13.0 c
	<1	10	4.8 c	10.0 d
Trial 2	>12	5	6.7 a	25.0 b
	7	10	11.6 b	30.0 a
	3	10	6.0 c	12.0 c
	2	10	6.0 c	12.0 c
	1	10	6.0 c	12.0 c
	<1	10	6.0 c	10.0 d

*Means separated by Duncan's multiple range test, $P > 0.0001$.
† Plant age in months.
‡ N = number of replicates (TSA plants).
§ Mean number of days from inoculation to first symptoms or plant death.
Note: Virus-free control plants in each age category remained healthy (data not shown).
The results were recorded 6 weeks after inoculation.

3.1.2 *Host-virus interaction and systemic hypersensitive necrosis in TSA*

The dot-blot assay (Table S1) revealed that the movement of TMGMV-U2 through TSA plant conformed to the previously described pattern of virus movement in plants.[29] The virus was detected in petioles of inoculated leaves and the main stem 1 day after inoculation. After 4 days, the virus was detected in roots in addition to the inoculated leaves and the main stem. After 6 days, it was detected in upper, younger, still expanding leaves. The lower, older leaves tested positive for the virus after the 7th day. The leaves started to abscise by the 6th day and continued until the plant was dead. Thus, TMGMV-U2 moved from the point of infection on the inoculated leaves through the stem, then to the roots, and moved back up to the actively growing points including the apical and axillary leaves. This pattern confirmed a rapid movement of the virus in the TSA plant as well as the rapid development of SHN. As the inoculated TSA plant started to wilt, its vasculature discolored, turning dark brown compared to the healthy control (Fig. 2). This indicates that TMGMV-U2 elicits necrosis of the phloem tissue that leads to the wilting and subsequent plant death.

3.1.3 *TMGMV-U2 titer in TSA from infection to plant death*

TMGMV-U2 did not reach high titers in TSA leaves compared to the leaves of the systemically susceptible Samsun nn tobacco (Table 4). The estimated mean virus concentrations in TSA were 70 to 80 µg per g of leaf tissue and 50 to 100 µg per g in leaves, stem, and roots combined. In comparison, in Samsun nn the virus concentration was 1.52 mg per g of leaf tissue or about 15 to 30 times higher than in TSA plant. Thus, the cause of TSA death is not from high virus titer in the plant. Rather it appears that TMGMV-U2 does not multiply to a high level because of the rapid host response that leads to the death of host tissues. The critical element in the death is likely the necrosis of the phloem tissue which would cause the infected TSA to wilt and die (Fig. 2).

3.1.4 *Effect of temperature on disease expression*

Inoculated TSA plants incubated at 18 °C and 32/22 °C in both trials developed typical TMGMV-U2 symptoms while symptoms failed to develop at continuous 32 °C. At 18 °C, foliar local lesions appeared first, followed by necrosis of stems and petioles about 1 week after lesion appearance. This was followed by high-density local lesions and foliar chlorosis (Fig. 3, Top left). The plants died about 1 month after inoculation in both trials while the control plants remained healthy. Inoculated plants under the 32/22 °C regime developed systemic wilt about 1 month after inoculation but did not die (Fig. 3, top right).

Table 3. Effect of TMGMV-U2 inoculation on TSA plants in field trials in Hawthorne, Florida

Percentage of TSA plants expressing symptoms					
Field trial 1*			Field trial 2†		
% Inoculated plants	N‡	Treatment	% Inoculated plants	N	Treatment
100.0a	30	0.5 g/L infiltrated	100.0a	32	1.5 g/L infiltrated
100.0a	30	Manual inoculation	97.0 a	32	3.0 g/L infiltrated
97.0 a	30	1 g/L infiltrated	97.0 a	30	Manual inoculation
0.00 b	30	Control	0.00 b	30	Control
Percentage of mortality of TSA plants					
% Inoculated plants	N	Treatment	% Inoculated plants	N	Treatment
93.0a	30	0.5 g/L infiltrated	97.0a	32	1.5 g/L infiltrated
90.0a	30	Manual inoculation	83.0a	32	Manual inoculation
87.0a	30	1 g/L infiltrated	94.0a	30	3.0 g/L infiltrated
0.00b	30	Control	0.00b	30	Control

Note: The results were recorded 6 weeks after inoculation.
*Based on Tukey's HSD test, ($P > 0.0001$).
† Based on Duncan's multiple range test, ($P > 0.0001$).
‡ Number of plants per treatment.

Figure 2. A healthy control (left) and a TMGMV-U2-infected TSA (right) plants and their respective split stems illustrating the state of wilting and discoloration of the vasculature 25 days after inoculation.

