

FITOPATOLOGIA

ALTERNARIA BROWN SPOT

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SUMMARY

The fungus *Alternaria alternata* “tangerine pathotype” causes the disease known as alternaria brown spot (ABS), which affects tangerines and their hybrids. The fungus produces the host-selective toxin (HST) ACT, which is responsible for the pathogenesis. The genes *ACTT-1*, *ACTT-2*, *ACTT-5* and *ACTT-6*, responsible for the toxin biosynthesis, are clustered into a single small dispensable chromosome with 1.9 to 2.0 Mb in size. ACT-toxin causes necrosis in susceptible plants, and the disease affects twigs, young leaves and fruits. The symptoms include brown to black necrotic spots surrounded by a yellow halo. Severe cases of ABS can lead to fruits and leaves abscission or death of the entire shoot. Foliar applications of copper-based fungicides are recommended for controlling and reducing the severity of ABS in field.

Index terms: *Alternaria alternata*, *Citrus reticulata*, tangerines, ACT toxin.

RESUMO

MANCHA MARROM DE ALTERNÁRIA

O patótipo tangerina do fungo *Alternaria alternata* causa a doença conhecida como mancha marrom de alternária (MMA), afetando tangerinas e seus híbridos. O fungo produz a toxina hospedeiro-seletiva (THS) ACT que é responsável pela patogenicidade. Os genes *ACTT-1*, *ACTT-2*, *ACTT-5* e *ACTT-6*, responsáveis pela biossíntese da toxina, estão agrupados em um único e pequeno cromossomo dispensável de tamanho entre 1,9 à 2,0 Mb. A toxina ACT causa necrose em plantas susceptíveis, e a

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doença afeta brotações, folhas jovens e frutos. Os sintomas incluem pontos necróticos de coloração amarronzada a negra, circundados por um halo amarelado. Aplicações foliares de fungicidas cúpricos são recomendadas para o controle e redução da severidade de MMA em campo.

Termos de indexação: *Alternaria alternata*, *Citrus reticulata*, tangerinas, toxina ACT.

1. INTRODUCTION

Alternaria brown spot disease (ABS), caused by the fungus *Alternaria alternata* “tangerine pathotype”, affects tangerines and their hybrids. The disease was first described in Australia in 1903 affecting Emperor tangerine (*Citrus reticulata* Blanco) (KIELY, 1964) and subsequently in Florida (USA) in 1974 affecting Dancy tangerine (WHITESIDE, 1976). It was also reported in Israel (SOLEL, 1991), South Africa (SWART et al., 1996), Spain (VINCENT et al., 2000), Italy (BELLA et al., 2001) and Argentina (PERES et al., 2003). In Brazil, it was reported in the States of Minas Gerais, Rio Grande do Sul, Rio de Janeiro and São Paulo, affecting mostly Murcott tangor, Ponkan and Dancy tangerines (GOES et al., 2001; PERES et al., 2003; SPÓSITO et al., 2003).

The high severity and the difficulty to control the disease, especially in high humidity regions, have led to the abandonment of orchards of highly susceptible varieties (TIMMER et al., 2000b). In the State of São Paulo, many citrus producers have eradicated their orchards of susceptible plants, mostly Murcott tangor, due to the increase in production costs related to the control and management of the disease.

The fungus *Alternaria alternata*

The genus *Alternaria* was first described in 1816 with *A. tenuis* as the type isolate (NEES, 1817). The genus is distributed worldwide as saprophytes, endophytes, plant pathogens and as emerging human pathogens, especially in immunocompromised patients (PEEVER et al., 2004; THOMMA, 2003).

Alternaria is easily identified by the shape of its conidia, which are large, ovoid to obclavate, dark-colored (melanized), multicellular with longitudinal and transverse septations (phaeodictyospores). Near the base, they are broader becoming taper to the apex, providing a club-like appearance. Conidia are produced in single or branched chains on short conidiophores (BARNETT & HUNTER, 1998).

Most *Alternaria* species do not have known teleomorphic phase. These species were usually classified into the division of the mitosporic fungi or the Phylum Fungi Imperfecti (THOMMA, 2003). Analysis of ITS, 18S rDNA, mitochondrial rDNA (mt SSU) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) sequences revealed close phylogenetic relationships among *Alternaria*, *Ulocladium*, *Embellisia*, *Nympha* and *Stemphylium* (teleomorph *Pleospora*). *Stemphylium* appears as a sister taxon of the monophyletic clade *Alternaria-Ulocladium-Embellisia-Nympha*. (BEERBE, 1996; CHOU & WU, 2002; MORALES et al., 1995; PRYOR & BIGELOW, 2003; PRYOR & GILBERTSON, 2000). Most species of *Stemphylium* have established teleomorphs in the genus *Pleospora*, which makes *Alternaria* spp. related to Pleosporaceae family (Phylum Ascomycota; subphylum Pezizomycotina; class Dothideomycetes; subclass Pleosporomycetidae; order Pleosporales).

