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A Leaf Disk Clearing and Staining Technique to Quantify Ascospores of *Mycosphaerella citri* in Young Citrus Leaves

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ABSTRACT

Quantitative information on ascospore level is very desirable for control of citrus greasy spot disease, especially in orchards with microsprinkler irrigation. We developed a novel leaf clearing and staining technique for ascospores of *Mycosphaerella citri*, using modifications of previous whole-leaf clearing and staining methods. The procedure provides more precise information on inoculum levels.

RESUMEN

La obtención de información cuantitativa sobre el nivel de ascosporas es bastante deseable para el control de la enfermedad mancha grasienta de los cítricos, especialmente en condiciones de irrigación con microaspersores. En este estudio se desarrolló una nueva técnica de aclaramiento y tinción de ascosporas de *Mycosphaerella citri* usando modificaciones de métodos previos para tinción y aclaramiento total de la hoja. El procedimiento brinda información más precisa sobre los niveles de inóculo, en comparación a las técnicas de muestreo de aire.

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Citrus greasyspot caused by the fungus *Mycosphaerella citri*, is a serious disease in citrus producing areas that have high temperatures and relative humidity (Whiteside, 1974). The fungus causes yellow mottling on the upper side and brown to black blisters on the lower side of the infected leaves. Severely affected trees often defoliate heavily causing up to 25% and 45% yield loss in sweet orange and grapefruit, respectively (Whiteside, 1982).

Fallen, decomposing leaves infected with *M. citri* produce fruiting bodies called ascocarps. Both rainfall and irrigation aid the ascocarps to rupture and release ascospores into the air. The ascospores are one-septate, straight or slightly curved, usually 2-3 by 6-12 micrometer. The disease cycles are initiated by ascospores which land on the under surface of young leaves when the relative humidity is nearly 100%.

Greasyspot disease severity is normally rated on a numerical scale based on the degree of foliar symptoms. For example, a zero means no symptoms and a 1 means one- to two shoots in a quadrant of a tree with greasyspot symptoms (Timmer *et.al.*, 1980). Ascospore trapping, using a Burkard 7-day recording spore trap (Burkhard Scientific Sales, Ltd., Rickmansworth, Herts, England) has been used as a quantitative assay to estimate inoculum levels. Seven-day tapes from the spore trap are cut into daily segments and mounted onto a slide. Tapes stained with cotton blue are read under a microscope to obtain relative total ascospores per week. Information on ascospore level is important for chemical control practices. Whiteside (1982) has shown that better control of greasyspot was obtained when the chemicals were applied during or after peak ascospore release than before major ascospore discharge. Ascospores of *M. citri* are released after irrigation or rainfall. In Texas, Timmer *et.al.* (1980) have shown that the peak ascospore release was between July and September. Ascospores that land on the lower surface of the leaf grow epiphytically, and enter the leaf through stomatal openings. Though quantitative data on ascospore release associated with rainfall and flood irrigation is known, there is no such information available from orchards with microsprinkler irrigation. Trees with microsprinkler irrigation receives more frequent water compared to trees with flood irrigation. Our objective was to develop a leaf clearing and staining method which offered a rapid and easier quantitative assay for ascospores of *M. citri* in citrus leaves. With modifications of previous whole-leaf clearing and staining procedures (Lee and Shaner, 1984; Shipton and Brown, 1962; White and Baker, 1954), we developed a novel leaf clearing and staining technique for ascospores of *M. citri*, in the lower surface of young citrus leaves.

MATERIALS & METHODS

Leaf samples: Leaf samples were obtained from a grapefruit orchard with severe greasyspot disease. The leaf samples were obtained between August and November, 1995. The leaf samples were collected from the mid-part of four quadrants (northeast, northwest, southeast, and southwest) from each of five trees. Two young leaves were collected from each of the quadrant of a tree and placed in a plastic bag

Table 1. Ascospores of Mycosphaerella citri counted on 7 mm diameter citrus leaf disks.

		Replicates over a period of four months			
Tree #	Tree quadrant	Aug. '95	Sept.'95	Oct. '95	Nov.'95
Tree 1	Northeast	179*	62	103	25
	Northwest	91	30	96	26
	Southeast	116	29	79	57
	Southwest	162	35	107	55
	Total	548	156	385	163
Tree 2	Northeast	105	29	57	71
	Northwest	96	37	40	79
	Southeast	85	15	42	53
	Southwest	78	222	58	25
	Total	364	303	197	228
Tree 3	Northeast	61	21	53	43
	Northwest	93	42	44	27
	Southeast	58	31	21	54
	Southwest	65	40	37	44
	Total	277	134	155	168
Tree 4	Northeast	88	35	38	56
	Northwest	86	20	54	29
	Southeast	98	28	38	23
	Southwest	117	33	33	23
	Total	389	116	163	131
Tree 5	Northeast	112	36	11	21
	Northwest	125	16	13	41
	Southeast	106	31	14	24
	Southwest	108	77	27	15
	Total	451	160	65	101

*individual readings in the table are averages of five disks read

and kept refrigerated until processing. Five leaf disks were cut from each leaf with a seven millimeter diameter cork borer.

Clearing and staining: Leaf disks were gently placed in a clearing agent containing two ml of 95% ethanol and one ml of lactophenol, for six days. Cleared sections were stained in either 70% chloral hydrate solution containing 0.1% trypan blue for two hours, or in 50% glycerine containing 0.1% trypan blue for 24 hours. Leaf disks were rinsed once for three minutes in water and stored in 50% glycerine. The ten leaf disks from one quadrant were mounted on a microscope slide with the lower side of the leaf facing up, and the ascospores were counted using a 10 X objective.

RESULTS & DISCUSSION

Since the clearing step made the leaves transparent, the spore counting was much easier. The one-septate ascospores were stained and they were easy to identify among other fungal spores, if any. Table 1 shows the total number of ascospores that were on the lower surface of a total of ten 7 mm-diameter leaf disks. There was considerable variation in the number of ascospores between the trees and between the sampling dates. However, the variation appeared to be less among the quadrants of a given tree.

Penetration of the germinating ascospores into the leaves occurs only through the stomatal opening. Therefore, it is important to make the spore counts on the lower surface of the disks. Care must be taken to keep the lower surface of the disks facing up in all of the steps involved in clearing, staining, and mounting process.

Compared to air sampling for ascospores using a Burkhard sampler (Timmer *et.al.*, 1980), the leaf disk clearing and staining procedure described above gives a direct quantitative assay of ascospores actually present on the leaves. This technique is easy to set up, and it has no additional capital investment. As chloral hydrate is a controlled substance, we prefer the use of glycerine and trypan blue. Though the process took a week, the quantitative data provides important information needed in making management decisions involving chemical control strategies. One week processing time may seemed long, however, there is sufficient time available for chemical application for greasyspot control.

This technique has already enabled us to follow the pattern of ascospores released as influenced by irrigation systems and the presence or absence of ascocarps in the leaf litter (Skaria and Tao, 1996). Also, it allows one to observe different stages of ascospore growth and penetration such as: the ramifying fungal growth over the leaf surface, hyphal tips growing into the sub-stomatal chambers, and the presence of an appressorium-like structure.

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