Plants incubated at continuous 32 °C in both trials were asymptomatic up to 17 days after inoculation, and no virus was serologically detected in the newly developed leaves (data not shown). When these plants were removed from 32 °C after 17 days and placed in a greenhouse at 25 ± 3 °C, they developed systemic, atypical, nonlethal symptoms suggesting an attenuated systemic hypersensitive response 5 or 6 days later (Fig. 3, bottom left). Several leaves abscised and older leaves still on the stems eventually died. Newly expanded leaves developed epinasty, yellowish green mosaic, stunting, and necrotic flecking. The stem and branches gradually became lignified and woody throughout (Fig. 3, bottom right). Fruiting on these plants was limited or

Table 4. TMGMV-U2 titers in inoculated TSA and Samsun nn tobacco in milligrams per gram of host tissues

TSA tissues: Study 1

		Milligrams of virus per gram of tissue						
	Harvest day	Leaf position[†]					Stem	Root
Time		1	2	3	4	5		
0	Day 0, control	0	0	0	0	NS[‡]	0	0
1	3 DAI*	0.03	0.03	0	0	NS	0	0.01
2	10 DAI	0.04	0.06	0	0	0.06	0	0.013
3	17 DAI	0.07	0.05	0	0.06	0.04	0.02	0.049
4	21 DAI	0.05	0.08	0.01	0.02	0.132	0.119	0.539
5	28 DAI	0.07	0.10	0.04	0.06	0.171	0.16	0.485
6	31 DAI	0.06	0.09	0.04	0.06	0.04	0.219	0.17
Mean titer, Times 1–6		0.05	0.07	0.02	0.03	0.09	0.09	0.21
Mean titer in leaves in mg (positions 1–5)		0.08						
Mean titer in leaves, stem, and roots in mg		0.05						

TSA tissues: Study 2

Time		1	2	3	4	5		
0	Day 0, control	0	0	0	0	NS[†]	0	0
1	3 DAI*	0	0	0	0	NS	0.03	0
2	10 DAI	0.07	0	0	0.09	NS	0.248	0.40
3	17 DAI	0.08	0.248	0.02	0.101	0.115	0.263	0.53
4	21 DAI	0.04	0.147	0.02	0.08	0.02	0.177	0.51
5	28 DAI	0.08	0.105	0.02	0.179	0.04	0.236	0.36
6	31 DAI	NS	0.251	NS	0.188	0.103	0.455	0.1
Mean titer, Times 1–6		0.05	0.13	0.01	0.09	0.07	0.24	0.32
Mean titer in leaves in mg (positions 1–5)		0.07						
Mean titer in leaves, stem, and roots in mg		0.10						

	Milligrams of TMGMV-U2 per g of Samsun nn tobacco leaves											
Production Run	1–3	4	5	6	7	8	9	10	11	12	13	Mean
Mg/g virus	2.40	2.25	2.20	2.12	1.83	0.60	1.63	1.56	0.80	1.22	1.60	1.52

*DAI = days after inoculation.
[†] Leaf position as described in Materials and Methods.
[‡] NS = not sampled.

Figure 3. Effect of three temperature regimes on TMGMV-U2 disease expression in TSA plants. Top left: High-density local lesions and chlorosis on inoculated leaves of a plant incubated at continuous 18 °C. Top right: Wilting of a plant incubated at 32/22 °C day/night temperatures. Bottom left: An attenuated systemic hypersensitive response approximately 30 days after removal of an inoculated plant from continuous 32 °C and further incubation at 25 ± 3 °C. Bottom right: Systemic lignification of a plant at continuous 32 °C, 7 months after inoculation.

nonexistent and fruits that developed gradually necrosed. The plants died in 6 to 12 months after removal from 32 °C. These results were consistent in both trials.

3.1.5 *Plant host range*

Results from the experimental host range of TMGMV-U2 from at least two trials are summarized in Table 5 and the complete data are presented in a sortable Excel file in the Supporting Information. Among all plants tested, more than half (58.2%) were immune to TMGMV-U2, 11.0% were resistant, and 8.5% were susceptible but asymptomatic. Of the remaining, 14.7% were systemically susceptible and 7.6% were highly susceptible. When only plants outside the Solanaceae (total 244) were considered, 87.3% were immune, 3.7% were resistant, and 6.2% were asymptomatic. Only 2.9% of the plants tested (seven species, Table 5 footnote) were susceptible and they developed only nonlethal, mild, mosaic symptoms.

Only plants belonging to the Solanaceae developed severe symptoms of partial or complete systemic necrosis and died although not all from SHN. Of the 191 plants in the Solanaceae, 20.9% were immune, 20.4% resistant, 11.5% asymptomatic, and 47.1% susceptible and symptomatic. Of the latter, the majority (66.0%) developed mild systemic mosaic symptoms, while 34.0% developed severe symptoms including partial or complete systemic necrosis (Table 5). Among the latter plants were *Capsicum* spp., *Nicotiana* spp., *Physalis pubescens* (groundcherry), and *S, viarum* (TSA).

Some *Capsicum* spp. are susceptible to TMGMV.[6] Of the 38 peppers we tested, *C. pubescens* 'Rocoto' was immune and *C. annuum* 'Pasilla Bajio' and 'Prairie Fire' and *C. frutescens* 'Tabasco' were resistant. The reaction of *C. baccatum* was inconclusive, while 14 others, including 13 *C. annuum* cultivars and one of *C. chinense* were susceptible with mild mosaic symptoms. Seventeen cultivars of *C. annuum* and two of *C. chinense* were highly susceptible and killed partially or fully from TMGMV-U2 infection. An example of severe host response in a *C. annuum* cultivar is illustrated in Fig. 4.