In 1992 it was proposed to organize the genus *Alternaria* into a number of species-groups (SIMMONS, 1992). Each group was typified by a representative species. For example, the *alternata* species-group include species with small catenate spores such as *A. alternata*. Others species-groups include the *brassicicola* group, the *cheiranthi* group, the *infectoria* group, the *porri* group, and the *tenuissima* group (SIMMONS, 1992, 1995).

Plant pathogenic species of *Alternaria* infect a broad range of economically important plants such as tangerine, apple, pear, tomato and potato. (PEEVER et al., 2004). In citrus, species of *Alternaria* cause the diseases known as *Alternaria* brown spot (ABS) of tangerines and their hybrids; *alternaria* leaf spot of rough lemon (*C. jambhiri* Lush.); black rot of citrus, a post harvest disease; and “*mancha foliar de los citricos*” citrus leaf spot of Mexican lime (*C. aurantifolia* [Christm.] Swingle), which occurs only in Western Mexico (AKIMITSU et al., 2003; PEEVER et al., 2004).

The phylogenetic classification of *Alternaria* citrus-associated species was unclear and confused along the time. Isolates causing ABS were originally classified as *A. citri* because their morphological similarity to isolates causing black rot (KIELY, 1964; PEGG, 1966). Further analysis, based on the descriptions of morphology and size of the conidia revealed their similarity to *A. alternata* (NISHIMURA & KOHMOTO, 1983). They were also referred as *A. alternata* f.sp. *citri* (SOLEL, 1991) to differentiate them from saprophytic isolates of *A. alternata*, and as *A. alternata* f.sp. *citri* tangerine (THOMMA, 2003) to differentiate them from isolates that infect rough lemon.

Analysis of sequences of mitochondrial ribosomal large subunit (mtLSU), beta-tubulin gene, endopolygalacturonase gene (endoPG) and two anonymous genomic regions (OPA1–3 and OPA2–1) of isolates causing ABS, *Alternaria* leaf spot and black rot of citrus was unable to detect significant genetic differences to classify them as distinct phylogenetic species (PEEVER et al., 2004). Considering that, it was proposed to classify all the small-spores citrus-associated isolates of *Alternaria* into the single phylogenetic species *A. alternata*.

In order to differentiate the isolates causing ABS on tangerines from those causing *Alternaria* leaf spot on rough lemon, the infrasubspecific classification of pathotypes is usually adopted. For example, isolates causing ABS are referred as *A. alternata* “tangerine pathotype” while those causing *Alternaria* leaf spot are referred as *A. alternata* “rough lemon pathotype”. Although they are morphologically similar, they produce distinct host-selective toxins (HSTs).

The host-selective toxin of *A. alternata* “tangerine pathotype”

Plant pathogenic species of *A. alternata* have several biological variants that produce distinct host-selective toxins (HSTs) (KOHMOTO & OTANI, 1991; NISHIMURA & KOHMOTO, 1983; THOMMA, 2003; MIYAMOTO, et al. 2008, 2009). HSTs are low-molecular weight secondary metabolites with toxicity toward distinct plant genotypes (YODER, 1980; NISHIMURA & KOHMOTO, 1983; WALTON, 1996; WOLPERT et al., 2003).

The tangerine pathotype of *A. alternata* produces the HST known as ACT-toxin, which is responsible for the pathogenesis to tangerines and their hybrids (AKIMITSU et al., 2003; KOHMOTO & OTANI, 1991; KOHMOTO et al., 1979, 1991; MIYAMOTO et al., 2008, 2009). The mode of action of ACT toxin is not clear, but some evidences suggest that the toxin initially affects the plasma membrane of susceptible cells (KOHMOTO et al., 1993). A small amount of the ACT-toxin ($2 \times 10^{-8} \text{M}$) causes veinal necrosis on leaves with a rapid loss of electrolytes from cells (KOHMOTO & OTANI, 1991; KOHMOTO et al., 1991, 1993).

