

Characterization of *Alternaria* isolates associated with Alternaria Rot of Citrus¹

Barry Pryor, Mike Matheron and Patricia Figuli

Introduction

Alternaria rot of citrus is a serious problem in citrus production world wide. In Arizona, the disease is most commonly found in Minneola tangelos and navel oranges grown in Maricopa County. Alternaria rot occurs primarily as a stem-end rot on fruit held in cold storage. However, under optimum conditions the disease occurs as a stylar-end rot in the orchards. In Arizona, the disease can significantly reduce yield, and annual fruit losses have been estimated at 0.5 box per tree. In terms of fruit quality, this disease can be a serious problem for the fresh fruit market as well as for the processing industry because only a small amount of rot imparts a bitter flavor and small black fragments of rotted tissue spoil the appearance of the juice.

The application of fungicides is the most common tactic used to reduce losses to this disease. However, to date, no consistent reduction in disease has been achieved through chemical applications. This suggests that additional information relating to the biology of the pathogen and the epidemiology of disease will be necessary for the successful development of a reliable disease management program.

Background and Objectives

In one of the first descriptions of Alternaria rot of citrus, the causal agent was identified as *Alternaria citri*. This fungus produces small spores aggregated in branching chains and produces several plant pathogenic toxins, which are specific to certain citrus cultivars. However, many *Alternaria* species also produce small spores aggregated in branching chains and collectively these species can be found in nearly all agricultural systems causing a variety of diseases. Moreover, only subtle morphological differences separate these species resulting in considerable difficulty differentiating these closely related fungi. More importantly, most plant pathology studies have not fully addressed the diversity of small-spored *Alternaria* species involved with the development of specific diseases. Although morphological differences may be subtle among small spored *Alternaria* species, the differences in pathogenicity may be great. Thus, for effective disease management of any disease caused by there fungi it is critically important to be able to separate the pathogenic species from the non-pathogenic species.

Previously, this separation has been difficult due to limited information on morphological characters useful for identification of small-spored *Alternaria* species. However, recent work by Dr. E. G. Simmons, recognized world-wide as the foremost authority on *Alternaria*, has resulted in the development of culture conditions and defined morphological criteria that permits the consistent and reliable identification of plant pathogenic small-spored *Alternaria* species. In specific work with citrus pathogens, Dr. Simmons has revealed that *A. citri* is actually a very uncommon fungus and that most of the isolates recovered from citrus and causing rot symptoms are not *A. citri*. In his recent work with citrus pathogens from Florida, South America, and the Middle East, studies revealed most fungi responsible for Alternaria rot of citrus more closely resembled other *Alternaria* species such as *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria*. Dr. Simmons goes on to describe 8 distinct *Alternaria* species associated with disease on citrus, determined from citrus samples sent to him from around the world. Most of these isolates could be placed into four different species-groups, the *A. alternata* group, the *A. tenuissima* group, the *A. arborescens* group, and the *A. infectoria* group. *Alternaria citri* appears to be a member of the *arborescens* group, although it is somewhat atypical for this group. DNA analysis, which would provide conclusive evidence for its taxonomic affiliations, has not been performed. It should be stressed that although these four groups have distinct

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names, they are morphologically very similar in many respects and identification is difficult for those not familiar with *Alternaria* taxonomy. The objectives of this study are to characterize *Alternaria* isolates recovered from citrus orchards in Arizona using morphological characteristics established by Simmons, examine the molecular diversity of these isolates using DNA fingerprinting techniques, and compare these isolates with known *Alternaria* species in order to definitively identify the pathogen responsible for Alternaria rot of citrus in Arizona.

Materials and Methods

Samples of fruit exhibiting disease lesions characteristic of symptoms of Alternaria fruit rot were collected from orchards near Phoenix, AZ, on 3/1/02. These included samples of tangelos, tangerines, navel oranges, and lemons. Samples of diseased fruit were collected from orchards near Wellton, AZ, on 3/5/02 and included samples of lemons, tangerines, and tangelos. Samples of diseased grapefruit were collected near Dateland, AZ, on 3/5/02. All samples were returned to the lab for the isolation of *Alternaria* fungi. Standard mycological techniques were used to isolate pure culture of fungi that were present both on the surface of the fruit lesion, directly underneath the surface of the fruit lesion, and 1/2" deep inside the fruit and underneath the lesion. All recovered isolates were stored on silica gel at 5 C for future studies.