Of the 38 tobaccos (*Nicotiana* spp.) tested including eight species and two experimental hybrids, none was immune, 19 were resistant, six were susceptible, one was susceptible but asymptomatic, and 12 were highly susceptible with partial or complete plant death. Of the plants that flowered while under observation, two, *N. tabacum* Samsun nn (Horrell JR, unpublished) and *Nicotiana* x *sanderae* cv. 'Avalon' developed color break from TMGMV-U2 infection.[33] Six cultivars of eggplant (*Solanum melongena*) were asymptomatic but ELISA-positive in at least one of two trials while six other cultivars were resistant and ELISA-negative in both trials. Two Petunias, *Petunia integrifolia* and *Petunia* x

Table 5. Response of plant species and cultivars screened against TMGMV-U2: Summary by host response

Host response category	Plants in this category	
	Number	Percentage
All plants		
Immune, no symptoms, no infection (ELISA negative)	253	58.2
Asymptomatic, ELISA positive	37	8.5
Resistant, hypersensitive local lesions in inoculated leaves	48	11.0
Susceptible (Systemic mosaic)	64	14.7
Highly susceptible, partial or complete systemic necrosis (severe damage or death)*	33	7.6
Total number of plants screened	435	100
Solanaceous plants		
Immune	40	20.9
Asymptomatic	22	11.5
Resistant	39	20.4
Susceptible	57	29.8
Highly susceptible	33	17.3
Total solanaceous plants screened	191	100
Non-solanaceous plants		
Immune	213	87.3
Asymptomatic	15	6.2
Resistant	9	3.7
Susceptible†	7	2.9
Highly susceptible	0	0.0
Total non-solanaceous plants screened	244	100

*Included seven species and one hybrid in four genera, all in Solanaceae (*Solanum viarum*, *Capsicum* spp., *Nicotiana* spp., and *Physalis pubescens*).
† Included are four species previously reported to be susceptible to TMGMV: *Echium plantagineum* (Boraginaceae),[30] *Eryngium planum* (Apiaceae),[31] *Tradescantia spathacea* and *Tradescantia zebrina* (Commelinaceae),[32] and three previously unreported hosts: *Gomphrena globosa* (Amaranthaceae), *Eryngium dorae* (Apiaceae), and *Richardia scabra* (Rubiaceae).

hybrida, were also susceptible with mosaic symptoms and were ELISA-positive.

Among the 10 *Eryngium* species (Apiaceae) screened, *E. dorae* and *E. planum* were susceptible to TMGMV-U2, *E. baldwinii* was asymptomatic and ELISA-positive in one of two trials, and the others were immune (Excel file in Supporting Information). Systemic mosaic was seen in *E. planum* and *E. dorae*; *E. baldwini* leaves were too small to observe symptoms but were ELISA positive and infective when back-inoculated. Of the other Apiaceae plants tested, *Coriandrum sativum* (coriander or cilantro) and *Daucus carota* (carrot) were asymptomatic but ELISA positive while four other species were immune and ELISA-negative (Excel file in Supporting Information).

Among the T&E species surveyed from state and federal listings, *Solanum donianum* occurs in Florida[26] but it was resistant and ELISA-negative in our tests. No other T&E species belonging to Solanaceae was found to occur in states where TSA was prevalent when the registration process was ongoing. Also, there were no published reports of susceptibility to TMGMV among the 713 Federal T&E plant species we reviewed.

There was no variability in host response to TMGMV-U2 inoculation among the 24 TSA accessions from Florida and Mississippi, USA or the 12 from New South Wales, Australia. All developed local lesions in 1–2 weeks and wilted and died by 38–40 days after inoculation whereas the non-inoculated control plants remained healthy. The results were consistent in two trials (Excel file in Supporting Information).

Excluding species of *Nicotiana*, many of which have been reported to be susceptible to TMGMV, we report here the following 29 species as new host records for TMGMV-U2: *Gomphrena globosa* (Amaranthaceae), *Eryngium dorae*, (Apiaceae), *Richardia scabra* (Rubiaceae), and *Browallia americana, Capsicum chinense, Physalis alkekengi, P. angulata, P. philadelphica* (Syn *P. ixocarpa*), *P. pubescens, P. walteri, Solanum acerifolium, S. aculeatissimum, S. aethiopicum, S. americanum, S. bahamense, S. capsicoides, S. caripense, S. diphyllum, S. jamaicense, S. mauritianum, S. nigrum, S. physalifolium, S. ptychanthum, S. rostratum, S. sarrachoides, S. sisymbriifolium, S. spinosissimum, S. suaveolens*, and *S. villosum* (Solanaceae). All developed foliar mosaic symptoms and tested ELISA positive in at least one of two trials.

3.2 TMGMV-U2 survival/persistence in dead TSA tissue, soil, water, and storage

3.2.1 Persistence in dead TSA tissue and potted soil

In both trials, all TSA plants inoculated with TMGMV-U2 died. In Trial 1, no local lesions were observed on Samsun NN plants inoculated with extract from soil collected prior to inoculation of TSA (the control soil). However, local lesions formed on all 12 (100%) of the Samsun NN plants inoculated with extracts from soil

Figure 4. Partial systemic necrosis in *C. annuum* cultivar 'Wizard' inoculated with TMGMV-U2 (left) compared to the total systemic hypersensitive necrosis (SHN) in TSA (right).