The structure of ACT toxin is closely related to AK- and AF-toxins, HSTs from Japanese pear and strawberry pathotypes of *A. alternata*, respectively. These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (KOHMOTO et al., 1993; NAKASHIMA et al., 1985; NAKATSUKA et al., 1986).

Heterologous probes based on the genes (*AKT-1* and *AKT-2*) responsible for the synthesis of the AKT-toxin in the Japanese pear pathotype, were used to identify the homologous *ACCT-1* and *ACTT-2* genes in the tangerine pathotype. These homologous genes are involved in the biosynthesis of the decatrienoic acid moiety of ACT-toxin (MASUNAKA et al., 2000). *ACTT-2* encodes a putative hydrolase and is present as multiple copies in the genome. The RNA silencing of the *ACTT-2* gene leads to the lost of ACT-toxin production as well as the fungal pathogenicity (MIYAMOTO et al., 2008).

Other two genes involved in the ACT-toxin biosynthesis were recently discovered. The genes *ACTT-5* and *ACTT-6* encode a putative acyl-CoA synthetase and enoyl-CoA hydratase respectively (MYIAMOTO et al., 2009). The genes *ACTT-1*, *ACTT-2*, *ACTT-5* and *ACTT-6* are clustered into a single small dispensable chromosome with a size of 1.9 to 2.0 Mb (MASUNAKA et al., 2005; MYIAMOTO et al., 2009). The cluster has been designated as ACT-toxin TOX cluster (ACTT) (MYIAMOTO et al., 2009).

Symptoms and disease cycle

The disease affects young twigs, leaves, fruits (Figure 1A) and leaves (Figure 1B). The symptoms include brown to black necrotic spots surrounded

by a yellow halo. Chlorosis and necrosis continue to expand along the veins due to the spread of the host-selective ACT-toxin produced (Figure 2D) by *A. alternata* (KOHMOTO et al., 1993). Severe cases of ABS can lead to fruits and leaves abscission or death of the entire shoot. Depending on the fungal strain and the plant susceptibility, symptoms can appear 16-48 hours after infection (TIMMER et al., 2003).

The disease cycle is simple (Figure 2) once no teleomorph has been related to *A. alternata*. Conidiophores emerge through the lesions and/or stomata of infected leaves (Figure 2G-F) that remain on the tree or have fallen to the grove floor. Conidia are released by rainfall events or sudden changes in relative humidity (TIMMER et al., 1998). They are easily dispersed by wind currents and rains drops, been deposited on the surface of susceptible fruits and leaves. Conidia germinate in the presence of moisture (Figure 2C) and the infection is as intense as the leaf wetness duration (CANIHOS et al., 1999).

The way the fungus penetrates the leaf depends on different hosts, environmental conditions and/or isolates of the pathogen. Penetration occurs through stomata or with the formation of appressoria in both sides of the leaf. Studies in Florida reported the penetration only through stomata without formation of appressoria (Bhatia, Peever and Timmer, *unpublished data* cited by AKIMITSU et al., 2003). In Israel, penetration was reported only with the formation of appressoria (SOLEL & KIMCHI, 1997). So far, our group observed penetration exclusively through appressoria formation on both adaxial and abaxial sides of Murcott tangor leaves (Figure 2D). After penetration, necrotic halos are observed surrounding appressoria due to the rapid spread of ACT-toxin in susceptible tissues. The toxin spread across leaf veins and necrosis hit all the tissues in contact to the toxin. The fungus grows on/into the lesions. Conidiophores produce conidia that are released and deposited on the surface of susceptible fruits and leaves, starting a new disease cycle.

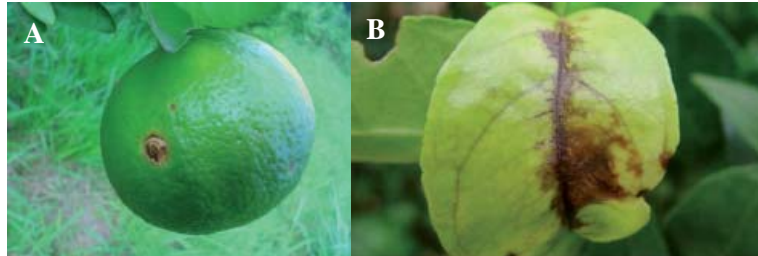


Figure 1. Typical disease symptoms of alternaria brown spot in fruits (A) and leaves (B) of Murcott tangor.

