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A Strain of Japanese holly fern mottle virus Infecting Leatherleaf Fern in the United States

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Abstract

Leatherleaf fern, a popular cut foliage plant for use in flower arrangements, was found showing virus-like symptoms at a local nursery in Baton Rouge, LA. The causal agent of the disease was determined to be a strain of Japanese holly fern mottle virus. The virus was identified by dsRNA, RT-PCR, and sequence analyses. The potential negative impact of this pathogen on leatherleaf fern production and its spread within the crop should be a concern for leatherleaf fern growers.

Leatherleaf fern (LLF, *Rumohra adiantiformis*) is native to sub-tropical and tropical climates and it is widely used for ornamental purposes, mainly as cut foliage for use in flower arrangements. It is grown commercially as a cash crop in the United States, Central America, and tropical South America. In 2005, the value at whole sales of LLF in the United States was 50.7 million dollars (5). Leatherleaf fern production is threatened by various factors, including plant pathogens. Anthracnose disease, caused by the fungus *Colletotrichum acutatum*, negatively affects the quality of fronds and makes them unmarketable (11). Plant parasitic nematodes have also been reported to affect LLF in Florida (7). Recently, Kloepper et al. (8) reported the association of endophytic fluorescent pseudomonads with fern distortion syndrome in Costa Rica. However, virus problems have not been reported with LLF production.

Very little research has been conducted on viral diseases affecting wild or cultivated ferns. Plant viruses have been reported to infect various fern species (3,4,9,10,13); however, only *Cucumber mosaic virus* (9) and a novel virus causing a disease of Japanese holly fern (*Cyrtomium falcatum*) (13) have been conclusively identified and characterized. Japanese holly fern mottle virus (JHFMoV) is a novel RNA virus widespread throughout the Southern United States and California. Symptoms caused by JHFMoV on Japanese holly fern (JHF), the only known natural host of this virus, consist of stunting, foliar necrosis, mosaic, and yellow mottling. In the past few years, there has been an increase in the incidence of this virus in some of these regions (13).

Potted LLF plants showing virus-like symptoms were observed at a local nursery in Baton Rouge, LA. Symptomatic and non-symptomatic plants were purchased and tested for RNA virus infection by double-stranded RNA (dsRNA) purification from foliar tissues followed by electrophoretic analysis in 5% polyacrylamide gels. dsRNA banding patterns obtained were similar to those reported for JHFMoV (12). Non-symptomatic plants did not yield dsRNAs. Due to the economic importance of LLF and the possibility that the virus was related to JHFMoV we decided to include the characterization of the virus affecting LLF in our research.

The primary material for this research consisted of eight symptomatic and four non-symptomatic plants purchased at the original nursery. One of the symptomatic plants was vegetatively propagated and used as source of material for graft transmission experiments, dsRNA extraction, molecular cloning, and virus purification. The other diseased LLF plants, together with the four healthy plants, were used for reverse-transcription polymerase chain reaction (RT-PCR) detection studies. A JHF plant infected with a JHFMoV-JM isolate (13) was used for comparative studies. dsRNA of additional JHFMoV isolates (DI from Mississippi and CA from California) were used in dsRNA analyses comparisons. Healthy JHF and LLF plants used in all experiments were kindly provided by Santa Rosa Tropicals Inc. (Santa Rosa, CA). These plants were symptomless and tested negative for the presence of dsRNAs.

Symptoms on LLF consisted of foliar necrosis in the form of oak leaf patterns (Fig. 1A) and yellow mottle (Fig. 1B and 1C). The necrosis was more prominent in the older leaves, and in all cases, the rest of the foliage was symptomless or exhibited only mottle or mild yellow mottle (Fig. 2).

Fig. 1. Leatherleaf fern showing foliar necrosis in the form of oakleaf patterns (**A**) and yellow mottle symptoms (**B**) and (**C**).

Fig. 2. Leatherleaf fern leaves from virus infected plants showing various degrees of yellow mottle symptoms.

Graft inoculations were conducted using unfolded leaves (scions) from symptomatic LLF and JHF plants (infected with JHFMoV-JM isolate). Scions from infected plants were grafted on the petioles of partially unfolded leaves of healthy LLF and JHF plants used as rootstocks (Fig. 3). Grafts were conducted on six plants in the case of LLF/LLF and JHF/JHF scion/graft combination and on four plants in the case of LLF/JHF and JHF/LLF scion/graft combination. Grafted plants were covered with a plastic bag and kept inside screen cages in the greenhouse. Ten days later, bags were removed and plants taken out of the cage. Grafted plants were evaluated for virus-like symptoms for a period of six weeks. Symptomatic and non-symptomatic plants were tested for dsRNA and JHFMoV-specific RT-PCR tests.

Fig. 3. Illustration of the graft inoculation of leatherleaf fern.

Successful virus transmission was obtained with four of six LLF plants grafted with LLF scions from symptomatic plants. Yellow mottle was observed six weeks after grafting, and these plants tested positive for JHFMoV infection by dsRNA and RT-PCR. Similarly, successful transmissions occurred with five of six JHF healthy plants grafted with JHFMoV infected scions. Transmissions were not obtained when four LLF were grafted with JHFMoV infected JHF scions or when four JHF plants were grafted with scions from symptomatic LLF. These results suggest that the virus strains may be host specific. However, graft compatibility could have been a factor since scions in homologous scion/rootstock combinations survived up to two weeks while scions in heterologous combinations wilted and collapsed considerably earlier.