Figure 5. Left: TMGMV-inoculated (dead, plots with brown plants) and noninoculated, control (healthy, plots with green plants) TSA plants in adjacent field plots. Plants in the inoculated plots were 100% dead while there were no diseased (wilted or dead) plants in the control plots. Right: The same plots as in the picture on the left (but photographed from the opposite direction) planted with Samsun nn tobacco (in the front) and *C. annuum* cultivar 'Wizard' (in the back, behind tobacco) after the TSA plants were tilled into the soil. These plants remained symptom-free despite the presumptive presence of residual TMGMV-U2 inoculum in the soil.

collected 1 month after the inoculated TSA plants had died. Local lesions were observed on two of the 12 (16.7%) Samsun NN plants inoculated with extracts from soil collected 6 months after inoculation of the TSA plants. No local lesions were observed on any of the 12 Samsun NN plants inoculated with extracts from dead TSA stem tissue collected 6 months after inoculation. No lesions developed on Samsun NN plants inoculated with extracts from the non-inoculated control TSA plant samples collected 6 months after inoculation. No symptoms were seen on Samsun nn plants (Trial 1), or *C. annuum* 'Enterprise' (susceptible) plants (Trial 2) grown in the same potted soil in which TMGMV-U2-infected TSA plants had grown and died.

3.2.2 *Persistence in field soil and spread at a research farm site*

In both trials, all TMGMV-U2-inoculated TSA plants died. No symptoms were observed in Samsun nn or pepper cultivars planted a day after the plots were mowed and grown for 5 months. Likewise, no symptoms were observed in Samsun nn or pepper cultivars planted 6 months after tilling and grown for 5 months. Soil samples tested by ELISA were negative for the virus. Likewise, no systemic mosaic was observed in the Samsun nn tobacco plants inoculated with the soil extracts.

Considering absorbance values 3× the average as positive for TMGMV-U2 presence in the samples, no virus was detected in peppers (Wizard or Camelot) or Samsun nn in either study (Table S2). Tissue samples of Samsun nn and pepper cultivars tested by ELISA were negative for the virus. In both trials, the control TSA plants remained disease-free proving the lack of spread of TMGMV-U2 to these plants despite their proximity to infected TSA plants and the large window of time during which biotic and abiotic factors could have caused the virus to spread (Fig. 5, picture on the left).

3.2.3 *Disease incidence in a susceptible pepper grown in TMGMV-U2-amended soil*

Neither the plant nor the fruit exhibited symptoms of TMGMV-U2 infection. The ELISA absorbance values for control leaf and root samples were virtually the same as those for plants exposed to the different virus concentrations (Table S3), confirming the lack of virus infection.

3.2.4 *Disease incidence in a sentinel pepper crop grown in the TMGMV-U2 production block*

The pepper crop grown in the sentinel block had higher yield at the first harvest and in both harvests combined compared to the control block (Table 6). None of the pepper plants in the sentinel block developed TMGMV-U2 symptoms. Likewise, none of the pepper plants in the control block were symptomatic. Pepper tissue samples were also negative for TMGMV-U2 in ELISA and back-inoculations. In Harvest 1, the total yield of Grade 1 pepper from the sentinel block was higher than from the control block whereas Grade 2 and Cull yields were lower in the former compared to the latter block. Yield by Grade in Harvest 2 followed the pattern in 1 but the total yield from Harvest 2 was lower compared to the control. The total yield from the combined 1 and 2 was higher from the sentinel block than from the control block, which was attributed to the weed pressure in the latter. Thus, even if TMGMV-U2 were present in the soil it did not affect pepper growth or yield, and no diseased pepper plants were observed in the sentinel or control plots.

3.2.5 *Stability of TMGMV-U2 at three storage temperatures and in three water samples*

TMGMV-U2 TGAI stored frozen at −20 °C or refrigerated at 5 °C remained infective for at least 1 year while the infectivity was lost after 1 month at room temperature. Our unpublished observations to date indicate that the freezer-stored virus has remained infective for 23 years and for at least 1 year in the refrigerator with no loss of activity (Charudattan, unpublished).

After 1 year, the number of local lesions caused by the water-stored virus ranged from 1 (stored in lake water) to more than 168 (stored in sterile deionized water). Lesion numbers ranged from 0 to 38 (tap water), 0 to 2 (lake water), and 1 to 168 (sterile deionized water) kept at 25 ± 2 °C (Table S4). Microbial activity was considered the probable cause of infectivity loss when stored in the eutrophic lake water.

3.3 Molecular characterization of the registered TMGMV-U2 isolate

Genomic sequence analysis and a comparison of the bioherbicide isolate of TMGMV-U2 (GenBank-GB EF469769)[34] indicated 99% identity with a published TMGMV sequence (GB M34077) by Solis and Garcia-Arenal.[35] The two genotypes of TMGMV described in

Table 6. Pepper yield from a field block previously cropped with TMGMV-U2-infected tobacco and a control block not previously cropped with tobacco

Harvest No.	Production block*			Control block		
	Yield by pepper grades[†,‡]					
	1	2	Cull	1	2	Cull
1	1170	266	155	716	344	242
Total yield 1			1591		1301	
2	116	64	41	100	118	69
Total yield 2			222		287	
Total of 1 and 2			1813		1588	

*A field block in which two TMGMV-U2-infected Samsun nn tobacco crops had been previously grown (details in Materials and Methods).
[†] Pepper grading based on the United States Standards for Grades of Sweet Pepper, USDA Agric. Marketing Serv., Fruit and Vegetable Programs, Fresh Produce Branch, Nov. 17, 2005.
[‡] Yield in kilograms.