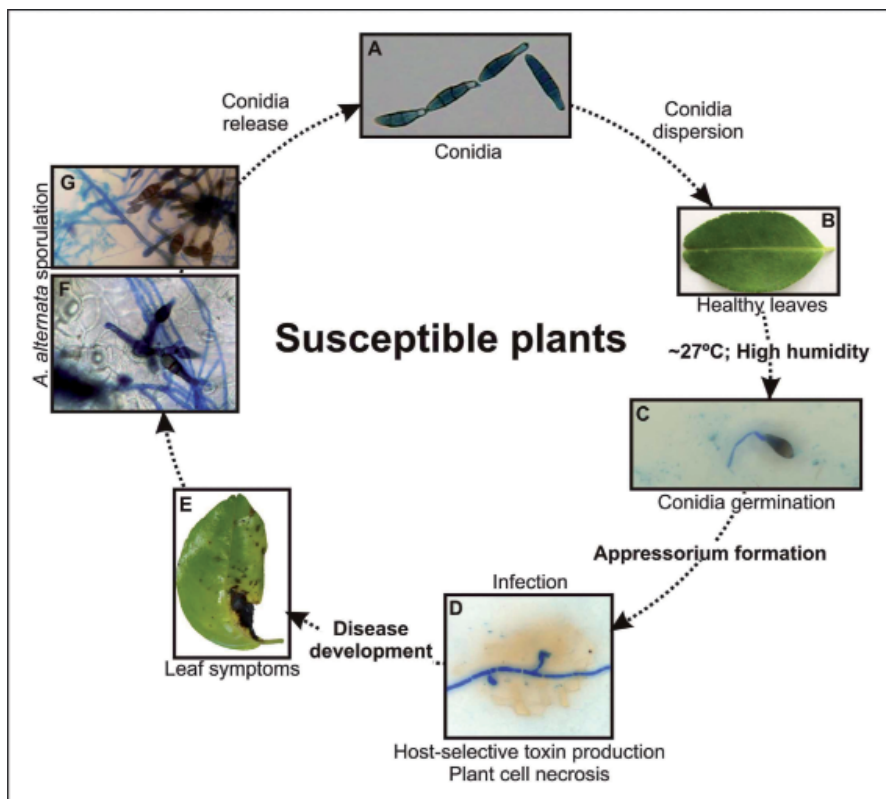


Figure 2. Disease cycle of alternaria brown spot. Conidia of *A. alternata* (A), susceptible leaves (B), conidia germination (C), appressorium formation and toxin production (D), leaf symptoms (E) and sporulation of the fungi (F-G).

Citrus susceptibility and resistance to alternaria brown spot

Alternaria brown spot affects mostly tangerines and their hybrids. The molecular mechanisms involved in disease resistance or susceptibility are still obscure. Direct and indirect hybrids of Dancy tangerine, some tangors (Murcott and Ortanique), and in some cases Redblush and Sunrise grapefruit are susceptible (TIMMER et al., 2003; SOUZA et al., 2009). The disease was also reported affecting Africa do Sul and Daisy tangerines, Nova tangelo and Ortanique tangor, as well as Temple x Dancy tangerine hybrids and Satsuma x Murcott 4 hybrids (FEICHTENBERGER et al., 2005; SOUZA et al., 2009).

In general, Clementine (*Citrus clementina* Hort. ex Tan.) and Cleopatra tangerine (*C. reshni* Hort. Tanaka), limes (*C. latifolia* Tanaka), lemons (*C. lemon* Burmann) and sweet oranges (*C. sinensis* L. Osbeck) are resistant to *Alternaria* brown spot in field. However, under laboratory conditions, some symptoms were induced in Valencia, Shamouti and Washington Navel oranges as well as in Volkameriano lemon after inoculating *A. alternata* “tangerine pathotype” obtained from Minneola tangelo (SOLEIL & KIMCHI, 1997).

Dancy tangerine is the parental of most of the hybrids and tangelos that are susceptible to the disease. It is speculated that the susceptibility is inherited from Dancy as a dominant characteristic, while resistance is thought to be recessive (KOHMOTO et al., 1991). Reciprocal crosses between resistant Clementine (ss) and its susceptible Clementine x Minneola tangelo (Ss) supported the hypothesis that resistance to *A. alternata* is controlled by a single recessive allele inherited from Clementine (DALKILIC et al., 2005). As demonstrated by the authors, an expected 1:1 (resistant:susceptible) segregation ratio was obtained in the Clementine x Clementine x Minneola backcross. However, the reciprocal crossing using Clementine as female parental generated a distorted 3:1 (resistant:susceptible) ratio, suggesting the influence of cytoplasmic genes on offspring resistance.