All fungal isolates were plated on a nutrient medium (weak potato dextrose agar) and incubated under fluorescent lights for 7 days to stimulate fungal sporulation. The characteristics of conidia and sporulation apparatus were recorded according to methods described by Simmons and by Pryor and Michailides. Fungal isolates were also grown on standard potato dextrose agar and incubated for 7 days in darkness for development of diagnostic colony morphology according to methods described by Pryor and Michailides. These characteristics were compared with those from *Alternaria* species of known identity (type cultures) obtained from E. G. Simmons, which were cultured simultaneously. Preliminary identifications were obtained if possible and recorded.

DNA was extracted from field isolates according to methods described by Pryor and Michailides in previous work with *Alternaria* species recovered from pistachio. Five different DNA primers specific for regions of repeating genomic DNA (microsatellite primers; T3B, GACA₄, AAG₈) were used in polymerase chain reaction (PCR) to produce genetic fingerprints specific to each isolate. Genetic fingerprints patterns were analyzed by recording the presence or absence of DNA fragments of specific sizes produced from each isolate. These results were organized into a data matrix and analyzed by cluster analysis to identify groups with similar patterns. These patterns were compared to those of *Alternaria* species of known identity (type cultures) to provide a second line of evidence in identifications. Data from morphological characterizations and genetic fingerprints were compared and combined for definitive identification of *Alternaria* isolates recovered from citrus orchards in Arizona.

Results

In these studies, 218 fungal isolates were isolated from fruit lesions on tangelos, tangerines, lemons, oranges, and grapefruit from areas around Phoenix and Yuma (Yuma, Wellton, and Dateland). The identifications of these isolates base upon morphological analysis are presented in Table 1. To summarize this data, of the isolates recovered, 97 resembled *A. alternata*, 66 resembled *A. tenuissima*, 3 resembled *A. arborescens*, and 3 resembled *A. infectoria*. None of these isolates resembled *A. citri* based upon morphology. In addition, 3 isolates represent two new, very unusual *Alternaria* species never before described. Also several isolates of *Colletotrichum gloeosporioides*, *Botryodiplodia rhodina*, and *Sphaeropsis* sp. were recovered, all of which are know to cause fruit rot of citrus. Several sabrobic fungi were also recovered such as *Bipolaris* and *Stemphylium*, but their occurrence is probably as secondary infections. A breakdown of isolates by host revealed that 58.5 % of all isolates recovered from Minneola tangelo were *A. alternata* and 41.5 % were *A. tenuissima*. A similar ratio can be found in navel oranges. The pathogenicity of all recovered isolates has not yet been determined. To date, no correlation between the presence of a particular isolate and host (type of citrus) or location has been evident.

Molecular characterization of the *Alternaria* isolates using microsatellite primers revealed that several distinct fingerprint patterns were apparent among the recovered *Alternaria* species. Representatives of these patterns are revealed in Fig. 1. Due to difficulties in statistically analyzing many fingerprint patterns, a comprehensive analysis

of fungal populations based upon fingerprint pattern has not been completed. However, based upon visual inspection of patterns, several important conclusions have been obtained to date. The most common patterns were most similar to those of *Alternaria alternata*, which was the most common fungus identified based upon similar morphological characters. However, even among these isolates significant variation can be found between citrus isolates and the type culture and even among the citrus isolates. This suggests that the population in Arizona citrus groves is not typical of the species and is quite variable. Moreover, individual fingerprint patterns correlated with several morphological variants of *A. alternata*, suggesting that morphology is variable within a particular genotype (fingerprint pattern). A more complete understanding of *Alternaria* diversity and population structure will be obtained with the completion of microsatellite fingerprint analysis, which is ongoing. These results will be critical for future epidemiological studies, as well as the potential development of molecular-based tools for population assessment and pathogen detection.