California[8,9] are distinguished by one (TMGMV-U5) being associated with a satellite TMV (STMV) and by the presence of repeated sequences in the 3′ untranslated region (UTR). Amplification of the 3′ coding region for our TMGMV-U2 bioherbicide isolate and that for a TMGMV-U5 resulted in products of different sizes when compared in a gel analysis (Fig. 6). The product for TMGMV-U5 was larger due to repeated pseudoknot sequences in its UTR. These results indicated that our bioherbicide isolate is a TMGMV-U2 and is distinct from TMGMV-U5 isolates.[9]

In an immunodiffusion test, Samsun nn leaf extracts containing our bioherbicide TMGMV-U2 isolate as antigen did not cross-react with antiserum to STMV (Fig. 7). Additional support that our isolate did not support STMV was indicated by double-stranded RNA extractions from infected virus tissues followed by gel analyses. The gel analysis of TMGMV-U2 did not show the double stranded RNA associated with STMV replication (Fig. 8).

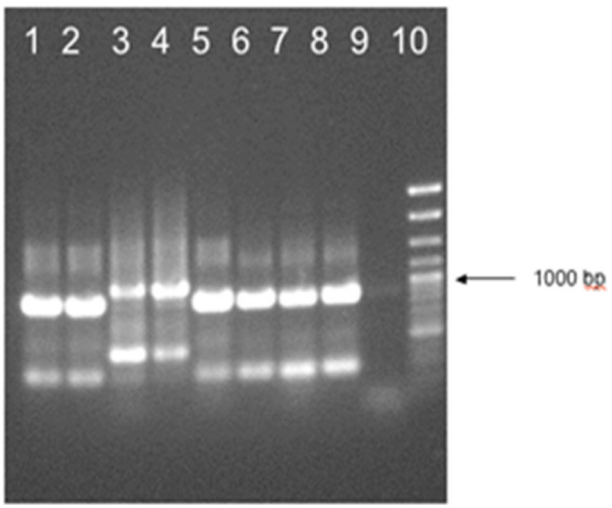

Figure 6. PCR probe analysis of TMGMV based on 3′ end untranslated region for variability in TMGMV-U5 *vs*. TMGMV-U2. Lanes 1 and 2: TGAI of the TMGMV-U2 bioherbicide isolate at 1 and 1/10th dilutions with its characteristic 'small-type' 3′ end (UTR). Lanes 3 and 4: a representative culture of TMGMV-U5 (the 'large type') from infected Samsun nn leaves at 1 and 1/10th dilutions. Lanes 5 and 6: a reference TMGMV-U2 culture of Zettler and Nagel[11,12] from infected *Eryngium planum* tissue at 1 and 1/10th dilutions. Lanes 7 and 8: the TMGMV-U2 bioherbicide isolate from infected TSA at 1 and 1/10th dilutions. Lanes 9: Blank. Lane 10: 100 bp ladder size marker. Primers used for PCR: #21 3′end rev, #145479–5488 forward. Expected PCR product size approximately 900 bp. 0.8% agarose gel. TAE buffer.

4 DISCUSSION

Unlike a classical biological control agent such as an exotic herbivorous insect that may provide a reduction in plant population density over time, the TMGMV-U2-containing bioherbicide that we have developed and commercialized, named SolviNix LC, can be used to kill and eradicate TSA infestations within a few weeks after application. As a bioherbicide, TMGMV-U2 is also an alternative to chemical herbicides for TSA management. The devastating SHN elicited by TMGMV-U2 is comparable to the effect of chemical herbicides registered for TSA control. The schematic in Fig. 9 describes the mode of action of TMGMV-U2 interaction with TSA leading to SHN of the host plant.

Figure 7. Lack of STMV in the bioherbicide isolate of TMGMV-U2 was confirmed from the absence of its cross-reaction with anti-STMV antiserum. Well A: 60 µL of undiluted anti-STMV antiserum. Outer wells contained infected Samsun nn tobacco leaf extracts as antigens. Wells 1 and 2: TMGMV-U5 + STMV; 3 and 4: the bioherbicide TMGMV-U2 isolate; and 5 and 6: uninfected tobacco extract.

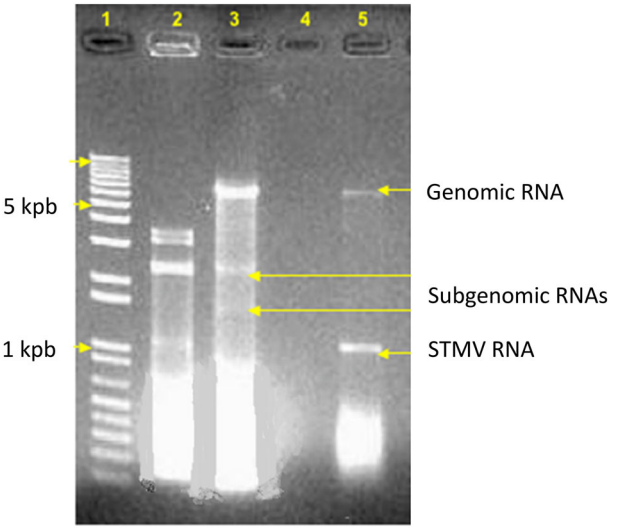

Figure 8. Lack of a satellite TMV (STMV) in the bioherbicide isolate TMGMV-U2 by gel analysis of double-stranded RNA extracted from infected plants. Lanes 1: 1 kB Invitrogen plus ladder molecular weight marker; 2: cucumber mosaic virus RNA; 3: TMGMV-U2 RNA; 4: blank; 5: RNA from a TMGMV-U5 + STMV isolate. In this picture, the gel's leading edge was digitally edited to demarcate lanes 2, 3 and 5.