Different degrees of resistance/susceptibility between close related hybrids suggests that other genes may be involved in the interaction *Citrus-A. alternata* (PEEVER et al., 2000). In fact, a quantitative resistance loci (QRL) explaining 30% of the phenotypic characteristics was identified in a citrus linkage map after analyzing 143 hybrids of susceptible Murcott tangor and

resistant Pera sweet orange showing a phenotypical 3:1 (susceptible:resistance) segregation ratio, considering percentage of affected twigs (BASTIANEL et al., 2005). This QRL is in accordance with the idea that another gene should be involved in the resistance of citrus against *A. alternata*.

Disease control

Foliar sprayings of copper-based fungicides (Table 1) are recommended for controlling and reducing the severity of ABS disease in field (TIMMER & ZITKO, 1994; SOLEL et al., 1997). Considering that the disease incidence is related to environmental conditions (humidity, heat and wetness), it should be necessary up to 15 fungicide applications to a proper control (TIMMER et. al, 2003).

Table 1. Effective fungicides against *Alternaria alternata*.

Group	Common name
Copper-based	Copper oxychloride
	Copper sulfate
	Copper hydroxide
	Cuprous oxide
Dithiocarbamates	Mancozeb
	Propineb
Dicarboximides	Iprodione
	Procymidone
Strobilurins	Pyraclostrobin
	Tryfloxystrobin
Conazoles	Difenoconazole
	Tebuconazole

Foliar copper-based fungicides have disadvantages once their activity is superficial, without penetration in foliar tissues. During rains, foliar copper-based fungicides are easily washed from leaves surfaces. Fungicides applications must be adjusted to those periods were the environmental conditions are the most favorable to the fungal dissemination (ALVA & GRAHAM, 1991).

Fungicides like dithiocarbamates, dicarboximides, strobilurins and conazoles are also effective against *A. alternata* (Table 1). In contrast, benzimidazoles are ineffective for controlling ABS due to the capability of *A. alternaria* to metabolize the compound (FEICHTENBERGER et al., 2005).

In order to determine the correct period for fungicide application, a system called *Alter-rater* was created in Florida based on mathematical models that daily predict the most favorable environmental condition for disease appearance (BHATIA et al., 2003). A point value from 0 to 11 is assigned to each day depending on the weather factors: rainfall more or less than 2.5 mm; leaf wetness more or less than 10 h; and average daily temperature below 20 °C, between 20 and 28°C, or greater than 28°C (TIMMER et al., 2000a). The point values are accumulated on a daily basis until the total reaches the predefined trigger value. Trigger values of 50, 75, 100, and 150 points for application of copper fungicides were suggested for groves with different cultivars and disease histories.

Although little is known about the effects of cultural practices on ABS severity, some practices might be helpful for reducing ABS incidence. Wider spacing, skirting and the use of under-tree irrigation systems seem to reduce disease severity in some groves in Florida (TIMMER et al., 2003). Avoidance of nitrogen fertilization reduces plant vegetative growth, limiting production of large amount of susceptible tissues.

2. CONCLUDING REMARKS

Alternaria brown spot represents a threat to tangerines future in Brazil. Disease control is difficult and expensive, forcing producers to replace tangerine groves with more profitable crops. The success of tangerine groves depends on better understanding the molecular mechanisms involved in ABS resistance together with the selection and production of resistant genotypes.

An intensive selection program performed by the partnership among Centro APTA Citros Sylvio Moreira and Unidade de Pesquisa de Desenvolvimento de Sorocaba (APTA Regional) is evaluating the field resistance of several varieties of tangerines and tangors. These varieties

are part of the Citrus Active Germplasm Bank of Centro APTA Citros Sylvio Moreira. Additionally, more than 1,000 hybrids of tangerines and sweet oranges have been evaluated to select resistant plants against ABS (SANTOS et al., 2009). Some of them are showing greater resistance in comparison to Murcott tangor and Ponkan tangerine. Varieties as Fremont and Thomas tangerines have shown resistance to ABS (AZEVEDO et al., 2009).

Associated to the selection program, molecular studies focusing on differential gene and protein expression of resistant and tolerant plants have been performed in order to identify the molecular mechanisms involved in resistance of different citrus genotypes. Projects performing microarray analysis and protein 2-Dimensional Electrophoresis (2DE) have been conducted and represent an important step to understand *Citrus-Alternaria* interaction (STUART et al., 2008; 2009).

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