Double-stranded RNAs were purified from 3.5-g leaf samples by phenol extraction and cellulose column chromatography (14). Purified dsRNAs were analyzed in 5% polyacrylamide gels. dsRNA analyses of the eight LLF plants showing symptoms yielded the same banding profile, which consisted of two

major bands of approximately 6.2 and 3.0 kbp, respectively, accompanied with several minor dsRNAs (Fig. 4, lane 3). The profiles obtained from all eight LLF resemble those reported for JHFMoV. Direct comparison of dsRNA profiles obtained from a symptomatic LLF and three JHF plants infected with various isolates of JHFMoV revealed only minor differences in sizes of putative subgenomic dsRNAs (Fig. 4). No detectable amounts of dsRNAs were obtained from any of the non-symptomatic LLF plants. Viral dsRNAs were readily detected in both symptomatic and non-symptomatic foliar tissues collected from infected LLF plants indicating systemic infections.

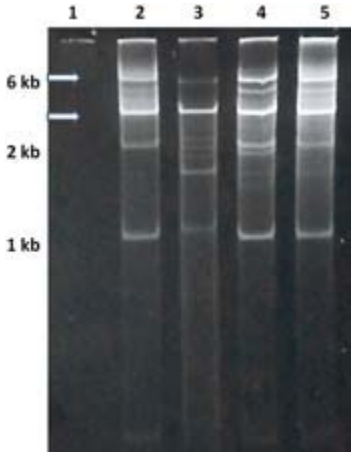

Fig. 4. Polyacrylamide gel electrophoresis (5%) of dsRNAs extracted from leatherleaf fern (LLF) and Japanese holly fern (JHF) plants. Healthy and infected LLF plants are in lanes 1 and 3, respectively. JHF plants infected with different isolates of JHFMoV are in lanes 2 (JHFMoV-JM), 4 (JHFMoV-DI) and 5 (JHFMoV-CA). Arrows point at the two major (6.2 and 3.0 kbp) dsRNA bands.

The virus was purified from symptomatic LLF leaves using alternate low- and high-speed centrifugations followed by 10% to 40% sucrose gradients as reported for purifying JHFMoV (13). Purified preparations were observed under the electron microscope and contained quasi-spherical virus particles that ranged from 30 to 40 nm in diameter, similar to the virions associated with JHFMoV (13).

The virus was detected in all eight symptomatic LLF plants by RT-PCR using a pair of primers designed from JHFMoV sequences (HFV-F: 5'-GGAGCATGATATGACTATGGT-3' and HFV-R: > 5'-GGAAAGACCGAAACATGGG-3') and procedures reported previously (13). RT-PCR products of expected size were cloned and sequenced. Obtained nucleotide and derived amino acid sequences were compared with sequences in the GenBank database using the Basic Local Alignment Tool (BLAST) (1). These analyses showed that the PCR products from several virus-infected LLF plants were uniform (98% to 100% nt identity) and shared 88% nucleotide sequence identity (97% amino acid identity) with JHFMoV-DI (GenBank Accession number NC_013133). These results definitively proved that the virus infecting LLF was an isolate of JHFMoV, which we designated as JHFMoV-LLF.

The identification of a plant virus infecting an economically important crop such as LLF is significant not only for the economic losses that the disease may cause in infected plants making them unmarketable, but also for the potential rapid spread of the virus within the crop. Although the natural vector of this virus is not known, vegetative propagation practices commonly used for LLF assure the spread of the virus if virus-infected mother plants are used.

Most newly emerged leaves from LLF plants infected with the virus did not show virus symptoms, and most mature leaves were symptomless, although the virus was detected from these symptomless leaves by RT-PCR and dsRNA analysis. As pointed out earlier, symptoms, particularly foliar necrosis, were consistently visible only on the older leaves. This particular feature of this strain of JHFMoV could make visual detection of the disease difficult and therefore allow the virus to be undetected.

The management for this potentially damaging LLF disease must involve the use of virus-free propagation materials. Virus-free fern plants can be produced by plant tissue culture from selected virus-free mother fern plants (2).

Pathogen-free ferns from selected spores through tissue culture have been reported (6). However, because JHFMoV is transmitted through spores, any selected mother plant obtained by this approach must be tested for the virus by dsRNA analysis and/or RT-PCR to ascertain the absence of the virus. These tests are extremely important because most newly emerged and upper leaves in infected LLF plants did not show obvious symptoms, making visual identification of the disease difficult and unreliable. Because symptoms were more consistently visible on the older leaves; these leaves should be the focus of visual survey of mother plants used for propagation.

Homeowners and landscape professionals are advised to purchase JHF and LLF plants that do not exhibit foliar necrosis or yellow mottle symptoms. Fern growers should test any plant showing virus-like symptoms or any plant used as source of vegetative propagation for JHFMoV and should destroy any infected plants.

Although the number of samples tested in this work was relatively small, the association of JHFMoV with all symptomatic plants and successful graft transmission of the virus followed by the symptom reproduction strongly support its involvement in the disease. In our previous study, we reported this virus from diseased JHF plants collected from different locations in Southeastern United States and California, whereas the research presented here clearly demonstrates that a strain of the same virus infects LLF.

The economic importance of JHFMoV-LLF for the leatherleaf fern industry and its geographic distribution are yet to be determined. Because of the vegetative method of propagation of LLF, it is reasonable to suspect that the virus may be widespread in fern producing regions and probably moved with infected plant material.

Note: Nucleotide sequence data generated in this work are available in the GenBank (Accession number HQ662604).

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