Although TMGMV-U2 elicited partial or complete plant death in several cultivars of *Nicotiana* spp., *Capsicum* spp., *and Physalis pubescence*, the TSA-TMGMV-U2 host-virus interaction is unique in that the virus first elicits a hypersensitive local response that is followed by the systemic hypersensitive host death that is consistent and predictable. A possible mechanism for this host-virus interaction is that TMGMV-U2 encodes an effector that is recognized by a resistance gene product in the host.[36] The recognition of the effector triggers a hypersensitive response (HR) that fails to contain the replicating virus to the local lesions at the infection sites but the response remains turned on as the virus moves through the plant. Unlike TMGMV-U2, TMV and ToMV did not trigger host resistance in TSA but instead caused systemic susceptibility (systemic mosaic). Hence, the TSA-TMGMV-U2 interaction reported in this paper has important significance to plant virology, plant molecular biology, and weed science as a model system to study virus-elicited systemic necrosis.

In the field, 85% to 100% TSA kill occurred when plants of uniform age and height were treated. When there was less than 100% kill (as shown in Table 3), it was generally from a failure of the inoculation. The infected plants invariably died, and the missed plants became diseased and died upon reinoculation. However, if the host physiology and metabolism are less than normal, the virus will not kill inoculated TSA plants. In our experience, nighttime temperatures of ≥10 °C, plants growing in a waterlogged condition, or old-growth plants from the previous year may not develop typical disease progression and die. As our data and discussions in Charudattan *et al.*[17] reveal, it was not easy or possible to obtain 100% TSA kill in large infestations containing hundreds or thousands of plants of different ages and heights. The application methods and tools usually missed smaller plants under larger canopies. Also, we found many examples in the field where the virus did not spread from infected to healthy TSA plants even when they were in physical contact.

The movement of TMGMV-U2 through the TSA plant followed the well-known model of virus movement in plants.[29] TMGMV-U2 moved from the point of infection, the inoculated terminal leaves, to the stem and then to the roots. From there it moved up to the actively growing points of the plant including the apical and axillary meristems. Throughout this progression, the virus was detected progressively in all parts of the plant. As it moved through the stem within days after infection, it destroyed the phloem tissue along the way causing the plant to wilt and die.

The TMGMV-U2 titer in inoculated TSA plants was low with an average of 0.08 mg per g in two trials. The titers in TSA leaf tissue were much lower compared to the roots and stem; the former averaged 0.08 mg per g from two trials and the latter 0.165 mg and 0.255 mg, respectively. The higher values observed for the dropped leaves, position #5, were due to the increase in the ratio of the virus to leaf weight caused by desiccation. Equal weights of tissue were processed for ELISA regardless of water content, resulting in higher values for the dropped leaves. In contrast to TSA, the titer in TMGMV-U2-infected Samsun nn leaf tissue was much higher, averaging 1.52 mg per g or nearly 20 times higher than in the TSA leaves. The fact that the virus does not reach high titers in TSA, coupled with the finding that the infected TSA plants invariably die, imply a lack of environmental risk from the use of TMGMV-U2 as a bioherbicide. The low titer assures that there will be negligible soil residue from infected TSA. This, and the fact that the virus does not persist for long periods in dead tissue or water, suggests there will be minimal, if any, virus residues in the environment from the bioherbicide's use. These were important findings that helped to get the virus registered as a bioherbicide.

The temperature effects study indicated that the virus will be effective under the subtropical temperatures of Florida and the Southeastern United States during TSA's growing season. Since registration and public use of the bioherbicide, we have found no instances of temperature-related loss of efficacy of TMGMV-U2.

We have reported here the most comprehensive data to date on the host range of TMGMV from our screening of 435 plant species including several cultivars of important crop plants, species in Solanaceae, weeds, and native plants. The testing was done under stringent conditions using the bioherbicide isolate and was repeated. The test plants were grown in a greenhouse and inoculated manually with 100 to 300 µg/mL of purified TMGMV-U2 a level that is much higher than the labelled rate of 10 µg per mL for the bioherbicide application. Therefore, the plants screened in the host range studies were subjected to a severe and acute exposure to the virus, unlike under natural field conditions.

Our results corroborate previous findings[6,7,25] that TMGMV is a pathogen adapted to the Solanaceae, as shown by the fact that 91% of the non-solanaceous plants tested were immune or resistant to TMGMV-U2. Of the highly susceptible plants, only peppers (*Capsicum* spp.) and tobacco (*N. tabacum*) have economic importance in TSA's range in the southeastern United States. While there were a few native Solanaceae species susceptible to TMGMV-U2, they are unlikely to be present at TSA-infested sites.