Discussion

Based upon results from the 2002 study, we now know that a complex of *Alternaria* species and strains is found on *Alternaria* fruit rot lesions in Arizona, and that *A. citri* is not part of this complex. However, it is not known whether or not these isolates are equally pathogenic, or if some are extremely pathogenic whereas others are simply saprobes. Additional studies involving a series of pathogenicity tests using the 218 fungal isolates recovered and different citrus fruit as hosts would be necessary to determine if the *Alternaria* isolates (and other fungi) recovered are equally pathogenic or are differentially pathogenic to different citrus fruit.

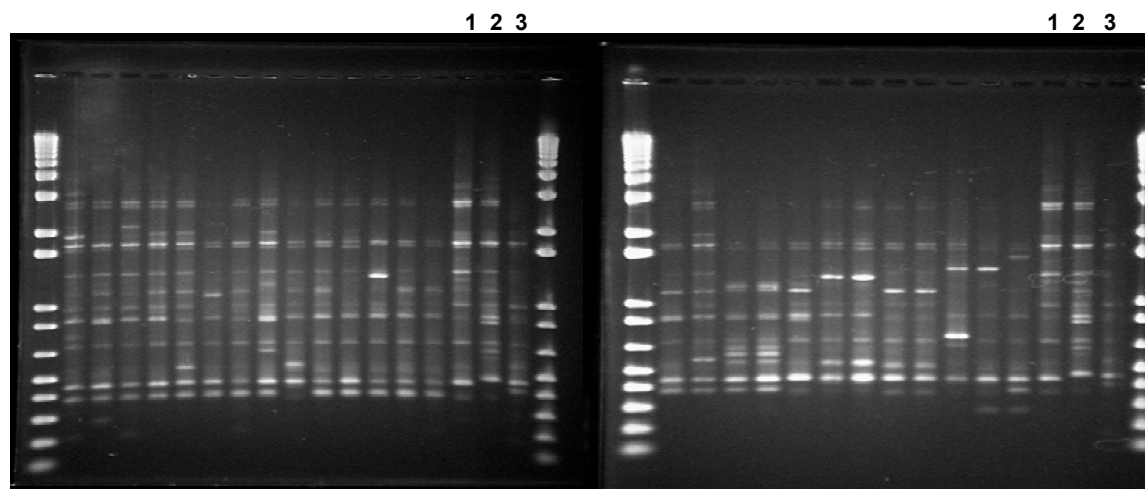
The importance of proper identification of the pathogen responsible for *Alternaria* rot of citrus cannot be overstated. It goes beyond simply providing the correct name of the fungus. First, it is critically important to be able to differentiate pathogenic *Alternaria* species from morphologically similar non-pathogenic *Alternaria* species. Both types are certain to occur in all citrus orchards, although the exact number of species may vary among geographic regions and among citrus varieties. It may very well be the case that more than one species is involved in disease development, which has recently been shown in the disease *Alternaria* late blight of pistachio (Pryor and Michailides). This information has direct applications in conducting periodic surveys of citrus orchards to determine pathogen populations, which may provide information on whether or not disease problems will occur later. If populations of pathogenic species are high, additional applications of protective fungicides may be required. If populations are low, growers may be able to reduce one or two applications during the season.

Proper identification of pathogenic *Alternaria* species will also have a direct impact on epidemiological studies and disease management. Different species have different life histories, such as growth rate, timing of sporulation, number of spores produced, and optimal conditions for spore germination and growth. All of these factors are important in the development of disease forecasting models, which are critical in optimizing effective and economical spray programs. In summary, the most successful management of *Alternaria* rot of citrus will be achieved only after a definitive assessment can be made of the diversity of small-spored *Alternaria* species that occur in citrus orchards, and the potential for these distinct species to cause disease.

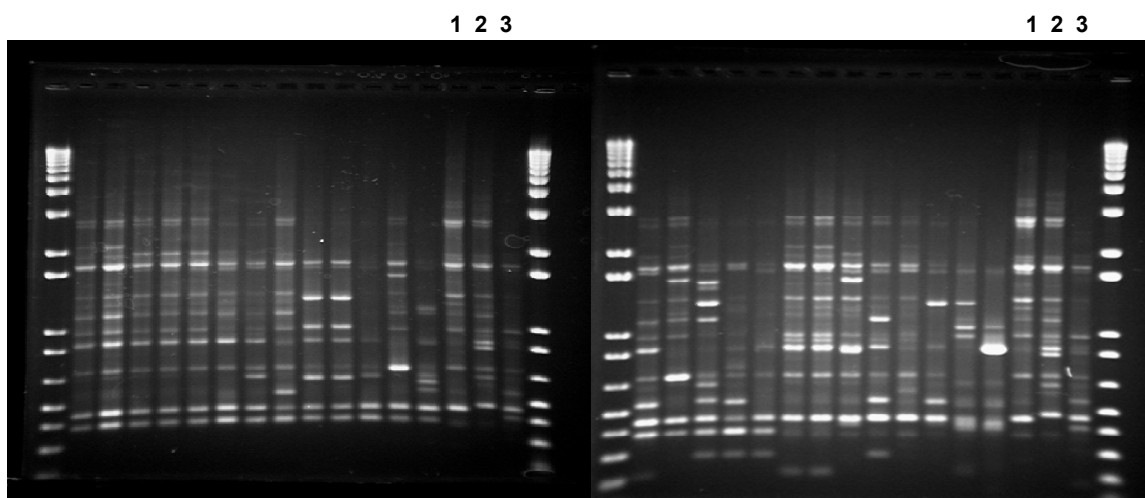
Table 1. Recoveries of fungi from citrus in Arizona exhibiting fruit rot symptoms. Identifications are based upon morphological characteristics.

Location	Fruit	Fungus	# of isolates
Phoenix	Minneola tangelo	<i>Alternaria alternata</i>	31
		<i>Alternaria tenuissima</i>	22
		<i>Stemphylium</i> sp.	1
	navel orange	<i>Alternaria alternata</i>	23
		<i>Alternaria tenuissima</i>	13
		<i>Alternaria arborescens</i>	1
		<i>Alternaria infectoria</i>	2
		<i>Alternaria new species</i>	1
		<i>Botryodiplodia</i> sp.	2
		<i>Sphaeropsis</i> sp.	1
		<i>Colletotrichum</i> sp.	1
	tangerine	<i>Alternaria alternata</i>	27
		<i>Alternaria tenuissima</i>	8
		<i>Colletotrichum</i> sp.	2
		<i>Sphaeropsis</i> sp.	2
		<i>Bipolaris</i> sp.	1
		<i>Alternaria new species</i>	1
	grapefruit	<i>Alternaria alternata</i>	7
		<i>Stemphylium</i> sp.	2
		<i>Sphaeropsis</i> sp.	1
		<i>Bipolaris</i> sp.	1
Yuma	tangerine	<i>Alternaria alternata</i>	8
		<i>Alternaria tenuissima</i>	15
		<i>Botryodiplodia</i> sp.	2
		<i>Sphaeropsis</i> sp.	5
		<i>Botryodiplodia</i> sp.	1
	grapefruit	<i>Alternaria alternata</i>	2
		<i>Alternaria tenuissima</i>	5
		<i>Alternaria arborescens</i>	2
		<i>Alternaria infectoria</i>	1
		<i>Alternaria new species</i>	2
	lemon	<i>Alternaria tenuissima</i>	3
		<i>Botryodiplodia</i> sp.	1

Fig. 1. Microsatellite fingerprints of select *Alternaria* isolates recovered from orchards in Phoenix and Yuma (including Wellton and Dateland). Fingerprint patterns were generated using PCR and the T3B microsatellite primer. PCR products were separated on 1% agarose gels. DNA size ladders flank each gel. Lanes labeled 1, 2, and 3 on each gel represent fingerprint patterns of *Alternaria* type isolates *A. alternata*, *A. tenuissima*, and *A. arborescens*, respectively.



Phoenix isolates



Yuma isolates