The lack of variation in the lethal susceptibility of TSA plants from the United States and Australia may be indicative of genetic homogeneity of this species (*S. viarum*). Alternatively, the same susceptible genotype may have been introduced into both countries. Although there is no clear evidence to pinpoint the origin of these TSA introductions, a limited genetic analysis of TSA accessions from the United States and Brazil by Kreiser *et al.*[37] suggests Brazil as the possible source of the U.S. plants. From the lack of genetic variation in the U.S. populations examined, a single introduction into the United States is plausible. The Australian TSA populations may be of the same genotype as in the United States.

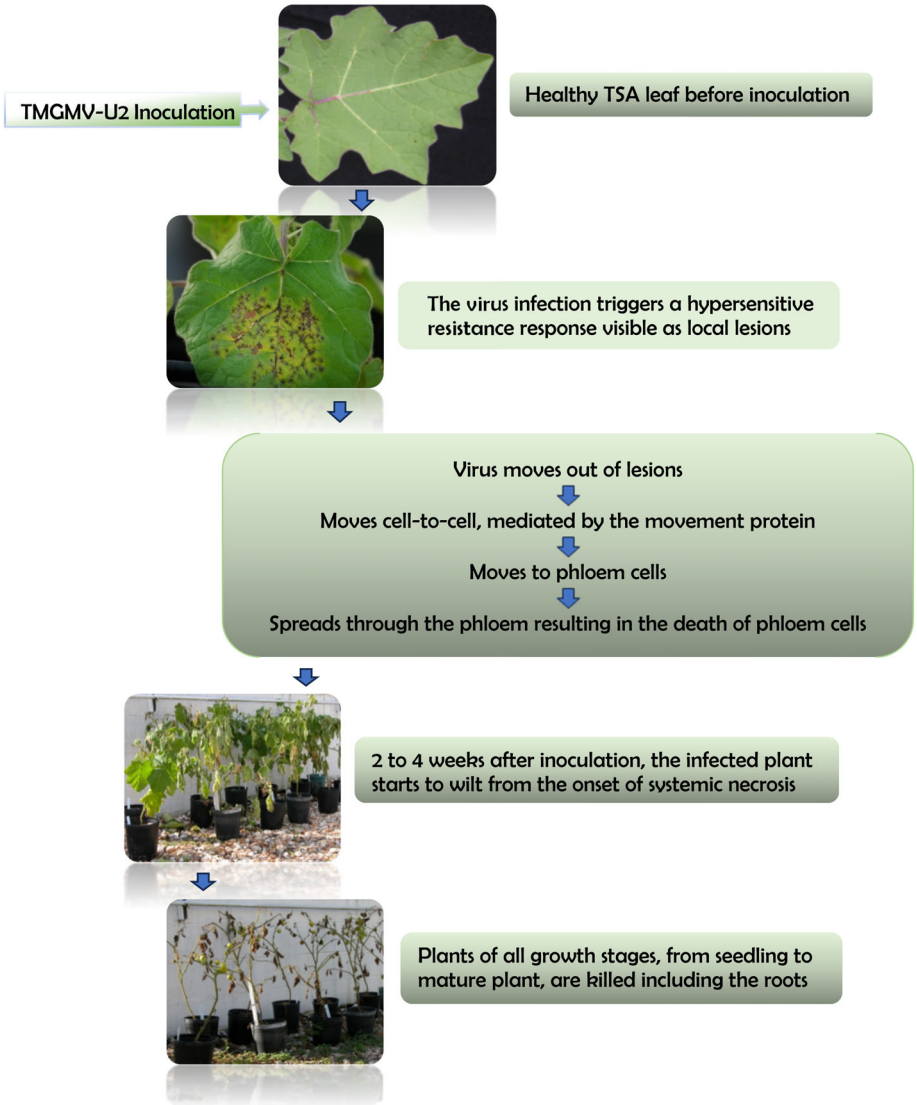

Figure 9. Mode of action: Sequence of TSA-TMGMV-U2 interaction resulting in plant death.

We are reporting here 29 new host records for TMGMV-U2. These host plants belong to four families, Amaranthaceae, Apiaceae, and Rubiaceae (one species in each), and Solanaceae (25 species). Recently, Surfinia trailing petunia (*Calibrachoa* or *Petunia* (Solanaceae),[38,39] *Osteospermum* (Asteraceae),[40] and *Tabernamontana* (Apocynaceae),[41] which are ornamental plants propagated in nurseries, have been reported as new hosts of TMGMV. Chai *et al.*[42] have reported finding TMGMV in eggplant in China from mixed infections with tomato mottle mosaic virus (Tobamovirus). In our studies, six of 12 cultivars of eggplant were asymptomatic but ELISA positive and another six resistant and ELISA negative. Alishiri *et al.*[43] have reported TMGMV infecting tomato in Iran. In our tests, all 13 cultivars of tomato were immune. According to Wetter[6] there was 'no infection' in tomato. Ishibashi *et al.*,[44] have reported that tomato is immune to TMGMV due to prevention of intracellular virus multiplication by the host tm-1 protein that binds to TMGMV replication proteins and inhibits RNA replication. In our registration application, we argued that tomato, a major crop in the United States, is immune and therefore will not be at risk from the TMGMV-U2-based bioherbicide.

Our results confirmed that TMGMV-U2 is likely to persist for a few months in dead roots, soil containing dead roots, and fallen leaves of TSA. It was present in the roots of infected TSA plants but was not detected in the soil in which infected TSA plants had grown. The length of time TMGMV-U2 remains infective in the soil is unknown. However, the chance of it spreading from infected roots appears to be small as indicated by the lack of symptom development in tobacco and pepper plants grown in soil in which TMGMV-U2-infected TSA had previously grown or when grown in TMGMV-U2-amended soil.

The lack of symptom development in the sentinel pepper plants or the fruit suggests that it is possible to grow a susceptible plant in soil containing TMGMV-U2 and that the presence of the virus in soil will not necessarily pose a risk to any nontarget susceptible hosts. Our results provided strong evidence against the risk of spreading TMGMV-U2 from the soil. Given the size of the TMGMV-infected production crops of tobacco (total 0.88 ha), the residual TMGMV-U2 in soil in the production plot could be construed to be significant enough to pose a risk to a subsequent susceptible crop, but this was not the case in our study. Also,

despite the increased chances for infection from using transplanted seedlings, there was no incidence of TMGMV-U2 disease in the sentinel pepper crop. There was also no evidence of asymptomatic infection, as determined by ELISA and back-inoculations.

Since satellite virus, STMV, can attenuate or otherwise modify the expression of the disease phenotype, at least in certain hosts,[14,15] we examined our bioherbicide isolate by using double-stranded RNA and serological analyses using anti-STMV serum. The results confirmed that the bioherbicide isolate did not contain a satellite virus. Molecular evidence also confirmed this isolate to be the U2 strain. Its genomic sequence was essentially identical to the sequences from Letschert *et al.*[28] and Solis and Garcia-Arenal.[35] Therefore, the bioherbicide isolate is not unique; other TMGMV-U2 isolates should have the same property of eliciting SHN in TSA but it could be worthwhile to screen several isolates from culture collections to determine possible differences in their host range.

5 CONCLUSIONS

- TMGMV-U2 was detected in a field soil studied for up to 6 months but is not likely to pose environmental risks from its use as a bioherbicide.
- Susceptible tobacco and peppers grown in soils that had TMGMV-U2-infected TSA were uninfected.
- Pepper plants raised from seeds in soil containing as much as 5 mg of TMGMV-U2 per 4-L-volume of soil did not become infected.
- By extension, when used as a bioherbicide, TMGMV-U2 is unlikely to pose a risk from soil-borne inoculum.
- TMGMV-U2 does not spread readily in the field and the risk of its persistence and spread are none or negligible.
- The potential for infecting susceptible plants from the bioherbicide use of the virus is remote.
- Cultivars of peppers and tobacco are at potential risk from TMGMV-U2, but the risk can be mitigated or prevented by prohibiting the bioherbicide's use near or in crops.
- The genomic sequence of the bioherbicide isolate is essentially identical to two published TMGMV sequences.
- TMGMV-U2 can be used safely and effectively as a bioherbicide to control TSA.

ACKNOWLEDGEMENTS

This paper is a companion to the review by the senior author that appears in this journal issue. Together the papers are based on an oral presentation given at the workshop on 'Overcoming the barriers to adoption of microbial bioherbicides,' in Bari, Italy on 26–28 September 2022. The senior author thanks The Organisation for Economic Co-operation and Development (OECD), Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems, the sponsor of the workshop, for the invitation and financial support that made it possible for him to participate in the workshop.

Reported in this paper are the discovery and proof-of-concept research done at the University of Florida, Department of Plant Pathology, Biological Control of Weeds Laboratory of R. Charudattan and the subsequent commercial development and registration of the virus through his company, BioProdex, Inc. The EPA registration application was helped and guided by Dr. Michael Braverman, Head, IR-4 Biopesticide and Organic Support Program, Princeton, NJ, who was key to the registration success and thus to the success of this bioherbicide development. Emeritus Professor Dr. F. W Zettler provided the isolates of TMGMV-U2, TMV, and ToMV and their antisera used in this research. Ms. Deborah M. Matthews, University of California, Riverside kindly provided anti-STMV antiserum.

We thank the University of Florida-IFAS Plant Science Research and Education Unit for the use of their fields and a greenhouse, the USDA-APHIS-PPQ for permitting the importation of the Australian TSA seeds, Chris Clausen, Darrin Heron, Peter James, Reece Luxton, and Ron Steen of Clarence Valley Council for collecting the Australian TSA seeds, and Russell Warner, S.J. Hampton and S.C. Navie from Technigro Pty. Ltd. Australia for organizing the seed collection and shipment.

The following provided research funding: University of Florida, College of Agricultural & Life Sciences; State of Florida Department of Agriculture and Consumer Services; USDA-NIFA-Small Business Innovation Research Grant (Award No. 2005-33610-15548), and an anonymous Angel Investor.

The opinions and arguments expressed in this paper are the sole responsibility of the senior author and do not reflect those of the OECD or of the governments of its member countries.

CONFLICT OF INTEREST

The authors as a team researched and gathered data to register the TMGMV-U2 as a bioherbicide. The principal author led the effort. His company, BioProdex, Inc., licensed this technology from the University of Florida Research Foundation, a minority shareholder in BioProdex, to develop and commercialize the bioherbicide. The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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